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Bacteria killer enzyme attached magnetic nanoparticles

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ABSTRACT

The objective of the present work was to develop immobilized lysozyme systems through adsorption on magnetic nanoparticles for potential usage in bacteria killing studies. For this, magnetic poly(HEMA-GMA) nanoparticles were prepared by surfactant free emulsion polymerization technique and functionalized with dye ligand Reactive Green 5. Synthesized magnetic nanoparticles were then characterized by FTIR, SEM, EDX and ESR studies. Particle size range of the polymers was found to be as 90–120 nm. Magnetic behavior was also demonstrated by ESR with the g value of 2.48. Maximum lysozyme loading was found to be as 1045.1 mg/g nanopolymer. Repeated usability of the magnetic nanoparticles was also studied. Immobilized form of lysozyme protected 85.85% of its initial activity at the end of the immobilization process. Bacteria killing capacity of the lysozyme immobilized magnetic nanoparticles were investigated by using *Micrococcus lysodeikticus* bacteria and it was demonstrated that all bacteria were successfully destroyed by the lysozyme immobilized magnetic nanoparticles within 5 min.

1. Introduction

Lysozyme is a small monomeric enzyme composed of 129 amino acid residues. The enzyme demonstrates glycoside hydrolase activity and contains four intact disulfide bounds [1–3]. It is an antimicrobial protein, plays an important role in the defense systems of living organisms, and naturally founds in body fluids especially in secretions such as saliva, tears and mucosa. Lysozyme also makes significant contribution to the anti-infectious immune system. Its antimicrobial spectrum is very large and kills bacteria by disintegrating the bacterial cell walls *via* simple enzymatic hydrolysis reaction of 1,4-β-linkages between the *N*-acetylmuramic acid and *N*-acetyl 1-D-glucoseamin residues, which are the main components of the peptidoglycan layer of the bacterial cell wall [4,5].

Because of its antibacterial behavior, lysozyme has been intensively applied as a preservative in various food products such as raw and processed meats, dairy products, fruits and vegetables. Thus, shelf lives of these food products are extended by using lysozyme as an additive [6]. Lysozyme has been also found diverse applications in pharmaceutics industry as a potential antimicrobial agent. The enzyme is also preferred in aseptic and therapeutic utilizations. Because of these important and unique applications of lysozyme, studies about its usage in different research areas have been intensively increased [7].

Magnetic nanoparticles have been attracted great attention for diverse technological and biotechnological applications with the direct proportion to the recent developments in nanotechnology. Magnetic nanoparticles have been found important positions for biotechnological applications such as magnetic resonance imaging (MRI), targeted drug delivery, fast biomolecule separation, molecular diagnosis, sensors works and hyperthermia practices [8–14]. Magnetic particles can be recovered and manipulated from their aqueous solution fast and easily by using an external magnetic field, because of their unique magnetic properties. Magnetic nanoparticles have been also applied as a new support material for various enzyme immobilization studies, recently [15–18]. These magnetic nano sized support materials exhibit good advantageous properties such as high surface areas to immobilize larger amounts of enzyme, lower mass transfer resistance, less fouling, and also demonstrates selective, fast, easy and unique separation of enzyme attached nanoparticles by applied external magnetic fields [15,19].

Polymeric magnetic particles have been intensively manufactured and preferred, because of the variety of surface functional groups located on the surface of the nanoparticles, which can be further modified in order to use for different practical applications [20–25].

In this presented study, surface of the magnetic nanoparticles was

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modified by a dye ligand Reactive Green 5. Dye ligands are very cheap and commercially available, and their attachment to support materials is very easy, especially *via* hydroxyl groups. These dye ligands are generally called as an affinity ligand, due to their molecular structures mimic the structure of the substrate or cofactor of some enzymes. These ligands can also easily interact with the active center of the enzymes [26]. Reactive Green 5, which also named as Procion Green H-4G, is a monochlorotriazine dye, and bears seven acidic sulfonate groups and three basic secondary amino groups. The dye also carries Cu(II) bonded phthalocyanine unit [27]. The main recognition interaction between the biomolecule and the dye ligand can be electrostatic, hydrophobic, and hydrogen bound, or a complex association of these interactions [27,28]. Lysozyme have been immobilized (or adsorbed) onto various kinds of dye affinity support materials from diverse sources [27–39].

In this presented work, magnetic nanoparticles were prepared by surfactant free emulsion polymerization technique and then used as a support material for lysozyme immobilization by the help of the dye ligand interaction. Additionally, bacteria killing capacity of immobilized form of lysozyme was also explored.

2. Materials and methods

2.1. Materials

Lysozyme (E.C. 3.2.1.17; from egg white), *Micrococcus lysodeikticus*, 2-hydroxyethyl methacrylate (HEMA), glycidyl methacrylate (GMA), magnetic nanopowder (Fe_3O_4; average diameter of 20–50 nm) and Reactive Green 5 were supplied from Sigma (St. Louis, USA). All other chemical were of analytical grade and used without any purification or clarification steps. Ultrapure deionized water (18.2 mΩ cm; Millipore, Simplicity®) was used in order to prepare all solutions used for experimental process.

2.2. Preparation and characterization of Reactive Green 5 attached magnetic nanoparticles

Preparation process of magnetic poly(HEMA-GMA) nanoparticles was carried out by using the surfactant free emulsion polymerization technique according to previous study [40]. Briefly, structural monomer HEMA and functional monomer GMA were mixed with PVA solution and then were shaken slowly. Fe_3O_4 and potassium peroxodisulfate were added to this initial polymerization solution, and then polymerization process was started by incubating the solution at 70 °C for 4 h. At the end of the polymerization process, synthesized nanoparticles were washed with ethanol and then further rinsed with distilled water to remove the unreacted reactive compounds. Dye ligand Reactive Green 5 was covalently immobilized onto the magnetic nanoparticles. For this, 1.0 mg/mL of magnetic nanoparticles were mixed with 20.0 mL of Reactive Green 5 solution (1.0 mg/mL, in 5.0 g of NaOH) at 80 °C for 2 h. At the end of the dyeing process, Reactive Green 5 attached nanoparticles were rinsed with distilled water and methanol, in order to remove the unbounded dyes.

Characterization studies of the synthesized magnetic nanoparticles have great importance to check the polymerization and modification is clear and successful. For this, dye attached magnetic nanoparticles were characterized by Fourier Transform Infrared Spectroscopy (FTIR), Scanning Electron Microscopy (SEM), Atomic Force Microscopy (AFM), Electron Spin resonance (ESR) and Energy Dispersive X-ray (EDX) analysis.

A FTIR spectrophotometer (Varian FTS 7000, USA) was used to evaluate and compare the characteristic IR bands of the synthesized mag-poly(HEMA-GMA) and Reactive Green 5 attached mag-poly (HEMA-GMA) polymers. For FTIR, 0.1 g of dried nanoparticles were mixed with 0.1 g of IR grade KBr, and then pressed into a pellet form mechanically. The pellet mounted in the FTIR spectrophotometer and then FTIR spectrum was recorded. Overall size and surface morphology of the magnetic nanoparticles were examined by SEM analysis. For SEM, dried nanoparticles were mounted on a SEM device (Philips XL-30S FEG, the Netherlands) and the SEM pictures of magnetic nanoparticles were photographed. Magnetism and the incorporation of the magnetic particles into nanoparticles were investigated by using an ESR spectrophotometer (JEOL JES-FA300, USA). Attached amount of Reactive Green 5 onto magnetic particles was examined by an EDX instrument (LEO, EVO40, Carl Zeiss NTS, Peabody, USA). Loaded amount of Reactive Green 5 was calculated by the data of EDX considering the sulfur stoichiometry.

2.3. Immobilization of lysozyme on dye attached magnetic nanoparticles

One of the intensively used immobilization methods is adsorption. In this work, lysozyme was immobilized onto magnetic nanoparticles by adsorption method by incubating the lysozyme solution with nanoparticles for 120 min at 25 °C. The effects of the initial lysozyme concentration, and the medium ionic strength on the lysozyme adsorption capacity of the nanoparticles were also investigated. Adsorbed amount of lysozyme was followed by monitoring the UV absorbance at 280 nm.

Adsorption isotherms are preferred to define the nature and type of an adsorption process. For this presented study, a simple plot was constructed by using the data of adsorbed amount of lysozyme (q_e) and equilibrium lysozyme concentration (C_e) in adsorption solution. Langmuir and Freundlich isotherms are the most preferred adsorption isotherms and they help to understand the adsorption process and type [26]. Langmuir model asserts that support material caries well-defined adsorption sites with equivalent affinity towards to target molecule. These adsorption sites are located far enough to avoid the cross-interaction of the binding sites, and thus these sites adsorb only one molecule for per adsorption region. For this reason, enthalpies and energies are equal, and Langmuir isotherm can be expressed as following equation (Eq. (1)):

$$q_{e=}\frac{q_m K_a C_e}{1+K_a C_e} \tag{1}$$

This equation can be transformed to linear form as demonstrated in Eq. (2):

$$\left(\frac{C_e}{q_e}\right)=\left(\frac{C_e}{q_m}\right)+\left(\frac{1}{q_m K_a}\right) \tag{2}$$

here, adsorption equilibrium constant is expressed as K_a; C_e is the concentration of the un-adsorbed target molecule; and q_e is the concentration of the un-adsorbed target molecule. Maximum adsorption amount of per unit weight of support material is shown as q_m.

The other intensively used adsorption model is Freundlich isotherm. This model suggests that, overall adsorption process is heterogeneous and target molecule distributes non-uniformly. The main difference of Freundlich model from Langmuir model is Freundlich model allows multi-layered adsorption.

Mathematical expression and linear form of Freundlich isotherm are given by Eq. (3) and Eq. (4), respectively.

$$q_e = K_f C_e^{1/n} \tag{3}$$

$$\ln q_e = \ln K_f + \frac{1}{n}\ln C_e \tag{4}$$

here, q_e is the equilibrium concentration of adsorbed species; C_e is the non-adsorbed amount at equilibrium; K_f and n are the constants for Freundlich model. K_f reflects the adsorption capacity, while n shows the intensity of adsorption.

Reusability of the magnetic nanoparticles for lysozyme adsorption was also investigated by repeating the adsorption process for 10 times using the same nanoparticles. In order to detach the adsorbed lysozyme from magnetic nanoparticles, 1.0 M of NaCl solution was used as a