Magnetic nanoparticles for novel biodetection assay techniques

Christopher Lundgren
Abstract

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Water-based suspensions of single-core iron-oxide particles with nominal sizes 15 - 50 nm were studied by AC and DC magnetometry techniques in order to determine their suitability for use in the volume amplified nanobead detection assay (VAM-NDA). The VAM-NDA can detect specific biomolecules, which have been magnetically tagged and volume amplified, by measuring the difference in the Brownian relaxation rate compared to a negative control sample. Single-core nanoparticles are expected to have a stronger low-field magnetic response, narrower size distributions and to better be described by the theoretical model constituting the foundation of the VAM-NDA, than particles consisting of a multi-grained cluster embedded in a non-magnetic coating, which previously have been used in the assay. Experiments showed that all particles exhibited signs of spin disorder, including a diminished magnetic response. The particles with nominal core diameters 25, 30 and 40 nm were superparamagnetically blocked at room temperature and very narrowly distributed in size. Therefore they would be useful for improving the assay, in spite of their diminished magnetic response.
1 Introduction

Nanoparticles and nanotechnology are currently a field of intense research. Understanding magnetism on the nanometre scale is essential, for instance, for overcoming the current theoretical limits for magnetic storage media. As it is currently possible to control the size of magnetic particles so that their dimensions can be made smaller than or comparable to the dimensions of the most important biological molecules, they can be used in different types of applications within the field of biomedicine.

Small particles could potentially be used in hyperthermia cancer treatment [1–4], where the particles then are dispersed throughout the target tissue and exposed to a high-frequency magnetic field. The high-frequency field will cause the particles to dissipate energy in the form of heat, thus destroying the cancerous tissue while sparing the healthy one, if should it be possible to sufficiently prolong the
Magnetic nanoparticles could also be used for target-specific drug delivery, which would be beneficial in treatment of certain medical conditions, such as cancer, when the treatment itself is likely to result in additional suffering of the patients, or less effective drug therapy when the patient is reluctant to continue the treatment due to side-effects. If the drug could be localized to a specific site of the body, both the side-effects and required dosage could be reduced. The drug would then be attached to a biocompatible carrier, which would consist of a magnetic particle core with a polymeric coating, and be prepared in form of a fluid, which would be injected into the bloodstream. A high-gradient magnetic field would then be used to direct the drug-carrier entities towards a specific site of the body.

2 Theory

2.1 Physical properties of magnetic nanoparticles

2.1.1 Superparamagnetism

Bulk materials are split up into magnetic domains, i.e. regions where the magnetisation is uniform in magnitude and direction. Each domain is separated by a domain wall, in which the magnetisation continuously changes its direction. The energy gained by splitting up the material into domains compared to that lost by forming domain walls diminishes as the overall volume becomes smaller. Therefore there is a critical size [5] below which the single-domain state is favoured. This size ranges from a diameter of about ten to a few hundreds of nanometres [5–7] dependent on which material is considered. The lower size limit for a single-domain particle is that with a diameter of 1 nm [7]. If the particle becomes any smaller than this there will not be enough atoms to constitute a domain.

A non-interacting ensemble of single-domain particles in thermal equilibrium are magnetised according to the classical theory of paramagnetism [8, 9]

\[ M = nm\mathcal{L}(\xi), \]

where \( nm = M_0 \) corresponds to complete alignment of the \( n \) magnetic moments per unit volume of magnitude \( m \). \( \mathcal{L}(\xi) = \coth(\xi) - 1/\xi \) is the Langevin function, with the argument \( \xi = \frac{nmH}{k_B T} \). \( H \) is the amplitude of the external field, \( k_B \) is Boltzmann’s constant and \( T \) is the temperature.

The susceptibility \( \chi = \frac{\partial M}{\partial H} \) is, to the accuracy \( \mathcal{O}(\xi^2) \), in the low-field linear approximation
\[ \chi_0 = \frac{nm^2}{3k_BT}, \quad (2) \]

which is the Curie law. Particles made out of magnetically soft materials have a magnetic moment of \( m = M_sV_m \), where \( M_s \) is the saturation magnetisation of the bulk material and \( V_m \) is the magnetic volume. The magnetic volume is, due to finite size effects (discussed more in section 2.1.2), smaller than the physical volume \( V \) for ferrite nanoparticles. The magnitude of \( m \) typically exceeds that of the Bohr magneton by several orders of magnitude, hence the prefix super in the word superparamagnetism, a term introduced by Néel in 1949 [10].

Particles with uniaxial symmetry, have the anisotropy energy

\[ E_a = -KV_m \cos^2 \theta, \quad (3) \]

where \( K \) is the anisotropy energy density and \( \theta \) is the angle between the magnetic moment direction and its easy axis of magnetisation. The energy barrier \( E_b = KV_m \) separating the two energy minima can be surmounted by thermal fluctuations according to the Néel-Brown theory [10, 11]

\[ \tau_N = \begin{cases} \tau_0\sigma^{-1/2}\exp\sigma, & \sigma \gtrsim 2 \\ \tau_0\sigma, & \sigma \ll 1, \end{cases} \quad (4) \]

where \( \tau_N \) denotes the Néel relaxation time and \( \sigma = \frac{KV_m}{k_BT} \) is a measure of the barrier height. \( \tau_0 \) is the time required for the magnetic moments to settle within the potential wells, and is often given a value in the range \( 10^{-12} \text{ - } 10^{-8} \text{ s} \).

For a specific set of particles and a particular experimental technique, there will be a temperature, \( T_B \), below which the particles cannot be seen to spontaneously reverse their magnetisation direction. This is called the blocking temperature, since the mechanism of internal moment reorientation will appear blocked on the time scale of the experimental technique.

When applied along the easy anisotropy axis, the field required to reverse magnetisation of a blocked single-domain particle by coherent rotation is the anisotropy field [12]

\[ H_K = \frac{2K}{M_s}, \quad (5) \]

where the ratio

\[ h = \frac{H}{H_K} = \frac{\xi}{2\sigma}, \quad (6) \]
2.1.2 Finite size effects

The smaller the particle size becomes, the more important will be the contribution from the atoms lying on the surface be to the magnetic properties of the particle. The surface atoms were initially believed to form a magnetic dead layer with a thickness of the order of the lattice parameter. Later it was shown that the lattice symmetry breaking due to vacancies and broken bonds at the surface sites lead to a non-collinear spin arrangement [13], which would reduce the magnetic moment in ferrite nanoparticles due to “canting” of the sublattice magnetisations. A simple model, which qualitatively describes the effect of surface symmetry breaking, is the core/shell model. A ferrimagnetic particle is then considered to consist of an ordered core, which is ferrimagnetic (FM), and a surface layer of disordered (paramagnetic, PM) spins.

Consider applying an external field \( \mathbf{H} \) to such a particle, above the ordering temperature \( T_g \), of the disordered shell. The FM core will then align its magnetisation along the external field direction, while the paramagnetic spins are oblivious to the applied field and will arrange themselves so that the exchange interaction at the core/shell interface is minimised. If the external field is left unchanged as the temperature is lowered below \( T_g \), the core and interfacial spin will maintain their orientation. If the external field direction is reversed, the FM core will try to change its magnetisation direction so that it aligns with the field, but the interfacial core/shell spins will be frozen in their initial field-cooling configuration due to exchange interactions. The overall preferred magnetisation orientation caused by the initial direction of the cooling field is an example of exchange anisotropy or exchange bias, which was first observed by Meiklejohn and Bean in 1957 [14]. They studied fine-particle Co compacts with a CoO coating and the effects induced on the low-temperature hysteresis curves by cooling the samples in fields of different magnitudes. The magnetisation curves were increasingly shifted in the opposite cooling-field direction as the magnitude of the cooling field increased. A field-induced shift, of a hysteresis loop driven to saturation, is a direct evidence of exchange bias. The disordered phase is not restricted to the particle surfaces [13, 15], but the core/shell model is meant here to serve as an example.

The field required to overcome the exchange interactions in an ensemble, where the individual particles are characterised by spin disorder, is much larger in magnitude than those experimentally available. The magnetisation curve will thus never completely reach saturation, and it is customary to add a linear term to equation (1)

\[
M = nmL(\xi) + \chi_{PM}H,
\]

where \( \chi_{PM} \) denotes the susceptibility of the non-saturating component.
2.1.3 Dynamic susceptibility

The frequency dependent susceptibility

\[ \chi(\omega, T) = \chi'(\omega, T) - i\chi''(\omega, T), \]  

of a set of non-interacting particles, can be described by the Debye formula [16]

\[ \chi(\omega, T) = \frac{\chi_0(T) - \chi_\infty(T)}{1 + i\omega\tau} + \chi_\infty(T), \]  

where \( \chi_0 \) and \( \chi_\infty \) denotes the low- and high-frequency susceptibilities and \( \tau \) the effective relaxation time. Equation (9) is valid when the measuring field \( H(t, x) \) with the Fourier space representation \( H_0(x)e^{-i\omega t} \), where \( t \) denotes the time coordinate, \( x \) the 3D-spatial coordinate vector and \( H_0 = |H_0(x)| \), fulfils the conditions

\[ \omega\tau_0 \ll 1 \]  
\[ \frac{mH_0}{k_BT} \ll 1. \]  

Ferrofluids are colloidal suspensions of magnetic particles. As the particles will have mechanical degrees of freedom, they will undergo relaxation due to Brownian motion according to [16, 17]

\[ \tau_B = \frac{3\eta V_{\text{hyd}}}{k_BT}. \]  

\( \eta \) is the viscosity of the carrier liquid and \( V_{\text{hyd}} \) is the hydrodynamic volume, i.e. the volume of a perfect sphere diffusing in the same manner as the particle.

The effective relaxation time is [18]

\[ \tau = \frac{\tau_N\tau_B}{\tau_B + \tau_N}. \]  

As a consequence of the exponential character of the of the high-barrier Néel relaxation time one of the limiting cases

\[ \tau = \begin{cases} \tau_N, & \tau_N \ll \tau_B \\ \tau_B, & \tau_N \gg \tau_B; \end{cases} \]  

are often fulfilled.
2.1.4 Polydisperse samples

Any sample volume used in an experimental situation will contain a large number of nanoparticles. All particles in the sample will naturally not be identical, and there will be a variety of sizes, shapes, crystalline and surface properties.

Whenever a measurement is sufficiently affected by a dispersion of those properties defining the quantity $x$ it is necessary to apply the averaging operator

$$\int_{x_{\text{min}}}^{x_{\text{max}}} dx g_x(x),$$

(15)

to the theoretical expression for the monodisperse ensemble, where $g_x(x)$ is a distribution function normalised by the condition

$$\int_{x_{\text{min}}}^{x_{\text{max}}} dx g_x(x) = 1.$$  

(16)

As the quantities most frequently occurring in theory are closely related to the particle volume, it is natural to choose $g_x(x)$ in accordance with its volume distribution, which is well-described by a log-normal distribution function [19]

$$g_x(x) = \frac{1}{\sqrt{2\pi \rho x}} \exp \left( \frac{-(\ln(x-\rho))^2}{2\rho^2} \right),$$

(17)

where $\rho$ is the standard deviation of $\ln x$. The integral (15) is then taken over $\mathbb{R}^+$, and it is necessary that the distribution function decays sufficiently fast outside the physically acceptable limits. The averaging operation is normally only carried out once, as the individual effect of each averaging procedure, when a sequence of such is performed, often neither are resolvable by theory nor motivated by experiment. The averaging operation will therefore be an effective such as the quantities appearing in product with $x$ implicitly will be absorbed into its distribution average.

The dynamic susceptibility averaged over a distribution of relaxation times, where the particular distribution function does not exist in closed form, was given a very simple form by Cole and Cole [20]

$$\chi(\omega, T) = \frac{\chi_0(T) - \chi_\infty(T)}{1 + (i\omega \tau)^{1-\alpha}} + \chi_\infty(T),$$

(18)

where $\alpha \in (0, 1)$ is a measure of the distribution width and approaches the Debye case in the limit $\alpha \to 0$. 


2.1.5 Interacting particles

Interparticle interactions in ferrofluids is a very complicated subject. Considering dipolar interactions in such systems it is useful to introduce the magnetodipolar interaction parameter [21]

$$\lambda = \frac{m^2}{r^3k_BT},$$

(19)

where $r$ is the distance between two particles of with equal magnetic moment $m$. It assumes its maximum when the interparticle distance becomes equal to the particles physical diameter $d$. Neglecting that $V_m < V$, its maximum possible value is [21]

$$\lambda_{\text{max}} = \frac{\pi M_s^2 d^3}{6k_BT}$$

(20)

As magnetic dipole-dipole interactions are the primary cause of particle aggregation, colloidal stability will be retained provided that [21]

$$\lambda_{\text{max}} \leq 4.45.$$  

(21)

The possibility to fulfil this criterion for a specific material decreases as the average particle size increases. In order to overcome this, particles are often coated with an organic layer, which is electrically charged. As the charges on the exterior of the surfaces are equal in sign for all particles, the organic coating provides both steric and electrostatic separation, the latter in the form of a diffusive electric double layer. The overall repulsive force between the particles can be quantified in terms of the zeta potential, which gives an additional measure of the colloidal stability.

If the particles are deprived of their mobility, i.e. the ferrofluid is frozen, and the particles are homogeneously separated, the interaction parameter attains the value

$$\lambda_{dd} = \frac{m^2}{k_BT}\phi,$$

(22)

where $\phi$ is the particle volume concentration. If $\sigma \gg \lambda_{dd}$, dipolar interactions can modify the expression for the high-barrier Néel relaxation time into the phenomenological Vogel-Fulcher law [22]

$$\tau = \tau_0^{\text{VF}} \exp \left( \frac{KV_m}{k_BT - T_0} \right),$$

(23)

where $\tau_0^{\text{VF}}$ is the average value of $\tau_0\sigma^{-1/2}$, which is assumed to be temperature independent, and $T_0 = \lambda_{dd}T$ [23] is a measure of the interaction strength. The
$T \to T - T_0$ scaling implies an increase of the energy barrier in the presence of dipolar interactions, which is most often seen in experiment. Theoretical considerations, however, suggest that weak dipolar interactions could lead to a decrease in the energy barrier [24–26], but only few experiments agree with this behaviour. The effect of dipolar interactions on the superparamagnetic relaxation time has led to some controversy and there is no simple answer within the framework of the simplest form of the model, which is the one presented here.

2.2 The VAM-NDA biodetection scheme

The volume amplified nanobead detection assay (VAM-NDA) [27, 28] is a biodetection assay which uses the Brownian relaxation of magnetic nanoparticles, magnetic beads, with blocked moments ($\tau_B \ll \tau_N$), to detect biomolecules [29].

The initial step is the target recognition [30, 31], where specially designed, synthetic linear oligonucleotides, short single-stranded DNA molecules, referred to as padlock probes, are used to find the target molecules. As the padlock-probe molecules are added to the sample they will bind to the target molecules by hybridisation and form circularised probe-target complexes as the ends of the padlock probe molecules are joined together by ligation.

The addition of an enzyme, RCA polymerase, promotes the replication of DNA sequences by rolling circle amplification (RCA) [32, 33], which results in long (several hundreds of nanometres up to micrometre-sized, dependent on the time allowed for the RCA to take place) random-coil single-stranded DNA molecules, with a periodically repeating sequence which is complementary to that of the padlock-probe.

The magnetic beads are prepared by biofunctionalisation, where oligonucleotides complementary to part of the RCA-coil DNA are attached onto the bead surfaces with the aid of a chemical-cross linker. When the oligonucleotide-functionalised beads are added into the sample, some of the beads will be immobilised when their surface oligonucleotides bind to the RCA-coil strand by base-pair hybridisation. The results is magnetically tagged RCA-coils with a significantly increased hydrodynamic diameter compared to the non-immobilised beads, which is then exploited by measuring the frequency dependent susceptibility of the sample.

A negative control sample, which always has to be used as reference, should have a well defined $\chi''$ maximum corresponding to the frequency of Brownian relaxation of the non-immobilised beads. A positive sample on the other hand will essentially have two $\chi''$ peaks, a high frequency peak (HFP) originating from non-immobilised beads and a low frequency peak (LFP) due to the relaxation of beads incorporated into the RCA-coils.

A positive sample is then identified either by turn-off detection, where a decrease in the amplitude of the HFP indicates that bead immobilisation has occurred, or by turn-on detection, where a LFP due to the magnetically tagged RCA coils...
3 Experiments and results

Six different ferrofluids were studied, all being manufactured by Ocean Nanotech [34] and being part of their SHP series. The samples consisted of single-core iron-oxide particles, prepared by the thermal decomposition method [35–40], which were coated with a layer of oleic acid and an additional layer of an amphiphilic polymer with carboxylic acid surface functional groups, and dispersed in deionised water. The particles had nominal core diameters 15, 20, 25, 30, 40 and 50 nm \(^1\) (each will be referred to by the prefix SHP- followed their nominal core diameter) and the thickness of the surface layers were approximately 2 nm each, adding a total of 8 nm to their respective physical diameters [41]. The zeta potentials for the specific batches, as determined by the manufacturer by dynamic light scattering measurements, ranged from -2.8 MabV to -7.6 MabV \(^2\). The iron concentrations, as deduced from X-ray fluorescence measurements made by Ocean Nanotech, were 5 mg per cm\(^3\), which corresponds to 6.9 mg Fe\(_3\)O\(_4\)/cm\(^3\), assuming only magnetite is formed. As magnetite has a density of 5.24 g/cm\(^3\) [8], the corresponding volume concentration is 0.13 %. All measurement data will be presented with respect to the iron-oxide content as estimated by this procedure.

Prior to all measurements, the samples were thoroughly shaken using a vortex mixer for at least one minute, in order to reverse any effects of sedimentation.

3.1 Magnetic characterisation

3.1.1 Static magnetisation measurements

A Quantum Design [42] MPMS-XL SQUID (Superconducting QUantum Interference Device) magnetometer was used to measure the effects of applying a static magnetic field.

Each sample, 30 µl of ferrofluid poured into a cylindrical cup made out of a polymeric material, which should be diamagnetic, was placed in the middle of a long colorless straw. The sample and straw were mounted onto a transport rod inside a low-pressure sample space, confined within a liquid-helium cryostat. The straw was placed inside the sample space with its longitudinal axis along the symmetry axis of a superconducting solenoid, the latter which provides the DC magnetic field. A superconducting wire constitutes the pick-up coil, which is wound in a second-order gradiometer configuration around the sample location. The pick-up coils are inductively coupled via a signal transformer

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\(^1\)Lot numbers were IB031110 (SHP-15), 122810 (SHP-20), 022411 (SHP-25), 120310 (SHP-30), 121010 (SHP-40) and 102809 (SHP-50).

\(^2\)The determined zeta potentials were -3.8 (SHP-15), -7.6 (SHP-20), -5 (SHP-25), -4.8 (SHP-30), -2.8 (SHP-40) and -4 (SHP-50) MabV.
circuit to an rf SQUID device, located inside a magnetic shield [43]. As the detection coils, signal transformer wires and SQUID input coil form a closed superconducting loop, magnetic flux changes in the pick-up coils produces a change in the detection coil system.

The Reciprocating Sample Option (RSO) [44] was used, where the sample response, as the sample is moved with a small-amplitude, periodic displacement inside the SQUID pick-up coil assembly, is recorded as a function of its position. By fitting the response curve to that of an ideal point dipole, using an algorithm which includes an extra fitting parameter to account for sample movement during the measurement procedure [45, 46], the magnetic moment is deduced and given by the instrument software in electromagnetic units (emu).

Background signal measurements were carried out by measuring the field dependence of the magnetic moment of the empty sample container at two different temperatures: 300 K and 100 K. The high-field response was fitted to a linear function of the external field $H$, and was subtracted from the subsequent measurements. At temperatures below 100 K, the 100 K background response was used. For a proper background subtraction however, the raw measurement signal from an empty sample holder measured in an identical manner as the sample should be subtracted in a specific way [47]. As the raw SQUID voltages from intermediate measurement scans were not saved, this approach could not be used for isolating the ferrofluid response, nor was it possible to otherwise treat the data.

**Magnetisation versus applied field** Magnetisation curves were recorded for all samples at temperatures 10, 100 and 300 K, by cycling an applied back and forth between $\pm 50$ kOe. The demagnetising field in the samples was neglected since magnetisation of the samples, due to low volume concentration of beads in the carrier liquid, were small and it would only result in a minor correction factor. As seen in fig. 1, the hysteresis curves, first measured at 300 K, all have the characteristic features of a superparamagnetic substance, including a negligible coercive field $H_c$. The magnetic moment is however significantly reduced compared to that of bulk magnetite [8], and only SHP-15 and SHP-20 have a tendency to saturate at high fields. The diminished magnetisation and absence of saturation is generally believed to be due to surface and finite size effects [13, 49–51], and appears to provide a reasonable explanation for these observations. In addition, coating the particles with an organic layer is also expected to cause a reduction of the magnetic moment [49], and might also contribute to the absence of a magnetically saturated state, which would consist of dipolar chain-like aggregates if the particles would be without imperfections. As the particle surfaces intentionally have been modified to provide electromagnetic repulsion, this might additionally aggravate the possibility to achieve a state of magnetic saturation.

The size dependence of the magnetisation, which is highest in the SHP-15 sample and decreases monotonically as the particle size increases up to the SHP-30 sample size, could be due to that the ability to manufacture uniform particles reduces with increasing particle size [52]. Particles larger than 30 nm could not
be synthesised while still maintaining a spherical shape and TEM images [41] show particles of rectangular shapes for sizes larger than 30 nm. The deviation from a spherical geometry could on one hand lessen the contribution from the Néel relaxation mechanism due to an increase in anisotropy. On the other hand, the larger particles will be more likely to contain defects due to incomplete synthesis. The slight increase in magnetisation compared to SHP-30 for the two larger particle sizes could possibly be due to a minor positive net effect of the aforementioned.

The hysteresis measurements were made in descending temperature order, with the magnet left on at 50 kOe between the temperature changes, thus making the hysteresis curves at the two lower temperatures field-cooled. At 100 K (fig. 2), a coercive field, defined as $H_c = (|H_c^+| + |H_c^-|)/2$, where $H_c^\pm$ denotes the loop intersect for positive and negative fields, respectively, of the order of 1 kOe was observed for particles with a nominal size larger than 20 nm. A significant horizontal loop shift $H_{EB} = (|H_c^+| - |H_c^-|)/2$ was also observed along with the non-negligible coercivity. These features are much less pronounced for the SHP-20 sample and are completely absent for SHP-15. The appearance of a coercive field indicates a deviation from a superparamagnetic state, which would imply that the particles larger than 20 nm owe their room-temperature superparamagnetism due to their ability to move in the ferrofluid liquid phase.

As the magnitude of $H_{EB}$ exhibits a strong correlation with the values of $H_c$, one could suspect that the whether the particles are superparamagnetic blocked or not in the liquid phase of the ferrofluid would be the primary cause of the shifted magnetisation curves. It is however not very obvious how to proceed in trying to explain the physical situation from this perspective. It is, however, also considered that the amount of both surface and internal spin disorder is directly mirrored by the magnitude of the loop shift [49], which makes sense with the knowledge that the larger particles were harder to synthesise without imperfections.

As the hysteresis loop, at least for SHP-40, does not close at 50 kOe it seems like this particle size follows a minor loop already at 100 K, which would indicate that the particles are very defective with regards to their crystallinity [49].

At 10 K (fig. 3) the observed characteristics are essentially enhanced, and the crossover between 20 and 25 nm becomes increasingly sharp. Now, however, SHP-15 also exhibits a small coercivity and the loop is also slightly shifted, which would indicate that a superparamagnetic blocking has occurred and possibly some internal freezing of a disordered-spin component. At 10 K neither of the loops measured for SHP-25, SHP-40 and SHP-50 close at the maximum applied field, which would indicate that these particles now follow a minor loop, implying that all three sets of particles are defective [49]. The SHP-30 particles show a similar tendency, but the loop closes at 35 kOe.

The values of $H_c$, $H_{EB}$ along with $M_0$ at the different temperatures are shown in table 1. The values of $M_0$ were obtained extrapolating the high-field part of the $M - 1/H$ curve to 0 and is not expected to be adequate from a physical point of view, but is primarily included to give an impression of the magnitude of $M_0$. 

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Table 1: **Data from magnetisation curves.** Data obtained from magnetisation measurements carried out in descending temperature order. The applied field was cycled back and forth between ±50 kOe with the magnet left on at 50 kOe between the temperature changes. The values of $M_0$ were obtained extrapolating the high-field linear part of the $M - 1/H$ curve to 0 and are not expected to be adequate from a physical point of view, but is primarily included to give an impression of the magnitude of $M_0$.

<table>
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<th>Sample</th>
<th>T (K)</th>
<th>$M_0$ (emu/g)</th>
<th>$H_c$ (Oe)</th>
<th>$H_{EB}$ (Oe)</th>
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It was possible to fit the 300 K data to equation (7) averaged over a log-normal distribution of magnetic volumes, but as the only values of $V_m$ that could produce a reasonable fit were much smaller than their nominal core volumes it did not appear that these fits would provide any further physical insight, and they are therefore not presented here. However, this indicates that the model implied by (7) does not give a correct description of the here investigated nanoparticles. The model does not account for interparticle interactions and magnetic anisotropy, which should be present in case of particle chain formation, and may also be that field dependence of the spin disordered part of nanoparticles is more complicated than a linear field dependence. However, none of the models presented in [9] which to account for anisotropy that would be applicable here even when adding a linear term to the expressions. As the particular expressions cannot be solved analytically, they hardly allow for an additional averaging over a size distribution. The common model which includes dipolar interactions [53] by employing a $T \to T + T^*$ scaling, where $T^*$ is interpreted as the rms dipolar interaction energy, yields much too high values of the dipolar interaction term to be physically meaningful if one requires $V_m$ to be close to the nominal particle size. Its theoretical justification can perhaps also somewhat be questioned.
Figure 1: **Magnetisation curves at 300 K.** Magnetisation curves measured at 300 K. All samples exhibit a superparamagnetic behaviour with the exception of a tendency not to saturate, something which is particularly evident in the samples with a nominal size over 20 nm.

**Zero field cool/field cool**  The zero field cooled (ZFC) and field cooled (FC) magnetisation was measured for SHP-20 by first heating the sample up to 345 K and then cooling it in zero field down to 10 K. At 10 K a small field of 20 Oe was applied and the magnetisation was measured as the sample was heated up to 345 K. The sample was then cooled down back to 10 K in the same measuring field. Measurements were performed while continuously sweeping the temperature with 3 K between measurement points, and a temperature sweep rate of 2 K/min. Temperature stabilisation was however used prior to measurements of the sample magnetic moment in the temperature interval 250 - 300 K.

As can be seen in fig. 4, the sample shows irreversible behaviour below approximately 260 K and the ZFC magnetisation exhibits a peak around 220 K. The FC magnetisation does not increase monotonically with decreasing temperature, as expected for a superparamagnetic system, but instead flattens out and exhibits a weak temperature dependence below the ZFC peak. At approximately 100 K a small but abrupt decrease in the FC magnetisation is seen, after which the FC magnetisation attains an almost constant value. A flattening out of the FC magnetisation curve has been attributed to the onset of glassy dynamics associated with a disordered phase below $T_g$ [50]. Assuming, however, a superparamagnetic system, a Wohlfarth-like expression [54] \(^3\) could be used to extract the blocking temperature distribution, shown in fig. 5. A log-normal distribu-

\[ g_T(T_B) \propto \frac{1}{T_B} \left( M_{FC} - M_{ZFC} \right) \]

\(^3\)In ref. [54] an expression for the blocking temperature distribution $g_T(T_B)$ is derived.
Figure 2: Magnetisation curves at 100 K. Magnetisation curves measured at 100 K after field-cooling from 300 K in a field of 50 kOe. The coercive field observed for particles with a nominal size over 20 nm indicates that they owe their room-temperature superparamagnetism due to their ability to move in the liquid phase of the ferrocolloid. A horizontal loop shift is also observed, and its magnitude exhibits a strong correlation with that of the coercive field. It is however believed that this correlation might be somewhat coincidental, and that the primary cause of the shifted magnetisation curves is due to poor crystallinity of the particles.
Figure 3: Magnetisation curves at 10 K. Magnetisation curves measured at 10 K after field-cooling from 100 K in a field of 50 kOe. The characteristics observed at 100 K are essentially enhanced, where there is a sharp distinction between the two smallest particle sizes, and the larger ones, where the latter exhibit blocked particle moments above the solidification temperature of the ferrofluid.
Figure 4: **Zero field cool/field cool.** Zero field cooled and field cooled magnetisation as a function of temperature as measured for SHP-20. The discontinuity in the curves around 270 K is due to freezing of the ferrofluid. The peak in the ZFC curve is related to the superparamagnetic blocking temperature and the low-temperature flattening of the FC curve has been attributed to the onset of glassy dynamics associated with a disordered phase below its ordering temperature.

A fitting function was used and the distribution mean and standard deviation were 173 K and 29 K, respectively.

### 3.1.2 Dynamic susceptibility measurements

**Frequency dependency of the AC susceptibility** An Imego [55] DynoMag AC susceptometer was used to measure the frequency dependency of the AC susceptibility at around 300 K in the frequency interval 1 Hz - 250 kHz. The measurement system is an induction-based one, and an excitation coil, surrounding the sample space, exposes the sample to an AC magnetic field of constant magnitude 5 Oe up to frequencies of 1 kHz, smaller in magnitude at frequencies above. As the system is configured as a first order gradiometer to minimise the signal from the excitation field, the response from the sample, 200 µl ferrofluid contained in a glass vial, is measured by two induction pick-up coils. The sample is moved to each of the two, one at a time, during the measurement sequence, and the difference in the induced voltage is used to obtain the susceptibility, which is given for the whole sample in SI units. The background signal is obtained by carrying out a measurement sequence, identical to that performed on the actual sample, on a vial containing only the dispersant, and is automatically accounted for by the instrument software. The total ferrofluid SI susceptibility was converted to the iron-oxide susceptibility in cgs units by dividing the given susceptibilities by $4\pi$ and multiplying them by the inverse iron-oxide volume concentration.

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Figure 5: **Blocking temperature distribution.** Distribution of blocking temperatures for SHP-20 extracted from ZFC/FC data. A log-normal distribution function was used and the distribution mean and standard deviation were 173 K and 29 K, respectively. The solid line constitutes a fit, and the squares are measurement data treated according to an expression derived in ref. [54] where the blocking temperature distribution
\[ \propto -\frac{T}{\partial T} (M_{\text{FC}} - M_{\text{ZFC}}). \]

Measurements on the undiluted samples showed Brownian relaxation characteristics for SHP-25 and larger sizes (fig. 6), while SHP-15 showed no relaxational behaviour, as shown in fig. 7. For the SHP-20 sample, a \( \chi'' \) peak broadening was observed, with a maximum at around 100 kHz, as indicated in fig. 7. This would correspond to a hydrodynamic diameter of around 15 nm in the case of pure Brownian relaxation, but the height of the peak is much too small in relation to its width and the magnitude of the in-phase component to be identified with the maximum of a single relaxation process.

The data for SHP-25, SHP-30 and SHP-40 were fitted to the Cole-Cole equation, by minimising the sum of squares error with respect to both real and imaginary parts of the susceptibility. Measurement data where \( \chi'' < 0 \) and \( \frac{\Delta \chi'}{\Delta T} > 0 \) (in the forward direction, which was the same as the measurement direction), were omitted in the fitting procedure, perhaps a bit too selectively choosing slightly different lower and upper cutoffs for the different samples, since these data points were likely not to improve the accuracy of the fit in the sense that they would not provide more information on the actual response from the ferrofluids. I.e. the high-frequency points where \( \chi'' \) shows an increase with increasing frequency are more likely to be either due a harder-to-subtract signal from the carrier liquid or due to resonant noise in the equipment electronics, rather than due to the occurrence of actual ferromagnetic resonance in the ferrofluids. Similarly, the data points at the lowest frequencies where \( \chi'' \) is negative and \( \chi' \) is increasing with increasing frequency is rather due to an improper choice of measurement points, since the instrument is dependent having a sufficient number of frequency cycles to average over to produce an accurate result.
Table 2: Brownian relaxation data. Fitting parameters and hydrodynamic sizes obtained from non-linear regression procedures. \( f_B \) denotes the peak frequency location for SHP-25, SHP-30 and SHP-40 while SHP-50 had an asymmetric relaxation spectrum which could not be accounted for by the Cole-Cole model, used for the smaller sizes. Instead a set of Debye relaxations, averaged over a generalised gamma distribution of relaxation times was used, and the value of \( f_B \) presented corresponds to the distribution mean. The hydrodynamic diameters \( D_{\text{hyd}} \) were calculated with respect to \( f_B \) assuming the viscosity of the medium was equal to that of water.

<table>
<thead>
<tr>
<th>Sample</th>
<th>( \chi'_{\infty} )</th>
<th>( \chi_0 )</th>
<th>( f_B ) (Hz)</th>
<th>( \alpha )</th>
<th>( D_{\text{hyd}} ) (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHP-25</td>
<td>0.13</td>
<td>0.44</td>
<td>26900</td>
<td>0.1</td>
<td>27</td>
</tr>
<tr>
<td>SHP-30</td>
<td>0.04</td>
<td>0.27</td>
<td>14300</td>
<td>0.04</td>
<td>33</td>
</tr>
<tr>
<td>SHP-40</td>
<td>0.03</td>
<td>0.63</td>
<td>4800</td>
<td>0.03</td>
<td>47</td>
</tr>
<tr>
<td>SHP-50</td>
<td>0.03</td>
<td>0.73</td>
<td>3600</td>
<td>-</td>
<td>53</td>
</tr>
</tbody>
</table>

The Cole-Cole parameters obtained from the curve fits were remarkably small, especially for SHP-30 and SHP-40. The slightly broader size distribution observed for SHP-25 could be due to partial contribution an internal relaxation process. The comparably high value of \( \chi'_{\infty} \) might be a spin-canting effect [15], or perhaps even more likely due to an overlapping Néel relaxation. The hydrodynamic diameters \( D_{\text{hyd}} \) were derived from the fitted peak frequencies \( f_B \) assuming the viscosity of the medium was equal to that of water. The resulting values along with the Cole-Cole fitting parameters can be seen in table 2.

The SHP-50 sample shows an asymmetric \( \chi'' \) peak broadening, possibly due to particle aggregation, which cannot be accounted for by a Cole-Cole equation. Instead, equation (9) was averaged over a distribution of relaxation times, where the distribution function chosen was a generalised gamma distribution [56] as it allows for arbitrary skewness. The resulting curves and fitting parameters are shown in fig. 6 and table 2, where the hydrodynamic size was derived from the distribution mean relaxation time.

Effects of varying the concentration. AC susceptibility measurements were also performed on SHP-25, SHP-30, SHP-40 and SHP-50 that had been diluted with deionized water by a factor of 2. In addition SHP-25 and SHP-50 were also diluted by a factor of 5. The resulting curves coincide almost perfectly when accounting for the dilution factor, which would imply that dipolar interactions are insignificant in these samples. For SHP-50 this would also suggest that if the particles have agglomerated, this cannot easily be reversed and that the distribution of aggregates is very homogenous. It could possibly also mean that the particles are not allowed to move in a three-dimensional way due to their non-spherical shapes.

High-frequency susceptibility measurements. The in-development Imego DynoMag HF AC susceptometer was used to measure the high-frequency (25 kHz - 10 MHz) relaxation spectrum for the three smallest sets of particles at
Figure 6: **Brownian relaxation.** Top panels: In-phase (left panel) and out-of-phase (right panel) components of the AC susceptibility for SHP-25 (triangles), SHP-30 (squares) and SHP-40 (circles). Solid lines are fits to the Cole-Cole equation. Lower panels: In-phase and out-of-phase components of the AC susceptibility (left and right panels, respectively) for SHP-50 (circles), where the solid lines are a fit to a set of Debye relaxations averaged over a generalised gamma distribution of relaxation times.
Figure 7: **SHP-15 and SHP-20.** In-phase (right panels) and out-of-phase (left panels) components of the AC susceptibility for SHP-15 (upper panels) and SHP-20 (lower panels). SHP-15 does not exhibit any relaxation in the measurement frequency range, while the out-of-phase peak seen at the uppermost frequencies for SHP-20 is much too small in relation to its width and the magnitude of the in-phase component to be identified with the maximum of a single relaxation process.
room temperature. The equipment and measurement procedure is the same as for the DynoMag, but the detection-coil system has been engineered to operate at a higher frequency range. In addition, the frequency dependent susceptibilities for SHP-20 and SHP-25 at frequencies 20 Hz - 200 kHz were measured using the same type of equipment as that used in the previous section, in order to obtain the full Brownian relaxation spectrum. As SHP-15 was not measured using this particular apparatus, the data from the previous section have been included together with the high-frequency data in fig. 8. As the previous measurements were conducted at temperatures a few degrees than the ones presented here, the small difference between the two measurement systems might appear be somewhat bigger than it actually is.

As indicated in fig. 8, SHP-25 shows only distinct features of Brownian relaxation, but the shift of the out-of-phase component of the susceptibility towards higher frequencies suggests a partially overlapping Néel relaxation, as already suspected. Both SHP-15 and SHP-20, however, are governed by Néel relaxation, and should, judging from the known data, have their characteristic relaxation peaks located at GHz frequencies.

**AC susceptibility vs temperature**  AC SQUID measurements were performed on SHP-15, SHP-20 and SHP-25 by observing the temperature dependent magnetisation under the influence of fixed-frequency probing field of magnitude 4 Oe. The AC measurement procedure is similar to that of the RSO, but the sample response is measured at two locations inside the SQUID pickup coil, and the AC field is provided by a copper coil which is concentric with

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**Figure 8: Extended relaxation spectrum.**
the superconducting DC magnet. A nulling waveform is used to cancel out the signal from the applied field, and the AC measurement procedure in general allows a sensitivity comparable to that of the RSO [57]. The measurements were preceded by following a sequence of steps [58] to minimise remnant fields in the sample chamber (to the order of $10^{-2}$ Oe). These include degaussing the magnetic shield, removing trapped currents from the superconducting magnet [59] and further the reducing the remaining field with the aid of a small field coil and a fluxgate magnetometer [60].

The temperature was varied from 10 K up to 250 and 270 K for SHP-15 and SHP-20, respectively, using the AC field frequencies 1.7, 17 and 170 Hz, and for SHP-25 the measurement temperature ranged from approximately 160 K up to 270 K using fields of frequencies 0.095, 0.95, 9.5 and 950 Hz. The measurements on SHP-15 and SHP-20 showed similar characteristics in the form of frequency dependent maxima of the $\chi'$ and $\chi''$ components, which both were shifted towards higher temperatures as the frequency of the applied field increased, as shown in fig. 9. The $\chi'$ components are nearly frequency independent above the cusp temperature, while exhibiting a frequency dependency below the $\chi'_{\text{max}}$ temperature, in consistency with a superparamagnetic blocking behaviour. The magnitude of the out of phase components decrease with increasing frequency, indicating that interparticle interactions are small in these samples [61].

SHP-25 on the other hand shows an increase in magnitude of the $\chi''$ components (fig. 9) with decreasing frequency, implying the existence of significant dipolar interactions in the sample. Furthermore, no in-phase maxima and only one out-of-phase maximum, at 260 K for the longest observation time, can be seen, suggesting that these particles to a large degree are superparamagnetically blocked at room temperature.

Trying to relate the experimental curves at the different frequencies for SHP-15 and SHP-20, noting that $\tau = 1/(2\pi f)$, by fitting the $\chi'/\chi''$ peak locations to equation (4) and neglecting the weak temperature dependence of the pre-exponential factor $\tau_0 N = \tau_0 \sigma^{-1/2}$, produced unphysically small values of $\tau_0 N (< 10^{-20}$ s.). Before proceeding by taking the logarithm of equation (23) and making a linear fit to the data, one must realise that the fitting parameters are not uniquely defined as the possible values of $T_0$ solely depend on the constraints imposed on the pre-factor $\tau_0 \nu F$ and vice versa. The constraint $\tau_0 \nu F \in (10^{-12}, 10^{-8})$ s resulted in such large values of $T_0$ that it would lead to a divergence of the relaxation time at a rate not supported by the experimental data. The number of data points available here are an absolute minimum to make any such fit meaningful and it can only be concluded that the data is in disagreement with the models used.

As a consequence of the fluctuation-dissipation theorem, the shape of the $\chi'' - T(\propto 1/\sigma)$ curve can be used to gain information about the distribution of energy barriers. The low temperature data for SHP-15 and SHP-20 have a non-exponential decay as $T \to 0$, a behaviour identified with the existence of a non-thermally activated relaxation process [62]. It has also been shown [15, 51] that non-collinear spin structures can cause an identical low-temperature behaviour, which should be the correct interpretation here.
Figure 9: **AC susceptibility vs temperature.** In-phase (top) and out-of-phase (bottom) components of the temperature dependent susceptibility of (left to right) SHP-15, SHP-20 and SHP-25. The magnitude of the out-of-phase components decrease with increasing frequency for SHP-15 and SHP-20, implying that interparticle interactions are insignificant in these samples. SHP-25 however show the opposite behaviour.
The high-temperature data show that all the samples are out of equilibrium at the highest measurement temperature as $\chi''$ is non-zero here (for proof see ref. [63]). This means that the internal (Néel) relaxation mechanism is effective to some degree when the samples are in the liquid phase, verifying the conclusions drawn from isothermal AC susceptibility measurements.

### 3.2 Comparison with particles from Micromod

Earlier experiments [27, 64–71] have mainly been made using magnetic beads consisting of a cluster of small superparamagnetic particles contained within a non-magnetic coating, manufactured by Micromod [72]. Raw measurement data, used for presenting the results in a recent publication [71] were obtained for comparison. The particles investigated were magnetic beads with nominal physical diameters 50 (BNF-starch, will be labeled BNF-50), 130 and 250 nm (Nanomag-D, will be labeled ND-130 and ND-250), consisting of smaller iron-oxide grains, mainly magnetite, with, for BNF-50, diameters 20 nm and for the latter two, diameters 5 - 15 nm. The iron-oxide grains were held together by a starch (BNF-50) or a dextran (Nanomag-D) casing, and all beads had amine surface functional groups. The particles were dispersed in deionized water, with an iron concentration of 5.1 mg/cm$^3$ (BNF-50) and 7.25 mg/cm$^3$ (Nanomag-D)\(^4\). Assuming as for the Ocean Nanotech particles, that 1 mg Fe corresponds to 1.38 mg Fe$_3$O$_4$\(^5\), the iron-oxide concentrations were 7.6 and 10 mg/cm$^3$ respectively.

**Static magnetisation** Hysteresis measurements were made at 300 K by SQUID magnetometry, using the same equipment and sample preparation as for the Ocean Nanotech particles. The field was however first varied from 0 Oe, to 50 kOe before being cycled back and forth between $\pm 50$ kOe.

The resulting curves, shown in fig. 10, have a typical superparamagnetic appearance with practically zero coercivity as well as a saturation tendency in the form of an almost zero high-field susceptibility. The background subtraction used was the same as for the Ocean Nanotech particles, i.e. that of a linear fit to the magnetic moment of an empty sample holder, which at some time during this work was found to be a more appropriate choice than that of a sample holder including the same amount of dispersant as the ferrofluid sample. The latter might in fact have been a better choice, and was used in ref. [71]. However, in order to make a proper comparison between the different types beads, the same background subtraction should be used, thus only the signal from the empty sample holder was subtracted. This choice might exaggerates the saturating tendency seen for the Micromod particles, but the magnetisations are higher and initial slopes are definitely steeper than for the room-temperature blocked particles from Ocean Nanotech.

\(^4\)Measured by Micromod, using a spectrophotometric method, for the specific batches of BNF-50 and Nanomag-D 250 used. The Nanomag-D 130 particles were purchased at the same occasion and had the same nominal iron content as ND-250, and it is assumed that using this iron concentration is sufficiently accurate.

\(^5\)This procedure was recommended by Micromod to estimat the iron-oxide content.
Figure 10: **Magnetisation curves, Micromod.** Magnetisation curves measured at 300 K for particles from Micromod. Triangles correspond to BNF-50, squares and circles to ND-130 and ND-250 respectively.

![Magnetisation curves](image)

**AC magnetisation**  The frequency dependent susceptibilities were also measured in the frequency range 1 Hz - 250 kHz, where the measurement procedure was the same as for the Ocean Nanotech particles. As seen in fig. 11, the particles have a large part of their characteristic relaxation spectrum at frequencies below those accessible by the DynoMag.

A fit to the Cole-Cole equation yielded fairly large values of the $\alpha$ parameter and for BNF-50 and ND-250 the sizes therefrom derived were much different than their nominal ones, as shown in table 3.

The data points at frequencies below 10 Hz, however, were discarded, even though the ND-250 sample showed a well-defined out-of-phase peak at 7 Hz. When using all measurement data it was impossible to produce an even remotely acceptable fit to any expression containing a unimodal (or even bimodal) distribution of sizes, in spite of trying every thinkable optimisation technique. Removing the data below 10 Hz, using a simple unconstrained minimisation algorithm (Levenberg-Marquardt method), turned out to be a rapidly convergent procedure when the sum of squares error was minimised with respect to a Cole-Cole expression, which what is shown in 3. The resulting fitting parameters for the other particle sizes were hardly affected by this and the data below 10 Hz were discarded for these particles too in order to achieve some kind of consistency.
Table 3: **Cole Cole fits for Micromod particles.** Fitting parameters and hydrodynamic sizes for particles from Micromod.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$\chi_\infty$</th>
<th>$\chi_0$</th>
<th>$f_B$ (Hz)</th>
<th>$\alpha$</th>
<th>$D_{\text{hyd.}}$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BNF-50</td>
<td>0.06</td>
<td>0.54</td>
<td>414</td>
<td>0.14</td>
<td>107</td>
</tr>
<tr>
<td>ND-130</td>
<td>0.06</td>
<td>0.28</td>
<td>184</td>
<td>0.2</td>
<td>140</td>
</tr>
<tr>
<td>ND-250</td>
<td>0.06</td>
<td>0.95</td>
<td>6</td>
<td>0.36</td>
<td>427</td>
</tr>
</tbody>
</table>

Figure 11: **Dynamic susceptibility for Micromod particles.** In-phase (left panel) and out-of-phase (right panel) components of the frequency dependent susceptibility for BNF-50 (squares), ND-130 (circles) and ND-250 (triangles). Solid lines are fits to a Cole-Cole equation.
4 Summary and conclusions

The particles from Ocean Nanotech with a nominal size over 20 nm were seen to have a blocked moment at room temperature, as well as very narrow size distributions, the only exception being the largest particles which showed signs of aggregation. A general characteristic observed for all the particles appeared to be a reduced magnetic moment compared to that of the bulk material.

The approximate $M_0$ values only correlate to a very limited degree with the low-field, low-frequency response observed in AC susceptibility measurements. In ref. [73] it is nicely shown how, for any particle size (defined as its distribution mean), both the low field static susceptibility and initial stepness of the magnetisation curve increase with increasing size-distribution width. This might very well turn out to be the best explanation for the essentially opposite behaviour seen in the Micromod samples, which all have wide size distributions and fairly high magnetic moments.

Although the magnetic moments of the Ocean Nanotech particles were smaller than expected, a too high magnetic moment is incompatible with a non-interacting monodisperse system of particles due to the ferrocolloidal stability criterion. This was already realised by Connolly and St. Pierre when they initially proposed this method of biodetection, as they required the use of particles with a low magnetic moment to avoid aggregation.

The theoretical justification for assuming that the multi-grain particles from Micromod behave as blocked single-domain particles is rather unclear as refs. [27, 64–69, 71] do not clarify this matter, nor do they cite any references which do. Per definition, all three fundamental requirements, which are single-domain, superparamagnetically blocked and non-interacting particles, will be violated to some degree when using particles from Micromod. Whether this is of any importance remains an open question.

Furthermore, the location of the characteristic relaxation peak should be another important point of consideration, as the more measurement points available, characteristic to a unique set of relaxation events, increase the certainty to which the initial size distribution can be determined. This speaks in favour of using the three blocked, non-aggregated sets of particles from Ocean Nanotech (i.e. SHP-25, SHP-30 and SHP-40). Also, the flatter the low-frequency spectrum is, the better the possibility to observe any contribution from immobilised beads in the event of a positive sample, and the ability to rule out the former should also significantly improve. As the Micromod particles have a lot of their relaxation characteristics located at frequencies below those accessible to the DynoMag, a curve fit, which already constitutes a very blunt and potentially misleading tool for determining a hydrodynamic size distribution, would begin to resemble a mere guessing procedure as an important set of data is unknown for these particles.

Another disadvantage of having a wide size distribution would be the non-negligible possibility of an actual multimodal distribution of sizes not resolvable by measurements on the surface-functionalised ensemble, which would thus ap-
pear unimodal. Any of the specific perturbations needed for completing the diagnostic procedure, which for instance could be changes in viscosity and alteration of the hydrodynamic mobility of the beads in the presence of RCA or the change of pH due to the addition of the buffer solution, the latter which would alter the displacement current induced in the carrying medium by an alternating magnetic field, could affect selections of the bead population in different ways. These differences could be sufficient to resolve the multimodal nature of the initial size distribution in a subsequent measurement, thus constituting a potential source of false detection. This might seen far-fetched and too hypothetical, but in relation to, for instance, the deviation from a Cole-Cole model for the Micromod particles considered here or the small differences in the relaxation spectrum identified with the detection of DNA for the lowest concentrations of RCA-coils, this seems like an appropriate concern.

Small particles, which even further should speak in favour for using particles from Ocean Nanotech, has been shown to more frequently hydridise into the interior of the RCA coils [74], which should provide a better possibility of obtaining a narrow distribution of immobilised beads in the biodetection procedure. Small particles should also reduce the time required for completing a diagnostic test, due to faster immobilisation kinetics [66].

The considerations so far has primarily been made with respect to the beads in their non-functionalised state. Some concerns with regards to perhaps very specific issues, might be confused with a critical point-of-view towards the previously made experiments. This requires emphasizing that much valuable work, combining the competence from several specialized areas of research, where the Micromod particles have served a useful tool, lie behind a series of important results. These works have demonstrated the practical achievability [27, 67, 68, 71, 75] of a potentially very powerful method of biodetection previously only described in theory, continuously showing technical improvements, including the extension of multiplex target detection.

Some questions raised regarding certain physical situations have also to some degree been considered and has partially verified by experiments, something which is well-summarised in ref. [28]. However striving for further detection sensitivity, reliability and reproducibility require fixing the most fundamental parameter to the highest possible degree, i.e. the non-manipulated beads should have a very well defined relaxation curve. This can essentially only be fulfilled by using a single-domain, monodisperse and non-interacting set of particles, requirements which are quite well met by the SHP-25, SHP-30 and SHP-40 particles from Ocean Nanotech.

Should the signal from the particles be too weak to acquire sufficient sensitivity, the signal read-out aspect of the assay must somehow be improved. This has already been considered [76] and is embodied in the development of a micro-resistive Hall sensor where the individual beads can be guided within a fluid channel towards the magnetic field sensor by use of the planar Hall effect [77]. The possibility to combine this method with the AC susceptibility technique, as well as other aspects regarding the possibility to transport individual beads is discussed in [76]. Yet another possibility to improve the assay would be to extend
the available frequency range by combining the currently used AC susceptometer with its high-frequency counterpart or to develop an AC susceptometer based on a toroidal technique described in [78], which was particularly considered in the initial proposal of the VAM-NDA.

In order to determine whether the Ocean Nanotech particles in fact are suitable for use in the biodetection assay requires verification by experiment, something that has not yet been possible. Particles from the SPP series (identical to SHP, but also having a 2 mm thick layer of polyethylene glycol) with nominal sizes 25, 30 and 40 nm were purchased from Ocean Nanotech along with a protein conjugation kit optimised for the SPP-40 particles. Following the conjugation protocol, along by varying different parameters in accordance with the recommendations made by Ocean Nanotech in private correspondence, yielded practically zero oligonucleotide bead-surface coverage as determined by fluorescence emission spectra. Ocean Nanotech were very cooperative with regards to this issue and offered to perform the desired surface functionalisation on SHP-30 instead. The particles sent back from them, however had almost equally poor oligonucleotide surface coverage. A different type of DNA conjugation than that previously attempted is considered for 30 nm-sized streptavidin-coated particles (SHS-30-05, 1 mg Fe/cm$^3$). The desired particles were sent as replacement, but have not yet been tried out for DNA conjugation.

Acknowledgments

Supervisor M. Sc. Rebecca Bejhed, Prof. Peter Svedlindh and Dr. Klas Gunnarsson have been helping out with measurements as well as ideas and advice. Dr. Teresa Zardán performed the DynoMag AC susceptibility measurements and have been working on the biofunctionalisation part together with Dr. Mattias Strömberg.

Thanks to Imego, in particular Andrea Prieto Astalan, Fredrik Ahrentorp, Jakob Blomgren, Christer Johansson and the other people involved in the DynoMag, for providing the possibility and help to perform the measurements using their new high-frequency AC susceptometer.

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