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Nanodiamond-Mediated Delivery of Water-Insoluble Therapeutics

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ABSTRACT A broad array of water-insoluble compounds has displayed therapeutically relevant properties toward a spectrum of medical and physiological disorders, including cancer and inflammation. However, the continued search for scalable, facile, and biocompatible routes toward mediating the dispersal of these compounds in water has limited their widespread application in medicine. Here we demonstrate a platform approach of water-dispersible, nanodiamond cluster-mediated interactions with several therapeutics to enhance their suspension in water with preserved functionality, thereby enabling novel treatment paradigms that were previously unrealized. These therapeutics include Purvalanol A, a highly promising compound for hepatocarcinoma (liver cancer) treatment, 4-hydroxytamoxifen (4-OHT), an emerging drug for the treatment of breast cancer, as well as dexamethasone, a clinically relevant anti-inflammatory that has addressed an entire spectrum of diseases that span complications from blood and brain cancers to rheumatic and renal disorders. Given the scalability of nanodiamond processing and functionalization, this novel approach serves as a facile, broadly impacting and significant route to translate water-insoluble compounds toward treatment-relevant scenarios.

KEYWORDS: nanodiamond · nanomedicine · chemotherapy · breast cancer · liver cancer

Several biomedically relevant compounds are difficult to solubilize in water, thus limiting their therapeutic potential.[1−5] These compounds have displayed remarkable therapeutic properties *in vitro* toward diseases such as liver and breast cancer.[1,2] However, since several of these therapeutics are soluble primarily in solvents generally regarded as unsuitable for injection, the realization of new routes to patient treatment enabled by these drugs has been hindered. As there remains a widespread need to package these compounds for facile delivery, a spectrum of polymeric and carbon-based nanomaterials has been explored.[6−15] For example, block copolymer-stabilized nanoemulsions have recently been explored as vehicles for polar and nonpolar agents.[6] Furthermore, lipid−polymer hybrid nanoparticles composed of lipid−PEG shells and a poly(lactic-*co*-glycolic acid) (PLGA) hydrophobic core have been developed for the release of drugs that are poorly water-soluble.[7] With regards to carbon-based strategies for the dispersal of poorly water-soluble drugs, PEGylated nanographene oxides have recently been explored for the delivery of an aromatic camptothecin (CPT) analogue.[15] The continued search for optimized nanomaterial-based strategies for the delivery of poorly water-soluble drugs has sought to develop a system that is scalably synthesized, noninvasive, and biocompatible and can be functionalized with nearly any type of therapeutic. The realization of an approach with the aforementioned properties would potentially generate significant impact across the entire spectrum of poorly water-soluble therapeutics and reveal a range of novel treatment paradigms previously precluded due to limitations in drug applicability.

Nanodiamonds (NDs) represent an important, emerging class of materials that possesses several medically significant properties.[16−36] To produce highly uniform particle diameters of 4−6 nm, NDs can be inexpensively processed *via* ultrasonication, centrifugation, and milling methodologies.[22,26] Furthermore, acid treatment to remove impurities can simultaneously result in carboxyl group surface functionalization, which can be harnessed toward subsequent drug interfacing. In addition, surface-bound carboxyl groups enable stable ND suspension in water. Therefore, these streamlined processes provide a rapid, inexpensive, and highly efficient approach toward making NDs scalable materials for medicine. Previous studies of NDs have demonstrated their carrier capabilities with Doxorubicin, cellular internalization *without* the need to coat the NDs with biocompatible or lipophilic agents, and preser-

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vation of drug efficacy upon murine macrophage and human colon cancer cell lines. Furthermore, comprehensive biocompatibility assays using quantitative real-time polymerase chain reaction (RT-PCR) interrogation of inflammatory cytokines have revealed their biocompatible properties.[26] In this study, we have shown that ND clusters are additionally capable of complexing with poorly water-soluble drugs to enhance their dispersive properties in water. To demonstrate the platform capabilities of the NDs, three drugs with important implications (Purvalanol A, 4-hydroxytamoxifen) or demonstrated relevance (dexamethasone) served as model systems. Demonstrated ND−drug interactions, enhanced dispersion in water, as well as preserved drug activity served as indicators for the broad applicability of the NDs toward delivering poorly water-soluble therapeutics to realize novel treatment routes.

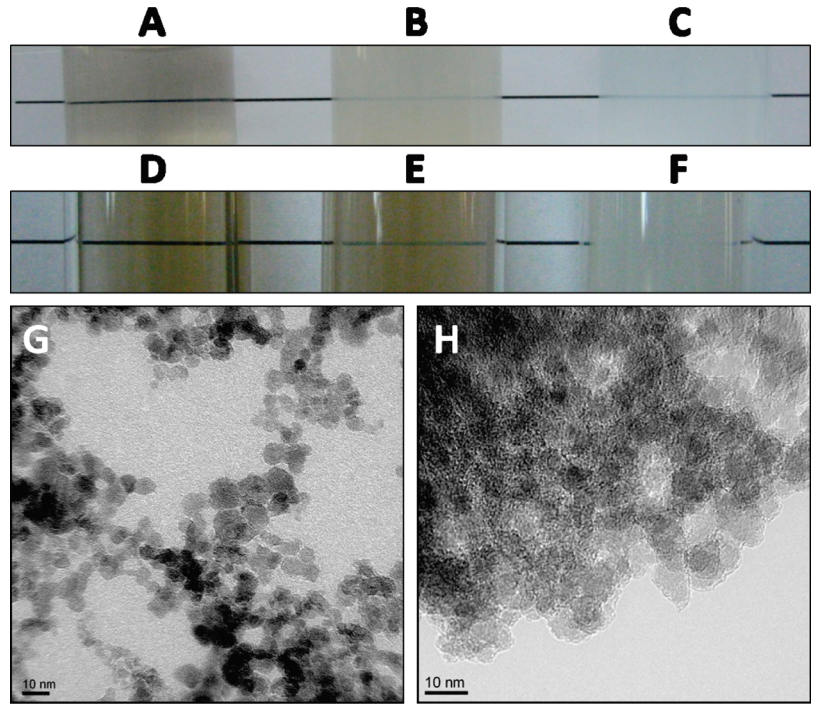

Figure 1. NDs enhance the ability to disperse Purvalanol A and 4-OHT in water. Vials were prepared against background, and the reduction in turbidity mediated by the NDs was confirmed under the following conditions: (A) 1 mg/mL ND in 5% DMSO in water; (B) 1 mg/mL ND, 0.1 mg/mL Purvalanol A in 5% DMSO in water; (C) 0.1 mg/mL Purvalanol A in 5% DMSO in water; (D) 1 mg/mL ND in 25% DMSO in water; (E) 1 mg/mL ND, 0.1 mg/mL 4-OHT in 25% DMSO in water; (F) 0.1 mg/mL 4-OHT in 25% DMSO in water. (G) TEM image of pristine NDs. (H) 4-OHT residue can be observed on the ND surface to confirm ND−drug interactions. Scale bars represent 10 nm.

RESULTS AND DISCUSSION

NDs were synthesized, purified, and processed as previously described.[22,26] Fourier transform infrared spectroscopy (FTIR) measurements confirmed the presence of carboxyl groups on the surface, which were deposited as a result of acid treatment during the purification process to remove contaminants.[26] The utility of the carboxyl groups was hypothesized to contribute to the ability to interface the NDs with drug molecules through physisorption or electrostatic interactions such that the drug could eventually be released upon external stimuli. In this study, this hypothesis was confirmed *via* a multitude of drug−ND imaging and characterization experiments and UV−vis analysis of drug−ND interfacing, in addition to functionality assays.

Due to its enormous potential as a chemotherapeutic for liver cancer, Purvalanol A was an ideal drug to complex with NDs. Soluble in DMSO, Purvalanol A is a cyclin-dependent kinase inhibitor capable of interrupting cell cycle progression. It has been shown to promote death in cell lines that overexpress *myc*, an oncogene that is often constitutively expressed in cancers. Due to the role of *myc* in cell proliferation, its overexpression or mutation often leads to cancer. 4-Hydroxytamoxifen (4-OHT), a water-insoluble breast cancer therapeutic, was selected as another model drug system due to its demonstrated efficacy against estrogen-relevant cancers. Last, dexamethasone (Dex) was selected as an additional drug model due to its broad clinical relevance as a steroidal anti-inflammatory, among other physiological conditions toward which it is applicable. All ND−drug complexes were demonstrated to be rapidly dispersible in water, indicating the potential applicability of ND platforms as scalable, water-insoluble therapeutic compound delivery agents.

In order to examine the solubility changes with the introduction of NDs, samples of NDs (20 mg/mL), ND:Purvalanol A (10:1 ratio of 20 mg/mL ND and 2 mg/mL Purvalanol A) and Purvalanol A alone (2 mg/mL) suspended in DMSO were compared. The DMSO mixtures were diluted 20-fold in water to create a 5% DMSO solution with the various mixtures of ND and drug (Figure 1A−C). Following dilution in water, Purvalanol A precipitated out of solution, producing a turbid liquid (Figure 1C). The presence of NDs significantly reduced the turbidity of Purvalanol A aqueous solutions, presumably through efficient drug adsorption to the ND surface, which implies a reduction in free Purvalanol A in solution. This surface interface between the NDs and therapeutics has been confirmed for numerous types of drugs in this study (*e.g.*, Doxorubicin, 4-hydroxytamoxifen, dexamethasone, *etc.*). It is hypothesized that physisorption is the main interaction between Purvalanol A and the NDs. We have previously demonstrated the potential for small molecule release by modulating this interaction with the addition and removal of salts.[26] Due to the reversible nature of the Purvalanol A−ND interface, the complexes served as a fa-

vorable platform for both initially dispersing the drug in water and facilitating its subsequent release.

4-Hydroxytamoxifen (4-OHT) was selected as the second therapeutic for ND−drug complexing given its importance as a triphenylethylene (TPE) treatment strategy for estrogen receptor (ER)-positive breast cancer. 4-OHT is soluble in ethanol and is often prescribed for its localized activity upon the breast even through systemic administration and therapy, which for other drugs can normally result in nonspecific effects. 4-OHT administration has been shown to reduce the risk of local recurrence, by preventing introduction of new primary tumors to the breast.[37−40]

ND-mediated enhancement of 4-OHT solubility in water was qualitatively examined and confirmed by observing degrees of visibility through vials which contained ND, 4-OHT, and ND:4-OHT samples in 25% DMSO similar to the interfacial test done with Purvalanol A (Figure 1D−F). In addition, to visually confirm ND:4-OHT interfacing, transmission electron microscope (TEM) images of NDs with and without bound 4-OHT were compared (Figure 1G,H). It was clearly observed that an amorphous 4-OHT residue was present upon drug addition to the ND solution (Figure 1H).

ND:4-OHT interfacing was further confirmed quantitatively *via* ND pulldown assays coupled with UV−vis spectrophotometric analysis (Figure 2). A wavelength scan of uncomplexed NDs revealed that the great majority of NDs pelleted upon centrifugation, leaving little ND remnants behind in the supernatant (Figure 2A). In contrast, a similar control assay with uncomplexed 4-OHT, before and after centrifugation, was performed. An insignificant change in the UV−vis absorbance demonstrated that, in the absence of NDs, the same amount of free 4-OHT resided within the supernatant despite centrifugation (Figure 2B). This reading served as a control to mark the changes in uncomplexed 4-OHT dispersion due to centrifugation. Therefore, it logically follows that ND:4-OHT complexes would pellet upon centrifugation, and uncomplexed 4-OHT would remain in the supernatant, resulting in a decrease in 4-OHT concentration in the supernatant. We tested this conjugation scheme by measuring UV−vis absorption for ND:4-OHT solutions before and after centrifugation at the same conditions and concentrations as the ND and 4-OHT controls (Figure 2B). This experiment revealed a marked change in absorbance between the uncentrifuged and centrifuged ND:4-OHT samples, which implied that a significant amount of 4-OHT was pulled down in conjunction with the NDs, possibly through ND:4-OHT physisorption and clustering. These data confirm our observation that NDs enhance the solubility of 4-OHT in 25% DMSO as compared to 4-OHT alone. The same clustering effect was observed in pulldown assays using FITC-labeled Dex−ND complexes (Figure 2C).

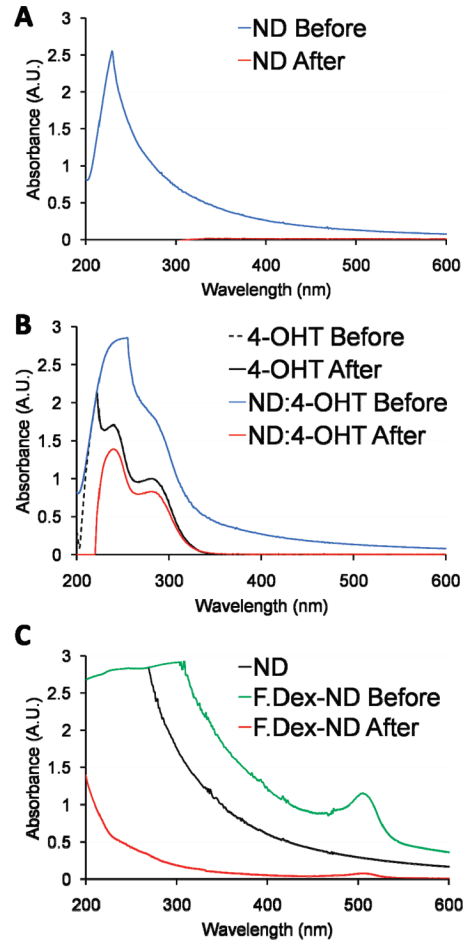

Figure 2. UV−vis spectrophotometric analysis of ND:4-OHT and Dex−ND complex pulldown. (A) UV−vis analysis of ND samples after centrifugation revealed a decrease in UV−vis absorbance, confirming the ability to utilize the NDs as agents to interface with the 4-OHT and draw the drug into the pelleted ND sample. (B) Comparative plot between the UV/vis absorbance of 4-OHT and ND:4-OHT demonstrates ND and 4-OHT interfacing. The free 4-OHT in solution decreased as a result of physisorption to NDs, which were removed from the aqueous solution *via* centrifugation. Note there is no observed effect on separating 4-OHT from the aqueous supernatant phase when NDs are absent, as illustrated by the overlapping dotted black and solid black lines representing 4-OHT before and after centrifugation, respectively. (C) Dex−ND complex formation was also confirmed as shown by the sequestering of Dex following centrifugation.

Similar to the interaction between Purvalanol A and ND, the interplay between 4-OHT and the NDs is also thought to be mainly attributed to physisorption and/or electrostatic in nature. As a result of potential dipoles that exist from the structure of 4-OHT, the presence of surface carboxyl groups could have contributed to the interfacing between the two components in order to preserve ND:4-OHT sequestering.

To determine the physical effects of the electrostatic interactions between NDs and respective therapeutics, the particle sizes and ζ potentials of the complexes were examined *via* dynamic light scattering (DLS) (Figure 3). The lack of solubility of the three drugs

is ultimately a result of particle aggregation upon titration with water from DMSO. In 5% DMSO, NDs had a mean diameter of 46.96 nm, and Purvalanol A, 4-OHT, and Dex aggregated into 340 μm, 485.1 nm, and 1.245 μm particles, respectively. Upon complexing with NDs, the average Purvalanol A, 4-OHT, and Dex particle sizes decreased to 556, 278.9, and 77.55 nm, respectively (Figure 3A−C). The decrease in particle size for all drugs tested is evidence of physisorption of drug molecules to the surface of the ND particles. These data demonstrate the ability for drug molecules to associate with NDs and, as a result, experience a significant decrease in particle size, in some cases by several orders of magnitude. Additionally, the ζ potentials of each drug were shown to become more positive upon association with NDs (Figure 3D−F). This increased ζ potential would contribute to the increased solubility of ND−drug complexes in water due to water molecules having a greater affinity for forming hydration shells around charged complexes compared to neutral molecules. Of note, larger particle sizes of NDs were observed upon titration with DMSO, an aprotic solvent. As demonstrated by the ζ potential measurements, increased neutral properties of the solution resulted in decreased solubility of the NDs and ND−drug complexes. Therefore, an ND/DMSO/water mixture and its associated potential would be capable of forming larger ND clusters compared to NDs in pure water.

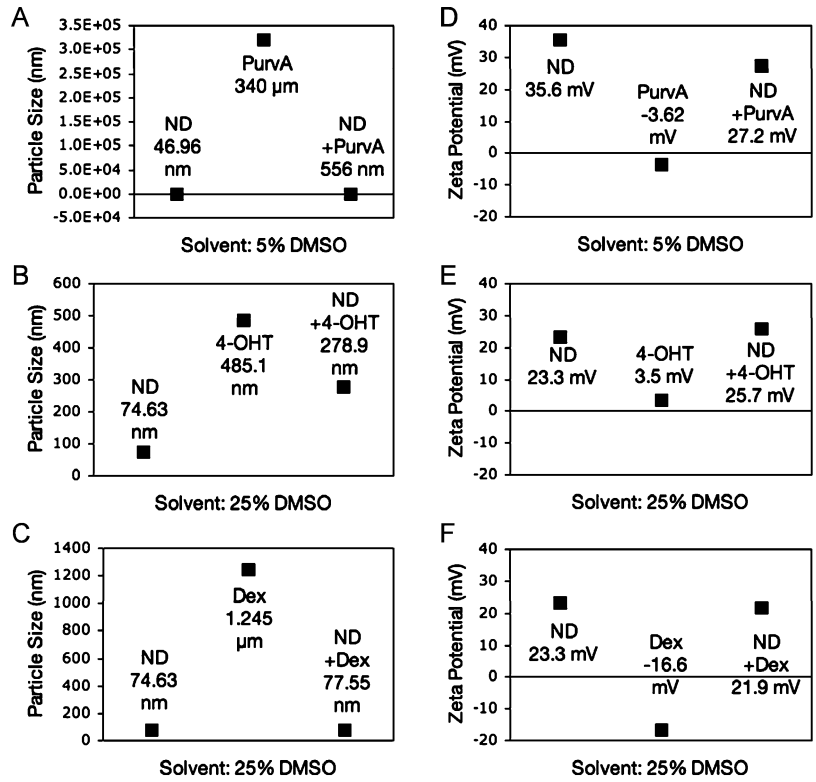

Figure 3. DLS analysis of particle size and ζ potential of ND−drug complexes. (A−C) Average particle size of all drugs decreased upon physisorption to NDs. (D−F) The ζ potential of all samples became more positive upon complexing with NDs.

Moreover, the increased drug solubility that we have demonstrated may also have potential clinical advantages pertaining to increased therapeutic efficacy as it has been shown that cellular internalization is enhanced when particles are both smaller and slightly positively charged.[41,42] Both properties are favorable for internalization across the negatively charged plasma membrane and may facilitate drug uptake *via* endocytosis and pinocytosis. Additionally, previous research has demonstrated successful cellular uptake of carriers with diameters in the hundreds of nanometers.[43−45] The NDs bring the submicrometer/micrometer size of insoluble drug aggregates into a particle size range that was conducive toward preserved therapeutic delivery and internalization.

To assess drug functionality following enhanced dispersion in water *via* ND complexing, DNA laddering assays were performed to confirm Purvalanol A-induced DNA fragmentation (Figure 4A). Fragmentation was evident in both ND:Purvalanol A and Purvalanol A samples,

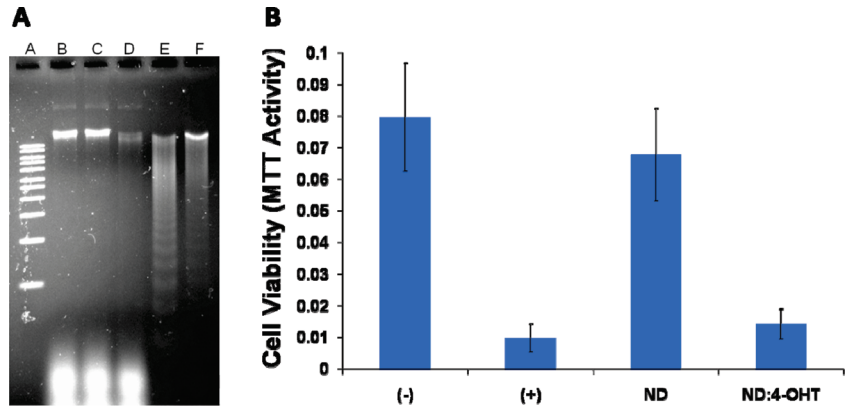

Figure 4. Therapeutic biofunctionality assays confirm maintained drug activity upon enhanced dispersion *via* ND complexing. (A) Preservation of Purvalanol A activity was confirmed *via* a DNA fragmentation assay with the following lane designations: (A) DNA marker; (B) negative control (nothing added); (C) 5% DMSO in water solution; (D) 1 mg/mL ND in 5% DMSO in water solution; (E) 1 mg/mL ND, 0.1 mg/mL Purvalanol A in 5% DMSO in water solution; (F) 0.1 mg/mL Purvalanol A in 5% DMSO in water solution. Lane E confirmed the potent activity of ND−Purvalanol A complexes. (B) MTT cell viability assays were performed to confirm the preserved therapeutic activity of 4-OHT following complex formation with the NDs. The following conditions were examined: (−) negative control; (+) positive control, 7.5 μg/mL 4-OHT; ND, 75 μg/mL ND; ND:4-OHT, 75 μg/mL ND, 7.5 μg/mL 4-OHT. All conditions were in culture media containing 1.31 mM acetic acid. Comparison of cell viability levels between the positive control and ND:4-OHT samples demonstrates preserved 4-OHT potency when complexed to NDs. One representative experiment of three is shown.

demonstrating the retained biological activity of Purvalanol after undergoing sequestration to and release from the NDs. As such, the assay attests to the capability of NDs not only to disperse a poorly water-soluble drug in an aqueous solution but also to maintain Purvalanol A therapeutic activity.

Additionally, the chemotherapeutic effects of the ND:4-OHT complexes were evaluated *via* MTT cell viability assays (Figure 4B). Figure 4B shows no significant difference in cell viability between MCF-7 cultures with and without NDs, which further confirms the reported biocompatibility of NDs. Moreover, comparison of cell viability between ND:4-OHT complexes and the 4-OHT positive control demonstrates that the ND:4-OHT complexes have the same magnitude of chemotherapeutic potency as the drug alone. Exposure to the ND:4-OHT complexes decreased cell viability over 7-fold compared to the negative control and ND cultures. Most importantly, these observations collectively confirm the ability for NDs to increase 4-OHT dispersion in water *via* formation of a water-soluble ND:4-OHT complex, while maintaining drug functionality.

This study has demonstrated the application of NDs toward enhancing water dispersion of poorly water-soluble therapeutics. Purvalanol A and 4-OHT/dexamethasone were selected as model drugs as they are characteristically soluble in DMSO and ethanol, respectively. Furthermore, due to the functionality of Purvalanol A as a broadly relevant cyclin-dependent kinase inhibitor/chemotherapeutic and 4-OHT as a potent breast cancer drug, their enhanced solubility in water is catalytic toward their continued translation to the clinical realm. NDs represent a class of medically significant nanomaterials that are potentially capable of enabling rapid and high-throughput complex formation with hydrophobic drugs to enable their suspension in water and clinically relevant applications. As such, NDs serve as scalable platforms that can facilitate facile delivery of these drugs with maintained biocompatibility.

EXPERIMENTAL METHODS

ND—Drug Complex Preparation. NDs produced *via* detonation synthesis and processed *via* ball milling and acid treatment resulting in surface-bound carboxyl groups were provided by the NanoCarbon Research Institute (Ueda, Nagano, Japan).[26] Samples of NDs (20 mg/mL), ND:Purvalanol A (10:1 ratio of 20 mg/mL ND and 2 mg/mL Purvalanol A), and Purvalanol A alone (2 mg/mL) suspended in DMSO were prepared. The DMSO mixtures were diluted 20-fold in water to create a 5% DMSO solution with the various mixtures of ND and drug.

To prepare the ND:4-OHT complexes, 1 mg of 4-OHT was solubilized in 174 mM acetic acid in deionized water. NDs (10 mg/mL) were sonicated for 4 h, added to the 4-OHT sample, and thoroughly vortexed to yield a ND:4-OHT conjugate solution (5 mg/mL ND, 0.5 mg/mL 4-OHT). Solvent only (174 mM acetic acid), ND only (5 mg/mL), and 4-OHT only (0.5 mg/mL) solutions were prepared as controls.

UV—Vis Spectrophotometric Characterization of Drug Adsorption/Desorption. Prior to scanning, all samples were diluted to concentrations of 50 and 500 μg/mL for 4-OHT and NDs, respectively. All samples underwent centrifugation at 14 000 rpm for 2 h at 25 °C, where the supernatant was then subsequently collected for spectroscopic scans from 200 to 600 nm. Drug loading concentrations were determined *via* ND complex pulldown experiments, which comprised an initial absorbance reading, then a 2 h centrifuge of all samples at 25 °C and 14 000 rpm followed by a final absorbance reading. The concentration of loaded drug was then calculated by measuring the difference between the initial and final readings.

Transmission Electron Microscopy. TEM was performed by sonicating the ND:4-OHT solution and then pipetting a droplet onto a carbon TEM grid (Ted Pella). Following 2 h of drying, a JEOL 2100F field emission gun TEM was used for high voltage 200 kV imaging. A pristine ND sample was also imaged *via* the same protocol.

Particle Size and ζ Potential Measurement. The particle size and ζ potential of the complexes were measured using a Zetasizer Nano (Malvern Instruments). ND:4-OHT and Dex—ND complexes were prepared in 25% aqueous DMSO as described previously. ND:Purvalanol A complexes were prepared in a similar manner in 5% aqueous DMSO as described previously. The final concentration of ND and therapeutic in all complexes was 1 and 0.1 mg/mL, respectively. All size measurements were performed at 25 °C at a 90° scattering angle. Mean hydrodynamic diameters were obtained *via* cumulative analysis of 11 measurements. The ζ potential measurements were performed using capillary wells at 25 °C, and the mean potential was obtained *via* cumulative analysis of 15 measurements.

DNA Fragmentation Assays. A 1:10 dilution of 5% DMSO in water, NDs in 5% DMSO in water (1 mg/mL), ND:Purvalanol A in 5% DMSO in water (10:1 ratio of 1 mg/mL ND and 0.1 mg/mL Purvalanol A), and Purvalanol A in 5% DMSO in water (0.1 mg/mL) were added to HepG2 tissue culture cells and grown for 24 h. The cultured cells were lysed in 500 μL lysis buffer (10 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% Triton X-100). Thirty minute incubations at 37 °C followed separate RNase A and proteinase K treatment. Following phenol chloroform extraction, nuclear DNA was isolated in isopropyl alcohol and stored at −80 °C overnight. The samples were then resuspended in DEPC water following a 70% ethanol wash and electrophoresed using a 0.8% agarose gel, and finally stained with ethidium bromide.

MTT Cell Viability Assay. MCF-7 cells were plated to 50% confluence in 96-well plates in pH 7.1 MEM/EBSS culture media containing 75 μg/mL NDs or ND:4-OHT complexes (75 μg/mL ND, 7.5 μg/mL 4-OHT), and 7.5 μg/mL 4-OHT was used as a positive control. All samples accounted for the 1.31 mM acetic acid associated with the 4-OHT ND complex solution. Cultures were maintained at 37 °C, 5% CO_2 for 44 h prior to performing the MTT-based cell viability assay according to the manufacturer's protocol (Sigma-Aldrich). Absorbances were determined at 570 nm using a Safire multiwell plate reader (Tecan) and Magellan software (Tecan). All samples were run in triplicate.

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