Magnetic Nanobeads Decorated with Silver Nanoparticles as Cytotoxic Agents and Photothermal Probes

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A versatile method for decorating magnetic nanobeads (being composite materials from polymers and superparamagnetic nanoparticles) with silver nanoparticles of 3–6 nm size is presented. Control over the silver nanoparticle coverage at the nanobead surface is achieved by changing the reaction parameters. Moreover, the silver-decorated magnetic nanobeads (Ag-MNBs) are studied with respect to their in vitro cytotoxicity on two distinct tumour cell lineages under different parameters, i.e., dose, incubation time, magnetic field applied during the culturing, silver ion leakage, and colloidal stability. It is found that enhanced magnetically mediated cellular uptake and silver ion leakage from the Ag-MNBs surface are the main factors which affect the toxicity of the Ag-MNBs and allow the half-maximal inhibitory dose of silver to be reduced to only 32 μg mL⁻¹. Furthermore, a synergic cytotoxicity induced by photo-activation of silver nanoparticles was also found.

1. Introduction

In the last decades, there has been a remarkable advancement in the preparation of metal nanoparticles, including silver or gold nanocrystals. Metallic nanoparticles have shown new properties, which have been exploited for different biomedical purposes including therapeutics,[1–6] diagnostics,[7] and bioimaging.[8–10] Among these, the antimicrobial activity of silver nanocrystals has also been studied.[11–13] Investigation in this field is of primary importance because of the emerging problems regarding antibiotic resistance. It has been found that only very few bacterial strains display resistance toward the antimicrobial effect of silver nanoparticles. Since then, silver nanoparticles in different formulations have been utilized in a wide range of commercially available products, including clothing, cosmetics, the food industry, sunscreens, etc.[14]

The widespread use of silver nanoparticles has raised in the scientific community an obvious question regarding the toxicity of silver nanocrystals towards eukaryotic cells. A consistent number of studies have indicated their toxicity not only towards bacteria but also towards a wide range of eukaryotic cells, including Paramecium[15,16] and mammalian cells.[17–22] Moreover, in eukaryotic organisms, such as Drosophila melanogaster or zebrafish, acute and chronic toxic effects of silver nanocrystals in vivo have also been observed.[23–25] In all those studies the dose-dependent toxicity of silver has been uncovered: the release of silver ions (Ag⁺) from AgNPs induces the formation of radical oxygen species (ROS) with subsequent mitochondrial dysfunction and DNA damage.[17,21,26,27] These cellular alterations are
considered to be the main factors involved in cell-cycle arrest. If the Ag⁺ released by the AgNPs persists, the cell activates mechanisms of apoptosis (controlled cell death). On the contrary, an interruption in the supply of Ag⁺ can be followed by cellular reactivation. [17,18] For these reasons, the local concentration of AgNPs is crucial in order to induce nanoparticle-mediated apoptosis on targeted cells, such as, for instance, cancer cells.

On the other hand, the association of cytostatic agents to a nanocarrier can limit the biological effects of the toxic agents only to specific sites. In order to achieve a controlled and localized administration of cytostatic agents to cancer cells, recently Sanpui and co-workers reported the preparation of biodegradable chitosan nanospheres decorated at their surface with AgNPs [29] A relevant apoptosis induction in human HT-29 colon cancer cells was demonstrated by using a much lower administered concentration of silver–chitosan nanostructures compared to the use of bare AgNPs. The chitosan nanospheres act as nanocontainers to confine AgNPs and locally enhance the Ag⁺ release.

As a further step in the development of engineered-nanocarriers, the combination of AgNPs with superparamagnetic probes – which can enhance the physical delivery towards specific cancer cell areas – is quite promising. Only few examples of hybrid magnetic silver nanostructures have been reported so far and they were mainly exploited as antimicrobial agents [29,30] Superparamagnetic nanoparticles (MNPs) have gained popularity as drug-delivery tools allowing magnetic manipulation under a magnetic field gradient, [31] as well as heat-therapeutic tools in hyperthermia treatment, even being combined with temperature-mediated drug release [32,33] Nanocarriers containing multiple MNPs are an appealing platform for the delivery of cytotoxic agents as they have shown a faster response to the magnet with respect to individual MNPs while keeping their superparamagnetic behavior [31,34,35].

Inspired by these works, we explore here the possibility of confining a large number of AgNPs on a magnetic carrier based on MNPs: we studied the cytotoxicity of such nanocomposites in vitro under different parameters, i.e., dose, incubation time, magnetic field applied during the culturing, and cell lineage. The comprehensive toxicity study carried out has revealed cytotoxicity effects which are the combination of features of both of the materials composing our hybrid silver-decorated magnetic nanobeads.

2. Results and Discussion

2.1. Preparation, and Structural, Optical and Colloidal Stability Investigation of the Ag-MNBs

We first clustered MNPs into a polymer matrix made of poly(maleic anhydride-alt-1-octadecene) using a procedure recently developed by us [36] In a second step, we nucleated AgNPs at the surface of the as-formed MNBs. At basic pH, the deprotonated carboxylic groups deriving from the hydrolysis of the polymer anhydrides could coordinate the Ag⁺ ions (silver nitrate, AgNO₃, was used as the Ag⁺ ion source). The addition of sodium borohydride (NaBH₄) promoted the silver reduction from Ag⁺ to Ag₀, which was detectable as a fast solution colour change from yellow to blue to brown, due to the nucleation of AgNPs onto the surface of the MNBs (Figure 1). To control the coverage of MNBs with AgNPs, different concentrations of AgNO₃ were tested. Transmission electron microscopy (TEM) performed on the Ag-MNBs showed a direct correlation between the number of AgNPs (of about 3–6 nm in size) at the bead surface and the amount of AgNO₃ (Figure 1b–c and S1b–e in the Supporting Information). Also, the gradual increase of the adsorption peak of the Ag-MNBs at 420 nm with increasing AgNO₃ concentration confirmed the higher coverage. The amount of AgNPs associated with the MNBs was also measured by elemental analysis. An increase of the Ag/(Fe+Mn) ratio from 0.69 to 2.4 was recorded when the amount of AgNO₃ was varied between 1 and 10 mM (Figure S2). The effect of the NaBH₄ on the nucleation was also studied. At 10–15 mM NaBH₄ concentration (and at 10 mM AgNO₃), the size of the AgNPs was assessed to be around 3–6 nm. At lower NaBH₄ concentrations the size of AgNPs increased up to 20 nm, but nucleation occurred only on few MNBs (Figure 1d–e and Figure S1f–j). This corresponded to the progressive red-shift of the Ag-MNBs absorption band from 420 to 440 nm when reducing the NaBH₄ concentration (Figure 1g). Although control over the coverage density and size of AgNPs was limited, an easy procedure was set to cover uniformly the surface of 100–150 nm MNBs with AgNPs of 5 nm. By dynamic light scattering (DLS), it was found that the Ag-MNBs dissolved in the culture media (provided with 10% FBS) were colloidal stable even for prolonged time and a slight increase of the DLS size of the Ag-MNBs was recorded when passing from water to the cell media (Figure S3).

2.2. Cytotoxicity Studies

The cytotoxicity of the as-prepared Ag-MNBs was assessed on two different cancer cells, HeLa and KB cells, by measuring the number of living cells after Ag-MNB treatment. A cell counting assay was carried out by an automated cell counter (Scepter™, Millipore) exploiting the Coulter principle of impedance-based cell-detection. At proper incubation times (6, 12, and 24 h), both cell types were exposed to different amounts of Ag-MNBs (referred as μg mL⁻¹ of silver measured by ICP). In presence of a magnet (0.3 T, with a magnetic field gradient of 36 T m⁻¹ at a 2 mm distance apart from the magnet) placed beneath the cell culture dish a clear time and dose dependence of cytotoxicity was found. The Ag-MNBs toxicity at 24 h on both cell lines was comparable (viability values around 20%–30%) with IC₅₀ doses (half-maximal inhibitory concentration) assessed respectively at 32 and 29 μg mL⁻¹. More significant is the comparison of those viability curves with the corresponding curves recorded in absence of the magnet. For the HeLa cells, the effect of the Ag-MNBs on the proliferation without magnet was negligible even after 24 h and at the highest dose tested (50 μg mL⁻¹). At 6 h the KB cells showed a viability above 80% at almost
all the concentration tested, while at longer exposure times (12 and 24 h) the Ag-MNBs became more toxic for doping concentrations higher than 20 μg mL⁻¹. As expected on samples of cells doped with MNBs not bearing AgNPs, no toxicity was recorded under any of the conditions studied (Figure S4 in the Supporting Information). Therefore, in summary these data show clear toxicity trend for HeLa and KB cells treated with Ag-MNBs, both in the presence and the absence of an applied magnetic field gradient; the toxicity was more remarkable when the magnet was placed beneath the cell dish, reaching in these conditions IC₅₀ doses of about 32 μg mL⁻¹ (Figure 2).

2.3. Intracellular Silver Uptake Experiments

The direct comparison of the curves with/without the magnet during culturing suggests that the toxicity is related not only to the amount of administered Ag-MNBs but also to the dose of Ag-MNBs taken up by the cells. To such purpose, we measured at a doping concentration of 32 μg mL⁻¹, the intracellular amount of Ag-MNBs for both cell lines. By looking at the absolute amount of Ag per cell (Figures 3a and b), the presence of the magnet on both cell lineages clearly induced a higher cellular uptake of the Ag-MNBs compared to the absence of the magnet (increasing from 10 to 80 pg-Ag cell⁻¹ for KB and from 9 to 50 pg-Ag cell⁻¹ for HeLa, at 12 h); this is responsible for the lower percentages of viable cells counted whenever the magnet was applied (Figure 2). Different uptake behaviors could also be distinguished between the two cell lineages. The HeLa cells have a faster uptake than the corresponding KB cells (at 6 h of incubation, the HeLa cells have taken up 50 pg-Ag cell⁻¹ versus 10 pg-Ag cell⁻¹ for the KB cells). At longer incubation time, the KB cells have internalized higher doses of Ag (80 pg-Ag cell⁻¹ for KB cells versus 50 pg-Ag cell⁻¹ for the HeLa cells). These data can explain well the higher acute toxicity (at 6 h of incubation) of HeLa with respect to KB cells when exposed to Ag-MNBs (at 32 μg mL⁻¹) in the presence of the magnet, while at longer incubation time the KB cells became more sensitive than the
HeLa cells to the Ag-MNBs due to the higher intracellular dose taken-up (lower percentage of viable cells are recorded for the KB cells with respect to the HeLa cells, Figure 2).

It is also worth analyzing the intracellular ion contents expressed as the ratio of Ag/(Fe\(^{2+}\)\(+\)Mn) per cell and to compare those values with the ratio of the as-prepared Ag-MNBs nanostructure externally administered to the cells (which corresponded to 2.6, the dashed line in Figures 3c and 3d). For the cells treated without the application of a magnet, this Ag/(Fe\(^{2+}\)\(+\)Mn) ratio is always higher than the initial one, while when the magnet guided the uptake of the Ag-MNBs, the intracellular ratio of Ag/(Fe\(^{2+}\)\(+\)Mn) was quite similar to

Figure 2. Viability assay for HeLa (a and b) and KB (c and d) cells when treated with Ag-MNBs at different doses in the absence (a and c) or the presence of a magnetic field gradient (b and d).

Figure 3. Intracellular uptake of Ag-MNBs on two cell lineages measured as the amount of Ag\(^+\) per cell (panels a and b) or as the ratio Ag/Fe\(^{2+}\)Mn per cell (c and d) in the presence or the absence of the magnet at different incubation times. All the experimental points were collected in triplicates. The dashed lines in panels c and d indicates the ratio of Ag/(Fe\(^{2+}\)\(+\)Mn) corresponding to the initial Ag-MNBs administered to the cells.
the initial one (externally administered to the cells). These results suggest a co-uptaking of Ag ions and Ag-MNBs when no magnet is present and a predominant uptake of Ag-MNBs when the magnet is applied. It is likely that the magnetic field gradient induces a homogeneous accumulation of Ag-MNBs at the cell surface, thus contributing to maintaining constant nanobead uptake. Overall these internalization data suggest a different nature of the silver form which provokes cytotoxicity when the cells are cultured in absence or presence of a magnet. Moreover, these data might also account for the high frequency of apoptosis observed by FACS after only 2 h of incubation and not detectable by the viability assay, as explained in the next paragraph.

2.4. Flow Cytometry Study

In order to gain more information on the status of the survived cells, a flow cytometry study was performed to identify apoptotic cells. Alexa Fluor®-labeled Annexin V was used for the detection of the phosphatidylserine on ongoing activated apoptotic cells. Both the cell types showed high levels of apoptosis at 32 μg mL⁻¹ of Ag-MNBs administered to the cells (65% of apoptotic cells for the Hela cells versus 44% for the KB cells after only 2 h). The percentages of the apoptotic cells increased with the incubation time and with the exposure to the magnet during the culturing, in accordance with the higher intracellular uptake/toxicity recorded (Figures 4 and S5 in the Supporting Information). It is noteworthy that almost negligible levels of induced apoptosis (17% for HeLa and 5% for KB) were found when the two cell lineages were incubated with MNPs that did not bear AgNPs (Figures 4 and S5).

2.5. Silver Release Assay from Ag-MNBs Surface

To elucidate the toxicity effect of the Ag-MNBs, we also studied the release of Ag⁺ on bare Ag-MNBs when kept at different conditions. The release of metallic ions depends on different parameters, including the capping agent on the silver nanoparticles, the pH of the medium, the incubation temperature, and the incubation time, which can induce the formation of ROS within the cells. While in water at pH 4 or 7 no significant release was detected over time; in complete cell-culture media after only 2 h about 6% (relative to the initial Ag concentration) of Ag⁺ from the Ag-MNBs was released, reaching a value of 16% at 72 h (Figure 5a). On Ag-MNBs, the AgNPs are not screened by any kind of ligands (Ag-NPs were nucleated only in presence of AgNO₃ and NaBH₄ and no specific stabilizer molecules were added) and likely the presence of some components in the media (especially the serum proteins, as reported by others[39,40]) might coat the Ag-NPs and promote the leakage of Ag⁺ released from the AgNPs. As expected in the latter case, the Ag⁺ measured in the supernatant also increased by increasing the initial concentration of Ag-MNBs in solution (16, 32, 40, and 50 μg mL⁻¹ of silver), leading to a constant release of Ag⁺ of up to 20% (Figure 5b).

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**Figure 4.** ApoAllert assay. a) the cell nuclei are stained in red by PI (propidium iodide), b) the DNA fragments present only inside the apoptotic cells are stained in green by means of fluorescein-dUTP, c) the apoptotic cell fraction on the total cells is shown in orange (all cells imaged were in apoptosis). FACS analysis: d) HeLa and KB cells when treated with MNBs or Ag-MNBs at 32 μg mL⁻¹ with or without magnet after 2 and 12 h at 37°C. As a positive control, H₂O₂ (5 μg mL⁻¹ for 500 μL of medium) was used.

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2.6. Adhesion Assay

We also looked at the effect of Ag-MNBs on the cell adhesion. The HeLa or KB cells were co-cultured with Ag-MNBs at different concentrations and the number of non-adherent cell was counted. In both cell lineages almost the totality of cells were not able to adhere to the substrate, indicating a severe interference of the Ag-MNBs on the adhesion process (Figure 6 and S6 in the Supporting Information). This phenomenon was still evident even when the lowest dose of Ag-MNBs was added. As a control trial, MNBs not bearing AgNPs on their surface were also used in the same experimental conditions and no remarkable effect on the adhesion was recorded at all the doping MNBs concentration tested.

2.7. Morphological Cell Study by Confocal Imaging

From a qualitative point of view, apoptosis was confirmed by the ApoAlert assay, by which apoptosis-induced nuclear DNA fragmentation (180–200 bp) via a TUNEL (terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling) fluorescence assay was detected. Figure 4 shows an optical merge of the green staining of nuclear DNA fragmentation of apoptotic cells with the red staining with propidium iodide (PI) of the nuclei of any cell, just revealed the fraction of apoptotic cells on the total cells imaged (in this image the cells labeled in red should correspond to healthy cells, but do not in this case, since all cells imaged were in apoptosis). To better evaluate the cytoskeletal modification occurring in cells treated with Ag-MNBs, the actin, one of the main proteins of the cytoskeleton, was first imaged via immunofluorescence.

The HeLa and KB cells used in this study showed a different morphology. HeLa cells were indeed more elongated and spread on the substrate, whereas KB cells maintained a more round shape and formed a close network when adherent in culture. After the incubation with Ag-MNBs, for HeLa, the actin filaments (in green) seemed less organized and also less cell interconnections were observed with respect to control cell samples. In treated KB cells, the actin filaments were confined to large cytoplasmic aggregates (Figure 7).

After incubation with Ag-MNBs, for both HeLa and KB cells, the actin filaments seemed less organized and also fewer cell interconnections were observed with respect to control cell samples (Figures 7a and d). This was the case not only for the cell culture in presence of the magnet (Figures 7c and f) but even for the cell culture when no external magnetic field gradient was applied during incubation (Figures 7b and d).

2.8. Morphological Cell Study by Both TEM and SEM Imaging

TEM images on sections of KB or HeLa cells treated with MNBs or Ag-MNBs confirmed the presence of nanostructures within large endosomes, thus suggesting in both cases that the nanobeads with or without Ag were internalized via endocytosis. Moreover, the AgNPs were still attached at the surface of the MNBs and no presence of free Ag nanoparticles was seen (Figures 8 and S7 in the Supporting Information). Scanning electron microscopy (SEM) provided an overview of the damages at the cell surface, as it revealed the presence of many apoptotic cells with their typical large apoptotic bodies together with several necrotic cells with breaks.
on their cell membranes (Figures 8c–d and S8). Obviously, in both cell types no sign of cellular stress was observed when cells were treated with MNBS not bearing AgNPs (Figures 8a, S7, and S9).

2.9. Laser Irradiation Trial and Viability Test

Heat generation by means of plasmonic NPs has been reported before. With the intent to study the laser irradiation on...
Cells treated with Ag-MNBs and MNBS, a set-up used for light-mediated heating of plasmonic nanoparticles inside cells was used.\cite{44} Cells were incubated either with Ag-MNBs or with silver polymeric nanobeads not bearing magnetic nanoparticles (Ag-NBs) or bare MNBS (not bearing AgNPs) and illuminated with a continuous near-infrared (NIR) laser for 5 min with a laser intensity of 7.7 mW μm$^{-2}$. The results clearly indicate that all AgNP-containing materials increased death of cells upon NIR irradiation, while no effect was seen on the MNBS (Figures 9 and S10). This effect correlates with a heavy nuclear fluorescent staining by DAPI due to a disruption of membrane integrity indicating an increase in phototoxicity of cells incubated with silver-containing nanobeads. Moreover, neither control cells (not treated with nanobeads) nor cells treated with the MNBS showed photoinduced damage as compared to the light unexposed cells. As expected for NIR light, toxicity due to the laser irradiation can be excluded and the effect on cell viability is only observed for the silver-containing nanobeads (both Ag-MNBs and Ag-NBs). In the context of these experiments, silver-containing beads were able to absorb the light of the laser and convert it into heat, likely due to the plasmon coupling of different AgNPs close to each other at the bead surface once inside the cell compartments. Though the plasmon resonance of the silver NPs peaks at 520 nm, there is sufficient absorption of light also at much higher wavelengths (as in our case at 830 nm), due to the close distance between individual NPs which results in coupling of plasmons of adjacent AgNPs.\cite{45} A similar effect has been described by other authors, mainly on gold NPs.\cite{41,46,47} The increase in the temperature was great enough

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Figure 9. Fluorescent and transmission images of cells in the presence and in the absence of the different nanobeads (MNBS, Ag-NBs, and Ag-MNBs) before and after light irradiation.
to kill the cells. Thus, the silver-containing nanobeads presented here are suitable for cellular hyperthermia. A certain degree of toxicity was observed in the cells incubated with both Ag-MNBs and Ag-NBs before light illumination. The rapid release of Ag⁺ from the surface of the nanobeads and the high degree of toxicity of these ions could be responsible for this increase in toxicity. However, the loss of viability was dramatically increased upon light irradiation of the Ag-MNBs and Ag-NBs-incubated cells.

3. Conclusion

A procedure to grow, in a controlled manner, AgNPs on top of polymeric magnetic nanobeads has been established. The total size of the silver-decorated magnetic nanobeads (Ag-MNBs) in water is still at the mesoscale (around 100 nm). The enhanced magnetically mediated cellular uptake strongly affects the toxicity of the Ag-MNBs. The intracellular accumulation of Ag-MNBs together with the release of Ag⁺ from the nanostructure surface are the factors which affect the cellular toxicity. The peculiar feature of accumulation of more AgNPs on top of nanoscale magnetic nanostructures allows for a drastically reduced dose of Ag-MNBs necessary to provoke toxicity to the cells; a dose of half-maximal inhibitory concentration of silver equal to 32 μg mL⁻¹ was found. This reduced dose was comparable to that achieved so far only on a few silver-based nanosystems and which did not show magnetic features. Additionally, a synergic and significant toxicity under laser irradiation has also been shown. As a future perspective, since toxicity related to the silver ion leakage from the Ag-MNBs surface was identified, upon providing a protecting layer to the Ag-MNBs; these hybrid materials might find application for the magnetic targeting and controlled release of toxic silver ions together with photothermal activation in cancer therapy.

4. Experimental Section

Chemicals: Milli-Q water (18.2 MΩ, filtered with filter pore size 0.22 μm) was from Millipore. All solvents used were of analytical grade and were purchased from Sigma-Aldrich. Poly(maleic anhydride-alt-1-octadecene), silver nitrate solution, sodium borohydride, glucose, Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), glutamine, penicillin/streptomycin, propidium iodide, GFP-phyaloxidin, glutaraldehyde, sodium cacodylate trihydrate, osmium tetroxide, and epoxy resin were purchased from Aldrich. Alexa Fluor®-labeled 647 Annexin V, ApoAlert kits were purchased from Invitrogen. RPMI-1640 without folic acid was purchased from Euroclone.

Synthesis of Silver-Magnetic Nanobeads (Ag-MNBs): The protocol for the preparation of the initial magnetic nanobeads was previously reported by our group. For the preparation of Ag-MNBs and the study of their toxicity on cells, it was necessary to enlarge the production of magnetic nanobeads. To such purpose, minor modifications were applied to the standard procedure. In details, MnFe₂O₄ nanoparticles of 9 nm in diameter were prepared according to the procedure reported by Sun et al. Briefly, a solution of MnFe₂O₄ nanoparticles in toluene (70 μL, 10 000 ppm of Fe-Mn concentration), was transferred in a 20 mL vial and mixed with a solution of poly(maleic anhydride-alt-1-octadecene) in tetrahydrofuran (THF, 150 μL, 50 mM). The mixture was dried completely under a flux of compressed air. After 20 min, THF (500 μL) was added and the vial was transferred on an orbital shaker at 1200 rpm. Soon after, ACN (1 mL) was added at a flow rate of 0.75 mL min⁻¹. The nanobeads obtained were collected by magnetic separation and the magnetic nanobeads (MNBs) were dissolved in ultrapure water (Figure S1a). This step was repeated three times. After the washing steps, around 500 μg of MNBs (referred as the sum of Fe+Mn) were usually obtained.

For the nucleation of AgNPs at the surface of the nanobeads, in a typical synthesis MNBs (140 μg, considering Fe+Mn) were dispersed in ultrapure water (20 mL, obtaining a suspension with a final concentration of 130 μg). NaOH (500 μL, 1 M) was added to the nanobeads and the vial was transferred on an orbital shaker, at a speed of 1200 rpm. Thereafter, silver nitrate (5 mL, 10 mM, aqueous solution) was added in 1 min and the solution was shaken for 10 min. After this time lapse, a solution of reducing agent (NaBH₄, 5 mL, 10 mM) was added at a flow rate of 2 mL min⁻¹. The reaction vial was shaken for 10 min and then centrifuged at 2500 rcf for 30 min to recover the Ag-MNBs. The precipitate, containing the Ag-MNBs, was recovered, redissolved in water and filtered on 1 μm syringe filters to remove large aggregates, followed by three steps of magnetic separation. The final product was dissolved in ultrapure water and stored at 4 °C until the cellular experiments were performed. In order to define the best conditions for the synthesis of the Ag-MNBs, different concentrations of AgNO₃ and NaBH₄ were tested. In the first series of experiments, the NaBH₄ solution was fixed at 10 mM and the AgNO₃ concentration was varied from 1 to 10 mM (1, 2.5, 5, and 10 mM) while keeping all the other parameters unchanged. In the second set of experiments, the NaBH₄ concentration was varied between 0.1, 1, 5, 10, and 15 mM while keeping the AgNO₃ fixed at 10 mM and all the other parameters unchanged.

Elemental Analysis: The concentrations of the silver, iron and manganese were measured by elemental analysis using an ICP-AES spectrometer (iCAP 6500, Thermo). The samples were digested in concentrated HCl/HNO₃ 3:1 (v/v) solution.

Absorption Spectra: The absorption spectra were recorded by a Cary300 spectrophotometer (Varian) in semi-micro PMMA cuvettes. The analyzed solutions contained the same amount of nanobeads (normalized by ICP-AES to amount of Fe and Mn).

DLS and Zeta Potential Measurements: Dynamic light scattering (DLS) and zeta potential measurements were performed using a Zeta Sizer (Malvern Instruments) equipped with a 4.0 mW He-Ne laser operating at 633 nm and an Avalanche photodiode detector.

Confocal Microscopy Characterization: Images of treated cells for the apop assay and the F-actin staining were acquired by using a Nikon confocal microscope (Nikon Optical Co., Ltd., Tokyo, Japan).

Transmission Electron Microscopy (TEM): Low-resolution TEM images on the Ag-MNBs and on the MNBs and on the cells treated with different nanostructures were recorded with a Jeol Jem 1011 microscope operating at an accelerating voltage of 100 kV.
Scanning Electron Microscopy (SEM): SEM images of Ag-MNBs treated cells were acquired using a Jeol JSM-6490LA scanning electron microscope.

Microscopy Set-up For Laser Irradiation: A continuous wave (CW) laser diode with 830 nm emission wavelength and with optical power up to 100 mW μm⁻² was used to induce hyperthermia. The laser was coupled by means of a beam-splitter (2P-Beamsplitter 725 DCSPXR from AHF Analysentechnik) to a microscope (AxioTech from Zeiss, Germany) and the collimated laser beam was focused onto the sample through the microscope objective (W Achroplan 10× magnification N.A. 0.25 from Zeiss, Germany) by a xy-stage. The sample was positioned in the field of view by a micrometer resolution motorized xyz-stage (SMI from Luigs & Neuman Feinmechanik und Elektrotechnik GmbH, Germany). All images were recorded with a microscope AxioTech (Zeiss, Germany) which was equipped with a phase contrast and a DAPI fluorescence (365 nm excitation and 445 nm emission) filter sets. Illumination in transmission mode was provided by a 100 W white light source (halogen lamp, HAL-100 from Zeiss, Germany) whereas in fluorescence mode, illumination was provided by an HBO-100 lamp (from Zeiss, Germany). Images were collected by a CCD camera (MRc AxioCam, Germany) connected to a PC and further analyzed with the software AxioVision (release 4.6 from Zeiss, Germany). The resulting power of the focused light beam (i.e., the light power of the light spot used for hyperthermia) was measured by a powermeter (Fieldmax II TO from Coherent) equipped with an optical sensor (Powermax PM3 from Coherent).

Cell Lineages: HeLa cells (ATCC CCL-2™), derived from cervical cancer cells and KB cells (ATCC CCL-17™), derived from epidermal carcinoma of the mouth, were respectively grown in DMEM and RPMI-1640 supplemented with 10% FBS, 2% essential amino acids and 1% each of non-essential amino acid, vitamins and penicillin-streptomycin at 37 °C in a humidified atmosphere with 5% CO₂.

In Vitro Cell Counting Assay: The cytotoxicity of the nanostructures was investigated by counting the number of living cells after treatment with Ag-MNBs. The cell-counting assay was carried out using a commercially available system, the Scepter™ micropette (Millipore). The Scepter™ micropette exploits the Coulter principle of impedance-based particle detection. Moving into the microfluidic path of the system, each cell produces a change in the measured impedance. This change is proportional to the size of the analyzed cells. For healthy cells this measure is quite different than those of unhealthy cells (necrotic cells are generally smaller in size than the healthy ones).

HeLa and KB cells were cultured in glass-bottom dish for 24 h and then incubated for 6, 12, and 24 h at 37 °C with concentration of Ag-MNBs fixed at 16, 32, 40, and 50 μg mL⁻¹ of silver. To understand the role of magnetic field gradient on cell uptake and toxicity, the exposure to Ag-MNBs was performed both in the presence and the absence of a magnet (0.3 T) placed beneath the dish culture. After the incubation, the cells were washed, detached and counted in suspension.

Intracellular Silver Uptake: The amount of Ag-MNBs uptaken by the cells was measured by elemental analysis. Both cell lineages were incubated with Ag-MNBs at 32 μg mL⁻¹ of silver for 6 or 12 h with or without the application of an external magnetic field gradient. After three washing steps, the cells were detached, counted and digested in concentrated HCl/HNO₃ 3:1 (v/v) solution.

FACS Analysis: Apoptotic cells expose phosphatidylserine on the outer cellular membrane. Annexin V is a Ca²⁺-dependent phospholipid-binding protein with high affinity for phosphatidylserine. Hence, this protein can be used as a sensitive probe for the detection, by FACS analysis, of phosphatidylserine at the membrane of apoptotic cells. 300 000 cells treated with Ag-MNBs (32 μg mL⁻¹ of silver) were incubated with or without the presence of magnet for 2 and 12 h. As positive control, apoptosis was induced using H₂O₂ (5 μL, 5%, Sigma Aldrich) in growth medium (500 μL). After the incubation period the cells were harvested and wash in cold phosphate-buffered saline (PBS). Annexin V Alexa fluor 647-labeled (5 μL, Invitrogen) was added to each 100 μL of cell suspension and incubated for 15 min. After the incubation period, annexin-binding buffer (400 μL) was added and the samples were analyzed using BD FACS Calibur (BD BIOSCENCES).

Silver Release Assay: In the first approach, Ag-MNBs were dispersed in ultrapure water (at pH 7 or 4, the latter acidified by HCl addition) or in DMEM medium supplemented with 10% of FBS at a final concentration of (32 μg mL⁻¹ of silver). The Ag-MNBs solutions were incubated for 2, 24, or 72 h at 37 °C under a 5% CO₂ atmosphere and soon after the Ag-MNBs were magnetically separated by the solution and the Ag concentration in the supernatant was measured by ICP. In the second trial, we tested the silver ion release from Ag-MNBs in DMEM medium supplemented with 10% of FBS at different concentrations of Ag-MNBs (16, 32, 40, and 50 μg mL⁻¹ of silver) and incubated for 24 and 48 h (37 °C, 5% CO₂).

Adhesion Study: 150 000 cells were cultured for 24 h in a 6-well dish, they were trypsinized, washed in PBS and mixed in suspension with Ag-MNBs at four different concentrations (16, 32, 40, and 50 μg mL⁻¹ of Ag). The cells were then reseeded in a new 12-well dish at 37 °C at 5% CO₂ for 6 h. Soon after the flasks were analyzed by optical microscope and the non-adherent cells in solution were counted using the cell counting system (Scepter™, Millipore).

Confocal Microscopy Staining of Actin Filaments: 300 000 HeLa and KB cells were cultured in a glass-bottom dish for 24 h. Cells were then treated with Ag-MNBs (32 μg mL⁻¹ of silver) for 12 h at 37 °C. Cells were treated with Phalloidin Green Fluorescent 688 conjugated (2 μg), while nuclei were stained using Propidium Iodine (12 μg mL⁻¹).

ApopAlert Assay: The ApoAlert DNA Fragmentation Assay Kit detects apoptosis-induced nuclear DNA fragmentation via a fluorescence assay. Terminal deoxynucleotidyl transferase (TdT) catalyzes the incorporation of fluorescein-dUTP at the free 3'-hydroxyl ends of the fragmented DNA. Fluorescein-labeled DNA can be detected via fluorescence microscopy thus indicating apoptotic cells. For the same images, the propidium iodine colors in red the nuclei of any living cells.

TEM on Treated Cells: For the characterization of the cells treated with MNBs and Ag-MNBs, after the incubation with the nanostructures, the KB or HeLa cells (6 × 10⁴) were washed three times in cacodylate buffer (0.1 M), fixed with glutaraldehyde in cacodylate buffer (2.5%) at 4 °C for 30 min and washed three additional times with cacodylate buffer. A solution of osmium tetroxide (1%) in cacodylate buffer was added for 1 h at room temperature. Following three washing steps in cacodylate buffer, the cells were dehydrated with 30%, 50%, 75%, 85%, 95%, and 100% (three times) absolute ethanol. Thereafter, the cells were infiltrated with Epon resin (two steps: 50% and 66% for resin in absolute
ethanol, 30 min each) and embedded in 100% resin at 60 °C for 2 d. Ultrathin sections (70 nm thick) were cut on a EM-UC6 (Leica Microsystems) ultramicrotome, stained with lead citrate and observed under a Jeol JEM 1011 operating at 100 KV.

**SEM Characterization on Treated Cells:** KB cells doped with Ag-MNBs both in adhesion and in suspension were fixed in glutaraldehyde (2%) in 0.1 M cacodylate buffer for 1 h, washed in the same buffer, post-fixed in a solution of osmium tetroxide (1%) in cacodylate buffer for 1 h and extensively washed in distilled water. The cells were then rapidly frozen in slush nitrogen to avoid the Leidenfrost effect. After immersion in the slush, the specimens were transferred to the cryo-chamber cold stage of a K750X Freeze Drier (Emitech Ltd., U.K.) and freeze-dried under vacuum overnight, allowing the temperature to gradually rise from −70 °C to room temperature. The specimens were then coated with 15 nm gold in a sputter coater (108auto/SE Cressington) and observed under SEM.

**Laser Irradiation Experiment and Viability Test:** 8 × 10^4 embryonic fibroblasts (3T3) were incubated in 1 mL growth medium (DMEM-F12 Ham’s supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% L-glutamine) in an ibidi dish over night to allow cell attachment. The day after the cells were incubated over night in growth medium with the different nanostructures: Ag-decorated NBs (Ag-NBs not bearing iron oxide nanoparticle inside), Ag-MNBs, and MNBs at a final concentration of 4 μg mL⁻¹ silver. After the treatment, cells were washed two times with PBS and were irradiated with an infrared laser emitting at 830 nm at a final intensity of 7.7 mW μm⁻² (20 mW and 2.6 μm² of laser spot area) for 5 min. To control possible laser-induced toxicity, cells in the absence of nanostructures were also illuminated following the same procedure. The cells were tested for viability before and after laser irradiation using an exclusion dye (DAPI), which tests the integrity of the cellular membranes (plasma and nuclear). Cells were incubated with DAPI (100 μM) in PBS for 5 min at room temperature then rinsed two times with PBS and incubated again in growth medium without phenol red. DAPI is only able to penetrate the cell if membrane damage are present, thus staining the cell nuclei. If the cells are healthy DAPI cannot diffuse through the cell membranes and the viable cells are not fluorescent.

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author. Additional details on structural characterization of the various nanostructures and characterization of cells are included.

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**References**

Supporting Information

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Magnetic Nanobeads Decorated with Silver Nanoparticles as Cytotoxic Agents and Photothermal Probes

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Supporting information

Magnetic nanobeads decorated with silver nanoparticles as cytotoxic agents and photothermal probes

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Figure 1S. TEM characterization of initial Ag-MNBs obtained using respectively 1, 2.5, 5 and 10 mM AgNO₃ solutions (from b to e) or alternatively by using respectively 15, 10, 5, 1, 0.1 mM NaBH₄ solutions (from f to j). In panel a) the characterization of bare magnetic nanobeads is reported. The scale bar for the insets is 20 nm. The Ag-MNBs showed in panels e and g have been prepared in the same conditions and correspond to the Ag-MNBs used in the cellular study. TEM characterization showed a higher surface coverage of AgNPs when gradually increasing the AgNO₃ concentration (b-e) while AgNPs with bigger diameters were nucleated at the bead surface when increasing the NaBH₄ concentrations however, a less homogeneous nucleation was also observed (f-j).
Figure 2S. Correlation between the initial AgNO₃ concentration used for the preparation of Ag-MNBs and the final Ag/(Fe+Mn) ratio measured by elemental analysis via ICP-AES. The initial MNBs had a diameter of 150 nm. These ratios have a certain variability related to the initial batch of MNBs. The cellular studies have been performed with Ag-MNBs having a ratio of 2.5±0.1. The Ag/Mn+Fe ratio provides a clear indication of a higher surface coverage of AgNPs at the bead surface when increasing the initial AgNO₃ concentration.
Figure 3S. Dynamic light scattering (panel a) and Zeta Potential measurements (panel b) of bare MNBs (blue line) and Ag-MNBs (red line). The hydrodynamic diameters given as intensity means of MNBs and Ag-MNBs in water just indicate the presence of individual beads in solution and the absence of aggregates. The stability is likely ascribed to the negatively surface charge of both MNBs and Ag-MNBs. The Ag-MNBs once dispersed in the complete cell media show a shift of the peak from 120-160 to 220 nm (green line), thus indicating the adsorption of some serum proteins at the bead surface.[1,2] Also in this case the absence of big aggregate can be assessed.

Figure 4S. Cell counting assay on HeLa cells and KB cells treated with MNBs. The amount of MNBs administered to the cells were 6.4, 12.8, 16 and 20 µg/mL of Fe+Mn which correspond respectively to the amount of Ag-MNBs of 16, 32, 40 and 50 µg/mL of Ag (considering a ratio of Ag /Fe+Mn equals to 2.5) All the experimental points have been repeated in triplicates. Panels a and c refer to the cell culturing in absence of the magnet, while panel b and d refer to the cell culturing in presence of the magnet.
Figure 5S. Flow cytometry analyses on HeLa (a-e: 2 hours incubation time, f-j: 12 hours incubation time) and KB (k-o: 2 hours incubation time) cells after staining with Alexa Fluor®-labelled Annexin V. Panels a, f and k refer to control cells. Panels b, g and l refer to incubation with MNBS. Panels c, h and m refer to cells treated with H₂O₂. Panels d, i and n refer to cells incubated with Ag-MNBs. Panels e, j and o refer to the corresponding experiments performed in presence of the magnet. In all the graphs the x-axis corresponds to the fluorescence intensity of Alexa Fluor®-labeled Annexin V, whereas the y-axis corresponds to the number of events. For the KB cells after 12 h of exposure the toxicity was so high that it was not possible to recover a sufficient amount of cells for the FACS analysis.
Figure 6S. Adhesion assay. Optical microscope images of the culture flasks are here reported. Panels a and d refer to control samples of respectively KB and HeLa cells; panels b and e refer to the same cells incubated with MNBs; panels c and f refer to same cells incubated with Ag-MNBs (16 μg/mL of Ag). Similar results were observed by increasing the concentration of Ag-MNBs (data not shown).

Figure 7S. TEM characterization of HeLa cells doped with MNBs (a) or with Ag-MNBs (b and d, refer to cells incubated in presence of the magnet). The scale bar for the insets is 200 nm.
Figure 8S. SEM image of KB cells not treated with any nanostructures.

Figure 9S. SEM images of KB cells doped with Ag-MNBs. a-c: KB cells after 4 h of incubation in suspension. a, control. Note the numerous long and thin cell filopodia (arrowheads); b, apoptotic KB cell with its characteristic apoptotic bodies (asterisks); c, necrotic KB cell. Note the numerous breaks on the cell membrane (arrowhead). d-f:

Laser irradiation experiment and viability test. Fluorescent images were analysed using the freelance software Image J (NIH). The pixel intensity was plotted against the frequency of appearance to obtain a histogram that showed the distribution of the data. Figure 9S provides clear evidences for photoinduced injury in cells treated with Ag-MNBs as well as with silver decorated nanobeads (Ag-NBs, not bearing
magnetic nanoparticles) by a significant shift of the intensity peaks as well as the area under the curve to higher values.

**Figure 10S.** Statistic for the viability before and after laser irradiation. Cells (3T3) were incubated with MNBs, Ag-NBs and Ag-MNBs. Untreated cells remained as control for photo-toxicity. Cells were tested for viability by nuclear DAPI staining of damaged cells. Fluorescent images were taken and analysed. The pixel intensity was plotted against the frequency to obtain a data distribution. The results for the viability of the cells in the absence or in the presence of the different nanobeads before (dot lines) and after (continuous lines) light irradiation, are reported.

**References**
