

Fe₃O₄ magnetic core coated by silver and functionalized with *N*-acetyl cysteine as novel nanoparticles in ferritin adsorption

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Abstract A novel metal-chelate affinity matrix utilizing *N*-acetyl cysteine as a metal chelating agent was synthesized. For this, magnetic Fe₃O₄ core was coated with silver by chemical reduction. Then, these magnetic silver nanoparticles were covered with *N*-acetyl cysteine, and Fe³⁺ was chelated to this modified magnetic silver nanoparticle. These magnetic nanoparticles were characterized by SEM, AFM, EDX, and ESR analysis. Synthesized nanoparticles were spherical and average size is found to be 69 nm. Fe³⁺ chelated magnetic silver nanoparticles were used for the adsorption of ferritin from its aqueous solution. Optimum conditions for the ferritin adsorption experiments were performed at pH 6.0 phosphate buffer and 25 °C of medium temperature and the maximum ferritin adsorption capacity is found to be 89.57 mg/g

nanoparticle. Ferritin adsorption onto magnetic silver nanoparticles was increased with increasing ferritin concentration while adsorption capacity was decreased with increasing ionic strength. Affinity of the magnetic silver nanoparticles to the ferritin molecule was shown with SPR analysis. It was also observed that the adsorption capacity of the magnetic silver nanoparticles was not significantly changed after the five adsorption/desorption cycles.

Keywords IMAC · Magnetic silver nanoparticles · Ferritin · *N*-acetyl cysteine

Introduction

In electron transfer systems within living organisms, one of the major metals is iron and it is essential for life. Although its physiologic importance, physico-chemical properties of iron make it difficult to use for aerobic organisms. At physiologic pH, iron molecules are collapsed and precipitated in aqueous solutions. Moreover, it generates reactive oxygen species by Fenton reaction and these species cause lipid peroxidation, protein oxidation, DNA mutations, and even cell damage (Briat et al. 1999). So that iron molecules in the metabolism must be kept down and iron homeostasis must be balanced. The major vehicle for iron balance in intracellular medium is ferritin. These proteins hold about 4500 iron atoms in the mineral form of ferrihydrite. Ferritin comprises 24 protein

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subunits of two different types, called as H and L (Orino et al. 2001). Ferritin is an almost spherical protein and its iron carrying core diameter is about 8 nm (Arosio and Levi 2002).

Recently, there has been intensive work for preparation of nanosized particles in different sizes and shapes. Because of the unique properties of nanoparticles, they have been wide used in various biotechnological applications for instance sensors and separation applications. At the same time, magnetic nanoparticles have also attracted great attention due to its various applications such as immobilization of proteins and enzymes, diagnostic, therapeutics, bio-separations, immunoassays, drug delivery, biosensors, magnetic resonance imaging, and so on (Mandal et al. 2005; Bao et al. 2007; Uygun et al. 2012a, b). One of the commonly used magnetic materials is Fe_3O_4 . Because of its good mechanic and magnetic properties, Fe_3O_4 magnetic materials are intensively used in biotechnology and medicine fields. So far, surface of the magnetic nanoparticles have been functionalized by coating with different metal ions such as Ag, Au or specific functional group ($-\text{NH}_2$, $-\text{SH}$) bearing molecules (XianXiang et al. 2009).

Immobilized metal-chelate affinity chromatography (IMAC) is one of the widely used chromatographic techniques for protein separations and purifications. In this technique, transition metal ions are used because they can form stable complexes with electron rich molecules carrying compounds. Electron rich molecules such as oxygen, nitrogen, and sulfur are interacting with metal ions via ion-dipole interactions (Özkara et al. 2003). Widely used metal ions for these purposes are first-row transition metals such as Zn^{2+} , Ni^{2+} , Cu^{2+} , and Fe^{3+} (Akgöl and Denizli 2004). In IMAC, separation and purification of proteins depend on their chemical affinity toward the metal ions which are bounded to solid stationary matrix (Riberio et al. 2008). Proteins interact with metal ions mainly through the imidazole group of histidine and, to a lesser extent, the indoyl group of tryptophan and the thiol group of cysteine. Aromatic side chains and the amino terminals of the protein also contribute this interaction. Metal ions used in IMAC are very cheap and these metal ligands are highly stable and have good capacity and selectivity properties over proteins. Also using of IMAC in protein separation and purification is very simple and IMAC supports can be reused hundreds of times without any detectable

adsorption capacity lost for proteins. Because of the above-mentioned features, IMAC may be preferable for purification of proteins (Akgöl et al. 2008; Erzengin et al. 2011; Uygun et al. 2011; 2012a; Baydemir et al. 2013).

Several techniques are used for characterization of the macromolecular interaction. One of the attractive methods used recently for this purposes is surface plasmon resonance (SPR) technique. SPR is an optical technique that uses the evanescent wave phenomenon to measure changes in refractive index very close to a sensor surface (McDonnell 2001). In SPR method, one component (affinity ligand) is immobilized on surface of the SPR chip, and interaction of this component with second one (target) is monitored. There are several advantages of this method: quantitative and simultaneous detection of binding, high sensitivity, easy for use, capability of determining binding, and dissociation rate constants and reusability (Diltemiz et al. 2008; Dongyun et al. 2003).

In this study, *N*-acetyl cysteine attached magnetic silver nanoparticles were synthesized to use in IMAC for ferritin. Magnetic core of the nanoparticles was synthesized by reaction of Fe^{2+} and Fe^{3+} in alkali conditions. Then, this magnetic core was coated by silver and functionalized with *N*-acetyl cysteine. Finally, Fe^{3+} ions were chelated on these magnetic silver nanoparticles. *N*-acetyl cysteine attached magnetic silver nanoparticles were characterized by scanning electron microscopy (SEM), atomic force microscopy (AFM), energy dispersive X-ray (EDX), and electron spin resonance (ESR). Then, *N*-acetyl cysteine attached magnetic silver nanoparticles were used for adsorption of ferritin from aqueous solutions with different conditions (ferritin amount, pH, ionic strength, and temperature). Affinity of the Fe^{3+} -chelated *N*-acetyl cysteine attached magnetic silver nanoparticles to the ferritin was demonstrated with SPR analysis. Desorption of ferritin and reusability of this *N*-acetyl cysteine attached magnetic silver nanoparticles were also investigated.

Materials and methods

Materials

Ferritin (from horse spleen), *N*-acetyl cysteine, silver nitrate, and ammoniac were supplied by Sigma

(Steinheim, Germany); iron(II)chloride, iron(III)chloride, potassium hydrogen phosphate, and sodium chloride were obtained from Merck (Darmstadt, Germany); acetic acid and sodium acetate were purchased from Riedel-de Haën (Seelze, Germany); All other chemicals were reagent grade and acquired from Aldrich (Milwaukee, USA). All solutions were prepared with deionized ultrapure Millipore Simplicity (18.2 MΩ cm) water.

Preparation of *N*-acetyl cysteine attached magnetic silver nanoparticles

Magnetic silver nanoparticles were synthesized using chemical reduction method. For this, 0.054 g of Fe(III)Cl₃·6H₂O was dissolved in 5.0 mL of distilled water and 0.00198 g of Fe(II)Cl₂·4H₂O was dissolved in 5.0 mL of distilled water. These solutions were mixed and then 1.0 mL of ammonia solution (26 %) was added to this final solution and mixed for 1 h. Prepared magnetic particles (Fe₃O₄) were separated magnetically and washed three times with distilled water (Gupta and Gupta 2005). In order to coat the magnetic particles, freshly prepared Fe₃O₄ magnetic particles were dispersed in 5.0 mL of water and mixed with 0.00426 g of AgNO₃ and reduced with 10 mg of sodium borohydride. Synthesized brownish magnetic silver nanoparticles were separated using a magnet and washed with distilled water (Xu et al. 2007). *N*-acetyl cysteine functionalized magnetic silver nanoparticles were synthesized as follows. Freshly prepared magnetic silver nanoparticles were dispersed in 5.0 mL of water and 0.02045 g of *N*-acetyl cysteine was added to this solution. After reduction with sodium borohydride (10 mg), solution was mixed with 30 min and separated magnetically and washed three times with distilled water. Schematic presentation of preparation of *N*-acetyl cysteine attached magnetic silver nanoparticles was demonstrated in Fig. 1.

Preparation of Fe³⁺-chelated *N*-acetyl cysteine attached magnetic silver nanoparticles

Chelation of Fe³⁺ ions to *N*-acetyl cysteine attached magnetic silver nanoparticles were described as follows: 0.6 g of *N*-acetyl cysteine attached magnetic silver nanoparticles were mixed with 100 mL of aqueous solution (at pH 5.0) containing 2.5×10^{-4} M Fe³⁺ for 24 h. Figure 1 also shows the

chelation of Fe³⁺ onto *N*-acetyl cysteine attached magnetic silver nanoparticles. Magnetic silver nanoparticles were separated magnetically and final Fe³⁺ solution was stored for ICP-OES (Inductively coupled plasma optical emission spectroscopy, Teledyne Leemans Labs, Prism, USA) analysis. The amount of chelated Fe³⁺ ions was calculated using the concentrations of Fe³⁺ ions in the initial solution and in the equilibrium. Fe³⁺ concentration was determined with ICP-OES using Fe³⁺ standard solution (1–4 ppm).

Characterization of Fe³⁺-chelated *N*-acetyl cysteine attached magnetic silver nanoparticles

Particle size, shape and the surface morphology of the magnetic silver nanoparticles were analyzed by SEM and AFM. For SEM analysis, magnetic silver nanoparticles were dried and SEM photographs were taken by a scanning electron microscope (Phillips, XL-30S FEG, the Netherland). For AFM analysis, dispersion of magnetic silver nanoparticles was covered onto a glass plate (1.0 × 1.0 cm). Dried magnetic silver nanoparticles was mounted in an AFM (Digital Instruments, MMSPM Nanoscope IV, USA) and scanned with tapping mode. *N*-acetyl cysteine attachment to magnetic silver nanoparticles was determined by EDX analysis instrument (LEO, EVO40, Carl Zeiss NTS, USA) and amount of attached *N*-acetyl cysteine was calculated from these data considering the sulfur stoichiometry. Magnetism of the magnetic silver nanoparticles was investigated with an electron spin resonance (ESR) spectrophotometer (Varian, EL 9, USA). For this, the sample was dried in a vacuum oven and then ESR spectrum of the sample was measured with the magnetic field of 1000–5000 G at room temperature.

Investigation of adsorption and desorption conditions of ferritin onto Fe³⁺-chelated *N*-acetyl cysteine attached magnetic silver nanoparticles

Ferritin adsorption onto synthesized Fe³⁺-chelated *N*-acetyl cysteine attached magnetic silver nanoparticles was studied batchwise. For this, magnetic silver nanoparticles were mixed with aqueous solutions of ferritin and magnetically stirred (100 rpm) for 2 h. Adsorbed ferritin onto magnetic silver nanoparticles was removed magnetically and final ferritin concentration of remaining solution was determined using UV–Vis spectrophotometer (Shimadzu, 1601, Japan)

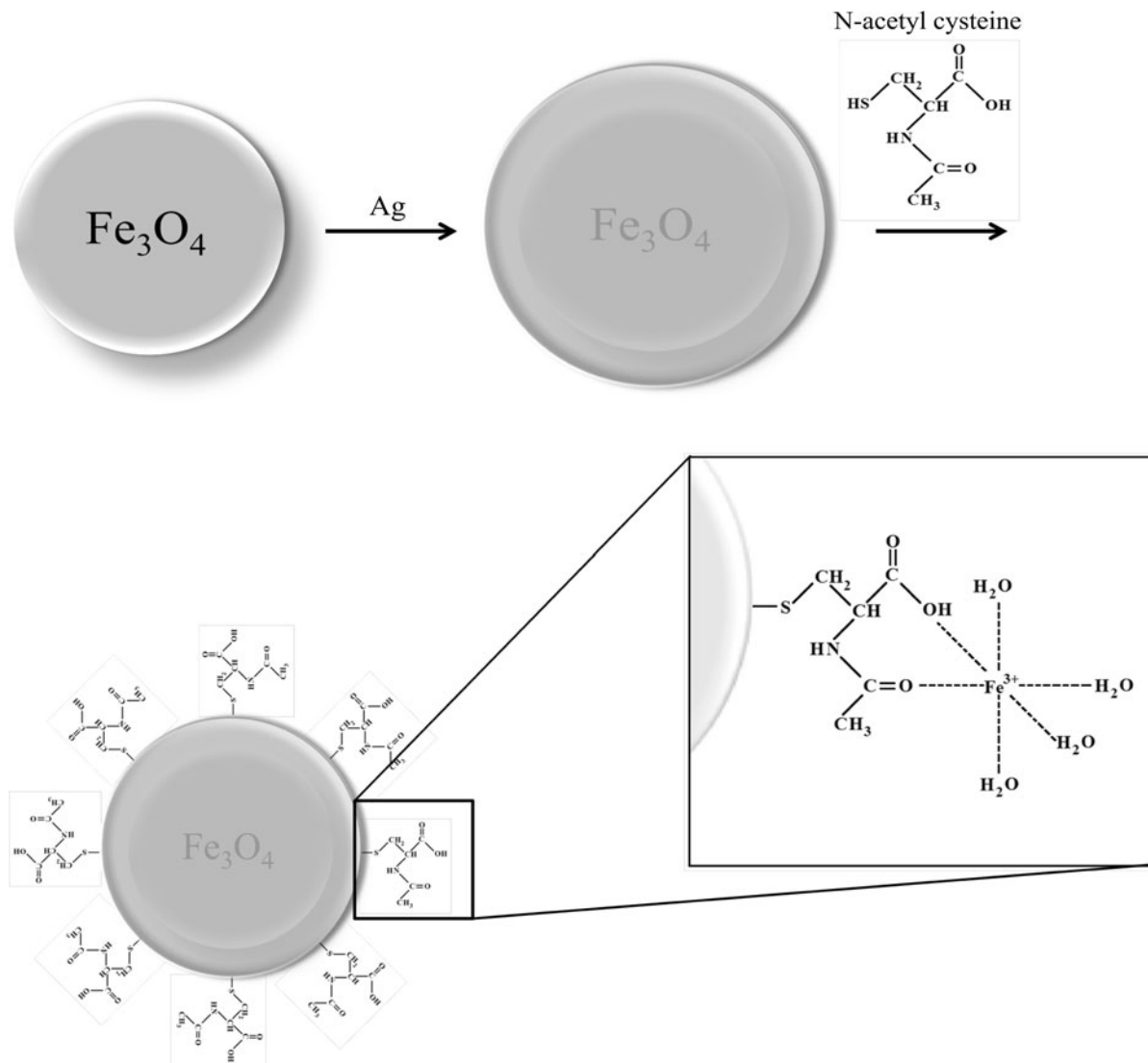


Fig. 1 Preparation and Fe^{3+} chelation of *N*-acetyl cysteine attached magnetic silver nanoparticles

at 280 nm. The amount of adsorbed ferritin was calculated as

$$Q = (C_0 - C)V/m$$

here, Q is the ferritin amount adsorbed on a unit mass of the magnetic silver nanoparticles (mg/g); C_0 and C are the ferritin concentration in the initial and final solutions, respectively (mg/mL); V is the volume of the ferritin solution (mL); and m is the mass of the magnetic silver nanoparticles.

In order to investigate the effect of medium pH on ferritin adsorption, adsorption experiments were carried out with different pH values. For this, acetate

(pH 4.0–5.0, 0.1 M) and phosphate (pH 6.0–8.0, 0.1 M) buffers were used. Initial ferritin concentration was changed between 0.01 and 0.15 mg/mL to determine the effect of ferritin concentration on adsorption. Ferritin adsorption experiments were carried out with different ionic strength (0–1.0 M, NaCl) to determine the effect of ionic strength on ferritin adsorption. Medium temperature was changed between 4 and 45 °C to show the effect of temperature on adsorption.

To examine the reusability of the magnetic silver nanoparticles, ferritin adsorption/desorption cycle was repeated for five times. Ferritin was desorbed from the

magnetic silver nanoparticles with 1.5 M of NaCl solution. Desorbed ferritin amount was determined spectrophotometrically at 280 nm. Desorption percentage was calculated as

$$\text{Desorption \%} = (\text{Desorbed ferritin} / \text{Adsorbed ferritin}) \times 100$$

After desorption, Fe^{3+} -chelated *N*-acetyl cysteine attached magnetic silver nanoparticles were reused for adsorption of ferritin. For this, magnetic silver nanoparticles were washed three times with water and equilibrate with appropriate buffer for next adsorption step.

SPR analysis

In order to show the affinity of the synthesized nanoparticles to the ferritin molecules, SPR analysis were carried out. For this, gold-coated SPR chips (GWC Technologies, SPR-1000-050) were coated with a layer of Fe^{3+} -chelated *N*-acetyl cysteine attached magnetic silver nanoparticles and then dried at 40 °C. The SPR chip was mounted in the SPR apparatus (GWC Technologies, SPR Imager II, USA) and washed with pH 6.0 phosphate buffer for 1 min. Then ferritin solution was injected to the chip and SPR intensity of the ferritin solution was read.

Result and discussion

Characterization of Fe^{3+} -chelated *N*-acetyl cysteine attached magnetic silver nanoparticles

Surface characteristics and shape of Fe^{3+} -chelated *N*-acetyl cysteine attached magnetic silver nanoparticles were investigated by SEM. As clearly seen in Fig. 2, synthesized silver nanoparticles are in spherical form and are in close contact with each other and agglomerated. Shape and the size of the prepared silver nanoparticles were also determined using AFM. Figure 3 demonstrates the AFM photographs of Fe^{3+} -chelated *N*-acetyl cysteine attached magnetic silver nanoparticles. The AFM illustrated in Fig. 3 shows that synthesized nanoparticles were spherical and average particle diameter was found to be 69 nm. EDX analysis of selected region of Fe^{3+} -chelated *N*-acetyl cysteine attached magnetic silver nanoparticles

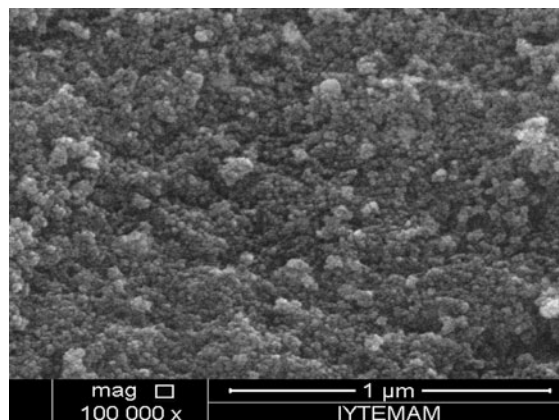


Fig. 2 SEM image of Fe^{3+} -chelated *N*-acetyl cysteine attached magnetic silver nanoparticles

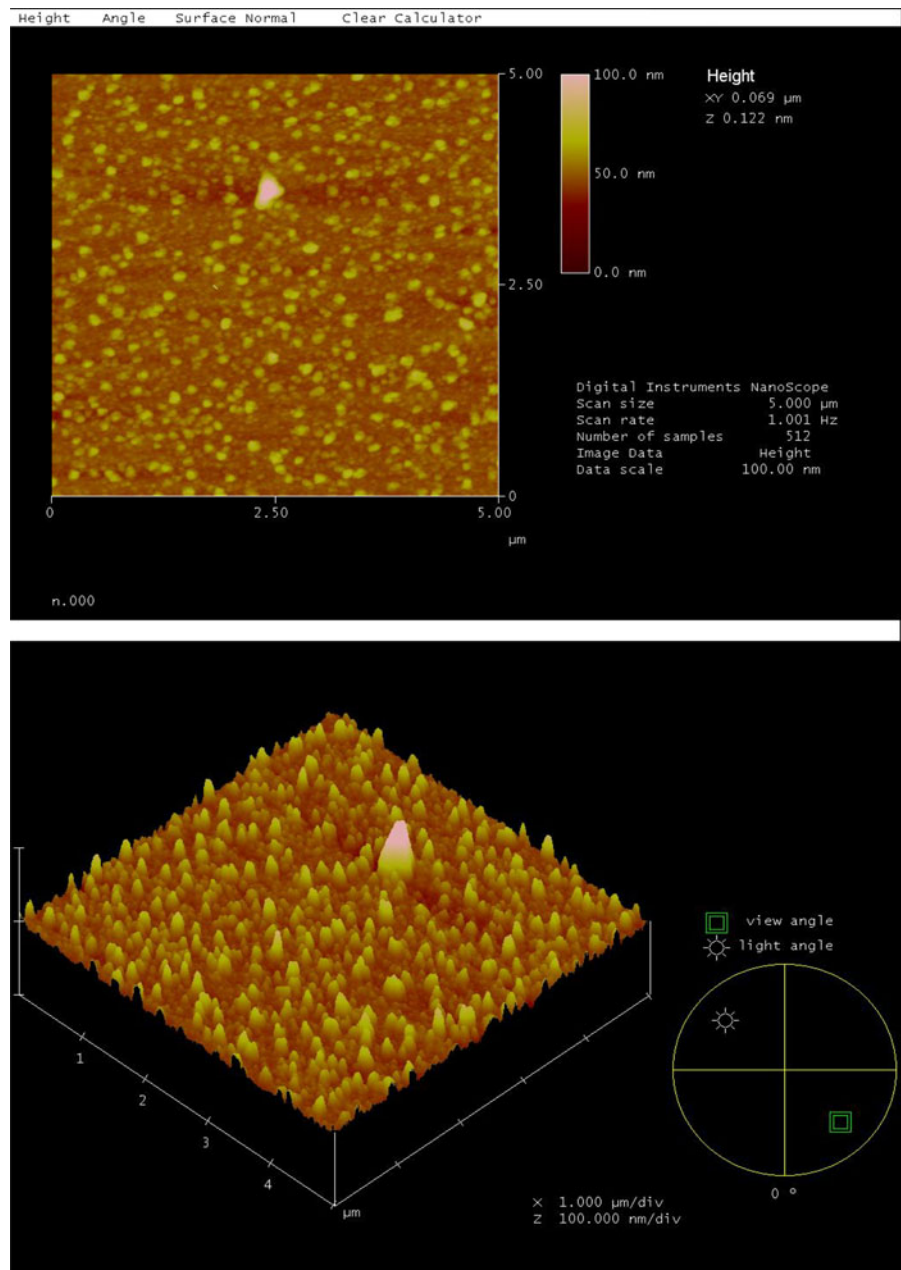
gave its chemical composition. Figure 4 shows the EDX analysis of magnetic silver nanoparticles. As seen here, prepared nanoparticles were composed of Fe, O, Ag, N, C, and S atoms and their mass percent was calculated as 55.95, 28.50, 8.78, 4.08, 1.54, and 1.16; respectively. Degree of *N*-acetyl cysteine incorporation onto silver nanoparticles was investigated with EDX analysis and attached *N*-acetyl cysteine amount was found to be 362.5 $\mu\text{mol/g}$ nanoparticle using sulfur stoichiometry. The magnetic behavior of Fe^{3+} -chelated *N*-acetyl cysteine attached magnetic silver nanoparticles was confirmed by ESR. Figure 5 shows the intensity of the magnetite peak against magnetic field. Magnetic quantity of the unpaired electrons carrying molecules were identified with *g* factor and it is calculated as

$$g = h\nu / \beta H_r$$

here, *h* is Planck's constant (6.626×10^{-27} erg/s); ν is the frequency ($9,707 \times 10^9$ Hz); β is the universal constant (9.274×10^{-21} erg/G), and H_r is the resonance of magnetic field (G). The *g* factor is also used for the identification of the origin of the signal. The *g* factor for the low and high spin complex of Fe^{3+} is determined in the range of 1.4–3.1 and 2.0–9.7, respectively (Swartz et al. 1972; Akgöl and Denizli 2004). The H_r value of the Fe^{3+} -chelated *N*-acetyl cysteine attached magnetic silver nanoparticles is 2,995 Gauss and the *g* factor for the Fe^{3+} -chelated *N*-acetyl cysteine attached magnetic silver nanoparticles was calculated to be 2.32 using the above equation.

The amount of chelated Fe^{3+} on the *N*-acetyl cysteine attached magnetic silver nanoparticles was

Fig. 3 AFM photographs of Fe^{3+} -chelated *N*-acetyl cysteine attached magnetic silver nanoparticles



measured as 41.7 μmol/g nanoparticle using ICP-OES.

Investigation of adsorption efficiency of ferritin

The effect of medium pH on the adsorption of ferritin onto Fe^{3+} -chelated *N*-acetyl cysteine attached magnetic silver nanoparticles was investigated in the acetate and phosphate buffers which pH's were varied

between 4.0 and 8.0, and the effect of pH over the ferritin adsorption is illustrated in Fig. 6. As seen in figure, maximum ferritin adsorption was observed with pH 6.0 phosphate buffer (82.75 mg/g nanoparticle). Above and below this pH values, ferritin adsorption capacity decreased. Isoelectric point of ferritin was reported to be 5.5 and at this pH value ferritin protein have no net charge (Chikkadi et al. 2011). In IMAC, protein adsorption is mainly based on

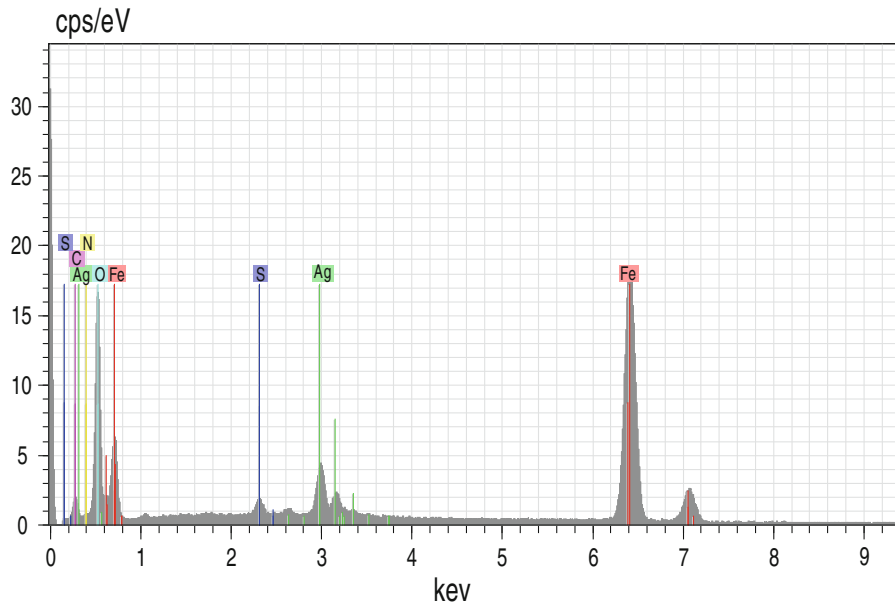


Fig. 4 EDX spectrum of Fe³⁺-chelated *N*-acetyl cysteine attached magnetic silver nanoparticles

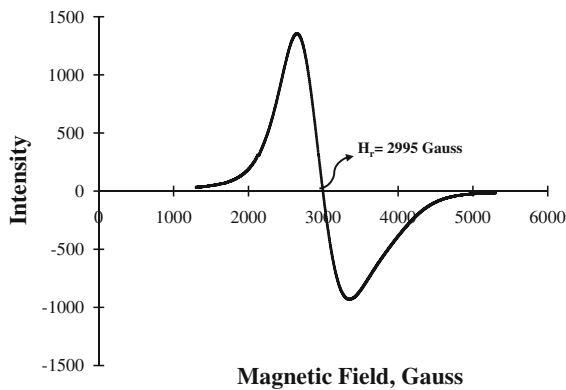


Fig. 5 ESR spectrum of Fe³⁺-chelated *N*-acetyl cysteine attached magnetic silver nanoparticles

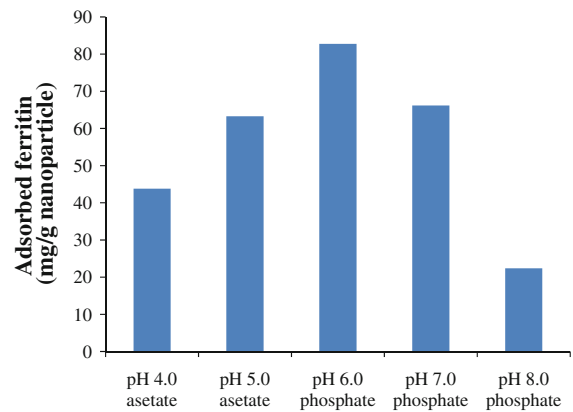


Fig. 6 Effect of pH on ferritin adsorption. Fe³⁺ loading: 41.7 μmol/g nanoparticle; initial ferritin concentration: 0.05 mg/mL; temperature: 25 °C

chelating bonding between metal ions and amino acid residues of protein. Above the isoelectric point, some amino acid residues of proteins are tending to be ionized and therefore at this pH values, protein adsorption onto IMAC matrixes was induced (Wong et al. 1991; Erzengin et al. 2011). pKa values of histidine residue are in the range of 6–7 and above isoelectric point of the ferritin histidine residues are in the ionized form. Therefore, maximum ferritin adsorption was observed over the isoelectric point of ferritin.

The effect of equilibrium ferritin concentration on the ferritin adsorption capacity onto Fe³⁺-chelated

N-acetyl cysteine attached magnetic silver nanoparticles illustrated in Fig. 7. As seen in figure, the ferritin adsorption capacity increased with increasing ferritin concentration and at 0.05 mg/mL of ferritin concentration adsorption capacity was saturated. It means that, all the active ferritin adsorption sites of the Fe³⁺-chelated *N*-acetyl cysteine attached magnetic silver nanoparticles bonded to ferritin molecules. Maximum ferritin adsorption was found to be 89.57 mg/g nanoparticle at 0.05 mg/mL concentration of ferritin.

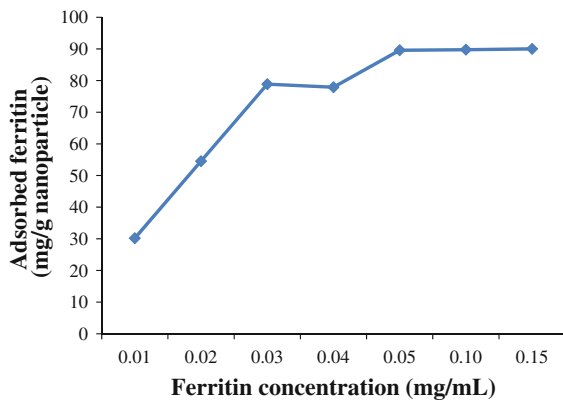


Fig. 7 Effect of ferritin concentration on ferritin adsorption. Fe^{3+} loading: 41.7 $\mu\text{mol/g}$ nanoparticle; pH: 6.0; temperature: 25 $^{\circ}\text{C}$

Table 1 Kinetic constants of Langmuir and Freundlich isotherms

Langmuir adsorption isotherm	Freundlich adsorption isotherm
$q_{max} = 96.93 \text{ mg/g}$	$K_F = 210.86 \text{ mg/g}$
$b = 162.52 \text{ mL/mg}$	$1/n = 0.322$
$R^2 = 0.994$	$R^2 = 0.7181$

$p < 0.05$ ($n = 3$)

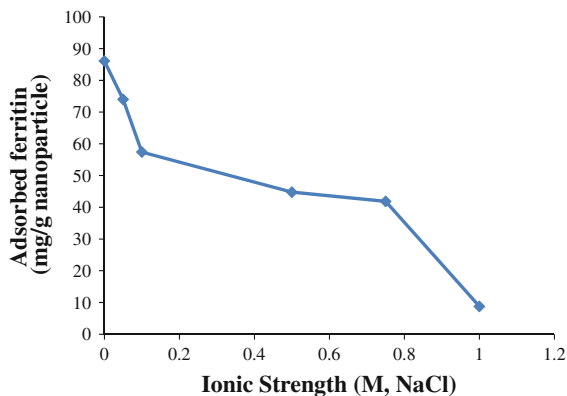


Fig. 8 Effect of ionic strength on ferritin adsorption. Fe^{3+} loading: 41.7 $\mu\text{mol/g}$ nanoparticle; initial ferritin concentration: 0.05 mg/mL; pH: 6.0; temperature: 25 $^{\circ}\text{C}$

Adsorption equilibriums are the important physicochemical aspects for evaluation of the adsorption process. Langmuir and Freundlich isotherms are the most commonly used equilibrium models (Zhu and Alexandratos 2005; Uygun et al. 2012c). The Langmuir model is based on the assumption of surface

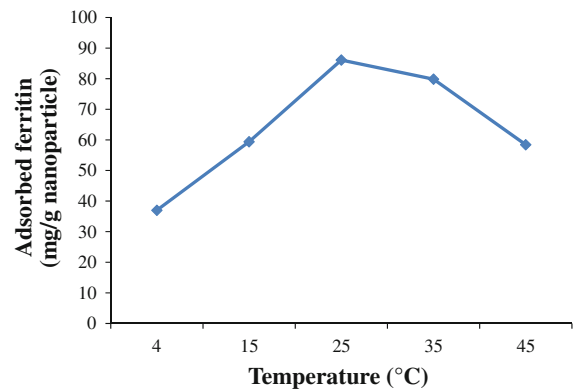


Fig. 9 Effect of temperature on ferritin adsorption. Fe^{3+} loading: 41.7 $\mu\text{mol/g}$ nanoparticle; initial ferritin concentration: 0.05 mg/mL; pH: 6.0

homogeneity such as equally available adsorption sites, monolayer surface coverage and no interaction between adsorbed species. The Freundlich isotherm is applicable to heterogeneous systems and reversible adsorption (Rauf et al. 2007; Uygun et al. 2009). Equations 1 and 2 represent the Langmuir and Freundlich isotherms, respectively.

$$1/q_e = (1/Q_{max}) + (1/Q_{max}b)(1/C_e) \quad (1)$$

$$\ln q_e = 1/n(\ln C_e) + \ln K_F \quad (2)$$

here, b is the Langmuir isotherm constant, K_F is the Freundlich constant, and n is the Freundlich exponent. $1/n$ is a measure of the surface heterogeneity ranging between 0 and 1, becoming more heterogeneous as its value gets closer to zero. The ratio of q_e gives the theoretical monolayer saturation capacity of the adsorption matrix. The kinetic constants of the Langmuir and Freundlich adsorption isotherms are summarized in Table 1. Comparison of all theoretical approaches used in this study shows that the Langmuir equation fits the experimental data best and these results suggest that Langmuir adsorption model is applicable to this system.

The effect of ionic strength on ferritin adsorption is demonstrated in Fig. 8. As seen in figure, ferritin adsorption capacity of the silver nanoparticles decreased with increasing ionic strength. The ferritin adsorption capacity decreased about 89.8 % when the NaCl concentration changed from 0 to 1.0 M. Repulsive electrostatic interactions between Fe^{3+} -chelated silver nanoparticles and ferritin molecules occurs when the ionic strength is increased so that the ferritin adsorption capacity decreased (Uygun et al. 2012d).

Fig. 10 SPR sensorgram of Fe^{3+} -chelated *N*-acetyl cysteine attached magnetic silver nanoparticles

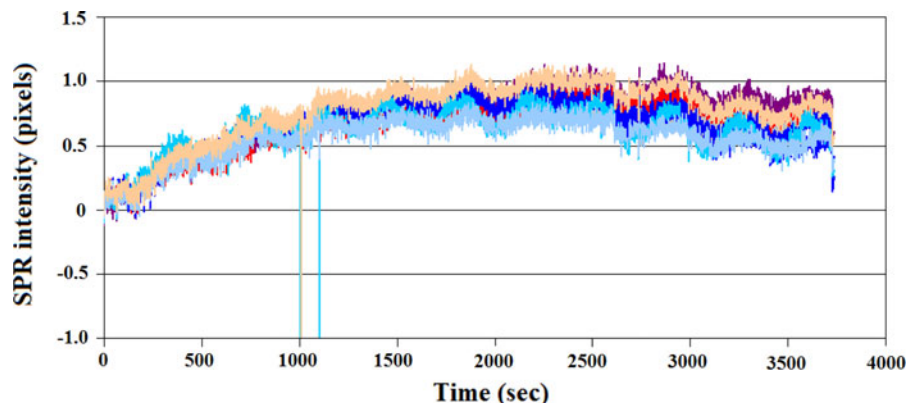


Figure 9 shows the effect of the medium temperature on the ferritin adsorption. As seen here, ferritin adsorption capacity increased when temperature increased to 25 °C and then, the adsorption capacity decreased when the temperature increased to 45 °C. This decrease can be attributed to thermodynamic influences which disrupt the interaction between nanoparticle and ferritin, and molecular three dimensional changes occur at high temperature. The effect of temperature on the adsorption of protein onto polymeric systems is based on the adsorption type. Because the adsorption is an exothermic process, adsorption capacity generally decreased with temperature (Altıntaş and Denizli 2006). However, when the hydrophobic interactions are efficient, adsorption capacity increased with increasing temperature (Akgöl et al. 2005).

Desorption and the repeated use of Fe^{3+} -chelated *N*-acetyl cysteine attached magnetic silver nanoparticles

The last step of the affinity separation is the fast and effective desorption of the adsorbed protein. For desorption, 1.5 M of NaCl (in pH 4.0 acetate buffer) solution was used and 98 % of the adsorbed ferritin was desorbed. After desorption process, nanoparticles were washed with distilled water and equilibrated with pH 6.0 phosphate buffer. To investigate the reusability of the Fe^{3+} -chelated *N*-acetyl cysteine attached magnetic silver nanoparticles, ferritin adsorption–desorption cycle was repeated with five times. At the end of the five adsorption–desorption cycles, ferritin adsorption capacity decreased only about 5.6 %.

SPR analysis

SPR sensorgram for the interaction between ferritin and Fe^{3+} -chelated *N*-acetyl cysteine attached magnetic silver nanoparticles was shown in Fig. 10. As seen in figure, while the chip was washed with buffer in the first 60 s, there was no significant signal. After 60 s, when the ferritin solution was injected to the SPR chip, SPR intensity began to increase and reached the value of 1.1 within 30 min. This study was repeated using seven different region of the chip and results were demonstrated by different colored line in the Fig. 10. This finding clearly showed that ferritin molecules were bounded to the silver nanoparticles selectively.

Conclusion

In this paper, synthesis of magnetic silver nanoparticles, their modification and chelation with Fe^{3+} and their usage for ferritin adsorption were reported. Characterization of the nanoparticles was carried out by SEM, AFM, EDX and ESR analysis. Synthesized nanoparticles were spherical and 69 nm in size. Ferritin adsorption conditions of this new IMAC support investigated, and optimum pH and temperature were determined as pH 6.0 and 25 °C, respectively. Adsorption efficiency of this silver nanoparticle decreased with increasing ionic strength. Affinity of the silver nanoparticles to the ferritin demonstrated with SPR analysis. Adsorption kinetics of the silver nanoparticle was also investigated and it is found that ferritin adsorption onto silver nanoparticle fits the

Langmuir model. Also, it is found that this IMAC supports remain stable for five adsorption–desorption cycles. As a result, these magnetic silver nanoparticles having good ferritin adsorption capacity and easy separation property may be a useful affinity matrix for the ferritin separation technology.

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