Published in final edited form as:

Acta Biomater. 2017 April 15; 53: 619–630. doi:10.1016/j.actbio.2017.02.003.

The influence of cell and nanoparticle properties on heating and cell death in a radiofrequency field

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Abstract

The use of non-invasive radiofrequency (RF) energy to induce mild thermal and non-thermal effects in cancer tissue is under study as an adjuvant to chemo, radio or immuno therapy. This study examines cell specific sensitivities to RF exposure and the potential of nanoparticles to elevate heating rates or enhance biological effects. Increases in the heating rate of water in an RF field operating at 13.56 MHz (0.004–0.028 °C/s) were positively correlated with concentration of hybrid nanoparticles (1–10 mg/ml) consisting of water soluble malonodiserinolamide [60]fullerene (C₆₀-ser) conjugated to the surface of mesoporous silica nanoparticles (SiO₂-C₆₀). The heating rate of highly conductive cell culture media (0.024 °C/s) was similar to that of the highest concentration of nanoparticles in water, with no significant increase due to addition of nanoparticles at relevant doses (<100 µg/ml). With respect to cell viability, anionic (SiO₂ and SiO₂-C₆₀) or neutral (C₆₀) nanoparticles did not influence RF-induced cell death, however, cationic nanoparticles (4–100 µg/ml) caused dose-dependent increases in RF-induced cell death (24–42% compared to RF only). The effect of cell type, size and immortalization on sensitivity of cells to RF fields was examined in endothelial (HUVEC and HMVEC), fibroblast (primary dermal and L939) and cancer cells (HeLa and 4T1). While the state of cellular immortalization itself did not consistently influence the rate of RF-induced cell death compared to normal cell counter parts, cell size (ranging from 7 to 30 µm) negatively correlated with cell sensitivity to RF (21–97% cell death following 6 min irradiation). In summary, while nanoparticles do not alter the heating rate of biologically-relevant solutions, they can increase RF-induced cell death based on intrinsic cytotoxicity; and cells with smaller radii, and thereby greater surface membrane, are more susceptible to cell damage in an RF field than larger cells.

Keywords

Nanoparticle; Fullerene; Silica; Cell radii; Hyperthermia; Radiofrequency; Cancer

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1. Introduction

As cells undergo malignant transformation they acquire unique physical attributes characterized in part by high glycolytic metabolism, altered surface elasticity, and changes in cell shape and size. Furthermore, Santini et al. [1] reported that transformed fibroblasts have higher cytoplasmic conductivity than normal fibroblasts. It was speculated that the higher conductivity could result from greater ionic flux in the cytoplasm or from the observed higher metabolic activity in transformed cells, the latter known as the Warburg effect [2]. Gascoyne and Shim reported that electrical properties of cells can be related to structural and composition attributes [3]. They define the cell as a high-conductivity aqueous object surrounded by a poorly conducting shell, with four dielectric parameters characterizing the cell: plasma membrane capacitance, conductance, interior conductivity, and permittivity [4].

The presence of the cell membrane enables high differential conductance between the interior and exterior of the cell. Applied electric fields cause disturbances in charge distribution, defined as electric polarization [5]. In the radio frequency (RF) range, cell suspensions exhibit β -dispersions due predominately to Maxwell-Wagner relaxation at the cell membrane [6]. Charging effects at the cell membrane, and differences in conductivities between the cytoplasm and the extracellular fluid, contribute to large and small dispersions, respectively [7]. Proteins, protein-bound water, and organelles also contribute small magnitude β -dispersions [8].

This study examines the potential for nanoparticles (NPs) to function as beacons that alter localized conductivity and thereby impact RF-induced heating rates. In solution, NPs with a net surface charge have an electrostatic potential based on the boundary between ions associated with the NP surface and counter ions in the dispersant. The ions form a double layer at the water-particle interface [6]. Schwartz [9] theorized that these counter ions are free to move transversally on the particle surface. Application of an electric field would displace the counter ions relative to the particle. re-establishment of the double ion layer after the electric field is removed would be dependent on diffusion, making the radius of the NP sphere directly related to the relaxation rate.

Previous studies have reported that gold nanoparticles with diameters below 10 nm heat in an RF field, with heating being attenuated by NP aggregation [10]. Other studies have reported that heat production in NP solutions is attributed to Joule heating due to ionic conductivity of the electrolyte solutions introduced with the NPs, rather than the NPs themselves [11]. In 1985, a group of Rice University chemists discovered a new form (allotrope) of carbon they called buckminsterfullerene, [60]fullerene or C_{60} [12]. The C_{60} molecule is about 1 nm in diameter, so it can be considered to bridge the gap between molecules and NPs. To further explore if particle size or surface charge impact solution conductivity or heating rates, we introduced purified water-soluble, neutral malonodiserinolamide-derivatized C_{60} (C_{60} -ser), both as free particles with hydrodynamic diameter between 2 and 3 nm and as surface functional groups on spheres with larger radii [200 nm mesoporous silica (SiO_2) NPs], into the RF-field in water or biologically-relevant solutions and measured the resulting heating rates. Furthermore, we examined the influence of NP radii and surface chemistry on cellular localization and cell viability in an RF-field

using C₆₀-ser, SiO₂, SiO₂-C₆₀, and cationic, β -ethanolamine fullerene-functionalized SiO₂ NPs (SiO₂-aminoC₆₀).

In addition to the effects of NPs on heating and viability, this study also explored the impact of cell properties, including cellular immortalization, cell type and cell radii on cell sensitivity to RF energy. Radiowaves were chosen based on higher tissue penetrance than infrared energy and the frequency of 13.56 MHz was chosen based on reports of NP heating and selective killing of cancer cells [13,14].

2. Material and methods

2.1. NP Fabrication

C₆₀-ser or 1,2:18,36:22,23:27,45:31,32:55,60-hexakis[di(2-hydroxy-1-hydroxymethyl-ethylcarbamoyl)-methano]-1,2:18,36:22,23:27,45:31,32:55,60-dihydro[60]fullerene, was synthesized according to the procedure published by Wharton and Wilson [15] with an exception being that the DBU base in step 4 was replaced with a DBU:phosphazene base P₁-*t*-Bu = 1:1 mixture to improve the overall yield of C₆₀-ser from 18% to 26%. Additional purification of C₆₀-ser was performed by dialysis of an aqueous solution using a cellulose membrane (molecular weight exclusion limit 2.0 kDa; Thermo Fisher Scientific Inc., Pittsburgh, PA, USA) up to the point where electrical conductivity of a purified C₆₀-ser became nearly equal to the conductivity of distilled water, and vacuum freeze-drying. C₆₀-serPF (C₆₀-418sm⁵-275¹ PromoFluor 633 conjugate) was synthesized according to our previously published procedure [16].

SiO₂-PF, SiO₂-C₆₀, SiO₂-C₆₀PF and SiO₂-aminoC₆₀PF were prepared by the following surface functionalization strategy using silica NPs with an average size of 200 nm and 4 nm pore size (Sigma-Aldrich, St. Louis, MO, USA). Initial surface modification was achieved using 2-[3-(triethoxysilyl)propyl]succinic anhydride, according to Russell et al. [17], as illustrated in Fig. 1. The carboxyl functionalized silica NPs (conjugate **1**) were then subjected to further functionalization to produce the following conjugates: SiO₂-PF (conjugate **2**, PF functionalized silica NPs), SiO₂-C₆₀ (conjugate **3**, C₆₀-ser functionalized silica NPs), SiO₂-C₆₀PF (conjugate **4**, PF and C₆₀-ser functionalized silica NPs), and SiO₂-aminoC₆₀PF (conjugate **5**, PF and aminoC₆₀ functionalized silica NPs). The following reagents were used to achieve the various surface functionalizations: PromoFluor-633, purchased from PromoCell GmbH, “aminoC₆₀-ser” and prepared and purified according to the procedure of Mackeyev et al. [18]), “aminoC₆₀” – described by Lee et al. [19]. Covalent functionalization was achieved using the water-soluble condensing reagent, N-(dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC·HCl, Sigma-Aldrich), in presence of catalytic amounts of 1-hydroxybenzotriazol hydrate (HOBt, Spectrum Labs), to form an amide bond between [60]-fullerene derivatives and 3-(2-succinic anhydride)propyl functionalized silica NPs, as shown in Fig. 1.

Optimal coupling conditions were achieved at room temperature in aqueous solution buffered to pH 6.5 with 10% 2-(N-morpholino)ethanesulfonic acid (MES) hemisodium salt (USB Corp.). The coated silica NPs (Fig. 1, steps 2–5) were separated from suspensions

using nylon 0.2 μm filters (Nylaflo™, Pall Corp.), further washed with distilled water and acetonitrile, and dried under vacuum.

Characterization of the resulting silica materials was performed using the following methods: 1) thermogravimetric analysis (TGA) on SDT 2960 Universal V3.4C TA thermogravimetric analyzer/ differential scanning calorimeter (TGA/DSC, analysis of weight loss, in Ar atmosphere, the 20–900 °C run at 10 °C/min heating rate); 2) fourier transform infrared spectroscopy (FTIR) on Nexus 670 Thermo-Nicolet spectrometer in the range of 500–4000 cm^{-1} with a Golden Gate diamond crystal attenuated total reflectance (ATR) device, with each spectrum being the result of 128 scans, with a resolution of 2 cm^{-1} ; and 3) fluorescence excited with a wavelength of 395 nm and observed at 660 nm on SPEX Fluorolog-2 spectrofluorometer (Jobin-Yvon Horiba) using quartz fluorometric cells (Starna, Inc., 1 cm optical path length).

2.2. Evaluation of particle surface potential and conductivity

The surface potential and conductivity of each particle formulation were determined using a Malvern Zetasizer (Worcestershire, UK). Particles were suspended in distilled water and each sample was measured 3–5 times, with data presented as averages and standard deviations.

2.3. NP internalization

Human HeLa cervical and murine 4T1 breast adenocarcinoma cancer cells were chosen for study to examine NP association and RF-field responses from two diverse cancer cell lines, both with respect to tissue and species of origin. Both cells lines were purchased from American Type Culture Company (ATCC; Manassas, VA, USA). HeLa and 4T1 cells were cultured in DMEM and RPMI, respectively. Primary human umbilical vein endothelial cells (HUVEC) and human dermal fibroblasts were cultured in EGM-2 and fibroblast basal medium, respectively, both supplemented with cytokine and serum kits. Immortalized [human microvascular endothelial cells (HMVEC) and mouse L929, were cultured in EGM and Minimum Essential Media, respectively, supplemented with cytokine and serum kits. Cell lines were either purchased from Lonza (HUVEC; Walkersville, MD, USA), ATCC (dermal fibroblasts and L929), or were a gift from Rong Shao at the University of Massachusetts. For cell internalization studies, cells were seeded into 12 well plates at 1×10^5 cells/well and 24 h later incubated with 10 $\mu\text{g}/\text{ml}$ $\text{SiO}_2\text{-C}_{60}\text{PF}$ or $\text{SiO}_2\text{-PF}$ NPs for an additional 2 or 24 h at 37 °C. Cell association was measured on trypsinized cells based on fluorescence using a Becton Dickinson LSR flow cytometer.

2.4. RF-induced heating of NP solutions

NP suspensions at 1–10 mg/ml were made using 18.2 MOhm distilled water. Phosphate buffered saline (PBS) was made in house using 10 mM phosphate buffer and 150 mM sodium chloride (NaCl). A quartz cuvette was placed in a custom Teflon holder and 1.3 ml of solution was added. Using an electronically driven device, the cuvette was placed at 5/16ths of an inch from the RF transmission head in the central region of the field with a 10 cm air gap between the transmitting and receiving heads. Samples were exposed to a high voltage RF field at 13.56 MHz at 600–900 W power using a Thermed LLC RF device (Erie,

PA, USA). Temperatures were recorded using an FLIR SC6000 infrared camera (FLIR Systems, Boston, MA) for a minimum of 120 s. The FLIR camera has a thermal sensitivity, or noise equivalent temperature difference, of 20 mK at 30 °C and range of –20 °C to 350 °C.

2.5. Cellular hyperthermia

Cells were maintained at 37 °C with 5% CO₂. 4T1 and HeLa cells were maintained in RPMI 1640 or Eagle's Minimum Essential Medium, respectively, containing 10% fetal bovine serum and 100 I.U./ml Penicillin and 100 µg/ml streptomycin (ATCC, Manassas, VA, USA). Cells were seeded into adjacent end wells of a 12-well plate at 1×10^5 cells/well with 2 ml of media per well. For all studies, the cell culture plate was positioned between the transmitting and receiving heads on a Teflon holder 3.5 cm (or as indicated) from the transmitting head and cells were exposed to RF energy at 13.56 MHz for 1 to 9 min at 900 W. This dose duration range was selected to cover lethal and non-lethal doses for the various cell types. To account for the influence of other factors on resonance conditions in the circuit, all data is presented as relative changes with respect to controls that differ by one variable. Since media conductivity has a large impact on heating rate, all cells were housed in the same media during RF treatment. Temperature was monitored using the FLIR infrared camera. Viability was monitored using a BD Biosciences LSR flow cytometer (San Jose, CA, USA) to measure uptake of propidium iodide and surface binding of annexin V 24 h after RF exposure by both detached and adherent cells. .

Alternatively, cell culture plates were suspended in a water bath at the indicated temperatures for 30 min. For cell imaging based on heat shock promoter 70 (HSP70)-driven expression of green fluorescent protein (GFP), 4T1 cells were transduced with HSP70-GFP lentivirus (Kerafast, Inc., Boston, MA, USA) using puromycin for selection.

2.6. Electron microscopy of NPs and cells

4T1 and HeLa cells were grown on silicon chips (Ted Pella, Redding, CA) in 24 well plates at 5×10^4 cells/well. Adherent cells were incubated with SiO₂-C₆₀ NPs for 2 h, and then washed in PBS to eliminate free NPs. The cell were then fixed in 2.5% glutaraldehyde, dehydrated in a series of ethanol solutions from 30–100% in distilled water, infiltrated with *t*-butanol, and dried in a desiccator. Cells were sputter-coated with 5 nm platinum-palladium prior to imaging with a Nova NanoSEM (FEI, Hillsboro, OR). Silica NPs were suspended in ethanol and dried on aluminum stubs, while flash frozen fullerene derivatives were coated onto 300 mesh TEM grids. Images were acquired using a Hitachi SU8230 scanning electron microscope (Hitachi High Technologies, Hillsboro, OR) equipped with secondary and scanning transmission electron detectors and the Bruker QUANTAX FlatQUAD detector (Bruker, Billerica, MA).

For transmission electron microscopy, cells were trypsinized and fixed in Trump's fixative (BBC Biochemical, Dallas, TX, USA). Post fixation with 1% buffered osmium tetroxide was followed by dehydration in ethanol and embedding in resin. Ultrathin sections were stained with 2% aqueous uranyl acetate and lead citrate and images acquired using an FEI Tecnai Spirit TEM (Hillsboro, OR, USA).

2.7. Confocal imaging of internalized NPs

4T1 and HeLa cells were seeded into 4-well chamber slides at a density of 7×10^4 cells per well. The next day, 10 $\mu\text{g/ml}$ NPs were added to the cell culture media for 24 h, and then cells were washed with PBS, followed by fixation with 4% paraformaldehyde in PBS for 20 min, rinsed twice with PBS, and permeabilized with 0.1% Triton-X in PBS for 10 min. Samples were then washed with PBS, incubated in 1% bovine serum albumin (BSA) in PBS for 20 min, and incubated in with fluorescein phalloidin (Thermo Fisher Scientific, Waltham, MA, USA) at one unit per slide in blocking buffer for 30 min. After rinsing with PBS, slides were mounted using Prolong Gold Antifade with DAPI (Life Technologies) as described by the manufacturer. Images were taken using a Nikon A1 confocal at $60 \times$ magnification.

To visualize intracellular C₆₀-serPF, HeLa cells were plated on No. 1.5 cover slips in 12 well plates at a density of 1×10^5 cells/well. After 18 h, cells were incubated with 10 $\mu\text{g/ml}$ C₆₀-serPF for two hours, followed by Molecular Probes® Celltracker™ Green CMFDA (5—Chloromethylfluorescein Diacetate; Life Technologies, Grand Island, NY, USA) for 30 min. Cells were washed in PBS, fixed with 3.7% paraformaldehyde, and mounted onto glass slides with ProLong® Gold Antifade Reagent containing 4',6-diamidino-2-phe nylindole (DAPI) (Life Technologies). Celltracker™ is a fluorescent probes that diffuses through the cell membrane and becomes a cell-impermeant reaction product after esterase cleavage. Superresolution imaging of cells was performed using Nikon's Structured Illumination Microscopy (N-SIM) technology. Images were acquired using the Nikon Ti inverted microscope equipped with the CFI Apo TIRF 100 $\times$ oil objective lens (N.A. 1.49).

2.8. Statistical methods

Data sets were compared using the excel T.Test (Student's t-Test) function assuming a two-tailed distribution with equal sample variance.

3. Results

3.1. Fabrication and characterization of NPs

For this study, we created a water soluble, traceable malonodi serinolamide-derivatized C₆₀ by covalently attaching C₆₀-ser to the fluorophore PromoFluor-633 (PF), with excitation/emission above 600 nm, to produce C₆₀-serPF particles, which are only few nm in size (MW circa 2.8 kDa) [20]. To create larger NPs with similar surface chemistry, C₆₀-ser and C₆₀-serPF were conjugated to the surface of 200 nm SiO₂ NPs (Fig. 1). Initial carboxy labeling of SiO₂ NPs was found to be 0.1 ± 0.012 mmol/g by performing TGA in Ar atmosphere (single-step decomposition taking place between 200 and 310 °C, Fig. S1b). SiO₂ NPs were either used as controls, or further functionalized to produce the following conjugates (illustrated in Fig. 1): SiO₂-PF, **2** (PF labeling at 0.002 mmol/g, Fig. S1c); SiO₂-C₆₀, **3** (C₆₀ at 0.03 ± 0.01 mmol/g or containing $7.0 \pm 1.5\%$ of C₆₀-ser w/w, Fig. S1d); SiO₂-C₆₀PF (**4**) and SiO₂-aminoC₆₀ (**5**) (PF and aminoC₆₀ at 0.002 and 0.03 mmol/g, respectively, Fig. S1e and f). TGA analyses of conjugates **2–5** show different decomposition pathways, with propyl-succinic acid moiety decomposition taking place within the 200–310 °C range, as it was mentioned above. Organic functional groups on the [60]fullerene core are responsible

for the weight loss at temperatures between 340 to 550 °C so two- or multi-step decompositions are observed, specific for each group type. Representative characterization data for the silica NPs is provided in Supplemental Figs. 1 and 2.

FTIR spectra of conjugates **1–5** (Fig. S2b-f) show several different features attributed to silica [21], including two main peaks characteristic of Si—O—Si bonds vibrational modes observed around 1070 and 800 cm^{-1} (weak band, due to Si—O—Si symmetric stretching, TO_2 mode). The highest frequency mode around 1070 cm^{-1} , which dominates in all spectra, is attributed to an asymmetric stretching (TO_3 mode). The TO_3 band is accompanied by an intense shoulder at the high frequency side. A band of medium intensity in Fig. S2a centered near 958 cm^{-1} is attributed to Si—OH stretching vibrations; it disappears in conjugates **2–5** due to following surface functionalization:

The band at 1717 cm^{-1} in spectrum of conjugate **1** is due to carboxylic acid carbonyl (hydrogen bonded) asymmetric stretching, also visible in spectra Fig. S2c-f. A very broad elevation in spectra of conjugates **3** and **4** (Figs. S2d and S2e) in the region between 3100 and 3600 cm^{-1} indicates the presence of exchangeable protons from alcohol groups of serinol and water molecules associated with them. The similar feature in spectrum of conjugate **5** indicates the presence of exchangeable protons from multiple amino groups.

It has been reported that C_{60} -ser forms highly dynamic aggregates, sized between 100–400 nm in diameter, which exist in equilibrium with free, hydrated C_{60} -ser molecules [22]. Scanning transmission and secondary electron microscopy imaging of C_{60} -ser showed the presence of aggregates of variable sizes built up of a single layer of C_{60} -ser molecules, with water locked inside a hollow core structure (Fig. 2a). The average radius (R_h) of individual C_{60} -ser molecules in Fig. 2a is 1.65 ± 0.19 nm.

Mixed low voltage secondary and backscatter electron images of SiO_2 and SiO_2 - C_{60} -ser NPs were acquired at 0.7 kV at 150–200 k magnification. Fig. 2a shows similar surface topography for SiO_2 and SiO_2 - C_{60} -ser NPs, including patches with parallel ridges. Electron dispersive X-ray spectroscopy (EDS) hypermaps of SiO_2 - C_{60} -ser particles, acquired at 3 kV and displayed in Fig. 2b, show silicon, carbon, or mixed element X-ray signals across the entire particle. Similar patterns of silicon and carbon were obtained for SiO_2 and SiO_2 - C_{60} -ser NPs, with EDS not able to detect differences in carbon content on the particle surfaces due to the presence of atmospheric carbon.

Surface charge and conductivity of NPs in water were measured using a Malvern Zetasizer. Surface charge was similar for SiO_2 , conjugates **1–4**, with mean values ranging from -34 to

–30 mV, respectively (Fig. 2c). SiO₂-aminoC₆₀PF (conjugate **5**) had a surface potential of 9.5 mV, while the neutral C₆₀-ser had a surface potential of –6.4 mV. Conductivity measurements, repeated in triplicate, showed that all NPs had statistically similar conductivities with mean values ranging from 0.005–0.011 mS/cm.

3.2. Cell association and internalization of NPs

Association with the cell surface, internalization and intracellular location influence the biological impact of NPs. Super-resolution imaging of Celltracker™ Green-labeled HeLa cells following incubation with C₆₀-serPF using N-SIM technology enabled sufficient resolution to detect subnuclear localization of C₆₀-serPF. Three-dimensional z-stacks (Fig. 3a and b) confirmed early localization of C₆₀-serPF within nucleoli. Internalized C₆₀-serPF (colored purple; ex/em: 640.5/700 nm) appears as punctate aggregates in the 100–500 nm range. Enhanced resolution based on grid pattern residing enabled extraction of data supporting colocalization of intranuclear C₆₀-serPF with Celltracker™ end products, the latter appearing as punctate green regions. The existence of C₆₀-ser aggregates within the nucleus of cells supports dynamic interactions among NPs, with larger aggregates undergoing assembly and disassembly under physiological conditions.

Fig. 3c shows abundant internalization of SiO₂-C₆₀PF NPs by primary dermal fibroblasts in fluorescent micrographs. Cells were stained for actin cytoskeletal components using fluorescein phalloidin and DAPI for nuclei. In the top row, two focal planes are presented to emphasize the perinuclear localization of the NPs. The middle image has been converted to grayscale and inverted so NPs for quantitation of internalized NPs within the focal plane. Using the Image J ITCN plug-in, the two cells contained 130 and 80 NPs, all localized in the perinuclear region of the cells 18 h after addition of NPs to the culture media.

Electron (Fig. 3d) and fluorescent (Fig. 3e) micrographs supported surface association and internalization of SiO₂-C₆₀PF NPs by murine 4T1 breast and human HeLa cervical carcinoma cells after 2 or 24 h at 37 °C, respectively. Scanning electron micrographs were pseudo-colored to emphasize the location and the nature of cellular associations with the NPs. The fluorescent images in Fig. 3e are projection images of z-stacks cropped to highlight the intracellular location of the NPs.

Surface ligands have the potential to increase or decrease NP association with cells. To compare uptake of SiO₂, with and without surface C₆₀, SiO₂ and SiO₂-C₆₀ NPs were surface labeled with PF and flow cytometry was used to compare labeling efficiency. Monomeric NPs, displayed in red in the histograms in Fig. 3f, had median intensities of 1154 and 990 for SiO₂ and SiO₂-C₆₀ NPs, and comprised 87% and 78% of the NP populations, respectively. When comparing NP uptake by 4T1 and HeLa cells at 24 h (Fig. 3g), both cell types displayed 2-fold greater fluorescence after incubation with SiO₂-C₆₀PF than their SiO₂-PF counterparts, indicating that cells either favored association with SiO₂-C₆₀PF over SiO₂-PF NPs, or that potential differences is particle presentation, such as aggregation influenced uptake. HeLa cells were consistently associated with more NPs than 4T1 cells, which may reflect the ability of larger cells to internalize more NPs. Similar trends in NP uptake were observed after 2 h incubation of cells with NPs (data not shown).

3.3. NP heating in an RF field

To study the ability of C₆₀-ser, free or conjugated to SiO₂, to accelerate the heating rate of aqueous solutions in an RF field, water containing 10 mg/ml SiO₂, SiO₂-C₆₀-ser or mass equivalents of C₆₀-ser, were suspended in a quartz cuvette and exposed to RF radiation operating at 13.56 MHz (Fig. 4a). The cylindrical quartz cuvette was chosen to minimize heating of the sample holder and a fixed sample height and location within the circuit were chosen to avoid or minimize differences in resonance conditions. Heating rates were calculated based on the initial 5–30 s of RF exposure using the FLIR SC6000 infrared camera to monitor temperature changes. The heating rate of PBS, a highly conductive buffer solution, increased from 0.20 to 0.37 °C/s with increasing power (600–900 W) with a linear fit that had a correlation coefficient of 0.92 (Fig. 4b). Water containing 0.08 mg/ml C₆₀-ser displayed a heating rate of 0.043 °C/s at 600 W, while 10 mg/ml SiO₂ or SiO₂-C₆₀, the latter including 0.08 mg/ml C₆₀-ser, displayed similar heating rates to that of PBS (0.16, 0.18, and 0.17, respectively; Fig. 4c). The heating rate of 2 ml of water containing SiO₂-C₆₀ NPs in a plastic cell culture plate was concentration (1–10 mg/ml) dependent, with a plateau at 5 mg/ml (Fig. 4d).

3.4. Influence of NPs on cell death in an RF field

For exposure of cells to the RF field, cell culture plates containing adherent cells in the three end wells were positioned on a Teflon stage in a consistent location within the circuit. Temperatures were monitored in the three wells using the FLIR infrared camera. SiO₂-aminoC₆₀ NPs were added to adherent HeLa cells at 0, 4, or 20 µg/ml for 24 h. NPs at the indicated concentrations did not alter the heating rate of media containing cells (Fig. 5a). Consistent with the known cytotoxicity of cationic NPs, SiO₂-aminoC₆₀ NPs caused dose-dependent cell death in HeLa cells after 24 h exposure based on flow cytometric analysis of propidium iodide uptake (Fig. 5b). Combined cationic NP and RF treatment (4–7 min) increased cell death above that caused by either agent independently, as measured 24 h after RF exposure (Fig. 5c). The combined treatment resulted in 24–42% higher cell death than that caused by RF alone, with the greatest disparity seen with 5 min RF irradiation. Cellular association with anionic or neutral NPs did not alter the RF heating rate of the housing media or the rate of HeLa cell death (data not shown).

3.5. Thermal and non-thermal RF effects on cell viability

To evaluate the contribution of thermal and non-thermal effects of RF to cancer cell death (in the presence and absence of internalized anionic NPs), 4T1 and HeLa cells were seeded into 12 well plates and incubated with and without SiO₂, C₆₀-ser, or SiO₂-C₆₀ at 10 µg/ml for 24 h. Cells were then either keep as NP-treated controls or exposed to heat using either a water bath (at 41 °C for 30 min; thermometer) or RF radiation at 900 W (exposure stopped when a temperature of 41 °C was reached based on infrared thermometry). While water bath-induced hyperthermia at 41 °C alone did not increase cell death, RF treatment to a high temperature of 41 °C, increased cell death in both control and NP-treated cells, despite the shorter duration exposure to the maximum temperature (Fig. 5d). Based on propidium iodide and annexin V staining of cells 24 h after RF exposure, 4T1 breast adenocarcinoma cells

died from a combination of necrosis and apoptosis, while HeLa cervical carcinoma cell death was predominately due to apoptosis.

To determine the thermal threshold for cell death, 4T1 cells, transformed with green fluorescent protein under the control of the heat shock protein 70 promoter (HSP70-GFP), were heated for 30 min using a water bath at 41, 43, 45 or 50 °C. As evident in fluorescent micrographs based on cell rounding and aggregation, temperatures of 45 °C or greater were sufficient to kill adherent 4T1 cells (Fig. 5e).

3.6. Impact of RF system set-up on heating rates

Based on differences in heating rates calculated using the quartz cuvette and plastic cell culture plate, we investigated whether the presence of plastic from the cell culture plate impacted temperatures recorded using the FLIR IR camera. Plastic located between the thermal camera and the targeted cell culture media was reduced by removing the outer rim of the plate as indicated in Fig. 6a. Intact and modified plastic plates containing media in the three end wells were then exposed to RF radiation for 5 min at 900 W (thermal images displayed in Fig. 6b). Both recorded temperatures and the derived heating rates were higher when non-essential (outer rim) plastic was removed from the plate (Fig. 6c and d), indicating that plastic interferes with thermal detection using the infrared camera. In addition, the distance of the cell culture plate from the transmitting head also impacted the heating rate, with 4 cm causing a significant decrease ($p < 0.005$) in the heating rate compared to 3.5 cm (with the plastic removed), and with increases in distance from 3 to 3.5 to 4 cm all causing a significant decreases in the heating rate in the presence of the plastic ($p < 0.005$). Overall, IR-based thermography of intact cell culture plates was 4-13 °C less than that determined using plates in which excess plastic was removed, with the disparity increasing with increasing RF dose duration (0–340 s).

Based on the finding that plastic from the cell culture plate attenuates IR heat detection, the thermal dose for RF-treated cells presented in Fig. 5d was at a minimum 4 °C higher than that recorded with the FLIR IR camera using an intact plate. Therefore, the RF thermal dose was at or above 45 °C (IR recorded temperature of 41 °C plus 4 °C or more). Based on our demonstration that temperatures of 45 °C or higher are sufficient to kill 4T1 cancer cells (Fig. 5e), the thermal contribution from RF exposure presented in Fig. 5d was sufficient to account for cell death.

3.7. Impact of cell immortalization on heating and RF-induced cell death

Primary and immortalized fibroblasts and endothelial cells differ in their sensitivity to the cytotoxic effects of cationic NPs, with primary cells being more sensitive [23]. To study differences in responses to RF hyperthermia, cells were treated for 3, 4, 5, or 6 min at 900 W. Average temperatures for primary dermal fibroblasts and immortalized L929 cells are presented in Fig. 7a, at 30 s intervals. The mean temperatures at each time point are presented in the final graph, showing similarities in temperatures for media in wells containing primary and immortalized fibroblasts.

RF-induced heating of media containing primary HUVEC and immortalized HMVEC endothelial cells were also measured over time, resulting in similar heating rates for

immortalized and primary endothelial cells in culture (Fig. 7b). Both immortalized and primary cells displayed a strong linear correlation between RF exposure time and temperature ($R^2 = 0.96$ and 0.98). The relationship between cell death and maximum temperature, shown in Fig. 7b (middle graph), was linear for HMVEC with a correlation of $R^2 = 0.92$. In the presented data, primary HUVEC are more resistant to cell death than HMVEC at lower temperatures, with overlapping cell death trends for HUVEC and HMVEC at temperatures above 45°C . The increased resistance to RF for HUVEC at low temperatures did not hold true in replicate experiments, with both cell types displaying overlapping linear relationships between cell death and duration of RF exposure (Fig. 7b, right graph).

Percent live cells as a function of RF duration are presented in Fig. 7c for all four cell types. Primary and immortalized endothelial cells show similar viability 24 h after RF exposure, with both cell types having extremely low viability (HUVEC 9% and HMVEC 14%) when treated for 8 min or greater with RF radiation. In contrast, L929 immortalized fibroblasts were unable to survive exposure to just 6 min RF radiation (95% cell death), despite the ability of primary fibroblasts to show no significant change in viability compared to no treatment controls at this same dose (21 and 15% cell death, respectively). Since similar relationships between cell immortalization and cell sensitivity to RF radiation did not exist for both fibroblasts and endothelial cells, the process of cell immortalization itself did not dictate sensitivity to RF radiation.

3.8. Impact of cell size on RF sensitivity

To examine the relationship between cell size and RF sensitivity, fibroblast, endothelial and cancer cells were imaged using transmission electron microscopy (TEM). Approximate cell diameters are shown for each cell type in Fig. 7d and are summarized in Table 1. Primary and immortalized endothelial cells are similar in size, while primary dermal fibroblasts are very large cells and immortalized L929 cells, which were most sensitive to RF radiation, are relatively small cells.

To further explore the impact of cell radii on RF-sensitivity, cell size and RF-induced cell death were compared using immortalized HMVEC, 4T1 and HeLa cells (Fig. 8). Flow cytometric forward scatter and scanning electron micrographs supported the following relative cell sizes: HMVEC > HeLa > 4T1 cells. Cells with the smallest radii (4T1 cells) were extremely sensitive to RF radiation, with 82% of cells dying at the lowest RF dose duration of 5 min. HeLa cells, with a larger cell radii than 4T1 cells, were more resistant to RF radiation, with only 20% cell death at the same dose. HMVEC cells were the most resistant of the three cell lines to RF energy, with cells requiring an RF dose of 7 min before exhibiting a significant loss in viability (63% cell death). Thus cell size, or radii, was negatively correlated with sensitivity to RF energy.

4. Discussion

While C_{60} itself is a water-insoluble carbon-based NP, derivatization of the [60]fullerene enables biological interactions and dictates subcellular localization, inclusive of lysosomes [24], mitochondria [25,26] and endoplasmic reticulum [27]. The serinol-functionalized

[60]fullerene derivatives (C_{60} -ser) are unique in that they are able to enter the nucleus through the nuclear pore complex [16]. Herein we demonstrated that C_{60} -ser NPs associate to form hollow spheres. We confirmed that C_{60} is able to localize within the nucleus and further demonstrated association with nucleoli.

NP attributes, such as surface functionalization, opsonization, charge, and size have all been shown to impact cellular associations and NP polarization in electric fields [28,29]. The ability to surface functionalize 200 nm silica NPs with C_{60} derivatives enabled us to study the impact of NP surface chemistry (SiO_2 vs SiO_2 - C_{60} -ser) and size (C_{60} -ser vs SiO_2 - C_{60} -ser) on cellular uptake, localization, RF heating and cellular sensitivity to the RF field. Cellular uptake of SiO_2 NPs was high in fibroblasts (>130 NPs/cell) and HeLa cells, with 1.9-fold lower uptake in 4T1 cells. Internalization was enhanced 2-fold for HeLa cells and 2.2-fold for 4T1 cells by the presence of C_{60} on the surface of SiO_2 NPs. In all cells studied, SiO_2 NPs were predominately localized in the perinuclear region of the cell, supporting localization within endosomes/ lysosomes. While C_{60} was also seen clustered in the perinuclear region of the cell (not shown), it was uniquely also seen in the nucleus. Cell lines used in this study and the relative robustness of NP uptake are summarized in Table 1.

Charged NPs caused a dose-dependent increase in the heating rate of water in an RF field operating at 13.56 MHz at concentrations ranging from 1–10 mg/ml (0.004–0.028 °C/s). The highest heating rate attained with water containing NPs was equivalent to that of PBS and thus as expected, NPs, ranging from 4–20 μ g/ml in cells and biologically-relevant, highly conductive solutions were unable to alter the RF induced heating rates. While anionic or neutral NPs did not increase cell death, either independently or in association with RF radiation, cytotoxic cationic NPs increased RF-induced cell death in an additive, dose-dependent fashion.

Sassaroli et al. [30] reported that induced electric dipoles and the double ion layer surrounding particles can increase energy absorption, however, in biological systems, the amount of absorption is small compared to that of the medium in the MHz range, resulting in no detectible enhancement of temperature change. They further reported that intracellular heating due to motion of NPs in the presence of an applied electric field is blunted due to confinement of particles within endosomes and lysosomes within cells. In this study, thermal doses from RF were sufficient to account for cell death compared to water bath related hyperthermia.

While immortalized L929 fibroblasts were more sensitive to RF radiation (95% cell death compared to 21% following a 6 min dose) than primary dermal fibroblasts, primary and immortalized endothelial cells displayed similar sensitivities at RF doses ranging from 5 to 9 min, negating a consistent link between immortalization and cell sensitivity to RF radiation. Sensitivity of cells to RF radiation did, however, mirror cell size (7–30 μ m), with the smaller cells being more susceptible to cell death (ranging from 21 to 97%) as summarized in Table 1. Since cell radius is inversely related to both the specific capacitance and conductance of the cell membrane [31], increased overall sensitivity of small radii cells to RF radiation and higher rates of necrotic cell death, as seen in 4T1 cells, may reflect greater membrane polarization per unit area and loss of membrane integrity.

While we demonstrated that cell radii is an important factor influencing cell sensitivity to RF radiation, total membrane content also needs to be taken into consideration. Shim et al. [32] plotted mean total membrane capacitance (C_{tot}) against the mean radius of 16 cell types and demonstrated that C_{tot} is proportional to $R^{3.01}$ of the cells. They reported that capacitance per unit area of cell plasma membrane (C_{mem}) varies between cell types and even within the same cell type depending on its state of differentiation, stage of cell cycle, and following exposure to toxins [33]. Factors such as membrane ruffling or folding impacted C_{mem} by expanding membrane volume above that of a smooth sphere (i.e. cell) of equivalent radius. [33]. Differentiation agents were found to cause changes in cell surface morphology that were accompanied by changes in specific capacitance of the plasma membrane [31]. Thus while cell size is correlated with sensitivity to RF radiation, extrinsic factors and cellular changes that alter cell attributes have the potential to impact cellular sensitivities as well. Unfortunately, cancer is a disease involving uncontrolled proliferation of heterogeneous cells, with each subtype having unique characteristics that have the potential to impact their response to electric energy.

5. Conclusions

Based on the high degree of heterogeneity in cancer cells present in solid tumors, it is unlikely that cell traits alone can be used for selective RF heating of malignant cells. The architecture of the tumor, with regions of hypovascularity, necrosis, and high cellularity or stromal content are more likely to influence localized heating. Factors such as distance to lesions and damage to normal tissue must also be taken into consideration when contemplating clinical translation. Advantages gained by the presence of NPs in enhancing RF-mediated cell death were predominately due to cationic NP cytotoxicity rather than accelerated heating of biological solutions. Future studies may benefit by exploring alternative frequencies or mechanisms to selectively alter conductivity and thereby RF heating rates and cell death.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.actbio.2017.01.086>.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors are grateful for technical support from Muto Atushi at Hitachi High Technologies; Joel Sederstrom and Brandon Saxton in the Baylor College of Medicine (BCM) Flow Cytometry Shared Resource Facility; Norman Lapin, Sarah Suki, and Jared Newton at BCM; and Jim Barrish in the Texas Children's Hospital Electron Microscopy Facility. Funding was provided by the National Institute of Health Physical Sciences in Oncology Center Grant U54CA143837; the Kanzius Cancer Research Foundation, Erie, PA, USA; and NeoTherma Oncology, Wichita, KS, USA. The sponsors did not participate in study design, data collection, analysis and interpretation, or writing of the report.

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Statement of Significance

The ability of nanoparticles to either direct heating or increase susceptibility of cancer cells to radiofrequency (RF) energy remains controversial, as is the impact of cell attributes on susceptibility of cells to RF-induced cell death. This manuscript examines the impact of nanoparticle charge, size, and cellular localization on RF-induced cell death and the influence of nanoparticles on the heating rates of water and biologically-relevant media. Susceptibility of immortalized or primary cells to RF energy and the impact of cell size are also examined. The ability to selectively modulate RF heating rates in specific biological locations or in specific cell populations would enhance the therapeutic potential of RF therapy.

Fig. 1.

Synthesis of NPs. Initial surface functionalization showing the synthesis of the carboxyl functionalized silica NPs (1) is illustrated at the top of the figure, followed by a hierarchical tree showing precursors for the synthesis of SiO₂-PF (2), SiO₂-C₆₀ (3), SiO₂-C₆₀PF (4), and SiO₂-aminoC₆₀ (5). Reagents included: (i) 1-(5-aminopentyl)-2-[(1*E*)-3-[(4*E*)-2-*tert*-butyl-7-[ethyl(3-sulfopropyl)amino]-4 H-chromen-4-ylidene]prop-1-en-1-yl]-3,3-dimethyl-3 H-indol-1-ium-5-sulfonate (PromoFluor-633, amino-modified label); (ii) aminoC₆₀-ser (3'-ethoxycarbonyl-3'-((2-aminiumethoxy)carbonyl)-3'', 3'', 3'', 3'', 3'', 3'', 3'', 3'', 3'', 3''-deca(2-hydroxy-1-(hydroxymethyl)ethylcaramoyl)-3'H, 3'H, 3'H, 3'H, 3'H, 3'H, 3'H, 3'H-hexacyclopropa [1,9 : 16,17 : 21,40 : 30,31 : 44,45 : 52,60] (C₆₀-I_h)[5,6]fullerene hydrochloride; (iii) aminoC₆₀-ser; and (iv) aminoC₆₀ (3',3',3',3',3',3',3',3',3',3'-dodeca(2-aminiummethyloxycarbonyl)-3'H,3'H,3'H,3'H,3'H,3'H,3'H,3'H-hexacyclopropa [1,9:16,17:21,40:30,31:44,45:52,60] (C₆₀-I_h)[5,6]fullerene dodecahydrochloride).

Fig. 2.

Physico-chemical characterization of NPs. Scanning and scanning transmission electron micrographs of C₆₀-ser, SiO₂ or SiO₂-C₆₀ NPs acquired using the Hitachi SU-8230 cold field emission SEM. a) Images of flash-frozen solutions of C₆₀-ser were acquired using bright field scanning transmission electron microscopy (upper left) and by detection of secondary electrons using the upper detector at 100,000 (upper right), 500,000 (lower left), and 1,000,000 (lower right) magnification at 30 kV. b) SiO₂ and SiO₂-C₆₀ mixed secondary and back scatter electron images acquired at 0.7 kV. c) Hyperspectral images of SiO₂-C₆₀ NPs acquired at 3 kV using the Bruker QUANTAX Flat energy dispersive X-ray spectroscopy (EDS) microanalysis system. d) Zeta potential and conductivity measurements for NPs.

Fig. 3.

Cellular internalization of nanoparticles by primary human dermal fibroblasts and cancer cells. A,B) N-SIM imaging of a HeLa cell incubated with C₆₀-serPF (purple) for 2 h. Cells were stained with DAPI (nucleus; blue) and Celltracker™ Green CMFDA prior to imaging. a) Z-stack projection image with crosshair. b) 3D renditions of the Z-stack. c) Primary dermal fibroblasts incubated with 10 µg/ml 200 nm SiO₂-C₆₀PF nanoparticles for 18 h, then stained with fluorescein phalloidin and DAPI. The top two images show two focal planes of the same field of view, emphasizing intracellular localization of the nanoparticles. A higher magnification micrograph of two fibroblasts is shown in the central images as fluorescent and grayscale images. Color inversion of the gray scale image enabled quantitation of nanoparticles within single focal planes using image J and the ITCN program with settings at 10,5, and 0.5 (bottom row). f,g) Pseudo-colored secondary electron (d) and confocal (e) micrographs showing SiO₂-C₆₀PF NP internalization by 4T1 murine breast and HeLa human cervical carcinoma cells after a 2 h (surface binding, d) or 24 h incubation (internalized, e). f,g) Flow cytometry analysis of SiO₂-C₆₀PF and SiO₂-PF internalization by 4T1 and HeLa cells after 2 h at 37 °C. f) Histograms display large particle clusters in blue and green, while the main particle cluster is shown in red. g) Mean fluorescent intensity of nanoparticles in HeLa and 4T1 cells after 24 h at 37 °C.

Fig. 4.

Nanoparticle heating in a radiofrequency (RF) field. a) Photograph showing a quartz cuvette, filled with 1.3 mL PBS, located in a Teflon sample holder between the RF transmitting (Tx) and receiving (Rx) heads. b) Heating rates of PBS in a quartz cuvette exposed to a 13.56 MHz field at 600, 700, 800 and 900 W. Heating rates were measured using a FLIR infrared camera and are expressed as temperature (°C) divided by time (seconds, s). c) RF heating rates of PBS or water doped with 10 mg/ml purified C₆₀-ser, SiO₂, or SiO₂-C₆₀ in a quartz cuvette (*p < 0.01). d) Heating rates of SiO₂-C₆₀ NPs in water at 1–10 mg/ml in a 12 well cell culture plate (*p < 0.01).

Fig. 5.

Impact of nanoparticles, radiofrequency (RF) radiation, or heat on cancer cell viability. a) Heating rate of media (2 ml per well, 12 well plate) containing cells after 24 h incubation with 0 (control), 4 or 20 $\mu\text{g/ml}$ SiO₂-aminoC₆₀PF nanoparticles. b) Percent cell death (PI uptake) is shown for HeLa cells incubated for 24 h with 0, 10, 50 or 100 $\mu\text{g/ml}$ SiO₂-aminoC₆₀PF nanoparticles (* $p < 0.01$). c) Cell death of HeLa cells shown as a function of nanoparticle dose and RF duration. d) 4T1 and HeLa cells were seeded in 12 well plates with and without SiO₂, C₆₀, or SiO₂-C₆₀ at 10 $\mu\text{g/ml}$ for 24 h. Cells were then either keep as nanoparticle-treated controls or treated with heat in water bath at 41 °C for 30 min, or with RF at 900 W up to a maximum temperature of 41 °C as determined using an infrared camera. Cell death is show as a function of necrosis (propidium iodide positive, orange) and early/late apoptosis (annexin V positive, gray). e) Fluorescent micrographs of HSP70-GFP-4T1 cells 24 h after 30 min water bath heat treatment at 41,43, 45 and 50 °C.

Fig. 6.

The impact of plastic and sample proximity to the transmission head on radiofrequency (RF) heating rates for cell culture media. a) RPMI media (2 ml per well) was placed in intact 12 well plates or plates in which the plastic edge was removed. Plates were then exposed to RF energy for 5 min at 900 W. b) Infrared images of the intact (top) and trimmed (bottom) plates. c) Heating rates of media in control and trimmed plates at variable plate distances from the transmitting head (* $p < 0.005$). d) Temperature of media in intact or trimmed plates during exposure to RF radiation at 13.56 MHz.

Fig. 7.

Impact of cell immortalization and size on heating and sensitivity of cells to radiofrequency (RF) radiation. a) Temperature of media as a function of RF dose duration in wells containing adherent immortalized (left) or primary (middle) fibroblasts. Each line represents a unique cell culture with the indicated total dose duration. Average heating rates at each RF dose for L929 and primary dermal fibroblasts are displayed in the graph to the right. b) Final maximum temperature for each well containing primary HUVEC or immortalized HMVEC endothelial cells at the indicated RF dose (left). Cell death as a function of RF-induced maximum temperature (middle) or as a function of RF dose duration (right). c) Cell viability (exclusion of propidium iodide uptake) of immortalized (red) and primary (blue) endothelial cells and fibroblasts (blue immortalized; red primary) 24 h following variable doses of RF exposure. d) Transmission electron micrographs of endothelial cells and fibroblasts with approximate cell diameters indicated.

Fig. 8.

Impact of cell size on sensitivity of cancer cells to radiofrequency (RF) radiation. Cell size is shown graphically based on flow cytometric forward scatter mean and median measurements. Representative SEM images support the relative sizes of each cell line (scale bars are 5, 4 and 5 μ m left to right). The bottom graph shows live (blue), necrotic (purple), early (red) or late (green) apoptotic HMVEC, HeLa, or 4T1 cells 24 h following variable doses of RF exposure. Error bars are standard deviations on 3 measurements, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Overview of cell lines and their biological responses to nanoparticles and radiofrequency irradiation. The summary includes results previously published in the Journal of Biomedical Nanotechnology by *McConnell et al. [1]; ND – no data

Table 1

Cell line	Species	Immortality	Cell type	Size EM μm (FSC)	RF Sensitivity (%Dead @6 min)	*Cationic NP Sensitivity	*NP Uptake
Dermal Fibroblast	Human	Primary	Fibroblast	30	21	78	+++
HUVEC	Human	Primary	Endothelial	15	28	80	++
HMVEC	Human	Immortalized	Endothelial	15 (86)	42	20	++
L929	Human	Tumorigenic	Fibroblast	10	97	47	+
Hel.a	Human	Cervical cancer	Epithelial	12 (69)	77	ND	+++
4T1	Mouse	Breast cancer	Epithelial	7 (58)	92	ND	+