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Immobilization of cholesterol oxidase to finely dispersed silica-coated maghemite nanoparticles based magnetic fluid

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ABSTRACT

In the recent years, the potential applicability of magnetic nanoparticles (MNPs) has witnessed a significant increase in interest towards the medical field, in particular, towards the usage of novel nanoparticles in diagnostics and disease treatment, respectively. In a present study, cholesterol oxidase (ChOx) was covalently immobilized to magnetic nanoparticles of maghemite (γ-Fe_2O_3) and further functionalized by silica (SiO_2) and amino-silane molecules. The activity of the bound enzyme was retained up to 60%, respectively. The binding of cholesterol oxidase was confirmed using FT-IR spectrophotometer. SEM analysis showed uniformly dispersed functional magnetic nanoparticles, which ranged in size from 22.5 to 50.8 nm, surrounded by amorphous silica. In this paper, the potential applications of chemically modified magnetic nanoparticles as carriers for cholesterol oxidase and other enzymes are discussed.

1. Introduction

Stability of the enzyme is very often improved by immobilization [1]. So far, many organic and inorganic substances have been used as materials for the fabrication of a suitable carrier for a diverse range of biomolecules, for instance, the enzyme. Among them, magnetic particles on nanometer scale receive considerable attention because of their widely increased use in the immobilization procedures of proteins and enzymes. Moreover, with the rapid development of nanostructured materials, the genesis of magnetic nanoparticles with improved characteristics brings the following advantages: (a) a significantly higher specific surface area is obtained for the attachment of a larger amount of enzyme per unit mass of nanoparticles, (b) lower mass transfer resistance is expected, and (c) the magnetically labelled immobilized enzymes can be selectively separated from a reaction mixture by the application of an external magnetic field [2–4]. Due to magnetic separation, it is possible to achieve very high efficiency of separation of many bioactive substances such as enzymes and proteins. Other applications of magnetic particles include immunoassays, drug targeting, drug transporting, and biosensing. Furthermore, the application of magnetic nanoparticles expands towards the removal of toxic elements from industrial wastes, respectively [5].

Modified magnetic materials are nowadays well-known and have been investigated intensively due to their potential applications in enzyme binding protocols [6]. Maghemite (γ-Fe_2O_3) is one of the famous magnetic materials in common use. Due to its magnetic character, γ-Fe_2O_3 nanoparticles can be attracted by a magnetic field and are easily separable in solution, thereby, enabling also separation of bioactive substances that are linked directly or indirectly to the surface of MNPs. For biological applications, it is very important to conjugate MNPs with appropriate reactive molecules, such as amino groups. The MNPs used in the present study were surrounded by amorphous SiO_2, i.e., Si–OH (silanol group) was located on the outer surface ready to be further modified. Surface modification with amino silane reactive groups is very common method for particle functionalization. Nevertheless, amino silane molecules that act as coupling agents are conjugate to the surface of magnetic nanoparticles after deposition of inorganic silica [7,8]. Adversely, direct attachment of amino silane molecules onto particle surface has also been already encountered [9]. High density of free amino groups ($-NH_2$) lying outwards the particle surface provides an excellent media for further chemical surface modification such as enzyme cross-linking with glutaraldehyde. Suitable functional coating of magnetic particles has many benefits. For instance, particle size is notably increased, resulted in the slight decrease of reactivity of the particles. Thus, in this fashion the problem of particle agglomeration could be thoroughly omitted.

Abbreviations: AEAPS, 3-(2-aminoethylamino)-propyl-dimethoxymethylsilane; ChOx, cholesterol oxidase; FT-IR, Fourier transform infrared spectroscopy; E, designation for enzyme; MNPs, magnetic nanoparticles; PBS, phosphate buffer solution; BSA, bovine serum albumin.

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It has been demonstrated that the silica coated superparamagnetic nanoparticles modified with amino silane molecules provide high chemical stability, thus, they are envisaged as very promising enzyme carriers in the enzyme immobilization technology, respectively.

Cholesterol oxidase (ChOx, EC 1.1.3.6) is a flavin enzyme and is the most commonly studied enzyme for the construction of biosensors for cholesterol assessment in biological samples. Thus, preliminary determination of cholesterol is clinically very important because abnormal concentrations of cholesterol in human beings lead to cholesterol related disorders such as hypercholesterolemia and other coronary diseases. Moreover, its commercial importance is foreseen also in agriculture as a potential insecticide. Its activity can be determined by monitoring the appearance of the conjugated ketones, the formation of hydrogen peroxide in a coupled assay with peroxidase, or by measuring the oxygen consumption polarographically [10].

Magnetic nanoparticles as solid carriers have several applications in medical and biotechnology fields. One of the important applications of the immobilized enzyme onto magnetic nanoparticles is in the treatment and diagnosis protocols [11,12]. Herein, it is considered that the cholesterol oxidase immobilized onto magnetic nanoparticles can be successfully used as a drug delivery media to the exact place of disease by simply using an external magnetic field and thereby eliminating the formed clots in the coronary system. Alternatively, a great potential of immobilized cholesterol oxidase onto magnetic nanoparticles could be foreseen as an analytical component in biosensors for the determination of cholesterol in various forms, as esterified cholesterol in serum, low and high density lipoproteins, and so on.

In this study, superparamagnetic silica nanoparticles of maghemite, additionally functionalized with amino silane organic molecules, were prepared for protein immobilization. Primarily, maghemite nanoparticles were prepared by the co-precipitation reaction of ferrous and ferric ions in alkaline medium with NH_4OH and then coated with silica layer, which was produced from sodium silicate solution directly. Primary coating of maghemite nanoparticles with silica gives biocompatibility and ulterior stability to the synthesized magnetic nanoparticles. Since the outer silica surface is covered with many hydroxide functional groups, this can be further functionalized by using a variety of known coupling agents with the aim to attain well stable and highly functionalized nanosized magnetic carriers. The present paper proposes to investigate the binding of cholesterol oxidase to γ-Fe_2O_3 magnetic nanoparticles. The size and structure of the particles were characterized using SEM spectroscopy. The binding of ChOx to magnetic carrier was confirmed by FT-IR analysis.

2. Experimental

2.1. Materials

All the chemicals used in the present study were generally of reagent grade obtained from commercial sources. Enzymes, cholesterol oxidase (ChOx, EC 1.1.3.6) with a specific activity of 30.1 U mg^{-1} solid was procured from Biozyme Laboratories, UK and bovine serum albumin (BSA) was purchased from Sigma–Aldrich, Germany, respectively. Chemicals including ferrous chloride tetrahydrate, ferric chloride hexahydrate, ammonia solution (25% (w/w)), sodium silicate and amino silane coupling agent, AEAPS, glutaraldehyde (GA, 25%) solution and cholesterol powder (95%) were of the highest purity and purchased from Sigma–Aldrich Chemicals Company, Germany. Aqueous solutions were prepared freshly with double distilled water before use.

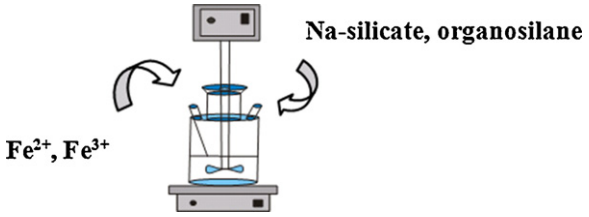

Fig. 1. Three-necked bottle reactor used for the synthesis of nanocomposites.

2.2. Procedures

2.2.1. Preparation of nanoparticles

Magnetic nanospheres of maghemite (γ-Fe_2O_3) with a mean diameter 13 nm were prepared by hydrothermal co-precipitation of Fe^{2+} and Fe^{3+} ions under alkaline conditions (NH_4OH) and harsh mechanical stirring. Thus, 6.22 g of $FeCl_3{\cdot}6H_2O$ and 5.37 g of $FeCl_2{\cdot}4H_2O$ were dissolved in 200 mL double deionized (miliQ) water and thoroughly mixed at room temperature. Ammonia solution was rapidly poured into solution of iron ions under vigorous stirring to increase a pH of ferric and ferrous solution to constant value, pH 11, respectively. The precipitate was extracted by a magnet and rinsed with copious amount of double distilled water, and ammonia solution, as well. Afterwards, the collected precipitate was dispersed in water. Upon addition of primary surfactant, citric acid (γ = 0.5 g/mL), in order to obtain finely dispersed magnetic nanoparticles, the particles were heated at 70 °C for 90 min. After completion of the reaction, the suspension of maghemite particles was cooled down and the pH was settled to a value of 10.1 ± 0.1. The particles attained in this work were brown in color and exhibited a strong magnetic response. Afterwards, chemical modification of the surface of γ-Fe_2O_3 nanoparticles proceeded in two-step reaction.

2.2.2. Surface functionalization of nanoparticles

First, the coating of γ-Fe_2O_3 nanoparticles surface with polymerizing silica (SiO_2) occurred, and then, silanization reaction by amino silane coupling agent took place in order to provide highly functionalized γ-Fe_2O_3 nanoparticles ready for subsequent surface treatment. The obtained sample was identified as maghemite surrounded by amorphous silica and additionally functionalized by AEAPS organosilane as confirmed by FT-IR measurement spectroscopy. Here, for the coating procedure of maghemite nanoparticles the three-necked bottle reactor equipped with a mechanical stirrer was used as shown in Fig. 1. Since there are large surface-to-volume atomic ratio, high surface activity, and amount of pendant bonds on nanoparticle surface, the atoms on the surface are prone to conjugate with other ions or molecules in solution. For a γ-Fe_2O_3/SiO_2 nanocomposite possesses an OH-rich surface, the coating process by silanization reaction on the surface of core/shell maghemite nanoparticle is therefore favoured by obtaining a covalent bonding between the γ-Fe_2O_3/SiO_2 nanoparticle and AEAPS molecule, respectively.

2.2.3. Activation by glutaraldehyde

Following this, the surface of chemically modified maghemite nanoparticles was further subjected to activation with the crosslinker, glutaraldehyde. The support crosslinking with glutaraldehyde is a two-step reaction. First, the terminated amino group of γ-Fe_2O_3/SiO_2 functionalized with AEAPS molecules reacts with glutaraldehyde to form a Schiff-base linkage and provides a terminal aldehyde, which can be then condensed with the free amino group in enzyme molecule to form a second Schiff-base bonding. Thus, in an earlier study [13], optimal reactive conditions for magnetic support activation were determined; the optimal pH