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Bioconjugation of trypsin onto gold nanoparticles: Effect of surface chemistry on bioactivity

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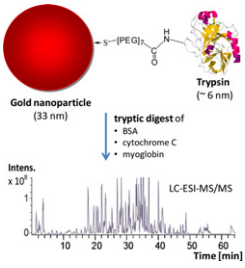
HIGHLIGHTS

- Size and spacer affect bioactivity of nanoparticulate trypsin reactor.
- Increase of GNP's size increases activity of bound trypsin.
- Increase of spacer length increases amount and activity of immobilized enzyme by factor 6.
- Decrease of digestion time up to less than 1 h when trypsin immobilized onto GNPs.
- Reduced auto-digestion compared to trypsin in-solution.

GRAPHICAL ABSTRACT



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ABSTRACT

The systematic study of activity, long-time stability and auto-digestion of trypsin immobilized onto gold nanoparticles (GNPs) is described in this paper and compared to trypsin in-solution. Thereby, the influence of GNP's size and immobilization chemistry by various linkers differing in lipophilicity/hydrophilicity and spacer lengths was investigated with regard to the bioactivity of the conjugated enzyme. GNPs with different sizes were prepared by reduction and simultaneous stabilization with trisodium citrate and characterized by UV/vis spectra, dynamic light scattering (DLS), ζ-potential measurements and transmission electron microscopy (TEM). GNPs were derivatized by self-assembling of bifunctional thiol reagents on the nanoparticle (NP) surface via dative thiol-gold bond yielding a carboxylic acid functionalized surface. Trypsin was either attached directly via hydrophobic and ionic interactions onto the citrate stabilized GNPs or immobilized via EDC/NHS bioconjugation onto the carboxylic functionalized GNPs, respectively. The amount of bound trypsin was quantified by measuring the absorbance at 280 nm. The activity of bound enzyme and its Michaelis Menten kinetic parameter K_m and v_{max} were measured by the standard chromogenic substrate N_α-Benzoyl-DL-arginine 4-nitroanilide hydrochloride (BApNA). Finally, digestion of a standard protein mixture with the trypsin-conjugated NPs followed by analysis with LC–ESI-MS and successful MASCOT search demonstrated the applicability of the new heterogenous nano-structured biocatalyst. It could be shown that the amount of immobilized trypsin and its activity can be increased by a factor of 6 using a long hydrophilic spacer with simultaneous reduced auto-digestion and reduced digestion time. The applicability of the new trypsin bioreactor was proven by digestion of casein and identification of α- as well as κ-casein by subsequent MASCOT search.

Abbreviations: GNP, gold nanoparticle; GNP-trypsin, GNP immobilized trypsin; DLS, dynamic light scattering; TEM, transmission electron microscopy; SPR, surface plasmon resonance; PEG, polyethylene glycol; MHA, 16-mercaptohexadecanoic acid; EDC, N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride; NHS, N-hydroxysuccinimide; BApNA, N_α-benzoyl-DL-arginine 4-nitroanilide hydrochloride; ZP, zeta potential.

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1. Introduction

Trypsin (E.C.3.4.21.4) is a serine protease commonly used in proteomics for digestion of proteins. However, drawbacks are long digestion times (up to 24 h), auto-digestion sub-products and poor enzyme-to-substrate ratio limiting high-throughput protein identification [1]. To overcome the main problems of in-solution digestion different approaches of enzyme immobilization strategies were developed to increase the enzyme-to-substrate ratio and thus, to reduce the digestion time by higher turn-over rate as well as to reduce the auto-digestion and thus, to increase the long-time stability of the enzyme. On-line and off-line approaches of enzyme reactors to increase the enzyme efficiency comprise co-polymerization in polymer matrices (sol–gel enzyme encapsulation) [2], immobilization onto monoliths [3–5], lab-on-a-chip platforms [6] as well as open tubular enzyme reactors [7] and immobilization onto micro- and nanobeads.

Breakthroughs in nanotechnology enforced the development of nanostructures for enzyme stabilization [8] and regulation of enzyme activity [9]. Thereby, pore size and volume, charge interactions, hydrophobic interactions and multiple attachments, surface chemistry and immobilization strategy are crucial points for engineering enzyme reactors on beads with regard to enzyme longevity and efficiency. Magnetic [10], silica coated magnetic [11], chitosan [12], and gold nanoparticles are the most common nanobeads for enzyme attachment and immobilization. In our study, we focused on the immobilization strategy of trypsin onto gold nanoparticles with regard to the influence of surface chemistry on enzyme stability and activity.

Gold nanoparticles (GNPs) in the range of 1–100 nm are used in a broad application field in chemistry, physics, medicine, pharmacy and biology [13]. They have been utilized for biosensors [14,15], for determination of ions (heavy metal cations and anions) [16] and small molecules [17], for DNA detection and peptide/protein analysis [18], and enrichment [19,20], for enzymatic activity assay [21,22] and cellular analysis [13] as well as in separation sciences [23,24] and in sample preparation [25]. In general, nanotechnology is getting more and more important in natural sciences.

GNPs can be easily prepared with low-cost by reduction of gold(III) chloride. The most common synthesis is established by Turkevich–Frens [26,27] by reduction and simultaneous stabilizing with citrate. Thereby, the size of the particles can be controlled by the molar ratio of gold(III)/reducing agent. Insights in the mechanisms of GNPs formation is given by Ojea-Jimenez et al. [28], Polte et al. [29] and Kumar et al. [30]. The size controlled synthesis generates nanoparticles with spherical-like appearance having unique physical, chemical and optical properties and large surface-to-volume ratio.

Additional advantages of GNPs compared to other nanoparticle materials is the surface plasmon resonance (SPR) band of GNPs at about 520 nm often utilized in chemical sensing and imaging. Furthermore, the size and concentration of GNPs can be determined from UV/vis and extinction spectra [31–34]. However, shifts of SPR maxima and shape changes of SPR curves are not only caused by particle size but also by formation of aggregates and/or adsorption of modifying molecules [24]. Thus, the UV/vis spectra provide a fast and easy control mechanism. Next to photometric assay, the size can be determined by microscopic techniques (TEM, SEM, AFM etc.), dynamic light scattering (DLS) [35], field flow fractionation and size exclusion chromatography [36]. Moreover, the zeta potential (ZP) of the nanoparticles is a measure for the stability of the colloidal GNP solution. The stability of a colloidal solution is described by the DLVO theory in the 1940s named after the scientists Deryagin, Landau, Verwey and Overbeek [37,38]. They suggest that the stability depends on the total potential energy function as a balance of attractive and repulsive forces (i.e. of van der Waals attraction and electrostatic repulsion) as well as of the potential energy due to solvational effects.

GNPs are also attractive because of their straightforward modification for sake of their functionalization, but also stabilization [39]. Functional groups can be introduced onto GNPs by dative binding and self-assembling of bifunctional ligands with terminal thiol groups and/or by ligand exchange with thiol containing molecules in a second step [40]. Biomolecules like proteins, enzymes and antibodies can either be attached directly to GNPs via ionic and hydrophobic interactions or by immobilization via linkers. The successful direct binding of trypsin onto GNPs is described by IR and Moessbauer spectroscopy measurements [41]. The attachment of trypsin was controlled by shift of absorbance maximum from 528 nm to 540 nm and change in shape of the UV/vis spectra with a slightly enhancement of the activity bound onto GNPs by 12% compared to in-solution [42]. The tryptic digest of GNP attached trypsin and analysis by micellar electrokinetic chromatography describes a change in enzyme specificity which was explained by change in conformation of attached enzyme [43]. The immobilization chemistry of trypsin onto gold nano-rods modified with a copolymer containing sulfonate and maleic acid groups via “click” chemistry, EDC coupling or electrostatical adsorption was compared by Gole and Murphy [44]. Polymer coating of nanoparticles is often used for stabilzation and prevent aggregation of GNPs and/or entrapping of enzymes e.g. for on-line proteolysis [45].

Promising advantages of GNP-conjugated biomolecules for specific recognition (enzyme–substrate, antibody–antigen, biotin–avidin etc.) are the increased concentration on the surface (high surface/volume ratio) resulting in higher recognition efficiency, the visualization due to SPR band, the ability of removal and/or extraction from solution by centrifugation and in case of the protolytic enzyme trypsin the reduced auto-digestion compared to enzyme in-solution digestion.

In this paper, we describe the effect of the bioconjugation chemistry of biomolecules on GNPs, in particular GNP size, surface chemistry and influence of linker and spacer length on their functionality (i.e. bioactivity). Thereby, trypsin was used as standard enzyme to control the amount of bound protein as well as the enzyme activity in dependence of the chemistry. The principle of the bioconjugation chemistry of trypsin onto GNPs used in our study is shown in Fig. 1.

2. Materials and methods

2.1. Materials and instruments

2.1.1. Chemicals

Trypsin (from hog pancreas, E.C.3.4.21.4, foreign activities: chymotrypsin $\leq 0.2\%$, 13,644 U mg^{-1}), cytochrome C (CYC_horse, from horse heart, E.C. 232-700-9, purity 97% based on water content of 5%, MW 12,384 Da), myoglobin (MYG_horse, from horse skeletal muscle, E.C. 309-705-0, 95–100%), casein, N_{α}-benzoyl-DL-arginine 4-nitroanilide hydrochloride (BApNA), tris(2-carboxyethyl)phosphine hydrochloride (TCEP), urea, gold(III) chloride trihydrate, trisodium citrate, 16-mercaptohexadecanoic acid (MHA), 11-mercaptoundecanoic acid, 3-mercaptopropionic acid, O-(2-carboxyethyl)-O′-(2-mercaptoethyl)heptaethylene glycol, N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS) and formic acid were all obtained from Sigma–Aldrich (Vienna, Austria). Thiol-$dPEG_4$™-acid was obtained from Celares (Berlin, Germany), the bovine serum albumin (BSA, ~99%, E.C. 232-936-2, protease free, essentially γ-globulin free) from SAFC (supplied by Sigma–Aldrich, Vienna, Austria) and ammonia and sodium bicarbonate from Merck KGaA (supplied by VWR, Vienna, Austria).