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Functionalization of γ-Fe_2O_3 nanoparticles through the grafting of an organophosphorous ligand

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ABSTRACT

An organophosphorous ligand terminated by an amine group has been used here for the first time to functionalize γ-Fe_2O_3 nanoparticles and to immobilize an enzyme onto their surface in a covalent way. The immobilization of β-glucosidase onto the maghemite nanoparticles was carried in this work out *via* a reductive amination reaction pathway which involves the terminal amine group on the ligand.

The enzymatic activity of the bound enzyme was evaluated in terms of the classical Michaelis–Menten kinetics. Indeed, the affinity of the bound enzyme for the substrate is preserved and is not affected by the high amount of enzymes onto the surface of the nanoparticles. Moreover, the related enzymatic activity slightly decreases compared to that of the free enzyme.

Such functionalized nanoparticles could help to improve the delivery and the recovery of biomolecules in biomedical applications by using a magnetic field. They could also provide a magnetic support which could be involved as a contrast agent, a biological label and a mediator for magnetic hyperthermia.

1. Introduction

Magnetic iron oxide nanoparticles (MIOP) have numerous applications in the biological field [1,2], especially as contrast agents for magnetic resonance imaging (MRI)[3] and mediators for magnetic hyperthermia [4,5]. Such nanoparticles can be dispersed in aqueous solution by grafting onto their surface some hydrophilic organic ligands [6,7] like carboxylate [8] or thiolate [9,10]. However, such ligands are not strong complexants of iron, and they may desorb with dilution [8,9,10,11]. Mutin et al. [12,13] showed that organophosphorus acids and their derivatives may be alternative and highly promising coupling molecules for the preparation of hybrid organic–inorganic materials, either by sol–gel processing or surface modification. Indeed, organophosphorous ligands (OPL) are actually known as excellent complexants of metallic ions and are frequently used in liquid–liquid extractions processes, particularly for concentration and purification of actinides, lanthanides and other polyvalent ions [14]. Recently, Yee et al. [15] have functionalized amorphous Fe_2O_3 particles with alkanesulfonic and octadecanephosphonic acids in ethanol, in order to study their magnetic properties towards functionalization. Very recently, Daou et al. [16] have successfully investigated the grafting of stilbene derivative with phosphonate on magnetite nanoparticles in tetrahydrofuran. Finally, Bellezza et al. [17] studied the catalytic properties of myoglobin (Mb) adsorbed on benzenephosphonate grafted-zirconia nanoparticles. All these results prompted us to use OPL instead of ligands containing oxygen or sulfur to functionalize MIOP for biological purposes. In this work, we have functionalized γ-Fe_2O_3 nanoparticles with an amine-ended OPL *via* a strong complexation surface reaction involving Fe—O—P bonds [13,16] and allowing to further couple in a covalent way an enzyme (the β-glucosidase) onto the surface of the MIOP. The activity of this enzyme was analyzed in terms of the Michaelis–Menten parameters. In this present study, we showed that OPL offer an attractive strategy in order to functionalize MIOP and to immobilize enzymes in a covalent way, with a good catalytic activity.

2. Experimental conditions

β-Glucosidase from almonds and 2-nitrophenyl-β-D-glucopyranoside were purchased from Fluka. Glutaraldehyde, salicylaldehyde, diethyl 4-aminobenzylphosphonate and sodium cyanoborohydride were purchased from Aldrich. Diisopropyl 4-

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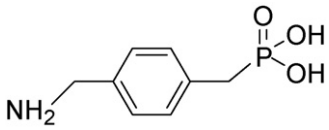

Fig. 1. Structure of the 4-(aminomethyl)benzylphosphonic acid ligand (**L**) used in this work.

(chloromethyl)benzylphosphonate was synthesized as previously described [18].

The UV–vis spectra were recorded on a Varian Cary 50 scan spectrophotometer in 1 mL quartz cells (optical length of 1 cm). The infrared (IR) spectra (DRIFT mode) were recorded on a Bruker Tensor 27 spectrophotometer and the samples for IR spectroscopy were dispersed in KBr. The zeta potentials were measured on a Malvern Instruments Zetasizer (nano-ZS) by using folded capillary cells. All transmission electron microscopy (TEM) images were obtained by using a JEOL 100 CX instrument (100 kV). The ^{1}H, ^{31}P, and ^{13}C NMR spectra were recorded in $CDCl_3$, with a Bruker AC 250 or a Bruker AC 400 spectrometers. The chemical shifts δ are expressed in ppm.

In order to synthesize 4-(aminomethyl)benzylphosphonic acid (**L** ligand), diisopropyl 4-(chloromethyl)benzylphosphonate (1 g, 3.28 mmol) in isopropanol (50 mL) with ammoniac (1 g, 55.6 mmol) were stirred during 18 h at room temperature. After evaporation of the solvent hydrobromic acid (9 mL, 48%) was added and the mixture was refluxed for 1 h. A white solid has appeared; after cooling, the solid, which corresponds to **L**, was obtained by filtration (yield 49%). ^{1}H NMR (D_2O, Na_2CO_3): 2.7 (d, J = 19.75 Hz, 2H, CH_2P); 4.45 (s, 2H, CH_2N); 7.16 (m, $4H_{ar}$). ^{31}P NMR (D_2O, Na_2CO_3): 18.8. ^{13}C NMR (D_2O, Na_2CO_3): 37.0 (d, J = 122.6 Hz, CH_2P); 63.8 (CH_2N); 126.7, 127.4, 129.2, 129.7 (C_{ar}). Melting point: 218 °C.

The maghemite nanoparticles were synthesized according to a procedure already described, superparamagnetic γ-Fe_2O_3 (maghemite) nanocrystals were prepared by alkaline co-precipitation of $FeCl_2$ and $FeCl_3$ salts [19]. γ-Fe_2O_3 nanocrystals were synthesized by oxidizing magnetite (1.3 mol) in nitric acid (2N, 1 L) containing iron nitrate (1.3 mol) with boiling. **L** (24,8 mg, 0.12 mmol) was dissolved in 15 mL alkaline solution (pH 9.8) and heated with the maghemite nanoparticles (0.3 mmol) for 30 min at 80 °C. The functionalization step occurred for a pH value of ca. 10.5. The functionalized nanoparticles were collected under magnetic field and washed twice with methanol. The recovered nanoparticles were dispersed in 5 mL of distilled water and were stable at room temperature for months. The amine-functionalized nanoparticles were mixed with an excess of glutaraldehyde (109 μL, 1.2 mmol) for 12 h under argon. The glutaraldehyde extensively washed nanoparticles were dispersed in 5 mL of 100 mM sodium acetate buffer (pH 5.0) and mixed with 800 μL of a β-glucosidase solution (10 mg/mL in 100 mM sodium acetate buffer at pH 5.0) for 6 h under argon, in the presence of $NaBH_3CN$ (10 mg, 0.16 mmol). After incubation, the nanoparticles carrying enzyme were collected by centrifugation (10 min at 7500 rpm), washed three times with 100 mM sodium acetate buffer (pH 5.0), and kept in 4 mL acetate buffer at 4 °C prior to use. The final iron content was checked by flame spectroscopy. The amount of non-adsorbed and free enzyme (ε = 76200 M^{-1} cm^{-1} at 278 nm) was determined spectroscopically as previously described [20].

3. Results and discussion

Fig. 1 shows the 4-(aminomethyl)benzylphosphonic ligand (**L**) that has been used in this work.

It consists of a phosphonic acid terminated by an aliphatic amine group. **L** has been deprotonated in alkaline solution and heated with a solution of γ-Fe_2O_3 nanoparticles which was prepared separately (Section 2). The resulting brownish powder, which was collected under magnetic field and washed to eliminate the uncoordinated ligand, corresponds to the γ-Fe_2O_3 nanoparticles functionalized by **L** (γ-Fe_2O_3**@L** nanoparticles). TEM image shows that the γ-Fe_2O_3**@L** nanoparticles (ca. 8.5 nm in diameter) are well dispersed in water at pH 5.3, as shown in Fig. 2.

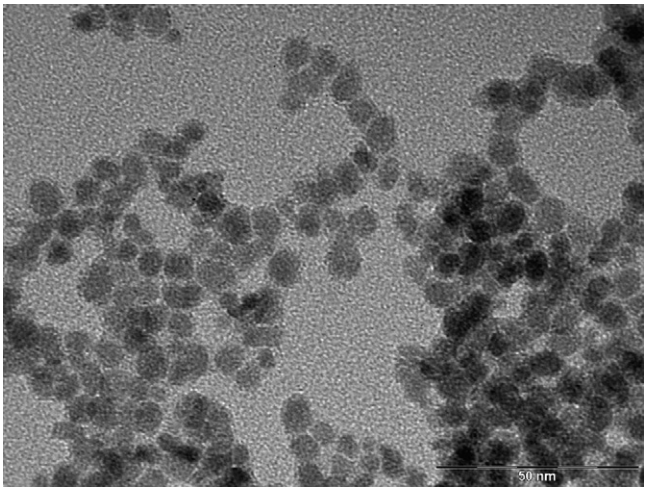

Fig. 2. TEM image of the γ-Fe_2O_3 nanoparticles functionalized with the **L** ligand.

At pH 5.15, the γ-Fe_2O_3**@L** particles exhibit a positive zeta potential of +6.3 mV. This positive zeta potential is probably due, in addition to the positive charge from the surface of the γ-Fe_2O_3 nanoparticles at pH 5.15, to the protonation, at acidic pH, of the amine groups available at the surface of the nanoparticles. This phenomenon may compete with the deprotonation of the phosphonic head (pKa ~ 3 and 6) [14] in the free ligands which are self assembled to the coordinated ligands *via* hydrogen interactions [21].

L and the γ-Fe_2O_3**@L** particles have been further characterized by IR spectroscopy (Supplementary material), as shown in Table 1.

The IR spectrum of **L** is essentially characterized by a strong band at 1226 cm^{-1} attributed to the P=O vibration band, and two strong bands at 997 and 943 cm^{-1}, attributed to the antisymmetric and symmetric P–OH vibration bands, respectively [14,22]. When going from **L** to γ-Fe_2O_3**@L** nanoparticles, the P–OH vibration bands disappear, and the P=O band is shifted from 1226 to 1090 cm^{-1} (Table 1), in line with the results published by Didi et al. [14] for similar ligands complexed with Fe^{3+} ions. In addition, a new and narrow IR band appears at 637 cm^{-1}, attributed to the P–O–Fe distortion vibration band, by comparison with the work of Didi et al. [14]. This is the proof that **L** is grafted onto the surface of the MIOP, probably in a tridentate mode involving some $P(OFe)_3$ units [22].

Another evidence of the grafting of the amine group onto the particles is provided by the action of salicylaldehyde (SA) on the γ-Fe_2O_3**@L** nanoparticles. Indeed, the dispersion of γ-Fe_2O_3**@L** nanoparticles with SA turns to a bright yellow color corresponding to the formation of an imine (maximum of absorbance at 385 nm) [23,24]. The γ-Fe_2O_3**@L** nanoparticles were separated from the

Table 1
IR bands of the free **L** ligand and the γ-Fe_2O_3 nanoparticles functionalized with **L**

	L[a]	γ-Fe_2O_3**@L**[a]
νP=O	1226	1090
νP–OH	997, 943	–
νP–O–Fe	–	637

[a] in cm^{-1}.