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Preparation and characterization of magnetic levan particles as matrix for trypsin immobilization

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ABSTRACT

Magnetic levan was synthesized by co-precipitating D-fructofuranosyl homopolysaccharide with a solution containing Fe^{2+} and Fe^{3+} in alkaline conditions at 100 °C. The magnetic levan particles were characterized by scanning electron microscopy (SEM), magnetization measurements, X-ray diffractometry (XRD) and infrared spectroscopy (IR). Afterwards, magnetic levan particles were functionalized by $NaIO_4$ oxidation and used as matrices for trypsin covalent immobilization. Magnetite and magnetic levan particles were both heterogeneous in shape and levan–magnetite presented bigger sizes compared to magnetite according to SEM images. Magnetic levan particles exhibited a magnetization 10 times lower as compared to magnetite ones, probably, due to the coating layer. XRD diffractogram showed that magnetite is the dominant phase in the magnetic levan. Infrared spectroscopy showed characteristics absorption bands of levan and magnetite (O–H, C–O–C and Fe–O bonds). The immobilized trypsin derivative was reused 10 times and lost 16% of its initial specific activity only. Therefore, these magnetic levan particles can be proposed as an alternative matrices for enzyme immobilization.

1. Introduction

Magnetic carrier technology has been applied as bioaffinity adsorbents for selective recovery from liquors containing other suspended solids; wastewater treatment via electrostatic adsorption; protein (enzyme) immobilization and in the preparation of immunological assays [1]. The magnetic properties of these particles play an important role in the effectiveness of the application and affect the behavior of the particles and ferrofluids in applied fields [2].

In biomedicine, the polymer shell ensures stability of the magnetic particles in physiological media providing non-toxicity by avoiding leakage of iron and enabling chemical modification for attachment of biologically active compounds [3]. The shells are biocompatible such as dextran, xylan, chitosan, PEG, etc. and possesses active groups, which can be conjugated to biomolecules such as proteins [4]. Magnetic particles are an attractive and efficient support for bioconversions using immobilized enzymes due to the following advantages: simplicity of the matrix synthesis and immobilization protocol and easy removal from the reaction medium by applying a magnetic field [5,6].

Magnetic carriers can be manufactured using inorganic materials or polymers. However, those based on polymers offers a variety of surface functional groups than can be tailored to specific applications [7]. The utilization of polysaccharides presents advantages due to a large number of derivable groups, wide range of molecular weights, low toxicity, biodegradability and high stability [8]. Most magnetic materials such as maghemite and magnetite are employed as the core of the supports [9]. The magnetite particles (Fe_3O_4) are preferred because of their greater saturation magnetization and no toxicity [10].

The application for biomolecules immobilization mainly based on the solid-phase magnetic feature which is able to achieve a rapidly easy separation and recovery from the reaction medium in an external magnetic field [11]. There are basically two main ways to immobilize protein on supports: physical adsorption and covalent immobilization. Comparatively, covalent immobilization presents the benefits of eliminating or reducing protein leakage (a stronger linkage is formed) and usually increases protein tertiary structure stability [12–16]. Immobilization of biomolecules onto

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insoluble supports is an important tool for the fabrication of a diverse range of functional materials or devices [17]. It provides many distinct advantages including enhanced stability, easy separation from reaction mixture, possible modulation of the catalytic properties, and easier prevention of microbial growth [18].

In our lab magnetic levan composite was previously used to purify using affinity binding lectins from *Canavalia ensiformis* (Con A) and *Cratylia mollis* seeds (Cramoll 1 and Cramoll 1, 4) [19]. Cramoll 1 was purified using this procedure in two steps instead of a preceding three-step protocol employing ammonium sulfate fractionation, affinity chromatography on Sephadex G-75, and ion exchange chromatography through a CM-cellulose column [19]. Here, these magnetic particles were characterized regarding structural, microstructural and magnetic properties and further used as matrix to immobilize trypsin.

2. Experimental

2.1. Materials

Levan from *Zymomonas mobilis* strain ZAG–12 (Molecular weight average equal to 300 kDa) was precipitated by addition of ethanol to 70% (v/v) at low temperature according to Calazans et al. [20]. Ferric chloride hexahydrate and ferrous chloride tetrahydrate were purchased from Merck (Germany) whereas ammonium hydroxide was from Vetec Chemical (Brazil). All other reagents were of analytical grade.

2.2. Preparation of magnetic particles of levan

An aqueous mixture with 5 mL of 1.1 M $FeCl_3.6\,H_2O$ and 5 mL of 0.6 M $FeCl_2.4\,H_2O$ were added to 50 mL of 2.0% w/v levan (prepared in distilled water). Ammonium hydroxide was then added to achieve a pH of 11. The mixture was then heated up to 85 ± 3 °C and kept for 30 min with vigorous stirring. The magnetic levan particles synthesized were thoroughly washed with distilled water to a neutral pH. The material was dried and kept at 25 °C. This procedure was according to Carneiro Leão et al. [21], except by incubation time (30 min), temperature (85 °C) and final pH of the mixture (11).

2.3. Matrix characterization

The particle size and morphology of the samples were established by scanning electronic microscopy (SEM), utilizing a JEOL Model JSN—5900 electron microscope. Magnetization measurements were obtained at 293 K and 313 K in magnetic fields from 0 to 5.0 T using a SQUID magnetometer (Quantum Design Model MPMS-5). The structural properties of the magnetic particles were characterized by X-ray powder diffraction, which was carried out in an X-ray diffractometer Siemens D5000. Representative powder samples were analyzed in the range $10^\circ < 2\theta < 90^\circ$ using CuKα radiation ($\lambda = 1.5406$ Å), in steps of 0.02°, and with a counting time of 1.0 s per step. Fourier transform infrared (FTIR) spectrum from the KBr pellet method in the range of 4000–400 cm^{-1} with particles-coated was recorded in a BRUKER instrument model IFS 66. Magnetite, magnetic levan and levan (2 mg each) were mixed with KBr (200 mg) and disks obtained under pressure at 490 atm. Scan (100 scans) was recorded with a 4 cm^{-1} resolution.

2.4. Magnetic levan particles Functionalization, trypsin immobilization and reuse of the enzymatic derivative

Magnetic levan particles (10 mg) and sodium metaperiodate (10 mg) were mixed in 3 ml distilled water [22]. This mixture was constantly stirred in the dark at 25° C for 7 h. The magnetic particles containing the partially oxidized levan were collected by a magnetic field of 0.6 T and washed with 0.1 M Tris-HCl buffer pH 8.0 (1.0 mL, 10 times). After this procedure, it was incubated with trypsin (0.2 mg/mL, 1.0 mL) for 16 h at 4 °C under mild stirring. The enzymatic derivative was collected by magnetic field of 0.6 T and supernatant and washings were collected for protein determination [23] using trypsin as standard. Sodium borohydride (0.03 M, 1 mL) was added to trypsin–magnetic levan particles and slightly mixed for 2 h at 4 °C. Then they were washed 10 times with the aforementioned buffer and kept in the buffer at 4 °C until use. The retained protein was estimated by the difference between the offered protein (200 μg) and that found in the supernatant and washings. The reuse was carried out by assaying of the same trypsin–magnetic levan particles preparation with BAPNA for 10 times intercalating each successive use by washing the immobilized enzymatic derivative 10 times with 0.1 M Tris-HCl buffer, pH 8. The activity of trypsin for the free and immobilized enzyme was measured as described by Amaral et al. [24]. The activity (unit) was defined as μmol BAPNA hydrolyzed during 1 min using an absorption coefficient of ε_{405} 9100 $M^{-1}\cdot cm^{-1}$. All of the experiments were carried out in duplicate.

3. Results and discussion

3.1. Preparation, size and morphology of the magnetic particles

The co-precipitation process to obtain magnetic levan was carried out in an alkaline aqueous medium and the final product obtained from this process yielded a dense, black and magnetic powder. These particles exhibited a magnetization in the presence of a magnetic field 0.6 T. SEM images shown in Fig. 1 reveal

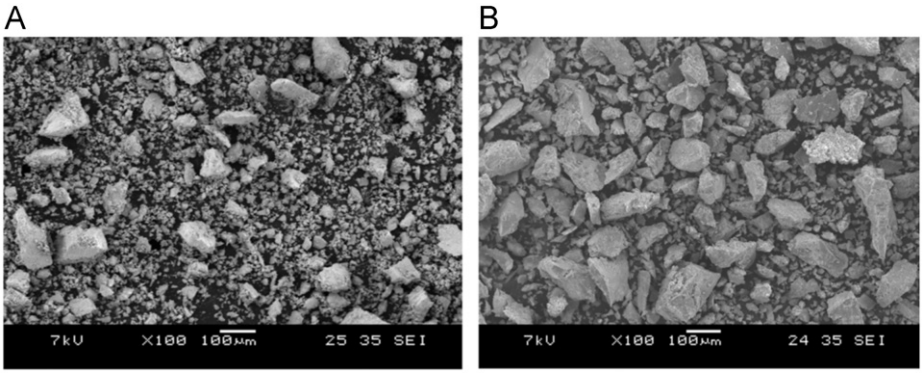

Fig. 1. Scanning electron microscopy images of magnetite (A) and magnetic levan (B) particles.

a heterogeneous morphology without porosity for both magnetite and magnetic levan particles. However, magnetic levan particles presented a bigger mean size than the magnetite ones. The particles can be considered as microparticles with sizes between 20–60 μm for magnetite and 100–200 μm for magnetic levan approximately. The coating produced changes in the size particle and according to Silva et al. [25] one can attribute to multi-core/shell structures.

3.2. Magnetization measurements

The magnetization of magnetite and magnetic levan particles (Fig. 2) indicated that there was neither remnant magnetization (Magnetization = 0 for Magnetic field = 0) nor coercivity. The saturation magnetization was found to be 10 times lesser for the magnetic levan as compared to magnetite. Neri et al. [26] using magnetic composite of polysiloxane coated with polyaniline also found the saturation magnetization lesser than magnetite and suggested that the presence of cations vacancy in maghemite could be responsible for this decrease. The coating decreases the force exercised by applied magnetic field because of the difficult alignment of magnetic dominions in the material, producing a smaller magnetization than that exhibited by particles-uncoated as well as observed by Xu et al. [27] and Ramanujam and Yeow [28]. Besides the presence of the polymer, the smaller amount of magnetic material (iron oxide core) and reduction in size of the particles (core-shell) should be considered, since magnetism arises because electrons have a property called spin. As dimensionality, and hence coordination, is reduced, more electrons are available to lead to magnetism [29].

3.3. X-ray analysis

Fig. 3 shows the XRD diffractograms for magnetite and magnetic levan particles. The magnetite was the dominant phase in the particles of magnetic levan with a primary scattering peak at around $2\theta = 35^{\circ}$. This result is according to Chen and Hu [30]. Magnetic levan particles showed small peak dislocations as compared to magnetite patterns. They probably happened due to tensions in the crystalline structure from magnetite because of modifications occurred during synthesis of the material. Furthermore, Fig. 3 shows that magnetic levan do not present sharp diffraction peaks. Instead, a broad band appears in each spectrum, which is characteristic for amorphous materials and also of ultrafine crystalline particles [27]. It suggests the presence of levan polysaccharide in the produced magnetic particles. Characteristic peaks of goethite (at $2\theta = 21.38^{\circ}$), hematite (at $2\theta = 33.15^{\circ}$), ferric hydroxide (at $2\theta = 26.38^{\circ}$) as well as other phases of iron oxide hydroxides were not detected [25,31]. However, another iron oxide (maghemite), difficult to distinguish from magnetite due to the proximity of the peaks, probably could have been formed as contaminant during the synthesis process [32].

3.4. Functional groups

Infrared spectroscopy (Fig. 4) showed that O–H groups are present in the levan polysaccharide, magnetite and magnetic levan near wavenumber of 3500 cm^{-1} with similar intensities. These O–H groups correspond to that present in organic compounds and to the OH^{-} groups adsorbed on the particle surface. The magnetic levan presented absorption bands in 2935.1 and 2878.5 cm^{-1} due to stretching vibration of C–H bond band in 1061.0 cm^{-1} and due to stretching vibration of C–O–C bond. These bonds are also present in the levan polysaccharide with bands in 2940.2 and 2886.2 cm^{-1} (stretching vibration of C–H bond), band in 1059.0 cm^{-1} (stretching vibration of C–O–C) indicative of the presence of polysaccharide in the magnetic particles. Previous studies [33] reported that the characteristic absorption bands of the Fe–O bond of bulk magnetite were in 570 and 375 cm^{-1}. However, Ma et al. [4] observed that these two bands shift of about 600 and 440 cm^{-1} respectively, and the band near 600 cm^{-1} is split into two peaks of 631.4 and 582.9 cm^{-1}. Here, (Fig. 4) is also shows a band near 600 cm^{-1} split in two peaks of 631.2 and 565.6 cm^{-1} for magnetite. However, levan–magnetite particles presented a single broad band at 583.6 cm^{-1}. This little difference can indicate that interactions

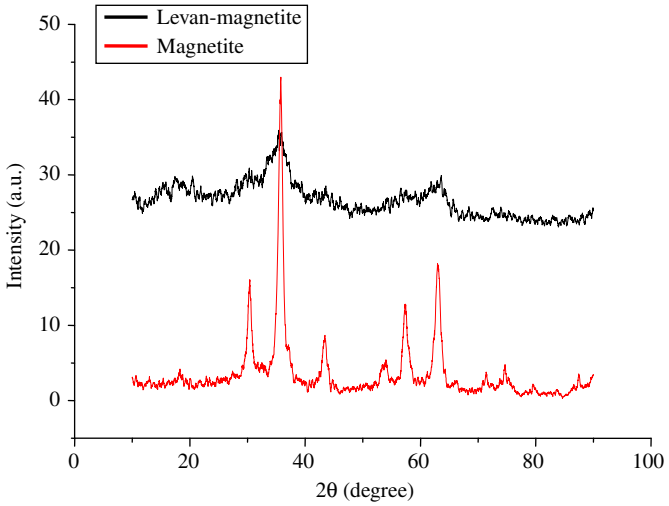

Fig. 3. X-ray powder diffraction patterns of magnetite and magnetic levan particles.

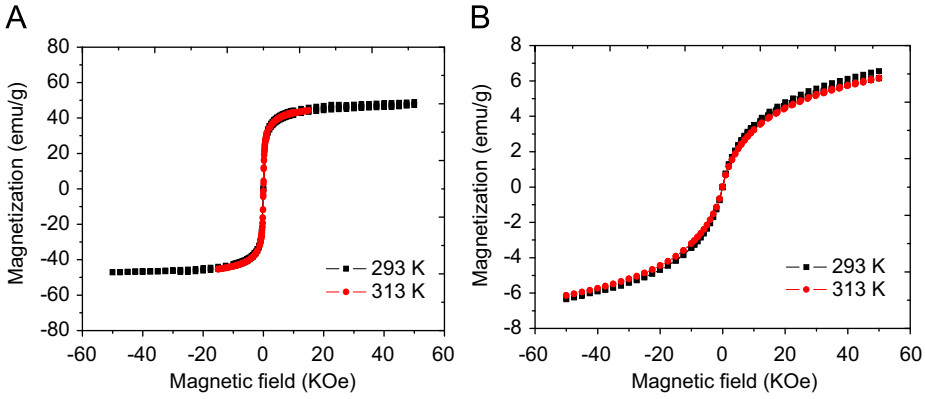

Fig. 2. Magnetization curves of magnetite (A) and magnetic levan (B) particles at 293 K and 313 K.

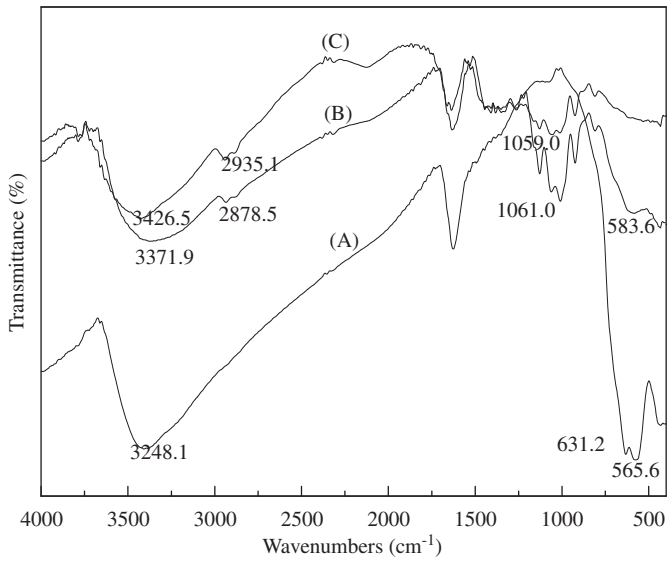

Fig. 4. FTIR spectra of the levan polysaccharide (A), magnetic levan (B) and magnetite (C).

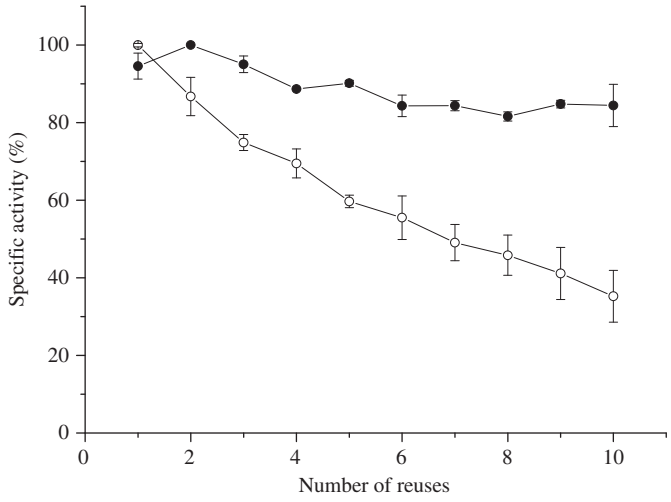

Fig. 5. Re-utilization of immobilized trypsin on magnetite (○) and magnetic levan (●) particles. The maximum specific activity of enzyme (100%) was 20.65 ± 0.69 mU/mg for trypsin immobilized on magnetic levan and 18.59 ± 0.08 mU/mg for trypsin immobilized on magnetite.

between coating (levan polysaccharide) and magnetite were intermolecular origins.

3.5. Application of the particles of magnetic levan for Bioprocessing

Trypsin was covalently immobilized on magnetic levan particles after partial oxidation of immobilized levan by the sodium periodate method (functionalization). Afterwards the trypsin was covalently bound to the oxidized levan [34]. The amount of immobilized trypsin was found to be 16.0 ± 2.8 μg/mg of matrix and the specific activity was 19.54 ± 1.34 mU/mg of protein when assayed under standard conditions using a low molecular weight substrate (BAPNA). The immobilized trypsin was reutilized 10 times and presented a mean activity equal to $84.43 \pm 5.42\%$ of the initial specific activity (Fig. 5). Meanwhile the adsorbed trypsin on magnetite (not coated) lost 64.75% of its initial specific activity after 10 reuses (Fig. 5). Therefore, coating [22] is important for immobilized enzyme performance providing higher stability. Neri et al. [35] using a different immobilized enzyme (β-galactosidase) and magnetic support (seminterpenetrating network of polysiloxane–polyvinyl alcohol composite) reported comparable retention after 10 reuses (84%). Similar results were also obtained by Amaral et al. [24] reusing immobilized trypsin on ferromagnetic Dacron (about 90%).

4. Conclusions

From the above displayed results one can conclude that magnetic levan particles presented larger size variation than magnetite particles due to the changes produced by coating. The presence of levan polysaccharide in the magnetic levan particles is suggested by FTIR characteristic absorption bands and by a broad band in each spectrum obtained from XRD diffractograms. The utilization of magnetic levan particles was shown to be efficacious for covalent enzyme immobilization as trypsin that can be reused several times without marked activity lost. Therefore, these magnetic levan particles can be proposed as alternative matrices for enzyme immobilization.

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