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Synthesis of Nanodiamond-Daunorubicin Conjugates to Overcome Multidrug Chemoresistance in Leukemia

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Abstract

Nanodiamonds (NDs) are promising candidates in nanomedicine, demonstrating significant potential as gene/drug delivery platforms for cancer therapy. We have synthesized ND vectors capable of chemotherapeutic loading and delivery with applications towards chemoresistant leukemia. The loading of Daunorubicin (DNR) onto NDs was optimized by adjusting reaction parameters such as acidity and concentration. The resulting conjugate, a novel therapeutic payload for NDs, was characterized extensively for size, surface charge, and loading efficiency. A K562 human myelogenous leukemia cell line, with multidrug resistance conferred by incremental DNR exposure, was used to demonstrate the efficacy enhancement resulting from ND-based delivery. While resistant K562 cells were able to overcome treatment from DNR alone, as compared with non-resistant K562 cells, NDs were able to improve DNR delivery into resistant K562 cells. By overcoming efflux mechanisms present in this resistant leukemia line, ND-enabled therapeutics have demonstrated the potential to improve cancer treatment efficacy, especially towards resistant strains.

Keywords

Nanodiamond; Drug Delivery; Nanomedicine; Leukemia; Chemoresistance

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Background

Leukemia is the sixth deadliest cancer in the United States as of 2011 [1]. While higher incidence cancer types, such as lung and prostate cancer, have experienced sharp declines in death rate, leukemia patients have not benefitted from a similar trend [1, 2]. A major challenge with treating leukemia is the development of resistance, particularly after exposure to chemotherapeutics [3]. While standard treatments, including chemotherapy drugs, tyrosine kinase inhibitors, and immunosuppressants [4, 5], can be effective in combating leukemia, response between patients can vary greatly due to the expression of multi-drug resistance (MDR) [5-7]. In fact, MDR is often attributed to relapse in leukemia patients who may initially exhibit promising response to therapy [6-8].

MDR is a type of resistance to a wide spectrum of cancer therapeutics that develops after exposure to only a single drug. This general type of resistance is often caused by the overexpression of adenosine triphosphate (ATP)-dependent efflux pumps called ATP-binding cassette (ABC) transporter proteins, which are normally responsible for the transport of a broad range of xenobiotics, lipids, and metabolic products across the cell membrane [9]. Attempts in the past two decades at neutralizing this effect have centered on developing competitive inhibitors, with limited success [10, 11]. First generation pharmacological inhibitors were challenged with high toxicity levels. Second and third generation inhibitors aimed to mitigate these problems, but have not been very promising in clinical trials [9]. Recent findings, however, have demonstrated that the use of nanoparticle-based drug carriers may be able to combat chemoresistance in many types of cancer [12, 13]. Nanoparticles offer a promising alternative for bypassing drug efflux mechanisms. By entering the cell through endocytosis, nanoparticle drug delivery can increase intracellular drug concentration and improve treatment efficacy (Figure 1, A). Further benefits conferred by nanoparticle delivery include protection of payload from both biotransformation and rapid systemic clearance. However, there are still hurdles with creating a nanoscale biomaterial for *in vivo* drug delivery that is biologically compatible, easily modified chemically to carry a variety of useful payloads, and able to combat drug efflux mechanisms [14].

A class of nanoparticles called nanodiamonds (NDs) offers a promising combination of high biocompatibility, scalability in production, and the capability to enhance therapeutic efficacy [15-18]. NDs have demonstrated outstanding compatibility in an array of biological environments at therapeutically relevant concentrations [19, 20]. NDs have been chemically modified to carry several key classes of cancer therapeutics including water insoluble drugs [21, 22]. Therapeutic gene delivery has also been demonstrated using ND platforms after surface functionalization with polymers [23-25]. The ND platform has been able to enhance the signal of MRI contrast agents by leveraging powerful ND surface interactions with water molecules [26]. NDs have also been incorporated into a variety of implantable devices for localized tumor therapy, demonstrating the capacity to improve drug release profiles [27, 28]. Notably, NDs have recently shown the ability to overcome drug efflux and increase apoptosis in liver and mammary tumors *in vivo*. In many examples, NDs not only facilitated the delivery but also enhanced the function of its payload [29].

In this study, we have demonstrated ND-enabled delivery and therapeutic enhancement in applications beyond solid tumors. NDs were engineered to reversibly bind and release the chemotherapeutic daunorubicin (DNR) *via* electrostatic interactions between the ND surface and DNR molecules. The loading of DNR was optimized by varying binding conditions such as pH and drug loading ratio. Subsequently, the ND-DNR (ND-R) conjugate was extensively characterized for size, surface charge, and loading efficiency. MDR was conferred to a K562 myelogenous leukemia cell line *via* incremental exposure to DNR.

After cellular resistance was confirmed by measuring gene expression, the efficacy of ND-R was compared with drug alone. Using the resistant leukemia cell line, the ability for NDs to enable the treatment of non-solid tumor cells exhibiting MDR was confirmed.

Materials and Methods

ND-R loading and optimization

To form ND-R conjugates, DNR was reversibly loaded onto ND platforms. ND and DNR were mixed at various ratios (w/w), followed by adjusting pH to basic conditions to promote binding. After incubation (5 min, 25° C), the solution was centrifuged (15 min, 2500 rpm, ~1450 g) to pellet bound ND-R. Unbound DNR remained in the supernatant and was subsequently removed. This supernatant was used to quantify unbound DNR to calculate the amount of DNR bound. The pelleted ND-R was resuspended in water using probe sonication (3x, 10 seconds).

A ND:DNR w/w ratio of 5:1 and 5:2 were tested. NaOH concentration was varied to adjust pH of binding conditions. 1.5, 2, 2.5, 3.75, 5 mM and 2, 3, 4, 5, 7, 10 mM of NaOH was tested for the 5:1 and 5:2 ratios respectively. For more details, see Supplementary Materials (available online at <http://www.nanomedjournal.com>).

ND-R characterization

The loading of DNR onto NDs for various binding conditions was quantified by measuring the unbound DNR in the supernatant after binding. DNR release from the ND platform was tested under various pH conditions by incubating ND-R in different pH solutions while subject to physiological conditions (37° C). pH environments were adjusted using NaOH and HCl for basic and acidic conditions respectively. ND-R was resuspended in water and pH 2, 4, 10, 12 conditions. At various time points (1, 2, 24, 48, 72 hrs), ND-R was repelleted (15 min, 2500 rpm, ~1450 g) and supernatant removed to quantify drug release. For more details, including information on the dynamic light scattering (DLS), transmission electron microscopy (TEM), and fourier transform infrared spectroscopy (FTIR) measurements, see Supplementary Materials.

Modeling ND-R using Molecular Dynamics

ND surface charge was determined using DFTB (Density Function Tight Binding) calculations as well as Henderson-Hasselbalch equation, which were described in the previous paper [30]. This ND model previously showed a good agreement with experiments in ND-PEI-siRNA bindings [30]. The atomic structure of DNR and charge distribution of DNR atoms was obtained using General Amber force field (GAFF). For more details, see Supplementary Materials.

Inducing resistance in K562 leukemia cells

A K562 myelogenous leukemia cell line was purchased (ATCC, VA, USA) and propagated according to ATCC guidelines. Resistance was conferred to the cell line *via* incremental exposure to DNR. Considering various procedures that have been previously established for conferring resistance [31, 32], we incubated K562 cells (5 ml total volume in 25 cm² flasks, VWR, PA, USA) with stepwise increments of DNR at 10 nM during each passage. A final DNR concentration of 150 nM was reached and a resistant K562 cell line was established, referred to in this manuscript as K562/DNR. For more details, including information on gene expression measurements and analysis, see Supplementary Materials.

Measuring Cell Viability

Cell viability was quantified using an MTT colorimetric assay (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). Treatment conditions ($n = 3$) were applied to different wells of the 96-well plate (1.5×10^4 cells) and incubated for an additional 48 hours. For testing ND biocompatibility (Figure 1, *B*), Sodium dodecyl sulfate (SDS) (Sigma Aldrich) was used as a positive control at a 1% concentration by volume. For more details, see Supplementary Materials.

Evaluating ND-R efficacy using IC-50 values

The effect of DNR and ND-R on the growth of K562 and K562/DNR was evaluated by calculating the IC-50 values for all 4 combinations. Cell viability was determined using MTT assays for a wide range of therapeutic concentrations. Equivalent drug concentrations for DNR and ND-R were used so that informed comparisons could be made. 0.1, 1, 10, 25, 50, 100, and 1000 $\mu\text{g/ml}$ of DNR were incubated with each cell line (Figure S2). The ND-R binding results were used to calculate the effective DNR concentration.

The cell viability results were converted to % cell death and plotted against logarithmic concentrations. Plots were fitted with a sigmoidal dose-response curve using Prism 5 software (Graphpad, CA, USA). The bottom and top of the curve fit were constrained to 0 and 100 percent cell death respectively for more consistent fitting results. IC-50 values were averaged over 3 trials of each combination of therapeutic and cell line to obtain mean $\pm$ SE values.

Results

The ND platform was engineered to form a delivery system capable of combating resistant leukemia by shuttling DNR. Requisite of effective delivery vehicles, NDs have demonstrated outstanding biocompatibility [33-35]. Figure 1, *B* shows the biocompatibility of varying ND concentrations exposed to K562 cells. Remarkably, cells exhibited >90% viability after exposure to 0.0005 - 0.5 mg/ml NDs, compared with <5% viability for positive control (SDS 1%). ND platforms also possess robust binding characteristics, able to load a wide range of biological components in addition to therapeutics [36-39]. Most intriguing of all is the demonstration that NDs are capable of treating resistant solid tumors *in vivo* [29]. ND-R conjugates were engineered to take advantage of these three crucial properties and tested against a resistant leukemia cell line.

DNR loading and optimization

DNR has been used extensively for treatment against leukemia [40]. This drug is similar to another anthracycline, DOX, which also intercalates DNA within cell nuclei to kill cancer cells. The ND-DOX (NDX) model has been proven to be very successful in treating resistant solid tumors in mouse models [29]. The established binding characteristics of DOX to NDs were leveraged to load DNR, which has similar chemical properties.

To optimize the loading efficiency of DNR, binding ratios of 5:1 and 5:2 (w/w) were tested. In previous studies, NDX was formed at 5:1 mixing ratio, in 2.5 mM NaOH binding conditions. Using this baseline, conditions were varied to maximize the loading of the novel drug combination of DNR and NDs. In an attempt to improve the loading efficiency of ND-R, a higher ratio of drug was testing, using a 5:2 ratio instead of 5:1. Figure 2, *A* depicts images of the binding process for mixing NDs and DNR (bottom row), with NDs alone (no DNR) subject to the binding process for comparison (top row). The red color is characteristic of DNR and the ND:DNR solution turns a deep purple color after 3mM of NaOH has been added, corresponding to a pH of 11.5. After pelleting the ND-R conjugates

through centrifugation, the supernatant is a light purple color in column (iii) bottom row, indicating the small amount of unbound DNR. Figure 2, *B* is a graph of the loading efficiency at various binding conditions. Two metrics were used to measure loading efficiency, the absolute DNR loading efficacy measuring the amount of DNR loaded per mg of NDs (dashed line) and procedural DNR loading efficiency measuring the amount of DNR loaded as a percentage of the initial DNR added during the ND-R synthesis process (solid line). The 5:2 conjugates are a substantial improvement in absolute DNR loading efficacy over the 5:1 conjugates (dashed line). In the former, ~ 0.3 mg of DNR was loaded per mg of ND, contains almost 2 times more DNR per mg of ND as the 5:1 conjugates, which loaded ~ 0.15 mg of DNR per mg of ND. Additionally, the DNR loading efficiency is very similar between the two, peaking at ~ 80% of initial DNR bound. Choosing the most effective binding condition yields the 5:2 binding ratio, with 3 mM of NaOH (red box), which represents both the highest amount of DNR bound per mg of ND and the best percentage of DNR bound. The 80% binding is used to calculate the effective drug content for a solution of ND-R conjugates.

ND-R characterization

After optimizing the loading conditions to bind DNR to NDs, the ND-R conjugates were characterized to verify successful loading and inform on platform characteristics such as particle size and DNR release. DLS was used to measure the average diameter of ND and ND-R, shown in Figure 3, *A*. NDs possess unique surface characteristics, with both positively and negatively charged facets that mediate strong interactions with water molecules and other ND particles [41]. NDs suspended in water formed aggregates that equilibrated at a diameter of 50.7 ± 3.3 nm. After binding with DNR, the conjugates equilibrated at 93.1 ± 8.2 nm, with the increase in size suggesting additional layers of bound DNR.

The Figure 3, *A* inset also depicts TEM images of ND and ND-R. Examining the visualization of ND and ND-R surfaces, there is a translucent coating present on the ND-R surfaces as compared with ND surfaces. The ND image clearly shows white boundaries between individual ND particles stacked together. However, the ND-R image shows a textured, bumpy layer covering the individual NDs, obscuring the white boundaries between individual ND particles. This coating suggests surface modification, which qualitatively indicates DNR loading.

For further evidence of chemical modification, FTIR spectra were taken comparing ND, DNR, and ND-R (Figure 3, *B*). ND surface analysis has determined that the dominant functional group on NDs is primarily carboxylic acid groups [42]. Consistent with this finding, the ND spectra contained peaks for the C=O stretch and O-H bend signals, the primary structures found in the carboxylic acid group (Figure 3, *B*, green arrows). Examining the DNR spectra, there were strong signals for peaks corresponding to the C=C stretch and C-O-C stretch (Figure 3, *B*, red arrows), characteristic of the abundance of C=C and C-O-C structures on the DNR molecule, and not present on the ND spectra. Confirming the binding of DNR, the ND-R spectra contained strong peaks that were characteristic of both the ND and DNR spectra, even those unique to only DNR.

It is important for successful drug delivery agents to not only load a variety of drugs, but to also be able to release the therapeutics in the proper biological setting. The drug release profile of ND-R was quantified for a variety of pH conditions (Figure 4, *A*). At basic pH values (pH 10, 12) close to the pH at binding, minimal drug release was observed. Less basic conditions of pH 7 and pH 4 resulted in steady DNR release over the first 24 hours, tailing off for the next 48 hours. At extreme acidic conditions (pH 2) most DNR is released

within the first 6 hours. The 6-hour time point for pH 2 had a high variance characteristic of burst release.

Modeling ND-R using molecular dynamics

The effective binding and release of DNR from ND platforms was confirmed by performing molecular dynamics (MD) simulations describing the effects of pH on the degree of DNR release in cellular environments. Figure 5, *C* shows the initial structure of a ND with 100 DNR molecules bound to its surface. In the figures, visualizations of water molecules were removed to clearly depict ND-DNR interactions. Figure 5, *D* shows the simulation results at pH 2 where 92% of DNR molecules (92 out of 100) were unbound from the ND. Figure 5, *E* shows the simulation results at pH 4 where 76% of DNR molecules are released from ND surface. Figure 5, *F* shows the simulation results at pH 7 where 13% DNR molecules are released from ND surface.

Inducing resistance in K562 leukemia cells

A resistant K562 cell line was necessary to test the efficacy of the ND-R conjugates. K562 cells were incubated with increasing doses of DNR until a 150 nM resistance was reached. Although there are numerous (> 48 genes) ABC transporters that have been discovered, the most extensively studied efflux proteins attributed to MDR are ABCB1, ABCC2, and ABCG2. Comparing between sensitive and resistant K562 strains, quantitative PCR was performed to measure the mRNA gene expression levels for these three genes (Figure 4, *B*). Consistent with previous studies [3, 7], ABCB1 and ABCC2 levels were significantly ($p < 0.05$) increased in the resistant line. While ABCG2 has been observed in some cases of resistant leukemia strains [3, 7], the correlation is not as strong [43]. This was the case for our K562/DNR cell line, with a moderate ($p > 0.05$) increase in ABCG2 expression.

Evaluating ND-R efficacy using IC-50 values

IC-50 values, the half maximal inhibitory concentration, quantify the effectiveness of an antagonist in inhibiting biological function. In this case, it is the amount of therapeutic necessary to cause 50% cell death. Dose response curves were constructed over a wide range of therapeutic concentrations and fitted to a sigmoid function (Figure 6). The data sets were well described by sigmoidal fits (avg. values of $r^2 > 0.94$ over all fits) (Table S1).

As indicated in Figure 6, *A*, K562/DNR cells were more than 4 times more resistant to DNR (51.7 ± 7.4 versus 12.1 ± 1.8 mM Table S2) than sensitive K562 cells. Proportional levels of resistance have been observed in previous studies [44], where a 500 nM K562 line (~4 times more resistant than our 150 nM K562/DNR) exhibited a 15 times decrease in IC-50 over sensitive K562. This result is further evidence for the successful development of resistance in K562/DNR.

Remarkably, ND-R conjugates were able to induce a 3-fold reduction in the IC-50 value of K562/DNR compared with DNR, from 51.7 ± 7.4 to 17.6 ± 0.9 mM (Table S2). ND-R treatment of K562/DNR was able to exhibit similar performance to DNR treatment of sensitive K562 (17.6 versus 12.1 mM), demonstrating the improved efficacy of ND-R towards combating drug efflux. ND-R was able to treat K562 only slightly better than DNR (8.1 versus 12.1 mM).

Discussion

Cancer therapies can fail due to increases in activity of cellular efflux mechanisms. Higher levels of chemotherapeutic efflux lead to reductions in intracellular drug concentration and subsequent progression of drug insensitivity [9]. The three most prominent MDR

transporters typically attributed to chemotherapeutic efflux, are ABCB1 (MDR1, P-glycoprotein), ABCC2 (MRP1), and ABCG2 (BCRP, MXR). Overexpression of these transporters has also been correlated with more aggressive phenotypes of cancer and malignant progression, such as cell migration and invasion [9]. Overcoming MDR is a serious problem that confronts many cancer therapies. To improve efficacy and reduce toxicity, current approaches are becoming increasingly specific, targeting the action of a single type of transporter protein. Not only do these approaches require precise diagnostic screening techniques, but they are also ineffective against multi-transporter resistance characteristic of MDR. Methods that utilize nanoparticles such as NDs as drug delivery vehicles may be useful towards developing a non-specific strategy to bypass efflux mechanisms in MDR.

Active drug efflux has been linked to causing resistance to anthracyclines, such as DOX and DNR. Classes of chemotherapeutics including anthracyclines are taken up by cells through passive diffusion. Upon entering cells, transporter proteins can detect and bind to these substrates and efflux them from the cell. Nanoparticles are able to shuttle their cargo into cells through different pathways, primarily endocytosis, which can bypass transport proteins and significantly improve drug efficacy by increasing intracellular drug concentration [12, 13]. DNR is an anthracycline that mediates cell death by entering the nucleus and intercalating DNA strands. It must be released from its transport vehicle in order to pass into the nucleus to take effect since nuclear pores are not large enough to accept nanoparticles larger than 40 nm [45]. Since ND-R is taken up by cells *via* endocytosis, ND-R will be subject to the acidic conditions of the endosome and lysosome, allowing DNR to release in the cell. The ND-R must not release DNR until it is in the cell, so that efflux is bypassed. The drug must leave the carrier because it has to enter the small nuclear pores and DNR can only take action when it is in the nucleus. The positive release characteristics of ND platforms, represented in Figure 4, is crucial towards the effective delivery of DNR.

In this study, ND-R has demonstrated the ability to improve treatment of resistant K562 leukemia cells. DNR was reversibly bound to ND platforms by leveraging the surface electrostatic properties of NDs. Sodium hydroxide surface treatment deprotonated surface carboxylic acid groups mediating substantial DNR loading. The resulting ND-R conjugate exhibited many crucial properties required of successful delivery agents, such as positive biocompatibility, narrow size distribution centered under 100 nm in diameter (93.1 nm), and readily reproducible and scalably processing techniques. DNR release profiles were stable and consistent in intracellular pH environments, as evidenced by its efficacy in treating resistant leukemia cells, and its steady release profile at pH 4 shown in Figure 4. This therapeutic system was able to outperform DNR alone for treating K562/DNR that exhibited significant overexpression for genes responsible for two major efflux proteins by reducing the IC-50 value 3-fold.

NDs have previously demonstrated the potential to treat resistant tumors in mouse models by delivering DOX. [29] This model served as a guide towards tuning the ND-R conjugate. The ND-R conjugate used in this study improved the drug loading amount and efficiency of DNR over DOX by increasing the mixing ratio from 5:1 to 5:2. Correspondingly, higher levels of NaOH chemical treatment were necessary to encourage binding.

Taking advantage of unique ND surface properties makes this procedure possible. The surfaces on an ND are highly charged and heavily functionalized by carboxylic acid groups [41]. At basic conditions, these groups become deprotonated and interact with protonated amines on the DNR molecule. The adsorption mechanism for loading DNR is a quick and simple process that leads to a reversibly bound drug conjugate. These strongly charged facets, however, promote polar interactions with other highly charged groups such as water

molecules or other NDs. As a result, NDs are prone to aggregating into larger particles in higher pH environments. However, ND-R particles had an average diameter of 93 nm after resuspension. It is encouraging that ND-R is able to remain under 100 nm in diameter *via* post-loading processing including probe sonication. There are many practical requirements to drug delivery for platforms to retain a tight size distribution within the range of 10-150 nm [13, 46]. Smaller particles under 10 nm are eliminated by the kidney through renal excretion. Larger particles are cleared by the mononuclear phagocytic system cells. Indeed, only particles less than 150 nm are able to cross the endothelial barrier (which may skew towards larger particles in leaky tumor vasculature). An added advantage towards treating leukemia, particles between 30-150 nm typically locate in bone marrow, the site where a majority of leukemia resides, because fenestrations in marrow endothelium measure 80-150 nm. It is important that platforms like ND-R are properly sized so that they can move safely and effectively through the body.

The size distributions in Figure 3 are taken from representative samples of ND and ND-R. A narrow size distribution, indicated by the width of the peak for the size distribution of ND-R (Figure 3, A), is also an important characteristic of a consistent and reliable processing technique. Utilizing a new therapeutic in a clinical setting requires scalability and reliability not only within samples, but also across different batches. A measure of consistency, the low standard deviation of ND-R (8.2 nm) was calculated across multiple batches produced separately.

IC-50 curves demonstrated the positive benefits of ND-platform DNR delivery. The over-expression of ABCB1 and ABCC2 in K562/DNR functionally manifested itself in its poor response to DNR. However, ABCG2 was not overexpressed in this resistant leukemia strain, which may have been a byproduct of this particular protocol. This particular efflux protein is most often attributed with mitoxantrone exposure. In past studies, correlation with leukemia relapse has not been absolute [43]. Improved IC-50 values for K562/DNR treated with ND-R versus DNR revealed that ND platforms were able to maintain binding while bypassing efflux pumps and still release drug within the cell. Shielding the drug before internalization is an advantage to using ND drug carriers with the added benefit of its sustained and steady release profile.

Ultimately, ND-R conjugates resulted in significantly better performance over DNR in killing resistant K562 cells. The IC-50 of K562/DNR dropped 3-fold with treatment from ND-R. This simple and scalable loading process requiring minimal preprocessing was able to achieve highly effective results. DNR treatment of resistant cells was highly variable, evidenced by the high standard deviation in IC-50 value, since it was severely affected by the constant action of efflux proteins. However, ND-R treatment obtained more consistent results since ND-R relied on endocytosis for internalization and avoided the variability caused by the drug efflux activity. Like other nanoparticles, NDs have been shown to primarily enter cells through endocytic uptake pathways [16]. The spectrum of pH environments in the endocytic pathway, from early stage endosome (~ 5) to late stage lysosome (~ 4.5), range from 4 – 5 [47]. The graph of DNR release in Figure 4, A shows that ND-R has a steady and sustained release profile at pH 4. Conversely, highly basic pH values cause negligible drug release while highly acidic pH values cause ND-R to exhibit burst release. Burst release can be a dangerous byproduct of drug delivery, and NDs have been able to demonstrate positive release profiles free from burst release in multiple studies [28, 29]. MD modeling has demonstrated efficacy in predicting and describing the behavior of nanoscale systems, especially for ND-based structures [30, 48]. Simulations have already been used to investigate the binding of NDs to a therapeutic with similar chemical structure as DNR (DOX) [48]. Instead, this study utilized MD simulations to confirm the effective release of DNR from ND carriers. Since binding occurs at basic pH values, acidic pH values

(2, 4, and 7) were tested. Confirming experimental results, DNR did not remain bound to NDs at acidic pH values during the simulations, exhibiting increased drug release at more acidic pH values. The reversible drug loading of the ND platform possesses release characteristics that match very well with the environments of its cellular uptake mechanism. K562 treated with both DNR and ND-R, on the other hand, yielded very similar IC-50 values since they were not affected by efflux and contained equivalent drug concentrations.

Further development of ND systems is still necessary to realize its clinical potential. Following this *in vitro* demonstration, studies must be continued in animal models to verify the safety of this delivery platform. Along with demonstrating excellent biocompatibility *in vitro*, including improved safety profiles compared with other similar carbon nanoparticles [19, 20], murine models have indicated excellent biological response and clearance [29]. Upon administration of therapeutically-relevant doses of NDs, inflammatory responses remained low and histological analysis did not reveal any significant changes in the tissue. Even more encouraging were the clearance results, which indicated that a vast majority of NDs were cleared from all major organs by day 10. The positive clearance profile of NDs is extremely relevant to leukemia treatment since high dosages may be required to reach cancerous blood cells throughout the body. However, introducing a targeting component may significantly reduce necessary dosages and increase the safety of treatment. Great progress has been shown in incorporating targeting capabilities using antibodies as capture ligands, while preserving the release profile mediated by the ND surface, using lipid encapsulation [49]. The successful development of these techniques may make possible the potential translation of ND-R therapy.

The diversity of the ND platform provides the possibility for added functionality. It is worth exploring if the electrostatic binding properties of NDs can be leveraged for other drugs, outside of anthracyclines. Multi-therapeutic systems have demonstrated improved performance [50, 51]. Combined with its aptitude for gene delivery, ND systems can be designed for combination drug/gene platforms. Introducing a gene therapy component would provide capabilities to not only bypass efflux mechanisms but also actively reduce the expression of the proteins causing it. The design of these new ND platforms will be aided by the advances in multiscale modeling, which has been shown to accurately inform the behavior of ND-PEI gene loading and release [30]. These ND models built from first principles have led to improvements in our ability to understand and manipulate NDs in experiments. Creating ND-enabled methods to combat MDR is a significant step in the process of ND development that has the potential of unlocking exciting new ways to improve cancer treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(A) An artistic rendering of the response from a resistant cancer cell to treatment from DNR versus ND-R. On the left, ABC transporter proteins are able to detect DNR within the cell and remove it *via* efflux pumps. On the right, ND-enabled delivery of DNR is able to bypass the efflux pumps. The reversibly-bound DNR is released in the cell and enters the nucleus, causing apoptosis. (B) Cell viability after exposure to varying concentrations of NDs, demonstrating the biocompatibility of NDs, even at higher concentrations.

Figure 2.

(A) Images depicting the DNR loading process. The top row shows a sample of NDs processed without DNR and the bottom row shows ND and DNR at a 5:2 mixing ratio. (i) The first panel shows ND and ND:DNR before any treatment. (ii) The second panel shows ND and ND:DNR after the binding environment has been adjusted to 3 mM of NaOH. (iii) The third panel shows the pelleting of ND and ND-R conjugates after centrifugation. (iv) The fourth panel shows the resuspended solution after supernatant from part (iii) was removed and replaced by an equal volume of water. (B) Graph of the DNR loading efficiency for various binding conditions. The red box indicates the optimal binding condition tested and the conditions used for the rest of the study.

Figure 3.

Characterization of the ND-R conjugates formed by the process outlined in Figure 2. (A) A graph of the size distribution of ND (solid line) compared with ND-R conjugates (dashed line) obtained using DLS. The increase in size of ND-R indicates the loading of DNR onto the ND surface. The inset images are taken using TEM, to visualize the differences in the surfaces of ND (top) and ND-R conjugates (bottom). The clear boundaries between ND particles are visible in the top image. The bottom image shows the textured surface present on the ND-R conjugates. This organic layer is indicative of the binding of DNR. (B) FTIR spectra of ND (gray line), DNR (red line), and ND-R (purple line). The spectra confirms the loading of DNR onto ND platforms because the ND-R spectra contains peaks that are characteristic of both ND and DNR spectras.

Figure 4.

(A) The release profile of DNR from ND-R conjugates under different pH environments. The graph tracks the DNR elution over a period of 72 hours. At pH 4, matching conditions ND-R particles would experience during the endocytic pathway, the elution profile is relatively steady and sustained. (B) A comparison of the gene expression (mRNA) of resistant versus sensitive versions of the K562 cell line for the three major efflux proteins in the ABC transporter family. These efflux proteins have been attributed to causing MDR in many cancers. Consequently, the resistant version of K562 has exhibited an increase in expression. ** $p < 0.05$

Figure 5.

Molecular dynamic simulations of ND and DNR to confirm DNR release from ND carriers. (A) The NDs considered in the model were 4.1 nm in diameter and consisted of 5795 carbon atoms. They were shaped like truncated octahedrons, with 8 hexagonal surfaces and 6 square surfaces with [111] and [100] facet planes respectively. (B) DNR molecules consist of atoms of carbon (cyan), hydrogen (gray), oxygen (red), and nitrogen (blue). (C) The initial structure of a simulated ND-R with 100 DNR molecules bound to the ND surface. (D) DNR release at pH 2. (E) DNR release at pH 4. (F) DNR release at pH 7.

Figure 6.

Examining the efficacy of DNR and ND-R by measuring the cellular viability of K562 and K562/DNR after exposure to range of drug concentrations. (A) A summary of the IC-50, or half maximal inhibitory concentrations of DNR and ND-R treatments. ND-R exhibited improved efficacy towards treating resistant K562 as compared with DNR. The resistance of K562/DNR is confirmed by the 4 fold increase over K562 in IC-50 value when exposed to DNR. $**p < 0.5$ (B) K562 + DNR (avg. $r^2 = 0.97$) (C) K562/DNR + DNR (avg. $r^2 = 0.96$) (D) K562 + ND-R (avg. $r^2 = 0.94$) (E) K562/DNR + ND-R (avg. $r^2 = 0.96$) dose response curves fitted to a sigmoid function. Each graph contains three trials with separate sigmoidal curves fitted to each trial. Mean IC-50 values are averaged over these three trials.