Supporting Information for

Magnetic One-Step Purification of His-Tagged Protein by Bare Iron Oxide Nanoparticles

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ABSTRACT: Magnetic separation is a promising alternative to conventional methods in downstream processing. This can facilitate easier handling, fewer processing steps, and more sustainable processes. Target materials can be extracted directly from crude cell lysates in a single step by magnetic nanoadsorbents with high-gradient magnetic fishing (HGMF). Additionally, the use of hazardous consumables for reducing downstream processing steps can be avoided. Here, we present proof of principle of one step magnetic fishing from crude Escherichia coli cell lysate of a green fluorescent protein (GFP) with an attached hexahistidine (His6)-tag, which is used as the model target molecule. The focus of this investigation is the upscale to a liter scale magnetic fishing process in which a purity of 91% GFP can be achieved in a single purification step from cleared cell lysate. The binding through the His6-tag can be demonstrated, since no significant binding of nontagged GFP toward bare iron oxide nanoparticles (BIONs) can be observed. Nonfunctionalized BIONs with primary particle diameters of around 12 nm, as used in the process, can be produced with a simple and low-cost coprecipitation synthesis. Thus, HGMF with BIONs might pave the way for a new and greener era of downstream processing.

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**Experimental procedures**

BIONs used in these experiments were characterized by transmission electron microscopy (TEM), X-ray diffraction (XRD), magnetometric measurements (SQUID) and Raman spectroscopy in an earlier investigation by Schwaminger et al.¹

For zeta potential measurements, BIONs (1 g L⁻¹) were incubated andtitrated in the range of pH 3 to 11. The zeta potential was measured with a Delsa Nano C (Beckman Coulter Inc., USA) where 0.1 M NaOH and 0.1 M HCl were used as titrants.

For HPLC analysis, a reverse phase C18 Kinetex EVO 5 μm, 150 x 3 mm (Phenomenex, CA USA) was used at a flowrate of 0.5 mL min⁻¹. Samples were diluted to a GFP content below 0.5 g L⁻¹ and filtered (0.2 μm) with a cellulose filter. A linear gradient from 40 to 80% acetonitrile (20 mM trifluoro acetic acid, TFA) with double distilled water (20 mM TFA) over 18 min was run for the separation of GFP from other proteins. The proteins were detected at 215 nm.

For dynamic light scattering (DLS) measurements the Delsa Nano C (Beckman Coulter Inc., USA) instrument was used as well and the samples were measured 100 times in a disposable cell with a 658 nm laser. Furthermore, DLS was conducted with a Zetasizer (Malvern Panalytical, UK) under similar conditions with. Here the density was adjusted to 5 g cm⁻³ and the refractive index to 2.4.

Differential centrifugal separation (DCS) was conducted with a Disc Centrifuge Model 24000 (CPS Instruments). 100 µL sample were injected at 18000 rpm and build gradient by injecting mixtures of 24%:8% sucrose solutions varying the sucrose gradient.

For optical centrifugation (OC), a LUMiSizer (LUM GmbH, Germany) was used. Here, the transmission at 870 nm of different centrifugation profiles from 750 to 4000 rpm were recorded and used for size distribution measurements. The density of magnetite (5.2 g cm⁻³) was used for the analysis of transmission profiles.

For the simulation FEMM was used to determine the magnetic flux and the magnetic field in the separator chamber.
Characterization of BIONs

**Figure S1.** TEM image of the as-synthesized nanoparticles (left top) and the corresponding particle size distribution (right top). XRD reflections (left bottom) and Raman spectrum (right bottom) of the freeze-dried magnetite nanoparticles. Reproduced from Schwaminger et al. (*Faraday Discuss.*, 2017, 204, 233-250 DOI: 10.1039/CF00105C) with permission from The Royal Society of Chemistry (RSC).

**Figure S2.** Magnetization loops of freeze-dried magnetite at 300 K (left). Zeta potential of magnetite nanoparticles (1 g L\(^{-1}\)) in the range of pH 3 to 11. Adapted from Schwaminger et al. (*Faraday Discuss.*, 2017, 204, 233-250 DOI: 10.1039/CF00105C) with permission from The Royal Society of Chemistry (RSC).
Characterization of proteins

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) analysis of the isolated fusion proteins was applied to verify the protein identity as well as to control and determine the molecular mass of GFP. An UltraFlex I MALDI-TOF/TOF mass spectrometer (Bruker) and the BioTools software package (Bruker) along with the MASCOT software (Matrix Science) and the SwissProt database was used for analysis. For identification the observed SDS-PAGE bands were excised and digested with trypsin following as described in more detail in Schäfer et al. Prior to analysis full length proteins were purified on C4 ZipTips (Merck Millipore) following the manufacturer’s protocol.

To control and determine the pI of our GFP isolate, isoelectric focusing experiments were used. The GFP was diluted to a concentration of 0.1 g L⁻¹ in a buffer with a low ionic strength (10 mM Tris-HCl buffer pH 8.25). 23 µL of the protein solution was loaded into each gel pocket on a SERVA FocusGel 3-10 24S. 5 µL of SERVA IEF 3-10 Marker was loaded in a separate pocket and empty pockets were filled with buffer. The gel was run with a Multiphor II electrophoresis device (Pharmacia Biotech Inc.). 1 to 2 mL of contact fluid was pipetted onto the device before placing the gel. The gel was cooled to 10 °C with a water bath. The current was held constant at 20 mA according to the manufacturer’s instructions. The gel was fixed for 15 min in a 20% (w/v) trichloroacetic acid solution and washed twice for 1 minute in wash solution (0.1% (w/v) CuSO₄, 10% (v/v) acetic acid, 25% (v/v) methanol). Then, the proteins were stained in a staining solution preheated to 50 °C (0.1% (w/v) CuSO₄, 10% (v/v) acetic acid, 30% (v/v) methanol, 0.02% (w/v) Coomassie G250) for 45 min. The gel was washed twice for 5 min and then destained for two to three times for 15 minutes in wash solution.

The GFP used has a His₆-tag attached directly to the N-terminus without spacer. The protein consists of 258 amino acids leading to the theoretical molecular weight of 27.7 kDa and the theoretical isoelectric point of 6.0. However, MALDI-TOF measurements of His₆-GFP indicate a size of 27.8 kDa. The isoelectric point (pI) of His₆-GFP is at pH 5.9 as derived from isoelectric focusing. The pI of the untagged protein was observed to be pH 5 and the size of this protein as determined with MALDI-TOF is around 27.0 kDa.

Sequence of His₆-GFP

MGSSHHHHHHSSGLVPRGSMSKGEELFTGVVPLVELDGDVGKHFSVSGEGEGDATYG KLTLCFTTGGKLVPVPTLVTTLTYGVOQCFRSRYPDHMKQHDFFKSAQMEQYQERTIFFK DDGNYKTRAEVKFEVDTLVNNIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGI KVNFKIRHNIDGVLADHYQQNTPIGDGPVLLPDNHYLSQRAKLADPNKRDHMVLLEFVTAAGITLGMDELYK

The purification of His₆-GFP was conducted with a Histrap IMAC and monitored with an AEKTA purifier system. The purification procedure was repeated three times in order to provide a sufficient amount of material. Figure S3 shows the conductance as well as the absorbance at 280 nm which corresponds to proteins due to tryptophane subunits in proteins and the absorbance at 485 nm which represents the characteristic absorption of GFP.
Figure S3. Chromatograms of a) run 1, b) run 2 and c) run 3.

Prior to IMAC, the concentration of the His<sub>6</sub>-GFP solution obtained from AEX was determined with a fluorescence measurements to be 9.42 g L<sup>-1</sup>. The purity of the His<sub>6</sub>-GFP containing sample after AEX obtained from fluorescence measurements is 77.4%. The samples were loaded on the column in AEX-buffer (TBS). A wash was also conducted with this buffer. The elution was conducted with IMAC buffer (50 mM phosphate buffer pH 7.5, 500 mM NaCl, 500 mM imidazole). In all fractions an overload of the column was observed. The elution yielded 100% purity as determined by fluorescence measurements as well as HPLC analytics and SDS-PAGE of His<sub>6</sub>-GFP which is illustrated in Table S4, Figure S5 and S6.

Table S4. Concentration and purity of His-GFP as determined with fluorescence measurements and HPLC, respectively.

| Samples       | Concentration of His<sub>6</sub>-GFP (g L<sup>-1</sup>) | Purity (%) | Monomer | Dimer |
|---------------|-------------------------------------------------------|------------|---------|-------|
| AEX-Eluate    | 9.42                                                  | 77.4       |         |       |
| Load 1        | 0.82                                                  | 60.9       |         |       |
| Load 2        | 2.02                                                  |            |         | -     |
| Load 3        | 1.4                                                   |            |         | -     |
Wash 1  0.74  49.7
Wash 2  1.86  -
Wash 3  0.81  -
IMAC Eluate 1  5.84  90.6  9.4
IMAC Eluate 2  9.74  89.4  10.6
IMAC Eluate 3  9.11  89.7  10.3
IMAC Eluate average  8.23  89.9  10.1

Figure S5. HPLC-chromatograms of run 1 to 3. Elution buffer (undiluted), eluate run 1 (1:10), eluate run 2 (1:20), eluate run 3 (1:20), load run 1 (1:2), wash run 1 (1:2).

SDS-PAGE clearly proves the purity of GFP as determined by HPLC but also demonstrates the existence of GFP dimers at around 60 kDa while the fraction around 27.7 kDa indicating a GFP monomer is the strongest band for all fractions.

Binding studies

Table S6. Overview of adsorption of GFP to BIONs

| Protein | Buffer | Load $q_{\text{max}}$ (g g$^{-1}$) | Affinity $K_D$ (g L$^{-1}$) | Affinity $K_D$ (µM) | Ligand density (GFP nm$^{-2}$) | Coverage |
|---------|--------|----------------------------------|-----------------------------|---------------------|-----------------------------|----------|
| His     | Ac     | 0.26                             | 0.01                        | 0.37                | 0.06                        | 0.45     |
| His     | MES    | 0.23                             | 0.02                        | 1.85                | 0.06                        | 0.43     |
| His     | Phos   | 0.27                             | 0.2                         | 7.41                | 0.06                        | 0.45     |
| His     | TBS    | 0.22                             | 0.01                        | 0.37                | 0.05                        | 0.38     |
| His     | Tris   | 0.26                             | 0.03                        | 1.11                | 0.06                        | 0.43     |
| Ref     | Tris   | 0.07                             | x                           | x                   | 0.02                        | 0.12     |
| Crude lysate | Tris | 0.27                             | 0.17                        | 6.18                | 0.07                        | 0.47     |
| Cleared lysate | Tris | 0.33                             | 0.07                        | 2.64                | 0.08                        | 0.56     |

S6
**Aggregation behavior**

![Graph showing aggregation behavior of different analysis methods.](image)

**Figure S7.** Comparison of different particle size analysis methods of 1 g L⁻¹ BIONs in 50 mM tris buffer at pH 7. The particles were analyzed by dynamic light scattering with a Beckman coulter Nano (BC), a Malvern Zetasizer (MZ) as well as by differential centrifugal separation (DCS) and optical centrifugation (OC).

**Untagged GFP binding**

![SDS-Gel of untagged GFP incubate with BIONs in tris buffer (50 mM, pH 7) for 1 hour, magnetically separated and washed two times (W1 and W2). A marker (M) is used to indicate the protein size (Colour Protein Standard Broad Range (10-250 kDa)).](image)

**Figure S8.** SDS-Gel of untagged GFP incubated with BIONs in tris buffer (50 mM, pH 7) for 1 hour, magnetically separated and washed two times (W1 and W2). A marker (M) is used to indicate the protein size (Colour Protein Standard Broad Range (10-250 kDa)).
Simulation of magnetic field

Figure S9: Simulated magnetic flux and magnetic field in the separator chamber for the HGMF process. a) Magnetic flux of $z$ and $r$ axis through the separator chamber and the aluminum coils. b) Magnetic field of $z$ and $r$ axis through the separator chamber and the aluminum coils. c) Magnetic flux through the separator chamber ($z$ axis). d) Magnetic field through the separator chamber ($z$ axis).

Separation process

Figure S10. Picture of collected wash fractions and eluate.
References
(1) Schwaminger, S. P.; Blank-Shim, S. A.; Scheifele, I.; Fraga-García, P.; Berensmeier, S. Peptide Binding to Metal Oxide Nanoparticles. *Faraday Discuss.* 2017, 204, 233–250.
(2) Schäfer, H.; Nau, K.; Sickmann, A.; Erdmann, R.; Meyer, H. E. Identification of Peroxisomal Membrane Proteins of Saccharomyces cerevisiae by Mass Spectrometry. *Electrophoresis* 2001, 22 (14), 2955–2968.