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Remotely Triggered Cisplatin Release from Carbon Nanocapsules by Radiofrequency Fields

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Abstract

The efficacy of nanoparticle-mediated drug delivery is limited by its peri-vascular sequestration, thus necessitating a strategy to trigger drug release from such intra-tumoral nanocarrier-drug depots. In our efforts to explore remotely-activated nanocarriers, we have developed carbon nanocapsules comprised of an ultrashort carbon nanotube shell (US-tubes) loaded with cisplatin (CDDP@US-tubes) and covered with a Pluronic surfactant wrapping to minimize passive release. We demonstrate here that non-invasive radiofrequency (RF) field activation of the CDDP@US-tubes produces heat that causes Pluronic disruption which triggers cisplatin release in an RF-dependent manner. Furthermore, release-dependent cytotoxicity is demonstrated in human hepatocellular carcinoma cell lines.

INTRODUCTION

Maximizing therapeutic efficacy and minimizing adverse effects remain the fundamental goals of drug delivery in cancer therapy. A vast majority of cancer chemotherapeutics, while potent agents, cannot be employed to full clinical benefit because of toxicity concerns. Over the past decade, drug-loaded nanocarriers have been widely fabricated and studied to enhance tumor specific delivery. Most strategies that involve either active or passive targeting have been successful in demonstrating tumor-specific accumulation of these nanocarriers to achieve anti-cancer toxicity in pre-clinical models. However, recent studies demonstrate that intra-tumoral distribution of nanocarriers is patchy rather than homogenous [1–4]. Nanocarriers extravasate from the vascular space and tend to accumulate in the peri-vascular space through fenestrations in tumor vasculature (also called enhanced permeation and retention effect) [5]. There are a multitude of factors that limit subsequent permeation of

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nanocarriers from the peri-vascular space to tumor cells, including the size of the nanocarriers, interstitial fluid pressure within the tumor, variations in pH in the microenvironment, composition of the extracellular matrix, and lymphatic clearance of nanocarriers [4, 6–9]. The spatial separation between drug-loaded nanocarriers and tumor cells often renders this strategy futile.

To take advantage of intra-tumoral drug depots that are formed by these nanocarriers, environmentally- or remotely-triggered drug release mechanisms are being developed. The premise is based on the notion that free drug, because of its smaller molecular size, can rapidly diffuse along its concentration gradient to cells distant from the nanocarrier depots. Environmentally sensitive nanocarriers exploit pH [10], temperature [11–13], or the presence of tumor-specific enzymes (such as matrix metalloproteinases [14]) to trigger drug release. This strategy is inherently limited by the inhomogeneous pH and enzyme concentrations in the tumor microenvironment [15]. Temperature-sensitive nanocarriers (such as certain liposomes) depend on a strategy that raises bulk tumor temperature above a critical transition temperature to accomplish a phase change [16]. However, raising bulk tumor temperature selectively and safely remains a significant challenge. Because of these limitations, nanocarriers that can be remotely activated or disrupted by focused ultrasound, light, or alternating magnetic fields to trigger drug release are under investigation. In each case, the nanocarrier is comprised of a nano-susceptor that absorbs energy and dissipates it locally to actuate release. Alternating magnetic fields offer full-body-penetration but acoustic and photo-triggered drug release are less ideal because of the non-uniformity and limited depth of penetration. These studies highlight the need for innovative strategies to enhance efficacy of nanocarriers to increase drug or biologic agent release after tumoral delivery.

In developing remotely-triggered nanocarriers, carbon nanotubes are of particular interest to us. Single-walled carbon nanotubes (SWCNTs) have been derivatized, functionalized, and loaded or conjugated with chemotherapeutics [17]. More importantly, SWCNTs exhibit unique properties in radiofrequency (RF) electric fields such as enhancement of local E-fields, absorption and dissipation of RF energy as heat, and alignment parallel to the incident E-field [18–20]. Because RF fields at low megahertz frequency offer the advantage of full-body-penetration, nanotube-RF coupling can potentially be exploited to triggered release. However, full-length SWCNTs have a very high aspect ratio and, therefore, may not be ideal for drug delivery applications. To address this problem, ultra-short carbon nanotubes (US-tubes, ~1.4 nm×20–80 nm) have been fabricated using a fluorination and pyrolysis process [21]. The defects created in the US-tube sidewalls by this procedure allow for easy loading of cytotoxic chemotherapeutics, e.g. cisplatin (CDDP), contrast agents (Gd^{3+} , I_2), or radioisotope agents ($^{211}AtCl$) [22–24]. Moreover, *in vivo* studies have demonstrated that US-tubes are non-toxic and well tolerated by mice even at very high doses (~0.5 mg/kg) making them a suitable candidate for nanocarrier development [25].

We hypothesized that we could produce US-tubes loaded with CDDP and wrapped with a Pluronic coating to prevent CDDP release from the US-tubes. We further hypothesized that heating of the surface of US-tubes by a non-invasive RF-field would lead to loss of Pluronic coating and CDDP release (Figure 1). Our studies were designed to confirm and characterize Pluronic-wrapped CDDP@US-tubes (W-CDDP@US-tubes), to measure CDDP release from W-CDDP@US-tubes following RF-field exposure, and to demonstrate RF-induced CDDP release at levels relevant to produce cancer cell cytotoxicity.

METHODS

Sample Preparation

Pluronic-F108-wrapped CDDP-US-tubes (W-CDDP-US-tubes) were prepared as described previously [24]. In brief, full-length SWCNTs (Carbon Solution Inc., Riverside, CA), produced by an electric-arc discharge method, were cut into US-tubes by fluorination followed by pyrolysis at 1000°C under an inert atmosphere. US-tubes were first purified in concentrated HCl by bath-sonication to remove amorphous carbon and metal catalyst impurities (nickel and yttrium), then chemically reduced by a metallic Na⁰/THF reduction procedure to produce individual US-tubes [26]. The debundled US-tubes were dispersed in deionized water via bath-sonication for 60 min, and then CDDP was added to the US-tube suspension and vigorously stirred for 24 hr. The reaction vessel was then left undisturbed overnight whereupon CDDP-US-tubes flocculated from solution. The CDDP-US-tubes were collected by filtration on a glass filter, washed with excess deionized water to remove all exterior CDDP from the outer surface of the US-tubes (as judged from Pt analysis on the filtrate aliquots by inductive-coupled plasma optical emission spectrometry [ICP-OES]), and dried at 80°C. W-CDDP-US-tubes were prepared by suspending dry CDDP-US-tube samples in a 0.17% (w/v) Pluronic-F108 solution via probe sonication for 2 min, followed by centrifugation at 3200 rpm for 10 min (3x) to remove unsuspended CDDP-US-tubes. Extensive characterization of this CDDP@US-tubes material has been reported previously [24].

Inductively-Coupled Plasma-Optical Emission Spectrometry (ICP-OES)

ICP-OES analyses were carried out by a Perkin Elmer Optima 4300 DV instrument with a CCD detector. The quantity of CDDP in each sample was determined by measuring the Pt concentration, which was detected at 265.95 nm. For all ICP-OES measurements, the collected samples were transferred into glass vials, then digested with 26% HClO₃. The samples were then diluted with 2% trace metal grade HNO₃. The Pt concentrations were determined from an average of five scans for each sample. Yttrium (371.029 nm) was used as the internal standard.

RF Generator Setup

We employed the Kanzius External RF (13.56 MHz) Generator System (ThermMed, LLC, Erie, PA) as described previously [27]. The generator utilizes a capacitively-coupled transmitter and receiver head to generate a uniform RF field within an air-gap of 10 cm. The generator input power was arbitrarily set at 950 W for all experiments. To demonstrate heating of US-tubes or CDDP-US-tubes, a suspension was created using 0.17% (w/v) Pluronic F-108 in de-ionized water or in PBS. Varying concentrations were loaded in a 1.3 mL quartz cuvette. The cuvette was placed in a Teflon holder and positioned at 5/16 inch from the transmitter head. The incident E-field intensity at this position was estimated to be 700kV/m. The temperature of the cuvette was recorded using an infrared thermal camera (FLIR Systems, Boston, MA). Heating rates were calculated between 5–120 seconds of RF exposure where the Time vs. Temperature plots were noted to be linear. In order to expose 12-well cell culture plates to the RF field for the CDDP release studies and cell studies, the 12-well plate was positioned at an arbitrary point in the RF field on a Teflon holder. At this point the ionic heating of 1 mL PBS in a well of a 12-well plate was noted to be (~1°C/min). This was used as an internal control before, during, and after RF field exposures. The 12-well plates loaded with samples were equilibrated to 37 °C in a cell culture incubator prior to each exposure. The plates were then removed from the incubator and placed on the Teflon holder at an ambient temperature of (20–22 °C). The plates were allowed to cool to 30 °C before starting the RF exposure experiments. This step was necessary to distinguish bulk from local heating effects.

CDDP release from CDDP-US-tubes

CDDP release studies from US-tubes were performed as previously described but with some modification [24]. Briefly, lyophilized CDDP-US-tubes were suspended in PBS with 0.17% (w/v) Pluronic F-108 at a US-tube concentration of approximately 700 mg/L. In order to expose the CDDP@US-tubes to various experimental conditions, 1 ml of sample was placed in each well of a 12- well plate. The plate was then exposed to the RF field or to constant temperature water bath hyperthermia at appropriate experimental parameters as detailed in the results section. The samples were then placed inside a dialysis-membrane (20,000 MW) cylinder, which was immersed in 800 mL PBS at room temperature (22–24 °C) for 168 hours. During immersion, the W-CDDP-US-tube sample in the dialysis-membrane cylinder was sampled periodically. The PBS solution in the container was under constant circulation and was replaced at least 4 hours before each sampling to maintain a favorable concentration gradient.

Fluorescence measurements

For fluorescence studies, samples were exposed to varying parameters in a 12-well cell culture plate and after exposure, were transferred to a 96-well culture plate at 0.2 mL per well. Fluorescence was measured using a Fluostar Omega (BMG Labtech, Ortenberg, Germany) apparatus with the appropriate excitation and emission filter sets for allophycocyanin. To demonstrate temperature-dependent Pluronic disruption from the W-CDDP-US-tube surface, fluorescence was measured using a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA).

Cell Studies

Both human liver cancer cell lines (Hep3B and HepG2) were purchased from American Type Culture Collection (Manassas, VA) and maintained as per the instructions of the supplier. Media, i.e., MEM (for HepG2 or Hep3B) was supplemented with 10% (v/v) fetal bovine serum. Additional supplementation was performed with sodium pyruvate, non-essential amino acids, Penicillin G and Streptomycin. Cells were cultured in T-75 or T-150 tissue culture flasks (Corning Inc., Corning, NY). For each cell line, the short tandem repeat fingerprint was confirmed by the Cell Line Characterization Core Service (M. D. Anderson Cancer Center, Houston, TX) within one year of all experiments. All media and supplements were purchased from Gibco (Life Technologies, Grand Island, NY). The cells were passaged approximately every three to five days before reaching confluency. Media was replaced every three days.

Release-dependent cytotoxicity was assessed in a 0.4-micron trans-well system (Corning Inc., Corning, NY). The W-CDDP@US-tubes were loaded in the top well and sub-confluent monolayers were grown in the bottom well. Aggregates of W-CDDP-US-tubes failed to pass through the filter as confirmed by UV-Vis (not shown). Released CDDP could, however, diffuse through the filter and enter the bottom compartment containing adherent cells with resultant CDDP-induced cytotoxicity. Viability was assessed 120 hours after drug exposure using a standard MTT assay.

Statistical Analysis

Unless otherwise stated, data points represent average from 3–6 independent experiments and error bars represent standard error of the mean. For inferential statistics, a p -value<0.05 was considered significant. Two group comparisons were made using two-tailed t-test for an unpaired sample. Multiple group comparisons were made using one-way analysis of variance (ANOVA) with Bonferroni post-hoc test.

RESULTS

RF absorption by US-tubes

We prepared US-tubes loaded with and without CDDP as detailed in the methods. Heating of full-length SWCNTs has been demonstrated and theoretically predicted under the influence of RF irradiation [18]. We wanted to determine if US-tubes dissipate heat when exposed to low-frequency radiowaves. The Kanzius RF generator was used for this purpose. US-tubes loaded with or without CDDP were subjected to gentle filter centrifugation through a 10 kDa centrifugation filter and resuspended in a solution of 0.17% (w/v) Pluronic-F108. This was repeated several times until the filtrate heating rate was about the same as a solution of 0.17% Pluronic-F108. This was necessary to eliminate ionic heating effects in the RF field that have been previously reported [27].

The heating rates for the two suspensions and their background are shown in Figure 2. Here, it can be noted that both US-tube and CDDP-US-tube suspensions heat in the RF field significantly above the background ionic heating. We also observed that the heating rates were concentration-dependent. Comparing the relative heating of US-tubes to CDDP@US-tubes, we found that drug loading somewhat attenuated the heating of US-tubes under RF field exposure.

Localized heating of US-tubes in a physiological environment

In order to understand the heating of CDDP-US-tubes in a more physiologically-relevant environment, CDDP-US-tubes in 0.17% (w/v) Pluronic-F108 were suspended in phosphate buffered saline (PBS, pH 7.4). Significant ionic heating under RF exposure was observed when the W-CDDP-US-tubes were suspended in PBS, however, there were no statistically significant differences in the heating rates of filtrate and suspension. We conclude that RF exposure of CDDP-US-tubes in PBS does not result in significant enhancement of the bulk temperature. Furthermore, these findings suggest that the ionic heating of PBS either overshadows or abrogates the heating contribution due to CDDP-US-tubes alone.

To address this hypothesis, an experiment was designed to probe localized heating of CDDP-US-tubes in PBS. It has previously been observed that uncoated SWCNTs have non-specific affinity for proteins and it is also known that Pluronic-wrapping attenuates the binding of proteins to the surface of SWCNTs [28]. Using this information, we utilized a relatively thermostable fluorescent protein (allophycocyanin (APC)) to probe the disruption of Pluronic on CDDP-US-tubes surfaces. The principle of this assay is demonstrated in Figure 3A. W-CDDP@US-tubes prevent APC binding and a fluorescent signal can be detected. Local heating of US-tubes or bulk ionic heating should disrupt Pluronic wrapping above the melting point of Pluronic-F108 (~57.5°C) allowing APC to bind to US-tube surfaces and thus produce quenching of APC fluorescence. Similar fluorescence quenching behavior has been described previously for other carbon-based nanomaterials such as carbon nanotubes [29], fullerenes [30], and nanographene sheets [31].

In order to test this hypothesis further, APC was added to a suspension of W-CDDP-US-tubes in PBS and a temperature-dependent decrease in fluorescence was observed. This was normalized to fluorescence measurements from a control solution of APC in 0.17% (w/v) Pluronic-F108 to exclude effects from photobleaching, temperature-dependent fluorescence quenching, or thermal denaturation of APC. The normalized curve demonstrated quenching of APC due to the presence of Pluronic-wrapped W-CDDP-US-tubes (Figure 3B). The first derivative of this curve revealed the peak transition at 59.7 °C (Figure 3C), which is consistent with the transition temperature of Pluronic-F108 (~57.5 °C). Therefore, we attribute the quenching of APC fluorescence to the temperature-dependent disruption of Pluronic-F108 on CDDP-US-tube surface due to the rise in bulk temperature.

Next, we used this system to study localized heating of W-CDDP@US-tubes in the RF field while suspended in PBS. The results, shown in Figure 3D, demonstrated that RF field exposure resulted in a dose-dependent quenching of fluorescence of APC, suggesting Pluronic disruption. We monitored the bulk temperature during this exposure, which reached a maximum of 42.5 °C after 5 min of RF field exposure. This experiment was then repeated by exposing the CDDP-US-tube suspension to 42.5 °C in a water bath for 5 min instead of exposing it to RF. Fluorescence measurements did not show significant quenching of APC in the water-bath experiment. This implies that the Pluronic disruption and APC fluorescence quenching is not due to a rise in bulk temperature. It is possible that fluorescence quenching of APC could be due to non-thermal denaturation of APC in the RF field but it was found that exposing APC in 0.17% (w/v) Pluronic-F108 solution to the RF field for 5 minutes does not result in significant fluorescence quenching, thus, excluding this possibility. We conclude that these data demonstrate localized heating at the surface of W-CDDP@US-tubes in a physiologic salt solution as well as disruption of Pluronic on the W-CDDP@US-tube surface.

RF-triggered release of CDDP from US-tubes

We have previously demonstrated that CDDP can be loaded into US-tubes through sidewall defects and wrapping the CDDP@US-tubes with Pluronic can significantly retard subsequent release of CDDP [24]. Thus, based on these previous as well as present studies, we reasoned that Pluronic disruption caused by localized heating of US-tubes should enhance release of CDDP from W-CDDP@US-tubes in a RF field-triggered fashion. In order to test this hypothesis, W-CDDP@US-tubes were suspended in PBS and subjected to varying durations of RF exposure. Samples not exposed to RF were used as a negative control. In order to differentiate release of CDDP due to bulk temperature and that due to localized heating of W-CDDP@US-tubes, water bath temperature controls were used. These samples were incubated in a water bath for a duration that matched the RF experiments. The incubation temperature for the water bath experiments was chosen as the final average temperature reached after 2.5 or 5 min of RF exposure. After the appropriate exposure, samples were immediately loaded in dialysis cassettes and CDDP release at room temperature was determined by ICP measurements.

Data shown in Figure 4 demonstrated that RF field exposure approximately doubles the release of CDDP from W-CDDP@US-tubes after 2.5 or 5 min of RF exposure at each time point measured (relative to no-RF controls). Relative area-under-curve (AUC) depicts cumulative release relative to the no-RF control. Regardless of the duration (2.5 or 5 min), RF exposure enhanced cumulative release of CDDP by 1.9 fold after 168 hours. Samples exposed to RF reached an average bulk temperature of 38.7 °C after 2.5 min and 49.5 °C after 5 min of RF exposure. We then exposed a W-CDDP@US-tubes suspension to these temperatures and durations in a water bath and measured the release at room temperature similar to RF-exposed samples. For the sample exposed at 49.5 °C for 5 min, we found significant enhancement of release that was similar to but less than the RF exposed samples (cumulative release, 1.7 fold), suggesting bulk temperatures lower than Pluronic's transition temperature of 57.5°C can allow release of CDDP. However, the temperature control sample exposed at 38.7 °C for 2.5 min demonstrated far less enhancement in cumulative release (1.3-fold). These results demonstrate and distinguish between CDDP release from W-CDDP@US-tubes caused by bulk and local (at the surface of the CDDP@US-tubes) temperature rise, when exposed to the RF-field.

RF-triggered CDDP release and cytotoxicity

To evaluate the significance of our findings, we exposed liver cancer cells to W-CDDP@US-tubes in a transwell system such that the CDDP@US-tubes nanocarriers were

spatially separated from the cellular compartment. Only released CDDP could diffuse through the trans-well membrane (pore size <400nm). This system is designed to model the limitations of *in vivo* drug delivery using nanocarriers where the drug-loaded carriers can be spatially separated from their intended site of delivery in cancerous tissue because of multi-scale barriers to transport. The experimental scheme is represented in Figure 5.

Approximately 5000 human liver cancer cells (Hep3B or HepG2) were plated on the bottom well of a 12-well trans-well system. After 24 hours, the cells were found to be adherent at which point a second compartment was placed on top and the cells were treated with PBS, W-US-tubes (13.7 mg/L) or W-CDDP@ US-tubes (CDDP 2.5 μ M, US-tube 13.7 mg/L). This concentration was the approximate IC_{50} for W-CDDP@US-tubes for both cells lines at 120 hours. The trans-well system was or was not subjected to a 3-min RF field exposure 1, 2, or 3 times at 24 hour intervals. Viability was assessed at 120 hours when the PBS treated control samples reached confluence. For both cell lines, it was noted that even the US-tubes alone are slightly cytotoxic at this concentration. We also noted that this slight cytotoxicity was not enhanced by multiple RF exposures. The data in Figure 5 demonstrated that Hep3B and HepG2 cells possess different sensitivities to RF-field exposure. As shown, Hep3B cells are not affected by multiple 3-min RF field exposures while HepG2 cells demonstrate slight toxicity to RF. Interestingly, this toxicity is not enhanced after repeated RF exposures. We found that the W-CDDP@ US-tube treatment reduced the viability of both cell lines to 50% due to the spontaneous release of CDDP. This cytotoxicity was significantly enhanced by a single 3-min RF exposure ($p < 0.01$). For Hep3B cells, repeated 3-min RF exposure demonstrated slightly higher cytotoxicity but this was not statistically significant. For the HepG2 cells, multiple RF-exposures did not further enhance the observed cytotoxicity. Overall, these data clearly demonstrated cytotoxicity attributable to RF-triggered CDDP release from CDDP@US-tubes.

DISCUSSION

Nanoparticle-mediated drug delivery is widely studied as it may help to overcome drug resistance and enhance tumor-selective toxicity. However, several challenges remain before this potential can be fully realized. While studies have demonstrated successful accumulation of nanocarriers within tumors, it can be argued that the therapeutic efficacy of these nanomaterials is limited by their sequestration in the peri-vascular space. The slow passive release of drugs from nanocarriers is therefore often not sufficient to allow therapeutic levels to be achieved within the tumor. Indeed, this argument is supported by recent studies that demonstrate patchy distribution of nanocarriers in the vascularized areas of the tumor, while certain avascular areas are completely devoid of the chemotherapeutic agent [2–4, 7]. The barriers to transport are incompletely understood and are likely to be multifaceted, involving both host and nanoparticle properties. While host parameters (interstitial fluid pressure, extracellular matrix composition, pH, and cellular stroma components) are difficult to modulate, nanoparticle design can be readily altered [8, 9]. It is well known that smaller nanoparticles permeate through the tumor interstitium better than larger nanoparticles [32]. However, small nanoparticles are less likely to take advantage of the EPR effect and could be readily cleared by the renal excretion. Hence, nanoparticle properties that govern optimal tumor accumulation are different from those that allow optimal tumor-wide permeation. An alternative approach provides a practical solution to this problem and utilizes remotely-triggered release of drugs after intra-tumoral accumulation of nanocarriers.

In this study, we have described a carbon nanocapsule-based system that can readily release its drug cargo in response to a non-invasive RF-trigger. Carbon nanotube interaction with an RF field has been previously studied [18]. It was noted that under RF field exposure, full-

length nanotubes dissipate energy as heat. Furthermore, this energy is sufficient to raise the bulk temperature of the surrounding environment allowing the use of SWCNTs as remotely-activated thermal agents. The heating of US-tubes has not been previously described. Like SWCNT, we have demonstrated remote heating of W-CDDP@ US-tubes and W-US-tubes in DI-water. However, we find that bulk heating of W-US-tubes or W-CDDP@US-tubes can only be observed in a non-ionic environment. When suspended in a physiologically relevant ionic environment (PBS), the bulk temperature rises due to ionic heating of PBS and the local heating of US-tubes cannot be measured. Theoretical studies have predicted that only metallic SWCNT can elevate bulk temperatures of ionic solvents through a “lightning-rod” effect where the metallic SWCNTs polarize in the presence of an E-field and enhance E-field strength by several orders of magnitude [20]. This can lead to a dissipation mechanism in the bulk ionic solvent that increases with increasing conductivity of the solvent. Contrary to metallic SWCNTs, the power dissipation by semi-conducting SWCNTs is thought to occur predominantly in the nanotube core and therefore is unlikely to enhance bulk temperature in a conductive host such as PBS. Defects created in the sidewalls of US-tubes during the fabrication process can alter the electronic properties of SWCNTs from which they are derived. Based on the experimental observations of this study, we infer that the US-tubes behave as a poor dielectric with predominant energy dissipation within the core of the US-tube. This heating can be somewhat abrogated by the CDDP loading, the reason for which is not clear at this time but could be due to basic changes in the dielectric properties of the US-tube.

Furthermore, we have demonstrated that despite the lack of contribution to temperature change in the bulk ionic media, W-CDDP@US-tubes can dissipate energy at the molecular level that is sufficient to disrupt the Pluronic coating in a RF dose-dependent manner, which subsequently allows CDDP release. Several studies have demonstrated remotely triggered drug release. For instance, ultrasound-triggered release has been demonstrated from acoustically-responsive gold nanocages, drug-loaded liposomes, or microbubbles [33–35]. While significant tumor regression was noted in these studies, small animal models do not recapitulate the limits imposed by depth and variability of penetration of ultrasound. Similarly, light has been extensively studied as a trigger to drug release with reports dating back to the 1980s. Photo-activation utilizes photoisomerization, photo crosslinking, photosensitization-induced oxidation, or photo-thermal activation of gold nanoparticles (See Ref. [36] for a review). Regardless of the mechanism of light-triggered drug release, penetration of light even at THz frequencies is limited to a few centimeters, and cannot be utilized for deep-seated tumors, thus necessitating alternative deep-penetrating strategies. Recently, Peiris *et al.* utilized an alternating magnetic fields (AMF) at radiowave frequencies (10 kHz) to trigger drug release from iron oxide nanoparticles cross-linked to drug-loaded liposome in a serial chain [1]. Similar to W-CDDP@US-tubes, they noted that drug release could be accomplished without raising bulk ionic temperature. They attributed this behavior to nanoscale vibration of magnetic iron-oxide particles in an AMF. In contrast, W-CDDP@US-tubes are a less magnetic material but a material that can be activated by the incident electric field of RF exposure as demonstrated by the present work. Similar to an AMF, RF electric fields can easily penetrate to deep-seated tumors and have well-documented safety. In the present study, we have demonstrated that RF exposure can significantly enhance drug release of CDDP from carbon nanocapsules to enhance cytotoxicity. We have also found that RF-triggered drug release from the carbon nanocapsules exhibits cytotoxicity in a drug release-dependent manner. Increasing the duration of RF exposure or frequency of RF exposure does not further enhance drug release suggesting an irreversible change in the Pluronic coating on the W-CDDP@US-tube surface. Unlike iron oxide cross-linked liposomes, US-tubes serve the dual purpose of an encapsulating agent and that of a susceptor in an RF-field, thereby eliminating the need for complex preparation methods. While we have demonstrated that a Pluronic coating

significantly retards drug release from the nanocapsules, up to 30% of CDDP can be passively released despite Pluronic coating. Future studies already underway are evaluating alternative temperature-sensitive coatings to allow stealth nanoparticles that only permit drug release in the presence of an RF field. In addition, preliminary data in our laboratory has demonstrated that the US-tube platform can be functionalized with antibodies, which will allow tumor specific targeting in conjunction with the known EPR effect.

CONCLUSION

We have developed a carbon-nanocapsule system that can be remotely triggered by RF irradiation to release CDDP. Mechanistic insight into the process has revealed thermal disruption of a Pluronic coating by US-tube heating in the RF-field without appreciable rise in bulk temperature at an RF dose that is minimally toxic to cancer cells. Furthermore, we have observed release-dependent cytotoxicity in human liver cancer cell lines in a trans-well system that mimics spatial limitations in delivery of chemotherapeutics by drug-loaded nanocarriers *in vivo*.

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1. Synthesis of US-tubes

2. Interior and exterior loading of CDDP with US-tubes

3. Extensive washing to remove exterior CDDP from CDDP@US-tubes

4. Pluronic wrapping to prevent (retard) CDDP release from W-CDDP@US-tubes

5. RF-triggered US-tube heating and pluronic disruption of W-CDDP@US-tubes

6. Slow passive release of CDDP over time from CDDP@US-tubes

Figure 1. Schematic describing synthesis, purification, and RF-triggered disruption of Pluronic F-108 and subsequent release of CDDP from W-CDDP@US-tubes.

Heating of CDDP±US-tubes in RF field

US-tube: 1x=725mg/L
 CDDP@US-tube: 1x=707mg/L
 CDDP conc = 117uM or 35mg/L

Figure 2.
 Heating rates of W-CDDP@US-tubes and W-US-tubes suspended in 0.17% pPluronic F-108 in comparison with filtrate alone as a function of concentration.

Figure 3.

Panel A. Schematic demonstrating the principle of the temperature-dependent Pluronic disruption assay.

Panel B and C. Temperature-dependent fluorescence quenching with a transition temperature close to the melting point of Pluronic F-108 is indicative of Pluronic disruption from the w-CDDP-US-tube surface.

Panel D. RF duration-dependent quenching of fluorescence demonstrates localized heating of W-CDDP@US-tubes in PBS. Water-bath temperature controls (HT) fail to demonstrate quenching of APC fluorescence suggesting lack of Pluronic disruption due to bulk temperature. The RF-dependent quenching is therefore not a result of non-thermal denaturation of APC.

CDDP release from CDDP@US-tubes $\pm$ RF

* $p < 0.001$, 38.7°C (2.5 min) versus RF (2.5 min)

$p < 0.001$, 49.5°C (5 min) versus RF (5 min)

Figure 4. CDDP release from W-CDDP@US-tubes with or without RF

Panel A. Release of CDDP from W-CDDP@US-tubes with or without RF exposure for 2.5 or 5 min is shown. In addition, bulk temperature controls are demonstrated to evaluate the contribution of bulk ionic heating to CDDP release from W-CDDP@ US-tubes
Panel B. Relative areas-under-curve depict cumulative CDDP release over 168 hours relative to untreated control.

Release-dependent cytotoxicity in a trans-well setup

A. Experimental Design

RF = 3min
CDDP = 2.5 μ M

*p<0.01 All groups vs. Untreated
#p<0.01 US-tubes vs. CDDP@US-tubes

B. Hep3B

C. HepG2

Figure 5. Release-dependent cytotoxicity of W-CDDP@US-tubes

Hep3B and HepG2 human liver cancer cells were plated in the bottom well of the trans-well plate and treated with PBS, W-US- tubes or W-CDDP@US-tubes followed with or without RF exposure for 3 min once, twice, or three times at 24 hour intervals. The cell viability was assessed 120 hours after treating with W-CDDP@US-tubes.