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Fixation of laccase enzyme into polypyrrole, assisted by chemical interaction with modified magnetite nanoparticles: A facile route to synthesize stable electroactive bionanocomposite catalysts

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ABSTRACT

Effective bio-electrocatalysts require stable immobilization of sufficient amounts of the bioactive component. In this study, a novel and efficient method for specific binding of laccase enzyme onto magnetite nanoparticles (NPs) is presented. The interaction between the chemically modified magnetite NPs and the enzyme was evidenced by both infrared (FT-IR) and X-ray photoelectron spectroscopy (XPS). Subsequently, the enzyme-coated magnetite NPs were successfully incorporated into polypyrrole (PPy) matrix during galvanostatic electropolymerization. The encapsulation of laccase covered NPs was proved by EQCN, TEM, and FT-IR spectroscopy; whereas the electrochemical behaviour of the formed bionanocomposite was characterized by cyclic voltammetry. In oxygen saturated solution a cathodic charge surplus was observed, related to the electrochemical reduction of oxygen. This surplus was two times higher in the case of the laccase containing layer compared to its only magnetite containing counterpart. Kinetic aspects of the oxygen reduction reaction (ORR) on the laccase containing films were investigated by hydrodynamic voltammetry, and the four-electron route was found to be exclusive, which is promising from the fuel cell perspective. Such synergistic combination of inorganic NPs and enzymes may open new avenues in the application of these bio-nanocomposite materials.

1. Introduction

Upon the continuously growing population, energy demand, and waste production; disciplines of Green Chemistry come more and more to the front. Therefore use of biocompatible, biodegradable materials, as well as employment of reactions involving relatively mild conditions is of prime importance. As the most selective and sometimes even considerably effective catalysts of Nature, enzymes are widely used in various catalytic processes and bio-sensing applications. Electroanalysis of different analytes (e.g., hydrogen-peroxide, glucose, and cholesterol), synthesis of organic molecules, and fuel-cell cathodes are equally important examples for practical utilization of enzyme based systems [1–3].

Although enzyme-activity can be exploited even in solution phase (as homogeneous catalysts), decay in performance, lack of reusability, and cost efficiency all hamper their use in such configuration [4]. On the other hand, through their immobilization on electrode surfaces, chemically-modified electrodes can be obtained, which can be used in various electrochemical procedures [5]. Most simply, adsorption of biomolecules can be facilitated by physical interactions, either on flat substrates as monolayers or in layer by layer assemblies [6–8]. Physisorption, however, sometimes harmfully affects the supramolecular structure and hence the activity of the enzyme [9,10]. One feasible approach to avoid such deactivation is the formation of specific (generally covalent) bonds between the substrate and the biomolecule (chemisorption). These interactions can be expedited by proper functionalization of the substrate, or by using a small molecule as linker [11]. Performance of such hybrids can be further enhanced by increasing the contact area of the enzyme with the analyte. In this vein, by replacing planar substrates by either nanoparticles [12,13], or nanostructured porous materials [14–16], electrocatalytic efficiency can be multiplied.

Note that direct immobilization of biomolecules on metal electrodes is hindered by different factors, most importantly by the distortion of their supramolecular structure. Therefore, to preserve the activity of an enzyme, their encapsulation into different matrices, mostly into conductive polymers is favoured. Nafion© and polydimethylsiloxane are also good examples [17,18], but using intrinsically conducting polymers (CPs) several additional benefits can also be harnessed [19,20]. In fact, CPs themselves are eminent

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candidates for different catalytic applications [21–23], while they are also efficient transducers, promoting the activity of the enzymes by fastening the electron transfer to/from the substrate [24,25].

Encapsulation of enzymes into CP-matrices can be performed on the basis of different interactions: either physisorption or specific chemisorption can take place between the monomer/polymer and the biomolecule. As for the preparation method, in situ chemical or electrochemical polymerization as well as layer by layer assembling approach (using pre-synthesized polymer) can be employed to obtain enzyme/polymer hybrids [26]. Finally even enzymes themselves can act as polymerization agent (oxidant), and this way get entrapped during the polymerization process [27–29].

Incorporation of enzyme-coated inorganic NPs (possibly also having intrinsic electrocatalytic activity) is an even more sophisticated variant of this method. This way, the NPs act not only as enzyme-carriers, but the catalytic activity and electrochemical performance of the individual components can be mutually reinforced. Such synergy was demonstrated for a large variety of CP-based hybrid assemblies [30].

Laccase enzyme, a copper containing oxidoreductase, can catalyse the oxidation of various compounds, while in the presence of molecular oxygen this later is reduced in a four electron process to water [31]. In fact, laccase is a prominent candidate for biofuel cell applications, as active component of the cathode [32–35]. In this manner, several attempts have been taken to immobilize laccase in CP matrices so far. Physical adsorption, chemical interaction, enzymatic polymerization as well as layer by layer method were applied [36]. A simple method demonstrated recently how to enrich laccase enzyme between two electrodeposited PEDOT layers (sandwich structure), with notable activity in ORR [37–40]. Moreover, laccase enzyme, immobilized on different particles (e.g., gold, glass) was incorporated into CPs [28,41].

Iron oxide nanoparticles, such as Fe_2O_3 or Fe_3O_4, are viable substrates for enzyme immobilization, based on the ease of surface modification [42–44]. In particular, laccase was immobilized on magnetite NPs by different methods [45–47]. Note, however, that these synthetic methods involve multiple steps (and coupling reagents) and often take several days to be implemented.

It was also demonstrated earlier that incorporation of iron-oxides can enhance catalytic activity of CPs, presumably because of the redox switching between Fe^{3+}/Fe^{2+} [48,49]. In this study we developed a quick and reproducible method to immobilize laccase enzyme on magnetite NPs, and for the subsequent incorporation of the coated NPs into a CP, namely into PPy. To the best of our knowledge, no report has been published on such system before.

2. Experimental

2.1. Materials

Magnetite (Fe_3O_4) NPs were synthesized by alkaline hydrolysis of iron(II) and iron(III) salts ($FeCl_2{\cdot}4H_2O$ and $FeCl_3{\cdot}6H_2O$, Sigma-Aldrich). The synthesis resulted in spherical shaped magnetite NPs with an average diameter of 11 nm (as confirmed by XRD and TEM results, not shown here). Details of the synthetic procedure as well as characterization of the particles were reported elsewhere [50,51].

Analytical grade pyrrole (Py) monomer, potassium tetraoxalate dihydrate (PTO) and laccase enzyme (Trametes versicolor; ≥10 U/mg) were purchased from Sigma-Aldrich, while analytical grade sodium chloride (NaCl) was purchased from VWR International. Pyrrole was freshly distilled under vacuum before use.

Oxalate-moieties were introduced to the magnetite surface by a 100 s treatment with 0.05 M PTO in deionized water (magnetite content was 10 g dm^{-3}). The surface-modified NPs were magnetically separated, and washed several times to reach neutral pH. Laccase enzyme was then added (0.6 g dm^{-3}) to the dispersion of the functionalized NPs (10 g dm^{-3}). After a given time of interaction (see below), the enzyme covered magnetite (Fe_3O_4/laccase) was magnetically separated from solution, and washed several times with deionized water to remove the excess, non-attached enzyme traces (Scheme 1.).

All polymerization solutions contained 0.1 M of the pyrrole monomer, 0.1 M NaCl and 0.001 M PTO in deionized water. The amount of magnetite NPs (either surface modified, or unmodified) was 10 g dm^{-3}. Polypyrrole (PPy), polypyrrole-magnetite (PPy/Fe_3O_4) and polypyrrole-magnetite-laccase (PPy/Fe_3O_4/laccase) composite films were deposited galvanostatically (j = 3 mA cm^{-2}) with a charge density of 300 mC cm^{-2}. For further voltammetric studies the solution was changed after the polymerization to phosphate buffer solution (pH = 7, c = 100 mM). $NaH_2PO_4{\cdot}H_2O$ and $Na_2HPO_4{\cdot}2H_2O$ were purchased from Reanal.

2.2. Methods

All electrochemical measurements were performed on a PGSTAT 10 (Autolab) instrument, in a classical three-electrode electrochemical cell, kept in a dark box. Glassy carbon disk (A = 0.07069 cm^{-2}) and Pt ring were used as working and counter-electrodes, respectively. The reference electrode was an Ag/AgCl/3 M NaCl electrode, having a potential of 0.200 V vs. SHE.

Electrochemical characterization was performed in a closed cell, in neutral phosphate buffer solution (c = 100 mM, pH = 7). Inert atmosphere was created by N_2 gas bubbling, while O_2-saturated atmosphere was ensured by O_2 gas purging through the solution before, and between the measurements for 30 min.

An Autolab Electrochemical Quartz Crystal Nanobalance (EQCN) module was employed for nanogravimetric measurements, using gold-coated quartz crystal electrode (f_0 = 6 MHz, A = 0.361 cm^2). The EQCN system was calibrated by electrochemical lead deposition using the standard procedure, and a coefficient value of 5.04 ng Hz^{-1} was obtained.

FT-IR studies were performed using a Bio-Rad Digilab Division FTS-65A/896 Fourier transform infrared spectrometer equipped with a Harrick's Meridian® SplitPea single-reflection diamond attenuated total reflectance (ATR) accessory. All infrared spectra were recorded between 400 and 4000 cm^{-1}, at 4 cm^{-1} optical resolution, by averaging 128 interferograms.

X-ray photoelectron spectra (XPS) were recorded on a Kratos Axis Ultra X-ray Photoelectron Spectrometer at room temperature, using an Al mono source. The gun was operated at 15 kV and 7 mA. Data was taken with 0.1 eV step size and 0.1 second dwell times. Pass energy for high resolution data (e.g., O1s, C1s, N1s, Fe2p) was 10. Data were calibrated using the standard value for C 1s at 285 eV.

Transmission electron microscopic (TEM) investigation of the composite layer after peeling it off from the electrode was performed using a FEI Tecnai G^2 20 X-Twin type instrument, operating at an acceleration voltage of 200 kV.

3. Results and discussion

3.1. Surface treatment and laccase immobilization

Laccase immobilization was carried out by following the sequence shown in Scheme 1. To form strong chemical bonds between the magnetite and the enzyme, surface of magnetite nanoparticles was modified by potassium tetraoxalate treatment for 100 s. The interaction between the as formed oxalate-functionalized oxide surface and the enzyme was monitored by ex situ ATR-FT-IR spectroscopy.