

Supporting Information For:

Fractionating Polymer Microspheres as Highly Accurate Density Standards

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Additional Experimental Details

MagLev

The MagLev setup used to characterize the distribution in density of the microspheres has been described previously.¹ Briefly, we place a cuvette containing aqueous manganese chloride (MnCl₂) between two permanent NdFeB magnets oriented with like poles facing. In this configuration there exists a nearly linear magnetic field gradient along the vertical center line. A diamagnetic object placed within the cuvette will levitate at a height, h , measured from the bottom face of the magnet to the centroid of the object, which correlates linearly with its volume-averaged density, $\bar{\rho}_s$ (kg/m³). This relationship can be described by Equation S1.

$$h = \frac{(\bar{\rho}_s - \rho_m)g\mu_o d^2}{(\bar{\chi}_s - \chi_m)4B_0^2} + \frac{d}{2} \quad (1)$$

In this equation, ρ_m (kg/m³) is the density of the paramagnetic medium, g (m/s²) is the gravitational acceleration, μ_o (T·m·A⁻¹) is the magnetic permeability of free space, d (m) is the spacing between the magnets, $\bar{\chi}_s$ is the volume-averaged magnetic susceptibility of the sample (SI, unitless), χ_m is the magnetic susceptibility of the medium (SI, unitless), and B_0 (Tesla) is the magnitude of the magnetic field at the surface of the magnets.

Paramagnetic solutions were prepared by dissolving 0.2 to 0.3 M MnCl₂ in water and adding diamagnetic zinc chloride (ZnCl₂) to approximately match the density of the solution to the density of the polymer microspheres. Polymer microspheres (10-15 at a time) were added to a plastic cuvette containing the paramagnetic solution and placed inside of the MagLev device with cylindrical magnets (2" diameter x 1" thick, axially

magnetized with $B_0 \sim 0.4$ T) and a distance of 45 mm between magnets. Three or more glass density standard beads were levitated simultaneously and used as references (see below for more information). A digital camera captured images once the objects reached equilibrium. We used ImageJ to determine the levitation height of the glass beads and polymer microspheres.

Comparison of Surface Tension and Buoyant Forces in Ficoll-Dextran-CsBr AMPS

To approximate the balance of surface tension and gravitational forces at the interfaces of our systems, we calculated an Eötvös Number.² Specifically, we analyzed the possibility of surface tension holding a bead at an interface despite that bead being less dense than the phase above or denser than the phase below. The dimensionless Eötvös Number, E_o , is defined as:

$$E_o = \frac{\rho g L^2}{\sigma} \quad (2)$$

where ρ is a characteristic density difference (in this case the difference in density between a bead and a phase), g is gravitational acceleration (or the relative centrifugal force for samples in a centrifuge), L is a characteristic length (in this case the diameter of the beads being separated) and σ is the surface tension. An Eötvös Number much greater than 1 indicates that gravitational forces are more dominant than forces from surface tension.³ We find an Eötvös Number of ~ 100 for $\rho = 0.0002$ g cm⁻³, $g = 3,210 \times 9.81$ m s⁻² (the separations in this paper were done at this relative centrifugal force), $L = 250$ μ m, and $\sigma = 4.14$ μ N m⁻¹ (as measured by spinning drop tensiometry (SITE100, Kruss) for a 9.84% (wt/vol) Ficoll, 6.16% (wt/vol) dextran AMPS).

An Eötvös Number of 100 is large enough to conclude that buoyant forces will likely dominate over surface tension at the interfaces in the AMPS used in this study. We

estimate, therefore, that contributions to imprecision in density measurements from surface tension in our systems should be negligible for the accuracy of density that we aim to achieve ($\sim 0.0002 \text{ g cm}^{-3}$).

Tuning a Ficoll-Dextran-CsBr AMPS

In this work, we used a two-phase AMPS composed of $\sim 10\%$ (wt/vol) Ficoll (a polysucrose, MW $\sim 400 \text{ kDa}$), $\sim 6\%$ (wt/vol) dextran (a polyglucose, MW $\sim 500 \text{ kDa}$), and different concentrations ($0\text{--}10\%$ [wt/vol]) of cesium bromide, CsBr. The least dense Ficoll-dextran-CsBr AMPS we obtained (measured at room temperature) was composed of 9.8% Ficoll, 6.2% dextran, 0% CsBr. This AMPS had a top phase density of approximately 1.0525 g cm^{-3} and a bottom phase density of approximately 1.0535 g cm^{-3} . Proportionally increasing the concentrations of Ficoll and dextran had the effect of (1) increasing the density of both phases and (2) increasing the difference in density between the two phases. **Table S1** demonstrates these effects. These AMPS all had roughly equal phase volumes (the top phase was approximately 20% smaller than the bottom phase by volume). Varying the ratio of Ficoll to dextran resulted in increasingly unequal phase volumes. To create denser AMPS, we added CsBr to increase the density of both phases. CsBr adds with a slight preference to the top phase, which has the effect of narrowing the difference in density between the two phases as CsBr is added, as shown in **Table S2**.

Table S1. Phase densities and the difference in density between the two phases for various Ficoll-dextran AMPS.

Ficoll Concentration (% wt/vol)	10%	11%	12%
Dextran Concentration (% wt/vol)	6.3%	6.9%	7.5%
Top Phase Density (g cm ⁻³)	1.0537	1.0595	1.0652
Bottom Phase Density (g cm ⁻³)	1.0562	1.0627	1.0687
Phase Density Difference (g cm ⁻³)	0.0025	0.0032	0.0035

Table S2. Phase densities and the difference in density between the two phases for AMPS composed of 10% (wt/vol) Ficoll, 6.3% (wt/vol) dextran, and various concentrations of CsBr, as measured by a DMA 35 U-tube densitometer (Anton Paar).

CsBr Concentration (% wt/vol)	0%	5%	10%
Top Phase Density (g cm ⁻³)	1.0537	1.0920	1.1302
Bottom Phase Density (g cm ⁻³)	1.0562	1.0940	1.1315
Phase Density Difference (g cm ⁻³)	0.0025	0.0020	0.0013

To compensate for the decrease in density between the two phases, higher concentrations of Ficoll and dextran were used in AMPS containing higher concentrations. The densest AMPS used in this paper was composed of 10.3% (wt/vol) Ficoll, 6.4% (wt/vol) dextran, and 10% (wt/vol) CsBr. We see no reason why denser AMPS could not be created with higher concentrations of Ficoll, dextran, and CsBr, but did not have a reason to create such systems for this paper. Because CsBr adds preferentially to the top phase, it is possible to invert the two phases such that the phase that would usually be the top phase is now denser than the phase that would usually be the bottom phase and becomes the bottom phase. As shown in **Table S3**, phase inversion is marked by the volume ratio between the two phases switching and additional CsBr increasing, rather than decreasing, the difference in density between the two phases.

We determined that when performing multiple fractionations at different densities, it was simplest to work from “bookend” AMPS (i.e. one high density AMPS and one low density AMPS) that could be mixed together to produce an AMPS of the desired density. We used a “lower” bookend composed of 9.8% (wt/vol) Ficoll, 6.2% (wt/vol) dextran, 1% (wt/vol) CsBr and an “upper” bookend composed of 10.3% (wt/vol) Ficoll, 6.4% (wt/vol) dextran, and 10% (wt/vol) CsBr. Due to uncertainties in mixing the AMPS from polymer powders, it is generally necessary to “tune” the bookends to have the desired difference in density. This is accomplished by centrifuging an aliquot of the AMPS, measuring its phase densities if it separated, and adding additional polymer or water to the AMPS to make adjustments. If an AMPS does not separate initially, we recommend adding 0.5% (wt/vol) Ficoll and 0.3% dextran as the first adjustment. If an AMPS initially separates with a larger difference in density between the phases than

desired, we recommend making a 5% dilution with water as a first adjustment. After the first adjustment, the subsequent adjustment can be extrapolated from the effects of the previous adjustments. Once the two bookend AMPS are created, they can be mixed to create AMPS at desired densities. As shown in **Table S4**, the average phase density of the two phases of an AMPS is a highly linear ($R^2 = 0.99998$) function of the mixing of the two bookends. As also shown in **Table S4**, however, mixtures of the two bookends tend to have a slightly large difference in density between the two phases than the bookends themselves. A slight dilution ($\sim 1\%$) of mixtures of the two bookends yields AMPS with differences in density between the two phases similar to the differences in density between the two phases of the bookends.

Measuring Density

The density of a sample is defined as its mass divided by its volume. One way to obtain a density measurement, therefore, would be to measure the mass and volume of a sample independently and divide to find a density. Highly accurate mass measurements can be made easily as commercially available balances can measure samples as small as 1 mg to an accuracy of 0.01% or better.^{4,5}

Accurately measuring a volume, particularly on small (< 50 mL) samples (both liquids and solids), is much more difficult. For example, while a 500 mL class “A” volumetric flask has an uncertainty in volume of 0.05% (by ISO 1042 standards), 10 and 1 mL “class A” volumetric flasks have uncertainties in volume of 0.25% and 2.5%, respectively.

Table S3. Phase densities and the difference in density between the two phases for AMPS composed of 12% (wt/vol) Ficoll, 7.5% (wt/vol) dextran, and various concentrations of CsBr, as measured by a DMA 35 U-tube densitometer (Anton Paar).

CsBr Concentration (% wt/vol)	10%	11%	12%	13%	15%
Top Phase Density (g cm ⁻³)	1.1426	1.1515	*	1.1676	1.1722
Bottom Phase Density (g cm ⁻³)	1.1437	1.1519		1.1681	1.1741
Phase Density Difference (g cm ⁻³)	0.0011	0.0004		0.0005	0.0019
Phase Volume Ratio (top : bottom)	2 : 2.5	2 : 2.5		2.5 : 2	2.5 : 2

* Did not separate.

Table S4. Phase densities for mixtures of two Ficoll-dextran-CsBr bookend AMPS, as measured by a DMA 35 U-tube densitometer (Anton Paar).

Percent Upper Bookend	Percent Lower Bookend	Top Phase Density (g cm ⁻³)	Bottom Phase Density (g cm ⁻³)	Average Phase Density (g cm ⁻³)	Phase Density Difference (g cm ⁻³)
0%	100%	1.0663	1.0676	1.0670	0.0013
20%	80%	1.0799	1.0817	1.0808	0.0018
40%	60%	1.0937	1.0955	1.0946	0.0018
60%	40%	1.1069	1.1092	1.1081	0.0023
80%	20%	1.1207	1.1233	1.1220	0.0026
100%	0%	1.1355	1.1361	1.1358	0.0006

Figure S1. Step-by-step protocol describing the fractionation of polymer microspheres using an AMPS. Black lines are added to highlight the pipette tip used to extract the beads and phases of the AMPS. Image contrast has been adjusted for clarity.

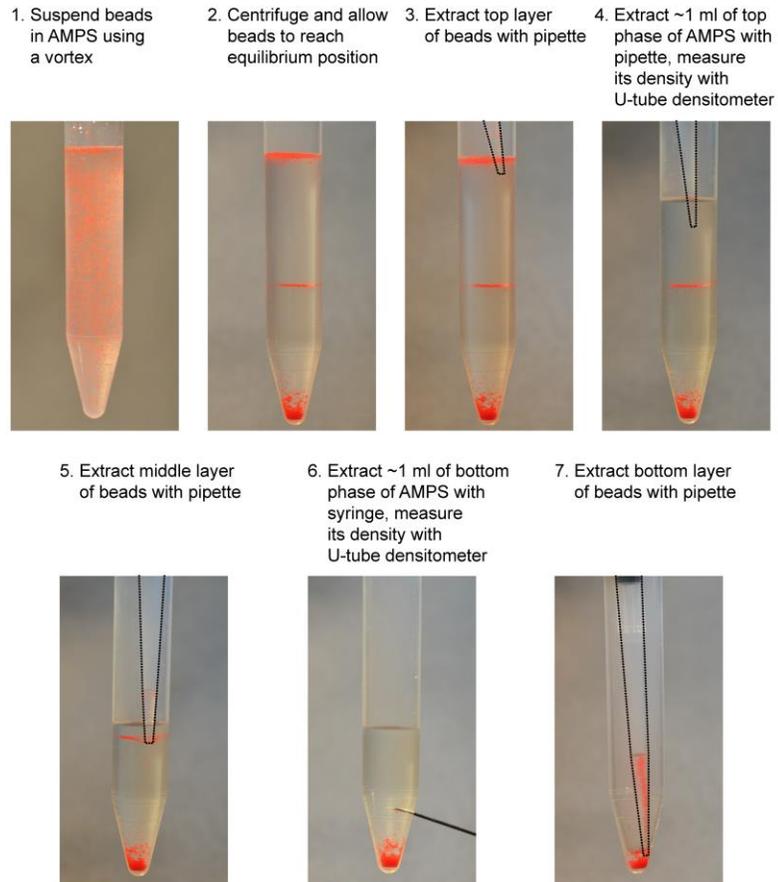


Table S5. Mean density and standard deviation in density for repeated measurements of aqueous solutions of dextran (MW = 500 kDa) and Ficoll (MW = 400 kDa) by a DMA 4500M U-tube densitometer (n = 10). As the concentration of the polymer increases, so does the standard deviation in density. The density value provided are the “viscosity corrected” values determined by the DMA 4500M. The “not viscosity corrected” values show the same standard deviations in density (± 0.00001).

% Polymer (wt/vol)	Dextran		Ficoll	
	Mean Density (g cm ⁻³)	Standard Deviation in Density (g cm ⁻³)	Mean Density (g cm ⁻³)	Standard Deviation in Density (g cm ⁻³)
5	1.01569	0.00024	1.01502	0.00002
10	1.03298	0.00016	1.03218	0.00005
20	1.07164	0.00059	1.06649	0.00024
30	1.11142	0.00085	1.10066	0.00028

Measuring the volume of a solid object is straightforward when the object has a simple geometry; its volume can be calculated from a few length measurements. Most solid objects, however, are too irregular in shape for this method. The most common method used to measure the volume of solid objects is by liquid displacement. In this method, an object is submerged in a liquid and the volume of displaced liquid – which is equal to the volume of the object – is measured.⁶ For small objects, however, using simple displacement to measure the density of a solid object is difficult as the volume of a small amount of liquid needs to be measured.

Below we discuss several methods for measuring the densities of small solid and liquid samples that avoid the challenges of trying to directly measuring the volume of a sample.

Measurement of the Densities of Liquids

U-Tube Densitometry

In many cases, the most accurate and reliable – and often simplest – method of measuring the density of a liquid is U-tube densitometry. In U-tube densitometry, a "U" shaped glass capillary is filled with the fluid whose density is to be measured. A vibration is then introduced to the "U" and the resulting resonant frequency is measured. The resonant frequency depends on the moment of inertia of the "U", which depends on density of fluid and the dimensions of the capillary. Because the resonant frequency is measured at a fixed point in the glass capillary (some distance from the fill and exit ports), as long as there are no air bubbles present and the "U" is completely filled with the fluid, the amount of excess fluid in the non-vibrating parts of the capillary does not

affect the measurement and there is no need for precise volumes to be used for each measurement.⁷

Handheld densitometers (Anton Paar, DMA 35) can measure 1 mL samples with an accuracy of $\pm 0.001 \text{ g cm}^{-3}$ and the most accurate bench top models (Anton Paar, DMA 5000M) can measure the density of 1 mL samples with an accuracy of $\pm 0.000005 \text{ g cm}^{-3}$.^{8,9} U-tube densitometers can be calibrated from two fluids of known density – most commonly air and water.¹⁰ Liquid density standards are discussed in detail below.

Other designs involving an oscillating capillary or another oscillating vessels have also been employed. The “U” tube design, however, is the most accurate.⁷

Measurement of the Densities of Solids

Pycnometry

One of the most common ways of measuring the density of a solid is pycnometry. A pycnometer generally consists of a sample chamber and a second chamber, both of known volume. The solid is added the sample chamber, which is then filled with a pressurized gas (usually helium) while the second chamber is evacuated. A valve is then opened between the two chambers and the change in pressure in the sample chamber is recorded. From the change in pressure and the volumes of the two chambers, the volume of the solid can be calculated. If a mass measurement is also made, the density of the solid can be calculated.¹¹⁻¹³

For solids with volumes on the order of $0.5 - 1 \text{ cm}^3$ or larger, commercial pycnometers can be used to make a volume measurement with an accuracy of approximately 0.03%. For smaller objects, however, the accuracy of pycnometry is greatly reduced. For samples with volumes smaller than 0.1 cm^3 , commercial

pycnometers can only make volume measurements with an accuracy of approximately 1%.¹¹⁻¹³

MagLev

As discussed above, MagLev is a method by which the densities of samples can be compared. With MagLev, the volume of an object does not need to be measured, as the only measured output is the height of an object, which is a direct function of density. MagLev can accurately (to $\pm 0.0002 \text{ g cm}^{-3}$) measure the densities of much smaller objects ($\geq 0.005 \text{ mm}^3$) than pycnometry can.¹ MagLev, therefore, is a powerful tool for measuring the densities of small solid objects.

MagLev measurements can only be as accurate as the density standards used to calibrate the system.¹ Three solid density standards are used typically as internal reference points to establish the relationship between height and density. These density standards are usually glass beads, which can be purchased commercially and have a density stated by the manufacture with an accuracy of $\sim 0.0002 \text{ g cm}^{-3}$, as measured by pycnometry.^{14,15}

Density Standards

Liquid Density Standards

Because density does not scale extrinsically with volume, the density of a large volume of a liquid can be measured and taken to be the same as the density of a smaller sample of that liquid. For this reason, liquids can be used as scale-independent density standards.

One of the most accurate and absolute liquid density standards is standard mean ocean water (SMOW), sometimes called Vienna standard mean ocean water (VSMOW).

SMOW is defined as being a sample of pure, gas-free water of a specific isotopic composition. This definition ensures that each sample of SMOW is identical to every other sample of SMOW, making it a universally consistent absolute density standard.¹⁶ During the late twentieth century, several standards laboratories performed experiments to precisely and accurately measure the density of ~1 L SMOW samples, and its density is now known to $\pm 0.0000001 \text{ g cm}^3$.¹⁷

Other liquids can also have their densities measured by hydrostatic weighing in large volumes in order to have smaller samples be used as density standards. Indeed, liquid density standards with densities known to $\pm 0.00001 \text{ g cm}^3$ are commercially available at a variety of densities (i.e. composed of a variety of materials).¹⁸ Without agreed upon conventions regarding factors such as isotopic composition, however, these density standards lack the universal consistency of SMOW. As shown in **Table S6**, variances in the relative isotope abundances of most elements result in uncertainties in the molar mass of pure samples as large as 0.14%. If 0.000,009 % accuracy is not required, crystals of silicon or other materials with more suitable densities could be grown to less rigorous specifications and used as density standards. If isotopic composition is not controlled for, however, the natural variations in atomic weights, as shown in **Table S6**, will limit the universal consistency of these density standards similarly to how the universal consistency of liquid standards is limited by these variations.

Table S6. Absolute and percent range in the molar mass of naturally occurring samples of select elements resulting from natural variances in their relative isotopic abundances.

Element	Range of Atomic Weight for Naturally Occurring Samples (g/mol)	Percent Difference Between Top and Bottom of Range
Hydrogen	1.0078 – 1.0082	0.040%
Boron	10.806 – 10.821	0.139%
Carbon	12.009 – 12.012	0.025%
Nitrogen	14.006 – 14.008	0.014%
Oxygen	15.999 – 16.000	0.006%
Silicone	28.084 – 28.086	0.007%
Sulfur	32.059 – 32.076	0.053%

Additionally, because a solid crystal often cannot be divided as easily as liquid without damaging its crystal structure while still ensuring a representative sampling of the original crystal (and therefore density), it is less practical to measure the density of a large sample of a solid in order to use smaller samples as density standards, than it is to do so with liquid density standards.

Rather than trying to control the precise isotopic composition of a material, a simpler method is to create a robust object and measure its density. Using this strategy, hollow glass beads (such as those used in this paper) are hand blown and have their densities measured with an accuracy of 0.0002 g cm^{-3} by pycnometry.¹⁹ The fractional thermal expansion of most glasses is on the order of 10^{-5} K^{-1} .^{17,20} Instability in temperature during measurement, therefore, should result in insignificantly small variations in density of glass density standard beads ($\sim 0.00005 \text{ g cm}^{-3}$ for a 4°C temperature change).

For solid density standards smaller than 4 mm in diameter, the only commercial products we are aware of are the polymer microspheres produced by CoSpheric. These products are recommended by GE in their Percoll gradient protocol for characterizing a density gradient.²¹ As shown in **Figure 2** of the results section, these microspheres have standard deviations in density from 0.006 g cm^{-3} to 0.021 g cm^{-3} . Fractionation of these beads by AMPS may increase their utility in applications requiring smaller standard deviations in density (see **Table 1**).

The density standards we describe in this paper can be fractionated such that they have small distributions in density and have their densities traced either to U-tube densitometry and air/water standard calibration or to MagLev and pycnometrically verified glass density standard beads.

Characterizing a Density Gradient

Density gradients are used to separate objects by their density. The position of an object in a gradient (i.e., its height) is related to the density of that object. In some cases, such as MagLev, the density gradient is linear, and in many cases, it is not. At a minimum, the density of the medium must be known at two points in order to fit a linear or other first order relationship between height and density. To determine a higher order fit or to verify the linearity of the relationship the density must be known at additional points.

Determining the density of the medium at a point can be done in two ways. Either a sample of the medium is extracted and its density measured, or a (usually solid) standard of known density is added to the gradient and its equilibrium height is assumed to be the height at which the gradient has a density equal to the density of the standard. The details, advantages, and disadvantages of both approaches are discussed below. In most cases, the use of density standards is a more practical approach.

Measuring the Densities of Samples of the Separation Medium

The greatest challenge to measuring the density of the separation medium at a point in space is that most U-tube densitometers require ~1 mL of solution.¹⁰ Such a sample is not collected from an infinitesimal point, but rather from a (hard to control) finite volume. A density value collected in this way is not the density at the desired point, but the average density of the volume collected. Since most experiments are performed in density gradients using 2–50 mL of gradient solution, on objects that occupy a small fraction of that volume, the uncertainty in the correlation of height to density will be significant. This challenge is, however, irrelevant for AMPS because these systems are

composed of steps in density; each phase in an AMPS is composed of one distinct density.

An additional challenge in sampling liquids to characterize points of a density gradient is that the collection of the sample can disturb objects in the gradient and can often destroy the gradient.

For these reasons, direct measurement of samples of the separation medium is usually not the best method to characterize a density gradient.

Using Density Standards to Characterize a Density Gradient

The use of density standards to characterize a density gradient is generally a much simpler and more convenient method than direct extraction of the medium, but there are several potential complications: (i) In order to relate the height of the standard to a density in the gradient, the standard must be assumed to be at a height determined solely by its density, rather than viscous or other forces. This assumption is usually valid when sufficient time can be allowed for the density standards to reach their equilibrium heights, but may not be valid in non-equilibrium gradients where sufficient equilibration time cannot be allowed. If the standards used are larger than the objects being separated, they can be assumed to settle to their density-determined heights at least as quickly as the objects being separated because larger objects move more quickly through viscous media.²² (ii) A density standard moving through the medium may introduce turbulences that prevent or disrupt the formation of a smooth gradient. The larger the density standard is, the more turbulence it will produce.³ Turbulence can be minimized in layered gradients by adding the standards at roughly their final heights during the layering process, and, thus, minimizing the distance the standards will need to travel. In time-

stable gradients, such as MagLev and AMPS, additional time may be needed to diminish disturbances from turbulence and reform equilibrium. (iii) A density standard may spatially exclude another object from its equilibrium height. This can be avoided by selecting a density standard that settles at a height (and density) far enough away from the height of the object of interest. (iv) The use of large density standards may introduce uncertainties in their measured heights if the standards are not perfectly spherical.¹

The use of density standards to characterize a density gradient, while requiring a careful experimental design, is generally the best means of characterizing a density gradient. The accuracy and precision of this method is, of course, ultimately dependent on the accuracy and precision to which the densities of the standards used are known. Selection of standards of an appropriate size is also important, as discussed above.

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