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Surface assembly on biofunctional magnetic nanobeads for the study of protein–ligand interactions

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ABSTRACT

One of the major challenges of proteomics today is to increase the power potential for the identification of as many proteins as possible and to characterize their interactions with specific free ligands (interactomics) or present on cell walls (cell marker), in order to obtain a global, integrated view of disease processes, cellular processes and networks at the protein level. The work presented here proposes the development of biofunctionalized magnetic nanobeads that might be used for interactomic investigations. The strategy consisted in immobilizing proteins via a non covalent technique that provides greater possibilities for the advent of faster, cheaper and highly miniaturizable protein analysis systems, in particular in situations where the amount of isolated protein is scarce (trace proteins). The advantage of the immobilization technique proposed here over more conventional covalent binding techniques is that it is versatile and universal (not protein specific) thus applicable to a wide range of proteins, in "mild" conditions that are non deleterious to the native structure and bioactivity of the immobilized protein. The feasibility of the technique was investigated using a model protein (streptavidin). The nanobeads were analyzed in size by light diffusion and transmission electronic spectroscopy, and in quantity of immobilized protein using a bioassay developed in the laboratory. Results are promising in that nanobeads exhibited good colloidal stability and surface concentrations in the monolayer range.

1. Introduction

The term "proteom" was used the first time in 1995, to describe the protein complement of a genome [1]; imperceptibly, the proteome was transmuted into a new discipline, "proteomics". As soon as the first complete genome sequence, of *Haemophilus influenzae*, was published [2], it became clear that many putative proteins encoded by the newly found genes had no known function and, of those with surmised function, many had functions attributed by analog only. This has been the case as more and more genomes have yielded to the massive sequencing efforts that are going on around the world. Yet the problem of making sense of the huge amount of sequence data that are being generated remains. Molecular biology has provided powerful techniques for high-throughput DNA analysis that are not yet reflected in the protein world. This has resulted in an emphasis on the "message" (mRNA or cDNA) rather than on the product of that message (protein). Assigning function to a novel protein requires the integration of many techniques and is currently a bottleneck in the drug-discovery process. To achieve this goal numerous tools have been developed, e.g. yeast two-hybrid system, immuno-coprecipitation, affinity chromatography, protein chips [3] but all exhibit a certain number of limitations (time consuming, expensive, not suitable for miniaturization and/or for the identification of trace proteins). We propose here the development of a new methodological tool in order to circumvent such limitations, and that is capable of identifying important partners of the protein of interest. The tool is based on the elaboration of magnetic nanobeads (NB) capable of identifying the various ligands of a protein. The primary goal of the work presented in this paper is to develop a new immobilization technique for this purpose that is versatile, universal and non protein dependent, applicable to a wide range of proteins and non covalent, since the latter exhibits a great

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number of drawbacks. Covalent grafting of proteins is often protein specific (the grafting protocol requires modifications from one protein to another), and can be deleterious to proteins (environmental factors such as the temperature required for efficient yield and the solvents used can denature or seriously harm the bioactivity of the immobilized protein). The method proposed here is not protein specific but applicable to a wide range of proteins, is performed under "mild" aqueous conditions (biocompatible environment, no organic solvents, and at room temperature), thus preserving the natural structure and bioactivity of the immobilized protein. To validate the feasibility of the method, a model protein (streptavidin) was immobilized. The advantage of this protein is that once immobilized in the appropriate conditions (monolayer), it can be used to "hook" a multitude of other proteins and biomolecules (e.g. membrane proteins, peptides, DNA fragments), provided the latter are biotinylated. This can be done for a multitude of biomolecules. Another option is that the technique proposed here can be applied for the direct grafting of the protein or biomolecule of choice. The magnetic character of the nanobeads allow for immediate separation and recovery of the protein or protein–ligand complex by simple application of a mild magnetic field.

The non covalent method used here, the "layer-by-layer" technique developed first by Decher et al. [1,2] and involving the immobilization of a bioactive protein (or peptide) by trapping it onto a previously "conditioned surface" obtained by alternatively adsorbing oppositely charged polyelectrolyte layers until a multiple charged layer is adsorbed onto the surface (conditioning layer), has been well characterized for flat surfaces [3–15]. The process was shown to maintain the native form [9] thus the bioactivity of the protein, as well as its availability even when "buried" under multiple bilayers of polyelectrolytes, for an active dynamic cell model (up to 10) [3]. The coating process was achieved here on nanobeads. However, our system being based on a passive biomolecular interaction model, our goal here, was on one hand to not burry the protein under multiple layers of PE, in order to maintain its availability to a potential ligand (biotin or biotin bound biomolecule such as a peptide, a lectin, an antibody or a macromolecule), and on the other hand to immobilize the protein on the nanobead surface in a stable form. This was ensured by a strong interaction with the PE conditioning film via its multiple electrostatic interaction sites (stemming from the numerous charges present on the PE film layer). Furthermore, we seek for minimal "capping" of the protein on the nanobead surface (either one or no PE layer, if possible, after protein immobilization), in order to avoid hindering the approach and capture of the ligand (e.g. biotin) or ligand-bound macromolecule (e.g. biotin-IgG) by the immobilized protein. Thus, the oppositely charged protein (pH far from pI) was deposited on the conditioning layer mainly by electrostatic attraction, then capped or not, by a polyelectrolyte layer of a charge different from that of the protein.

The modified nanobeads were analyzed in size and zeta surface potential (which gives an idea of the electrical surface charge), and using a biological assay developed by our group, based on a "ligand depletion method" involving measurements of the concentration decrease of a fluorescently labeled ligand after exposure to given amounts of protein immobilized nanobeads, in order to study the stability and homogeneity (polydispersity) of the nanocolloidal suspension after protein modification, as well as the quantity of immobilized proteins (mono- or multiple layers), respectively. The purpose of the paper was to validate the feasibility of the technique with the model protein. If so, the technique could be applicable to the immobilization of a wide variety of proteins to nanobeads for protein analysis. The results are promising since stable and homogeneous suspensions could be obtained, showing high levels of immobilized protein on the nanobead surface.

2. Materials and methods

2.1. Materials

Magnetic ferric oxide nanobeads (Fe_2O_3) were obtained from Aldrich–Sigma in a powdered state (iron oxide nanopowder 98%). The nanobeads sizes were in the 10–20 nm range, as deemed by the Sigma–Aldrich Company. These nanobeads (NB) were suspended in ultrapure water (milliQ), initially filtered through a 0.45 μm millipore filter, to obtain a final initial 1%, w/w, stock suspension. The polyelectrolytes (PE) used, PAH (poly (allylamine hydrochloride), Mw = 15,000) and PSS (poly (sodium 4-styrene sulfonate), Mw = 70,000) were purchased from Sigma–Aldrich. The fluorescently labeled streptavidin ligand was purchased at Sigma–Aldrich (Biotin 4-Fluorescein).

2.2. Surface treatment

2.2.1. Nanobead coating

The initial 1%, w/w, nanobead stock suspension was homogenised under mechanical stirring for 15 min (600 rpm), sonicated for 15 min, and then stirred again for 7 min. The nanobeads were then coated with an initial "preconditioning" layer of PAH. Then a layer of PSS of opposite charge was deposited to form the first PE bilayer ($n = 1$). The particles were further coated sequentially with PEs, in the alternating order PAH/PSS until three bilayers of PAH/PSS were obtained (NB-(PAH-PSS)$_n$ with n spanning from 1 to 3). Each PE coating step was performed in aqueous solutions of filtered ultrapure water (milliQ) at 5 mg/mL of PE, under similar stirring conditions during 15 min. The PE/nanobead suspensions were sonicated for 15 min, and stirred again under similar conditions for another additional 10 min to avoid aggregate formation. The nanobeads were then recovered by applying a mild magnetic field (using a small magnetic stir bar). The PE rich supernatant phase was then eliminated and the beads were rinsed twice in filtered ultrapure water under mechanical stirring during 5 min, and separated again in a mild magnetic field. This latter rinsing step was performed twice in filtered ultrapure water, after which the particles were resuspended in filtered ultrapure water to obtain a final 0.5%, w/w, suspension of PE coated nanobeads. Some material loss was observed during the coating/wash cycles and was in the 1–5% range NB weight loss.

2.2.2. Protein immobilization

The globally positively charged protein (streptavidin, pI = 5.5, purchased at Sigma–Aldrich) was immobilized to the 3 bilayer negatively charged PE coated nanobeads (NB-(PAH-PSS)$_3$) by electrostatic interaction in a 1 mg/mL protein/0.01 M acetate buffer (acetic/acetate buffer, pH 3.8). In order to stabilize the assembly structure, the protein coated particles were then capped with a final PE layer, consisting of the negatively charged PE (PSS). The "capping" procedure was performed by separating the particles from the PE solution, rinsing in ultrapure filtered water, separating them via magnetic field application, according to the previous procedure, then by resuspending them in the same acetate buffer, in order to stabilize the final structure (Fig. 1).

2.3. Surface characterization

2.3.1. Nanobead characterization

The treated and non treated NBs were characterized for size (and eventual aggregate formation) by dynamic light scattering (DLS) and for superficial charge accumulation by the determination of the zeta potential. Light scattering and zeta potential measurements were performed on a Malvern® Nanosizer Nano ZS. TEM