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**PAPER**

# Ultrasensitive detection of phenolic compounds based on a spin-labeled luminescent lanthanide complex

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Herein we propose a novel method for ultrasensitive detection of phenolic compounds. This method was developed based on a spin-labeled terbium complex $Tb^{3+}$/cs124-DTPA-TEMPO (**1**). This spin-labeled terbium complex is a weakly luminescent compound and shows strong off-on luminescent response to phenolic compounds in the presence of horseradish peroxidase (HRP), glutathione (GSH) and hydrogen peroxide. The analyte recognition and signaling mechanism are discussed and the factors affecting the off-on luminescence have been explored. Detection limits of 1.1 nM for phenol, 1.1 nM for resorcine, 0.6 nM for *m*-cresol, 3 nM for *p*-cresol, and 0.5 nM for 2,4-dichlorophenol were obtained, respectively. The practicability of the proposed method has been tested in detection of the concentration of spiked nearshore seawaters, and recoveries of 77.4–80.4% with relative standard deviations (RSDs) of 1.0–2.2% were obtained.

## Introduction

Natural phenolic compounds, used as antioxidants either in the form of natural plant materials such as fruits, vegetables, grains, nuts, oilseeds and herbs or as doses of isolated compounds, play important roles in preventing and fighting disease. Natural phenolic compounds have been also found to be harmful to organisms including humans depending on the concentration and chemical function.[1,2] Industrial phenolics are inherently toxic with regard to their effect on organisms,[3] and the toxic effect is dependent on the structure of phenolic compounds.[4] Wide use of phenolic compounds in pharmacy, preservatives, dyestuffs, pesticides and cosmetics as industrial raw and processed materials, usually released into the environment, has induced more and more environmental concerns. The quantification of phenolic compounds for controlling their abuse in pharmacy and the food industry and probing the biological activities of phenolic compounds and their metabolites in biosystems have received increased attention in recent years.[2] Conventional methods for determination of phenolic content are the Folin and Ciocalteu method,[5] the antipyrine dye method,[6] and chromatography.[7,8] Other methods include electrochemistry,[9] chemiluminescence[10,11] and immunochemical methods.[3]

Results have demonstrated that phenolic compounds with electron-withdrawing substituents display phenoxyl radical intermediated deleterious pro-oxidant activity by oxidizing essential thiols, such as glutathione (GSH), and generate reactive oxygen species that damage lipids, proteins and DNA. Such phenoxyl radicals may also react directly with DNA to form covalent DNA.[4] Peroxidase-catalyzed reactions, such as those catalyzed by myeloperoxidase (MPO) or cyclo-oxygenase (COX) have proved to be a highly efficient way to provide phenoxyl radicals. Horseradish peroxidase (HRP), a heme-containing peroxidase, has demonstrated sensitivity for a great number of phenolic compounds.[12–15] A two-electron oxidation of the ferri-heme in the active site of HRP produces an enzyme intermediate, compound I, in the presence of hydrogen peroxide. Two following one-electron reduction steps return the peroxidase to its native state in the presence of phenolic substrates and phenoxyl radicals are produced in the meantime. Within the intracellular milieu, these detrimental phenoxyl free radicals are primarily quenched by antioxidant glutathione (GSH)—a poor substrate of peroxidase, and result in the formation of more stable and easily trapped thiyl radicals (glutathionyl radical, GS·); at the same time, the phenols regenerate (Chart 1).[16] In the above reaction, the phenolic compounds play an important role in promoting GS· production. Glutathionyl radicals have

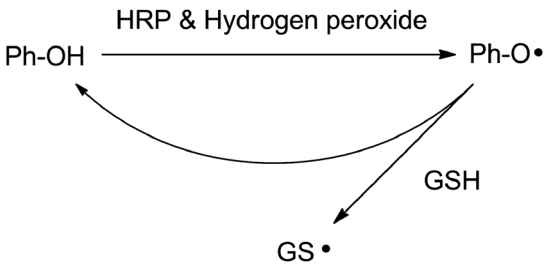


**Chart 1**

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proved to react effectively and irreversibly with nitroxides to produce secondary amines.[16,17] By exploring new methods that have the characteristics of high sensitivity, selectivity, ease of operation and high speed for quantification of phenolic compounds, we found that the GS· radical related phenol-phenoxyl radical recycling gave us a good chance to develop a radical recognition and luminescence signaling-based method for phenolic compounds detection, and furthermore, the phenol-phenoxyl radical recycling would induce a signal amplification and show ultrasensitive signaling properties.

For the thiyl radical recognition, we chose, based on our previous work, a water-soluble spin-labeled terbium complex $Tb^{3+}$/cs124-DTPA-TEMPO (**1** in Chart 2), where TEMPO = 2,2,6,6-tetramethylpiperidinyloxy, a nitroxide radical that is responsive to the thiyl radical, cs124 = 7-amino-4-methyl-2(1H)-quinolinone and DTPA = diethylenetriamenepentaacetic.[18] **1** shows a high electron paramagnetic resonance (EPR) signal of the nitroxide radical moiety and weak luminescence, as a result of the intramolecular quenching effect of the nitroxide radical on the sensitized terbium luminescence. The intensity of both the EPR and luminescence can be modulated by eliminating the paramagnetism of the nitroxide radical through recognition of an analyte of reductant, carbon-centered radical, or thiyl radical, and thus respond to an analyte or a radical by off-on luminescent and on-off EPR bimodal signaling. Nitroxide radicals are a familiar tool to recognize thiyl radicals by EPR spectroscopy. Nitroxide radicals can trap the thiyl radicals rapidly with a rate constant of about $10^9$ $M^{-1}$ $s^{-1}$.[16] In recent years, nitroxide covalently labeled fluorophores have received increasing attention, and a review on the use of tethered nitroxide–fluorophore molecules as probes of oxidative change and free radical generation and reaction has been given.[19] Compared with a spin-labeled organic fluorophore, the spin-labeled lanthanide-based complex fluorophore shows unique properties. Luminescent lanthanide complexes have been increasingly employed in the detection of biologically important analytes due to their long emission lifetimes, which enable the removal of light scattering and short-lived background autofluorescence with a time-resolved luminescence technique.[20–23] Sensitive and selective time-gated luminescence detection of hydroxyl radicals in water by using a terbium probe has also been reported.[24] Herein, we demonstrate that **1** shows a sensitive response to thiyl radicals, and that the off-on luminescent and on-off EPR signal is related stoichiometrically to phenolic compounds. A luminescent method with remarkable detection limits has been developed. And this thiyl trapping-based method may also be favorable for a time-resolved luminescence technique and show great potential in probing phenolic compounds and their metabolites in biological systems.

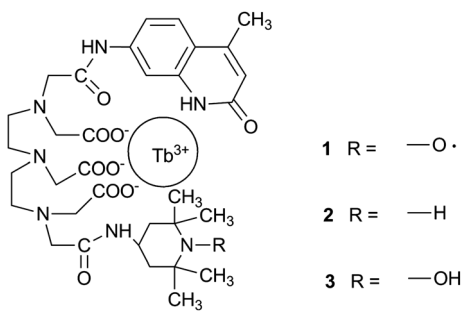



Chart 2

## Experimental section

### Chemicals

**1** was synthesized following the route set up by this lab's previous work and the detailed procedure for preparation and characterization of **1** has been described in our previous published paper.[18] The glutathione (GSH) reduced, phenol, *m*-cresol, resorcine, 2,4-dichlorophenol, 2,6-dimethylphenol, 2-nitrophenol, quinol, 4-aminophenol, catechol, phloroglucinol and hydrogen peroxide were purchased from Sangon Biotech (Shanghai). Horseradish peroxidase (HRP), *p*-cresol and L-tyrosine were purchased from Sigma. All the chemicals were used as received without further purification. Solutions were prepared with demineralized water which was obtained from a Millipore Direct-Q purification system (18.2 MΩ). All experiments were carried out in 10 mM Tris-HCl buffer solution at pH 7.4 and at room temperature.

### Apparatus

All luminescence measurements were taken with a RF-5301PC spectrofluorophotometer (Shimadzu). The excitation wavelength was set at 340 nm, and the intensities of Tb luminescence were acquired at 546 nm. EPR measurements were recorded on a Bruker EMX X-Band EPR spectrometer. All microwave parameters were kept constant, approximately 9.8 GHz microwave frequency, 20 mW microwave power, 10 dB attenuator, 100 kHz modulation frequency, 1.0 G modulation amplitude and 1.280 ms time constant.

## Results and discussion

### HRP catalyzed phenol-dependent off-on luminescence and on-off EPR bimodal signaling

The recognition and signaling mechanism of the spin-labeled lanthanide complex probe **1** to phenolic compounds is shown in Chart 3.

In the presence of $H_2O_2$ and HRP, phenolic compounds were oxidized and transformed to the corresponding phenoxyl radicals. The produced phenoxyl radicals subsequently reacted with GSH, with the formation of GS·, and recycling between phenolic compounds and phenoxyl radicals took place. On reaction with

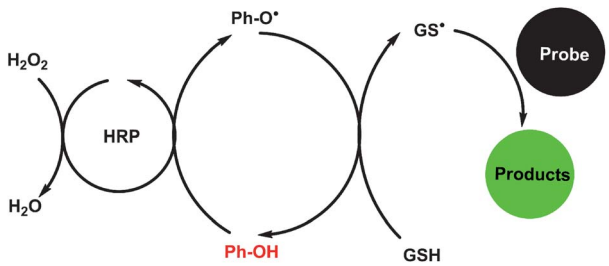



Chart 3

the thiyl radical, the nitroxide radical in **1** changed to its diamagnetic derivatives and a strong off-on luminescent increase was observed. Concomitant with the off-on luminescent increase was an on-off EPR signal decrease (Fig. 1). The highly luminescent derivatives were supposed to be the piperidine derivative (**2** in Chart 2) and the hydroxylamine derivative (**3** in Chart 2), two major products of the nitroxide radical trapping thiyl radical,[16] which would also lead to the paramagnetic signal vanishing. For control, experiments with the same conditions except in the absence of phenol, GSH, hydrogen peroxide and HRP, respectively, were carried out. The results demonstrated that a much slower increase of luminescence was observed for the control systems lacking phenol, GSH and hydrogen peroxide, respectively, and no noticeable changes in luminescence were detected in the absence of HRP (Fig. 2). Results given by the control systems suggest that **1** hardly reacts directly with the phenoxyl radical. We have previously noticed that the nitroxide radical covalently linked to a sulfonated poly(phenylene ethylnylene) backbone (PPE-$SO_3$) reacted with GSH and L-cysteine (CySH) and off-on fluorescence was observed.[25] Different from the spin-labeled polymer, this spin-labeled lanthanide compound is not sensitive to GSH.

### Phenol concentration dependent off-on $Tb^{3+}$ luminescence

For quantification of phenol, a relative luminescent intensity, $\Delta L/L_0$, was used as the indicator for depressing the background resulting from the control system, where $\Delta L = L - L_0$, $L$ and $L_0$ represent luminescence in the presence and absence of phenol, respectively. Fig. 3 shows luminescent intensities as a function of reaction time. The results demonstrate that the reaction rates are dependent on the concentrations of phenolic compounds, which act as quasi-catalysts in producing thiyl radicals. Measuring the luminescent intensities at a fixed-time $t$, the relative luminescent intensities give linear responses to phenolic compounds (Fig. 4). The sensitivity increased with a increasing fixed-time $t$ when the data were measured. In practical application, the fixed-time $t$ can be set accordingly (Table 1).

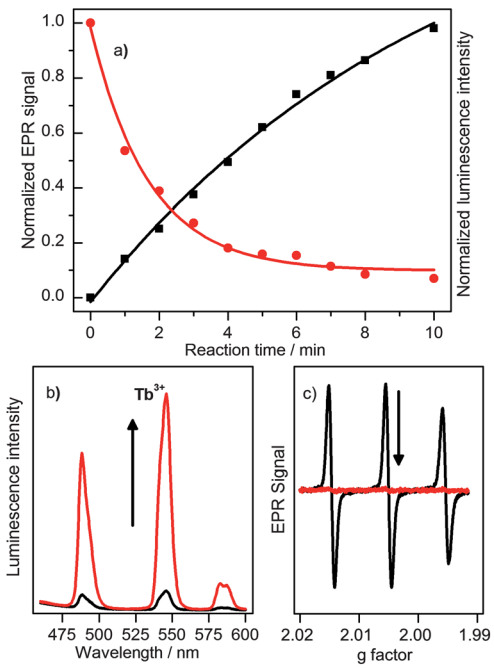



**Fig. 1** (a) Time-dependent TEMPO EPR signal (red, centered at $g$ = 2.006) and Tb luminescence (black, $\lambda_{ex}$ = 340 nm, $\lambda_{em}$ = 546 nm) following addition of 5 μM phenol, 10 μM hydrogen peroxide, 10 μM GSH and 1.25 mg $L^{-1}$ HRP to a solution of 10 μM of **1** in 10 mM Tris-HCl buffer at pH 7.4. (b) Comparison of luminescence spectra ($\lambda_{ex}$ = 340 nm) and (c) EPR spectra before (black line) and after reaction (red line).

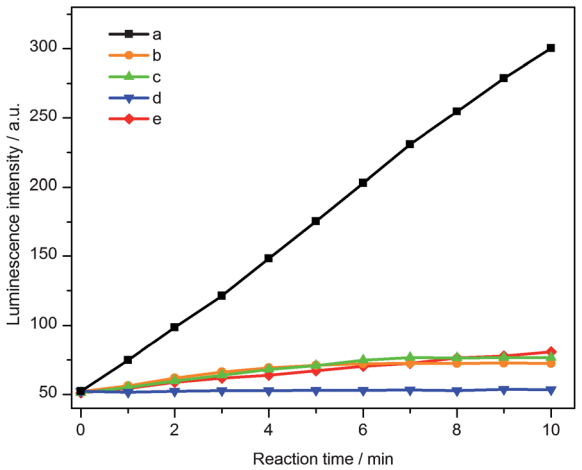



**Fig. 2** Luminescent intensities ($\lambda_{ex}$ = 340 nm, $\lambda_{em}$ = 546 nm) as a function of reaction time for the reaction system of 0.05 μM phenol, 0.1 μM hydrogen peroxide, 0.1 μM GSH, 0.125 mg $L^{-1}$ HRP and 0.1 μM of **1** in 10 mM Tris-HCl buffer at pH 7.4 (a), and those in the absence of GSH (b), hydrogen peroxide (c), HRP (d) and phenol (e) respectively.

### Factors affecting the off-on $Tb^{3+}$ luminescence

Phenolic compounds are typical substrates of peroxidases. Peroxidases, such as HRP or MPO containing phenol-binding sites, oxidize phenolic compounds efficiently to the

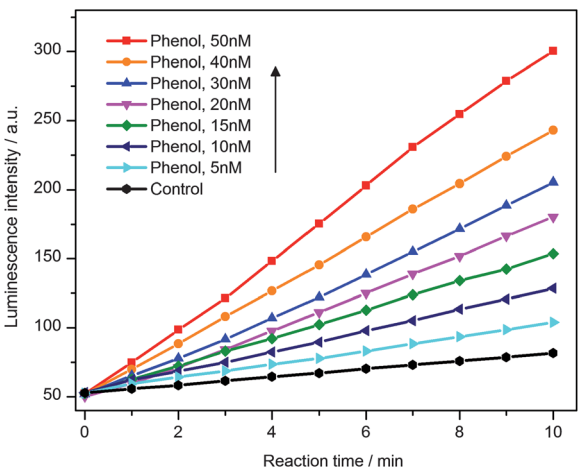



**Fig. 3** The luminescence intensities ($\lambda_{ex}$ = 340 nm, $\lambda_{em}$ = 546 nm) as a function of reaction time in the presence of various concentrations of phenol from 0 (bottom) to 50 nM (top) within 10 min following addition of 0.1 μM hydrogen peroxide, 0.1 μM GSH and 0.125 mg $L^{-1}$ HRP to a solution of 0.1 μM of **1** in 10 mM Tris-HCl buffer at pH 7.4.

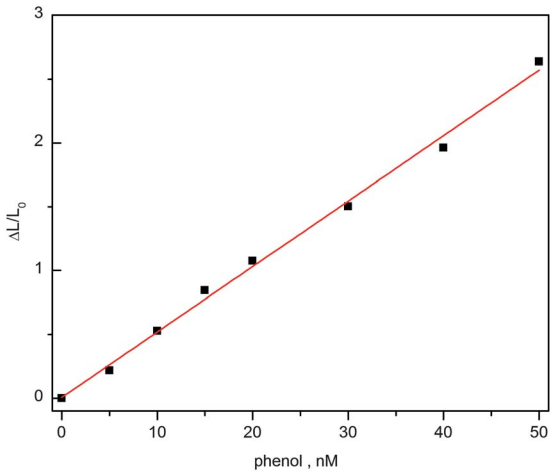



**Fig. 4** The plot of $\Delta L/L_0$ measured with a fixed-time $t$ of 10 min against the concentration of phenol ($\lambda_{ex}$ = 340 nm, $\lambda_{em}$ = 546 nm) following addition of 0.1 μM hydrogen peroxide, 0.1 μM GSH and 0.125 mg $L^{-1}$ HRP to a solution of 0.1 μM of **1** in 10 mM Tris-HCl buffer at pH 7.4.

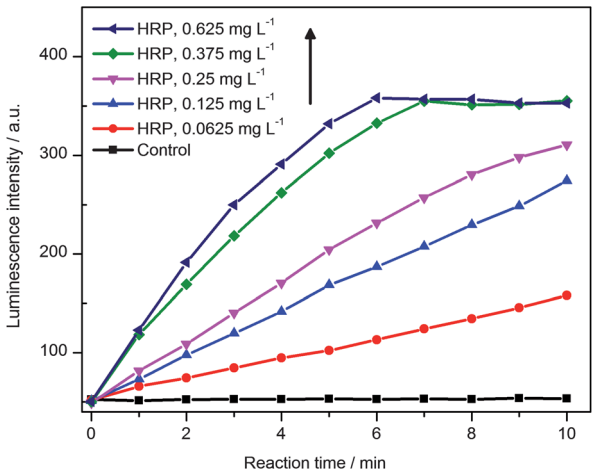



**Fig. 5** Luminescent intensity as a function of reaction time for reaction systems with varying HRP concentrations from 0 (bottom) to 0.625 (top) mg $L^{-1}$.

corresponding phenoxyl radicals with rate constants of $10^6$ to $10^8$ $M^{-1}$ $s^{-1}$.[14] Among these peroxidases, HRP is relatively cheap and commercially available in different purities. The concentration of HRP, functioning as a catalyst in the phenol-phenoxyl recycling, is an important factor in controlling the reaction rate. Luminescent intensities were measured as a function of reaction time after various amounts of HRP were added into the pH 7.4 Tris-HCl buffer solutions containing phenol, GSH, $H_2O_2$ and **1**. The results demonstrate that luminescence increases faster at a higher HRP concentration (Fig. 5).

When HRP is used as a catalyst for oxidation of phenolic compounds, hydrogen peroxide is demanded as an oxidant. The overall apparent reaction rate was dependent on $H_2O_2$ concentration when the concentration of $H_2O_2$ was relatively low and other conponents were kept constant. The overall apparent reaction rate became relative steady when the concentration of $H_2O_2$ varied from 0.1–1 μM. The catalytic reaction was stoped immediately when catalase was added in the reaction system and the luminescence from then on kept constant (data not shown).

### Detection limits, linear ranges and selectivity for detection of phenolic compounds

Based on the mechanism mentioned above, the optimized experiment conditions were: 0.1 μM of **1** was mixed with GSH (0.1 μM), hydrogen peroxide (0.1 μM) and phenol (in varying concentration), then HRP (0.125 mg $L^{-1}$) was added into the mixture solution, and the luminescent intensity was measured at 10 min after the addition of HRP. The results show that the plot of the relative luminescent intensity ($\Delta L/L_0$) *versus* the concentration of phenol is linear in the range of 5–50 nM ($R$ = 0.998) with a detection limit of 1.1 nM based on a signal-to-noise ratio (S/N) of 3, indicating that **1** can serve as a quantitative and sensitive probe for phenol.

The luminescence response of **1** to other phenolic compounds was also tested, and ascending luminescence was observed for resorcine, *m*-cresol, *p*-cresol and 2,6-dichlorophenol present, as shown in Table 1. But no obvious off-on luminescence response was observed for 2,6-dimethylphenol, 2-nitrophenol, 4-aminophenol, quinol, catechol, phloroglucinol and L-tyrosine, even at much higher concentration (Fig. 6). All phenols mentioned above are shown in Scheme 1.

A different reactivity of phenolic compounds as the substrate of HRP has been observed in early researches. The difference may be ascribed to several factors. Some literatures claimed phenolic compounds with electron-donor substituents in an *ortho*-position and electron-acceptor substituents in a *meta*-position show no response to the catalytic action of HRP.[26–28]

The practicability of the proposed method was tested in determination of the concentration of 2,4-dichlorphenol in environmental waters. The water samples were collected at the surface of nearshore seawaters in the south of Xiamen. The water samples were stored in tin foil packaged bottles to avoid light. Freshly obtained seawater samples were filtered with a 0.45 μm filter membrane and then kept at 4 °C. The samples were

**Table 1** Determination of phenolic compounds

| Phenolic compound | Fixed-time $t$ (min) | Linear range for determination (nM) | Detection limit (nM) | $R$ ($n$ = 7) | Standard curve equation (nM) |
|---|---|---|---|---|---|
| Phenol | 10 | 5 to 50 | 1.1 | 0.998 | $y = 0.00574 + 0.0513x$ |
| Resorcine | 10 | 5 to 50 | 1.1 | 0.998 | $y = -0.0210 + 0.0516x$ |
| *m*-Cresol | 5 | 5 to 50 | 0.6 | 0.982 | $y = 0.471 + 0.0840x$ |
| *p*-Cresol | 5 | 5 to 50 | 3.0 | 0.995 | $y = 0.0572 + 0.0173x$ |
| 2,4-Dichlorophenol | 3 | 5 to 50 | 0.5 | 0.998 | $y = 0.0832 + 0.0781x$ |

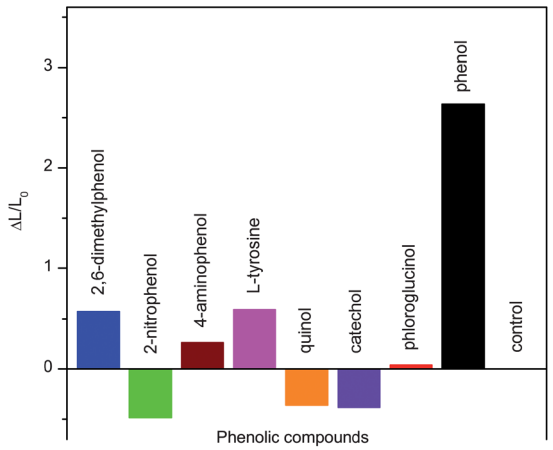
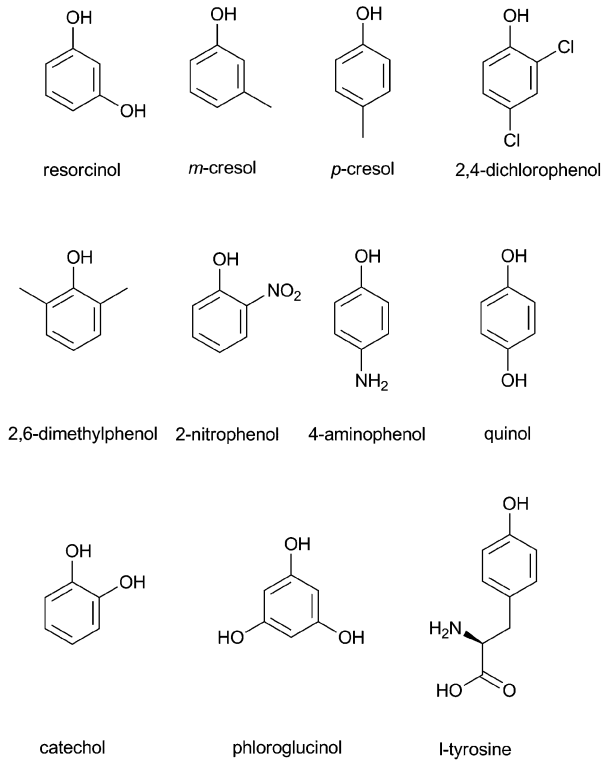



**Fig. 6** Comparison of the off-on luminescent responses of **1** to different phenolic compounds at a fixed-time *t* of 10 min, following addition of 0.1 μM hydrogen peroxide, 0.1 μM GSH and 0.125 mg $L^{-1}$ HRP to a solution of 0.1 μM of **1** in 10 mM Tris-HCl buffer at pH 7.4; 5 μM of 2,6-dimethylphenol, 2-nitrophenol, 4-aminophenol, quinol, catechol, phloroglucinol and L-tyrosine; 0.05 μM of phenol.




**Scheme 1** The structures of phenolic compounds.

analysed directly without further pretreatment. No phenolic compounds were detected in any of the samples. The sample was spiked with a standard solution of 2,4-dichlorophenol, and recoveries of 77.4–80.4% with relative standard deviations (RSDs) of 1.0–2.2% were obtained (Table 2). The proposed method has proved simple, fast and suitable for analyzing phenolic compounds at low nanomole per liter level in seawater samples.

**Table 2** Determination of 2,4-dichlorophenol in water samples (spiked water samples, $n = 3$)

| 2,4-Dichlorophenol (nM) | RSD (%) | Recovery (%) |
|---|---|---|
| 10 | 2.2 | 79.9 |
| 30 | 1.0 | 80.4 |
| 50 | 2.1 | 77.7 |

## Conclusions

Phenolic compounds, as good substrates of HRP, can be easily oxidized by hydrogen peroxide and transform to phenoxyl radicals. The phenoxyl radicals can react with GSH and transform back to its parent phenolic compounds. Concomitant with the regeneration of the phenolic compounds is the formation of thiyl radicals. The novel spin-labeled terbium complex-based bimodal probe $Tb^{3+}$/cs124-DTPA-TEMPO (**1**) has proved to be a sensitive probe in recognizing and signaling the as-produced thiyl radical. On reaction with the thiyl radical, the nitroxide radical in **1** transforms to highly luminescent derivatives, and shows sensitive off-on luminescence and on-off EPR response to phenolic compounds indirectly. In the HRP-$H_2O_2$ mediated phenol-phenoxyl radical and GSH-GS· reaction cycle, phenolic compounds act as a quasi-catalyst to promote the production of thiyl radical and amplify the luminescent signaling. The thiyl radical recognition-based method has shown great potential in the detection of water-soluble phenolic compounds in environmental waters. Furthermore, one may expect that this probe would also be a suitable tool for recognizing thiyl radicals, which play a crucial role in the metabolizing process or give a measurement of oxidative and nitrosative species in cells and tissues.[29–34]

## Acknowledgements

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