Self-Assemblies of Magnetic Nanoparticles (MNPs) and Peroxidase Enzymes: Mesoporous Structures and Nanoscale Magnetic Field Effects (nano-MFEs) for Enhanced Activity BioNanoCatalysts (BNCs).

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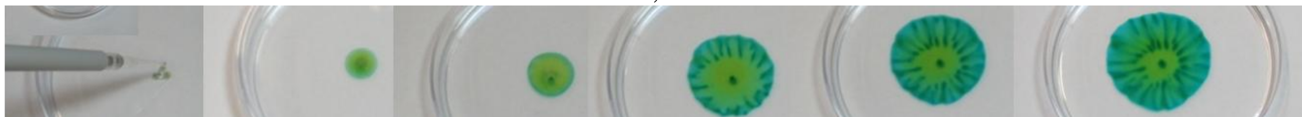

ABSTRACT

Bionanocatalysts (BNCs) are self-assembled mesoporous clusters of magnetic nanoparticles (MNPs) embedding enzymes functioning with small diffusible substrates and reaction products, which includes oxido-reductases and biochemical systems of thereof . Oftentimes immobilization of these valuable enzymes with high biotechnological relevance by adsorption, complexation or covalent linking results in the alteration of native structures leading to drastic loss of catalytic activities. In contrast, within the nanopores of the BNCs, these enzymes are showing increased activities compared to their free counterpart due to faster turnover and/or lower inhibition. In addition, they can be readily recovered and reused as catalysts for free-radical mediated reactions.

Keywords: bioproducts, bioremediation, green catalysts, green chemistry, magnetic nanoparticles, peroxidases.

1 INTRODUCTION

Dedicated enzymes, and enzymatic systems, for medical, industrial and environmental applications are key to the development of novel processes that can meet the requierements for more efficient, milder, economical, and overall greener chemistry. Although tremendous progress has been made in the fields of genetics, protein engineering and expression systems to deploy relevant enzymes as chemical reagents, many biochemical systems are eluding their full large-scale potential due to cost, inhibition and efficiency limitations. Oxido-reductases, especially peroxidase, are among the most interesting enzymes for industrial applications because of the diversity of biochemical reactions they can perform (polymerization and depolymerization) and the broad array of aromatic substrates they can use. Increasing their activities and efficiency, reducing their inhibition, broadening the range of conditions to use (and re-use) them constitute critical steps to develop new high efficiency biocatalysts within the paradigms of Green Chemistry and Sustainability [1].

Our work has previously demonstrated that the activity of peroxidase enzymes can be increased in presence of magnetic nanoparticles [2] likely due to Magnetic Field Effects localized at the the nanoscale (nano-MFE); we recently demonstrated that the mesoporous nature of this self-assembled structure dictates their unique properties [3]. We are engineering new ultrastuctures to target relevant applications in the fields of bioremediation (phenolic contaminated water) and production of aromatic monomers as chemical feedstocks from lignocellulosic materials.

2 MATERIAL AND METHODS

2.1 Synthesis of magnetic nanoparticles

MNPs were synthesized by co-precipitation of Fe^{2+} and Fe^{3+} under alkaline conditions in a bubbling nitrogen atmosphere. Temperature was controlled to control the size of the nanoparticles. An acidic solution (25 ml) of the iron salts (2 g of $FeCl_2 \cdot 4H_2O$ and 5.2 g of $FeCl_3 \cdot 6H_2O$) was added dropwise to NaOH (250 ml, 1.5 M) under constant stirring. Non-oxidizing conditions were achieved by bubbling all solutions with nitrogen for 15 minutes prior to reaction. The instantaneous black precipitation of Fe_3O_4 was captured with a neodymium magnet, washed and neutralized, and kept in distilled water until further use.

2.2 MNPs functionalization and gold coating

The coating of magnetite nanoparticles was achieved by mild reduction of gold tetrachloroaurate ions onto O-phosphorylethanolamine (OPEA) functionalized MNPs under constant sonication. The coating procedures were performed under nitrogen using a modified rotary evaporator apparatus coupled to a sonic bath. Briefly, 20 mg of MNPs (1mg/ml) were sonicated for 30 min. OPEA (2g) was added and allow reacting with the MNPs (40 ml final volume) under sonication. MNPs@OPEA were rinsed and resuspended in milliQ water. Gold tetrachloroaurate was added to the MNPs@OPEA and the temperature was raised to 85 °C at which point citric acid/citrate (pH5.5)

was added. Upon addition of the citric acid, the batch turned deep red then purple indicating the formation of the gold shells.

2.3 Enzymes and Bionanocatalyst self-assemblies

Horseradish Peroxidase (HRP, E.C. 1.11.1.7, type VI-A) was purchased from Sigma Aldrich. Its Reinheitszahl index (OD A_{403}/A_{280}) was found to be around 2.9. Lignin Peroxidase (LiP, E.C. 1.11.1.14), Manganese Peroxidase (MnP; E.C. 1.11.1.13) and Versatile Peroxidase (syn. hybrid peroxidase, manganese-lignin peroxidase: VeP EC 1.11.1.16) were purchased from Jena Bioscience (Germany) and further purified by FPLC (AKTA Explorer, GE Bioscience) using an anionic exchange column (Resource Q, GE Bioscience). BNCs were synthesized by mixing the magnetic nanoparticles with the peroxidase enzymes at specific ratio. Nanoparticles were sonicated for 20 min prior to use. BNCs were formed overnight under constant agitation at 4°C unless indicated otherwise. Quantification of entrapped enzymes within magnetite nanoparticles was carried out using quantitative high-throughput FTIR (HTS-XT-Vertex70, Bruker, Germany).

2.4 Biochemical assays

The peroxidase activity of native HRP and BNC was monitored using the chromogenic phenol/AAP assay that generates phenoxy radicals reacting with aminopyrene to form the pink-colored quinoneimine dye. An automated plate-reader (Synergy 4, Biotek) with injection capabilities and temperature-controlled chamber was used to record the absorbance of the solution at 510 nm in 96-well plates (4 replicates) for 30 min. Hydrogen peroxide was used in concentrations ranging between 10^{-7} M and 1 M.

High-troughput colorimetric assays for fungal peroxidases were performed in 96-well UV transparent microplates (Falcon) using standardized colorimetric protocols in sodium tartrate or malonate (LiP: Veratryl alcohol, pH 3, 310 nm; MnP: 2,6 dimethoxyphenol, pH 4.5, 468 nm and 270 nm for the formation of Mn^{3+}-organic acid complex; VeP: veratryl alcohol or 2,6 methoxyphenol, pH 4.5, 310 nm, 468 nm, 270 nm).

2.5 Lignin depolymerization and phenolic polymerization methods

A Kraft lignin depolymerization assay was conducted using HRP, LiP, VeP and MnP, and BNCs thereof. MnP and VeP assays were performed in presence of manganese. Assays were performed in triplicates in sodium tartrate or sodium malonate buffer, pH 5.5. Kraft lignin slurries (10mg/ml) were incubated for 1 or 4 hours then filtered through 0.2 µm pore membrane filter to remove particulates. The UV-Vis spectra of the solutions were acquired with a Biotek Plate Reader. The release of aromatic molecules from lignin depolymerization was monitored at 280 and 310 nm. The spectra were corrected for the background.

The phenol removal assay was a two-step procedure. The first step consisted in forming polyphenols with Horseradish Peroxidase in 1 mM PBS buffer. Reaction volumes were fixed at 2 ml or 10 mL. The final concentration of phenol was fixed at 1 mM and HRP at 30 nM. The BNCs formed with M90 were varying in enzyme-to-nanoparticles ratio. The second step was the precipitation of these polyphenols by adding sodium chloride (500 mM). The samples were centrifuged at 12,000 g for 20 minutes and the supernatant collected. The soluble phenol in solution was measured at 280 nm with a Biotek Plate Reader.

2.6 Kinetic Parameters

The specific activity, A ($mmol_{product}$. s^{-1}.$mmol_{HRP}^{-}$1), was calculated as the ratio V/$mmol_{HRP}$ using the extinction of the respective products formed at the wavelength monitored. A H_2O_2 substrate inhibition model derived from the ping-pong bi-bi 2 substrate inhibition model was used to extract the kinetic parameter of the reaction The modified equation from the model is:

$$V = \frac{V_{max}\,[H_2O_2]}{K_m + [H_2O_2](1 + \frac{[H_2O_2]}{K_i})} \quad (1)$$

where V_{max} is the maximum enzyme velocity ($mmol.s^{-1}$), the maximum rate the enzyme reaction can achieve, expressed in the same units as V, K_m is the Michaelis-Menten constant (mM), K_i is the inhibition constant for H_2O_2 (mM).

3 RESULTS AND DISCUSSION

3.1 MNPs and BNCs characterization

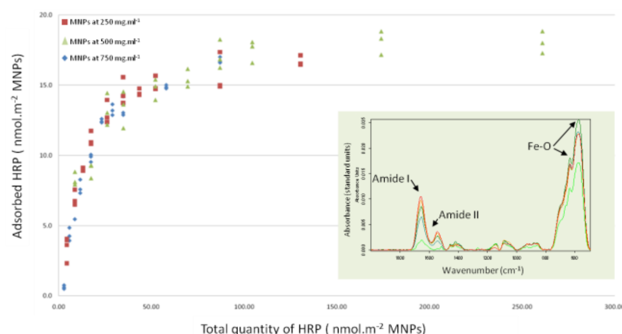

Figure 1: Quantification of entrapped Horseradish Peroxidase (HRP) by QHT-FTIR for M90 expressed as immobilized HRP standardized per total surface area. Insert: FTIR spectra of M90-BNCs used for the standardization .

MNP synthesis requires low cost reagents and can be easily tailored for size and magnetic characteristics by controlling the temperature during the precipitation. M25 MNPs (formed at 25°C) are smaller and mostly monodispersed forming small clusters and free nanoparticles whereas M90 (formed at 90°C) are bigger and form large clusters of 100 nm in diameter on average with high affinity for the enzymes (Fig. 1) Their superparamagnetic properties allow for fairly monodispersed MNPs in water. The presence of the enzyme triggers the self-assembly of the mesoporous BNCs by electrostatic charge compensation (Fig. 2).

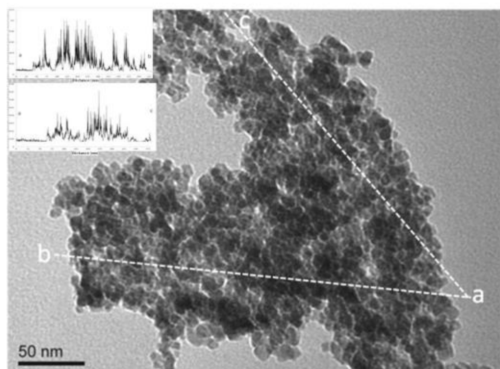

Figure 2: Representative TEM of M90-BNCs clusters (1nM HRP) at 4 μg.ml^{-1} of MNPs, Transects (background corrected pixel intensity) are presented to illustrate nanopore distribution within the MNPs cluster.

The size and magnetization of the MNPs affect the formation of the BNCs the size and mesoporous volume of the clusters after enzyme entrapment. The aggregates formed with M90 MNPs contain more enzyme than the ones formed with M25 MNPs. Under 10 nmol.m^{-2} of enzyme in solution, 100% of the enzymes are entrapped in the clusters formed with M90, while about 50% are trapped in the M25 clusters.

3.2 Peroxidase activities of BNCs

Initial reaction velocities were used to estimate K_m, V_{max}, K_i from Eq. 1 (Fig.3). The V_{max} of M25 or M90 MNPs was several orders of magnitude lower than those of the free HRP and BNCs. The turnover rates, k_{cat}, were very consistent with free HRP, M25-BNC, and M90-BNC datasets. BNC formed with M25 and M90 both had a higher K_m. M25-BNC had V_{max}, and k_{cat} two to three times greater than the free enzyme at the same concentration. Also, the k_{cat} of M25-BNCs increased with the fraction of bound enzyme while the K_i was in the same range than the free HRP. M90-BNC had a K_i about 10 times greater than the free HRP, while its V_{max} was similar. The higher K_i for M90 indicated the lower extent of substrate inhibition from H_2O_2 compared to the free enzyme and M25-BNCs.

Similar results were achieved with fungal peroxidases using gold-coated nanoparticles resistant to acidic conditions and organic chelating agent (Fig. 4).

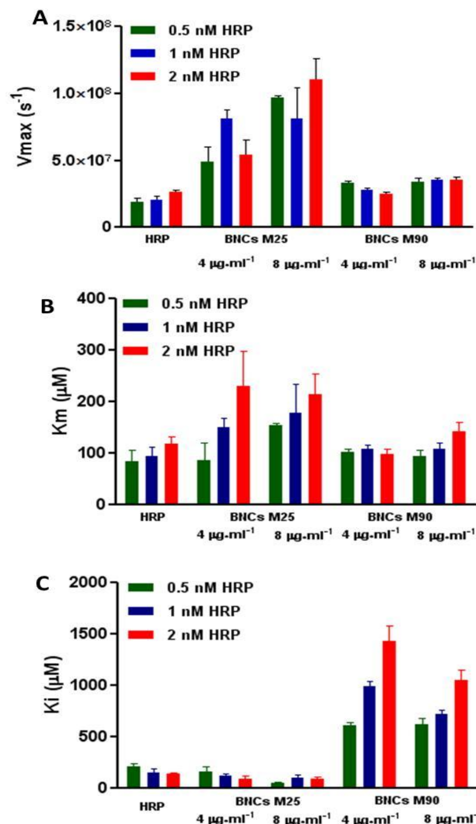

Figure 3: Kinetic parameters of free HRP and BNCs formed with HRP and M25 or M90 :A- Maximal Velocity (Vmax), B- Substrate affinity (Km) and C- substrate inhibition (Ki). (Phenol/AAP assay)

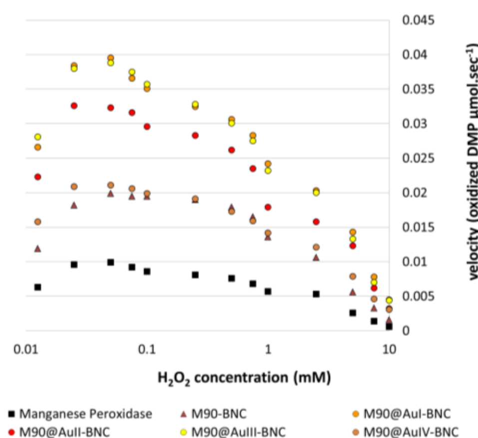

Figure 4: Velocity plot of manganese peroxidase and M90 gold-coated BNCs for the oxidation of dimethoxyphenol (2.5 nM MnP, DMP and Mn^{2+} at 1 mM in 50mM malonate)

3.3 BNCs for lignin depolymerization

Lignin depolymerization using chemically resistant gold coated BNCs was demonstrated. A lignin depolymerization method was developed in order to detect the production of soluble aromatics (e.g., coniferyl, sinapyl, and coumaryl alcohols or derivatives thereof). Increased absorbance was observed at characteristic wavelengths of aromatic compounds with the fungal peroxidase system (Fig. 5). The BNCs formed with horseradish peroxidase (plant peroxidase) did not release any aromatic molecules from lignin in the conditions of the assays.

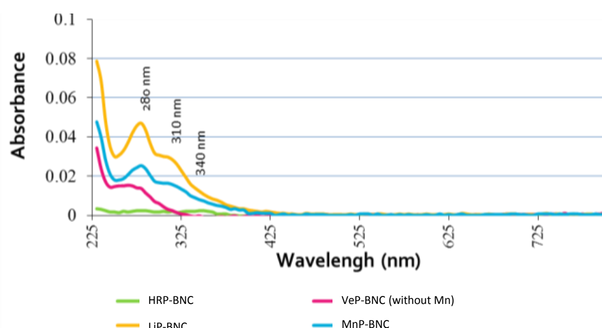

Figure 5: Increase in release of soluble aromatics from Kraft lignin by BNcs of magnetite and Horseradish peroxidase, Versatile Peroxidase (VeP), Lignin Peroxidase (LiP) and Manganese Peroxidase (MnP)

3.4 BNCs for remediation of phenolics

Phenol removal using HRP-BNCs was demonstrated. Phenol polymerization assays were conducted using a two-step process: (i) enzymatic polymerization to polyphenols, and (ii) condensation of the polyphenol polymers by sodium chloride.

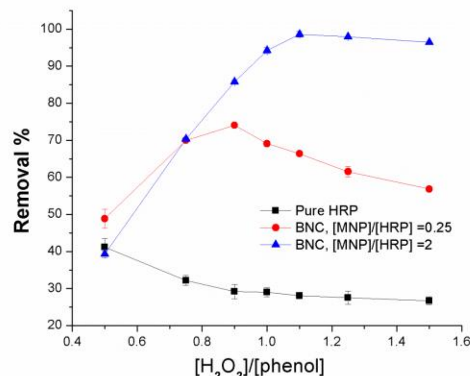

Figure 6: Effect of Peroxide to Phenol ratio on phenol removal by magnetite M90-BNCs formed with Horseradish peroxidase at different MNPs to Enzyme ratio.

As shown by the activity plot in Fig. 6, the BNCs formed with HRP and M90 MNPs were more efficient at removing the phenol than the free enzyme. The foregoing result demonstrates a marked improvement in phenol removal when using BNCs rather than a free HRP system.

Besides the increased extent of phenol removal, there was an optimal H_2O_2 concentration shift for maximal removal (or polymerization) with the BNC system. This indicated that BNCs have a lower inhibition from H_2O_2 compared to the free enzyme a sexpected. Moreover, the BNC system offers a broader H_2O_2 concentration range. These features demonstrate that BNCs can be used in unstable and harsher process conditions than their free enzyme counterpart.

4 CONCLUSIONS

Entrapped peroxidase enzyme systems within the mesoporous clusters of MNPs offer powerful alternatives to exploit free-radicals driven secondary biochemical reactions. Interestingly, BNCs also increases the range of conditions the enzymes can be used under. Once formed, the clusters are stable and the increase in activities is maintained at different pHs, temperatures and ionic strengths. This protective effect against detrimental reaction conditions is observed for BNCs with higher clusterization and mesoporous volume.

Since the BNCs showed surprising resilience in various reaction conditions and can be accommodate a diversity of enzymatic systems, they pave the way towards new hybrid biocatalysts that can be designed and engineered from the bottom-up towards biotechnological to industrial-scale applications. New ultrastructures for re-usability, synthetic catalytic systems, and applications will be presented based on the concepts of mesoporous magnetic bionanocatalysts.

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