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Papain-functionalized gold nanoparticles as heterogeneous biocatalyst for bioanalysis and biopharmaceuticals analysis

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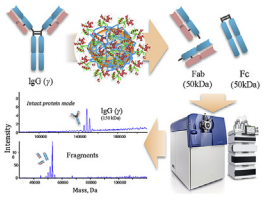
HIGHLIGHTS

- An immobilized enzyme reactor was synthesized with gold nanoparticles (GNPs) as support.
- Polyelectrolyte layer-by-layer GNP modification with subsequent papain coupling.
- Resonant mass measurement technology for determination of surface coverage.
- Enhanced catalytic efficiency of immobilized papain compared to free papain.
- Successful digestion of human IgG with papain-GNP verified by HPLC-μESI-QTOF-MS in intact protein mode.

GRAPHICAL ABSTRACT



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ABSTRACT

Surface-modified gold nanoparticles (GNPs) were synthesized via layer-by-layer process with alternating cationic polyallylamine and anionic poly(acrylic acid) polyelectrolyte layers leading to a highly hydrophilic biocompatible shell supporting colloidal stability. Afterwards, papain was covalently immobilized on the modified GNPs via amide coupling between the amino groups on papain and the terminal carboxylic groups of the modified GNPs by using *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide and *N*-hydroxysulfosuccinimide sodium as coupling agents. The resultant papain-functionalized gold nanoparticles were characterized by surface plasmon resonance, dynamic light scattering and zeta potential measurements. The new technology resonant mass measurement was applied for determining the average number of papain molecules immobilized per GNP by measurement of the single nanoparticle buoyant mass in the range of femtograms. The activity of the immobilized enzyme was estimated by determination of the kinetic parameters (K_m, V_{max} and k_{cat}) with the standard chromogenic substrate N_α-benzoyl-DL-arginine-4-nitroanilide hydrochloride. It was found that K_m of immobilized and free enzyme are in the same order of magnitude. On contrary, turnover numbers k_{cat} were significantly higher for GNP-conjugated papain. Further, the gold nanobiocatalyst was applied for digestion of polyclonal human immunoglobulin G to yield protein fragments. The resultant fragment mixture was further analyzed by high-performance liquid chromatography-microelectrospray ionization-quadrupole-time-of-flight mass spectrometry, which demonstrated the applicability of the bioreactor based on papain functionalized

Abbreviations: GNP, gold nanoparticle; papain-GNP, papain-functionalized GNP; DLS, dynamic light scattering; SPR, surface plasmon resonance; RMM, resonant mass measurement; EDC, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide; Sulfo-NHS, *N*-Hydroxysulfosuccinimide sodium; PAH, Poly(allylamine hydrochloride); PAA, Poly(-acrylic acid, sodium salt); BApNA, N_α-Benzoyl-DL-arginine-4-nitroanilide hydrochloride; ζ-potential, zeta potential; IMER, immobilized enzyme reactor.

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GNPs. The immobilized papain not only has higher catalytic activity and better stability, but also can be easily isolated from the reaction medium by straightforward centrifugation steps for reuse.

1. Introduction

Enzymes with a number of excellent properties (high activity, selectivity and specificity) are extensively used to catalyze a wide range of commercially important processes [1]. However, some limitations exist for the applications of enzymes, such as low stability and high sensitivity to the employed conditions. Therefore, immobilization techniques of enzymes (leading to immobilized enzyme reactors, IMERs) emerged as a powerful strategy to overcome some of these limitations [2–10]. They received particular interest due to some advanced properties such as high catalytic efficiency, improved stability, elimination of self-digestion, flexible control of the reaction, easy removal after reaction, no contamination of the product with enzyme and repetitive usage [4,11–14]. Enzymes immobilized on nanomaterials possess considerable prospect in various fields, because the catalytic properties of enzymes can be flexibly combined with the unique features of nanomaterial structures [12,15–19].

Silica nanoparticles [20], polystyrene [4] and magnetic nanoparticles [21] have been frequently utilized for the enzyme immobilization by covalent binding, entrapment, adsorption, ionic binding, affinity binding and so on [22–24]. A growing number of studies suggested that immobilizing enzyme on nanomaterials can enhance reaction rates while improving enzyme stability [25–28]. Amongst the nanomaterials, gold nanoparticles (GNPs) have received great attention in biology, biochemistry and biomedical research areas due to controlled geometrical, excellent optical and flexible surface chemical properties [29]. GNPs can be synthesized in a straightforward and low-cost method by reduction of gold (III) chloride. Due to the chemical inertness of gold they possess excellent chemical stability and due to charged capping groups on their surface also a high colloidal stability. They can be easily further functionalized with appropriate organic or biological ligands which form the basis for their extremely broad applications. Like for other metal nanoparticles, a surface plasmon resonance (SPR) band can be observed in the visible spectrum which is the result of the collective oscillation of electrons in the conduction band of gold nanoparticles in resonance with a specific wavelength of incident light [30]. For gold nanoparticles it results in a strong absorbance band in the visible region (around 525 nm). The exact wavelength maximum as well as width of the absorption band depends on the nanoparticle size and shape, size distribution, and morphological uniformity of GNPs [31,32]. SPR measurement is therefore a straightforward and useful tool to characterize GNPs and monitor the success of surface modification as well as to evaluate the aggregation status of GNP suspensions. If a second peak can be observed in the red-shifted region of the UV-VIS spectrum, it implies that aggregation of GNPs occurred. The large surface-to-volume ratio of GNPs provides considerable promise for the bioconjugation with various receptor molecules (e.g. proteins, DNAs, aptamers, antibodies, or lectins) [33]. The bioconjugation chemistries used for the immobilization are generally derived from the protein labeling chemistries by using various commercial crosslinkers based on maleimides, succinimidyl esters and so on. In addition, carboxylic and amino groups of proteins are widely used as the reactive sites for conjugation through amide bond formation [34].

Papain, a cysteine protease with broad specificity present in the latex of *Carica papaya*, catalyzes the hydrolysis of peptide, ester and amide bonds. Therefore, it is extensively employed in food, pharmaceutical, biology and biomedical researches [35–40]. In recent years, preparing Fab fragments with papain via specific digestion above the hinge region (the site of the disulfide bonds which connect the two heavy chains) of the whole antibody attracted some attention. The Fab fragment is a monovalent antibody structure harboring the CDR (complementarity-determining region) without Fc portion. It can still specifically recognize and bind to antigens. In biopharmaceuticals research and quality control, Fab fragments are prepared to characterize the protein on an intermediate level instead of the whole intact immunoglobulin (middle up and middle down). Middle-up refers to the analysis of an antibody after its cleavage into large fragments, e.g. by reduction or limited proteolytic cleavage. Proteins of such smaller size are easier to analyze than larger proteins like intact antibody and can be detected with better sensitivity. Middle-down refers to the mass measurement of the gas phase fragmentation of antibody fragments, in analogy to the mass measurement of the gas phase fragmentation of intact protein in top-down analysis [41]. An antibody digested by papain generates two Fab fragments (about 50 kDa) and one Fc fragment (also about 50 kDa). Pure Fab fragment can be obtained after purification of digests by affinity chromatography (with protein A to remove the Fc part), ion exchange, and size exclusion chromatography [39,40]. The main goal of this study was to prepare stable immobilized papain on GNPs for use in sample preparation protocols of antibody analytics. For this purpose, it is necessary that the obtained immobilized papain nanobiocatalyst has sufficient colloidal stability and satisfactory bioactivity to yield useful Fab fragments for analytical characterization by mass spectrometry via enzyme digestion. In this research, surface-modified GNPs with highly hydrophilic and biocompatible shell were first prepared from oppositely charged polyelectrolytes via their alternating deposition by a layer-by-layer process (LBL) onto citrate stabilized GNPs [42–44]. The immobilization process was carried out by using cross-linker agents to link the carboxylic group on the surface of carboxy-modified GNPs with the amino group on papain [42–44]. The covalent (instead of adsorptive) bonding was selected for irreversible immobilization to avoid enzyme leaching from the support surface [45]. The number of immobilized papain molecules on GNPs is an important figure for the determination of the immobilization efficiency. Resonant mass measurement (RMM) was applied herein to measure the mass increase of the nanoparticles upon immobilization of papain and determine the surface coverage [46]. It is well-known that papain, like other proteases, is prone to auto-digestion. The resultant peptides will cause the contamination of the protein digestion products. Therefore, immobilization of proteases like papain on a solid support is a good solution to eliminate this kind of contamination produced by auto-digestion [47]. In addition, the papain-functionalized GNPs can be easily removed from the reaction solution by a simple centrifugation process, which also terminates the digestion. Therefore, using immobilized papain makes it easy to control the reaction time without using any stop reagents which normally introduce contamination to the product solution.