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The on-bead digestion of protein corona on nanoparticles by trypsin immobilized on the magnetic nanoparticle

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ABSTRACT

Proteins interacting with nanoparticles would form the protein coronas on the surface of nanoparticles in biological systems, which would critically impact the biological identities of nanoparticles and/or result in the physiological and pathological consequences. The enzymatic digestion of protein corona was the primary step to achieve the identification of protein components of the protein corona for the bottom-up proteomic approaches. In this study, the investigation on the tryptic digestion of protein corona by the immobilized trypsin on a magnetic nanoparticle was carried out for the first time. As a comparison with the usual overnight long-time digestion and the severe self-digestion of free trypsin, the on-bead digestion of protein corona by the immobilized trypsin could be accomplished within 1 h, along with the significantly reduced self-digestion of trypsin and the improved reproducibility on the identification of proteins by the mass spectrometry-based proteomic approach. It showed that the number of identified bovine serum (BS) proteins on the commercial Fe_3O_4 nanoparticles was increased by 13% for the immobilized trypsin with 1 h digestion as compared to that of using free trypsin with even overnight digestion. In addition, the on-bead digestion of using the immobilized trypsin was further applied on the identification of human plasma protein corona on the commercial Fe_3O_4 nanoparticles, which leads the efficient digestion of the human plasma proteins and the identification of 149 human plasma proteins corresponding to putative critical pathways and biological processes.

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1. Introduction

Engineered nanosized materials have been widely applied in drug/gene delivery, disease diagnosis and biosensing etc. [1–5]. When nanoparticles are introduced into biological systems, proteins would interact with nanoparticles and lead to the formation of so called "protein corona" on the surface of the nanoparticles. As reported previously, the protein corona on nanoparticles would critically impact the biological identities of nanoparticles [6–9] and/or result in the physiological and pathological consequences of the macrophage uptake, blood coagulation, complement activation and cellular toxicity, etc. [10–16]. The elaborate investigation on protein corona would accordingly make great sense for the extensive application of nanomaterials.

To characterize proteins adsorbed on the surface of nanoparticles, technologies such as the dynamic light scattering (DLS) [15,17,18], transmission electron microscopy (TEM) [18], circular dichroism (CD) [18,19], size exclusion chromatography (SEC) [20,21], isothermal titration calorimetry (ITC) [20,22], fluorescence quenching technology [23] and surface plasmon resonance (SPR) [21,22], and mass spectrometry (MS) etc. have been performed to determine the thickness, density, arrangement, conformation, affinity and identification of proteins on nanoparticles [9]. From which, the identification of proteins involved in a protein corona on the surface of nanoparticles would be one of the key issues for the understanding of biological effects and/or responses of nanoparticles with protein corona [11]. Generally, protein corona formed on nanoparticles should be eluted and then enzymatically digested for the following analysis of mass spectrometry (MS). Generally, detergent could be applied to elute proteins from nanoparticles as the denaturation of proteins by SDS [24]. However, SDS detergent existed in elution process would interfere the following enzymatic digestion and identification of protein corona by mass spectrometry. The isolation of proteins from SDS elution was thus required usually via the precipitation of using organic solvent such as acetone overnight [24] and/or with a further SDS-PAGE separation [17,25,26], which unfortunately were time-consuming and

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laborious. Moreover, the sample loss for low abundant proteins, the low digestion efficiency and the cross-contamination were the nerve-racking problems during protein isolation and digestion, which would bring the great adverseness to the determination of proteins, especially for minute biological samples. Rather than the isolation of protein corona from nanoparticles via SDS elution and the following precipitation with organic solvents, the direct digestion of protein corona on nanoparticles (also called on-bead digestion) might be an efficient approach to avoid the interference of SDS. With free trypsin, the protein corona on nanoparticles could be directly digested, which could simplify the procedures of the isolation and digestion of protein corona from nanoparticles [27]. However, the long time digestion of free trypsin is required to complete the digestion of proteins from nanoparticles for above-mentioned on-bead digestion or SDS elution assay. Moreover, the self-digestion of the applied free trypsin in solution would bring the interference in the identification of protein corona by MS and the followed protein database searching.

Trypsin immobilized on stationary phases has demonstrated the highly efficiency as well as the correspondingly reduced self-digestion in the digestion of proteins [28–30]. To avoid the limitation of using SDS elution as well as to improve the digestion of protein corona from nanoparticles with a high efficiency, in this work, the on-bead digestion of protein corona on nanoparticles was carried out by using the trypsin immobilized on magnetic Fe_3O_4 nanoparticles. It showed that the digestion of protein corona on the surface of nanoparticles could be efficiently completed within 1 h rather than the usual long time (overnight) digestion, with the significantly reduced self-digestion as compared to the severe self-digestion of free trypsin. In addition, the reproducibility for the digestion and identification of protein corona on nanoparticles was improved obviously due to the absence of SDS as well as the reduced self-digestion of trypsin after the immobilization on nanoparticles. Also, with the help of the magnet property, the trypsin immobilized on the magnetic nanoparticles could be simply isolated from the digestion solution.

2. Materials and methods

2.1. Chemicals and materials

Fe_3O_4 nanoparticle (~30 nm), Dithiothreitol (DTT), iodoacetamide (IAA), TPCK-treated trypsin, bovine serum albumin (BSA), formic acid (FA), tetraethyl orthosilicate (TEOS) and (3-aminopropyl) triethoxysilane (APTES), sodium cyanoborohydride ($NaCNBH_3$) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (ACN, HPLC grade) and ammonium solution (25%) was from Merck (Darmstadt, Germany). Iron (III) chloride hexahydrate ($FeCl_3.6H_2O$), sodium acetate anhydrous, ethylene diamine, isopropanol, ethylene glycol (EG), glutaraldehyde and ethanol were from Tianjin Kermel plant of chemical reagent (Tianjin, China). SDS-PAGE marker and loading buffer were obtained from Thermo Scientific (San Jose, CA). Water used in experiments was doubly distilled and purified by a Mill-Q system (Millipore, Bedford, MA, USA). Bovine serum (BS) was bought from Tianhang Biological Technology Co. (Zhejiang, China). The human plasma used in experiments was obtained from the Second Affliated Hospital of Dalian Medical University (Dalian, China) and stored at −80 °C before use.

2.2. Synthesis of the immobilized trypsin

Fe_3O_4 nanoparticle with the size of 250 nm was firstly synthesized according to a literature procedure [31]. Then the synthesized Fe_3O_4 nanoparticle was coated by a silica shell via sol-gel process (noted as $Fe_3O_4@SiO_2$ nanoparticles) and followed by the reaction with APTES to yield the amine-functionalized $Fe_3O_4@SiO_2$ nanoparticles (noted as $Fe_3O_4@SiO_2@NH_2$ nanoparticles) [29]. The amine functionalized magnetic Fe_3O_4 nanoparticles were then reacted with glutaraldehyde in PBS solution to introduce the aldehyde groups on [32]. After that, the Fe_3O_4 nanoparticles were reacted with TPCK treated trypsin to immobilize the trypsin on Fe_3O_4 nanoparticles, followed with a capping procedure of the remaining aldehyde groups on Fe_3O_4 nanoparticles by glycine. The obtained trypsin immobilized Fe_3O_4 nanoparticles were preserved in PBS buffer containing 0.02% sodium azide for later use to digest protein corona on nanoparticles (the detailed synthesis procedures were illustrated in supporting information).

2.3. The digestion of standard BSA on Fe_3O_4 nanoparticles by the immobilized trypsin

Standard BSA solution (2 mg/mL) was prepared by dissolving 6 mg BSA in 3 mL NH_4HCO_3 solution (50 mM, pH 8). 100 μL of 30 nm Fe_3O_4 nanoparticles (0.2% w/w in PBS, pH 7.4) suspension were mixed with 100 μL of standard BSA solution, and incubated by shaking at 1400 rpm for 1 h at 37 °C to prepare the composite of BSA and Fe_3O_4 nanoparticle. The resulting composite of BSA and Fe_3O_4 nanoparticle was held by a magnet, washed with PBS for at least 3 times, resuspended in a solution of 8 M urea and 50 mM NH_4HCO_3, and then reduced with 10 mM DTT at 37 °C for 2 h and followed by alkylation with 20 mM IAA at room temperature with shaking in dark for 30 min. Then the solution was diluted to ~1 M urea and the prepared BSA-Fe_3O_4 complexes were then digested by the immobilized trypsin (10 μg) with digestion time of 15 min, 30 min and 1 h, respectively. After that, the immobilized trypsin were retained by a magnet, and the tryptic digests of BSA from the BSA-Fe_3O_4 complexes were collected, acidified (pH 2~3), desalted, and finally lyophilized to dryness for LC-MS/MS analysis. For comparison, the BSA-Fe_3O_4 complexes were also digested by free trypsin (4 μg) for 15 min, 30 min, 1 h and overnight, respectively.

2.4. The digestion of protein corona of BS on Fe_3O_4 nanoparticles by immobilized trypsin

To evaluate the digestion efficiency of immobilized trypsin, the BS protein corona was prepared on the 30 nm Fe_3O_4 nanoparticles. Briefly, 200 μL of BS was incubated with 200 μL of Fe_3O_4 nanoparticles suspension (0.2% w/w in PBS) with shaking at 1400 rpm on a multi-thermo-shaker (Hangzhou Allsheng Instruments Co., Ltd.) at 37 °C for 1 h to yield the composite of BS protein corona@Fe_3O_4 nanoparticles. The as-formed composite of BS protein corona@Fe_3O_4 nanoparticles was washed with PBS for 3 times with a help of a magnet, resuspended in a 200 μL of 8 M urea, 50 mM NH_4HCO_3 solution, and followed with the reduction by 10 mM DTT at 37 °C for 2 h and the alkylation by 20 mM IAA at room temperature with shaking in dark for 30 min subsequently. Then the solution was diluted to ~1 M urea and the digestion of the BS protein corona bound on Fe_3O_4 nanoparticles was carried out by incubating the composite with immobilized trypsin (20 μg) at 37 °C for 1 h. For comparison, the same amount of BS protein-Fe_3O_4 complexes were also digested by free trypsin (8 μg) for 1 h and overnight, respectively. The digested peptides of the BS protein corona from the composite of BS protein corona@Fe_3O_4 nanoparticles were collected by removing the residual Fe_3O_4 nanoparticles as well as the magnetic immobilized trypsin by a magnet, which were acidified to pH 2–3, desalted, lyophilized and stored at −30 °C.

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