Absence of enhanced uptake of fluorescent magnetic particles into human liver cells in a strong magnetic field gradient

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We investigated whether we can detect enhanced magnetic nanoparticle uptake under application of a large magnetic force by tagging the particles with a fluorescent dye. Human liver cells were cultured in a micro-channel slide and exposed to two types of magnetic nanoparticles with a diameter of 100 nm at a concentration of 10,000 particles/cell for 24 hours. Even though we achieved a magnetic force that exceeded the gravitational force by a factor of 25, we did not observe a statistically significant increase of immobilised particles per cell.

Keywords:

I. INTRODUCTION

The interaction of micro- and nanoparticles with cells is a challenging research area of major importance. Micro- and nanoparticles, which may be toxic [1], can enter our body by accident, or they can be administered intentionally in biomedical procedures such as drug delivery [2, 3] and in vivo imaging [4, 5].

Interaction studies between nanoparticles and cells are mainly performed using in vitro cell cultures in multi-well plates [6]. In the case of small molecules, diffusion ensures that the molecule concentration is reasonably constant over the volume of the well. However, micro- and nanoparticles are subject to sedimentation. This has two implications. First, sedimentation gradually increases particle concentration at the cell membrane, the rate of which depends strongly on the particle diameter. Secondly, the particles exert a force on the cell membrane, which may affect particle incorporation [7].

Magnetic micro- and nanoparticles have the advantage that they can be manipulated by external magnetic fields, which is exploited in targeted drug delivery [8, 9], mechano-stimulation [10] and hyperthermia treatment [11]. The magnetic forces that one can apply are orders of magnitude greater than gravitational forces. Therefore, by magnetically attracting nano-particles towards the bottom of the well, we can accelerate sedimentation and increase particle incorporation.

There have been a few reports on the increase of particle uptake. For example, Prijic and colleagues elegantly demonstrated the increased uptake of superparamagnetic particles [12]. They found that the total iron content in the cell, measured by inductively coupled plasma atomic emission spectroscopy, increased by a factor of 3–8. Unfortunately, this method requires one million or more cells. Moreover, one cannot discriminate between the uptake of particles or iron ions in the solution. The same researchers also observed particle uptake by transmission electron microscopy. This method requires fewer cells, but is very labour-intensive. It is also difficult to discern between particles on the cell membrane and those incorporated in the cell.

Rather than observing the particles themselves, one can observe their effect on the cell. One elegant option is to use magnetic particles to transinfect cells, a process called magnetofection [13, 14]. Pickard and Chari [15] demonstrated this by attaching green fluorescent protein (GFP) plasmids to Neuromag SPI-ONs. When the particles entered the cell, the plasmids were reproduced. The subsequent generation of GFP determined whether cells are transfected. The application of force by means of magnetic field gradients enhanced the uptake by a factor of 5. A slow oscillation seemed to have a positive effect.

Particles in a cell can be identified with optical microscopy by means of fluorescence, for which Dejardin and colleagues used ScreenMag-Amine magnetic particles tagged with fluorescein [16]. The particles were treated with activated penetratin to increase their uptake. By integrating the intensity of the emitted light over the sample area, an increase in uptake of about 30% was observed. The magnet used in this experiment was only 13 mm in diameter, leading to particle accumulation in the center of the observation area and subsequent loss of information on particle concentration. A similar approach was taken by Venugopal and colleagues [17] as well as by Park and colleagues [18], who additionally showed by flow cytometry an increase in uptake ranging from a factor of 0.5 (Venugopal) to 7 (Park).

Encouraged by these fluorescence experiments, we investigated whether one can use optical microscopy to observe individual fluorescent magnetic nanoparticles in in vitro cell cultures to detect an increase in
magnetic particle uptake under application of a magnetic force. For this experiment, we designed a system with a large magnet of 70 mm diameter, on top of which Ibidi µ-Slide channel slides could be mounted (Figure 1). Using such a big magnet ensured that the forces are strong and uniform. Human liver cells from a HepG2 cell line in the channel slides were exposed to fluorescent magnetic nanoparticles of 100 nm diameter. The number of particles per area served as a metric to study the influence of the magnetic force. We conclude that our experimental configuration showed no significant effect of the magnetic field.

II. THEORY

The force on a magnetic object that is small compared to the spatial variation of the externally applied field \( B \) [T] can be approximated from its total magnetic moment \( m \) [A/m]

\[
F = -\nabla (mB). \tag{1}
\]

The magnetic moment of a magnetic object in a fluid is generally a function of strength, direction and history of the applied field. The applied field is a combination of the external field and the field of all other magnetic particles in the fluid. Moreover, very small particles will be subject to Brownian motion. Therefore, in principle, the calculation of forces on magnetic particles in a magnetic field gradient is complex. To obtain first approximations, we consider a single particle that is either a permanent magnetic dipole or a soft magnetic sphere.

In the case of the permanent magnetic dipole approximation, we assume a particle with a permanent magnet moment \( \mu_0 m_p = I_r V_p \), where \( I_r \) [T] is the remanent magnetisation of the particle with volume \( V_p \) [m³]. We further assume that field changes are slow such that particles can rotate against viscous drag into the direction of the field. In this case, equation (1) reduces to

\[
F = m_p \nabla B, \quad B = |B|. \tag{2}
\]

For the second approximation, we assume a soft magnetic sphere with a susceptibility of \( \chi \), which is the ratio between the magnetisation \( I \) [T] in the particle and the internal field \( B_{in} \) [T]. As a sphere has a demagnetisation factor of \( 1/3 \), the internal magnetic field is

\[
B_{in} = -B + \frac{1}{3} I = -B + \frac{1}{3} \chi B_{in}
\]

and all fields are (anti-)parallel. In this approximation, the energy and resulting force are

\[
U = -\frac{1}{2} V_p \frac{3\chi}{\mu_0(3 + \chi)} B^2
\]
\[
F = \frac{1}{2} V_p \frac{3\chi}{\mu_0(3 + \chi)} \nabla B^2 \tag{3}
\]
\[
= V_p \frac{3\chi}{\mu_0(3 + \chi)} (B \nabla)B.
\]

The factor \( 1/2 \) originates from integrating from \(-\infty\), where the energy is 0, and we used the vector identity \( \nabla B^2 = 2(B \nabla)B \).

III. EXPERIMENTAL

To maximise uniformity, we based the magnetic field system on the largest NdFeB magnet we could readily obtain (Supermagnete.de). This magnet has a diameter of 70 mm, a height of 35 mm, and is made of N45, which is specified to have a remanent magnetisation of 1.3T. These magnets can seriously injure the experimentalist if accidentally brought too close together. Therefore we encased them in PVC (70 mm (121605), 100 mm (121457) and 125 mm (121620) reducer rings from Wildkamp, Netherlands). The position of and distance between the µ-Slide channel and the magnet were accurately fixed using a 3D-printed nylon plastic top holder (see Figure 1). The source files for the 3D-printed holder are available as additional material.

To calculate the magnetic field and forces generated by the magnet, we integrated the magnetic charge densities. In contrast to finite-element methods, the field is calculated only at the points of interest, which is much faster at high precision. The resulting equations are generated automatically by the MagMMEMS package, which is a preprocessor for Cades [19]. The input files are available in the supplementary material.
The magnetic field above the magnet is measured with a MetroLab THM1176 three-axis Hall sensor attached to a microscope glass slide to obtain the field components at the same height as the \( \mu \)-Slide channel. To visualise the particle density, we used Ibidi \( \mu \)-Slide I Luer channels (Ibidi 80176) filled with bare iron-oxide nanoparticles of 5 nm diameter (EMG304 by FerroTec).

For cell studies, human hepatoma HepG2 cells (ATCC, HB-8065) were cultured in Eagle’s minimal essential medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin in an incubator at 37 °C and 5% CO\(_2\) atmosphere. The cell concentration was determined by means of a hemocytometer and diluted to \( 5 \times 10^4 \) cells/mL. Of this solution, 30 \( \mu \)L was introduced into Ibidi \( \mu \)-Slide VI 0.4 channels with an Ibireat surface coating to promote cell adhesion (Ibidi 80606). The dimension of the channels is 0.4 mm \( \times \) 3.8 mm \( \times \) 17 mm, hence the maximum cell density is 23 cells/mm\(^2\).

The Ibidi channels were left inside the incubator for 24 hours on top of the holder with and without a magnet before analysis. To assess cell viability, we used a live/dead double-staining assay (Sigma-Aldrich, 04511). Analysis was performed on 10 images chosen randomly over the channel area. Average and standard deviations were calculated from three independent experiments (30 images in total).

We studied the interaction between cells and magnetic nanoparticles with an average diameter of 100 nm. For this we used red fluorescent cross-linked dextran iron-oxide cluster-type particles (94-00-102 from Micromod) with a specified iron concentration of 6 mg/mL and a particle concentration of \( 6 \times 10^{12} \) particles/mL. In addition, we used green fluorescent (510 nm) iron oxide incorporated conjugated polymer particles (905038 from Sigma-Aldrich) with a specified iron concentration of 100(10) \( \mu \)g/mL. The composition of the polymer and the particle concentration are not specified by the manufacturer.

Both suspensions were first diluted by a factor of 235 and added to the cell culture medium in a volume ratio of 1:51. The final particle concentration for the MicroMod particles in the cell culture is therefore \( 5 \times 10^8 \) particles/mL with an iron concentration of 0.5 \( \mu \)g/mL. Hence, there is an average of \( 1 \times 10^4 \) particles per cell in the medium. The final iron concentration for the cell culture with Sigma-Aldrich particles is 8 mg/mL.

Images were taken with a Leica DMi8 fluorescence microscope at a size of 2048 pixel \( \times \) 2048 pixel. Both a 20× and a 40× lens were used, calibrated at 324 and 162 nm/pixel, respectively.

Particles were counted using ImageJ software (Wayne Rasband, NIH, USA). The image taken with a red (Micromod particles) or green (Sigma-Aldrich particles) filter was converted into 8-bit greyscale. An intensity threshold was applied to create a 1-bit mask.

The “Analyze Particles ...” script was run to count the number of particles using a lower size threshold. Both the intensity threshold and size threshold were varied. The ImageJ script that automates this process and generates the overlay image is available as supplementary material.

### IV. RESULTS AND DISCUSSION

#### A. Field and forces

The force field above the permanent magnet varies with distance to the magnet surface in both strength and direction. For experiments with magnetic nanoparticles inside the channel slide, we want the lateral forces in the plane of the channel to be as small as possible, yet the vertical force to be as high as possible and very uniform over the area of interest. Using the soft sphere model of equation (II), we determined that the optimal height of the channel slide for both conditions is 10.5 mm above the magnet.

Figure 2 shows the calculated magnetic field components perpendicular to the channel (\( B_x \)) and along the channel (\( B_z \)) at this optimum height, compared with field measurements in the center and at the edge of the magnet. A fit of the calculation to the measure-
FIG. 3: Calculated force densities in vertical (red) and lateral (black) direction as a function of the position over the length of the channel slide. The top curve indicates the case where the particle is a permanent magnet with remanent moment $I_r$ (equation 2). To obtain the force, multiply by $V_p I_r$. The bottom curve indicates the case where the particle is a sphere of volume $V_p$ and susceptibility $\chi$ (equation II). To obtain the force, multiply the value on the vertical axis by $3V_p \chi/(3 + \chi)$.

Figure 3 shows the forces on particles for both the permanent dipole magnet (top) and soft sphere (bottom) models. For the permanent dipole model, the forces are normalised to the particle magnetic moment $I_r V_p$ [Tm$^3$]. A typical particle has a magnetisation in the range from 0.1 to 1 T, so force densities are on the order of 1 to 10 MN/m$^3$. For comparison, the gravitation force density on a typical iron-oxide particle is 40 kN/m$^3$ (gravitation acceleration times mass density difference with water).

For the soft sphere model, the forces are normalised to $3V_p \chi/(3 + \chi)$, which ranges from 0 to 3$V_p$. A typical iron-oxide particle has a susceptibility greater than 1 [20], meaning that force densities are in the same range as for the permanent dipole approximation.

Both models show a variation of 14% in the vertical component of the force over the length of the 30 mm channel. The soft sphere model has a much weaker lateral force. At the entry of the channel, the force only tilts about 1° inward, whereas for the permanent dipole model, it tilts at 10°.

The large magnet produces a very uniform distribution of magnetic particles. Figure 4 shows a channel slide filled with a diluted suspension of 5 nm iron-oxide particles. Immediately after filling, no concentration gradient can be observed. After several hours, a slight reduction in concentration is observed close to the channel entries, yet the concentration appears optically uniform over the area of interest. Therefore, this magnet configuration applies forces that are at least 25 times higher than the gravitational force, perpendicular to the surface and uniform over most of the channel slide.

### B. Cell viability

Two different fluorescent magnetic particles were used: cross-linked dextran iron-oxide composite particles (from Micromod) and iron oxide incorporated conjugated polymer particles (from Sigma-Aldrich). Both particles have an average diameter of 100 nm. These particles are expected to be non-toxic as they were developed especially for cell studies. To confirm this, we performed a cell viability test on HepG2 cells exposed for 24 hour to approximately 10,000 particles per cell. Experiments were performed both with and without application of a magnetic field. Figure 5 shows a typical result with a very small number of dead cells (red). Analysis of thirty images from three independent measurements shows that the survival rate is higher than 90%, which is equal to the control without particles within measurement uncertainty.

### C. Particle counting

Only the fluorescent particles can be imaged by means of an optical filter. To eliminate observer bias, and for practical reasons, particle counting was automated using ImageJ software (see Section III). The image analysis procedure has two adjustable param-
FIG. 5: Composite images of a live/dead cell viability assay of $5 \times 10^4$ HepG2 cells/mL exposed to $5 \times 10^8$ nanoparticles/mL for 24 hours. Dead cells are displayed in red, live cells in green. Top: Cells exposed to 100 nm diameter cross-linked dextran iron-oxide composite particles (Micromod). Bottom: 100 nm diameter iron oxide incorporated conjugated polymer particles (Sigma-Aldrich). The right-hand images are of cultures that were placed on the magnet. The cell survival for both types of particles and with or without field is higher than 90\%, which is equal within measurement error to the control. Therefore, we conclude that these particles are non-toxic for a period of 24 hours, as expected.

FIG. 6: Number of detected red fluorescent particles as a function of the minimum size in pixels (top, using an intensity threshold of 25) and detection threshold (bottom, using a size threshold of 9 pixels). The number of detected particles is very sensitive to these settings, especially to the intensity threshold level.

eters. The first is the threshold size of the observed particle in pixels. This value should be sufficiently high to avoid false detection due to noise, yet small enough not to miss particles. With a 20× lens, the pixel size is 324 nm, which is lower than the wavelength of the emitted light, so the minimum threshold size should be larger than 2 pixel × 2 pixel. Figure 6, top image, shows that the number of detected red fluorescent particles (Micromod) decreases with increasing threshold size. Above 3 pixel × 3 pixel, the decrease is linear. We assume that the rapid drop for small threshold sizes is caused by noise. A reasonable value of 3 pixel × 3 pixel yields a value that is about half the maximum value.

The second parameter is the intensity threshold, which discriminates between the background and the particles. This value lies in the range of 1–255. It should not be so low that it avoids false detection by noise, yet not so high that it misses faint particles. In contrast to the size threshold, the amount of particles detected is extremely sensitive to the intensity threshold, see the bottom graph of Figure 6. This is illustrated in the ImageJ results of the particle detection algorithm shown in Figure 7. At a threshold of 24, the non-uniform illumination disturbs the image, whereas the output seems reasonable from 26 to 28. However, the particle count varies by a factor of 10 over this small range.

The total number of particles expected from the concentration of $5 \times 10^8$ particles/mL is approximately $1 \times 10^5$ in the field of view. It is clear that, whatever the settings, the method grossly underestimates the number of particles. This is very likely due to particle agglomeration, but one should not rule out that particles lose their fluorescent properties. This does not render the method entirely useless. By using an identical method to detect particles in different images, a relative comparison can be made between the case with and without the magnet. Therefore, we analysed images with a size threshold of 3 pixel × 3 pixel and an intensity threshold as that value at which the non-uniform illumination disappears (e.g. 25 in Figure 7).
D. Cell and particle count

The image analysis method was applied to human liver cell cultures with Micromod and Sigma-Aldrich particles, both with and without magnetic field. Figure 8 shows composite images of typical results for these four cases. We superimposed the location of detected particles in red (Micromod particles) and green (Sigma-Aldrich particles) on top of the greyscale bright field images. The cell and particle densities appear more or less similar. We performed statistical analyses of 61 images (for Micromod 17 and 7, for Sigma-Aldrich 23 and 14, away from and on top of the magnet, respectively). Figure 9 shows there is no clear difference in particle density among the four cases. There is a hint that the particle density is lower in the experiment with the MicroMod particles (left) on the magnet (red). Given the uncertainty in the particle detection algorithm, however, we refrain from drawing a definite conclusion.

In addition to particles, we also analysed the number of cells. Figure 10 shows the observed cell density for the four different cases. As the particles do not affect cell viability, we do not expect major differences. Indeed, the cell density does not exhibit a statistically significant dependency on particle type or field condition. The cell density is in agreement with the cell concentration in the administered solution (Section III), indicated by the black line labeled “theory”.

We also counted the number of particles registered with cells in the bright field image. Figure 11 shows that, again, there is no clear difference between the cells that were away from and those on top of the magnet. However, it is surprising that we observed less than ten particles on top of cells, which is far too few. From the cell and particle densities shown in Figures 9 and 10, one would expect approximately 40 particles per cell. A possible explanation could be that particles do not adhere well to the cell membrane. Nevertheless, one should not rule out that particles may be incorporated into the cell and lose their fluorescent properties.

From these observations we conclude that the type of particles used in this experiment is not very suitable to determine whether there is an enhanced uptake of magnetic particles under application of a strong field gradient. Compared to the uncertain-
FIG. 9: Particle density obtained from image analysis for samples with two different magnetic particles, away from (grey) and on top of (red) the magnet. A box is drawn around the region between the first and third quartiles, with a horizontal line at the median value. Whiskers extending from the box encapsulate two-thirds of the data points. Data outside that range is indicated by points. There is no statistically significant difference in the four cases. For the Micromod particles, there is a hint of a reduction in particle density when the sample was on the magnet.

FIG. 10: Observed cell density for samples with two different magnetic particles, away from (grey) and on top of (red) the magnet. The cell density is on the order of the expected value (black line labeled “theory”) and there is no significant influence of the magnetic field.

FIG. 11: Number of particles that coincide with a cell for two different magnetic particles, away from (grey) and on top of (red) the magnet. The differences are not significant. As in the particle count for the Micromod particles (Figure 9), there might be some reduction in particles per cell under application of a field.

By using a cylindrical magnet with a diameter of 70 mm, the vector components of the field and force in the vertical direction were dominant, with a strength in excess of 300 mT and 6 MN/m³, respectively. Over the length of the 30 mm channel, the force strength remains within 14% and the force direction varies by less than 1°.

HepG2 human liver cells exposed for 24 hours showed no significant change in cell viability when exposed to cross-linked dextran iron-oxide composite particles (Micromod) or iron oxide incorporated conjugated polymer particles (Sigma-Aldrich), both of 100 nm diameter and with a concentration of 10 000 particles/cell.

The number of fluorescent particles detected by optical microscopy depends by at least one order of magnitude on the settings of the automated image detection algorithm. The number of detected particles is especially sensitive to the intensity level threshold.

Analysis of over 60 images did not show an increase in the number of observed magnetic particles overlapping with cells when a magnetic force is applied. On the contrary, the particle density seems to be a factor of 40 lower on cells than in regions without cells.

From these measurements, we conclude that using fluorescent magnetic nanoparticles to demonstrate enhanced particle uptake in magnetic fields is far from trivial, and may not be the optimal approach.

V. CONCLUSIONS

We constructed a magnetic system that exerts a strong force on fluorescent magnetic nanoparticles inside a channel slide with a human liver cell culture.
