Evaluation of In-Situ Magnetic Signals from Iron Oxide Nanoparticle-Labeled PC12 Cells by Atomic Force Microscopy

Lijun Wang1,∗, Yue Min1, Zhigang Wang1, Cristina Riggio2,†, M. Pilar Calatayud3,†, Josephine Pinkernelle4, Vittoria Raffa2, Gerardo F. Goya3, Gerburg Keilhoff4, and Alfred Cuschieri1

1Institute for Medical Science and Technology (IMSaT), College of Medicine, Dentistry and Nursing, University of Dundee, 1 Wurzburg Loan, Dundee DD2 1FD, UK
2Scuola Superiore Sant’Anna, Piazza Martiri della Libertà, 33 56127 Pisa, Italy
3Instituto de Nanociencia de Aragón, Universidad de Zaragoza, 50018 Zaragoza, Spain
4Otto-von-Guericke University, Institute of Biochemistry and Cell Biology, D-39120 Magdeburg, Germany

The magnetic signals from magnetite nanoparticle-labeled PC12 cells were assessed by magnetic force microscopy by deploying a localized external magnetic field to magnetize the nanoparticles and the magnetic tip simultaneously so that the interaction between the tip and PC12 cell-associated Fe3O4 nanoparticles could be detected at lift heights (the distance between the tip and the sample) larger than 100 nm. The use of large lift heights during the raster scanning of the probe eliminates the non-magnetic interference from the complex and rugged cell surface and yet maintains the sufficient sensitivity for magnetic detection. The magnetic signals of the cell-bound nanoparticles were semi-quantified by analyzing cell surface roughness upon three-dimensional reconstruction generated by the phase shift of the cantilever oscillation. The obtained data can be used for the evaluation of the overall cellular magnetization as well as the maximum magnetic forces from magnetic nanoparticle-labeled cells which is crucial for the biomedical application of these nanomaterials.


INTRODUCTION

Labeling of mammalian cells with superparamagnetic nanoparticles has been increasingly used in biomedicine, e.g., cell homing and tissue regeneration, cell manipulation and separation, molecular detection, magnetic hyperthermia, drug delivery, magnetic resonance imaging (MRI) and tracking;1,2 and all these applications rely on the magnetic properties of the nanomaterials or their interaction with an external magnetic field. There are a number of approaches that can be applied to evaluate cell labeling by magnetic nanoparticles (MNPs). These include at least optical microscopy, MRI, inductively coupled plasma mass spectrometry (ICP-MS), magnetometry, electron paramagnetic resonance (EPR), and transmission electron microscopy (TEM). While all these methods have certain advantages, they have a common drawback, i.e., none of those approaches, except MRI, can be used to assess the in-situ magnetic signals from the nanoparticles following uptake by cells. Although usually fully characterized prior to cell labeling, the MNPs may lose or change their physicochemical and hence magnetic properties after cellular uptake especially as the majority of nanomaterials are internalized by endocytic pathways, and depending on their intrinsic stability, they are prone to degradation in the acidic environment present within the endosome/lysosome compartments.3,4 Hence, there exists a need for reliable information on the fate of cell-associated MNPs and their residual magnetic properties by re-evaluating the magnetic signals following their binding with the cells or internalization under the physiological conditions. Although MRI is ideal to monitor the bio-distribution of magnetically
In the present study, we used Fe$_3$O$_4$ nanoparticles that can be detected by this technique by specific tip-sample interaction (magnetic force microscopy, MFM) enabling the study of macro-scale magnetic domains in materials and more recently, in analyzing free magnetic nanoparticles.\textsuperscript{8–11} With the combined AFM/MFM technique, the topography is first acquired by the main scan using the dynamic mode AFM, with the MFM imaging being obtained after the tip is lifted (lift mode) at certain distance from the sample (lift height). This technique provides information on cell topographic features and other properties including magnetic force and localization with high spatial resolution. MFM is hence particularly useful in assessing the magnetic properties of cell-bound MNPs. Reliable identification of the magnetic signals of MNPs in labeled cells is however difficult because of the very weak signals from the nano-scale particles or micro/submicron-scale particle clusters bound to the cell membranes or internalized into the cells. For this reason reported studies on MFM of cells are scant.\textsuperscript{12–14} For efficient detection of weak magnetic signals the distance between the MFM tip and the sample surface (lift height) is commonly kept 20–30 nm.\textsuperscript{12,13} This lift height in MFM studies of cell samples is, however, probably not large enough for selective magnetic detection due to the very irregular texture of the cell surface, which further confounds the weak magnetic signals with non-magnetic interfering forces such as electrostatic, hydrophobic and van de Waals forces. Lift heights of 20–30 nm have been reported to be insufficient even for detection of specific interaction between the MFM tip and free MNP.\textsuperscript{8,11,15} Thus magnetic characterization of cell-bound MNPs remains a challenging problem. In addition, cell MFM images are very difficult to analyze due to the lack of objective parameters. At present there is no established microscopic method which provides a reliable assessment of the in-situ magnetic signals of cell-bound MNPs.

Magnetite iron oxide (Fe$_3$O$_4$) nanoparticles are the most commonly used magnetic nanomaterial in biomedical applications due to the ease of synthesis and high biocompatibility.\textsuperscript{1} Fe$_3$O$_4$ nanoparticles are superparamagnetic by virtue of their small size; hence they exhibit a detectable magnetic force only in the presence of an applied external magnetic field at room temperature.\textsuperscript{8–11,14} In the present study, we used Fe$_3$O$_4$ nanoparticles that were functionalized with nerve growth factor-β (NGF-β) conjugated with a fluorescent tag Alexa Fluor488 as an exemplar of MNP application in biomedicine, to load differentiated rat pheochromocytoma PC12 cells, a commonly used cell culture model for nerve regeneration study. We analyzed the resulting magnetically labeled cells by MFM under a vertical external magnetic field (parallel to the axis of the MFM tip) by placing and shielding a permanent magnet underneath the sample thereby undertaking tip-sample magnetic interaction studies in the presence or absence of the applied magnetic field. We show that by magnetizing the tip and sample simultaneously and by controlling the orientation of the fields of both the tip and the sample by the external magnetic field, the scan lift height for detection of cell-bound iron oxide MNPs by MFM can be increased to more than 100 nm. We further demonstrate that by re-constructing the magnetic phase signals in 3-dimensions (3D) and by performing surface roughness analysis\textsuperscript{15} used in cell topographic study,\textsuperscript{5,16} a semi-quantitative evaluation of average and localized maximum forces of tip-MNP interaction in MNP-labeled cells is possible.

**MATERIALS AND METHODS**

**Materials**

Dulbecco’s minimal essential medium (DMEM), horse serum, fetal bovine serum (FBS), Trypsin-EDTA, L-Glutamine, Penicillin/Streptomycin (Penstrep), Alexa Fluor488, anti-rabbit Alexa Fluor546 secondary antibody were purchased from Invitrogen. Bovine serum albumin (BSA), poly-L-lysine, and NGF-β were purchased from Sigma Aldrich. All chemicals, unless otherwise stated, were purchased from Sigma Aldrich.

The Fe$_3$O$_4$ nanoparticles used in this study were synthesized and characterized as previously reported and were coated with polyethyleneimine (PEI).\textsuperscript{17}

**Functionalization and Fluorescent Labeling of the MNPs**

NGF-β was first conjugated with Alexa Fluor488 by the reaction between the tetrafluorophenyl (TFP) ester of the dye with the primary amines of the protein. The conjugate (NGF$_\text{fluo}$) was purified through a size exclusion resin. The concentration of the purified fluorescence labeled NGF-β protein was evaluated by UV-vis analysis. Functionalization and labeling of the MNPs by NGF$_\text{fluo}$ were carried out by addition of the fluorescent NGF-β (35 μg ml$^{-1}$) to PEI-coated MNPs (500 μg ml$^{-1}$) and stir of the suspension at room temperature for three hours. Unbound NGF$_\text{fluo}$ was removed by magnetic separation (three washing steps) and the functionalized MNPs were re-suspended in water. The amount of NGF$_\text{fluo}$ bound to the surface of MNPs was calculated by subtraction, i.e., by measuring the absorbance at 280 nm of the supernatant derived from the each washing steps. The NGF$_\text{fluo}$ concentration was estimated by using a calibration curve obtained with known concentrations.
The resultant NGF_\text{fluor} functionalized nanoparticles (referred as MNPs) were used in all the following experiments unless otherwise stated. More detailed functionalization of the MNPs was described elsewhere.\textsuperscript{18}

**Cell Culture and MNP Labeling for Fluorescence and Scanning Force Microscopy**

PC12 cells were cultured under standard conditions in DMEM media containing 15% horse serum and 1% Pen-strep. Cells were seeded on the poly-L-lysine pre-coated coverslip (0.13–0.16 mm thickness, VWR) in 12-well plate to reach the confluence of 60–70% before induced to neuronal phenotype in serum-reduced (2%) media supplemented with 50 ng ml\(^{-1}\) NGF-\text{beta} for 4 days. Differentiated PC12 cells were then incubated with the functionalized MNPs (25 \(\mu\)g ml\(^{-1}\)) for 14–16 hrs. For confocal fluorescence microscopy, cells were washed with PBS at 4 \(^\circ\)C for three times and then fixed with 4\% paraformaldehyde for 15 min prior to permeabilization and block with 10\% FBS and 0.3\% Triton X-100. Cells were then incubated overnight with rabbit anti-\beta-III-tubulin (Covance) (1:1000 in PBS + 1\% FBS + 0.3\% Triton X-100). After washing with PBS cells were incubated with anti-rabbit Alexa Fluor546 secondary antibody (1:200 in PBS) for 1 hr. Cell nuclei were counterstained with 1 \(\mu\)g ml\(^{-1}\) of 4',6-diamidino-2-phenylindole (DAPI) in PBS (Roche Applied Science). After washing with PBS coverslips were embedded with Immu-Mount (Thermo Scientific). Microscopic analysis was done with a TCS SPE DMI4000 confocal microscope (Leica, Wetzlar Germany) and edited in the Leica Application Suite 2.3. For AFM/MFM study, the MNP-loaded cells were washed by PBS and fixed with 2.5\% glutaraldehyde for 15 min. The coverslip was washed by deionized water and mounted face up on a glass slide (1.0–1.2 mm thickness, VWR). The sample was air dried and ready for AFM/MFM scan.

**Set Up the Magnetic Force Microscopy System**

All SFM experiments were conducted with the BioScope Catalyst AFM system (Bruker, UK) incorporated with an inverted optical microscope (IX71, Olympus UK). Commercially available standard MFM tips (MESP-RC) coated with a thin layer of magnetic Co/Cr alloy were applied for the MFM measurement (Bruker, UK), and the coercivity of the tips provided by the manufacturer was 400 Oe (31.5 kA/m). To generate the external magnetic field during MFM scanning, a 12 mm diameter/3 mm thickness neodymium magnet (www.first4magnets.com) was installed under the stage of the optical microscope (Fig. 1(A)). The position of the magnet was realized by fixing the magnet on an unused objective under the stage of the inverted optical microscope. The presence or absence of the external magnetic field were controlled by positioning in or turning away the magnet-bound objective by rotating the nosepiece as generally practiced during microscopy. The distance from the MFM tip or sample to the surface of the magnet was estimated (Fig. 1(A)). The vertical distribution of the magnetic field generated

---

**Figure 1.** Schematic representation (not drawn to scale) of the MFM experimental system and magnetic field distribution and direction. A magnet was positioned under the stage of the inverted optical microscope (A). The magnetic field strength was measured as described in the Materials and Methods and plotted as the function of distance from the center of the disc magnet top end (B). The direction of the local magnetic fields (dashed arrowed lines) of the MFM tip and the MNP sample orientated by the external magnetic field (M, solid arrowed line) is shown in (C).
from the magnet top surface upward was measured by a magnetometer (Model #DCM 2320, AlphaLab, Inc. Salt Lake City, USA) and a field strength-distance curve was then plotted as shown in Figure 1(B). The external magnetic field acted on the sample and MFM tip could then be estimated according to the field strength-distance curve, however, the actual applied external field strength when glass slide and coverslip were placed on the microscope stage was also measured by the magnetometer.

Assessment of the Effect of the Installed Permanent Magnet on the Magnetic Force Scanning

The influence of the external magnetic field on the sensitivity and specificity in our experimental setup of magnetic interaction between the tip and the MNPs was first evaluated in free MNPs. MNPs were diluted in water and dispersed on the glass slide. The sample was dried in air before MFM scan using scan rate 0.334 Hz and 256 scan lines for various lift heights (100, 200, and 400 nm).

Detailed lift height analysis of PC12 cell samples was then performed by the MFM setup using lift heights of 60, 80, 100, 140, 200, 300, and 400 nm. The reason to choose such a wide range of lift heights was that magnetic detection depends on the size of MNP clusters, which was the commonly observed forms of membrane-bound or internalized MNPs, and the size of cell-bound MNP clusters often varied greatly. The bigger clusters would produce stronger signal thus could be sensed from longer distance by the tip. By studying a wide range we could optimize a lift height that could be used for most samples. As PC12 cells have a size in the range of 10–30 μm and most cell samples have a size in the range of 10–30 μm with scan rate of 0.334 Hz and 256 scan lines.

Surface Roughness Analysis of Magnetic Force Images of MNP-Loaded PC12 Cells as a Tool to Assess Magnetically Labeled Cells

3D surface texture parameters have been used in topographic analysis in material science and the concept of cell surface roughness has been applied in our previous studies in assessing the topographic properties of the cell membrane in response to different environments. Recently, Nenadović and colleagues introduced the surface roughness parameters for the optimization of lift height in MFM imaging of thin cobalt and nickel films. Here we introduced a variety of statistics to characterize the “magnetic surface roughness” of the cells. It should be noted that only the statistic surface parameters limited to the z-axis representing the change in the oscillation phase of the cantilever due to the magnetic forces between the tip and the sample were used to define MFM images in our study. The parameters we analyzed include \( R_q \), the calculated root-mean-square (RMS) value within the scanned area representing the standard deviation of height data from the mean plane; the skewness \( R_{sk} \), a measure of the asymmetry of the surface data about the mean line profile with a negative skew indicating predominance of valleys resulted from attractive interaction and vice versa positive \( R_{sk} \) representing peaks by repulsive force between the tip and the sample; the kurtosis \( S_k \), the measure of the flatness/sharpness of the surface data about the mean with a “spiky” surface being \( S_k > 3 \) and a “bumpy” surface being \( S_k < 3 \); \( R_p/R_s \), absolute peaks (\( R_p \), positive value) and valleys (\( R_s \), negative value) in the 3D images. The maximum profile peak and valley heights \( R_p \) and \( R_s \) should be assumed as the measures of the maximum repulsive and attractive interactions between the tip and the sample.

In order to avoid non-specific information from areas that was not covered by the cells (i.e., the surface of cell culture cover slip), an area of 10 μm \( \times \) 10 μm in each scanned area (30 μm \( \times \) 30 μm) of MFM images was randomly selected on cells. “Magnetic surface roughness” parameters of the cells were then calculated for the selected areas using NanoScope Analysis software (NanoScope V8.20, Bruker, UK) in which the following equations were applied for the calculation:

\[
R_q = \sqrt{\frac{\sum_{i=1}^{n} (Z_i - \bar{Z})^2}{N}} \tag{1}
\]

\[
R_{sk} = \frac{1}{n R_p^3} \sum_{i=1}^{n} (Z_i - \bar{Z}) \tag{2}
\]

\[
R_{ku} = \frac{1}{n R_p^4} \sum_{i=1}^{n} (Z_i - \bar{Z})^4 \tag{3}
\]

In addition to the above calculated parameters, the maximum vertical distance between the highest and lowest data points \( R_{max} (R_p + |R_s|) \) was also included.

RESULTS

Size Distribution and Magnetic Properties of PEI-Coated MNPs and Their Functionalization with Fluorescent NGF-β

The PEI-coated Fe₃O₄ nanoparticles have octahedral morphology and an average size of 25 ± 5 nm as determined by TEM (Fig. 2). The hydrodynamic diameter of the nanoparticles obtained by dynamic light scattering (DLS) measurement is 73 ± 17 nm, indicating a low degree of aggregation in the colloidal state. Detailed magnetic properties were described in our previous report. The hysteresis loops \( M(H) \) of the synthesized nanoparticles at room temperature show a small but measurable coercivity of \( H_c = 6.1 \) kA/m and a magnetic saturation \( M_s = 58 \) Am²/kg Fe₃O₄.

The conjugation of NGF-β with Alexa Fluor488 (NGF_{Alexa}) was confirmed by the absorbance spectrum in
Figure 2. Characterization of Fe₃O₄ nanoparticles by TEM, (A) Fe₃O₄-PEI and (B) Fe₃O₄-PEI-NGF flour. The images show the magnetic nanoparticles (MNPs) have octahedral morphology and an average size of 25±5 nm.

which two peaks are present: 280 nm typical for proteins and 494 nm specific to Alexa Fluor488 (data not shown). The mole ratio of Alexa Fluor488 to NGF-β was 1.5 (n = 4 replicates). The amount of NGF₅₉₅adsorbed on the MNP surface was 10(±5) μg per mg of MNPs (n = 6 replicates). Detailed absorption analysis results were described elsewhere.18

MNP Cellular Uptake by PC12 Cells Documented by Fluorescence Microscopy

The fluorescence labeling enabled observation of the MNPs by fluorescence microscopy. The confocal images of the anti-β-III-tubulin stained PC12 cell indicated localization of the fluorescent MNPs (Fig. 3(B)) on and inside the cell when analysed by z-levels (data not shown) and rotation from frontal to side view (Fig. 3(C)). The cellular localization of the MNPs was also evaluated by TEM and by SEM-FIB-EDX in previous study.18 Non-functionalized nanoparticles (Fe₃O₄-PEI) could not be visualized by the fluorescence microscopy (Fig. 3(A)). However, the integrity and magnetism of these nanoparticles could not be assessed by the fluorescence imaging as we observed similar images when cells were incubated with Alexa Fluor488-NGF-β conjugate (NGF₅₉₅) (data not shown).

Figure 3. Confocal microscopic images of differentiated PC12 cells. (A) A single PC12 cell loaded with non-fluorescent MNPs (Fe₃O₄-PEI). (B) PC12 cells incubated with functionalized MNPs (Fe₃O₄-PEI-NGFflour). Green: MNPs; Red: tubulin staining; Blue: nucleus staining. The rotation slide of the image reveals various localizations of MNPs on cell membrane or inside the cell (C). White arrow in (B) points to MNPs possibly on or in the neurite. Bars = 25 μm.

Thus fluorescence microscopy does not reveal the real magnetic state of the MNPs following their uptake by cells; i.e., whether the MNPs were degraded with resultant changes/loss of their magnetic properties.

Magnetic Field Distribution of the Permanent Magnet and Its Effect on Tip-Sample Interaction

The distance from the MFM tip or sample to the surface of the magnet was estimated within 3.2–4.4 mm during MFM operation (Fig. 1(A)). So the corresponding magnetic field strength at the tip and the sample was approximately 1020–1390 gauss (81–110.6 kA/m) (Fig. 1(B)). In addition, if the magnet was turned away from the vertical axis of sample/MFM tip under the microscope stage, its effective magnetic field, as measured by the magnetometer, on the sample and tip was reduced to the background level (data not shown). Therefore this experimental system provides a robust MFM setup for studies on superparamagnetic nanoparticle-labeled cells.

Since the coercivity of the MFM tip is 31.5 kA/m (400 Oe), the magnetic intensity at the tip provided by the applied external magnetic field was significantly large, suggesting that the magnetic tip in this experimental set up may not need magnetization, a common prerequisite in MFM studies. As the coercivity and saturation magnetization of the PEI-coated MNPs were $H_C = 6.1 \text{ kA/m and } M_S = 58 \text{ Am}^2/\text{kg Fe}_3\text{O}_4$, respectively, these are unlikely to increase upon functionalization,
the magnetic moments of both the sample and the MFM tip were presumed to be dominated by the much stronger external magnetic field.

MNPs on supporting glass surface were clearly shown in AFM height (Fig. 4(A)) and AFM phase (Fig. 4(B)) scans, however, without the applied external magnetic field, MFM phase contrast of MNP aggregates could just be observed at lift height of 100 nm (Fig. 4(C)). Scans with lift heights of 60 and 80 nm showed similar results (data not shown). When the magnetic field was applied, the MFM phase contrast was clearly observed at lift height 100 nm (Fig. 4(D)). The signal (degree of phase change) was lift height-dependent as it attenuated with increasing lift heights (Figs. 4(D)–(F)). When a non-magnetic tip, i.e., a standard AFM tip (ScanAsyst in air, Bruker UK) was used (in lift mode) to scan the free MNPs on glass surface in the absence and presence of the external magnetic field, no phase contrast was detected (data not shown). These data demonstrate the specific detection of magnetic interaction between the tip and the nanoparticle aggregates. As the polarization of both the tip and the sample by the static magnetic field was maintained consistently opposite to each other as illustrated in Figure 1(C), the attractive interaction hence dark (negative) phase shift between the tip and sample predominated over the repulsive force-induced bright phase contrast in the MFM imaging (Figs. 4(D)–(F)) (see more in the Discussion).

Detection of Magnetic Forces Between the MFM Tip and MNP-Loaded PC12 Cells

It was previously reported that the influence of a substrate and its texture on MFM imaging from thin magnetic film or free MNPs, although depending on the sample topography, scan speed, feedback loop optimization, scan size, etc (see more in the Discussion), can be ignored if the lift height exceeds above 40–45 nm.\(^{8,15}\) We performed the MFM experiments with differentiated PC12 cells at lift height of 60 nm or above to take into consideration the more complex nature of cell surface properties and its rough uneven topography. Extremely weak, almost negligible, phase contrast of cell-bound MNPs was observed in the absence of the magnetic field at lift height of 60 nm (Fig. 5, middle row left). In sharp contrast, exposure to the external magnetic field induced strong MFM phase signals at the same scan lift height (Fig. 5, bottom row left). With increasing lift heights (to 80 and 100 nm) the MFM phase contrast was only slightly weakened (Fig. 5, bottom row) and the phase contrast in the images obtained without the applied magnetic field was negligible (Fig. 5, middle row). Again, attractive forces between tip and the sample were dominant and caused negative (dark) phase contrast in the MFM images of the cells.

In order to evaluate the sensitivity of the magnetic detection, a nanoparticle aggregate on the cell membrane was analyzed. The force sensitivity of the MFM cantilever is...
determined by the ratio of the change in the phase of cantilever oscillation to the randomly fluctuating change in oscillation due to thermal noise. In another experimental system, the minimum detectable MFM signal of MNPs was shown to occur when the signal to noise ratio (SNR) was 1 and the phase noise of the MFM phase images was estimated to be $\pm 1^\circ$. In our experiments, the level of random fluctuation of the MFM cantilever, as implied by the phase shift in the absence of the external magnetic field in Figures 6(C) and (E), may be well within $1^\circ$, and the MFM phase change of the MNPs, when magnetized, was significantly larger than the basal level of thermal noise (Figs. 6(B) and (D)).

These data demonstrate that it is possible to use an external magnetic field to enable analysis of magnetically labeled cells by MFM with a lift height that avoids all non-magnetic interactions between the tip and the sample. Data in both Figures 4 and 5 show not only the lift height-dependence of the magnetic signals detected by the MFM system but also the importance of the lift height in removing artifacts often experienced with AFM imaging. There were no major artificial scratches observed when the lift height exceeded 100 nm in most samples studied. We therefore focused on experiments using lift height larger than 100 nm to investigate the optimal MFM conditions for the assessment of magnetic signals in PC12 cells.

**Optimal MFM Scan Lift Height and “Magnetic Texture” of Cell Surface for the Evaluation of Magnetic Signals from MNPs-Labeled Cells**

These experiments explored lift heights of 100 nm, 140 nm and 200 nm in the evaluation of cell surface texture.
Figure 6. Force sensitivity of the MFM cantilever. A large particle aggregate was scanned and AFM phase (A) and MFM phase images were obtained with lift height 100 nm in the presence (B) and absence (C) of the external magnetic field. The MFM phase shift was analyzed by section plotting as indicated by the horizontal lines in B and C and was shown in D and E, respectively. Note the scale difference in D (') and E (m'). The MFM phase shift in the absence of the applied magnetic field (thermal noise) was within 1°. Bars = 2 μm.

properties generated from magnetic signals. Table I shows the influence of MFM lift height on the measurement of the “magnetic texture” parameters based on the MFM phase contrast on the cell surface.

Overall, all the absolute values of the magnetic roughness parameters increased when MNP-loaded PC12 cells were scanned with the magnet underneath compared with absence of the external magnetic field or non-labeled control cells exposed to the static magnetic field. The data indicated that attractive interaction is predominant since the $R_{sk}$ is negative, with its absolute values being much larger (and also lift height-dependent) in MNP-labeled cells exposed to the magnetic field compared to no such exposure. Labeled cells without the magnetic field or non-labeled samples under the magnetic field produced close to zero values of $R_{sk}$, a profile of relatively even plane with similar amounts of peaks and valleys. The $R_{sk}$ obtained with lift height 140 nm and 200 nm from groups with no magnetic field was however slightly deviated from zero but the reason for this is unknown.

Since the cell surface has a much more complex texture and roughness than previously studied thin films or other inorganic materials, and because the MNPs or MNP agglomerates are randomly distributed in the cells or on the cell surface, we analyzed the maximum valley depth and peak height values ($R_v$ and $R_p$) in order to assess the strongest attractive and repulsive forces between the tip and the cell-bound MNPs, i.e., the maximal magnetic signals associated with the cells under the test conditions. Both $R_v$ and $R_p$ in MNP-loaded cells showed a clear dependence on the MFM lift height in the presence of the external magnetic field and were significantly larger than both control groups, further confirming the close association of the roughness profile with the specific magnetic interaction between the MFM tip and the sample. The $R_v$ and $R_p$ in control samples were very similar, representing a “basal level” of influence of the cell membrane on the measurement. $R_{ku}$ indicates the “spikiness” of the membrane surface.15 Stronger magnetic interaction caused the $R_{ku}$ further away from 3 as shown in the table. The $R_{ku}$ of non-labeled control cells (3.076) was very similar to that of MNP-loaded samples with tip-sample distance of 140 nm in the absence of magnetic field (2.990), suggesting that, in the absence of an external magnetic field, the surface magnetic environment of MNP-loaded cells that could be sensed by the MFM tip at lift height 140 nm may have reached the “background level” as represented by the non-labeled cells (when exposed to the external magnetic field). We then examined MNP-labeled PC12 cells with the lift height at 140 nm and compared the various
Table I. The effect of tip lift height of MFM imaging of PC12 cells on cell surface “magnetic roughness” measured in the presence (+M) or absence (−M) of the external magnetic field and calculated using NanoScope Analysis software as described in the Materials and Methods.

<table>
<thead>
<tr>
<th>Lift height (nm)</th>
<th>MNP–loaded cells (+M)</th>
<th>MNP–loaded cells (−M)</th>
<th>Unloaded cells (+M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0.311</td>
<td>0.154</td>
<td>0.124</td>
</tr>
<tr>
<td>140</td>
<td>0.291</td>
<td>0.182</td>
<td>0.208</td>
</tr>
<tr>
<td>200</td>
<td>0.244</td>
<td>0.162</td>
<td>0.124</td>
</tr>
</tbody>
</table>

| Skewness $R_{sk}$ | 0.196                  | 0.034                  | 0.096               |
| Kurtosis $R_{ku}$ | 0.747                  | 0.483                  | 0.217               |
| Max. peak height $R_{p}$ | 2.59                   | 2.06                   | 2.72                |
| Max. valley depth $R_{v}$ | 1.690                  | 1.062                  | 0.629               |
| Max. roughness $R_{max}$ | 2.947                  | 1.199                  | 1.216               |

With the optimized lift height we investigated if the chosen “magnetic roughness” parameters could be used to semi-quantify the overall magnetic strength or strongest magnetic forces at certain points of the labeled cells. As shown in Figure 8, all studied parameters were significantly larger when obtained under exposure to the external magnetic field compared to that without the field ($p < 0.05$). $R_q$ is calculated as RMS value and reflects the general cell magnetism. As only a very small portion of area is “occupied” by the MNPs relative to the entire analyzed cell surface, the increase in $R_q$ in magnetically labeled cells is much smaller than that of other studied parameters (0.109 degree). The $R_v$ and $R_p$ on the other hand, were found to have larger rises in externally magnetized samples as these are related to the maximum interactive magnetic forces in the analyzed area. The increase in negative and positive MFM phase contrasts as described by $R_v$ and $R_p$ in labeled cells were 1.024 and 0.726 degrees, respectively. The difference in the total absolute value of MFM phase change as reflected by the $R_{max}$ was 1.748 degree. These results demonstrate the feasibility of reliable detection and analysis of magnetic responses of cell-bound MNPs.

Figure 7. Optical and MFM phase image overlay (top) and corresponding 2D (middle) and 3D (bottom) MFM phase images of the same sample area demonstrating the amplitude of magnetic signals in MNP-labeled PC12 cells in the absence (left) and presence (right) of the applied external magnetic field. The optical image (phase contrast 20×) and MFM phase image were overlaid using MIRO Software (Bruker, UK). MFM imaging was performed with lift height 140 nm.

Figure 8. “Magnetic roughness” of Fe$_3$O$_4$ nanoparticle-labeled PC12 cells. Roughness analysis was carried out at randomly selected 10 μm × 10 μm area in each scanned sample using lift height 140 nm in the absence or presence of magnetic (M) field as represented in Figure 7 and as described in the Materials and Methods. Values = mean ± SD, n > 8.
DISCUSSION

In the present study, by applying an external static magnetic field, we were able to obtain for the first time, reliable magnetic signals from nanoparticle-labeled cells by MFM using a lift height larger than 100 nm. Due to the superparamagnetic nature of the MNPs, detectable magnetic signals of the MNPs by MFM can only occur in the presence (i.e., exposure) to an external magnetic field under ambient conditions. This MFM setup has enabled us to analyze sequentially the same cell/area of the samples in the presence and absence of the static magnetic field. We were able to optimize the distance between the MFM tip and the cell sample in our experiments based on use of a permanent magnet underneath the sample but also for other MFM set ups where, for instance installation of an electromagnetic apparatus is possible.

Section plotting (by drawing a line across the phase image to show the phase shift) is commonly used for the analysis of magnetic signals as shown in our previous image to show the phase shift) is commonly used for the analysis of magnetic signals as shown in our previous experimental conditions. This MFM setup has enabled us to analyze magnetization of the MNPs with the newly formed neurite. In Figure 3(B), showing the possible interaction and magnetic signals from nanoparticle-labeled cells by MFM can only occur in the presence of the external magnetic field, the intrinsic magnetism of MNPs, and the size, formation and cellular localization of MNPs or their agglomerates. Cell-bound MNPs are often seen as clusters on the cell surface or inside the cells due to the intrinsic physicochemical properties of MNPs and because of cellular endocytosis. We were able to observe by MFM some large cell-bound MNP clusters even with lift height exceeding 400 nm (data not shown). Single MNPs are probably not detectable by MFM imaging with lift height 140 nm under the experimental conditions described, and small MNP clusters beneath the cell membrane and away from the cell surface (e.g., a couple of hundreds nanometers) may not be detectable. Owing to the adherent sticky nature of the polymer used for the coating of the MNPs (PEI), the MNPs became attached to either the cell membrane or the bottom of the cell culture dishes even after extensive wash before any assay as shown in previous reports including our own. This explains why a large number of fluorescent particles are randomly stuck to the substrate surface and on top of cells (Figs. 3(B) and (C)).

Differentiation of the membrane-bound and internalized MNPs is also important in MFM studies. Although in the present study we focused on all cell-associated MNPs including membrane-bound and those inside the cells; but did not differentiate between them, however, this issue had been investigated in a previously reported study in which the AFM topography (height) and phase images were analysed in combination with MFM images of same area on the cell surface by section plotting. Membrane-bound MNPs produced an AFM signal change as well as a MFM phase shift, while internalized MNPs typically caused only changes in MFM phase but not in AFM height/phase signals. In this way, a spatial localization of MNPs (inside or outside cell membranes) could be obtained.

There are complex interactions between the magnetic properties of the tip, sample and the externally applied field used in the current study. Since the tip and sample were both magnetized by the applied external magnetic field, the magnetism of the tip is opposite to the raster scanned surface of MNPs (see Fig. 1(C)). As a result, the negative phase shift from attractive interaction was dominant in our experiments (Figs. (4)–(8), Table I). On the other hand, the artificial scratches in MFM images of MNP-loaded cells are completely avoided in the surface roughness analysis as they can cause significant false interpretation. The artifacts in biological samples are closely associated with a number of parameters during AFM scanning including raster scanning rate of the tip. The scan rate used in present MFM study was 0.334 Hz. The artificial scratches disappeared in most MFM scans when lift height exceeded 100 nm.

The factors determining the lift height of MFM include at least the magnetization of the MFM probe, the strength of the external magnetic field, the intrinsic magnetism of MNPs, and the size, formation and cellular localization of MNPs or their agglomerates. Cell-bound MNPs are often seen as clusters on the cell surface or inside the cells due to the intrinsic physicochemical properties of MNPs and because of cellular endocytosis. We were able to observe by MFM some large cell-bound MNP clusters even with lift height exceeding 400 nm (data not shown). Single MNPs are probably not detectable by MFM imaging with lift height 140 nm under the experimental conditions described, and small MNP clusters beneath the cell membrane and away from the cell surface (e.g., a couple of hundreds nanometers) may not be detectable. Owing to the adherent sticky nature of the polymer used for the coating of the MNPs (PEI), the MNPs became attached to either the cell membrane or the bottom of the cell culture dishes even after extensive wash before any assay as shown in previous reports including our own. This explains why a large number of fluorescent particles are randomly stuck to the substrate surface and on top of cells (Figs. 3(B) and (C)).

Differentiation of the membrane-bound and internalized MNPs is also important in MFM studies. Although in the present study we focused on all cell-associated MNPs including membrane-bound and those inside the cells; but did not differentiate between them, however, this issue had been investigated in a previously reported study in which the AFM topography (height) and phase images were analysed in combination with MFM images of same area on the cell surface by section plotting. Membrane-bound MNPs produced an AFM signal change as well as a MFM phase shift, while internalized MNPs typically caused only changes in MFM phase but not in AFM height/phase signals. In this way, a spatial localization of MNPs (inside or outside cell membranes) could be obtained.

There are complex interactions between the magnetic properties of the tip, sample and the externally applied field used in the current study. Since the tip and sample were both magnetized by the applied external magnetic field, the magnetism of the tip is opposite to the raster scanned surface of MNPs (see Fig. 1(C)). As a result, the negative phase shift from attractive interaction was dominant in our experiments (Figs. (4)–(8), Table I). On the other hand, the artificial scratches in MFM images of MNP-loaded cells are completely avoided in the surface roughness analysis as they can cause significant false interpretation. The artifacts in biological samples are closely associated with a number of parameters during AFM scanning including raster scanning rate of the tip. The scan rate used in present MFM study was 0.334 Hz. The artificial scratches disappeared in most MFM scans when lift height exceeded 100 nm.

The factors determining the lift height of MFM include at least the magnetization of the MFM probe, the strength of the external magnetic field, the intrinsic magnetism of MNPs, and the size, formation and cellular localization of MNPs or their agglomerates. Cell-bound MNPs are often seen as clusters on the cell surface or inside the cells due to the intrinsic physicochemical properties of MNPs and because of cellular endocytosis. We were able to observe by MFM some large cell-bound MNP clusters even with lift height exceeding 400 nm (data not shown). Single MNPs are probably not detectable by MFM imaging with lift height 140 nm under the experimental conditions described, and small MNP clusters beneath the cell membrane and away from the cell surface (e.g., a couple of hundreds nanometers) may not be detectable. Owing to the adherent sticky nature of the polymer used for the coating of the MNPs (PEI), the MNPs became attached to either the cell membrane or the bottom of the cell culture dishes even after extensive wash before any assay as shown in previous reports including our own. This explains why a large number of fluorescent particles are randomly stuck to the substrate surface and on top of cells (Figs. 3(B) and (C)).

Differentiation of the membrane-bound and internalized MNPs is also important in MFM studies. Although in the present study we focused on all cell-associated MNPs including membrane-bound and those inside the cells; but did not differentiate between them, however, this issue had been investigated in a previously reported study in which the AFM topography (height) and phase images were analysed in combination with MFM images of same area on the cell surface by section plotting. Membrane-bound MNPs produced an AFM signal change as well as a MFM phase shift, while internalized MNPs typically caused only changes in MFM phase but not in AFM height/phase signals. In this way, a spatial localization of MNPs (inside or outside cell membranes) could be obtained.

There are complex interactions between the magnetic properties of the tip, sample and the externally applied field used in the current study. Since the tip and sample were both magnetized by the applied external magnetic field, the magnetism of the tip is opposite to the raster scanned surface of MNPs (see Fig. 1(C)). As a result, the negative phase shift from attractive interaction was dominant in our experiments (Figs. (4)–(8), Table I). On the other hand, the artificial scratches in MFM images of MNP-loaded cells are completely avoided in the surface roughness analysis as they can cause significant false interpretation. The artifacts in biological samples are closely associated with a number of parameters during AFM scanning including raster scanning rate of the tip. The scan rate used in present MFM study was 0.334 Hz. The artificial scratches disappeared in most MFM scans when lift height exceeded 100 nm.
other hand, noticeable positive phase change from repulsive forces was also encountered in our study, and this usually occurred at the edge of the nanoparticles (clusters), being observed as bright contrast (Figs. 4–7), which are significantly stronger compared with the control ($R_p^*$ in Fig. 8). Similar phenomenon was observed in previous reports. This observation may also imply the existence of a component of magnetism, possibly from the Co/Cr coated MFM tip, which was not orientated by the applied field. The remnant magnetization of the tip could therefore be dynamically affected by the local magnetization of the scanned sample and produced positive phase shift.

CONCLUSIONS

Magnetic characterization of in-situ MNPs in biological samples is of great importance due to the rapid development in biomedical applications of these nanomaterials. In this study the detection and semi-quantitative evaluation of magnetic signals from super paramagnetic nanoparticles in cultured cells has been realized by magnetic mode of scanning force microscopy. This technique has great potential in providing information on cell physical and topographic features as well as magnetic localization with high spatial resolution. The use of a localized external magnetic field significantly increased the magnetic interaction between the scanning tip and the MNPs hence the signal/noise ratio. The description of magnetic surface roughness analysis for the cells enabled evaluation not only of the overall average magnetic forces but also the maximum magnetic interaction the cells could produce in response to an external magnetic field. The complex interactions between the external magnetic field, the MFM probe and the magnetic nanomaterial, however, have not been clarified and need substantial amount of work both theoretically and experimentally. In addition, as current MFM technology has not been able to overcome the interference of raster scan and oscillation of the AFM cantilever in liquid to the measurement of the magnetic interaction between the MFM tip and the sample, MFM phase or frequency shift can only be measured in ambient environment in fixed cells. Future breakthrough in magnetic detection technology would be essential in realizing MFM imaging in living biological samples.

Acknowledgments: The authors wish to thank Drs. Paul Campbell and Paul Prentice for their valuable help in initiating the AFM/MFM study in Dundee. We are also grateful to Dr. Dun Liu and Mr. Donald McLean for their support in setting up the facility and carrying out the reported study. This work was financially supported by the MARVENE (ERA-NET NanoSci-E + NAN092) EU project and the Engineering and Physical Sciences Research Council (EPSRC) UK (EP/H007040/1 and EP/H010033/1).

REFERENCES


