Original article:

Cytochrome c adsorption in a continuous flow system by using Cu(II)-chelated magnetic affinity particles

Recep AKKAYA[1], Birnur AKKAYA[2]

Abstract:

Background and methodology: In the current study magnetic poly(ethylene glycol dimethacrylate-N-methacryloyl-(L)-histidine methyl ester) poly(Egdma–Mah)) was used as a metal chelated affinity particles. Cu^{2+} ions loaded directly to MAH ligand of magnetic particles for the adsorption of cytochrome c (Cyt c) in a continuous flow system. ***Results:*** The maximum Cyt c adsorption capacity on the magnetic particles and Cu^{2+}-chelated magnetic particles were 42 mg/g and 197 mg/g in phosphate buffer (pH 8.0), respectively. Cu^{2+} loading increased the Cyt c adsorption capacity, significantly. Cyt c adsorption capacity decreased with increased temperature and with increasing magnetic field. According to reusability studies Cyt c molecules could be reversibly adsorbed and desorbed five times. The binding isotherm was determined by scatchard analysis followed by application of Hill equation to the data obtained, then binding constant and n_H Hill coefficient were calculated.

Keywords: Adsorption; Biophysics; chromatography; cytochrome c; hill plots; magnetic particles.

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Introduction

The links between molecular biophysics and biochemistry and human diseases have been the subject of considerable scientific research effort for a number of decades. Among the affinity chromatography methods immobilized metal affinity chromatography (Imac) is a great one for seperation of biomacromolecules[1–4]. It is widely used for seperation of biomolecules based on analytical, laboratory and pilot-scale [5-7]. First-row transition metal ions (Zn^{2+}, Ni^{2+}, Cu^{2+} and Fe^{3+}) make chelation with compound containing O, N and S. Imac prove an useful technique for seperation of biomolecules by using affinities of materials which contain N, O, S as an electron donor atoms to chelated first row transition metal ions as a electron pair acceptor molecule [8-10]. Proteins interact by using their effective functional groups such as the imidazol group, the indoyl group and thiol group, respectively. Low cost of metals and reusability performans of metal chelated adsorbents are the advantages of Imac[11]. Metal ions, support, length of spacer arm, ligand concentration, pH and buffer type affect the adsorption of proteins[12]. Because of the magnetic character of the beads, sampling and collecting the adsorbed protein are easier and faster, but when magnetic field is removed, their magnetization will be dissappear. The conventional fluidized bed systems and chromatographic seperation and chromatographic seperation have got some problems. Magnetic beads can solve these problems because magnetic adsorbents provide low pressure drop, high feed-stream solid tolerance, absence of particle mixing, high mass transfer rates and good fluid-solid contact[13-14].

In the present study, Mah was used as a immobilized ligand for cupper chelating for adsorption of Cyt c because of its imidazole group which interact with Cu^{2+} ions. Then, Cu^{2+} ions were immobilized on the magnetic particles. Then Cyt c adsorption on to the metal-coupled affinity particles from aqueous solution in a continuous flow system were performed under different concentrations of Cyt c, at different pH, magnetic field and flow rates. Desorption of Cyt c and reusability of the mag poly(Egdma–Mah)

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particles were also tested. The binding isotherm was determined by scatchard initially and followed by application of hill equation to the data obtained, then binding constant and hill coefficient (n_H) were estimated.

Materials and Methods

Materials

L-histidine methyl ester and methacryloyl chloride, poly(vinyl alcohol) (MW: 100.000, 98% hydrolyzed), Fe_3O_4, (diameter <1 μm) and protein, cytochrome c from horse heart, were purchased from Sigma-Aldrich (USA). Egdma and benzoyl peroxide was obtained from Fluka A.G. (Switzerland).

Preparation of Mah

Mah preparation was summarized in elsewhere[15]. The brief experimental procedure was given as follows: L-histidine (5.0 g) hydrochloride and hydroquinone (0.2 g) were dissolved in 100 ml of dichloromethane solution and it was cooled down to 0 °C. Triethylamine (12.7 g) was poured in to this solution. Methacryloyl chloride (5.0 ml) was mixed slowly with this solution which was stirred magnetically at RT during 2 h. Unreacted methacryloyl chloride and hydroquinone were extracted by 10% NaOH solution. A rotary evaporator was used for aqueous phase evaporation. For Mah crystallization ether–cyclohexane mixture was used and then MAH crystals were dissolved in ethyl alcohol.

Synthesis of magnetic poly(Egdma–Mah) particles

Preparation of magnetic particles was described details in previous article[11]. It could be summarized as follows: 200 mg of poly(vinyl alcohol) as a stabilizer was dissolved in 50 ml deionized water for the preparation of the continuous phase. For preparation of dispersion phase, 8.0 ml of Egdma, 1.0 g of Mah, 1.0 g of Fe_3O_4 and 12.0 ml of toluene were mixed in a beaker. Then 100 mg of benzoyl peroxide as a initiator was aded. The dispersion phase was added to the continuous medium. The polymerizaton was carried at 65 °C for 4 h and at 90 °C for 2 h by stirring at 600 rpm. At the end of polymerization, the reactor content was cooled at room temperature. The particles were washed after polymerization.

Continuous flow system studies

Experiments of the magnetic particles for Cyt c was performed in a continuous flow system. The magnetic particles were stirred with Cyt c solution during 2 h by magnetic field application. To investigate concentration effect of Cyt c on adsorption capacity, concentrations between 0.5–2.5 mg/ml were used. For determination of maximum pH on Cyt c adsorption capacity following buffers were used: phosphate- buffer (pH 6.0-8.0) and carbonate buffer (pH 9, 10). Effect of flow rate was investigated with in the range of 1-4.5 mL/min. Effect of magnetic field was investigated under magnetic field of 0-6-13-20 mT.

Reusability

Reusability experiments of magnetic metal-chelated particles were carried in a 2.0 M NaCl containing buffer solution under continuous flow system by stirring (at a stirring rate of 150 rpm) during 1 h at RT. The Cyt c concentration in desorption medium was determined by spectrophotometry for every repeated cylcle. For checking leakage of Cu^{2+} ions, the amount of Cu^{2+} ions in desorption medium were determined by an atomic absorption spectrometry.

Ethical clearence: The sudy does not need ethical clearence.

Results and discussion

Time effect on adsorption capacity

Fig. 1 shows the contact time curves which were obtained by decreasing the concentration of Cyt c within the protein solution by increasing contact time. Because of high driving force, adsorption rate increased with the increasing Cyt c concentration. Because of high Cyt c concentration the beginning of adsorption process a fast adsorption process occurs and then reaches equilibrium in 120 min due to low Cyt c concentration.

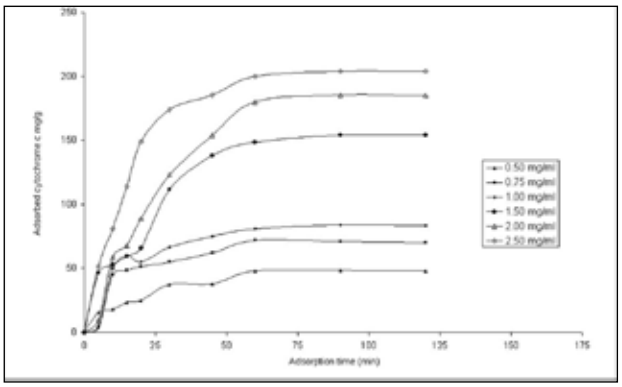

Fig.1. Effect of contact time on adsorption capacity.

pH studies

Adsorbtion of Cyt c on the magnetic particles and the Cu^{2+}-chelated magnetic particles as a function of pH was given in Fig. 2. The figure shows maximum

absorption peak at pH 8.0 (phosphate buffer). At the pH 8.0 value there was electrostatic interactions and coordination complex formation between cytochrome c and chelated-Cu^{2+} ions may result both from the ionization states of several groups on amino acid side chains in cytochrome c structure, and from the more folded structure of Cyt c molecules at this pH [11].

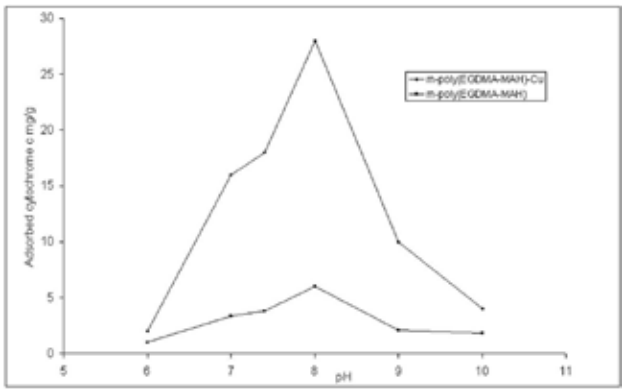

Fig.2. pH effect on adsorption capacity.

Adsorption isotherms

Figure 3 gives linear increasing which represents a specific affinity between Cyt c (histidine residiue) and Cu^{2+}-complexed groups at low concentrations.

Above 1.5 mg/mL increase less rapidly and achieves saturation value. It becomes constant at 2.0 mg/mL Cyt c concentration. There was also adsorption between Cyt c and plain m-poly(Egdma-Mah) because of nonspecific interactions.

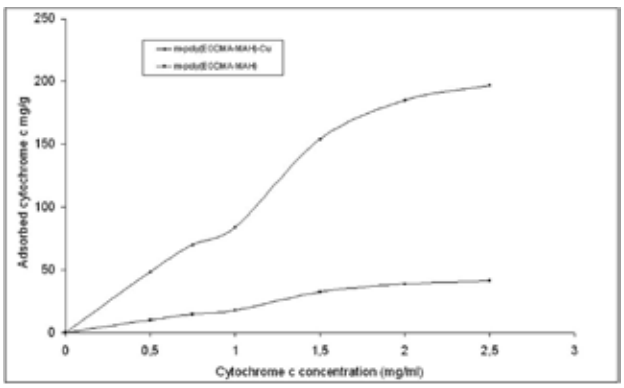

Fig.3. Cyt c concentration effect on adsorption capacity.

As seen from fig. 4(b) scatchard plot shows a positive cooperativity[16]. Noteworthy, cooperativity stuffes are calculated by using the hill plot. The slope of these plot give the hill coefficient (n_H) which shows the degree of cooperativity (Figure 5). In the present

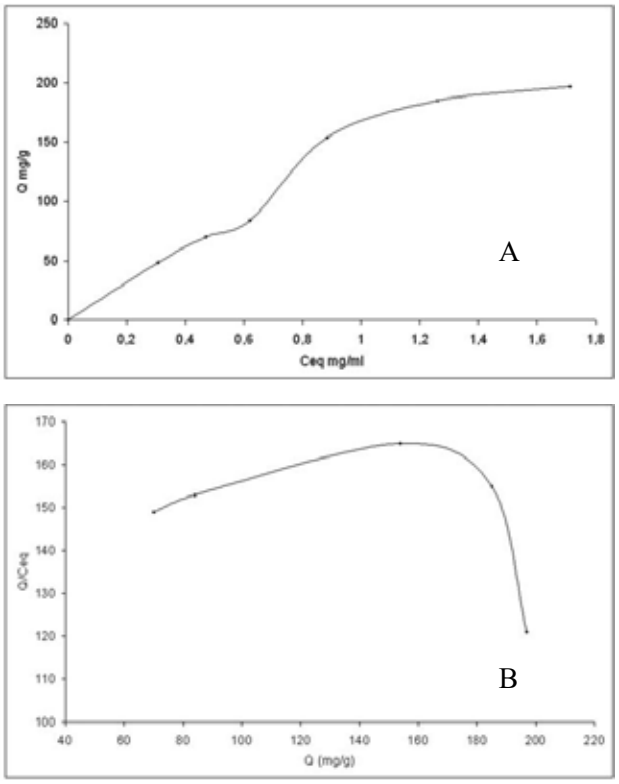

Fig.4. (a) Hypothetical binding plot (b) Scatchard plot

study association constant and intrinsic association constant were found 2.25 10^4 M^{-1} and 1.8 × 10^4 M^{-1}, respectively[17].

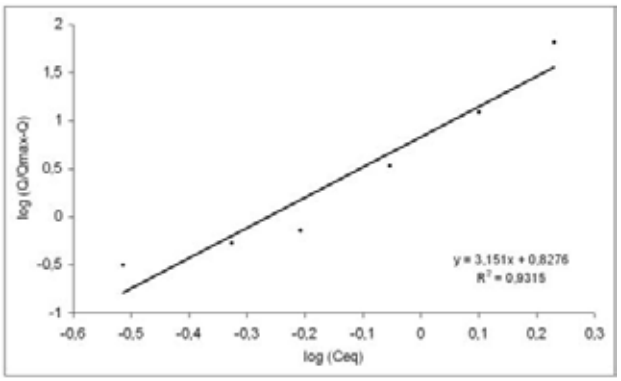

Fig. 5. Hill Plot. n_H= 3 and K= 0,55 (4.44 10^{-5} M)

Magnetic Field effect on adsorption capacity

Figure 6 shows effect of magnetic field on Cyt c adsorption capacity and increasing magnetic field caused to decrease of adsorbed Cyt c. Increasing the magnetic field lieds to strong agglomeration of magnetic particles and makes fluidization of particles more difficult[18].

Flow rate effect on adsorption capacity

Cyt c adsorption capacity of m-poly(Egdma-Mah)-Cu^{2+} particles at different flow rates (Figure 7). The

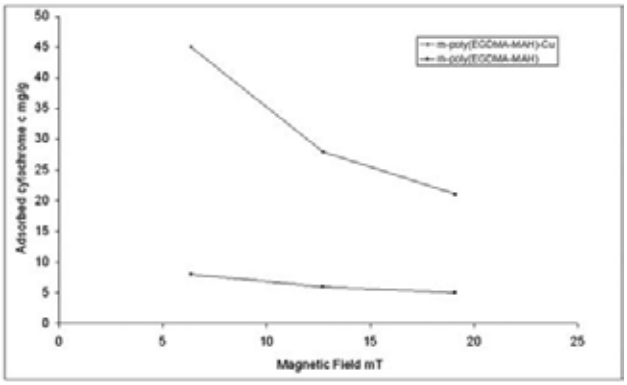

Fig.6. Magnetic field effect on adsorption capacity.

adsorption capacity decreased from 90 mg/g to 23 mg/g with increase of flow rate from 1 mL/min to 4.5mL/min. When the flow rate is decreased, better adsorption capacity is achieved because of the longer contact time in the column, Cyt c has more time to interact with chelated Cu^{2+} ions.

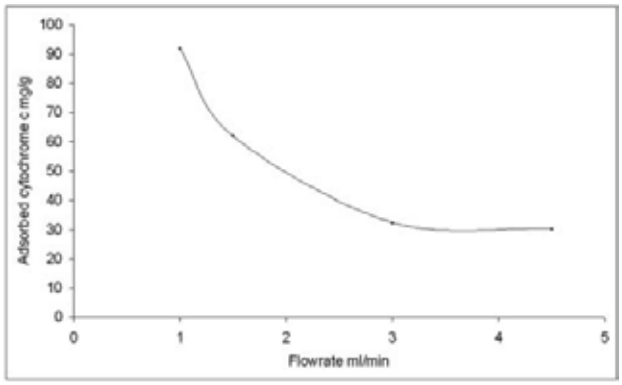

Fig.7. Flow rate effect on adsorbtion capacity.

Regeneration of the particles

Regeneration of the adsorbed Cyt c must be done in the shortest time and the highest amount of adsorbed Cyt c must be desorbed. Thus the regeneration efficiency of the magnetic particles are important. In the current study, more than 95% of the adsorbed Cyt c molecules were regenerated easily from the Cu^{2+} chelated mag-poly(Egdma–Mah) particles only in 30 min. There was not significant lost in the adsorption capacity of the magnetic particles (Fig.8).

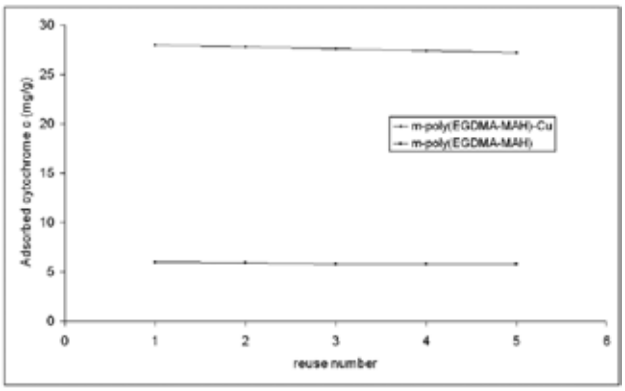

Fig.8. Reusability of magnetic particles.

Conclusions

In this study m-poly(Egdma-Mah) was produced and metal-chelating ligand, Mah, and the active functional group, L-histidin is incorporated in the polymeric structure. This material has magnetic behaviour and it is used as an adsorbent to investigate Cyt c adsorption capacity in continuous flow system. Cyt c adsorption studies were investigated by other researchers[19-21]. Thus adsorption studies were performed succesfully by using the affinity of m-poly(Egdma-Mah)-Cu^{2+} particles toward Cyt c and desorption studies was also performed under MSFB system. In this study scatchard analysis was researched and positive cooperation was found. Cyt c, an electron carrier mitocondirial protein, localizes in intermembrane space and has been identified as molecule that trigger apoptosis . That is why Cyt c has an important role in apoptosis, cancer and tumor[22-25]. This paper provides an overview of the rapidly expanding, nascent field of research that deals with the biomechanics and biophysics of cancer cells

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Authors's contribution: B.A and R.A developed the idea and designed the study. R.A gathered the data and performed the experiments. B.A analyzed the results. R.A wrote and submitted the manuscript, B.A and R.A edited and approved of final draft.

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