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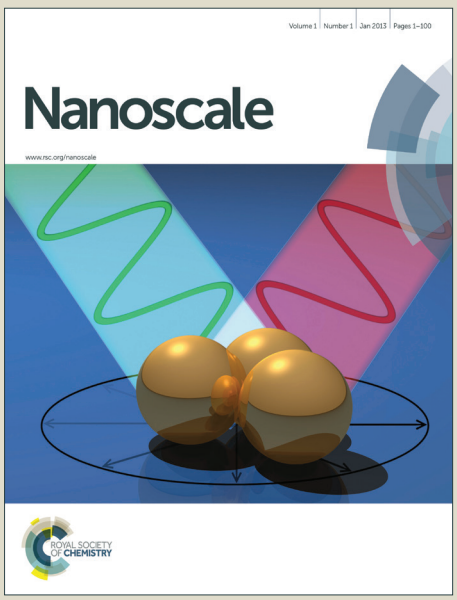

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ARTICLE

Effect of Drug Release Kinetics on Nanoparticle Therapeutic Efficacy and Toxicity

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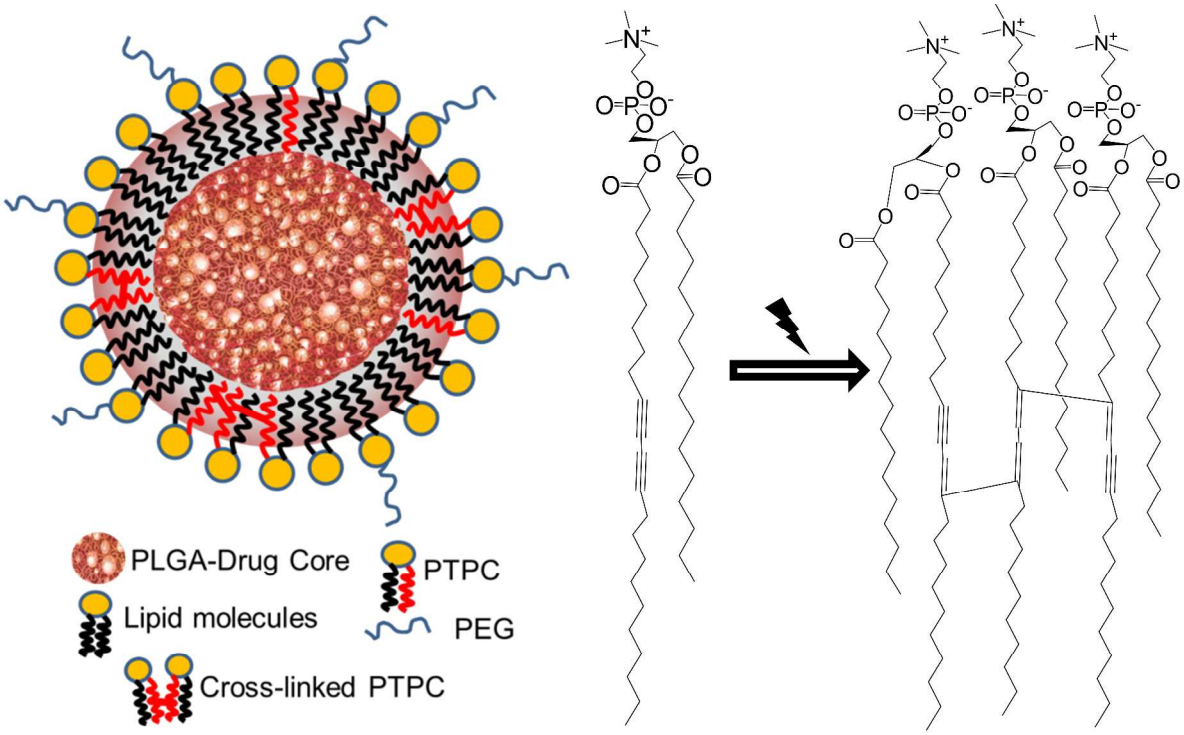
The effects of nanoparticle (NP) properties, such as size, shape and surface charge, on their efficacy and toxicity have been studied extensively. However, the effect of controlled drug release on NP efficacy and toxicity has not been thoroughly evaluated *in vivo*. Our study aims to fill this knowledge gap. A key challenge in characterizing the relationship between drug release and therapeutic ratio is to fabricate NPs that differ only in their drug release profile but are otherwise identical. To overcome this challenge, we developed crosslinkable lipid shell (CLS) NPs, where the drug release kinetics can be modulated without changing any other NP property. Using CLS NPs with wortmannin and docetaxel as model drugs, we determined the relationship between the release and therapeutic efficacy and toxicity of the drugs. We have determined that drug release kinetics can affect the therapeutic efficacy of NP docetaxel and NP wortmannin *in vitro* and *in vivo*. Our study also demonstrates that a decrease in drug release kinetics can result in a decrease in the hepatotoxicity of CLS NP wortmannin. Using two model drugs, the current findings provide the first direct evidence that NP drug release profile is a critical factor in determining the NP therapeutics' efficacy and toxicity *in vivo*.

Introduction

Advances in nanomedicine have led to the rapid clinical translation of nanoparticle (NP)-based therapeutics.[1-3] NPs possess several unique properties, such as preferential tumor accumulation or at sites of inflammation and low distribution in normal tissue, which make them well suited for the treatment of diseases such as cancers.[1, 2, 4, 5] Preclinical research has studied and established that many NP characteristics, such as size, shape, surface properties and drug loading, can affect therapeutic efficacy, biodistribution, pharmacokinetics and toxicity of NP-drug formulations.[6-12] Such knowledge has facilitated the optimization of NP drug delivery systems and the clinical translation of NP-based drugs.[2, 13, 14] However, the effect of controlled drug release, a property that is shared by many NP drug delivery systems, has not been thoroughly evaluated. To date, there has been no *in vivo* study comparing the therapeutic efficacy as well as toxicity of NPs that differ only in their drug release kinetics. Given that many of the NP therapeutics currently under clinical and preclinical investigation have controlled drug release profile, understanding the relationship between drug release kinetics and NP therapeutic efficacy and toxicity can be critical to the success of these formulations.[15-22] In this study, we aimed to address this by examining the effect of drug release profile from the NP formulations of two model drugs on their toxicity and therapeutic efficacy *in vitro* as well as *in vivo*.

A key challenge in studying the effects of drug release kinetics has been the ability to compare NPs that have different release kinetics but are otherwise identical. Most NP platforms do not allow changes to drug release without changes to other NP properties such as the shape, size or surface charge. Keeping most parameters identical is critically important in order to minimize the number of variables for the accurate determination of the effect of drug release kinetics. To overcome this challenge, we developed a novel strategy to independently control drug release without changing other NP properties. We have engineered NPs that only differ in release kinetics by incorporating a cross-linkable lipid into an existing lipid-polymer NP platform. The lipid-polymer NP is comprised of a hydrophobic polymeric core of poly (lactic-co-glycolic acid) (PLGA) and an outer lipid shell. The lipid shell is a self-assembled monolayer of lipids (lecithin) and lipid-polymer conjugates (2-distearoyl-sn-glycero-3-phosphoethanolamine – poly(ethylene glycol) or DSPE-PEG).[3] The PEG molecules on DSPE-PEG form the outer layer of NP and are thus largely responsible for the NP's surface properties.[21] Drug release kinetics from these NPs is a two-step process: first is the simple diffusion, which is controlled by the lipid monolayer interface and second is the combined effect of diffusion and degradation of the hydrophobic polymer core.[23] We theorized that we can independently vary the NP drug release kinetics by altering the lipid-monolayer interface without changing NP size, shape and its outer surface. To modify the drug diffusion across lipid monolayer, we incorporated a polymerizable lipid (1-palmitoyl-



Scheme 1. Schematic representation of CLS NPs showing intermolecular cross-linking between PTPC side chains at the NP interface **(left)**, PTPC structure **(center)** and one of the putative cross-linking reactions among the side chains **(right)**.

2-(10,12-tricosadiynoyl)-*sn*-glycero-3-phosphocholine (or PTPC)) into the lipid interface layer of the NPs (**Scheme 1**). The side chain of the PTPC contains triple bonds that can cross-link under ultraviolet light (UV)-irradiation with the adjacent PTPC molecules as also shown in Scheme 1. We have termed these modified NPs the cross-linkable lipid shell (CLS) NP. We hypothesized that the CLS NPs with different PTPC content would have differential drug release kinetics. Furthermore, given that the PTPC molecules are incorporated in the lipid-monolayer interface and not exposed to NP surface, we also theorized that NPs with different PTPC content would have the same physical properties.

To study the relationship between NP drug release kinetics and the therapeutic efficacy and toxicity of the drugs they carry, two chemotherapeutic agents, docetaxel (Dtxl) (M.W= 807.9 g/mol) and wortmannin (Wtmn) (M.W= 428.4 g/mol), were utilized. Dtxl was selected as it is one of most extensively studied chemotherapeutic in nanomedicine.[24] It is a highly hydrophobic drug that reversibly binds to microtubles with high affinity. We have recently shown that at sub-therapeutic dose levels Dtxl NPs can have a differential cellular response compared to free Dtxl and this difference can potentially be due to controlled drug release.[25] Wtmn is another highly hydrophobic drug that selectively and irreversibly inhibits PI-3 kinases.[26] Wtmn was chosen because we had recently observed that a polymeric NP formulation of Wtmn has lower hepatotoxicity than free Wtmn, despite the high accumulation of NP Wtmn in the liver.[26] To study the effects of drug release kinetics of Dtxl and Wtmn on their efficacy and toxicity, we prepared CLS NP formulations of each therapeutic. The drug release profiles of the CLS NP formulations were determined. The effect of drug release kinetics on the efficacy and toxicity of CLS NP Dtxl and CLS NP Wtmn were investigated *in vitro* and further validated *in vivo*.

Experimental

1. Materials

1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-carboxy (polyethylene glycol) 2000 (DSPE–PEG2000–COOH) and the cross-linkable lipid (1-palmitoyl-2-(10,12-tricosadiynoyl)-sn-glycero-3-phosphocholine (PTPC) were obtained from Avanti Polar Lipids (Alabaster, AL). Docetaxel (Dtxl) and wortmannin (Wtmn) were purchased from Sigma-Aldrich (St. Louis, MO). PLGA (poly(D,L-lactide-co-glycolide)) with a 50:50 monomer ratio, ester terminated, and viscosity of 0.72–0.92 dl/g was purchased from Durect Corporation (Pelham, AL). Soybean lecithin consisting of 90–95% phosphatidylcholine was obtained from MP Biomedicals (Solon, OH). 200 Proof Ethanol (Molecular Biology Grade) and Acetonitrile (HPLC Grade) were purchased from Fisher Scientific (Hampton, NH). Dulbeco's Phosphate Buffer Saline (PBS) – 1X was purchased from Gibco by Life Technologies (Carlsbad, CA). H460 (lung cancer) was purchased from UNC's Lineberger tissue culture facility and KB (head and neck) was purchased from ATCC.

2. Formulation and characterization of NP with cross-linking lipid:

PLGA-Lecithin-PEG-core-shell NPs were synthesized from PLGA, soybean lecithin, and DSPE-PEG-COOH using a previously reported nanoprecipitation technique.[3] Lecithin (1 mg/mL in 4% ethanol) and DSPE-PEG-COOH (1 mg/mL in 4% ethanol) (7:3 molar ratio) were dissolved at 15% of the PLGA polymer weight and heated to 55.0 °C. Different molar percentages of PTPC of the total lipid content were added, depending on the specific particle desired (0%, 5%, or 10% CLS NP). Dtxl or Wtmn were dissolved at a dosage of 10% (wt/wt) of the polymer into the PLGA/acetonitrile solution (10 mg/mL) before nanoprecipitation. The PLGA/Dtxl (or Wtmn) acetonitrile solution was then added dropwise to the heated

aqueous solution under vigorous stirring followed by 3 min of vortexing. The NPs were allowed to self-assemble for 1 hour with continuous stirring under vacuum. The NPs were then photo-cured under UV light (365nm) using a UV lamp (UVP LLC., Upland, CA) for 25 minutes. The NP solution was washed twice using an Amicon Ultra-4 centrifugal filter with a molecular weight cut-off of 30 kDa and then resuspended in PBS to obtain a final desired NP concentration of 1 mg/mL.

3. Characterization of NP with cross-linker lipid:

CLS NP size (hydrodynmic diameter in nm), polydispersity, and surface charge (ζ-potential in mV) were obtained with Zetasizer Nano ZS System (Malvern, Inc.) by using 1 : 10 dilution of NPs (1 mg/mL) with PBS at 25˚C. For the analysis, Malvern's proprietary default algorithms (general purpose and auto mode) were used to determine the Z-ave (average diameter) and the ZP (average surface charge), respectively. TEM images were obtained at the Microscopy Services Laboratory Core Facility at the University of North Carolina-Chapel Hill, School of Medicine. NP samples were diluted (5X) in deionized water before mixing with 2% aqueous uranyl acetate solution (1:1) that was used as a negative stain. Separately, 400 mesh formvar-carbon filmed copper grids were glow discharged followed by the addition of a 20 µl droplet of the particle suspension. After a few minutes, the suspension was carefully drawn using an edge of a filter paper and then air dried. Micrographs were obatined using a LEO EM910 TEM at 80 kV (Carl Zeiss Microscopy, LLC, Thornwood, NY), and digital images were taken using a Gatan Orius SC1000 digital camera with Digital Micrograph 3.11.0 software (Gatan, Inc., Pleasanton, CA).

CLS NPs	Size (d.nm)	Zeta Potential (mV)
0%	49.3 ± 1.7	-21.4 ± 0.9
5%	49.5 ± 0.9	-24.1 ± 0.3
10%	50.1 ± 0.4	-20.7 ± 0.9

Table 1: 0%, 5% and 10% CLS NPs showing similar surface charge and hydrodynamic diameters.

4. NP drug release analysis:

Drug release studies of CLS NP formulations of Dtxl and Wtmn were conducted using our previously described technique.[27] To measure the release profile, NP solutions at a concentration of 1 mg/mL were split into Slide-ALyzer MINI dialysis microtubes with molecular cutoff of 10 kDa (Pierce, Rockford, IL) and subjected to dialysis against 4 L phosphate buffer saline (PBS) with gentle stirring at 37°C. PBS was changed periodically during the process. At the indicated times, 30 µL of solution was removed from the microtubes and mixed with 200 µL acetonitrile to dissolve the NPs. Dtxl or Wtmn content was subjected to quantitative analysis using an Agilent 1100 HPLC (Palo Alto, CA) equipped with a C18 chromolith flash column (Merck KGaA Darmstadt, Germany). Dtxl absorbance was measured by a UV–VIS detector at 228 nm and a retention time of around 7.5 min in 0.25 mL/min acetonitrile/water gradient mobile phase of 11 min; Wtmn absorbance was measured at 254 nm and a retention time of around 8.1 min. Wilcoxon two group test was performed for statistical analysis.

5. Cell Culture:

Cancer cell lines were maintained in house after purchasing from their respective sources. KB cells were cultured using Eagle's Minimum Essential Medium supplemented with 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin. H460 cells were cultured using RPMI-1640 medium supplemented with 10% (v/v) FBS, 2.0 mM glutamine, 1.5 g/L sodium bicarbonate, 10 mM HEPES buffer (Corning), 1.0 mM sodium pyruvate, 1% (v/v) penicillin/streptomycin and 4.5 g/L glucose (Sigma). Everything was manufactured by Gibco unless otherwise specified and purchased from UNC's tissue culture facility.

6. *In vitro* Cytotoxicity:

In a 96-well plate, 1 x 10^4 H460 or KB cells were plated 24 h prior to treatment with CLS NP Dtxl, CLS NP Wtmn or free drugs. The cells were treated with 2.0 µM of Dtxl or 5.0 µM of Wtmn either without NPs or encapsulated in 0% or 10% CLS NPs for 1.0 h. The cells were subsequently washed with PBS and allowed to grow for another 24 h. Cell viability was then analyzed using an MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)] assay. Briefly, the cells were washed with PBS, followed by the addition of a 120 µL mixture of their respective culture medium containing 20% MTS reagent (Promega) and 1% phenazine methosulfate (PMS) as the electron coupling reagent (Promega), directly to culture wells. H460 cells were incubated for about 30 min and KB cells were incubated for about 1.0 hour after which the plates were read at the absorbance value of 490 nm using a 96-well plate reader (BioTek, Synergy 2) Student t-test was performed for statistical analysis.

7. *In vivo* tumor efficacy:

H460 cells (1 x 10^6 cells in 200µL 1:1 RPMI-1640 and matrigel) were subcutaneously inoculated into left flank of 6-8 week-old male nude (*nu/nu*) mice. Ten days after inoculation, the mice were randomly distributed into different groups for subsequent treatment. Mice (n=7 per group) were administered either saline, free Wtmn (70 µg/kg), free Dtxl (0.5mg/kg), 0% or 10% CLS NP Wtmn or Dtxl at 70 µg/kg (Wtmn) or 0.5mg/kg (Dtxl) dose via a tail vein injections. The tumors were then irradiated with two doses (3 Gy) of radiation at 6.0 h and 24 h post injections with XRAD 320. Mice were shielded with a specially designed lead shield allowing radiation of the tumor site and minimal radiation to other organs. The tumor volumes were measured every 2 days, and relative change in tumor volume was calculated using the relation V/Vo, where V is the volume calculated and Vo is the initial volume on day 0 (ten days after the inoculation). For statistical analysis, wilcoxon two group test was performed.

8. Hepatotoxicity Study:

NP Wtmn with 0% or 10% PTPC was tail vein i.v. injected at a dose of .7 mg/kg into male CD1 mice (n=5 per group). Blood was collected from the mice 24 hours post-injection via submandibular bleed. The blood samples were centrifuged at 3000 rpm for 10 minutes to separate the plasma. The plasma was then submitted to the Animal Clinical Laboratory Core Facility at UNC School of Medicine for analysis of AST and ALT levels, which studied the samples provided using an automated chemical analyzer (VT 350, Ortho Clinical Diagnostics, Rochester, NY). Student t-test was performed for statistical analysis.

Results and discussion

Results

CLS NP formulations of Dtxl and Wtmn were synthesized that contained 0%, 5% and 10% molar concentration of PTPC. NPs were formulated based on a previously developed

nanoprecipitation method.[3] As seen table 1, physical properties of CLS NPs such as size and surface charge were determined using dynamic light scattering (DLS) and zeta potential analyses, respectively. The hydrodynamic diameters of 0%, 5% and 10% CLS NPs were found to be 49.3 ± 1.7 nm, 49.5 ± 0.9 nm and 50.1 ± 0.4 nm while the surface charge values were -21.4 ± 0.9 mV, -24.1 ± 0.3 mV and -20.7 ± 0.9 mV, respectively. The size, morphology and dispersity of the NPs were also validated using transmission electron microscopy (TEM). Figure 1 shows that the CLS NPs are monodisperse with similar spherical shape and size. Further, TEM micrographs were also subjected to quantitative analysis by using ImageJ software such that the diameters of at least 300 NPs per sample were analysed, showing a very similar NP size distribution profile (Supporting information, Figure S1) Further, all the NPs were found to be stable over a period 4 days when dispersed in 10% fetal bovine serum (FBS) and analyzed using DLS (Supporting information Figure S2). Based upon the high performance liquid chromatography (HPLC) results, the drug loading was found to be 1% w/w for Dtxl and 2% w/w for Wtmn compared to NPs.

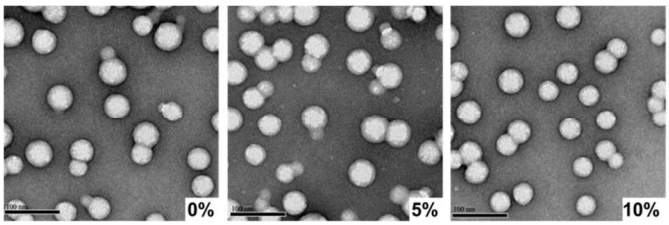


Figure 1: TEM images of 0%, 5% and 10% CLS NPs (left to right). Scale bar = 100 nm.

CLS NP formulations of Dtxl and Wtmn were subjected to drug release studies and the release curves of each NP were then plotted (Figure 2). As seen in Figure 2a, about 65% of the Dtxl is released in first 9.0 h from 0% CLS NPs while only about 30% of the drug is released from 10% CLS NPs in the same time period. The difference was slightly less pronounced in case of Wtmn (Figure 2b), where about 55% of the drug is released in first 9.0 h from 0% CLS NPs, while 30% is released in same period from 10% CLS NPs. Rate of diffusion of free Wtmn and Dtxl dissolved in DMSO in equivalent concentrations as their NP counterparts is shown in Figure S3 for comparison, which demonstrates that in each case of the free drug, about 75% of the drug is lost in first 6.0 h. Also seen in Figure S3, is the release rate 5% CLS NPs, which shows an intermediate rate between 0% and 10% CLS NPs, with no statistical difference as compared to 0% CLS NPs and thus was not used in the further study. Further, higher concentrations of PTPC (≥ 20%), also did not show any significant decrease in rate of drug release compared to 10% CLS NPs.

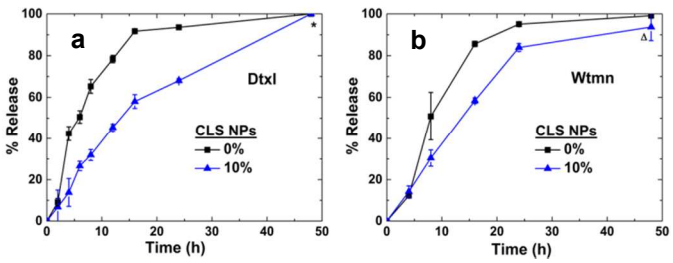


Figure 2: Drug release profiles of 0% and 10% CLS NPs using **(a)** Dtxl and **(b)** Wtmn. (* p= 0.002, $^{\Delta}$ p= 0.05, between 0% and 10% CLS NPs)

To study the effect of difference in release kinetics of different formulations on the *in vitro* efficacy of CLS NP Dtxl and CLS NP Wtmn, the NPs were incubated for one hour with two different human cancer cell lines (H460 (a non-small cell lung cancer) and KB (a head and neck cancer)). The cytotoxic effects of 0% and 10% CLS NP Wtmn as well as 0% and 10% CLS NP Dtxl on the cell lines were determined. Cell viability was analyzed using an MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)] assay. As seen in Figure 3a, 62% H460 cells and 53% KB cells remained viable after treatment with 10% CLS NP Dtxl while 68% H460 cells and 60% KB cells remained viable after treatment with 0% CLS NP Dtxl. As seen in Figure 3b, 0% and 10% CLS NP Wtmn showed 2% and 11% cytotoxicity in H460 cells, while 6% and 15% cytotoxicity in KB cells, respectively. The effects of free drugs and empty NPs are shown in supporting information (Figures S5 and S6).

To confirm our *in vitro* findings, we compared the therapeutic efficacy of 0% and 10% CLS NP Wtmn as a radiosensitizer in *nu/nu* mice bearing the H460 cells (subcutaneously on their left flank). As seen in Figure 4b, the NP formulations of Wtmn (0.07 mg/kg) are more effective than free Wtmn or radiation only. More importantly, we observed a significant difference in tumor growth between 0% and 10% CLS NP Wtmn with the latter being more effective. Similarly, Dtxl (0.5mg/kg) was administered in mice bearing subcutaneous flank xenografts of H460 cells, where 10% CLS NPs were also found to be significantly more effective in inducing cytotoxicity than 0% CLS NPs (Figure 4a). All the appropriate controls with free drugs or empty NPs show no significant difference in the efficacy when compared to saline or radiation only and are presented in the supporting information, Figure S7. To detemine the effect of drug induced hepatotoxicity, higher doses of Wtmn (0.7 mg/kg) or its NP counterparts were injected into the mice. As seen in table 2, both CLS NP formulations of Wtmn led to elevations in ALT but not in AST, consistent with drug-induced hepatotoxicity. More importantly, the 10% CLS NP Wtmn was found to possess a considerably lower hepatotoxicity (64 ± 30.25) than 0% CLS NP Wtmn (133 ± 37.90).

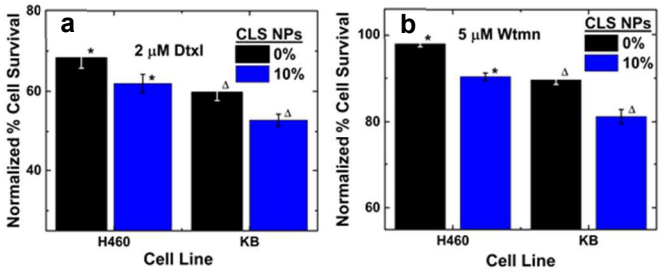


Figure 3: In vitro cytotoxicity of **(a)** 2 μM Dtxl and **(b)** 5 μM Wtmn comparing 0% and 10% CLS NPs in H460 and KB cells.(*, $^{\Delta}$ p < 0.05)

Discussion

The two main objectives of this research are: (a) to devise a strategy to formulate NPs that only differ in drug release kinetics, and (b) to study the effects of rate of drug release on the therapeutic efficacy and toxicity of NP formulations. To accomplish these objectives, CLS NPs were synthesized by incorporating different concentrations of PTPC molecules. As shown Figure 1, changing the PTPC concentration did not alter

the surface charge, size, shape or morphological properties nor the drug loading capacity of the CLS NPs. This is likely due to the fact that PTPC has a very similar molecular structure to lecithin (Scheme 1), the main component of the NP surface. Based upon the overall structure of the NPs, the lipid side chain of PTPC gets incorporated into the interface between the hydrophobic core and the hydrophilic shell of the NPs, resulting in no change in their surface properties. However, we observed a slower drug release profile for both Dtxl and Wtmn with an increase in the molar composition of PTPC. As seen in Figure 2a, PTPC's effect on release kinetics is more dramatic in Dtxl NPs as compared to Wtmn NPs. While the difference in the release profile of 0% and 5% CLS NPs containing Wtmn is not as pronounced (Figure S3), the trend of higher PTPC content leading to slower release profile is preserved. Further, we observed a significant difference between 0% and 10% CLS NPs Wtmn. These observations confirm that the drug release kinetics of the CLS NPs can be modified simply by controlling the concentration of and thus, the degree of cross-linking between PTPC side chains. Overall, we were able to engineer the NPs with differential drug release properties that are otherwise identical in size, shape and surface properties. However, when the percentage of PTPC was increased to more than 10%, we did not observe any further change in the drug release rate, suggestive of a saturation limit of PTPC incorporation into the NPs.

1/3 Maximum Tolerated Dose	Normal Range	0% CLS NP	10% CLS NP
ALT U/L	40-50	133 ± 37.90*	64 ± 30.25*
AST U/L	40-50	27.50 ±11.12	29.25 ± 9.94

Table 2. Hepatotoxicity of Wtmn in 0% and 10% CLS NPs at one-third maximum tolerated dose. *p<0.05

The development of CLS NPs enabled us to evaluate the effect of drug release kinetics of the NPs on therapeutic efficacy and toxicity of the encapsulated drugs. The *in vitro* cytotoxic effects of 0% and 10% CLS NP Wtmn or Dtxl on two different cell lines shows that 10% CLS NPs are significantly more cytotoxic than 0% CLS NPs, in both H460 cells and KB cells. These observations are consistent with previous findings, which show that a sustained release of chemotherapeutics is more effective in inducing cytotoxicity.[15-17, 22, 28-32] Further, we observed that the difference in the cytotoxicity of 0% and 10% CLS NP Wtmn in H460 was not as pronounced as in KB cells. This could be largely due to the relative insensitivity of H460 cells to NP Wtmn (Supporting information Figure S4). *In vitro* analysis of free Wtmn, Dtxl or empty CLS NPs shows significantly lower cytotoxicity compared to 0% and 10% CLS NPs as seen in Figures S5 and S6. These results, consistent with previous literature, demonstrate that rate of drug release can directly affect the NP therapeutics' efficacy *in vitro*.

In order to further develop our understanding of a relationship between drug release kinetics and its therapeutic efficacy *in vivo*, we compared the effects of different CLS NP formulations using mice bearing flank tumor xenografts. For both Dtxl and Wtmn, 10% CLS NPs were more effective (in conjunction with radiotherapy) than 0% CLS NPs. Further, the biodistribution of NPs was similar at 24 h among the different NP formulations (Supporting information Figure S8). These results suggest the difference in therapeutic efficacy is primarily due to difference in drug release. Pegylated NPs of average diameters of 50-80 nm have relatively long blood circulation half lives.[33] While this is beneficial, this can also lead to an increased drug loss in circulation. As such, the slower drug release profile can thus lead to increased bioavailability of a drug at the site of tumor, leading to a better efficacy. While we think that the radiation helped in obtaining a better curve separation by providing a synergistic effect with a slower drug release profile, this nevertheless, shows that drug release kinetics can have a direct effect on therapeutic efficacy of NPs.

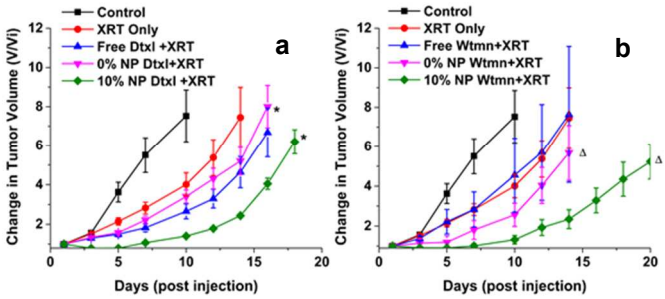


Figure 4: *In vivo* efficacy of **(a)** Dtxl demonstrating the radiosensitization effect of 0% and 10% CLS NPs Dtxl compared to free Dtxl and radiation only in H460 cells. **(b)** Wtmn demonstrating the radiosensitization effect of 0% and 10% CLS NPs Wtmn compared to free Wtmn and radiation only in H460 cells (*, $^{\Delta}$ $p < 0.05$ between 0% and 10% CLS NPs)

We have previously reported that NP formulation of Wtmn can reduce its toxicity profile, specifically, the dose-limiting hepatotoxicity.[26] In this study, we evaluated whether changes in drug release profile can further lower the hepatotoxicity of Wtmn. Our results showed that there is significant difference in hepatotoxicity between 0% and 10% CLS NPs with 10% CLS NP being less toxic. This implies that reducing the NP Wtmn's drug release kinetics can lead to a further reduction in NP Wtmn's hepatotoxicity. Such results are intriguing as NPs in general are cleared by the liver (Supporting information Figure S8). The lower hepatotoxicity of 10% CLS NP Wtmn is likely the result of slowed Wtmn release which leads to less insult to hepatocytes at a given time. However, studies confirming this hypothesis are currently underway. Together with the *in vivo* efficacy data, our findings provide the first direct evidence of an increased NP efficacy and reduced toxicity through the modification of drug release kinetics.

It is important to note that while our studies have shown that slower drug release leads to higher therapeutic efficacy, such results are produced within a range of drug release kinetics. It is highly likely that if drug release kinetics are reduced further, the therapeutic efficacy may eventually diminish as the drug exposure to tumor cells would be too low to produce effective cytotoxicity. These findings have strong implications for the clinical development of NP therapeutics. Based upon these results, we believe that the clinical development of a NP therapeutic should include an optimization process for the drug release kinetics. The current study can be employed for the synthesis of NP-based chemotherapeutics that may provide novel therapeutic capabilities and a higher therapeutic ratio based on their unique controlled drug release profiles. We also acknowledge that our work is based on only two therapeutics and more detailed/systematic evaluations are necessary to fully characterize the relationship between drug release kinetics and therapeutic efficacy and toxicity of NP formulations.

Conclusions

In summary, our research has examined the effects of drug release kinetics on the therapeutic efficacy and toxicity of NP based chemotherapeutics. Using Dtxl and Wtmn as model drugs, we found that drug release can directly affect therapeutic efficacy and toxicity of nanoformulations. While previous studies have explored changing NP composition to alter the drug release profiles,[15-17, 22, 28-32] this is the first report that has provided direct evidence of a relationship between drug release kinetics and NP therapeutic efficacy and toxicity *in vivo*.

Acknowledgements

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Notes and references

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Electronic Supplementary Information (ESI) available: [NP size distribution, DLS analysis showing NP stability, rate of diffusion of free Wtmn and Dtxl, in vitro cytotoxicity of different concentrations of Wtmn, in vitro cytotoxicity of free Wtmn, Dtxl and empty NPs, in vivo efficacy of free Wtmn, Dtxl and empty NPs and NP biodistribution are available.]. See DOI: 10.1039/b000000x/

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For Table of Contents:

Effect of Drug Release Kinetics on Nanoparticle Therapeutic Efficacy and Toxicity

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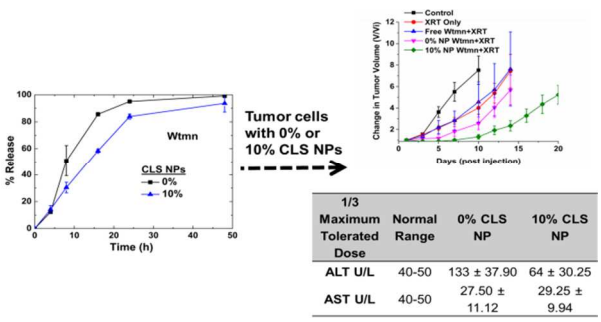
Graphical Abstract



1/3 Maximum Tolerated Dose	Normal Range	0% CLS NP	10% CLS NP
ALT U/L	40-50	133 ± 37.90	64 ± 30.25
AST U/L	40-50	27.50 ± 11.12	29.25 ± 9.94

Identical nanoparticles that differ only in their drug release kinetics demonstrate that it directly affects drugs' therapeutic efficacy and toxicity.