Biomaterials 35 (2014) 7860–7869

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Enhancing therapeutic efficacy through designed aggregation of nanoparticles

Tanmoy Sadhukha [a], Timothy S. Wiedmann [a], Jayanth Panyam [a, b, *]

[a] Department of Pharmaceutics, College of Pharmacy, University of Minnesota, Minneapolis, MN 55455, USA
[b] Masonic Cancer Center, University of Minnesota, Minneapolis, MN 55455, USA

ARTICLE INFO

Article history:
Received 16 April 2014
Accepted 28 May 2014
Available online 16 June 2014

Keywords:
Superparamagnetic iron oxide
Magnetic hyperthermia
Aggregation
Necrosis
Apoptosis
Autophagy

ABSTRACT

Particle size is a key determinant of biological performance of sub-micron size delivery systems. Previous studies investigating the effect of particle size have primarily focused on well-dispersed nanoparticles. However, inorganic nanoparticles are prone to aggregation in biological environments. In our studies, we examined the consequence of aggregation on superparamagnetic iron oxide (SPIO) nanoparticle-induced magnetic hyperthermia. Here we show that the extent and mechanism of hyperthermia-induced cell kill is highly dependent on the aggregation state of SPIO nanoparticles. Well-dispersed nanoparticles induced apoptosis, similar to that observed with conventional hyperthermia. Sub-micron size aggregates, on the other hand, induced temperature-dependent autophagy through generation of oxidative stress. Micron size aggregates caused rapid membrane damage, resulting in acute cell kill. Overall, this work highlights the potential for developing highly effective anticancer therapeutics through designed aggregation of nano delivery systems.

1. Introduction

Nano drug delivery systems are of considerable interest in cancer therapy because of their ability to accumulate passively in tumors [1–3]. A key area of research is the effect of size of the delivery system on therapeutic effectiveness [4–6]. Several reports suggest that the optimal size of nanoparticles for tumor therapy is around 100 nm [3]. However, the presence of proteins can lead to aggregation of nanoparticles in biological environment [7–10]. For example, depending on the surface properties, nanoparticles can form sub-micron to micron size aggregates in the presence of serum proteins [11,12]. The effect of aggregation on the therapeutic performance of nanoparticles has not been previously investigated.

In our studies, we examined the consequence of aggregation on superparamagnetic iron oxide (SPIO) nanoparticle-induced magnetic hyperthermia (MH). Superparamagnetic materials generate heat when placed in alternating magnetic field [13,14]. Depending on the size of the material, heat is generated through either Néel or Brownian relaxation or a combination of the two [15,16]. Heat generated and the associated temperature change from such relaxation phenomena dissipate over short distances due to the high thermal conductivity of water and can therefore be used for highly focused heating [17,18]. This concept is utilized in MH, where an external magnetic field is applied to SPIO nanoparticles trapped in tumor tissue that results in local heating and tumor cell kill [18].

We hypothesized that the aggregation state of SPIO nanoparticles will affect their magnetic properties, which, in turn, will affect their tumor cell kill effectiveness. To test this hypothesis, we fabricated SPIO nanoparticle aggregates of various sizes and evaluated their ability to induce MH in cell culture models as well as in a mouse subcutaneous model of lung cancer.

2. Materials and methods

2.1. Materials

Ferrous chloride tetrahydrate, ferric chloride hexahydrate, myristic acid, Pluronic F127, ascorbic acid, potassium hydroxide, 1,10-phenanthroline and sodium acetate were purchased from Sigma (St. Louis, MO). Penicillin/streptomycin, fetal bovine serum, Dulbecco's phosphate buffered saline, F-12K (Kaighn's modification) and trypsin-EDTA solution were obtained from Invitrogen Corporation (Carlsbad, CA). Cytotox 96® non-radioactive cytotoxicity assay and CellTiter 96® AQueous one solution cell proliferation assay kit were purchased from Promega (Madison, WI). The commercial SPIO nanoparticles (Nanomag®) were purchased from Micromod Partikeltechnologie GmbH (Rostock, Germany). For Western blotting, antibody against HSP70 was from Enzo Life Sciences (Farmingdale, NY), anti-GAPDH-HRP and anti-β-actin-HRP were from Sigma, anti-PARP1, anti-cleaved PARP1, anti-LC3B and HRP-linked anti-rabbit and anti-mouse IgG were from Cell Signaling Technology (Danvers, MA).

* Corresponding author. Department of Pharmaceutics, College of Pharmacy, University of Minnesota, Minneapolis, MN 55455, USA. Tel.: +1 612 624 0951; fax: +1 612 626 2125.
E-mail addresses: jpanyam@gmail.com, jpanyam@umn.edu (J. Panyam).

http://dx.doi.org/10.1016/j.biomaterials.2014.05.085
0142-9612/

2.2. Methods

2.2.1. Synthesis of SPIO nano and micro aggregates

SPIO nanoparticles were synthesized by condensation of 0.82 g ferric chloride hexahydrate and 0.33 g ferrous chloride tetrahydrate dissolved in 30 ml of degassed and nitrogen-purged water with 3 ml of 5 M ammonium hydroxide. The reaction was allowed to continue for 30 min under magnetic stirring (200 G magnetic stir bar, 1600 rpm) and nitrogen gas bubbling through the reaction mixture. Iron oxide formed was washed three times with 30 ml of nitrogen-purged water, followed my magnetic separation and heating on a hot (80 °C) oil bath. About 100 mg of myristic acid was added to the heated mixture, which was stirred for another 30 min at 1600 rpm using a 200 G magnetic stir bar. Excess myristic acid was removed by two washes with acetone, followed by two washes with water to remove excess acetone. Each wash was followed by magnetic separation of nanoparticles. The myristic acid-coated particles were then suspended in 30 ml water using a water-bath sonicator and 100 mg of Pluronic F127 was added and stirred overnight. The final dispersion was fractionated by centrifugation at 800 *g* for 6 min. The supernatant contained nano aggregates. The pellet was dispersed in 15 ml of water and 20 mg Pluronic F127 by sonication for 1 h and was comprised of micro aggregates.

Coumarin-6 labeled SPIO nanoparticles were synthesized by incorporating coumarin-6 into the fatty acid layer of SPIO nanoparticles dispersed in water [19]. One ml of 500 μg/ml coumarin-6 in ethanol was added to 100 mg magnetite equivalent of SPIO NP in 10 ml of water and stirred overnight. The coumarin-6 labeled SPIO nanoparticles were washed three times in water followed by magnetic separation of nanoparticles. The final product was dispersed in 10 ml of water and 5 mg Pluronic F127 and inspected under a fluorescent microscope to confirm the presence of dye-label on particles.

2.2.2. Characterization of SPIO nanoparticles

2.2.2.1. Particle size. The hydrodynamic diameter of SPIO nanoparticles was determined by dynamic light scattering. About 1 mg of SPIO nanoparticles was dispersed in 2 ml of deionized water by sonication, and the dispersion was subjected to particle size analysis using a Delsa™ Nano C Particle Analyzer (Beckman, Brea, CA). Measurements were performed at 25 °C and at a 165° scattering angle. Mean hydrodynamic diameter was calculated based on size distribution by weight, assuming a lognormal distribution. For transmission electron microscopy (TEM), a drop of an aqueous dispersion of SPIO nanoparticles was placed on a Formvar®-coated copper grid (150 mesh, Ted Pella Inc. Redding, CA). Excess liquid was removed using a filter paper, and the grid was allowed to air-dry. Particles were imaged using a JEOL JEM-1210 transmission electron microscope (Peabody, MA). The diameters of at least 100 different particles were measured using ImageJ software and averaged to determine the mean Feret's diameter.

2.2.2.2. Composition. The form of iron oxide was assessed by Fourier-transformed infrared spectroscopy (FT-IR). About 5 mg of SPIO nanoparticles was analyzed using Vertex 70 FT-IR spectrophotometer (Bruker Optics Inc., Billerica, MA). Scans were performed from 4000 cm^{-1} to 400 cm^{-1} and each spectrum was obtained by averaging 16 interferograms at a resolution of 2 cm^{-1}. The data was collected and analyzed using OPUS software (Bruker Optics Inc., Billerica, MA). Magnetic properties were determined with an accurately weighed sample of SPIO nanoparticles, which was sprinkled on a lightly greased silicon wafer and placed in a vibrating sample magnetometer (Micromod Model 3900, Princeton, NJ) operating at room temperature. Magnetization curves were recorded in magnetic fields ranging from −1 T to 1 T, at increments of 0.002 T. The curve was normalized to the mass of magnetite to obtain the saturation magnetization per gram of magnetite. The composition of SPIO nanoparticles was estimated using 1,10 phenanthroline-based iron assay [20]. SPIO nanoparticles were dissolved in 12 N hydrochloric acid. The solution was then diluted with distilled water to obtain a final acid concentration of 0.2 N. To this acid solution, 10 mg/ml ascorbic acid, 1.2 mg/ml 1,10 phenanthroline, 22.4 mg/ml potassium hydroxide and 123 mg/ml sodium acetate were added in a volume ratio of 1:1:1:1:5. Absorbance of the resultant solution was measured at 490 nm using a microplate reader (ELx800 Absorbance Microplate Reader, Biotek, Winooski, VT). Solutions of commercial iron oxide powder and ferric chloride (hexahydrate) in 0.2 N hydrochloric acid were used as standards.

2.2.2.3. Heating rate. The heating rates for different formulations were determined by placing 0.5 ml of the formulation (2 mg/ml or 1 mg/ml magnetite equivalent) in AMF. Temperature measurement was performed every 2 s using a fluoroptic probe inserted in the middle of the sample. Sample temperature was monitored for 30 s to ensure temperature stability, after which AMF was turned on. Specific loss power (SLP) was calculated from the initial heating rate (60 s) as the product of magnetite concentration, temperature change and the specific heat capacity of water. The contribution of the polypropylene sample tube (0.6 ml volume) to heat loss was ignored in SLP calculations.

2.2.3. Cell culture studies

A549 (human lung adenocarcinoma) and A549-Luc (stable luciferase expressing) cells were used in the study. Both cells were propagated using F-12K medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic solution and maintained at 37 °C and in 5% carbon dioxide.

2.2.3.1. Cell death after magnetic hyperthermia. About 1 million cells were suspended in 500 μl of F-12K (with 10% FBS) and mixed with 500 μl SPIO nano or micro aggregates in saline to make a final concentration of 1.75 mg/ml magnetite. The suspension was placed in AMF (6 kA/m, 386 kHz) for 5, 15 or 30 min, maintaining the temperature of the cell suspension between 43 and 46 °C. Cells not exposed to AMF, with or without SPIO nanoparticles, served as control. Additionally, cells were kept in a water bath for 30 min at 46 °C, to serve as conventional hyperthermia controls. Magnetic hyperthermia induced using a commercial formulation of dextran-coated SPIO (Nanomag®-D-SPIO) was also used for comparison of *in vitro* efficacy. Cells were pelleted 2 h following treatment, and the amount of LDH released by the cells in the supernatant was determined. LDH released by same number of freeze-thaw lysed cells was used to calculate 100% cell death. In addition, 50,000 treated cells were plated per well in a 24-well plate and assayed for cell survival after 24 h by MTS assay.

2.2.3.2. Instantaneous PI uptake during AMF exposure. About 1 million cells were suspended in 500 μl of F-12K (with 10% FBS) and incubated with 500 μl nano or micro aggregates or Nanomag® in saline (final concentration of 1.75 mg/ml magnetite) at 4 °C for 30 min. To each tube, 10 μM PI was added, and the treated cells were subjected to 30 min of AMF, while maintaining the cell suspension temperature below 5 °C with an ice bath. Cells not exposed to AMF, with or without SPIO nanoparticles, were used as controls. Following AMF exposure, cells were immediately washed with buffer, resuspended in medium and kept on ice. PI present in cells was detected in the FL-3 channel of the flow cytometer (BD, FACSCalibur™). Data from 10,000 cells in each group were analyzed using Cyflogic software.

2.2.3.3. Reactive oxygen species (ROS) generation. Immediately prior to the addition of SPIO formulations, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA) (7.5 μM) and PI were added to the cell suspension. Cells were subjected to flow cytometric analysis after 2 h. The deacetylated and oxidized product, 2′,7′-dichlorofluorescein, formed due to ROS generation in the cells, was detected in the FL-1 channel while PI fluorescence was detected in the FL-3 channel.

2.2.3.4. Western blot analysis. Cells were lysed using RIPA buffer containing protease inhibitor cocktail (Sigma) on ice. Cell lysates were centrifuged at 13,000 rpm for 10 min to pellet the insoluble cellular debris, and the supernatant was analyzed. Protein concentration in the supernatant was determined by the bicinchoninic acid assay (Thermo Scientific). Protein samples (15 μg) were loaded onto a 4–15% Tris-hydrochloride gel (Bio-Rad Laboratories) and, after electrophoresis for 40 min at 100 V, transferred onto a nitrocellulose membrane (Whatman) using a Criterion blotter (Bio-Rad Laboratories). The membrane was blocked with 5% non-fat dry milk in tris-buffered saline containing Tween 20 (TBST) for 1 h. The membrane was then incubated overnight at 4° C with primary antibodies against PARP1, cleaved PARP1, HSP70, LC3B, GAPDH-HRP or β-actin-HRP, diluted in either 5% non-fat dry milk in TBST or 5% BSA in TBST. After three 8-min washes with TBST, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit (for PARP1, cleaved PARP1 and LC3B) or anti-mouse (for HSP70) IgG in 5% non-fat dry milk in TBST for 1 h and then washed three times with TBST. Protein bands were then visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). For densitometric quantification, immunoblots were digitized on a flatbed scanner and the signal intensities of the visualized bands were quantified using ImageJ (NIH) and OriginPro 8 software (OriginLab Corporation, Northampton, MA).

2.2.3.5. Confocal microscopy. Following incubation with coumarin-6 labeled SPIO nanoparticles and AMF exposure, cells were stained with LysoTracker® red DND-99 (Invitrogen) for 30 min at 37 °C or Texas red® -X conjugated wheat germ agglutinin (Invitrogen) for 10 min at 37 °C and washed with phosphate buffered saline. Ten μl of the cell suspension was added to a glass slide, mounted by a cover glass and sealed with clear nail polish. The slides were imaged using a 40×/1.30 numerical aperture oil-immersion objective in an Olympus FluoView FV1000 BX2 upright confocal microscope (Olympus Corporation). The images were analyzed using FV1000 Viewer software (Olympus Corporation) and ImageJ (NIH).

2.2.3.6. Effect of aggregate size on cell kill by magnetic hyperthermia. To further investigate the effect of aggregate size on cell kill by magnetic hyperthermia, we performed additional fractionation of SPIO aggregates. The final dispersion containing myristic acid-coated and Pluronic-stabilized SPIO nanoparticles was fractionated by centrifugation at 400 *g* for 6 min. The residue from this step was termed Fraction A. The supernatant was further centrifuged at 600 *g* for 6 min, 800 *g* for 6 min and 1200 *g* for 6 min, and the residue was separated from the supernatant following each fractionation. The residues from each of the centrifugation runs were labeled Fractions B-D, and the final supernatant was labeled Fraction E. The hydrodynamic size of the fractions A, B, C, D and E was 1000 nm (1003 ± 90 nm), 750 nm (748 ± 44 nm), 405 nm (406 ± 17 nm), 345 nm (344 ± 8 nm) and 220 nm (221 ± 2 nm), respectively.

About 1 million cells were suspended in 500 μl of F-12K medium (with 10% FBS) and mixed with 500 μl of SPIO Fraction A, B, C, D or E in saline, resulting in a final concentration of 1.75 mg/ml magnetite. The suspension was placed in AMF (6 kA/m, 386 kHz) for 15 min, maintaining the temperature of the cell suspension between 43 and 46 °C. Cells not exposed to AMF, with or without SPIO nanoparticles, served as controls. Cells were pelleted 2 h following treatment, and the amount of LDH