Conjugates of Magnetic Nanoparticle-Enzyme for Bioremediation

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ABSTRACT

Enzymes are proteins that function as biocatalysts in bioremediation. One of the major concerns in environmental applications of enzymes is their short lifetime. Enzymes lose their activity due to oxidation, which results in less stability and a shorter lifetime thereby rendering them less efficient. An effective way to increase the stability, longevity, and reusability of the enzymes is to attach them to magnetic iron nanoparticles. If enzymes are attached to the magnetic iron nanoparticles then we can easily separate the enzymes from reactants or products by applying a magnetic field. With this aim, two different catabolic enzymes, trypsin and peroxidase, were attached to uniform core-shell magnetic nanoparticles (MNP's), produced in our laboratory. Our study indicates that the lifetime and activity of enzymes increases dramatically from a few hours to weeks and that MNP-Enzyme conjugates are more stable, efficient, and economical. We predict that MNPs shield the enzymes preventing them from becoming oxidized. This results in an increased lifetime of the enzymes. Because of the high magnetization (~140 emu/g) of our MNPs, nanoparticle-enzyme conjugates can efficiently be magnetically separated, making enzymes more productive. We also found that the enzyme structure plays a major role in efficient attachment of MNPs

Keywords: enzyme, magnetic nanoparticle, conjugate, bioremediation, environmental application

1 INTRODUCTION

Small size, high surface area and low toxicity has made magnetic iron nanoparticles most promising element for various fields such as biomedical and environmental applications. Since the mid-1970s, MNPs have been widely studied for their applications in various areas of biomedical science from therapeutic agent targeting and magnetic resonance imaging (MRI) to cell separation and purification [1]. Drug targeting and delivering, gene therapy, and hyperthermia are active biomedical research areas with great promise for disease treatment. But much less effort has been applied to developing MNPs in the environmental research field. Recently, a field demonstration was performed in which nanoscale (100-200 nm in diameter) bimetallic (Fe/Pd) particles were gravity-fed into groundwater contaminated by chlorinated aliphatic hydrocarbons [2]. The nanoparticles were found to be uniquely suited for subsurface delivery and dispersion and rapidly degraded chlorinated contaminants such as trichloroethylene (TCE) by reductive dehalogenation during oxidation of the zero-valent metals.

Contaminations in soil and water are major concerns of environment. For example, 2, 4, 6-trinitrotoluene (TNT) is anthropogenic pollutants present in environment. It's found in solid form and it slowly dissolves, gradually polluting soil and groundwater. Pure iron Fe(0) nanoparticles can biotransform one or more nitro group of TNT into amino group detoxifying the area, which is contaminated because of TNT [7, 9]. Presence of enzymes in a biochemical reaction accelerates the rate of reaction. During reaction enzyme retain their property, thus they can be cost effective if we could reuse them [14]. Enzymes can be reused if we can immobilize them by attaching them on a solid surface, this will make it easier to separate enzymes from the solution. If enzymes are attached to the magnetic iron nanoparticles then we can easily separate the enzymes from reactants or products by applying a magnetic field. Short lifetime of enzymes outside the living cell also limits their applications [9]. Attempts have been made repeatedly to increase the stability of enzymes by encapsulating biomolecules in silica gels but repeatability and long term stability still remains a concern [16]. Lack of stability of enzymes during storage is also one of the issues with enzymes.

The magnetic nanoparticle-enzyme conjugates (MNP-Es) will have a major advantage over metal-only particles such as those described by Elliot and Zhang [2] that react stoichiometrically with substrates in equimolar reactions rather than catalytically; zero-valent metals are quickly consumed by water passivation and/or contaminant reduction. In contrast, particle-bound enzymes, when stabilized to prevent protein degradation, can act as true catalysts, turning over many moles of substrate molecules before ultimate enzyme inactivation. Moreover, immobilization of bioactive molecules on the surface of magnetic nanoparticles is of great interest because the magnetic properties of these bioconjugates promise to greatly improve the active delivery, recovery, and control of biomolecules in environmental and other applications.

Rossi et al. [3] covalently conjugated the enzyme glucose oxidase to 20-nm (diameter) magnetite (Fe_3O_4) nanoparticles in their development of glucose sensors. The iron oxide nanoparticles were derivatized with amino groups using 3-(aminopropyl)triethoxysilane, and glucose

was attached to the amino linkers. The amount and activity of the immobilized enzyme was increased relative to that with only physical adsorption processes. Covalent immobilization also increased the stability of the enzyme. The nanoparticles maintained their bioactivity at 4°C for up to 3 months. The direct binding of an *Aspergillus niger* glucose oxidase via a carbodiimide linkage to magnetic nanoparticles was found to be very effective, resulting in bound enzyme efficiencies between 94-100% and increased enzyme resistance to thermal and pH-dependent denaturation [4]. The same reaction was used to examine cholesterol oxidase (CHO) properties after binding to Fe_3O_4. Stability and activity of CHO was enhanced after attachment to magnetic nanoparticles, improving the potential for use of this enzyme in various biological and clinical applications [5]. Ohobosheane et al. [6] demonstrated biochemical modification of silica-based nanoparticles whose surfaces were linked to glutamate dehydrogenase and lactate dehydrogenase allowing them to function as biosensors and biomarkers. The immobilized enzyme molecules were shown to retain excellent enzymatic activity in respective reactions.

Here we reported a new method to cross linking enzymes with bifunctional reagents which help in increasing the life time of enzyme; this process involves the crystallization of enzymes Proteins sometimes don't get efficiently crystallize or proteins are locked during crystallization making them inactive [17]. We have found an efficient way of binding enzymes. Attaching enzymes to magnetic iron nanoparticles extends their lifetime from few hours to weeks. In this paper, we described how surface area plays a major role in attaching enzymes to the nanoparticles and how iron nanoparticles help in immobilizing enzymes.

2 EXPERIMENT

Monodispersive core-shell iron nanoparticles were produced using third generation cluster deposition apparatus [10-12]. The size of the nanoparticles was controlled by varying the growth distance, power, and helium and argon gas ratio. Uniform ~20 nm size iron nanoparticles were deposited on a plastic substrate. The nanoparticles were then removed from the plastic surface and collected in the solution of pH 7. Magnetic moment of the iron oxide nanoparticles produced in our lab is ~140 emu/g (4). Fig. 1(a) shows a TEM image of ~20 nm iron nanoparticles before enzymes were attached, and Fig. 1(b) is the image of nanoparticles uniformly dissolved in the solution.

Two catabolic enzymes were attached to the nanoparticles namely trypsin and horseradish peroxide C (HRP). To prevent denaturation and leaching nanoparticles were coated with 3-aminopropyl triethoxy silane, thus prolonging the stability of the magnetic nanoparticle. The first reaction shown in Fig. 2 is an example of silanization of MNP passivated with ferric-oxyhydroxy-polymer with 3-aminopropyltriethoxysilane. Commercially available cross-linking agents were used to attach activated enzymes to nanoparticles. Four different coupling reagents: **SANH** (succinimidyl 4-hydrazinonicotinate acetone hydrazone); **C6-SANH** (C6-succinimidyl 4-hydrazinonicotinate acetone hydrazone); **SFB** (succinimidyl 4-formylbenzoate) and **C6-SFB** (C6-succinimidyl 4-formylbenzoate) were used. These reagents prevent homopolymerization of MNP's and enzymes, and provide variability in spacer-arm length from 5.8 to 14.4 Å.

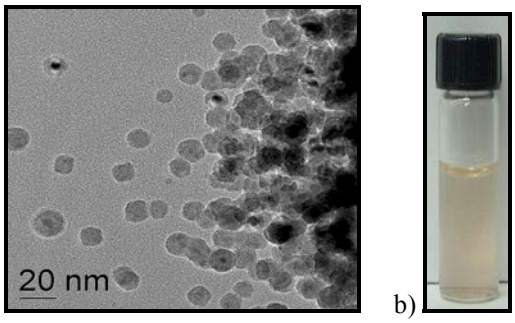

Fig. 1: TEM image of ~20 nm iron nanoparticles, (b) is the image of the nanoparticles uniformly dissolved in the solution of pH 7.

This length variability results in MNP-E conjugates with different rotational properties, which in turn influences enzyme active center accessibility and directly affect MNP-Es enzymatic activity. The SANH converts primary amines to hydrazinopyridine moieties and SFB converts primary amines to benzaldehyde moieties. These two moieties cross-link producing final MNP-E congeners. The random polymerization of multiple enzyme molecules or MNP particles is avoided. The C6-SANH and C6-SFB contain six-carbon aliphatic linkers between functional groups.

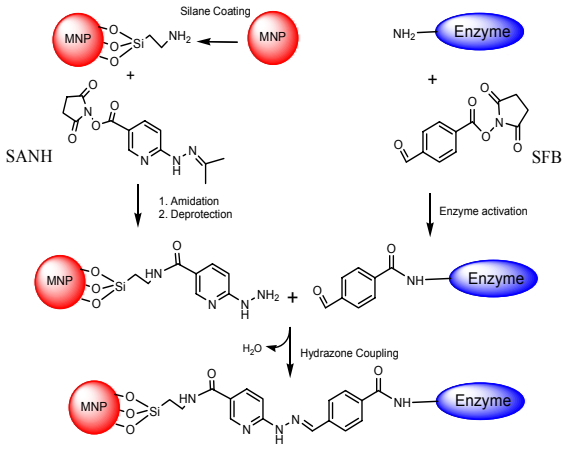

Fig. 2: Reaction scheme used for producing MNP-Es using amino-silane-coated MNP's.

The enzymes were covalently linked with nanoparticles by reacting them separately with amino-silane or peptide coated nanoparticles. After the modifications the MNP's

were purified of excess reagent. The hydrazine/hydrazide-modified MNP's were reacted with the aldehyde-modified molecule to yield the desired MNP-E conjugates (Fig. 2). Both reaction mixtures (enzyme + SANH and MNP's + SFB) were incubated for 3 hours at room temperature with no shaking in buffer (pH=7.3). The concentration of SFB and SANH were in 10 molar excess of protein or MNP's. After activation the excess of the heterobifunctional coupling agent and the buffer replacement (to the conjugation buffer, pH=4.7) was performed in single chromatographic step using a Sephadex G25, PD10 column.

3 RESULTS AND DISCUSSION

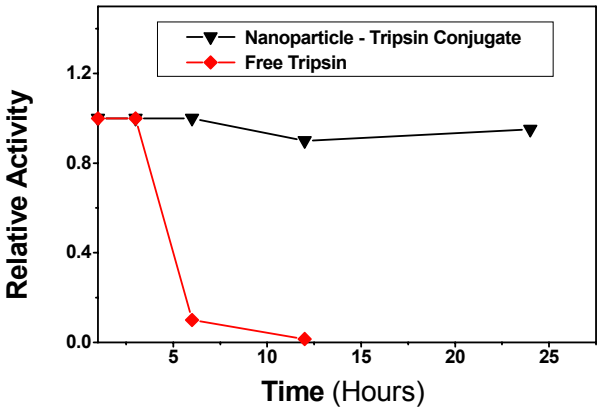

Fig. 3: Stability of MNP-trypsin at pH=7 at 5 ^{o}C.

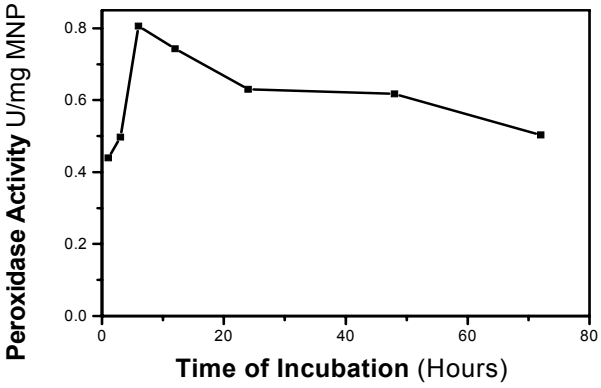

Fig. 4: Optimization MNP-Peroxidase coupling reaction time (Conjugate Peroxidase—SANH/ MNP's- SFB)

During coupling reaction activated MNP and enzyme solution were agitated using rocking shaker. The optimal molar ratio of enzyme to MNP was 10:1 and optimal pH was 4.7. We attached MNP's to trypsin, and found the stability of the trypsin is very good. Fig. 3 shows the relative activity of free trypsin and nanoparticle-trypsin conjugate. It can be clearly seen that free trypsin looses its activity after about six hours (1) in comparison to the MNP-E conjugate which is active for more than twenty five hours. We also attached MNP to peroxidase and checked the activity of the peroxidase after every three hours. As seen in Fig. 4 peroxidase was active for more than seventy hours. Stability of enzymes was now tested by incubating them for 5 weeks.

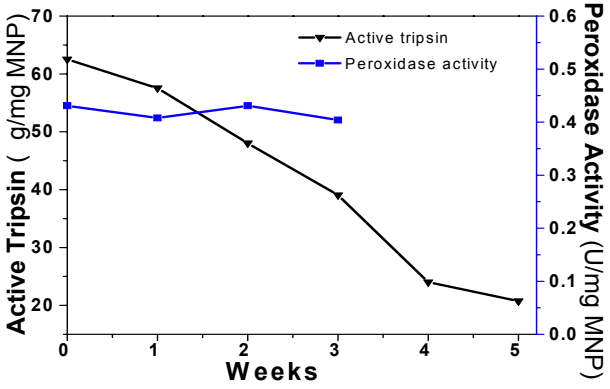

Fig. 5: Stability of MNP-Es in weeks.

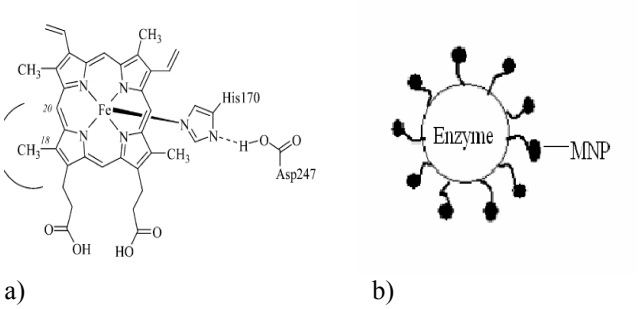

Fig. 6: a) Structure of horseradish peroxidase b) Enzyme with the envelope of MNP

Fig 5 shows the stability of enzyme with MNP. MNP's and enzymes were joined to each other by covalent bond using a heterobifunctional cross linkers. We used SANH and SFB as cross linkers to attach MNP's and Enzymes. These cross linkers enveloped the MNP's on enzymes. We estimated the density of enzymes conjugated to the MNP's using protein concentration measurement techniques. Knowing available surface area, protein amount bonded, and enzyme dimensions, we were able to calculate the fraction of surface covered by a given enzyme; trypsin covered 20% of the available surface of MNP and peroxidase covered 25% of the available surface area of MNP. Horseradish peroxidase C has the iron atom at the center Fig. 6a. Experiments are performed with iron immobilized on the surface of HRP for biosensors and biochip applications [15]. Theorell in 1942 published that

HRP and some of its derivative has the magnetic properties. Magnetic moment of our iron oxide nanoparticles produced is ~140 emu/g [10-12]. Presence of iron atom at the center of enzyme and high magnetic moment of nanoparticles resulted in increased attachment of peroxidase to nanoparticles in comparison to trypsin. Enzyme looses its activity due to the process of oxidation or other processes. The enzyme starts self digesting which results in the loss of the activity. We predict that magnetic nanoparticle envelope's the HRP and trypsin enzyme thus completely shielding it as seen in Fig 6b. This slows down the process of oxidation which results in the increase in lifetime as seen in Fig. 4 & Fig. 5. The productivity and cost efficiency of enzymes could be increased if we could reuse them. Iron nanoparticles being magnetic, we are able separate MNP-E conjugates after the reaction and immobilize enzymes making them more productive. We did this by applying a magnetic field of 45 gauss to MNP-E conjugates in vile [Fig. 1b]. MNP-E conjugates were effectively separated in solution in about 30 seconds.

4 CONCLUSION

Iron nanoparticles significantly increase the lifetime of enzymes thus making enzymes more productive. Immobilizing enzymes by attaching to iron nanoparticles makes it more stable this is because iron nanoparticles cage the enzymes by preserving or improving their activity. Size of the nanoparticle plays a very important role in attachment of enzymes. The higher rate of enzyme attachment to the nanoparticle was due to large surface area offered by iron nanoparticle of size ~20 nm. The fact that trypsin and peroxidase are active for more than five weeks implies that our iron nanoparticles are not toxic at this condition. MNP-E conjugates are also efficiently immobilized by using an external magnetic field.

5 ACKNOWLEGEMENT

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