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# 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 ($C_{60}$-ser) conjugated to the surface of mesoporous silica nanoparticles ($SiO_2$-$C_{60}$). 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 ($SiO_2$ and $SiO_2$-$C_{60}$) or neutral ($C_{60}$) 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.

**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.



## 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

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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 $C_{60}$-ser, $SiO_2$, $SiO_2$-$C_{60}$, and cationic, β-ethanolamine fullerene-functionalized $SiO_2$ NPs ($SiO_2$-amino$C_{60}$).

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

$C_{60}$-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 $P_1$-*t*-Bu = 1:1 mixture to improve the overall yield of $C_{60}$-ser from 18% to 26%. Additional purification of $C_{60}$-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 $C_{60}$-ser became nearly equal to the conductivity of distilled water, and vacuum freeze-drying. $C_{60}$-serPF ($C_{60}$_418sm$^5$_275$^1$ PromoFluor 633 conjugate) was synthesized according to our previously published procedure [16].

$SiO_2$-PF, $SiO_2$-$C_{60}$, $SiO_2$-$C_{60}$PF and $SiO_2$-amino$C_{60}$PF 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 (conjugage **1**) were then subjected to further functionalization to produce the following conjugates: $SiO_2$-PF (conjugate **2**, **PF** functionalized silica NPs), $SiO_2$-$C_{60}$ (conjugate **3**, $C_{60}$-ser functionalized silica NPs), $SiO_2$-$C_{60}$PF (conjugate **4**, PF and $C_{60}$-ser functionalized silica NPs), and $SiO_2$-amino$C_{60}$PF (conjugate **5**, PF and amino$C_{60}$ functionalized silica NPs). The following reagents were used to achieve the various surface functionalizations: PromoFluor-633, purchased from PromoCell GmbH, "amino$C_{60}$-ser" and prepared and purified according to the procedure of Mackeyev et al. [18]), "amino$C_{60}$" – 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