Multifunctional Magneto-Polymeric Nanohybrids for Targeted Detection and Synergistic Therapeutic Effects on Breast Cancer**

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High-resolution molecular and cellular imaging is one of the most promising applications of nanoparticles, and a number of nanostructured materials, especially of cadmium-containing II–VI semiconductors, have been developed to suit this purpose.[1] Recently, it was demonstrated that magnetic nanoparticles can be utilized as excellent magnetic resonance imaging (MRI) probes for noninvasive in vivo monitoring of molecular and cellular events.[2] We recognized the presence of a number of biocompatible polymers suitable for targeted drug delivery[3] and came upon an idea to combine the magnetic nanocrystals as ultrasensitive MR contrast agents, anticancer drugs as chemotherapeutic agents, and biodegradable amphiphilic block copolymers as stabilizers into a multifunctional hybrid nanosystem. The prepared multifunctional magneto-polymeric nanohybrids (MMPNs) further modified by antibodies would seek cancerous parts and allow simultaneous MR imaging and treatment (Scheme 1).

Herein, we report a novel protocol for the preparation of MMPNs and characterization to evaluate the sensitivity and stability of prepared nanohybrids. Furthermore, targeted detection ability by MRI and synergistic tumoricidal efficacy on breast cancer cells were investigated for cellular and animal models.

Monodispersed magnetic nanocrystals, soluble in organic solvents, were synthesized as reported (Figure 1a,b).[4] To obtain the required water solubility, the hydrophobic nanocrystals and anticancer drug (doxorubicin, DOX) were simultaneously encapsulated with the amphiphilic block copolymer by a nanoemulsion method to give MMPNs.[3e] The structures of MMPNs examined by TEM are shown in Figure 1c,d, and the sizes of MMPNs are consistent with the results from laser scattering: 69.7 ± 8.7 nm (MnFe₂O₄) and 72.8 ± 9.3 nm (Fe₃O₄), respectively (see the Supporting Information).

**Scheme 1. Schematic illustration for the fabrication of MMPNs.

**Figure 1. TEM images of magnetic nanocrystals of a) MnFe₂O₄ and b) Fe₃O₄ and MMPNs containing c) MnFe₂O₄ or d) Fe₃O₄ nanocrystals. e) Graph of relaxivity versus the concentration of MMPNs; MnFe₂O₄ (○), Fe₃O₄ (▲). f) Magnetic hysteresis loops of magnetic nanocrystals (solid line) and MMPNs (dashed line); MnFe₂O₄ (black) and Fe₃O₄ (gray). Mₛ is a standard magnetization (80.77 emu g⁻¹); Mᵣ is the magnetization of each of the magnetic nanocrystals and MMPNs.
MnFe$_2$O$_4$ nanoparticles were conjugated with MMPNs in the cell lines, MMPNs with clustering individual magnetic nanocrystals. The hysteresis reported that the MR sensitivity is greatly enhanced by NIH3T6.7 cells compared with MDA-MB-231 cells, respectively (Figure 2a). It was also reported that the MR sensitivity is greatly enhanced by clustering individual magnetic nanocrystals. The hysteresis loops of MMPNs were observed using a vibration sample magnetometer at 300 K (Figure 1f). Our results showed that the MMPNs exhibited superparamagnetic behavior without magnetic hysteresis. The saturation of magnetization values for MMPNs were 48.78 emu g$^{-1}$ (MnFe$_2$O$_4$) and 40.65 emu g$^{-1}$ (Fe$_3$O$_4$) at 1.5 T, which are smaller than those of corresponding magnetic nanoparticles, 80.77 emu g$^{-1}$ (MnFe$_2$O$_4$) and 73.98 emu g$^{-1}$ (Fe$_3$O$_4$), owing to the presence of organic components.

As MR probes in aqueous phase, the MMPNs demonstrated excellent colloidal stability at a high concentration (up to 1 mM of MMPNs), in a wide range of salt concentrations (NaCl, ca. 5 M), and within various pH ranges (from about pH 4 to about pH 12; Supporting Information).

The targeting efficacy of the MMPNs and their detection by MRI were investigated in breast cancer cell lines. The human epidermal growth factor receptor 2 (HER2) was used as a tumor-targeting marker for the treatment of patients with metastatic breast cancer. Fibroblast NIH3T6.7 cells, which highly express the HER2/neu cancer markers, were compared with MDA-MB-231 cells, which express low levels of the cancer markers. To compare the performance of MMPNs in these cell lines, MMPNs with MnFe$_2$O$_4$ nanoparticles were conjugated with anti-HER antibody (HER, herceptin) by utilizing the carboxyl group on the surface of the MMPNs. The difference of $\Delta R_2/R_2$ (normalized $R_1 = T_2$) in cells treated with HER-conjugated MMPNs (HER-MMPNs) compared to those of MMPNs conjugated with an irrelevant antibody (IRR, human IgG; IRR-MMPNs) were about 320% (NIH3T6.7) and about 57% (MDA-MB-231), respectively (Figure 2a). In addition, HER-MMPNs exhibited specific affinity (4.4 times) for NIH3T6.7 cells compared with MDA-MB-231 cells, demonstrating the efficient targeted delivery of MMPNs for the HER2/neu receptor. IRR-MMPN-treated cells, however, showed partial enhancement against the MR signal intensity owing to nonspecific binding.

The fluorescence intensity of NIH3T6.7 cells treated with HER-MMPNs was larger than that of MDA-MB-231 cells (Supporting Information). In addition, the confocal microscopic images showed that NIH3T6.7 cells incubated with HER-MMPNs presented excellent uptake efficiency compared to other cases (Figure 2b–e). In the case of NIH3T6.7 cells treated with HER-MMPNs, a bright green color was observed in the microscopic image, whereas faint green fluorescence was noted for the IRR-MMPNs. These flow cytometry analysis and microscopic results demonstrated that HER-MMPNs successfully bind to NIH3T6.7 cells and are subsequently taken into the cells.

To assess the therapeutic potential for the combination of therapeutic antibodies and chemotherapeutic drugs, the degree of drug release and the inhibition of cell growth were investigated. The amount of DOX in the HER-MMPNs and entrapment efficiency were 3.3 wt% and 71.4%, respectively. These results were similar to those of the MMPNs: 3.5 wt% (amount of DOX in the MMPNs) and 73.5% (entrapment efficiency). Drug-release data from the prepared MMPNs and HER-MMPNs are shown in Figure 2f. The release test was performed in triplicate, and the obtained values were used to calculate a mean value and standard deviation. After 3 and 12 days, 60% and 80% of the encapsulated DOX was released from the MMPNs and HER-MMPNs, respectively, owing to polymer degradation.

We next determined the in vitro differential cytotoxicity of HER-MMPNs, IRR-MMPNs, and HER-MNPs (HER-MNPs, IRR-MNP...
conjugated with a non-drug-loaded magnetic nanoparticle–polymer hybrid) using an MTT assay on NIH3T6.7 and MDA-MB231 cells, compared to those of DOX + HER, free HER, and free DOX. Cells were incubated for 4 h with those prepared samples. After the initial incubation period, the cells were washed and incubated further for 72 h. The cell viabilities are presented as the ratio of the number of live cells incubated under the above conditions to the number of nontreated control cells (Figure 2g,h). Our data show that HER-MMPNs are remarkably more cytotoxic than the nontreated control cells (Figure 2g,h). Our data show that viabilities are presented as the ratio of the number of live cells because of minimal vascular supplying parts. Consequently, nanometer-sized MMPNs could be taken up by tumor cells in comparison with normal tissue, and IRR-MMPNs (77.3 ± 6.2 nm) could exhibit inhibition effects owing to an EPR effect. Interestingly, HER-MMPNs (79.3 ± 7.9 nm) demonstrated more effective tumor growth inhibition than IRR-MMPNs. These noticeable results suggested that injected HER-MMPNs were target-specifically delivered to overexpressed HER2/neu receptors of NIH3T6.7 cells in the mouse model and were taken up by a receptor-mediated endocytosis process. Release of DOX from the HER-MMPNs demonstrated exceptional therapeutic efficacy.

In summary, we have developed multifunctional magnetopolymeric nanohybrids (MMPNs) composed of magnetic nanocrystals and anticancer drugs encapsulated by an amphiphilic block copolymer. The antibody-modified MMPNs (HER-MMPNs) demonstrated ultrasensitive targeted detection by MRI in vitro and in vivo models. In addition, the HER-MMPNs showed excellent synergistic effects for the inhibition of tumor growth. This study may be used as a foundation for the development of novel nanodrugs for the simultaneous diagnosis and treatment of various types of cancers.

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Supporting Information

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Supporting Information

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Scheme S1. Preparation scheme of PLGA-PEG block copolymers.

Figure S1. 1H- NMR spectrum of PLGA-PEG block copolymers
Figure S2. FT-IR spectrum of PEG-PLGA block copolymers.

Figure S3. Size distribution of MnFe$_2$O$_4$-MMPNs (solid line) and Fe$_3$O$_4$-MMPNs (dashed line) using laser scattering.
Figure S4. TEM images of MMPNs containing MnFe$_2$O$_4$.

Figure S5. Thermogravity analysis of MMPNs; MnFe$_2$O$_4$ (solid line) and Fe$_3$O$_4$ (dashed line).

Figure S6. T2-weighted MR images of MMPNs and their color maps in aqueous solution.
Figure S7. Colloidal stability test in serum (a) and NaCl solution (b). (c) Colloidal stability test against various pH ranges.

Figure S8. Flow cytometry results of non-treated control cells (a) and HER-MMPNs treated cells; MDA-MB-231 (b) and NIH3T6.7 (c) cells.

Figure S9. MR images of cancer-targeting events of HER-MMPNs (a-d) and IRR-MMPNs (e-h) in NIH3T6.7 cells implanted in mice at various time intervals: (a, e) preinjection, (b, f) immediately (c, g) 1 h, and (d, h) 12 h after injection of the MMPNs.
Experiments

1. Synthesis and characterization of block copolymers.

Carboxyl-terminated PEG-PLGA copolymers were synthesized by bioconjugation techniques (Scheme S1). PLGA (MW: 5k, 0.4 mM) dissolved in 10 mL methylene chloride was activated by adding 2.0 mM of 1,3-dicyclohexylcarbodiimide and 2.0 mM of n-hydroxysuccinimide. The reaction was carried out for 24 h at room temperature under a nitrogen atmosphere. The resultant products were filtered and purified with excess ether. The precipitates were dried under vacuum and stored until later use. To synthesize the PEG-PLGA copolymer, 134 mg of NH2-PEG-COOH (MW: 3.4k) and 200 mg of activated PLGA were dissolved in 4 mL of dimethyl formamide and then, 50 µL of triethylamine was added to the solution at 4°C. This reaction was carried out under a nitrogen atmosphere for 6 h. The purification process and storage method were performed in the same manner as previously described. The yield of final products was 72.6%. The resulting PEG-PLGA was characterized at 400 MHz on a Varian INOVA400 NMR spectrometer with a sample spinning rate of 5 kHz at 25°C; δ values of 3.62 (hydrogen of PEG backbone), 1.54 (methyl group of lactide chain), 5.21 (hydrogen of lactide backbone), 4.83 (hydrogen of glycolide chain). The FT-IR spectrum of the block copolymers via Excalibur™ series showed characteristic bands at 1,612 cm⁻¹ and 1,729 cm⁻¹ indicating the presence of amide and ester bonds, respectively.

2. Synthesis and characterization of MMPNs.

Magnetic nanocrystals (30 mg) and chemotherapeutic drugs (DOX, 5 mg) were dissolved in 10 mL of dichloromethane. The organic phase was added to 20 mL of an aqueous phase containing PEG-PLGA (200 mg). After mutual saturation of the organic and continuous phase, the mixture was emulsified for 10 min with an ultrasonicator (ULH700S, Ulsohitech) operated at 600 W. After solvent evaporation, the products were purified with three cycles of centrifugation at 20k rpm. The precipitated nanoparticles were redispersed in a 10 mM sodium phosphate buffer (2 mL, pH 7.4). The size and size distribution of the MMPNs were analyzed by laser scattering (ELS-Z, Otsuka electronics). The morphology and presence of magnetic nanocrystals were evaluated with a transmittance electron microscope using a negative staining method. The quantity of magnetic nanocrystals in the MMPNs was analyzed with a thermo-gravimetric analyzer (SDT-Q600, TA instrument).

3. Determining of the drug release profile.

10 mg of MMPNs was suspended in 2 mL of phosphate buffered saline (10mM, pH 7.4) and sealed in dialysis tubing and then immersed in 20 mL of buffer solution at 37.5°C. The system was shaken at a moderate speed, and at regular time intervals, the amount of released drug was monitored by absorbance (480 nm) using a UV spectrophotometer (Optizen 2120UV, MECASYS Co). In addition, drug loading contents and entrapment efficiency were also measured in the same manner.
4. HER conjugation with MMPNs.

In order to conjugate the antibody with prepared MMPNs, 1 mg of HER (Herceptin®, Roche Pharma Ltd.) was dissolved in 10 mM sodium phosphate buffer (400 µL, pH 7.4) and mixed with 100 µL of the MMPNs solution (5 mg/mL). Next, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (2.0 mM) and n-hydroxysuccinimide (2.0 mM) were added to the previous solution. After 4 h, HER-MMPNs were purified with a Sephacryl S-300 column (Amersham Biosciences). 10 equivalent HER were conjugated on surface of HER-MMPNs, as evaluated using BCA kit. The affinities of antibody conjugated MMPNs against target cells were investigated using flow cytometry and fluorescence microscope. Target cells (NIH3T6.7 and MDA-MB-231 cells, 10^6 cells/mL) were incubated and treated with the MMPNs for 30 min. Then, FITC conjugated Anti human IgG antibody (ICN) was added into the well for binding with HER. The resultant was washed 3 times with 0.2% FBS and 0.02% NaN3 in PBS. These samples were resuspended in 400 µL, 4% paraformaldehyde, and scanning of the cell-associated fluorescence was performed using a FACScalibur (Beckton-Dickinson, Mansfield, MA) at the wavelength of λ = 488 nm.

5. MR imaging procedure.

All MR imaging experiments were performed with a 1.5 T clinical MRI instrument with a micro-47 surface coil (Intera; Philips Medical Systems, Best, the Netherlands). R2 relaxivities of two sets of MMPNs containing each MnFe2O4 and Fe3O4 magnetic nanocrystal were measured by the Carr-Purcell-Meiboom-Gill (CPMG) sequence at room temperature: TR = 10 sec, 32 echoes with 12 msec even echo space, number of acquisition = 1, point resolution of 156 × 156 µm, section thickness of 0.6 mm. R2 defined as 1/T2 with units of sec⁻¹. The relaxivity coefficient (mM⁻¹sec⁻¹) equals to ratio of R2 to MMPNs concentration. For T2-weighted MR imaging of in vitro cells at 1.5 T, the following parameters were adopted: point resolution: 156 × 156 µm, section thickness of 0.6 mm, TE = 60 ms, TR = 4000 ms, number of acquisitions = 1. For T2 mapping of in vitro cells, the following parameters were adopted: point resolution of 156 × 156 µm, section thickness of 0.6 mm, TE = 20, 40, 60, 80, 100, 120, 140, 160 ms, TR = 4000 ms, number of acquisitions = 2. For T2-weighted MR imaging of a live mouse, the following parameters were adopted: resolution of 234 × 234 µm, section thickness of 2.0 mm, TE = 60 ms, TR = 4000 ms, number of acquisitions = 1. For T2 mapping of a live mouse, the following parameters were adopted: point resolution of 234 × 234 µm, section thickness of 2.0 mm, TE = 20, 40, 60, 80, 100, 120, 140, 160 ms, TR = 4000 ms, number of acquisitions = 2.


Cell viability was determined in NIH3T6.7 and MDA-MB-231 cells maintained in DMEM (Dulbecco’s Modified Eagle’s Medium) in a 5 % of CO2 atmosphere. Cytotoxicity of free DOX, free HER, DOX+HER, HER-MNPs, IRR-MMPNs and HER-MMPNs were evaluated by measuring the inhibition of cell growth using the MTT assay. Briefly, cells were plated at a
density of $4\times10^3$ cells/ml in 96-well plates and treated with the above compounds for 4 h up to equivalent concentration; HER (72 nM) and DOX (80 nM). After treatment, the cells were washed and incubated for an additional 72 h. MTT assay was performed using Cell Proliferation kit (Roche, USA). Cell viabilities were presented as the ratio of the number of cells treated to the number of non-treated control cells. Cell viability graphs were plotted as DOX concentration.

7. Animal experiments

Investigation of tumor growth rate was progressed using NIH3T6.7 cell ($1 \times 10^7$ cells per animal) xenografted nude mice (body weight = ~25 g) at the proximal thigh. After the implantation, tumors had developed to ~90 mm$^3$ and we performed comparative efficacy studies by dividing animals into seven groups (n=3). Free HER, free DOX, HER+DOX, HER-MPNs, IRR-MMPNs, and HER-MMPNs (an equivalent dose of DOX= 3 mg/kg) suspended in PBS were injected into the tail vein of animals at day 0, 7, and 14. At predetermined time points, length of the minor axis (2a) and a major axis (2b) of tumors were measured using a caliper. Tumor volume was then calculated using the formula for a prolate spheroid: \(\frac{4}{3}\pi a^2b\).\textsuperscript{S4}

Reference


