Ferrophasing: Magnetically Induced Gel-Like Behavior in Superparamagnetic Iron Oxide Nanoparticles
I. Preliminary Results and Observations

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Date: August 2018

Introduction:

Highly magnetic biocompatible colloidal nanoparticles like those developed by Liberti et al.[1] have many properties in common with classical ferrofluids[2]. For example, solutions of both exhibit increases in density and viscosity under the influence of magnetic fields (Liberti, unpublished observation). Classical ferrofluids are formed in non-polar solvents typically by ball milling magnetite with kerosene in the presence of simple detergents. On the other hand, those of Liberti et al are aqueous solutions. Those materials, hereafter called Ferrofluids [FF] are formed by proprietary methods involving peptizing slurries of magnetite crystals and subjecting them to high-power sonication, resulting in colloidal quasi- stable crystalline cores which can be coated in that transient state with polymers or proteins to yield stable, colloidal nanoparticles. Depending on processing conditions, cores with a mean diameter from 20 – 170 nm can be obtained. The resulting nanoparticles, even with multilayered coatings, are > 84% magnetic mass. They have been used commercially for cell separation, immunodiagnostics, and as MRI contrast agents [3-7]. They are quite dissimilar to other magnetic nanoparticles that can be synthesized in the same size range, e.g. those of Molday and Molday[8] which have been commercialized by Miltenyi Biotec for cell separation. Molday materials are about 8% magnetic mass and require very high magnetic gradients for separation[9]. In contrast our FF or cells labeled with FF separate in gradients as low as 4 – 6 kGauss/cm[10].

FF have previously been reported to act as stable phases in the presence of magnetic gradients[11]. The original observations of phase behavior were made from the following experiments: 100 µL of an 8 µg/mL solution (ca. 2.4×10^8/µg for 135nm particles) tinted blue for visualization by the addition of a small amount of food coloring and made dense by the addition of 0.5% w/v sucrose was placed into a microtiter well, which was overlaid with buffer to form a stable two-layer system. When that microtiter well was placed into a quadrupole magnet[12] of appropriate bore, a blue-colored annular cylinder immediately formed on the periphery of the well surrounding a clear central cylinder of buffer. Removing the well from the magnet immediately caused the layers to revert to their original positions, and that could be repeated several times. On the other hand, if the well was left in the magnetic gradient, material could be observed collecting evenly on the walls of the well within 2 – 5 min. Within 8 – 11 min, the separation was complete, leaving behind a blue-colored, thinner annular cylinder that eventually diffused throughout the well. For similar experiments where cells were included in the bottom layer, cells therein appeared to completely move with that layer. For both the dye and included cells, it was shown that there was no significant interaction with these nanoparticles. From these experiments, where ferrofluids act like a phase under the influence of magnetic gradients such that molecules or cells within them transport with them, the term “Ferrophasing” was coined. We report here more recent additional observations of Ferrophasing, as well as how this phenomenon can be overcome which gives some insights into this novel phenomenon and these unusual materials.

Materials and Methods:

Ferrofluids were prepared essentially by steps described above and by methods detailed in Liberti et al[1]. The coat protein was human serum albumin [HSA] crosslinked by proprietary methods. In all physical aspects, these materials behave identically to those used earlier coated with BSA. As these methods can produce controlled size

distributions, for these experiments the median size was 135 nm with a distribution from about 55 to 165 nm. From that preparation and using proprietary magnetic fractionation methods two preparations of median size 135 +/- 8 nm and 68 +/- 6 nm were prepared. Size and size distributions were determined with a Brookhaven Zeta 90 Plus Particle size analyzer (Holtsville, NY). The 68 nm preparation could not be separated when left overnight in a quadrupole magnetic device (Liberti, unpublished observation) with maximum gradient of 12 kGauss/cm. That is consistent with historical results in our laboratory.

To create an experimental arrangement where phases could be visually observed better than would be the case than peering through micro titer wells in a quadrupole, a bucking magnetic device was created. The device was made by taking two 100 x13 x 13 mm N52 grade rare earth block magnets, magnetized through a 13 mm dimension and affixing their 100 x 13mm like pole sides to the 100 x 13 mm sides of a 100 x 13 x 7 mm soft iron bar. Thus the magnet$_1$ - Iron bar – magnet$_2$ linear arrangement was as follows: **S<-N** [**Iron bar**] **N->S**, where the underlines represent the block magnets. With such arrangements extremely high (≅ 12-14 kGauss/cm at the surface) and uniform gradients can be produced on both side of the soft iron spacer. When this device, 100 mm high x 33 mm wide and 13 mm thick) is positioned vertically on a bench top and a curette filled with a FF solution placed adjacent to 7 x 100 mm iron bar, FF will collect uniformly on the inside wall nearest the device. Similarly, if the device is laid flat and a cuvette placed atop the iron bar, FF collects uniformly on the floor of the vessel.

To observe and study Ferrophasing with this device, a trough with inner dimensions 1.0 cm wide x 4.0 cm long and 1.0 cm high was used to layer solutions of buffers and FF solutions with or without different concentrations of added sucrose. FF solutions were stained pale blue with minute additions of blue food coloring (Patent Blue V, Sigma-Aldrich). A peristaltic pump was used for layering.

Results and Discussion:

To confirm and extend our earlier observations[12], the bucking magnet – trough arrangement described in methods was employed. In Table I the results of experiments done with three concentrations of the 68 and 135 nm FF samples are tabulated where FF concentrations in the upper layer are varied and concentrations of sucrose in the lower buffer layer are varied as well. For these experiments, 2 mL of buffer with different concentrations of sucrose was placed in troughs and with a peristaltic pump 2 ml of FF tinted blue with food coloring layered on top. These stable layered solutions were placed on top of the bucking magnet, described, so that they experienced a strong downward magnetic gradient. The observations on the movement of these layers are tabulated in Table I.

Table I. Downward movement of Ferrofluid layer through non-ferrofluid layer as function of [FF] and [sucrose] in the opposing layer

A. 135 nm HSA-Ferrofluid **a** ≤ 0.5 sec **b** ≤ 1.0 sec

% Sucrose w/v	[FF] 10 µg/mL	[FF] 15 µg/mL	[FF] 20 µg/mL
0.5	immediate	Immediate	Immediate[a]
1.0	Partial	Rapid[b]	Immediate
2.0	Layer distortion/partial	Partial	Rapid
3.0 and above	No movement	No movement	No movement

B. 68 nm HSA-Ferrofluid

% Sucrose w/v	[FF] 10 µg/mL	[FF] 15 µg/mL	[FF] 20 µg/mL
0.5	Rapid	ND	Immediate
1.0	Partial	ND	Rapid
3.0 and above	No movement	No movement	No movement

For 10, 15 or 20 µg/ml FF solutions layered over 0.5% sucrose containing buffer, the FF containing layer for both the 68 and 135 nm samples moved intact to the bottom of the vessel and the buffer layer to the top, confirming the original report. However, the rapidity with which the 'switching' occurs as shown in the Table, is affected by both FF concentration and FF size as evidenced by the 68 nm FF compared with the 135 nm preparation at 10 µg/mL. The rates are also affected by the magnetic gradient as they could be diminished by placing 1.0 mm spacers between the bottom of the trough and the bucking magnet. These results were reproducible and were compiled by multiple observers. They demonstrate that dilute solutions of these ferrofluids move as a phase under the influence of a magnetic gradient and that this phasing is related to their concentration and their size, i.e. magnetic moment. Similarly, to some extent, stability of such phases is related to both FF size and concentration. That the effect can be negated by sucrose solutions > 3% w/v is surprising as opposing solutions only have a density increase of less than 2%. On the other hand, the internal density of ferrofluid solutions does increase in the presence of a magnetic gradient. Hence, a density phenomenon could be operative.

The observations of experiments when the layering and magnetic force configuration was reversed, i.e. ferrofluid layer on bottom, buffer on top and the bucking magnet placed on top creating an upward pulling force are tabulated in Table II.

Table II. Upward movement of Ferrofluid layer through non-ferrofluid layer as function of [FF] and [sucrose] in the Ferrofluid layer

A. 135 nm HSA-Ferrofluid a = <0.5 sec b=<1.0 sec

% Sucrose w/v	[FF] 10 µg/mL	[FF] 20 µg/mL
0.2	Immediate[a]	Immediate
0.4	Rapid[b]	Immediate
1.0	No movement	No Movement

B. 68 nm HSA-Ferrofluid

% Sucrose w/v	[FF] 10 µg/mL	[FF] 20 µg/mL
0.2	Rapid	Rapid
0.4	ND	Rapid
1.0	No movement	No Movement

It should first be noted that for the experiments in Table II the sucrose is added to the Ferrofluid solutions and not to the opposing solutions as was the case for the previous results. That was required because it is not possible to layer buffer solutions over low concentrations of FF without them mixing. None the less, both FF

preparations showed Ferrophasing at low concentrations of sucrose added to the lower FF layer and for both the blue color moved with the FF phase. When the concentration of sucrose in FF layers was increased to 1.0% and above, Ferrophasing was completely inhibited. For the 68 nm FF, the layers remained stable over time. For the 135 nm FF, after 3-4 min FF visibly moved upwards to the interface and continued to the meniscus where it could be lifted off on a glass slide placed between the trough and the bucking magnet. In those cases, the blue dye remained in the lower layer.

In spite of the fact that the experiments of Table I and II seem reciprocal, they are significantly different. For Table I, the FF layer is opposed by solutions of differing density and in Table II the sucrose is in the FF layer. This suggests that more than one principle is operative in Ferrophasing. To gain some insight into Ferrophasing, consider that if one uses Maxwell's equations to calculate the magnetic force required to capture a single 135 nm magnetic particle of appropriate density and magnetic moment, the magnetic gradient required is in excess of 90kGauss/cm[1]. Yet these materials readily separate in magnetic gradients as low as 4– 6 kGauss/cm. This led the author to propose that these magnetic nanoparticles likely form North->South pole chains under the influence of a magnetic field and accordingly, their magnetic moment and effective mass changes; thus, this gives rise to erroneous results for such calculations. When this idea was shared with John Ugelstad many years ago, he subsequently demonstrated that his 2µm magnetic Dynal beads, visible by microscopy, do indeed form chains in a magnetic field[13, 14].

For 10 µg of 135nm FF at ≈3 x 10^8 particles/µg, it can be shown that 8 x 10^6 'chains' of 5 mm length could be formed in the experimental arrangements used here. Given the highly hydrophilic nature of these BSA- or HSA-coated materials, it seems reasonable to suggest that under the influence of a magnetic field, these chains interact with one other laterally and with water molecules to form a gelatin-like structure, thus leading to the entrapment of elements contained therein. Based on that notion, the inhibition of Ferrophasing by the higher level of sucrose addition to the FF layer experiments of Table II could be explained by competition of FF-chains with sucrose for H_2O.

In experiments similar to those of Table II, it was found that red blood cells could be transported by Ferrophasing. That was done by adding 10% RBC by volume to a 15 µg/mL solution of 135 nm FF (no sucrose) to the bottom layer, overlaying with buffer and subjecting that to an upward pulling gradient. Ferrophasing occurred and all RBC were visually observed to stay in the ferrofluid layer as that phase moved to the top of the vessel. Removal of the gradient caused the layers to return to their original positions and that could be repeated for 3 cycles with little mixing of the layers. It was determined that there no interaction between RBC and these FF.

Based on the apparent stability of Ferrophases, it seemed worthwhile to determine if stable Ferrophases could be established in vessels and be controlled externally. To that end, the following experiments were performed. Aliquots (1 – 3 mL) of blue-tinted ferrofluid solutions (15 – 25 µg/mL) were pumped through micro-bore (0.5 mm ID) tubing submerged in 500 mL graduated cylinders filled with buffer. The outlet of the tubing was positioned approximately 2 cm from the vessel wall, against which externally an N52-grade rare-earth block magnet (70x13x13 mm) was placed. As the ferrofluid solution exited the tubing, it remained in a remarkably intact stream (compared to a rapidly diffusing stream in the absence of a magnet) and moved towards the magnet, forming a blue-tinted bolus against the vessel wall adjacent to the magnet. Boluses so formed were routinely maintained overnight at RT with no change in visual structure.

To test the robustness of these Ferrophase boluses, they were formed as above but instead of maintaining the position of the external block magnet fixed, they were moved up or down or side to side on the cylinder surface (velocity about ca. 5 cm/s). In all cases, the boluses – and the dye contained therein – moved with the

magnet. If the magnet was removed whilst the bolus was near the midpoint of the vessel, it would begin to fall in the absence of the magnetic field, but could be restored by bringing the magnet back to the vessel wall surface near the bolus.

When RBC at concentrations of $5x10^6$/mL were included in buffered ferrofluid solutions in experiments identical to the above, RBC remained within the phase and could be held in position without lysis for as long as 72 hr. at RT. Alternatively, if an isotonic solution containing ferrofluid and RBC was pumped into a vessel containing deionized water, the color of the bolus reddened markedly indicating after about 6 h indicating red cell lysis.

Based on these results in combination with the FF layering experiments, we conclude that highly magnetic colloidal nanoparticles form structures under the influence of magnetic fields that can entrap cells and macromolecules, and that those entities can be moved or held in position near a surface by an external magnet. Because RBC lysis occurred when phases were established in deionized water, it appears that small ions can diffuse in or out of these phases. On the other hand, molecules like blue food coloring (molecular mass = 582 g/mol) appear to be retained within the phase. It is also noteworthy that by employing smaller magnetic nanoparticles (e.g., the 58 nm preparation), phases that are stable over long periods of time can be formed as the magnetic nanoparticles cannot be separated due to their Brownian motion and smaller magnetic moment. Alternatively, phases formed with larger magnetic nanoparticles (e.g., the 135 nm preparation) can be stabilized merely by placing the external magnet sufficiently far from the vessel wall, thus lowering the magnetic gradient.

There are several potential applications where Ferrophasing – or conversely, the ability to overcome this phenomenon – can be exploited. As an example of the latter, we have demonstrated that by maintaining a ferrofluid-containing layer in place via density alteration, immunomagnetically labeled target cells can be pulled out of the ferrofluid phase, through the buffer phase, and recovered with high yield and at high purity (manuscript in preparation). Other possible uses of Ferrophasing could be in drug delivery, whereby an injected bolus of ferrofluid admixed with a drug could be magnetically transported through a patient's circulatory system to the target site (i.e., by injecting near an externally placed magnet and moving the magnet to the target site). The Ferrophasing phenomenon could be further leveraged by allowing for localization of the drug at the target site – either with an external magnet or a small implanted magnet–and modulating its release (e.g., by decreasing the magnetic gradient from an external magnet over time). In that connection, it was demonstrated that a small magnet (ca. 1.5x0.4x 0.4mm) placed in a test tube is capable of creating a stable Ferrophase with the 58 nm ferrofluid preparation. Due to the size of that FF, the phase will not collapse because it does not separate with that gradient. Another potential application is the ability to position and hold cells, macromolecules, and small molecules (ca. 500 g/mol) in a given location within a vessel where certain reactions or manipulations might take place. The ability to do that by external means as described here could be useful in a variety of fields, but certainly could find application in biotechnology and bioprocessing.

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