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Supramolecular assembly of β-cyclodextrin-modified gold nanoparticles and Cu, Zn-superoxide dismutase on catalase

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Abstract

A bienzymatic supramolecular nanoassembly containing catalase and Cu, Zn-superoxide dismutase is reported. Catalase was hydrophobically modified with 1-adamantanecarboxylic acid and then immobilized on β-cyclodextrin-coated gold nanospheres via supramolecular associations. The bienzymatic nanocatalyst was further prepared by co-immobilization of β-cyclodextrin-modified superoxide dismutase. Supramolecularly immobilized catalase and superoxide dismutase retained 73 and 35%, respectively, of their initial specific activity. The range of optimum pH for catalase was increased from 7.0–7.5 to 6.5–7.5, and the thermal stability improved by 7 °C after co-immobilization. Superoxide dismutase was 90-fold more resistant to inactivation by 100 mM H_2O_2 after bienzymatic immobilization with catalase on metal nanoparticles.

Keywords: Catalase; Superoxide dismutase; Gold nanoparticles; β-Cyclodextrin; Enzyme immobilization; Nanocatalyst

1. Introduction

Recently, a great effort has been devoted to prepare nanoscale materials containing biological macromolecules [1–3]. These bio-functionalized nanodevices have relevant uses in sensing and imaging nanotechnology, drug delivery systems and electronic applications [4,5] and constitute the basis of future highly efficient nanorobots.

A special interest has been conferred to construct novel water-soluble nanocatalysts by immobilizing enzymes on colloidal particles [6–11]. Thus far, noble metal colloids have been the nanomaterials most extensively employed as three-dimensional scaffolds for enzyme immobilization [6,8–10] due to their unique optical and electronic properties. In general, such colloidal structures have been coated with enzyme monolayers through covalent immobilization methods, mainly based on the sulfur-noble metal chemistry [6,8,10]. However, to the best of our knowledge, no attention has been paid to the use of supramolecular interactions for preparing multilayer enzyme nanocatalysts.

Cyclodextrins (CD), cyclic oligosaccharides containing six (α-CD), seven (β-CD) or eight (γ-CD) α-1-4-linked D-glucopyranose units, have been used to stabilize gold nanoparticles in their perthiolated form [12]. On the other hand, we have conjugated CD units to enzymes in order to increase their stability and favour immobilization [13–15].

In a previous work we have shown that adamantane-modified proteins can be non-covalently immobilized on β-cyclodextrin (CD)-coated electrodes via supramolecular associations [16]. Specifically, Cytochrome *c* (Cyt *c*)-modified with adamantane (ADA) was immobilized at the surface of a silver electrode through the formation of stable

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host–guest complexes between ADA units located at the surface of the modified protein and perthiolated CD chemisorbed on the electrode surface. It was further demonstrated that most of the ADA units remained free after immobilizing the protein at the electrode surface (unpublished results).

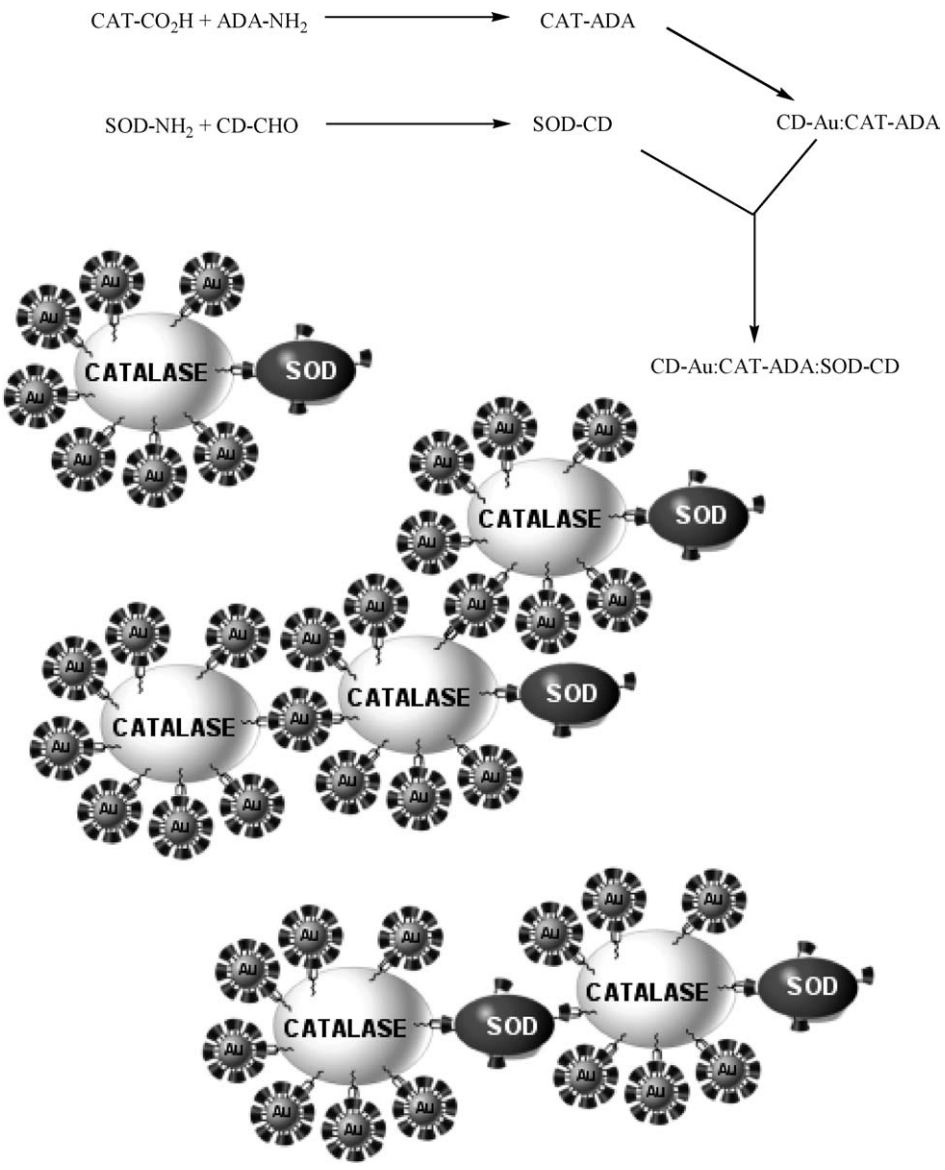
From these observations, we expected that such CD–ADA host–guest interactions could also be used for the co-immobilization of a second protein previously modified with CD. Here we report the preparation of a bienzymatic supramolecular assembly between catalase–adamantane (CAT–ADA) and Cu, Zn-superoxide dismutase-β-cyclodextrin (SOD–CD) conjugates on gold nanoparticles capped with perthiolated β-cyclodextrin (Scheme 1).

Co-immobilization of catalase and Cu, Zn-superoxide dismutase by covalent links has been previously reported as a method for protecting the latter enzyme against inactivation with H_2O_2 [17].

2. Materials and methods

2.1. Materials

Catalase (beef liver, EC 1.11.1.6, 10.3 U/g) and superoxide dismutase (bovine erythrocytes, EC 1.15.1.1, 3500 U/mg) were purchased from Roche Molecular Biochemistry (Mannheim, Germany). Xanthine, xanthine oxidase (cow milk, EC 1.1.3.22), $HAuCl_4$, nitroblue tetrazolium chloride and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) were purchased from Merck (Darmstadt, Germany). β-Cyclodextrin was purchased from



Scheme 1. Supramolecular assemblies of catalase and superoxide dismutase on β-cyclodextrin-coated gold nanospheres.

Amaizo (Indiana, USA). All other chemicals were of analytical grade.

2.2. Determination of nanoparticle size

High-resolution electron microscopy (HREM) images were obtained using JEOL 4000-EX instrument, operating at 400 kV. The particle size distribution was obtained from digitalized amplified micrographs by averaging the larger and smaller axis diameters measured in each particle.

2.3. Preparation of the CD-capped gold nanoparticles (Au–CD)

The β-cyclodextrin-capped gold nanoparticles were prepared as previously reported [12] using perthiolated β-cyclodextrin as capping molecules and a CD:$AuCl_4^-$ molar ratio of 1:5. The average diameter of these particles was determined by high-resolution electronic microscopy (HREM), and the amount of oligosaccharides attached was estimated by the redissolution method [18], assuming that the nanoparticles are perfect spheres.

2.4. Preparation of the enzyme conjugates

2.4.1. CAT–ADA

A reaction mixture containing 10 mg of catalase, 10 mg of 1-adamantanecarboxylic acid and 10 mg of EDAC, dissolved in 5 ml of 100 mM sodium phosphate buffer pH 6.0, was stirred for 1 h at room temperature and then for 16 h at 4 °C. The solution was further dialyzed against 20 mM sodium phosphate buffer pH 7.0.

2.4.2. SOD–CD

$NaBH_4$, 10 mg, were added to a reaction mixture containing 10 mg SOD and 10 mg mono-6-formyl-β-cyclodextrin [13] dissolved in 5 ml of 100 mM sodium phosphate buffer pH 7.0. The solution was stirred for 6 h in the dark at 4 °C and further dialyzed against 20 mM sodium phosphate buffer pH 7.0.

2.5. Enzymes co-immobilization on cyclodextrin-modified gold nanospheres

For the co-immobilization of the conjugated enzymes, a gold nanoparticle solution (3 mg/ml in 20 mM sodium phosphate buffer pH 7.0) was first mixed with CAT–ADA, for a final protein concentration of 0.30 mg/ml. The solution was gently stirred at 4 °C during 6 h, then mixed with a NaCl solution (10% m/v final concentration) and kept at 4 °C for 4 h. The mixture was further centrifuged to remove the non-immobilized enzyme. The pellet was 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 second layer was assembled by further mixing the former nanoparticle with a SOD–CD solution (40 μg/ml) as previously described for catalase. Protein concentration was estimated by the Lowry method using bovine serum albumin as standard [19].

2.6. Assays

Catalase activity was determined spectrophotometrically by the rate of decomposition of H_2O_2 at 25 °C in 20 mM sodium phosphate buffer pH 7.0 [20]. SOD activity was determined at 25 °C in 20 mM sodium phosphate buffer pH 7.0 by the xanthine/xanthine oxidase method using nitroblue tetrazolium chloride as indicator [21]. Total carbohydrate content was determined by the phenol/sulfuric acid method using glucose as standard [22]. The degree of modification of amino groups was determined by measuring the amount of free amino groups with *o*-phthalaldehyde using glycine as standard [23].

2.7. Thermostability

Native, modified and co-immobilized enzyme preparations were incubated in 20 mM sodium phosphate buffer, pH 7.0 at the stated temperatures for 10 min. Samples were removed, chilled quickly and assayed for enzymatic activity.

2.8. pH optimum

The catalytic activities of native, modified and co-immobilized enzyme preparations were measured at 25 °C in different buffer solutions: 20 mM sodium acetate, pH 5.0–6.0; 20 mM sodium phosphate, pH 6.5–8.0; 20 mM sodium borate, pH 8.5–9.0.

2.9. Stability of the co-immobilized SOD in the presence of 100mM H_2O_2

Native and co-immobilized SOD preparations were incubated at 30 °C in 100 mM H_2O_2 in 20 mM sodium phosphate buffer, pH 7.0. Aliquots were removed at scheduled times, treated with 1 μg of catalase, and further assayed for SOD activity.

3. Results and discussion

The gold nanoparticles, stabilized by a self-assembled monolayer of perthiolated β-cyclodextrin (Au–CD), served as scaffolds to obtain the bienzymatic nanocatalyst. These gold nanoparticles were obtained using a CD:$AuCl_4^-$ molar ratio of 1:5. The structures and size distribution of the naked gold nanoparticles were characterized by high-resolution electronic microscopy (HREM), revealing an average diameter of 3.5 nm (Fig. 1A).

The small size of the obtained nanoparticles is a result of the effect of the β-cyclodextrin molecules over the growing gold colloids, as nanovessels that control the cluster diameter.

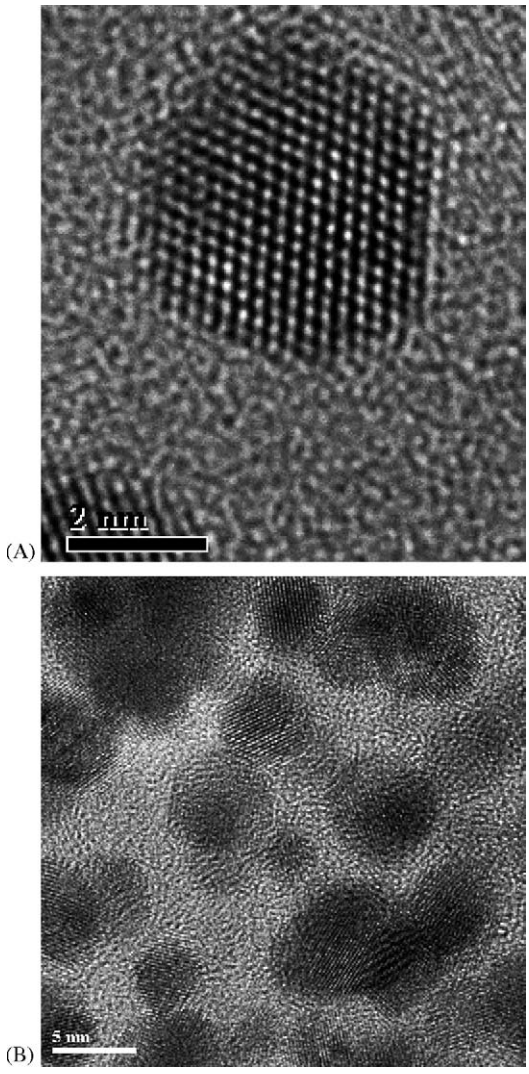


Fig. 1. HRTEM images of Au–CD nanoparticles before (A) and after immobilization of CAT–ADA and SOD–CD (B).

Larger nanoparticles would not be water-soluble. On the other hand, it is very important to use small nanoparticles in order obtain a nanodevice as small as possible that could easily diffuse through blood vessels and cell membranes.

Each nanoparticle contains, on average, 10 molecules of CD. Such as unit constitutes an excellent scaffold for supramolecular associations with modified enzymes. CD is capable of forming highly stable inclusion complexes with adamantane derivatives in aqueous solution [24]. In addition, it has been reported that CD receptors chemisorbed on the surface of gold nanoparticles maintain the host properties that they exhibit in homogeneous aqueous solution [12].

The first layer to be assembled on the Au–CD nanoparticle was catalase modified with adamantane moieties. For this, bovine liver catalase was chemically modified with 1-adamantanecarboxylic acid through a water-soluble carbodiimide-catalyzed condensation reaction. As a result, 87 amino groups out of 112 (78%) of catalase were modified with adamantane. Interestingly, a more active and more stable catalase was obtained after modification with adamantane and neutralization of the positively charged lysine residues on the surface of the enzyme. The specific activity of catalase was increased from 10.3 to 15.2 U/g after these modifications.

The adsorption isotherm for CAT–ADA on CD-modified gold nanoparticles, at 4 °C and pH 7.0, is depicted in Fig. 3A. The amount of associated CAT–ADA increased rapidly with increasing enzyme concentration up to 0.30 mg/ml. This saturating level of associated CAT–ADA corresponded to 87% of the added enzyme. It could be expected that the architecture of the immobilized CAT–ADA should be formed by a protein core covered by the CD-modified gold colloids, considering both the average size of the nanoparticles prepared, as well as the high molecular weight of catalase.

The second step in the preparation of bienzymatic nanoparticle was performing by treating the Au-catalase colloids with SOD modified with CD. It was assumed that some of the adamantane moieties of CAT–ADA would be free and could serve as guests of CD units conjugated to SOD. The SOD–CD conjugate was synthesized by attaching mono-6-formyl-β-cyclodextrin to the free amino groups of the enzyme through a reductive alkylation process with $NaBH_4$ [13]. An average of 12 mol oligosaccharides were linked to each mole of dimeric protein, as revealed by quantification of both the carbohydrate content and the transformed amino groups in the neoglycoenzyme. SOD retained 97% of its original activity after this conjugation. The high amount of CD attached, as well as the high degree of catalytic activity retained by the conjugate, proved the effectiveness of the synthetic method used.

An adsorption behavior, similar to that of CAT–ADA on Au–CD nanoparticles, was observed for the glycosidated SOD (Fig. 2B). The main difference between the two adsorption behaviors is that the saturation value was reached at a much lower protein concentration for SOD–CD. In this latter case, only 68% of the conjugated enzyme was immobilized. After this bienzymatic construction, modified catalase and superoxide dismutase retained 73% (7.5 U/g) and 35% (1225 U/mg) of their initial enzyme specific activity, respectively.

The obtained supramolecular-mediated bienzimatic nanocatalyst of CAT–ADA and SOD–CD on Au–CD nanoparticles presents an approximate (Au–CD):(CAT–ADA):(SOD–CD) molar ratio of 9:1:0.8. The relatively high proportion of Au–CD nanoparticles in the composition of the bienzymatic nanoparticle was expected according to the 9:1 molar ratio of adamantane moieties in CAT–ADA with respect to the CD units in Au–CD. This must provoke a great steric hindrance surrounding CAT–ADA, which explains the relatively low immobilization of SOD–CD. At the same time, this steric effect should affect the conformation of both conjugated enzymes, which could be the reason for the observed decrease in the enzymatic activities.

It should be highlighted that the final preparation could actually correspond to a mixture of protein–metal aggregates

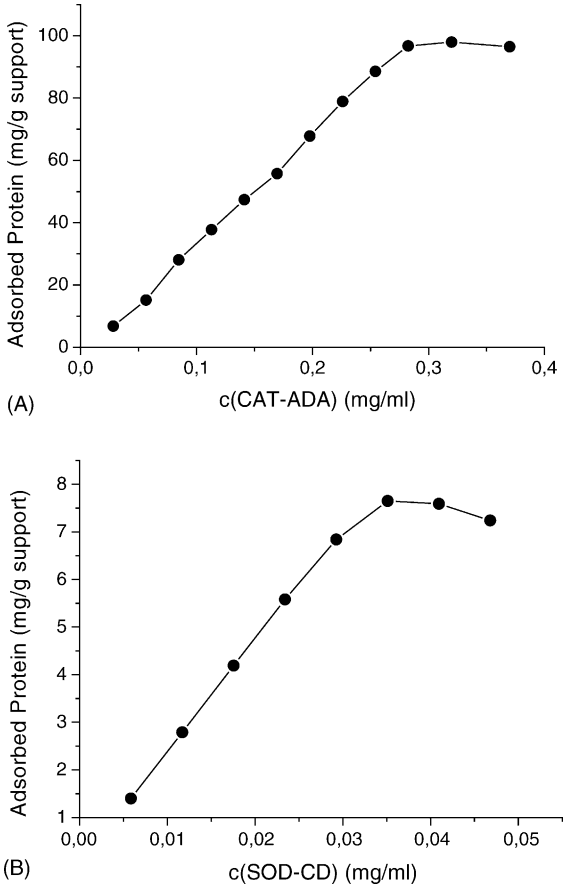


Fig. 2. Adsorption isotherm for CAT–ADA on Au–CD nanoparticles (A) and SOD–CD on CAT–ADA immobilized on CD-modified gold colloids (B) at 4 °C and pH 7.0.

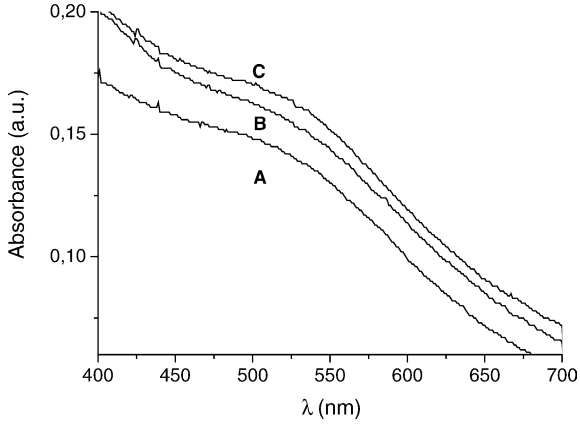


Fig. 3. VIS spectra of Au–CD nanoparticles (3 mg/ml) at 25 °C in 50 mM sodium phosphate buffer, pH 7.0 before (A) and after immobilization of CAT–ADA (B) and co-immobilization of CAT–ADA and SOD–CD (C).

with a random association mode, in which the SOD–CD conjugates and the high excess of Au–CD nanoparticles may surround each CAT–ADA unit (Scheme 1). This suggestion was confirmed by the presence of aggregates in the HRTEM images of the bienzymatic nanocatalyst (Fig. 1B). This consideration could also justify the unexpected lower enzymatic activity expressed by the SOD–CD conjugate after its inclusion in the nanostructured preparation.

A very important role in this random distribution of nanoparticles and modified enzymes is played by the fact that the mean diameters of the latter (CAT: 11.4 nm, SOD: 6.8 nm), both larger than that corresponding to the former (3.5 nm, Fig. 1A). Several metal nanoparticles surrounding a biomolecule is a common and expected situation and has been reported before [25].

The supramolecular association of catalase and superoxide dismutase on the cyclodextrin-capped nanoparticles was studied by electronic spectroscopy (Fig. 3). Native nanoparticles showed a broad absorption band, characteristic of this type of CD-coated gold nanostructures [12]. Upon immobilization of catalase, the intensity of the surface plasmon resonance increases for the gold nanoparticle, indicating interaction of the enzyme protein molecules with the metal surface of the colloids. Similar increased absorption has been observed after the interaction of gold nanoparticles with other biomolecules [10,26] and has been explained by an increase of the local refractive index in the vicinity of the colloid surface [26]. On the other hand, the intensity of the surface plasmon resonance was only slightly increased after co-immobilization of SOD.

The bienzymatic nanocatalyst was characterized according to its optimum pH and stability properties against thermal and H_2O_2 treatment. The pH–activity profile of catalase co-immobilized in this supramolecular nanoassembly is depicted in Fig. 4A. The optimum pH range of enzymatic activity was increased for catalase from 7.0–7.5 to 6.5–7.5 by co-immobilization with SOD on CD-coated Au nanospheres. It was further demonstrated that the thermal stability for co-immobilized catalase was increased in about 7 °C (Fig. 4B). These results suggest that the microenvironment of the conjugated enzyme was changed by the supramolecular assembly. Considering that CAT is a tetrameric enzyme [20], the supramolecular cross-linking of its protein chains with the CD-modified gold nanoparticles through host–guest interactions should reinforce its folding and, therefore, improve its functional stability. On the other hand, no significant changes were observed for optimum pH and thermal stability of SOD either after CD-modification and co-immobilization with catalase on the gold particles (data not shown). The main reason of this behavior is due to the fact that SOD is a highly thermostable enzyme with a broad range of optimum pH [27]. Our results suggest that these properties were not affected by glycosidation-immobilization. In addition, no changes in the pH–activity profile and thermal stability of a bienzymatic nanocatalyst prepared with CAT–ADA and SOD–CD without Au nanospheres was observed (data not shown). This fact suggests that the improvement in the functional properties of these co-immobilized enzymes was mediated by changes in their microenvironment, mediated by the presence of the metal nanoparticles.

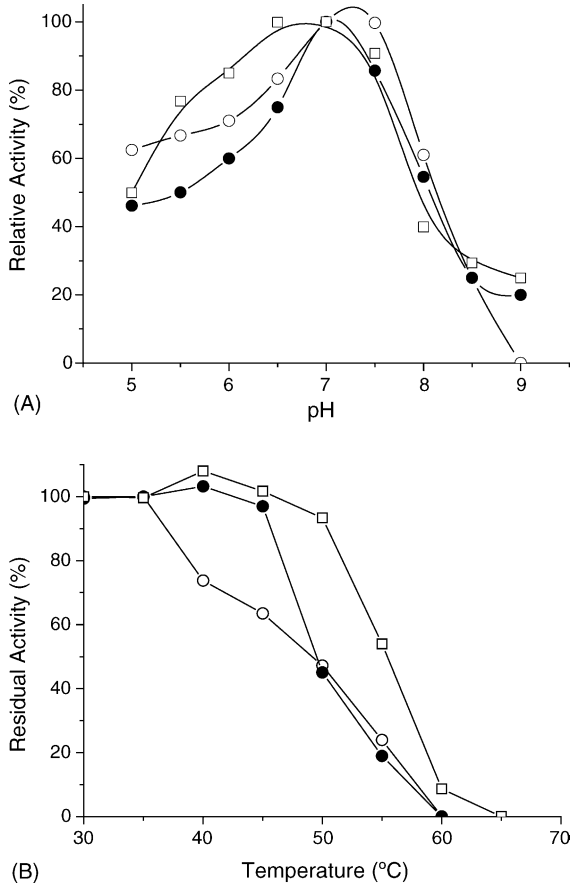


Fig. 4. Optimum pH (A) and thermal stability profile (B) of native (◯), ADA-modified (●) and co-immobilized (□) catalase forms.

Biomedical and analytical applications of SOD are currently limited by the inactivation of the enzyme by its own reaction product, H_2O_2 [28]. The time-course of inactivation of free, CD-modified and co-immobilized SOD by incubation in 100 mM H_2O_2 at pH 7.0 and 30 °C are depicted in Fig. 5. Native and CD-modified enzyme preparations were rapidly inactivated by H_2O_2, but its CD conjugate co-immobilized form retained about 90% of the initial activity after 2 h incubation. The half-life of SOD was increased from 13 min to 19.5 h, demonstrating that co-immobilization of SOD with catalase on gold nanoparticles conferred significant stabilization of this enzyme. This suggests that H_2O_2 inactivation of SOD is prevented by the presence of catalase in the context of the bienzymatic nanoparticle.

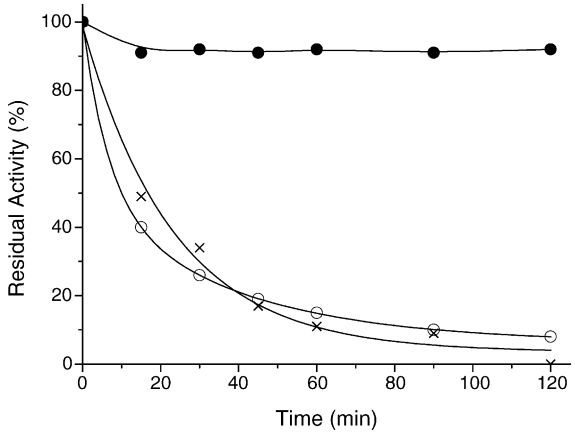


Fig. 5. Kinetics of inactivation of native (◯), CD-modified (X) and co-immobilized (●) SOD forms against 100 mM H_2O_2.

Improved resistance against inactivation with H_2O_2 has been previously described for SOD covalently linked with CAT, but the stabilization towards H_2O_2 treatment shown by this conjugate was significantly lower than the bienzymatic nanocatalyst described here [17]. On the other hand, the specific activity of catalase was noticeably reduced after chemical conjugation with SOD [17].

It should be highlighted that the improved stabilization of SOD against H_2O_2 should be directly mediated by the proximity of the active centre of catalase, as a consequence of our strategy of immobilizing one enzyme over the other. This fact constitutes an advantage of this described method over those based on the co-immobilization of both enzymes on the same surface. On the other hand, the stability towards H_2O_2 shown by the bienzymatic nanocatalyst prepared via supramolecular associations was noticeably higher than those prepared via to SOD against H_2O_2.

It has been previously reported that modification of enzymes with metal nanoparticles improved electroanalytical response of biosensors constructed with this kind of biocomposite [29]. According to this fact, our results open new possibilities for preparing more effective bienzymatic sensor systems based on this type of supramolecular nanostructures.

4. Conclusions

In this work a new strategy for preparing a CAT–SOD bienzymatic nanocatalyst was established through the formation of supramolecular nanoassemblies. The effectiveness of this method has been demonstrated by the improved functional properties showed by the co-immobilized enzymes. Experiments are now in progress to generalize this method of bienzymatic nanoimmobilization.

Acknowledgments

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