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In Vitro Binding of Heavy Metals by an Edible Biopolymer Poly(γ-glutamic acid)

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An edible biopolymer poly(γ-glutamic acid) (γ-PGA) was evaluated for possible use as an chelating/binding agent in the treatment of metal intoxication in humans. *In vitro* binding of the toxic heavy metals lead and cadmium as affected by pH, contact time, metal concentration, γ-PGA dose, and essential metals was carried out in a batch mode. A maximum binding occurred in the pH range 5−7, corresponding to the gastrointestinal pH values except for the stomach. Binding isotherms at pH 5.5 were well described by the heterogeneous models (Freundlich and Toth), while the lead isotherm at pH 2.5 showed a S-type curve, which was fitted as multiple curves with the Langmuir model and a shifted-squared Langmuir model. However, no adsorption occurred for cadmium at pH 2.5. The maximum binding capacities of lead and cadmium at pH 5.5 were 213.58 and 41.85 mg/g, respectively. A curvilinear biphasic Scatchard plot signified a multisite interaction of metals. Binding was extremely rapid with 70−100% of total adsorption being attained in 2 min. Kinetics at low and high metal concentrations obeyed pseudo-first-order and pseudo-second-order models, respectively. The γ-PGA dose−activity relationship revealed a low dose of γ-PGA to be more efficient in binding a large amount of metals. Incorporation of Cu, Zn, Fe, Mg, Ca, and K showed only a minor influence on lead binding but significantly reduced the binding of cadmium.

KEYWORDS: Adsorption; poly(γ-glutamic acid); biodegradable polymer; heavy metals; isotherms; kinetics; metal selectivity

INTRODUCTION

Heavy metals such as lead, cadmium, and mercury are nonessential nutrients, which are potentially toxic at very low concentrations due to their nonbiodegradable nature and prolonged biological half-life. Several reports have shown that the accumulation of heavy metals in humans can cause severe damage to kidney and liver as well as impair the immune and central nervous systems, resulting in vital pathological changes and functional abnormalities (cognitive and behavioral), gastrointestinal toxicity, and chronic renal failure (*1*−*3*). As heavy metals are effective competitors and possess some chemical similarities with essential metals (Zn^{2+}, Fe^{2+}, Ca^{2+}, etc.), they interact with a number of divalent metal transporters, disrupting various physiological functions (*1*, *2*). Therefore, it is important to develop effective and safe detoxifying agents to prevent metal accumulation in humans.

Chelation therapy is one of the most common methods in the treatment of metal intoxication. Ethylenediaminetetraacetic acid (EDTA), 2,3-mercaptopropanol (BAL), dimercaptosuccinic acid (DMSA), diethylenetriaminepentaacetic acid (DTPA), and penicillamine are the chelating agents widely used for removal of metal toxicity (*4*−*6*). However, these chelating agents have been reported to cause potential side effects (*4*, *6*). For instance, DMSA, the most acceptable antidote in lowering lead levels in humans, may result in essential metal deficiency, alteration in mineral metabolism, mucocutaneous eruption, nausea, and developmental toxicity (*4*). Consequently, for over a decade, dietary fibers from various food products including rice bran, wheat bran, and oat bran as well as fruit fibers (*7*−*9*) were evaluated as an alternative to chelation therapy with the aim to overcome toxic side effects. In recent years, natural polymeric materials have also been gaining interest as potential adsorbents of heavy metals because of their nontoxic and biodegradable nature (*10*). For example, alginate, an algal polysaccharide, has been shown to be effective in scavenging food and chemical mutagens under both *in vitro* and *in vivo* conditions (*10*).

Poly(γ-glutamic acid) (γ-PGA), an edible and biodegradable biopolymer, is synthesized extracellularly by *Bacillus* species by either the *de nova* method in solid state fermentation or the salvage bioconversion pathway in submerged fermentation (*11*, *12*). It is commercially available in different ionic forms (Ca, Na, Mg, etc.) and molecular masses (10,000

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to 2 million Da). γ-PGA consists of repetitive glutamic acid units connected by γ-amide linkages between α-amino and γ-carboxyl functional groups, leaving the α-carboxyl groups available for conjugation to a variety of compounds including metal cations (−[−NH−CH(COOH)−$(CH_2)_2$−CO−]$_n$−)(*11*,*12*). Because of its biocompatible nature, γ-PGA has been used as a drug carrier in various biomedical preparations to enhance drug targeting specificity, lower systemic drug toxicity, improve drug absorption, and protect drugs from biochemical degradation, and their therapeutic activities have also been evaluated (*13*). Recently, Tanimoto et al. (*14*) reported that γ-PGA could enhance the calcium absorption in the small intestine. Besides, γ-PGA also finds potential application in a wide variety of fields including food, agriculture, cosmetics, medicine, and water treatment, which have been well reviewed (*11*, *12*). Our earlier studies explored the application of γ-PGA as adsorbent for cationic dyes (*15*) and heterocyclic amines (*16*), as well as carrier for encapsulation of lycopene (*17*) and soy isoflavones (*18*). Previous studies on binding of heavy metals by γ-PGA have been restricted to metals ions such as Ni^{2+}, Cu^{2+}, Mn^{2+}, Al^{3+}, Cr^{3+}, Hg^{2+}, and U^{4+} for water treatment (*19*−*21*). However, its application in binding lead and cadmium is still lacking and the possible use as a chelating/binding agent in the treatment of metal intoxication in humans has to be explored. Therefore, the objectives of the present work were to study the binding characteristics of lead and cadmium by γ-PGA as a function of pH, contact time, metal concentration, γ-PGA dose, and several essential metals (Cu, Zn, Fe, Mg, Ca, and K) in a batch mode under *in vitro* conditions.

MATERIALS AND METHODS

Poly(γ-glutamic acid). The extracellular biopolymer poly(γ-glutamic acid) produced from *Bacillus subtilis* var. Natto was supplied by Vedan Enterprise Corporation (Taichung, Taiwan). Detailed procedures for fermentation, cultivation, extraction, and purification of γ-PGA have been reported by the manufacturer elsewhere (*11*). The synthesis of γ-PGA was achieved by the salvage bioconversion pathway through submerged fermentation, accumulating γ-PGA largely during the stationary phase of cell growth (*11*). The physicochemical characteristics of γ-PGA were reported in our previous article (*16*). Briefly, physical appearance = white, granulate, free-flow powders; MW = 1230 KDa (GPC/HPLC); purity = 95% (HPLC); particle size = 1−150 μm; clarity (OD_{400}) = 0.18; moisture content = 5% (automatic infrared analyzer); bulk density = 0.32 g/mL; decolorizing power (methylene blue dye) = 135.00 mg/g; total heavy metals (as Pb) = 15 mg/L (includes Pb = 5 mg/L, Cd = 2 mg/L, As_2O_3 = 2 mg/L); *E.Coli* and *Salmonella* = not detectable/1 g; pK_a value = 4.09 (potentiometric titration); elemental analysis (%) = Found: C, 44.86; H, 5.91; N, 10.49; S, 0. Calculated: C, 46.51; H, 5.43; N, 10.85; S, 0 (Perkin-Elmer CHN-2400).

Binding Experiments. Initially, stock solutions (1000 mg/L) of lead and cadmium were prepared from their respective metal salts lead(II) nitrate ($Pb(NO_3)_2$) and cadmium(II) sulfate ($3CdSO_4 \cdot 8H_2O$), both of which were procured from Sigma (St. Louis, MO). Then, suitable dilutions were made with deionized water (Millipore, Bedford, MA) to obtain working solutions of desired concentration. The *in vitro* binding experiments were carried out by taking 50 mL of metal solutions in 125 mL volumetric flasks and adjusting to the desired pH using a Suntex 701 pH meter (Suntex Instruments Co. Ltd., Taipei, Taiwan), equipped with a Horiba 9611-10D glass electrode (Horiba Instruments Inc., CA). Next, a weighed amount of activated γ-PGA was added and the mixture was shaken at 120 rpm in a water bath shaker at 37 °C until equilibrium or defined time intervals. The solutions were then filtered through a 0.2-μm membrane filter and the filtrates were analyzed for metal concentration. Control experiments were also performed to monitor any metal adsorption on volumetric flask and/or membrane filter.

Metal Analysis. Both lead and cadmium were analyzed by using a GBC 932 atomic absorption spectrophotometer (AAS) (GBC, Victoria, Australia) connected to a corresponding hollow cathode lamp (GBC) with wavelength at 283.3 and 228.8 nm, slit width at 0.2 and 0.2 nm, and lamp current at 5 and 3 mA, respectively. An oxidizing flame of an air−acetylene mixture at a ratio of 5:1 was employed. After initial instrumental optimization, a calibration curve was developed using four concentrations each of lead (5, 20, 35, and 50 mg/L) or cadmium (0.5, 1, 2, and 3 mg/L) prepared from their respective stock solutions. Then, the concentration of lead or cadmium in the unknown sample aliquots was automatically determined based on the calibration curve.

pH Variation Study. Lead or cadmium solutions at 50 mg/L were adjusted to different pH values (1.5, 2, 2.5, 3, 3.5, 4, 5, 6, and 7), and each was shaken with a γ-PGA dose of 0.4 g/L at 37 °C for 2 h. Both HNO_3 and NaOH procured from Laboratory-scan Analytical Sciences (Bangkok, Thailand) and Riedel-de-Haën (Seelze, Germany), respectively, were diluted and used to adjust the pH of metal solutions.

Binding Isotherm Study. Binding isotherm curves were prepared by taking 13 lead concentrations of 5, 10, 20, 30, 40, 50, 60, 80, 100, 125, 150, 175, and 200 mg/L or 10 cadmium concentrations of 5, 10, 15, 20, 30, 40, 50, 60, 80, and 100 mg/L, adjusting each to pH 5.5, and agitating with 0.4 g/L of γ-PGA separately at 37 °C for 2 h. In addition, a similar experiment was done for lead at pH 2.5, which corresponds to a typical pH of the stomach. However, no adsorption occurred for cadmium at pH 2.5.

Binding Kinetic Study. Four concentrations of lead (10, 25, 50, and 100 mg/L) or cadmium (5, 10, 25, and 50 mg/L) were each adjusted to pH 5.5 and agitated separately with 0.4 g/L of γ-PGA at 37 °C to different time intervals of 0.5, 1, 2, 3, 5, 8, 12, 16, 20, 30, 60, and 120 min to determine the rate of metal binding by γ-PGA.

γ-PGA Dose−Activity Relationship. To establish the γ-PGA dose−activity relationship, a total of 12 γ-PGA doses of 0.04, 0.08, 0.1, 0.2, 0.3, 0.4, 0.6, 0.8, 1, 1.2, 1.6, and 2 g/L were added separately to each of the lead solutions at 25, 50, and 100 mg/L or cadmium solutions at 10, 25, and 50 mg/L with pH at 5.5, and shaken at 37 °C for 2 h.

Selectivity Study−Effect of Essential Metals. The selectivity of lead or cadmium by γ-PGA in the presence of several biologically essential metals such as copper, zinc, iron, magnesium, cadmium, and potassium was studied. Metal salts of copper(II) sulfate and zinc(II) sulfate heptahydrate were purchased from Sigma (St. Louis, MO), calcium(II) chloride dihydrate was purchased from J. T. Baker (Phillipsburg), potassium chloride and magnesium(II) sulfate heptahydrate were purchased from Nacalai Tesque (Kyoto, Japan), and iron(II) sulfate heptahydrate was purchased from Ajax Chemicals (Australia). Metal solutions (50 mL) containing a 1:1 binary mixture of lead or cadmium and an essential metal were prepared at 5, 25, 50, and 100 mg/L. The solution mixtures were then adjusted to pH 5.5 and agitated separately with 0.4 g/L of γ-PGA at 37 °C for 2 h.

Binding Calculation and Data Modeling. Experiments were carried out in duplicate and the arithmetic mean values showed a maximum deviation of only ±2%. The metal binding capacity (q, mg/g) at different time intervals was calculated using the mass balance equation $q = (C_i - C)(V/W)$, where C_i (mg/L) is the initial metal concentration, C (mg/L) is the metal concentration remaining in solution after adsorption for time t (min), V (L) is the volume of metal solution, and W (g) is the weight of γ-PGA. For metal binding at equilibrium ($C = C_e$, $q = q_e$), the amount of metal removed can be calculated using the same equation described above. On the other hand, the amount of metal binding at different γ-PGA doses was expressed in a percentage (R, %) to facilitate the modeling of experimental data according to the equation $R = 100(C_i - C_e)/C_i$.

Experimental data on binding kinetics and isotherms were modeled with several theoretical models by using a GNUPLOT software program (version 4.0 for Windows), which employed a nonlinear regression method based on the Marquardt−Levenberg algorithm. Two error indicators, namely the coefficient of determination (r^2) and the chi-squared statistic (χ^2), were used to determine the degree of fitness by theoretical models. A Microsoft Excel function provided the r^2 values directly, while χ^2 was calculated by applying the equation

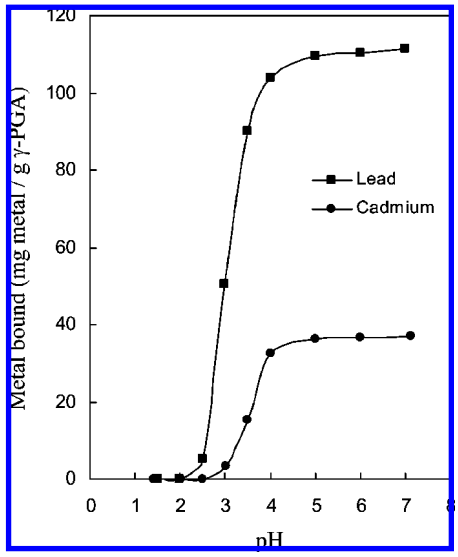

Figure 1. Effect of solution pH on lead and cadmium binding by γ-PGA: initial metal concentration, 50 mg/L; γ-PGA dose, 0.4 g/L; temperature, 37 °C; contact time, 2 h.

$\chi^2 = \Sigma((q_{exp} - q_{mod})^2/q_{mod})$, where q_{exp} and q_{mod} (mg/g) are the experimental and modeled amounts of metal bound on γ-PGA, respectively.

RESULTS AND DISCUSSION

pH Variation Study. From the physiological point of view, the effect of pH on metal binding is imperative, as the gastrointestinal pH varies widely from 2.5 in the stomach to 7 in the small intestine, due to the presence of a complex mixture of different varieties of minerals and secretions (*16*). In addition, for solution systems involving multifunctional macromolecules such as γ-PGA and metals, pH can affect the dissociation of cation exchange groups, conformational changes in molecular structure, stability of metal complexes, and speciation of metals (*20*). **Figure 1** illustrates the binding trend for lead or cadmium by γ-PGA, as affected by pH ranging from 1.5 to 7. Adsorption was practically nil until pH reached 2 for lead and 2.5 for cadmium. However, a small amount of metal adsorption did occur at pH 2.5 for lead and pH 3 for cadmium, followed by a sharp rise, reaching a plateau at pH 5. With the exception of the stomach (pH 2.5), the binding of heavy metals by γ-PGA was appreciable under the pH conditions of the small intestine (pH 7), the cecum/ascending colon (pH 5.5), and the distal colon (pH 6.8). Although only a poor adsorption occurred at the stomach pH, it is inappropriate to judge the detoxifying capacity of γ-PGA merely under the stomach pH. Instead, the small intestine is a more important organ responsible for absorption of minerals, foods, and toxic materials.

For adsorbent/chelating materials rich in carboxyl groups, the cation binding strongly depends on the solution pH and pK_a of the material. Accordingly, at pH < pK_a of γ-PGA (4.09), the carboxyl groups of γ-PGA remain primarily in the nonionized form and thus prevent the metal from binding for the solution at pH < 3. Moreover, the tendency of γ-PGA to form intramolecular hydrogen bonds at low pH with a more compact α-helix conformation (*11, 12*) may diminish the functional groups available for metal binding. However, a more pronounced adsorption at pH > 2.5 for lead and pH > 3 for cadmium was probably due to ionization of carboxyl groups with a concurrent conformational change from α-helix to random coil. It should also be pointed out that although only a small fraction of carboxyl groups are ionized at pH < pK_a, an appreciable metal adsorption did occur at pH 3−4, revealing the compact helical structure of γ-PGA to generate a significant electrostatic field to facilitate metal binding at pH < pK_a.

With a rise in pH from 2.5 to 5 for a 50 mg/L solution, the amount of lead and cadmium binding onto γ-PGA increased by 104.30 and 36.35 mg/g, respectively. This large difference in binding can be accounted for by the difference in electronegativity (2.33 for lead and 1.69 for cadmium). Furthermore, lead at increasing concentrations may form a number of labile hydrolysis and polymeric species (*22*) such as $Pb(OH)^+$, $Pb(OH)_2$, $[Pb_3(OH)_5]^+$, and $[Pb_4(OH)_4]^{4+}$, all of which are more prone to binding/retention onto γ-PGA, resulting in a binding capacity greater than normally expected by simple ion exchange rules. The interaction between metal and γ-PGA can also cause significant changes in the helix−coil transition as a function of pH, which in turn may affect the overall binding capacity of metals (*23*).

Thus, lead (as Pb^{2+} and hydrolyzed species) and cadmium (as Cd^{2+}) may interact with carboxylate anions of γ-PGA. Besides, they may also bind with amide groups of γ-PGA, which could be substantiated based on the hard soft acid base (HSAB) principle proposed by Pearson (*24*), who postulated that hard acids prefer to bind a hard base through ionic interactions, while soft acids prefer a soft base through covalent interactions. As Pb^{2+} and Cd^{2+} were classified as a borderline acid and a soft acid, respectively, their interaction with a hard base like carboxylate anions on γ-PGA could be possible only in association with some other functional groups such as amides, as suggested by Rivas and Maureira (*25*). Classification of Pb^{2+} as a borderline acid may also justify its higher binding capacity over cadmium.

Binding Isotherms. As the maximum binding of lead and cadmium occurred in the pH range 5−7, the binding isotherms obtained at pH 5.5 should be representative for the gastrointestinal pH. In addition, the isotherm curves were obtained for lead binding at pH 2.5, which represents the typical stomach pH, but the adsorption of cadmium did not occur at this pH. **Figure 2** shows the respective isotherm curves of lead and cadmium at pH 5.5, which were characterized by an increased uptake following a rise in metal concentration. With an increase of concentration from 5 to 200 mg/L, the amount of lead binding onto γ-PGA rose by 205.43 mg/g and cadmium by 31.31 mg/g. Yet, a plateau was not reached, indicating the heterogeneous nature of metal interaction with γ-PGA. On the contrary, the lead isotherm at pH 2.5 showed three distinct portions, signifying a S-type curve according to the general classification of solution isotherms (*15, 16*) (**Figure 3**). Initially, a low saturation region was observed for the metal concentration of 5−20 mg/L, followed by a sharp rise in lead adsorption, resulting in a convex shape, and finally the adsorption slowed down to reach a maximum of 12.20 mg/g at 200 mg/L. The S-type curve with a low binding capacity at pH 2.5 may be caused by protonation of carboxyl groups on γ-PGA with a compact helical structure at pH < 3, as mentioned in the preceding section.

Modeling of Isotherms at pH 5.5. Modeling of isotherm data is vital in deriving meaningful information on binding characteristics, including maximum binding capacity and binding constant. Therefore, several theoretical isotherm equations, including those of Freundlich, Langmuir, and Toth (*26*), were employed to evaluate which could best describe the experimental data. Generally, the Freundlich and Toth models are applicable to heterogeneous systems, while the Langmuir model is based on a homogeneous monolayer adsorption (*15, 16, 26*). The nonlinear forms of these models can be represented as below:

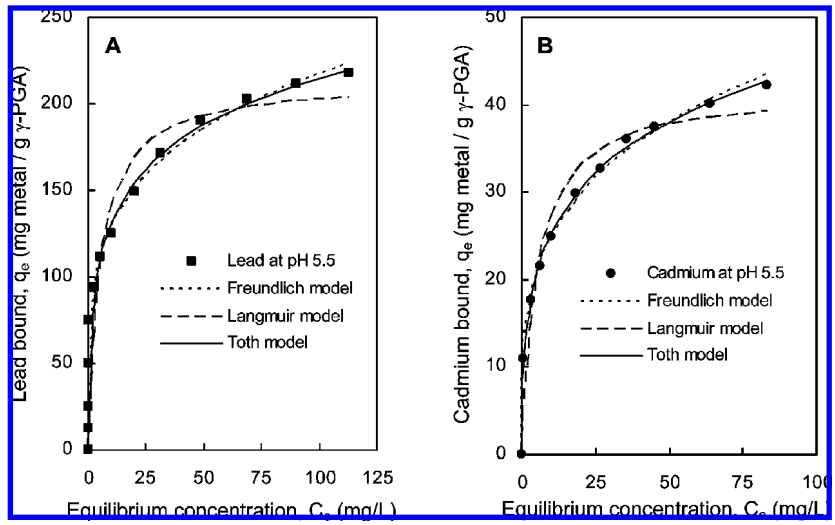

Figure 2. Binding isotherms for lead (A) and cadmium (B) binding by γ-PGA at pH 5.5. Initial concentration range: 5−200 mg/L for lead and 5−100 mg/L for cadmium; γ-PGA dose: 0.4 g/L; pH: 5.5; temperature: 37 °C; contact time: 2 h; isotherm equations used for modeling are given inside the text as eqs 1−3.

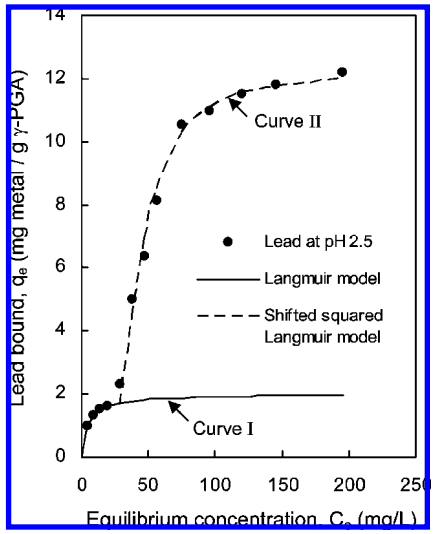

Figure 3. S-type isotherm for lead binding by γ-PGA at pH 2.5. Experimental conditions are the same as those given under **Figure 2**. Curve I indicates the initial low saturation region covering four experimental points, and curve II denotes the convex shape of a S-type isotherm representing a sudden rise in metal binding to reach a plateau. Curve I is fitted by the Langmuir model (eq 2), and curve II is fitted by a shifted squared Langmuir model (eq 5).

$$q_e = K_F C_e^{1/n} \quad (1)$$

$$q_e = \frac{q_m b C_e}{1 + bC_e} \quad (2)$$

$$q_e = \frac{AC_e}{(1 + (b_T C_e)^{\gamma})^{1/\gamma}} \quad (3)$$

where K_F (L^n mg^{1-n}/g) and n are the Freundlich constants denoting binding capacity and binding intensity, respectively; q_m (mg/g) and b (L/mg) represent the maximum binding capacity and binding constant, respectively; and A (L/g), b_T (L/mg), and γ are the Toth constants. Among the three models tested, both Freundlich and Toth precisely fitted the lead and cadmium isotherms at pH 5.5, implying again the heterogeneous nature of adsorption (**Figure 2**). In other words, more than one type of binding site with different affinities was involved in lead and cadmium binding by γ-PGA. This is evident from the close-to-unity r^2 values and low χ^2 values obtained for these models compared to the Langmuir model (**Table 1**). To further confirm the multisite interaction, the isotherm data were subjected to Scatchard plot analysis (*16*) according to the following equation:

Table 1. Isotherm Parameters for Lead and Cadmium Binding by γ-PGA at pH 5.5

	modeled isotherm parameters	
isotherm model	lead	cadmium
Freundlich[a]		
K_F (L^n mg^{1-n}/g)	76.57	13.48
n	4.42	3.77
r^2	0.994	0.994
χ^2	0.64	0.22
Langmuir[b]		
q_m (mg/g)	213.58	41.85
b (L/mg)	0.187	0.186
r^2	0.899	0.945
χ^2	18.96	11.32
Toth[c]		
A (L/g)	661.47	249.50
b_T (L/mg)	0.507	0.528
γ	0.179	0.141
r^2	0.992	0.998
χ^2	1.08	0.08

[a] $q_e = K_F C_e^{1/n}$, where K_F and n are the Freundlich constants denoting binding capacity and binding intensity, respectively. [b] $q_e = (q_m b C_e)/(1 + bC_e)$, where q_m and b represent the maximum binding capacity and binding constant, respectively. [c] $q_e = (AC_e)/(1 + (b_T C_e)^{\gamma})^{1/\gamma}$, where A, b_T, and γ are the Toth constants.

$$\frac{q_e}{C_e} = q_m K_b - q_e K_b \quad (4)$$

where K_b (L/mg) is a binding constant related to the affinity between γ-PGA and metals. The Scatchard plot showed a curvilinear biphasic plot (**Figure 4**), indicating a heterogeneity and positive cooperativity between binding sites during metal adsorption on γ-PGA. This is apparent, as the plotting of q_e/C_e versus q_e theoretically eliminates the effect of concentration on the shape of the Scatchard plot and hence any observed deviation from linearity can be attributed to multisite affinity (*16*). Thus, both carboxyl and amide groups may be responsible for Pb^{2+} or Cd^{2+} binding, with a predominant contribution of the former at low metal concentrations. However, not all heavy metals

Table 2. Isotherm Parameters for Lead Binding by γ-PGA at pH 2.5

model parameters	curve I—Langmuir model	curve II—shifted-squared Langmuir model[a]
q_m(mg/g)	2.00	12.22
b (L/mg)	0.206	0.002
r^2	0.999	0.989
χ^2	4.79×10^{-6}	0.41

[a] $q_e = (q_m b(C_e - C_s)^2)/(1 + b(C_e - C_s)^2)$, where q_m and b denote the maximum binding capacity and binding constant, respectively, C_e is the equilibrium concentration and C_s is the shifted concentration along the *x*-axis in **Figure 3**.

could interact with both carboxyl and amide groups of γ-PGA as reported by Hikichi et al. (*27*), who used NMR spectra to demonstrate that Cu^{2+} ions interacted with the carboxyl groups and nitrogen of the amide groups, whereas Mn^{2+} interacted only with the former.

Modeling of Lead Isotherm at pH 2.5. On the other hand, several isotherm models, including the Freundlich, Langmuir, Toth, Redlich−Peterson, Sips, Flory−Huggins, Tempkin, Radke−Prausnitz, and Vieth−Sladek models, gave a poor correlation for the lead isotherm at pH 2.5. Therefore, the isotherm curve at pH 2.5 was divided into two curves with the initial low saturation region covering four experimental points to be curve I, while the remaining to be curve II. Each curve was modeled separately with various isotherm models, and the Langmuir isotherm (eq 2) was found to fit curve I with high r^2 (0.999) and low χ^2 (4.79×10^{-6}) values (**Figure 3**, **Table 2**). However, none of the above isotherm models fitted curve II. So a modified form of the Langmuir equation, the shifted-squared Langmuir model (SSLM) proposed for S-type curves by Grant et al. (*28*), was applied to curve II. The nonlinear expression of the SSLM is given below:

$$q_e = \frac{q_m b(C_e - C_s)^2}{1 + b(C_e - C_s)^2} \quad (5)$$

where C_s is the shifted concentration along the *x*-axis. Compared to all the other models, the SSLM provided a precise fit of curve II with a high correlation ($r^2 = 0.989$, $\chi^2 = 0.41$) of the experimental data (**Figure 3**, **Table 2**).

Discussion on Model Variables. The maximum binding capacity q_m values predicted by the Langmuir model (213.58 and 41.85 mg/g) were close to the experimental data (217.93 and 42.25 mg/g) for lead and cadmium at pH 5.5 (**Table 1**). These q_m values were higher than those reported for lead and cadmium binding at pH 7 by wheat bran dietary fibers, which amounted to 31.04−128.80 mg/g and 15.55−70.88 mg/g, respectively (*7*). However, a higher q_m value was reported by Sergushchenko et al. (*29*), which equaled to 492.90 and 328.80, 597.00 and 5.00, 521.00 and 57.80, 17.70 and 3.20, 10.80 and 4.00, and 6.00 and 3.50 mg/g for *in vitro* binding of lead and cadmium at pH 5−5.5, respectively, by therapeutic drugs such as sodium thiosulfate, unithiol, trilon B, penicillamine, activated carbon, and polyphepan. The binding constant *b* value for lead (0.187 L/mg) was almost the same as that for cadmium (0.186 L/mg), implying their identical binding stability with γ-PGA under single-solute conditions. However, the binding intensity *n* derived from the Freundlich isotherm was higher for lead (4.42) than for cadmium (3.77), which is consistent with a larger q_m value (213.58 mg/g) of the former. Also, the *n* value lying in the range of 1−10 denotes a favorable binding (*30*) by γ-PGA. For the lead isotherm at pH 2.5, the SSLM predicted a q_m value of 12.22 mg/g (experimental $q_m = 12.20$ mg/g), with a very low *b* value (0.002 L/mg), signifying a poor binding stability of lead by γ-PGA under the stomach pH conditions (**Table 2**).

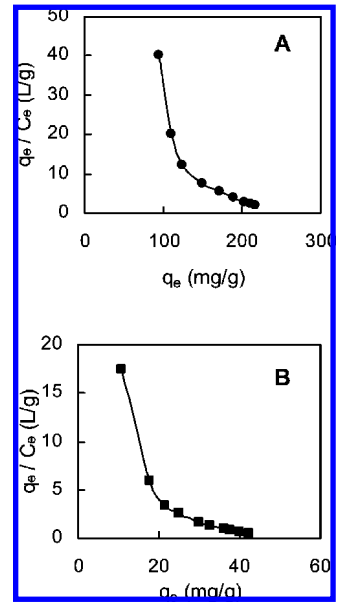

Figure 4. Scatchard plot isotherm analysis of lead (A) and cadmium (B) binding by γ-PGA at pH 5.5. The plot is according to the Scatchard equation $q_e/C_e = q_m K_b - q_e K_b$, where q_e (mg/g) is the amount of metal bound at equilibrium time, C_e is the metal concentration in solution at equilibrium, and q_m (mg/g) and K_b (L/mg) represent the maximum binding capacity and binding constant related to the affinity between γ-PGA and metal, respectively.

Binding Kinetics. Determination of the kinetics of metal adsorption is critical in elucidating the reactivity of γ-PGA and evaluating its application in the treatment of acute and/or chronic metal poisoning. The kinetics of metal binding monitored at four concentrations of lead (10, 25, 50, and 100 mg/L) or cadmium (5, 10, 25, and 50 mg/L) showed a rapid adsorption, with equilibrium binding of 25.00, 62.50, 108.35, and 169.55 mg/g for lead and 10.97, 17.46, 27.39, and 36.00 mg/g for cadmium being achieved within the same time of 2, 5, 8, and 12 min, respectively (**Figure 5**). About 82−100% and 70−99% of total adsorption was attained in 2 min for lead concentrations ranging from 10 to 100 mg/L and cadmium concentrations from 5 to 50 mg/L, respectively. This rapid adsorption behavior of γ-PGA is important for treatment of acute metal intoxication. To derive the rate constant and binding capacity, the kinetic data were modeled with two rate equations, pseudo-first-order and pseudo-second-order (*15*, *21*, *26*), which are expressed in their nonlinear forms as

$$q = q_e[1 - \exp(-k_1 t)] \quad (6)$$

$$q = \frac{t}{1/k_2 q_e^2 + t/q_e} \quad (7)$$

where k_1 (L/min) and k_2 (g/mg·min) are pseudo-first-order and pseudo-second-order rate constants, respectively. Fitted kinetic models are shown in **Figure 5**, and the model variables optimized by nonlinear regression are presented in **Table 3**. The pseudo-first-order model showed a better fit for lead at 10, 25, and 50 mg/L and cadmium at 5 and 10 mg/L. However, the binding kinetics of lead at 100 mg/L and cadmium at 25 and 50 mg/L were precisely described by the pseudo-second-order model. This outcome indicated that metal adsorption at low concentrations may involve a dominant ion exchange mechanism

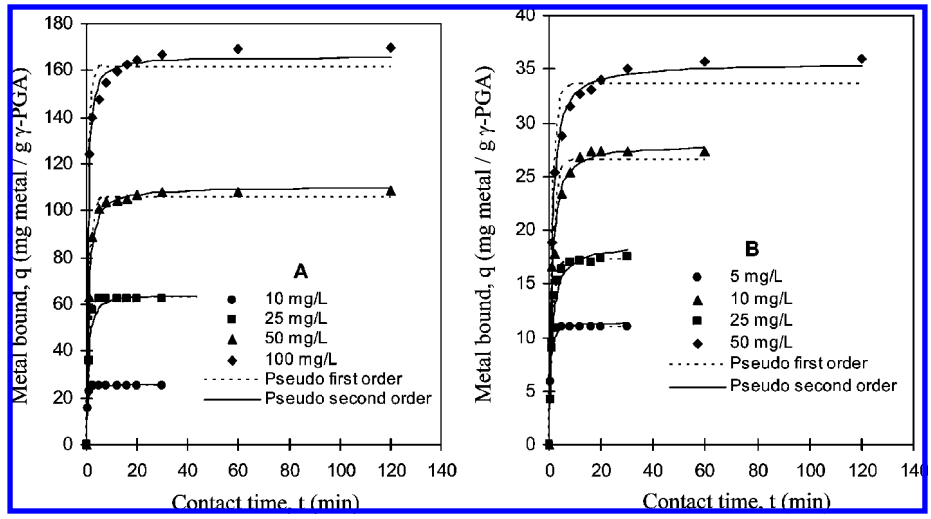

Figure 5. Kinetic curves for lead (A) and cadmium (B) binding by γ-PGA. Initial metal concentrations: 10, 25, 50, and 100 mg/L for lead, and 5, 10, 25, and 50 mg/L for cadmium; γ-PGA dose: 0.4 g/L; pH: 5.5; temperature: 37 °C; the kinetic equations employed for modeling are given inside the text as eqs 6 and 7.

Table 3. Kinetic Parameters for Lead and Cadmium Binding by γ-PGA

		pseudo-first-order model parameters[a]				pseudo-second-order model parameters[b]			
metal conc (mg/L)	experimental q_e (mg/g)	q_e (mg/g)	k_1 (L/min)	r^2	χ^2	q_e (mg/g)	k_2 (g/mg·min)	r^2	χ^2
Lead									
10	25.00	25.09	2.075	0.983	0.08	25.95	0.166	0.840	0.64
25	62.50	62.75	0.973	0.964	0.51	64.92	0.029	0.837	2.12
50	108.35	105.81	0.903	0.979	0.36	110.27	0.015	0.968	0.72
100	169.55	161.31	1.331	0.733	3.38	166.64	0.016	0.941	0.74
Cadmium									
5	10.97	11.01	1.750	0.973	0.09	11.46	0.286	0.836	0.46
10	17.46	17.20	0.726	0.991	0.29	18.72	0.054	0.950	1.11
25	27.39	26.52	0.716	0.853	1.32	28.18	0.041	0.954	0.37
50	36.00	33.56	0.729	0.872	1.11	35.59	0.031	0.984	0.15

[a] $q = q_e[1 - \exp(-k_1 t)]$, where q and q_e are the amounts of metal bound at time t and equilibrium, respectively, and k_1 is the pseudo-first-order rate constant. [b] $q = t/((1/k_2 q e^2) + (t/q_e))$, where k_2 is the pseudo-second-order rate constant.

Table 4. Modeled Parameters of the γ-PGA Dose−Activity Relationship for Lead and Cadmium Binding

	modeled parameters[a]		
metal conc (mg/L)	a	b	r^2
Lead			
25	2.03×10^{-4}	9.22×10^{-3}	0.989
50	7.01×10^{-4}	9.57×10^{-3}	0.997
100	1.65×10^{-3}	1.04×10^{-2}	0.993
Cadmium			
10	1.21×10^{-3}	1.15×10^{-2}	0.989
25	3.01×10^{-3}	1.31×10^{-2}	0.987
50	5.41×10^{-3}	1.67×10^{-2}	0.980

[a] Using the equation $R\ (\%) = m/(a + bm)$, where R = percentage of metal removal and m = γ-PGA dose (g/L).

through carboxyl groups, as the pseudo-first-order expression is the same as the ion exchange model proposed by Boyd (*15, 26*). Conversely, at higher metal concentrations, chemical forces through participation of amide groups may be predominantly involved, and hence, the kinetic data obeyed the pseudo-second-order model. Obviously, the pseudo-second-order model is applied to chemical reactions involving valence forces through sharing or exchange of electrons (*15, 21, 26*). Following a rise in metal concentration, the rate constants decreased, with lead being adsorbed at a much faster rate than cadmium. The faster rate of lead binding over cadmium may be ascribed to the same reasons as discussed before for the difference in their binding capacity.

γ-PGA Dose−Activity Relationship. Figure 6 depicts the γ-PGA dose−activity curves for lead and cadmium, respectively. Under the same γ-PGA dose, a higher percentage of lead removal was observed than that of cadmium, as shown by a steep curve for the former. Following a rise in γ-PGA dose from 0.04−0.3, 0.05−1.0, and 0.1−2.0 g/L for lead concentrations at 25, 50, and 100 mg/L, the percentage of lead removal increased by 30.6, 56.5, and 50.3, respectively. Similarly, for cadmium concentrations at 10, 25, and 50 mg/L, the percentage of removal rose by 49.1, 50.7, and 35.5, respectively, for a γ-PGA dose raised from 0.04−0.6, 0.05−1.6, and 0.1−2.0 g/L. Nevertheless, the amount of metal adsorbed per unit weight of γ-PGA declined following the increase of γ-PGA dose (shown as dotted lines). For example, with a rise of γ-PGA from 0.04 to 0.3 g/L, the amount of lead binding at 25 mg/L dropped from 433.75 to 83.33 mg/g. This reveals that the metal binding was not proportional to γ-PGA at high doses, which may be attributed to a lower binding capacity utilization of γ-PGA caused by its aggregation or agglomeration, which in turn may diminish the vicinity of binding sites. In addition, the particle interaction at high γ-PGA dose may result in desorption of some weakly bound metal ions from the γ-PGA surface. Since the binding response was more pronounced at low γ-PGA concen-

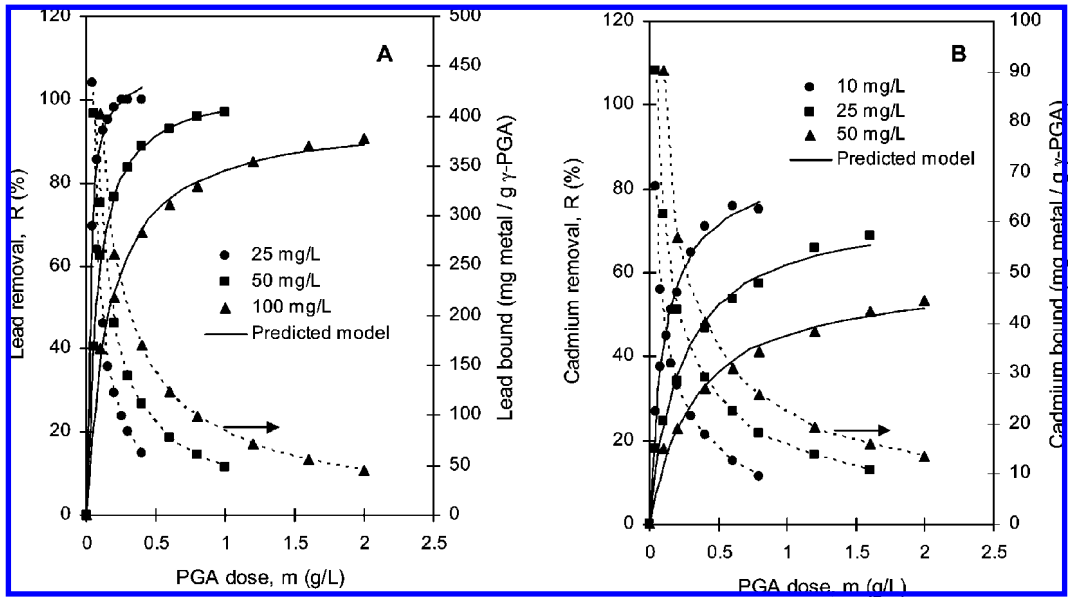

Figure 6. γ-PGA dose−activity relationship for binding of lead (A) and cadmium (B). Initial metal concentrations: 25, 50, and 100 mg/L for lead, and 10, 25, and 50 mg/L for cadmium; pH: 5.5; temperature: 37 °C; contact time: 2 h; curves with dotted lines denote the amount of metal bound in mg/g of γ-PGA; the predicted model is according to the equation $R = m/(a + bm)$, where R is the percentage of metal removal and m (g/L) is the γ-PGA dose.

Table 5. Effect of Essential Metals (Cu, Zn, Fe, Mg, K, and Ca) on Lead and Cadmium Binding by γ-PGA

	lead binding (mg/g) in a 1:1 mixture of lead−essential metal				cadmium binding (mg/g) in a 1:1 mixture of cadmium−essential metal			
essential metal	5 mg/L	25 mg/L	50 mg/L	100 mg/L	5 mg/L	25 mg/L	50 mg/L	100 mg/L
control	12.50	62.50	109.20	165.35	10.73	23.38	34.40	43.54
Cu[a]	12.50	51.33	81.10	142.78	7.11	3.32	1.18	0
Zn[a]	12.50	58.38	97.95	172.40	7.84	10.28	14.19	9.59
Fe[a]	12.50	58.03	98.03	186.70	7.50	10.04	13.25	1.55
Mg[a]	12.50	56.98	99.93	180.40	6.37	6.90	7.49	0
Ca[b]	12.50	58.03	95.28	149.55	5.46	6.80	3.96	0
K[b]	12.50	62.50	107.40	162.03	10.72	22.96	26.00	4.32

[a] As sulfate salt. [b] As chloride salt.

tration, small doses of γ-PGA should be efficient in the treatment of metal intoxication. The relationship between the percentage of metal removal (R, %) and γ-PGA dose (m, g/L) can be expressed in the form of a mathematical equation as given below:

$$R\,(\%) = \frac{m}{a + bm} \tag{8}$$

Fitting eq 8 with the experimental data showed a high r^2 value (0.980−0.997) for various concentrations of lead and cadmium, proving the validity of this equation, and the fitted variables (a and b) are presented in **Table 4**.

Selectivity Study—Effect of Essential Metals. The application of chelating agents in the treatment of metal poisoning may affect the bioavailability of some other essential metal ions such as Cu^{2+}, Zn^{2+}, Fe^{2+}, Mg^{2+}, Ca^{2+}, and K^+ (*4*, *7*−*9*). This adverse effect could be prevented by appropriate supplementation of these essential minerals. Nonetheless, the binding of essential metal ions may also affect the detoxifying effect of chelating agents on target heavy metals. Thus, the influence of essential metals on binding of lead or cadmium by γ-PGA was investigated as a bisolute system to evaluate the metal selectivity of γ-PGA.

Effect on Lead Binding. Among the six essential metals ions studied, Cu^{2+} ions showed a maximum effect in decreasing the binding of lead, while K^+ ions exhibited a low (≤2%) or nil effect (**Table 5**). At 5 mg/L, no interference occurred; however, when the concentration was raised to 25 and 50 mg/L, the lead binding capacity dropped by 17.9 and 25.7% for Cu^{2+}, 6.6 and 10.3% for Zn^{2+}, 7.2 and 10.2% for Fe^{2+}, 8.8 and 8.5% for Mg^{2+}, and 7.2 and 12.8% for Ca^{2+}, respectively. In contrast, compared to the control at 100 mg/L, a synergistic effect was shown for Zn^{2+}, Fe^{2+}, and Mg^{2+} ions, resulting in an increase in lead binding by 4.3, 12.9, and 9.1%, respectively, whereas both Cu^{2+} and Ca^{2+} ions reduced lead binding by 13.7 and 9.6%, with the effect being lower than that at 25 or 50 mg/L. The synergistic effect may be caused by conformational change or structural modification of γ-PGA in the presence of Zn^{2+}, Fe^{2+}, and Mg^{2+} ions at 100 mg/L. It is also possible that the extra mobility acquired by lead ions during competition may facilitate binding of additional lead ions onto remote sites in γ-PGA.

Effect on Cadmium Binding. The level of cadmium binding drastically decreased as the concentration of essential metals rose from 5 to 100 mg/L (**Table 5**). Potassium ions showed no interference in cadmium binding at 5 and 25 mg/L, but a significant reduction of 24.4 and 90.1% occurred at 50 and 100 mg/L, respectively. On the contrary, the other metals ions decreased cadmium binding at all concentrations, with Cu^{2+}, Ca^{2+}, and Mg^{2+} ions showing a greater influence than Zn^{2+} and Fe^{2+} ions. At a concentration level of 5, 25, and 50 mg/L, the cadmium binding dropped profoundly by 33.7, 85.8, and 96.6% for Cu^{2+}, 49.1, 70.9, and 88.5% for Ca^{2+}, and 40.6, 70.5, and 78.2% for Mg^{2+}, respectively, but declined to zero at 100 mg/L.

However, both Zn^{2+} and Fe^{2+} ions at 5, 25, 50, and 100 mg/L reduced cadmium binding by 26.9 and 30.1%, 56.0 and 57.1%, 58.8 and 61.5%, and 78.0 and 96.4%, respectively.

Comparatively, γ-PGA showed a higher selectivity toward lead than cadmium in the presence of essential metals, implying the greater stability of the $Pb^{2+}-\gamma$-PGA complex. A large interference by Cu^{2+} ions for both lead and cadmium binding may be due to its high electronegativity (1.90) compared to the other essential metals. Another contributing factor can be the tendency of copper (like lead) to form labile hydrolyzed species (*22*), which are more prone to binding by γ-PGA. Ou et al (*7*). also observed Cu^{2+} ions to be more effective than Zn^{2+}, Fe^{2+}, and Ca^{2+} in interfering with heavy metal binding (Pb^{2+}, Cd^{2+}, and Hg^{2+}) by wheat bran dietary fibers. On the other hand, the low interfering effect by K^+ ions on lead binding should be due to its low positive charge. The pronounced effect of Ca^{2+}, Mg^{2+}, K^+, Fe^{2+}, and Zn^{2+} on cadmium binding can be explained based on the HSAB principle (*24*). Accordingly, compared to cadmium (soft acid), the metal ions Ca^{2+}, Mg^{2+}, and K^+ being hard acids should more readily bind with the hard base like the carboxyl groups on γ-PGA. Similarly, Fe^{2+} and Zn^{2+}, both of which belong to borderline acids, can interact more easily with carboxyl groups. However, for lead binding, only <13% interference was shown by the essential metal ions except Cu^{2+}. Though Pb^{2+} ion is only a borderline acid, the interaction of lead with γ-PGA may be stronger enough to prevent any major interference by essential metals, because of its high electronegativity (2.33) and the tendency to form several hydrolyzed and polymeric species (*22*). The high selectivity of lead should be an advantage, as DMSA, a chelating agent currently used in lead detoxication treatment, was reported to induce essential metal deficiency (*4*).

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