Aqueous Multiphase Systems of Polymers and Surfactants Provide Self-Assembling Step-Gradients in Density

Charles R. Mace,[†] Ozge Akbulut,[†] Ashok A. Kumar,[‡] Nathan D. Shapiro,[†] Ratmir Derda,[†] Matthew R. Patton,[†] and George M. Whitesides*,[†,§]

[†]Department of Chemistry & Chemical Biology, [‡]School of Engineering and Applied Sciences, and [§]Wyss Institute for Biologically Inspired Engineering, Harvard University, Cambridge, Massachusetts 02138, United States

S *Supporting Information*

ABSTRACT: This Communication demonstrates the generation of over 300 phase-separated systems—ranging from two to six phases—from mixtures of aqueous solutions of polymers and surfactants. These aqueous multiphase systems (MuPSs) form self-assembling, thermodynamically stable step-gradients in density using a common solvent, water. The steps in density between phases of a MuPS can be very small ($\Delta\rho \approx 0.001$ g/cm^3), do not change over time, and can be tuned by the addition of co-solutes. We use two sets of similar objects, glass beads and pellets of different formulations of Nylon, to demonstrate the ability of MuPSs to separate mixtures of objects by differences in density. The stable interfaces between phases facilitate the convenient collection of species after separation. These results suggest that the stable, sharp step-gradients in density provided by MuPSs can enable new classes of fractionations and separations based on density.

All matter has density, and density can be used to differentiate sub-populations of complex mixtures. Separations based on differences in density have applications in fields as varied as archeology (e.g., fossils),[1] forensics (e.g., trace materials transferred by contact),[2] and hematology (e.g., cells in blood).[3] Two sub-populations of different densities in a mixture can be separated by a liquid medium that provides a step in density (e.g., a sink/float assay). Separating multiple sub-populations based on density may require multiple liquids and steps. One method to prepare fluid systems having multiple layers with different densities is to overlay miscible solutions of a solute in a common solvent (e.g., sucrose or polysucrose in water) in increments of increasing concentration.[4] This approach is not ideal because the profiles of density that are produced by this method are not sharp and change as the components diffuse. Self-assembling, thermodynamically stable, molecularly sharp step-gradients in density would offer new approaches to separations based on density.

When mixed, many aqueous solutions of polymers, surfactants, and salts form insoluble phases. Common examples of such two-component mixtures (aqueous two-phase systems, ATPSs) are solutions of poly(ethylene glycol) (PEG) and Ficoll (a polysucrose),[5] dextran (a polyglucose) and Ficoll,[6] or PEG and Triton (a surfactant).[7] The two phases of those ATPSs order spontaneously according to density and provide three stable interfaces—air/phase I, phase I/phase II, phase II/container—for the collection of species based on density. The interfacial surface energy between the phases of an ATPS is astonishingly low (100 nJ/m^2–100 μJ/m^2)[8] due to the use of water as the common solvent for the system. A low interfacial surface energy minimizes the effect of the interface on the equilibrium position of an object within an ATPS (Supporting Information (SI)). Furthermore, using water as the common solvent in phase-separated systems enables the investigation of biological mixtures.[9]

Systems that generate more than two phases in a common solvent would provide additional interfaces and enable the collection of more than three populations that differ in density. Step-gradients in density produced by phase separation are superior to systems based on concentration gradients: (i) They have distinct (sharp on the molecular scale) steps in density at phase boundaries. (ii) The composition and properties of the phase-separated layers do not change with time. (iii) The difference in densities between adjacent phases can be very small ($\Delta\rho \approx 0.001$ g/cm^3). (iv) They can be prepared well in advance of their use and stored until needed. (v) They re-form spontaneously if perturbed or agitated. Stable, sharp step-gradients in density thus provide the basis for new types of fractionations, classifications, and separations.

This Communication demonstrates the use of immiscible mixtures of aqueous solutions of polymers and surfactants to generate multiple thermodynamically stable steps in density through phase separation. We describe a library of 311 new aqueous multiphase systems (MuPSs), ranging from two to six phases. These systems can be used to generate self-assembling step-gradients in density. Each phase of a MuPS contains multiple solutes: the immiscible phases of a MuPS are not solutions of single components but mixtures of components, and the net composition of each phase determines its density. The densities of the phases of MuPSs can also be adjusted using water-soluble additives (e.g., salts) and water-miscible solvents (e.g., D$_2$O). We demonstrate the use of MuPSs in density-based applications using two model systems: a mixture of six glass beads in a five-phase system, and a mixture of four different formulations of Nylon in a three-phase system.

We investigated 23 polymers and 11 surfactants for their ability to induce phase separation in aqueous solutions (Table S1). The criteria for selection were (i) commercial availability

(to make them conveniently available to users), (ii) low cost (< $2/g; we required multigram quantities to complete preliminary miscibility assays), (iii) solubility in water at pH ~7 (to limit the number of parameters for this initial screen), and (iv) chemical diversity (to provide a range of potentially useful functionality). Several types of polymers and surfactants did not satisfy our criteria for selection, and we did not include them in our screen for MuPSs. For instance, we did not include polypeptides (high cost) or linear polyethyleneimine (PEI; low solubility in water ~pH 7). Branched PEI, however, is more soluble and was included in the screen. We focused on compositional diversity. We did not investigate the effect of temperature on phase separation nor systematically examine the influence of molecular weight or branching of the polymers on the properties of the MuPSs; others have studied these effects in the context of ATPSs.[5,6,10]

The densities of the phases of MuPSs can be adjusted by changing the concentrations of the polymers or the surfactants that are used to generate them.[6] These densities can be tuned further by incorporating co-solutes into MuPSs (here, D_2O and CsBr). We chose to investigate D_2O instead of other water-miscible solvents (e.g., ethanol) because D_2O—as an isotope of water—does not affect the solubility of polymers or surfactants (SI). It provides a maximum 11% increase in the density of an aqueous solution and is relatively inexpensive for solutions that are not highly enriched isotopically (<$0.35/mL for a solution with 70% D_2O enrichment). We used CsBr as an additive to increase the densities of the phases of MuPSs. We selected this salt for five reasons: (i) Polymers with coordinating functional groups (e.g., poly(methacrylic acid); PMAA) may chelate multivalent cations and precipitate; they do not, however, chelate monovalent cations like Cs^+. (ii) CsBr is commercially available and inexpensive. (iii) Solutions of CsBr span a range of densities that is useful for biological separations (the density of a 1 M CsBr solution is 1.161 g/cm^3). (iv) CsBr is very soluble in water at room temperature (>5 M). (v) Solutions of CsBr at high concentrations are transparent at visible wavelengths.

We prepared every two-component combination of the 34 polymers and surfactants we selected for this screen (561 mixtures in total; Figure 1). For each combination, we thoroughly mixed equal volumes of two selected stock solutions of a polymer or a surfactant by vortexing for 30 s to allow the components of each phase to reach their equilibrium concentrations. Mixtures of immiscible solutions form emulsions and thus appear turbid. If these emulsions are left to equilibrate under ambient conditions, separation of the phases into distinct layers occurs over the course of minutes to days; the time required depends on the differences in density and viscosity of the phases ($\Delta\rho \approx 0.001-0.100$ g/cm^3; $\Delta\eta \approx 1-1000$ cP). To accelerate phase separation, we subjected the emulsions to centrifugation: typically, complete separation is observed after <5 min at 2000*g*.

A visually discernible interface characterized those two-component mixtures that separated into discrete phases (i.e., formed an ATPS); miscible mixtures produced homogeneous solutions. A number of mixtures formed either gels or precipitates; these systems are not immediately useful in density-based applications, and we did not consider them further. We confirmed that phase separation occurs for a number of previously characterized ATPSs and identified 91 previously unreported systems (Table S2). These new ATPSs
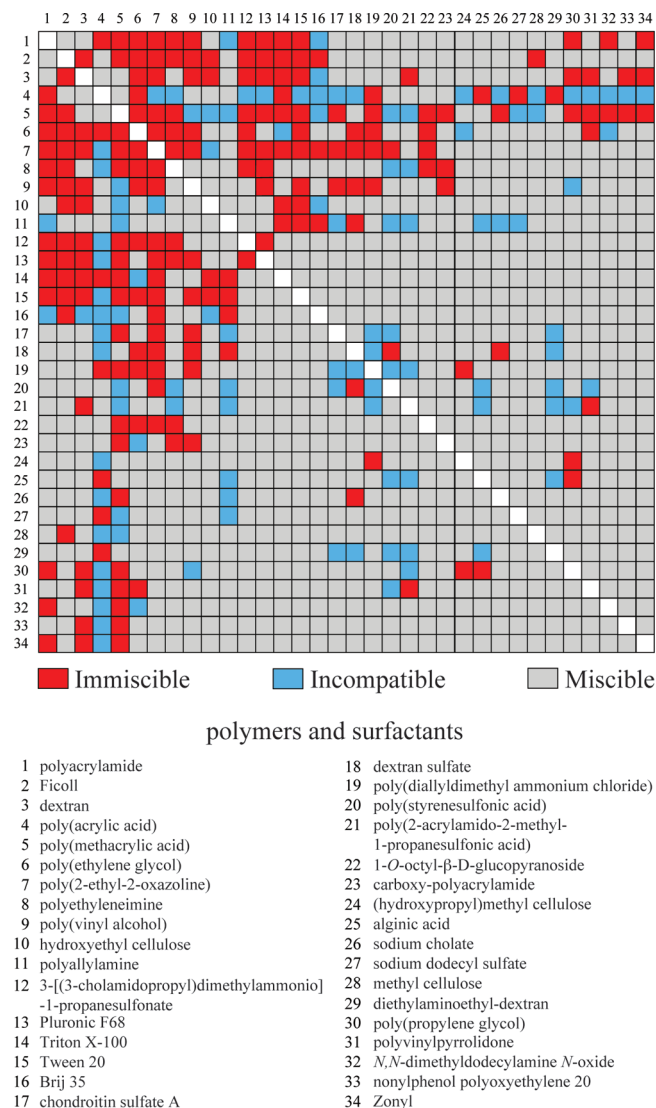

Figure 1. Phase separation in two-component mixtures of aqueous solutions of polymers and surfactants. Two-component mixtures of 23 aqueous polymer solutions and 11 aqueous surfactant solutions (561 mixtures in total) produced three outcomes: no phase separation (*miscible*, gray), formation of a precipitate or gel (*incompatible*, blue), or phase separation (*immiscible*, red).

Legend: ■ Immiscible ■ Incompatible ■ Miscible

polymers and surfactants

1 polyacrylamide	18 dextran sulfate
2 Ficoll	19 poly(diallyldimethyl ammonium chloride)
3 dextran	20 poly(styrenesulfonic acid)
4 poly(acrylic acid)	21 poly(2-acrylamido-2-methyl-
5 poly(methacrylic acid)	1-propanesulfonic acid)
6 poly(ethylene glycol)	22 1-*O*-octyl-β-D-glucopyranoside
7 poly(2-ethyl-2-oxazoline)	23 carboxy-polyacrylamide
8 polyethyleneimine	24 (hydroxypropyl)methyl cellulose
9 poly(vinyl alcohol)	25 alginic acid
10 hydroxyethyl cellulose	26 sodium cholate
11 polyallylamine	27 sodium dodecyl sulfate
12 3-[(3-cholamidopropyl)dimethylammonio]	28 methyl cellulose
-1-propanesulfonate	29 diethylaminoethyl-dextran
13 Pluronic F68	30 poly(propylene glycol)
14 Triton X-100	31 polyvinylpyrrolidone
15 Tween 20	32 *N,N*-dimethyldodecylamine *N*-oxide
16 Brij 35	33 nonylphenol polyoxyethylene 20
17 chondroitin sulfate A	34 Zonyl

include systems produced from mixtures of two polymers, one polymer and one surfactant, or two surfactants.

We wrote an algorithm using Matlab (SI) that used the results of the two-component screen to predict the multi-component mixtures that would produce multiple phases. For example, if mixtures of solutes A/B, B/C, and A/C each result in two-phase systems, then we expected a mixture of solutes A/B/C to produce a three-phase system.[6] Using this approach, we predicted the existence of MuPSs with as many as six phases from combinations of solutes used in the two-component miscibility screen. Based on the ATPSs listed in Table S2, the algorithm predicted 247 systems with three or more phases. We were able to prepare 221 of these systems, included five six-phase MuPSs (details in Tables S3–S6). Prior to this study, only one of the three-phase systems that we found had been described in the literature.[11] Including new ATPSs identified from our two-component screen, we have discovered 311

mixtures of aqueous solutions of polymers or surfactants that result in phase separation.

Each phase of an ATPS contains water and some amount of both solutes used to form the ATPS.[8] We expected each phase of a MuPS would contain a mixture of solutes as well, and the evaluation of a five-phase MuPS by ^{1}H NMR spectroscopy confirmed this hypothesis (SI). Each phase of a MuPS can be described by the solute that exists at the highest concentration in the phase (e.g., a PMAA-rich phase).

To demonstrate the use of MuPSs in density-based applications, we added a group of six density standard beads to an emulsion of an aqueous solution containing 6.4% (w/v) PEG, 5.6% (w/v) poly(2-ethyl-2-oxazoline) (PEOZ), 9.0% (w/v) poly(vinyl alcohol) (PVA), 4.8% (w/v) dextran, and 6.4% (w/v) Ficoll (Table S8). This emulsion behaved as a sink/float medium for the added density standard beads: three beads floated and three beads sank (Figure 2). Upon centrifugation,
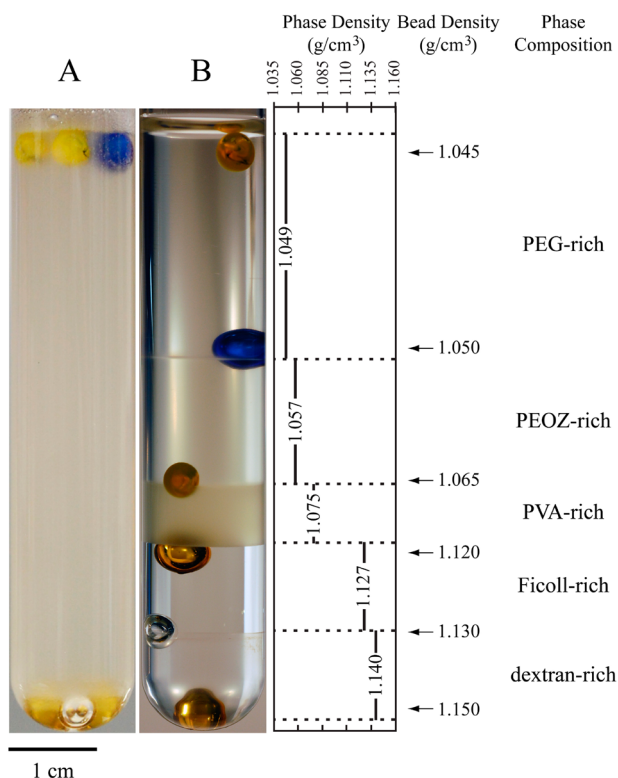

Figure 2. Preparation and demonstration of MuPSs in density-based separations. We prepared a five-phase MuPS by mixing aqueous solutions of PEG, PEOZ, PVA, dextran, and Ficoll. We added a set of six density standard beads to this mixture: three beads floated and three beads sank in the emulsified aqueous medium (A). The mixture of polymers separated into five phases upon centrifugation, and each bead sedimented under the applied centrifugal field until it reached an interface whose densities straddled the density of the bead (B).

this mixture produced a five-phase MuPS, and the beads sedimented to positions based on their densities: one at each of the four interfaces between the five phases, one at the interface between air and the top phase, and one at the interface between the bottom phase and the container. The position of the center of mass of the bead at an interface gives additional information about its density (SI).

We reproduced this separation using an identical MuPS that we prepared prior to the addition of beads rather than *in situ*.

After 12 months at room temperature, the step-gradient in density produced by this MuPS remained unchanged, and we were able to reproduce the bead separation experiments successfully after this prolonged storage period. This stability is, of course, entirely different from that observed in layered solutions of sucrose of different concentrations.

Co-solutes and co-solvents can be added to MuPSs to tune the densities of the phases. We prepared a series of two-phase MuPSs that contained 10% (w/v) PEOZ and 15% (w/v) PEG in aqueous solutions that contained H_2O/D_2O ranging from 100% H_2O to 70% D_2O/30% H_2O. Each MuPS had identical polymer compositions and differed only in the total concentration of D_2O. After phase separation by centrifugation, we measured the densities of isolated samples of each phase (Figure 3A). The relationship between the concentration of
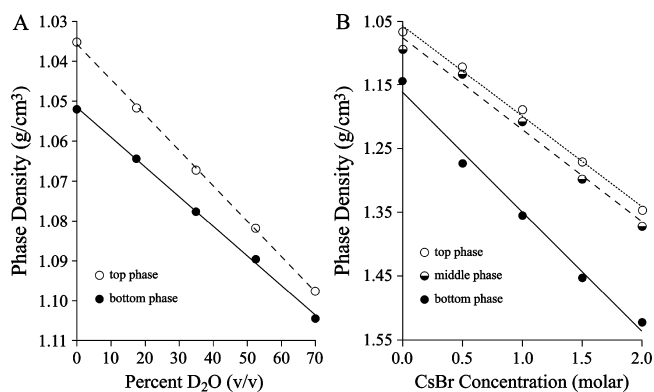

Figure 3. Tuning the density of the phases of MuPSs with additives. A two-phase MuPS was prepared from mixtures of PEG and PEOZ in aqueous solutions with a range of isotopic purity (0−70% D_2O by volume) (A). A three-phase MuPS was prepared from mixtures of PEG, PEI, and PMAA in aqueous solutions that included CsBr over a range of concentrations of salt (B). In both cases, the densities of the phases increased linearly with the concentration of the additive.

D_2O added to the mixture of polymers and the density of each phase was linear ($R^2 > 0.998$), but the slopes of the linear fit to each set of data were not equal: 9×10^{-4} and 7×10^{-4} g/cm³/%D_2O for the top and bottom phases, respectively. These results are consistent with the observation that the concentrations of water differ between phases of ATPSs.[6] The phase with lower water content will incorporate less *total* D_2O at equal concentrations, and the density of this phase will thus increase at a lower rate compared to the density of a phase with higher water content (SI).

We also examined the addition of water-soluble salts as a method to increase the densities of the phases of a MuPS. We prepared a series of three-phase MuPSs that contained 11.7% (w/v) PEG, 8.3% (w/v) PEI, and 10.7% (w/v) PMAA and included up to 2.0 M CsBr. Although CsBr is soluble in water at concentrations >5.0 M, the presence of high concentrations of polymers in solution lowered the solubility of the salt to ~2.0 M. The relationship between the concentration of salt added to the MuPSs and the density of each phase was linear, but the slopes were not equal (Figure 3B). Using LiBr instead of CsBr produced a similar result but with lower densities at equal concentrations of salt (Figure S3).

For the three-phase system with added CsBr, we used ^{1}H NMR to quantify the concentrations of water and polymer in each phase and to calculate the amount of salt in each phase (Figure S4). As expected, CsBr is not distributed equally among

the three phases; the phase to which CsBr partitions preferentially—in this case, the bottom phase—increases in density more than the other phases.

The ability to tune the densities of the phases of MuPSs using a number of methods—changing the combinations of solutes, changing the concentrations of solutes, or adding co-solutes—provides options to identify compositions of systems that are useful for separations. For example, we prepared a three-phase MuPS from a mixture of 10% (w/v) Ficoll, 13.3% (w/v) PEOZ, and 10% (v/v) Brij 35 and tuned the density of this system by adding 25.5% (v/v) D_2O. Following centrifugation, this system separated four different formulations of Nylon—Nylon 12 ($\rho = 1.032$ g/cm^3), Nylon 6 ($\rho = 1.057$ g/cm^3), Nylon 6/66 ($\rho = 1.120$ g/cm^3), and Nylon 6/12 ($\rho = 1.147$ g/cm^3)—by differences in their densities (Figure 4). In
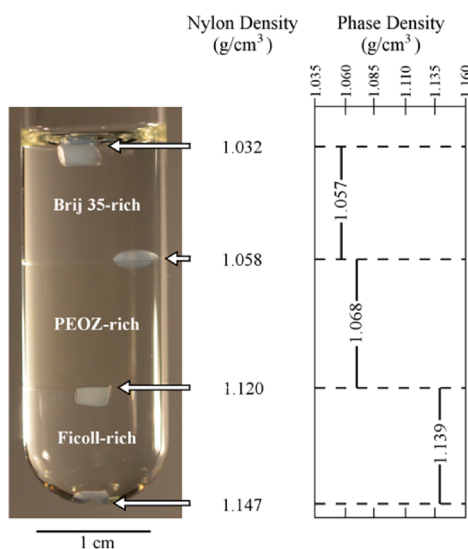

Figure 4. Separation of Nylon beads based on density using a MuPS. We prepared a three-phase MuPS from a mixture of aqueous solutions of Ficoll, PEOZ, and Brij 35. The water used to prepare this MuPS contained ~25.5% (v/v) D_2O. We used this MuPS to separate four formulations of Nylon based on density.

the analogous three-phase system containing 100% H_2O, however, the separation of Nylon species was incomplete: two Nylon beads (Nylon 6 and Nylon 6/66) were captured at the same interface between the middle and the bottom phases. The addition of D_2O to the MuPS increased the densities of the phases to appropriate values for separation of the Nylon species (Figure S6).

Density, as a common property of all matter, often serves as a convenient and efficient marker for the differentiation and separation of species from mixtures. MuPSs provide a means to produce self-assembling, thermodynamically stable step-gradients in density; these systems should find use in separations and analyses based on density. MuPSs can be prepared by mixing immiscible aqueous solutions of polymers and surfactants.

The number of immiscible components used to produce the system determines the final number of phases and, therefore, the number of interfaces: a MuPS with N phases has $N+1$ interfaces, and each interface is capable of isolating species based on density. Herein, we describe a library of over 300 MuPSs that range from two to six phases and demonstrate that the steps in density produced by MuPSs can be tuned by

incorporating water-miscible additives (e.g., co-solvents or co-solutes).

Unlike the step-gradients in density produced by layered media, the interfaces between the layers of a MuPS are sharp on a molecular scale and stable. Species collected at sharp interfaces are easier to visualize, separate, and isolate. MuPSs enable the creation of small steps in density ($\Delta\rho \approx 0.001$ g/cm^3) that remain stable because the composition and properties of the phase-separated layers do not change with time. This characteristic makes MuPSs useful when the storage and long-term stability of the separation media are important. Although multi-solvent systems can also produce stable, immiscible phases, only MuPSs can be used to separate objects that are compatible with a single solvent. Specifically, the use of water as the common solvent should enable the separation and analysis of multiple sub-populations from complex biological mixtures (e.g., cells from whole blood) based on density.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Detailed materials and methods, description of ordering of solutes in two-component mixtures, description of algorithm to predict MuPSs, tables of MuPSs, and quantification of ^1H NMR experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

gwhitesides@gmwgroup.harvard.edu

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

■ REFERENCES

(1) Munsternan, D.; Kerstholt, S. *Rev. Palaeobot. Palyno.* **1996**, *91*, 417.

(2) Petraco, N.; Kubic, T. *J. Forensic Sci.* **2000**, *45*, 872.

(3) English, D.; Andersen, B. R. *J. Immunol. Methods* **1974**, *5*, 249.

(4) Glinski, W.; Gershwin, M. E.; Budman, D. R.; Steinberg, A. D. *Clin. Exp. Immunol.* **1976**, *26*, 228.

(5) Albertsson, P.-Å. *Biochim. Biophys. Acta* **1958**, *27*, 378.

(6) Albertsson, P.-Å. *Partition of Cell Particles and Macromolecules*; Wiley Interscience: New York, 1986.

(7) Yamazaki, M.; Ohsika, M.; Ito, T. *Biochim. Biophys. Acta* **1991**, *1063*, 175.

(8) Hatti-Kaul, R. *Mol. Biotechnol.* **2001**, *19*, 269.

(9) (a) Fisher, D. *Biochem. J.* **1981**, *196*, 1. (b) Lutwyche, P.; Norris-Jones, R.; Brooks, D. E. *Appl. Environ. Microb.* **1995**, *61*, 3251. (c) Kamei, D. T.; Liu, C.-L.; Haase-Pettingell, C.; King, J. A.; Wang, D. I. C.; Blankschtein, D. *Biotechnol. Bioeng.* **2002**, *78*, 190. (d) Frampton, J. P.; Lai, D.; Sriram, H.; Takayama, S. *Biomed. Microdevices* **2011**, *13*, 1043.

(10) (a) Albertsson, P.-Å.; Cajarville, A.; Brooks, D. E.; Tjerneld, F. *Biochim. Biophys. Acta* **1987**, *926*, 87. (b) Johansson, H.; Karlström, G.; Tjerneld, F.; Haynes, C. *J. Chromatogr. B* **1998**, *711*, 3.

(11) (a) Albertsson, P.-Å. *Biochemistry* **1973**, *12*, 2525. (b) Ruan, K.; Wang, B.-H.; Xiao, J.-X.; Tan, J.-N. *J. Disper. Sci. Technol.* **2006**, *27*, 927.