Magnetic nanoparticles-induced endosomal rupture for cytosolic antibody delivery and intracellular labeling

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Abstract

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Antibodies (Ab') and their derivatives are powerful tools for intracellular diagnostics and targeting. But the cell membrane barrier limited the intracellular applications of antibodies. Many strategies have been developed to solve these problems, but a vast majority of them may bring about safety concerns or show poor clinical prospect. Here we report a cytosolic antibody delivery strategy by the combination of magnetic nanoparticles (MNPs) and commercial permanent magnets. Firstly, the positive-charged MNPs and antibodies were premixed to form MNP-Ab' complex. After cell incubation of the complex and magnet enrichment, cell uptake of the complex was considerably enhanced. The static parallel magnetic field (MF) treatment was then realized by placing two parallel commercial magnets onto the culture plate. Robust endosomal rupture and antibody

endosomal escape were induced due to this treatment. Finally, the antibody molecules were effectively released into the cytosols for live-cell intracellular labeling. This strategy also exhibits superb performance for the co-labeling of intracellular targets. MNP-induced endosomal rupture is a promising strategy for cytosolic antibody delivery, intracellular diagnosis and specific targeting in live cells.

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Chapter 1. Introduction

Proteins are polymers of amino acids that self-organize into three-dimensional, tertiary structures with specific biological functions. They catalyze biochemical reactions, transmit signals, form receptors and transporters in membranes, and provide intracellular and extracellular structural support. The variety of these functional biomacromolecules provides researchers with a real treasure house for potential biomedical applications. There are now more than 200 FDA-approved protein therapeutics since the advent of human recombinant insulin in 1982[1]. And it is worth noting that nearly half of these therapeutic proteins are monoclonal antibodies (mAbs), that can bind mono-specifically to certain cells or proteins. These mAbs can inhibit interactions, activate pathway, or alter cell fate. Under certain treatment conditions, patients' immune systems would be stimulated to attack cells labeled with these mAbs. More recently antibodies have been used to bind to molecules involved in T-cell regulation to remove inhibitory pathways that block T-cell responses. This is known as immune checkpoint therapy[2]. In addition to the regulation functions of mAbs, their targeting abilities could also be applied in direct therapy. For instance, in radioimmunotherapy, radioactively conjugated mAbs against cellular antigens would be used. They could localize a target cell line, delivering lethal chemical doses[3]. Another therapeutic system Antibody-drug conjugates (ADCs), that consist of antibodies and linked drug molecules, are also common in use[4]. Typically, when the ADC meets the target cell (a cancerous cell e.g.), the drug is released to kill it.

In addition to therapeutic applications, with the help of the specific binding between mAbs and their corresponding antigens, they could also be applied in diagnostic tests and subcellular structure labeling. Once monoclonal antibodies for a given substance have been produced, they

can be used to detect the presence of this substance. The interested proteins can be detected using the Western blot and immuno dot blot tests. In immunohistochemistry, monoclonal antibodies can be used to detect antigens in fixed tissue sections, and similarly, immunofluorescence can be used to detect a substance in either frozen tissue section or live cells. More recently, antibodies and their functional derivatives are becoming supreme molecules for intracellular diagnostics and targeting[5], especially for the recognition of "undruggable" cancer-associated targets like Ras, c-Myc and androgen receptor variant 7[6].

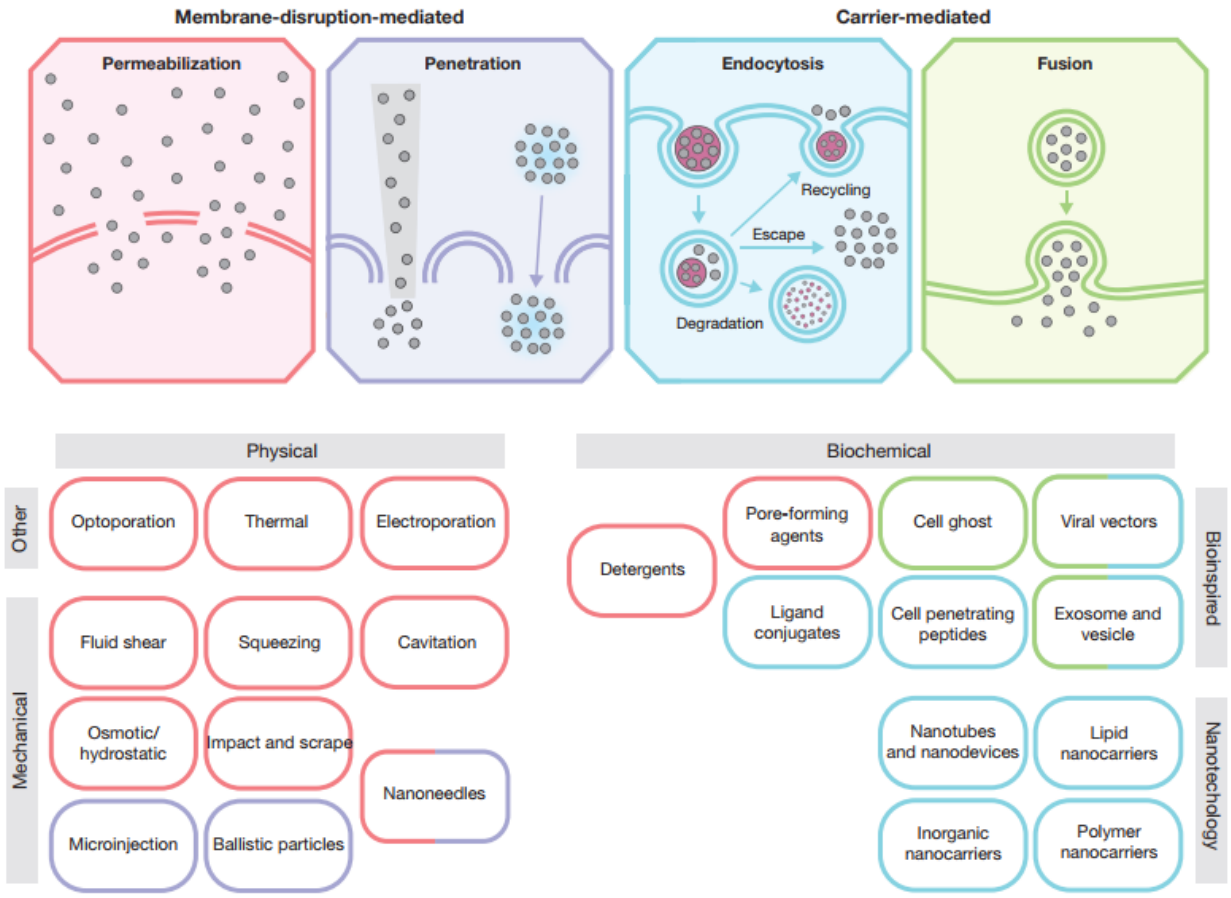

Figure 1. Map of the relationship between intracellular delivery approaches, basic mechanism and conventional physical and biochemical categorizations[7].

However, the biomedical application of biomacromolecules, antibodies e.g., is still restricted to permeabilized cells or extracellular targets. One of the major concerns is that these molecules would be uptaken by live cells through endocytosis, trapped inside endosomes, and finally degraded[8-9]. Thus, a variety of strategies, that are defined as intracellular delivery, have been developed to solve this problem. Intracellular delivery can be classified into two main categories: carrier-based and membrane-disruption-based techniques (**Figure. 1**)[7] . Membrane-disruption modalities mainly aim for creating temporal discontinuities in cell membrane. The main two ways membrane disruption is accomplished are through direct penetration or permeabilization. In direct penetration, a conduit or vehicle is utilized to directly break through the cell membrane, creating a passage for the cargo. Microinjection was the first intracellular delivery method to be invented and represents a classic case of a direct penetration strategy[10-11]. A miniaturized syringe-needle equipment is applied to disrupt cell membrane and pump fluid containing the molecule of interest inside the cell. In contrast to direct penetration, permeabilization strategies make the cell transiently permeable to cargo present in the extracellular solution. The plasma membrane is considered permeable when membrane disruptions are of sufficient size and lifetime to permit passage of the cargo molecules or materials. Many different permeabilization strategies have been attempted. They range from mechanical and laser-based to electrical and chemical[12-14].

Carrier-based approaches comprise various biochemical assemblies, mostly of molecular to nanoscale dimensions. The purpose of carriers is threefold: (1) to package the cargo and protect it from degradation, (2) to gain access to the intended intracellular compartment, and (3) to release the payload with the appropriate spatiotemporal dynamics. These carriers could be basically classified into viral and non-viral carriers. The viral vectors exploit the viral infection pathway to enter cells but avoid the subsequent expression of viral genes that leads to replication and

pathogenicity[15]. At present, viral vectors are the most clinically advanced nucleic acid delivery agents owing to their high efficiency and specificity. However, their weaknesses are also obvious: (1) labor-intensive and expensive protocols, (2) safety issues, (3) risk of causing immune/ inflammatory responses, (4) integration into the genome with recombinant vectors. And more importantly, in protein intracellular delivery, viral carrier-mediated strategy is indirect. They can only serve as the carrier of nucleic acids and the corresponding functional proteins, e.g. antibodies, would be expressed after the transfection[16]. The risk of insertional genotoxicity could be caused in this way. Motivated by the limitations of their viral counterparts, hundreds of non-viral carriers have been designed, using vast combinations of lipid, polymer, and inorganic nanomaterials, sometimes featuring functionalization with ligands, cell-penetrating peptides and other targeting or stabilizing agents[17-20]. In contrast to viral carriers, non-viral carriers could be applied in not only nucleic acid delivery, but also direct intracellular delivery of proteins. The dose could be controlled better, and the genotoxicity risk could be avoided in this way. Nearly all non-viral carriers are taken up via endocytosis[21-23]. To reach the intended intracellular target, the cargo must escape endosomal progression, which otherwise leads to degradation in lysosomes or regurgitation back to the cell surface[24]. Many methods have been developed for overcoming this problem, but they still have certain limitations. Fusing antibodies with cell penetrating peptides can promote the transmembrane efficiency of antibody molecules and cytosolic delivery, but the common issue of this technology is that the mechanism of cell internalization and endosomal escape process are poorly understood, and the activity of antibodies may be extensively affected after fusion[25-26]. Cholesterol-tag is a promising platform for protein/peptide delivery into live cells, but its delivery effect is dominated by the molecular weight of proteins[27]. For lipid nanocarriers, which are considered the most advanced non-viral vectors for nucleic acid delivery, quantitative studies

reveal that approximately 1% of the nanocarriers escape from endosomes[28]. However, the delivery mediated by lipid nanocarriers are still spontaneous. The uptake and release of cargo proteins cannot be well controlled.

Therefore, developing a cytosolic antibody delivery strategy with superb biocompatibility, transporting efficacy, controlled delivery manner and clinical translation prospect is of great importance. As customized drug delivery platforms, nanocarriers can obtain effective antibody delivery into the cytosol of live cells with high throughput[5]. However, nanocarriers are predominately internalized by cells via endocytosis. Controllable endosomal escape, antibody function maintenance and low immunogenicity are the major challenges of nanocarriers. Here we report an effective strategy for cytosolic antibody delivery and intracellular labeling via the combination of magnetic nanoparticles (MNPs) and remote parallel static magnetic field. This technology reveals a novel mechanism for the endosomal escape of antibodies with antibody function maintenance and provides an effective strategy for live-cell intracellular labeling.

Chapter 2. Materials and methods

2.1 Materials

Unless otherwise mentioned, all chemicals were purchased from Sigma-Aldrich. The positive-charged magnetic nanoparticles (MNPs) were purchased from Micromod Partikeltechnologie GmbH Company. Anti-ki67 antibodies and anti-GAPDH antibodies were bought from Biolegend Incorporated. Cyanine3 (cy3) NHS ester and Alexa Fluor 488 (AF488) NHS ester were purchased from Lumiprobe Corporation. Neodymium magnets were purchased from K&J Magnetics Incorporated. Hoechst 33258, Lysotracker Green® and BCA protein Assay Kit were purchased from ThermoFisher Scientific. CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) was obtained from Promega Corporation.

2.2 Antibody conjugation

To visualize the distribution of antibodies inside cells, the antibody molecules were chemically conjugated with cy3. Briefly, the antibody was dissolved in PBS, followed by the addition of cy3 (dissolved in anhydrous DMSO). The reaction molar ratio of antibody to probe was 1: 3. After overnight reaction and stirring at room temperature, cy-3 labeled antibodies (Ab') were purified by desalting column according to manufacturer's instruction. Ab' conjugated with AF488 were obtained by the same procedure.

2.3 Preparation of the MNP-Ab' complex

Briefly, 100 μL MNPs solutions (1 mg mL^{-1}) were mixed with 182.5 μL Ab' solutions (0.3 mg mL^{-1}) by pipetting, and were kept in 37 °C water bath for 30 min. Then the mixture was sonicated

in an ultrasonic water bath for 1 min. After further 8-min water bath incubation at 37 °C, the MNP-Ab' complex was ready for use.

2.4 Cell culture

Hela human cervical cancer cells (ATCC# CCL-2) were purchased from American Type Culture Collection (ATCC), and were cultured in Eagle's minimal essential medium supplemented with 10% fetal bovine serum and penicillin (100 unites mL^{-1}) and streptomycin (100 μg mL^{-1}). Cells were grown in a cell incubator supplied with 5% CO_2.

2.5 Cell incubation

HeLa cells were planted on 24-well glass-bottom plates with 10^4 cells per well. After overnight culturing, cells were treated with various treatments: bare Ab' (0.03 mg mL^{-1}), MNP-Ab' complex (0.1 mg mL^{-1} Fe and 0.03 mg mL^{-1} Ab'), bare Ab' (0.03 mg mL^{-1}) plus magnet enrichment, and MNP-Ab' complex (0.1 mg mL^{-1} Fe and 0.03 mg mL^{-1} Ab') plus magnet enrichment. After 4 h incubation, cell medium was replaced, and cells were stained by Hoechst 33258. The distribution of antibody molecules within cells was observed on an Olympus fluorescence microscope equipped with a true-color CCD, Qcolor 5.

2.6 Endosomal rupture after magnetic field (MF) treatment

HeLa cells were planted on 24-well glass-bottom plates with 10^4 cells per well. After overnight culturing, cells were treated with bare Ab', MNP-Ab' complex, bare Ab' plus magnet enrichment, or MNP-Ab' complex plus magnet enrichment for 4 h. Then cell medium was replaced before exposure to the magnetic field (MF). After 1.5 h MF treatment, cells were immediately stained by Hoechst 33258 and Lysotracker Green for microscopy.

2.7 Intracellular ki67 labeling via MNP-mediated cytosolic antibody delivery

Cells were planted on 24-well plates and cultured overnight. Cells were treated with bare Ab', MNP-Ab' complex, bare Ab' plus magnet enrichment, MNP-Ab' complex plus magnet enrichment for 4 h, followed by medium replacement and 1.5 h MF treatment. After that, the treated cells were cultured for another 3 h, and were counterstained by Hoechst 33258 before microscopy.

2.8 Cytotoxicity of MNP-mediated cytosolic antibody delivery

Cells were planted on 96-well plates with 5,000 cells per well. After overnight culturing, cells were treated with MNP-Ab' containing various concentrations of Fe. Then cells medium was also replaced, and the cells received 1.5 h MF treatment and 3 h further culturing. Treated cells were added with MTS solution for cell viability detection. For long-time cytotoxicity analysis, cells were treated with 4 h MNP-Ab' incubation, 1.5 h MF treatment and 24 h further culturing before cell survival analysis.

Chapter 3. Results and discussion

3.1 Enhanced cell uptake of magnetic nanoparticle-antibody complex

The basic characterization of bare MNPs and MNP-Ab' complex was first implemented. Changes in both size and surface potential were observed. Due to the electrostatic adsorption of negatively charged Ab', the size was increased from 28.97 nm to 36.03 nm, and the surface potential was decreased from 5.66 mV to -10.20 mV (**Figure 2**).

To investigate the efficiency of MNP-mediated cytosolic antibody delivery, the antibody molecules internalized by cells were observed. As shown in **Figure 3a**, bare anti-ki67 Ab' (labeled with cy3) can hardly be uptaken by cells. But after premixing with the positively charged MNPs, the formed MNP-Ab' complex could be internalized by cells through endocytosis after incubation (**Figure 3b**). By adding one magnet plate at the bottom of cell culture plate during incubation (magnet enrichment), the fact that bare Ab' were difficult to be internalized by cells was not changed (**Figure 3c**). On the other side, with the help of magnetic field, the amount of MNP-Ab' complex on cell surface were greatly increased. Therefore, the complex was uptaken by cells with higher efficiency compared with the incubation without magnet enrichment (**Figure 3b, d**). The results demonstrated that incubating cells with MNP-Ab' plus magnet enrichment could obtain an optimized endocytosis for potential intracellular labeling.

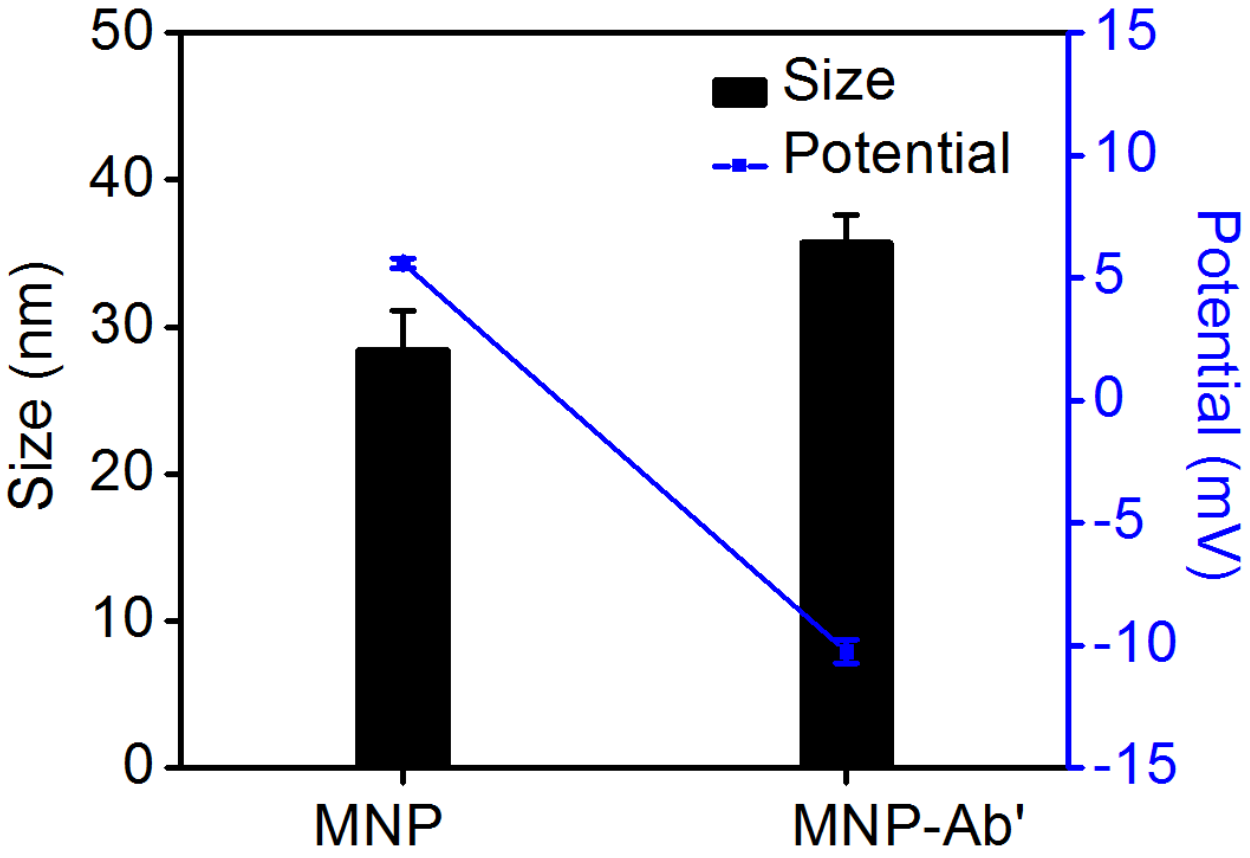

Figure 2. Size and surface potential of MNP and MNP-Ab' complex.

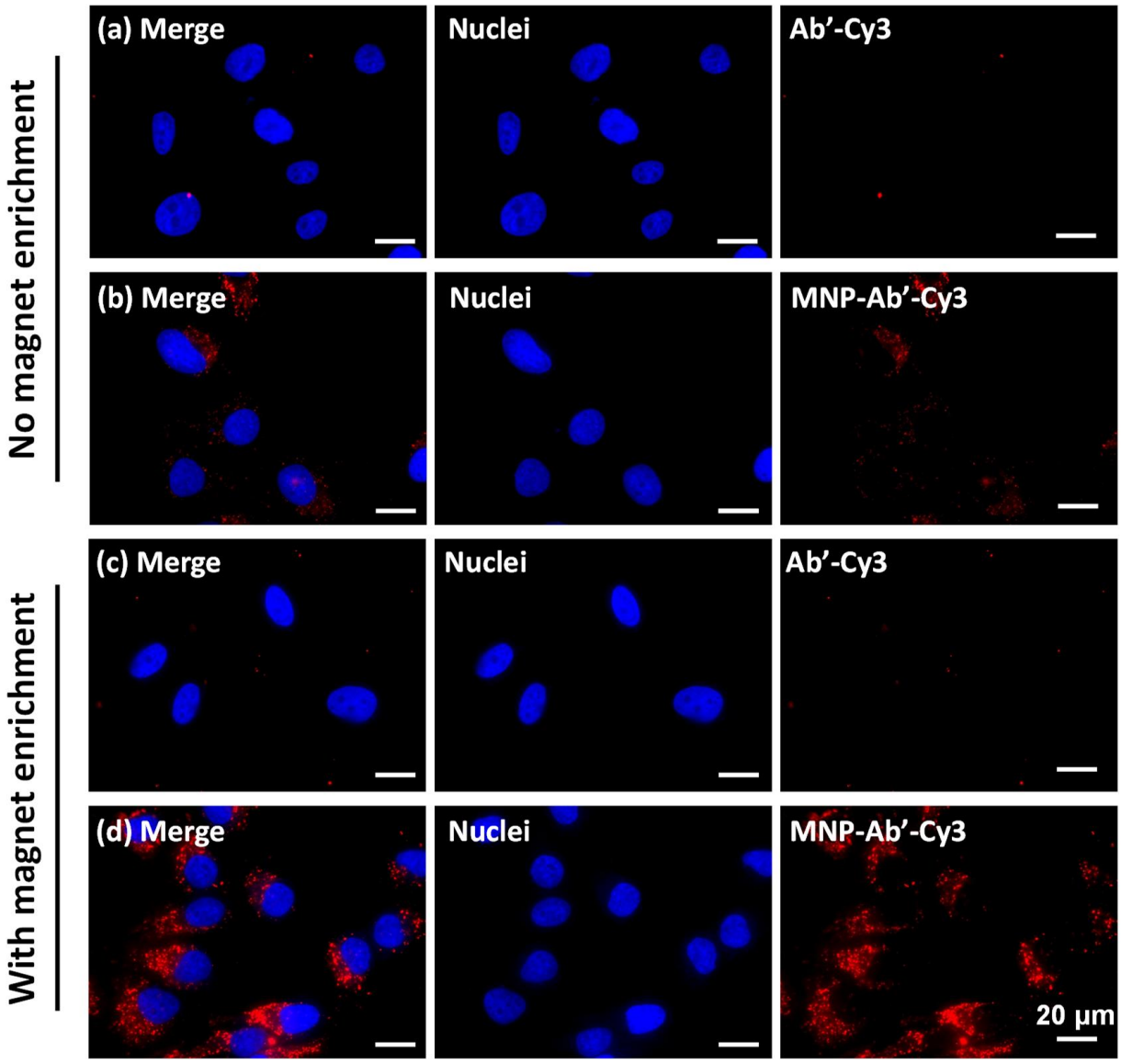

Figure 3. Bare anti-ki67 Ab' and MNP-Ab' complex internalized by HeLa cells after 4 h cell incubation. (a, b) Antibody molecules internalized by cells after incubation with bare Ab' or MNP-Ab' complex. (c, d) Antibody molecules internalized by cells after incubation with bare Ab' or MNP-Ab' complex with magnet enrichment. Ab' was labeled by cy3 (red), and cell nuclei were counterstained by Hoechst 33258 (blue).

Cell membrane, which consists of lipids, proteins, and other components, is the first interface contacting with nanoparticles during the endocytosis process. This initial adhesion is followed by the activation of an energy-dependent uptake mechanism[29-31], which allows the NPs to be internalized into the cell and further trafficked to different subcellular locations, typically ending in lysosomal accumulation[29, 32-33]. Therefore, increasing the initial adhesion of nanoparticles (NPs) would be an effective strategy for enhancing endocytosis. Unfortunately, when delivering proteins with nanoparticle carriers through electrostatic adsorption, biomolecular corona that effectively screens the bare NP surface would form[34-35]. The formation of a NP corona will lower the surface energy and unspecific interactions between the NP−corona complex and the cell membrane are much reduced, therefore suppressing adhesion and uptake[36]. In our method for solving this problem, MNPs and static magnetic field were combined to provide extra force directing MNP-Ab' complex towards cell membrane (**Figure 4**). In this way, the adhesion of the MNP-Ab' complex could first be improved. Moreover, The extra mechanical force generated by the MNP-MF system has the same order of magnitude as the actin force[37]. Therefore, the magnetic force can further assist the actin force in the processes of membrane deformation and pit creation, enhancing the endocytosis.

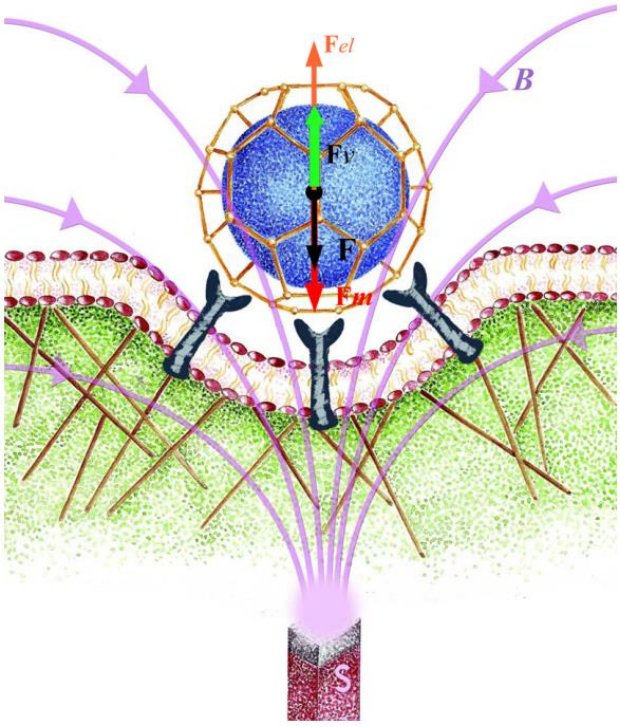

Figure 4. (Color online) Scheme of magnetically controlled endocytosis[37].

3.2 Magnetically induced endosomal escape

To investigate whether endosomal escape could be induced by the combination of MNP and magnetic field (MF) treatment, cells incubated with bare Ab' or MNP-Ab's complex were further divided into two groups according to whether they had been treated with magnet enrichment or not. 1.5 h treatment of 2-side MF (two same magnets were placed at the top and bottom of the culture plate) was then added onto cells in these two groups. As shown in **Figure 5a**, without preparatory magnet enrichment, after bare Ab' incubation and MF treatment, no Ab' distribution was observed within the cytoplasm, since bare Ab' were difficult to get into live cells. After MNP-Ab' complex incubation and MF treatment, Ab' mainly distributed in the endosomes, and Ab' distribution outside endosomes were observed in a few cells (white arrows) (**Figure 5b**). In

contrast, benefitted from the magnet enrichment during cell incubation, Ab'-MNP complex obtained enhanced internalization. And notably, almost all cells showed significant Ab' distribution outside endosomes after the MF treatment, indicating that the intracellular endosomes were disrupted after MNP-Ab' incubation and MF treatment so that the antibody molecules could escape from endosomes for cytosolic antibody delivery (**Figure 5d**).

In addition, after magnet enrichment, the release manners of MNP-Ab' complex with different durations of MF treatment were also observed. According to **Figure 6**, it was observed that the MF-mediated endosomal escape of Ab' is a gradual procedure. During the first 10 min of MF treatment, no obvious endosome rapture was observed. After 30 min treatment, the fluorescent signal of MNP-Ab' complex started defusing from endosomes and formed fog-like signal inside the cytosol, indicating the beginning of endosomal escape. Then, uniformly distributed fluorescent signal could be observed after 60 min MF treatment. The endosomal escape condition at this time point was very close to the phenomenon in **Figure 5d**, which means considerable release of MNP-Ab' complex had already been finished.

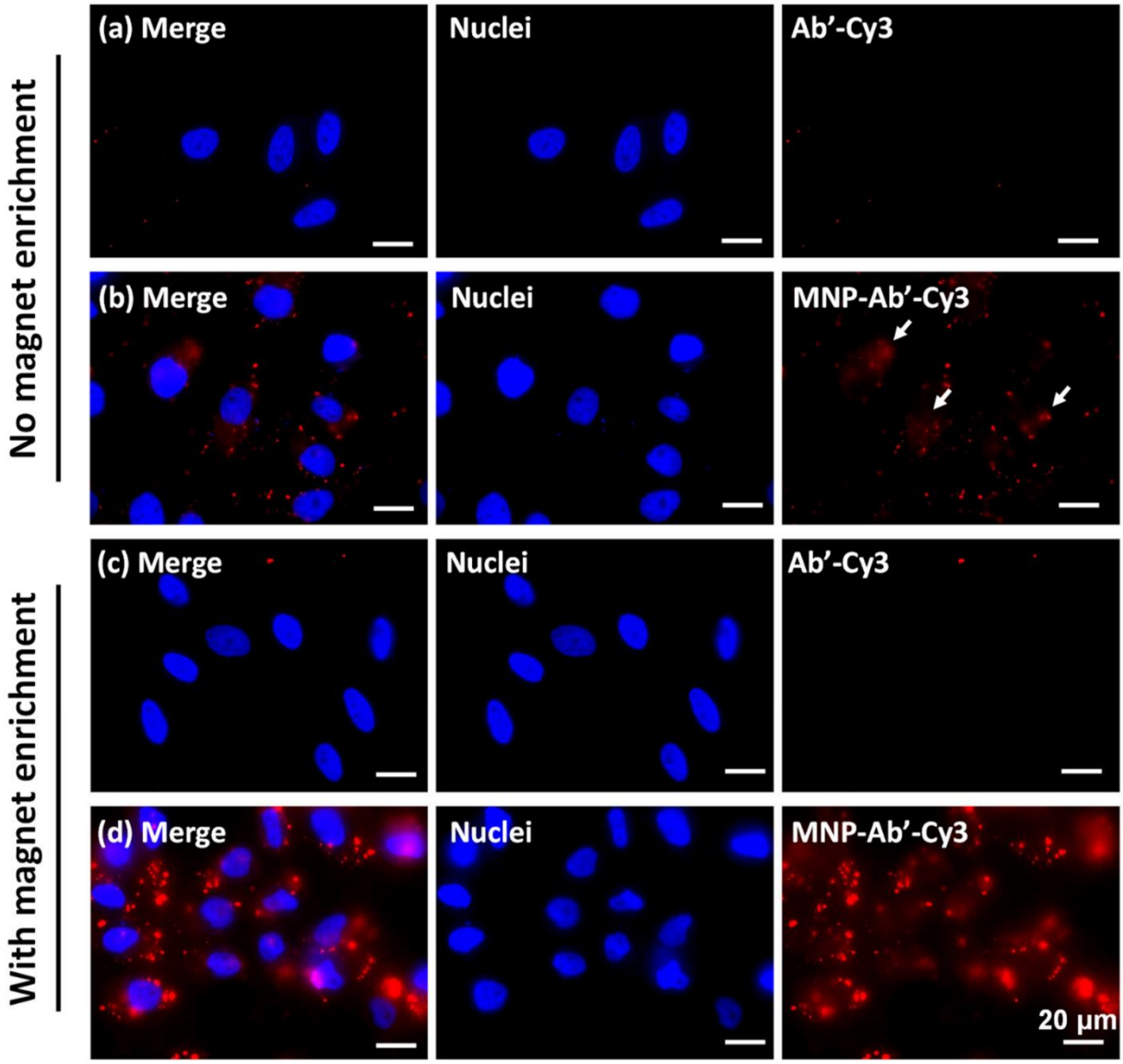

Figure 5. Anti-ki67 antibodies released from endosomes after 4 h cell incubation and 1.5 h MF treatment. Antibody molecules released from intracellular endosomes after incubation with (a) bare Ab', (b) MNP-Ab' complex, (c) bare Ab' plus magnet enrichment, or (d) MNP-Ab' complex plus magnet enrichment, followed by MF treatment. White arrows indicated the distribution of antibody molecules outside endosomes.

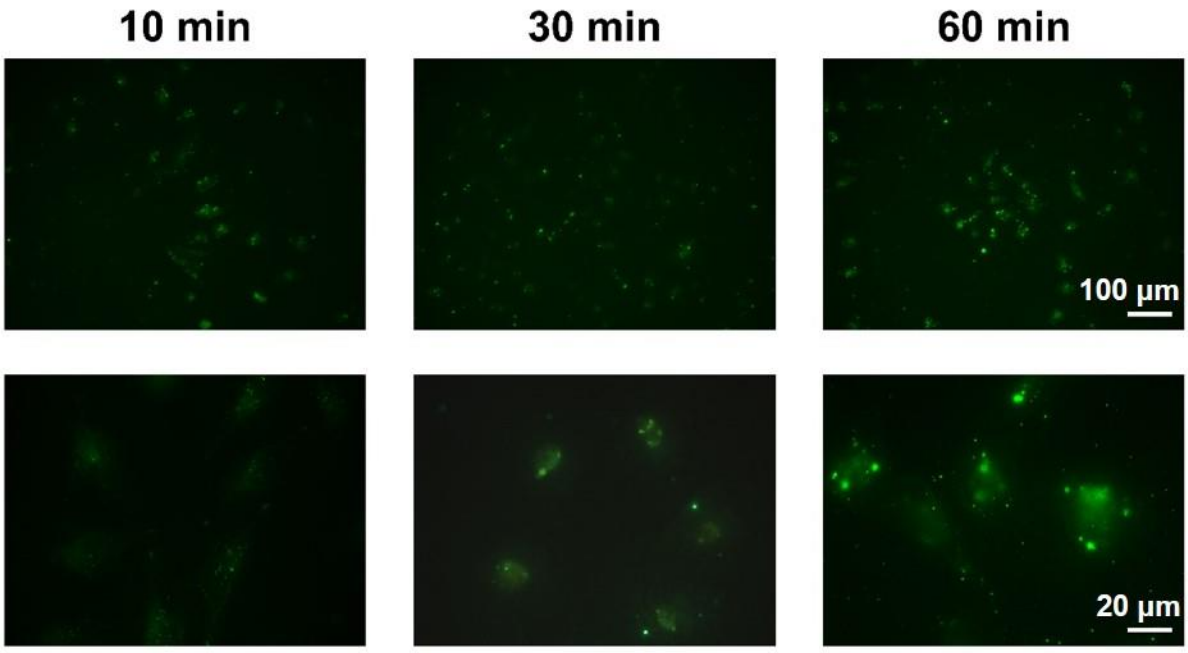

Figure 6. Short-term release of anti-ki67 antibodies (labeled with AF488) after 4 h cell incubation and different durations (10 min, 30 min, 60 min) of 2-side magnet treatment.

The mechanism of MNP-induced endosomal rupture was investigated by observing the co-localization of endosomes and antibody molecules (**Figure 7**). After bare Ab' incubation plus MF treatment, cell endosomes remained intact. MNP-Ab' incubation (no magnet enrichment) plus MF treatment induced successful delivery of antibody molecules into cells, but most of the endosomes were unharmed and antibody molecules were still trapped inside endosomes. Notably, MNP-Ab' incubation (with magnet enrichment) followed by MF treatment induced the rupture of almost all the intracellular endosomes. The results demonstrated that the combination of MNPs and MF treatment indeed induced endosomal rupture: MNP-Ab' complex transported large amounts of antibody molecules into cells after cell incubation and magnet enrichment, and the internalized MNPs could smash endosomes during the MF treatment to induce endosomal rupture. MNP-

mediated endosomal rupture realized successful endosomal escape and cytosolic delivery of antibody molecules for potential intracellular labeling.

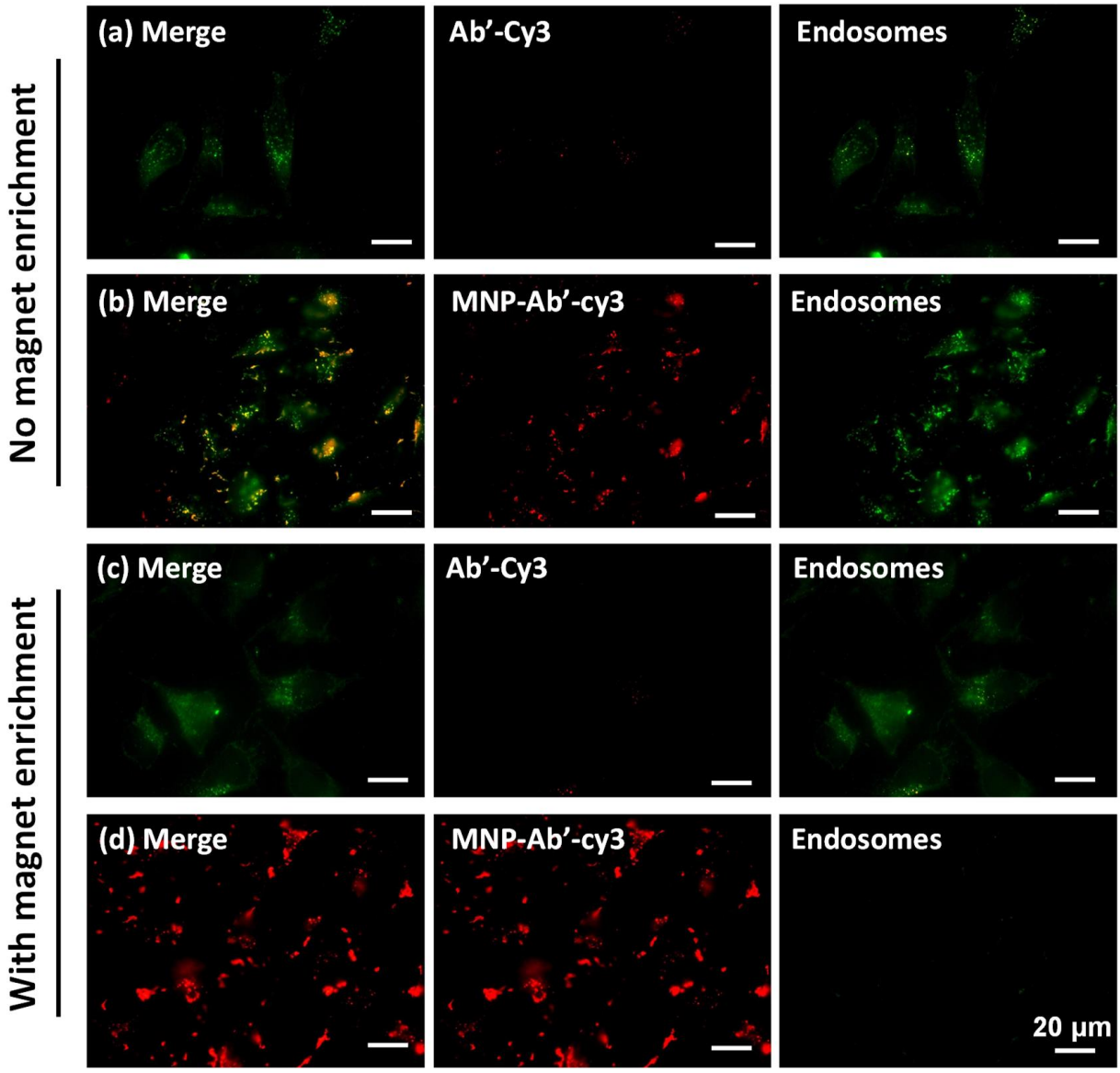

Figure 7. The mechanisms of endosomal rupture in live cells induced by the combination of MNPs and MF treatment. (a, b) The distribution of intracellular endosomes and antibody molecules after incubation with bare Ab' or MNP-Ab' complex for 4 h, followed by magnetic field treatment for 1.5 h. (c, d) The distribution of intracellular endosomes and antibody molecules after incubation with bare Ab' or MNP-Ab' complex for 4 h with magnet enrichment, followed by MF treatment

for 1.5 h. Cy3-labeled anti-ki67 Ab' were shown as red, and endosomes were counterstained by Lysotracker Green (green). Intracellular labeling achieved by MNP-mediated cytosolic antibody delivery was investigated.

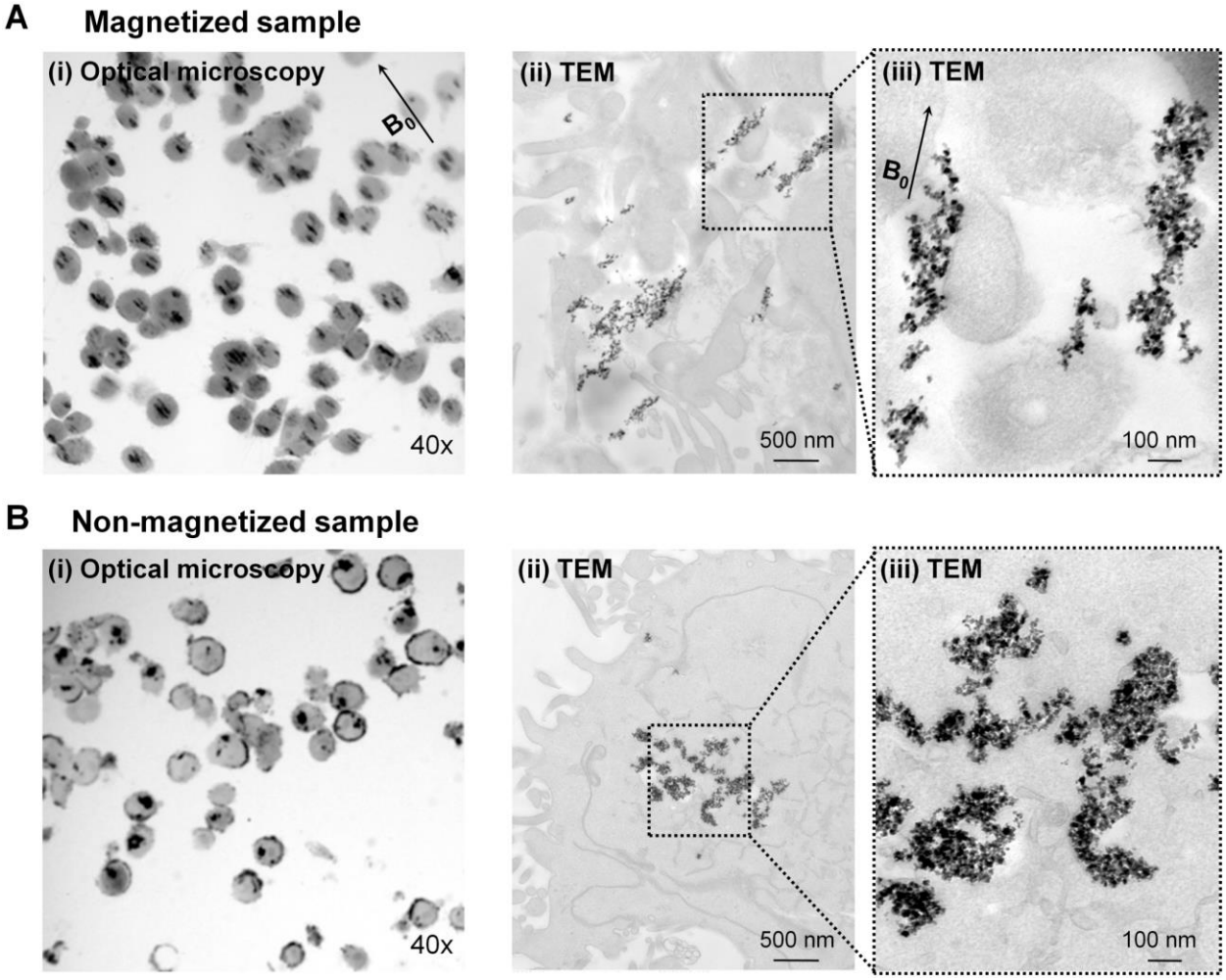

Figure 8. Microscopic images of MNP aggregates orientation in MDA-MB-231 cells. Optical microscopy and TEM micrographs of the orientation of MDA-MB-231 cells incubated (A) at B0 = 4.7T magnetic field (i) optical image at 40x, (ii) TEM at 17,500x (scale bar, 500 nm), and (iii) TEM at 65,000x (scale bar, 100 nm), or (B) at a non-magnetic condition (i) optical image at 40x, (ii) TEM at 17,500x (scale bar, 500 nm), and (iii) TEM at 65,000x (scale bar, 100 nm)[38].

Nanomagnetic materials are very useful in cancer therapy. Except using the hyperthermia properties, their mechanical response to MF is also an important clue for damaging cancer cells, creating discontinuity on cell substructure. It is worth noting that the strength of this therapeutic effect could be controlled for intracellular delivery as well. In response to MF, nanomagnetic materials could disrupt cell membrane through spin-vortex-mediated oscillation[39], or smash endosomes by forming one-dimensional superstructures (**Figure 8** e.g.)[38, 40]. The proposed mechanism of our intracellular delivery method is similar to the second way mentioned above. Under the MF, nanomagnetic materials could aggregate into one-dimensional superstructures, from 100 nm needle-shaped clusters[41-42] to micrometer-scale fibrous structures[43]. Both the shapes and sizes of these magnetically formed superstructures are favorable to pierce endosomes, triggering endosomal escape of co-delivered cargo proteins.

3.3 Intracellular labeling ability of released antibodies

To evaluate whether antibody molecules released from endosomes could recognize and combine with their targets, the cells were cultured for another 3 h after the MF treatment for specific antigen-antibody combination. Surprisingly, superb intracellular ki67 labeling after the cytosolic antibody delivery was achieved. The cell nuclei, in which ki67 proteins exist, were clearly labeled (**Figure 9**). However, MNP-Ab' incubation (no magnet enrichment) plus MF treatment could not induce successful labeling of intracellular ki67 proteins since the insufficient internalization of MNPs could not induce general endosomal rupture for antibody cytosolic delivery. Bare Ab' incubation plus MF treatment also could not induce intracellular ki67 labeling because bare Ab' molecules could not enter cytosol efficiently. Altogether, by integrating the MNP-Ab' incubation plus magnet

enrichment and the MF treatment, excellent cell internalization and endosomal rupture were realized to induce successful cytosolic antibody delivery and intracellular ki67 labeling.

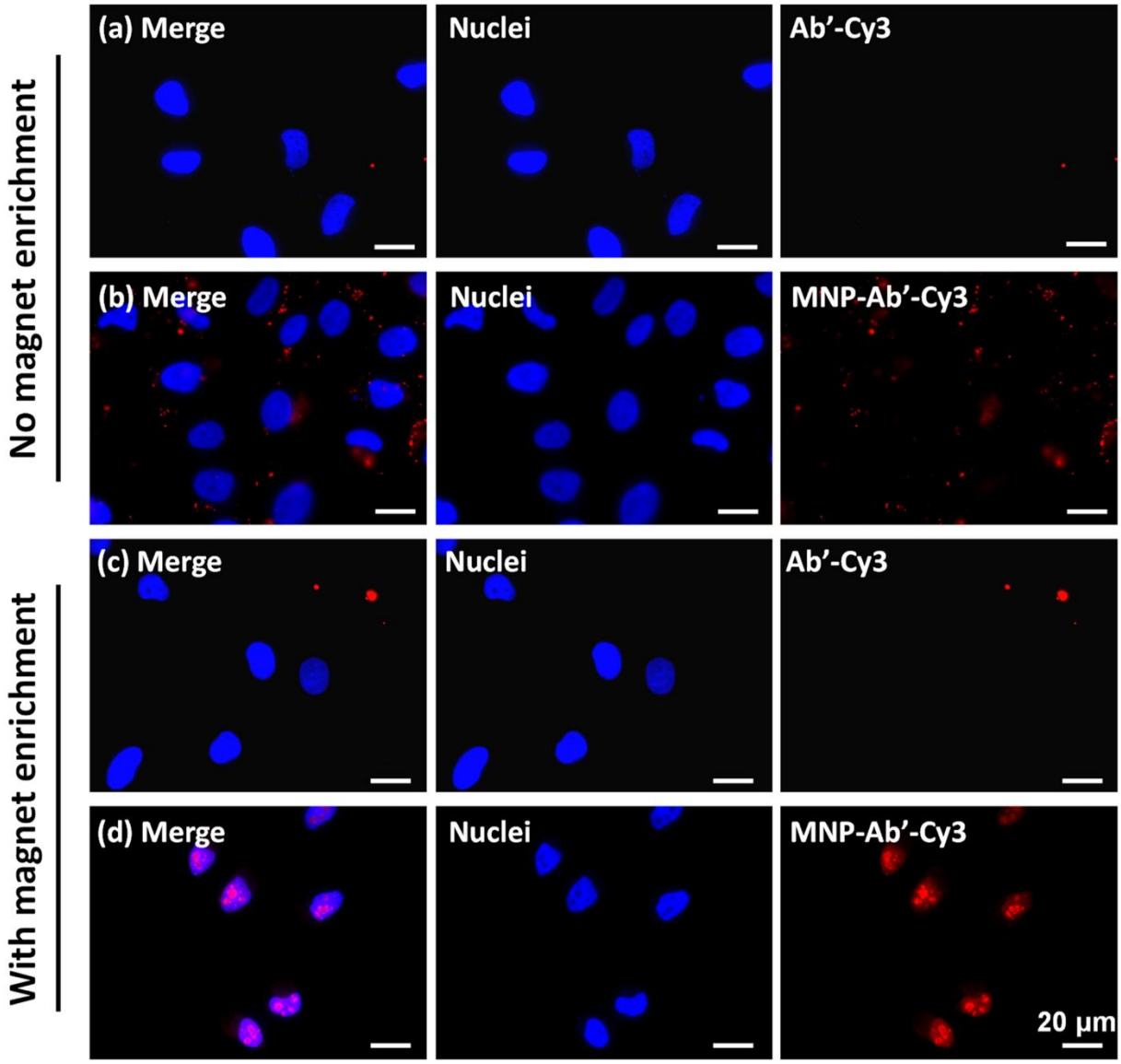

Figure 9. Intracellular ki67 labeling realized by MNP-mediated cytosolic antibody delivery. Cells were incubated with (a) bare Ab', (b) MNP-Ab' complex, (c) bare Ab' plus magnet enrichment, and (d) MNP-Ab' complex plus magnet enrichment for 4 h, followed by MF treatment for 1.5 h and further culturing for 3 h.

The availability of MNP-mediated cytosolic antibody delivery for intracellular labeling was also investigated on a commonly used and widely distributed biomarker – glyceraldehyde-3-phosphate dehydrogenase (GAPDH)[44]. MNPs were premixed with anti-GAPDH antibodies to prepare the MNP-Ab' complex, and cells were incubated with MNP-Ab' complex (with magnet enrichment), treated with MF, and cultured for another 3 h in the same way. As shown in **Figure 10**, the cytosolic antibody delivery obtained by MNP-induced endosomal ruptures successfully labeled intracellular GAPDH in live cells. The results demonstrated that cytosolic antibody delivery via MNP-mediated endosomal rupture could be utilized as a universal strategy for live-cell intracellular labeling.

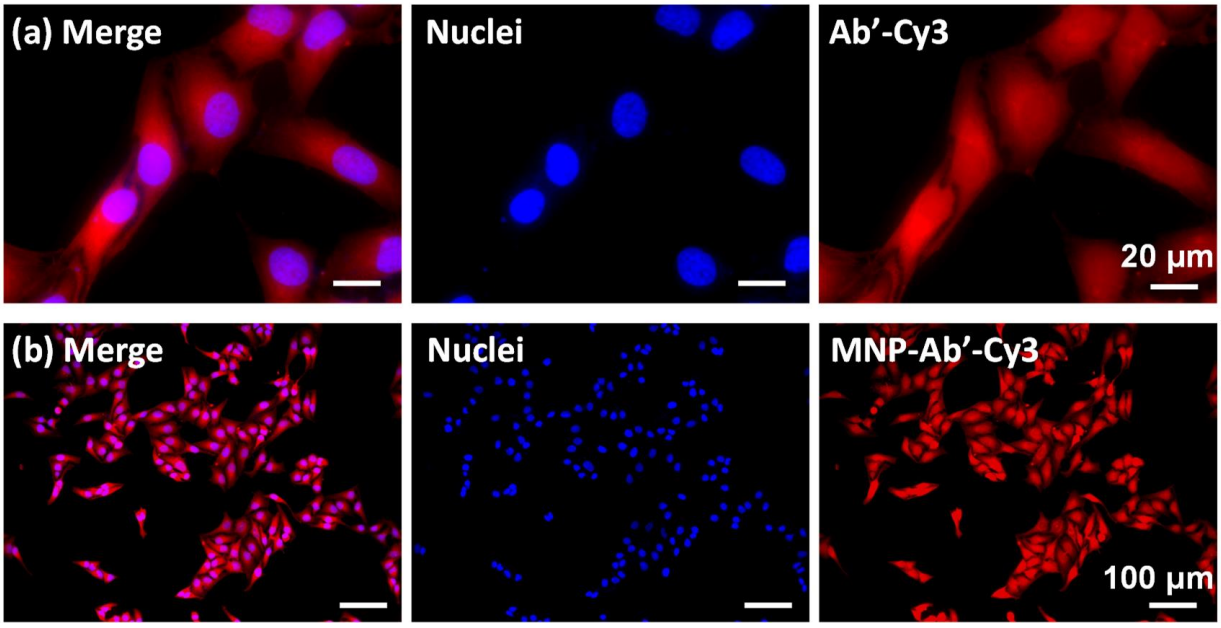

Figure 10. Intracellular GAPDH labeling realized by MNP-mediated cytosolic antibody delivery. Cells were incubated with MNP-Ab' complex plus magnet enrichment for 4 h, followed by MF treatment for 1.5 h and further culturing for 3 h. The cells were observed with (a) 600 × magnification and (b) 100 × magnification. Anti-GAPDH antibody was labeled with cy3 (red) and cell nuclei were marked by Hoechst 33258.

The intracellular labeling specificity obtained by MNP-mediated cytosolic antibody delivery was investigated. As shown in **Figure 11a, b**, the MNP-mediated cytosolic antibody delivery in live cells and the immunofluorescent staining in permeabilized cells showed consistent labeling of intracellular ki67/GAPDH, proving that MNP-mediated endosomal rupture and cytosolic antibody delivery could induce superb intracellular labeling with high specificity. By premixing MNPs with both anti-ki67 antibody and anti-GAPDH antibody and incubating cells with this MNP-Ab' complex mixture, MNP-mediated cytosolic antibody delivery also exhibited perfect co-labeling of intracellular ki67 and GAPDH (**Figure 11c**), which proved the availability of our strategy for the once-for-all labeling of multiple intracellular targets. Moreover, our strategy also showed high compatibility to live cells. Treated with high dose MNPs (containing 200 μg/mL Fe) with magnet enrichment, MF treatment and 3 h further culturing, less than 20% of the cells were killed (**Figure 11d**).

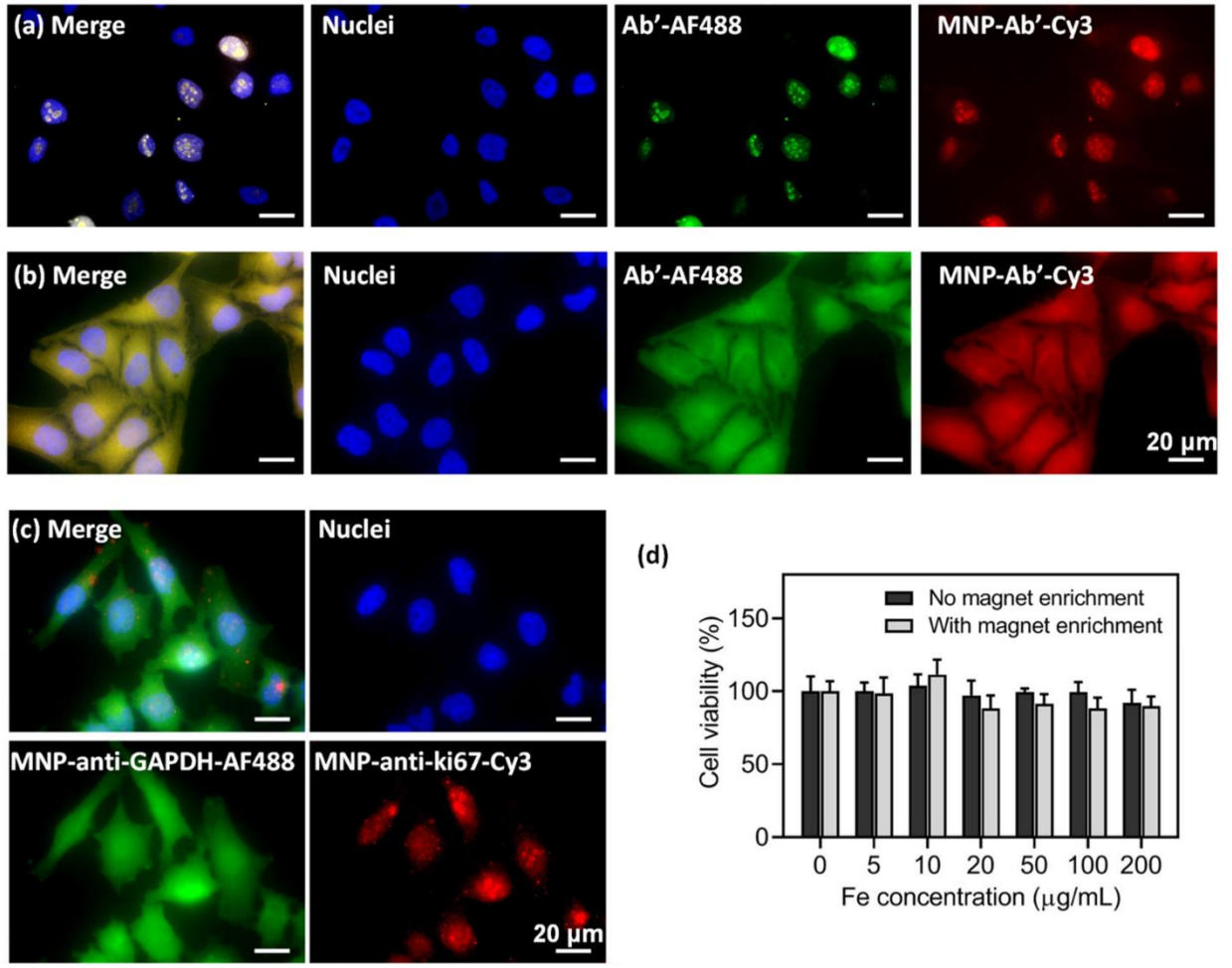

Figure 11. The specificity of intracellular cytosolic protein labeling realized by MNP-mediated cytosolic antibody delivery. The specificity of MNP-mediated cytosolic antibody delivery for intracellular (a) ki67, (b) GAPDH labeling, by investigating the co-localization of MNP-mediated intracellular labeling in live cells and immunofluorescent staining in permeabilized cells. (c) Co-labeling cytosolic ki67 and GAPDH via MNP-mediated cytosolic antibody delivery. (d) Cytotoxicity of MNP-mediated antibody delivery after 4 h cell incubation, 1.5 h MF treatment and 3 h further culturing (n = 6).

3.4 Long-term effects of the MNP-mediated delivery

As a novel strategy for cytosolic antibody delivery and intracellular labeling in live cells, the long-term toxicity induced by MNP-mediated endosomal rupture was investigated. As shown in **Figure 12a**, 24 h after MNPs incubation and MF treatment, approximately half of the cells were killed, indicating that many cells were injured after the MF treatment. The cell death was probably caused by MNP-induced endosomal rupture, which induced the release of endosomal contents (like protons, enzymes and cathepsins) into cytosol and cell death[45-46]. However, this phenomenon raised another opportunity of our technology - continuously observing intracellular cell activities after cell injury. The distribution of ki67 proteins in the injured cells was observed after the MNP-Ab' incubation plus MF treatment, since injured cells usually showed nuclei segmentation[47]. As shown in **Figure 12b**, the distribution of ki67 in the injured cell was dramatically changed 8 h after the MF treatment, indicating that the nuclei morphology was changed 8 hours after cell injury instead of immediate segmentation.

In addition, the decay of delivered antibodies was evaluated using anti-GAPDH antibodies. After premixing with MNPs, magnet enrichment and MF treatment, cells were observed at different time points in long term: 3 h, 1 day, 2 days after MF treatment. According to **Figure 13**, the fluorescence signal of AF488-labeled anti-GAPDH antibodies obviously decreased after 1 day culture. And the signal almost disappeared after 2 days culture. The decay of fluorescence signals was caused by the dilution of intracellular antibody concentrations during the continuous cell division. Meanwhile, it also indicated that our delivery method would not generate long-term residue of cargo proteins, that might cause potential risks.

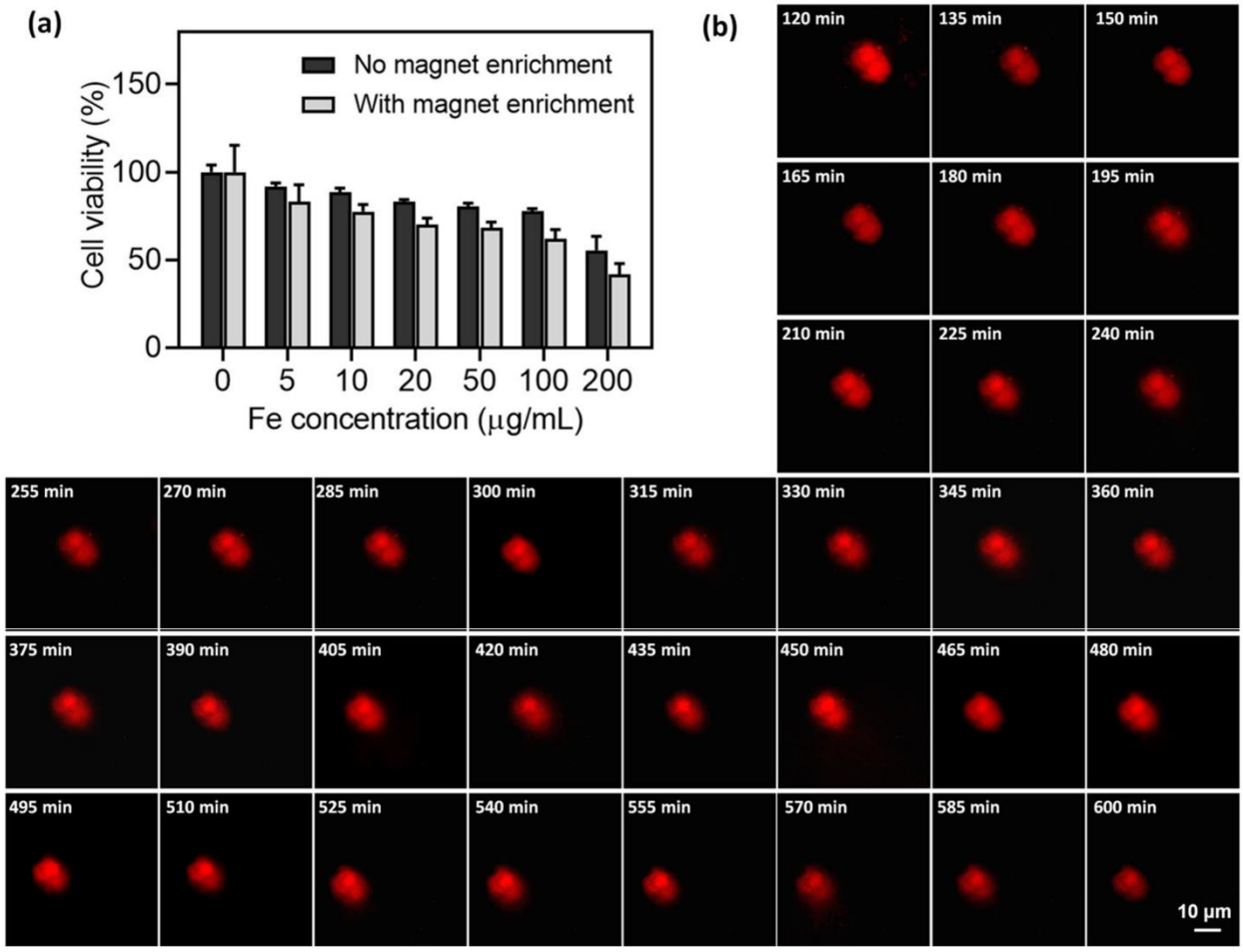

Figure 12. Real-time tracking ki67 distribution in living cells via MNP-mediated cytosolic anti-ki67 antibody delivery. (a) Long-term cytotoxicity of MNP-mediated cytosolic antibody delivery. Cell survival were detected after 4 h cell incubation, 1.5 h MF treatment and 24 h further culturing (n = 6). (b) Tracking intracellular ki67 distribution via MNP-mediated cytosolic antibody delivery. Anti-ki67 Ab' was labeled by cy3 (red). Ki67 distribution in cell nuclei was apparently changed 495 min after the MF treatment.

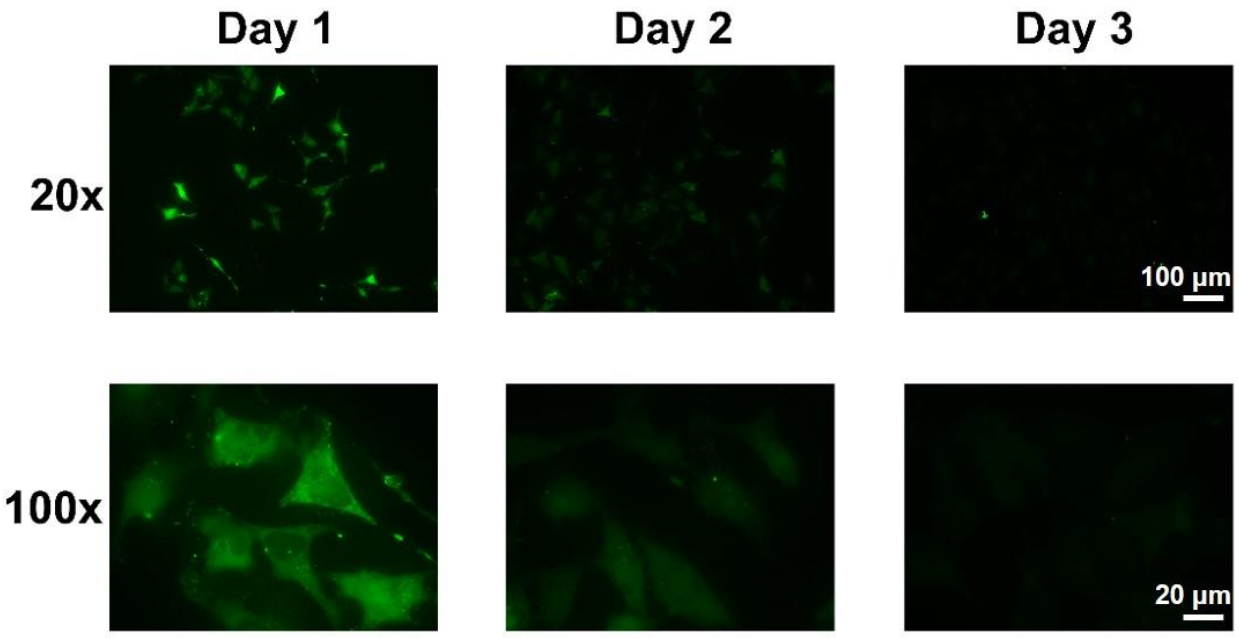

Figure 13. Decay of intracellular antibody concentrations during long-term culture. These 3 time points correspond to 3 h, 1 day, 2 days after MF treatment, respectively.

Chapter 4. Conclusions

In this work, a remarkably effective cytosolic antibody delivery strategy is developed via integrating the biocompatible magnetic nanoparticles (MNPs) and the commercial magnets, and this strategy raises an opportunity for intracellular protein labeling in live cells. The key procedures for successful live-cell intracellular labeling is: (1) premixing antibody molecules with MNPs to develop the MNP-Ab' complex; (2) incubating cells by the MNP-Ab' complex with magnet enrichment to obtain optimized Ab' internalization; (3) execute MF treatment to induce endosomal rupture so that sufficient Ab' could be released to cytosol for intracellular protein labeling. MNP-mediated cytosolic antibody delivery can also achieve once-for-all labeling of multiple intracellular targets, real-time intracellular environment surveillance of injured cells. In all, our strategy is a powerful tool for intracellular diagnostics and targeting in live cells and is a promising strategy for supervising vital intracellular activities and treating the "undruggable" diseases.

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