Influence of Hyperthermia on Efficacy and Uptake of Carbon Nanohorn-Cisplatin Conjugates

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Single-walled carbon nanohorns (SWNHs) have significant potential for use in photothermal therapies due to their capability to absorb near infrared light and deposit heat. Additionally, their extensive relative surface area and volume makes them ideal drug delivery vehicles. Novel multimodal treatments are envisioned in which laser excitation can be utilized in combination with chemotherapeutic-SWNH conjugates to thermally enhance the therapeutic efficacy of the transported drug. Although mild hyperthermia (41–43 °C) has been shown to increase cellular uptake of drugs such as cisplatin (CDDP) leading to thermal enhancement, studies on the effects of hyperthermia on cisplatin loaded nanoparticles are currently limited. After using a carbodiimide chemical reaction to attach CDDP to the exterior surface of SWNHs and nitric acid to incorporate CDDP in the interior volume, we determined the effects of mild hyperthermia on the efficacy of the CDDP-SWNH conjugates. Rat bladder transitional carcinoma cells were exposed to free CDDP or one of two CDDP-SWNH conjugates in vitro at 37 °C and 42 °C with the half maximal inhibitory concentration (IC₅₀) for each treatment. The in vitro results demonstrate that unlike free CDDP, CDDP-SWNH conjugates do not exhibit thermal enhancement at 42 °C. An increase in viability of 16% and 7% was measured when cells were exposed at 42 deg compared to 37 deg for the surface attached and volume loaded CDDP-SWNH conjugates, respectively. Flow cytometry and confocal microscopy showed a decreased uptake of CDDP-SWNH conjugates at 42 °C compared to 37 °C, revealing the importance of nanoparticle uptake on the CDDP-SWNH conjugate's efficacy, particularly when hyperthermia is used as an adjuvant, and demonstrates the effect of particle size on uptake during mild hyperthermia. The uptake and drug release studies elucidated the difference in viability seen in the drug efficacy studies at different temperatures. We speculate that the disparity in thermal enhancement efficacy observed for free drug compared to the drug SWNH conjugates is due to their intrinsic size differences and, therefore, their mode of cellular uptake: diffusion or endocytosis. These experiments indicate the importance of tuning properties of nanoparticle-drug conjugates to maximize cellular uptake to ensure thermal enhancement in nanoparticle mediated photothermal-chemotherapy treatments. [DOI: 10.1115/1.4026318]

Introduction

Cancer is a leading cause of mortality worldwide and while new technologies are continually being developed, the rates of incidence and survival remain relatively unchanged [1]. Urinary bladder cancer in particular is gaining attention from clinicians and researchers since it is the fourth most common noncutaneous malignancy [2]. Currently, transurethral resection of bladder tumors is the primary treatment. However, patients that undergo conventional resection often experience high rates of recurrence and can suffer from poor quality of life following complete resection [3]. Therefore, the development of new therapies to improve patient morbidity and prognosis of superficial and invasive urinary carcinomas is of the utmost importance.

Therapies that utilize mild hyperthermia (41–43 °C) and a wide range of chemotherapeutic drugs, including antineoplastic agents such as cisplatin (CDDP), have been explored as potential urinary cancer treatments [4–7]. Both in vitro and in vivo studies have investigated the enhancement of CDDP with hyperthermia and have shown promising results, including not only additive efficacy but also synergism between the heat and drug [7]. Specifically, CDDP has been investigated for use in bladder cancer as an adjuvant to thermal therapies due the discovery of its potential for high enhancement [8]. Compared to chemotherapy alone, heat in

addition to chemotherapy can significantly decrease tumor progression and recurrence in bladder cancer and other malignancies, can diminish collateral damage to healthy tissue, and minimize patient discomfort [9–11]. While the mechanism for thermal enhancement of antitumor drugs remains controversial, an effective treatment using CDDP results only when the drug is transported into tumor cells and accumulates in the nucleus where it can react with deoxyribose nucleic acid (DNA), resulting in DNA adducts [12]. The formation of these adducts subsequently results in apoptosis due to the inability to replicate DNA [5,13]. The two prevailing hypothesized mechanisms for the increase in drug efficacy are enhanced drug diffusion into the cell in response to hyperthermia caused by a more fluid membrane or increased intercalations with the DNA as a result of unknown cellular changes [14]. In vitro studies have shown the combination of heat and CDDP results in a higher efficacy of treatment, with the viability of tumor cells decreasing by as much as 30% in the combinatorial treatment compared to CDDP alone [7]. However, the practicality of simultaneous application of the two modalities at a regional level is limited due to the inability to achieve thermal selectivity within the desired tumor region, thus limiting the overall utility of the combination [15,16].

Nanotechnology could provide the missing link between a theorized application of hyperthermia for drug enhancement and a potential clinically viable treatment option with both heat and drug delivery that decreases residual toxicity and damage to healthy tissue [17]. Nanoparticle-assisted photothermal therapies enable highly localized and selective heating and targeted delivery of drugs [18]. Therapies are currently being developed that utilize

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unique nanostructures, including gold nanorods and spheres [19,20], gold nanoshells [21], and carbon nanomaterials such as single and multiwalled nanotubes [22] and single-walled carbon nanohorns that absorb light strongly in the near-infrared (NIR region) and convert the light into heat [23,24]. Despite the promise of nanoparticle-assisted therapies, they often produce uneven and insufficient heating of the tissue due to a heterogeneous distribution of nanoparticles in a nonuniform tumor that can lead to incomplete destruction, resulting in reoccurrences [25,26] and motivating the need for multimodal therapies with higher efficacy.

While nanoparticles are primarily used to deliver only heat for tumor ablation, they can also be utilized to selectively induce mild hyperthermia in the desired treatment region to enhance the therapeutic efficacy of a chemotherapeutic drug [19]. Specifically, the use of CDDP with gold nanorods or graphene oxide in combination with near infrared light to produce a synergistic effect of heating with a chemotherapeutic agent has recently been explored. These studies determined that nanoparticles are capable of producing a thermal effect needed for enhancement with decreases of viability as much as 25% in vitro [25,27,28]. Utilizing nanoparticles for photothermal enhancement of chemotherapeutic drugs is, therefore, a clinically feasible option due their ability to generate temperatures required for enhancement that are lower than the temperatures required for single-modal tumor destruction.

In addition to the heat generating capability of nanoparticles, they can also be utilized as drug delivery vehicles. The nanoscale (~100 nm) size of these nanoparticles allows for passive targeting to the tumor site through exploitation of the enhanced permeability and retention (EPR) effect that results from a leaky vasculature and poor lymphatic network in the tumor microenvironment [29]. While free drugs can passively diffuse out of the majority of blood vessels in the body, the leaky vasculature of the tumor may permit a nanoparticle carrying a drug to extravasate selectively in the tumor space [30]. Additionally, the dysfunctional lymphatic drainage in tumors aids in retention of the extravasated nanoparticles, thereby promoting accumulation in the desired region and enabling drug localization and treatment [31]. In addition, it has been shown that mild hyperthermia, which could be delivered by nanoparticles via laser irradiation, aids in the extravasation of larger nanoparticles to the tumor site in a size-dependent manner, resulting in enhanced accumulation of nanoparticles reaching their targeted destination, further motivating hyperthermia as an adjuvant to nanoparticle drug delivery [32]. Overall, the ability to conceal a drug within a nanosized device capable of producing heat leads to possibly the greatest passive targeting to the tumor through the EPR effect. This treatment can be further enhanced with addition of active targeting through ligands conjugated to the surface of the nanoparticle [33].

Single-walled carbon nanohorns (SWNHs) stand out as a promising nanoparticle for multimodal therapies for delivery of heat and chemotherapy to produce a synergistic effect [23]. We have previously demonstrated the ability of SWNHs to be used with clinically relevant laser parameters and nanoparticle concentrations to generate lethal temperature elevations and cause tumor cell death or temperatures required for thermal enhancement [2,24,34–37]. SWNHs also offer many advantages over other nanoparticles, including low inherent toxicity due to lack of a metal catalyst during synthesis. SWNHs have substantially greater surface area compared to spherical particles and high interior volume making them excellent drug transport vehicles in addition to their photothermal properties [38]. While carbon nanotubes aggregate in micron-sized particles, SWNHs form a spherical or dahlia-like shape (Fig. 1) on the order of 100 nm, which allows for passive tumor targeting and significant cellular uptake [39]. We have previously shown the capability to reproducibly synthesize SWNHs of uniform size and capability to conjugate quantum dots (QDs) to SWNHs in order to monitor cellular uptake such that the overall size of the nanohorn conjugate does not increase significantly, thereby minimizing interference with measurement of cellular uptake [40]. Additionally others have shown the ability to

Fig. 1 (a) Depiction of single-walled carbon nanohorns in their aggregated state of many single-walled cones forming ~150 nm diameter dahlia shape. Two types of CDDP-SWNHs conjugates were developed to determine their potential use as photothermal enhancers of the drug. The first SWNH-CDDP is the incorporated CDDP (b) in which the SWNHs are opened and CDDP is loaded into the internal space of the nanoparticle. The second conjugate (c) utilizes covalent attachment of the CDDP to the exterior surface of the SWNH. In order to visualize uptake kinetics, Zn/S shell Cd/Se quantum dots were attached to the SWNHs creating the SWNH-QD (d).

incorporate CDDP inside of SWNHs and have verified efficacy in vitro [41,42]. Overall, the high capacity for both photoabsorptive heat delivery and drug loading provides SWNHs with many advantages for multimodal therapies.

In this paper, we exploit the substantial surface area and interior volume of SWNHs to create conjugates with CDDP loaded on the exterior surface or interior volume of SWNHs as depicted in Fig. 1. The conjugate with CDDP incorporated freely in the interior of the SWNH allows for leaching of drug over time at a slow rate, while the surface-conjugated CDDP-SWNH concentrates drug to the location of SWNHs, as it remains bound to the nanoparticle [43]. We explore the influence of mild hyperthermia (42 °C) on thermal enhancement and cellular uptake of these two CDDP-loaded SWNHs in comparison to free CDDP. The overall efficacy of the CDDP-SWNH conjugates was examined in vitro using a rat bladder transitional carcinoma cell line. Additionally, we investigated release kinetics of the CDDP-SWNH conjugates under normal physiological and in response to hyperthermic conditions. We have shown for the first time that the degree of thermal enhancement is influenced by the mode of incorporation of the drug in the nanoparticle and its corresponding size and can be highly dependent on cellular uptake of the nanoparticle, which is affected by hyperthermia.

Methods

Cell Culture. The effects of hyperthermia on the efficacy of SWNHs as drug delivery vehicles were evaluated in N-(4-[5-nitro-2-furyl]-2-thiazolyl) formamide induced rat bladder transitional cell carcinoma cells, AY-27 cells, originally developed by Dr. S. Selman and Dr. J Hampton (Medical College of Ohio, Toledo, OH). AY-27 cells were cultured in RPMI Medium 1640 (Invitrogen; Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Sigma Aldrich; St. Louis, MO), 1% penicillin and streptomycin (Sigma Aldrich), and were maintained in a humidified atmosphere at 37 °C with 5% CO₂ and 95% air. Cells were passaged every four days prior to reaching 80% confluency. Passage numbers 37–42 were used for all experiments.

Preparation of CDDP-SWNHs. SWNHs were obtained from Dr. David Geohagan from Oak Ridge National Laboratory. These pristine SWNHs were oxidized by the addition of 8 M nitric acid and heating to 120 °C under reflux. Oxidized SWNHs were reprotonated using a 1 M hydrochloric acid solution to yield particles with protonated carboxylic acid groups. For the purposes of this paper, oxidized SWNHs will be referred to as SWNHs.

Two types of CDDP-SWNH nanoconjugates were investigated to determine the most suitable system for delivery of drugs to the cancer cells under hyperthermic conditions. The first nanodrug conjugate was an SWNH with cisplatin (cis-diamminedichloroplatinum(II), CDDP; Sigma Aldrich; St. Louis, MO) incorporated freely within its interior. Following the method used by Ajima et al., CDDP was incorporated into the interior of the SWNH by first dissolving CDDP in dimethyl sulfoxide (DMSO) to make a 1 mg/mL solution [42]. This solution was then sonicated for 1–2 min to dissociate aggregates and disperse the CDDP. After stirring for 2 h, SWNHs were added to make a 1:1 w/w ratio of CDDP to SWNH, sonicated for 15–30 min until dispersed and stirred for an additional 2 h. Following stirring, the solution was put under nitrogen flow until completely dry (up to two days). Before use, the particles were resuspended in phosphate buffered saline (PBS; ThermoScientific; Logan, UT) and sonicated gently to ensure a homogenous mixture. This CDDP-SWNH formulation will be referred to as CDDP_INC_SWNH and can be seen in Fig. 1(b).

The second CDDP-SWNH conjugate used in this study involved CDDP covalently attached to the exterior surface of the SWNH. Modified from a study in which CDDP was attached to single-walled carbon nanotubes, these particles were created via a carbodiimide reaction [44]. First, SWNHs were sonicated until dispersed in dH₂O at a concentration of 0.1 mg/mL. The solution was transferred to a round bottom flask and N-(3-Dimethylamino propyl)-N'-ethylcarbodiimide hydrochloride (EDC; Sigma Aldrich; St. Louis, MO) was added while stirred. After 5 min of stirring, N-hydroxysulfosuccinimide sodium salt (sulfo-NHS; Sigma Aldrich; St. Louis, MO) was added to the solution and stirred for an additional 5 min. The final concentrations of EDC and sulfo-NHS were 0.1 M and 0.005 M, respectively. CDDP was dissolved in dimethyl sulfoxide (DMSO; Fisher Scientific; Fair Lawn, NJ) to make a 1 mg/mL solution, and this solution was added to the SWNH solution such that the ratio of CDDP to SWNHs was 1:1 w/w. The solution was warmed gently by setting the hot plate to 37–38 °C and allowed to stir at 300 rpm for 1 h. This final solution was filtered through a 0.1 µm nylon membrane over vacuum filtration. The solid product was washed 2–3 times with water, once with ethanol to sterilize and an additional 2–3 times with water in order to remove the ethanol and any residual CDDP not attached to SWNHs. To resuspend the particles, the membrane was transferred to a small vial containing PBS. The membrane in PBS was sonicated for 1–2 min until the particles were suspended in solution. This CDDP-SWNH complex will be referred to as CDDP@SWNH and can be seen in Fig. 1(c).

Preparation of SWNH-QDs. SWNHs like many nanoparticles utilized for photothermal therapies or delivery vehicles lack an inherent fluorescent signal, making visualization of the particles even in vitro nearly impossible. To overcome this shortcoming and to facilitate monitoring SWNH uptake as a function of temperature, quantum dots (QDs) (CdSe/ZnS core shell quantum dots) were attached to the SWNHs using an EDC/NHS reaction as previously published by our group [40]. SWNHs were dispersed in dH₂O at a final concentration of 0.1 mg/mL and were sonicated for 1 h to reduce aggregations. SWNHs were then added to a round bottom flask containing 0.1 M EDC and stirred for 5 min after which 5 mM of sulfo-NHS was added to the solution. Cysteamine hydrochloride (AET; Sigma Aldrich; St. Louis, MO) was then added to the flask at a concentration of 0.2 M and the overall solution was adjusted to a pH of 6.0 using 1.0 M HCl to prevent disulfide formation. After 24 h of stirring, commercially

available QD powder (Ocean Nanotech LLC; Springdale, AR) was dissolved in chloroform (1.0 mg/mL) and added to the SWNHs in a weight ratio of 4:1 w/w QD to SWNH. The pH of the solution was then adjusted to 7.0 for enhanced particle stability and the solution was stirred for 24 h. The high affinity to thiol of the ZnS coating on the QDs causes them to move from the chloroform phase to the SWNHs, creating the SWNH-QD complex. A summary of SWNH shape and the nanoparticle conjugates used in this paper can be seen in Fig. 1.

Characterization of Nanoparticles. Transmission electron microscopy (TEM) was used to evaluate the presence and localization of CDDP or QDs on different SWNH complexes (INC, @, and QD) using a Zeiss 10CA TEM equipped with AMT Advantage GR/HR-B CCD camera system (Carl Zeiss AG; Oberkochen, Germany). To prepare TEM grids, samples of SWNHs, SWNH-QD, CDDP@SWNH, and CDDP_INC_SWNH were diluted in methanol to approximately 0.05 mg/mL. This sample was introduced to a 200 nm Lacey carbon mesh TEM grid (Electron Microscopy Sciences; Hatfield, PA), washed twice with hexane, and dried for 2 min before being washed again with hexane.

Viability Studies. To investigate the thermal enhancement properties of mild hyperthermia (42 °C) on the different CDDP-SWNH complexes described above and on free CDDP, AY-27 cells were seeded on 12-well tissue culture polystyrene dishes at 20,000 cells per well and were incubated for 24 h to reach their exponential growth phase. Following the incubation period, cells were exposed independently to each type of the SWNH-CDDP conjugates or free CDDP suspended in cell media at 37 °C or 42 deg for 1 h. The concentrations of SWNHs and CDDP were selected based on clinically relevant doses. The concentration of free CDDP studied was 50 µM (0.015 mg/mL), which was selected after performing a parametric analysis on a concentration of CDDP for 1 h that resulted in approximately 50% viability to achieve IC₅₀ for all studies. Concentrations of 0.01 mg/mL of CDDP_INC_SWNHs and CDDP@SWNHs were chosen as this concentration results in the same order of magnitude of CDDP exposure and is achievable in vivo at the tumor site [35,42]. As mentioned previously, SWNHs were selected due to their ability to act as both drug delivery vehicles and photothermal heat generators under NIR. However, in this study an isothermal water bath (Lab RTE and water bath system; Thermo Scientific; Albuquerque, NM) set either to 37 °C or 42 °C was used to heat the samples to minimize spatial temperature variation within wells (compared to laser heating) and between samples, which would confound interpretation of the influence of a specific temperature on cell viability and uptake. We have shown SWNHs at concentrations of 0.01 mg/mL can easily be used to generate temperatures of 42 deg when irradiated with a laser. Immediately following the 1 h exposure of either the CDDP-SWNHs or free CDDP, the samples were washed with ice cold PBS three times to remove any residual SWNHs or CDDP from the cell surface and to halt endocytosis processes that could result in additional nanoparticle uptake. Cells were then cultured in fresh RPMI 1640 supplemented medium for 72 h and viability was assessed using an Alamar blue metabolism assay (Life Technologies, Grand Island, NY) according to the standard manufacturer's procedure. Briefly, a 10% Alamar blue solution was added to each well of the 12-well plates and the samples were incubated for 3 h to allow for colorimetric change. Then, 100 µL aliquots were taken from each sample in triplicate and placed into a clean 96-well plate, totaling 24 measurements per group ($n=8$). Absorbance was then measured with a Spectra-MAX M2e microplate reader (Molecular Devices; Sunnyvale, CA) at 570 and 600 nm. The resulting viability was expressed as a mean value $\pm$ sample standard deviation. The significance of the results was verified with a Student's t-test and a 95% or 99% confidence was used to signify statistical significance between groups.

Fig. 2 (a) TEM image showing dahlia shape of pristine SWNHs with an average diameter of 80 nm. (b) TEM image showing high contrast QDs (dark aggregations) on an aggregate of SWNHs attached to lacey carbon mesh. (c) TEM illustrating successful incorporation of CDDP (dark aggregations) in SWNHs and (d) TEM showing dark attachment of CDDP to SWNH.

Cellular Uptake. In order to measure the influence of hyperthermia on the thermal enhancement of drug-loaded SWNHs, the effect on uptake must be elucidated. AY-27 cells were seeded in tissue culture treated polystyrene six-well dishes at 200,000 cells per well for each time point (0, 30, and 60 min) and were incubated for 48 h to reach a confluency of greater than 50%, during which the medium was changed every 24 h. SWNH-QDs were diluted in RPMI 1640 medium to a final concentration of 0.01 mg/mL and were sonicated for approximately 15 s prior to all experiments. The cells were then exposed to SWNH-QD in culture medium for 0, 30, and 60 min at either 37°C or 42°C in an isothermal water bath. After treatment, cells were rinsed three times with ice cold PBS to remove any unbound SWNH-QDs. Cells were then trypsinized and centrifuged at 120 G for 5 min at 4°C. The cell pellet was then resuspended in fresh culture media at a final cell concentration of $1 - 10^6$ cells/mL. Cell suspensions were then maintained on ice until they were analyzed with a BD Biosciences FACS Aria cell sorter (San Jose, CA). A 488 nm excitation source was used with a 610/620 nm emission filter and a 595 nm long pass filter. Cellular debris was excluded with a forward versus side scatter gate and aggregations were excluded. Gating was utilized using a negative sample and the data were expressed as median fluorescence intensity along with a percent positive cell population. In addition to this experiment, another cellular uptake study was performed using a preheat stress in which the cells were exposed to 42°C for either 2 or 4 h and then allowed to return to 37 deg for 2 h prior to exposure of SWNH-QD at 37°C. This preheat study eliminates the effects of thermally enhanced transport on cellular uptake and instead focuses on cellular changes that could affect endocytotic processes.

Confocal microscopy was used to evaluate the cellular distribution of SWNH-QDs to confirm that nanoparticles were being internalized by the cells and not merely attached to the cell membrane. To accomplish this, cells were seeded in an identical manner as the flow activated cell sorting (FACS) study, and cellular distribution at 60 min was characterized. Following ice cold PBS washes, the cells were fixed in 3.7% paraformaldehyde (MP Biomedicals; Solon, OH) for 15 min. Cells were then rehydrated and their membranes were permeabilized with 0.5% Triton-X (Sigma Aldrich; St. Louis, MO) for 15 min and blocked with 1% BSA (Santa Cruz Biotech;

Santa Cruz, CA) for 1 h. Oregon Green 488 phalloidin at 20 nM (Invitrogen; Grand Island, NY) was then used to image the cytoskeleton. 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) (Invitrogen; Eugene, OR) was added for 5 min prior to imaging in order to view the nucleus with a Zeiss LSM510 confocal microscope (Carl Zeiss AG; Oberkochen, Germany). The cellular localization of nanoparticles were imaged with regard to the nucleus and cytoskeleton to confirm internalization.

Release of CDDP From CDDP-SWNH Conjugates. The release kinetics of CDDP from the two CDDP-SWNH conjugates was determined using a size-exclusive dialysis-based experiment. For both the CDDP_INC_SWNHs and the CDDP@SWNHs, 2 mg of the conjugate formed with a 1:1 w/w CDDP was dispersed in 10 mL of dH₂O inside of a dialysis membrane cellulose tube (Spectra/Por, Spectrum Laboratories Inc.; Rancho Dominguez, CA; MWCO~10,000D). The molecular weight cutoff of the tubing allowed for free CDDP to diffuse across the membrane but prevented diffusion of SWNHs or CDDP-SWNH conjugates. The dialysis membranes containing the CDDP-SWNH conjugates were immersed in 400 mL of dH₂O. Twenty milliliters of the solution outside of the tubing was taken for each time point (0–12 h) and the CDDP concentration was determined by measuring Pt concentration using Inductively coupled plasma mass spectrometry (ICP-MS) (Spectro ARCOS SOP, Spectro Analytical Instruments, Kleve Germany). The release percentage compared to the 12 h time point was expressed as a function of time.

Results

The SWNH conjugates illustrated in Fig. 1 were examined using high contrast TEM imaging to confirm the size of the nanoparticles and the presence of QDs attached to the SWNH surface and cisplatin either within the interior or on the surface of the SWNH for their respective experiments. Figure 2(a) shows the unique dahlia shape of the SWNHs, which is utilized for high drug loading by harnessing both the extensive surface area and large internal volume. This figure also shows the average diameter of the SWNHs to be approximately 80 nm, which corresponds with the appropriate size range (50–100 nm) of particles amenable

Fig. 3 Proliferation (viability) data for AY-27 cells exposed to one of three (a–c) CDDP regimens and the effect of mild hyperthermia on the efficacy of the treatment. (a) Viability for free CDDP exposed to the cells for 1 h for three heating profiles explained in methods and compares to the projected additive of mild hypothermia and CDDP. (b) Similar data for CDDP@SWNHs and (c) shows the viability when the cells are exposed to CDDP_INC_SWNHs. (d) Plots the difference in viability between 42 °C and 37 °C exposure for the three types and highlights the changes in thermal enhancement.

to the EPR phenomena resulting in the greatest uptake in the tumor microenvironment [29]. Figure 2(b) demonstrates our ability to attach QDs to the surface of the SWNHs, which enabled imaging and uptake quantification of the SWNH-QD conjugates. As we have previously shown, the average diameter of the SWNH did not increase significantly with the addition of QDs to the SWNH surface, resulting in an increase of less than 20 nm and only a slight change in the ζ potential. Therefore, the SWNH-QD can serve as a representative structure for determining the uptake of CDDP-SWNH conjugates. Figure 2(c) illustrates the aggregates of CDDP inside of the SWNHs, which confirms our methods used for incorporating the CDDP into the interior of the SWNHs to determine thermal enhancement properties. Figure 2(d) reports our ability to chemically attach the CDDP onto the surface of the SWNH. Overall the results from this study confirm the existence of both the fluorescent beacons (QDs) and the drug payload (CDDP), which is critical for our analysis of the influence of hyperthermia on drug efficacy and uptake in AY-27 rat bladder cells.

We determined the viability of AY-27 bladder cells following exposure to two types of CDDP-SWNH conjugates, incorporated and attached CDDP, and as a control for thermal enhancement, free CDDP, in AY-27 rat bladder cancer cells to determine the potential of thermal enhancement with these nanoparticle-drug conjugates.

Figure 3(a) plots the viability of three different treatment groups using a concentration of 50 μ M CDDP 72 h after exposure for 1 h. The first group shown was exposed to free CDDP at 37 °C and the average viability of the groups ($n = 8$) was 54%. As mentioned in the methods, the concentration was chosen to be clinically relevant and induce approximately 50% cell death at 37 °C. Since CDDP is a chemotherapeutic drug that is known to have synergy with heat, an additional group of cells exposed at 42 °C was studied and the resulting viability was determined to be 26%. The projective additive, which is the viability expected if there was no interplay between heat and CDDP, is calculated for this treatment by multiplying the survival fractions of exposure to

42 °C without CDDP with the survival fraction of cells exposed to CDDP at 37 °C [6]. The projected additive interaction was surpassed in this study, proving synergism between the mild hyperthermia and CDDP ($p < 0.01$). Additionally, the cells preheated at 42 °C for 2 h showed mild synergism between the CDDP exposure and heat with an overall viability of 38%, which was significantly lower than the projected additive ($p < 0.05$).

Figure 3(b) shows the viability of cells following exposure to CDDP@SWNH conjugates under identical thermal conditions as Fig. 3(a). The measured viability for the cells exposed to CDDP@SWNHs at 37 °C was 56%. Unlike the free CDDP group, the CDDP@SWNHs exposed at 42 °C resulted in a viability of 72%, a significant increase in viability compared to 37 °C ($p < 0.01$). The preheated samples showed no significant difference between the cells exposed to 37 °C and each group resulted in a higher viability than the projected additive implying there was no synergism observed with heat compared with the free CDDP studies.

The viability of the CDDP_INC_SWNHs under identical thermal conditions is plotted in Fig. 3(c). Cells exposed to the incorporated conjugates at 37 °C had an average viability of 52%. Cells exposed to 42 °C had a slightly, nonsignificantly, higher viability of 59% that was greater than the 47% viability shown in the preheated samples. Like the CDDP-SWNH conjugates, the internalized groups showed no thermal enhancement when compared to the projected additive.

When examining the viability of samples after exposure to CDDP@SWNHs and CDDP_INC_SWNHs, the thermal enhancement observed with free CDDP was not only minimized but also reversed when combined with heat, even though they had similar IC_{50} compared to the CDDP-SWNH conjugates at 37 °C. The combined results are shown in Fig. 3(d), with the viability difference between 42 °C and 37 °C indicating a -28% for the free CDDP. In contrast to this decrease in viability, a positive, or increase in viability, of 15.88% for CDDP@SWNH and 7.42%

Fig. 4 Uptake of SWNH-QDs by AY-27 cells under normal physiological conditions (37 °C) and hyperthermia(42 °C) determined by FACS cytometry. (a) Median fluorescent intensity of the cells from 0–60 min exposure of heat and SWNH-QD. (b) Plots the percent positive cells determined by gating the fluorescent signal from (c) a control sample. (d) and (e) are representative data from 37 °C and 42 °C for 1 h, respectively, showing a sharp decrease in both fluorescence intensity and percent gated positive.

for CDDP_INC_SWNH is seen when comparing 42 °C and 37 °C exposures.

In order to elucidate the mechanism causing the difference in viability observed for hyperthermia in combination with SWNH-CDDP conjugates as compared to free CDDP, we investigated whether these phenomena were due to decreased cellular uptake of the nanoparticles. Cells exposed to 0.01 mg/mL SWNH-QDs for 0, 30, and 60 min at either 37 °C or 42 °C were measured via flow cytometry to determine quantitatively the amount of nanoparticles uptaken and the influence of hyperthermia on cellular uptake. Figure 4 displays the results of this study as both a median fluorescence signal (Fig. 4(a)) and percent positive gated cells (Fig. 4(b)). It is apparent from the data that mild hyperthermia causes a statistically significant ($p < 0.01$) decrease in uptake at 42 deg when compared to 37 °C, which is determined from the decrease of 219.7 relative fluorescent units down to 145, a change in almost 35% ($n = 6-8$). Additionally, a negative sample without exposure to SWNH-QDs was used (Fig. 4(c)) for gating purposes to allow for a percent of population containing the SWNH-QDs to be calculated (Figs. 4(d) and 4(e)). The viability decline from 66.2% to 45% when exposed to 42 °C for 1 h (Fig. 4(b)) may be attributed to the decrease in treatment efficacy of these SWNH-CDDP conjugates.

While the FACS system provides valuable quantitative data, it lacks the ability to determine the intracellular distribution of nanoparticles to confirm uptake. In order to determine the cellular distribution of SWNH-QDs following exposure, immunofluorescence staining of the nucleus and cytoskeleton was performed and imaged with confocal microscopy. Figure 5 shows representative images of cellular uptake of SWNH-QDs at 37 °C (Figs. 5(a)–5(c)) and under mild hyperthermia (42 °C) (Figs. 5(d)–5(f)). Cell nuclei were stained with DAPI (Figs. 5(c) and 5(f)) and the

cytoskeleton (F-actin) was stained with phalloidin and shown in Figs. 5(b) and 5(e) in order to visualize the localization of the nanoparticles. Figures 5(a) and 5(d) provide brightfield images that show the localization and aggregation of SWNH-QDs inside of the cell after numerous cold PBS washes and 24 h incubation in fresh medium. The larger black dots observed in Figs. 5(a) and 5(d) infer strongly that the SWNH-QDs are endocytosed and aggregate within the cytoplasm. The F-actin stain seen in Figs. 5(b) and 5(e) confirm location of the particles inside the cell, not just attached to the membrane, confirming uptake. Nuclear stains (Figs. 5(c) and 5(f)) illustrate the location of the SWNH-QDs after 24 h of incubation and show that the delivered cisplatin should be capable of interacting with the nucleus due to its close proximity after 24 h.

The cell viability following exposure to 42 °C for 2 h, followed by a 2 h relaxation period, and then subsequent exposure to 37 °C was accomplished as shown in Figs. 6(a)–6(c). These data provide information regarding cellular changes due to hyperthermia that may not be active during transport kinetics such as protein expression and cytoskeleton changes that may have ultimately affected the efficacy of the drug. It was determined for CDDP@SWNHs and CDDP_INC_SWNHs that the preheat stress caused no change in treatment efficacy. In order to determine if this may also hold true for the uptake, cells underwent a similar heating regimen followed by exposure of the SWNH-QDs to 37 °C. The data for median fluorescence intensity were plotted for a control sample of cells exposed to 37 °C and a 1 and 2 h preheat group. Results from this study show that there was not a significant difference in uptake in the three samples (~50% uptake).

The difference in viability observed between the CDDP@SWNHs and CDDP_INC_SWNHs when exposed to 42 °C was noted from the viability studies and implies a change in

Fig. 5 Images of AY-27 cells exposed to SWNHs for 1 h taken 24 h after exposure. Brightfield images(a) and (d) 24 h after exposure to 0.01 mg/mL SWNH at 37 °C and 42 °C, respectively, showing nanoparticle aggregation within the cell after uptake(b) and (e) are stained for the cytoskeleton, showing internalization of the nanoparticles(red dots) at 37 °C and 42 °C, respectively, and (c) and (f) are DAPI stained to show internalization of the nanoparticles(red dots) reaches the nucleus after 24 h.

Fig. 6 Median fluorescent intensity for two different preheat stresses of 42 °C are compared to the control nonheated samples. Cells were allowed to rest for 1 h then were exposed to 0.01 mg/mL SWNH-QD for 1 h and fluorescent intensity was measured via FACS.

mechanism of action. This difference cannot be explained by a difference in uptake since the size and charge of the particles remains the same and the data from the SWNH-QD experiments should hold true for both of these groups. To determine potential differences between the samples, release rates of CDDP were measured over a 12 h time period for both of the CDDP-SWNH conjugates and the results were plotted in Fig. 7. The greatest difference observed exists for the two sets of conjugates. The CDDP@SWNHs possess an immeasurable amount of CDDP released from the SWNHs for all time points, most likely due to the strong covalent bond that was not broken within the 12 h period. The CDDP_INC_SWNH exposed to both 37 °C and 42 °C exhibit a time-dependent release profile, with majority of the CDDP being released within the first 4 h. It should also be noted that an increase in drug release in the first hour is evident in the sample exposed to 42 °C compared to the 37 °C ($p < 0.05$).

Fig. 7 Release of CDDP from CDDP_INC_SWNH (red and blue lines) and CDDP@SWNH(red dots along x-axis). The quantities of CDDP released into 400 mL water were measured via ICP-MS for platinum concentration. Results showed sufficient release of incorporated CDDP within 12 h, while no release of CDDP from the CDDP@SWNH was measured, showing stability of the bond.

Discussion

While other groups have previously explored photothermal-heat-generating nanoparticles to enhance the delivery and efficacy of chemotherapeutic agents such as cisplatin or doxorubicin, their experimental designs only utilize the nanoparticles as heat sources and not as a drug delivery vehicle [27]. This results in potentially higher efficacies as cellular uptake of the nanoparticles, and more importantly how the photothermal generated hyperthermia affects the uptake of nanoparticles, is excluded. Our objective was to utilize SWNHs as both heat generators and drug delivery vehicles to study the efficacy and transport kinetics of the drug-nanoparticle conjugate in response to hyperthermia. We conducted experiments to determine the effect of hyperthermia on CDDP-SWNH conjugates by evaluating the degree of cell death, uptake, and drug

release. While our study does neglect many of the important steps in vivo prior to the nanoparticle interacting with the cancer cell such as interaction with blood, extravasation into the tumor site, and drug clearance, this study provides insight into the effects of nanoparticle and drug size in order to enhance thermal-mediated chemotherapy for alternate drug conjugate designs.

After TEM imaging was utilized to confirm the creation of the CDDP-SWNH conjugates, the capability of heat to enhance the efficacy of free CDDP in media was determined by measuring the cell viability following treatment using the AY-27 bladder cancer cell line. We were able to show that the viability of the AY-27 cells when exposed to free CDDP at 42 °C compared to 37 °C was nearly 30% lower (Fig. 3(d), CDDP). These values are similar to prior reported literature for the breast cancer cells MCF7, in vitro that were similarly exposed to hyperthermia of 41 °C for similar durations. The decrease in viability observed in response to elevated temperatures likely corresponds to an increase in diffusion of the cisplatin into the cells due to greater membrane fluidity at the higher temperatures seen in moderate hyperthermia treatments (41–43 °C) [7]. As can be noted from Fig. 4(a) there is a significant decrease in viability in response to the 42 °C exposure compared to the projected additive, which further proves synergy in the treatment. Additionally, data from the preheat experiments imply synergism is possible even prior to exposure, which has been shown before in clinical studies involving human bladders in which the timing was shown to be important for drug enhancement with heat [45].

We then investigated whether thermal enhancement occurred when CDDP@SWNHs (CDDP covalently attached to the surface of SWNHs) and CDDP_INC_SWNHs (CDDP was located in the interior space of the SWNH) were exposed to 42 °C for 1 h. Compared to the thermal enhancement of free CDDP, we observed an increase in viability when cells were exposed to 42 °C and either CDDP@SWNHs or CDDP_INC_SWNHs ($p < 0.01$) (Fig. 3(b)). Furthermore, cells preheated to 42 °C and exposed to CDDP@SWNHs or CDDP_INC_SWNHs exhibited a similar viability as the cells maintained at 37 °C, indicating the sensitivity of cellular uptake and drug loading during concurrent heating. These results contradict other in vitro studies that show greater efficacy of CDDP when exposed to gold nanorods and NIR heating (viability decrease of almost 30% for MCF-7 cells) compared to CDDP alone, resulting in noted thermal enhancement [27]. These studies only incorporate the drug and nanorods into the culture medium without complexation of the two. Therefore, the gold nanorods were not used to transport the drug into the cells and only employed as a localized heat source in response to NIR irradiation. While these studies can show efficacy in vitro, they do not provide a system that is capable of delivery of both heat and drugs in vivo. This effect of temperature on cellular uptake of the nano-drug carrier for these multimodal therapies provides valuable information that can eventually be optimized for an in vivo setting to maximize uptake of the drug by the cells.

The difference between our lack of enhancement and enhancement previously shown with work by others with nanoparticle-mediated photothermal-chemotherapy in which the drug was not complexed with the heat generating nanoparticle [6,25,27] led us to examine the mechanism causing thermal enhancements of free CDDP as opposed to the decreased toxicity observed for CDDP-SWNH conjugates in combination with heat. We noted a large size difference between free CDDP (4 Å) and CDDP-SWNH conjugates (~100 nm). The size of free CDDP should permit simple diffusion across the cell membrane [14] while the CDDP-SWNH conjugates may necessitate endocytosis in order to enable uptake. Chithrani et al. found that spherical gold nanoparticles of 50 nm diameters, which are of similar size to our CDDP-SWNH conjugates, were endocytosed, which implies that endocytosis should be the main mechanism of intracellular transport [46]. With this in mind, we investigated the influence of hyperthermia on cellular uptake of the nanoparticles and, thus, the ultimate intracellular concentration of the drug through endocytosis. Studies of QD-

labeled SWNH conjugates confirmed that hyperthermia, while allowing for greater diffusion of smaller particles, may diminish endocytosis and halt the larger nanoparticle from entering, which is not seen with the smaller moieties. These results imply that a delivery system that utilizes a heating mechanism, but requires entrance of the nanoparticle into the cells for the drug to function, may encounter an additional barrier to treatment efficacy. This decrease in uptake almost certainly explains why the treatments at 42 °C with the CDDP-SWNH conjugates resulted in lower efficacy compared to free CDDP since these nanoparticles did not enter cells and the drug was unable to act during the 72 h incubation period. The results from our microscopy study (Fig. 5) confirm the assumptions of uptake in the quantitative data that SWNHs were internalized since they are evident in the same focal plane as the cytoskeleton and nucleus and do not correspond to only surface attached SWNHs, which would have no effect on drug efficacy. It should be noted that the nanoparticles utilized in the uptake studies were SWNH-QD conjugates that varied slightly in size from pristine SWNHs with the addition of the quantum dots. However, we have previously shown that the size changes (<20 nm) and ζ potential changes (<-18 mV) between the SWNH-QD and SWNHs allow the use of these fluorescent beacons to visualize and quantify uptake [40] as a model for pristine SWNHs.

In addition to the uptake studies in Fig. 4, the preheat stress studies help confirm that the decrease in efficacy of the CDDP-SWNH conjugates is due to reduced internalization. Figure 6 shows that for both preheat stress periods (1 and 2 h), the amount of CDDP-SWNH conjugate uptake is unaffected. Likewise, the viability of the preheated samples compared to the samples maintained at 37 °C when exposed to CDDP-SWNH conjugates was statistically insignificant. This likely indicates that the preheat stress did not affect nonhyperthermic cellular events and subsequent endocytotic uptake. It has been shown that a preheat stress is capable of inducing unknown cellular changes that result in synergism with CDDP [47,48]. By studying the uptake of CDDP-SWNH conjugates as a function of the preheat stress, the amount of drug exposed to the cells is unchanged because of a lack of modification in internalization. The preheat stress experiment confirms that the decrease in uptake occurring in response to hyperthermia results in a decreased activity of the CDDP-SWNH conjugates.

While the uptake data of the SWNH-QDs provided an explanation for the decreased efficacy of the CDDP-SWNH conjugates in response to mild hyperthermia, it did not explain the differences in viability observed after exposure to the two SWNH drug conjugates. CDDP@SWNHs exhibited a greater decrease in thermal enhancement and had a statistically significant thermal hindrance, or increase in viability. The drug release studies shown in Fig. 7 provide an explanation for the differences observed in the efficacy of the two CDDP-SWNH conjugates at 42 °C. Because the attached drug in the case of CDDP@SWNHs is covalently linked to the SWNH, CDDP is only capable of entering the cell whenever the SWNH enters. The attached CDDP was not released in the 12 h time period during exposure to either 37 °C or 42 °C. This indicates that the internalization of the CDDP@SWNH would have a larger role in the overall efficacy of treatment. The CDDP_INC_SWNHs exhibit a release profile in which most of the drug that exits the complex does so in the first 4 h. This means that during the 1 h exposure, the drug is free to enter the cell via both diffusion and endocytosis, thus minimizing the effect of uptake on treatment efficacy. Further supporting the difference in viability between the two CDDP-SWNH conjugates at 42 °C is the increased release profile in the first hour at 42 °C compared to 37 °C for the CDDP_INC_SWNH. The results from the interior-loaded CDDP follow similar trends to Ajima's group in which cisplatin was incorporated within SWNHs in a similar method [42]. However, this group did not explore the effect of hyperthermia on release. While our group envisions laser irradiating the nanoparticles, we expect there to be minimal differences between NIR

heating and water bath heating on the release kinetics of the drug. NIR photothermal heating of SWNHs occurs due to vibrations in the chemical structure that might result in an increase in detachment of the CDDP@SWNHs; however, we hypothesize this to be negligible due to the lack of detachment seen in isothermal heating. Likewise, minimal changes should be seen in NIR heating for drug leaching as diffusion is temperature dependent and minimal changes in temperature are hypothesized for the two heating methods. Our results imply that if more CDDP can interact with the cell and capitalize on hyperthermic conditions to enter the cell through increased diffusion prior to being uptaken, efficacy can be increased. Therefore, a treatment that utilizes heat generation through the nanoparticle to release more drug load at the target site prior to cell uptake would achieve the thermal enhancement associated with the free drug through increased diffusion, while additionally harnessing the benefits of nanohorn delivery such as targeting, and lower systemic side effects, and increased carrying capacity due to the large surface area of SWNHs.

Based upon the results from this study, we believe that the most efficacious thermochemotherapy treatments would selectively release the payload at the tumor site prior to uptake of the particle while additionally supplying the heat for increased diffusion. One particular method for this to occur is through a thermally responsive polymer that acts as a linker between the heat-generating nanoparticle core and the reactive drug species such as CDDP. Currently there is research utilizing thermally responsive polymers to trigger a drug release at certain temperatures, but there is little work into combining these polymers to the photoabsorbing, heat-generating nanoparticles capable of localized heating, as most of these still rely on more traditional regional heating [49,50]. The combination of these two technologies may provide the optimal platform for combined delivery of heat and drug, while additionally allowing thermal enhancement.

Conclusion

We have compared two methods to conjugate cisplatin with single-walled carbon nanohorns for use in photothermal-chemotherapy treatments. CDDP was successfully loaded into the interior and attached to the surface of SWNHs. The capability of heat to enhance the efficacy of the CDDP-SWNH conjugates was examined. Unlike free CDDP, no thermal enhancement of the efficacy of the drug in the CDDP-SWNH conjugates around the IC₅₀ of the drug in vitro was observed. FACs and microscopy were used to confirm a decrease in uptake of the conjugates at 42°C, which was most likely responsible for the decrease in efficacy. Release profiles of CDDP from the two nanoparticle conjugates indicate that careful consideration should be made to ensure the drug payload is released prior to uptake by the tumor cells to allow for full thermal enhancement. We envision a combination therapy that is capable of localized delivery of heat and chemotherapeutic drugs to the extracellular space of a tumor that will allow for optimized synergism between the modalities, impacting the treatment of bladder cancer and other malignancies after careful considerations for the implications of the drug release and uptake results from this study.

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