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Modeling and experimental validation of covalent immobilization of *Trametes versicolor* laccase in gold nanoparticles

Deborah González Abradelo [1] https://orcid.org/0000-0002-74671478
Bessy V. Cutiño Ávila [1]
Darío González Abradelo [2] https://orcid.org/0000-0002-9431-8647
José M. Guisán Seijas [3] https://orcid.org/0000-0003-1627-6522
Jorge González-Bacerio [1,4] https://orcid.org/0000-0002-7155-9165
Alberto del Monte-Martínez [1] * https://orcid.org/0000-0001-9608-5448

[1] *Centro de Estudio de Proteínas, Facultad de Biología, Universidad de La Habana. La Habana, Cuba*
[2] *Laboratorio de Bioinorgánica, Facultad de Química, Universidad de La Habana. La Habana, Cuba*
[3] *Departamento de Biocatálisis, Instituto de Catálisis y Petroleoquímica. Madrid, España*
[4] *Departamento de Bioquímica, Facultad de Biología, Universidad de La Habana. La Habana, Cuba*

Corresponding author: adelmonte@fbio.uh.cu

Keywords

*covalent immobilization on gold nanoparticles through amide bonds; immobilized derivative functional competence; protein load for immobilization; rational design of immobilized derivatives strategy; **Trametes versicolor** laccase*

ABSTRACT

Introduction. Protein immobilization is one of the most used technologies to increase the enzyme operational stability and improve their usage in biotechnological applications. Usually, the synthesis of immobilized derivatives is performed by the trial and error method. Our strategy for the rational design of immobilized derivatives (RDID) allows to select the optimal conditions for their synthesis. Gold nanoparticles (AuNPs), as non-porous solid supports, represent excellent systems for protein immobilization, with successful applications in nanomedicine, biocatalysis and electronics. **Methods.** The objective of this work was to validate the RDID strategy for covalent immobilization of the enzyme laccase from the basidiomycete white-rot fungus *Trametes versicolor* on AuNPs, activated with mercaptoundecanoic acid (AuNP-MUA) and MUA-ethylenediamine (AuNP-MUAen), for the potential use of the resultant biocatalysts in bioconversion processes. **Results.** As a result, the laccase-AuNP-MUA immobilized derivative is better than laccase-AuNP-MUAen in predicted immobilized derivative functional competence (42,8 % *vs* 26,1 %, respectively). In addition, all clusters predicted for laccase binding on AuNP-MUA resulted in immobilized enzymes with accessible active sites. In contrast, the half clusters predicted for interaction of laccase with AuNP-MUAen resulted in enzymes with poorly accessible active sites. Activity prediction was confirmed by an experimental expressed enzymatic activity of 45 % in laccase-AuNP-MUA system. Experimental maximal protein load matches the theoretical value ($12,7 \pm 0,7 \times 10^{-12}$ *vs* 12×10^{-12} protein mg / activated AuNP mL). The results obtained in this research suggest the possibility of using this biocatalyst on a large scale.

Modelación y validación experimental de la inmovilización covalente de la lacasa de *Trametes versicolor* en nanopartículas de oro

Palabras clave

carga proteica para la inmovilización; competencia funcional del derivado inmovilizado; estrategia de diseño racional de derivados inmovilizados; inmovilización covalente en nanopartículas de oro mediante enlaces amida; lacasa de ***Trametes versicolor****.*

RESUMEN

Introducción. La inmovilización de proteínas es una de las tecnologías más utilizadas para incrementar la estabilidad operacional de las enzimas y potenciar su uso en aplicaciones biotecnológicas. Usualmente, la síntesis de derivados inmovilizados se realiza mediante el método de ensayo-error. Nuestra estrategia de diseño racional de derivados inmovilizados (DRDI) permite seleccionar las condiciones óptimas para su síntesis. Las nanopartículas de oro (AuNPs), como soportes sólidos no pororosos, representan sistemas excelentes para la inmovilización de proteínas, con aplicaciones exitosas en nanomedicina, biocatálisis y electrónica. **Métodos.** El objetivo de este trabajo fue validar la estrategia DRDI para la inmovilización covalente de la enzima lacasa del hongo basidiomiceto de la podredumbre blanca *Trametes versicolor* en AuNPs activadas con ácido mercaptoundecanóico (AuNP-MUA) y MUA-etiléndiamina (AuNP-MUAen), para el uso potencial de los biocatalizadores resultantes en procesos de bioconversión. **Resultados.** Como resultado, el derivado inmovilizado lacasa-AuNP-MUA es mejor que lacasa-AuNP-MUAen en la competencia funcional del derivado inmovilizado predicha (42,8 % ***vs***. 26,1 %, respectivamente). Además, todas las agrupaciones de residuos superficiales predichas para la unión de la lacasa a AuNP-MUA resultaron en enzimas inmovilizadas con sitios activos accesibles. Por el contrario, la mitad de las agrupaciones predichas para la interacción de la lacasa con AuNP-MUAen resultaron en enzimas con sitios activos pobremente accesibles. La predicción de la actividad se confirmó por una actividad enzimática expresada experimental de 45 % en el sistema lacasa-AuNP-MUA. La carga proteica máxima experimental coincide con el valor teórico (12,7 ± 0,7 x 10^{-12} ***vs*** 12 x 10^{-12} mg de proteína / mL de AuNP activadas). Los resultados obtenidos en este trabajo sugieren la posibilidad de utilizar este biocatalizador a gran escala.

INTRODUCTION

Protein immobilization is probably the most used technology to increase the operational stability of these molecules and improve their usage in the synthesis of affinity matrices, biocatalysts for enzymatic bioconversion processes and other analytical and biomedical applications. [1-6] The traditional immobilization methods frequently produce low enzyme activities, due to the overlapping or destruction of the active sites by the binding to the support. [7] Usually, immobilization techniques involve the trial and error method, which is unfavorable due to the high cost of proteins and supports. Today, the design of immobilized systems is very complex and it is usually empirically performed (experimental screening or protein engineering). The empirical optimization of immobilization requires many experiments, and the discrimination among the different variables that are affecting the system is hard.

For this reason, it is necessary to rationalize and optimize the processes of obtainment of immobilized derivatives, on the basis of their physical-chemical characteristics and applications. Our strategy of rational design of immobilized derivatives (RDID) is directed to predict the behavior of the immobilized derivative before its synthesis, by the usage of mathematic algorithms and bioinformatics tools. [8] In this manner, it is possible to select the optimal conditions for its synthesis.

The industrial development in the last decades has implicated the release of high amounts of highly toxic residual chemical compounds, such as colorants, to the environment. [9] The microorganisms traditionally used to remove the colorants in textile wastes are the basidiomycete white-rot fungi. [10,11] The fungal species from the *Trametes* genus produce the ligninolytic enzyme laccase in high concentrations. This monomeric enzyme has low substrate specificity, being able to catalyze the oxidation of aromatic compounds with similar structure to lignin and, therefore, to remove colorants. [12,13]

On the other hand, gold nanoparticles (AuNPs), as non-porous solid supports, represent excellent systems for protein immobilization, with successful applications in nanomedicine, biocatalysis and electronics. [14,15] For this reason, the objective of this paper was to validate our RDID strategy for covalent immobilization of the enzyme laccase from Trametes versicolor on AuNPs, activated with mercaptounde-

canoic acid (AuNP-MUA) and MUA-ethylenediamine (AuNP-MUAen), for the potential use of the resultant biocatalysts in bioconversion processes. The following immobilization parameters were calculated: most probable configuration, functional competence of the immobilized derivatives and maximal quantity of protein to be immobilized. The laccase enzyme was immobilized on both supports, according to the immobilization parameters predicted by the RDID1.0 software. Last, experimental parameters: expressed enzymatic activity, immobilized enzymatic activity and practical maximal protein load were determined.

METHODS

Enzyme and reagents

The laccase enzyme from ***T. versicolor***, as well as the reagents $H[AuCl_4]$, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), MUA, sodium citrate, sodium dodecyl sulphate (SDS), acetone, ethyl ether, ethylenediamine, and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) are from Sigma-Aldrich (MO, USA). Laccase was used at 0,11 mg/mL in water.

Computational methodology

The experimentally obtained laccase 3D structure was retrieved from the protein data base RCSB Protein Data Bank (http://www.rcsb.org/pdb; PDB code: 1GYC). Structural analyses were performed using the molecular visor PyMol. [16] The deviation angle (DA) parameter was calculated using the plug-in eMovie from the PyMol software.

The software RDID1.0© (Enzyme Technology Group) [17] was used to predict the possible clusters in the laccase surface and to calculate the parameter covalent configuration probability (cCP) for each cluster. This software was also used to calculate the DA, the percentage of functional competence (% FC; catalytic competence in the particular case of enzymes), the percentage of functional competence in all population (% FCP), and the immobilized derivative functional competence (ImmDer. FC). DA is the angle between the active site entrance of the immobilized enzyme and the normal straight line to the support. % FC is the percentage of functionally (catalytically) competent enzymes, regarding all the molecules immobilized through the same cluster of reactive groups. It is calculated according to: % FC = 100 % - DA x 1.11 % (the functional competence of the cluster is penalized in 1.11 % for each deviation angle). % FCP is the percentage of functionally competent enzymes in each cluster, regarding the whole immobilized derivative. It is calculated according to: % FCP = cCPx% FC. ImmDer. FC is the percentage of functionally competent enzymes in the whole immobilized derivative (adding up the % FCP of all clusters). It is calculated according to: ImmDer. FC = Σ % FCP.[18]

In this work, we developed the following algorithms for nanoparticles. The theoretical maximal quantity of protein to immobilize in the nanoparticle (tMQ_{NP}) was determined. It is calculated according to: tMQ_{NP} = (NPN x MW x ANPSA) / (N_A x LIA_{NP}), where NPN is the total nanoparticle number in 1 mL of the immobilization solution, MW is the molecular weight of the protein to be immobilized, ANPSA is the surface area of activated nanoparticles, N_A is the Avogadro number and LIA_{NP} is the interaction area of the protein with the nanoparticle. ANPSA is calculated according to ANPSA = $4\pi r^2$, where r is the ratio of activated nanoparticle (for AuNP of 7,5 nm of diameter, *ANPSA* is approximately 176,715 x 10^{-81} m^2). LIA_{NP} is calculated according to LIA_{NP} = $2\pi rh$, where h is the height of the spherical cap occupied by the protein (figure 2).

Synthesis of AuNP-MUA

Sodium citrate (0,1103 g; 3,24 x10^{-4} mol) was refluxed in 100 mL distilled water and 5,2 µL 30 % $H[AuCl_4]$ solution were added. It was waited until getting a bright red color and the reflux was removed. The system was cooled and 0,2113 g SDS was added. Afterwards, 16 mg (7,5x 10^{-6} mol) MUA were added. Reaction was strongly shaken for 24 h and the liquid was concentrated in rotoevaporator. Citrate, MUA and SDS remains were removed by filtration of AuNP solution in polypropylene 1K Amicon ultracentrifugal filters (Millipore, Darmstadt, Germany).

Synthesis of AuNP-MUAen

Ethylenediamine (2 mL; 100 mg) was added to 2 mL of AuNP-MUA. The mixture was shaken for 20 min and adjusted at pH 7,0. In the case that AuNP-MUA precipitated, the same amount of SDS was added to solubilize the AuNP-MUA. After, 10 mg EDC were added for 15 min in ultrasonic and ice bath. This step was repeated. The synthesis was continued for 15 min in the same conditions. AuNP-MUAen was washed with acetone and ethyl ether several times by centrifugation until pelleted and dry AuNP-MUAen was observed. Synthesized AuNP-MUAen was solubilized in 8 mL distilled water and kept at room temperature.

Assessing the activated AuNPs size and concentration

Activated AuNPs size was determined from the maximal wavelength, and concentration from the molar extinction coefficient, both parameters available in the Sigma-Aldrich safety data sheet, 2013 (http://www.sigmaaldrich.com/united-states.html). UV-visible spectrum of the synthesized activated AuNPs is shown in figure 1.

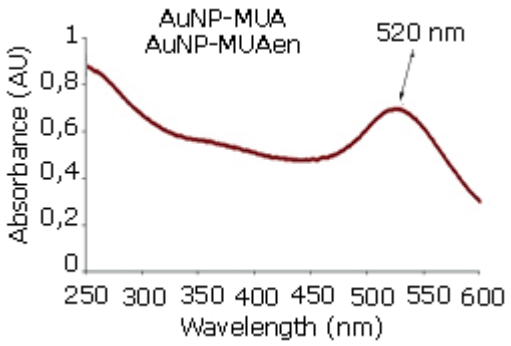

Fig. 1. UV-visible spectrum of the synthesized activated AuNPs. The plasmonic resonance band is shown by an arrow. AU: arbitrary units. AuNP-MUA: Mercaptoundecanoic acid-gold nanoparticle. AuNP-MUAen: Ethylendiamine-mercaptoundecanoic acid-gold nanoparticle.

Immobilization of *Trametes versicolor* laccase on AuNP-MUA and AuNP-MUAen

Activated AuNPs (100 μL; 4 mg/mL) was added to 200 μL distilled water pH 5,0. Temperature was maintained lower than 10ºC. EDC (1 mg) was added on two occasions and the mixture was sonicated for 10 min. Afterwards, 100 μL of enzyme solution (1 mg/mL) were prepared. The initial sample for enzyme activity and protein quantification by the Bradford method [19] was taken. Activated AuNPs solution was added slowly to the enzyme solution. The mixture was shaken, controlling temperature and pH, and sonicated for 5 min. The mixture was shaken for 2 h in cold and centrifuged for 15 min at 10,000 rpm and 4 ºC. The final sample (supernatant) for enzyme activity and protein quantification was taken. Finally, the pellet of activated AuNP was washed with water and centrifuged in the same conditions. Distilled water (1 mL) at pH 5,0 was added to the activated AuNP pellet and it was kept in cold.

Immobilization was controlled by the parameter differential protein immobilization grade [diff. IG (prot)]. [20] The experimental parameter practical maximal quantity of protein to immobilize (pMQ) was calculated from the obtained diff. IG (prot) values. [8] Immobilized enzymatic activity was determined by the differential method (soluble initial activity - activity in washes after immobilization), and it is expressed in units of enzymatic activity per support mL. Expressed enzymatic activity was determined by the same differential method, but it is expressed in percentage taking as 100 % the soluble initial enzymatic activity.

Assessment of laccase enzymatic activity

Laccase activity was assessed monitoring the absorbance at 420 nm, related with the oxidation of 1 mM ABTS in 0,1 M sodium acetate buffer pH 5,0. The ABTS molar extinction coefficient is 36 M^{-1} cm^{-1}. [21] Reactions were performed in 3 mL cuvettes at 25 ºC with 100xL of enzymatic solution (1 mg/mL in water). The experiments were performed by triplicate. One unit (U) of enzymatic activity was defined as the enzyme amount able to oxidize 1xmol ABTS per min under the assay conditions.

RESULTS

New mathematic algorithms for protein immobilization in nanoparticles

In this work, new mathematic algorithms in the RDID strategy for optimization of the maximal protein load to be immobilized on non-porous solids supports (nanoparticles) were developed. Protein molecules are located only in the nanoparticles surface, because these particles are non-porous supports. The nanoparticle diameter is very small, comparable to the protein diameter. Therefore, the protein-nanoparticle interaction cannot be considered protein-flat surface, as in the case of porous solid supports. Hence, the protein-nanoparticle interaction area (LIA_{NP}) was calculated in a different manner, according to the equation shown in Materials and methods section (figure 2).

The equation for LIA_{NP} is $LIA_{NP} = 2\pi rh$. h is calculated according to h = r – a (figure 2). a is calculated according to $\alpha = r \times sen(\alpha)$. The angle a is calculated according to $\alpha = arctan[(r+(d/2)] / [(d/2)]$. d is the protein diameter (figure 2).

Prediction of theoretical parameter *tMQ* for covalent immobilization of laccase from *Trametes versicolor* on activated AuNP supports

To optimize the protein load to immobilize, the parameter tMQ was calculated. For AuNP of 7,5 nm of diameter, tMQ value was 12 x 10^{-12} protein mg / support mL.

Prediction of the most probable configuration and immobilized derivative functional competence

Two activated AuNPs of 7,5 nm of diameter were used as supports for laccase immobilization in this work: AuNP-MUA and AuNP-MUAen. These supports contribute to laccase immobilization with two types of groups in the nanoparticle surface: acid (AuNP-MUA) and amino (AuNP-MUAen). These support groups interact with the opposite clusters of residues in the protein surface: AuNP-MUA with protein amino groups and AuNP-MUAen with protein acid groups. To predict the most probable configuration of the laccase-activated AuNP systems, the cCP of each cluster was calculated (table 1). With the aim of estimating the functionality of each immobilized derivative and selecting the best support, the ImmDer. FC was calculated (Table 1).

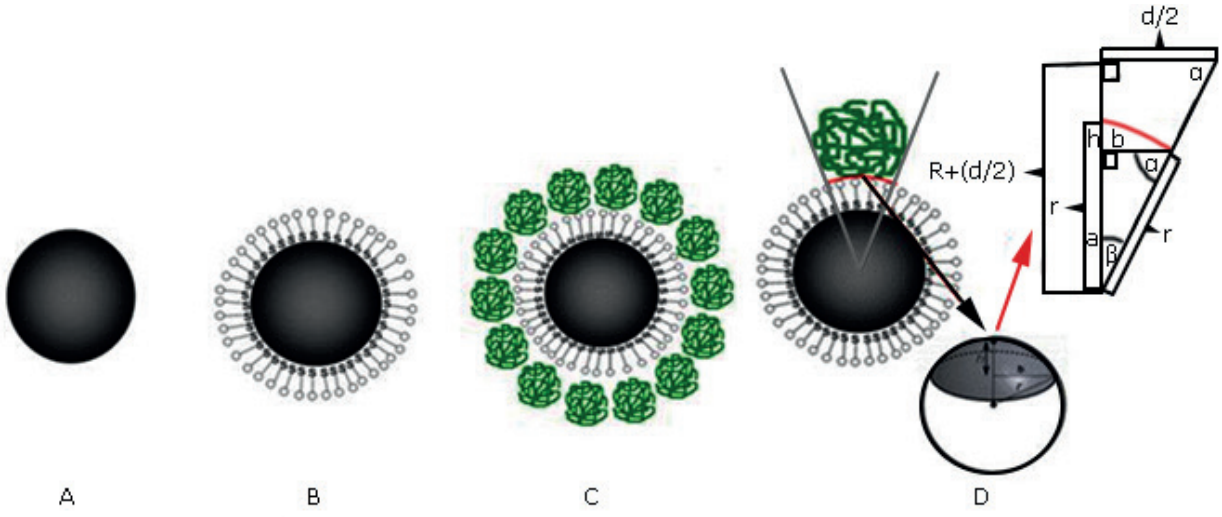

Fig. 2. Structural and geometrical considerations to calculate the protein-nanoparticle interaction area. A) Gold nanoparticle. B) Functionalized gold nanoparticle (spacer arm). C) Laccase from ***Trametes versicolor*** immobilized on functionalized gold nanoparticle. D) Calculus of the laccase-nanoparticle interaction area by application of trigonometric functions.

Table 1. *$RDID_{1.0}$* predictions of the possible clusters, covalent configuration probabilities and functional competence for immobilization of *Trametes versicolor* laccase enzyme on AuNP-MUA and AuNP-MUAen supports

Immobilized derivative	Cluster number	Protein residues	DA (°)	*cCP*	*ImmDer. FC* (%)
laccase-AuNP-MUA	1	N-term, K^{39}, K^{40}, K^{130}, K^{194}	30	0,50	42,8
	2	K^{39}, K^{40}, K^{130}, K^{475}, K^{482}	41	0,00	
	3	N-term, K^{59}, K^{482}	73	0,50	
laccase-AuNP-MUAen	1	D^{42}, D^{96}, D^{128}, D^{138}, E^{142}	43	0,15	26,1
	2	D^{42}, D^{96}, D^{128}, D^{323}, D^{419}, D^{470}, D^{473}	29	0,22	
	3	D^{364}, D^{486}, D^{492}, E^{496}	111	0,12	
	4	D^{96}, D^{323}, D^{364}, D^{470}, D^{473}, D^{486}, D^{492}	104	0,22	

DA: Deviation angle. cCP: Covalent configuration probability. ImmDer. FC: Immobilized derivative functional competence. AuNP-MUA: Mercaptoundecanoic acid-gold nanoparticle. N-term: Amino terminal group. AuNP-MUAen: Ethylendiamine-mercaptoundecanoic acid-gold nanoparticle. For immobilization through protein amino groups, three clusters were predicted (table 1). In this case, only two clusters share the most reactive amino group: the amino terminal group. For this reason, they have the highest cCP. The three clusters are catalytically competent, with DA < 90º (Table 1 and figure 3), although cluster 3 has the highest DA (73º; table 1 and figure 3B3). This fact diminishes its catalytic competence. For this reason, the ImmDer. FC does not reach 50 %.

In contrast, for immobilization through protein acid groups, six clusters were predicted. Three clusters (3, 4 and 6; table 1) are not catalytically competent, because the *DA* > 90º (active site overlapped with the support surface; figure 4). Since the sum of *cCP* corresponding to these three clusters are close to 0,5, their affectation of the immobilized derivative catalytic competence is significant (table 1). As a result, the ImmDer. FC is very low (26,1 %; table 1).

Immobilization of *Trametes versicolor* laccase enzyme on AuNP-MUA and AuNP-MUAen supports

T. versicolor laccase enzyme was immobilized on AuNP-MUA and AuNP-MUAen supports of 7,5 nm of diameter. As is shown in table 2, the pMQ value was similar to the tMQ one. On the other hand, expressed enzymatic activity (taking as 100 % the soluble enzymatic activity), experimentally determined, is similar to the predicted ImmDer. FC (table 1) for each immo-

bilized derivative. In this sense, the highest retention of functional activity was obtained for the laccase-AuNP-MUA immobilized derivative (45 %; table 2). The total immobilized enzymatic activity of this biocatalyst is 1,7 times that of laccase-AuNP-MUAen (402 *vs.* 239 U / support mL; table 2). For this reason, the recommended support for the enzyme immobilization is AuNP-MUA.

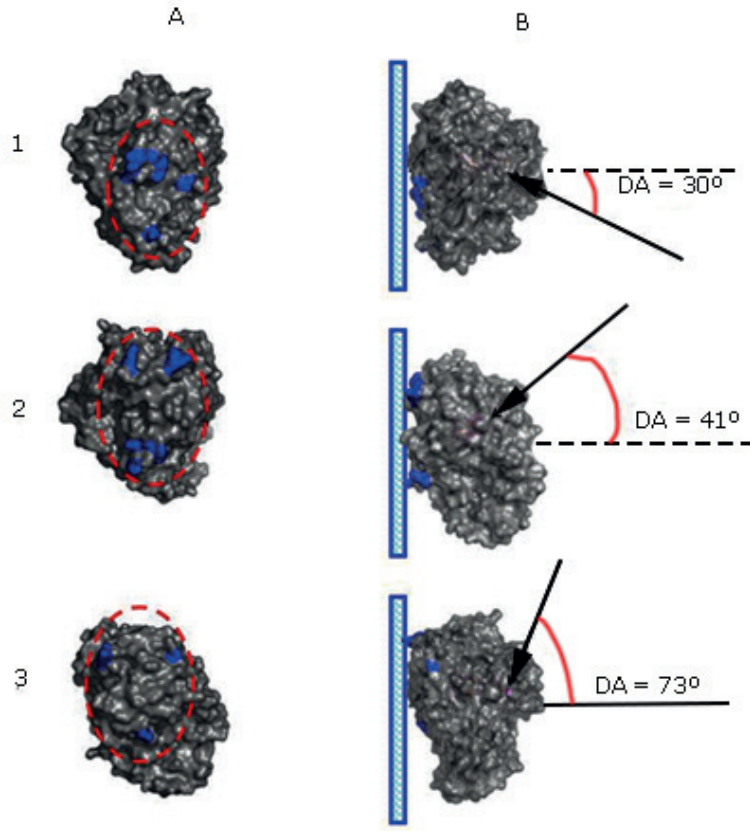

Fig. 3. Graphical representation of the different possible configurations of ***Trametes versicolor*** laccase-AuNP-MUA support system, predicted by $RDID_{1,0}$ software from the predicted clusters**.** (1-3) Clusters 1-3. (A) Possible clusters in laccase (surface representation) for covalent immobilization on AuNP-MUA support. (B) Most probable configuration of the immobilized derivative by each cluster. Grey: Enzyme surface area. Blue: Residues with amino groups on the enzyme surface. Discontinuous red circles: Cluster delimitation. ***DA***: Deviation angle. Arrow: Active site entrance. Black discontinuous line: Normal of the nanoparticle surface.

DISCUSSION

The mathematic algorithms developed in this work to calculate the maximal protein load to be immobilized on nanoparticles allow for extending the spectrum of RDID strategy applications to novel support types, such as non-porous ones. The main new consideration in this case is that the interaction is not produced between the protein and a flat surface, as in porous supports, due to the comparable diameter between proteins and nanoparticles. For porous supports, the theoretical maximal amount of protein to be immobilized, tMQ, is calculated assuming ideal conditions and not taking into account diffusional restrictions.[8] For this reason, the theoretical and practical maximal amount of protein to be immobilized could be different in these supports.[8] However, for nanoparticles, considering that they are not porous solid supports, the practical maximal quantity of protein to be immobilized should be close to the theoretical amount.

T. versicolor laccase has few superficial lysines; then, only three clusters were predicted (table 1). The reactivity of the lysines in the cluster 2 is not enough to reach the cCP values of clusters 1 and 3. On the other hand, as can be inferred from Table 1, acid residues are abundant and randomly distributed over the protein surface. This result in several clusters and the impossibility of orientating the immobilization, due to the cCP of all clusters, is close enough. Three clusters (3, 4 and 6; Table 1) produce enzyme-support configurations with high *DA* and low predicted catalytic activity. Therefore, the laccase-AuNP-MUAen biocatalyst has 59 % immobilized enzymatic activity, in comparison with laccase-AuNP-MUA immobilized derivative (239 *vs*. 402 U / support mL, respectively; table 2). Curiously, we immobilized covalently the laccase enzyme from *T. maxima* (another species from the *Trametes* genus) on a porous solid support, and the highest expressed enzymatic activity was obtained when the immobilization was perfor-

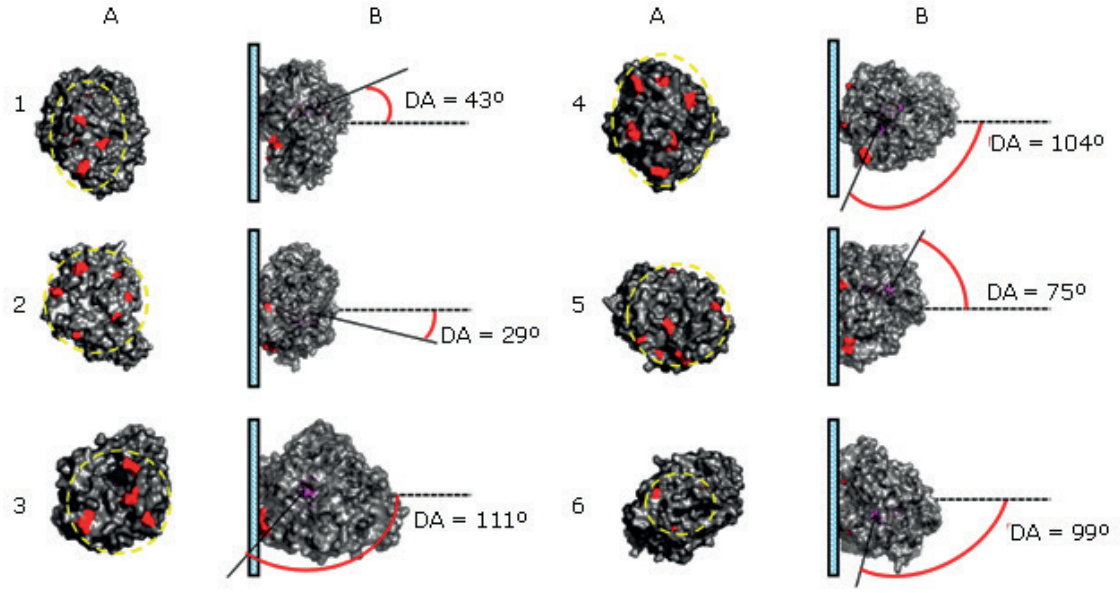

Fig. 4. Graphical representation of the different possible configurations of *Trametes versicolor* laccase-AuNP-MUAen support system, predicted by $RDID_{1.0}$ software from the predicted clusters. (1-6) Clusters 1-6. (A) Possible clusters in laccase (surface representation) for covalent immobilization on AuNP-MUAen support. (B) Most probable configuration of the immobilized derivative by each cluster. Grey: Enzyme surface area. Red: Residues with acid groups on the enzyme surface. Discontinuous yellow circles: Cluster delimitation. *DA*: Deviation angle. Arrow: Active site entrance. Black discontinuous line: Normal of the nanoparticle surface.

Table 2. Experimental values of protein load and enzymatic activity in the immobilized derivatives laccase-AuNP-MUA and laccase-AuNP-MUAen

Immobilized derivative	*pMQ* (protein mg / support mL)	Immobilized enzymatic activity (U / support mL)	Expressed enzymatic activity (%)
laccase-AuNP-MUA	$12,7 \pm 0,7 \times 10^{-12}$	402	45
laccase-AuNP-MUAen	$16,5 \pm 0,8 \times 10^{-12}$	239	27

AuNP-MUA: Mercaptoundecanoic acid-gold nanoparticle. AuNP-MUAen: Ethylendiamine-mercaptoundecanoic acid-gold nanoparticle. *pMQ*: Practical maximal quantity of protein to immobilize. U: enzymatic activity unit.

med through protein acid groups at pH 5,0 rather than through protein amino groups at pH 7,0. [22]

In this work, the experimental expressed enzymatic activity (45 and 27 % for laccase-AuNP-MUA and laccase-AuNP-MUAen, respectively; Table 2) was similar to predicted *ImmDer. FC* (42,8 and 26,1 % for laccase-AuNP-MUA and laccase-AuNP-MUAen, respectively; table 1). This result demonstrates the validity of our RDID strategy to model protein immobilization on nanoparticles.

The two activated AuNPs tested in this work were evaluated for the potential use of the resultant biocatalysts in bioconversion processes. Immobilization of soluble enzymes on solid supports is essential for the development of industrial processes based on biotransformations catalyzed by these enzymes. [1-6,23] Immobilization allows for recovery, and hence enzyme reuse in discontinuous reactors, since it permits enzyme separation from the remaining reaction mixture. On the other hand, the development of continuous processes of catalyzed enzyme reactions is only possible if the enzyme is immobilized inside the reactor.

Conclusions

In this work, we focused on the development of new algorithms for the strategy of rational design of immobilized derivatives. These algorithms permit calculating and optimizing the maximal protein load to be immobilized on non-porous

solid supports, especially AuNPs. These new algorithms and the RDID strategy in general were successfully applied to the optimization of immobilization of *T. versicolor* laccase enzyme on nanoparticles.

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Conflict of interests

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Authors contribution list

- Conceptualization: Alberto del Monte Martínez
- Data curation: Alberto del Monte-Martínez,
- Formal analysis: Jorge González Bacerio, Alberto del Monte Martínez
- Funding acquisition: Alberto del Monte Martínez
- Investigation: Deborah González Abradelo, Bessy V. Cutiño Avila, Darío González Abradelo
- Methodology: Jorge González Bacerio, Alberto del Monte Martínez
- Project administration: Alberto del Monte Martínez
- Resources: José M. Guisán Seijas, Alberto del Monte Martínez
- Software: Alberto del Monte Martínez
- Supervision: Jorge González Bacerio, Alberto del Monte-Martínez

- Validation: Alberto del Monte Martínez
- Visualization: Alberto del Monte Martínez
- Writing-original draft: José M. Guisán Seijas, Jorge González-Bacerio, Alberto del Monte Martínez
- Writing-review and editing: José M. Guisán Seijas, Jorge González Bacerio, Alberto del Monte Martínez

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