

Controlling the partitioning behavior of magnetic micro-particles via hydrophobization with alkylamines: Tailored adsorbents for continuous bioseparation



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ABSTRACT

The purpose of this work was the synthesis of functional particles for use in biotechnological applications such as protein purification and enzymatic conversions. The process applied combined magnetic micro-particles and aqueous micellar two-phase systems. Hydrophilic magnetic micro-particles were coupled with alkylamines of various lengths, thus improving partitioning in this switchable aqueous two-phase system. The newly introduced covalent bonds on the particle surface were verified by FTIR measurements. Different ratios of alkylamine to surface groups were tested and the surface group density was determined by titration and a novel method of considerably reduced analysis time. Finally, the chosen surface modified particles were successfully applied in an exemplary protein purification process reaching a purity of 87% and more.

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1. Introduction

Continuous magnetic extraction (CME) is a new process concept that allows for the continuous purification of proteins using a combination of functional magnetic particles and aqueous micellar two-phase systems (AMTPS). CME has been applied successfully for protein purification [1,2], and recently, the extension of its use to enzymatic conversion processes was proposed [3]. For the CME process, the capability of AMTPS to form two phases above the so-called lower critical solution temperature (LCST) is an important feature, allowing to switch the system between a single phase and a two phase regime by means of a small temperature shift.

The combination of magnetic adsorbents or carriers and such temperature switchable two-phase systems has a number of advantages. In case of protein purification, these are, e.g., suitability for crude feedstocks, direct combination of the capture and

purification step, avoidance of large storage vessels and product degradation through continuous processing, fast phase separation supported by an external magnetic field, and high selectivity as well as purity through selective binding of the target to the adsorbent particles. Enzymatic conversion processes combined with CME prevent the product from being contaminated with the enzyme. The reaction can be controlled easily by fast separation of the enzyme from the suspension. The immobilized enzyme is more stable (regarding temperature, pH, etc.) and can be reused several times by continuous recovery of the immobilisates, thus utilizing their lifetime to the best possible extent.

A major factor for the applicability of CME is the partitioning behavior of the magnetic particles into the surfactant rich top phase formed during temperature induced phase separation. Because this condition often is not met per se by functional magnetic particles, the purpose of our work was to systematically modify their surface in order to attain a reliable partitioning behavior. The following requirements were defined for particles ideally suitable for CME: They need to be suspended well in the single-phase state, they have to partition into the top phase of the AMTPS in the two-phase state and they have to have a specific surface functionality (enzyme binding sites, ion exchange, etc.) depending on the application.

While many studies deal with surface modification of magnetic particles and its effect on hydrophobicity [4–9], directed partitioning of the particles has not been investigated. An exception can be

Abbreviations: AMTPS, aqueous micellar two-phase systems; ATR, attenuated total reflection; CD, coupling density; CME, continuous magnetic extraction; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; FTIR, Fourier-transform infrared spectroscopy; IP, inflection point; LCST, lower critical solution temperature; PVA, polyvinyl alcohol; SCD, surface charge density; TOC, total organic carbon.

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found in studies on Pickering emulsions [10], where the partitioning behavior of particles at the phase boundary plays an important role. It defines which component makes up the dispersed phase and which the continuous phase [11]. Therefore, applications and methods in this field [12–16] are focused on the stabilization of multi-phase systems. However, in contrast to our goal, complete partitioning of particles into one-phase or the other is not suitable in the case of Pickering [12].

Partitioning of magnetic silica particles in an AMTPS was investigated and explained earlier [17]. This study is aimed at directing and modifying the partitioning behavior of magnetic polyvinyl alcohol (PVA) micro-particles as needed. Alkyl groups are introduced to the particle surface, which previously were used to render magnetic particles hydrophobic [6,7].

2. Experimental

2.1. Materials

Magnetic micro-particles (diameter approx. $2\ \mu\text{m}$) with a hydrophilic polymer coating were used in all experiments. They disperse easily in aqueous suspensions and have a carboxylic functionality on the surface (M-PVA C22, by PerkinElmer Chemagen, Baesweiler, Germany). The carboxy groups act as ion exchangers and binding sites for surface modification in this application. Previously, they were used for enzyme immobilization via EDC [3]. The micro-particles are made up of nanometer sized magnetite crystals which are embedded in a matrix of crosslinked polyvinyl alcohol. A magnetization curve of a particle sample is given in Fig. 1. It can be seen that the particles show a very low remanent magnetization ($<0.5\ \text{Am}^2/\text{kg}$) which means that they do not become permanently magnetized. Therefore, for the intended use the particles can be looked at as superparamagnetic, permitting easy redispersion once the magnetic field is removed. Additionally, they have a high saturation magnetization (approx. $40\ \text{Am}^2/\text{kg}$) which allows fast separation from suspension.

The non-ionic surfactant Eumulgin ES (Cognis, part of BASF SE, Ludwigshafen, Germany) was used to generate a thermoresponsive two-phase system. Eumulgin ES has a molecular weight of $696\ \text{g/mol}$ [18] and an LCST of approx. $25\ ^\circ\text{C}$. A 10% surfactant system in a $0.01\ \text{M}$ phosphate buffer (pH 7.0) was used for protein purification (adsorption and wash step). For desorption of the protein, $1\ \text{M}$ NaCl was added to this buffer in the elution step.

Originally, Eumulgin ES, the phase-forming component itself, was considered as a possible candidate for covalent surface modification of the particles (data not shown). However, alkylamines appeared to be more suitable for coupling reactions onto the carboxylated particle surface. They have the general structure of $\text{CH}_3(\text{CH}_2)_x\text{NH}_2$. In our case, $x=3, 5, \dots, 17$, i.e., *n*-butylamine to octadecylamine, was used. Since higher alkyl lengths lead to a decrease in water solubility, solutions of the target molecules in ethanol were prepared.

2.2. Coupling of alkylamines with EDC and sulfo-NHS

The surface modified particles are synthesized by a two-step coupling method with EDC (1-ethyl-3-(3-dimethylamino-propyl)carbodiimide hydrochloride, Merck, Germany) and sulfo-NHS (*N*-hydroxysulfosuccinimid, Sigma–Aldrich). In the first step the particles are activated with EDC/sulfo-NHS in $0.1\ \text{M}$ MES buffer (pH 5.0) for 30 min at $11\ ^\circ\text{C}$ in a shaker. Sulfo-NHS is added to stabilize the resulting intermediate structure. After activation, the particles are washed two times. The respective alkylamine is added to the activated particles suspended in $0.1\ \text{M}$ phosphate buffer (pH 7.5) and the mixture is incubated for 2.5 h at $25\ ^\circ\text{C}$. The resulting surface modified particles are washed three times in ethanol and twice in $0.02\ \text{M}$ phosphate buffer (pH 7.0). Subsequently, they are dispersed in $0.01\ \text{M}$ NaOH before $0.1\ \text{M}$ HCl is added to the suspension to remove adsorbed alkylamines. Finally, they are washed again twice in phosphate buffer, in which they are stored afterwards until use.

2.3. Determination of the partitioning behavior

The partitioning behavior of the particles was an essential criterion for assessing the quality of the new surface modified material and comparing it to the unfunctionalized particles. Only $0.2\ \text{mg}$ of particles are necessary for this analysis procedure. In a $2\ \text{ml}$ tube, the particles are dispersed in a 10% AMTPS ($100\ \mu\text{l}$ of Eumulgin ES in $900\ \mu\text{l}$ of $0.02\ \text{M}$ buffer). Depending on the pH, a citrate, phosphate, or TRIS buffer is used. After addition of the surfactant, the pH is set to the desired value. The suspension is mixed well and the mixture is incubated at $30\ ^\circ\text{C}$ for 15 min. Partitioning is then observed visually and documented with a digital camera.

2.4. FTIR measurements

A Fourier-transform infrared spectrometer (FTIR) with an attenuated total reflection (ATR) unit (Tensor 27 with Platinum ATR, Bruker Optics, Germany) was used for all measurements. The particles are first washed three times in MES buffer (pH 5.0) and twice in ultrapure water before they are dried in the oven at $60\ ^\circ\text{C}$ for at least 48 h. After an initial baseline measurement against air, the samples are measured. Approx. $10\ \text{mg}$ of dried, ground particles are applied to the crystal and compressed with the stamp of the ATR unit. All sample measurements are performed three times at least. Prior to measuring a new sample, the crystal is cleaned with isopropanol and the baseline is checked for impurities. The FTIR spectra are processed with the software OPUS (Version 7.2, Bruker Optics). They are corrected for atmospheric influences (atmospheric compensation), vector normalized, averaged and baseline-corrected.

2.5. Calculation of coupling density

One of the factors varied in this work was the coupling density of the target molecules (alkylamines) on the particle surface. The coupling density CD (%) can be calculated directly from the

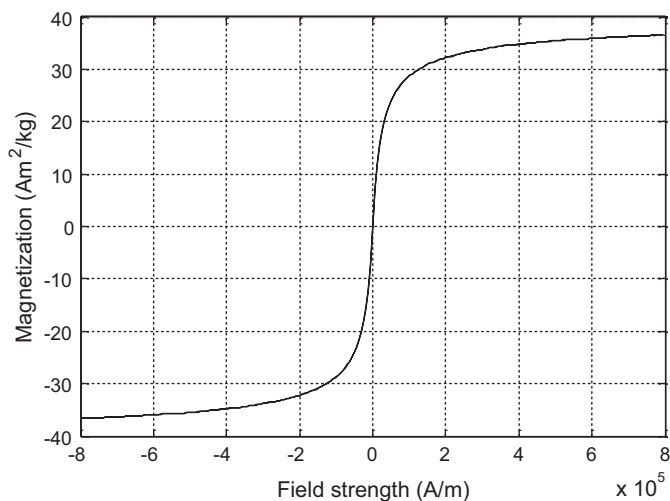


Fig. 1. Magnetization curve of magnetic micro-particles (M-PVA C22, chemagen) recorded using an alternating gradient magnetometer. The particles show nearly superparamagnetic behavior, i.e., very low remanence ($<0.5\ \text{Am}^2/\text{kg}$).

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