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Arsanilic acid modified superparamagnetic iron oxide nanoparticles for Purification of alkaline phosphatase from hen's egg yolk

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ABSTRACT

Recent studies of magnetic carrier technology have focused on its applications in separation and purification technologies, due to easy separation of the target from the reaction medium by applying an external magnetic field. In the present study, Fe_3O_4 superparamagnetic nanoparticles were prepared to utilize a chemical co-precipitation method, then the surfaces of the nanoparticles were modified with arsanilic acid derivatives which were used as the specific nanocarriers for the affinity purification of alkaline phosphatase from the hen's egg yolk. The six different types of magnetic nanocarriers with varied lengths of the linkers were obtained. All samples were characterized step by step and validated using FTIR, SEM, EDX, VSM and XRD analysis methods As the results were shown, the use of inflexible tags with long linkers on the surface of the nanocarrier could lead to better results for separation of alkaline phosphatase from the hen's egg yolk with 76.2% recovery and 1361.7-fold purification. The molecular weight of the purified alkaline phosphatase was estimated to be 68 kDa by SDS–PAGE. The results of this study showed that the novel magnetic nanocarriers were capable of purifying alkaline phosphatase in a practically time and cost effective way.

1. Introduction

Iron oxide (Fe_3O_4) nanoparticles widely utilized in a variety of the fields including biotechnology and biomedicine, enzyme and protein separation and purification [1–5], DNA and RNA purification [6], immunoassay [7,8], magnetic resonance imaging (MRI) [9], and targeted drug delivery [10–13]. Magnetic nanoparticles (MNP) could be modified through coating with suitable polymers [14] and silane [15,16] coupling agents. This could provide a matrix for the binding of functional groups required to prepare ligand immobilized MNP for the affinity separation and purification of specific biomolecules [17–23]. Enzyme separation and purification are greatly needed in bio-medicine, bio-science, food science, drug design, organic synthesis and industrial applications. Techniques that are typically being used in enzyme purification are chromatography, precipitation, ultrafiltration, centrifugation, and dialysis [24]. These techniques suffer from a number of limitations such as being time-consuming, pre-treatments requirements, expensive instrumentations, and skilled operators [25]. Magnetic nanocarriers used in separation technology have advantages over common separation procedures including a) rapid operation, b) significant reduction in the operation cost, c) simple separation and recovery procedure, d) easy and selective removal of target analytes captured to magnetic materials from the sample [26,27]. Affinity ligands used in the modification of magnetic nanoparticle surfaces include molecules such as antibodies, dyes, and metal ions. These modifications in most cases are expensive, toxic or unstable. Alkaline phosphatase (EC 3.1.3.1, ALP) is a nonspecific phosphormonoesterase found extensively in many mammalian tissues and dairy products [28]. It is involved in various metabolic functions such as fat absorption, mucosal defense, and skeletal mineralization, also is used to catalyze the hydrolysis of phosphomonoesters, and release of free inorganic phosphate and alcohol. ALP is often used as a major target in analytical chemistry, biochemistry and medical chemistry for probing causative disease mechanisms [29,30]. The hen's egg yolk ALP is a metalloprotein (Zn^{2+}) composed of two identical inactive subunits. The ALP hydrolyzes phosphomonoester non-specific compounds at alkaline pH [31,32]. Specific binding of the ALP to *p*-arsanilic acid or 4-(*p*-aminophenylazo)-phenylarsonic acid involves at least a positively charged

Abbreviations: TEO, stetraethyl orthosilicate; TEA, triethylamine; DCC, *N,N-dicyclohexylcarbodiimide*; APP, 4-aminophenylarsonic acid; APTES, 3-aminopropyltrimethoxysilane; CPTES, 3-chloropropyltrimethoxysilane; MNP, superparamagnetic nanoparticles; EC 3.1.3.1, ALP, alkaline phosphatase; AAOh, 1-ammonium-3-aza-4-oxo-7-heptanoat; AAPP, 4-(4-Aminophenylazo) phenylarsonic acid; AAOh, 1-ammonium-3-aza-4-oxo-7-heptanoate

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group in the active site. The active site of ALP which include arginine amino acid should interact with the arsenic acid. A hydrophobic section in the active site of ALP, could interact with the aromatic portion of the 4-(*p*-aminophenylazo)-phenylarsonic acid or the substrate [33]. The inhibition effects of 4-(*p*-aminophenylazo)-phenylarsonic acid (I_{50}; 2.48 ± 0.54 mM) is stronger than *p*-arsanilic acid (I_{50}; 11.5 ± 2.5 mM) as the affinity tags [34]. Several sources were used for ALP purification such as milk [35], rat liver [36] and calf intestinal [37] by the conventional purification methods. These methods with various techniques such as centrifugation, precipitation, and gel filtration [38], ion-exchange and affinity chromatography also were used for ALP purification from hen's egg yolk [34]. However, these purification methods are usually complicated, time consuming, low recovery and expensive in terms of equipment and experimental reagents. The aims of the current research were developed reasonably fast and low-cost method for the preparation of novel magnetic nanocarriers with facile application for the isolation and purification of ALP from biological samples using MNP technology. To evaluate the effectiveness of the prepared nanocarriers, arsanilic acid derivatives were applied for modification of MNP with different lengths of the linkers. Herein, we used TEOS, CPTES, and AAOh to prepare four different types of magnetic nanocarriers, named ClMNP@APP (1), ClMNP@AAPP (2), ClMNP@AAOh@APP (3) and ClMNP@AAOh@AAPP (4) with different lengths of the linkers. Additionally, MNP was modified by TEOS and APTES to further preparation of two different types of magnetic nanocarriers with different lengths of the linkers namely CMNP@APP (5) and CMNP@AAPP (6). These products were utilized as the magnetic nanocarriers specifically to purify ALP from hen's egg yolk. The findings of this study show that the novel magnetic nanocarriers were capable of purifying ALP by providing a practical approach which was time and cost efficient.

2. Experimental

2.1. Materials

Iron (III) chloride hexahydrate ($FeCl_3{\cdot}6H_2O$, 97%), Iron (II) chloride tetrahydrate ($FeCl_2{\cdot}4H_2O$, 99%), ammonia solution (28%), tetraethyl orthosilicate (TEOS, 98%) triethylamine (TEA, 99%), *N,N*-dicyclohexylcarbodiimide *(DCC 99%),*succinic anhydride (99%), and 4-aminophenylarsonic acid (APP) were purchased from Sigma-Aldrich Co. 3-aminopropyltrimethoxysilane (APTES, 95%), 3-chloropropyltrimethoxysilane (CPTES, 97%), toluene, dimethyl sulfoxide and other solvents were purchased from Merck Co and used as received.

2.2. Instrumentation

The melting points were determined on a Büchi B-545 apparatus in open capillary tubes and are uncorrected. The chemical structures of the nanoparticles were analyzed by Fourier transform infrared (FTIR) spectroscopy. The FTIR spectra were recorded on a Bruker Tensor 270 spectrometer. The surface morphology and size of the nanoparticles were evaluated by scanning electron microscopy (SEM) MIRA3 TESCAN. The elemental analysis of the nanoparticles was carried out by energy dispersive X-ray spectroscopy (EDX) MIRA3 TESCAN. The samples were spread on a SEM stub and sputtered with gold. XRD measurements were performed on Bruker (model D 8 advance, Germany) diffractometer with Cu Kα radiation. A vibrating-sample magnetometer was used to probe the magnetic properties of MNPs at room temperature (VSM; AGFM, Kashan, Iran).

2.3. Preparation of magnetic nanocarriers 1–6

2.3.1. Preparation of MNP

13.60 g of $FeCl_3{\cdot}6H_2O$ and 5.0 g of $FeCl_2{\cdot}4H_2O$ were mixed with a magnetic stirrer in a round bottom flask containing 600 mL of deionized water under argon atmosphere at room temperature. Then 140 mL of ammonia solution was dropwise added to the mixture with vigorous stirring. After the color of the solution turned to black, the magnetite precipitates were magnetically separated and washed several times with deionized water and ethanol. The final black color of MNP were vacuum dried at 50 °C for 24 h [39].

2.3.2. Surface modification of MNP with TEOS (SMNP)

5.0 g of dried MNP were suspended in 500 mL of ethanol/deionized water (4:1) mixture by ultrasonication and the pH of the solution was adjusted to 10 through applying ammonia solution. Then, 10 mL of TEOS was dropwise added into the above solution and the mixture was mixed by a mechanical stirrer under argon atmosphere at 60 °C. The stirring was continued for another 5 h, and then silica coated MNP (SMNP) was separated by an external magnet and washed three times with deionized water and two times with ethanol. The final dark brown SMNP was vacuum dried at 50 °C for 18 h.

2.3.3. Surface modification of SMNP with CPTES (ClMNP)

3.0 g dried of SMNP was added to 300 mL dry toluene, then an excess of CPTES (7.5 mL) was added followed by 3 mL of TEA. The mixture was stirred by a mechanical stirrer under argon atmosphere at 90 °C for 48 h. Then SMNP was coated with chloropropyl functional group. Afterward, ClMNP was magnetically separated and washed two times with toluene, two times with deionized water, and two times with ethanol and dried at 50 °C overnight.

2.3.4. Preparation of magnetic nanocarrier 1 and 2

As descried previously, APP affinity tag were purchased from Sigma-Aldrich Co. and AAPP affinity tag was prepared according to the following procedure: 4.34 g APP was dissolved in 5 mL concentrated HCl and diluted with 25 mL water, diazotization is effected at 0 °C using 1.5 g sodium nitrite in 12 mL water, excess nitrous acid is consumed through adding 0.2 g urea, and then after 15 min a solution of 1.86 g aniline in 1.6 mL of concentrated HCl which is diluted with 12 mL water is added at one time at 0 °C, and the mixture was stirred for an hour at 0 °C and then was allowed to remain two hours at 25 °C. Dilute sodium hydroxide is added until all the precipitate is formed, the mixture is filtered until the filtrate is turned slightly acidic with dilute acetic acid; the bright red precipitate washed with water, and sucked dry on the filter paper and crystallized from (1:9) water/ethanol, which yields bright pale red crystals (m.p > 300 °C) [40].

2.3.4.1. ClMNP@APP as a magnetic nanocarrier (1). In brief, 1.0 g of dry ClMNP was added to 150 mL absolute ethanol, then an excess of APP (1.0 g) was added to the flask followed by 0.68 mL of TEA. The mixture was stirred by a mechanical stirrer under argon atmosphere at 60 °C for 24 h. At last, ClMNP@APP was magnetically separated and rinsed several times with deionized water and ethanol and dried at 50 °C for 24 h.

2.3.4.2. ClMNP@AAPP as a magnetic nanocarrier (2). 1.0 g of dry ClMNP was added to 150 mL absolute ethanol, then an excess of AAPP (1.60 g) was added to the flask followed by 0.68 mL of TEA. The mixture was stirred by a mechanical stirrer under argon atmosphere at 60 °C for 24 h. Then ClMNP@AAPP was separated by an external magnet and washed with deionized water and ethanol to remove unreacted materials. Finally, the resulted ClMNP@AAPP was vacuum dried at 50 °C for 24 h.

2.3.5. Surface modification of ClMNP with AAOh (ClMNP@AAOh)

Firstly, 1-ammonium-3-aza-4-oxo-7-heptanoate (AAOh) as a linker was prepared according to the following procedure: 5.0 g (50 mmol) of succinic anhydride dissolved in 50 mL of dry THF. The above solution was slowly added to the solution of 1, 2-diaminoethane (3.0 g; 50 mmol) in 20 mL of THF at room temperature. The mixture was