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# Lipid crosslinking enhances the stability of nanolipoprotein particles in serum by multiple orders of magnitude

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# Lipid crosslinking of nanolipoprotein particles substantially enhances serum stability and cellular uptake

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*nanoparticles, drug delivery, rHDL, NLP, DiynePC, serum stability, cross-linking*

## Abstract

Nanolipoprotein particles (NLPs) consist of a discoidal phospholipid lipid bilayer confined by an apolipoprotein belt. NLPs are a promising platform for a variety of biomedical applications due to their biocompatibility, size, definable composition and amphipathic characteristics. However, poor serum stability hampers the use of NLPs for *in vivo* applications such as drug formulation. In this study, NLP stability was enhanced upon the incorporation and subsequent UV-mediated intermolecular crosslinking of photoactive DiynePC phospholipids in the lipid bilayer forming crosslinked nanoparticles (X-NLPs). Both the concentration of DiynePC in the bilayer and UV exposure time significantly affected the resulting X-NLP stability in 100% serum, as assessed by size exclusion chromatography (SEC) of fluorescently labeled particles. Crosslinking did not

significantly impact the size of X-NLPs as determined by dynamic light scattering and SEC. X-NLPs had essentially no degradation over 48 hours in 100% serum, which is a drastic improvement compared to non-crosslinked NLPs (50% degradation by ~10 minutes). X-NLPs had greater uptake into the human ATCC 5637 bladder cancer cell line compared to non-crosslinked particles, indicating their potential utility for targeted drug delivery. X-NLPs also exhibited enhanced stability following intravenous administration in mice. These results collectively support the potential utility of X-NLPs for a variety of *in vivo* applications.

## Introduction

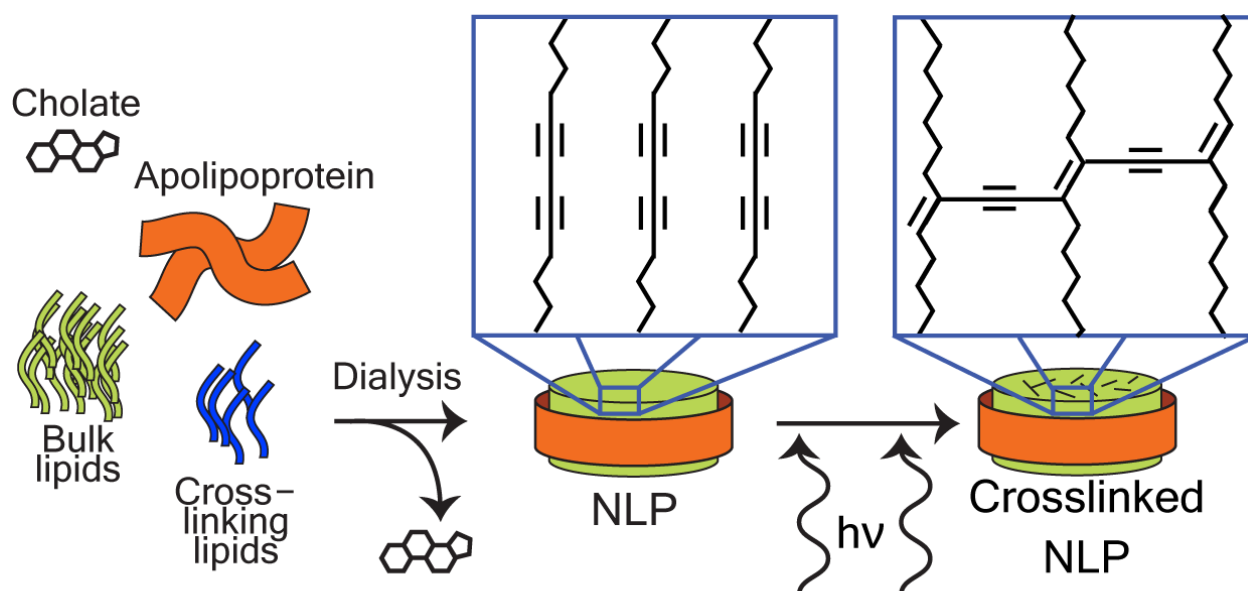
Transformative advances in the field of nanotechnology are making possible the improved formulation and delivery of therapeutic agents. In particular, nanoparticle-mediated drug delivery has the potential to address several limitations of conventional drug delivery systems, including nonspecific biodistribution, low water solubility, poor oral bioavailability, poor pharmacokinetics, and low therapeutic indices.<sup>1</sup> Many disparate types of nanoparticles are currently being explored to address these limitations and improve targeted therapeutic delivery *in vivo*.<sup>2-9</sup> Nanolipoprotein particles (NLPs), also referred to as nanodiscs and reconstituted high density lipoproteins (rHDLs), represent a promising nanoparticle system for *in vivo* delivery applications as their components are biocompatible and their diameters can be tuned between 6 and 25 nm. NLPs are mimetics of endogenous high-density lipoproteins (HDLs) found naturally existing in human biological serum,<sup>10</sup> and are comprised of lipids and proteins that self-assemble into a discoidal lipid bilayer that is constrained in size by a belt of apolipoprotein. The biomimetic nature of NLPs potentially constitutes a distinct advantage for improving drug delivery *in vivo* without compromising safety. NLP composition and self-assembly protocols have already been optimized to solubilize membrane proteins,<sup>11-16</sup> protein pore complexes,<sup>17</sup> and hydrophobic drugs.<sup>18-20</sup> Due to the versatility in assembly components, NLPs may also be tailor-made for a variety of applications, including targeted drug delivery, antigen delivery<sup>20-21</sup> and immune stimulation.<sup>22</sup> Importantly, NLPs have been reported to exhibit no overt toxicity and immunogenicity *in vivo*,<sup>23</sup> critical attributes for any *in vivo* delivery platform. To date, the stability of individual, discrete NLP particles in serum is low.<sup>23</sup> However, this issue may be addressed through screening lipid and protein combinations and selecting for optimized stability.<sup>22, 24</sup> Despite the potential advantages of this platform, there have been no systematic

studies focused on optimizing the *in vivo* stability of NLPs or other HDL mimetics. Nanoparticles often display superior pharmacokinetics, such as an enhanced area under the curve and volume of distribution, when compared to free drugs.<sup>25-26</sup> However, for drug delivery applications, nanoparticles must be stable under biological conditions in order for the drug formulation to take advantage of these pharmacokinetic properties.

It is clearly desirable to optimize the composition of NLPs in order to meet or exceed the serum stability of other nanoparticle platforms for drug-delivery.<sup>23</sup> For example, polymeric micelles have a half-life in serum of 9 hours.<sup>27</sup> In contrast, NLPs exhibit half-lives of several hours in media supplemented with 20% fetal bovine serum (FBS) at 37°C. However, half-lives in 100% FBS at 37°C are on the order of minutes.<sup>23</sup> This low stability is likely due to components of NLPs being held together predominantly through van der Waal forces rather than covalent bonds and are thus readily disrupted through interactions with lipids and other serum components *in vivo*. The poor stability of NLPs in serum may be due to lipid remodeling as described previously for natural HDL and LDL particles in serum over time, with varying rates dependent on the specific lipoprotein construct.<sup>28</sup> While a number of studies have demonstrated that HDL constituents can be monitored *in vivo* for days,<sup>28-30</sup> these are indirect measures of HDL stability as only a single component of the macromolecular complex was monitored with no indication if the particle was still intact. In one of the earliest reports on HDL stability, for example, circulation half time of the HDL was based on tracking <sup>125</sup>I-labeled apolipoprotein.<sup>28</sup> Similarly, Daerr et al. evaluated the circulation time of HDLs by tracking <sup>14</sup>C-labeled apolipoprotein.<sup>29</sup> While these approaches provide important information on clearance of the apolipoprotein, and potentially the dynamics of HDL populations *in vivo*, no information is provided as to whether the labeled nanoparticle remained intact upon administration. The fate of individual discrete HDL particles, with regards to maintaining the structure and composition as originally formulated, has not been clearly elucidated. Given that natural lipoproteins exchange content with other lipoproteins in serum, it is expected that synthetic constructs composed of lipids and scaffold proteins, such as NLPs, could undergo a similar process. This type of apolipoprotein and lipid exchange with natural HDLs would be highly undesirable for targeted delivery of drugs as the localization and pharmacokinetics of the drug formulation could be significantly altered due

to particle instability. Thus, optimizing the NLP composition in order to enhance stability of the intact particle *in vivo* is imperative for targeted drug delivery.

Chemical crosslinking of NLPs is one approach to enhance stability. Crosslinking of the individual apolipoprotein scaffold proteins within an NLP has been shown to enhance particle stability and integrity under non-physiological conditions (e.g. stored in PBS at 4 °C).<sup>10</sup> Alternatively, stabilization of other lipidic structures has been demonstrated using lipid-based crosslinking approaches,<sup>31-33</sup> whereby, lipids with specialized hydrophobic chains are included in lipid mixtures. These lipids, such as 1,2-bis(10,12-tricosadiynoyl)-sn-glycero-3-phosphocholine (DiynePC), possess reactive diacetylene groups that are incorporated into the acyl chains. When exposed to specific wavelengths of UV light (Figure 1), adjacent diacetylene groups, located within the core of the lipid membrane, are able to crosslink.



**Figure 1. Schematic showing preparation of X-NLPs.** An aqueous preparation of cholate-solubilized lipids and apolipoprotein is dialyzed to initiate NLP self-assembly. Assembled NLPs are subsequently crosslinked by exposure to a UV light source.

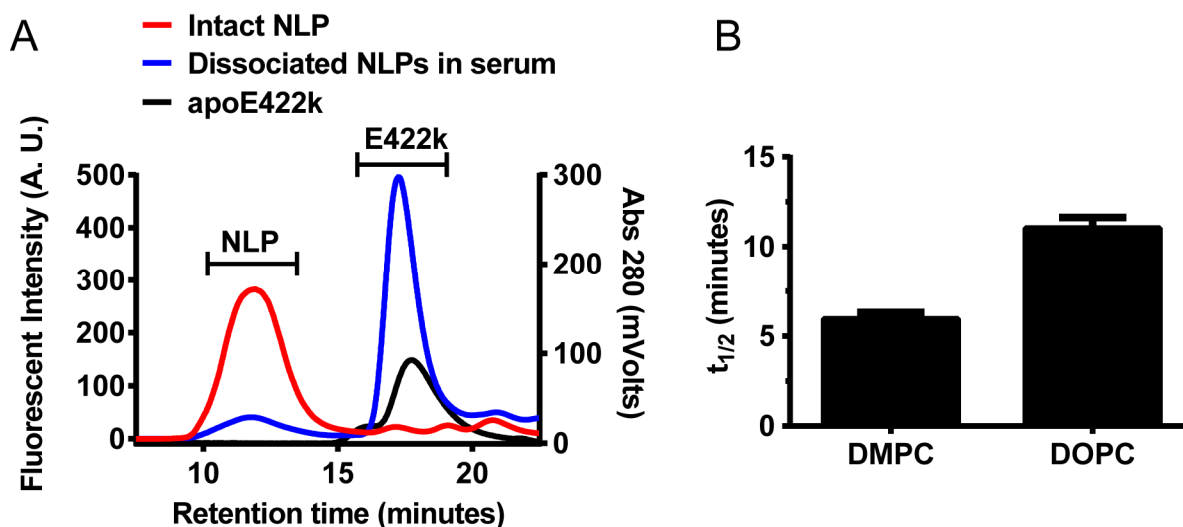
The goal of this study was to evaluate the effect the incorporation of a polymerizable lipid, capable of crosslinking, on NLP stability. To assess the effect that this type of crosslinking has on NLP stability, we developed a simple assay based on monitoring intact NLPs by SEC to more

accurately assess the effect of acyl chain cross-linking on NLP stability under *in vivo* conditions (100% FBS at 37°C). To further assess the effects of stability with regards to *in vivo* delivery applications, we examined the effect of crosslinking on cellular uptake *in vitro* and particle stability *in vivo*.

## Results and Discussion

### Characterization of NLPs formulated with a photo-polymerizable lipid

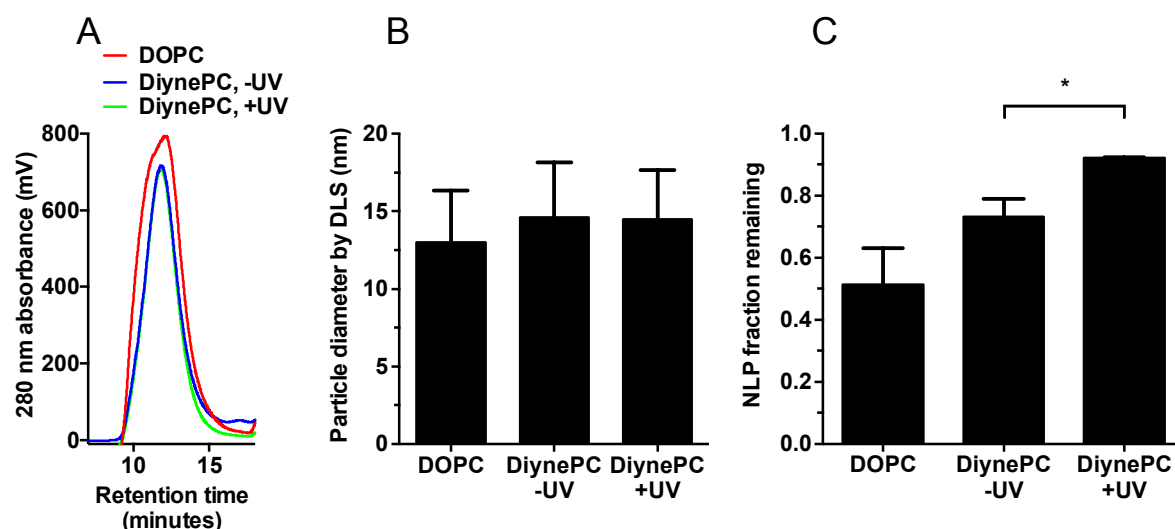
Analytical size exclusion chromatography (aSEC) was used to measure the half-life of the NLPs under different conditions. For these experiments, lysine residues on the apoE422k (E4) scaffold protein were labeled using AlexaFluor 488 NHS esters (AF488). Elution of the labeled scaffold protein was monitored by fluorescence detection (Excitation = 497 nm; Emission = 520 nm). This approach provided a spectrofluorometric elution signature unique to the apoE422k (and thus the integrity of the NLP) whose signal intensity (fluorescence quantum yield) was negligibly perturbed by the presence of serum proteins and other constituents that typically preclude optical absorbance monitoring under these experimental conditions. The large difference in size (and hence retention time,  $t_r$ ) between the NLP and unbound E4 provides convenient interrogation of NLP integrity by comparing the fraction of intact NLPs ( $t_r$  ~9-14 min) versus unbound apoE422k scaffold protein released upon NLP dissociation ( $t_r$  ~17.5 min). Representative aSEC chromatograms of intact (red line), dissociated NLPs (blue line), and purified apoE422k protein are shown in Figure 2A.



**Figure 2. Use of aSEC to measure serum stability of NLPs.** *A*, SEC traces of fluorescent intact (red trace) and degraded (blue trace) NLPs (non-crosslinked) in 100% FBS. Elution of NLPs ( $t_r$  ~12 min) are clearly distinguishable from dissociated E422k scaffold protein ( $t_r$  ~17.5 min). The scaffold protein retention time ( $t_r$  ~17.5 min) is verified using purified, unlipidated apoE422k (black trace, measured by absorbance).  $\lambda_{ex}$  = 495 nm,  $\lambda_{em}$  = 520 nm. Samples were analyzed using a Superdex 200 PC column (3.2/300 mm) at a 0.1 mL/min flow rate. *B*, a bar graph comparing the  $t_{1/2}$  (time at which 50% of NLPs degraded) of NLPs consisting of phospholipids DMPC and DOPC in 100% serum. Error bars represent standard error.

Dissociation of the NLPs clearly resulted in a substantially reduced peak area at elution times associated with intact NLP, along with a concomitant increase in the area of the peak assigned to unbound E4. By analyzing the NLP and serum mixtures at varying times, we are able to calculate the time at which 50% of the NLP population has degraded ( $t_{1/2}$ ) under these conditions. For lipids such as DMPC and DOPC, which are commonly used in the preparation of NLPs, half-lives of ~5 and ~11 minutes were obtained (Figure 2B). These results are consistent with those previously reported.<sup>23</sup> Similar analytical protocols were used to measure the  $t_{1/2}$  of a variety of NLP and X-NLP formulations under these simulated *in vivo* conditions (100% FBS at 37°C).





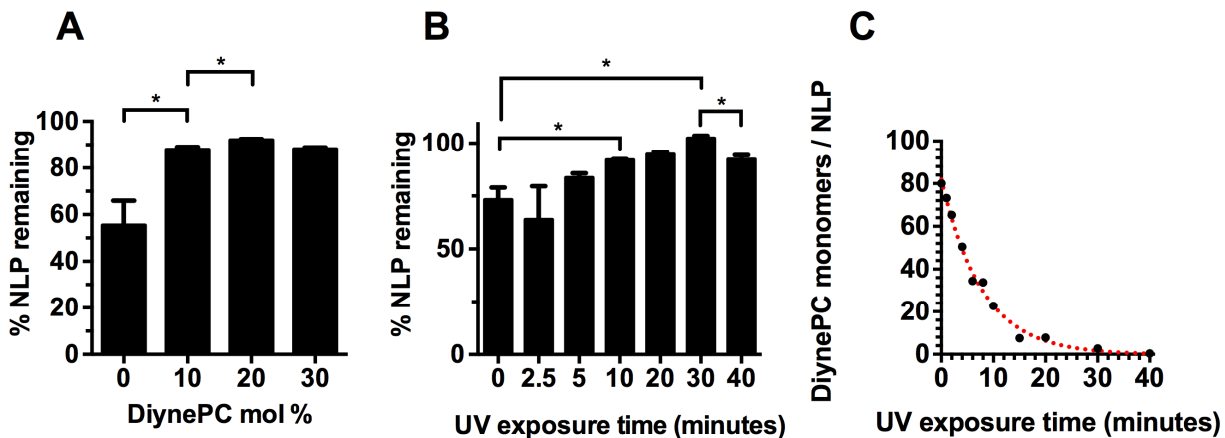
**Figure 3.** aSEC NLP or X-NLP peak area, particle size and serum stability. **A** aSEC traces of DOPC NLPs and DOPC-DiynePC NLPs with and without UV exposure. Samples were analyzed using a Superdex 200 PC column (3.2/300 mm) at a 0.1 mL/min flow rate. **B**, average particle size of DOPC NLPs and DOPC-DiynePC NLPs with and without 10 minutes of UV exposure obtained through dynamic light scattering. **C**, stability data of DOPC NLPs and DOPC-DiynePC NLPs with and without UV exposure (10 minutes) in sera for 10 minutes, expressed as fraction of NLP peak remaining. (\*  $p = 0.045$ ). Error bars represent standard error.

To improve upon the very low stability of the NLPs in 100% serum as observed from previous work, we evaluated the effect of adding a photopolymerizable lipid, DiynePC, to the NLP assembly to facilitate lipid cross-linking.<sup>34-35</sup> DiynePC is a phospholipid bearing reactive diacetylene groups on each acyl chain. When exposed to UV light ( $\lambda_{\text{max}} = 254$  nm), adjacent diacetylene moieties polymerize, resulting in intermolecular crosslinking within the lipid bilayer. In this study, we assessed the ability of DiynePC crosslinking to enhance the stability of DOPC-based NLPs, as DOPC NLPs are relatively stable and exhibit low immunogenicity and toxicity.<sup>23</sup>

DOPC-based NLPs were assembled with increasing concentrations of DiynePC, and assessed for particle formation, size, and stability. For initial assessment, NLPs were assembled with 20 mol% DiynePC and 80 mol % DOPC. DiynePC NLPs (+/- crosslinking) exhibited aSEC retention profiles that were comparable to DiynePC-free (neat) DOPC NLPs (Figure 3A). UV

crosslinking (10 min at 254 nm) of DiynePC NLPs had no effect on their size and polydispersity relative to neat DOPC NLPs as assessed by aSEC (Figure 3A) and dynamic light scattering (Figure 3B). To evaluate the effect of DiynePC on NLP stability before and after crosslinking, DOPC were incubated in 100 % serum for 10 minutes and the fraction of the NLP peak remaining relative to non-serum controls was measured by aSEC (Figure 3C). While the addition of 20 mol % DiynePC did not lead to a significant increase in NLP stability relative to DOPC NLPs ( $p = 0.16$ ) (Figure 3C), UV-exposed DiynePC NLPs exhibited significantly greater stability relative to both non-UV-exposed NLPs ( $p = 0.045$ ) and neat DOPC NLPs ( $p = 0.040$ ) (Figure 3C). Further structural characterization of NLPs with DiynePC was carried out using small angle X-ray scattering (SAXS).<sup>36</sup> We analyzed three NLP formulations DOPC alone, DiynePC (20 mol %) NLP without UV exposure, and DiynePC (20 mol %) NLPs exposed to UV for 10 minutes. All three constructs provide similar SAXS profiles (Supplementary Information Figure 1). In particular, no significant perturbations of the DiynePC NLP SAXS profile were observed upon UV crosslinking. These data indicate that the NLP size and structure are maintained after crosslinking, mirroring the results obtained by aSEC and DLS.

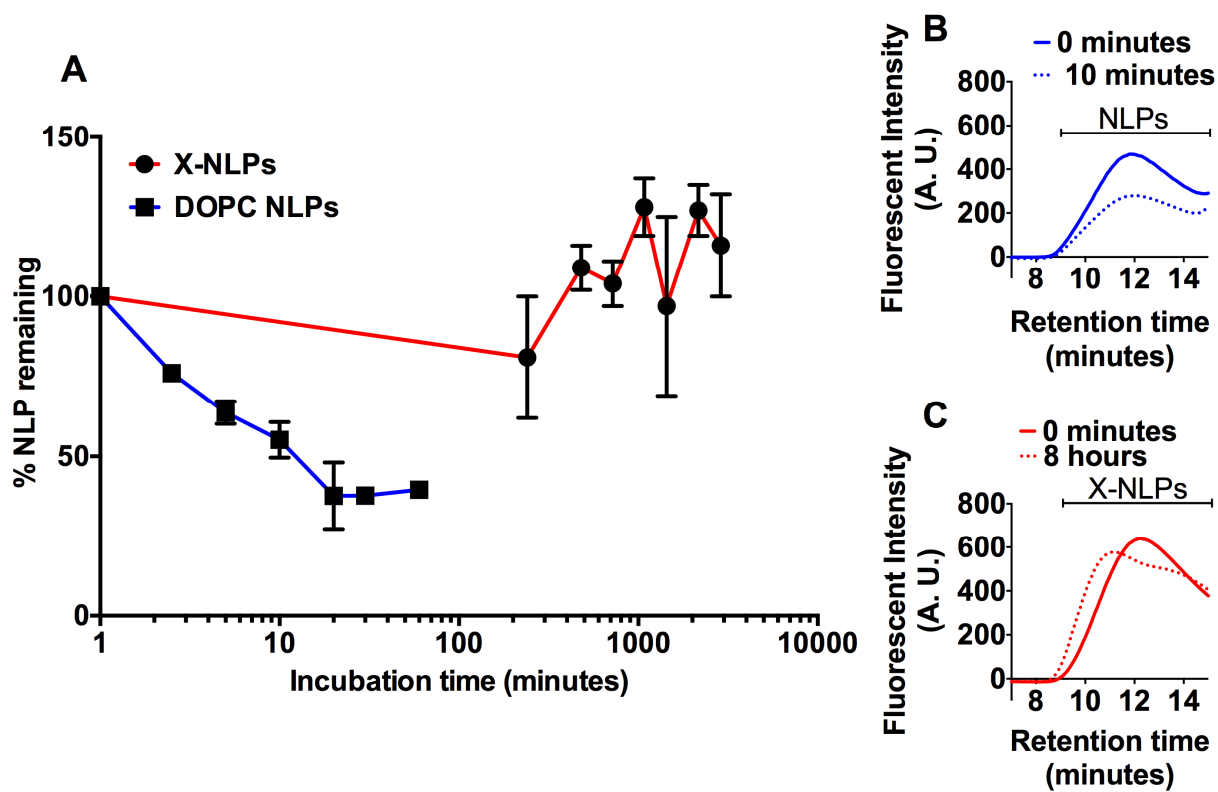
To better assess the relationship between mol % DiynePC and NLP stability, NLPs were assembled with 10, 20 and 30 mol % DiynePC, UV crosslinked (10 min at 254 nm), and incubated in 100% serum at 37°C for 10 minutes. The fraction of the original NLP peak remaining after this 10 minute incubation period is shown in Figure 4A. A consistent and stable increase in stability (NLPs remaining) was observed with an increase in DiynePC concentration from 0% to 20 mol %, with a significant difference between 0 % and 10 % ( $p = 0.036$ ) and 10% and 20% ( $p = 0.018$ ); however, no significant increase was observed between 20% and 30%. As such, 20 mol% DiynePC was chosen for all subsequent experiments.



**Figure 4.** NLP stability and UV exposure optimization. Stability of particles formulated with DiynePC. **A**, the percent of NLP remaining after 10 minutes in 100% FBS at 37°C for different ratios of DiynePC and DOPC exposed to UV (254nm) for 10 minutes. **B**, a plot showing the fraction of NLP population remaining after 10 minutes in FBS at 37°C as a function of UV-C exposure with 20% DiynePC, 80% DOPC (\*  $p < 0.05$ ). NLPs were crosslinked for the indicated times, and were analyzed by aSEC before and after incubation in serum to calculate. The difference between these aSEC traces is represented as % NLP remaining after serum incubation. **C**, a plot showing the number of free DiynePC monomers per NLPs after varying lengths of UV exposure. An exponential decay curve (red) was fit to the data with an  $R^2$  of 0.992. Error bars represent standard error of the mean.

To evaluate the effect of UV irradiation time on DiynePC NLP stability, DiynePC NLPs (20 mol %) were assembled and exposed to UV for different lengths of time. The fraction of the NLP area was then measured before and after a 10-minute incubation in 100% serum (Figure 4B). An increase in the amount of intact NLP remaining was observed for preparations with UV exposure times up to 30 minutes ( $p = 0.012$ ) (Figure 4B). Interestingly, a significant decrease in stability was observed when the exposure time was increased from 30 minutes to 40 minutes ( $p < 0.01$ ) (Figure 4B). These results imply that shorter irradiation times yield fewer DiynePC-DiynePC crosslinks, whereas longer irradiation times may compromise the integrity of the NLP. To minimize the risk of the potential negative effects of the UV exposure on NLP components that may be incorporated in future iterations (e.g. for drug delivery applications), an exposure time of 10 minutes was selected for all experiments as this was the shortest time that yielded a

significant difference in performance over NLPs with no UV exposure. To confirm that DiynePC was crosslinked as a result of UV exposure, NLP composition was analyzed using reverse phase HPLC to quantify the amount of DiynePC lipid in each NLP prior to cross-linking, and monitoring the loss of monomer (as an indirect measure of intermolecular crosslinking) as a function of exposure time. DiynePC detection was achieved using an evaporative light scattering detector, providing a means to quantify the DiynePC in our NLPs. Using these data and the known lipid:protein ratio for each NLP formulation, the expected number of DiynePC monomers remaining in a single NLP as a function of UV exposure was calculated (Figure 4C). As expected, increased UV exposure times resulted in decreased amounts of free DiynePC (Figure 4C). By 40 minutes, less than 0.4% of the free DiynePC was detected, suggesting complete intermolecular crosslinking of the DiynePC monomers. Based on these results, all subsequent experiments were conducted with X-NLPs consisting of 20% DiynePC, and irradiation times of 10 minutes, as this exposure time yielded the first significant difference over the non-exposed group ( $p = 0.045$ ) (Fig. 4B).



**Figure 5.** Long-term stability in FBS at 37°C of DOPC NLPs compared to X-NLPs. **A.** NLP and X-NLP samples were incubated for indicated times and analyzed by aSEC. Percent NLP remaining reflects integrated areas of samples after incubation relative to  $t=0$ . Error bars represent standard error. **B.** Individual aSEC traces of neat DOPC NLPs at 0 minutes (solid trace) and 10 minutes (dashed trace), demonstrating rapid degradation of NLP peak. **C.** Individual aSEC traces of X-NLPs at 0 minutes (solid trace) and 8 hours (dashed trace), demonstrating NLP peak integrity and slight shift to larger size over time.

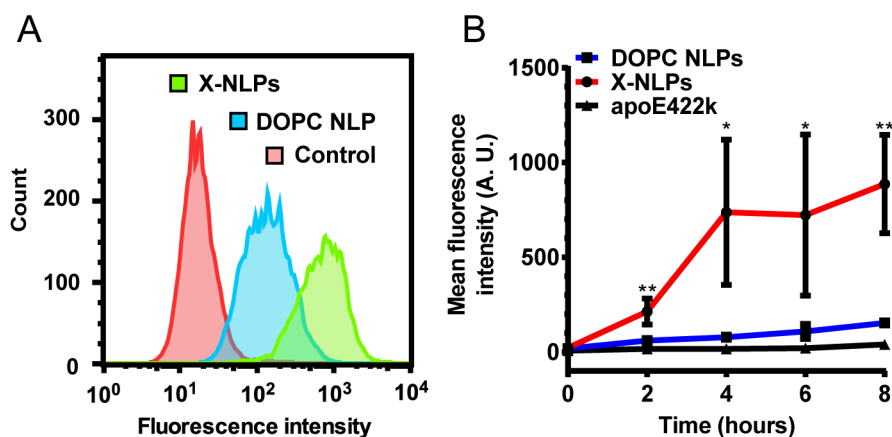
To assess the long-term stability of X-NLPs in 100% serum, X-NLPs were incubated in 100% serum at 37°C and analyzed over a 48 hour period (Figure 5A). Over the course of 48 hours, we observed no apparent loss in NLP integrity, which was surprising given that DOPC NLPs demonstrate significant dissociation within 10 minutes (Figure 5B). Interestingly, there was a slight increase in X-NLP particle size over several hours, presumably due to interactions with serum proteins (Figure 5C). This contributed to higher overall signal intensity over time for the X-NLPs relative to  $t=0$ . These results are a clear demonstration that crosslinking the bilayer core significantly enhances overall NLP stability. DiynePC has been shown to reduce detergent solubility of supported lipid bilayers,<sup>31</sup> and liposomes formulated with crosslinked DiynePC were found to be more stable in serum compared to controls.<sup>33</sup> However, an increase in stability of X-NLPs spanning 3 orders of magnitude was unexpected. While crosslinking DiynePC is known to stabilize lipid structures, it was unclear if this would prevent the NLP scaffold protein from dissociating from the particles. These results suggest that the instability of the non-crosslinked particles can be attributed to the lipid bilayer, rather than the scaffold protein. While we do not definitively understand the internal lipid bilayer structure of the NLPs after crosslinking, we were able to show that even with 10 minutes of UV exposure, the majority of the DiynePC monomers are crosslinked (Figure 4C). However, it is unclear whether the crosslinked DiynePC lipids are organized into small groups inside the NLP bilayer, or if there is a continuous network of crosslinked lipids throughout the particles.

## **Uptake of NLPs into ATCC 5637 human bladder cancer cells**

Efficient delivery of therapeutics via nanoformulation requires either controlled drug release during circulation or efficient cellular uptake of NLPs containing a drug “cargo”. We previously demonstrated that NLPs are rapidly taken up by mouse macrophages.<sup>22</sup> We hypothesize that maximizing NLP stability will further increase their cellular uptake by reducing or eliminating NLP dissociation in the culture media or in vivo. Furthermore, apolipoproteins such as apoE422k possess a binding domain specific for the extracellular LDL receptor (LDLR), which is overexpressed by a variety of cancer cell types and is considered a potential target for selective drug delivery.<sup>37-39</sup> Therefore, the NLP itself may already have inherent cancer cell targeting abilities. To explore the applicability of targeted NLP-based drug delivery in a cancer model, we assessed uptake of neat DOPC NLP and X-NLP constructs by human bladder cancer type-II carcinoma cells (the ATCC 5637 cell line), as they represent a model of a viable target for chemotherapeutic delivery. The active uptake of NLPs is understood to be facilitated by the LDL receptor protein, which binds to a domain on lipidated apolipoproteins or truncated apolipoprotein fragments used in these experiments.<sup>40</sup> To monitor cellular uptake, cells were incubated with AF488-labeled NLPs (for 0, 2, 4, 6 and 8 hours), trypsinized, and analyzed by flow cytometry (Figure 6A). As shown in Figure 6B, uptake of crosslinked NLPs was significantly higher at every time point tested. In control experiments, uptake of labeled E422k apolipoprotein alone (not associated with lipid or NLPs) was shown to be negligible (black curve). Cellular internalization of NLPs in 5637 cells was confirmed by flow cytometry in the presence and absence of fluorescence quenching antibodies and with fluorescence microscopy (Supporting Information S2). These combined results indicate that uptake of E422k is only possible when it is associated with an NLP or lipid-rich environment, and that particle dissociation will effectively deplete the media of particles competent in cellular uptake. When stability of the NLP is enhanced through crosslinking, this leads to greater NLP uptake into cells. These results are consistent with LDLR mediated uptake, although additional experiments will be required to confirm that NLP uptake is indeed mediated by LDLR.

Previous work has shown that NLPs formulated with DOPC do not have any detectable cytotoxicity.<sup>23</sup> Since X-NLPs comprise a new NLP formulation containing a reactive lipid, cytotoxicity in 5637 cells was assessed. Cells were dosed with DOPC NLPs, NLPs with

DiynePC (no UV exposure), or NLPs with DiynePC (10 minutes of UV exposure) (X-NLPs) at concentrations ranging from 0-100 micrograms per milliliter of complete media. (Supporting Information Figure S3). No cytotoxicity was detected with any of the three formulations, indicating that the DiynePC component does not impart any cytotoxicity to the NLP formulation at the concentrations tested.



**Figure 6.** Flow cytometry analyses showing enhanced uptake of crosslinked NLPs into human bladder cancer cells. **A**, representative ( $n \geq 5$  for each NLP group) fluorescence histograms for control cells not dosed with NLPs, cells dosed with DOPC NLPs, and cells dosed with X-NLPs after 8 hours. **B**, a time course showing mean fluorescent intensities of 5637 cells dosed with fluorescently-labeled DOPC NLPs, X-NLPs, and labeled apoE422k. Statistical comparisons are represented for X-NLP and DOPC NLP comparisons at indicated times (\*  $p < 0.05$ , \*\*  $p < 0.01$ ). Error bars represent standard error.

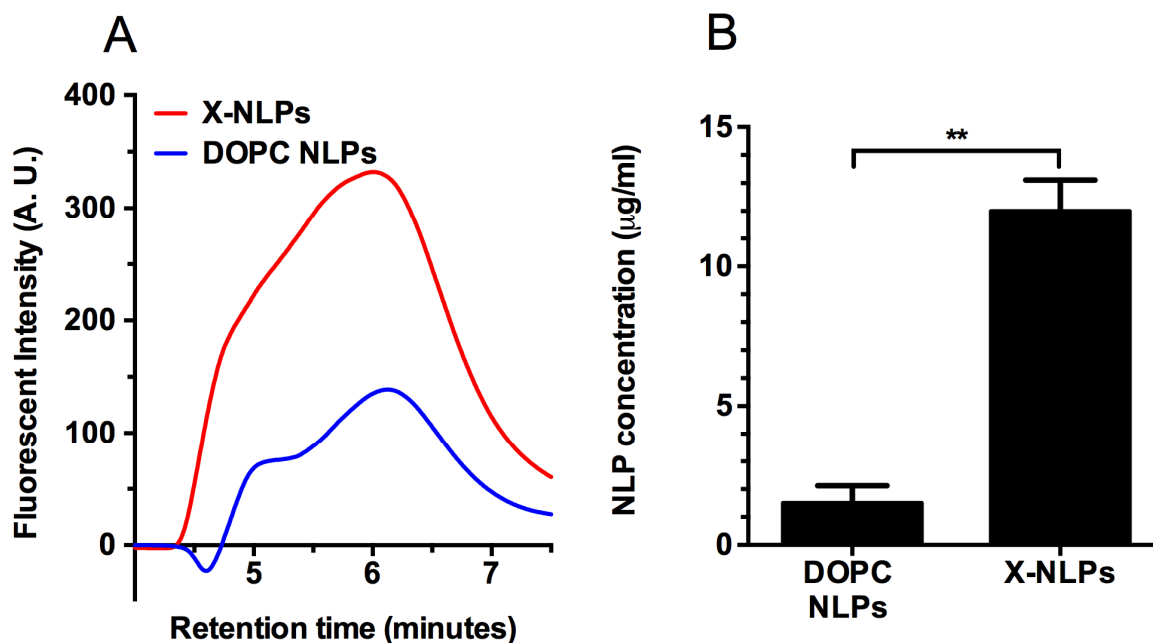
### Stability assessment of NLPs and X-NLPs *in vivo*

To determine if the improved stability *in vitro* translates to an *in vivo* model, C57BL/6 mice were injected with AF488-labeled NLPs via the intravenous (i.v.) route. For these *in vivo* experiments, a 10-minute time point was chosen to provide sufficient time for the non-crosslinked NLPs to begin degrading in the serum while avoiding significant clearance of NLPs from the blood stream and/or uptake by cells in the blood.<sup>23</sup> Serum was isolated from blood collected after i.v. injection and analyzed by aSEC to evaluate NLP integrity. Serum from mice injected with

DOPC NLPs exhibited a clear fluorescence signal above background (Figure 7A – blue line). Importantly, mice injected with X-NLPs had a significantly stronger fluorescence NLP signal (Figure 7A – red line). To determine the actual serum NLP concentration, a standard curve using fluorescent standards diluted in serum were used to measure NLP and X-NLP peak areas. When this analysis was performed, a significantly higher serum concentration ( $0.012 \pm 0.001 \mu\text{g}/\mu\text{l}$  versus  $0.0015 \pm 0.0006 \mu\text{g}/\mu\text{l}$ ,  $p < 0.01$ ) was observed in the mice receiving the X-NLPs (Figure 7B). These results suggest that enhancing NLP stability in vivo is feasible using Diyne crosslinking lipids.

It is worth noting that although these preliminary studies demonstrated that the X-NLPs were more stable than the NLPs, we could only assess NLP stability over a very short time period (~10 min) since analysis of these results could be confounded by NLP loss from serum due to cell uptake and removal from the blood. Furthermore, the fluorescence-based detection scheme is not sensitive enough to measure blood samples taken over longer times. Thus, the methods described in this study cannot be utilized to adequately measure pharmacokinetics (PK) or biodistribution of intact NLPs. Due to the significantly improved stability of X-NLPs, it would be interesting to compare the PK and biodistribution profiles of X-NLPs vs NLPs. As discussed above, previous studies have attempted to evaluate the PK and biodistribution of HDL mimetics by monitoring the apolipoprotein. Although these studies proved valuable in terms of understanding how long these proteins remain in circulation, they provide no information on whether the particle remains intact throughout this process. One potential approach to achieving such measurements would be utilizing near-IR FRET pairs in the lipid component and apolipoprotein component of the NLP and assessing the fluorescent emission spectra of tissue, blood and urine; such an approach would allow one to evaluate the pharmacokinetics and biodistribution of the lipid component, apolipoprotein component and intact NLP independently.





**Figure 7.** Enhanced stability of crosslinked NLPs in vivo. **A**, aSEC traces of serum collected from individual mice injected with fluorescent DOPC NLPs (blue line) and X-NLPs (red line). Background fluorescence was subtracted from each trace. Samples were analyzed using a Superdex 200 Increase column (3.2/300mm) at a 0.2 mL/min flow rate. **B**, DOPC NLP ( $n = 3$ ) and X-NLP ( $n = 4$ ) concentration in serum based on standard curves for NLPs in serum. (\*\*  $p < 0.01$ ). Error bars represent standard error.

## Conclusions and Outlook

The objective of this study was to develop a novel formulation for NLPs that exhibited superior stability in biological media. Incorporation of the photo-polymerizable lipid DiynePC provided a ready means of greatly increasing NLP stability upon UV exposure. This enhanced stability was observed in 100% serum, and translated to enhanced performance both in vitro and in vivo. The effect of crosslinking on particle stability was evident by a significant increase in uptake by human bladder cancer cells compared with non-crosslinked NLPs. This is attributed to the presence of more intact crosslinked particles being available in the cell culture media for longer periods of time relative to the less stable, non-crosslinked particles. Importantly, in vivo

evaluation of the crosslinked particles in mice also showed more intact particles present after 10 minutes of circulation. These experiments reveal that NLPs have the potential to be a long-circulating nanoparticle suitable for drug delivery applications.

## Acknowledgements

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## Supporting Information

Raw data from SAXS and derived structural data on NLPs from SAXS. (Figure S1). Flow cytometry data and fluorescence micrographs indicating internal fluorescence (Figure S2). Cytotoxicity data for DOPC NLPs and NLPs with DiynePC (Figure S3). Comparison of fluorescence intensities of NLPs before and after UV exposure (Figure S4).

## Materials and Methods

### Materials

1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), and 1,2-bis(10,12-tricosadiynoyl)-*sn*-glycero-3-phosphocholine (DiynePC) were purchased from Avanti Polar Lipids (Alabaster, AL). All other reagents were ordered from Sigma-Aldrich (St. Louis, MO). RPMI-1640 was purchased from ATCC. Fetal bovine serum, Penicillin/Streptomycin, and Alexa Fluor 488 NHS Ester (AF488) were obtained from Invitrogen (Carlsbad, CA).

### Protein expression and purification

The expression clone for the 22 kDa N-terminal fragment of human apolipoprotein E4 (apoE422k, kindly provided by Dr. Karl Weisgraber) featuring a cleavable His-tag<sup>41</sup> was expressed and purified as previously described.<sup>42-43</sup>

### **NLP assemblies**

NLPs were assembled according to a previously reported procedure,<sup>42-43</sup> with slight modifications. Briefly, lipids were either prepared or obtained in chloroform and aliquoted into glass vials. Chloroform was then removed using a stream of N<sub>2</sub> under agitation to form a thin lipid film. Lipids were solubilized in PBS buffer (137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM phosphate buffer, pH 7.4) using 80 mM sodium cholate. After addition of the apoE422k (150  $\mu$ M in final assembly volume), samples were incubated at 22°C for at least 1 hour. Assemblies with DiynePC were heated to 37°C for 30 minutes and then cooled to 22°C for thirty minutes after the addition of PBS buffer containing 80 mM cholate in order to fully solubilize the solution. Assemblies were dialyzed overnight against PBS to remove cholate.

### **Labeling the NLPs with Alexa Fluor dyes**

NLPs were labeled with either AF488 by incubating the NLPs with the reactive dye for at least 2 hrs (5:1 dye:NLP molar ratio). The reaction was performed in PBS buffer containing 5 mM sodium bicarbonate, pH 8.2. After completion of the reaction, 10 mM Tris pH 8.0 was added to quench any unreacted dye and incubated for 30 minutes. Free dye in the NLP solution was removed by using a dye-removal column kit, as directed (Thermo Fisher, Rockford, IL).

### **NLP Purification**

Samples were subsequently analyzed and purified by SEC (Superdex 200, 10/300 GL column, GE Healthcare, Piscataway, NJ) in PBS buffer (0.5 mL/min flow rate). The exclusion limit of the column was determined with Blue Dextran 2000. SEC fractions (500  $\mu$ l) were collected every 60 s. SEC fractions containing homogeneous NLP populations were concentrated using 50 kDa MWCO spin concentrators (Sartorius). NLP concentrations were determined by using a

Nanodrop ND-1000 spectrophotometer (ThermoScientific, Lafayette, CO) at an absorbance of 280 nm. The concentrated NLP samples were then stored at 4°C until further use. In these experiments, the NLP concentration was calculated based on the apoE422k concentration by assuming that each NLP contained 6 apoE422k scaffold proteins.<sup>42, 44</sup>

### **Polymerization of NLPs containing DiynePC**

NLPs that contained DiynePC were treated with UV-C following purification. NLP solutions in polypropylene tubes were placed in a Stratalinker 2400 UV crosslinker (Stratagene, La Jolla, CA) and exposed to 254 nm light for the specified time.

### **SEC analysis of NLP stability in complex biological fluids**

NLP samples were incubated in FBS and 10% FBS in RPMI-1640 and subsequently analyzed by SEC (Superdex 200 PC 3.2/30 column, GE Healthcare) in PBS buffer. A flow rate of 0.1 ml/min was used to ensure no overlap in the elution of disassembled apoE422k and intact NLP. The NLPs labeled with AF488 were monitored using a RF-20 fluorescence detector (Shimadzu) set to excite at 497 nm and to measure fluorescence at 520 nm to avoid interfering absorbance from serum proteins and constituents. The raw chromatograph obtained from the fluorescence detector was further analyzed by fitting a series of Gaussian functions to the trace through code written in Python using the lmfit library. Peaks centered between 9 and 14 minutes were considered to be NLP populations, while free apoE422k was found to elute at approximately 17.5 minutes under these conditions. The NLP Gaussian functions were then integrated to assess NLP disassembly as a function of time. These peak areas, from independent samples incubated in media or serum for varying times, were then arranged into a time series, and exponential decay functions were fit to each combination of peak areas. For each fit, the function was normalized around time zero, and the half-life of the function was recorded. Extreme outliers were then discarded.

### **Particle size measurements using dynamic light scattering**

Purified NLP solutions were diluted to approximately 0.2 mg/ml. The size distribution of the particles was analyzed using a Zetatrac (Microtrac). Each sample was analyzed three times in

sequence to obtain an average measurement. The analysis chamber was rinsed with DI water between samples.

### **SAXS measurements and analysis**

SAXS data were collected at the SIBYLS beamline (12.3.1)<sup>45</sup> at the Advanced Light Source at Lawrence Berkeley National Lab. SIBYLS has implemented a high throughput data collection strategy coupling synchrotron light with liquid handling robotics.<sup>46</sup> Samples of 18 $\mu$ L volumes are transferred one at a time from a 96 well plate to the sample holder which is then exposed to a focused beam of X-rays. The sample to detector distance is fixed at 1.5 meters. The wavelength was 1 $\text{\AA}$ . The flux was  $10^{13}$  photons/second. The beam size at the sample was 4x1mm converging to a 100 $\mu$ m spot at the detector. The sample thickness was fixed at 1mm. Four exposures were collected from each sample; 0.5, 1, 2 and 5 second exposures. Temperature of data collection was 20 C. A MAR 165 detector was used resulting in the collection of q values ranging  $0.01 < q < 0.32\text{\AA}^{-1}$ . Buffer with no protein was collected both before and after the sample enabling two subtractions for reduced error in subtraction.<sup>47</sup> The MAR 165 area detector was concentric with the beam providing multiple measurements of equivalent q values. Each image collected was circularly integrated and normalized for beam intensity during collection. The 1 dimensional profiles were buffer subtracted using beamline software specific for 12.3.1.<sup>48</sup>

Three concentrations were collected per sample. The highest concentration sample was 1 mg/ml, which was diluted to 0.66 mg/ml and 0.33 mg/ml for measurements that probed concentration dependence. SAXS data were merged for highest signal to noise and minimized influence from radiation damage. For merging we utilized the program Scatter. The python based program WillItFit<sup>49</sup> was used to fit the data. WillItFit was implemented for applying algorithms designed to fit a variety of NLPs.<sup>50</sup> We utilized the model of an elliptical NLP with tags. While the algorithm has 12 fitted parameters (and more if neutron data is used simultaneously), we report 5 of the most robust parameters determined (Supplemental Information 1). Regardless of the search method used (Genetic, Levenberg-Marquardt, Swarm) or the starting values, the algorithms returned nearly identical values for high quality fits.

### **Quantification of DiynePC monomers**

Compositional analysis of NLPs was carried out on a Shimadzu Prominence reverse phase HPLC system. A Kinetex 2.6  $\mu$ m XB-C18 column (Phenomenex), heated to 30°C, was used to analyze the injected NLP samples. The solvent was run as a gradient from a mixture of 30% methanol and 70% water to 100% isopropanol. All solvents had 0.05% trifluoroacetic acid. Known quantities of NLP (by scaffold protein concentration) were injected and detection of the lipid component was accomplished using an evaporative light-scattering detector, which used a supply of ultra-pure nitrogen to nebulize the eluent from the HPLC (40°C). Quantification of DiynePC was accomplished through the use of a standard curve generated with known quantities of DiynePC. This curve was used to translate DiynePC peak area to concentration, which was used to calculate the amount of DiynePC monomer per NLP particle based upon the known lipid:protein ratio assuming 6 E422k proteins per NLP.

### **Culture of human bladder cancer type II carcinoma cells**

The 5637 human bladder cancer cell line was obtained from ATCC. Cells were grown in T-75 vented tissue culture flasks in RPMI-1640 with 1% penicillin/streptomycin and 10% FBS. Cells were incubated at 37°C and 5% CO<sub>2</sub> until confluent. Cells were then removed from the flasks using trypsin and plated into 24 well plates for dosing experiments. Cells were allowed to grow to confluence prior to dosing experiments.

### **NLP uptake into human bladder cancer cells**

Prior to dosing, cells were placed into new media for one hour. Fluorescently labeled NLPs (2.5 micrograms/well) were then pipetted into the wells and the cells were returned to the incubator following the completion of dosing. To measure the uptake at various time points, cells were removed from the wells using trypsin, and then pelleted through centrifugation at 4k RPM for five minutes. The supernatant was discarded and the cells were resuspended in 0.5 ml of PBS using a pipette to disrupt the cell pellet. The cell suspension was analyzed using a FACScalibur (Becton Dickinson, Franklin Lakes, NJ). The mean fluorescence intensity was obtained for the population of cells.

Cellular internalization of NLPs was determined by flow cytometry and fluorescence microscopy. For the flow cytometry experiments, cells were plated onto a 96 well plate and

dosed with 1  $\mu\text{g}$  of NLP in 100  $\mu\text{l}$  of media for 3 hours. Cells were then prepared for flow cytometry as described above. Prior to analysis, Alexa Fluor 488 antibody was added to half the cells dosed with fluorescent NLPs at a concentration of 1  $\mu\text{g}/\text{ml}$  and allowed to incubate for an hour. Control cells that were not dosed were also analyzed to measure the background, which was then subtracted out from the measurements for cells dosed with the fluorescent NLPs. For fluorescence microscopy, cells were grown on glass-bottom petri dishes (MatTek Inc., Ashland, MA) that was coated with poly-d-lysine prior to use. Cells were then dosed with 10  $\mu\text{g}$  of NLP (in 2 ml of media) and incubated for 6 hours. Cells were washed with PBS and 1X solution of background suppression dye (Thermo-Fisher, Rockford, IL) was added. Cells were imaged with a fluorescence microscope at 20X magnification using a GFP filter set.

### **Cytotoxicity assay**

Human bladder cancer 5637 cells were plated onto a 96 well plate at a density of 50,000 cells per well. Cells were dosed with NLPs in triplicate at the specified concentrations in a mixture of 5:1 fresh media and PBS. Cells were incubated at 37°C for 24 hours. A cytotoxicity assay kit (Promega, Sunnyvale, CA) was used according to the manufacturers instructions. Final absorbance measurements at 490 nm were performed on a BioTek H1M plate reader (BioTek, Winooski, VT).

### ***In vivo* NLP stability**

NLPs and X-NLPs were labeled with AF750 as described above. Groups of four C57BL/6 mice received an intravenous injection with 50  $\mu\text{g}$  of NLP and X-NLP through the tail vein. 10 minutes after administration, the mice were euthanized and blood was collected via cardiac puncture. The blood was immediately subjected to centrifugation and serum collected. The serum was then run on aSEC to assess NLP stability, using a Superdex 200 Increase column (3.2/300 mm) at a 0.2 mL/min flow rate to decrease sample run time and minimize delay between serum collection and analysis. All experiments involving animals were conducted after review and approval by the Institutional Animal Care and Use Committee at Lawrence Livermore National Laboratory (IACUC protocol number 2012-002).

### **Statistical analysis**

Statistical comparisons between datasets was carried out in Microsoft Excel (2011). To compare datasets and generate p values, the built in T-test function (two-sample unequal variance) was used. All distributions were assumed to be two-tailed.

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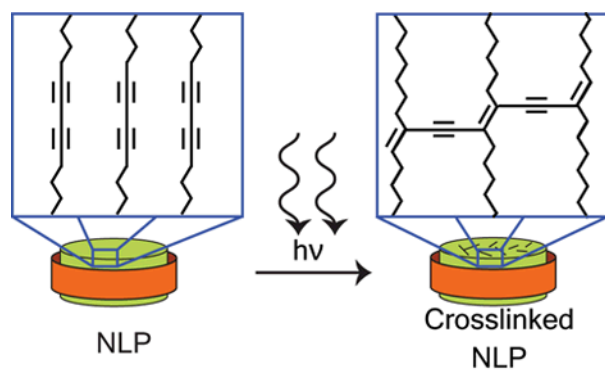
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