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Continuous protein purification using functionalized magnetic nanoparticles in aqueous micellar two-phase systems

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ABSTRACT

A novel technique for technical-scale continuous purification of proteins is presented. It is based on the combined use of functionalized magnetic nano-particles and an Aqueous Micellar Two-Phase System featuring the non-ionic surfactant, Eumulgin ES, which undergoes temperature induced phase separation at ~25 °C. In the first step, conducted below the transition temperature (i.e. 15 °C), the magnetic sorbent particles are added into the single dispersed phase and bind the protein of interest. Next, on raising the temperature to 30 °C the protein-laden magnetic particles partition strongly into the micelle-rich top phase of the micellar two-phase system that's formed. The magnetically susceptible top phase is then continuously separated from the micelle-poor phase in a flowthrough tailor-made magnetic extractor featuring a permanent magnet providing an upwardly acting magnetic force. This separation device was shown to be effective for continuous separation of a wide range of differently sized magnetic particle sorbents (i.e. from 2 μm diameter to as small as 25 nm) from a 10% (w/w) Eumulgin ES system; high separation efficiencies were recorded for the phase-forming surfactant (87 to >98%), and all magnetic sorbent particles tested (95–99.9%). Finally, protein purification by continuous magnetic extraction was demonstrated at 15 L scale for the recovery of an antibody fragment, A33 Fab′, from a crude extract of *Escherichia coli* periplasm. Nearly 70% of the A33 Fab′ initially present in the extract at 15.6% of the total protein content was recovered in a 2-fold concentrated and highly purified (>98%) state. Further, the amounts of magnetic sorbent and phase-forming surfactant lost in the process were very small; thus recycling of both components into subsequent rounds of continuous magnetic extraction is highly feasible.

1. Introduction

Preparative chromatography, a core technology within the biopharmaceutical sector for several decades, plays critical roles in the downstream processing of modern biopharmaceuticals. However, against increasing pressures (rocketing product titers, increasing size and complexity of emerging bio-products to name but a few), it is increasingly being viewed as a serious bottleneck in manufacturing [1,2]. Against this backdrop, the modification/improvement of existing traditional fixed column chromatography systems [3], or their replacement with integrated and/or continuous separation processes represent increasingly important challenges to solve. Aqueous two-phase systems (ATPS) have long been regarded as possible alternatives ever since Albertsson's classical demonstration in the mid 1950s [4] of selective partitioning of (bio)molecules between two aqueous phases of a mixture of polymers and/or salts. Aqueous Micellar Two-Phase Systems (AMTPS) introduced by Bordier [5] some 25 years later differ considerably from classical ATPS. AMTPS feature non-ionic surfactants which undergo temperature-induced phase separation resulting in two phases, i.e. one 'micelle-rich' (or 'coacervate-rich') and the other 'micelle-poor' [6]. Numerous applications and combinations of phase-forming polymers have been proposed since the early 1980s for the selective enrichment of a protein in one of the aqueous phases (also described as aqueous two-phase extraction, ATPE, or cloud point extraction, CPE) [6,7]. A striking advantage of ATPE is the ease with which its operation can be up-scaled and made continuous, and as consequence several process schemes demonstrating the feasibility of continuous bioprocessing exploiting ATPS [8–10]

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and AMTPS [11,12] have been published within the last decade. The high cost and loss of phase-forming components in the target protein-depleted phase represent a significant economic hurdle to overcome, but the main reasons for ATPE's perceived lack of viability are that the physical principles underlying the partitioning behavior of a given target protein between the phases remain unclear; and that very considerable experimental effort is required to identify an economically attractive system [13]. Affinity-enhanced ATPS were introduced [14] in order to circumvent the above issues. In affinity ATPS the phase-forming polymer is either chemically modified, or alternatively ligands are 'introduced', by one of a variety of ways, into the two-phase system to enhance the partitioning of a molecule of interest into one of the two phases [15]. The introduction of ligands into ATPS by adding functionalized magnetic particles constitutes a particularly attractive class of affinity-enhanced ATPS [16–18]. The combination of ATPS with magnetic particles can dramatically enhance the rate of phase separation following application of an external magnetic field cf. traditional affinity ATPS, provided that the particles partition strongly into the dispersed phase [19–21].

Previously, in our laboratories we introduced the new concept of 'Magnetic Extraction Phases (MEPs)' [22], illustrated by the successful combination of magnetic cation exchange micro-sorbents with a Triton X-114 based AMTPS, to separate lysozyme from ovalbumin. Here, sorption of the target molecule, lysozyme, was performed at a temperature in the single-phase regime (T_S). Phase separation was subsequently induced by raising the temperature (T_T, denoting two-phase regime). The protein-loaded particles accumulated in the micelle-rich phase of the resulting AMTPS and the micelle-poor phase was removed. In the following step, an AMTPS was set up using an appropriate elution buffer and the micelle-rich phase that was obtained post sorption. Previously bound protein species were desorbed from the particles and partitioned variously within the AMTPS according to their individual partition coefficients (K), whilst all of the magnetic sorbent particles moved into the micelle-rich phase. More recently, we extended the MEP concept to nano-scaled magnetic sorbents and an AMTPS based on the non-ionic surfactant, Eumulgin ES [23]. The 'Eumuglin ES AMTPS' represents a significant improvement over 'Triton X-114 AMTPS'. For instance, it simultaneously reduced surfactant costs, afforded the use of a moderate phase separation temperature, and importantly, yielded extreme differences in the partitioning coefficients of the target protein and the magnetic sorbent particles. As a direct consequence of extreme K-values, on elution the target protein partitioned exclusively to the micelle-poor phase, whereas the magnetic sorbents accumulated entirely in the micelle-rich phase. The MEP experiments described above using the new improved combination (Eumuglin ES AMTPS plus magnetic nano-particle sorbents) were performed at laboratory scale. In this study, we detail the systematic transfer of lab-scale manual MEP operation to a technical-scale 'Continuous Magnetic Extraction' (CME) process. We demonstrate continuous separation of cation exchange functionalized magnetic nano-particles from the micelle-poor phase at flow rates of several liters per hour; and subsequently apply CME for the continuous capture and purification of an antibody fragment from a crude *Escherichia coli* cell extract.

2. Materials and methods

2.1. Materials

Four different functionalized magnetic particles were employed in this study. 'Poly(NIPA-*co*-AAc)' particles (200 nm mean diameter by dynamic light scattering), received as a gift from Dr. Rodica Turcu (National Institute for Research and Development of Isotopic and Molecular Technologies, NIIMT, Cluj-Napoca, Romania), featured magnetite cores embedded in a matrix of poly(*N*-isopropylacrylamide-*co*-acrylic acid). 'MagPrep Silica 25' and 'MagPrep SO_3 100' particles were kindly donated by Dr. Johannes Bauer (Merck KGaA, Darmstadt, Germany). Both particles comprised magnetite crystals coated with a thin layer of silica, however, the surface of the latter was further functionalized with sulfonate (SO_3^-) moieties analogous to those commonly employed for strong cation exchange (CEX) chromatography media. Scanning electron microscopy revealed single particles of narrow size distribution, measuring 25 nm and 100 nm across for the base silica coated and sulfonate derivatized particles respectively. Chemagen M-PVA-DEAP particles were supplied by PerkinElmer chemagen Technologie GmbH (Baesweiler, Germany). This material is a spherical beaded 'polyvinyl alcohol (PVA)-magnetite' composite of ca. 2 μm, functionalized with diethylaminopropyl (DEAP) groups. The strong CEX beaded chromatography medium, Fractogel® EMD SO_3 (M) was a further donation from Dr. Johannes Bauer (Merck KGaA, Darmstadt, Germany), and the HiTrap rProtein G column was from GE Healthcare (Uppsala, Sweden). *E. coli* strain W3110 expressing the A33 Fab′ [24] and protocols for its cultivation were acquired from UCB Celltech (Slough, UK) under a Material Transfer Agreement. The non-ionic surfactant, Eumulgin ES (PPG-5-Laureth-5, CAS-No.: 68439-51-0), was purchased from Cognis (Düsseldorf, Germany). The chemicals, sodium tetraphenylborate (ACS reagent, ≥99.5%), barium chloride (99.9% trace metal basis), Tris(hydroxymethyl)aminomethane (ACS reagent, ≥99.8%), bicene (BioXtra, ≥99% by titration), glycine (for electrophoresis, ≥99%), acetone (≥99.8%), methanol (≥99.9%), glacial acetic acid (ACS reagent, ≥99.7%), were all obtained from Sigma-Aldrich (St. Louis, MO, USA). Phosphate buffered saline (Dulbecco 'A' tablets) was purchased from Oxoid Limited (Basingstoke, UK), disodium hydrogen phosphate and sodium chloride were supplied by Carl Roth (Karlsruhe, Germany) or Sigma–Aldrich, and citric acid monohydrate and Coomassie Brilliant Blue R250 (C.I. 42660) were from Merck Millipore (Darmstadt, Germany). Pre-cast 15% mini-PROTEAN® TGX™ gels and Precision Plus Protein™ All Blue Standards were supplied by Bio-Rad Laboratories Inc. (Hercules, CA, USA). Quant-iT™ PicoGreen® dsDNA kit was acquired from Invitrogen Life Technologies (Carlsbad, CA, USA), and the bicinchoninic acid (BCA) protein assay kit and bovine serum albumin (BSA; fraction V powder, ≥96% pure by electrophoresis) were both obtained from Sigma–Aldrich. All other chemicals not stated above were from Sigma–Aldrich or Merck Millipore. The water used in all experiments was deionized and purified using a Millipore Milli-Q Ultrapure system.

2.2. Determination of AMTPS phase diagrams

The location of the curve defining the two-phase and single-phase regimes within a T–x diagram (where x is the mass fraction of the surfactant in the mixture) was determined as follows. Different fractions of surfactant and the corresponding buffers were weighed into 15 mL centrifuge tubes. The centrifuge tubes were tempered in a temperature-controlled water bath (Model RC 20 S, Lauda Brinkmann, Deran, NJ, USA), and incubated for a minimum of 12 h until complete phase separation had been achieved. After this period the top and bottom phases of the resulting two-phase system were quickly separated and retained for subsequent analysis of the surfactant concentrations in each phase (see Section 2.9).

2.3. Magnetic extractor for CME

The tailor-made magnetic extractor constructed for the CME studies described in this work (Fig. 1a) features a separation chamber, housed within a permanent magnet yoke (627 mm