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Supramolecular-mediated immobilisation of L-phenylalanine dehydrogenase on β-cyclodextrin-modified gold nanospheres

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Native and adamantane-modified L-phenylalanine dehydrogenase was immobilised on β-cyclodextrin coated gold nanospheres via supramolecular associations. The amount of immobilised protein was estimated to be about 100–110 μg per milligram of support. The nanocatalyst retained high catalytic activity and showed increased affinity for the substrate. Both immobilised enzymes showed fluorescence emission spectra similar to that of the native protein counterpart. The range of optimum pH for catalytic activity was increased from 11.5 to 11.5–12.5 for the native and adamantane-modified enzymes after adsorption on gold nanospheres. Immobilised native and modified L-phenylalanine dehydrogenase retained about 20% and 41%, respectively, of the initial activity after 10 cycles of reuse. These results open a new perspective to the possible application of cyclodextrin-modified gold nanospheres as water-soluble carriers for enzyme immobilisation.

Keywords: Phenylalanine dehydrogenase; Cyclodextrin; Gold nanospheres; Supramolecular immobilisation

1. Introduction

In recent years, there has been extensive interest in nanoscience due to the many potential applications in the fields of nanoelectronics and ultrasensitive biomolecular detection [1–3]. In this sense, special attention has been conferred to construct novel water-soluble nanocatalysts by immobilising enzymes on nanosized particles [4–7] in order to prepare efficient biocatalysts for fine organic chemistry, drug delivery systems and biosensor devices [8, 9]. Noble metal colloids have been the nanomaterials most extensively employed as three-dimensional scaffolds for enzyme immobilisation [5, 6, 10, 11], due to their unique physical and chemical properties owing to their extremely

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small sizes and large specific surface areas [12]. In general, such colloidal structures have been functionalised with enzymes through covalent immobilisation methods, mainly based on the sulphur-noble metal chemistry [6, 10, 11]. However, the use of host-guest supramolecular interactions constitutes an excellent and interesting approach for preparing enzyme nanocatalysts [13, 14]. This method has been also employed for preparing bienzymatic nanocatalysts [15].

Kaifer *et al*. [16] reported the preparation of water-soluble β-cyclodextrin (CD)-coated gold nanospheres by treating the growing metal colloids with the perthiolated oligosaccharide derivatives, demonstrating that CD retained its host properties after attaching to gold nanoparticles. On the other hand, supramolecular associations have been successfully used for immobilising adamantane-modified proteins on CD-coated electrodes. A CD monolayer behaved as an interface that avoided direct interaction of cytochrome c with the electrode surface. Adamantane moieties modifying the protein served as hosts for the supramolecular association to the CD monolayer in a multivalence fashion [17]. The stabilisation of non-derivatised enzymes by supramolecular interactions with CD-branched polymeric materials in aqueous media has been also described [18, 19].

Proteins tend to denaturise by their interaction with metal surfaces, especially nanoparticles. The process is typical for those cases where the surface is charged [20]. Nevertheless, several reports indicate that this is by no way a rule and an enzyme associated to a metal nanoparticle may maintain its activity [21, 22]. Apparently, the possibility of denaturation of a protein when associated to a metal nanoparticle depends on the nature of protein, the size of the nanoparticle and its charge. The characteristic of the capping molecules that form an interface between the nanodevice and the enzyme is very important. For example, when BSA was bound to citrate-coated gold nanoparticles no denaturation was observed while that process took place when the protein was bound on bare nanoparticles [23]. The conformation of cytochrome c was studied when immobilised on gold nanoparticles capped with negative, positive and neutral species. When neutral poly(ethyleneglycol)thiol was used as capping agent no denaturation was observed on cytochrome c, while with the major effect was observed with gold nanoparticles capped with positively charged aminoethanethiol [24].

Neutrally charged CDs as capping agents should serve to form interfaces able to avoid any undesirable denaturation process of an immobilised enzyme on hard nanoparticles [13–15]. This immobilisation process should be of supramolecular nature and with a high specificity on how the enzyme is associated. Moreover, CDs are able to assist refolding of proteins as ''molecular chaperones'' [19].

On these basis, our hypothesis is that both native and adamantane-modified enzyme structures could associate with CD-gold nanospheres through host-guest interactions. In this sense, the present paper deals with the hydrophobic modification of *Bacillus badius* L-phenylalanine dehydrogenase (PheDH, EC 1.4.1.20) with 1-adamantane carboxylic acid and the use of CD-coated gold nanoparticles as support for the immobilisation of to supramolecularly interact with both modified and non-modified enzyme forms.

PheDH is a NAD^+-dependent enzyme that catalyzes the reversible oxidation-reduction reactions for L-phenylalanine [25]. This enzyme has been selected for our

study due to its wide use in biomedical analysis and the enantioselective synthesis of Phe and related L-amino acids from their keto analogs [26, 27].

2. Materials and methods

2.1. *Materials*

Bacillus badius PheDH, recombinantly expressed in *E. coli*, was prepared as previously described [18]. L-Phenylalanine, α-nicotinamide adenine dinucleotide (NAD^+), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) and 1-adamantane carboxylic acid were obtained from Wako Pure Chem. (Osaka, Japan). βCD was obtained from Amaizo (Indiana, USA) and used as received. All other chemicals were analytical grade.

2.2. *Preparation of CD-modified gold nanospheres*

The nanospheres were prepared by the method developed by Kaifer and co-workers using perthiolated-βCD as capping molecule [13], and [CD]/[$AuCl_4^-$] ratio of 0.20. The amount of oligosaccharides attached to the nanoparticles was estimated by the redissolution method [28] to be 10 molecules of βCD, assuming that the nanoparticles are perfect spheres.

The size of the CD-coated gold nanoparticles was determined by high-resolution transmission electron microscopy using a JEOL 4000-EX instrument, operated at 400 kV. The particle size distribution was obtained from digitalised amplified micrographs by averaging the larger and smaller axis diameters measured in each particle.

2.3. *Preparation of PheDH-adamantane (Phe-ADA) derivative*

A reaction mixture containing 5 mg of PheDH, 5 mg of 1-adamantanecarboxylic acid and 5 mg of EDAC, dissolved in 3 ml of 100 mM sodium phosphate buffer pH 6.0, was stirred for 1 h at room temperature and for 16 h at 4°C. The solution was further purified by exhaustive dialysis at 4°C against 10 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA.

2.4. *Enzyme immobilisation on CD-modified gold nanospheres*

For the immobilisation of native and modified enzyme forms, 500 µl of a gold nanoparticle solution (10 mg/ml in 20 mM sodium phosphate buffer pH 7.0) was first mixed with 500 µl of either enzyme solutions (0.60 mg/ml in the same buffer). The solutions were gently stirred at 4°C during 6 h, then mixed with a concentrated NaCl solution (10% m/v final concentration) and kept at 4°C during 4 h. The mixtures were further centrifuged to remove the non immobilised enzymes. The powders so obtained were rinsed several times with a cool 10% NaCl solution and then resuspended in 1 ml of 20 mM sodium phosphate buffer pH 7.0. The amount of adsorbed enzyme was estimated by difference after measuring the non-immobilised protein.

2.5. *Analytical determinations*

The enzymatic activity of PheDH was determined at 25°C in 100 mM glycine/KCl/KOH buffer, pH 10.4, containing 2.5 mM NAD^+ and using 10 mM L-Phe as substrate [26]. One unit of phenylalanine dehydrogenase activity is defined as the amount of enzyme that catalyzes the formation of 1.0 μmol NADH per minute under the described conditions. Michaelis-Menten parameters were calculated from Eadie-Hofstee plots. Protein concentration was estimated by the Bradford method using bovine serum albumin as standard [29]. The amount of adamantane residues attached to the protein was quantified by estimating the amount of free amino groups in PheDH before and after modification [30].

The fluorescence emission spectra of native and modified PheDH before and after immobilisation were measured with 0.3 nM enzyme solutions in 10 mM sodium phosphate buffer, pH 7.0, containing 1 mM EDTA using a RF-1501 Shimadzu spectrofluorophotometer. The protein solution was excited at 280 nm and emission scanned between 300 nm and 400 nm.

2.6. *Optimum pH, thermostability and reusability assays*

The optimum pH for PheDH preparations was measured by determining the catalytic activity at 25°C in 100 mM glycine/KCl/KOH buffer at different pH values, ranging from 8.0 to 12.5.

To investigate the thermal stability of the enzyme preparations, all PheDH forms were incubated at 55°C in 20 mM sodium phosphate buffer, pH 7.0 during 1 h. Aliquots were withdrawn at scheduled times and the remaining activities were measured as described above.

In addition, the reusability of the immobilised PheDH preparations was determined by measuring the enzymatic activity retained after several precipitation/redissolution processes. After each reaction run, the enzyme–gold nanoparticles were precipitated by treating with 10% NaCl solutions at 4°C during 4 h, further removed by centrifugation and washed with NaCl solution to remove any residual substrate or unbound enzyme within the nanoparticle matrix. They were then dissolved in 10 mM sodium phosphate buffer, pH 7.0, containing 1 mM EDTA and reintroduced into fresh reaction medium and enzyme activities were detected at optimum conditions.

3. Results and discussion

Dark red and water soluble gold nanoparticles were prepared by *in situ* modification of growing metal colloids with perthiolated-βCD [16]. These particles showed spherical geometry slightly distorted at the surface, as determined by HRTEM (figure 1A). This distortion should be caused by the attachment of the CD moieties to gold during the colloid formation process. The average diameter of the nanospheres was determined as 3.4 ± 1.6 nm, and the size distribution is provided in figure 2.

For preparing PheDH-gold nanocatalysts, two different association strategies were used. In the first one, native enzyme was directly treated with the CD-coated metal colloids. In the second approach, the enzyme was first modified with 1-adamantane

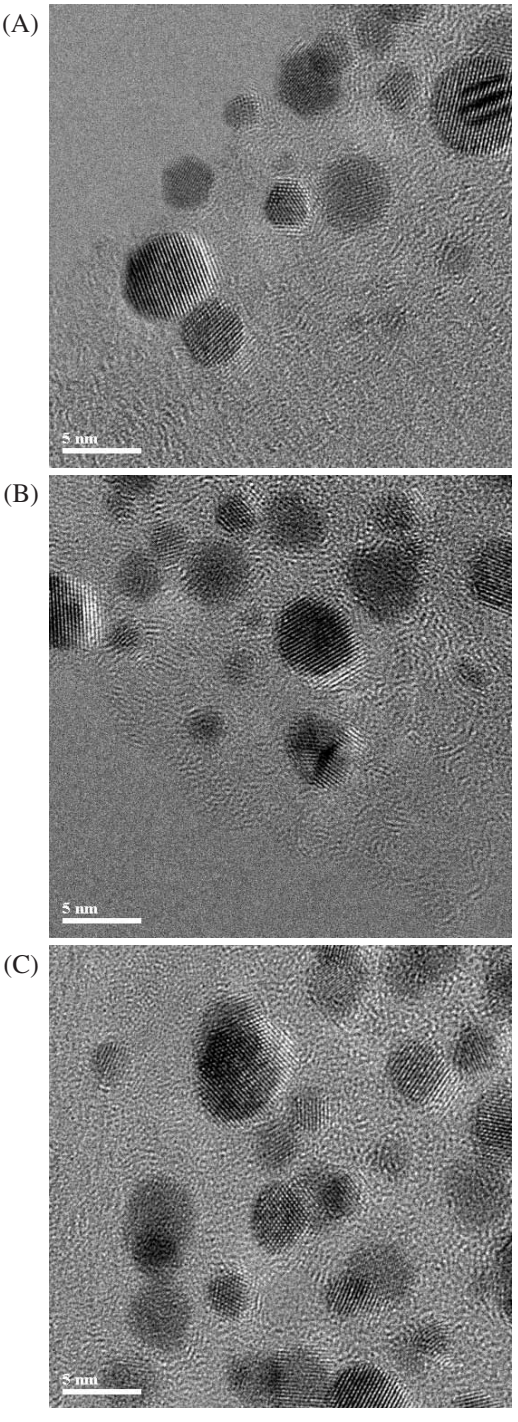


Figure 1. HRTEM images of Au-CD nanoparticles before (A) and after immobilisation of native (B) and adamantane-modified PheDH (C).

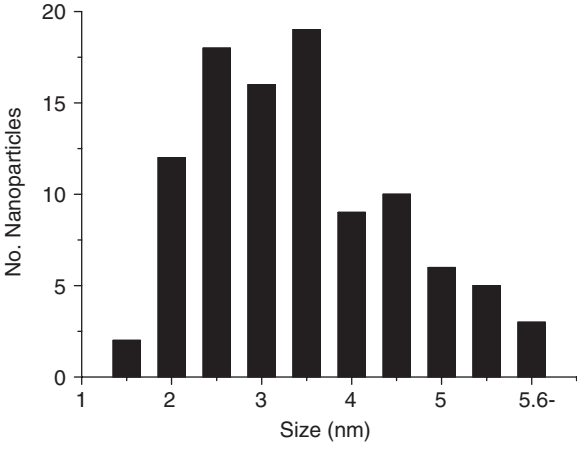
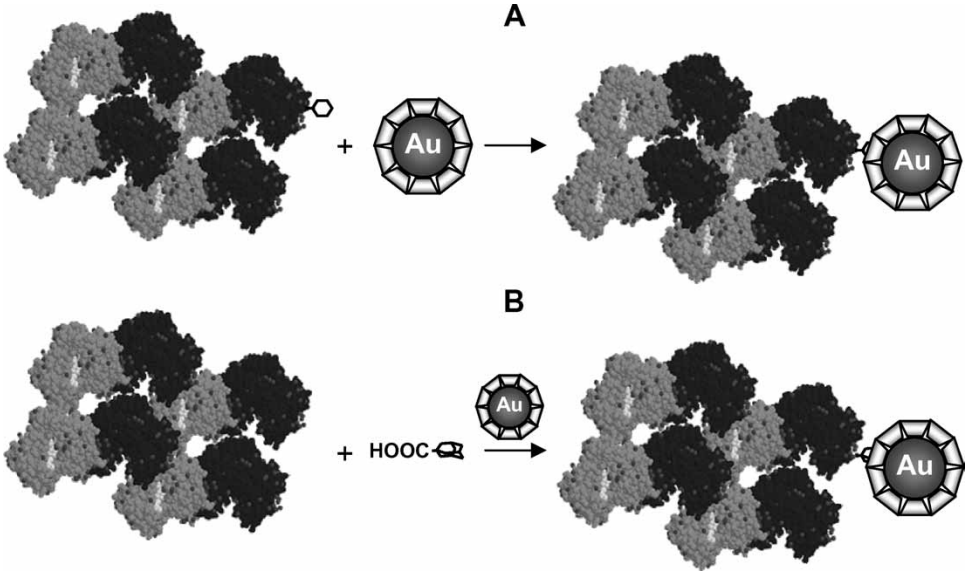


Figure 2. Size distribution of Au-CD nanoparticles.



Scheme 1. Preparation of supramolecular assemblies of native (A) and adamantane-modified PheDH (B) on β-cyclodextrin-coated gold nanospheres.

carboxylic acid in order to increase the amount of hydrophobic moieties at the protein surface, and consequently favoring its interaction with the CDs at the metal nanoparticles (scheme 1). It should be highlighted that CDs form highly stable inclusion complexes with adamantane derivatives in aqueous solution [31]. On the other hand, CDs attached at the surface of gold nanoparticles maintain the high affinity toward adamantane residues that they exhibit in homogeneous aqueous solution [16].

In the present work, and average of 13 mol of adamantane residues were attached to each mol of octameric protein, as revealed by colorimetric analysis.

The architecture of both immobilised enzymes (native and modified) could be a mixture of protein-metal aggregates with undefined structure, in which of two or more molecules of PheDH could be associated by using CD-Au nanoparticles as bridges, as suggested by the presence of colloid aggregates in the HRTEM images (figures 1B,C).

The immobilisation process of PheDH on CD-capped nanoparticles was studied by electronic spectroscopy. The CD-coated gold nanospheres showed the characteristic surface plasmon resonance due to gold with a maximum absorption around 510–520 nm (figure 3). Upon association of the native enzyme, the intensity of the surface plasmon resonance increased for the gold nanoparticle. In addition, a red shift of the maximum was observed for this band. These effects were slightly higher for the supramolecular nanodevice prepared with the modified enzyme. These facts indicate an interaction of both enzyme protein molecules with the metal surface of the colloids. Similar effects have been observed after the interaction of gold nanoparticles with other biomolecules [6, 32] and have been explained by an increase of the local refractive index in the vicinity of the colloid surface [32].

In table 1 the properties of the prepared nanocatalysts are reported. Although the enzymatic activity of PheDH was reduced to 77% after hydrophobic derivatisation, the amount of modified protein adsorbed in the nanoparticles was slightly higher when comparing with the native counterpart. This reduction in the catalytic activity could be caused by the transformation of several lysine residues located in the microenvironment of the catalytic site of PheDH, affecting both the local charge and hydrophilic topology of this site. On the other hand, both native and modified enzyme forms showed a slight increase in activity after immobilisation as well as a higher affinity for substrate. This fact could be explained by a local increase of substrate in the microenvironment of the immobilised enzymes, caused by the interaction of L-Phe with neighboring CD hosts. Similar behaviors have been previously observed for enzymes acting toward hydrophobic substrates after modification or immobilisation with CD

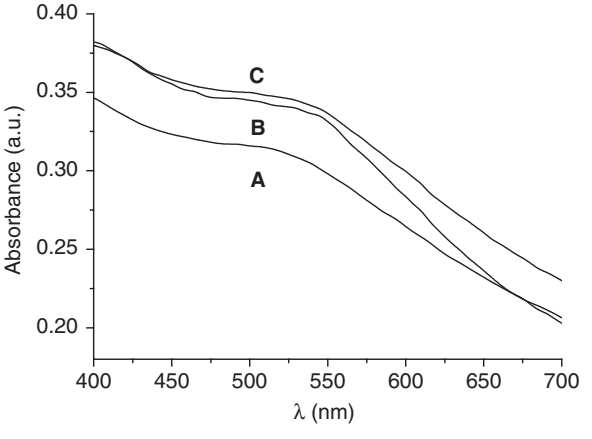


Figure 3. VIS spectra of the CD-modified gold nanospheres at 25°C in 20 mM sodium phosphate buffer pH 7.0 before (A) and after immobilisation of native (B) and modified PheDH (C).

derivatives [33–35]. It is also possible that a most active enzyme conformation is induced for PheDH by interaction with the CD-coated nanospheres.

Native PheDH also showed a noticeable improvement in the catalytic efficiency after immobilisation, as is reported in table 1 for the corresponding value of k_{cat}/K_m. On the contrary, the catalytic efficiency of the adamantane-modified enzyme preparation, both in immobilised and free forms, was noticeably lower than the corresponding to native counterpart.

The fluorescence spectra of native and modified enzyme forms before and after immobilisation on the nanoparticles are depicted in figure 4. The spectra of the native

Table 1. Properties of PheDH immobilised on CD-modified gold nanospheres.

Parameter	PheDH	PheDH + AuCD	Modified PheDH	Modified PheDH + AuCD
Immobilised activity (U/mg CD-Au)	–	1.1	–	0.9
Protein immobilised (μg/mg CD-Au)	–	100	–	110
Specific activity (U/mg PheDH)	10.4	11.0	8.0	8.2
$K_{m\,(app.)}$ (μM)	215	201	82	106
$k_{cat\,(app.)}$ (s^{-1})	816	865	105	170
$k_{cat}/K_{m\,(app.)}$ ($\mu M^{-1}s^{-1}$)	3.8	4.3	1.3	1.6

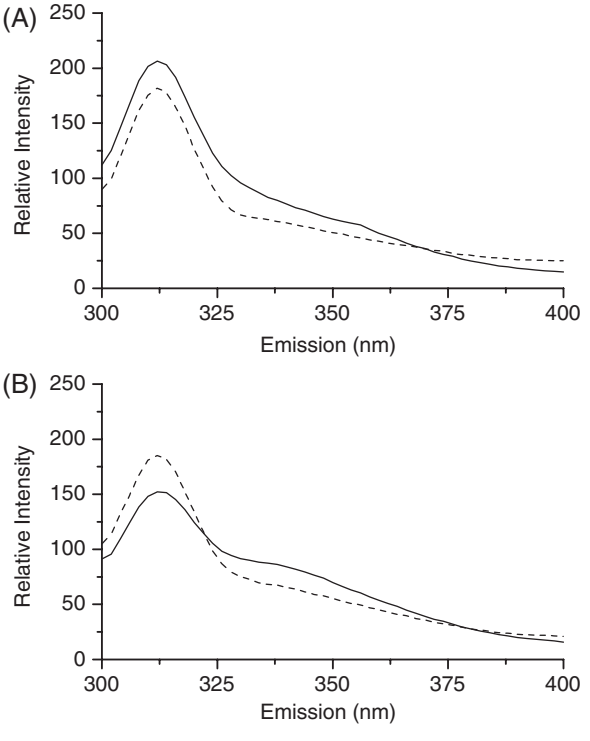


Figure 4. Fluorescence emission spectra of native (A) and adamantane-modified PheDH (B) before (——) and after (·····) immobilisation on CD-coated gold nanospheres.

and immobilised PheDH preparations upon excitation at 280 nm showed an identical pattern with an emission maximum at 313 nm, characteristic of tryptophan residues buried into the hydrophobic protein core. The fact that no red shift of emission maximum was observed suggests that the conformational structure of the enzyme was retained after immobilisation on the CD-coated nanoparticles. Indeed, the fluorescence emission spectrum of the native enzyme presents a maximum at 327 nm after thermal denaturation [36]. However, derivatisation of PheDh with adamantane residues yields a partially unfolded enzyme form, as evidenced by the decrease in the fluorescence intensity and the appearance of a secondary band shifted to the red zone of the spectrum. Interestingly, the conformational structure of the protein was partially recovered for the modified enzyme after immobilisation on the nanoparticles, as can been deduced from its fluorescence spectrum. This kind of "chaperone-like activity" over protein structures has been previously reported for CDs [19] as well as for CD-coated nanosized supports [13].

The pH-activity profile for the enzymes immobilised in this supramolecular nanoassembly is depicted in figure 5. The optimum pH range of enzymatic activity was increased for both native and modified PheDH forms after immobilisation on CD-coated Au nanospheres. In this sense, immobilised enzymes showed higher catalytic activity at alkaline values of pH (11.5–12.5) than the native counterpart

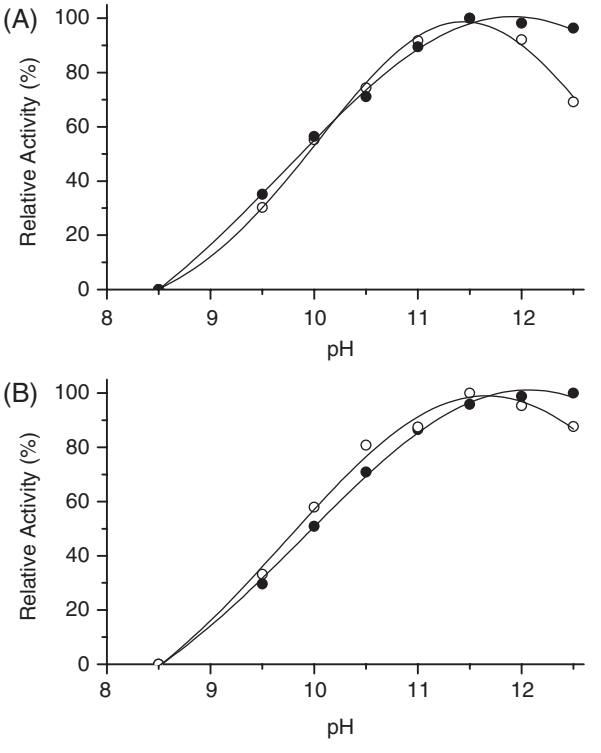


Figure 5. pH-activity profile of native (A) and adamantane-modified PheDH (B) before (——) and after (·····) immobilisation on CD-coated gold nanospheres.

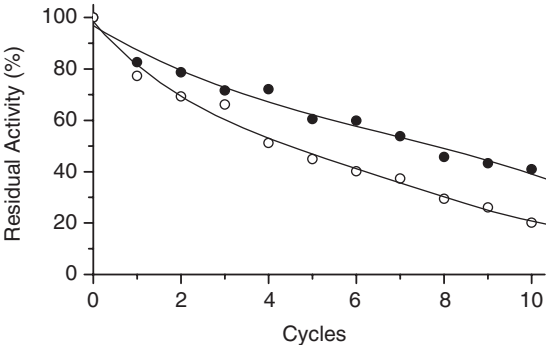


Figure 6. Reusability of native (○) and adamantane-modified PheDH (●) immobilised on CD-coated gold nanospheres.

(optimum pH 11.5). This result suggests that the microenvironment of the enzyme was changed by the supramolecular assembly as considered above. It should be important to point out that the thermal stability of the enzyme was not improved by the immobilisation process, and also that derivatisation with adamantane residues significantly reduced the thermal resistance of the enzyme (data not shown). This fact suggests that the improvement in the catalytic properties of the immobilised enzymes was induced by changes in their microenvironment, mediated by the presence of the CD-metal nanoparticles covering the protein structure.

The reusability of the nanocatalysts was assessed by adding concentrated NaCl solution to precipitate the immobilised preparations, dissolving again in storage buffer solution and assaying for enzymatic activity. It was seen that both enzyme preparations lost activity with each precipitation/redissolution cycle according to a first-order process (figure 6). However, the immobilised adamantane-modified PheDH form showed lower value of the constant for this process (0.09 $cycles^{-1}$), in comparison with those corresponding to the immobilised native enzyme (0.15 $cycles^{-1}$). Consequently, the nanocatalyst prepared with the modified and non-modified enzyme preparations retained about 41% and 20% of the initial activity after 10 cycles of reuse. This higher reusability properties of the modified enzyme adsorbed on the CD-coated gold nanoparticles was expected, considering the higher stability of the CD-adamantane host-guest complexes [31].

4. Conclusions

In this paper, we reported the supramolecular immobilisation of native and adamantane-modified PheDH on CD-coated gold nanoparticles in aqueous solution. This immobilisation process improved the catalytic properties of PheDH and conferred reusability to both enzyme nanocatalysts. Our results open a new perspective to the possible application of CD-modified gold nanospheres as water-soluble carriers for enzyme immobilisation.

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References

[1] D.L. Klein, R. Roth, A.K.L. Lim, A.P. Alivisatos, P.L. McEuen. A single-electron transistor made from a cadmium selenide nanocrystal. *Nature*, **389**, 389 (1997).
[2] C.M. Niemeyer. Nanoparticles, proteins, and nucleic acids: biotechnology meets materials science. *Angew. Chem. Int. Ed.*, **40**, 4128 (2001).
[3] T.A. Taton, G. Lu, C.A. Mirkin. Two-color labeling of oligonucleotide arrays via size-selective scattering of nanoparticle probes. *J. Am. Chem. Soc.*, **123**, 5164 (2001).
[4] C.V. Kumar, G.L. McLendon. Nanoencapsulation of cytochrome *c* and horseradish peroxidase at the galleries of α-zirconium phosphate. *Chem. Mater.*, **9**, 863 (1997).
[5] J. Li, J. Wang, V.G. Gavalas, D.A. Atwood, L.G. Bachas. Alumina-pepsin hybrid nanoparticles with orientation-specific enzyme. *NanoLett.*, **3**, 55 (2003).
[6] Y. Xiao, F. Patolsky, E. Katz, J.F. Hainfeld, I. Willner. "Plugging into enzymes": nanowiring of redox enzymes by a gold nanoparticle. *Science*, **299**, 1877 (2004).
[7] M.H. Liao, D.H. Chen. Immobilization of yeast alcohol dehydrogenase on magnetic nanoparticles for improving its stability. *Biotechnol. Lett.*, **23**, 1723 (2001).
[8] C. Schuler, F. Caruso. Preparation of enzyme multilayers on colloids for biocatalysis. *Macromol. Rapid Commun.*, **21**, 750 (2000).
[9] D.A. Schultz. Plasmon resonant particles for biological detection. *Curr. Opin. Biotechnol.*, **14**, 13 (2003).
[10] A.L. Crumbliss, S.C. Perine, J. Stonehuerner, K.R. Tubergen, J. Zhao, J.P. O'Daly. Colloidal gold as a biocompatible immobilization matrix suitable for the fabrication of enzyme electrodes by electrodeposition. *Biotechnol. Bioeng.*, **40**, 483 (1992).
[11] J. Zhao, J.P. O'Daly, R.W. Henkens, J. Stonehuerner, A.L. Crumbliss. A xanthine oxidase/colloidal gold enzyme electrode for amperometric biosensor applications. *Biosens. Bioelectronics*, **11**, 493 (1996).
[12] A.S. Edelstein, R.C. Cammarata. *Nanomaterials: Synthesis, Properties and Applications*, Institute of Physics Publishing, Bristol, UK (1996).
[13] R. Villalonga, A. Fragoso, R. Cao, P.D. Ortiz, M.L. Villalonga, A.E. Damiao. Supramolecular-mediated immobilization of trypsin on cyclodextrin-modified gold nanospheres. *Supramol. Chem.*, **17**, 387 (2005).
[14] R. Cao, A. Fragoso, R. Villalonga. Towards nanomedicine with a supramolecular approach. *IEE Proceedings-Nanobiotechnology*, **152**, 159 (2005).
[15] R. Villalonga, R. Cao, A. Fragoso, A.E. Damiao, P.D. Ortiz, J. Caballero. Supramolecular-mediated bienzymatic immobilization of catalase and superoxide dismutase on β-cyclodextrin-modified gold nanospheres. *J. Mol. Catalysis B. Enzymatic*, **35**, 79 (2005).
[16] J. Liu, W. Ong, E. Román, M.J. Lynn, A.E. Kaifer. Cyclodextrin-modified gold nanospheres. *Langmuir*, **16**, 3000 (2000).
[17] A. Fragoso, J. Caballero, E. Almirall, R. Villalonga, R. Cao. Immobilization of adamantane-modified cytochrome C at electrode surfaces through supramolecular interactions. *Langmuir*, **18**, 5051 (2002).
[18] M. Fernández, M.L. Villalonga, J. Caballero, A. Fragoso, R. Cao, R. Villalonga. Effects of β-cyclodextrin-dextran polymer on stability properties of trypsin. *Biotechnol. Bioeng.*, **83**, 743 (2003).
[19] M. Fernández, M.L. Villalonga, A. Fragoso, R. Cao, R. Villalonga. Effect of β-cyclodextrin-polysucrose polymer on the stability properties of soluble trypsin. *Enzyme Microb. Technol.*, **34**, 78 (2004).
[20] A. Verma, J.M. Simard, J.W.E. Worrall, V.M. Rotello. Tunable reactivation of nanoparticle-inhibited β-galactosidase by glutathione at intracellular concentrations. *J. Am. Chem. Soc.*, **126**, 13987 (2004).

[21] A. Gole, C. Dash, C. Soman, S.R. Sainkar, M. Rao, M. Sastry. On the preparation, characterization, and enzymatic activity of fungal protease-gold colloid bioconjugates. *Bioconjugate Chem.*, **12**, 684 (2001).
[22] I.D.G. MacDonald, W.E. Smith. Orientation of cytochrome c adsorbed on a citrate-reduced silver colloid surface. *Langmuir*, **12**, 706 (1996).
[23] S.H. Brewer, W.R. Glomm, M.C. Johnson, M.K. Knag, S. Franzen. Probing BSA binding to citrate-coated gold nanoparticles and surfaces. *Langmuir*, **21**, 9303 (2005).
[24] M.-E. Aubin-Tam, K. Hamad-Schifferli. Gold nanoparticle-cytochrome c complexes: the effect of nanoparticle ligand charge on protein structure. *Langmuir*, **21**, 12080 (2005).
[25] Y. Asano. Phenylalanine dehydrogenase. In *Encyclopedia of Bioprocess Technology: Fermentation, Biocatalysis and Bioseparation*, M. Flickinger, S.W. Drew (Eds), pp. 1955–1963, John Wiley & Sons, Inc., New York (1999).
[26] Y. Asano, A. Yamada, Y. Kato, K. Yamaguchi, Y. Hibino, K. Hirai, K. Kondo. Phenylalanine dehydrogenase of Bacillus badius. Purification, characterization and gene cloning. *Eur. J. Biochem.*, **168**, 153 (1987).
[27] Y. Asano, A. Yamada, K. Kato, K. Yamaguchi, Y. Hibino, K. Hirai, K. Kondo. Enantioselective synthesis of (s)-amino acids by phenylalanine dehydrogenase from Bacillus sphaericus: use of natural and recombinant enzymes. *J. Org. Chem.*, **55**, 5567 (1990).
[28] J. Liu, J. Alvarez, W. Ong, E. Román, A.E. Kaifer. Phase transfer of hydrophilic, cyclodextrin-modified gold nanoparticles to chloroform solutions. *J. Am. Chem. Soc.*, **123**, 11148 (2001).
[29] M.M. Bradford. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.*, **76**, 248 (1976).
[30] D. Bruneel, E. Schacht. Chemical modification of pullulan: 1. Periodate oxidation. *Polymer*, **34**, 2628 (1993).
[31] W.C. Cromwell, K. Byström, M.R. Eftink. Cyclodextrin-adamantane carboxylate inclusion complexes: studies of the variation in cavity size. *J. Phys. Chem.*, **89**, 326 (1985).
[32] N. Nath, A. Chilkoti. A colorimetric gold nanoparticle sensor to interrogate biomolecular interactions in real time on a surface. *Anal. Chem.*, **74**, 504 (2002).
[33] M. Fernández, A. Fragoso, R. Cao, M. Baños, R. Villalonga. Chemical conjugation of trypsin with monoamine derivatives of cyclodextrins. Catalytic and stability properties. *Enzyme Microb. Technol.*, **31**, 543 (2002).
[34] M. Fernández, A. Fragoso, R. Cao, R. Villalonga. Improved functional properties of trypsin modified by monosubstituted amino-β-cyclodextrins. *J. Mol. Catalysis B. Enzymatic*, **21**, 133 (2003).
[35] M. Fernández, M.L. Villalonga, A. Fragoso, R. Cao, R. Villalonga. Stabilization of α-chymotrypsin by modification with β-cyclodextrin derivatives. *Biotechnol. Appl. Biochem.*, **36**, 235 (2002).
[36] R. Villalonga, S. Tachibana, Y. Pérez, Y. Asano. Increased conformational and thermal stability properties for phenylalanine dehydrogenase by chemical glycosidation with end-group activated dextran. *Biotechnol. Lett.*, **27**, 1311 (2005).