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Stability of antibody-conjugated gold nanoparticles in the endo-lysosomal nanoenvironment: Implications for non-invasive radiofrequency-based cancer therapy

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Abstract

The use of non-invasive radiofrequency (RF) electric fields as an energy source for thermal activation of nanoparticles within cancer cells could be a valuable addition to the emerging field of nano-mediated cancer therapies. Based on investigations of cell death through hyperthermia, and offering the ability for total body penetration by RF fields, this technique is thought to compliment and possibly out-perform existing nano-heat-treatments that utilize alternative heat production via optical or magnetic stimuli. However, it remains a challenge to understand fully the complex RF-nanoparticle-intracellular interactions before full system optimization can be engineered. Herein we have shown that liver cancer cells can selectively internalize antibody-conjugated gold nanoparticles (AuNPs) through receptor-mediated endocytosis, with the nanoparticles predominantly accumulating and aggregating within cytoplasmic endo-lysosomes. After exposure to an external RF field, non-aggregated AuNPs absorbed and dissipated energy as heat causing thermal damage to the targeted cancer cells. We also observed that RF absorption and heat dissipation is dependent on solubility of AuNPs in the colloid, which is pH dependent. Furthermore, by modulating endo-lysosomal pH it is possible to prevent intracellular AuNP aggregation and *enhance* thermal cytotoxicity in hepatocellular cancer cells.

Keywords

pH; radiofrequency; gold nanoparticles; lysosomotropics; hyperthermia; cancer

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SUPPORTING INFORMATION

Additional results including heating of citrate-capped AuNPs, epidermal growth factor receptor expression on liver cancer cell lines, intracellular localization and stability of AuNPs, internalization kinetics of C225-AuNPs with or without preincubation with lysosomotropic agents and pH dependent fluorescence spectra of C225-AuNP-FITC are presented in supporting information.

BACKGROUND

Spherical gold nanoparticles (AuNPs) have many potential biological applications, such as diagnostic imaging agents^{1–4}, drug or gene delivery vectors^{5–8}, and thermal actuators for cancer therapy^{9, 10}. AuNPs are prototypical nanomaterials that have excellent biocompatibility and offer ease of conjugation to various biological molecules of interest. Peptides, aptamers, antibodies, and their fragments can provide the molecular recognition necessary to target some types of cancer cells where differentiation between malignant and normal tissue is important. The most widely used scheme for targeted AuNP delivery to cancer cells involves conjugating antibodies to AuNPs, which are then selectively internalized by cancer cells that express the cell surface target for that antibody¹¹.

The remote thermal activation of intra-cellular AuNPs by non-invasive, non-ionizing radiation forms the basis of delivering targeted hyperthermia to cancer cells. 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. An example of such a system employs near-infrared (NIR) laser (808nm) irradiation which heats untargeted gold nanoshells that predominantly accumulate in tumor tissue on the basis of the enhanced permeation and retention (EPR) effect¹⁰. The heating is based on the known optical plasmon resonance of gold in the near infrared region. This therapy is currently in phase 1 clinical trials for the treatment of refractory and/or recurrent head and neck cancer. While highly effective for superficial tumors, NIR energy is not suited to target deeper cancers because of its limited penetration depth through human tissue (<3–5 cm)¹². An alternative approach that heats magnetic nanoparticles, such as dextran-coated iron oxide, in an inductively coupled magnetic field has been reported^{13, 14}. However, the high concentrations of iron oxide needed for adequate heat therapy can only be achieved by direct intra-tumoral injection which limits its practical use^{15, 16}.

Non-invasive radiofrequency field (13.56MHz) induced heating of AuNPs offers several advantages over existing nano-strategies. RF energy has low tissue specific absorption rates (SAR) and therefore has excellent whole body tissue penetration with documented safety in humans^{17, 18}. Furthermore, it has been previously shown that selective hyperthermic cytotoxicity can be achieved *in vitro* and *in vivo* after systemic delivery of directionally-conjugated AuNPs targeted to pancreatic cancer xenografts without harming normal tissues in an animal model⁹. However, there are several challenges in optimizing non-invasive RF-based heating of AuNPs before their utility in cancer therapy can be exploited.

We have observed that aggregation of AuNPs in a colloid abrogates nanoparticle heating in a non-biological system, as is discussed below. It has also been shown that antibody-conjugated AuNPs targeted to cell surface receptors are predominantly internalized by energy-dependent receptor-mediated endocytosis^{19, 20}. These studies have shown that, upon internalization, these nanoparticles form intracellular aggregates and fall out of colloidal suspension within the endo-lysosomal vesicles. A precise understanding of interaction of surface modified AuNPs with the endo-lysosomal nano-environment is therefore necessary. Two major factors that can influence colloidal stability within endosomes include antibody degradation by proteolytic enzymes and progressive acidification of internalized cargo by vacuolar specific proton-ATPase pumps²¹. Recently, Se'e *et al.* investigated the fate of peptide layers on the AuNP surfaces targeted to HeLa cervical cancer cells²². They found that non-specific L-cathepsin protease within the endosomes is responsible for degradation of the peptide layer on the AuNP surface. Another report has implicated B-cathepsin protease degradation of cross-linked iron oxide nanoparticles²³. Cathepsin proteases are pH

dependent enzymes that have optimum activity at pH 3.5–5²⁴. However, in these experiments, the direct effect of pH on the stability of gold nanoconjugates was not investigated.

We hypothesize that endo-lysosomal pH is a key determinant of stability and solubility of antibody-conjugated AuNPs in the intracellular nano-environment. We further hypothesize that progressive acidification of the internalized cargo, in itself, can cause aggregation of AuNPs in the endo-lysosomal compartment. Finally, we demonstrate that RF field-induced hyperthermic cytotoxicity in cancer cells can be significantly enhanced by strategies that modulate the endo-lysosomal pH.

METHODS

AuNP conjugation and characterization

Bioconjugation of antibody to AuNPs is of fundamental importance in designing high specificity targeting of AuNP to malignant cells. It is known that antibodies will adsorb on the gold surface by ionic and hydrophobic interactions when pH is maintained around their isoelectric point²⁵. This method has several drawbacks that include a requirement for high concentrations of antibody for conjugation, random orientation of the antibody molecule on the AuNP surface with subsequent loss of affinity for the target due to conformational change in antibody structure, and replacement of antibody by other non-specific biological molecules *in vivo*. In our studies we have used an alternative method previously described by Kumar *et al.*, which takes advantage of the predominant glycosylation of the Fc portion of the antibody¹¹. Glycosylated residues on the Fc portion of an anti-epidermal growth factor receptor (EGFR) antibody (C225) were conjugated to a heterofunctional alkane linker using an amide linkage. The other end of the linker has a monothiol tether, which was then used to attach the antibody-linker complex to AuNPs, using Au-S interactions (Supplemental Information). Specifically, AuNPs (10nm) 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 region¹¹. Briefly, a solution of 10 nm AuNPs (50µg/ml) was 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 DI-H₂O. A small shift (<10nm) in the peak plasmonic absorbance of the AuNPs (NS1, Applied NanoFluorescence, Houston, TX) was indicative of a non-aggregated conjugation state after challenge with equivolume 10% sodium chloride. Dynamic light scattering (DLS, Horiba, Ltd., Irvine, CA) determined the average hydrodynamic diameter of the constructs (500 measurements per sample in triplicate). Protein concentrations were determined using Bio-rad colorimetric assay based on the Bradford method. Briefly, Bio-rad reagent was added to the protein solutions in 96-well plates. Absorbance was measured at 595nm using a microplate reader as per manufacturer's instructions (Bio-rad Laboratories, Hercules, CA). Concentrations were calculated from a standard curve of known protein concentrations.

Cell lines

All cell lines (Panc-1, Hep3B and SNU 449) were purchased from American type culture collection (ATCC) and maintained as per the instructions of the supplier. To generate a 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).

TEM imaging

Cell pellets were fixed with a 3% glutaraldehyde/2% paraformaldehyde solution in 0.1M 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).

RF generator and heating experiments

Colloidal solutions of C225-AuNPs were prepared as described in the text. Buffers were extracted using a 10kD Amicon Ultra-15 centrifugation filter (Millipore, Billerica, MA). Colloids or their suspension buffers (extracted) were placed in a 1.3 mL quartz cuvette placed on a custom design Teflon holder under open air conditions at ambient room temperature as described previously²⁶. The cuvette was placed 5/16th of an inch from the transmission head located at an arbitrary point on the X-Y plane (3', 4') identical to other experiments using different parameters (sample variation, power etc). The solutions were then exposed to the high voltage RF field (12.4kV.m⁻¹) at 600W generator power (13.56 MHz operating frequency, 10 cm air gap between transmission and receiver heads, ThermMed, LLC, Inc., Erie, PA), which equates to ~205 kW/m² using the standard equations for electromagnetic irradiance. This is different than the set-up described by Li *et al.* (25W, 13.56MHz, head-spacing of 30.5cm with a distance of 5 cm from the transmission head to the cuvette) resulting in an electric-field strength of 2.5 kV.m⁻¹²⁷. Temperatures were recorded every 0.1625 seconds with an infrared camera (FLIR SC 6000, FLIR Systems, Inc., Boston, MA) for a total duration of 120 seconds or until the sample reached 70 °C (to prevent electrical arcing due to excess water evaporation) Heating rates were calculated along the linear portion of the heating curve as equations for the steady-state rate of heat flow would only begin to follow an exponential curve towards the last few seconds of their 120 s exposure (See Supplementary Data).

For *in vitro* experiments, 10⁵ SNU449 cells were plated in 3 adjacent wells of a 12-well plate. The plates were positioned on a Teflon holder in the RF field such that there was a uniform RF field across the three wells. Bulk media temperature remained between 30°C and 41°C as measured by an infrared camera (FLIR SC 6000, FLIR Systems, Inc., Boston, MA). Viability was measured with flow cytometry (LSRII, BD Biosciences, Franklin, NJ) 24 hours after 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 propidium iodide (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.

Intracellular pH determination

First, calibration was performed. 10^5 SNU449 cells were incubated with FITC-C225-AuNP conjugates for 30 minutes at $200\mu\text{g/ml}$ at 0°C . This allowed binding of the conjugates to the cell surface without internalization. The unbound conjugates were removed by washing the cells with PBS. This was then followed by incubation at 37°C for 30 minutes to start the internalization process. This time was chosen because most of the conjugates are internalized by this time. An aliquot of $50\mu\text{L}$ was removed and cells were mixed with $250\mu\text{L}$ of NaN_3 and NH_4Cl at varying pH. This allowed equilibration of intracellular (unknown) and extracellular pH (known). Fluorescence ratio was then calculated using flow cytometry and plotted against pH to obtain a standard curve.

Protein denaturation assay

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. Cycloheximide ($10\mu\text{g/ml}$) was added 10 min prior to RF exposure in order to block translation of newly transcribed luciferase mRNA. The cells were then treated with RF for a varying duration. Immediately after RF 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 using a bioluminescence reader.

RESULTS

Stability of C225-AuNP nanoconjugates in an acidic environment

Directional conjugation of 10nm AuNPs to C225 via Au-S bonding was confirmed by a small shift ($<10\text{nm}$) in peak plasmonic absorption (Figure 1A). The hydrodynamic diameter of C225-AuNP was measured by dynamic light scattering and was found to be 32.6 ± 0.7 nm, which also supports successful antibody conjugation. Stability of C225-AuNP nanoconjugates in an acidic environment was investigated by incubating samples at varying pH for 4 hours. A red shift in maximal absorbance wavelength of the UV-Vis spectra in comparison to naked AuNPs ($\Delta\lambda_{\text{max}} > 10\text{nm}$) was regarded as evidence of aggregation. C225-AuNP nanoconjugates were found to aggregate below pH 5.5 (Figure 1B). This was consistent with the visible change in color of the gold colloid from deep red to purple and increased hydrodynamic diameter by DLS (data not shown). It is well known that a pH-dependent change in protein conformation can alter stability of gold nanoconjugates by altering the surface charge²⁸. We investigated if the change in stability of gold nanoconjugates can be explained in part by antibody dissociation from the surface of AuNPs. To this end, C225-AuNPs were incubated at $100\mu\text{g/ml}$ at varying pH for 4 hours. The samples were centrifuged at 13,200 RPM for 2 hours until all the gold had precipitated. The supernatant was collected and protein concentration was determined. As shown in Figure 1C, C225 dissociates from the AuNP surface as pH decreases below pH 6. Similar results were seen with 15 minutes of incubation (data not shown). From these experiments, it is evident that in a non-biological system an exposure to acidic pH can result in aggregation of gold protein nanoconjugates. In contrast, a C225-AuNP colloidal suspension was stable and non-aggregated for more than 3 days at a physiologic pH of 7.4.

Heating of C225-AuNPs in the RF field is dependent on colloidal stability

We investigated if C225-AuNP heating was affected by colloidal stability. Non-aggregated suspensions were created by use of an in-house proprietary technique that enabled us to achieve very high concentrations ($\sim 1500\text{ ug/ml}$) of non-aggregated AuNPs (Supporting

Information not for publication). The stability of these colloids was confirmed by UV-Vis and zeta-potential measurements, which depicted a minute UV-Vis peak-shift with a zeta-potential reduction to -23 mV from -31 mV. Using our high-power RF system (13.56 MHz at 600 W), we then evaluated the heating rates of the re-suspended AuNPs. These heating rates were found to be concentration-dependent, reaching a heating rate of 0.07 °C.s⁻¹ for a concentration of 1000 µg/ml (Supporting Information, data taken from²⁹), which is significantly smaller than that reported in prior studies which failed to account for an ionic-buffer heating contribution^{26, 30, 31}. These AuNP heating rates were then compared to C225-AuNP conjugates under different pH environments. To that end, C225-AuNP (100 µg/ml) samples were first incubated at pH 5.5 and pH 7.4 for 4 hours. The samples were then centrifuged in a microcentrifuge and the pellet was resuspended in DI water. This was repeated multiple times to remove excess buffers without aggregation in pH 7.4 samples (Note: pH 5.5 samples aggregate secondary to a low pH and were re-dispersed using brief sonication). The C225-AuNP colloids in DI water, stable (pre-treated with pH 7.4) and unstable (pre-treated with pH 5.5), were divided each into two halves. The heating rates of the first were determined as is. Buffers were extracted from the other half and heating rates of buffer solutions were recorded. As shown in Figure 1D, for similar concentrations of AuNPs (100 µg/ml) the addition of C225 antibody increased the heating rates of the AuNPs provided the nanoparticles were conjugated and at physiological pH. Pre-exposure to an acidic environment (pH 5.5) reduces heating due to aggregation. This data suggests that the stability of the colloid is central to the heating of conjugated and unconjugated AuNPs.

C225-AuNPs undergo intracellular aggregation secondary to endo-lysosomal acidification

In order to assess the intracellular fate of C225-AuNP, we incubated three human cancer cell lines (Panc-1, pancreatic cancer; Hep3B, hepatocellular cancer and SNU449, hepatocellular cancer), having variable EGFR expression with C225-AuNP at a concentration of 100 µg/ml. Samples were fixed after 4 hours of incubation and TEM images were obtained. In all cell lines, intracellular AuNP aggregates were observed. These aggregates were invariably localized in membrane bound endo-lysosomal compartments. No nanoparticles were identified in other organelles, the cytoplasm, or the nucleus. Occasional, non-aggregated nanoparticles were observed at the cell surface (Supplemental Information). The findings suggest that intracellular AuNP aggregation is a ubiquitous phenomenon seen across multiple cell lines after receptor-mediated endocytosis.

Based on the above experiments, we hypothesized that intracellular aggregation occurs because of progressive acidification of the endo-lysosomal compartment. The acidification of the endo-lysosomes has been well characterized and is known to be due to the intravesicular accumulation of protons by the action of the membrane-bound V-type H⁺-ATPase pump³². A pH-sensitive fluorescence-based method was used to determine pH in the nano-environment of the AuNPs. C225 was labeled with fluorescein isothiocyanate (FITC) at a ratio of six molecules of dye per molecule of antibody (determined by UV-Vis) using a FITC-antibody conjugation kit, as per manufacturer's instructions (Invitrogen, Carlsbad, CA). FITC-labeled antibody was then conjugated to AuNPs using a linker as described above. Unconjugated antibody was removed after centrifugation. It is known that AuNPs can quench fluorescence if the fluorophore is in close approximation to the gold core. Fluorescence measurements were therefore performed using a fluorospectrometer. No difference was noted in the fluorescence of AuNP bound and unbound antibody on a per mole basis (data not shown). FITC-labeled C225-AuNP conjugates were then incubated at varying pH, and the emission spectra were recorded after excitation with a blue LED diode (Supplemental Information). A pH-dependent fluorescence could be observed at 505–570 nm and pH-independent fluorescence at 600–630nm³³. The ratio of fluorescence at 505–570

nm to that at 600–630 nm could therefore be used to measure pH in the gold nano-environment as detailed below.

Intracellular pH measurements were performed using flow cytometry (Method detailed in Supplemental information). A calibration curve is shown (Figure 2A). Approximately 10^6 SNU449 cells were incubated with FITC-C225-AuNP 100 $\mu\text{g/ml}$ conjugates for 30 minutes at 0°C . This resulted in binding of the conjugate to the cell surface without internalization. The unbound conjugates were removed by centrifugation and cells were washed and resuspended in media at pH 7.4 at 37°C . Fifty microliter aliquots were removed and added to 200 μL s of ice-cold PBS (pH 7.4) or PBS (pH 2.5) to measure the total and intracellular fluorescence of the conjugate, respectively. Extracellular fluorescence can be eliminated by incubating samples at pH 2.5 either because of antibody stripping from the cell surface or fluorescence quenching by acidic pH, allowing intracellular fluorescence measurements³⁴. It was observed that within 30 minutes C225-AuNPs were in a compartment with a pH of 5.8 ± 0.4 (mean $\pm$ standard deviation) (Figure 2B). This is consistent with internalization studies on C225 that indicate localization of C225 in early endosomes with a pH of 6–6.5³⁵. There was progressive acidification of the endo-lysosomes as the gold nanoconjugates progressed from early endosomes to late endosomes (pH 5.6 ± 0.3) and finally to lysosomes (pH 5.3 ± 0.2). Intracellular pH measurements in the AuNP nano-environment below pH 5 cannot be accurately performed as we have shown that C225 dissociation increases as pH is decreased below 5.

Lysosomotropics prevent endolysosomal-aggregation of C225-AuNPs by modulating pH

In a set of three different experiments SNU449 cells were pretreated for 1 hour with concanamycin A (10nM), a highly specific V-type H^+ -ATPase blocker; or chloroquine (200 μM), a lysosomotropic agent that accumulates in late endosomes and lysosomes subsequently raising pH; or NH_4Cl , (30mM) a weak base that raises the pH of all cellular compartments. The cells were washed and then incubated with FITC-C225-AuNPs followed by pH measurements as described above. Concanamycin A completely blocked the acidification of the vesicular compartments (Figure 2B). Partial response was seen with NH_4Cl and chloroquine. The pH measurements after 120 minutes were recorded to be 7.5 ± 0.1 , 6.2 ± 0.3 and 5.9 ± 0.3 for concanamycin A, NH_4Cl , and chloroquine, respectively. These data suggest that progressive acidification around the AuNP nano-environment can be reversed by lysosomotropic agents. Pre-treatment with these agents did not alter the C225-AuNP internalization kinetics as determined by flow cytometry (Supplemental information). TEM images before and after pre-treatment with lysosomotropics demonstrate isolated nanoparticles with the absence of aggregation in the vesicular compartments suggesting intra-cellular aggregation is a pH dependent process (Figure 3). Vesicular disruption was not observed, however, a few isolated nanoparticles could be identified in the cytosol.

Lysosomotropics enhance thermal cytotoxicity of C225-AuNPs in RF field

If lysosomotropics can increase the intracellular pH and prevent intracellular AuNP aggregation, it is plausible that hyperthermic cytotoxicity can be enhanced by treatment with these agents. We incubated SNU449 cells with concanamycin A, or chloroquine, or NH_4Cl at the concentrations used in previous experiments for 4 hours, along with C225-AuNPs at 200 $\mu\text{g/ml}$. After 4 hours, media was removed and adherent cells were washed with PBS. The media was replaced and the cells were exposed to an RF field (13.56 MHz, 600W) for 9 minutes. Cell viability was assessed 24 hours after RF exposure using a FACS Annexin-V and PI assay as described elsewhere⁹. It was observed that concanamycin A and chloroquine enhanced hyperthermic cytotoxicity of C225-AuNP treated cancer cells, which supports our hypothesis (Figure 4.). A similar response was not observed for NH_4Cl . In fact, NH_4Cl diminished the hyperthermic cytotoxicity observed with C225-AuNP. This is likely because

of the non-specificity of NH_4Cl to raise the pH of all cellular compartments. An acidic cytosolic pH is required for activation of caspases, which are the key effectors of apoptotic cell death. An alkaline cytosolic milieu, therefore, affords protection to cancer cells against thermal activation of apoptotic cascades³⁶.

Since methods to measure intracellular temperature have not been developed and validated, we used intracellular protein denaturation as a surrogate to measure intracellular thermal dose. It is widely known that protein denaturation is among the first of many changes after exposure of cells to hyperthermia³⁷. A luciferase-based bioluminescence assay was established to measure protein denaturation in SNU449 cells. Cells were transfected with a plasmid expressing luciferase that localized to the cytosol. Functional firefly luciferase can convert luciferin to oxyluciferin with generation of photons. In contrast, thermal denaturation of luciferase can decrease the amount of light generated in a dose-dependent manner. Transfected SNU449 cells were, therefore, treated with or without lysosomotropic agents and were then exposed to RF field treatment (13.56MHz 600W) at a sub-lethal dose of 3 minutes. Cells were immediately lysed using a cell lysis buffer on ice. This was necessary to prevent induction of heat shock protein expression that can subsequently increase renaturation of denatured proteins. As shown in Figure 5, protein denaturation was potentiated by all three lysosomotropic agents, suggesting that the observed RF-induced cytotoxicity is preceded by thermal denaturation of proteins.

DISCUSSION

The use of radiowave-irradiated nanoparticles to produce hyperthermia within biological tissues has exciting potential implications in imaging and therapy. Several manuscripts thus far have misattributed the ionic heating of buffers to the heating of AuNPs^{9, 26, 30, 31, 38}. Recent experiments argue that there is negligible absorption of radiowaves by AuNPs²⁷. In biological systems however, cytotoxicity has been reported using multiple assays and two different RF generators^{9, 26, 30, 38}. Further, RF-mediated targeted hyperthermia has also been reported after systemic administration and targeted delivery of AuNPs in pre-clinical models⁹. Clearly, the apparent discrepancy requires further study.

Measuring the heating of radiowave-irradiated AuNPs in a colloidal system presents a challenge. We have observed that colloidal buffer heating rates can preclude and, indeed, overshadow the inherent heating properties of AuNPs themselves. These artifacts have been also observed by others using a smaller scale RF system²⁷. In their recent study, AuNP pellets were isolated from colloids by centrifugation and resuspended in water to evaluate their heating properties under a low-power (25W) RF field (13.56MHz). Although no AuNP heat production was observed, it is not clear if the lack of heating was due to low RF power, low AuNP concentration (~ 30–56 ug/ml), the *aggregated state* of the AuNPs, or simply due to the removal of ionic buffers. We therefore designed an alternative experiment to enable re-suspension of highly concentrated, *non-aggregated* AuNPs in MilliQ water, even after the removal of ionic buffers. The difference in heating rates between the re-washed AuNP-MilliQ water suspension and the background MilliQ water demonstrated the total heat contribution from the freely-suspended AuNPs themselves. Our findings suggest that citrate-capped AuNPs certainly absorb RF energy, a finding that is abrogated upon aggregation²⁹. The heating rates reported in this study are several orders of magnitude lower than prior studies^{9, 31}. Since aggregation is caused by excessive removal of citrate from the gold surface, this suggests that citrate capping is the predominant mechanism contributing to energy loss in the colloid and not the gold core itself. Similarly, we found that antibody-conjugated AuNPs also absorb RF energy, which is abrogated when the protein coating is lost due to a decrease in pH. As C225-AuNPs invariably localize to acidic vacuoles after cellular uptake, this provided us with a cell-based model to investigate the contribution of

antibody coating in RF absorption by C225-AuNPs. We found that consistent with *in vitro* data, agents that increase endolysosomal pH and therefore preserve the protein coating on the AuNP surface enhance RF-mediated cytotoxicity. In contrast, the loss of antibody coating due to acidic pH also resulted in loss of hyperthermic cytotoxicity. Of note, we were limited in our methods to measure real-time temperature changes inside the cells during RF exposure. The findings of hyperthermic cytotoxicity were confirmed, however, using a bioluminescence-based protein-denaturation assay as an indirect measure of thermal cytotoxicity.

Our findings are partly supported by the recent theoretical analyses of electromagnetic absorption in metal spheres by Hanson *et al.* that suggest that the RF absorption cross section in a coated spherical gold nanoparticle is dominated by the electric-dipole contribution of the surface coating with negligible contributions from the gold core^{39, 40}. However, the authors also note that despite the contribution of RF absorption by the surface coating, AuNPs do not enhance RF absorption above that of ionic media surrounding the AuNPs. Conversely, from the experimental data presented in this study, it seems plausible that by modulating the surface coating on the AuNPs, hyperthermic cytotoxicity can be achieved in the ionic environment of human cancer cells. Several factors, including the nature of surface coating, compartmentalization within the cell leading to high volume fractions, and aggregation of nanoparticles with preserved surface coating may account for the differences in experimental data and theoretical models. These differences in actual measured results compared to theoretical predictions require further study.

In summary, we have shown that surface coating plays a vital role in absorption of radiowaves both *in vitro* and in a cell-based biological system. These findings highlight the unique opportunities and challenges in the design of next generation RF absorbers for cancer therapy and imaging.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Effect of pH on C225-AuNP (10nm) nanoconjugates. A, B) UV-Vis spectra of C225-AuNP at different pH values. A red-shift ($\Delta\lambda_{\max} > 10\text{nm}$) is observed as the pH is decreased from 6 to 5 suggesting aggregation. C) C225 dissociation from AuNP surface increases as the pH is decreased. D) A higher heating rate difference is observed for C225-AuNP pre-incubated at pH 7.4 in comparison with pH 5.5. (*Unpaired one-sided t-test*; $p=0.039$)

Figure 2.

Endo-lysosomal pH in the C225-AuNP nanoenvironment. C225 was labeled with a pH sensitive fluorophore, FITC, and then conjugated to AuNPs (10nm). A) Standard calibration curve: SNU449 cells were incubated with FITC-C225-AuNP nanoconjugates at 100 μ g/ml for 30 minutes. Endo-lysosomal pH was equilibrated to a preset extracellular pH using membrane permeabilization with NaN₃ and NH₄Cl. B) SNU449 cells were pretreated with or without (●) lysosomotropic agents, ▲ concanamycin A (10nM), ◆ NH₄Cl (30mM), or ■ chloroquine (200 μ M) for 1 hour followed by 30 minutes incubation with FITC-C225-AuNPs (100 μ g/ml) on ice for 30 minutes. Excess nanoconjugate was removed and endo-lysosomal pH was measured over time and is reported as mean with standard deviation.

Figure 3. Intracellular localization and stability of AuNPs imaged by electron microscopy. SNU449 cells were incubated with C225-AuNPs (100 $\mu\text{g/ml}$) in the absence (A) or presence of concanamycin A 10nM (B), chloroquine 200 μM (C) and NH_4Cl 30mM (D) for 4 hours. Left Panel: 7500 $\times$, Middle Panel: 50,000 $\times$, shows the intracellular localization of AuNPs, Right Panel: 100,000 $\times$, shows aggregated or non-aggregated state of AuNPs within the ELVs. ELV=endo-lysosomal vesicle, M=mitochondria, N=Nucleus

A. Controls

B. Concanamycin A

C. Chloroquine

D. NH₄Cl

Figure 4.

Hyperthermic cytotoxicity secondary to RF field (13.56MHz, 600W) treatment. SNU449 cells were incubated with C225-AuNP (200 μ g/ml) in absence or presence of concanamycin A 10nM (B), chloroquine 200 μ M (C), or NH₄Cl 30mM (D) for 4 hours immediately followed by RF exposure for 9 minutes. Cell viability was assessed by flowcytometry-based assay (Annexin-V & PI staining) 24 hours after RF exposure. A) Control cells without RF exposure. (ns= not significant, *p-value<0.05, **p-value<0.01, all comparisons vs. untreated controls).

Figure 5.

Thermal denaturation of luciferase after RF field exposure. SNU449 cells were incubated with (white bars) or without (black bars) C225-AuNPs (200 µg/ml) in absence or presence of concanamycin A 10nM, chloroquine 200 µM, or ammonium chloride 30mM for 4 hours immediately followed by RF exposure for 3 minutes. Non-denatured luciferase concentration was measured using a bioluminescence assay and the data was normalized to the total protein concentration of the lysate. (error bars represent standard deviation, ns=not significant, **p-value<0.01, *p-value<0.05, unpaired independent sample t-test)