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Spectroscopic investigation of sulfonate phthalocyanine to probe enzyme reactions for heavy metals detection

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ABSTRACT

Optical absorption and Raman spectra of the sulfonated copper phthalocyanine (CuTsPc) layer were exploited for detection of cadmium (Cd) contaminants in water. Acetylcholine esterase was immobilized by freely suspending them in calcium alginate microbeads and this gel was then spincoated on the drop cast sulfonated copper phthalocyanine film on a glass substrate to form a bilayer. The inhibition of catalytic reaction between acetylcholine chloride and enzyme due to Cd contaminants was monitored by recording changes in spectra of drop cast CuTsPc as an indicator. The detection limit of cadmium content in water was found to be 1 ppm.

1. Introduction

The heavy metal contamination of water poses hazardous risk to humans and aquatic life. Pollution can occur in a variety of ways. For example, food products including vegetables, grains and fruits become contaminated by accumulating metals from pullulated soil and water. In a recent study on weekly spatial and temporal fluctuations of metal concentrations over two years in the Ten Mile Creek (South Florida), the surface runoff from agricultural lands and urban wastewater, geological backgrounds and tidal flow were identified as the principal sources of river sediments pollution. The heavy metal contents in this region were found to be well over the U.S. Environmental Protection Agency prescribed limits [1]. Similar studies were undertaken in Thailand to investigate the effect of cadmium concentrations in water and sediments in Chao Phraya River on fish industries [2]. The effects of long-term storage of heavy metals within the river sediments were determined by examining contaminants in the groyne fields built along the River Odra (Poland) during the industrialisation in the coal mine districts. It is reported that long period sediment contamination levels could be 60 times larger than local geochemical background [3]. Mining and smelting also increase heavy metal contaminations of soils. For example, the target hazard quotients and estimated daily intakes for cadmium (Cd) and lead (Pb) of rice and vegetables grown around the Dabaoshan mine, South China were found to exceed the FAO/WHO permissible limit of 0.001 mg/l calculated at hardness 100 for Warm Water Aquatic Habitat [4,5].

Using the atomic absorption spectrometry, it was possible to determine the concentrations of heavy metals as low as 1 ng L^{-1} of heavy metals. The technique is based upon adsorption of trace metals on the polymeric resin MCI GEL CHP 20Y after treatment with 2-(2-quinolinil-azo)-4-methyl-1,3-dihydroxidobenzene [6]. The limit as low as 1 ppb was achieved for detection of cadmium (Cd^{2+}) and lead (Pb^{2+}) ions by employing the technique of total reflection at the interface between the planar silicon nitride waveguide and sensing membrane containing composite polyelectrolyte self-assembled films of urease or acetylcholine esterase and cyclotetrachromotropylene as enzyme and indicator, respectively. The catalytic activities of enzymes were inhibited by the presence of metal ions. Individual enzyme reactions as well as their inhibition by metal ions were studied by monitoring the intensity of light output from the planar waveguide [7,8].

Calcium alginate beads were successfully used to immobilize α-Amylase enzyme [9] and *Oryza sativa* L. peroxidase [10]. In the present investigation, acetylcholine esterase (AChE) enzyme was freely suspended in porous spun films containing uniformly dis-

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tributed calcium alginate beads. The purpose of this article is to present an experimental investigation into the effectiveness of sulfonated copper (II) phthalocyanine (CuTsPc) as an indicator for enzyme reactions with acetylcholine chloride (AChCl) solution. The phthalocyanine molecules are known to exhibit different molecular organisations in the aggregates depending on the neighborhood pH value [11,12]. The split Q absorption bands and the Raman peaks were chosen to monitor enzyme reactions during the initiation and inhibition processes for heavy metals detection.

2. Experimental

In order to fabricate the bilayer structure in Fig. 1(a), a 20 nm thick CuTsPc layer was deposited by drop casting of a very small volume of CuTsPc (chemical structure shown in Fig. 1(b)) dissolved at 1 mg/ml concentration in deionized water of 18 MΩ cm on an ultrasonically cleaned glass substrate. The AChE enzyme was dissolved in a 1 mg/ml concentration in a 0.05 M Trizma base buffer at pH 7.4 and a 4% (w/v) solution of sodium alginate was added to the enzyme solution in deionized water of 18 MΩ cm. This gel was then spin coated on the CuTsPc film using the Chemat technology spin coater KW-4A at the speed of 1000 rpm. The sodium alginate layer was then exposed to the 2% (w/v) calcium chloride solution in order to obtain calcium alginate micro beads with enzymes freely suspended inside pores. The membrane was estimated to be approximately 80 nm thick.

Using the PerkinElmer Lambda 950 spectrophotometer within the range 450–800 nm at 1 nm resolution, UV–vis absorbance spectra were recorded for CuTsPc/(calcium alginate-AChE) bilayer at room temperature. A monochromatic beam of intensity I_0 was allowed to be incident perpendicular to the plane of the films. An uncoated substrate was used as a reference for absorption, and a surface silvered mirror for reflection, so that the outputs I_t were solely in terms of the transmission characteristics of the films. Using Beer's law, values of $\ln(I_0/I_t)$ was recorded as absorbance [13]. Raman spectra were recorded on a Nicolet Almega XR dispersive Raman spectrophotometer, equipped with a green argon 514 nm laser accumulated 128 number of scans and an exposure time of 2 s at 4 cm^{-1} resolution. The spectra were recorded in the range of 300–1600 cm^{-1}. In order to investigate the enzyme activities, the membrane was dipped in acetylcholine chloride solution (pH 7.4 by diluted sodium hydroxide aqueous solution) for 15 min at 37 °C in an incubator and initial enzyme activity was then measured by recording both UV–vis absorption and Raman spectra for the sample. The bilayer membrane was subsequently exposed to water contaminated with cadmium of four different concentrations varying from 100 ppm to 1 ppm for 15 min. Residual enzyme activity was finally determined by repeating optical absorption and Raman spectroscopic measurements, A Digital Nanoscope III, Atomic force microscope was used to study the surface morphology of the spun calcium alginate films on silicon substrate. Films were imaged in tapping mode AFM along with silicon cantilevers [14].

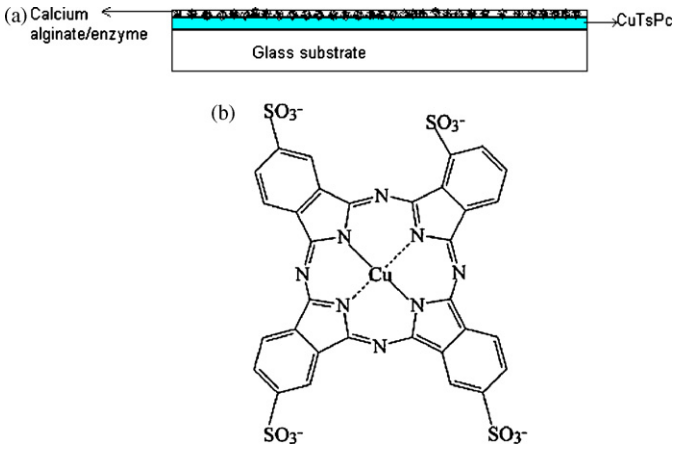

Fig. 1. (a) Schematic diagram of CuTsPc/(calcium alginate-AChE) bilayer and (b) chemical structure of copper phthalocyanine tetrasulfonate (CuTsPc).

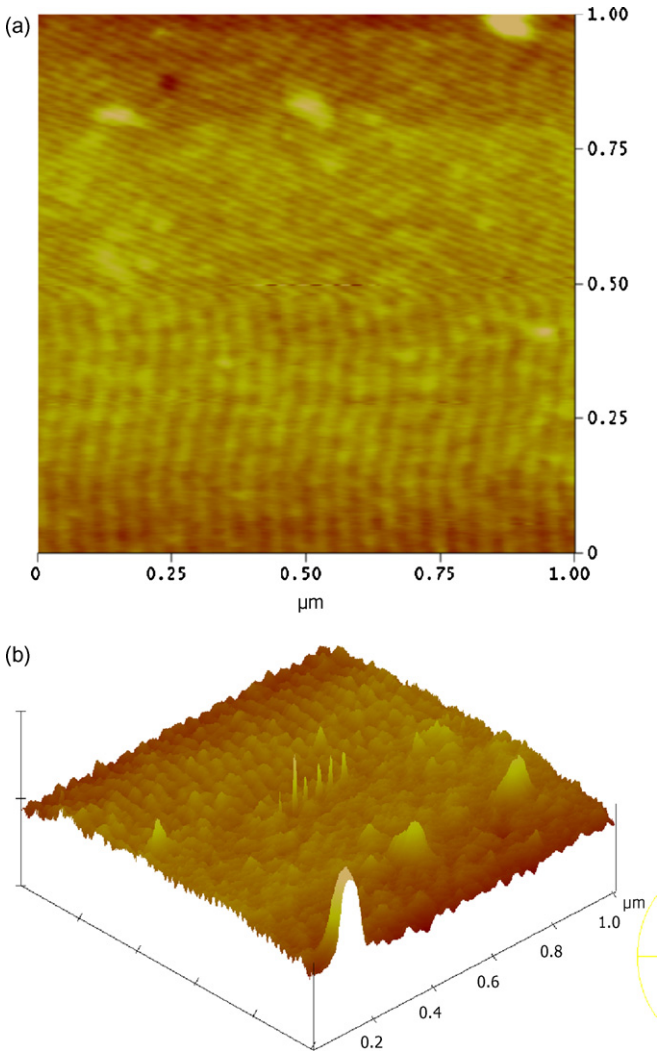

Fig. 2. (a) Two and (b) three dimensional AFM micrograph of top surface of calcium alginate on the silicon substrate.

3. Results and discussions

The AFM micrographs in Fig. 2 show that the top surface of the calcium alginate on the silicon substrate was covered with spherical beads, largely self-organised, of 40–50 nm in diameter without intervening spaces. The surface was found to be uniform and the average roughness was estimated to be 8 nm.

As shown in Fig. 3(a), essential characteristics of the UV–vis absorption spectra of the CuTsPc/(calcium alginate-AChE) bilayer on the glass substrate are similar to those reported for CuTsPc film the appearance of the Soret band at ~350 nm due to the transition between $\pi(b_{2u})$ and $\pi^*(e_g^*)$ levels and the split Q bands at ~550 nm (Q_1) and 678 nm (Q_2). Q_1 represents the dimerisation while monomers are associated with the peak Q_2 [15]. The spectra were not expected to be influenced by the presence of the top layer since the absorption peak is reported to have occurred at 250 nm for calcium alginate which is transparent in the range between 350 nm

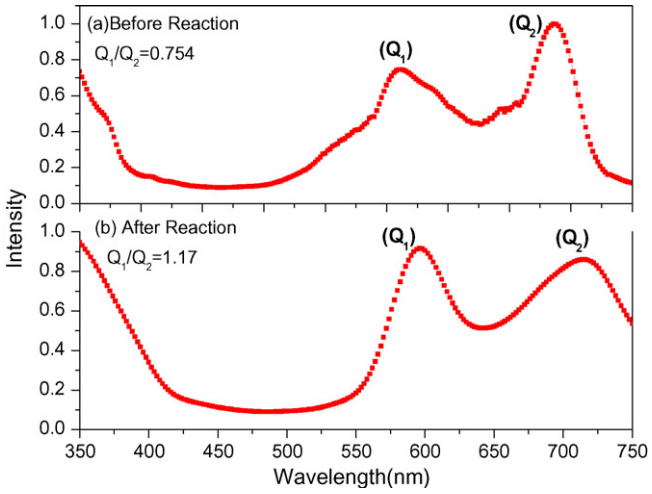

Fig. 3. UV–vis absorption spectra of CuTsPc/(calcium alginate-AChE) bilayer (a) before and (b) after reaction with AChCl. The numbers on the graphs give the dimer/monomer ratio determined from the intensities of Q bands.

and 800 nm [16]. When the CuTsPc/(calcium alginate-AChE) bilayer was treated in the acetylcholine chloride solution, the characteristic features of the UV–vis spectra remained unaltered but the positions of both Q bands underwent redshifts. Both peaks were also found to be relatively broad. A simple monomer-dimer equilibrium model is, therefore, believed not to be adequate for explaining the CuTsPc aggregation due to interaction and inhibition processes [17,18]. The peak intensity was, on other hand, higher for Q_1 than Q_2 band implying a possible increase in the concentration of dimmer species due to the acetylcholine reaction with acetylcholine esterase [19]. The acetic acid and choline were produced as a result of the reaction between enzyme acetylcholine esterase and acetylcholine chloride. The acidic medium is believed to have caused protonation of sulfonated groups of CuTsPc molecules leading to decrease in the net negative charge on each molecule. The molecules will experience less electrostatic repulsion and thus tend to molecular aggregation arising from reduced electrostatic repulsion was therefore associated with the changes in Q_1 band characteristics [20].

Systematic changes in the intensities and positions of monomer and dimmer peaks are observed in the UV–vis spectra (Fig. 4) due

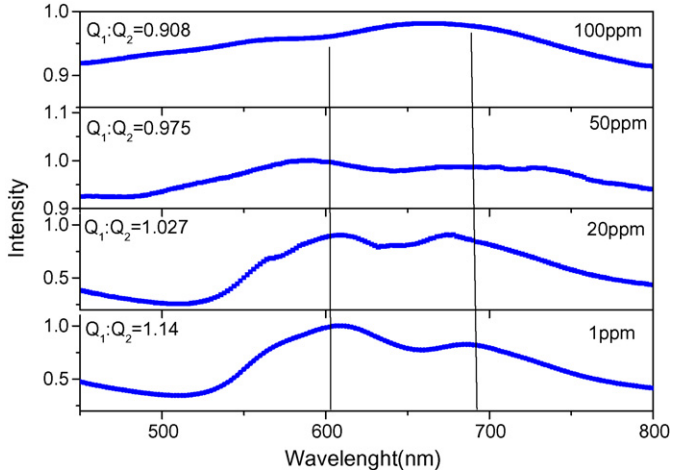

Fig. 4. UV–vis absorption spectra to study residual activity of CuTsPc/(calcium alginate-AChE) bilayer after reaction with AChCl and exposed to (a) 100 ppm (b) 50 ppm (c) 20 ppm (d) 1 ppm of Cd^{++} ions. The numbers on the graphs give the dimer/monomer ratio determined from the intensities of Q bands.

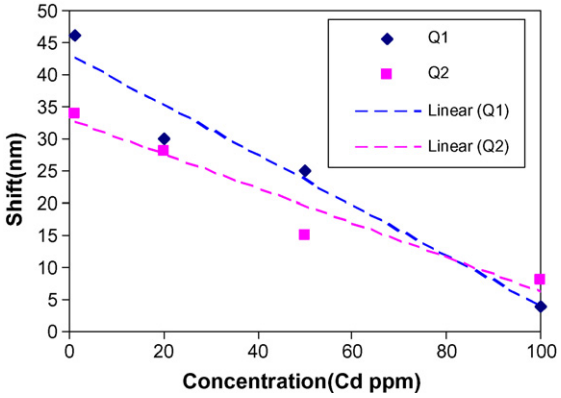

Fig. 5. Dependence of the wavelength shifts of Q bands relative to their positions in Fig. 3(a) on concentrations of Cd^{++} ions.

to the inhibition by cadmium (Cd^{++}) ions. Values of the ratio of the intensities of Q_1 to Q_2 were found to decrease with the increase in the concentration of Cd^{++} ions. For 100 ppm exposure, the residual enzymatic activity was found to be significantly small and the spectrum resembled the original membrane structure with dominating monomer structure. This is expected as enzymatic reaction would be increasingly inhibited by the rise in the concentration of Cd^{++} ions bound to the active sites. The resulting change in local pH in the neighborhood of the CuTsPc indicator layer became gradually smaller with greater inhibition due to lower production of acetic acid. As shown in Fig. 5, values of wavelength shifts of Q bands due to Cd^{++} ions exposure were found to linearly dependent upon the concentrations. The sensitivity σ is estimated to be 1.6%/ppm using the value of the slope s_i in the following equation:

$$\sigma = \frac{1}{2}\sum_{i=1}^{2}\frac{s_i}{\lambda_{0i} - \lambda_{ci}} \quad (1)$$

where λ_{0i} = position of Q_i peak recorded for the CuTsPc/(calcium alginate-AChE) bilayer before reaction with AChCl in Fig. 3(a) and λ_{ci} = position of Q_i peak recorded for the acetylcholine chloride treated CuTsPc/(calcium alginate-AChE) bilayer in Fig. 3(b). The factor 1/2 takes into account of the effect of differing slopes.

The inhibition of enzymatic activities by Cd^{++} was further examined by the Raman spectroscopy in Fig. 6 over the range of the wavenumber from 400 cm^{-1} to 1500 cm^{-1}. The spectra were obtained for the CuTsPc/(calcium alginate-AChE) bilayer before and after interaction with AchCl. The summary of observations is given in Table 1. The CuTsPc thin film is known to exhibit several inter-molecular and intramolecular vibrations [21]. These vibrational modes of observed Raman bands mainly originate from

Table 1
Analysis of Raman spectra in Fig. 6.

Peak position (cm^{-1})		Interpretation
Prior to enzyme reaction	Post enzyme reaction	
354	Disappear	Cu–N stretch
485	500	Isoindole ring deformation
	510	Isoindole ring deformation
550	Disappear	Isoindole ring deformation
597	600	Isoindole ring deformation
680	680	16 membered inner ring breathing
700	700	16 membered inner ring breathing
760	760	C–N stretching
830	830	C–N stretching
1500	Disappear	Monomer
	1540	Dimer

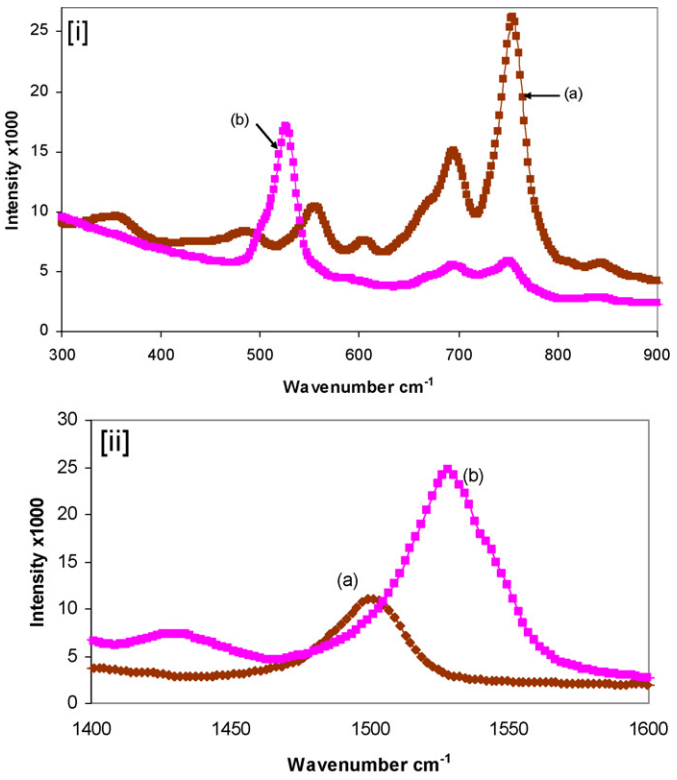

Fig. 6. Raman spectra of CuTsPc/(calcium alginate-AChE) bilayer (i) from 300 cm^{-1} to 900 cm^{-1} and (ii) from 1400 cm^{-1} to 1600 cm^{-1} (a) before and (b) after reaction with AChCl.

the vibrations of the macrocycle, isoindole moieties, C–H bendings and metal–nitrogen stretch [22,23]. The Raman peak at about 1500 cm^{-1} was attributed to the υ_3 vibrational mode. The peak was recorded at 1510 cm^{-1} for sample before interaction with AChCl and is believed to be associated with the monomeric form of CuTsPc whereas the peak was blue shifted to 1540 cm^{-1} after the AchCl treatment [24]. The peak at 1540 cm^{-1} was due to the aggregation of CuTsPc molecules.

In addition to the UV–vis absorption studies, the Raman spectra support the explanation of aggregation of CuTsPc molecules due to change in local pH due to enzyme reaction. When the CuTsPc/(calcium alginate-AChE) bilayer was exposed to the solutions containing cadmium at different concentrations, the Raman spectra in Fig. 7 showed a systematic shift with the increasing concentration. The peak close to 1520 cm^{-1} in Fig. 7(a) after the interaction

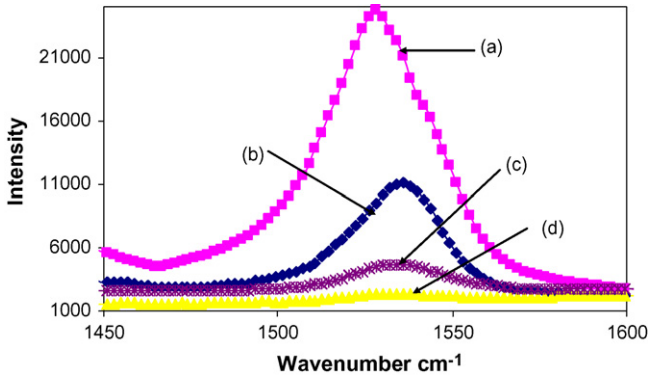

Fig. 7. Raman spectra to study residual activity of membrane after reaction with AChCl and then exposed to (a) 100 ppm (b) 50 ppm (c) 20 ppm (d) 1 ppm of Cd^{++} ions.

with 100 ppm Cd ion solution was identified monomer phthalocyanine structure whereas the peak close to 1540 cm^{-1} in the spectrum (d) obtained after 1 ppm Cd exposure indicates the dimmer configuration. The value for sensitivity σ is found to be 1.8%/ppm using 1500 cm^{-1} and 1540 cm^{-1} as reference wavenumbers λ_{0i}^{-1} and λ_{ci}^{-1}, respectively. This value is close to one obtained previously from optical absorption spectra.

4. Conclusions

A bilayer sensing membrane structure was fabricated consisting of spun AChE immobilized in calcium alginate beads and indicator CuTsPc layer. The physical adsorption of an acetylcholine esterase enzyme onto calcium alginate microbeads proves to be a simple way to prepare immobilized enzymes. The method relies on non-specific physical interaction between the enzyme protein and the surface of the matrix offering a route to stabilizing adsorbed enzymes. Once the enzyme is immobilized, however, it finds itself in a drastically different microenvironment from one in free solution. The new microenvironment is responsible for the enzymatic reaction and detection of heavy metals. When AChE reacted with acetylcholine chloride, the pH in the indicator layer vicinity changed due to the acidic product of the reaction. The pH induced aggregation of phthalocyanine molecules produced significant absorption and Raman spectra. This method can be further developed to meet the standard of cadmium hazard limit of detection 0.01 mg/l by ICP/MS; 2 mg/l by FAAS prescribed by WHO [25]. It is possible to detect cadmium ion in environmental media where other divalent cations are removed by different chelating agents. The membrane is insoluble in the test solution and the immobilisation process is also irreversible. The bilayer sensing membrane is, therefore, suitable for use in a disposable sensor. The method is cost-effective, easily adaptable for practical implementation and less disruptive to the enzymatic protein than other chemical attachment.

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