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Can Hg(II) be Determined via Quenching of the Emission of Green Fluorescent Protein from *Anemonia sulcata* var. *smaragdina*?

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Abstract *Anemonia sulcata* var. *smaragdina* is a widely distributed Cnidarian species along Turkish coastlines. It is also a well-known example of a facultative symbiotic life form in sea ecosystems. Green fluorescent proteins (GFPs) in *Anemonia sulcata* var. *smaragdina* have vital roles in this symbiotic form. The fluorescence quenching by Hg(II) in the supernatants obtained from *A. sulcata* var. *smaragdina* was shown in this study. According to results, there was a statistical significant relationship (R^2=0.9913) between increased Hg(II) concentration and decreased fluorescence intensity of GFP supernatants obtained from *A. sulcata* var. *smaragdina*. Mn(II), Fe(II), and Al(II) showed no interference effect and did not change the fluorescence intensity of GFP supernatants obtained from *A. sulcata* var. *smaragdina.* In conclusion, the fluorescence quenching of GFPs by Hg(II) can be a novel method to determine the Hg(II) levels in aqueous solution. Therefore, further researches are strongly warranted because of the possible potential applications of the fluorescence quenching of GFPs by Hg(II).

Keywords *Anemonia sulcata* var. *smaragdina* · Hg(II) · Fluorescence · Quenching · Heavy metals

Introduction

Anemonia sulcata var. *smaragdina* is one of the well-known morphotypes of *Anemonia viridis*. It is easily distinguished from another morphotype, *Anemonia rustica*, with its pink tips in the tentacles. *Anemonia sulcata* var. *smaragdina* contains green fluorescent proteins (GFPs). This species is a well-known facultative symbiotic life form between a cnidarian and

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an alga known as zooxanthellae. While the animal provides a hosting place and protection for alga, the alga provides oxygen and carbohydrate-based products to animal by photosynthesis. However, over photosynthetic activity results the increased oxygen concentration and also increased reactive oxygen species (ROS) in this symbiotic life [1–4]. The symbiosis of *Anemonia*/*Symbiodinium* is an obligatory one. If the animals lose the zooxanthellae completely, they will die after a while. One of the well-known events observed in *A. viridis* called "bleaching" can be explained with the overproduced ROS in this symbiotic life. In this event, the animal does not want to continue to live together with algae inasmuch as the overproduced ROS causes the DNA breaks, protein oxidation, and lipid peroxidation [5]. Therefore, the animal expels the algae from its body dependent on the light conditions or zooxanthellae lose their photosynthetic pigments [6–9]; then, the whole color of *A. sulcata* var. *smaragdina* transforms into white [1, 10–16]. One of the important sources of ROS is exogenous pollution. There are many papers in the literature reported that some heavy metals increase types of the ROS *in vivo* condition in aerobic cell metabolism [17–21]. On the other hand, the existence of GFPs in *Anemonia* species has provided a light to many important studies in many different fields such as medicine [22–24]. So far, GFPs were applied in many studies as intracellular calcium, chloride, and pH indicators [25]. Moreover, Rahimi et al. [26] showed that the other GFP-like proteins called "red fluorescent proteins, namely native DsRed and its variants" had an ability to bind the Cu^{2+} ions in the presence of other divalent cations and this binding caused the fluorescence quenching of the proteins that they studied [27–30].

The determination of traces and ultra-traces of Hg(II) ions is becoming increasingly important, particularly due to their toxicological effects. Environmental contamination by Hg(II), a widely known toxic heavy metal, can result in death or severe damage to the brain. The most widely used techniques for the quantification of Hg(II) are based on cold vapor atomic absorption spectrometry. Other published methods include anodic stripping voltammetry, X-ray fluorescence spectrometry, neutron activation analysis, inductively coupled plasma mass spectrometry, and atomic fluorescence spectrometry.

Molecular fluorescence is attracting a great deal of interest in environmental monitoring since it is inherently more sensitive than other molecular spectroscopies and so it can be used for the analysis of very low trace concentrations. As far as mercury is concerned, only a few methods have been described so far [31–36] for its fluorimetric determination (Table 1).

Table 1 Comparison of fluorimetric methods for the determination of Hg(II).

Reagent	Procedure characteristics	DL[a] (ng ml^{-1})	Effect	Reference
Green fluorescent proteins in *Anemonia sulcata* var. *smaragdina*	The complex formation between Hg(II) and ligand	8.2	Quenching	This paper
Oxacarbocyanine and tetraphenylborate	The reagents are immobilized on plasticized polyvinyl chloride	20	Quenching	[31]
N-9-Anthrylmethyl-*N*-methyl-*N*′-benzoylthiourea	Formation of a fluorescence complex; micellar medium is used	11.6	Increase	[32]
Porphyrin derivative	Formation of a fluorescent complex	1.4	Increase	[33]
Rhodamine 6G	Use of iodide as ligand	10	Quenching	[34]
Thiamine	Oxidation of the reagent to yield thiocrome	3.6	Increase	[35]
6-Mercaptopurine	The luminescent reaction between Hg(II) and ligand	1.4	Quenching	[36]

[a] Detection limit (defined as the concentration of Hg(II) which corresponded to three times of the noise)

Moreover, the majority of these methods present problems of sensitivity, selectivity, reaction time, or procedure complexity.

In this paper, we have answered below the research questions:

✓ Does Hg(II) quench the emission of green fluorescent protein from *A. sulcata* var. *smaragdina*?

✓ If so, can it be a novel method to determine the Hg(II) levels in aqueous solution?

Materials and Methods

A. sulcata var. *smaragdina* was collected from Gümüldür—Izmir, Turkey. The geographical coordinates are 38°04′01.62″N and 26°59′46.39″E. The specimens were transferred to the laboratory with plastic aquarium. Volume of the aquarium was 24 l. Since the laboratory was not far away from Gümüldür, the samples reached the laboratory within 1 h after sampling. The tentacles were cut from their bases. Ten tentacles were put in each Eppendorf tube. The samples were homogenized with IKA-JANKEL homogenizator in 10-s intervals. In order to remove the cell debris, the homogenates were centrifuged in a refrigerated centrifuge at 12,000 rpm for 20 min at +4°C. The UV–VIS spectrum was obtained from Shimadzu UV–VIS 1601 spectrophotometer. The excitations and emissions of supernatants were done in Varian spectrofluorometer.

Results

The response of GFP supernatants obtained from *A. sulcata* var. *smaragdina* to Hg(II) concentration was aimed to be investigated in the present study. To characterize the existence of GFP in the supernatant, it was excited at 425 nm and emission spectrum was recorded at 520 nm. Since the best results were obtained in these wavelengths, they were selected (Fig. 1). According to Fig. 2, the emission at 520 nm gave very good accordance

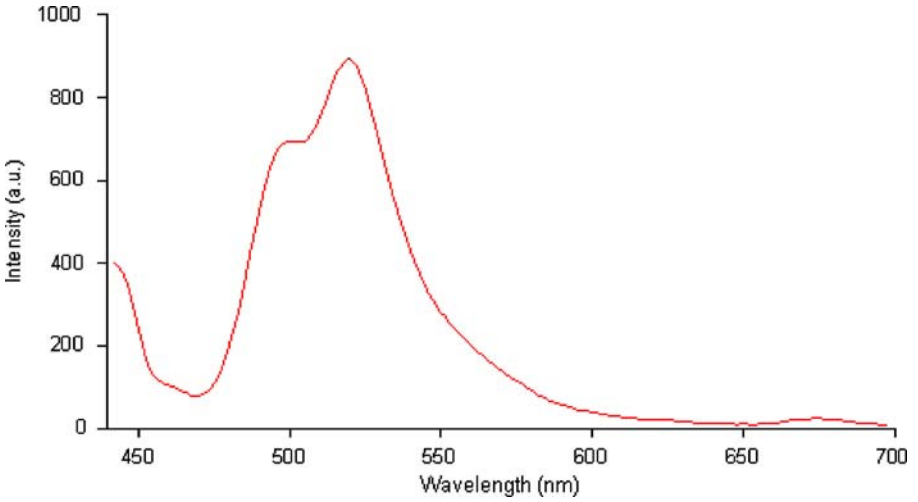

Fig. 1 The emission spectrum of GFP supernatants obtained from *Anemonia sulcata* var. *smaragdina* ($\lambda_{excitation}$=425 nm; $\lambda_{emission}$=520 nm; slit width=10 nm)

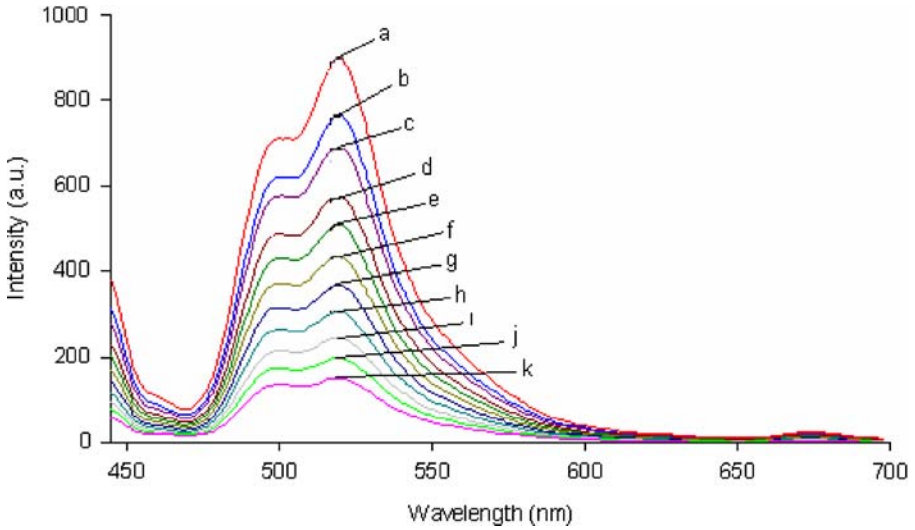

Fig. 2 The response of emission of GFP supernatants obtained from *Anemonia sulcata* var. *smaragdina* to various concentrations of Hg(II). *a* 0 μg ml^{-1}, *b* 2 μg ml^{-1}, *c* 5 μg ml^{-1}, *d* 10 μg ml^{-1}, *e* 15 μg ml^{-1}, *f* 20 μg ml^{-1}, *g* 25 μg ml^{-1}, *h* 30 μg ml^{-1}, *i* 35 μg ml^{-1}, *j* 40 μg ml^{-1}, *k* 45 μg ml^{-1} (λ_{ex}=425 nm, λ_{em}=520 nm)

with increased Hg(II) concentrations. The range of Hg(II) studied was 2.0–45.0 μg ml^{-1}. In order to show the relationship between Hg(II) concentration and fluorescence intensity, Fig. 3 was plotted. As can be seen from Fig. 3, there was a statistical significant relationship (R^2=0.9913) between increased Hg(II) concentration and decreased fluorescence intensity of GFP supernatants obtained from *A. sulcata* var. *smaragdina.* In order to see the effects of other interferent metals on this interaction, more experimental data were collected in the existence of the other interferent metals in the same conditions. For this experiment, the concentration of Hg(II) was fixed at 5 μg ml^{-1} and the concentrations of other interferent metals were selected as 10 μg ml^{-1}. The data for this experiment was depicted in Fig. 4. Mn (II), Fe(II), and Al(II) showed no interference effect and did not change the fluorescence intensity of GFP supernatants obtained from *A. sulcata* var. *smaragdina.* On the other hand, lower fluorescence intensities were observed in the existence of other interferent metals.

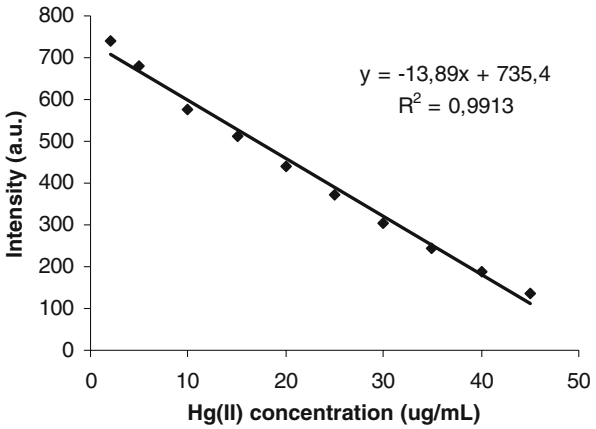

Fig. 3 The relationship between Hg(II) concentration and emission intensity obtained from *Anemonia sulcata* var. *smaragdina* ($c_{Hg(II)}$=2.0–45 μg ml^{-1})

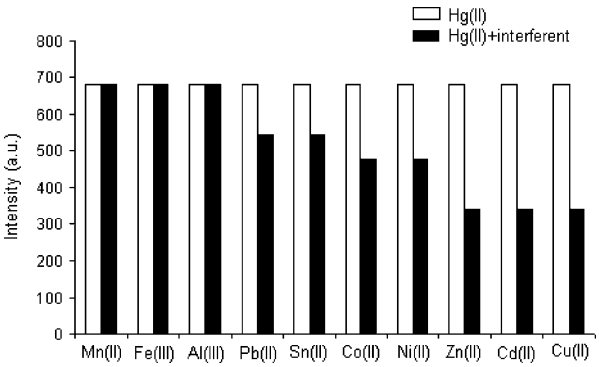

Fig. 4 The effects of interferent metals on the fluorescent intensity of GFP supernatants obtained from *Anemonia sulcata* var. *smaragdina* (λ_{ex}=425 nm, λ_{em}= 520 nm). The concentration of Hg(II) is fixed at 5 μg ml^{-1}. The concentrations of interferent metals are 10 μg ml^{-1}

The order of fluorescence intensities in the existence of other interferent metals was Pb(II)= Zn(II)>Co(II)=Ni(II)>Zn(II)=Cd(II)=Cu(II).

Discussion

Sea anemones are important facultative symbiotic life forms of marine ecosystems. As a well-known example of sea anemones, *A. sulcata* var. *smaragdina* lives with single-cell algae. The role of the single-cell algae which contains photosynthetic pigments in this symbiotic life is to produce oxygen and also photosynthesis products. On the other hand, these algae are protected by anemone from their predators. *A. sulcata* var. *smaragdina* provides a hosting place to these algae and takes the products of these algae. Under normal conditions, this relationship is well protected; however, some unwanted events such as pathogenic contamination and exposure to excessive UV radiation damage this relationship. For example, excessive subjecting to UV radiation results in the overproduction of reactive oxygen species by algae in the anemones, and then the relationship is broken and this event is known as "bleaching". On the other hand, cold shock, pathogenic infections, and reduced salinity might also be other factors for bleaching [1, 37–44]. In the present study, fluorescence quenching related to green fluorescent proteins via Hg(II) was shown in *A. sulcata* var. *smaragdina*. The fluorophor structure in GFP (Fig. 5) is based on the three

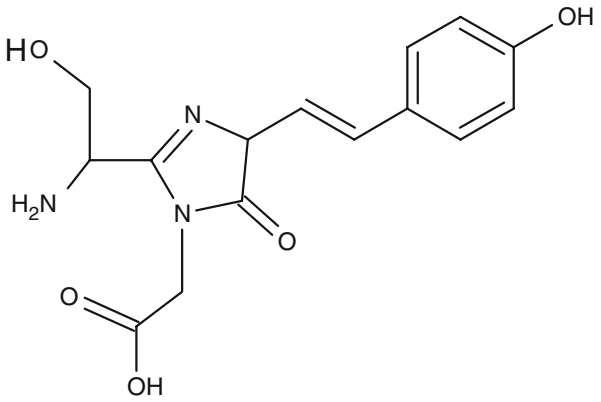

Fig. 5 Fluorophor part of GFP

special amino acid sequence: Ser_{65}-Tyr_{66}-Gly_{67} [45]. In this study, Hg(II) with nitrogen and oxygen atom in GFG molecule interact and the fluorescence quenching was observed at the increasing concentration of Hg(II) ions. The photostability time of interaction was determined with a steady-state spectrofluorimeter in time-based mode. The data were acquired at 520 nm. The photostability time of interaction was 75 min. After this time, the interaction decomposed and the fluorescence intensity of GFP reached the value in the beginning. Therefore, GFPs from *A. sulcata* var. *smaragdina* could be used in biosensor researches. On the other hand, mycosporine-like amino acids (MAAs) can also be considered as fluorescence source in the *A. sulcata* var. *smaragdina* since these structures have similar absorption and fluorescence properties. According to Banaszak, cnidarians have a higher amount of MAAs compared to other invertebrate phyla [46]. When the papers related to fluorescence quenching by Hg(II) are considered, there are few researches. These structures are based on lab-synthesized molecules [47–53]. He et al. developed a fluorescent chemical sensor for Hg(II) determination by using a corrole derivative [47]. Chan et al. also developed a Hg(II)-selective optical sensor based on fluorescence quenching of 5,10,15,20-tetraphenylporphyrin [54]. However, their methods require extraction of Hg(II) from aqueous solutions. Zhang et al. studied the synthesis of a novel fluoroionophore, 5-*p*-[[4-(10′,15′,20′-triphenyl-5′-porphinato) phenyloxyl]-1-butyloxyl]phenyl-10,15,20-triphenylporphine (DTPP) and its fluorescence quenching against Hg(II) to develop mercury-sensitive optical fiber chemical sensors [55]. Plaschke et al. also showed the determination of Hg(II) from aqueous solution by developing a water-soluble porphyrin and porphyrin-doped sol–gel films [56]. These exclusive attempts on Hg(II) determination via fluorescence quenching were examined; it can be easily seen that there are difficulties in the preparation of these chemical sensors because of the materials used [47, 54–56]. The materials in the abovementioned studies might have high cost compared to our proposed material. Therefore, in our study, we want to take the attention of scientists for use of naturally occurring green fluorescent proteins for the preparation of chemical or optical sensors for the determination of Hg(II) from aqueous solution. Hg(II) ion, which is well known for its toxicity, shows strong affinity for the ligands containing nitrogen, oxygen, and sulfur atoms. In this study, Hg(II) also showed electrostatic interaction with nitrogen and oxygen atom in GFG molecule. Thus, the fluorescence of GFP molecule decreased at the increasing concentration of Hg(II) ions. The detection limit (defined as the concentration of Hg(II) which corresponded to three times the noise) [57] was estimated to be 8.2 $\mu g\ l^{-1}$ for Hg(II). The results in this study promise the possible application of the green fluorescent proteins in the development of optical sensors for heavy metal determinations. Therefore, further study on the development of a novel optical sensor by using GFP for Hg(II) determination from aqueous solution is strongly warranted.

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