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Luciferase-based protein-denaturation assay for quantification of radiofrequency field-induced targeted hyperthermia: developing an intracellular thermometer

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Abstract

Background—Several studies have reported targeted hyperthermia at the cellular level using remote activation of nanoparticles by radiofrequency waves. To date, methods to quantify intracellular thermal dose have not been reported. In this report we study the relationship between radio wave exposure and luciferase denaturation with and without intracellular nanoparticles. The findings are used to devise a strategy to quantify targeted thermal dose in a primary human liver cancer cell line.

Methods—Water-bath or non-invasive external RF generator (600W, 13.56 MHz) was used for hyperthermia exposures. Luciferase activity was measured using a bioluminescence assay and viability was assessed using Annexin V-FITC and Propidium iodide staining. Heat shock proteins were analyzed using western-blot analysis

Results—Duration-dependent luciferase denaturation was observed in SNU449 cells exposed to RF field that preceded measurable loss in viability. Loss of luciferase activity was higher in cetuximab-conjugated gold nanoparticle (C225-AuNP) treated cells. Using a standard curve from water-bath experiments, the intracellular thermal dose was calculated. Cells treated with C225-AuNP accumulated 6.07 times higher intracellular thermal dose than the untreated controls over initial 4 minutes of RF exposure.

Conclusions—Cancer cells when exposed to an external RF field exhibit dose-dependent protein denaturation. Luciferase denaturation assay can be used to quantify thermal dose delivered after RF exposures to cancer cells with and without nanoparticles.

Keywords

Radiofrequency; Hyperthermia; Intracellular; Luciferase; Gold nanoparticles

Background

Hyperthermia has been used in the treatment of cancers for hundreds of years(1). However, the translation of this modality to the clinic is an ongoing challenge as improvements are made in clinical hyperthermia delivery and thermography systems. Recently, there is

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Declaration of Interest

The authors report no declaration of interest

renewed interest in devising strategies to deliver hyperthermia with the advent of nanotechnology. The rationale for such therapies is based on the observation that metallic, semi-conducting, or magnetic nanoparticles can be physically tuned to absorb electromagnetic energy from a remote source outside the body and dissipate it as heat within the tissue bearing the nanoparticles (2–4). The use of radiofrequency (RF) waves to heat naked and antibody-targeted gold nanoparticles (AuNPs) and carbon nanotubes has been reported *in vitro* and *in vivo* (2, 5, 6). Thermal dosimetry in these experiments was based on bulk media temperatures. It is important to note (as will be discussed in this study) that localized heating of nanoparticles within the intracellular environment can cause protein denaturation and cell death without appreciable changes in the bulk media temperature above normal(4). Strategies to quantify intracellular temperature are therefore needed to understand temperature-dependent biological effects of non-ionizing electromagnetic radiation delivered with or without nanoparticles.

To date, several fluorescence-based methods to measure intracellular temperature have been reported (7–9). For example, a temperature-dependent transient change in fluorescence intensity of various fluorophores has been used to develop hydrogel-based nanoprobe that can monitor real-time intracellular temperature. These methods require microinjection of nanoprobe into the cell and/or a microscope mountable hyperthermia delivery system that is not readily available, therefore, limiting its general utility.

The aim of this study was to develop an alternative strategy that uses temperature-dependent protein denaturation to quantify intra-cellular heat delivered after radio wave exposure. To that end, a hepatocellular carcinoma cell line, SNU449, was stably transfected to express firefly luciferase and its denaturation was studied. Luciferase catalyzes a reaction where light is produced by converting the chemical energy of luciferin oxidation through an electron transition, forming oxyluciferin. If all the reactants for the reaction are provided in saturating concentrations, the light intensity is directly proportional to and dependent on the active luciferase in the cell lysate at room temperature(10). Transfected cells that constitutively express firefly luciferase, when exposed to heat, show loss of function of luciferase due to denaturation (10–13). In water-bath experiments, this loss of function was dependent on the incubation temperature and the duration of incubation (11, 14). Given these findings, we hypothesize that RF field exposure will cause intracellular temperature-dependent luciferase inactivation that can be quantified using a commercially available bioluminescent assay. We further validate this system by quantifying intracellular thermal dose of antibody-targeted gold nanoparticles upon remote activation by a non-invasive RF field.

Methods

Generation of firefly luciferase expressing cell line

Recombinant human lentivirus expressing green fluorescent protein together with firefly luciferase under the control of a CMV promoter (pCMV-GFP/Luc plasmid) was acquired from (Providential Biotech LLC, Chamblee, GA). The pCMV-GFP/Luc vector was transfected into NIH293T cells to generate GFP/Luc-expressing lentivirus. This was then used to infect SNU449 cells. GFP/Luc-transduced stable SNU449 cells were obtained by sorting GFP-positive cells for green fluorescence with a FACScan (BD biosciences, Boston, MA).

AuNP conjugation and characterization

AuNP (10 nm) were purchased and used as is (Ted Pella, Inc., Redding, CA). C225 (Bristol-Myers Squibb, New York, NY) was conjugated via a covalent linker SPT-0012 (Sensopath

Technologies, Inc., Bozeman, MT) from a previously published protocol with slight modifications based on glycosylation of the Fc region (15). Briefly, a solution of 10 nm AuNPs (50 µg/ml) were twice washed in a borate buffer solution at pH ~ 8. C225-linker was slowly added to the modified AuNP colloid at w:w ratio of 3:5 respectively. The solution was placed on a continuous mixer and incubated at room temperature for 4 hours. Next, the conjugate was centrifuged at 13,000 rpm for 40 minutes. Supernatant containing excess C225-linker was removed and the C225-AuNP pellet was resuspended in PBS. A small shift (<10 nm) in the peak plasmonic absorbance of the AuNPs (NS1, Applied NanoFluorescence, Houston, TX) was indicative of a non-aggregated conjugation state.

AuNP internalization

C225-AuNP internalization was assessed using transmission electron microscopy imaging (TEM) and inductively coupled plasma mass spectroscopy (ICP-MS). For TEM analysis SNU449 cells (5×10^4) were plated in 12-well plates. For ICP-MS analysis SNU449 cells (1×10^6) were plated in a T75-flask. The cells were allowed to become adherent over a 24 hour period. The media was exchanged and C225-AuNP conjugate was added at a final concentration of 100 mcg/ml for 1, 4 or 24 hours at 37°C. The media was removed and cells were washed with PBS x2. Subsequently, cells were harvested after trypsinization, washed and centrifuged.

For TEM analysis cell pellets were fixed with a 3% glutaraldehyde/2% paraformaldehyde solution in 0.1 M cacodylate buffer at pH ~ 7.4. Samples were washed with 0.1% cacodylate buffered tannic acid, treated with 1% buffered osmium tetroxide, and stained with 1% uranyl acetate. The samples were ethanol dehydrated and embedded in LX-112 medium. After polymerization, the samples were cut with a Leica Ultracut microtome (Leica, Deerfield, IL), double stained with uranyl acetate/lead citrate in a Leica EM stainer, and imaged with a JEM 1010 TEM (JEOL, USA, Inc., Peabody, MA) at an accelerating voltage of 80 kV. Images were acquired with AMT Imaging System (Advanced Microscopy Techniques Corp., Danvers, MA). For ICP-MS analysis, cells were counted and cell pellets were dissolved in aqua regia over 24 hours and Au concentration measured against known standards.

RF heating experiments

Non-invasive radiofrequency-based hyperthermia was delivered using a 600W power, 13.56 MHz RF-field generator (Therm Med LLC, Erie, Pennsylvania). The setup is described elsewhere (16). Briefly, the RF generator is connected to a high Q coupling system with a Tx head (focused end-fired antenna circuit) and reciprocal Rx head. The distance between the two heads was adjusted to 10 cm.

For *in vitro* experiments, 10^5 SNU449 cells were plated in 3 adjacent wells of a 12-well plate. The plates were positioned 1.5 inches from the Tx head on a Teflon holder in the RF field such that there was a uniform RF field across the three wells. Bulk media temperature was measured continuously by an infrared camera (FLIR SC 6000, FLIR Systems, Inc., Boston, MA).

Bioluminescence and Viability measurements

Bioluminescence measurements were performed using a luciferase assay kit (Promega, Madison, WI). SNU449 cells were plated and treated in 12-well plates as described above for TEM experiments. After incubation for a variable duration at 37°C with or without C225-AuNP, cells were washed with PBS twice and media was replaced (1 ml/well). Cyclohexamide (10 µg/ml) was added 10 min prior to RF or water-bath exposure in order to block translation of newly transcribed luciferase mRNA. The cells were then exposed to the

RF field or water-bath for a varying duration. Immediately after exposure, cells were placed on ice and lysed using lysis buffer as per manufacturer's recommendation. The lysates were briefly centrifuged at 13000 rpm for 15 seconds to separate insoluble cellular debris. The supernatant was collected and luciferase activity was measured.

Viability was measured with flow cytometry (LSRII, BD Biosciences, Franklin, NJ) 24 hours after RF or water-bath exposures. Cyclohexamide was not added for viability studies prior to RF exposure. Briefly, cell media (i.e., dying cells that were floating) was collected and the adherent cells were collected after trypsinization. Each sample was washed and stained with Annexin-V-FITC and PI without fixation or permeabilization. Annexin V is a protein that binds to phosphatidylserine, which is externalized in apoptotic cells. Propidium iodide (PI) fluoresces when it is bound to DNA in membrane-damaged cells. Cells that were negative for both markers were characterized as viable.

Immunoblotting

Heat shock protein expression (Hsp 27, 70 and 90) was determined by Western blot analysis. Protein extracts acquired from adherent cells (as described above) were electrophoresed on Bis-Tris protein gel, transferred to a PVDF membrane, and sequentially incubated in 5% dry milk and mouse monoclonal primary antibodies anti-Hsp70 (3A3, SantaCruz Biotechnology), or anti-Hsp90 (4F10, SantaCruz Biotechnology), or anti-Hsp27 (G3.1, Neomarkers). Next, the membranes were washed and incubated with HRP-conjugated goat anti-mouse secondary antibody (Jackson Laboratories). Images were acquired by a high-resolution photo scanner (CanoScan 4400F, Canon, Inc., Lake Success, NY) after the bands were detected with a peroxide solution (GenDEPOT, Inc., Barker, TX).

Thermal dose calculations

Thermal dose defined as cumulative equivalent minutes at 43°C (CEM43) was calculated from time-temperature plots as detailed elsewhere (17, 18) using the relationship: $CEM43 = tR^{43-T}$, where t = duration of hyperthermia exposure (minutes), $R = 0.25$ for temperature $< 43^{\circ}C$ and $R = 0.5$ for temperature $\geq 43^{\circ}C$, and T is the temperature of incubation ($^{\circ}C$). For exposures with varying temperature over a period of time, the thermal dose was calculated for 1-minute increments and total dose is reported as the cumulative sum.

Results and Discussion

Radiofrequency absorption by SNU449 monolayer exceeds that of bulk media

In order to evaluate the relationship between cell death and duration of RF field exposure, SNU449 cells expressing luciferase were plated in 12-well plates. After 24 hours, cells formed sub-confluent adherent monolayers at which point they were exposed to the RF field (13.56 MHz, 600W) for a varying duration. The RF field causes ionic heating in the bulk media. In order to separate the bulk heating effects from RF-induced intracellular hyperthermia, all RF exposures were started with cells at 30°C. Cell viability assessed 24 hours after RF exposure by Annexin-V-FITC/PI assay demonstrates that the LD₅₀ for RF exposure was 12.13±0.6 min with a corresponding bulk media temperature of 38.7°C (i.e. CEM43=0.004 min) as shown in figure 1A. A 20-minute RF exposure resulted in complete loss of viability with bulk media temperatures reaching a maximum of 39.8°C (CEM43=0.09 min). Next, we investigated if the maximum bulk media temperature attained during RF exposure could explain the loss of viability in SNU449 monolayers.

The effects of heating of bulk media on cell viability were evaluated using a series of water-bath experiments and a similar protocol as described above. SNU449 monolayers in 12-well plates were exposed to varying temperatures and cell viability was assessed 24 hours after

heat exposure (Figure 1B). Media temperature in a well without cells, submerged in a water-bath was measured with an alcohol thermometer as an internal control. Again, temperature-dependent loss of viability was observed but this required significantly higher temperature for a much longer duration (LD_{50} at 49.7°C for 30 min, $CEM43=3117$ min) as compared to radiofrequency field-exposed cells (LD_{50} at 38.7°C for 12.13 min, $CEM43=0.004$ min). We noted that incubation at 40°C for 30 minutes ($CEM43=0.47$ min) did not decrease cell viability compared to control cells at 37°C . These findings suggest that heating of bulk media temperature during RF exposure cannot completely account for the loss in viability of SNU449 monolayers and that the cell monolayers accumulate heat at a faster rate than the bulk ionic media.

Quantification of intracellular thermal dose after RF exposure

A ubiquitous phenomenon and earliest change induced by hyperthermia on a biological system is protein denaturation(1). In order to quantify the thermal dose delivered within SNU449 monolayers we monitored levels of luciferase activity after varying the duration of RF exposure. Immediately after RF exposure, cells were lysed on ice and luciferase activity was measured. The results are reported as percent of untreated controls (after subtracting background). The data demonstrates that luciferase undergoes RF exposure duration-dependent denaturation (Figure 1C). The IC_{50} for luciferase activity was 4.7 ± 0.6 min at a bulk media temperature of 34.8°C . Control cells kept at 37°C in a water bath for 10 min, however, did not have loss in luciferase activity suggesting that inactivation of luciferase by RF exposure in this experiment is unrelated to changes in bulk media temperature.

Since heat distribution in a water-bath system is by convection, bulk media temperatures should therefore correspond to intracellular temperatures. We sought to investigate what would be the equivalent water-bath thermal dose that would produce the same level of loss in luciferase activity after an RF exposure. To that end, SNU449 cells were exposed to varying temperature for a varying duration in a water-bath, and luciferase activity immediately recorded following exposure (Figure 1D). Luciferase activity was then plotted against log CEM and was found to be inversely correlated ($R^2=0.81$) as shown in figure 1E.

Using this standard curve, an equivalent thermal dose ($CEM43$) that would correspond to the level of luciferase denaturation after RF exposure was calculated. As shown in figure 1F, intracellular thermal dose ($CEM43$) after 4 minutes of RF exposure was 0.22 ± 0.08 min. The bulk temperature did not exceed 37°C during this time ($CEM43 = 0$ min) and therefore could not have delivered the thermal dose calculated within the SNU449 cells.

Intracellular gold nanoparticles enhance luciferase denaturation upon RF exposure

Radio wave absorption and heat dissipation by gold nanoparticles (AuNPs) inside cancer cells has been reported in prior studies(2, 19). We evaluated if cells with internalized AuNPs accumulate higher thermal dose in comparison with cells without AuNPs. To that end, RF experiments were conducted using the protocol described above. Epidermal growth factor receptor (EGFR)-expressing SNU449 cells were treated with antibody (anti-EGFR/C225)-conjugated gold nanoparticles for 4 hours prior to RF exposure. AuNP internalization was confirmed by TEM images as shown in Figure 3. Luciferase denaturation was measured after varying the duration of RF exposure. As shown in Figure 2A, luciferase denaturation was higher in cells treated with C225-AuNPs as compared to untreated cells (no AuNPs).

Luciferase denaturation measurements over the dynamic range (0–4 min) of the dose response curve from figure 2A were used to calculate the intracellular thermal dose of C225-AuNP treated and untreated cells. The data (Figure 2B) demonstrates that over the initial 4 minutes of RF exposure, C225-AuNP treated cells accumulated thermal dose 6.07 times that

of untreated controls ($p < 0.05$). The contribution of bulk media heating to intracellular thermal dose was negligible. There was no difference in the bulk media temperature of C225-AuNP treated and untreated cells demonstrating that intra-cellular hyperthermia is not enough to cause measurable changes in bulk temperature.

To determine if there is a correlation between uptake of gold nanoparticles and thermal dose accumulation within cancer cells, we measured the amount of gold nanoparticles internalized after a varying duration of incubation with C225-AuNP in SNU449 cells. Figure 4A demonstrates that the amount of AuNPs per cell increase with the duration of incubation. Next, we determined if there is a correlation between increasing intracellular accumulation of C225-AuNPs with time and subsequent thermal dose enhancement after RF exposure. We treated SNU449 cells for 1, 4 or 24 hours with C225-AuNPs and after replacing media exposed the cells to RF for a duration of 3.6 min (IC_{75} of luciferase activity in untreated cells, Figure 1C). It was noted that the loss of luciferase activity and calculated intracellular thermal dose corresponds with the duration of incubation up to 4 hours (Figure 4B and 4C). There was no difference in the luciferase activity of cells incubated with C225-AuNP for 4 hours or 24 hours even though ICP data demonstrates a higher amount of C225-AuNPs in cells incubated for 24 hours compared to 4 hours. We take these findings to represent progressive acidification of endolysosomal vesicles carrying C225-AuNPs leading to their aggregation and inactivation, thereby limiting the pool of C225-AuNPs that can be activated beyond 4 hours of incubation (unpublished data).

Next, we sought to investigate if thermal dose enhancement observed in these experiments has any significant biological effects. Incubation of SNU449 cells with C225-AuNPs for 24 hours followed by RF exposure up to 5 minutes (to match the experiments above) did not demonstrate significant changes in viability (data not shown). We reason that at this exposure even C225-AuNP enhanced thermal dose is below the threshold ($CEM_{43} > 678.82$ min from figure 1B) to observe significant changes in viability.

The findings of this study suggest that a luciferase denaturation assay can serve as a valuable tool to monitor intracellular hyperthermia delivered through electromagnetic radiation. Moreover, heating of nanoparticles after remote electromagnetic activation can also be studied. There are several parameters that need to be controlled during the assay to allow reliable thermal dosimetry. Transfected cells that constitutively express firefly luciferase, when exposed to heat, show immediate inactivation (loss of function) of luciferase (10–14). A proportion of luciferase undergoes terminal degradation and cannot be recovered. However, a portion may undergo heat-shock protein-mediated renaturation (10–12). It is plausible that differences in the amount of heat shock proteins in different experimental conditions could confound our measurements. Although induction of heat-shock protein expression after heat shock takes several hours, we studied that possibility. Heat-shock protein levels were measured for cells heated in a water-bath for 30 minutes at varying temperatures or for cells treated with or without C225-AuNPs before and immediately after RF in the cell lysates (Figure 4D). No significant differences were observed in the relative amounts of heat shock proteins at the time luciferase activity measurements were performed for the thermal exposures utilized in this study ($CEM_{43} < 100$). In addition to heat-shock protein expression, renaturation of luciferase is dependent on the incubation time and temperature of cells after heat-shock (10, 12). Friedland et al. reported less than 5% recovery in bacterial luciferase activity 20 min after heat shock when the samples were kept at 3°C (10). Similarly, we observed negligible recovery of luciferase activity up to one hour after RF exposure when the samples were placed on ice (data not shown).

There are several limitations for intracellular measurement of thermal dose using this method. The measurements are not real-time and are carried out in retrospect. Therefore

only cumulative effects can be studied for varying RF, or other thermal exposures. Since different cell lines differ in their level of heat shock protein expression, a standard calibration curve for each cell line being tested must be calculated. The measurement of luciferase activity is facilitated by the availability of commercially available kits in which reactants are optimized for maximum light generation for a given quantity of functional luciferase. Since the relationship between loss of luciferase activity and CEM43 is exponential, the accuracy of this method is high at low thermal exposures and decreases with increasing CEM43. The assay is not suitable to quantify thermal dose (CEM43) in excess of 100 min. Finally, there is a possibility that the observed protein denaturation could be due to non-thermal effects of RF exposure on luciferase. However, this is unlikely, as several studies have failed to show non-thermal effects of RF field on protein denaturation(20, 21). It was also reported that any change in protein conformation and loss of function even from high power RF sources could be explained by local temperature changes in the medium surrounding the proteins(22). From these studies together with our findings we conclude that the changes in luciferase activity observed in this study most likely represent the changes in intracellular temperature.

Despite the limitations stated above, this is the first method that can be used to quantify intracellular thermal dose after RF field exposure in cancer cells. It can therefore serve as a valuable platform to assess heating of remotely activated nanoparticles in an RF field. With further validation this method may be adapted to other forms of electromagnetic radiation, such as alternating magnetic field, when fluorescence-based methods are not readily available. Ultimately, the findings also hold promise for employing luciferase-based protein denaturation assay for *in vivo* thermal dosimetry. Implanted xenograft tumor models in mice expressing luciferase and *in vivo* bioluminescence imaging systems are readily available. Reliable thermal dosimetry *in vivo* will depend on accurate calibration of the standard curve, delivery of substrate to the tissue at a saturating concentration and standardization of measurement conditions. Bioluminescence-based thermometry unlike fluorescence-based methods is expected to have a very high signal-to-noise ratio because of minimal signal attenuation and lack of intrinsic bioluminescence. Indeed, *in vivo* bioluminescence imaging using an enhanced firefly luciferase has been performed for fewer than 10 cells (23) suggesting that even small tumors or micro metastases may be amenable to temperature measurements using this method.

Conclusion

Cancer cells, when exposed to an external RF field, exhibit dose-dependent protein denaturation and cytotoxicity due to intracellular hyperthermia. Bulk media temperature measurements can underestimate intracellular thermal dose. We have demonstrated that luciferase denaturation can be used to quantify the early effect of intracellular heat delivered after RF exposures. The method was also successfully used to assess enhanced heating of remotely activated C225-targeted AuNPs in cancer cells.

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Figure 1. Quantification of thermal dose in cells treated with RF without C225-AuNPs

A. Loss of cell viability in SNU449 cells exposed to an RF fields (13.56 MHz, 600W) for a varying duration with the corresponding bulk media temperature. ($n=3$, symbols represent mean $\pm$ SEM).

B. Loss of cell viability in cells exposed to water-bath at varying temperatures for 30 minutes with corresponding calculated thermal dose. ($n=3$, symbols represent mean $\pm$ SEM)

C. Loss of luciferase activity in SNU449 cells with increasing duration of RF exposure. ($n=3$, symbols represent mean $\pm$ SEM)

D. Time and temperature-dependent loss of luciferase activity after thermal exposure of SNU449 cells in a water-bath. ($n=3$, symbols represent mean $\pm$ SEM)

E. Thermal dose (CEM43) was calculated from data shown in figure 1D and plotted against measured luciferase activity to generate a standard curve.

F. Using the standard curve (Figure 1E), intracellular thermal dose, CEM43 (T_i) was calculated for data shown in figure 1C. Extracellular thermal dose (T_e) due to bulk media

temperature was calculated from measured bulk temperatures and was found to be negligible. ($n=3$, symbols represent mean $\pm$ 95% CI)

Figure 2. Quantification of thermal dose in cells treated with C225-AuNPs

A. Preincubation of SNU449 cells with C225-AuNPs (100 $\mu\text{g/ml}$) for 4 hours followed by RF exposure, enhances thermal denaturation of luciferase.

B. Intracellular thermal dose (T_i) was quantified using the standard curve in Figure 1 E. Extracellular thermal dose (T_e) due to bulk media temperature was calculated from measured bulk temperatures and was found to be negligible. ($n=3$, symbols represent mean $\pm$ 95% CI).

(All p-values are from unpaired two-tailed t-test.)

Figure 3. Gold nanoparticle internalization by SNU449 cells

Transmission electron microscopy images demonstrating uptake of C225-AuNPs (100 μg/ml) by SNU449 cells after 4 hours of treatment.

Figure 4. Uptake of C225-AuNPs and their biological effects

A. Intracellular amount of Au in each SNU449 cell as determined by ICP-MS increases with increasing duration of exposure to C225-AuNPs (100 μ g/ml). ($n=3$, bars represent mean $\pm$ SEM)

B. Loss of luciferase activity in SNU449 cells incubated with or without C225-AuNPs (100 μ g/ml) for varying duration followed by exposure to RF field for 3.6 min. ($n=3$, bars represent mean $\pm$ SEM)

C. Relationship of duration of SNU449 cells incubation with C225-AuNPs (100 μ g/ml) and intracellular thermal dose (same data as figure 4B after intracellular thermal dose calculation using the standard curve in Figure 1E). ($n=3$, symbols represent mean $\pm$ 95% CI)

D. Heat shock proteins were analyzed using western-blot immediately after thermal exposure in a water-bath (1) or radiofrequency field (2). (All p -values are from unpaired two-tailed t -test)