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High-gradient magnetic separation for technical scale protein recovery using low cost magnetic nanoparticles

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ABSTRACT

Downstream processing still lacks efficient and integrated separation techniques. We present a high-gradient magnetic separation (HGMS) process for the successful purification of recombinant histidine-tagged Green Fluorescent Protein (His-GFP) from an *E. coli* cell lysate by means of superparamagnetic iron oxide nanoparticles functionalized with a pentadentate chelate ligand. The separator is an improved rotor stator prototype with 1 L chamber volume. Using 100 g of carrier, a purification performance of approx. 12 g His-GFP per hour could be achieved with an eluate purity of 96% and a yield of 93% for the whole process. We demonstrate how varying processing parameters enhances the final results and provide evidence of the potential of HGMS to become a real alternative to conventional downstream processes. These pilot scale experiments show that the combination of high performance nanocarriers and optimized separator design offers an attractive system for technical implementation. Almost no nanoparticle loss took place during the experiments. The demonstration of liter scale processing with nanoparticles is important because, due to their higher surface-to-volume ratio in comparison to microparticles, it is now possible to achieve higher capacities. Furthermore, the applied nanoparticles can be seen as low-cost, very stable carriers compared to common polymer microparticles.

1. Introduction

Nowadays, as in many other industrial sectors, biotechnological production volumes are continuously increasing while product prices are falling. Therefore, large-scale sustainable bioprocessing requires maximizing efficiency together with reducing energy consumption and waste [1]. The lack of revolutionary advances in downstream processing is slowing biotechnological progress, preventing adequate responses to the increasing demand for biotechnological products. This situation has long been recognized [2], but the processing chain following fermentation remains a time and cost intensive sequence with many steps. The concentration of biomolecules and the removal of impurities are critical, decisively influencing the efficiency of the entire processing chain [3]. Highly-integrated separation processes and innovative concepts are necessary to provide a strong impetus to this industrial sector, making it more advanced and competitive [4]. New alternatives are needed at all stages and have to compete economically with the unit operations currently applied, e.g. solid/liquid separation and subsequent chromatographic purification. In this context, high-gradient magnetic separation (HGMS) is a quite promising fast and comparably simple technology, which enables the direct isolation of target biomaterial from raw biological suspensions by means of functionalized magnetic carriers [5]. This technology, also called high-gradient magnetic fishing [6], allows a significant reduction in the number of processing steps while increasing total product yield. Even though the advantages of magnetic separation for bioprocessing applications are obvious [7], nowadays it is primarily employed only at a laboratory scale, mainly due to the lack of adequate liter-scale separators, the high cost of the industrial systems and the complex design [8].

The working principle of HGMS is the application of large static magnetic fields (approx. 1 T) to columns containing ferrous matrices. Magnetic gradients as high as 10^4 T/m create forcefields capable of capturing even weakly magnetic particles in a flow stream [9]. These processes have been applied in some industrial sectors, such as wastewater treatment [10], food processing or steel production for more than 50 years [11]. Nowadays, other uses like removal of contaminants from mineral oils are also being explored [12]. In most cases, the magnetic separators are primarily used in continuous flow processes. Their use for biotechnological purposes began in the early 1970s [13] and is still common today

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though generally in the form of batch separators [14,15], although in a recently published work Lindner and Nirschl report on the combination of HGMS and centrifugation to generate a continuous process [16]. The number of published biotechnological large-scale HGMS applications is still low [17–21]. Specifically Franzreb and different collaborators have been working intensively in the field, reporting on HGMS methodology and parameters [6] as well as concrete application examples with micron beads as carriers e.g. for lactoferrin capture [22], superoxide dismutase recovery [23] as well as the integrated multiple use of immobilized lipase [24]. More recently reports on other areas, such as algae separation, have been the aim of studies [25].

Within the broad pool of magnetic beads available for biomolecular capture by magnetic forces, smaller iron oxide magnetic nanoparticles (MNPs) are a special group which is being intensively studied nowadays. They are highly interesting due to their large surface-to-volume ratio and their biocompatibility. However, there are some difficulties with their use in HGMS: (1) large magnetic field gradients are needed to manipulate the small particles; (2) flow conditions have to be optimized to retain all magnetic material; (3) the applied MNPs for the separation process must first be functionalized with the appropriate chemistries, a task that usually requires intensive developing processes and high costs. One classical method for bioseparation, and more specifically for histidine (His)-tagged proteins, is the use of chelate ligands decorated with immobilized metal ions [26]. This technique, called immobilized metal ion affinity (IMA), employs typically tri- or tetradentated ligands. In this paper we present a separation method based on MNPs functionalized with a pentadentate chelate ligand (ethylene diamine tetraacetic acid, EDTA) later loaded with copper ions. The characterization of the applied Cu-EDTA-MNP system as well as the separation results on a laboratory scale were recently published by our group [27]. While IMA techniques are established for the purification of proteins, the application on an industrial scale is quite new because of worries regarding the effects of the His-tag as well as metal ion leaching [28]. In our previous paper, we reported on the lack of metal ion leaching with EDTA as a chelate ligand. This system yields promising results with very good efficiency also at a pilot-plant scale, as we show with the present report on the isolation of His-tagged green fluorescent protein (His-GFP) as a model biomolecule by means of high-gradient magnetic separation from an *E. coli* cell lysate.

The work presented here was carried out on a HGMS rotor stator prototype [13]. First results from a pilot plant similar to ours were published very recently and dealt with the multicycle purification of equine chorionic Gonadotropin (eCG) using microparticles [29]. Previous publications had already reported on studies carried out with a smaller rotor stator system: Brown and collaborators showed the suitability of HGMS for a technical application [30], whereas Müller and co-authors isolated eCG and compared the HGMS process with the technically established fixed-bed chromatography [31]. Our focus lies on the application of nanoparticles (instead of micro-) and also on the optimization of relevant processing parameters to improve final purification and concentration factors. Although Moeser and collaborators [32] demonstrated that MNPs coated with a bifunctional polymer or a phospholipid layer were retained by the magnetic field despite their small size, the HGMS experiments in their work were run at a mL scale. To the best of our knowledge, the only previous report on the suitability of large-scale HGMS for nanoparticulate systems in bioseparation processes is the one by Holschuh and Schwämmle [33]. They applied Protein A coated beads of about 100 nm in diameter to purify antibodies and showed the high potential of the method for the bioprocessing of up to 100 L. Their device included four Neodyn® magnetic rods which were manually driven. In our work, we have used even smaller particles than Holschuh and Schwämmle (about 30 nm in diameter). The fact that HGMS with small nanoparticles has now been successfully applied at an industrial level is of great importance because the downstream industry can then benefit directly from the current enormous developments in nanoresearch. Moreover, the applied nanoparticles are low-cost carriers thanks to the easier and shorter synthesis in comparison to common polymer microparticles; another advantage is their better long term stability because no degradation takes place.

Our example demonstrates that HGMS offers a real, promising alternative for the recovery field in an industrial environment with the additional advantages of being a fast, robust and comparably simple processing technique.

2. Materials and methods

2.1. Materials

The plasmid vector pET-28a (Novagen, Madison, USA) was used for the cloning and overexpression of His-GFP (model protein) from an *E. coli* BL21 (DE3) strain. After fermentation, the cell suspension was concentrated in a disk separator (GEA Westfalia Separator Group, Germany), then disrupted by high-pressure homogenization in two passages at 900 bar (GEA Niro Soave, Italy) and finally stored at −20 °C. The dry biomass content was between 0.8% and 1.5%. The target protein concentration in the lysate was approx. 8.5 g L^{-1}; this system was chosen as model for practical reasons although the initial target protein concentration is significantly higher than in common industrial suspensions. The His-GFP standard was purified from the lysate and showed a purity >98% as determined by 12% SDS PAGE and analytical reversed phase chromatography. The purification steps included (1) immobilized metal ion affinity chromatography using a HisTrap FF crude column (5 mL, GE Healthcare, Sweden), (2) anion exchange chromatography using a column packed with Unosphere Q (10 mL, BioRad laboratories, Germany) and (3) size exclusion chromatography (HiLoad 16/600 Superdex 75 pg, GE Healthcare, Sweden).

The lysate solution for processing was kept in standard 0.05 M Tris (Tris-(hydroxymethyl)aminomethane) buffer, pH 8.5. As the washing buffer a solution of 0.05 M sodium dihydrogen phosphate ($NaH_2PO_4 * 2H_2O$) and 0.5 M NaCl, pH 7.9 was used. The elution buffer contained 0.05 M $NaH_2PO_4 * 2H_2O$, 0.5 M NaCl and imidazole (0.05 M, 0.25 M or 0.5 M), pH 7.9. Imidazole and Tris were obtained from AppliChem, Germany. All other reagents were supplied by Carl Roth, Germany. All chemical agents used were of analytical grade or higher.

The applied core-shell MNPs are described elsewhere [27]. Briefly, they comprise a magnetite core coated with a silica layer, a spacer, EDTA as pentadentate affinity ligand and copper ions as the binding site for protein capture. The EDTA-MNPs were provided by Merck KGaA. The metal ion loading step was carried out by our group. In laboratory scale experiments a high binding capacity for His-GFP, high selectivity, good recyclability and long term stability were proven. The Langmuir isotherm constants for the His-GFP adsorption in the presence of host cell proteins at pH 8.5 in 0.05 M Tris buffer solution were determined as K_d = 9.2 mg L^{-1} and q_{max} = 245 mg g^{-1}. Furthermore, main physical properties as a saturation magnetization of 59 A m^2 kg^{-1} (remanence < 0.08%), a primary particle diameter of 22 ± 4 nm and a specific surface area of 89 m^2 g^{-1} were determined.

2.2. Analytical instrumentation

Qualitative analyses of proteins included SDS-PAGE and Coomassie staining with Brillant Blau R250 provided by Carl