Cancer Hot Paper

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Magnetically Triggered Dual Functional Nanoparticles for Resistance-Free Apoptotic Hyperthermia**

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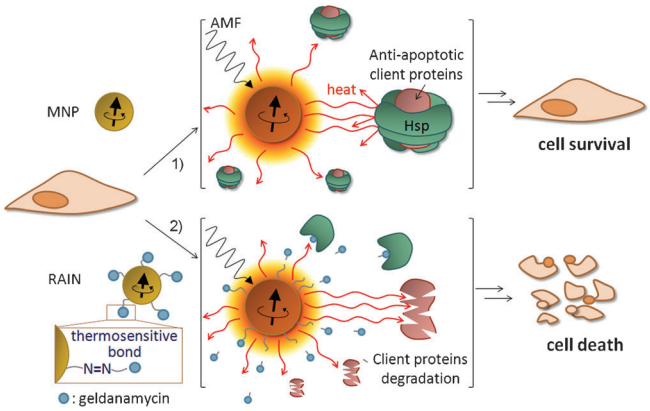
Therapeutic resistance is one of the major clinical problems and remains a persistent hurdle for disease treatments.[1] For example, both bacteria and cancer cells gradually increase their ability to shield themselves from treatments such as chemotherapy and radiotherapy. Some of the reported protective mechanisms for resistance involve chemodrug efflux and bypassing targeted signaling feedback loops.[1c] As a consequence of these problems, researches for circumventing therapy resistance are intensively pursued in the biomedical fields including pharmaceutics and cancer biology.

Recently, innovative therapeutic approaches beyond conventional orthodox therapies, such as chemical drugs, have been actively developed and one of them is cancer hyperthermia, in which thermal treatments of cancer cells at mild temperature (40–45 °C) preferentially eliminate them through an apoptotic cell death process without damaging normal tissues.[2] With the exceptional capability to generate thermal energy at targeted areas, nanomaterials such as gold, iron oxide, and graphene have been investigated for use in hyperthermia treatment of cancer.[3] Unique advantages of hyperthermia using nanomaterials include spatiotemporally controlled treatments of targeted diseases in a noninvasive manner. Compared with other heat generation nanomaterials such as gold and graphene which use light as the trigger, magnetic hyperthermia can be advantageous for targets that reside even deep inside the biological system without penetration depth problem.[4] In addition, the fact that magnetic field causes no adverse effect on biological tissues serves as a distinctive benefit for noninvasive in vivo applications. Despite all these potential advantages, however, hyperthermia for apoptosis, in general, is ineffective because heat-treated cells readily acquire resistance to thermal stress and markedly increase their survival rate, which is called thermoresistance.[5] As a result, harsh (e.g. high temperature over 48 °C) thermal treatment is usually required to bring about a useful level of therapeutic efficacy, but it adversely initiates an undesirable cell dying process called necrosis which can potentially induce inflammatory disease and cancer metastasis.[6] Therefore, effective execution of apoptosis at low temperature without inducing thermoresistance has been challenging.

In this study, we introduce a new type of resistance-free apoptosis-inducing magnetic nanoparticle (RAIN) that can promote thermoresistance-free apoptosis. The RAIN consists of two functional subunits of 1) heat shock protein (Hsp) inhibition and 2) heat generation from magnetic nanoparticle (MNP) in which these two functions are designed to be triggered only by the application of an alternating magnetic field (AMF). We demonstrate that the RAIN successfully promotes exclusive apoptosis and obstructs cell survival by inhibiting Hsp not only in vitro but also in vivo under low-temperature (ca. 43 °C) hyperthermia conditions (Scheme 1).



Scheme 1. Resistance-free apoptosis-inducing magnetic nanoparticle (RAIN) for effective apoptotic hyperthermia. 1) Heat-treated cancer cells launch a protective mechanism for cell survival by overexpression of heat shock protein (Hsp) that acts as a molecular chaperone for the protection of client proteins. As a consequence, conventional apoptotic hyperthermia shows only a marginal efficacy. 2) The RAIN simultaneously generates heat and releases a Hsp inhibitor to block the protective function of Hsp upon application of an alternating magnetic field (AMF). The RAIN is effective for apoptotic hyperthermia.

As the heat generator for hyperthermia, a 15 nm $Zn_{0.4}Fe_{2.6}O_4$ MNP,[7] which has a relatively high heat-generating capability (SLP = 471 W g^{-1}), is used. Because thermoresistance is closely associated with Hsp, which shields cells from apoptosis by reducing unfolding and denaturation of Hsp client proteins subjected to thermal stress,[8] a Hsp inhibition strategy is employed to minimize the stress-mediated chaperonic functional responses. Hsp90 is targeted because its client proteins are known to play key roles in cell survival, regulation, and oncogenic signaling.[9] First, for the inhibition of Hsp90, geldanamycin (GM), a benzoquinone ansamycin known as an inhibitor for Hsp90,[9d,e] is conjugated to the MNP as shown in Figure 1 a. 4,4′-Azobis(4-cyanovaleric acid) is attached to the MNP to yield azo-MNP. Then, 17-(3-

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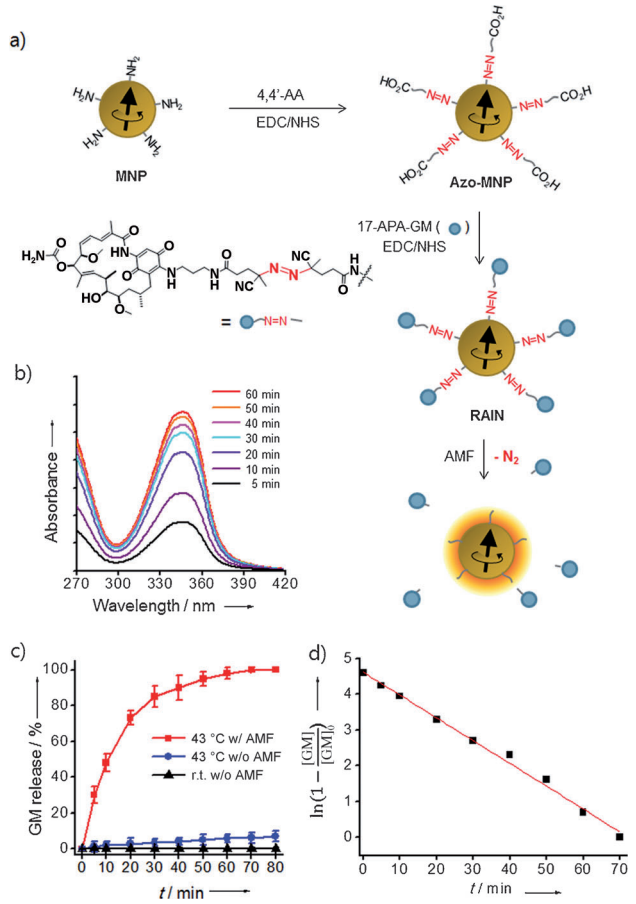

Figure 1. Synthesis of RAIN and its thermoresponsive features under an AMF. a) Synthesis of RAIN. The amine-functionalized magnetic nanoparticle (15 nm $Zn_{0.4}Fe_{2.6}O_4$, MNP) is connected with GM by using 4,4′-AA as a thermolabile crosslinker. The thermolabile azo linker is cleaved with the loss of nitrogen gas upon exposure to an AMF. b) UV/Vis absorption spectra of released GM under controlled AMF. c) Time-dependent cumulative GM release profile with and without application of an AMF. d) Time-dependent release kinetics. A plot of $\ln(1-[GM]/[GM]_0)$ versus time (in minutes) shows a linear relationship which indicates that the azo bond cleavage follows first-order kinetics. 4,4′-AA = 4,4′-azobis(4-cyanovaleric acid), AMF = alternating magnetic field, 17-APA-GM = 17-(3-aminopropylamino)geldanamycin, DMSA = dimercaptosuccinic acid, EDC = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, GM = geldanamycin, MNP = magnetic nanoparticle, NHS = *N*-hydroxysuccinimide.

aminopropylamino)geldanamycin (17-APA-GM)[10] is conjugated with the azo-MNP to construct the RAIN. In this design, GM is linked to the surface of the MNP through a thermally cleavable azo linker,[11] which allows the release of GM through the heat generation from the MNP upon the application of an AMF (Figure 1a). The thermal release profile of GM from the RAIN (0.1 mg mL^{-1}) is explored by applying an AMF (500 kHz, 37.4 kA m^{-1}) to reach the apoptotic hyperthermia temperature range (43 ± 1 °C).

GM release is monitored by using an UV/Vis absorption spectrometer at 345 nm (Figure 1b). The release of GM is observed to be proportional to the heat exposure time (Figure 1b) and complete GM release from the RAIN occurs at about 60 minutes, at which the concentration of GM is 0.01 μM (red line, Figure 1c). In contrast, at room temperature without the application of an AMF, no GM is released from the RAIN (black line, Figure 1c). Interestingly, very little (< 7%) GM is released from the RAIN when it is heated externally to 43 °C using a hot water bath (blue line, Figure 1c), although the solution temperature is identical to the case of AMF application (Figure 1c). This finding indicates that nanoscale local heating[12] at the surface of the MNP, estimated as 90 °C, is critical to promote release of GM by dissociation of the azo bond on the MNP surface. The kinetic plot displayed in Figure 1d shows that GM release from the RAIN through azo bond cleavage follows first-order kinetics.[13] Such localized heating upon the application of an AMF is crucial for effective triggering of the dual functions of heat generation and GM release of the RAIN.

The apoptotic hyperthermia efficacy of the RAIN is explored using an in vitro study. A mixture of MDA-MB-231 breast cancer cells (1.0×10^4 cells per well) and 0.1 mg mL^{-1} of the RAIN (or an equivalent amount of the MNP) are incubated for 5 h. Then, an AMF (500 kHz, 37.4 kA m^{-1}) is applied to maintain the temperature of the solution at 43 °C for 80 minutes using a temperature probe and an AMF transducer (Figure 2a), and Figure 2b shows the temperature profile. The RAIN is designed to release GM and inhibit Hsp90 upon application of an AMF, which gives rise to a significant enhancement of hyperthermia-mediated apoptosis (Figure 2a). The cell death percentage is measured by CCK-8 assay. For the sample with the MNP only, cell death reaches 25% (black line, Figure 2c) upon hyperthermia treatment for 80 minutes at 43 °C. In contrast, cell death is significantly increased to 89% at 60 minutes and 100% at 70 minutes with the RAIN under identical hyperthermia conditions (red line, Figure 2c). This in vitro study clearly demonstrates that inhibition of Hsp90 makes the cells more vulnerable to apoptotic hyperthermia. Furthermore, the expression levels of Hsp90 are examined by using Western blot analysis (Figure 2d).[9e] Compared with control cells that are not exposed to heat, a strong expression of Hsp90 is clearly observed for cells treated with MNP hyperthermia (Figure 2d(i)). In contrast, cells treated with RAIN hyperthermia under identical conditions shows a much lower expression level of Hsp90 (Figure 2d(i)). Hsp90 expression levels are quantified by using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a loading control (Figure 2d(ii)). Compared with control cells, MNP hyperthermia treated cells exhibit three times higher Hsp90 expression level, while RAIN hyperthermia treated cells show comparable expression of Hsp90 to the control (Figure 2d(ii)). As seen in Figure 2c, cancer cells are completely dead upon exposure to RAIN hyperthermia for 70 minutes while only a marginal amount of cancer cells (< 25%) are dead with MNP hyperthermia for the same time period. This observation indicates that inhibition of thermoresistance process is critical for effective apoptotic hyperthermia.

Cell death pathways[14] induced by the RAIN are examined by using annexin V-fluorescein isothiocyanate (annexin V-FITC) and propidium iodide (PI) staining (Figure 2e). The assay detects changes in the plasma membrane of the cells. Apoptosis initially induces phosphatidylserine exposure with-

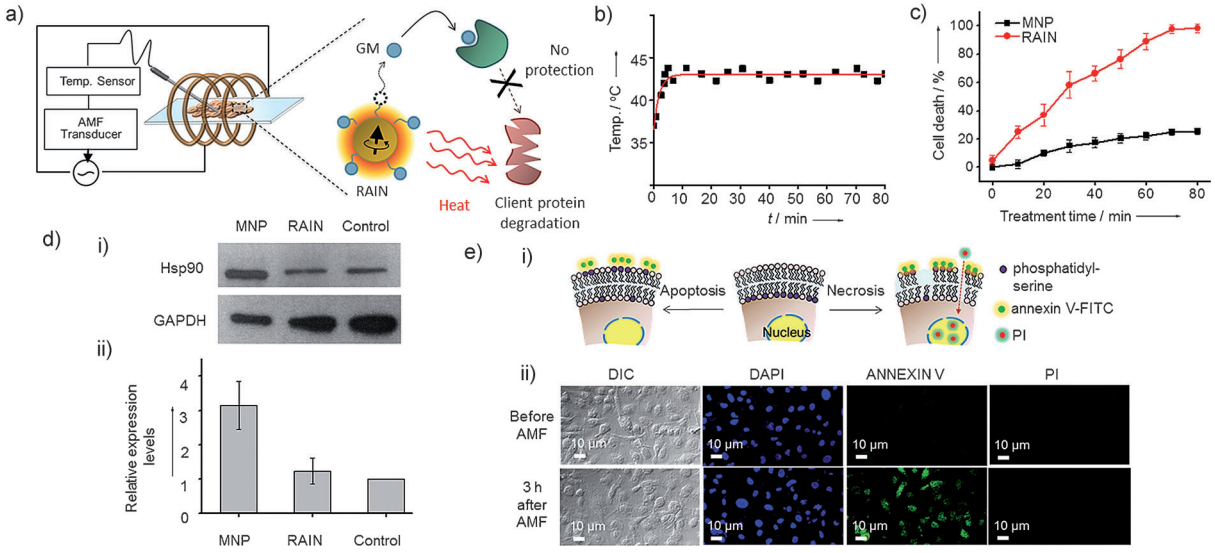

Figure 2. In vitro apoptotic magnetic hyperthermia studies using the RAIN for MDA-MB-231 cancer cells. a) Illustration of the in vitro experimental set-up. RAIN-treated MDA-MB-231 cells are incubated and then an AMF is applied to reach a temperature of 43 °C. Dual functions of the RAIN are heat generation and GM release for Hsp90 inhibition. b) Temperature profile. A temperature of 43 °C with a deviation of 1 °C is maintained under an AMF of 37.4 kA m^{-1} at 500 kHz. c) RAIN and MNP hyperthermia treatment efficacy at 43 °C. d) Western blot results: i) Western blot of MDA-MB-231 cells treated with the MNP, and the RAIN under an AMF. ii) The expression of Hsp90 quantified by normalization to GAPDH. e i) Different cell death pathways (apoptosis versus necrosis). Apoptosis is initiated by phosphatidylserine inversion without cell membrane permeablization. In case of necrosis, phosphatidylserine exposure and membrane rupture occur simultaneously. e ii) Optical images of RAIN-treated MDA-MB-231 cells before and 3 h after an AMF application. Nuclei are stained with DAPI (4′,6-diamidino-2-phenylindole; blue). Membrane inversion without rupture is detected by annexin V-FITC (annexin V-fluorescein isothiocyanate; green) without staining of PI (propidium iodide, red), which confirms apoptotic cell death promoted by RAIN hyperthermia.

out causing cell membrane permeabilization. This process enables annexin V-FITC to bind to phosphatidylserine, but PI is unable to enter into cells owing to the intactness of the cell membrane at the initial stage of apoptosis (Figure 2 e(i)). In contrast, annexin V-FITC and PI interact with the surface and DNA inside the cell, respectively, when the membrane ruptures upon onset of necrosis (Figure 2 e(i)). Before magnetic hyperthermia, neither annexin V-FITC nor PI-stained cells are detected (Figure 2 e(ii)). At 3 h post-hyperthermia treatment, only annexin V-FITC stained cells are detected and no PI stained cells are observed, indicating the inversion of phosphatidylserine and no permeabilization of the cell membrane, which are hallmarks of early apoptosis stage. These results thus support that hyperthermia-induced cell death by the RAIN occurs predominantly through apoptosis.

We further examine the efficacy of RAIN promoted apoptotic magnetic hyperthermia in vivo. Figure 3 a illustrates the schematic diagram of the magnetic hyperthermia set-up. MDA-MB-231 breast cancer cells are first xenografted to the right hind legs of an experimental group of nude mice ($n = 3$). The RAIN (50 μg), dispersed in normal saline (35 μL), is then directly injected into the tumor (100 mm^3) in each mouse. The mouse is placed in a magnetic induction coil and the temperature of the tumor is measured in real time by using an optical fiber probe. AMF (500 kHz, 37.4 kA m^{-1}) is applied by using a transducer to maintain the temperature of the tumor at about 43 °C during the entire 30 minutes application time and Figure 3 b shows the measured temperature profile. After a single hyperthermia treatment, the change in the tumor volume is monitored over a period of 14 days (Figure 3 c). In the untreated control group of mice, the tumor size is found to increase approximately eightfold over this period (black line, Figure 3 c). In contrast, the tumors of the group of mice receiving RAIN hyperthermia are eliminated by day 8 (red line, Figure 3 c). For comparison purposes, another group of mice are treated with MNP hyperthermia only and the changes of their tumor sizes are monitored in the same way. Although their sizes are found to decrease initially, the tumors in this group of mice start to grow back at day 10 and the sizes eventually increase about 2.5 fold at day 14 (blue line, Figure 3 c). The pictures given on the right side of each plot in Figure 3 c clearly show the tumor size changes in each group of mice. The tumor tissues treated with RAIN hyperthermia are then analyzed by using immunofluorescence histology (Figure 3 d). The bright green fluorescence observed in an untreated control shows the presence of cancer cells (Figure 3 d(i)) while the absence of fluorescence indicates elimination of tumor (Figure 3 d(ii)).

This study has demonstrated a new approach for resistance-free apoptotic magnetic hyperthermia, the success of which has been validated by using both in vitro and in vivo experiments. Considering therapeutic efficacy of conventional apoptotic hyperthermia is limited by the occurrence of induced resistive thermotolerance, apparently disparate two goals have been incompatible: 1) the usage of mild temperature (about 43 °C) for inducing apoptosis of cancer cells without affecting normal cells and 2) high therapeutic efficacy. However, the RAIN allows these two goals to be achievable simultaneously where Hsp-inhibition is the key to achieve high efficacy of apoptotic hyperthermia. The concept

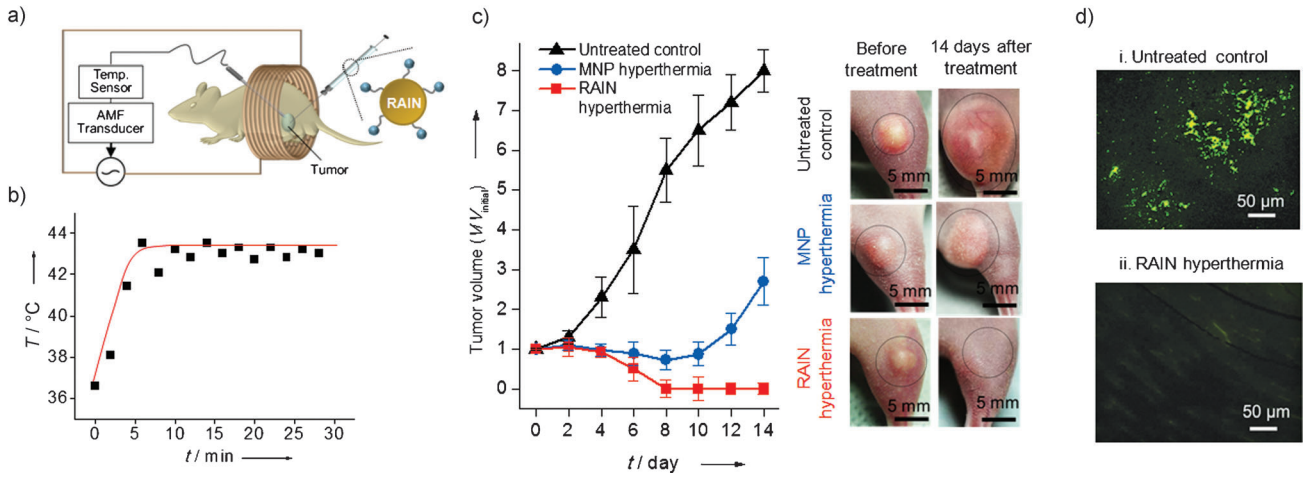

Figure 3. In vivo RAIN-mediated apoptotic magnetic hyperthermia. a) Schematic illustration of the magnetic hyperthermia set-up. The RAIN (50 μg) are directly injected into the tumor (100 mm^3, $n = 3$) of a mouse and subjected to an AMF (37.4 $kA\,m^{-1}$, 500 kHz) for the hyperthermia study. b) Temperature profile at tumor site. 43 °C is maintained and monitored by using an optical fiber temperature probe. c) A plot of tumor volume ($V/V_{initial}$) versus the number of days after single hyperthermia treatment. RAIN hyperthermia shows complete tumor elimination at day 8 (red line) whereas initial slight reduction followed by regrowth of the tumor is observed in the group of MNP hyperthermia (blue line). Images of xenografted tumors (MDA-MB-231) on nude mice before treatment and 14 days after treatment are displayed on the right side of the plot. d) Immunofluorescence histological images of the untreated control tumor (top image, i), and the tumor after RAIN hyperthermia treatment (bottom image, ii).

developed in this study is simple and should be readily applicable to a wide range of disease treatments especially for hyperthermia with heat-generating nanomaterials by minimizing treatment resistance and maximizing treatment efficacy.

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