The development of diagnostic and therapeutic nanomaterials (1-8) for drug/gene co-delivery and ultrasonic/magnetic resonance imaging (MRI) contrast enhancement has been progressing rapidly towards various cancer cell types, especially in brain (9), liver (10-13), and other sites (14-22). In particular, ultrasmall superparamagnetic iron oxide (SPIO) nanoparticles (NPs) offer cell tracking, targeting, and substrate delivery to specific target site(s) (23-25). Linear polyethylenimine (PEI) polymers of low or high molecular weights have been employed to deliver genes with enhanced transfection efficiencies and possibly reducing cytotoxicities (26,27). In particular, ultrasmall deferoxamine-coated SPIO-NPs were first reported in our group (9,12) and studied for their biomedical properties. Deferoxamine, also known as desferal, is a clinically approved drug to treat iron poisoning. Slow degradation of iron oxide NPs in vivo will result in soluble iron ions which will, in turn, capture by deferoxamine layer at the nanoparticle’s periphery. Eventually, the deferoxamine-iron complexes will be excreted in the urine, thus reducing the in vivo toxicity especially in the heart and liver.

In view of delivering genes and facilitating MRI towards hepatocellular carcinoma (HCC) HepG2 cells with enhanced cellular uptake or transfection efficiencies, we report herein the use of deferoxamine-coated ultrasmall (8-10 nm) Fe₃O₄ SPIO-NPs (23-25), hybridizing with circular plasmid DNAs (pEGFP-C1), and branched PEI (25 kDa, PDI =2.5) to furnish ternary composites (9,12,13,26-32) for MRI and fluorescence imaging. The
biocompatibility of the ternary complex was evaluated by agarose gel retardation assay. The cellular uptake of the ternary complex is proposed by the receptor-mediated endocytosis (13,33,34) of HCC cells. Circular plasmid DNA pEGFP-C1 (~4.7 kb, Clontech) encodes a red-shifted variant of wild-type green fluorescence protein (GFP) in mammalian cells. The plasmid was prepared by using the QIAprep Spin Miniprep Kit (QIAGEN) with $A_{260}/A_{280}$ ratio larger than 1.8. The fluorescence intensity is directly proportional to the amount of GFP expressed in the cells. By the strong, enhanced and constitutive expression of the reporters, the signals can be easily detected. They are optimized so that the reporters can be expressed in a variety of cell types/lines. It is envisaged that after receptor-mediated endocytosis of the composites, the NPs in the composites would be cleaved and localized in the cytoplasm, which is responsible for generating MRI dark contrast signal. On the other hand, the pDNA of the composites would be further imported into the nucleus, which is responsible for expressing the fluorescence.

NPs with a deferoxamine coating could be self-assembled with negatively charged pDNA and positively charged branched PEI to furnish the ternary composites (200-300 nm) (9,12,13), thereby stabilizing by multiple electrostatic interaction and hydrogen bonds (35,36). The morphology and surface functional groups of the composites were characterized by transmission electron microscopy (TEM) and Infrared (IR) absorption spectroscopy, respectively, which were reported previously in the literature. To evaluate the pDNA condensation ability of the PEI, agarose gel retardation assay was performed. The samples were then loaded onto 1% agarose gel containing 1x RedSafe Nucleic Acid staining solution (iNtRon Biotechnology). Free DNA (naked DNA) and commercially available transfecting agent Lipofectamine (Life Technologies) were used as controls. After electrophoresis (Figure 1) carried out at 110 V in tris-acetate-EDTA buffer (pH 7.4), uncomplexed (free) pDNA will migrate into the gel. RedSafe staining dye will stain the pDNA and DNA bands can then be visualized under a UV transilluminator. On the other hand, PEI and PEI/NP can retard the pDNA migration toward the cathode.

Figure 1 Gel electrophoresis on 1% agarose gel whereas the DNA (pEGFP-C1) concentration is fixed at 0.5 μg/well.
and exclude the dye from staining the nucleic acid. By observing the presence or absence of band and band intensity with the use of NPs, the pDNA packaging can be monitored, which is a critical step before transfection by the assay.

HCC HepG2 cells (ATCC, Manassas, VA) were cultured with DMEM (Life Technologies) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 °C and in a humidified 5% CO₂ atmosphere. Subsequently, about 50,000 cells were seeded onto each well of the 24- or 96-well plates. After 24 h, the culture medium was replaced with the serum-free DMEM containing different composites. After incubation for 5 h, the medium was aspirated and refreshed with complete DMEM. The cells were further incubated for 24 h at 37 °C. Colorimetric method was used to study the iron concentration for the cells that were transfected with the composites. The cells were washed, collected, and counted for the intracellular iron content quantification. After centrifugation (4,500 g) for 5 min, the collected cell pellets were dispensed in 100 µL 12% HCl solution and incubated at 60 °C for 4 h. After incubation, the suspension was centrifuged (12,000 g) for 10 min, whereas the supernatants were collected for iron concentration quantification. A sample solution (50 µL) was added into the wells of a 96-well plate, and then ammonium persulfate (50 µL, 1%) was added to oxidize the ferrous ions into ferric ions. Finally, potassium thiocyanate (100 µL, 0.1 M) was added to the solution and incubated for 5 min to form the red color of iron-thiocyanate. The absorption at 490 nm of the sample was observed on a microplate reader (Bio-Rad, Model 3550).

In vitro MRI was performed with HepG2 cells 24 h after transfection. After washing with PBS, the cells were trypsinized and counted. Different numbers (12.5, 25, 50, and 100 k) of cells were placed in an Eppendorf tube (1.5 mL) separately. After a centrifugation at 3,000 g for 5 min, the Eppendorf tubes were placed perpendicular to the main magnetic induction field (B₀) in a 20 cm × 12 cm × 8 cm water bath. MRI was performed with a 3.0-T clinical whole-body magnetic resonance unit (Achieva, Philips Medical Systems), using a transmit-receive head coil. T₂ relaxation times were measured by using a standard Carr–Purcell–Meiboom–Gill pulse sequence [repetition time (TR) =2,000 ms, echo time (TE) range =30-960 ms, 32 echoes, field-of-view (FOV) =134×67 mm², matrix =128×64, slice thickness =5 mm, number of excitations =3]. The magnetic resonance sequence was a two-dimensional gradient-echo sequence with TR/TE =400/48 ms, flip angle =18°, matrix =512×256, resolution =0.45×0.45 mm, slice thickness =2 mm, and number of excitations =2. Sagittal images were obtained through the central section of the bottom tips of the Eppendorf tubes. HepG2 cells were transfected separately with ternary complexes of varying NP concentrations and analyzed by in vitro MRI. Substantial negative (dark) contrast MRI signals with “ballooning” effect are observed in Figure 2 with the cells that were centrifugated at the bottom of Eppendorf tube. Under fixed amounts of PEI (0.2 ng) and DNA (0.5 µg) per well, HepG2 cells that were transfected with higher NP concentrations possessed stronger MRI dark contrast signals. For ternary complexes containing 0.1 and 1.0 µg NP, MRI signals were detectable and visually observable at cell number of 100, 50, 25, and 12.5 k, respectively. T₂ relaxation times were calculated by fitting the logarithmic region of interest signal amplitudes (1,600 pixel) versus TE. The T₂ relaxivities (r₂) were determined by a linear fit of the inverse relaxation times as a function of the iron concentrations used. The in vitro r₂ of the two composites 0.2 ng PEI/0.5 µg DNA/0.1 µg NP and 0.2 ng PEI/0.5 µg DNA/1.0 µg NP were determined to be 1.46 and 2.20 s⁻¹ mM⁻¹ Fe, respectively. Prussian blue staining
(9,12,17-19,23-25) is an alternative method to quantify the \textit{in vitro} iron content. These results suggest that the ternary hybrid nanocomposites hold promise as effective MRI contrast agents and are potentially suitable for magnetic targeting to cancer sites.

About 50,000 cells were seeded onto each well of the 24-well plates for GFP observation. The typical GFP green fluorescent images of HepG2 cells which have been separately transfected with (A-C) different ternary composites 0.1 ng PEI/0.5 µg DNA/NP with varying amounts of NP (A: 0.1 µg, B: 1.0 µg, and C: 2.5 µg), (D) 0.1 ng PEI/0.5 µg DNA, and (E) Lipofectamine/0.5 µg DNA, are shown in Figure 3. The green fluorescence of the transfected HepG2 cells was visualized by a Nikon TE2000 fluorescence microscope 24 h after transfection. According to the visual assessment of these images, the cellular uptake efficiencies of the pDNA in the ternary complexes (Figure 3A-C) to the HepG2 cell nucleus generally lower than that using Lipofectamine or PEI (Figure 3C,D). However, the cellular uptake efficiencies of the pDNA in the ternary complexes (Figure 3A-C) to the HepG2 cell nucleus will be greatly enhanced 24 h after transfection (12). It is reasonably to consider that the cellular uptake efficiency depends on the surface charge density and stability of the composites during the receptor-mediated endocytosis and further cleavage of the composite with NP localized in cytoplasm and pDNA in nucleus. The transfected composites would eventually be dissociated into separate components and subject to an efflux mechanism. Moreover, cytotoxities of cells incubated with the composites in working concentrations that were examined by methylthiazolyldiphenyl-tetrazolium (MTT) bromide assay in HepG2 cells, have been reported. The cell viabilities range from 70% to 85%, which are higher than using Lipofectamine or PEI (12).

In conclusion, ternary composites based on PEI/DNA/deferoxamine-NP have been prepared by tuning the PEI/NP ratios and with a fixed DNA amount, for transfection towards HCC HepG2 cells. The cell transfection efficiencies involving NP uptake and gene expression with the ternary composites could be altered by tuning the PEI/NP ratios in the composite, which have been characterized by \textit{in vitro} MRI and GFP fluorescence. From the MRI assessments, the \textit{in vitro} $r_2$ values of ternary complexes 0.2 ng PEI/0.5 µg DNA/0.1 µg NP and 0.2 ng PEI/0.5 µg DNA/1.0 µg NP were determined to be 1.46 and 2.20 s$^{-1}$ mM$^{-1}$ Fe, respectively. The as-prepared composites or other nanostructured magnetic composites (37) offer potential biomedical applications in simultaneous gene delivery, imaging contrast enhancement, and metabolism study for the next generation \textit{in vivo} carcinoma nano-theranostic purpose.
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