NJC

Accepted Manuscript

This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/njc

Journal Name

RSCPublishing

ARTICLE

Association of *pseudomonas putida* formaldehyde dehydrogenase with superparamagnetic nanoparticles: an effective way of improving the enzyme stability, performance and recycling

Cite this: DOI: 10.1039/x0xx00000x

Caterina G. C. M. Netto[a], Leandro H. Andrade[a] and Henrique E. Toma[a*]

Received ooth January 2012,
Accepted ooth January 2012

DOI: 10.1039/x0xx00000x

www.rsc.org/

The association of formaldehyde dehydrogenase from *Pseudomonas putida* (FalDH) with two different magnetic nanoparticles, led to distinct enzyme responses depending upon the chemical modification process applied. The magnetite nanoparticles coated with aminopropyltriethoxysilane (MagNP-APTS) exhibited a lower activity and stability, while magnetite coated with a silica shell and APTS (MagNP@SiO_2APTS) afforded excellent results, improving the stability and performance of FalDH in relation to the free enzyme. Such differences were ascribed to unfavorable conformational changes in the MagNP-APTS/FalDH system, as indicated by the kinetics and Raman spectroscopy data, not discarding the possible interference from the exposed Fe(II)/III) ions. The MagNP@SiO_2APTS/FalDH catalyst could be successfully recycled by using an external magnet, keeping its highest performance, close to 100%, during the first four cycles, and decaying slightly up to 70% after the 10^{th} cycle.

Introduction

Enzyme immobilization is currently employed in biotechnological processes for improving the stability and recycling the very expensive biocatalyst. In general, most of the enzyme supports are typically macroscopic. The use of Fe_3O_4 nanoparticles can be particularly rewarding for allowing the exploitation of relevant aspects associated with the nanoscale, such as a large global surface area, high mobility and a strong magnetization effect. Fe_3O_4 nanoparticles exhibit single magnetic domains which are randomly oriented under normal conditions, but in the presence of a magnetic field they respond rapidly by aligning their magnetic moments, leading to a strong magnetization and no hysteresis. Such aspects characterize the superparamagnetic behaviour. The superparamagnetic materials introduce a differential aspect in relation to the existing supports, which is the possibility of recovering and recycling the enzymes in a rather simple way, by applying an external magnet field.[1]

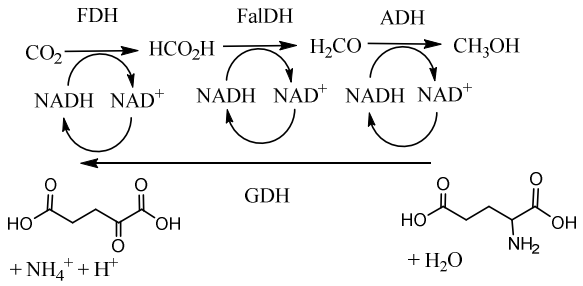
As a consequence of their natural complexity, enzymes exhibit peculiar protein compositions and structures influencing the properties of the active sites. Therefore, the enzyme immobilization on superparamagnetic nanoparticles cannot be treated as a trivial question. As we reported before,[1-5] the characteristics of the nanoparticles and their chemical functionalization play a dramatic role in the performance of the immobilized enzymes. For this reason, every modified enzymatic system should be regarded as a new one.

Nanoparticles and enzymes are nanoscale size species. The associated enzyme-nanoparticle species behaves as an entity displaying intrinsic mobility and distinct physical and chemical properties in relation to the free species, or in comparison with the

New Journal of Chemistry Accepted Manuscript

macroscopic systems. Their assembly involves a supramolecular design, starting from the appropriate functionalization of the nanoparticles and ending up with the successful binding of the enzyme *via* a coupling agent. As commonly observed in supramolecular systems, a wide range of effects resulting from the molecular assembly can influence the activity of the modified enzymes, changing, for instance, the conformation and the environment around the active sites. Therefore, by focusing on the nanoscale, new properties and possibilities can arise, especially on a molecular scale, generating a quite distinct scenario from the conventional immobilization of enzymes on macroscopic supports.[1]

Among the enzymes of great interest, there is a special group of dehydrogenases known as FDH (formate dehydrogenase), FalDH (formaldehyde dehydrogenase), ADH (alcohol dehydrogenase), and GDH (glutamate dehydrogenase), involved in the conversion of CO_2 into methanol (scheme 1). Such enzymes are not particularly stable in aqueous solutions when kept apart from their natural environment, and their association with superparamagnetic nanoparticles seem rather interesting, particularly for future biotechnological applications.



(scheme 1)

In this paper we focused on the association of magnetite nanoparticles (MagNP) with formaldehyde dehydrogenase from *Pseudomonas putida* (FalDH), a non glutathione dependent enzyme responsible for the oxidation of formaldehyde to formate. It requires NAD+ as acceptor, and belongs to the formaldehyde:NAD^+ oxidoreductase family,[6]

$$HCHO + NAD^+ + 3H_2O \rightleftharpoons HCOO^- + NADH + 2\ H_3O^+$$

The enzyme exhibits a homotetramer structure, comprising 398 amino acids per subunit, with a global molecular mass of 170 kDa.[7] In each subunit there are seven cysteine residues and two zinc ions. It has been used for formaldehyde detection and many efforts have been dedicated to the immobilization of FalDH in order to improve its stability.[8] Among the commonly employed supports are: nafion,[9-12] chitosan,[10] CdS,[13] dextran,[14] glass beads,[15] polymer beads,[16,17], silica,[18-19] and sepharose.[20]

Our focus was concentrated on the performance of FalDH associated with superparamagnetic nanoparticles. Initially, the MagNPs were functionalized with aminopropyltriethoxysilane (APTS) in order to provide an external distribution of aminopropyl residues anchored on the Fe_3O_4 core by means of Si-O groups (Figure 1). Such amino groups act as anchoring sites for the enzyme, using glutaraldehyde as the coupling agent.

It should be noticed that in the case of MagNP-APTS nanoparticles, the thin Si-O binding layer cannot effectively protect the Fe_3O_4 core from its interaction with the solvent and external groups. Depending upon the type of enzymatic catalysis, this aspect can be quite relevant. In order to improve the shielding, the MagNPs should be coated with a silicate shell, before applying the APTS treatment, as shown in Figure 1. This type of particle was here denoted MagNP@SiO_2APTS. The comparison between these two modified nanoparticles is quite important, since it can reveal the influence of the Fe_3O_4 core in the enzymatic process, and on the mechanism involved.

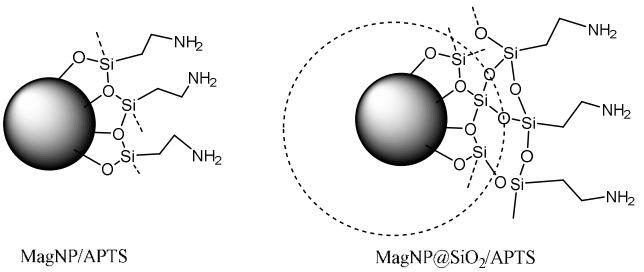


Figure 1. Pictorial representation of MagNP-APTS and MagNP@SiO_2APTS.

Experimental section

Formaldehyde dehydrogenase from *Pseudomonas Putida,* NADH/ NAD^+, glutaraldehyde (1.2% w/v in water), formaldehyde (37% w/v in water) and TRIS were purchased from Sigma-Aldrich and used without previous purification.

New Journal of Chemistry Accepted Manuscript

This journal is © The Royal Society of Chemistry 2012

The preparation of Fe_3O_4 and $Fe_3O_4@SiO_2$ nanoparticles, and their coating with APTS have been described elsewhere.[2-5,21] Their magnetic characterization has also already been reported.[2-5,21] The average (core) sizes were evaluated by TEM measurements, as 10 ± 2 nm.

The association of FalDH with MagNP-APTS was carried out according to the following optimized procedure: In a 2 mL flask, 0.8 mg of a MagNP-APTS suspension in the buffer solution of Tris-HCl 0.1 mol dm^3, pH 7.5 was added to 1 mL of FalDH solution (0.063 mg/mL) prepared with the same buffer. Then, 3 x 10^{-5} mol glutaraldehyde was added, and the reaction was allowed to proceed at 0 °C for 20 min. Finally, with the help of an external magnet the particles were confined at the bottom, and washed 3 times with 100 µL of 0.1 mol dm^3 Tris-HCl, pH 7.5 buffer solution.

In the case of MagNP@SiO_2APTS, the optimum procedure was the following: 0.3 mg of the superparamagnetic nanoparticles was dispersed on 100 µL of 0.1 mol dm^3 Tris-HCl buffer solution (pH 7.5), and mixed with 1 mL of a FalDH solution containing 0.063 mg/mL in 0.1 mol dm^{-3} Tris-HCl buffer solution. Then 1x10^{-5} mol of glutaraldehyde was added, and the reaction allowed to proceed at 0 °C for 20 min. The MagNP@SiO_2APTS/FalDH material was confined with a magnet, and washed 3 times with 100 µL of 0.1 mol dm^{-3} Tris-HCl, pH 7.5 buffer solution.

Under this condition, in both cases, based on the Bradford method,[22] the amount of FalDH associated with the MagNPs was essentially 100%.

The kinetics of conversion of formaldehyde to formic acid was monitored according to the following procedure: To a conventional quartz spectrophotometric cuvette, 30 µL of formaldehyde (37%, 13 mol L^{-1}) , 20 µL of NAD^+ (7.5 mmol dm^{-3}) and the free or immobilized FalDH were mixed in 1 mL with buffer solution (0.1 mol dm^{-3} Tris-HCl, pH 7.5). The reaction was monitored by the formation of NADH, with λ_{max} at 340 nm. All the variables were kept constant, except one, in order to evaluate its specific influence on the kinetics.

UV–visible spectra were recorded on a Hewlett Packard 8453-A diode-array spectrophotometer. Fluorescence measurements were recorded on a LS-100 fluorometer from Photon Technology Inc. Confocal Raman spectroscopy measurements were recorded in a WITec alpha 300R microscope equipped with a Nd:YAG laser (λ = 532 nm) and a Nikon objective (20x NA = 0.8).

Results and Discussion

Enzyme immobilization was carried out using glutaraldehyde to connect the APTS amino group to the enzyme available amino groups, e.g. from lysine, by forming imine bonds, as shown in Figure 2. Such a procedure was here preferred, because of the best results obtained from a recent comparison involving several distinct binding procedures for lipases and superparamagnetic nanoparticles.[21] Despite the complexity involved in the glutaraldehyde mechanism, the process has been widely employed in biochemistry, because of its excellent performance under mild conditions.[23]

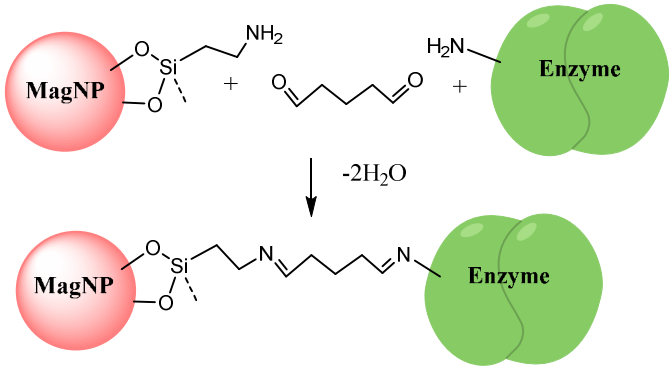


Figure 2. Enzyme binding to the APTS modified MagNPs *via* glutaraldehyde.

The binding procedure was optimized for the MagNP-APTS and MagNP@SiO_2APTS systems, by monitoring the enzyme activity as a function of the nanoparticle amount, glutaraldehyde concentration and incubation time.

Keeping the FALDH constant (0.063 mg or 0.37 x 10^{-9} mol), and adding 1 x 10^{-5} mol glutaraldehyde, the appropriate amount of MagNP was varied from 0.2 to 1.2 mg, and the enzymatic activity for the conversion of formaldehyde to formic acid was evaluated, as shown in Figure 3. In the case of MagNP-APTS, the maximum activity stabilized above 0.8 mg of the nanoparticles, showing a maximum formaldehyde conversion of 36%. For MagNP@SiO_2APTS, the maximum activity was obtained using 0.3 mg of nanoparticles, yielding practically 100% of formaldehyde conversion.

New Journal of Chemistry Accepted Manuscript

A previous estimate of the analytical composition of MagNP-APTS was close to $Fe_3O_4(APTS)_{0.29}$. Since magnetite exhibits a cubic crystallographic cell of 0.59 nm^3 encompassing eight Fe_3O_4 units,[24] a typical 10 nm nanoparticle (core) would contain 13,560 Fe_3O_4 units,[2] leading to global molecular mass of about 3.6 x 10^6 Da incorporating 2.5 x 10^3 APTS molecules. Accordingly, 0.8 mg of nanoparticles would contain approximately 0.21 x 10^{-9} mol of $Fe_3O_4(APTS)_{0.29}$ nanoparticles, leading to a FalDH:MagNP-APTS proportion close to 2:1. In the case of the MagNP@SiO_2APTS particles, such proportion would be larger, because of the volume expansion due to the SiO_2 shell.

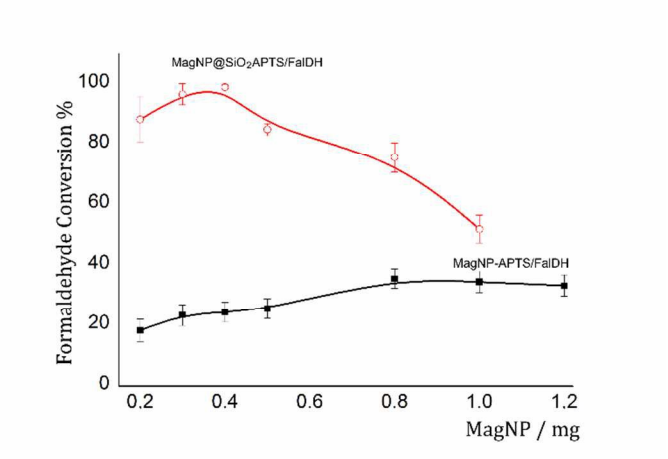


Figure 3. Influence of the amounts of magnetic nanoparticles in the FalDH enzymatic activity after the binding process.

After evaluating the best MagNP amounts, the influence of the amount of glutaraldehyde was investigated by using 1 mg of nanoparticles, as shown in Figure 4. MagNP-APTS required 3 x 10^{-5} mol glutaraldehyde to reach a maximum formaldehyde conversion (40%), while MagNP@SiO_2APTS required 3 times less (1 x 10^{-5} mol) to reach the maximum conversion at 60%, under similar conditions. In both cases, the proportion of glutaraldehyde in relation to the enzyme was > 10^4, more than that necessary to react with FalDH and all the available amino groups from APTS. Perhaps an excess of glutaraldehyde would be required to prevent the hydrolysis of the reactive diimine bonds generated from the aldehyde and amino group reaction. However at the present time, the exact mechanism involved in the gutaraldehyde method remains somewhat controversial and poorly understood.[23]

In Figure 4, one can see that above the optimum concentration of the coupling agent, there is a decrease in the conversion yield, probably originated from cross-linking reactions and possible degradation of the enzyme induced by the excess of reactive aldehyde groups.

The influence of the binding time was also investigated (Figure 5), keeping the previous optimum amounts of nanoparticles and glutaraldehyde. In both cases, the maximum activity was attained after 20 min of reaction. The formaldehyde conversions for MagNP-APTS and MagNP@SiO_2APTS were 50 and 100%, respectively.

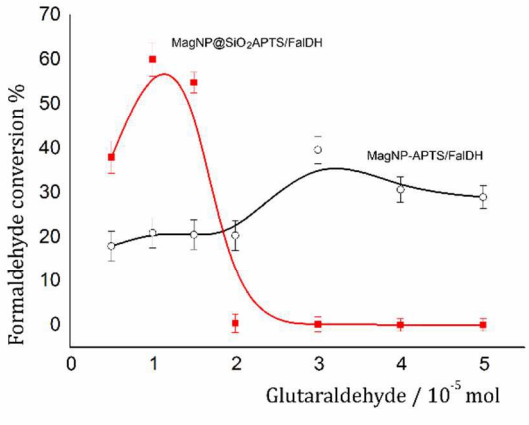


Figure 4. Effect of the glutaraldehyde amount in the performance of MagNP-APTS/FalDH and MagNP@SiO_2APTS/FalDH in the formaldehyde conversion.

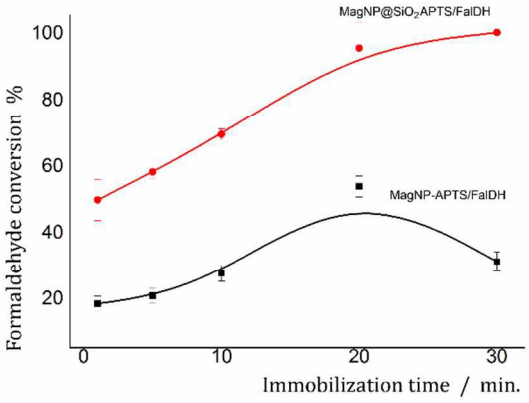


Figure 5. Influence of the incubation time on the formaldehyde conversion to formic acid for MagNP-APTS/FalDH, MagNP@SiO_2 APTS/FalDH.

New Journal of Chemistry Accepted Manuscript

This journal is © The Royal Society of Chemistry 2012

After the initial optimization process, the pH influence on the enzymatic conversion of formaldehyde to formic acid has been evaluated, as shown in Figure 6.

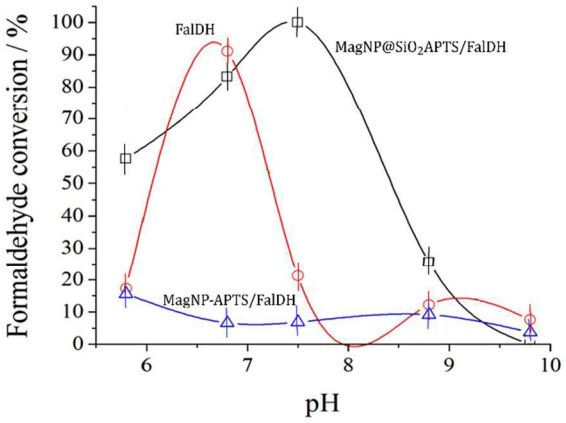


Figure 6. Influence of the pH on the formaldehyde conversion to formic acid for free FalDH (○), MagNP-APTS/FalDH (△), MagNP@SiO_2APTS/FalDH (□).

Free FALDH exhibited an optimum activity (> 90%) at pH 6.8, while for MagNP-APTS/FalDH the optimum activity was observed close to pH 5.8, but showing a dramatic decrease of efficiency (< 20%). In the case of MagNP@SiO_2APTS/FalDH, the activity remained quite high (> 90%) exhibiting an optimum performance at pH 7.5.

The temperature influence on the catalysis was also analyzed, revealing two conversion maxima at 32 and 42 ^oC for free FALDH (Figure 7), with typical efficiencies around 45%. MagNP/APTS-FalDH also exhibited a two maxima profile, at 27 and 42 ^oC, but with a large decay of activity (< 25%). In contrast, the activity of MagNP@SiO_2/APTS-FalDH remained quite high in the temperature range from 27 to 42 ^oC, yielding formaldehyde conversions above 90%.

Recycling experiments have been carried out for the MagNP-APTS/FalDH and MagNP@SiO_2APTS/FALDH systems, under optimized conditions, as summarized in Figure 8. As already observed for MagNP-APTS/FalDH, the activity was dramatically reduced, dropping to half of its initial value in the second cycle, and decreasing systematically in the successive cycles. In contrast, MagNP@SiO_2APTS/FalDH kept the efficiency above 90% during the first four cycles, decreasing only slowly, up to 70% in the 10th cycle.

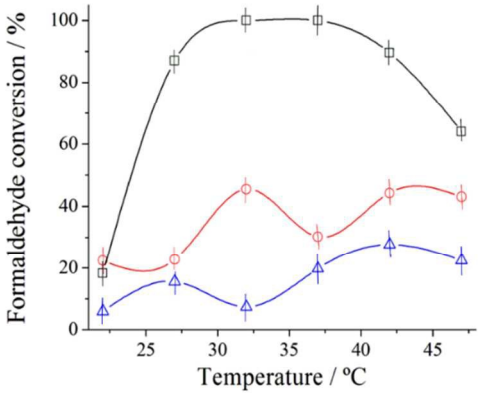


Figure 7. Influence of the temperature on the formaldehyde conversion to formic acid for free FalDH (○,); MagNP-APTS/FalDH (△), MagNP@SiO_2APTS/FalDH (□)

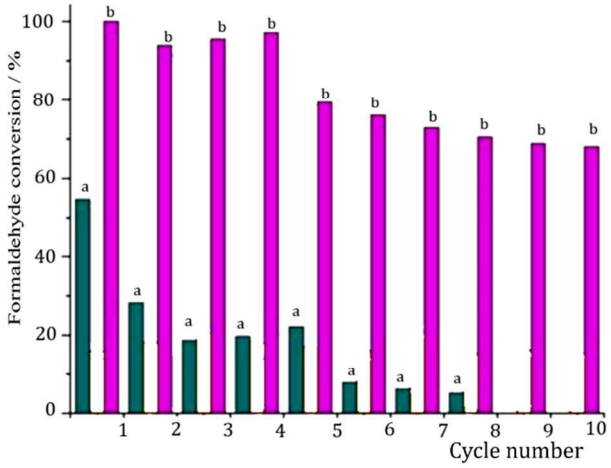


Figure 8. Recycling behaviour of a) MagNP-APTS-FalDH and b) MagNP@SiO_2APTS/FalDH.

A mechanistic discussion is necessary in order to explain the contrasting kinetic results observed for the free and MagNP associated FalDH enzyme. In general, the enzymatic kinetics can be described by the Michaelis-Menten formalism, involving the association of the substrate with the enzyme/cofactor species, followed by the activation and formation of the products.

New Journal of Chemistry Accepted Manuscript

In the case of the enzymatic conversion of formaldehyde to formic acid, the corresponding Michaelis-Menten constant, K_M, can be determined from the rates (v) measured as a function of the formaldehyde concentrations [c], according to the Lineweaver-Burk equation (1)

$$\frac{1}{v} = \left[\frac{K_M}{v_{max}}\right]\frac{1}{[c]} + \frac{1}{v_{max}} \quad (1)$$

The K_M value for free FalDH was determined as 0.3 μmol dm^{-3} at 0.1 mol dm^{-3} phosphate buffer, pH 6.8, and 32 ºC. In the case of MagNP-APTS/FalDH, K_M increased to 0.65 μmol dm^{-3} (0.1 M Tris-HCl buffer, pH 8.8 and 42ºC), while for the MagNP@SiO_2APTS/FalDH system, K_M decreased considerably to 0.05 μmol dm^{-3} (0.1 M Tris-HCl buffer pH 7.5 at 32 ºC). The result is excellent, considering that a smaller K_M indicates a higher substrate affinity, and this is a highly desirable point in enzymatic catalysis.

Therefore, the observed differences in the Michaelis–Menten constants are consistent with the low performance observed for the MagNP-APTS/FalDH system, and with the improved activity observed for MagNP@SiO_2APTS/FalDH, in relation to the free enzyme. The most plausible explanation for these differences should be the distinct chemical interaction promoted by the functionalized nanoparticles, changing the conformational properties of the polypeptide chains.

Formaldehyde dehydrogenase from *Pseudomonas putida* is a homotetrameric enzyme, displaying a molecular mass of 170 kDa.[7] The active site in each subunit is composed by the histidine-67, cysteine-46 and aspartate-169 amino acids coordinated to a Zn(II) ion, as illustrated in Figure 9. The histidine and cysteine amino acids are closely related to the protein β structures. The interaction between the enzyme and MagNP can perturb the subunits interaction, changing the intermolecular β structures, and the corresponding active sites.

In order to evaluate this hypothesis, confocal Raman studies have been carried out for these systems, before and after the addition of formaldehyde. The discussion will mainly focused on the amide I band associated with the predominant of intermolecular β structures.[25,26] The amide I band exhibits a predominant contribution of C=O stretching (80%), and minor contributions from C-N stretching (10%) and N-H bending (10%).[27]

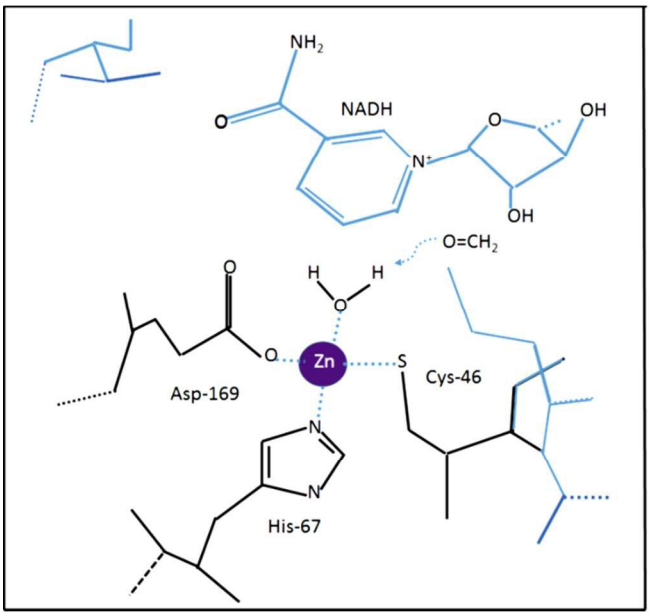


Figure 9. Pictorial representation of the FALDH active site.

The Raman spectra in the amide I region, for the FALDH, MagNP-APTS/FalDH and MagNP@SiO_2APTS/FalDH systems, can be seen in Figure 10. The amide I band from free FALDH is observed at 1695 cm^{-1}, and when formaldehyde was added to the enzyme, this band shifted slightly to 1733 cm^{-1}. According to Carey et al.[28,29] when the amide carbonyl group is located inside a hydrophobic environment, its Raman band usually appears around 1725 cm^{-1}. Therefore, in free FALDH, it is possible that the carbonyl groups near the active site are moving into a more hydrophobic environment after the formaldehyde binding.

When FalDH is associated with MagNP-APTS, the amide I band is observed at 1620 cm^{-1}, which is a region associated with the vibrational frequencies of the intermolecular β structures.[26] Presumably, the inductive effect from the interaction between the iron oxide and the carbonyl groups can be responsible for the large shift observed for the vibrational frequency, in relation to the free enzyme. After the addition of formaldehyde, the amide I band

New Journal of Chemistry Accepted Manuscript

This journal is © The Royal Society of Chemistry 2012

shifted to 1637 cm^{-1}, revealing more pronounced conformational changes in the protein chain. For the MagNP@SiO_2APTS/FalDH system, the amide I band is located at 1638 cm^{-1} in the region of intramolecular β structures, shifting slightly to 1644 cm^{-1} after the addition of formaldehyde. This small shift reflects only minor conformational changes associated with the binding of formaldehyde.[28]

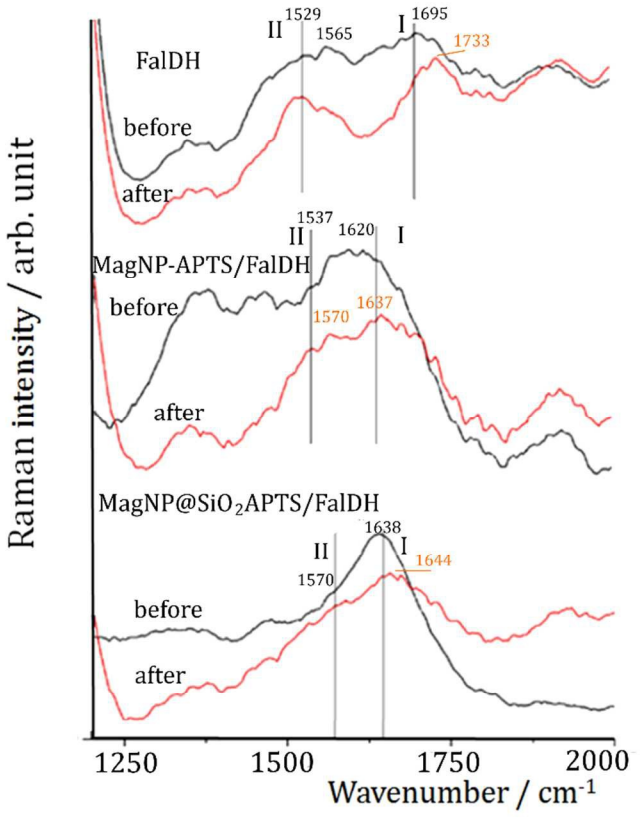


Figure 10. Confocal Raman Spectra for (A) free FalDH, (B) MagNP-APTS/FalDH, (C) MagNP@SiO_2APTS/FalDH before and after the addition of formaldehyde.

The amide II bands are usually observed around 1560 cm^{-1} involving 60% contribution from N-H bending and 40% contribution from C-N stretching.[27] In Figure 10 one can observe a band at 1529 cm^{-1} in free FalDH associated with $\delta(NH_3^+)$ and another one at 1565 cm^{-1} ascribed to ν(CC) of the imidazole ring. In the case of the MagNP-APTS/FalDH and MagNP@SiO_2APTS/FalDH systems, these bands are very poorly defined, showing some changes around 1570 cm^{-1} after the interaction with formaldehyde, possibly associated with the imidazole ring.

The amide III bands are located around 1300 cm^{-1}, involving a rather complex composition of C-N stretching (30%), N-H bending (30%), C=O stretching (10%) and other vibrational modes.[27] They can be observed practically at the same region (1350 cm^{-1}) for the three systems in Figure 10, however with a much larger intensity in the case of MagNP-APTS/FalDH. This band is dramatically perturbed after the addition of formaldehyde. By exploring the enzyme structure from PFDH (PDB code, 1KOL) with the Discovery Studio 2.5 program (Accelerys, San Diego, CA), it was possible to see that very close to the catalytic zinc ion there is a phenylalanine residue (PHE93) facing one carbonyl group from ASP169. When formaldehyde binds to the active site, this carbonyl group can get closer to the aromatic ring from phenylalanine, inducing conformational changes probably related with the amide III bands. .

In general, the conformational changes, as evidenced by the changes in the amide I and III bands seem more drastic for the MagNP-APTS/FalDH system. In addition, the partially exposed Fe(II)/Fe(III) ions can also exert some influence on the enzyme, by interfering in the electron transfer process. This has already been demonstrated in our previous work on the thioredoxin catalysis,[30] and is consistent with the fact that the formaldehyde dehydrogenase activity is inhibited when the sulfhydryl group becomes unavailable.[31]

In the case of the MagNP@SiO_2APTS/FALDH system, conformational changes induced by the magnetic nanoparticles should also be taking place, but leading to minor changes in the amide III band after the interaction with formaldehyde, as observed for free FalDH. Such conformational changes are not influencing the active sites. Actually they seem to improve the enzyme stability and performance, allowing its successive recycling with minor decay of activity for at least 10 successive recycles.

Conclusions

The association between the superparamagnetic nanoparticles and FalDH can lead to dramatic changes in the enzyme activity, depending upon the chemical modification applied to the system.

Highly active formaldehyde dehydrogenase was obtained by combining the enzyme with MagNP@SiO_2APTS, using the

New Journal of Chemistry Accepted Manuscript

glutaraldehyde method, exhibiting 100% of efficiency for formaldehyde conversion at mild temperatures and physiological pH.

MagNP-APTS led to an opposite result, decreasing the general activity of FalDH, and its stability.

The kinetics and Raman spectroscopy data indicated substantial conformational changes in the MagNP-APTS/FalDH system. The lack of the silica protective coating can also contribute for the decay of enzymatic activity, by exposing the Fe(II)/Fe(III) ions at the surface, capable of interfering in the redox processes.

A successful recycling performance has also been obtained with for the MagNPS@SiO_2APTS/FalDH system, showing a great improvement in the relation to the free enzyme.

Acknowledgements

The support from Fundação de Amparo à Pesquisa do Estado de São Paulo and Conselho Nacional de Desenvolvimento Científico e Tecnológico is gratefully acknowledged.

Notes and references

*Corresponding author, E-mail: henetoma@iq.usp.br

[a]Instituto de Quimica, Universidade de São Paulo, São Paulo, 05508-000, Brazil.

1. C. G. C. M. Netto, H. E. Toma and L. H. Andrade, L. H.; *J. Mol. Cat. B: Enzymatic*, 2013, **85-86**, 71.
2. C. G. C. M. Netto, L. H. Andrade and H. E. Toma, *Tetrahedron: Asymmetry,* 2009, **30**, 2299.
3. C. G. C. M. Netto, M. Nakamura, L. H. Andrade and H. E. Toma, *J. Mol. Cat. B: Enzymatic*, 2012, **84,** 136.
4. L. H. Andrade, L. P. Rebelo, C. G. C. M. Netto and H. E. Toma, *J. Mol. Cat. B: Enzymatic,* 2010, **66**, 55.
5. L. P. Rebelo, C. G. C. M. Netto, H. E. Toma and L. H. Andrade, *J. Braz. Chem. Soc*. 2010, **21**, 1537.
6. B. Persson, J. Hedlund and H. Jornvall, *Cel. Molec. Life Sci,* 2008, **65**, 3879.
7. N. Tanaka, Y. Kusakabe, K. Ito, T. Yoshimoto and K. T. Nakamura, *J. Mol. Biol.*, 2002**, 324**, 519.
8. M. Badihi-Mossberg, V. Buchner and J. Rishpon, *Electroanalysis*, 2007, **19,** 2015.
9. P. K. Addo, R. L. Arechederra and S. D. Minteer, *Electroanalysis*, 2010, **22**, 807.
10. T. L. Klotzbach, M. Watt, Y. Ansari and S. D. Minteer, *J. Membrane Sci.,* 2008, **311**, 81.
11. N. L. Akers, C. M. Moore, S. D. Minteer, *Electrochim. Acta,* 2005, **50**, 2521.
12. C. M. Moore, N. L. Akers, A. D. Hill, Z. C. Johnson and S. D. Minteer, *Biomacromolecules,* 2004, **5**, 1241.
13. W. Vastarella and R. Nicastri, *Talanta*, 2005, **66**, 627.
14. Y. I. Korpan, O. O. Soldatkin, O. F. Sosovska, H. M. Klepach, E. Csoregi, F. Vocanson, N. Jaffrezic-Renault and M. V. Gonchar, Microchimica Acta, 2010, **170**, 337.
15. Y. Sekine, *Anal. Chim. Acta,* 1993, **280**, 179.
16. B. El-Zahab, D. Donnelly, and P. Wang, *Biotech. and Bioengineering,* 2008, **99**, 508.
17. N. Kiba, S. Yokose, M. T. Kazue and T. T. Suzuki, *Anal. Chim. Acta,* 1999, **378**, 169.
18. T. Shimomura, T. Itoh, T. Sumiya, F. Mizukami and M. Ono, *Sensors and Actuators B-Chemical*, 2008, **135**, 268.
19. T. Itoh, T. Shimomura, Y. Hasegawa, J. Mizuguchi, T. Hanaoka, A. Hayashi, A. Yamaguchi, N. Teramae, M. Ono and F. Mizukami, *J. Mat. Chem*., 2011, **21**, 251.
20. F. Vianello, L. Zennaro and A. Rigo, *Biosens. Bioelectron.* 2007, **22**, 2694.
21. M. Yamaura, R. L. Camilo, L. C. Sampaio, M. A. Marcelo, M. Nakamura and H. E. Toma*, J. Mag. Mag. Mat*., 2004, **279**, 210.
22. M. M. Bradford, *Anal. Biochem*., 1976, **72**, 248.
23. I. Migneault, C. Dartiguenave, M. J. Bertrand and K. C. Waldron, *BioTechniques*, 2004, **37**, 790.
24. R. M. Cornell and U. Schwertmann, The Iron Oxides, Wiley, Weinheim, 2003.
25. C. W. Wharton, *Biochem. J*., 1986, **233**, 25.
26. R. Tuma, *J. Raman Spec.*, 2005, **36**, 307.
27. B. Stuarty, Infrared spectroscopy: fundamentals and applications, John Wiley & Sons, N.Jersey, 2004.
28. P. R. Carey and A. C. Storer, *Ann. Rev. Biophys. Bioeng*. 1984, **13**, 25.
29. P. R. Carey and P. J. Tonge, *Acc. Chem. Res*., 1995, **28**, 8.
30. C. G. C. M. Netto, E. H. Nakamatsu, L. E. S. Netto, M. A. Novak, A. Zuin, M. Nakamura, K. Araki and H. E. Toma, *J. Inorg. Biochem.,* 2011, **5**, 738.
31. S. Ogushi, M. Ando and D. Tsuru, *Agricultural.and Biological Chem.,* 1986, **50,** 2503.

New Journal of Chemistry Accepted Manuscript

 This journal is © The Royal Society of Chemistry 2012