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Structural and functional aspects of trypsin–gold nanoparticle interactions: An experimental investigation

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ABSTRACT

Trypsin (Trp) is arguably the most important member of the serine proteases. Constructs made up of gold nanoparticles (GNP) with trypsin have been known to exhibit increased efficiency and stability in various experiments. Here we report simple Trp–GNP constructs mixed in different trypsin-to-GNP ratios which exhibit higher efficiencies in biochemical assay, varying resistance to autolysis and higher ability in cell trypsinization. Trp–GNP constructs in different trypsin-to-GNP ratios exhibit prolonged and sustained activity compared to native trypsin in N-α-p-benzoyl-p-nitroanilide (BAPNA) assay as monitored by UV-Visible spectroscopy. The activity was monitored as a function of decreasing rate of linear release of p-nitro aniline (resulting from the cleavage of BAPNA by trypsin) with time during the assay, whose absorbance was measured at 410 nm (λ_{max} p-nitro aniline). We have done extensive studies to understand structural basis of this trypsin GNP interaction by using atomic force microscopy (AFM), transmission electron microscopy (TEM) and circular dichroism (CD) techniques. Our findings suggest that on interaction, the gold nanoparticles probably form an adherent layer on trypsin that effectively changes the morphology and dimensions of the nanoconstructs. However, trypsin-to-GNP ratio is extremely important, as higher concentration of GNP might damage the conformation of protein. Stability studies related to denaturation show that 1:1 Trp–GNP constructs exhibit maximum stability and high efficiency in all assays performed.

1. Introduction

Trypsin is arguably the most important member of the Serine Protease clan. It has wide implication in different biochemical techniques like limited proteolysis, residual enzymatic activity and proteolytic assays [1–6]. Besides, trypsin is a key player in the digestive system of different species from mammals to reptiles, Pisces and even plants. The structure of trypsin from different sources is well established [7,8]. Apart from some minor sequence difference, the overall structure is almost the same. The structure of trypsin consists of mainly antiparallel β-sheets and loops with sporadic presence of two small α-helices. Asp 189 residue in the trypsin pocket is believed to determine its high substrate specificity and strong biochemical property [9]. In spite of its importance, trypsin has many drawbacks especially autolysis (self-digestion in solution), poor enzyme-to-substrate ratio that often becomes problematic during protein identification, and extremely small half life in biochemical assay disallowing long-time studies with minimum reagent quantity [10,11]. Multi-dimensional approaches have been implemented to increase the enzyme efficiency and reduce the auto-digestion. Various developments in this field include the sol–gel encapsulation, immobilization of enzyme on monolith surface, and construction of open tubular enzyme reactor [12–14].

Interaction of nanoparticles (NPs) with proteins nowadays has become immensely interesting for researchers considering the biological importance of proteins in every living system. The nano-protein complex is generally referred to as nanoparticle-protein

Abbreviations: GNP, gold nanoparticle; NPs, nanoparticles; Trp, trypsin; NP-PC, nanoparticle-protein corona; BAPNA, n-benzoyl p-nitro anilide.

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corona (NP-PC), and to understand its mode of interaction, considerable impetus has to be given to the initial adsorption of the NPs on the protein surface and the conformational and morphological changes of the protein and NP, respectively [15]. However, advent of nanotechnology has provided a new paradigm in stabilizing enzyme assembly through interaction with inorganic nanostructures [16,17]. Many parameters like charge and hydrophobic interactions, surface characteristics, single and multiple attachments and most importantly, the pore size and volume have to be considered to understand the true aspects of nanoparticle-protein interaction. Among all the enzymes, trypsin–NP interaction is the most extensively studied till now. Magnetic, silica-coated magnetic, chitosan and gold nanoparticles (GNP) are the most common types that have been used for immobilization on trypsin surface [18–21]. Hinterwart et al. have reported an efficient GNP conjugated trypsin system that shows enhanced activity and reduced auto-digestion [22]. Gold nanoparticles (GNPs) are widely used in physics, chemistry, pharmacy and biological field predominantly due to their variable size (1–100 nm) and ease as well as cost effectiveness of their synthesis [23]. The utility of GNPs has already been well established in the field of biosensors, DNA and protein detection, enriching enzymatic activity, nanoalloys, etc. [24–28]. Synthesis of gold nanoparticles using biological templates has grabbed exceptional attention due to their unique properties such as biosensing, determination of ions and small molecules, optical imaging, DNA detection, etc. The presence of green templates provides good stability to the nanoparticle and makes them less toxic for biological interactions. The interest in GNPs is largely due to the relative ease of their synthesis, with good control of their shape and sizes and their good biocompatibility [29,30].

However, the actual mode of interaction of NP in protein surface, the change in the morphology of NP and the change in protein conformation needs to be investigated in a more detailed manner to understand the possible reasons behind the higher activity exhibited by the protein molecules while interacting with NPs. In this article we have reported an enhanced activity of trypsin in biochemical assay with N-α-p-benzoyl-p-nitroanilide (BAPNA), enhanced activity in cell trypsinization and reduced autolysis on simple incubation with GNPs (in different v/v ratios) without any biological and chemical conjugations. We have employed a two-pronged approach to understand the structural effects of nanoparticle–protein interaction. Instrumental techniques like TEM and AFM are utilized to visualize the change in morphology of native nanoparticles and nanoparticle-protein constructs. Our findings clearly suggest that on interaction with trypsin, GNPs undergo substantial change in their dimensions and morphology especially with respect to size, shape and height. Considering the other paradigm of the interaction, we focussed on the conformational change of trypsin that takes place during this interaction. An approach using circular dichroism (CD) spectroscopy helped us to identify the change in the secondary structural elements of GNP-adsorbed trypsin constructs with respect to the native protein. Stability of different Trp–GNP constructs [1:1, 1:2, 1:3 (v/v)] was also measured using guanidium chloride (GdmCl) dependent denaturation combined with their respective CD profiles. Objective of the study was to understand the proper mechanism of Trp–GNP interaction and also to assess the limiting ratio of Trp–GNP constructs for most effective output.

2. Materials and methods

2.1. Materials

All chemicals were procured from Sigma–Aldrich and used without further purification.

2.2. Synthesis of gold nanoparticles on starch template and conjugating trypsin on the gold nanoparticles

Gold nanoparticles were prepared by the reduction of chloroauric acid ($H[AuCl_4]$) (5 mM), after addition of reducing agent cum stabilizing agent starch (2.0 wt%) as biological template (molecular weight 190 kDa as estimated through intrinsic viscosity method) and the solution was intensively stirred for 60 min. Then the resultant solution was taken for microwave heating for 120 s at 900 W power and 2450 MHz frequency. This leads to the reduction of trivalent gold ion Au^{3+} to neutral Au atom. The microwave heating will lead to the formation of nanoparticles with uniformity in size and shape in the presence of starch template. The gold nanoparticles thus prepared were stable for 6 months without any change in its surface plasmon resonance as indicated from the absorption spectra taken using UV-Visible spectroscopy. Trypsin solution was prepared at a concentration of 5 mg/ml and simple incubation with GNPs (in different v/v ratios, 1:1, 1:2, 1:3) for about 1 h was done at room temperature without any biological and chemical conjugations.

2.3. Physicochemical characterization of trypsin conjugated gold nanoparticles

2.3.1. Atomic force microscopy (AFM)

The AFM observations were performed with an Agilent Technologies, 5500 Pico Plus AFM system. All the images were obtained with the Aquastic mode using cantilevers having resonance frequency 150–300 kHz, tip height 10–15 μm and tip length 225 μm. Mica was chosen as a solid substrate and used immediately after cleavage in a clean atmosphere. During this characterization experiment, the probe and cantilever were immersed completely in the water solution. The nanocapsule suspension on mica was dried in air (65% humidity) for 30 min. Images were captured and analyzed using Picoscan 5.33 software of Molecular Imaging Corporation.

2.3.2. Transmission electron microscopy (TEM)

The nanoparticle suspension was ultrasonicated properly by a probe-type sonicator and a drop was put on a copper grid (300 mesh) coated with a layer of amorphous carbon kept on filter paper and allowed to air dry. About 15 min after nanoparticle deposition, the grid was tapped gently with filter paper to remove excess water. TEM was performed at an accelerating voltage of 300 kV. Photomicrograms were obtained using TEM (Tecnai G2 30, FEI, The Netherlands).

2.3.3. Measurement of zeta potential

The nanoparticle suspension was sonicated properly and the surface charge was measured using a zeta sizer (Zetasizer Nano ZS; Malvern Instruments, Malvern, UK).

2.4. Proteolytic experiments on trypsin gold nanoparticle constructs

2.4.1. Assay of enzymatic activity of trypsin using BAPNA

The synthetic substrate N-α-p-benzoyl-p-nitroanilide (BAPNA) is an effective reagent for any type of trypsin assay [31]. Trypsin cleaves BAPNA to produce p-nitroaniline that has λ_{max} at 410 nm. Therefore, trypsin activity can be measured by measuring the decreasing rate of linear release of the p-nitroaniline at 410 nm with time (as the absorption measured at 410 nm decreases with time due to continuous consumption of BAPNA by trypsin and leading to less and less release of p-nitroaniline with time). The reagents, bovine b-trypsin, BAPNA, Tris(hydroxymethyl)aminomethane (commonly referred as tris), and $CaCl_2$, were bought from Sigma, and dimethyl sulphoxide