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

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Materials and Methods

Materials. The following chemicals were purchased from Sigma-Aldrich: alginic acid sodium salt, chondroitin sulfate A, dextran sulfate sodium salt, Ficoll, (hydroxypropyl)methyl cellulose, poly(2-acrylamido-2-methyl-1-propanesulfonic acid), poly(2-ethyl-2-oxazoline), polyacrylamide, poly(diallyldimethylammonium chloride), poly(ethylene glycol), polyethyleneimine, poly(methacrylic acid sodium salt), poly(propylene glycol), polyvinylpyrrolidone, Brij 35, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), cetyl trimethylammonium bromide (CTAB), Pluronic F68, sodium chloate, Tween 20, Triton X-100, Zonyl, lithium bromide (LiBr), cesium bromide (CsBr) and benzene-1,2-disulfonic acid dipotassium salt. The following chemicals were purchased from Polysciences: poly(acrylic acid), poly(allylamine hydrochloride), poly(styrenesulfonic acid sodium salt), and poly(vinyl alcohol). Dextran was purchased from Spectrum Chemical. Diethylaminoethyl-dextran hydrochloride was purchased from MP Biomedicals. Carboxy-modified polyacrylamide, hydroxyethyl cellulose, methyl cellulose were purchased from Scientific Polymer Products. N-octyl-β-D-glucopyranoside was purchased from Calbiochem. N,N-dimethyldodecylamine N-oxide was purchased from Fluka. Sodium dodecyl sulfate was purchased from J.T. Baker. All polymers were used without further purification.

We purchased a series of glass density standard floats ("beads") from American Density Materials, the densities of which spanned 1.0400 g/cm^3 to 1.1500 g/cm^3. The vendor certified that the density of each bead was calibrated to ± 0.0002 g/cm^3 at 23 °C. We purchased pellets of four different formulations of nylon (i.e., Nylon 6, Nylon 12, Nylon 6/12, and Nylon 6/66) from Sigma Aldrich. The pellets were irregularly shaped with dimensions on the order of 2–5 mm. The densities of the Nylon formulations were determined by magnetic levitation (*vida infra*).

Preparation and Characterization of Stock Solutions. We prepared stock solutions of polymers in Milli-Q water (pH 5.5) at high concentrations, between 1–50% (w/v) or 1–50% (v/v) without adding salts or titrating the pH. We characterized the density of each stock solution (Table S1) using an oscillating U-tube densitometer (Anton Paar DM35N). Each measurement requires approximately 800 µL. The viscosities of the stock solutions of some polymers were too viscous to be analyzed directly by the densitometer (e.g., polyacrylamide and poly(2-acrylamido-2-methyl-1-propanesulfonic acid)). We calculated these densities by measuring the densities of three lower concentrations and determining the linear fit to the data.

Experimental Details

Phase Separation in Two-Component Mixtures of Aqueous Solutions of Polymers and Surfactants. To perform the initial two-component immiscibility screens, we added equal volumes (150 µL) of each polymer or surfactant solution in Table S1 into a microcentrifuge tube, we vortexed the tube for 30 seconds to thoroughly mix the solutions, and we accelerated phase-separation by centrifugation at 2000g for five minutes in a VWR Galaxy Mini centrifuge. In some cases, although each experiment began with equal volumes of stock solutions, we observed that the final aqueous two-phase system (ATPS) was characterized by phases with unequal volumes. This is a well-understood phenomenon that results when the initial concentrations of the system are near the node of the tie-line that characterizes the phase diagram of the system.[1] From our investigation of 34 polymers and surfactants, we identified a number of unreported ATPSs and confirmed those that have been previously described by others (Table S2).

Table S1. Properties of the aqueous stock solutions of polymers and surfactants: average molecular weight (Da), concentration (% w/v or % v/v), and density (g/cm^3). An asterisk (*) refers to densities that were calculated, rather than measured.

	polymer or surfactant	avg. MW (Da)	concentration	density (g/cm^3)
1	polyacrylamide	10,000	40% (w/v)	* 1.149
2	Ficoll	400,000	40% (w/v)	1.130
3	dextran	500,000	30% (w/v)	1.101
4	poly(acrylic acid)	450,000	10% (w/v)	1.035
5	poly(methacrylic acid)	5,000	40% (w/v)	1.279
6	poly(ethylene glycol)	20,000	40% (w/v)	1.069
7	poly(2-ethyl-2-oxazoline)	200,000	35% (w/v)	1.059
8	polyethyleneimine	25,000	30% (w/v)	1.037
9	poly(vinyl alcohol)	3,000	10% (w/v)	1.022
10	hydroxyethyl cellulose	90,000	2% (w/v)	1.004
11	polyallylamine	60,000	20% (w/v)	1.052
12	CHAPS	614	25% (w/v)	1.042
13	Pluronic F68	8,400	44% (w/v)	1.049
14	Triton X-100	625	20% (w/v)	1.017
15	Tween 20	1,228	45% (v/v)	1.067
16	Brij 35	1,198	30% (v/v)	1.025
17	chondroitin sulfate A	25,000	10% (w/v)	1.044
18	dextran sulfate	500,000	20% (w/v)	1.103
19	poly(diallyldimethylammonium chloride)	400,000	20% (w/v)	1.044
20	poly(styrenesulfonic acid)	75,000	30% (w/v)	1.100
21	poly(2-acrylamido-2-methyl-1-propanesulfonic acid)	2,000,000	15% (w/v)	* 1.042
22	1-*O*-Octyl-β-D-glucopyranoside	292	10% (w/v)	1.011
23	carboxy-polyacrylamide	200,000	6% (w/v)	1.018
24	(hydroxypropyl)methyl cellulose	10,000	2% (w/v)	1.003
25	alginic acid	240,000	2% (w/v)	1.010
26	sodium cholate	408	43% (w/v)	0.997
27	sodium dodecylsulfate	288	35% (w/v)	0.998
28	methyl cellulose	86,000	1% (w/v)	1.001
29	diethylaminoethyl-dextran	500,000	10% (w/v)	1.028
30	poly(propylene glycol)	425	40% (w/v)	1.029
31	polyvinylpyrrolidone	55,000	20% (w/v)	1.038
32	*N,N*-dimethyldodecylamine *N*-oxide	229	24% (w/v)	1.123
33	nonylphenol polyoxyethylene (20)	13,420	40% (v/v)	1.035
34	Zonyl	N/A	50% (v/v)	1.037

Table S2. List of new and previously reported ATPS found by this study.

	Previously Reported ATPS		REF
1	dextran	Ficoll	2
6	dextran	poly(ethylene glycol)	3
3	dextran	poly(propylene glycol)	3
7	dextran	poly(vinyl alcohol)	3
2	dextran	polyvinylpyrrolidone	3
4	dextran	Triton X-100	4
5	dextran	Tween 20	4
8	dextran sulfate	poly(ethylene glycol)	2
9	dextran sulfate	poly(styrene sulfonic acid)	5
10	Ficoll	poly(ethylene glycol)	2
11	hydroxyethyl cellulose	Triton X-100	6
12	poly(acrylic acid)	poly(ethylene glycol)	7
15	poly(ethylene glycol)	poly(vinyl alcohol)	3
13	poly(ethylene glycol)	polyvinylpyrrolidone	3
14	poly(ethylene glycol)	Tween 20	4

	New ATPS	
16	1-O-octyl-β-D-glucopyranoside	poly(2-ethyl-2-oxazoline)
17	1-O-octyl-β-D-glucopyranoside	poly(ethylene glycol)
18	1-O-octyl-β-D-glucopyranoside	poly(methacrylic acid)
19	1-O-octyl-β-D-glucopyranoside	polyethyleneimine
20	alginic acid	poly(acrylic acid)
21	alginic acid	poly(propylene glycol)
22	Brij 35	Ficoll
23	Brij 35	poly(2-ethyl-2-oxazoline)
24	Brij 35	polyallylamine
25	carboxy-polyacrylamide	poly(methacrylic acid)
26	carboxy-polyacrylamide	poly(vinyl alcohol)
27	carboxy-polyacrylamide	polyethyleneimine
28	CHAPS	dextran
29	CHAPS	Ficoll
30	CHAPS	Pluronic F68
31	CHAPS	poly(2-ethyl-2-oxazoline)
32	CHAPS	poly(ethylene glycol)
33	CHAPS	poly(methacrylic acid)
34	CHAPS	polyacrylamide
35	CHAPS	polyethyleneimine
36	chondroitin sulfate A	poly(2-ethyl-2-oxazoline)
37	chondroitin sulfate A	poly(methacrylic acid)
38	chondroitin sulfate A	poly(vinyl alcohol)
39	dextran	hydroxyethyl cellulose
40	dextran	nonylphenol polyoxyethylene (20)
41	dextran	Pluronic F68
42	dextran	poly(2-ethyl-2-oxazoline)
43	dextran	poly(2-acrylamido-2-methyl-1-propanesulfonic acid)
44	dextran	Zonyl
45	dextran sulfate	poly(2-ethyl-2-oxazoline)
46	dextran sulfate	poly(vinyl alcohol)

Table S2, cont.

		New ATPS
47	dextran sulfate	polyallylamine
48	dextran sulfate	sodium cholate
49	diethylaminoethyl-dextran	poly(acrylic acid)
50	Ficoll	methyl cellulose
51	Ficoll	Pluronic F68
52	Ficoll	poly(2-ethyl-2-oxazoline)
53	Ficoll	poly(methacrylic acid)
54	Ficoll	poly(vinyl alcohol)
55	Ficoll	polyethyleneimine
56	Ficoll	Triton X-100
57	Ficoll	Tween 20
58	hydroxyethyl cellulose	Ficoll
59	hydroxyethyl cellulose	Tween 20
60	(hydroxypropyl)methyl cellulose	poly(diallyldimethyl ammonium chloride
61	(hydroxypropyl)methyl cellulose	poly(propylene glycol)
62	*N,N*-dimethyldodecylamine *N*-oxide	poly(methacrylic acid)
63	*N,N*-dimethyldodecylamine *N*-oxide	polyacrylamide
64	nonylphenol polyoxyethylene (20)	poly(methacrylic acid)
65	Pluronic F68	poly(2-ethyl-2-oxazoline)
66	Pluronic F68	poly(methacrylic acid)
67	Pluronic F68	poly(vinyl alcohol)
68	Pluronic F68	polyacrylamide
69	Pluronic F68	polyethyleneimine
70	poly(2-acrylamido-2-methyl-1-propanesulfonic acid)	polyvinylpyrrolidone
71	poly(2-ethyl-2-oxazoline)	poly(diallyldimethyl ammonium chloride
72	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)
73	poly(2-ethyl-2-oxazoline)	poly(methacrylic acid)
74	poly(2-ethyl-2-oxazoline)	poly(styrene sulfonic acid)
75	poly(2-ethyl-2-oxazoline)	poly(vinyl alcohol)
76	poly(2-ethyl-2-oxazoline)	polyacrylamide
77	poly(2-ethyl-2-oxazoline)	polyethyleneimine
78	poly(2-ethyl-2-oxazoline)	Triton X-100
79	poly(2-ethyl-2-oxazoline)	Tween 20
80	poly(acrylic acid)	poly(diallyldimethyl ammonium chloride
81	poly(acrylic acid)	polyacrylamide
82	poly(acrylic acid)	sodium dodecyl sulfate
83	poly(acrylic acid)	Triton X-100
84	poly(diallyldimethyl ammonium chloride	poly(ethylene glycol)
85	poly(diallyldimethyl ammonium chloride	poly(methacrylic acid)
86	poly(diallyldimethyl ammonium chloride	poly(vinyl alcohol)
87	poly(ethylene glycol)	poly(methacrylic acid)
88	poly(ethylene glycol)	polyacrylamide
89	poly(ethylene glycol)	polyethyleneimine
90	poly(methacrylic acid)	poly(propylene glycol)
91	poly(methacrylic acid)	polyacrylamide
92	poly(methacrylic acid)	polyethyleneimine
93	poly(methacrylic acid)	polyvinylpyrrolidone
94	poly(methacrylic acid)	sodium cholate
95	poly(methacrylic acid)	Triton X-100

Table S2, cont.

	New ATPS	
96	poly(methacrylic acid)	Tween 20
97	poly(methacrylic acid)	Zonyl
98	poly(propylene glycol)	polyacrylamide
99	poly(vinyl alcohol)	Tween 20
100	polyacrylamide	poly(vinyl alcohol)
101	polyacrylamide	polyethyleneimine
102	polyacrylamide	Triton X-100
103	polyacrylamide	Tween 20
104	polyacrylamide	Zonyl
105	polyallylamine	Triton X-100
106	polyallylamine	Tween 20

There are several notable exceptions to our screen: we did not investigate kosmotropic salt solutions (e.g., ammonium sulfate) that are known to form ATPS with poly(ethylene glycol),[8] and we did not supplement the aqueous solutions with sodium chloride or other salts that are known to facilitate polymer–polymer phase separation.[9] Furthermore, we observed that several additional polymers were either only sparingly soluble or did not phase separate with the solutes listed above. These included xanthan gum, chitosan, gelatin, linear polyethyleneimine, poly(N-isopropylpolyacrylamide), and a fourth generation hyperbranched bis-MPA polyester-64-hydroxyl dendrimer. Sodium carboxymethyl cellulose and total Yeast RNA (as a polyphosphate source), while soluble, did not phase separate with any of the chemicals listed in Table S1.

Clustering of Two-Phase Systems. Flory-Huggins Theory has been used to describe phase separation in several aqueous two-phase systems.[10] The Flory interaction parameters are dependent on the specific polymers and solvent used. The relationship between key variables, such as temperature and molecular weight, is deeply embedded in the free energy equations; general intuition about which conditions lead to phase separation is difficult to establish without either rigorous computation over several parameters or many simplifying assumptions.[11]

While establishing a general set of solubility parameters for aqueous solutions is beyond the scope of this work, we suggest an ordering system that is both based on solubility and consistent within the set of chemicals that we include in this screen. We assigned a 34-component vector to each of the twenty-three polymers and eleven surfactants, which comprised arbitrary values that we used to characterize the result of each two-component mixture: '0' for mixtures that resulted in homogeneous solutions (miscible), '1' for mixtures that resulted in a precipitate or a gel (incompatible), and '2' for mixtures that resulted in phase separation (immiscible). For example, the matrix for poly(vinyl alcohol), M_{PVA}, and Brij 35, M_{B35}, are:
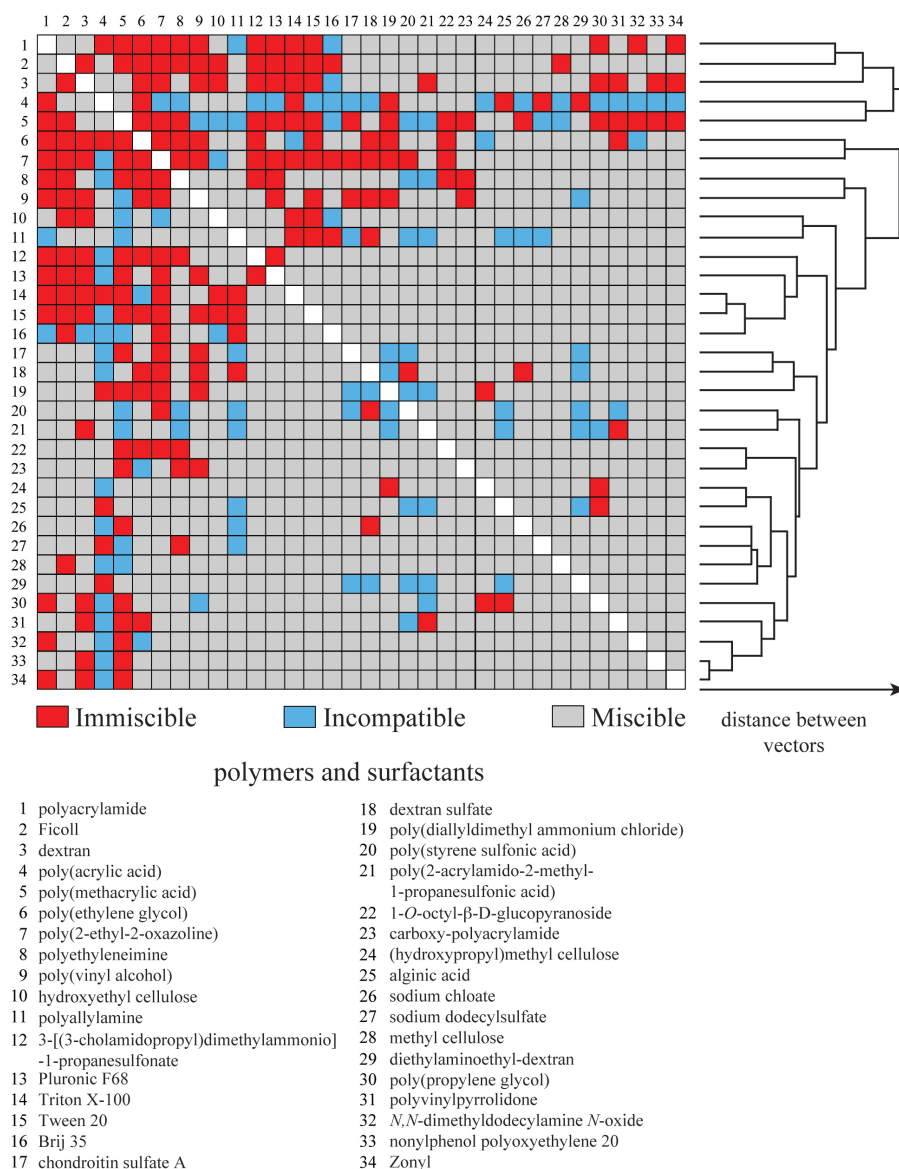
$$M_{PVA} = [1\ 0\ 0\ 2\ 2\ 2\ 2\ 2\ 2\ 2\ 2\ 0\ 0\ 1\ 0\ 0\ 0\ 0\ 0\ 2\ 0\ 0\ 0\ 2\ 0\ 0\ 0\ 2\ 0\ 0\ 0\ 0]$$

$$M_{B35} = [1\ 1\ 0\ 2\ 0\ 1\ 2\ 1\ 0\ 0\ 0\ 0\ 0\ 0\ 0\ 0\ 2\ 0\ 0\ 0\ 1\ 0\ 0\ 0\ 0\ 0\ 0\ 0\ 0\ 0\ 0\ 0]$$

In this 34-dimensional space, we can compare the miscibility profiles of clusters of polymers by analyzing the Euclidean distances between vectors. By this method, larger distances between vectors describe polymers that are likely to be components of phase-separated systems within this series, while shorter distances imply miscibility. We ordered the polymers in our ATPS matrix according to these vector distances (Figure S1). Using this approach to ordering, we identified several patterns based on similarities in miscibility in two-component mixtures: neutral polysaccharides (numbers *2* and *3*), acrylic acids (*4* and *5*), sulfated polysaccharides (*17* and *18*), sulfonic acids (*20* and *21*), and some non-fluorinated surfactants (numbers *12–16*) can be clustered by patterns of miscibility. Other species that might be expected to cluster, such as the cellulose derivatives (numbers *10, 24, 28*), have dissimilar patterns of miscibility.

Algorithm for the Prediction of Multiphase Systems. Albertsson observed, in many cases, that sets of mutually immiscible polymers that formed ATPS could be used to generate systems with more than two phases.[2] Applying this principle, we used our list of ATPS to generate a list of systems with more than two phases. For example, we predicted that a combination of solutes A, B and C would produce a three-phase system if two-component combinations of these solutes (i.e., [A B], [A C], and [B C]) phase separate. We developed an algorithm to generate a list of all possible systems of *N* phases given all possible systems of *N-1* phases. For example, we examine a set of component polymers A, B, C, and D. For each candidate multiphase system, there are *N* unique combinations of *N-1* components (i.e., for each

Figure S1. Results of two-component mixtures of aqueous solutions of polymers and surfactants: "miscible" (grey) corresponds to mixtures that produced no phase separation, "incompatible" (blue) corresponds to mixtures that generated a precipitate or a gel, and "immiscible" (red) corresponds to mixtures that resulted in phase separation between the two solutions. The polymers are ordered according to the distances between the vectors that describe their miscibility within this series, from top to bottom with decreasing distance (i.e., increasing similarity), and are illustrated by a miscibility similarity tree.

polymers and surfactants

1	polyacrylamide	18	dextran sulfate
2	Ficoll	19	poly(diallyldimethyl ammonium chloride)
3	dextran	20	poly(styrene sulfonic 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 chloate
11	polyallylamine	27	sodium dodecylsulfate
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

four-phase system there should be *four* three-phase systems).   [A B], [A C], and [B C] are the

combinations for the candidate [A B C], and [A B], [A D], and [B D] are the combinations for

the candidate [A B D]. If the *N* combinations formed a subset of the known systems of *N-1*

phases, the candidate system was labeled as a predicted system until all candidates were

evaluated and a complete list of predicted systems was formed. In this example, if combinations

[A B], [A C], [B C], and [A D] were observed experimentally, but [B D] was not observed, then,

[A B C] is a predicted system, and [A B D] is not a predicted system. We applied this algorithm

to our list of systems of two phases to predict multiphase systems. We then iteratively applied

the algorithm to the predicted multiphase systems until we could not generate a higher order

system. In this way, we predicted systems composed of up to six phases based on our initial

screen of two-component mixtures.

Aqueous Multiphase Systems of Polymers and Surfactants. We investigated each

multiphase system predicted by our algorithm systematically. We were able to generate 112 of

the 122 predicted three-phase MuPSs (Table S3), 73 of the 87 predicted four-phase MuPSs

(Table S4), 31 of the 33 predicted five-phase MuPSs (Table S5), and all 5 of the predicted six-

phase MuPSs (Table S6).

We added equal volumes (150 µL) of the solutions of each component (unless otherwise

noted) to a microcentrifuge tube, vortexed the mixture for 30 seconds, and accelerated phase

separation by centrifugation for 5 minutes at 16000*g* (VWR Galaxy 1816). For several predicted

MuPSs, we observed that phase separation only occurred following the increase of the

concentration of a polymer component used with respect to the other polymers (e.g., three-phase

system #36 required twice the volume of the hydroxyethyl cellulose solution (300 µL), which

increased the overall concentration of this solute in the MuPS mixture). Since phase separation

Table S3. List of prepared three-phase MuPSs. A reference accompanies the previously reported system that we confirmed. Components that were not added at equal volumes are noted by the factor of the increase (i.e., **[2X]** for twice the volume of each of the other components).

	Three-Phase MuPSs			**REF**
1	1-*O*-Octyl-β-D-glucopyranoside	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)	
2	1-*O*-Octyl-β-D-glucopyranoside	poly(2-ethyl-2-oxazoline)	poly(methacrylic acid)	
3	1-*O*-Octyl-β-D-glucopyranoside	poly(2-ethyl-2-oxazoline)	polyethyleneimine	
4	1-*O*-Octyl-β-D-glucopyranoside	poly(ethylene glycol)	poly(methacrylic acid)	
5	1-*O*-Octyl-β-D-glucopyranoside	poly(ethylene glycol)	polyethyleneimine	
6	1-*O*-Octyl-β-D-glucopyranoside	poly(methacrylic acid)	polyethyleneimine	
7	Brij 35	Ficoll	poly(2-ethyl-2-oxazoline)	
8	carboxy-polyacrylamide	poly(methacrylic acid)	polyethyleneimine	
9	CHAPS	dextran	Ficoll	
10	CHAPS	dextran	Pluronic F68	
11	CHAPS	dextran	poly(2-ethyl-2-oxazoline)	
12	CHAPS	dextran	poly(ethylene glycol)	
13	CHAPS	Ficoll	Pluronic F68	
14	CHAPS	Ficoll	poly(2-ethyl-2-oxazoline)	
15	CHAPS	Ficoll	poly(ethylene glycol)	
16	CHAPS	Ficoll	poly(methacrylic acid)	
17	CHAPS	Ficoll	polyethyleneimine	
18	CHAPS	Pluronic F68	poly(2-ethyl-2-oxazoline)	
19	CHAPS	Pluronic F68	poly(methacrylic acid)	
20	CHAPS	Pluronic F68	polyacrylamide	
21	CHAPS	Pluronic F68	polyethyleneimine	
22	CHAPS	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)	
23	CHAPS	poly(2-ethyl-2-oxazoline)	poly(methacrylic acid)	
24	CHAPS	poly(2-ethyl-2-oxazoline)	polyacrylamide	
25	CHAPS	poly(2-ethyl-2-oxazoline)	polyethyleneimine	
26	CHAPS	poly(ethylene glycol)	poly(methacrylic acid)	
27	CHAPS	poly(ethylene glycol)	polyacrylamide	
28	CHAPS	poly(ethylene glycol)	polyethyleneimine	
29	CHAPS	poly(methacrylic acid)	polyacrylamide	
30	CHAPS	poly(methacrylic acid)	polyethyleneimine	
31	CHAPS	polyacrylamide	polyethyleneimine	
32	chondroitin sulfate A	poly(2-ethyl-2-oxazoline)	poly(methacrylic acid)	
33	chondroitin sulfate A	poly(2-ethyl-2-oxazoline)	poly(vinyl alcohol)	
34	dextran sulfate	poly(2-ethyl-2-oxazoline)	poly(vinyl alcohol)	
35	dextran sulfate	poly(ethylene glycol)	poly(vinyl alcohol)	
36	dextran	Ficoll	hydroxyethyl cellulose **[2X]**	
37	dextran	Ficoll	Pluronic F68	
38	dextran	Ficoll	poly(2-ethyl-2-oxazoline)	
39	dextran	Ficoll	poly(ethylene glycol)	2
40	dextran	Ficoll	poly(vinyl alcohol)	
41	dextran	Ficoll	Triton X-100	
42	dextran	Ficoll	Tween 20	

Table S3, cont.

		Three-Phase MuPSs		REF
43	dextran	hydroxyethyl cellulose	Triton X-100	
44	dextran	hydroxyethyl cellulose	Tween 20	
45	dextran	Pluronic F68	poly(2-ethyl-2-oxazoline)	
46	dextran	Pluronic F68	poly(vinyl alcohol)	
47	dextran	poly(2-acrylamido-2-methyl-1-propanesulfonic acid) **[2X]**	polyvinylpyrrolidone	
48	dextran	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)	
49	dextran	poly(2-ethyl-2-oxazoline)	poly(vinyl alcohol)	
50	dextran	poly(2-ethyl-2-oxazoline)	Triton X-100	
51	dextran	poly(2-ethyl-2-oxazoline)	Tween 20	
52	dextran	poly(ethylene glycol)	poly(vinyl alcohol)	
53	dextran	poly(ethylene glycol)	polyvinylpyrrolidone	
54	dextran	poly(ethylene glycol)	Tween 20	
55	dextran	poly(vinyl alcohol)	Tween 20	
56	Ficoll	hydroxyethyl cellulose	Triton X-100	
57	Ficoll	hydroxyethyl cellulose	Tween 20	
58	Ficoll	Pluronic F68	poly(2-ethyl-2-oxazoline)	
59	Ficoll	Pluronic F68	poly(methacrylic acid)	
60	Ficoll	Pluronic F68	poly(vinyl alcohol)	
61	Ficoll	Pluronic F68	polyethyleneimine	
62	Ficoll	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)	
63	Ficoll	poly(2-ethyl-2-oxazoline)	poly(methacrylic acid)	
64	Ficoll	poly(2-ethyl-2-oxazoline)	poly(vinyl alcohol)	
65	Ficoll	poly(2-ethyl-2-oxazoline)	polyethyleneimine	
66	Ficoll	poly(2-ethyl-2-oxazoline)	Triton X-100	
67	Ficoll	poly(2-ethyl-2-oxazoline)	Tween 20	
68	Ficoll	poly(ethylene glycol)	poly(methacrylic acid)	
69	Ficoll	poly(ethylene glycol)	poly(vinyl alcohol)	
70	Ficoll	poly(ethylene glycol)	polyethyleneimine	
71	Ficoll	poly(ethylene glycol)	Tween 20	
72	Ficoll	poly(methacrylic acid)	polyethyleneimine	
73	Ficoll	poly(methacrylic acid)	Triton X-100	
74	Ficoll	poly(methacrylic acid)	Tween 20	
75	Ficoll	poly(vinyl alcohol)	Tween 20	
76	*N,N*-dimethyldodecylamine *N*-oxide	poly(methacrylic acid)	polyacrylamide	
77	Pluronic F68	poly(2-ethyl-2-oxazoline)	poly(methacrylic acid)	
78	Pluronic F68	poly(2-ethyl-2-oxazoline)	polyacrylamide	
79	Pluronic F68	poly(2-ethyl-2-oxazoline)	polyethyleneimine	
80	Pluronic F68	poly(methacrylic acid)	polyacrylamide	
81	Pluronic F68	poly(methacrylic acid)	polyethyleneimine	
82	Pluronic F68	poly(vinyl alcohol)	polyacrylamide	
83	Pluronic F68	polyacrylamide	polyethyleneimine	
84	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)	poly(methacrylic acid)	
85	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)	poly(vinyl alcohol)	
86	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)	polyacrylamide	
87	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)	polyethyleneimine **[2X]**	
88	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)	Tween 20	
89	poly(2-ethyl-2-oxazoline)	poly(methacrylic acid)	polyacrylamide	
90	poly(2-ethyl-2-oxazoline)	poly(methacrylic acid)	polyethyleneimine	

Table S3, cont.

	Three-Phase MuPSs			REF
91	poly(2-ethyl-2-oxazoline)	poly(methacrylic acid)	Tween 20	
92	poly(2-ethyl-2-oxazoline)	poly(vinyl alcohol)	Pluronic F68	
93	poly(2-ethyl-2-oxazoline)	poly(vinyl alcohol)	polyacrylamide	
94	poly(2-ethyl-2-oxazoline)	poly(vinyl alcohol)	Tween 20	
95	poly(2-ethyl-2-oxazoline)	polyacrylamide	polyethyleneimine	
96	poly(2-ethyl-2-oxazoline)	polyacrylamide	Triton X-100	
97	poly(2-ethyl-2-oxazoline)	polyacrylamide	Tween 20	
98	poly(acrylic acid) **[2X]**	poly(ethylene glycol)	polyacrylamide **[2X]**	
99	poly(ethylene glycol)	poly(methacrylic acid)	polyacrylamide	
100	poly(ethylene glycol)	poly(methacrylic acid)	polyethyleneimine	
101	poly(ethylene glycol)	poly(methacrylic acid)	polyvinylpyrrolidone	
102	poly(ethylene glycol)	poly(methacrylic acid)	Tween 20	
103	poly(ethylene glycol)	poly(vinyl alcohol)	polyacrylamide	
104	poly(ethylene glycol)	poly(vinyl alcohol)	Tween 20	
105	poly(ethylene glycol)	polyacrylamide	polyethyleneimine	
106	poly(ethylene glycol)	polyacrylamide	Tween 20	
107	poly(methacrylic acid)	poly(propylene glycol)	polyacrylamide	
108	poly(methacrylic acid)	polyacrylamide	polyethyleneimine	
109	poly(methacrylic acid)	polyacrylamide	Triton X-100	
110	poly(methacrylic acid)	polyacrylamide	Tween 20	
111	poly(methacrylic acid)	polyacrylamide	Zonyl	
112	poly(vinyl alcohol)	polyacrylamide	Tween 20	

Table S4. List of prepared four-phase MuPSs. Components that were not added at equal volumes are noted by the factor of the increase (i.e., **[2X]** for twice the volume of each of the other components).

	Four-Phase MuPSs			
1	1-*O*-Octyl-β-D-glucopyranoside	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)	polyethyleneimine
2	CHAPS	dextran	Ficoll	Pluronic F68
3	CHAPS **[2X]**	dextran	Ficoll	poly(ethylene glycol)
4	CHAPS	dextran	Pluronic F68	poly(2-ethyl-2-oxazoline)
5	CHAPS	dextran	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)
6	CHAPS	Ficoll	Pluronic F68	poly(methacrylic acid)
7	CHAPS	Ficoll	Pluronic F68	polyethyleneimine
8	CHAPS **[2X]**	Ficoll	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)
9	CHAPS	Ficoll	poly(2-ethyl-2-oxazoline)	poly(methacrylic acid)
10	CHAPS **[2X]**	Ficoll	poly(2-ethyl-2-oxazoline)	polyethyleneimine
11	CHAPS	Ficoll	poly(ethylene glycol)	poly(methacrylic acid)
12	CHAPS	Ficoll	poly(ethylene glycol)	polyethyleneimine
13	CHAPS	Ficoll	poly(methacrylic acid)	polyethyleneimine
14	CHAPS **[2X]**	Pluronic F68	poly(2-ethyl-2-oxazoline)	poly(methacrylic acid)
15	CHAPS	Pluronic F68	poly(2-ethyl-2-oxazoline)	polyethyleneimine
16	CHAPS	Pluronic F68	poly(methacrylic acid)	polyacrylamide
17	CHAPS	Pluronic F68	poly(methacrylic acid)	polyethyleneimine
18	CHAPS	Pluronic F68	polyacrylamide	polyethyleneimine
19	CHAPS **[2X]**	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)	poly(methacrylic acid)
20	CHAPS	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)	polyacrylamide
21	CHAPS	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)	polyethyleneimine
22	CHAPS	poly(2-ethyl-2-oxazoline)	poly(methacrylic acid)	polyacrylamide
23	CHAPS **[2X]**	poly(2-ethyl-2-oxazoline)	polyacrylamide	polyethyleneimine
24	CHAPS	poly(ethylene glycol)	poly(methacrylic acid)	polyacrylamide
25	CHAPS	poly(ethylene glycol)	poly(methacrylic acid)	polyethyleneimine
26	CHAPS	poly(ethylene glycol)	polyacrylamide	polyethyleneimine
27	CHAPS	poly(methacrylic acid)	polyacrylamide	polyethyleneimine
28	dextran	Ficoll	hydroxyethyl cellulose	Triton X-100
29	dextran	Ficoll	hydroxyethyl cellulose	Tween 20
30	dextran	Ficoll	Pluronic F68	poly(2-ethyl-2-oxazoline)
31	dextran	Ficoll	Pluronic F68	poly(vinyl alcohol)
32	dextran	Ficoll	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)
33	dextran	Ficoll	poly(2-ethyl-2-oxazoline)	poly(vinyl alcohol)
34	dextran	Ficoll	poly(2-ethyl-2-oxazoline)	Tween 20
35	dextran	Ficoll	poly(ethylene glycol)	poly(vinyl alcohol)
36	dextran	Ficoll	poly(ethylene glycol)	Tween 20
37	dextran	Ficoll	poly(vinyl alcohol)	Tween 20
38	dextran	Pluronic F68	poly(2-ethyl-2-oxazoline)	poly(vinyl alcohol)
39	dextran	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)	poly(vinyl alcohol)
40	dextran	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)	Tween 20
41	dextran	poly(ethylene glycol)	poly(vinyl alcohol)	Tween 20
42	dextran sulfate	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)	poly(vinyl alcohol)
43	Ficoll	Pluronic F68	poly(2-ethyl-2-oxazoline)	poly(methacrylic acid)
44	Ficoll	Pluronic F68	poly(2-ethyl-2-oxazoline)	poly(vinyl alcohol)
45	Ficoll	Pluronic F68	poly(2-ethyl-2-oxazoline)	polyethyleneimine
46	Ficoll	Pluronic F68	poly(methacrylic acid)	polyethyleneimine
47	Ficoll	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)	poly(methacrylic acid)

Table S4, cont.

		Four-Phase MuPSs		
48	Ficoll	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)	poly(vinyl alcohol)
49	Ficoll	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)	Tween 20
50	Ficoll	poly(2-ethyl-2-oxazoline)	poly(methacrylic acid)	polyethyleneimine
51	Ficoll	poly(2-ethyl-2-oxazoline)	poly(methacrylic acid)	Tween 20
52	Ficoll	poly(2-ethyl-2-oxazoline)	poly(vinyl alcohol)	Tween 20
53	Ficoll	poly(ethylene glycol)	poly(methacrylic acid)	polyethyleneimine
54	Ficoll	poly(ethylene glycol)	poly(methacrylic acid)	Tween 20
55	Ficoll	poly(ethylene glycol)	poly(vinyl alcohol)	Tween 20
56	Pluronic F68	poly(2-ethyl-2-oxazoline)	poly(methacrylic acid)	polyacrylamide
57	Pluronic F68 **[2X]**	poly(2-ethyl-2-oxazoline)	poly(methacrylic acid)	polyethyleneimine
58	Pluronic F68	poly(2-ethyl-2-oxazoline)	poly(vinyl alcohol)	polyacrylamide
59	Pluronic F68	poly(2-ethyl-2-oxazoline)	polyacrylamide	polyethyleneimine
60	Pluronic F68	poly(methacrylic acid)	polyacrylamide	polyethyleneimine
61	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)	poly(methacrylic acid)	polyacrylamide
62	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)	poly(methacrylic acid)	polyethyleneimine
63	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)	poly(methacrylic acid)	Tween 20
64	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)	poly(vinyl alcohol)	polyacrylamide
65	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)	polyacrylamide	polyethyleneimine
66	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)	polyacrylamide	Tween 20
67	poly(2-ethyl-2-oxazoline)	poly(methacrylic acid)	polyacrylamide	polyethyleneimine
68	poly(2-ethyl-2-oxazoline)	poly(methacrylic acid)	polyacrylamide	Triton X-100
69	poly(2-ethyl-2-oxazoline)	poly(methacrylic acid)	polyacrylamide	Tween 20
70	poly(2-ethyl-2-oxazoline)	poly(vinyl alcohol)	polyacrylamide	Tween 20
71	poly(ethylene glycol)	poly(methacrylic acid)	polyacrylamide	polyethyleneimine
72	poly(ethylene glycol)	poly(methacrylic acid)	polyacrylamide	Tween 20
73	poly(ethylene glycol)	poly(vinyl alcohol)	polyacrylamide	Tween 20

Table S5. List of prepared five-phase MuPSs. Components that were not added at equal volumes are noted by the factor of the increase (i.e., **[2X]** for twice the volume of each of the other components). A sixth phase formed in System 14 (noted with an *) after sitting for over two hours at ambient conditions.

Five-Phase MuPSs				
1	CHAPS	dextran	Ficoll	Pluronic F68	poly(2-ethyl-2-oxazoline)
2	CHAPS	dextran	Ficoll	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)
3	CHAPS	Ficoll	Pluronic F68	poly(2-ethyl-2-oxazoline)	poly(methacrylic acid)
4	CHAPS	Ficoll	Pluronic F68	poly(2-ethyl-2-oxazoline)	polyethyleneimine
5	CHAPS **[2X]**	Ficoll	Pluronic F68	poly(methacrylic acid)	polyethyleneimine
6	CHAPS	Ficoll	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)	poly(methacrylic acid)
7	CHAPS	Ficoll	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)	polyethyleneimine
8	CHAPS	Ficoll	poly(2-ethyl-2-oxazoline)	poly(methacrylic acid)	polyethyleneimine
9	CHAPS **[2X]**	Ficoll	poly(ethylene glycol)	poly(methacrylic acid)	polyethyleneimine
10	CHAPS	Pluronic F68	poly(2-ethyl-2-oxazoline)	poly(methacrylic acid)	polyacrylamide
11	CHAPS	Pluronic F68	poly(2-ethyl-2-oxazoline)	poly(methacrylic acid)	polyethyleneimine
12	CHAPS	Pluronic F68	poly(2-ethyl-2-oxazoline)	polyacrylamide	polyethyleneimine
13	CHAPS	Pluronic F68	poly(methacrylic acid)	polyacrylamide	polyethyleneimine
14*	CHAPS	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)	polyacrylamide	polyethyleneimine
15	CHAPS	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)	poly(methacrylic acid)	polyacrylamide
16	CHAPS	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)	poly(methacrylic acid)	polyethyleneimine
17	CHAPS	poly(2-ethyl-2-oxazoline)	poly(methacrylic acid)	polyacrylamide	polyethyleneimine
18	CHAPS	poly(ethylene glycol)	poly(methacrylic acid)	polyacrylamide	polyethyleneimine
19	dextran	Ficoll	Pluronic F68	poly(2-ethyl-2-oxazoline)	poly(vinyl alcohol)
20	dextran	Ficoll	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)	poly(vinyl alcohol)
21	dextran	Ficoll	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)	Tween 20
22	dextran	Ficoll	poly(2-ethyl-2-oxazoline)	poly(vinyl alcohol)	Tween 20
23	dextran	Ficoll	poly(ethylene glycol)	poly(vinyl alcohol)	Tween 20
24	dextran	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)	poly(vinyl alcohol)	Tween 20
25	Ficoll	Pluronic F68	poly(2-ethyl-2-oxazoline)	poly(methacrylic acid)	polyethyleneimine
26	Ficoll	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)	poly(methacrylic acid)	polyethyleneimine
27	Ficoll	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)	poly(methacrylic acid)	Tween 20
28	Ficoll	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)	poly(vinyl alcohol)	Tween 20
29	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)	poly(methacrylic acid)	polyacrylamide	polyethyleneimine
30	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)	poly(methacrylic acid)	polyacrylamide	Tween 20
31	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)	poly(vinyl alcohol)	polyacrylamide	Tween 20

Table S6. List of prepared six-phase MuPSs. Components that were not added at equal volumes are noted by the factor of the increase (i.e., **[2X]** for twice the volume of each of the other components).

Six-Phase MuPSs				
1	2	3	4	5
CHAPS **[2X]**	CHAPS **[2X]**	CHAPS	CHAPS	dextran
Ficoll	Ficoll	Pluronic F68	poly(2-ethyl-2-oxazoline)	Ficoll
Pluronic F68	poly(2-ethyl-2-oxazoline)	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)	poly(2-ethyl-2-oxazoline)
poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)	poly(methacrylic acid)	poly(methacrylic acid)	poly(ethylene glycol)
poly(methacrylic acid)	poly(methacrylic acid)	polyacrylamide	polyacrylamide	poly(vinyl alcohol)
polyethyleneimine	polyethyleneimine	polyethyleneimine	polyethyleneimine	Tween 20

is a function of concentration, this is not an unexpected result. Interestingly, there were several ATPSs that did not combine to produce MuPSs; for example, a carboxy-polyacrylamide–poly(vinyl alcohol)–poly(methacrylic acid) mixture results in the precipitation of some polymers even though each of the three precursor two-component mixtures were immiscible. If the concentration of a component was less than the concentration required to induce phase separation with other components in the system, the result would be a MuPS with fewer phases than anticipated. This result is likely dependent on individual solutes and combinations of solutes. Failed attempts at producing MuPSs typically resulted in a system comprising one fewer layer than anticipated; these systems are listed in Table S7. In one case (Table S5, #14), a mixture of five components—originally five phases after centrifugation—formed six phases with well-defined interfaces after sitting at ambient conditions for over two hours. One component could be the primary constituent of two different phases if micelles are formed and the phases differ in the concentrations of micelles.[12]

Characterization of Layers (i.e., phases) of Multiphase Systems: Density and Composition. We used oscillating U-tube densitometry to measure the final densities of each layer of a MuPS after phase separation. We performed experiments in plastic 15-mL conical tubes because we could pierce the side of the tube with a needle at the specific site of a desired layer in order to remove a sample by syringe. Since the densitometer requires roughly 750 µL of sample to completely fill the U-tube, we used 1–5 mL of stock solutions of solutes to ensure that we could isolate an adequate volume of pure solution after phase separation.

The phase diagrams of aqueous two-phase systems are characterized by a binodal curve and tie-lines.[1] The binodal curve delineates the concentrations where it is either thermodynamically favorable for the two-component mixture to form a homogeneous solution or

Table S7. List of three-phase, four-phase, and five-phase MuPSs that we could not prepare experimentally, but were predicted by our algorithm.

Multiphase Systems					
1	dextran sulfate	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)		
2	dextran sulfate	poly(2-ethyl-2-oxazoline)	poly(styrene sulfonic acid)		
3	poly(2-ethyl-2-oxazoline)	poly(diallyldimethyl ammonium chloride	poly(ethylene glycol)		
4	poly(2-ethyl-2-oxazoline)	poly(methacrylic acid)	Triton X-100		
5	poly(acrylic acid)	poly(diallyldimethyl ammonium chloride	poly(ethylene glycol)		
6	poly(acrylic acid)	polyacrylamide	Triton X-100		
7	poly(diallyldimethyl ammonium chloride	poly(2-ethyl-2-oxazoline)	poly(methacrylic acid)		
8	poly(diallyldimethyl ammonium chloride	poly(2-ethyl-2-oxazoline)	poly(vinyl alcohol)		
9	poly(diallyldimethyl ammonium chloride	poly(ethylene glycol)	poly(methacrylic acid)		
10	poly(diallyldimethyl ammonium chloride	poly(ethylene glycol)	poly(vinyl alcohol)		
11	1-*O*-Octyl-β-D-glucopyranoside	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)	poly(methacrylic acid)	
12	1-*O*-Octyl-β-D-glucopyranoside	poly(2-ethyl-2-oxazoline)	poly(methacrylic acid)	polyethyleneimine	
13	1-*O*-Octyl-β-D-glucopyranoside	poly(ethylene glycol)	poly(methacrylic acid)	polyethyleneimine	
14	CHAPS	dextran	Ficoll	poly(2-ethyl-2-oxazoline)	
15	CHAPS	Ficoll	poly(2-ethyl-2-oxazoline)	Pluronic F68	
16	CHAPS	Pluronic F68	poly(2-ethyl-2-oxazoline)	polyacrylamide	
17	CHAPS	poly(2-ethyl-2-oxazoline)	poly(methacrylic acid)	polyethyleneimine	
18	dextran	Ficoll	poly(2-ethyl-2-oxazoline)	Triton X-100	
19	dextran	poly(2-ethyl-2-oxazoline)	poly(vinyl alcohol)	Tween 20	
20	Ficoll	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)	polyethyleneimine	
21	Ficoll	poly(2-ethyl-2-oxazoline)	poly(methacrylic acid)	Triton X-100	
22	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)	poly(vinyl alcohol)	Tween 20	
23	poly(diallyldimethyl ammonium chloride	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)	poly(methacrylic acid)	
24	poly(diallyldimethyl ammonium chloride	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)	poly(vinyl alcohol)	
25	1-*O*-Octyl-β-D-glucopyranoside	poly(2-ethyl-2-oxazoline)	poly(ethylene glycol)	poly(methacrylic acid)	polyethyleneimine
26	Pluronic F68	poly(2-ethyl-2-oxazoline)	poly(methacrylic acid)	polyacrylamide	polyethyleneimine

to phase separate. The shape of the binodal is defined by a set of parallel tie-lines, which connect nodes along the binodal. Different points along a tie-line correspond to systems that differ in total composition and volume ratio, but equivalent final concentrations of solutes.

It is apparent from these phase diagrams that, while conditions exist for the formation of separate phases, each layer of an ATPS is a heterogeneous mixture of both components (i.e., a polyacrylamide–polyethyleneimine mixture produces *polyacrylamide-rich* and *polyethyleneimine-rich* phases). Mixtures composed of three or more mutually immiscible solutes should behave similarly, where each layer of a MuPS could contain multiple solutes. Higher dimensional phase diagrams characterizing the behavior of mixtures that lead to a MuPS from several linked ATPS would explain why the initial conditions used in our screen (e.g., concentration and volume) resulted in either (i) phase separation or (ii) the formation of fewer phases than anticipated. We chose to survey the final layer compositions of MuPSs by density and by ^{1}H-NMR spectroscopy in order to support this premise. For the ^{1}H-NMR experiments, 200 μL samples of each phase and of each stock solution were diluted in D_2O containing a known concentration (70 mg/mL) of the NMR internal standard benzene-1,2-disulfonic acid dipotassium salt. Comparisons of the peak integrations (relative to the internal standard) in the ^{1}H-NMR spectra of the stock solutions and the samples from each phase provided estimates of the concentration of each polymer in each phase (Table S8).

Density-Based Separation Using MuPSs. We prepared a five-phase MuPS from a mixture of poly(vinyl alcohol), poly(2-ethyl-2-oxazoline), poly(ethylene glycol), Ficoll, and dextran (Table S9). While we utilized poly(2-ethyl-2-oxazoline) with an average molecular weight of 200,000 Da for screening phase separation, we observed that a shorter chain polymer

Table S8. Measured densities and compositions of each phase of a five-phase MuPS generated from a mixture containing 6.4% (w/v) poly(ethylene glycol) (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.

Phase	Density (g/cm^3)	Final Solute Concentration (w/v)				
		PEG	PEOZ	PVA	Ficoll	Dextran
1 (top)	1.049	16	1.4	0.4	< 1	< 1
2	1.057	0.6	19	3.2	< 1	< 1
3	1.075	0.4	9.2	10	< 1	< 1
4	1.127	< 1	< 1	< 1	39	< 1
5 (bottom)	1.140	< 1	< 1	< 1	< 1	36

Table S9. List of polymers and conditions used to create a five-phase MuPS for separating density

standard beads based on density.

Polymer	avg. MW (Da)	stock % (w/v)	volume added (mL)	total % (w/v)
poly(vinyl alcohol)	3,000	25	4.5	9.0
poly(2-ethyl-2-oxazoline)	50,000	35	2.0	5.6
poly(ethylene glycol)	20,000	40	2.0	6.4
Ficoll	400,000	40	2.0	6.4
Dextran	500,000	30	2.0	4.8

with an average molecular weight of 50,000 Da had an identical ATPS miscibility profile to the longer polymer, but its mixtures were characterized by different binodal curves (data not shown). Complete phase separation occurred after centrifugation in a swinging bucket rotor at 3200g (Sorvall RT6000B) for 90 minutes. We removed aliquots of each layer and measured their densities by oscillating U-tube densitometry. From least dense to most dense (i.e., top to bottom), the layer densities were 1.049 g/cm^3, 1.057 g/cm^3, 1.075 g/cm^3, 1.127 g/cm^3, and 1.140 g/cm^3. As an alternative to the method we describe in the manuscript, we introduced six density standard beads (American Density Materials) to an identical pre-formed MuPS and sedimented them through the gradient in density in a swinging bucket centrifuge at 1000g for 15 minutes. Each of the six beads (densities of 1.0450 g/cm^3, 1.0500 g/cm^3, 1.0650 g/cm^3, 1.1200 g/cm^3, 1.1300 g/cm^3, and 1.1500 g/cm^3 ± 0.0002 g/cm^3) sedimented to a position within the MuPS based on their densities: one at either 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.

Position of the Bead at the Interface. The position of the center of mass of the bead at an interface gives additional information about the density of the bead; this position is a function of the difference in buoyant density between the bead and each of the adjacent phases, the magnitude of the interfacial surface tension, and the contact angle of the phases with the surface of the bead.[13] Ignoring slight differences in wetting, if the buoyant density of the bead is equal to the average of the densities of the two phases that meet at the interface, the center of mass of the bead will lie at the interface. If, however, the density of the bead is different than the average, the center of mass will lie above or below the interface. For example, in the five-phase system shown in Figure 2, the center of mass of a bead with a density of 1.050 g/cm^3 is above the

interface between the PEG-rich phase (ρ = 1.049 g/cm^3) and the PEOZ-rich phase (ρ = 1.057 g/cm^3).

Limits to Separations Based on Density. As noted in the manuscript, the interfacial surface energy between the phases of ATPSs is very low, and we expect this characteristic to be true of MuPSs with more than two phases. Interfacial surface energy, however, does provide an inherent limit on the ability of such systems to separate objects by density. A simple dimensional analsysis reveals the relationship between interfacial surface energy, an objects' size, density, and the relative centrifugal force under which a separation is carried out. To a first approximation, the gravitational energy required to remove an object of characteristic size R from an interface will be: $E_g = \Delta\rho R^4 \cdot RCF$, where $\Delta\rho$ is the difference in density between the object and the phase below the interface and RCF is the relative centrifugal force in units of g's. The interfacial surface energy will be: $E_s = \gamma R^2$, where γ is the interfacial surface energy between the phases. When these two energies are of the same order of magnitude, density alone will not determine whether an object remains at an interface. Using these equations we can estimate the minimum size of a particle that could be separated with density (Equation S1).

$$R_{\min} = \sqrt{\frac{\gamma}{RCF \cdot \Delta\rho}} \qquad \text{(S1)}$$

For a desired resolution in density, $\Delta\rho$, we can increase the centrifuge speed to overcome interfacial surface energy effects and separate small objects. For example, even with γ as high as 5 μJ/m^2, micron-sized objects can be separated to a density resolution of 0.05 g/cm^3 if an RCF greater than 10000g is appplied.

Separation of Micron-Sized Particles Based on Density Using MuPSs. We demonstrated this effect—increasing RCF to account for interfacial surface energy effects on

small objects—by separating particles with equal sizes (d = 2.8 µm) but a difference in density: polystyrene microspheres (ρ = 1.05 g/cm^3) and Dynabeads (ρ = 1.14 g/cm^3). We added a suspension containing ~250000 particles (polystyrene, Dynabeads, or a mixture of particle types) in water into a plastic capillary that contained a poly(ethylene glycol)–Ficoll ATPS prepared from a 1:1 mixture of 15% (w/v) poly(ethylene glycol) and 20% (w/v) Ficoll (ρ_{top} = 1.025 g/cm^3, ρ_{bottom} = 1.074 g/cm^3). After two minutes of centrifugation at 13700g in a microhematocrit centrifuge (CritSpinStatSpin; Iris Sample Processing, Inc.), (i) polystyrene particles sedimented through the top phase and concentrated at the interface between phases, (ii) the denser Dynabeads passed through both phases and concentrated at the interface between the bottom phase and the container, and (iii) a mixture of both types of beads sedimented by density independently, resulting in homogenous populations at separate interfaces (Figure S2).

Tuning the Densities of Phases in MuPSs Using D$_2$O. We prepared stock solutions of 20% (w/v) poly(2-ethyl-2-oxazoline) and 30% (w/v) poly(ethylene glycol) in aqueous solutions containing 100% H$_2$O to 70% D$_2$O/30% H$_2$O. We generated a series of two-phase MuPSs from mixtures of these solutions. After phase separation by centrifugation (five minutes at 2000g), we used an oscillating U-tube densitometer (Anton Paar DMA 35) to measure the densities of isolated samples of each phase. In addition, from the two-phase system containing only H$_2$O, we diluted 200 µL samples of each phase in a solution of D$_2$O (99.9% D) containing a known concentration (70 mg/mL) of the NMR internal standard benzene-1,2-disulfonic acid dipotassium salt. We measured the concentration of H$_2$O in each phase using ^{1}H-NMR. The top phase consisted of 69% H$_2$O (v/v), while the bottom phase contained only 57% H$_2$O (v/v). The ratio of these percentages (69/57 = 1.21) is nearly identical, within experimental error, to the

Figure S2. Density-based separation of particles using a MuPS. The density step produced by a poly(ethylene glycol)–Ficoll MuPS selectively filters particles of equivalent size ($d = 2.8$ μm) based on their differences in density. We added ~250000 particles to a microhematocrit tube containing the MuPS. After centrifugation, the density step produced by the MuPS separated solutions containing: (A) superparamagnetic microspheres only (Dynabeads (Dy); $\rho = 1.14$ g/cm^3), (B) polystyrene only (PS; $\rho = 1.05$ g/cm^3), and (C) a mixture of Dynabeads and polystyrene (Dy/PS).
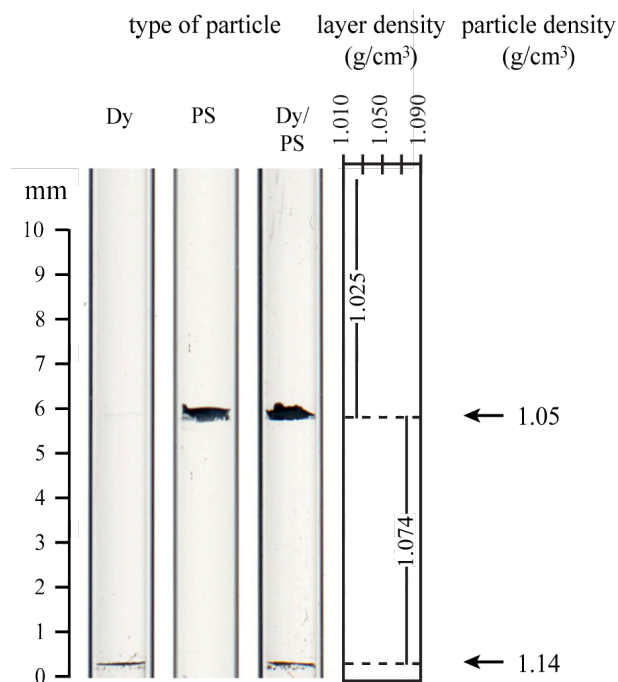
ratio of slopes obtained in the graph of density versus the % D_2O added to the system

$(8.85\times10^{-4}/(7.43\times10^{-4}) = 1.19)$. These data are consistent with the hypothesis that adding D_2O to

a MuPS will increase the density of each phase at a rate that is dependent on the amount of water

in that phase.

Tuning the Densities of Phases in MuPSs Using Salts. We used two alkali bromide

salts, LiBr and CsBr, as additives to MuPSs. Both alkali bromide salts should have similar

characteristics when added to MuPSs (e.g., optical transparency and solubility), but their

densities should be different at equal concentrations (e.g., densities of LiBr and CsBr solutions at

1 M are 1.055 g/cm^3 and 1.161 g/cm^3, respectively). We prepared a series of three-phase MuPSs

from mixtures equal volumes of 35% (w/v) poly(ethylene glycol), 25% (w/v) polyethyleneimine,

and 32% (w/v) poly(methacrylic acid) that also included LiBr or CsBr at a concentration from

0.0 M to 2.0 M. After phase separation, we removed samples from each phase and measured

their densities using densitometry. Similar to the trends in Figure 3 with CsBr, the densities of

the phases increased linearly with the addition of LiBr (Figure S3).

In addition, we diluted samples (of known volume) of each phase in a solution of D_2O

(99.9% D) containing a known concentration (70 mg/mL) of the NMR internal standard

benzene-1,2-disulfonic acid dipotassium salt. We measured the concentration of H_2O and of each

polymer in each phase using ^{1}H-NMR, and calculated the percentage of salt in each phase by

subtraction. This procedure results in relatively large errors in the calculated concentration of

CsBr, but still provides data that demonstrate the expected trends. Figure S4 demonstrates that

the relative amount of CsBr in each phase (i.e., the partitioning of CsBr between the phases) is

constant over the measured concentrations of CsBr.

Figure S3. A three-phase MuPS was prepared from mixtures of PEG, PEI, and PMAA in aqueous solutions that included LiBr over a range of concentrations of salts. The densities of the phases increased linearly with the concentration of the added LiBr.
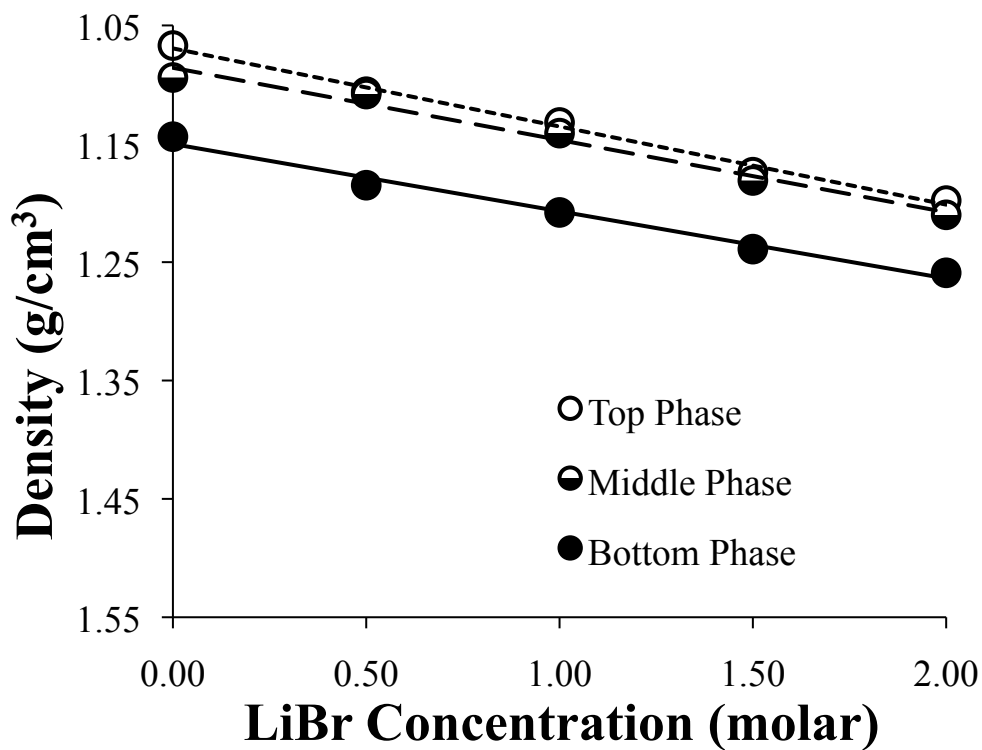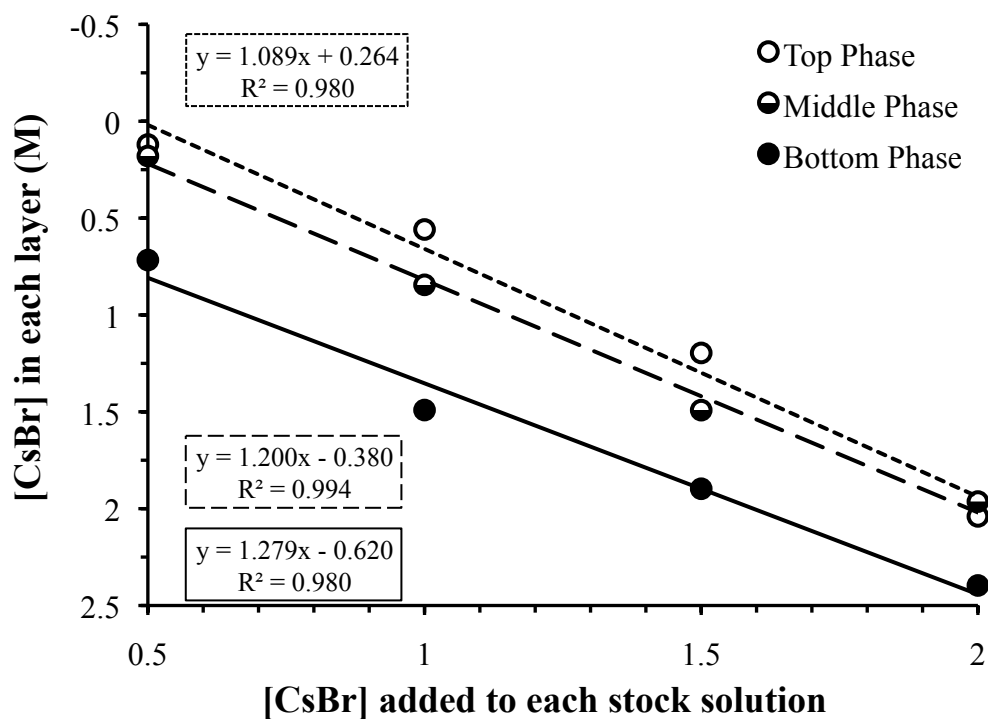
Figure S4. Partitioning of CsBr between each phase of a three-phase system consisting of mixtures of equal volumes of 35% (w/v) poly(ethylene glycol), 25% (w/v) polyethyleneimine, and 32% (w/v) poly(methacrylic acid). The amount of CsBr in each layer of this three phase system is not the same; the preferential partitioning of CsBr to the bottom phase is, however, consistent.

Tuning the Densities of Phases in MuPSs Using Methanol. We evaluated the use of methanol as a co-solvent to decrease the density of phases. We prepared a four phase MuPS consisting of 12% (v/v) Tween-20, 6% (w/v) poly(ethylene glycol), 9% (w/v) poly(2-ethyl-2-oxazoline), and 5% (w/v) polyacrylamide with a range of methanol concentrations (0–20% (v/v)). All phases showed a decrease in density with the addition of methanol. The top three phases fit to lines ($R^2 > 0.99$), but the polyacrylamide-rich bottom phase did not ($R^2 < 0.80$); the density of that phase saturated near a methanol concentration of 10% (v/v) (Figure S5). To avoid this non-linearity, we did not use methanol or similar organic solvents (e.g., ethanol) to tune the densities of MuPSs in this study.

Use of Magnetic Levitation in Measurements of Density. In order to design a MuPS that can separate materials in a mixture based on density, the densities of each material must be known. Unfortunately, the density of a material provided by a manufacturer may not always be accurate. We used magnetic levitation (MagLev) to measure the densities of pellets of four different formulations of Nylon. The MagLev technique has been described in detail elsewhere.[14,15] Briefly, MagLev involves placing diamagnetic samples into a container filled with a paramagnetic fluid, which is then placed into an apparatus comprising two permanent magnets with like poles opposed that are held apart at a fixed distance. The vertical position of the sample within the paramagnetic medium (i.e., "the levitation height") correlates with its density. The resolution of a density measurement by MagLev—and the range of densities of objects that can be levitated within a single paramagnetic medium—is related to the magnetic susceptibility of the paramagnetic medium, and, therefore, the concentration of the paramagnetic

Figure S5. Non-linear effects of the addition of methanol on the density of phases of MuPSs. A four-phase MuPS consisting of 12% (v/v) Tween-20, 6% (w/v) poly(ethylene glycol), 9% (w/v) poly(2-ethyl-2-oxazoline), and 5% (w/v) polyacrylamide was prepared in water with a range of methanol concentrations (0 – 20% (v/v)). Although the addition of methanol reduced the density of the phases of MuPS, the effect on the density of the polyacrylamide-rich bottom phase were non-linear.
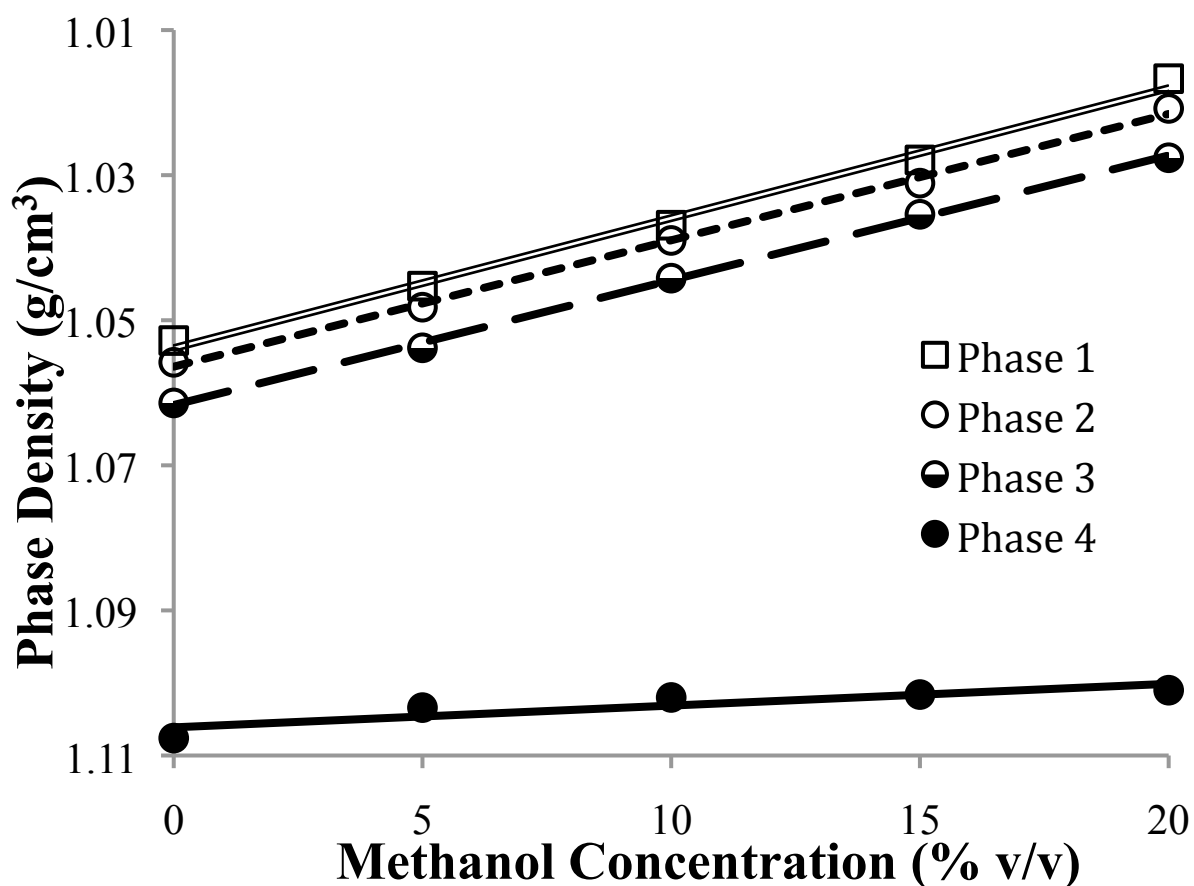
salt dissolved into the medium. To measure the densities of Nylon pellets, we used aqueous solutions of $MnCl_2$ at 0.5 M and 1.0 M. The range of densities available for analysis at 0.5 M $MnCl_2$ is 1.016 – 1.087 g/cm^3, and the range of densities available for analysis at 1.0 M $MnCl_2$ is 1.031 – 1.173 g/cm^3. The accuracies of these measurements are $\pm$ 0.001 g/cm^3 and $\pm$ 0.002 g/cm^3, respectively. We used density standard beads with densities of 1.0500 g/cm^3 and 1.1000 g/cm^3 to calibrate these two levitation media. Table S10 compares the densities measured by MagLev to those provided by the manufacturer in the product information sheet.

Separation of Nylon Beads. To separate the four different formulations of nylon by density, we used a three-phase MuPS. We mixed stock solutions of 40% (w/v) poly(2-ethyl-2-oxazoline), 30% (w/v) Brij 35, and 30% (w/v) Ficoll at equal volumes to produce a three phase system. The poly(2-ethyl-2-oxazoline) and the Brij 35 were both in 100% H_2O. We made the Ficoll in solutions of varied isotopic purity, from 100% H_2O to 30% H_2O and 70% D_2O—contributing a total concentration of up to 25.5% D_2O (v/v) when mixed in equal volumes with the other solutions. The final D_2O content was measured by ^{1}H-NMR spectroscopy. The densities of the phases increased linearly as more D_2O was added (Figure S6A). At 0% H_2O, the phase densities were not in the right range to separate four formulations of Nylon (i.e., Nylon 6, Nylon 12, Nylon 6/12, and Nylon 6/66) (Figure S6B). By tuning the density with D_2O, we were able to identify a system capable of separating all four formulations of Nylon: a the three-phase MuPS that used the stock solution of Ficoll mixed in 30% H_2O and 70% D_2O (for a final estimated concentration of D_2O of 25.5%) (Figure S5C).

Table S10. Densities of Nylon pellets. We compare the values as provided by the manufacturer

(Sigma-Aldrich) to those we measure by magnetic levitation (MagLev).

	Densities (g/cm^3)	
Pellet Type	**Manufacturer**	**MagLev**
Nylon 12	1.01	1.032
Nylon 6/12	1.3	1.058
Nylon 6/66	1.3	1.120
Nylon 6	1.084	1.147

Figure S6. Tuning density of a three-phase MuPS with D_2O to separate different formulations of nylon. The MuPS was formed with a final concentration of 13.3% (w/v) poly(2-ethyl-2-oxazoline), 10% (w/v) Brij 35, and 10% (w/v) Ficoll in solutions with varying isotopic purity of water. The densities of the phases increased linearly as the concentration of D_2O increased (A). The horizontal lines depict the densities of Nylon 12, Nylon 6/12, Nylon 6/66, and Nylon 6. At 0% D_2O, nylon 6 and nylon 6/66 both reside at the same interface at the bottom of the system (B). By increasing the D_2O concentration, we reached a regime where the four different formulations of nylon collect at separate interfaces (C).

A.

B. 0% D₂O

C. 25.5% D₂O

Nylon 12

Nylon 6/12

Nylon 6/66

Nylon 6
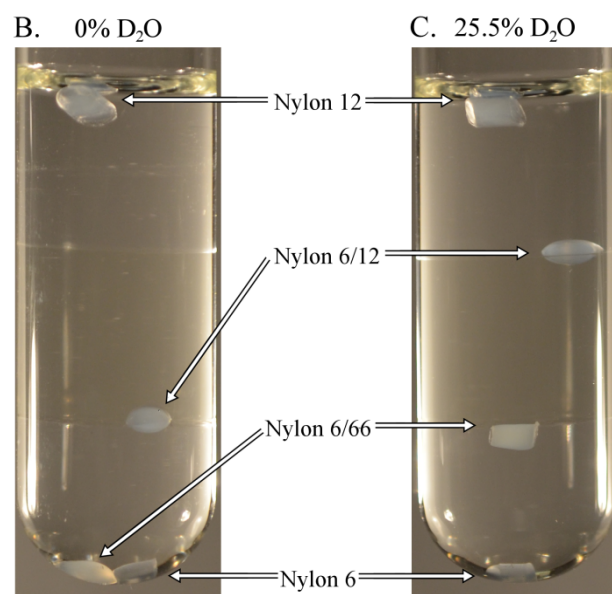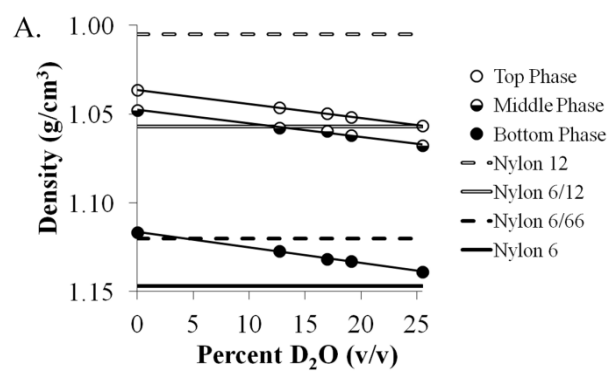
References

(1) Albertsson, P.-Å. *Biochem. Pharmacol.* **1961**, *5*, 351–358.

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

(3) Albertsson, P.-Å. *Biochim. Biophys. Acta* **1958**, *27*, 378–395.

(4) Sivars, U.; Tjerneld, F. *Biochim. Biophys. Acta* **2000**, *1474*, 133–146.

(5) Piculell, L.; Lindman, B. *Adv. Colloid Interface Sci.*. **1992**, *41,* 149–178.

(6) Robb, I. D.; Williams, P. A.; Warren, P.; Tanaka, R. *J. Chem. Soc. Faraday Trans.* **1995**, *91*, 3901–3906.

(7) Saravanan, S.; Reena, J. A.; Rao, J. R.; Murugesan, T.; Nair, B. U. *J. Chem. Eng. Data* **2006**, *51*, 1246–1249.

(8) Gao, Y.-L.; Peng, Q.-H.; Li, Z.-C.; Li, Y.-G. *Fluid Phase Equilibr.* **1991**, *63*, 173–182.

(9) Eisenberg, H.; Felsenfeld, G. *J. Mol. Bio.* **1967**, *30*, 17–37.

(10) Brooks, D. E.; Sharp, K. A.; Fisher, D. In *Partitioning in Aqueous Two-Phase Systems*; Brooks, D. E., Fisher, D., Eds.; Academic Press: Orlando, FL, 1985.

(11) Johansson, H.; Karlström, G.; Tjerneld, F.; Haynes, C. *J. Chromatogr. B* **1998**, *711*, 3–17.

(12) Kamei, D.T.; Liu, C.-L.; Haase-Pettingell, C.; King, J.A.; Wang, D.I.C.; Blankschtein, D. *Biotechnol. Bioeng.* **2002**, *78*, 190–202.

(13) Rapacchietta, A. V.; Neumann, A. W. *Journal of Colloid and Interface Science* **1977**, *59*, 555–567.

(14) Mirica, K. A.; Phillips, S. T.; Shevkoplyas, S. S.; Whitesides, G. M. *J. Am. Chem. Soc.* **2008**, *130*, 17678–17680.

(15) Mirica, K. A.; Shevkoplyas, S. S.; Phillips, S. T.; Gupta, M.; Whitesides, G. M. *J. Am. Chem. Soc.* **2009**, *131*, 10049–10058.