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Comparison of the antioxidant activity of catalase immobilized on gold nanoparticles via specific and non-specific adsorption

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ABSTRACT

In this study, we present a comparison of the antioxidant activity of catalase immobilized on gold nanoparticles (AuNPs) by two methods: i) directly on the surface of AuNPs (non-specific immobilization), and ii) via chemical bonding using a linker (specific immobilization). Quantification of the enzyme amount adsorbed on the nanoparticle surface was determined by native-polyacrylamide gel electrophoresis (native-PAGE). Colloidal stability of AuNPs before and after the enzyme immobilization was monitored with dynamic light scattering (DLS) and UV–vis spectroscopy. The size of the metallic core was determined by scanning-transmission electron microscopy (STEM). The enzymatic activity of catalase immobilized on AuNPs was investigated by antioxidant tests and compared with free (non-immobilized) catalase. It was found that the activity of catalase immobilized on AuNPs is affected by the immobilization method. Moreover, it was found that the non-specific immobilization decreased the antioxidant activity while the specific immobilization of catalase allowed the catalase activity to remain at the same level as that of free catalase.

1. Introduction

Nanobiotechnology has recently been a major area of scientific and technological interest in the development of novel applications such as bio-sensing [1–4], drug delivery [5–7] or catalysis [8–11]. A wide range of nanomaterials, especially nanoparticles (NPs), have been used as materials suitable for bio-functionalization [12–19] mainly due to their size, high surface area to volume ratio, unique chemical and physical properties, high particle mobility during reaction, and also because they offer a wide range of surface modification possibilities. Gold nanoparticles (AuNPs) serve as an excellent matrix for biomolecule conjugation (e.g. catalase [15], His-tagged horseradish peroxidase and ferredoxin-NADP + reductase [16], glucose oxidase [20], malate dehydrogenase and citrate synthase [21]). AuNPs rapidly react with amino and thiol groups of proteins, which results in high protein loading, and also due their biocompatibility and colloidal stability.

Catalase (CAT) is an enzyme that catalyzes the conversion of harmful hydrogen peroxide to water and molecular oxygen [15,22]. Thus far, the antioxidant properties of catalase have been studied extensively for potential effectiveness in wound healing [23], antioxidative therapy [24], chemotherapy [16] and preventive medicine. However, catalase undergoes rapid elimination from the blood stream, demonstrates poor intracellular delivery [25]. Moreover, catalase as is typical of some enzymes, exhibits a short shelf life, poor operational stability and reusability, thus limiting its potential application. An idea to overcome these problems is the immobilization of catalase on solid supports e.g. on NPs. Immobilization of catalase or other proteins on solid supports is an efficient way to increase its operational stability. Moreover, it can be an effective method for preparation of hybrid bio-conjugates with new properties. A combination of the unique properties of AuNPs (biocompatibility, relative non-toxicity) with proteins can result in new materials that can exhibit new or enhanced biological properties of both the components e.g. antioxidant, antifungal, anti-inflammatory, antiviral and antibacterial properties, among others. Moreover, NPs can also be used as delivery systems for proteins and can be applied as therapeutics. Hence, an immobilization could also be an interesting method in the context of preparing new types of materials with unique properties.

Several methods of protein immobilization on NPs have been studied so far [15–18,26]. In general, they can be divided into two main

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categories: i) non-specific and ii) specific immobilization [14]. Non-specific methods generally include adsorption (based on van der Waals forces, ionic and hydrogen bonding, and hydrophobic interactions [27,28]), non-specific covalent bonding [29,30], entrapment [31] and encapsulation [32]. In the specific methods of enzyme immobilization two groups can be distinguished: i) non-covalent methods, and ii) covalent ligation approaches. The non-covalent method include interactions between streptavidin-biotin [15] or polyhistidine and bivalent metal ions (Ni^{2+}) [16]). Covalent ligation include "click" reaction [13] or immobilization through cysteine residues [14]. The immobilization method affects the protein structure and hence defines the final function and enzymatic activity of the protein. The main problem with protein immobilization is the selection of a proper immobilization procedure (simple and effective) that allows functional bio-conjugates to be obtained. Most of the research revealed a decrease in protein activity after the immobilization compared with the control sample (free enzyme) [15]. Chirra and co-workers [15] presented the comparison of two chemical methods of catalase coupling on AuNPs: biotinylation and carbodiimide chemistry. The first involves the biotinylation of both the AuNPs and catalase and then coupling them together using a streptavidin crosslinker. The second method, uses carbodiimide chemistry to form amide bonds between carboxylic acid coated AuNPs and the amino groups of the catalase. Both methods result in active protein on the surface of AuNPs, though with a lower activity compared to the control sample (free catalase). The other drawbacks of the presented methods are that they are multistage procedures and are based on the chemical modification of both bio-conjugate components.

The other problem connected with the protein immobilization is the determination of the exact number of proteins present on the NPs. The immobilization of proteins on nanomaterials or other support should be designed so that in the solution only protein-NPs bio-conjugates are present and there are no free un-immobilized proteins that influence the activity results. In the literature, there is still an absence of works that present the activity results of bio-conjugates in relation to the amount of protein present on single NPs. Typical procedures used for the determination of the protein amount present on NPs contain centrifugation and washing steps [13]. This stages may affect the structure and consequently protein functionality. Hence, the elaboration of a simple and effective procedure for protein immobilization on nanomaterials along with a method for determining the exact number of proteins present on their surface, without the need of removal, seems to be justified.

In this paper, we present a comparison of the antioxidant activity of the model enzyme catalase immobilized on AuNPs by two methods: i) non-specific and ii) specific immobilization. The first method involves protein adsorption directly onto the surface of the NPs. The second method is based on a two-step chemical bonding of the enzyme through specific interactions between the imidazole histidine groups of His-tag-catalase and Ni^{2+} ion, complexed by the nitrilotriacetic acid residues present on the surface of the NPs. All experiments were performed on samples with a strictly defined amount of protein present on single NPs. The surface coverage of NPs with protein was determined by native polyacrylamide gel electrophoresis (native-PAGE). As the protein amount present on NPs is known, the presented method of immobilization does not require the discarding of free enzyme before activity tests. Moreover, no enzyme modification before its immobilization was performed. Characterization of bio-conjugates was completed using dynamic light scattering (DLS), UV–vis spectroscopy, and scanning transmission electron microscopy (STEM). The enzymatic activity of immobilized catalase was investigated in a long-term experiment, focusing on the effect of different orientations of protein molecules on the surface of the AuNPs.

2. Materials and methods

2.1. Synthesis of gold nanoparticles (AuNPs)

Synthesis of gold nanoparticles (13 nm) was carried out in water by the chemical reduction method as described previously [33]. Briefly, chloroauric acid water solution ($1.81 \cdot 10^{-2}$ % wt., 95.416 g) was boiled under stirring, followed by addition of sodium citrate (1% wt., 4.584 g) for the $HAuCl_4$ reduction. The reaction was continued for 15 min and then cooled down to room temperature. The final concentration of AuNP was determined with ICP-MS and is equal 96.7 ppm (theoretical concentration is equal 100 ppm). During synthesis deionized water (18.2 MΩ cm, Millipore-Q) was used.

2.2. Linker synthesis

A 5 ml volume of methanol was cooled in a 20 ml round bottom flask to −20 °C and 1.0 ml (11.4 mmol of thionyl chloride ($SOCl_2$) was added dropwise. The cooling bath was removed and to the formed solution of hydrogen chloride in methanol, 0.5 g (1.9 mmol) of N,*N*-bis (carboxymethyl)-L-lysine (NTA) was added. The flask was tightly stoppered and the contents was stirred with gentle heating (ca. 40 °C) for 3 h. After that time no starting amino acid was visible on thin-layer chromatography (TLC). The methanol was removed in vacuo and the crystalline residue was rinsed with diethyl ether, filtered and dried in open air at room temperature. Yield 0.5 g (1.5 mmol, 77.2%).

Hydrochloride of trimethyl ester of NTA 0.5 g (1.5 mmol was suspended in methylene chloride (10 mL) and diisopropylethylamine (0.7 mL, 4 mmol) was added at ambient temperature followed by addition of liponic acid (LA) 0.31 g (1.5 mmol) and N,N,N′,N′-tetramethyl-O-(benzotriazol-1-yl)uronium tetrafluoroborate (TBTU) (0.5 g, 1.5 mmol). The reaction mixture was stirred overnight, diluted with methylene chloride (20 mL) and washed twice with 1 M aqueous NaOH, twice with 2 M aqueous HCl, brine and dried over anhydrous $MgSO_4$. The solvent was evaporated and the product was purified using column chromatography with an ethyl acetate/hexane solvent system. Yield 0.66 g (1.3 mmol, 89.3%).

The trimethyl ester of N2,N2-bis(carboxymethyl)-N6-[5-(1,2-dithiolane-3-yl)pentanoyl]-*L*-lysine (LA-NTA) (0.66 g, 1.3 mmol) was dissolved in methanol (20 mL) and 1 M NaOH (5 ml, 5 mmol) was added. The mixture was stirred for 5 h at room temperature and monitored with TLC (chloroform:methanol, 9:1). When the reaction was complete, methanol was evaporated under reduced pressure. After being acidified to pH ~3 with 6 M aqueous HCl the LA-NTA was extracted with ethyl acetate, dried over $MgSO_4$ and concentrated under vacuum to give 0.35 g (0.78 mmol, 59.7%) of final product. A scheme of the synthetic procedure of LA-NTA linker is shown in Fig. 1.

In the next step, a complex of the nickel (II) with LA-NTA linker (LA-NTA-Ni) was formed by the reaction of LA-NTA linker with an excess of nickel (II) sulfate heptahydrate in aqueous solution (molar ratio 1:1.1 mmol). The obtained LA-NTA-Ni complex was then used for the specific immobilization of catalase on the AuNPs.

2.3. Catalase preparation (CAT-His-tag)

2.3.1. Construction of the plasmid containing recombinant catalase

Two primers were designed to amplify the human cDNA of catalase. The forward primer: 5′ CGAACAAGCTTCCATGGCTGACAGCCG 3′ and reverse primer: 5′ GACTTACGCGTCAGATTTGCCTTC 3′ containing HindIII and MluI restriction sites, respectively (underlined), were used in PCR reactions, the final product was a 1581 bp fragment. The obtained product was cloned into pEX-C-His (OriGene) under the control of the viral T7 promoter with Hind III and *Mlu* I restriction enzymes. The final product was a plasmid containing human catalase cDNA with a His-tag on the C-terminus of the protein. The constructed plasmid (pEX-CAT) was transformed into *Escherichia coli* XL-blue. The ligation

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