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Accurate and reliable quantification of the protein surface coverage on protein-functionalized nanoparticles

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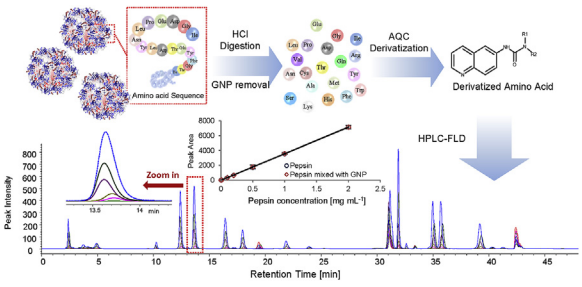
HIGHLIGHTS

- Accurate analysis of the surface coverage of protein-functionalized nanoparticles.
- Direct determination of bound protein by protein hydrolysis and amino acids analysis.
- Sensitive HPLC assay with fluorescence detection of AQC-derivatized amino acids.
- Calibration with protein or amino acids (if protein sequence is known).
- Application to pepsin-coated gold nanoparticles.

GRAPHICAL ABSTRACT



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ABSTRACT

The ability to accurately quantify the protein coverage on nanoparticles is critical for assessing the quality of the surface chemistry and the success of the functionalization process of protein-nanoparticle conjugates. Surface coverage determination is therefore an integral part in the quality control of protein-modified nanoparticles in industrial nanotechnology. In this work, a novel and conventional method was established for direct quantification of the protein surface coverage on metallic nanoparticles. Different concentrations of pepsin were conjugated to gold nanoparticles (GNPs) by a straightforward adsorptive immobilization process as a model system, and a protein quantitation methodology based on the amino acid analysis of the hydrolysate of the protein-GNP conjugates was established. For this purpose, pepsin functionalized GNPs (pepsin-GNP bioconjugates) were processed via *in situ* hydrolysis with 6N HCl and subsequent derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC reagent). Direct quantitative amino acid analysis was performed based on measuring the intensity of AQC-glycine derivative by high-performance liquid chromatography with fluorescence detection (HPLC-FLD). The method allows for detection of surface coverages as low as 0.1 μg mL^{-1} pepsin (corresponding to 2.89×10^{-9} mol L^{-1}) in the colloidal solution. Method imprecision for replicated surface coverage determinations was <5% RSD and accuracies, as determined by % recoveries, were always in the 98–118% range. This method allows precise and accurate quantification of protein coverages, even when less than 1% of the protein in the reaction mixture is immobilized. It was found that the degree of surface coverage of adsorptively bound pepsin on GNPs correlated with the pepsin concentrations in the conjugation reaction mixtures. Washing with phosphate buffer removed weakly bound proteins, i.e. the soft protein corona. The adsorption behavior could be described by a Freundlich isotherm model. This

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direct and reliable method promises great potential for the accurate quantification of protein coverages of various protein-nanoparticle bioconjugates.

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1. Introduction

Nowadays, nanoparticles are being extensively used in biomedical and biotechnological research. For instance, nanoparticle-antibody conjugates have been employed as affinity carriers to extract specific biomarkers for mass spectrometric analysis from complex biological samples [1–5]. Due to their controlled geometrical and flexible surface chemical properties, nanomaterials have also acted as excellent solid supports for enzyme immobilization [6]. For all these applications, the assay performance of protein-nanoparticle bioconjugates depends mainly on the interactions between the nanoparticle and protein, including the protein surface coverage and orientation on the nanoparticle surface [7–10]. Therefore, in order to control assay quality and optimize the surface chemistry, it is critical to develop a method for the accurate quantitation of the surface coverage on nanoparticles in order to evaluate the success and reproducibility of nanoparticle-protein conjugation reactions. In addition, accurate quantification of the protein concentration on nanoparticles is a key requirement for the valid assessment of the kinetic performance of enzyme-nanoparticle conjugates, and also for their quality control to make it possible that the enzyme-nanoparticle bioconjugate can be successfully and reproducibly applied as heterogeneous catalyst in industrial processes.

The accurate determination of the protein surface coverage on protein-nanoparticle conjugates is still challenging. To date, only a few studies directed some attention towards the determination of the protein concentration on nanomaterials [11–18]. However, compared to the vast number of scientific articles on nanoparticle synthesis, modification and enzyme immobilization methods, research on protein surface coverages and methods for this purpose are highly underrepresented in nanoscience. Typically, total protein quantification assays (Lowry assay, Bradford assay and bicinchoninic acid BCA assay) are employed which allow detection of non-immobilized proteins in the supernatant of the binding/reaction mixture with low concentrations [12,13,19,20]. The immobilized protein content can be calculated by mass balance considerations. Thus, these approaches are therefore so-called indirect assays. A validation of these assays is rarely carried out in the context of protein surface coverage determination of such bioconjugates. It is assumed that accuracies may suffer with these indirect protein quantitation assays. For example, the protein content is usually determined in the reaction mixture after removal of the nanoparticles. However, during the following washing steps protein loosely adsorbed to the surface might be desorbed and the protein coverage will hence be altered. Loss of protein, e.g., due to adsorption on other surfaces during the sample preparation might lead to erroneous results as well [7]. Furthermore, these protein quantification assays have limited precision, which may cause problems, if only a small percentage of the protein in the reaction mixture is bound to the nanoparticle surface. For instance, if the imprecision of the assay is 10% (RSD) and only 1% of the protein in the reaction mixture gets immobilized, it can rarely be imagined that the protein coverage can be determined with high accuracy by these indirect assays. Besides, these assays are prone to interferences from various chemicals. Therefore, the direct analysis of bound protein is considered highly advantageous in terms of assay accuracy. ELISA assays have been used for the determination of bound proteins (e.g. antibodies) on gold nanoparticles [1,21]. If such ELISA assays are directly used to detect immobilized proteins, the effect of the matrix (nanoparticle carrier) on the determined protein concentration will remain unclear. Furthermore, ELISA kits are expensive. Fluorescence methods have also been proposed to directly analyze protein concentrations on nanoparticle surfaces [7]. This method involves fluorescence labeling of the protein in order to achieve sufficient sensitivity. Other techniques such as dynamic light scattering (DLS) and nanoparticle tracking analysis (NTA) measure the increase in hydrodynamic diameter after adsorption in order to derive information on surface coverages [14–18,22,23]. However, the thickness of the bound protein layer does not directly provide accurate information on bound proteins per nanoparticles. Yet, models have been derived which allow to correlate the average number of protein molecules bound to the nanoparticle to the hydrodynamic radius [24,25]. Such a model has also been exploited to convert hydrodynamic radii measured by Taylor dispersion analysis (TDA) into protein surface coverages [26]. Recently a technique called resonant mass measurement (RMM) has been proposed to determine the protein mass on single nanoparticles [14,27–29]. It actually measures the buoyant mass of nanoparticles in a microfluidic channel by frequency shifts of a resonator when a nanoparticle passes by. The buoyant mass can be converted to dry mass, which allows then to calculate the number of proteins per nanoparticle due to knowledge of the numbers of particles, which are counted as well. Another sophisticated method uses an electrospray-differential mobility analyzer (ES-DMA) for the separation of NP and protein-NP conjugates which are coupled to an aerosol particle mass analyzer (APM) that determines mass by a balance of electrical and centrifugal forces. The differential analysis of unmodified carrier and protein-modified NPs allows the quantification of ligand densities [30]. Very recently, a targeted mass spectrometry-based method has been developed to directly measure the amount of antibody covalently bound to magnetic particles [11]. This method involves an on-bead digestion and analysis of a few characteristic tryptic peptides by isotope-dilution liquid chromatography-tandem MS, using selected reaction monitoring acquisition. Unfortunately, all these latter techniques require equipment, which is not widely available in analytical laboratories.

Hence, the aim of this study was to establish a novel and generally applicable strategy for directly quantifying protein coverages on gold nanoparticles based on the reliable method of amino acid analysis following acidic hydrolysis of the protein. In this work, pepsin was employed as the model protein, and different concentrations of protein-GNP bioconjugates coated with different concentrations of pepsin were prepared by a straightforward adsorptive immobilization process with subsequent washing steps to remove unbound and loosely bound proteins. Protein-GNP bioconjugates were chemically digested *in situ* with 6N HCl to generate free amino acids and GNPs mixtures. After GNP removal and amino acid derivatization, direct quantitative analysis was performed based on measuring the intensity of the AQC-glycine derivative by using HPLC-FLD (Fig. 1). An *in situ* digestion procedure of the bioconjugates was adopted without prior protein elution and isolation steps from nanoparticles, which extended the application ranges to protein-nanoparticle conjugations with different bonding

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