Macrophage entrapped silica coated superparamagnetic iron oxide particles for controlled drug release in a 3D cancer model

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Abstract

Targeted delivery of drugs is a major challenge in treatment of diverse diseases. Systemically administered drugs demand high doses and are accompanied by poor selectivity and side effects on non-target cells. Here, we introduce a new principle for targeted drug delivery. It is based on macrophages as transporters for nanoparticle-coupled drugs as well as controlled release of drugs by hyperthermia mediated disruption of the cargo cells and simultaneous liberation of nanoparticle-linked drugs. Hyperthermia is induced by an alternating electromagnetic field (AMF) that induces heat from silica-coated superparamagnetic iron oxide nanoparticles (SPIONs). Here, we show proof-of-principle of controlled release by the simultaneous disruption of the cargo cells and the controlled, AMF induced release of a toxin, which was covalently linked to silica-coated SPIONs via a thermo-sensitive linker. Cells that had not been loaded with SPIONs remain unaffected. Moreover, in a 3D co-culture model we demonstrate specific killing of associated tumour cells when employing a ratio as low as 1:40 (SPION-loaded macrophage : tumour cells). Overall, our results demonstrate that AMF induced drug release from macrophage-entrapped nanoparticles is tightly controlled and may be an attractive novel strategy for targeted drug release.

Keywords:

Cell based drug delivery; macrophages; magnetic silica nanoparticles; hyperthermia; controlled drug delivery, 3D tumor model

Introduction

Strategies for controlled release of drugs are being developed to facilitate a local and/or on time delivery of therapeutic compounds thereby avoiding toxic side effects emerging from systemic and continuous drug applications. In the last years, a variety of different cues have been proposed to achieve controlled release[1, 2]. Generally, while physiological triggers such as pH[3], temperature[4], redox activation and enzymatic degradation[5] provide the opportunity for self-reactive and autonomous liberation of therapeutic compounds, they pose a challenge with regard to limited specificity of the trigger. Thus, drug release systems that are activated in response to externally induced and temporally and/or locally controlled triggers represent an attractive alternative. Such more specific triggers include light, non-physiological temperature, ultrasound as well as electric and magnetic triggers.

Various materials have been proposed as carriers for drugs. Among others, mesoporous silica nanoparticles (MSNs) have emerged as a promising platform because of their large surface area, tunable pore size and permanent pore system as well as controllable particle size and shape, and versatile functionalization of the particle surface[6-10]. Mesoporous silica shows good biocompatibility and low toxicity[11-13]. Therapeutic drugs can be either encapsulated within MSNs or attached to the surface via a functional group or linker[14]. MSNs have been used in medical research as drug delivery vehicles or carriers of growth factors, nucleic acids, cell markers or other molecules. Different molecules, such as folate or other specific ligands as well as antibodies have been attached to the surface of MSNs with the aim to target the particles to specific environments, i.e. cancer[15]. However, it remains to be shown that these strategies are viable[16, 17].

Core-shell nanoparticles based on a superparamagnetic core and a biocompatible inert shell offer additional options. In an oscillating electromagnetic field, such particles can reach the characteristic supermagnetic threshold, once the core-shell size falls well below 100 nm and iron oxide core sizes fall below 25 nm and 30 nm for magnetite (Fe_3O_4) and maghemite ($\gamma\text{-Fe}_2\text{O}_3$), respectively. In this case, Néel and Brown relaxation leads to heat dissipation[18]. Because of their specific physical properties these superparamagnetic iron oxide nanoparticles (SPIONs) gained high interest for various biomedical applications. SPIONs have been exploited as contrast agents in magnetic resonance imaging (MRI) already for nearly three decades[19]. They can in principle be guided to a target with the help of an externally applied magnetic field, but this approach is limited to superficial areas of the body[20]. Directing SPIONs to an implant site (implant-directed magnetic drug targeting), where the implant itself

can locally enhance an externally applied magnetic field, appears to be a viable option[21]. Similar to MSNs also SPIONs have been functionalized with various molecules including antibodies or receptor ligands with the aim to facilitate visualization of tumors[22-31]. A combination of SPION cores with mesoporous silica shells appears especially attractive due to the easy functionalization of silica and the high cargo load the porous shell can carry[32, 33]. Another interesting property of SPIONs is their ability to induce local heat when exposed to an external alternating magnetic field (AMF).

Various strategies have been developed to exploit AMF-induced heat to locally increase the temperature or to trigger the release of encapsulated drugs from SPIONs, either by attaching these molecules via thermosensitive linkers or by disrupting the integrity of the SPIONs[34]. Already a minor elevation of temperature of few degrees is sufficient to alter cellular functions and to provoke cell death as a consequence of various mechanisms, including apoptosis, protein degradation, DNA damage as well as reduced fluidity and integrity of the cellular membranes[35]. The capacity of SPIONs to generate local heat if exposed to AMF has been exploited to induce hyperthermia in cancer tissue. First studies could show tumor reduction upon direct injection of SPIONs into the tumor sites[36-41]. More recently, it was explored if SPIONs could be targeted to the tumor tissue with the help of tumor-infiltrating cells. Intravenous injection of such SPION-loaded mesenchymal stem cells resulted in enrichment of SPIONs in tumor tissue and subsequent application of AMF was sufficient to induce hyperthermia and cell necrosis to a certain extent. However, no significant reduction in tumor size was observed, indicating that the therapeutic potential of hyperthermia induced by such an approach might be limited[42].

In the last years, macrophages emerged as another/an interesting cell type for take up of nanoparticles [43, 44]. However, controlled drug release from macrophage entrapped nanoparticles remains to be explored. Here, we explore macrophages as carriers for SPIONs carrying a silica shell. We show that macrophages efficiently take up the silica-coated SPION nanoparticles. AMF-induced hyperthermia can induce death of loaded macrophages in a cell type specific manner. Using silica-coated SPION nanoparticles functionalized with a toxin via a thermosensitive linker (T-SPIONs) we demonstrate that AMF is sufficient to liberate the toxin from T-SPION loaded cells, with negligible spontaneous release. The release of the toxin could specifically induce the death of Kaposi's sarcoma cells in 3D spheroid co-cultures, thereby potentiating the efficiency of heat-induced killing of tumour cells.

Materials and Methods

Cell culture

The mouse macrophage cell lines IC21, J774a.1, RAW264.7 as well as the human breast carcinoma cell line MCF-7 and the human monocyte cell line U937 were obtained from DSMZ (Braunschweig, Germany). IC21 and U937 cells were cultured in RPMI media supplemented with 10% fetal calf serum, 60 µg/mL penicillin and 100 µg/mL streptomycin. J774a.1 and RAW264.7 cells were cultured in DMEM media supplemented with 10% serum, 60 µg/mL penicillin and 100 µg/mL streptomycin, MCF-7 cells were cultured in DMEM media supplemented with 10% serum, 0.01 mg/mL human recombinant insulin, 60 µg/mL penicillin and 100 µg/mL streptomycin.

K-EC represent a KSHV infected human endothelial cell line (designated rKSHV-HuARLT elsewhere[45]). The cells were cultivated on plates coated with 0.5% gelatin (G1393-100ML, Sigma) in endothelial growth medium (EGM, CC-3124, Lonza) in the presence of 2 µg/mL doxycycline. Maintenance cultures of rKSHV-HuARLT cells additionally contained 5 µg/mL puromycin, while all the experiments were performed in the absence of the selection drug. Note that K-ECs express virus encoded eGFP.

Primary monocytes were isolated from mouse using a protocol described elsewhere[46]. The cells were differentiated for 3 days in IMDM media (Gibco™ 12440061) supplemented with 10% fetal calf serum, 60 µg/mL penicillin and 100 µg/mL streptomycin and 20% M-CSF containing supernatant from recombinant L929 cells for three days and subsequently cultured in absence of M-CSF for additional 4 days. Macrophages were harvested on day 7 for the indicated experiments.

All cells were cultured at 37 °C in a humidified normoxic atmosphere with 5% CO₂.

SPIONs, T-SPIONs and FITC-SPIONs

As non-modified SPIONs we used nanostructured silica coated superparamagnetic iron oxide particles (MAGSILICA®) obtained from Evonik Industries AG, Essen, Germany.

For the formation of T-SPIONs nanostructured silica coated superparamagnetic iron oxide particles MAGSILICA® (Evonik Industries AG, Essen, Germany) were covalently modified with a toxic maytansinoid to yield T-SPIONs. The synthesis and characterization of these drug-nanoparticle conjugates are described in detail elsewhere [47, 48]. Briefly, for the

formation of T-SPIONs SPIONs (MAGSILICA®, Evonik Industries AG, Essen, Germany) were covalently modified with a maytansin derivative [49]. This derivative containing a modified ester side chain at C3 for attachment to functionalized MAGSILICA® was obtained by a combination of mutasynthesis using a mutant strain of *A. pretiosum* blocked in the biosynthesis of AHBA and semisynthesis[50]. The thermosensitivity is associated with a retro Diels Alder concept (see supplementary Figure 3A for details). The preparation and the physical as well as biological properties of the nanostructured particle-drug conjugate employed were described previously in detail[48].

The payload of the toxin was found to be 0.4 mg per 100 mg MAGSILICA®, the release efficiency was 0.06 mg payload per 100 mg MAGSILICA® (~15% of theoretical loading) (see Seidel et al., 2017 for further details). The specific adsorption rate was calculated to be 33.94 W/g in the presence of the magnetic field with an amplitude of 4.8kA/m at 0.2mg/ml (0.12mg in 0.6ml) concentration (see Supplementary Information II for details of the calculation).

Fluorescein isothiocyanate-loaded SPIONs, designated FITC-SPIONs, were synthesized using hydrophilic superparamagnetic iron oxide nanoparticles as core material[21]. The iron oxide nanoparticles were coated with silica and labelled with FITC[32]. The synthesis and the characterization are described in detail in Supplementary Information II and Figures S4-6.

Uptake of SPIONs by macrophages and microscopy

Unless otherwise stated, 30 µg SPIONs were used for loading 10⁵ cells. Loading was confirmed by phase contrast microscopy.

For uptake studies, cells were seeded at a concentration of 40,000 cells/cm² per well of a 48 well plate (Costar Corning) and grown in indicated media overnight. The cells were incubated with 30 µg of SPION or FITC loaded SPIONs for 2 hours in serum free media. Afterwards, the cells were washed 3 times with PBS to get rid of non-loaded particles. Uptake of FITC-loaded and unloaded SPIONs was analyzed using flow cytometry (LSR II, BD Biosciences), confocal microscopy (Zeiss 510 Laser Scanning confocal microscope) and transmission microscopy of resin embedded ultrathin sections (Zeiss EM910, Oberkochen, Germany). To further assess apoptosis, we performed scanning electron microscopic imaging with aldehyde fixed, critical-point dried and gold-palladium sputter coated samples (Zeiss Merlin, Oberkochen, Germany).

178

179 **Biocompatibility**

180 Cells were cultured in 48 well plates (Costar Corning) at a concentration of 40,000 cells/cm²
181 per well and incubated with varying amounts of SPIONs (20, 50 and 100 µg per 10⁵ cells) in
182 serum free media for 2 hours. Afterwards, the cells were washed and suspended in growth
183 media supplemented with FCS. The cells were incubated for 48 hours and viability of cells
184 was analyzed using flow cytometry based Annexin V apoptosis assay (ab14058, Abcam) and
185 WST-1 assay (5015944001, Roche).

186

187 **AMF induced hyperthermia**

188 10⁵ cells were loaded with 30 µg SPIONs as described above. Cells were detached from the
189 wells by trypsinization and re-suspended in culture media. According to the previously
190 evaluated release kinetics (Seidel et al., 2017) a magnetic field was applied for 40 minutes
191 with a constant field amplitude H_0 (4.8 kA/m, 6 mT or 60 Oe) and constant frequency f (779
192 kHz) using a magnetic field inductor (Hu5000+, Himmel, Germany). This procedure did not
193 result in measurable increase of cell culture media indicating that AMF induced heat is local.
194 Afterwards, the cells were incubated in complete culture media for 24 hours and then
195 subjected to AnnexinV and WST-1 assays. Raptinal, an apoptosis inducing agent, was used at
196 a concentration of 2 µM.

197

198 **Toxin release upon AMF**

199 Transwell (Costar Corning, 3470) with a pore size of 0.4 µm were used to allow diffusion of
200 the soluble media ingredients including the toxin but not cells and SPIONs. Briefly, the upper
201 chamber contained either cell-free T-SPIONs or T-SPION-loaded J774a.1 macrophages.
202 AMF exposure or mock treatment of cell-free T-SPIONs and T-SPION-loaded J774a.1
203 macrophages was done directly before transfer to the upper chamber. As controls, unloaded
204 J774a.1 cells or the free toxin were added to the upper chamber. The lower chamber carried
205 the target cells, MCF-7 human breast carcinoma cells or K-EC. Viability of MCF-7 cells and
206 K-EC was analyzed using WST-1 post 72 hours of incubation.

207

208   **Spheroid production**

209   4000 K-EC cells per well were seeded on a 0.5% agarose-coated 96-well plate. After 24 or 48
210   hours, the formed aggregates (spheroids) were harvested and subjected to AMF in 1 mL tubes
211   with 200 μ L media. Afterwards, the spheroids were cultured on agarose coated 96 well plates
212   for 48 hours before proceeding to either confocal microscopy or viability assays. Image J
213   software was used to reduce agarose dependent background signal of light microscope
214   pictures.

215   For the co-culture, K-EC cells and J774a.1 cells were employed in a ratio of 40:1 and handled
216   as described before. For confocal microscopy, J77a.1 cells were labelled with cell tracker red
217   (Invitrogen C34552).

218

Results

Efficiency of SPION uptake by macrophages and biocompatibility

To assess uptake efficiency and consequences of SPIONs for macrophages, cells were incubated with FITC-labelled SPIONs, excessive particles removed and analyzed by flow cytometry and confocal microscopy. To assess the uptake in different cells various macrophage cell lines were compared to primary bone marrow derived mouse macrophages. Uptake efficiencies varied between >95% and 80% (Figure 1A, 1B). Confocal microscopy (Figure 1C) and TEM analysis (Figure 1D) demonstrated internalization of SPIONs in intracellular compartments. Biocompatibility of SPION loading was determined after 2 days of culture by quantification of metabolic activity and apoptosis. We employed both assays to distinguish between reversible cell damage and terminal killing. Raptinal, a control apoptosis inducer significantly decreased the viability of most types of macrophages with exception of IC21 cells that show rather high resistance to this drug (Figure 1E). No induction of apoptosis was observed even with the highest loading of SPIONs (Figure 1E) while metabolic activity was reduced maximally by 20% (Figure S1). In conclusion, these results indicate high compatibility of the tested macrophages upon internalization of SPIONs.

Cell killing by Alternating Magnetic Field (AMF) induced hyperthermia

To release nanoparticles from loaded macrophages we employed targeted killing by hyperthermia. Since exposure of SPIONs to an alternating electromagnetic field (AMF) results in hyperthermia[51] we evaluated the efficiency of AMF to induce cell death in SPION loaded cells. Loaded macrophages were placed inside of a 2-coil inductor, exposed to an AMF and further cultivated for 24 hours (Figure 2A). Cell viability was determined by WST-1 analysis and Annexin V based apoptosis assay. Unloaded macrophages subjected to AMF and SPION-loaded macrophages without AMF exposure were used as controls. The exposure of SPION-loaded primary mouse macrophages, RAW264.7 and J774a.1 cells to AMF resulted in a dramatic decrease of metabolic activity in a dose dependent manner (Figure 2B, S2) and a comparably strong apoptotic reaction (Figure 2C). In contrast, unloaded cells exposed to AMF or SPION-loaded macrophages without AMF application did not show a notable drop in metabolism and apoptosis. The application of the AMF to SPION loaded IC21 cells neither changed their metabolic activity nor induced apoptosis (Figure 2B, 2C) suggesting a higher resistance of these cells to apoptosis induction. Scanning electron microscopy was then used to visualize the effect of AMF application on the morphological

characteristics of the surface of SPION-loaded J774a.1 cells (Figure 2D). Control cells and SPION-loaded cells exhibited distinct lamellipodial extensions and an amoeboid shape, while AMF exposure induced characteristic apoptotic tags such as cell shrinkage and membrane blebbing.

In essence, these results indicate that the exposure of SPION loaded cells to AMF induces cell killing while the efficacy of this process can be modulated by SPION-dosing.

Magnetic field induced drug release

Next, we evaluated if AMF can induce the release of drugs from cell entrapped SPIONs. To this end we used maytansin as a highly potent toxin with antiproliferative properties in the nanomolar range. Previous work showed that maytansinoids covalently coupled to SPIONs via a heat sensitive linker[48] can be released by an external AMF. Such toxin-conjugated SPIONs (called T-SPIONs, see Figure S3A) were loaded to macrophages. First, we examined if the conjugated toxin would affect cell viability upon internalization of particles. Compared to cells loaded with drug-free SPIONs we determined a minor drop of metabolic activity of J774a.1 cells (Figure S3B), indicating a non-significant level of unspecific toxin-release. Next, we tested whether AMF-induced toxin-release leads to effective cell killing. Therefore, we selected IC21 cells which are highly resistant to AMF-induced hyperthermia, thus allowing to test exclusively the effect of the conjugated drug (Figure 3A). Exposing the T-SPION loaded macrophages but not cells containing the drug-free SPIONs to AMF induced a significant cell killing (78%). In accordance with the results obtained in J774a.1 cells, uptake of T-SPIONs did not decrease IC21 cell viability without AMF application. This demonstrates an AMF induced toxin release from the T-SPIONs.

While these data show the effect of AMF on T-SPION-loaded macrophages, we investigated whether upon AMF induced hyperthermia the toxin released from the T-SPION inside the carrier macrophages would induce cell killing of neighbouring target cells. To this end we employed T-SPION loaded J774a.1 cells and exposed them to AMF. Since these cells undergo apoptosis when exposed to AMF induced hyperthermia a combination of cell killing and toxin-release can be studied. Compared to cells loaded with unconjugated SPIONs we determined a non-significant drop of metabolic activity before AMF (Figure S3B). As target cells we selected human breast cancer MCF-7 cells as well as Kaposi's sarcoma associated herpes virus (KSHV) infected K-ECs[45]. MCF-7 cells and K-ECs were sensitive to the unconjugated toxin with an LC50 of 24 nM and 15 nM, respectively (Figures S3C and S3D). To separate carrier macrophages and target cells we used transwell inserts which allow

diffusion of the toxin through the membrane pores (Figure 3B). MCF-7 and K-EC target cells were seeded in the lower chamber, while the upper chamber contained either cell-free T-SPIONs or T-SPION-loaded J774a.1 macrophages previously exposed to AMF or left untreated. As controls, the unloaded J774a.1 cells or the released toxin was used. Activity of target cells was analyzed after 72 hours in transwell co-culture. As expected, addition of unconjugated toxin into the upper chamber reduced the activity of the MCF-7 cells and K-ECs to 20% and 30%, respectively, compared to the control (unloaded J774a.1 cells) (Figures 3C, D). When T-SPIONs alone or J774a.1 cells loaded with T-SPIONs were added to the upper chamber without prior application of AMF, a slight reduction of MCF-7 cells and K-ECs activity (85% and 80% of control) was detectable. Importantly, the activity of target cells strongly dropped to 40% when free T-SPIONs or T-SPION-charged J774a.1 macrophages were subjected to AMF before loading onto transwell inserts (Figures 3C, D). These results indicate that toxin release from conjugated SPIONs can be induced by AMF application. Of note, AMF induced release of the toxin from free and cell-entrapped T-SPIONs resulted in a comparable reduction of the target cells' viability, both for MCF-1 cells and for K-ECs. This indicates that the toxin was released from the carrier cells and that its activity was not altered by the cellular environment. All in all, this experiment proves the AMF induced release of the toxin from T-SPION entrapped cells and subsequent death of exposed tumour cells.

AMF induced release of toxin in 3D tumour co-cultures

Finally, we intended to examine the effect of AMF-released toxin from loaded carrier cells in an *in vitro* system that mimics the physiological properties of solid tumours. In 3D culture conditions, K-ECs form compact spheroids which show invasive properties, emphasizing the tumorigenic status of the cells[45]. We tested if AMF induced release of the toxin would allow to kill tumour cells in such 3D spheroids. First, tumour spheroids were cultivated in the presence of different concentrations of toxin (Figure S3A) for 48 hours. Microscopic examination revealed that toxin concentrations of 20 nM and higher led to a loss of spheroid integrity indicating death of cells (Figure 4A).

To test if AMF induced release of toxin from T-SPION-loaded macrophages could induce effective cell death within a 3D solid tumour spheroids we created co-cultures of loaded macrophages and tumour cells. This set-up should mimic the situation in tumours upon infiltration of macrophages. To this end, spheroids were generated from mixtures of K-ECs and J774a.1 macrophages in a 40:1 ratio. Confocal microscopy was used to localize the GFP

expressing K-ECs and J774a.1 macrophages labelled by cell tracker red. Optical sectioning indicates that J774a.1 macrophages are able to integrate into the spheroids formed by the endothelial cells, thereby mimicking tumour infiltrating macrophages (Figure 4B). To evaluate if release of toxin would compromise spheroid integrity, spheroid formation was induced by mixing J774a.1 macrophages loaded with SPIONs or T-SPIONs. Control spheroids contained K-ECs or K-ECs cells plus unloaded J774a.1 macrophages. Spheroids were subjected to AMF and re-cultivated for 48 hours. First, we compared spheroid integrity by microscopy. Spheroids containing only K-ECs as well as spheroids of K-ECs in combination with unloaded or SPION loaded J774a.1 macrophages showed comparable spheroid structures in the absence of an external oscillating electromagnetic field (Figure 4C). However, AMF application to SPION containing spheroids resulted in partial loss of the compact spheroid structure with some outgrowing cells, presumably as a result of heat-induced killing of macrophages and/or a bystander effect within the spheroids. Importantly, T-SPION containing spheroids were completely disrupted after AMF application while cultivation upon mock treatment led to a partial loss of spheroid structure with outgrowing cells (Figure 4C). To quantify the toxic effect, we determined the metabolic activity after 48 hours of re-cultivation. A low but significant reduction of metabolic activity (90%) was observed in control co-cultures with SPION-loaded macrophages after AMF application confirming a low level of heat induced affection of cells in the spheroids (Figure 4D). However, a strong reduction of cell metabolic activity and thus viability was observed in spheroids containing T-SPION loaded macrophages after AMF application, indicating that toxin release from macrophages in the 3D spheroids induces death of the tumour cells (Figure 4D).

Discussion

In this study, we exploited AMF induced hyperthermia for both, release of a toxin from a heat-sensitive drug/SPION conjugate as well as killing of macrophages loaded with these functionalized particles. We demonstrate that this strategy provides on-demand release of drugs thereby rendering SPION-loaded macrophages as a potent drug delivery systems.

Recently, we introduced a superparamagnetic drug release system (T-SPIONs) which relies on a thermolabile covalent linker that connects the anticancer drug maytansin with superparamagnetic nanostructured SPION particles[47, 48]. In this paper we extended this strategy by employing macrophages as cellular vehicles for carrying these nanoparticles. Importantly, we show that the release of the cytotoxic cargo depends almost entirely on magnetic hyperthermia and that no or negligible non-specific release was observed in cells loaded with T-SPIONs (Figure 3). Noteworthy, when applying an oscillating electromagnetic field we induced apoptosis in SPION-loaded macrophages that was directly linked with hyperthermia and the thermally-induced linker cleavage and liberation of the SPION-bound toxin.

Drug delivery systems have a crucial impact on the pharmacokinetic profile of a drug, as they can influence the rate of drug release as well as the site and duration of drug action. Expecially, this can help to reduce side effects of a drug, especially of toxins employed in cancer therapy when the therapeutic window is narrow and the difference between toxic and therapeutic levels is small.

So far, various organic or inorganic delivery systems have been developed, showing specific advantages and disadvantages with regard to biocompatibility, drug loading and drug release capacity[52]. Selected studies investigated the temperature dependent release from a thermoresponsive polymer covering mesoporous silica nanoparticles or with a thermoresponsive group on the exterior surface[7, 53-56]. A rapid release of drugs upon raising the temperature above 37°C was reported. However, these systems still struggle with the problem of spontaneous drug release or drug release induced by minor, non-pathological fluctuations of the physiological body temperature. In the present study, we employed a highly toxic maytansin derivate that was covalently attached to core-shell iron oxide / silica nanoparticles. The superparamagnetic property of the iron oxide core allows to induce magnetic hyperthermia and controlled drug release. Hyperthermia induced by this type of particles can reach local temperatures between 42-50 °C in the environment of the particle which is well above the pathophysiological temperatures of 36-42 °C.

In this study, we combined this drug release system with a drug delivery concept that utilizes macrophage carriers. Macrophages have the intrinsic ability to efficiently take up 'biological micro- and nanoparticles' such as pathogenic bacteria and viruses. We observed efficient uptake of SPIONs as well as lack of adverse toxicity in primary mouse BMDM as well as all the tested macrophages and monocytes cell lines which is in agreement to previous studies[52, 57]. When we subjected the SPION loaded macrophages to AMF we observed a drop in cell viability to at least 40% (based on the WST-1 assay) while primary mouse macrophages revealed a much higher susceptibility to AMF-induced magnetic hyperthermia (Figure 2B). This suggests that primary cells are most vulnerable towards temperature increase, while the immortalized cell lines are less sensitive for apoptotic triggers.

In particular, the macrophage cell line IC21 turned out to be largely resistant towards magnetic hyperthermia. Various mechanisms could contribute to this property[58-61]. As a consequence, IC21 represent a cell system of choice for evaluating the consequences of intracellular release of the cargo coupled to SPIONs via a heat-sensitive linker.

We evaluated the specificity of magnetic hyperthermia mediated controlled toxin release in in vitro cell systems. We used two conditions, namely, a transwell and spheroid system to assess the efficacy of the approach. The transwell system allowed assessing the toxin release on neighbouring cells. Both MCF-7 breast cancer and KSHV transformed K-EC tumour cells were employed as indicator cells. A high specificity was observed, both, upon AMF induced release of the toxin from T-SPIONs but also from macrophages loaded with these nanoparticles. More than 70% drop in viability of cancer cells was recorded while there was no effect on cells if the loaded macrophages were not exposed to an AMF.

In the spheroid system mimicking the physiological properties of solid tumours, we observed only a minor effect of hyperthermia when using toxin-free SPIONs which is in line with a previous study [42]. In contrast, upon hyperthermia induced release of the toxin, the integrity of the tumour spheroids was significantly affected and cells died, indicating that the hyperthermia the induced release of the toxin (but not hyperthermia alone) is sufficient to kill tumor cells.

These results indicate that controlled drug release from drug-linked SPIONs entrapped in carrier cells can be achieved using magnetic hyperthermia and that the problems associated with nonspecific release are minimized.

An effective nanoparticle-based drug delivery system should be able to recognize and deliver its cargo to specific pathologic tissues, and minimize negative side effects to healthy tissues. The combination of cell based drug delivery systems with a tightly controlled release system can fulfill these specifications. A larger number of immune cells, mainly macrophages and T cells, are recruited into inflamed tissue, attracted and guided by gradients of cytokines. Inflammation is a critical component in the progression of tumors and infections. Thus, some studies have exploited the homing property of immune cells to design cell-based drug delivery system. Pang et al. exploited the potential of macrophages as biocarriers to deliver nanoparticles into brain tumor sites[43]. They demonstrated that the tumor-targeting efficiency of nanoparticles was enhanced after loading into macrophages, including a deeper permeation at the glioma parenchyma. Macrophage-based delivery of therapeutic nanozymes containing redox enzymes to the brain was shown by Brynskikh et al[62]. Combining these cellular delivery systems with an on demand release of cargo will allow to rise the therapeutic dose at the site of action in a defined time window. Efficient killing of carrier cells and heat-controlled release of the cargo due to AMF-induced hyperthermia afford this prediction.

Conclusion

We report on a (comprehensive) magnetic hyperthermia controlled drug release system using a thermolabile linker attached on one position to a model anticancer drug derived from maytansin and on the other terminus to SPIONs. This system is only activated upon exposure to an external AMF. No nonspecific release of the drug was observed. Our present study provides the first proof of principle and paves the way for in vivo translation.

Data availability

There are no restrictions whatsoever on the data reported in this work.

Acknowledgements

This work was supported by grants from Deutsche Forschungsgemeinschaft (DFG, WI2648/3-1, BE1664/21-1), the Cluster of Excellence REBIRTH (From Regenerative Biology to Reconstructive Therapy) as well as from the Biofabrication for NIFE initiative,

436 which is financially supported by the ministry of Lower Saxony and the Volkswagen Stiftung
437 (BIOFABRICATION FOR NIFE: VWZN2860). Further support came from the Ministry of
438 Science and Culture of Lower Saxony (MWK; graduate program Hannover School of
439 Nanotechnology hosted by the Laboratory of Nano and Quantum Engineering (LNQE),
440 Leibniz University Hannover). S.U. wishes to acknowledge the HZI graduate school and
441 DAAD for support. S. T. was supported by the DAAD. We thank I. Schleicher for assistance
442 in the electron microscopy studies. Further thanks go to the LNQE for the use of their TEM
443 equipment.

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Figure Legends

Figure 1: Uptake of SPIONs from macrophages and biocompatibility.

Cells were exposed to SPIONs for 2 hours. Excess SPIONs were removed by repeated washing and analyzed after 48 hours. (A) Flow cytometry histogram from J774a.1 cells upon incubation with $20 \mu\text{g}/10^5$ SPIONs, FITC-SPIONs or untreated control cells. (B) Quantitative analysis of FITC-SPIONs uptake by indicated cell lines and primary macrophages (BMDM) as determined by flow cytometry. The data represent mean $\pm$ SEM. (C) Confocal microscopy image of J774a.1 cells upon uptake of FITC-SPIONs. (D) J774a.1 cells with and without SPION loading were evaluated by transmission electron microscopy. (E) Viability of indicated macrophage cell lines as well as primary bone marrow derived macrophages (BMDM) after treatment with the indicated amount of SPIONs (in $\mu\text{g}/10^5$ cells) as measured by flow cytometry based Annexin V assay. C: untreated cells; R: Raptinal treated. The data represent mean $\pm$ SEM.

Figure 2: Magnetic hyperthermia induced cell death.

(A) Experimental outline. (B, C) Viability of the indicated cell lines and primary macrophages (BMDM) was assessed by Annexin V assay (B) or WST-1 assay (C). C: untreated cells, R: Raptinal-treated, AMF: alternating magnetic field. (D) Cell morphology was visualized by scanning electron microscopy (SEM). Electron micrographs of J774a.1 cells indicate cell shrinkage and the formation of membrane blebs upon AMF exposure and Raptinal treatment.

Figure 3: AMF induced toxin release.

(A) IC21 cells were loaded with SPIONs or T-SPIONs ($20 \mu\text{g}/10^5$ cells) and exposed to AMF or were mock treated. Metabolic activity of cells was measured after 48 hours using the WST-1 assay. (B) Experimental outline to assess the toxicity of neighbouring indicator cells using a transwell assay. The upper chamber was supplied with the maytansin derivative (Toxin), T-SPIONs or the J774a.1 cells loaded with T-SPIONs or unloaded. The application of AMF is indicated. The activity of target cells in the lower chamber was measured using the WST-1 assay for MCF-7 (C) and for K-ECs cells (D).

Figure 4: Induced killing of 3D tumour spheroids.

(A) Loss of spheroid integrity upon exposure of K-EC spheroids with indicated concentration of soluble toxin (maytansin derivative) for 48 hours. Representative pictures are shown. (B) Spheroids of macrophage/tumour cell co-cultures in a ratio of 1:40. K-EC express GFP and are indicated in green, J774a1 were labelled with cell tracker dye (red) (C) Macrophage/tumour spheroids (1:40) were generated from K-EC and J77a.1 cells that were previously loaded with SPIONs, T-SPIONs or were left unloaded. The pictures show representative spheroids before or 48h after AMF treatment. (D) The metabolic activity of spheroids from (C) was measured by WST-1 assay. Data represent mean $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (t-test).

References

- [1] S. Senapati, A.K. Mahanta, S. Kumar, P. Maiti, Controlled drug delivery vehicles for cancer treatment and their performance, *Signal Transduct Target Ther*, 3 (2018) 7.
- [2] M.D. Norris, K. Seidel, A. Kirschning, Externally Induced Drug Release Systems with Magnetic Nanoparticle Carriers: An Emerging Field in Nanomedicine, *ADVANCED THERAPEUTICS*, 0 (2018) 1800092.
- [3] J. Liu, Z. Luo, J. Zhang, T. Luo, J. Zhou, X. Zhao, K. Cai, Hollow mesoporous silica nanoparticles facilitated drug delivery via cascade pH stimuli in tumor microenvironment for tumor therapy, *Biomaterials*, 83 (2016) 51-65.
- [4] B. Sanz, M.P. Calatayud, T.E. Torres, M.L. Fanarraga, M.R. Ibarra, G.F. Goya, Magnetic hyperthermia enhances cell toxicity with respect to exogenous heating, *Biomaterials*, 114 (2017) 62-70.
- [5] B. Kumar, S. Kulanthaivel, A. Mondal, S. Mishra, B. Banerjee, A. Bhaumik, I. Banerjee, S. Giri, Mesoporous silica nanoparticle based enzyme responsive system for colon specific drug delivery through guar gum capping, *Colloids Surf B Biointerfaces*, 150 (2017) 352-361.
- [6] C. Bharti, U. Nagaich, A.K. Pal, N. Gulati, Mesoporous silica nanoparticles in target drug delivery system: A review, *Int J Pharm Investig*, 5 (2015) 124-133.
- [7] H. Omar, J.G. Croissant, K. Alamoudi, S. Alsaiani, I. Alradwan, M.A. Majrashi, D.H. Anjum, P. Martins, R. Laamarti, J. Eppinger, B. Moosa, A. Almalik, N.M. Khashab, Biodegradable Magnetic Silica@Iron Oxide Nanovectors with Ultra-Large Mesopores for High Protein Loading, Magnetothermal Release, and Delivery, *J Control Release*, 259 (2017) 187-194.
- [8] L. Sun, D. Wang, Y. Chen, L. Wang, P. Huang, Y. Li, Z. Liu, H. Yao, J. Shi, Core-shell hierarchical mesostructured silica nanoparticles for gene/chemo-synergetic stepwise therapy of multidrug-resistant cancer, *Biomaterials*, 133 (2017) 219-228.
- [9] K. Möller, T. Bein, Talented Mesoporous Silica Nanoparticles, *Chemistry of Materials*, 29 (2017) 371-388.
- [10] Y. Zhou, G. Quan, Q. Wu, X. Zhang, B. Niu, B. Wu, Y. Huang, X. Pan, C. Wu, Mesoporous silica nanoparticles for drug and gene delivery, *Acta Pharm Sin B*, 8 (2018) 165-177.
- [11] F. Tang, L. Li, D. Chen, Mesoporous Silica Nanoparticles: Synthesis, Biocompatibility and Drug Delivery, *Advanced Materials*, 24 (2012) 1504-1534.
- [12] N. Ehlert, P.P. Mueller, M. Stieve, T. Lenarz, P. Behrens, Mesoporous silica films as a novel biomaterial: applications in the middle ear, *Chemical Society Reviews*, 42 (2013) 3847-3861.
- [13] S. Williams, A. Neumann, I. Bremer, Y. Su, G. Dräger, C. Kasper, P. Behrens, Nanoporous silica nanoparticles as biomaterials: evaluation of different strategies for the functionalization with polysialic acid by step-by-step cytocompatibility testing, *Journal of Materials Science: Materials in Medicine*, 26 (2015) 125.
- [14] L. Xiong, J. Bi, Y. Tang, S.Z. Qiao, Magnetic Core-Shell Silica Nanoparticles with Large Radial Mesopores for siRNA Delivery, *Small*, 12 (2016) 4735-4742.
- [15] Y. Qie, H. Yuan, C.A. von Roemeling, Y. Chen, X. Liu, K.D. Shih, J.A. Knight, H.W. Tun, R.E. Wharen, W. Jiang, B.Y. Kim, Corrigendum: Surface modification of nanoparticles enables selective evasion of phagocytic clearance by distinct macrophage phenotypes, *Sci Rep*, 6 (2016) 30663.
- [16] S. Wilhelm, A.J. Tavares, Q. Dai, S. Ohta, J. Audet, H.F. Dvorak, W.C.W. Chan, Analysis of nanoparticle delivery to tumours, 1 (2016) 16014.
- [17] M. Torrice, Does Nanomedicine Have a Delivery Problem?, *ACS Cent Sci*, 2 (2016) 434-437.

- [18] A. Kirschning, L. Kupracz, J. Hartwig, New Synthetic Opportunities in Miniaturized Flow Reactors with Inductive Heating, *Chemistry Letters*, 41 (2012) 562-570.
- [19] R. Weissleder, G. Elizondo, J. Wittenberg, C.A. Rabito, H.H. Bengel, L. Josephson, Ultrasmall superparamagnetic iron oxide: characterization of a new class of contrast agents for MR imaging, *Radiology*, 175 (1990) 489-493.
- [20] T. Neuberger, B. Schöpf, H. Hofmann, M. Hofmann, B. von Rechenberg, Superparamagnetic nanoparticles for biomedical applications: Possibilities and limitations of a new drug delivery system, *Journal of Magnetism and Magnetic Materials*, 293 (2005) 483-496.
- [21] N. Angrisani, F. Foth, M. Kietzmann, S. Schumacher, G.L. Angrisani, A. Christel, P. Behrens, J. Reifenrath, Increased accumulation of magnetic nanoparticles by magnetizable implant materials for the treatment of implant-associated complications, *Journal of Nanobiotechnology*, 11 (2013) 34.
- [22] D. Artemov, N. Mori, B. Okolie, Z.M. Bhujwalla, MR molecular imaging of the Her-2/neu receptor in breast cancer cells using targeted iron oxide nanoparticles, *Magn Reson Med*, 49 (2003) 403-408.
- [23] R.E. Serda, N.L. Adolphi, M. Bisoffi, L.O. Sillerud, Targeting and cellular trafficking of magnetic nanoparticles for prostate cancer imaging, *Mol Imaging*, 6 (2007) 277-288.
- [24] G. Kou, S. Wang, C. Cheng, J. Gao, B. Li, H. Wang, W. Qian, S. Hou, D. Zhang, J. Dai, H. Gu, Y. Guo, Development of SM5-1-conjugated ultrasmall superparamagnetic iron oxide nanoparticles for hepatoma detection, *Biochemical and biophysical research communications*, 374 (2008) 192-197.
- [25] T.J. Chen, T.H. Cheng, C.Y. Chen, S.C. Hsu, T.L. Cheng, G.C. Liu, Y.M. Wang, Targeted Herceptin-dextran iron oxide nanoparticles for noninvasive imaging of HER2/neu receptors using MRI, *J Biol Inorg Chem*, 14 (2009) 253-260.
- [26] L. Yang, H. Mao, Y.A. Wang, Z. Cao, X. Peng, X. Wang, H. Duan, C. Ni, Q. Yuan, G. Adams, M.Q. Smith, W.C. Wood, X. Gao, S. Nie, Single chain epidermal growth factor receptor antibody conjugated nanoparticles for in vivo tumor targeting and imaging, *Small*, 5 (2009) 235-243.
- [27] K.L. Vigor, P.G. Kyrtatos, S. Minogue, K.T. Al-Jamal, H. Kogelberg, B. Tolner, K. Kostarelos, R.H. Begent, Q.A. Pankhurst, M.F. Lythgoe, K.A. Chester, Nanoparticles functionalized with recombinant single chain Fv antibody fragments (scFv) for the magnetic resonance imaging of cancer cells, *Biomaterials*, 31 (2010) 1307-1315.
- [28] L. Yang, H. Mao, Z. Cao, Y.A. Wang, X. Peng, X. Wang, H.K. Sajja, L. Wang, H. Duan, C. Ni, C.A. Staley, W.C. Wood, X. Gao, S. Nie, Molecular imaging of pancreatic cancer in an animal model using targeted multifunctional nanoparticles, *Gastroenterology*, 136 (2009) 1514-1525 e1512.
- [29] C. Leuschner, C.S. Kumar, W. Hansel, W. Soboyejo, J. Zhou, J. Hormes, LHRH-conjugated magnetic iron oxide nanoparticles for detection of breast cancer metastases, *Breast Cancer Res Treat*, 99 (2006) 163-176.
- [30] C. Zhang, M. Jugold, E.C. Woenne, T. Lammers, B. Morgenstern, M.M. Mueller, H. Zentgraf, M. Bock, M. Eisenhut, W. Semmler, F. Kiessling, Specific targeting of tumor angiogenesis by RGD-conjugated ultrasmall superparamagnetic iron oxide particles using a clinical 1.5-T magnetic resonance scanner, *Cancer Res*, 67 (2007) 1555-1562.
- [31] F. Sonvico, S. Mornet, S. Vasseur, C. Dubernet, D. Jaillard, J. Degrouard, J. Hoebeke, E. Duguet, P. Colombo, P. Couvreur, Folate-conjugated iron oxide nanoparticles for solid tumor targeting as potential specific magnetic hyperthermia mediators: synthesis, physicochemical characterization, and in vitro experiments, *Bioconjugate chemistry*, 16 (2005) 1181-1188.

- [32] Y.-S. Lin, C.L. Haynes, Synthesis and Characterization of Biocompatible and Size-Tunable Multifunctional Porous Silica Nanoparticles, *Chemistry of Materials*, 21 (2009) 3979-3986.
- [33] C. Tao, Y. Zhu, Magnetic mesoporous silica nanoparticles for potential delivery of chemotherapeutic drugs and hyperthermia, *Dalton Transactions*, 43 (2014) 15482-15490.
- [34] C.S. Kumar, F. Mohammad, Magnetic nanomaterials for hyperthermia-based therapy and controlled drug delivery, *Adv Drug Deliv Rev*, 63 (2011) 789-808.
- [35] B. Hildebrandt, P. Wust, O. Ahlers, A. Dieing, G. Sreenivasa, T. Kerner, R. Felix, H. Riess, The cellular and molecular basis of hyperthermia, *Crit Rev Oncol Hematol*, 43 (2002) 33-56.
- [36] A. Ito, K. Tanaka, H. Honda, S. Abe, H. Yamaguchi, T. Kobayashi, Complete regression of mouse mammary carcinoma with a size greater than 15 mm by frequent repeated hyperthermia using magnetite nanoparticles, *Journal of bioscience and bioengineering*, 96 (2003) 364-369.
- [37] J. Motoyama, N. Yamashita, T. Morino, M. Tanaka, T. Kobayashi, H. Honda, Hyperthermic treatment of DMBA-induced rat mammary cancer using magnetic nanoparticles, *Biomagn Res Technol*, 6 (2008) 2.
- [38] S. Balivada, R.S. Rachakatla, H. Wang, T.N. Samarakoon, R.K. Dani, M. Pyle, F.O. Kroh, B. Walker, X. Leaym, O.B. Koper, M. Tamura, V. Chikan, S.H. Bossmann, D.L. Troyer, A/C magnetic hyperthermia of melanoma mediated by iron(0)/iron oxide core/shell magnetic nanoparticles: a mouse study, *BMC Cancer*, 10 (2010) 119.
- [39] A. Jordan, R. Scholz, K. Maier-Hauff, F.K. van Landeghem, N. Waldoefner, U. Teichgraeber, J. Pinkernelle, H. Bruhn, F. Neumann, B. Thiesen, A. von Deimling, R. Felix, The effect of thermotherapy using magnetic nanoparticles on rat malignant glioma, *J Neurooncol*, 78 (2006) 7-14.
- [40] A. Jordan, R. Scholz, P. Wust, H. Fahling, J. Krause, W. Wlodarczyk, B. Sander, T. Vogl, R. Felix, Effects of magnetic fluid hyperthermia (MFH) on C3H mammary carcinoma in vivo, *Int J Hyperthermia*, 13 (1997) 587-605.
- [41] M. Johannsen, B. Thiesen, A. Jordan, K. Taymoorian, U. Gneveckow, N. Waldofner, R. Scholz, M. Koch, M. Lein, K. Jung, S.A. Loening, Magnetic fluid hyperthermia (MFH) reduces prostate cancer growth in the orthotopic Dunning R3327 rat model, *The Prostate*, 64 (2005) 283-292.
- [42] T.L. Kalber, K.L. Ordidge, P. Southern, M.R. Loebinger, P.G. Kyrtatos, Q.A. Pankhurst, M.F. Lythgoe, S.M. Janes, Hyperthermia treatment of tumors by mesenchymal stem cell-delivered superparamagnetic iron oxide nanoparticles, *International journal of nanomedicine*, 11 (2016) 1973-1983.
- [43] L. Pang, J. Qin, L. Han, W. Zhao, J. Liang, Z. Xie, P. Yang, J. Wang, Exploiting macrophages as targeted carrier to guide nanoparticles into glioma, *Oncotarget*, 7 (2016) 37081-37091.
- [44] J. McMillan, E. Batrakova, H.E. Gendelman, Cell delivery of therapeutic nanoparticles, *Progress in molecular biology and translational science*, 104 (2011) 563-601.
- [45] C. Lipps, M. Badar, M. Butueva, T. Dubich, V.V. Singh, S. Rau, A. Weber, M. Kracht, M. Köster, T. May, T.F. Schulz, H. Hauser, D. Wirth, Proliferation status defines functional properties of endothelial cells, *Cellular and Molecular Life Sciences*, 74 (2017) 1319-1333.
- [46] X. Zhang, R. Goncalves, D.M. Mosser, The isolation and characterization of murine macrophages, *Curr Protoc Immunol*, Chapter 14 (2008) Unit 14 11.
- [47] K. Seidel, A. Balakrishnan, C. Alexiou, C. Janko, R.M. Komoll, L.L. Wang, A. Kirschning, M. Ott, Synthesis of Magnetic-Nanoparticle/Ansamitocin Conjugates-Inductive Heating Leads to Decreased Cell Proliferation In Vitro and Attenuation Of Tumour Growth In Vivo, *Chemistry*, 23 (2017) 12326-12337.

- [48] L. Mancuso, T. Knobloch, J. Buchholz, J. Hartwig, L. Moller, K. Seidel, W. Collisi, F. Sasse, A. Kirschning, Preparation of thermocleavable conjugates based on ansamitocin and superparamagnetic nanostructured particles by a chemobiosynthetic approach, *Chemistry*, 20 (2014) 17541-17551.
- [49] G.D. Tobias Knobloch, Wera Collisi, Florenz Sasse, Andreas Kirschning, Unprecedented deoxygenation at C-7 of the ansamitocin core during mutasynthetic biotransformations, *Beilstein Journal of Organic Chemistry*, 8 (2008) 861-869.
- [50] A. Kirschning, F. Taft, T. Knobloch, Total synthesis approaches to natural product derivatives based on the combination of chemical synthesis and metabolic engineering, *Organic & Biomolecular Chemistry*, 5 (2007) 3245-3259.
- [51] R. Di Corato, A. Espinosa, L. Lartigue, M. Tharaud, S. Chat, T. Pellegrino, C. Menager, F. Gazeau, C. Wilhelm, Magnetic hyperthermia efficiency in the cellular environment for different nanoparticle designs, *Biomaterials*, 35 (2014) 6400-6411.
- [52] C. Fu, T. Liu, L. Li, H. Liu, D. Chen, F. Tang, The absorption, distribution, excretion and toxicity of mesoporous silica nanoparticles in mice following different exposure routes, *Biomaterials*, 34 (2013) 2565-2575.
- [53] Y.-H. Deng, C.-C. Wang, J.-H. Hu, W.-L. Yang, S.-K. Fu, Investigation of formation of silica-coated magnetite nanoparticles via sol-gel approach, *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 262 (2005) 87-93.
- [54] S.Z. Zhengyang Zhou, Di Zhanga Grafting of thermo-responsive polymer inside mesoporous silica with large pore size using ATRP and investigation of its use in drug release, *J. Mater. Chem*, 17 (2007) 2428-2433.
- [55] E. Yu, A. Lo, L. Jiang, B. Petkus, N. Ileri Ercan, P. Stroeve, Improved controlled release of protein from expanded-pore mesoporous silica nanoparticles modified with co-functionalized poly(*n*-isopropylacrylamide) and poly(ethylene glycol) (PNIPAM-PEG), *Colloids Surf B Biointerfaces*, 149 (2017) 297-300.
- [56] K.K.K. Ye-Zi You, Stephanie L. Brock, David Oupický, Temperature-Controlled Uptake and Release in PNIPAM-Modified Porous Silica Nanoparticles, *Chem. Mater*, 20 (2008) 3354-3359.
- [57] A. Gallud, O. Bondarenko, N. Feliu, N. Kupferschmidt, R. Atluri, A. Garcia-Bennett, B. Fadeel, Macrophage activation status determines the internalization of mesoporous silica particles of different sizes: Exploring the role of different pattern recognition receptors, *Biomaterials*, 121 (2017) 28-40.
- [58] R. Ludwig, F.J. Teran, U. Teichgraeber, I. Hilger, Nanoparticle-based hyperthermia distinctly impacts production of ROS, expression of Ki-67, TOP2A, and TPX2, and induction of apoptosis in pancreatic cancer, *Int J Nanomedicine*, 12 (2017) 1009-1018.
- [59] K.A. Court, H. Hatakeyama, S.Y. Wu, M.S. Lingegowda, C. Rodriguez-Aguayo, G. Lopez-Berestein, L. Ju-Seog, C. Rinaldi, E.J. Juan, A.K. Sood, M. Torres-Lugo, HSP70 Inhibition Synergistically Enhances the Effects of Magnetic Fluid Hyperthermia in Ovarian Cancer, *Mol Cancer Ther*, 16 (2017) 966-976.
- [60] M.Y. Sherman, V.L. Gabai, Hsp70 in cancer: back to the future, *Oncogene*, 34 (2015) 4153-4161.
- [61] E. Gozal, L.A. Ortiz, X. Zou, M.E. Burow, J.A. Lasky, M. Friedman, Silica-induced apoptosis in murine macrophage: involvement of tumor necrosis factor-alpha and nuclear factor-kappaB activation, *Am J Respir Cell Mol Biol*, 27 (2002) 91-98.
- [62] A.M. Brynskikh, Y. Zhao, R.L. Mosley, S. Li, M.D. Boska, N.L. Klyachko, A.V. Kabanov, H.E. Gendelman, E.V. Batrakova, Macrophage delivery of therapeutic nanozymes in a murine model of Parkinson's disease, *Nanomedicine (Lond)*, 5 (2010) 379-396.

Figure 1

Figure 1

Figure 2

Figure 2

Figure 3

Figure 3

Figure 4

Figure 4

Supplementary Material

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