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# Chelation of Copper(II) by Natural and Edible Biopolymer Poly(γ-glutamic acid) in Aqueous Solution

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**Abstract**

The naturally occurring edible biopolymer poly(γ-glutamic acid) (γ-pga) was shown to be efficient chelating agent of copper(II) ($Cu^{2+}$) ions. The complexation between $Cu^{2+}$ ions and γ-pga in aqueous solutions (pH 3–7) has been studied by uv-visible absorption and FTIR techniques. Formation of [Cu(γ-pga)] complex was confirmed by the observation of blue shift of the absorption band in the visible region and the changes in FTIR spectrum of solid complex. The equatorial coordination sphere of $Cu^{2+}$ ion is proposed to be [2×carboxylate and $H_2O$ (3O)–Cu–N (peptide)]. The effect of solution pH on the binding kinetics was studied. Kinetic data obtained from different batch experiments was modeled using both pseudo first- and second-order kinetic equations. The best results were achieved with the pseudo second-order kinetic model.

**Keywords:** Poly (γ-glutamic acid), copper(II) , chelation , binding kinetics

## Introduction

Naturally occurring biopolymers have attracted considerable interest from polymer researchers in recent year [1-4]. This interest arose as a result of an increased awareness of the environment and a desire to produce environmentally safe materials. Poly(γ-glutamic acid) (γ-pga) meets the demands of the times with respect to the new biomaterial industry that came up from well-being issues for a healthy life [5-7]. γ-pga naturally exists in the traditional Japanese foods, e.g., *natto* (fermented soybeans) [8,9]. It is produced by *Bacillus natto*, as a component of *natto* mucilage (sticky substance). Therefore, γ-pga is a promising biopolymer for use as a health food, a thickener, an osteoporosis-preventing factor, and a stabilizer in the food industry; as a moisturizer in cosmetics; as a hydrogel (especially super absorbent polymer, SAP) for environmental and agricultural; as a tissue engineering material, as a biodegradable packing material, and in many other possible applications including liquid crystal displays (LCDs) and conductive display material, gene vector, curative biological adhesive, dispersant, and enzyme-immobilizing material [10-21].

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The high proportion of carboxylic functions in γ-pga (Fig. 1) has been found to provide novel binding properties for metal ions, and thus a number of studies using this polymer for the removal of metal ions from wastewater have been conducted [22-25]. Recently, Tanimoto et al reported that γ-pga increases the calcium absorption in the small intestine. [26]. As a drug carrier, γ-pga has been used in the preparation of biomedical products, and their therapeutic activities have also been evaluated [27-28].

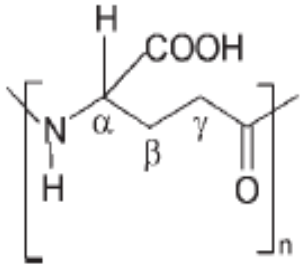


**Figure 1:** Chemical structure of γ-pga.

However, the kinetics and the binding constant of metal-γ-pga complex, and its medicinal activity have not yet been studied extensively. Recently, we found that oxovanadium(IV) ion ($VO^{2+}$) coordinated with γ-pga to form [VO(γ-pga)] complex in aqueous solution, which exhibited a potent antidiabetic activity for the treatment of not only streptozotocin (STZ)-induced diabetic mice—a type 1 diabetic model—but also type 2 diabetic $KKA^{y}$ mice when introduced by oral gavage [29-32].

We have also found that copper-poly(γ-glutamic acid) [Cu(γ-pga)] complex, in which copper(II) coordination is $Cu(O_4)$, showed potential *in vitro* insulin-mimetic activity [33]. The *in vitro* insulin-mimetic activity of [Cu(γ-pga)] complex was examined by determining both inhibition of free fatty acid release and glucose uptake in isolated rat adipocytes treated with epinephrine. This important finding prompted us to investigate the kinetics and the binding constant of [Cu(γ-pga)] complex in aqueous solution. For this reason, it seemed worthwhile to study the chelation reaction in aqueous solution at various pHs, initial concentrations of $CuSO_4$ and temperatures, which would give an insight into the kinetics and binding constant of [Cu(γ-pga)] complex.

In this paper, the [Cu(γ-pga)] complex was characterized by uv-visible and IR spectroscopic methods. The kinetics of [Cu(γ-pga)] complexation in aqueous solution was investigated by dialysis technique at various pHs. The concentration of copper in the complex was determined by atomic absorption spectroscopic method.

## Experimental

### Materials

The γ-pga having molecular weight $4.8\times10^5$ Da was used in this work without further purification. $CuSO_4\bullet5H_2O$ was purchased from Wako Pure Chemical Industries (Osaka, Japan). The dialysis membranes (Medicell international Ltd. London; size 46/81) were used with a molecular weight cut off not greater than 14000 Da. All other reagents were commercially available in the highest grade of purity and were used without further purification.

### Preparation of [Cu(γ-pga)] complex in aqueous solution.

The [Cu(γ-pga)] complex was prepared in aqueous solution at various pH by mixing $CuSO_4$ (1 M) with γ-pga (0.1–1% w/v) solutions . The final concentration of $CuSO_4$ was 0.1–10 mM and that of γ-pga was 0.1–1% w/v depending on the measurement conditions. The pH of the reaction mixtures was determined by using pH meter (410A Orion). The pH of the samples was adjusted by adding micro liter quantities of 1 M NaOH or 1 M HCl.

### Preparation of [Cu(γ-pga)] complex in solid state

[Cu(γ-pga)] complex was prepared by mixing excess amounts of $CuSO_4$ (1 M) and 10 mL of γ-pga (1% w/v) solutions under stirring at room temperature. A large amount of acetone was added to the remaining solution to complete precipitation of [Cu(γ-pga)] complex. The resulting precipitate was washed with distilled water and acetone, and dried on silica gel under a vacuum condition at room temperature.

### Preparation of Membrane

The dialysis membranes were prepared by immersing the membranes in hot water at 70°C. At every one hour interval the water was changed by fresh deionized distilled water for efficient removal of sulfur and other

soluble materials. These membranes were then stirred for an hour to cool and to bring to at room temperature. Stirring was continued with 70% (v/v) methanol for 30 minutes. The membranes were then stored overnight in 50% methanol.

**Binding Equilibrium**

The binding equilibrium of γ-pga with $CuSO_4$ was measured by a batch dialysis technique with the pH ranging from 3.0 to 7.0. The pH of the samples was adjusted either by adding micro liter quantities of 1 M HCl or 1 M NaOH. Aqueous solution of the polymer (0.1% w/v, 3 mL) in a dialysis membrane tube was placed in a 122 mL stoppered bottle containing 60 mL of $CuSO_4$ (1 mM) solution and shaken using a shaking thermostat machine at a speed of 120 rpm and at a constant room temperature (30 ± 0.2 °C). The sample was withdrawn at desired time intervals. The amount of $Cu^{2+}$ ions bound was determined from the difference in copper concentration in aqueous sample before and after treatment with γ-pga. The copper concentration in the solution was measured by atomic absorption spectrometer (AA-7000, Shimadzu Corporation, Japan) equipped with auto sampler ASC-7000. The concentration of copper was calculated using a calibration curve at a concentration range of 5–100 ppm for the standard copper solutions (Wako Pure Chemical Industries Ltd., Osaka, Japan). The correlation coefficient of linear regression was r = 0.999 for a total of four metal concentrations.

The amount of $Cu^{2+}$ ions bound at time *t*, $q_t$ (mmol/g) was determined by

$$q_t = V(C_0 - C_t)/m \quad (1)$$

where $C_0$ and $C_t$ (mmol/L) are the concentrations of copper before and after treatment with γ-pga at any time *t*, respectively; *V* is the volume of the $CuSO_4$ solution (L) and *m* is the amount of γ-pga used (g).

**Results and Discussion**

**Characterization of [Cu(γ-pga)] complex by uv-visible spectroscopy in aqueous solution**

$CuSO_4$ showed an absorption maximum in the visible range at 780 nm (Fig. 2). The absorption maximum of the [Cu(γ-pga)] complex in aqueous

solution (pH 6) was observed in the visible range around 720 nm when the complex was prepared by mixing $CuSO_4$ (1 M) and γ-pga (1% w/v) solutions (final concentration of $CuSO_4$: 1 mM and γ-pga: 1% w/v). However, free γ-pga absorbed only in the UV region. Thus the blue shift (60 nm) of the absorption band around 720 nm in the visible region must be attributed to the transition of electrons correlated with the complex formation. This is typical of the replacement of water molecules by higher field ligands such as carboxyl or amide groups in the coordination sphere of copper.

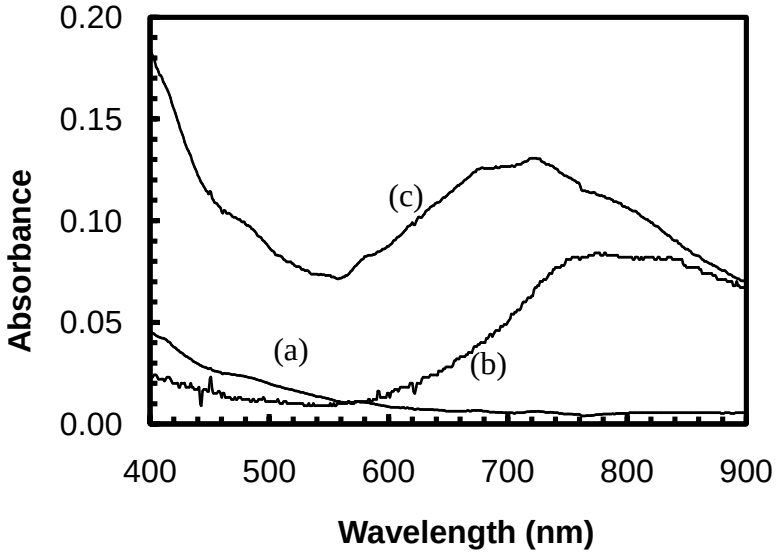


**Figure 2:** Visible absorption spectra of (a) 1% w/v γ-pga; (b) 1 mM $CuSO_4$ and (c) [Cu(γ-pga)] complex in aqueous solution at pH 6.

## Characterization of [Cu(γ-pga)] complex by FTIR spectroscopy

The FTIR spectrum of γ-pga (Fig. 3) exhibited the following absorption bands: the strong N–H stretching band at 3295 $cm^{-1}$, the strong stretching vibration band due to the C=O in the COOH group at 1734 $cm^{-1}$, the bands at the region 1593 $cm^{-1}$ for amide I (C=O stretch) and 1568 $cm^{-1}$ for amide II (N–H bend). On the other hand, the FTIR spectrum of solid [Cu(γ-pga)] complex prepared at pH 6 showed no band at 1734 $cm^{-1}$ (Fig. 4) for the

C=O vibration of the carboxylic group indicating that the COOH group has been deprotonated and coordinated to the metal center. The strong absorption bands of the asymmetrical and symmetrical valency vibrations due to the $COO^-$ group were not clearly observed at around 1600 and 1400 $cm^{-1}$, respectively. However, the absorption bands were observed at around 1660 and 1402 $cm^{-1}$, respectively. The relatively large value of $\Delta[\upsilon_{as}(COO^-) - \upsilon_s(COO^-)]$ = 258 $cm^{-1}$ was indicative of monodentate carboxylate coordination to Cu(II).[29] Similar phenomena was observed in the case of [VO(γ-pga)] complex in which the carboxylic groups of γ-pga were coordinated to the vanadyl ion ($VO^{2+}$).[29] Moreover, a new band appeared at around 470 $cm^{-1}$ which is due to Cu–N bond stretching. These results suggest that the both carboxylate oxygen and peptide nitrogen atoms of γ-pga have been bound to copper ion ($Cu^{2+}$). The equatorial coordination sphere of $Cu^{2+}$ ion is proposed to be [2×carboxylate and $H_2O$ (3O)–Cu–N (peptide)].

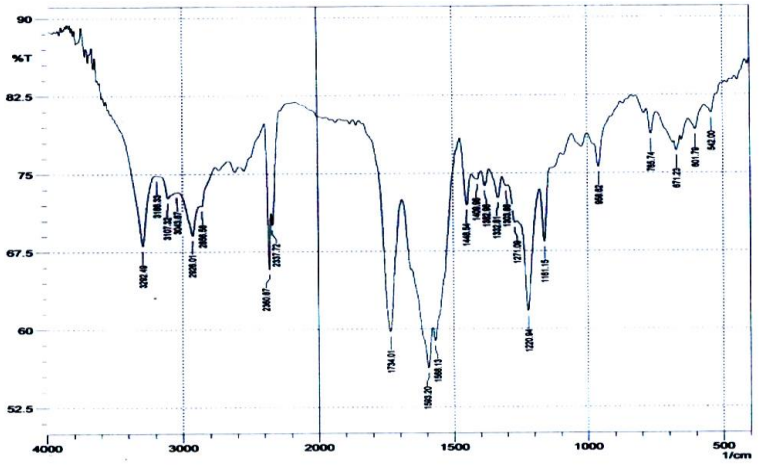


**Figure 3:** FTIR spectrum γ-pga in KBr

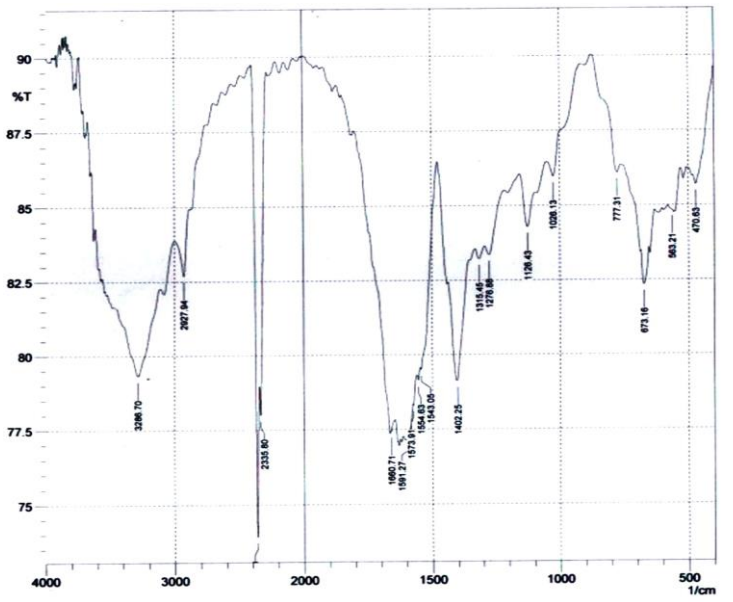


**Figure 4:** FTIR spectrum [Cu(γ-pga)] complex in KBr.

## Kinetics of binding equilibrium

To evaluate the kinetics of binding equilibrium, the following assumptions were made by taking experimental conditions into account: (1) there is no interaction between free metal ions and the membrane; (2) permeation of the polymer and polymer-metal complex are completely rejected, and the complex concentration is in equilibrium with the concentration of free metal ion which is going to permeate through the membrane; and (3) amount of metal hydroxide is neglected.

The effect of pH on the kinetics of $Cu^{2+}$ ions binding by γ-pga at 30 $^{o}$C is shown in Fig. 5a, where the initial concentration of $CuSO_4$ was 1 mM. The rate of uptake of $Cu^{2+}$ ions on the adsorbent material indicated that about 420 min was taken to reach the equilibrium time for all pHs. However, the data was taken for 500 min to make sure that complete equilibrium was established. Before the equilibrium time, it indicates that the initial rate of binding ($dq/dt$) increases significantly with increasing solution pH (Fig. 5b(i)). It can be seen that the pH of aqueous solution plays an important role on the rate of $Cu^{2+}$ ions binding onto γ-pga and

the suitable pH is 6.0 among the observed pH ranging from 3.0 to 7.0 (Figure 5b(ii)).

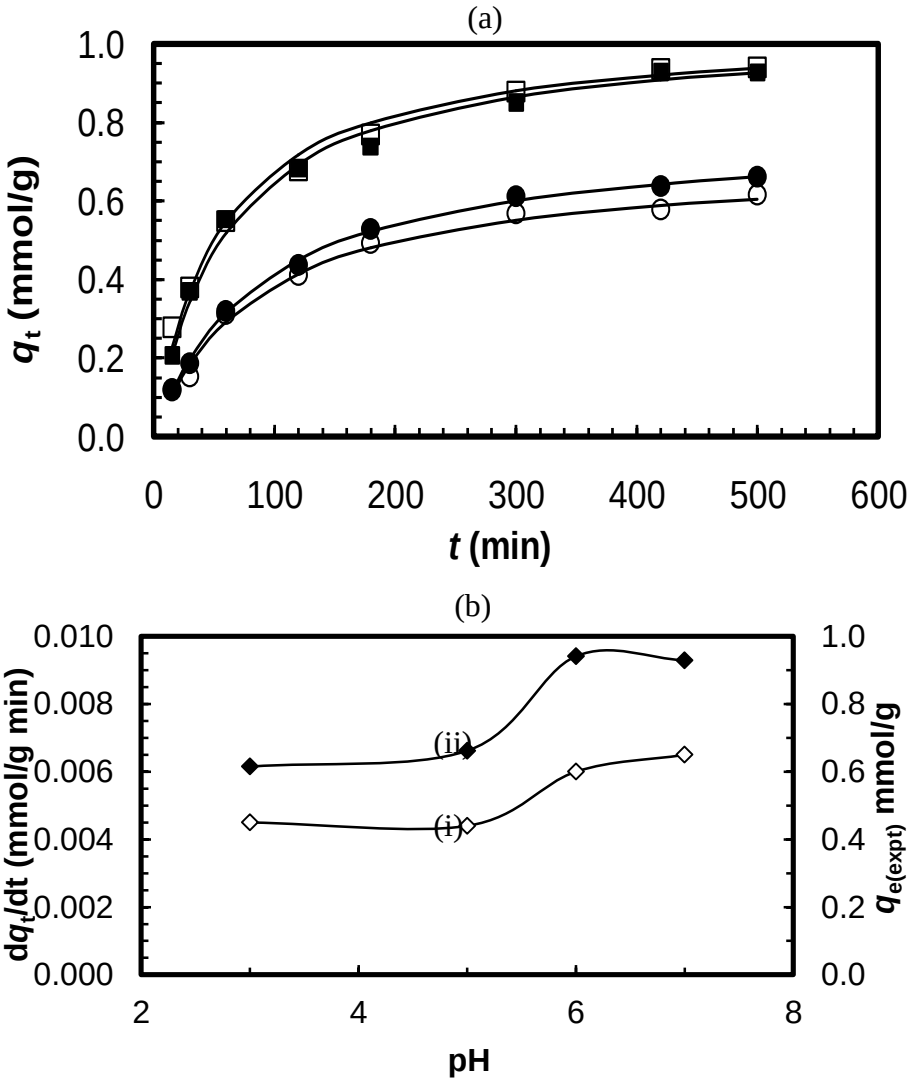


**Figure 5:** (a) Binding kinetics of $Cu^{2+}$ ions onto γ-pga at different pHs opened circles: pH 3; closed circles: pH 5; opened squares: pH 6; closed squares: pH 7). All solid lines are simulated binding kinetics of $Cu^{2+}$ ions onto γ-pga at respective

pHs. (b) The changes of (i) initial binding rate ($dq_t/dt$) and (ii) equilibrium binding capacity ($q_{e(exp)}$) with pH.

In order to investigate the mechanism of binding kinetics, the pseudo first-order and pseudo second-order equations were used to test the experimental data. In 1898, S. Lagergren[34] expressed the pseudo first-order rate expression for the liquid-solid adsorption system and the most popular linear form is expressed as follows:

$$\log(q_e - q_t) = \log q_e - (k_1/2.303)t \quad (2)$$

where $q_e$(mmol/g) and $q_t$ (mmol/g) are the amounts of $Cu^{2+}$ ions bound onto γ-pga at equilibrium and at any time $t$, respectively, and $k_1$ (1/min) is the rate constant of pseudo first-order binding. A straight line of $\log(q_e - q_t)$ versus $t$ suggests the applicability of this kinetic model to fit the experimental data. The equilibrium binding capacity ($q_e$) is required to fit the data, but in many cases $q_e$ remains unknown due to slow binding processes. Also, in many cases, the pseudo first-order equation does not fit well to the whole range of contact time and is generally applicable over the initial stage of the adsorption processes.[34,35]

The pseudo second-order kinetic model is expressed as:[35,36]

$$q_t = k_2 q_e^2 t/(1+ k_2 q_e t) \quad (3)$$

where $k_2$ (g/mmol min) is the rate constant of pseudo second-order reaction and can be determined from a linearized form of this equation, represented by Eq. (4):

$$t/q_t = 1/k_2 q_e^2 + (1/q_e)t \quad (4)$$

If second-order kinetics is applicable, the plot of $t/q_t$ versus $t$ should show a linear relationship. There is no need to know any parameter before hand and the equilibrium binding capacity ($q_e$) can be calculated from Eq. (4). Contrary to the other model, it predicts the behavior over the whole range of binding and is in agreement with a binding mechanism being the rate-controlling step,[34,35] which may involve interactions between $Cu^{2+}$ ions and γ-pga.

The slopes and $y$-intercepts of plots of $\log(q_e - q_t)$ versus $t$ were used to determine the pseudo first-order rate constant ($k_1$) and equilibrium

binding capacity ($q_e$) (figure not shown). These results are shown in Table 1. A comparison of results with the correlation coefficients ($R^2$) is also shown in Table 1. The values of $R^2$ for the pseudo first-order kinetics model were low. Also, the calculated $q_{e(cal)}$ values obtained from the pseudo first-order kinetic model do not give reasonable values, which are low compared with experimental $q_{e(exp)}$ values (Table 1). These results suggest that the binding kinetics of $Cu^{2+}$ ions onto γ-pga is not a pseudo first-order process.

The slopes and *y*-intercepts of plots of $t/q_t$ versus $t$ were used to calculate the pseudo second-order rate constant ($k_2$) and $q_e$, respectively. The straight lines in plot of $t/q_t$ versus $t$ showed a good agreement of experimental data with the pseudo second-order kinetic model for various pHs (Fig. 6).

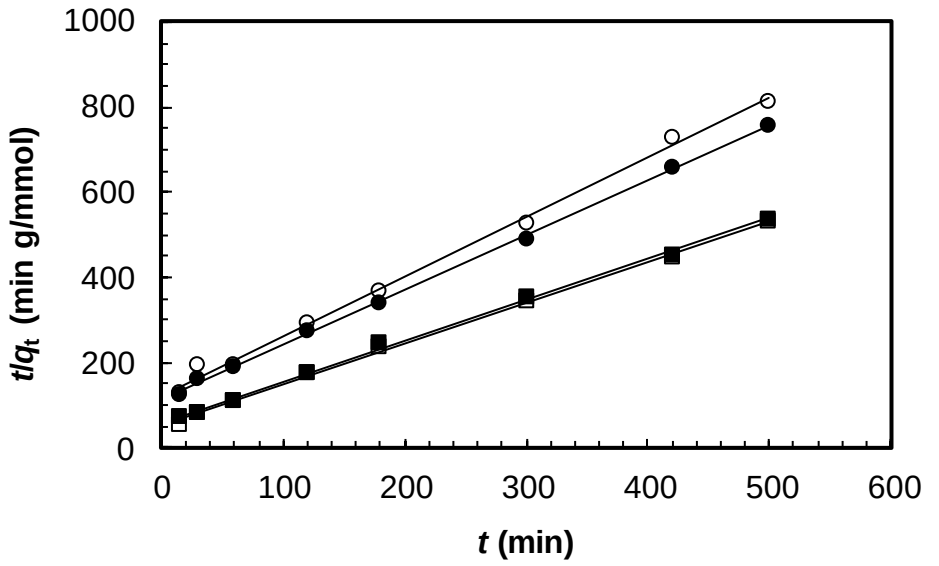


**Figure 6:** Plot of the pseudo second-order model ($t/q_t$ versus $t$) at different pHs (opened circles: pH 3; closed circles: pH 5; opened squares: pH 6; closed squares: pH 7).

**Table 1:** Comparison of the pseudo first- and second-order rate constants, and calculated and experimental $q_e$ values at various pHs

| pH | $q_{e(exp)}$ (mmol/g) | First-order kinetic model | | | Second-order kinetic model | | |
|---|---|---|---|---|---|---|---|
| | | $k_1$ (1/min) | $q_{e(cal)}$ (mmol/g) | $R^2$ | $k_2$ (g/mmol min) | $q_{e(cal)}$ (mmol/g) | $R^2$ |
| 3 | 0.62 | 0.0088 | 0.53 | 0.986 | 0.0165 | 0.71 | 0.996 |
| 5 | 0.66 | 0.0088 | 0.58 | 0.942 | 0.0146 | 0.78 | 0.999 |
| 6 | 0.94 | 0.0099 | 0.72 | 0.937 | 0.0179 | 1.04 | 0.998 |
| 7 | 0.93 | 0.0097 | 0.74 | 0.957 | 0.0160 | 1.04 | 0.998 |

Moreover, the experimental binding kinetic profiles (Fig. 5a) are perfectly reproduced in the simulated data (each solid line in Fig. 5a) obtained from numerical analysis on the basis of pseudo second-order kinetic model (Eq. 3) using the values of $k_2$ and $q_{e(cal)}$ listed in Table 1. These results confirm that the studied binding kinetics belongs to the pseudo second-order kinetic model.

## Conclusion

Formation of [Cu(γ-pga)] complex was confirmed by the observation of blue shift of the absorption band in the visible region and the changes in FTIR spectrum of solid complex. The equatorial coordination sphere of $Cu^{2+}$ ion is proposed to be [2×carboxylate and $H_2O$ (3O)–Cu–N (peptide)]. The pH of aqueous solution plays an important role on the rate of $Cu^{2+}$ ions binding onto γ-pga and the suitable pH is 6.0 among the observed pH ranging from 3.0 to 7.0. Kinetic data obtained from different batch experiments was modeled using both pseudo first- and second-order kinetic equations. The best results were achieved with the pseudo second-order kinetic model.

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