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Studies toward stereoselective bionanocatalysis on gold nanoparticles

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ABSTRACT

As yet, different enzymes were immobilized on gold nanoparticles both through adsorption and covalent binding. However, there is only one evaluation if such immobilization influenced enzyme enantioselectivity, which is an essential parameter in biocatalysis. Therefore systematic studies with enzymes immobilized on gold nanoparticles through covalent binding and embedded through adsorption were performed. Adsorption was not efficient method and it significantly lowered enantioselectivity of enzymes. In turn, covalent binding was in most cases very good method of immobilization, especially for *Pseudomonas cepacia* lipase, where conversion and enantioselectivity were even slightly better than for native enzyme. It was also evaluated that in case of adsorption size of nanoparticles did not influence enantioselectivity, but in case of covalent binding small nanoparticles gave much better results than big ones.

1. Introduction

Stereoselectivity is one of a key parameter in biocatalysis and in biosensors based on enzymatic reactions. Such biosensors have been improved by adding enzymes immobilized on gold nanoparticles on an electrode surface. Sensors of glucose, fundamental in diagnostics and food testing, had lower detection limit and were more selective, if enzymes on AuNPs in silicate [1], mesoporous carbon [2] or composite [3] were placed on electrodes. Sensor of hydrogen peroxide were more stable when horseradish peroxidase was immobilized on AuNPs, instead of direct immobilization on gold electrode [4]. L-lactate concentration is measured indirectly by measuring concentration of NADH. NADH is reduced during oxidation of lactate to pyruvate catalyzed by lactate dehydrogenase. Gold electrode is not sensitive and selective for NADH, but gold electrode covered with silicate gel and AuNP with dehydrogenase was more sensitive [5].

Gold nanoparticles have influence on activity of enzymes used as biocatalysts. Wu et al. [6] found out that *Candida rugosa* lipase had three times lower K_m parameter (Michaelis constant) in reaction of hydrolysis of *p*-nitrophenyl palmitate when enzyme was immobilized on AuNPs. Maximal velocity of reaction was not changed. Smaller AuNPs (13 nm diameter) gave better results than big ones [7]. Aspartyl protease [8] and pepsin [9] after immobilization on gold nanoparticles had similar activity in reaction of protein hydrolysis as native enzymes. In addition, enzymes with AuNPs embedded on zeolite were active in wider temperature and pH range. In these experiments stereochemical aspect of bionanocatalysis was not investigated.

Aside from non-thiolated AuNPs, also nanoparticles capped with thiols were used in biocatalysis. Lysine-tagged aminopeptidase II from *Bacillus stearothermophilus* was covalently bound to gold nanoparticles thiolated with 16-mercaptohexadecanoic acid [10]. Amount of immobilized enzyme was 50 and 12.5 times bigger for enzyme tagged with respectively 9 and 3 lysine residues than for native enzyme. In case of enzyme modified with 3 Lys, 64% of immobilized enzyme was active and its pH and temperature stability was improved. Enzyme was reused a few times and was active in 5 catalytic cycle (24% of the initial activity in 5th cycle).

Interactions of gold nanoparticles with proteins change conformation and structure of proteins, e.g. hemoglobin [11] and albumin [12]. Amount of α-helices decrease whereas β-sheets and β-turns increase. In case of enzymes such interactions, mainly near active site, should cause changes in substrate specificity or stereoselectivity of biocatalysts. All cited examples of enzymes immobilized on gold nanoparticles were kinetic studies. But there is only one case of studies where stereoselectivity of such enzyme was mentioned, and it turned out that immobilization on gold nanoparticles

Abbreviations: AuNPs, gold nanoparticles; c, conversion; *C. antarctica*, *Candida Antarctica*; *C. rugosa*, *Candida rugosa*; CDI, carbonyldiimidazole; DLAP, duck liver acetone powder; E, enantioselectivity; ee_s, enantiomeric excess of substrate; NADH, nicotinamide adenine dinucleotide; *M. javanicus*, *Mucor javanicus*; PBS, phosphate buffer saline; PLE, pig liver esterase; PPL, porcine pancreatic lipase; *P. cepacia*, *Pseudomonas cepacia*; TLAP, turkey liver acetone powder.

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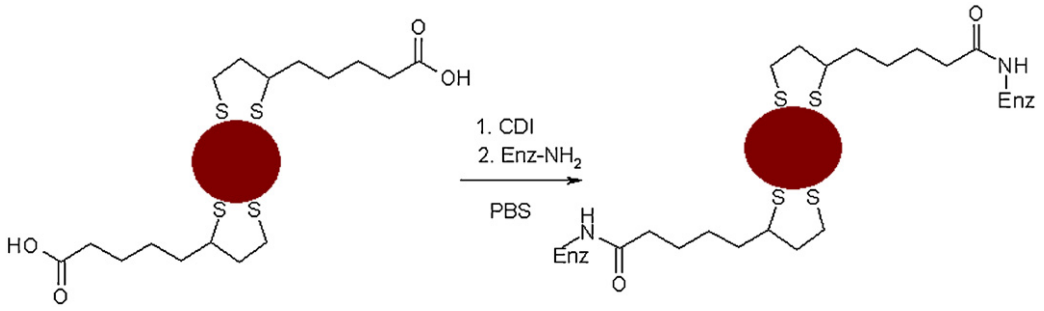


Fig. 1. Covalent immobilization of enzymes (CDI – carbonyldiimidazole, Enz-NH_2 – enzyme, PBS – phosphate buffer saline).

did not change enantioselectivity of alcohol dehydrogenase [13]. We decided to investigate in systematic way how gold nanoparticles of different kinds and sizes affect stereoselectivity of selected hydrolytic enzymes.

2. Experimental

Chemicals (except solvents) and enzymes were purchased from Sigma–Aldrich and used without further purification. Solvents were purchased from POCh and purified with distillation. Liver acetone powders were prepared [14] and enzymes were purified [15] in our laboratory by standard protocols. Thin layer chromatography was performed on TLC Plates 60 F-254 from Merck. Silica gel 60 230–400 Mesh was from Merck. UV–vis spectra were recorded on Hitachi U-1900 Spectrophotometer. High performance liquid chromatography was performed with Varian ProStar (with UV Varian ProStar 330 detector and Chromatopac C-R6A analyzer). Centrifugation: Eppendorf Centrifuge 5804 R.

Protein was measured with the Bradford dye calibrated against BSA [16], according to procedure attached to the Bradford reagent.

2.1. Nanoparticles

Non-thiolated nanoparticles were prepared according to known procedure: 3.5 ± 0.7 nm AuNPs were obtained using sodium borohydride [8], 13 ± 1.5 nm and 20 ± 4 nm AuNPs were obtained using sodium citrate [6,10]. Concentrations of nanoparticles solutions were 200 nM, 12 nM and 18 nM respectively. Observed absorption maxima were 514 nm, 520 nm and 522 nm, which was in agreement with literature data.

Thiolated AuNPs: To 70 mL 10.3 mM methanolic solution of $HAuCl_4 \cdot H_2O$, 1 mL 0.24 M methanolic solution of α-lipoic acid was added. Next, 35 mL 32 mM water solution of $NaBH_4$ was added. After 2 h of mixing at ambient temperature, the mixture was centrifuged (20 min, 13,000 rpm), the pellet was washed 4 times with methanol and dried. 120 mg of gold nanoparticles of 5 ± 1 nm diameter were obtained.

2.2. Immobilization

Adsorption: solution of non-thiolated nanoparticles (0.2 mL, initial concentration) was centrifuged (20 min, 13,000 rpm) and redispersed in 1.1 mL of distilled water. 0.1 mL enzyme solution (10 U) was added. After 30 min incubation at 23 °C, the probe was centrifuged and the pellet was rinsed with buffer (0.01 M PBS pH 7.4) until the supernatant had no catalytic activity, and re-dispersed in 0.3 mL of PBS.

Covalent binding: 8 mg AuNPs thiolated with α-lipoic acid were dissolved in 1.4 mL 0.02 mM CDI in PBS and shaking 2 h at 23 °C. Concentration of α-lipoic acid was 1.4 mM. Next, 0.1 mL enzyme solution (10 U) was added and shaking another 2 h. After this time the mixture was centrifuged (20 min, 13,000 rpm), the pellet was rinsed 3 times with buffer and re-dispersed in 0.3 mL of PBS.

2.3. Ethyl 3-hydroxy-3-phenylpropanoate

Synthesis of racemic ethyl 3-hydroxy-3-phenylpropanoate was performed according to known procedure [17]. HPLC method for resolution of ethyl 3-hydroxy-3-phenylpropanoate enantiomers: Chiralcel-OB column, eluent was hexan/isopropanol 96:4; flow 0.8 mL/min. Retention times: 19.08 min (*R*-enantiomer), 21.81 min (*S*-enantiomer).

2.4. Kinetic resolution

10 mg of selected enzyme was incubated 45 min at 23 °C with 0.2 mL solution of 3.5 nm AuNPs, 0.1 mL solution of 20 nm AuNPs or 0.2 mL of water. Enzyme prepared in this way was added to 1 mL of PBS with 0.1 mmol of 3-hydroxy-3-phenylpropanoate. The mixture was shaking at 23 °C until conversion was about 50% (monitored with TLC). The mixture was extracted with diethyl ether and washed with saturated solution of sodium bicarbonate. Extract was dried with anhydrous magnesium sulfate, filtered and solvent was evaporated under reduced pressure. Recovered substrate was purified with column chromatography.

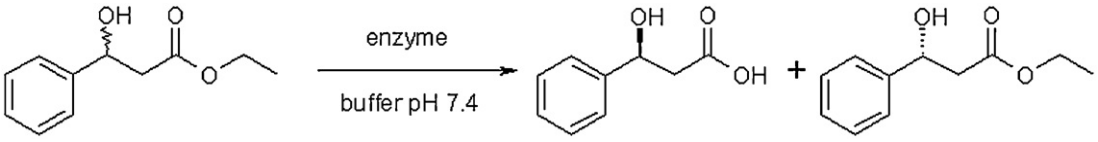
In case of immobilized enzymes 0.3 mL of enzyme on AuNPs in PBS was added instead of 10 mg of enzyme incubated with AuNPs.

Selected experiments were performed 5 times. Differences in conversion and enantiomeric excess were less than 5%.

3. Results and discussion

3.1. Kinds of nanoparticles

Nanoparticles have dimensions similar to proteins, therefore their size should play crucial role in interactions with enzymes. This problem was examined only for *C. rugosa* lipase [7]. For our studies a few selected kinds of AuNPs were generated. Non-thiolated AuNPs reduced with sodium citrate [6,10] or sodium borohydride [8] were obtained as water solutions. Reaction progress and growing of nanoparticles was followed up by spectroscopic methods [8]. Absorption maxima indicated that nanoparticles of relevant sizes were formed. To obtain 5 nm thiolated nanoparticles procedure known for 11-mercaptoundecanoic acid was used [18] with α-lipoic acid as a thiol. Reduction was performed in water–methanol solution and reducing agent was sodium borohydride. Some modifications of procedure, including changing of thiol amount, were brought in to form larger nanoparticles, but effective formation of nanoparticles was observed only for original conditions. Obtained nanoparticles, were next used as a base to immobilization of selected enzymes.



Scheme 1. Kinetic resolution of ethyl 3-hydroxy-3-phenylpropanoate.

3.2. Immobilization

Non-thiolated AuNPs of various sizes were used to embedding selected enzymes by adsorption [7]. Due to the great affinity of thiol and amino groups, which are present in proteins, to gold such procedure was reasonable. Water solutions of enzymes were added to water solutions of nanoparticles, mixed, centrifuged and re-dispersed in PBS (0.01 M phosphate buffer saline pH 7.4). Absorption maximum showed small red shift related to bigger size of conjugates than nanoparticles alone. Aggregation of AuNPs was not observed.

Covalent immobilization (Fig. 1) was formation of amide bond between carboxylated gold nanoparticles and amino groups of enzymes using carbonyldiimidazole as a coupling agent. AuNPs capped with α-lipoic acid were added to solution of CDI in PBS. After shaking, enzyme solution was added and the mixture was shaking 2 h. Next, immobilized enzyme was centrifuged, washed with PBS and re-dispersed in PBS. Deep gray-black color of solution indicated that good dispersion was obtained. For nanoparticles bigger than 5 nm immobilization was less effective.

3.3. Kinetic resolution

Aim of our research was to investigate various immobilization procedures and their influence on enantioselectivity of reaction, and not to work out a new optically pure compound. Therefore racemic ethyl 3-hydroxy-3-phenylpropanoate was picked out which is well-examined compound. Reaction conditions of its kinetic resolution were optimized and the best native biocatalyst was found [19,20]. In our laboratory, this class of compounds is also well-known [21]. The hydrolysis reaction was chosen because non-thiolated nanoparticles are stable in water solutions. Ester was obtained by reduction of ethyl benzoylacetate with procedure reported by Hattori et al. [17].

Firstly, kinetic resolution reactions were catalyzed by native enzymes and by mixtures of enzymes and nanoparticles (Scheme 1). Catalytic effect was a sum of adsorbed and free enzyme in solution. Results are presented in Table 1. Among over twenty hydrolases used, nine were good catalysts of the model reaction.

Nanoparticles did not affect conversion of the reaction. In case of pig liver esterase (PLE), porcine pancreatic lipase (PPL) and *Candida antarctica* lipase adding of AuNPs changed enantioselectivity of the reaction. For PLE and PPL enantioselectivity was improved and effect depended on size of nanoparticles. For majority of enzymes influence of nanoparticles was non-significant. In case of *Pseudomonas cepacia* lipase, which is the best biocatalyst of examined reaction, enantioselectivity did not decrease which was very important and promising result (entries 13–15, Table 1).

In the second step, enzymes embedded on gold nanoparticles were used to the kinetic resolution reaction. Two enzymes were active after adsorption, and only *P. cepacia* lipase catalyzed the reaction stereoselectively (Table 2). Enantioselectivity was lower because of long reaction time and increasing participation of autohydrolysis.

Size of nanoparticles had rather small influence on enantioselectivity of the reaction. Also time of incubation of enzyme with nanoparticles (results not showed) was not essential. The process of adsorption was very fast (it took a few minutes).

Table 1
Kinetic resolution: native enzymes with or without nanoparticles.

Entry	Enzyme	Time [h]	Conversion [%]	ee_s [%]	E^a
1	Turkey liver acetone powder	5	40	9	1.4
2	TLAP + 3.5 nm AuNPs	5	40	8	1.4
3	TLAP + 20 nm AuNPs	5	40	10	1.6
4	Wheat germ lipase	3	40	1	–
5	Wheat germ lipase + 3.5 nm AuNPs	3	40	3	–
6	Wheat germ lipase + 20 nm AuNPs	3	40	4	–
7	Pig liver esterase	8	45	38	3.9
8	PLE + 3.5 nm AuNPs	8	45	50	6.7
9	PLE + 20 nm AuNPs	8	45	44	5.0
10	Porcine pancreatic lipase	2	50	29	2.3
11	PPL + 3.5 nm AuNPs	2	50	37	3.1
12	PPL + 20 nm AuNPs	2	50	43	3.8
13	*P. cepacia* lipase	5	55	>99	72
14	*P. cepacia* lipase + 3.5 nm AuNPs	5	55	>99	117
15	*P. cepacia* lipase + 20 nm AuNPs	5	55	>99	117
16	*C. antarctica* lipase	1.5	55	28	2
17	*C. antarctica* lipase + 3.5 nm AuNPs	1.5	55	29	2.1
18	*C. antarctica* lipase + 20 nm AuNPs	1.5	55	18	1.6
19	Duck liver acetone powder	16	40	10	1.5
20	DLAP + 3.5 nm AuNPs	16	40	7	1.3
21	DLAP + 20 nm AuNPs	16	40	10	1.5
22	*Rhizopus arrhizus* lipase	2.5	40	12	1.6
23	*Rhizopus arrhizus* lipase + 3.5nmAuNPs	2.5	40	11	1.6
24	*Rhizopus arrhizus* lipase + 20nmAuNPs	2.5	40	10	1.5
25	*Rhizopus niveus* lipase	3	40	13	1.6
26	*Rhizopus niveus* lipase + 3.5 nm AuNPs	3	40	12	1.6
27	*Rhizopus niveus* lipase + 20 nm AuNPs	3	40	12	1.6

[a] Calculated from $E = [\ln((1-c)\times(1-ee_s))]/[\ln((1-c)\times(1+ee_s))]$.

Table 2
Kinetic resolution catalyzed by enzymes adsorbed on gold nanoparticles.

Entry	Enzyme	Time [d]	Conversion [%]	ee_s [%]	E^a
1	*P. cepacia* lipase on 3.5 nm AuNPs	2	20	8	2.1
2	*P. cepacia* lipase on 13 nm AuNPs	2	20	10	2.7
3	*P. cepacia* lipase on 20 nm AuNPs	2	30	14	2.2
4	PLE on 3.5 nm AuNPs	2	25	*rac*	–
5	PLE on 13 nm AuNPs	2	25	*rac*	–
6	PLE on 20 nm AuNPs	2	25	*rac*	–

[a] Calculated from $E = [\ln((1-c)\times(1-ee_s))]/[\ln((1-c)\times(1+ee_s))]$.

Table 3
Kinetic resolution catalyzed by enzymes immobilized covalently.

Entry	Enzyme	Time [d]	Conversion [%]	ee_s [%]	E^a
1	*P. cepacia* lipase – native enzyme	2	47	68	15.6
2	*P. cepacia* lipase on thiol-AuNPs	2	48	72	16.7
3	PPL – native enzyme	3	28	13	2.3
4	PPL on thiol-AuNPs	3	23	9	2.0
5	PLE – native enzyme	2	19	2	–
6	PLE on thiol-AuNPs	2	10	*rac*	–
7	Wheat germ lipase – native enzyme	5	23	2	–
8	Wheat germ lipase on thiol-AuNPs	5	19	4	1.5
9	*C. antarctica* lipase – native enzyme	2	22	*rac*	–
10	*C. antarctica* lipase on thiol-AuNPs	2	28	7	1.5
11	*M. javanicus* lipase – native enzyme	3	16	*rac*	–
12	*M. javanicus* lipase on thiol-AuNPs	3	11	*rac*	–

[a] Calculated from $E = [\ln((1-c)\times(1-ee_s))]/[\ln((1-c)\times(1+ee_s))]$.

Table 4
P. cepacia lipase – 5 catalytic cycles.

Entry	Enzyme	Time [d]	Conversion [%]	ee_s [%]	E^a
1	Native enzyme	2	47	68	15.6
2	Enzyme on thiol-AuNPs – 1st cycle	2	48	72	16.7
3	Enzyme on thiol-AuNPs – 2nd cycle	2	42	53	11.2
4	Enzyme on thiol-AuNPs – 3rd cycle	2	37	40	8.0
5	Enzyme on thiol-AuNPs – 4th cycle	2	25	16	3.3
6	Enzyme on thiol-AuNPs – 5th cycle	2	22	7	1.9

[a] Calculated from $E = [\ln((1-c)\times(1-ee_s))]/[\ln((1-c)\times(1+ee_s))]$.

Adsorption as a method of immobilization was inefficient and enantioselectivity was very low. Therefore we decided to use covalently immobilized enzymes (Table 3). Amount of immobilized enzyme was measured as a difference between concentration of enzyme in the probe and in supernatant after centrifugation. The same amount of native enzyme was used in reference reaction. Reactions were performed with nanoparticles of various sizes, but only for small 5 nm AuNPs effective biocatalysts were obtained.

It turned out that in most cases conversion and enantioselectivity of the reaction with immobilized enzymes were similar to results obtained for the same amount of native enzymes. Lipases loose at most 30% of initial activity, only esterase loose 50%.

Results obtained for *P. cepacia* lipase were significantly better than for any other enzyme. We checked structure of this enzyme in Protein Data Bank (Fig. 2) [22]. There are seven lysine residues near the protein surface. Therefore immobilization through the amide bond was effective for this enzyme. The structure of enzyme is an explanation of the fact that only small nanoparticles were good base for immobilization. Small AuNPs had size similar to the enzyme and therefore they could connect through one or two lysine residues, which did not cause significant deformation of the lipase. Bigger nanoparticles could bind more lysine residues of one enzyme molecule and it could deactivate the lipase.

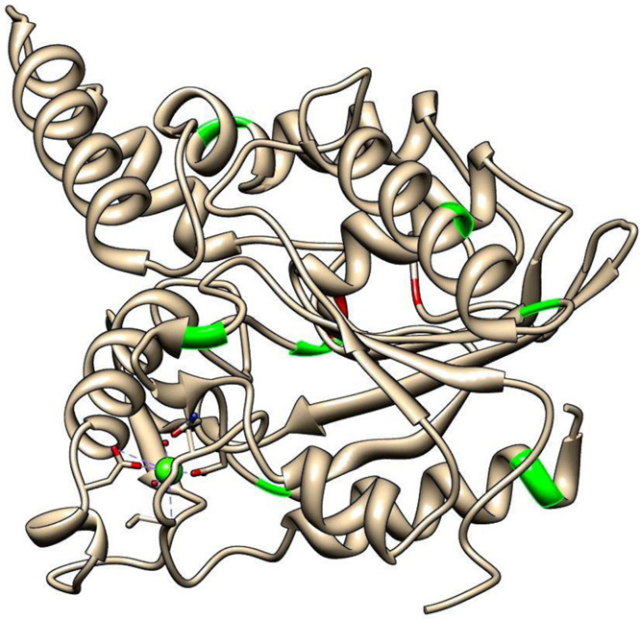


Fig. 2. Structure of *P. cepacia* lipase (red – cysteine, green – lysine). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.4. Reusability

The main advantage of immobilized enzymes is possibility to recycle them. Enzymes embedded on nanoparticles through

adsorption were difficult to recover and were inactive in second cycle. For *P. cepacia* lipase, which is the best catalyst of the model reaction, five catalytic cycles with the same portion of the enzyme immobilized covalently on gold nanoparticles were performed (Table 4).

Conversion and enantioselectivity were gradually lower because of a slight release of the enzyme from AuNPs. This effect is especially important in biosensors, where enzyme must be permanently bound with electrode to ensure stable measurement conditions. Lipases are not typical enzymes for biosensors but cases of their usage are known [23,24]. Gold nanoparticles have not been used in biosensors with lipases yet, but having in mind that amide bond is not very stable in water conditions, usage of other bonds should be considered in the future.

However, covalent immobilization on gold nanoparticles does not influence activity and selectivity of the enzyme.

4. Conclusion

This work describes systematic studies on influence of gold nanoparticles on enzyme enantioselectivity. Two different approaches toward stereoselective bionanocatalysis were combined based on enzyme immobilized and embedded on gold nanoparticles. Over twenty different hydrolases were tested and only in two cases model reaction was indeed catalyzed by embedded enzymes. While the yields of reaction were similar, only *P. cepacia* lipase embedded on AuNPs behaved as stereoselective biocatalyst. Attempts to reuse this embedded enzyme were performed, unfortunately were unsuccessful. Size of nanoparticles had negligible influence on enantioselectivity.

We have found that adsorption was inefficient method and had negative influence on enantioselectivity of the reaction. Therefore enzymes were covalently immobilized on gold nanoparticles capped with α-lipoic acid. Only enzymes immobilized on small nanoparticles were active biocatalysts. Bigger AuNPs were poor base for immobilization and caused deactivation of enzymes. Catalytic activity of enzymes immobilized covalently was comparable with their native forms. Obtained biocatalysts were more efficient and enantioselectivity of reaction was significantly better than for embedded enzymes. Especially for *P. cepacia* lipase, catalytic abilities were greatly improved. This immobilized enzyme was also used in five catalytic cycles and retained 50% of its activity.

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