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# Protein separation with magnetic adsorbents in micellar aqueous two-phase systems

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ABSTRACT

We introduce a new concept for the purification and concentration of macromolecules such as proteins based on the use of magnetic adsorbents in concert with micellar aqueous two-phase systems. Aqueous solutions of certain nonionic surfactants can, depending on the prevailing temperature, separate into two co-existing phases, one enriched, the other depleted in surfactant. These are known as micellar aqueous two-phase systems. Hydrophilic proteins are normally excluded from the 'surfactant-rich' phase of micellar aqueous two-phase systems. We show here that by introducing magnetic microadsorbents into micellar aqueous two-phases the phase selectivity of a hydrophilic protein can be effectively inverted. The magnetic adsorbents adsorb the target protein and pull it out of the 'surfactant-depleted' phase into the 'surfactant-rich' phase from which it is normally excluded. Starting from a model solution containing a 1:1 ratio of the hen egg white proteins lysozyme and ovalbumin, the application of magnetic cation exchange micro-adsorbents combined with the nonionic surfactant Triton X-114 resulted in a target lysozyme yield of 74% and purity of >80%. The combination of selective magnetic adsorbents with micellar aqueous two-phase systems yields a highly tunable extraction system. Potentially, these so-called 'Magnetic Extraction Phases' provide the basis for flexible and easily scaled fractionation processes for hydrophilic proteins.


## 1. Introduction

Liquid–liquid extraction using aqueous two-phase systems (ATPS), is a reliable separation method for proteins and other biomolecules. ATPS integrates clarification and concentration with partial purification, and is thus eminently applicable to the early stage processing of complex unclarified bioprocess feedstocks [1–7]. APTS are usually formed when two incompatible polymers, such as polyethyleneglycol (PEG) and dextran, or a polymer and a salt, e.g. PEG and $Na_2SO_4$, are mixed in water. More recently, two-phase aqueous micellar systems (AMTPS), which form when certain classes of surfactants dissolve in water, have been proposed as especially attractive, if not superior, systems for bioseparation use [8–13]. Two-phase aqueous micellar systems usually split above or below the so-called 'Cloud-Point Temperature ($T_{CP}$)' into two distinct phases, one 'surfactant-rich', the other 'surfactant-poor'. At technical scales of operation the recycling of the phase forming polymer is likely to be easier in AMTPS *cf.* traditional ATPS, because only one phase, the 'surfactant-rich'-phase, must be processed.

A difficult task when working with ATPS lies in defining operating conditions under which the target molecule is recovered in sufficient yield and purity. Since the mid-1970s the use of affinity ligands in ATPS to increase both the capacity and specifity for a given target molecule has been studied extensively [14–17]. This approach employs ligands either in free form or attached to one of the phase-forming polymers [18,19]. In our view, the use of affinity principles becomes especially attractive when applied to micellar ATPS. The use of micellar ATPS to extract hydrophobic proteins has been explored extensively since Bordiers initial report over 25 years ago [20], and in more recent times these systems have, through the introduction of affinity functions, been adapted very successfully to the separation of hydrophilic proteins [21–23].

Re-use of phase-forming polymers and affinity ligands is an essential prerequisite for industrial use of affinity ATPS. Among methodologies addressing these issues are the selective precipitation of ligands [19], and the use of ligands bound to polymers [24], or solid particles [25–31] To date this latter approach has only been applied to traditional and not micellar ATPS.

Another important issue especially pertinent to industrial scale ATPS is the phase separation rate, which usually determines the required process time. Accelerated phase separation in ATPS has been achieved by centrifugation in disc stack centrifuges [32,33],

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**Nomenclature**

| | |
|---|---|
| $a$ | parameter used to solve a quadratic equation |
| $b$ | parameter used to solve a quadratic equation |
| $c$ | concentration ($g\,L^{-1}$) |
| CR | dimensionless capacity ratio |
| $f$ | help function |
| $K$ | partitioning coefficient |
| $K_L$ | Langmuir-parameter ($mg\,L^{-1}$) |
| $q$ | loading of adsorbent surface ($mg\,g^{-1}$) |
| $q_{max}$ | maximum loading ($mg\,g^{-1}$) |
| $R$ | volume ratio |
| $T$ | temperature (°C) |
| $V$ | volume (L) |

*Subscripts*

| | |
|---|---|
| 0 | initial value |
| B | bottom-phase |
| CP | cloud point |
| Lys | lysozyme |
| Ova | ovalbumin |
| P | particle |
| Succ | successive equilibrium |
| Sync | synchronous equilibrium |
| T | top-phase |

*Abbrevations*

| | |
|---|---|
| AMTPS | aqueous micellar two-phase systems |
| ATPS | aqueous two-phase systems |
| HGMF | high gradient magnetic fishing |
| MEP | magnetic extraction phases |

and using external microwave, acoustic, electrical [34,35] and magnetic fields [36–39]. The employment of an external magnetic field becomes possible, when the ATPS phases are 'seeded' with small magnetically responsive particles, which partition preferentially into the dispersed phase of the system. The first investigators to successfully combine magnetic affinity adsorbents with ATPS were Suzuki et al. [40].

The application of magnetic supports for the purposes of bioseparation is very widely practiced and is well-established at small scale. At larger scales of operation magnetic separation is currently best achieved by 'High-Gradient Magnetic Fishing (HGMF)' [41,42]. HGMF is a fast, robust and scalable approach to recover macromolecules directly from crude bioprocess feedstocks, which fuses three classical steps – clarification, concentration and initial purification – into just one unit operation composed of a few sub-steps. In HGMF initial adsorption of the molecule of interest onto functionalized micro- or nanoscaled magnetic support particles is integrated with subsequent collection of 'product-loaded' support particles from the suspending feedstock using a highly-magnetized stainless steel filter matrix. Following washing, the adsorbed species are eluted from support into a smaller volume.

Despite bright future prospects HGMF suffers from a number of problems requiring further attention [41,42]. Of these, achieving complete recovery of the adsorbents out of the magnetic filter at the end of the process remains a considerable challenge. An interesting alternative to capturing 'product-loaded' magnetic particles in a magnetic filter is to extract them into a liquid phase. Here, building upon Suzuki et al.s' earlier study [40], we introduce a new process idea, i.e. combining micellar ATPS with HGMF to generate highly tunable 'Magnetic Extraction Phases (MEPs)'. This MEP concept unites several advantages of each process while simultaneously eliminating some of their major drawbacks. The system chosen to illustrate the feasibility of this new magnetic extraction process is the adsorption and partitioning of two globular proteins from chicken egg white, lysozyme and ovalbumin, in an MEP composed of superparamagnetic cation exchange micro-adsorbents [43–45] and the extensively studied nonionic surfactant, Triton X-114.

## 2. Materials and methods

### 2.1. Materials

Superparamagnetic base particles (type M-PVA 012; supplied as 50 mg/mL suspension) with a diameter between 1 and 3 μm were purchased from chemagen Biopolymer-Technologie AG (Baesweiler, Germany). The chemicals, acrylic acid (anhydrous, >99%, cat no. 01730), ammonium cerium(IV) nitrate (99.99+%, cat no. 229547-106) and Triton X-114 (pure, cat no. X-114) were supplied by Sigma Aldrich Corporation (St. Louis, MO, USA). Chicken egg white derived proteins, ovalbumin (grade V, ≥98%, A 5503) and lysozyme (E.C. 3.2.1.17, 78643 U/mg, cat no. 62971), were obtained from the same company and were used without further purification. Novex NuPAGE® pre-cast Bis-Tris 10% (w/v) polyacrylamide gels (1.0 mm thick, 15-well) were purchased from Invitrogen Corporation (Carlsbad, CA, USA), and Coomassie Brilliant Blue R250 (cat no. 1.12553.0025) was supplied by Merck (Darmstadt, Germany). All other chemicals used were of analytical grade, and Milli-Q water was employed throughout.

### 2.2. Methods

#### 2.2.1. Functionalization of magnetic supports with cation exchange groups

Immediately prior to functionalization with weak cation exchange moieties the surfaces of the M-PVA starting material were washed extensively at room temperature (20–22 °C) using the following 'acetone–methanol–water' series: 100% acetone; 50% acetone/50% methanol; 100% methanol; 50% methanol/50% MilliQ water; 100% MilliQ water. Briefly, this involved suspending portions (equivalent to 0.25 g dry weight) of M-PVA particles with 50 mL volumes of each washing solution. After 300 s of vigorous vortex mixing the particles were recovered with the aid of a strong (0.4 T) NdFeB bar magnet. The washings were discarded and the wet magnetic particle cake subsequently resuspended with the next solution in the wash series. At the end of the washing procedure, the particles were washed three times with water and then stored at 4 °C until further use.

Magnetic cation exchange adsorbents featuring a polyacrylic acid brush surface were prepared by a Cerium(IV) initiated 'graft from' polymerization procedure similar to that described by Brown et al. [45]. Wet magnetic particles (0.15 g dry weight) were suspended in 9 mL of $N_2$ purged water. Acrylic acid monomer (0.45 mL) was subsequently added, and after shaking vigorously under nitrogen for 300 s, the graft reaction was initiated by the addition of 0.45 mL of a solution containing 76 $mg\,mL^{-1}$ ammonium cerium(IV) nitrate in 2 M nitric acid. After 3 h of incubation at room temperature with shaking under a nitrogen blanket, polyacrylic acid grafted magnetic supports were magnetically separated from suspension, and then washed successively with 20 mL portions of the following solutions – once with 0.2 M $Na_2SO_3$ in 10 (w/v) acetic acid (stop solution); twice with water; once with 1 M NaCl; and twice again with water – before storing in water at 4 °C until required.