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Research Paper

Fast and sensitive detection of mercury (II) using fluorescent Phycocyanin in aqueous system

Mogos Girmatsion [a, b], Awet Adhanom [b, c], Henok Gebremedhin [b, c], Abdu Mahmud [a, b], Yunfei Xie [a], Yuliang Cheng, [a,] Hang Yu [a,] Weirong Yao [a], Yahui Guo [a*,] He Qian [a]

a. State Key Laboratory of Food Science and Technology, School of Food Science and Technology, Jiangnan University, Wuxi 214122, China.
b. Department of Marine Food and Biotechnology, Massawa College of Marine Science and Technology, Massawa, Eritrea.
c. Quality control laboratory, Ministry of Marine Resources, Massawa, Eritrea
** Correspondence: Yahui Guo*

ABSTRACT
Mercury (Hg^{2+}) toxicity is one of the most common chemical poisonings, that mainly occurs from drinking polluted water. In the current work, Phycocyanin (PC) was exploited as a fluorescent sensor for sensitive and selective detection of Hg^{2+} in an aqueous system. PC-Hg^{2+} interaction was monitored using steady-state fluorescence measurement. Using Tris-buffer, pH 6.5 as a diluent and, the fluorescence intensity of PC showed a potent decline upon the attendance of increasing Hg^{2+} concentrations (50-1200 nM). Under controlled conditions of experimentation, the current sensor showed a good linear relationship and the limit of detection was 18 nM. Besides, Hg^{2+} could be easily discriminated against other 9 metals ($Cu^{2+,}$ $Zn^{2+,}$ Pb2+, $Mg^{2+,}$ Mn^{4+}, Li^{+}, $Fe^{3+,}$ Co^{2+}, and Al^{3+}) in the attendance of 900 nM of each metal. The other metals showed observable effects only when the concentration of PC increased to 3.5 umol/L. Moreover, the current fluorescent detection assay was also tested in real samples of pond water and good recoveries and RSD were recorded.

KEYWORDS: *Phycocyanin; Mercury (Hg^{2+}); detection; quenching; diluent; interaction; intensity; fluorescence; effect; emission*

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I. INTRODUCTION

Inorganic mercury (H^{2+}) is a type of heavy metal known for its potential risk of causing serious toxic effects in the human [1,2] The water environment, literally referring to underground and drinking waters is the major reservoir for mercury (H^{2+}) and other heavy metals and from it metals become part of the food chain through absorption. Food commodities such as fish, leafy vegetables, and rice are often contaminated with heavy metals [3]. Presence of mercury ions (Hg^{2+}) in water and foods is very common. Mercury (II) toxicities resulting from drinking and consumption of contaminated animal products are linked with significant effects in the human body that can eventually lead to incidents of morbidities and mortalities. In order to prevent the public from the eminent dangers of these chemicals, implementing rapid, effective and more importantly simple and practicable analytical approaches is critically important. Many works related to Hg^{2+} investigation have been reported since the past several decades with an increasing trend of using advanced methodologies for the detection of $Hg^{2+,}$ especially, in aqueous environments. For instance, Childress et al. [4] conjugated polymer nanoparticles (CPNs) doped with a mercury-responsive Rhodamine derivative to detect Hg^{2+} up to 0.7 ppb. Chen et al. [5] also developed a fluorescence sensor with a complex of a cationic oligopyrene derivative and oligothymine for the detection of Hg^{2+} in aqueous media and the LOD was 5 µmol/L.

More recently Tao et al. [6] established a fluorescent probe based on a simple coumarin derivative and was able to determine Hg^{2+} of up to 8 µmol/L. These are only some of the many great works related to Hg^{2+} detection. Broadly speaking, most of the techniques reported on Hg^{2+} detection is complicated to develop and they can't be easily exploited in real-world applications. Besides, the materials used for the development of the probes are not friendly to the environment, the LODs are not in harmony with reguLation and the molecules

used are expensive or have other issues such as insolubility. Therefore, the development of environment-friendly, simple, sensitive, and more importantly practicable detection probes is an ultimate criterion of an ideal method.

Proteins are among the most abundantly employed biomaterials in analytical chemistry as well as in many other disciplines because of their exclusionary three-dimensional configuration, distinct biological properties, and versatile nature in recognition and assembly [7, 8]. Moreover, proteins are considered safe materials, a characteristic that makes their application, especially in laboratories quite fascinating and acceptable. Phycocyanin is a globuLar type of Phycobiliproteins member, which is among today's very interesting molecules in scientific applications. Phycocyanin (PC) is a molecule that functions as an accessory and support in photosynthesis in specialized structures such as prokaryotic blue-green algae and eukaryotic red algae. Phycocyanin contains tetrapyrrole molecules called phycocyanobilins that are capable of emitting fluorescence [9,10] This property makes it a candidate for various applications such as fluorescent detection, immunodiagnostics, biotechnology, nutraceuticals, cosmeceuticals, and pharmaceuticals [11,12, 10].

The objective of the current work, is to developed a fast, simple and easily implementable protein sensor using Phycocyanin for sensitive and selective detection of Hg^{2+} without the employment of any complicated nanomaterials or probe system. PC, it is highly unstable and sensitive to external environments such as pH, type of medium, temperature, and light [14,12]. Similarly, metals have also been identified to be sensitive and show variability with changes in pH [15]. Hence, the current fluorescent probe was proposed based on establishing an ideal aqueous environment for the PC-Hg^{2+} interaction. The interaction between Phycocyanin (PC) and Hg^{2+} was analyzed based on the effect of four different pH-regulated aqueous media (Tris-buffer, PBS, HEPES, and PB) having pH ranging from 6.5 to 8.5 and ionic concentration 0.02 M. The quenching effect of Hg^{2+} was found to be more notable when Tris-buffer, pH-6.5 was used as a diluent. The sensitivity and selectivity of Hg^{2+} as well as applicability of the probe in real water sampleswas studied. In the sensitivity assay, carried out under Tris-buffer, pH-6.5, the fluorescence response of PC was quenched in the presence of Hg^{2+} concentrations ranging from 50 to 200 nM. The limit of detection (LOD) reached down to 18 nM. In the selectivity study to compute Hg^{2+} with 9 different metals ($Cu^{2+,}$ $Zn^{2+,}$ Pb2+, $Mg^{2+,}$ Mn^{4+}, Li^{+}, $Fe^{3+,}$ Co^{2+}, and Al^{3+}), it was revealed that Hg^{2+} can be easily distinguished among the mixtures. Further**,** the current strategy was tested for the detection of Hg^{2+} in pond water to confirm its applicability in real samples and showed a good promise.

II. MATERIALS AND METHODS

Reagents and chemicals

Purified Phycocyanin (40 Kda) was purchased from Zhejiang Binmei Biotech. Co., Ltd. (Linhai, China). All the reagents used for the preparation of Tris-base, PBS, PB and HEPES buffer solutions were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Double-distilled water was collected from a Milli-Q A10 filtration system (Millipore, Billerica, MA, USA) and was used throughout the experiments. All the other chemicals were analytical grade and used without

Instrumentation

UV2450 Spectrophotometer and Spectro-fluorophotometer RF-6000, Shimadzu Inc., (Kyoto, Japan) both were used for the absorbance and fluorescence measurements respectively. Transilluminator imaging system (Tanon, 5200), Tianlong equipment factory (Shanghai, China) was used to acquire fluorescence images under UV light of 365 nm. ResuLts of all the optical measurements were analyzed using origin pro9 software.

Preparation of solutions

Aqueous PC stock solutions were prepared during each experiment using distilled water. Then working solutions were further prepared freshly by making different dilutions for instant use according to needs. Stock solutions of Hg^{2+} and the other metals ions used in this work ($Cu^{2+,}$ $Zn^{2+,}$ Pb2+, $Mg^{2+,}$ Mn^{4+}, Li^{+}, $Fe^{3+,}$ Co^{2+}, and Al^{3+}) were prepared as follows; for Hg^{2+},1000 µM was prepared in dilute nitric acid and from it, further dilutions were made available for use; for the rest of metals standard solutions were prepared in double-distilled water and further dilutions were also made accordingly. Tris-base buffer, Phosphate Buffer Saline (PBS), PB & HEPES, all of which with pH 6.5,7, 7.5, 8, 8.5 and ionic strength 0.02 M were carefuLly prepared using double distilled water and other required reagents.

Optical measurements

All spectroscopic measurements corresponding to PC and PC-Hg^{2+} were conducted at room temperatures. Every experiment was carried out in triplicates and the average resuLts of absorbance and fluorescence tests were used for analysis and discussions well as to present the conclusions. UV-Vis measurements of PC were carried out in the absence and presence of Hg^{2+} to assess the properties of PC and PC-Hg^{2+} in terms of the nature of absorbance intensity and absorption maxima; Spectra measurements were taken in

the range 400-700 nm and baseline correction was done using ul-trapure water (Type 1). Fluorescent measurements were conducted in the range of 450 – 800 nm. The slit width and scan speed for all fluorescence measurements were maintained regularly at 1 nm and 2000 nm/s respectively during all experiments. Generally, fluorescence evaluations include examining the effect of the pH-regulated aqueous systems for best PC-Hg^{2+} interaction among the four diluents; analyzing the fluorescence intensity and fluorescence maxima of Hg^{2+} - free PC with a simultaneous increase in its densities; as well as studies on the sensitivity, selectivity, and applicability of the current probe.

Detection of Hg^{2+}

For the detection of Hg^{2+}, 100 ul of 2.5 mg/mL PC pre-prepared in distilled water was diluted with 350 ul Tris, pH 6.5 followed by the addition of 50 ul of Hg^{2+} aliquots of different concentrations. All solutions were vigorously shaken for 1 minute in a vortex. After several minutes of incubation, solutions were transferred to a quartz cuvette for fluorescence measurement.

III. RESULTS AND DISCUSSIONS

Characterization of the optical properties of PC

The current work relies on utilizing PC as a fluorescence probe for ultrasensitive and selective detection of Hg^{2+} in aqueous media. As shown in Figure 1**, i**n the absence of Hg^{2+}, PC has absorption, excitation, and emission maxima located at 620 nm, 532 nm, and 647nm respectively, all in PC solution prepared by diluting 2.5 mg/mL aqueous solution of PC with Tris-buffer, pH-6.5 which makes the PC final concentration ~0.5 mg/mL. Depending on the above maxima wavelengths, the absorbance and fluorescence properties of PC after the addition of Hg^{2+} and/or the other metals are analyzed. The values of absorption, excitation, and emission maxima are in agreement with previous reports [16-18] respectively.

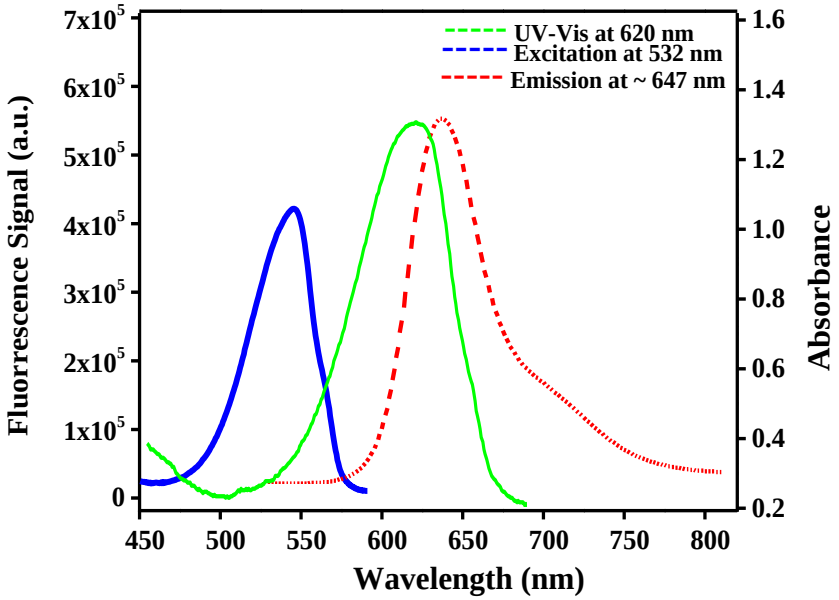


Figure 1. UV–visible absorption spectrum (green), fluorescence excitation spectrum (blue) and emission spectrum of PC solution (Red).

Figure 2, depicts the UV – visible absorbance spectra of PC in the presence of Hg^{2+} and the other metals. It is apparent from the figure that the absorbance intensity of PC declined in the presence of Hg^{2+}, while the other metals not. Also, the spectrum of PC in the presence of Hg^{2+} is wider than the other spectra. Such alterations in the spectral pattern of PC delineate the effect of Hg^{2+} on PC presumably due to the interaction between the chromophores of PC and Hg^{2+} [20]. No shift in peak position was noticed in all the spectra.

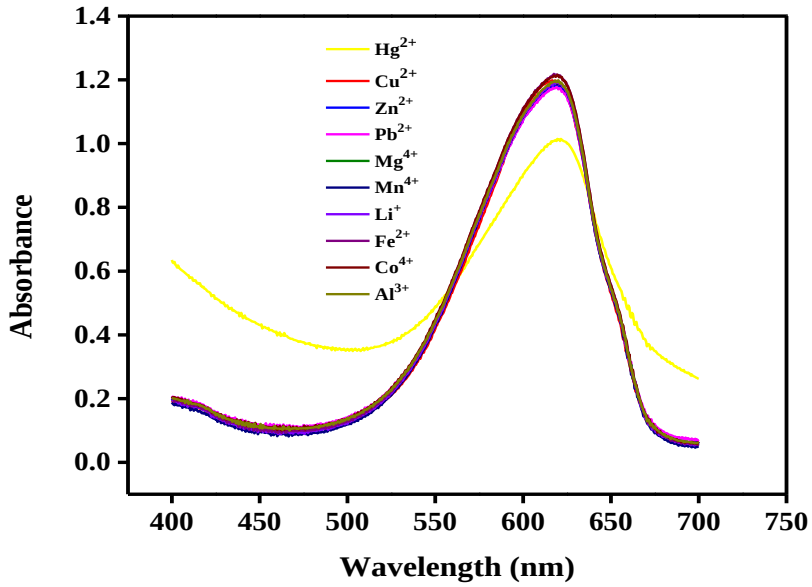
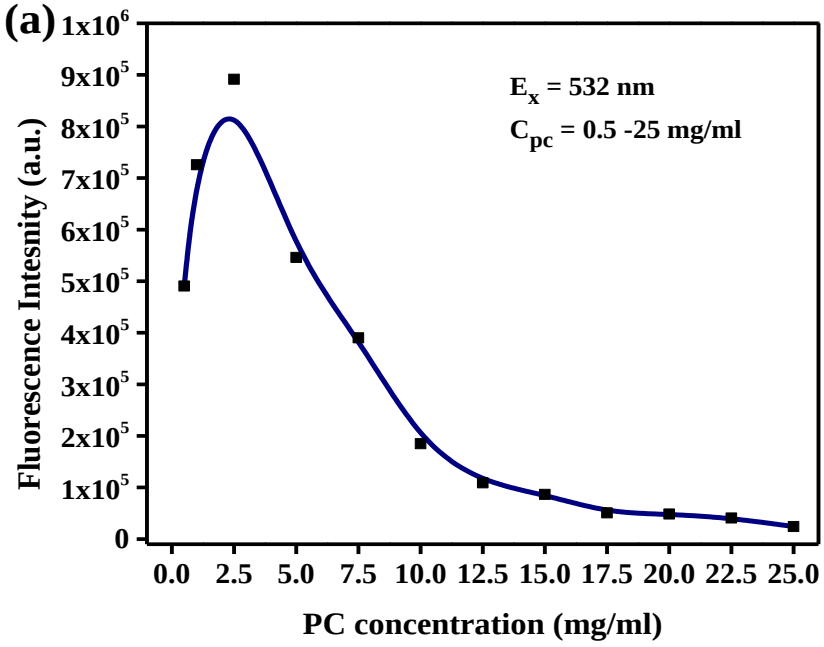


Figure 2. UV-Vis spectra of PC in the presence of different metals

The fluorescence intensity and fluorescence maxima of PC were studied in the absence of Hg^{2+}. Both the intensity and maxima were examined carefully against the following range of PC 0.5, 1, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20,22.5, and 25 mg/mL prepared in distilled water. As illustrated in Figure 3a**,** at low concentrations, the intensity tends to increase till it reaches the emission maxima at 2.5 mg/mL then it declines at PC concentrations (Cpc) beyond it. The increment in intensity is attributed to the reason that monomer chromophores of PC are well-provoked and animated at low and/or moderate concentrations and on the contrary, the declination is caused by aggregation of the protein at higher concentrations which resulted from the interactions of chromophores of one monomer with the chromophores of others within the protein [21,17]. Figure 3b, portrays alterations in fluorescence maxima as a function of concentration. It is shown that as the concentration goes up, so does the shift in the fluorescence maxima. Such behavior is attributed to factors such as energy transfer between excited chromophores, aggregation, or clustering of PC monomer as well as self-quenching [17, 22]



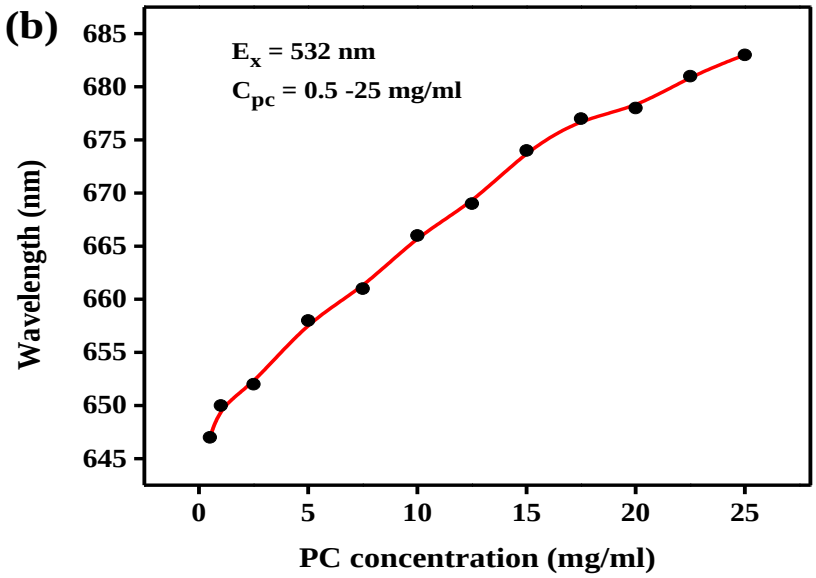


Figure 3. (a) shows the pattern of fluorescence intensity of PC as concentration increases (b) Fluorescence maxima of PC elevates as the concentration increases

Optimization of the fluorescence sensor

Effect of buffer on Hg^{2+} quenching efficacy

As we stated in the introduction, in this work PC is used as a sensor to detect Hg^{2+} without being functionalized or treated with special molecuLes. The efficiency of the PC-Hg^{2+} interaction was essentially dependent on the effect of the diluting medium. Figure 4, shows the characteristic quenching impact of Hg^{2+} induced on the fluorescence intensity of PC with respect to different buffered solutions (Tris-base, PBS, PB, HEPES) used as diluting media. In the presence of 1200 nM Hg^{2+}, the quenching effect is seen to be more notable in the case of Tris-buffer, pH 6.5. Thus, Tris-buffer, pH 6.5 was, used for conducting further experiments. According to previous reports PC is generally stable within the pH range of 5 to 7 whereas, at alkaline pH the chromophores of Phycocyanin tend to decompose to a variety of colored products due to denaturation [23]. Here, drastic effect of alkalinity started from pH 8 as in the case of PBS and HEPES as well as all buffers at pH 8.5, where the fluorescence intensity of PC is suppressed in the absence of Hg^{2+}, and contrarily enhanced in the presence of 900 nM Hg^{2+} (Figure 4). The cause of enhancement up on Hg^{2+}. addition is not clear here, however, this type of behavior may provide a new insight to exploit these aqueous environments for the detection of Hg^{2+} or other metals using PC based on enhancement effect rather than on quenching effect

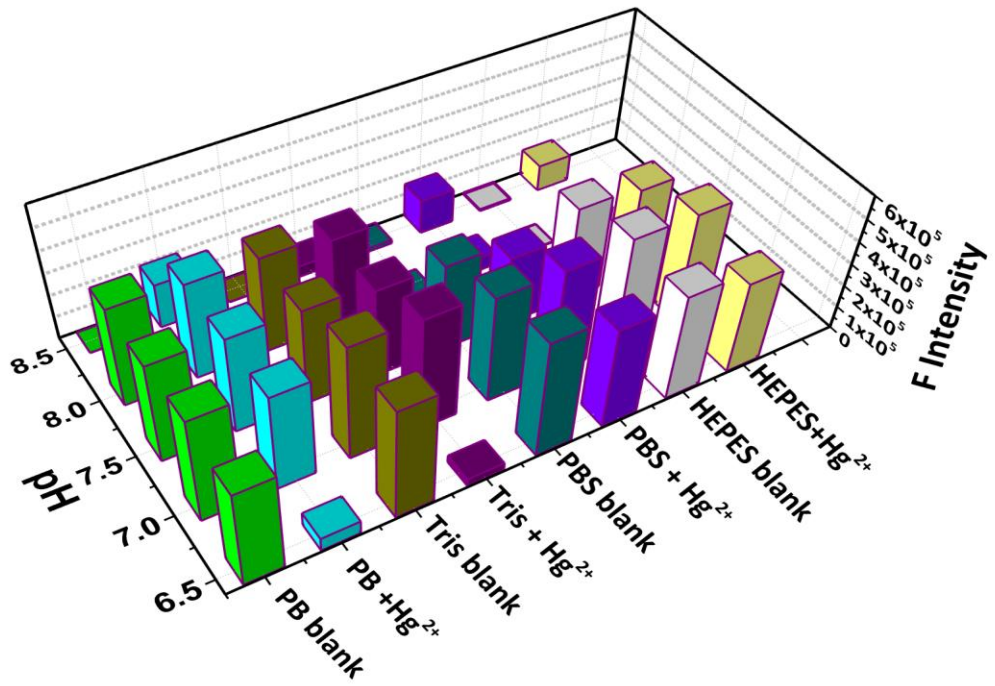


Figure 4. Effect of different buffers on the fluorescence quenching functionality of Hg^{2+}: starting from pH 8, the intensity of PC started to decline even in the absence of Hg^{2+} due to the alkalinity of the pH

Optimization of PC concentration

Before proceeding to conducting further studies to detect the contaminants using PC as a sensor, it was important to examine the optical properties of PC at varying concentrations and determine the appropriate one with in the domain where a linear increment in intensity is seen. PC concentrations of 0.5, 1,2, 3, 4.5, 5, 7.5, 9, 10, 12.5, 15, 17.5, 20,25, 30, 35, 40, 45, and 50 mg/mL were dissolved in aqueous solutions based on the general procedure described in the methodology section and the nature of the fluorescence intensity and peak positions of were studied. Fluorescence measurement were recorded at excitation wavelength (532 nm) and intensities corresponding to each concentration were taken in accordance to their maximum wavelengths. As shown in figure 3.2 the fluorescence intensity increased generally until the maximum intensity was attained (20 mg/ml). Beyond this level, the intensity showed declining trend. However, a linear pattern is seen more between 0.5 and 10 mg/ml PC and this means that a PC concentration for further studies should be considered with in this region. This is associated with the fact that, the concentration should be high enough to provide strong fluorescence signal and low enough to avoid inner filter effect [24]. At low and moderate concentrations there is adequate opportunity for the PC phycocyanobilins to be easily provoked and emit efficient light wavelength. However, at higher concentration, saturation of the solution tends to occur and the phycocyanobilin pigments fail to produce light. This can be caused due to aggregation as the molecules (chromophores) within each protein molecule (Phycocyanin) tend to interact with each other [25] Therefore, 2.5 mg/ml was chosen as suitable concentration for fluorescence detection of $Hg^{2+,}$ where the final concentration is 0.5 mg/ml in the 500 ul final volume.

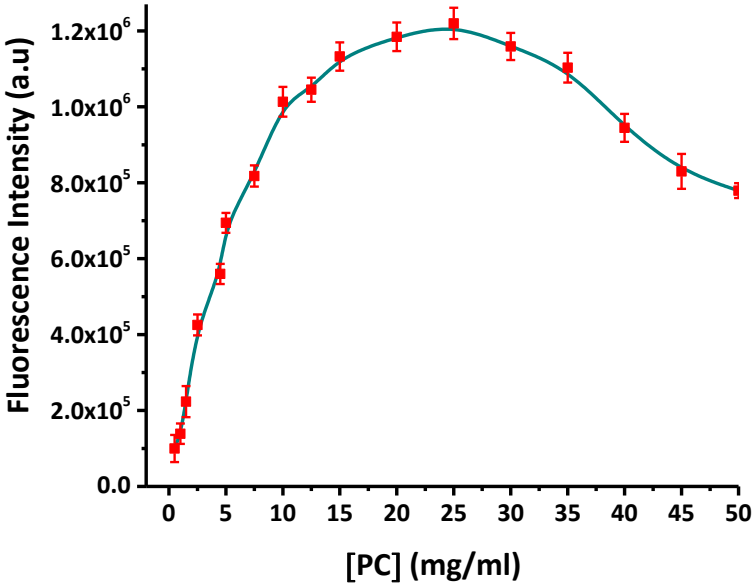
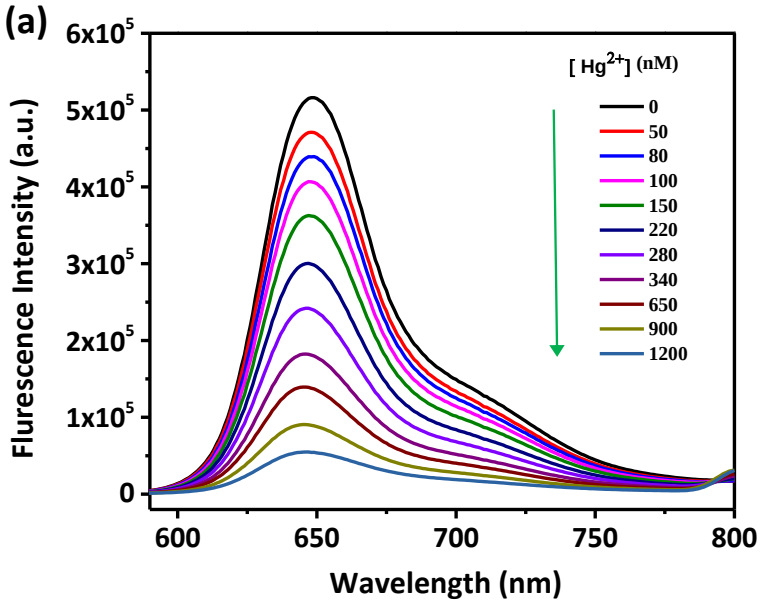


Figure 5 A graph displaying the nature of PC fluorescence intensity at different concentration (0.5 – 50 mg/ml), Tris, pH 6.5.

Sensitivity and selectivity studies

Spectral changes in the fluorescence emission intensities of PC- Hg^{2+}in the presence of different concentrations of Hg^{2+} ions (0, 50, 80, 100, 150, 220, 280, 340, 650, 900 &1200 nM) were studied to assess the sensitivity of Hg^{2+} towards PC under the treatment of Tris-buffer, pH 6 as a suitable diluent. Figure 6a, shows that the PC suspension induced remarkable quenching on the fluorescence intensity of PC is quenched upon the **a**ddition of Hg^{2+}. The emission maxima blue shifted by 4 nm. The. This indicates that the type of quenching induced by Hg^{2+} is a static quenching that occurs due to a complex formation between PC and Hg^{2+} before excitation or in the ground state. Therefore, the quenching effect is not related to the concentration of Hg^{2+} but rather to the interaction or sensitivity between PC and Hg^{2+}. Figure 6b, presents a linear graph of the sensitivity test; inset depicts a good linear correlation between Hg^{2+} and PC with R^2 = 0.9949. The limit of detection (LOD)was estimated to be 18 nM based on the standard error of the intercept and slope.



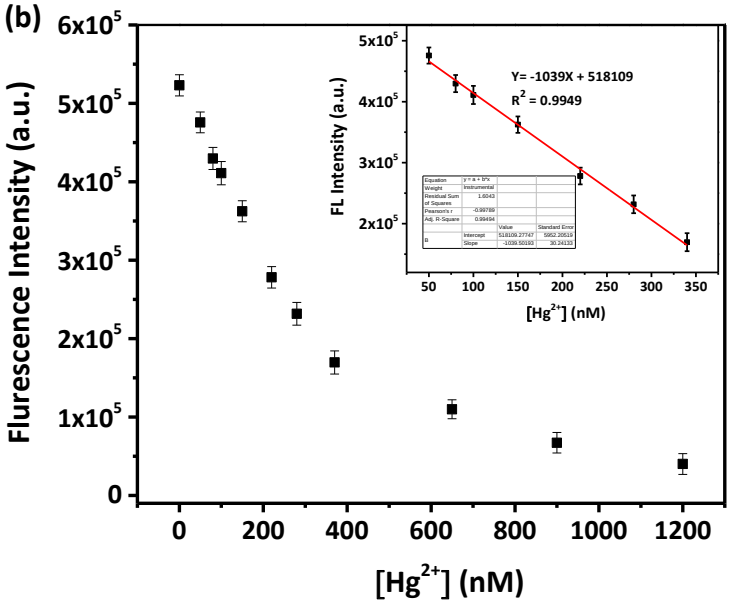


Figure 6. (a) Fluorescence emission response of PC in the presence of different concentrations of Hg^{2+} ions (0, 50, 80, 100, 150, 220, 280, 340, 650, 900 &1200 nM). **(b)** A linear relationship between PC and $Hg^{2+.}$ constructed using 50 to 340 nM Hg^{2+}

In order to validate the selectivity of the current fluorescent assay, the behavior of $Cu^{2+,}$ $Zn^{2+,}$ Pb2+, $Mg^{2+,}$ Mn^{4+}, Li^{+}, $Fe^{3+,}$ Co^{2+}, and Al^{3+} was computed against that of Hg^{2+} using a spectro-fluorophotometer and a Transilluminator imaging system, under UV light (365 nm). As the bar graphs in Figure 7a point out, except $Hg^{2+,}$ the other metals did not induce a noticeable impact on the fluorescence intensity of the PC at a concentration of 900 nM. This means that Hg^{2+} can be distinguished in a mixture containing all these metals very easily at the defined concentration. When the concentration of all the other metals was reinforced to 35 umol/L.

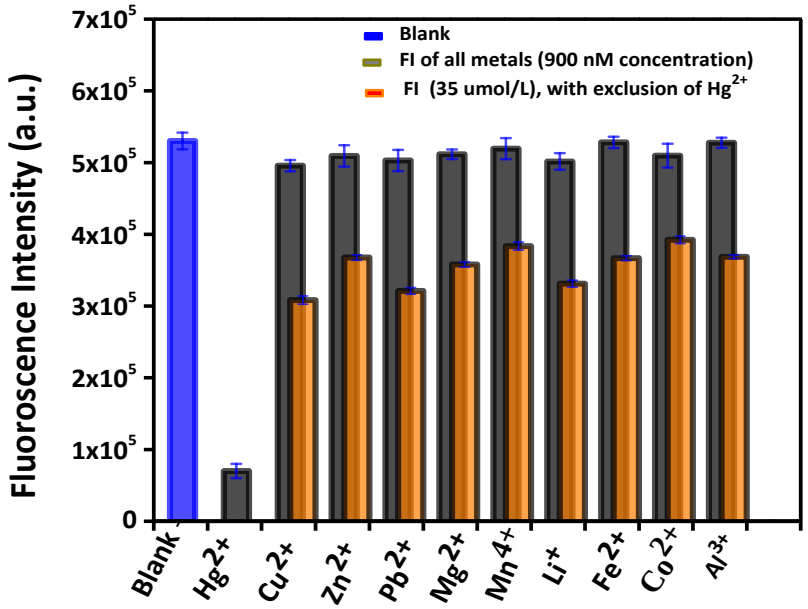


Figure 7. (a) Fluorescence emission intensities of different heavy metal ions at concentrations of 900 nM and 35 umol/L, **FI**-fluorescence intensity

Application of the protein sensor for the detection of Hg^{2+} in real water samples

To evaluate its applicability, the proposed protein sensor was used to detect Hg^{2+} in real water samples (pond water). Pond water samples were collected from the water reservoirs in Jiangnan university campus and filtered using 0.22 μm pore filter and buffered using Tris buffer, pH 6.5. A recovery assay was conducted on samples spiked with different Hg^{2+} concentrations (80 and 100 nM) and obtained resuLts are presented in Table 1. The recovery (98.3 &105.5%) and relative standard deviation (RSD) resuLt (5.94 & 6.11%) are within the acceptable limit. In addition to validation of the performance of the proposed methods in real samples, we also contrasted the level of sensitivity based on the LOD with some previously reported fluorescence-based methods for Hg^{2+} detection as illustrated in Table 2 and the comparison shows that the present work has a comparatively better performance than most of the methods presented.

Table 1. Detection of Hg^{2+} in pond water samples (n=3)

Sample	Hg^{2+} added (nM)	Hg^{2+} detected (nM)	Recovery (%)	RSD (%)
Pond water	80	79.8	105.5	5.94
	100	102.5	102.5	6.21

Table 2. Comparison between previous reported works with the current sensor for Hg^{2+} detection

Type of detection method	Matrix	Detection limit	References
Colorimetric phosphorescent Chemosensor	Aqueous solution	25 nM	[26]
Fluorescent Probe and Micelle Systems	Water	9.1 nM	[27]
Fluorescence sensor	Water	5 *10 $^{-9}$ M	[5]
Fluorescent sensor (based DNA Functionalized Carbon Dots)	Aqueous media	1.02 nM	[28]
Fluorescence sensor (based on magnesium and nitrogen co-doped carbon quantum dots)	Water	0.02 M	[29]
Fluorescence detection based on resonance energy transfer	Water	0.7 ppb	[4]
Fluorescence sensor based on coumarin derivative	Aqueous solution.	8 μmol/L	[6]
A Label- and Enzyme-Free Fluorescent Assay	Tobacco	0.2 nM	[30]
Quantum Dot Fluorescence Quenching Assay	drinking water	0.1 nM	[31]
Current method	Aqueous medium.	18 nM	----

Mechanism of the PC-Hg^{2+} interaction

Phycocyanin is formed from two alike polypeptides: one called α, having a phycocyanobilin (chromophore) attached at cysteine 84 and the other called β, having two phycocyanobilins attached at cysteine 84 and 155. The alterations that occur in the optical behaviors of PC after its conjugation with Hg^{2+} are mainly associated to the presence of a cysteine residue resided in the 84th position of the β-unit of PC. The chromophore that is attached to the cysteine at the 84th position is believed to have fluorescence emitting property [20]. Thus, an interaction between PC and Hg^2 takes place through the fluorescent chromophore and the binding locations found in Hg^{2+}. Another important proposition on the interaction between PC and Hg^{2+} is associated with the electrostatic field of PC. The isoelectric point (PI) of PC is 5.18. Under an aqueous environment with pH above the PI, the electrostatic force in PC is dominated by negative charges, probably due to the electronegative oxygen atoms (in the carbonyl group) of the amino acids [26,32]. Such charge state provides a PC- Hg^{2+} interaction at a protein level instead of at a prosthetic group (chromophore) level. Thus, the quenching effect of Hg^{2+} upon PC is as a resuLt of the interaction of the metal with the protein itself and with its chromophore through the cysteine residue attached by way of thioester group. Both the binding site and the electrostatic based interactions are highly determined by the symmetrical arrangement of the chromophore in the apoprotein.

Figure 8. Illustration of the presumed mechanism of interaction between PC and Hg^{2+}; the pigmented chromophore of PC in its ground state is provoked by laser light; followed by emission of red light after excitation. After the addition of Hg^{2+}, PC's fluorescence intensity is suppressed. Graph; shows fluorescence spectra of PC before and after addition of Hg^2

IV. CONCLUSIONS

In the current work, we have shown that Hg^{2+} can be successfuLly detected in an aqueous system, using Phycocyanin without further treatment. The interaction between PC and Hg^{2+} is found to be greatly affected by the nature of the environment surrounding it as results of fluorescent measurements revealed. Using Tris-buffer, pH 6.5 as an ideal diluent, PC as a fluorescent probe has shown high sensitivity and selectivity in the aqueous system. Under maintained experimental conditions, the fluorescence intensity of PC is suppressed in the attendance of increasing Hg^{2+} concentrations (50-1200 nM) with a good linear relationship and a limit of detection of 18 nM. In addition, Hg^{2+} couLd be easily discriminated among the other 9 metals in the attendance

of 100 nM. Moreover, application of the current fluorescent detection sensor in real samples of pond water showed good recoveries and RSD. Therefore , the current probe can be a good candidate to be exploited for further applications such as in other biological samples to detect Hg^{2+} ions for its highly sensitive, selective, as well as simple advantage.

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Disclosure statement

The authors declare that there are no conflict of interest.

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