

Surface-Modified Melphalan Nanoparticles for Intravitreal Chemotherapy of Retinoblastoma

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PURPOSE. The goal of this work was to design and assess the ability of unmodified and surface-modified poly(lactic-co-glycolic acid) (PLGA) nanoparticles (NPs) to enhance cell association, provide efficacy in retinoblastoma cells, and overcome current administration challenges, including hydrolysis and precipitation, of intravitreal administration.

METHODS. A single emulsion method was used to encapsulate Coumarin 6, to enable NP visualization via fluorescence microscopy. Melphalan NPs were synthesized using an adapted double-emulsion method to reduce melphalan loss during fabrication. Melphalan loading and release were quantified against a free melphalan standard. The cellular association and internalization of unmodified and surface-modified NPs were determined using flow cytometry, and the efficacy of melphalan NPs was quantified in retinoblastoma cells.

RESULTS. The highest cell association was observed with TET1 and MPG-NPs after 24 hours administration; however, a significant fraction of NPs were associated with the cell surface, instead of undergoing internalization. MPG-NPs fabricated with the low saturation process were most efficacious, while all surface-modified NPs improved efficacy relative to unmodified NPs when formulated using the highly saturated process. Similar effects were observed as a function of NP dose, with TET1 and MPG-NPs particularly efficacious.

CONCLUSIONS. Surface-modified NPs achieved enhanced association and efficacy in retinoblastoma cells relative to unmodified NPs, with MPG and surface-modified NPs exhibiting the strongest efficacy relative to other NP groups. In future work we seek to assess the ability of these NPs to improve transport in the vitreous, where we expect a more dramatic impact on efficacy as a function of surface modification.

Keywords: nanoparticles, cell penetrating peptide (CPP), retinoblastoma, ocular cancer, vitreous, nanoparticle transport

Retinoblastoma is the most common pediatric intraocular tumor and occurs in young children under the age of five. Retinoblastoma occurs in 1 of every 16,000 to 18,000 live births in the global population, with approximately 8000 children developing retinoblastoma annually.¹ In developed countries, the survival from this disease is greater than 95%, but unfortunately in low and medium income countries the mortality is as high as 70%.

The treatment of retinoblastoma has evolved from the primary treatments of enucleation and external beam radiation, to more globe conserving treatments. While external beam radiation has salvaged vision, major complications include the increased risk of secondary cancer and detrimental side effects that promote facial anomalies in the developing bone. Moreover, enucleation has often resulted in partial or total vision loss. To abrogate these severe complications, systemic chemotherapy protocols were developed in the 1990s that revolutionized retinoblastoma management by conserving eyes and vision without the adverse effects of radiation.

The last few decades have seen a paradigm shift in retinoblastoma management with the introduction of intra-arterial chemotherapy. Melphalan is the preferred chemotherapeutic for intra-arterial treatment because of its proven efficacy against retinoblastoma.² By selectively injecting chemotherapy, such as melphalan, into the ophthalmic artery via a catheterization procedure, the systemic side effects of chemotherapy have been mitigated. Yet, even with the advent of intra-arterial chemotherapy, vitreous seeding, or the establishment of hypovascularized cluster of tumor cells in the vitreous humor, is still considered a primary hurdle to obtaining efficacious delivery. Due to the avascular structure of the vitreous, inadequate chemotherapeutic concentrations are delivered, minimizing tumoricidal effect. To address these challenges, the use of intravitreal melphalan for vitreous seed control in retinoblastoma³ was pioneered, significantly improving the efficacy in patients to 60% to 100%, relative to previous cases in which nearly all patients with vitreous seeds underwent enucleation.



Despite the effectiveness of intravitreal melphalan injections, often multiple injections at weekly or monthly intervals are required, depending on the tumor load. This frequent administration contributes to an increased risk of side effects (mainly retinal toxicity from chemotherapy), risk of tumor dissemination with the injection, and the need for repeated anesthesia.^{4,5}

As the field of intraocular drug delivery evolves, new therapeutic delivery platforms are emerging to address the needs and limitations of current treatment options. Although different delivery platforms have been investigated for the treatment of intraocular diseases, polymeric nanoparticles (NPs) offer a platform that can provide targeted delivery to specific tissues, thereby eliminating drug exposure to surrounding healthy tissue.^{6,7} Moreover, delivery systems made from US Food and Drug Administration-approved polymers such as poly(lactic-co-glycolic) acid (PLGA) are biocompatible and show favorable degradation kinetics. PLGA NPs provide high encapsulation of active agents and exert prolonged delivery and residence time, thereby minimizing the number and frequency of administrations.^{7,8} For some active agents, like melphalan, stability is also a major concern, due to its lack of aqueous solubility, short half-life, and tendency to precipitate (within 30 minutes) in an aqueous solution. By encapsulating melphalan in NPs, the half-life and stability of the drug may be increased, thus prolonging the therapeutic window of the drug *in vivo*.

Recent advances in NP design have integrated two foci to advance intraocular delivery approaches. One is the incorporation of surface-functionalized ligands to improve cell uptake and active agent release. The other addresses delivery challenges to less amenable physiological environments (e.g., those with mucous membranes or fluid-filled spaces like the vitreous). For drug and gene delivery, the modification of carriers with positively charged molecules, such as cell penetrating peptides (CPPs), significantly enhances cell association and internalization.⁹⁻¹¹ In contrast, small (<500 nm) or neutrally charged “stealth” carriers more rapidly and effectively penetrate the tightly spaced and negatively charged vitreous or mucosal environments.¹²⁻¹⁴ In previous work, we have assessed the binding and internalization mechanisms of unmodified, MPG, and poly(ethylene glycol) (PEG)-modified NPs in cells¹⁵⁻¹⁷ and found that surface-modification with MPG results in higher cell association and binding, likely due to the positive zeta-potential, relative to more negatively charged unmodified and neutrally charged PEG NPs. Regardless of surface-modification (unmodified, MPG-modified, or PEG-modified), cell internalization occurred via clathrin-mediated endocytosis.¹⁵ Moreover, surface-modification with PEG has been observed to improve NP transport from the anterior to posterior segment of the eye as well as provide increased intravitreal NP stability. Therefore, the design of efficacious therapeutic delivery vehicles must consider the trade-offs between neutral/negatively charged carriers that typically improve distribution and positively charged carriers that enhance cell internalization.

In the study presented here, we sought to design and test new formulations of surface-modified melphalan PLGA NPs to provide an efficacious delivery alternative for the treatment of retinoblastoma. Long-term, we envision that surface-modified PLGA NPs have the potential to maintain drug stability, enhance delivery and tissue transport, and may offer a platform to eliminate the need for repeated intravitreal injections, thereby reducing adverse effects, minimizing the need for anesthesia, and reducing the risk of tumor dissemination. Clinically, these formulations may also be useful by reducing administration frequency and associated costs in developing

countries, while improving patient compliance with follow-up care.

METHODS

Synthesis of Avidin Palmitate Conjugates

Avidin palmitate was synthesized for conjugation to NPs as previously described.^{15,16,18,19} Briefly, 40 mg avidin (A9275, Sigma-Aldrich Corp., St. Louis, MO, USA) was dissolved in 4.8 mL 2% sodium deoxycholate (NaDC) in phosphate-buffered saline (PBS) warmed to 37°C. Palmitic acid-NHS (PA-NHS, Sigma-Aldrich Corp.) was dissolved in 2% NaDC to a final concentration of 1 mg/mL and sonicated until well-mixed. PA-NHS solution (3.2 mL) was added dropwise to the avidin NaDC solution, and reacted overnight at 37°C. The following day, the reaction was dialyzed in 1200 mL 0.15% NaDC in PBS heated to 37°C. Free PA-NHS was dialyzed overnight at 37°C using 3500 molecular weight cut-off (MWCO) tubing to remove free palmitic acid. After overnight dialysis, the dialysis tubing contents were transferred to a glass vial and stored at 4°C until use.

NP Synthesis

PLGA NPs were synthesized using the emulsion solvent evaporation method, with two different adaptations. First, a single emulsion synthesis method was used to encapsulate the fluorescent dye Coumarin 6 (C6), to enable visualization via fluorescence microscopy. Briefly, NPs were synthesized using an oil-in-water (o/w) single emulsion technique.^{6,12,15,16,19} Carboxyl-terminated poly(lactic-co-glycolic acid; PLGA) (0.55–0.75 dL/g, LACTEL) was used to synthesize 100 to 200 mg batches. C6 was dissolved in methylene chloride (DCM) overnight at a concentration of 15 µg C6 per mg of PLGA. The following day, the PLGA/C6/DCM solution was added dropwise to a 5% polyvinyl alcohol (PVA) solution of equal volume, vortexed, and sonicated. The resulting NPs were hardened in 0.3% PVA during solvent evaporation for 3 hours.

To incorporate the drug, melphalan, PLGA NPs were synthesized using a double-emulsion method. The double emulsion method was altered to reduce melphalan release during the fabrication process (Fig. 1). For this adapted double emulsion process, PLGA was dissolved in DCM overnight prior to synthesis. The following day, melphalan was solubilized in Tris-EDTA buffer at a concentration of 50 µg per mg PLGA and incorporated into the PLGA/DCM solution followed by subsequent sonication. This resultant solution was added dropwise to 5% PVA, and this PLGA/DCM/melphalan/PVA solution was vortexed and sonicated (Fig. 1A). The resulting NPs were hardened in a 10-mL sink of 9% PVA that was either unsaturated (no additional melphalan) or saturated with melphalan at 1 or 10 mg/mL to prevent unwanted melphalan diffusion from the NPs during fabrication (Fig. 1B).

For NPs without surface modification, NPs were washed after hardening and centrifuged at 4°C, three times in deionized water (diH₂O) to remove residual solvent. NPs were frozen, lyophilized, and stored at –20°C until use. A similar protocol was followed to synthesize NPs modified with the biotinylated peptides: MPG (3177 Da, GenScript, Piscataway, NJ, USA), PEG (5000 Da, Nanocs Inc.), and TET1 (1873 Da, GenScript), with the addition of avidin palmitate (1 mg/mL) to the 5% PVA solution (Fig. 1C). Surface-modified NPs were collected after the first wash and incubated for 30 minutes with biotinylated ligands at a molar ratio of 3:1 ligand to avidin in PBS. After the conjugation, the NPs were washed two more times with diH₂O, centrifuged, frozen, and lyophilized. All lyophilized NPs were stored at –20°C after synthesis.

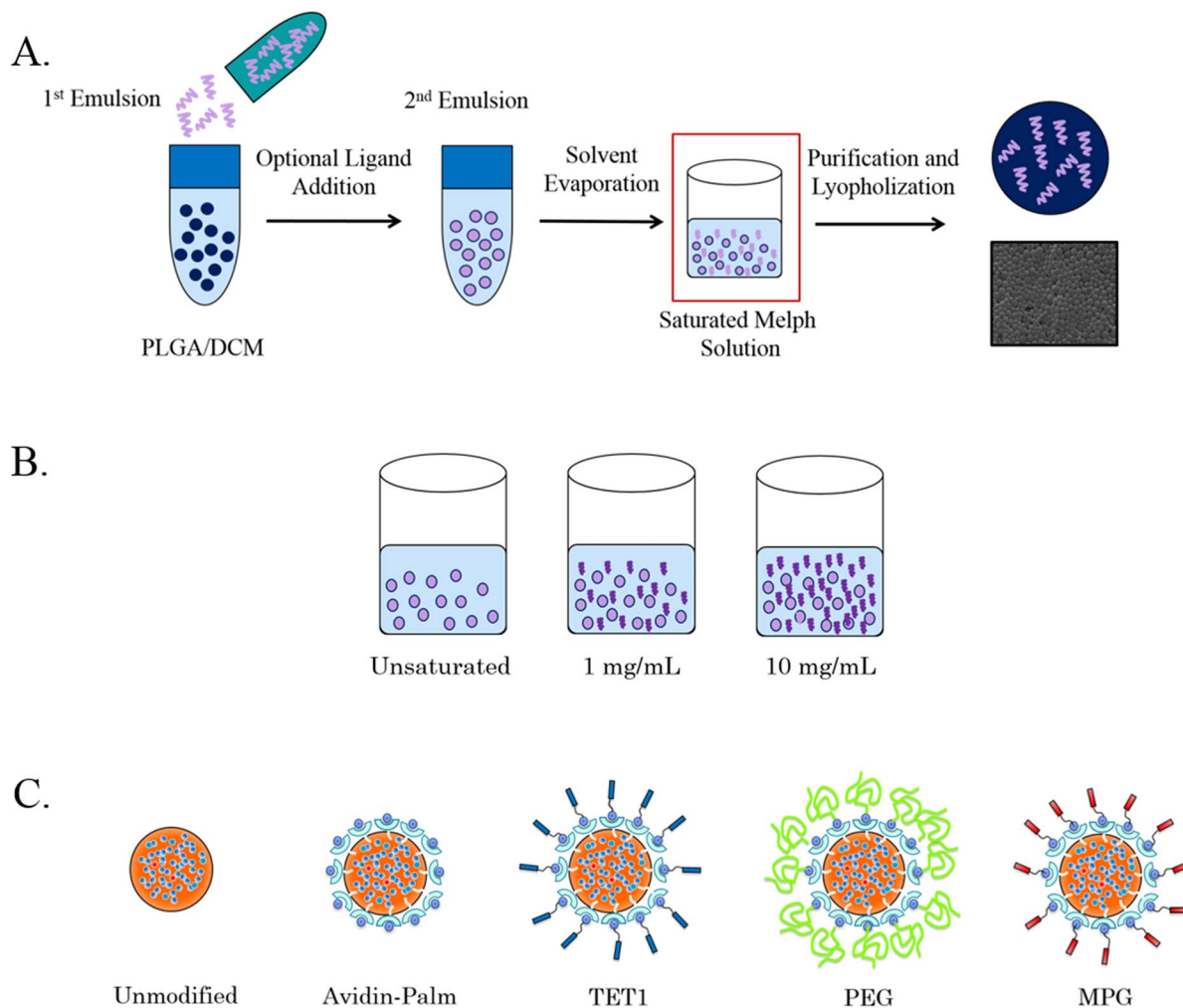


FIGURE 1. (A) Schematic of the fabrication process using an adapted double emulsion technique, with (B) different saturated melphalan solutions used during the evaporation stage, and (C) depicting unmodified and surface-modified NP groups used in this study.

NP Characterization

NP morphology was characterized via scanning electron microscopy (SEM; Zeiss SUPRA 35VP, Thornwood, NY, USA), as we have previously done.¹⁸ To quantify NP loading and encapsulation efficiency, approximately 3 to 5 mg melphalan NPs were incubated for 14 days in PBS to allow complete release of melphalan from the NP, as organic dissolution methods formed melphalan degradation products. Sample eluates were collected at 7 and 14 days and centrifuged for 10 minutes at 20,440g, followed by supernatant collection. The quantity of melphalan was determined by reading the absorbance of sample supernatants at 260 nm and comparing to a free melphalan standard. Melphalan release with respect to time was assessed using the same methodology. Melphalan NPs were suspended in 1 mL of 1× PBS (pH 7.4) at a concentration of 2 mg/mL and incubated with gentle agitation at 37°C. At fixed time points (1, 2, 4, 6, 24, and 48 hours), the supernatant was collected and the amount of melphalan released from the NPs was quantified using absorbance. Free melphalan standards were also incubated for 1, 7, and 14 days, and compared to the absorbance of freshly prepared melphalan standards to ensure that degradation did not affect melphalan detection.

NP Binding and Internalization

The human retinoblastoma cell line, Y79, was purchased from ATCC (Manassas, VA, USA). Y79 cells were maintained in RPMI medium, supplemented with 20% fetal bovine serum and 1% penicillin-streptomycin. All cells were maintained in 5% CO₂ at 37°C and were grown to 80% confluence prior to experiments.

The association and internalization of NPs with Y79 cells were determined using flow cytometry, as performed in our previous studies.^{16,17} The four C6 NP formulations, unmodified, TET1, PEG, and MPG groups, were added (50 µg/mL) to Y79 cultures plated in six-well plates at a concentration of 3×10^5 cells per well. After 1.5 and 24 hours administration, the cells were transferred to fluorescence-activated cell sorting (FACS) tubes and centrifuged at 400g for 5 minutes to sediment the cells, and the supernatant was removed. The cells were resuspended in 1 mL FACS buffer, centrifuged again, the supernatant was removed, and the cells were resuspended in 500 µL FACS buffer. Half of these samples were incubated on ice, to assess total binding and internalization, while the other half were prepared to assess internalization. To determine internalization, 100 µL 0.4% trypan blue was added, and samples were incubated at room temperature for 5 minutes.¹⁶

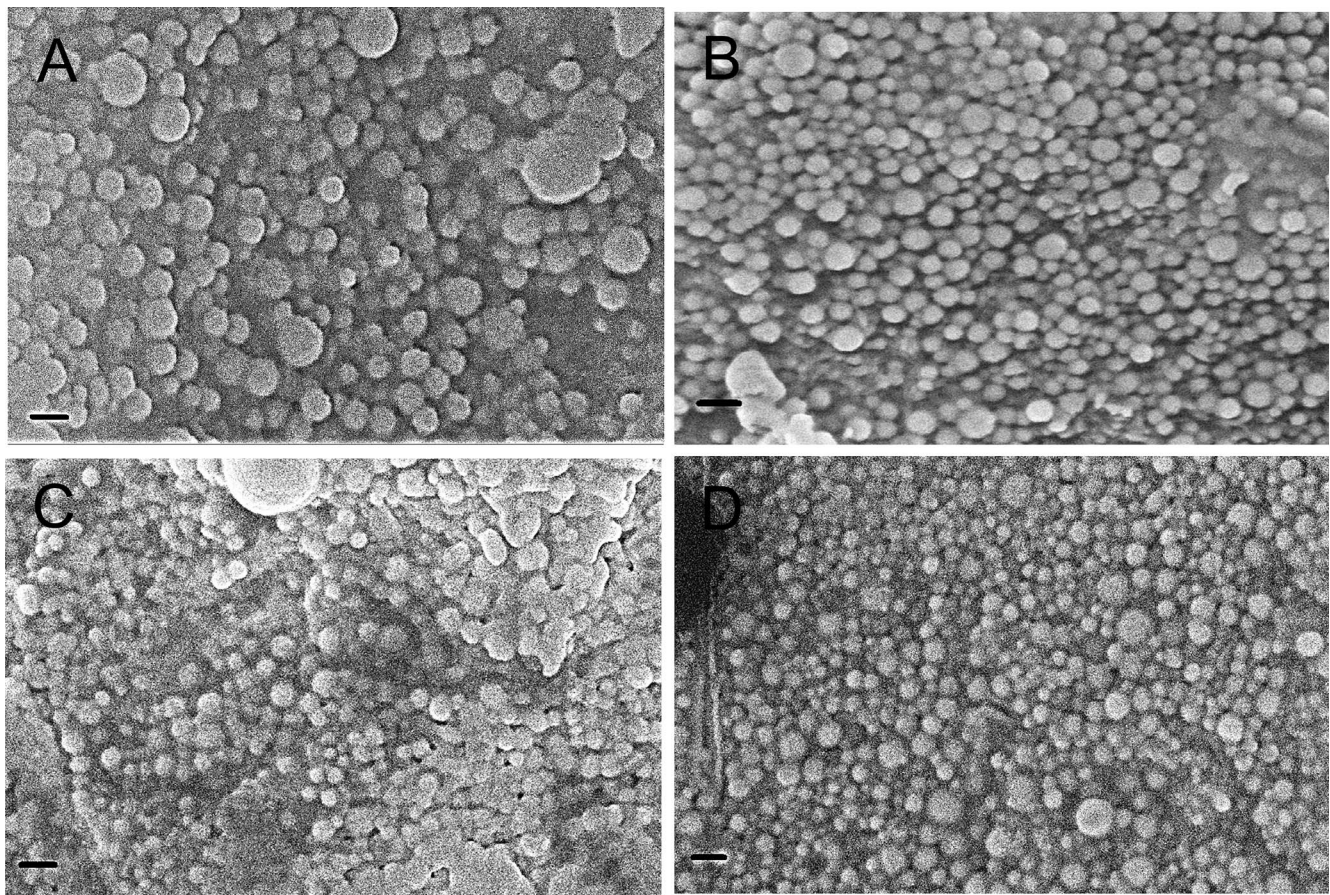


FIGURE 2. SEM images of (A) unmodified, (B) TET1, (C) PEG, and (D) MPG surface-modified NPs fabricated using a PVA solution saturated with 10 mg/mL melphalan. Scale bar: 200 nm.

Trypan blue has been used by our lab and others^{15-17,20,21} to differentiate between NPs bound to (associated with) the surface and those internalized by the cell, due to its inability to penetrate live cell membranes. As such, trypan blue has been used to quench extracellular fluorescence signals from NPs bound to the cell surface.^{15-17,20,21} After incubation, the cell solutions were centrifuged, the supernatant removed, followed by resuspension in 800 μ L FACS buffer. Centrifugation and washes were repeated twice, and the cells were resuspended in 500 μ L FACS buffer for analysis. Flow cytometry was then conducted on both groups of samples to determine the total cellular association (binding and internalization), relative to internalization only.

NP Efficacy

The chemotherapeutic efficacy of unmodified and TET1, PEG, and MPG-modified melphalan NPs in monolayer cell cultures was determined using the MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) cell proliferation assay. Retinoblastoma Y79 cells were seeded at a density of 1.5×10^3 cells per well and allowed to grow overnight. Cells were then treated with surface-modified melphalan NPs at a maximum NP concentration of 5 mg/mL, dependent on the amount of melphalan released at 24 hours. Untreated cells, cells treated with blank NPs, and cells treated with 10% dimethylsulfoxide were used as controls. Cells were then incubated under normal cell culture conditions for 24 hours. Following this 24 hours treatment, 10 μ L MTT reagent was added to each well in the

absence of light, and the plate was incubated under normal cell culture conditions for 4 hours. Following incubation, 50 μ L lysis buffer (10% SDS in 0.01 M HCl) was added to each well and placed in the incubator overnight for cell lysis. On the following day, sample absorbance was quantified at a wavelength of 570 nm. NP-treated cells were normalized to cells treated with blank unmodified NPs to account for any variation in efficacy due to polymer-cell interactions. To account for the possibility of NP presence affecting cell viability, blank NPs were administered as an additional control, alongside untreated cells.

Statistical Analysis

All experiments were conducted with a minimum sample size of $n = 3$. Data were analyzed by applying Tukey's test with significance $P \leq 0.05$. Figure error bars represent the standard deviation (SD) of measurements, with statistical significance between groups depicted with an overbar.

RESULTS

NP Characterization

The impact of different NP synthesis adaptations on NP morphology, melphalan loading, and encapsulation were assessed. The morphology of NP batches was evaluated using SEM (Fig. 2), and all NP formulations were observed to have spherical morphology. Additionally, the diameters of unhy-

TABLE 1. Characterization of NP Melphalan Loading

NP Formulation	Melphalan Loading, µg Melphalan per mg NP
Unsaturated	7.3 ± 0.4
Saturated, 1 mg/mL	41.1 ± 0.7
Saturated, 10 mg/mL	627.0 ± 15.3

drated NPs were determined using ImageJ (V1.50B) (National Institutes of Health, Bethesda, MD, USA), with unmodified, MPG, PEG, and TET NPs exhibiting diameters of 145 ± 54 , 129 ± 38 , 107 ± 64 , and 123 ± 33 nm, respectively. No statistically significant differences were observed in NP diameter as a function of surface modification, with the exception of unmodified and PEG NPs (145 vs. 107 nm, $P \leq 0.001$).

To determine melphalan loading, NPs were dissolved in NaOH, and melphalan concentration was quantified via absorbance relative to a free melphalan standard. The melphalan loading increased as a function of solution saturation, providing respective values of 7.3 ± 0.4 , 41.1 ± 0.7 , and 627.0 ± 15.3 µg melphalan per mg NP for the unsaturated, 1 mg/mL saturated, and 10 mg/mL saturated unmodified NP formulations (Table 1). Surface-modified melphalan NPs formulated using the saturated method (both 1 and 10 mg/mL) were characterized similarly. Melphalan loading for the 1 mg/mL saturated batches was 135.7 ± 7.8 , 244.5 ± 8.6 , and 212.7 ± 4.5 µg melphalan per mg NP for TET1, PEG, and MPG-modified NPs, respectively (Fig. 3). Surface-modified NPs saturated with a higher concentration of melphalan (10 mg/mL) incorporated 458.4 ± 3.5 , 320.0 ± 3.7 , and 197.8 ± 12.1 µg melphalan per mg NP for TET1, PEG, and MPG NPs, respectively (Fig. 3). All NP batches demonstrated statistically significant differences in melphalan loading as a function of surface-modification type for a given saturation solution.

Cumulative release experiments were conducted to determine the amount of melphalan released from NPs with respect to time. For unmodified NPs, the unsaturated formulation released 7 ± 0.4 µg melphalan/mg NP over 24 hours, while unmodified NPs fabricated with 1 or 10 mg/mL melphalan saturation released 33.2 ± 1.2 and 286 ± 3.5 µg melphalan/mg NP, respectively, over 24 hours (Fig. 4). For TET1, PEG, and MPG-modified NPs saturated with 1 mg/mL melphalan, the cumulative release was 150.9 ± 45.6 , 111.2 ± 0.8 , and 167.8 ± 48.3 µg melphalan/mg NP, respectively (Fig. 5A), while TET1, PEG, and MPG-modified NPs saturated with 10 mg/mL melphalan demonstrated cumulative release of 272.7 ± 95 , 225.7 ± 61 , 206.5 ± 49 µg melphalan/mg NP after 24 hours (Fig. 5B).

NP Efficacy

NP Formulations Saturated With 1 mg/mL Melphalan.

The efficacy of melphalan NPs on retinoblastoma cells varied as a function of both melphalan (µM) and NP (mg/mL) concentrations (Figs. 6, 7; Supplementary Figs. S1, S2). MPG NPs were the most effective treatment group, as a function of melphalan concentration, demonstrating a statistically significant decrease in the 50% inhibitory concentration (IC_{50}) value, relative to unmodified NPs (IC_{50} s 52.6 ± 11 and 80.2 ± 0.5 µM, for MPG and unmodified NPs, respectively). The IC_{50} values of TET1, PEG, and unmodified NPs were higher than free melphalan, while MPG NPs demonstrated statistically similar efficacy to free melphalan (IC_{50} s 52.6 ± 11 and 36.2 ± 2.8 µM, respectively, $P \leq 0.05$) (Figs. 6, 8A; Table 2).

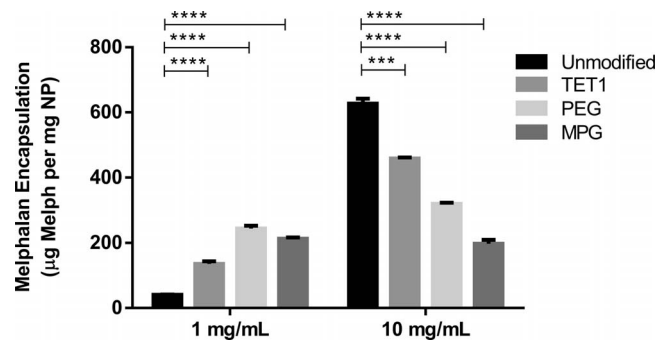


FIGURE 3. Melphalan loading in unmodified, TET1, PEG, and MPG-modified NP formulations fabricated with the adapted 1 or 10 mg/mL saturation process. Statistical significance of loading is shown with respect to the unmodified NP formulation. Loading values are shown as the mean \pm SD, with statistical significance. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

As a function of NP dose, all surface-modified NPs were more efficacious than unmodified NPs ($P \leq 0.0001$). Unmodified NPs were the least efficacious NP formulation (IC_{50} 1.02 ± 0.13 mg NP/mL), relative to TET1, PEG, and MPG-modified NPs, with IC_{50} s of 0.35 ± 0.06 , 0.38 ± 0.07 , and 0.23 ± 0.04 mg NP/mL, respectively (Supplementary Fig. S1; Fig. 8B; Table 2).

NP Formulations Saturated With 10 mg/mL Melphalan. For NP formulations saturated with 10 mg/mL melphalan, all surface-modified NPs were more efficacious than unmodified NPs (Figs. 7, 8A; Table 2) as a function of melphalan dose. TET1, PEG, and MPG NPs all demonstrated similar IC_{50} s of 75.3 ± 4.2 , 79.1 ± 12.3 , and 63.4 ± 7.4 µM that were less efficacious than free melphalan.

As a function of NP dose, TET1 and MPG-modified NPs were more efficacious than unmodified NPs (IC_{50} s 0.22 ± 0.05 and 0.24 ± 0.04 mg NP/mL, relative to 0.39 ± 0.04 mg NP/mL respectively, $P \leq 0.05$) (Supplementary Fig. S2; Fig. 8B; Table 2). Additionally, no differences were observed between untreated cells and cells treated with blank NPs, indicating that the presence of blank NPs, at the concentrations tested, does not affect cell viability.

NP Binding and Internalization

The effect of surface modification on cell association and internalization was determined via flow cytometry. Overall, surface-modified NPs exhibited increased cell association and internalization with respect to unmodified NPs. After 1.5 hours, MPG NPs demonstrated the highest levels of cell association and internalization with mean fluorescence intensities (MFIs) of 8866 ± 2213 and 2471 ± 318 , respectively, and were found to have a statistically significant increase in cell association (total binding plus internalization), relative to TET1 and unmodified NP groups (6006 ± 379 and 4592 ± 918 MFI, respectively) (Fig. 9). After 24 hours, total NP association increased for the TET1-modified NP group, while association decreased for unmodified, PEG, and MPG-modified NP groups, relative to the 1.5 hours time point. Overall, after 24 hours, TET1 and MPG-modified NPs had the highest levels of cell association (9555 ± 1858 and 7803 ± 4062 MFI) and were statistically significant with respect to PEG-modified (for TET1) and unmodified NPs (TET1 and MPG). Cell internalization also increased with respect to time for the TET1 and MPG NP groups, although a significant fraction of NPs were externally associated with the cell surface.

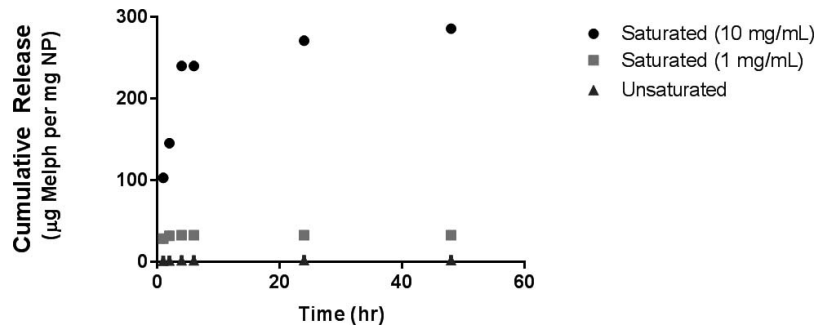


FIGURE 4. Cumulative release of melphalan from NPs that were fabricated with the conventional unsaturated (0 mg/mL) or adapted 1 or 10 mg/mL melphalan-saturated double emulsion process.

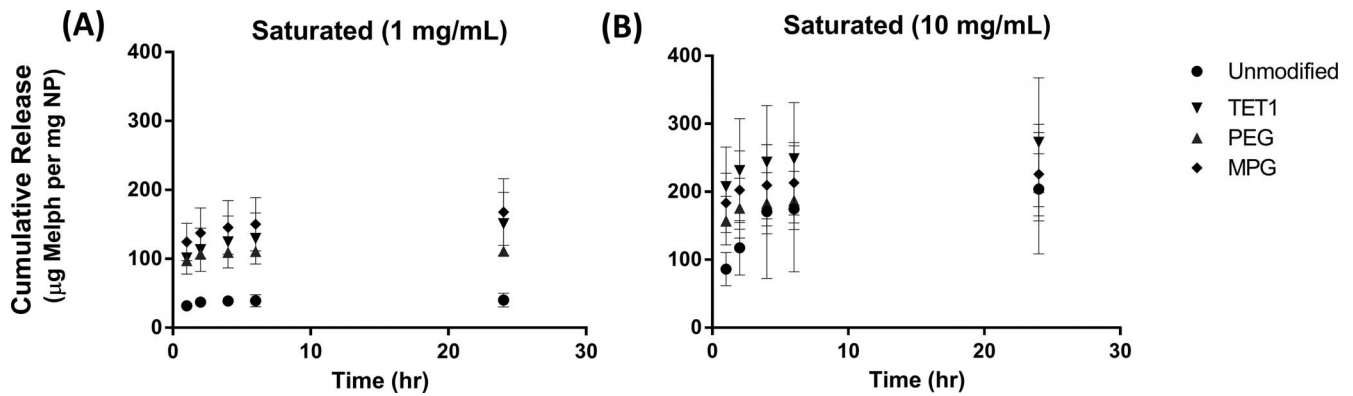


FIGURE 5. The cumulative release of melphalan from surface-modified NPs formulated with the adapted (A) 1 mg/mL and (B) 10 mg/mL saturation processes.

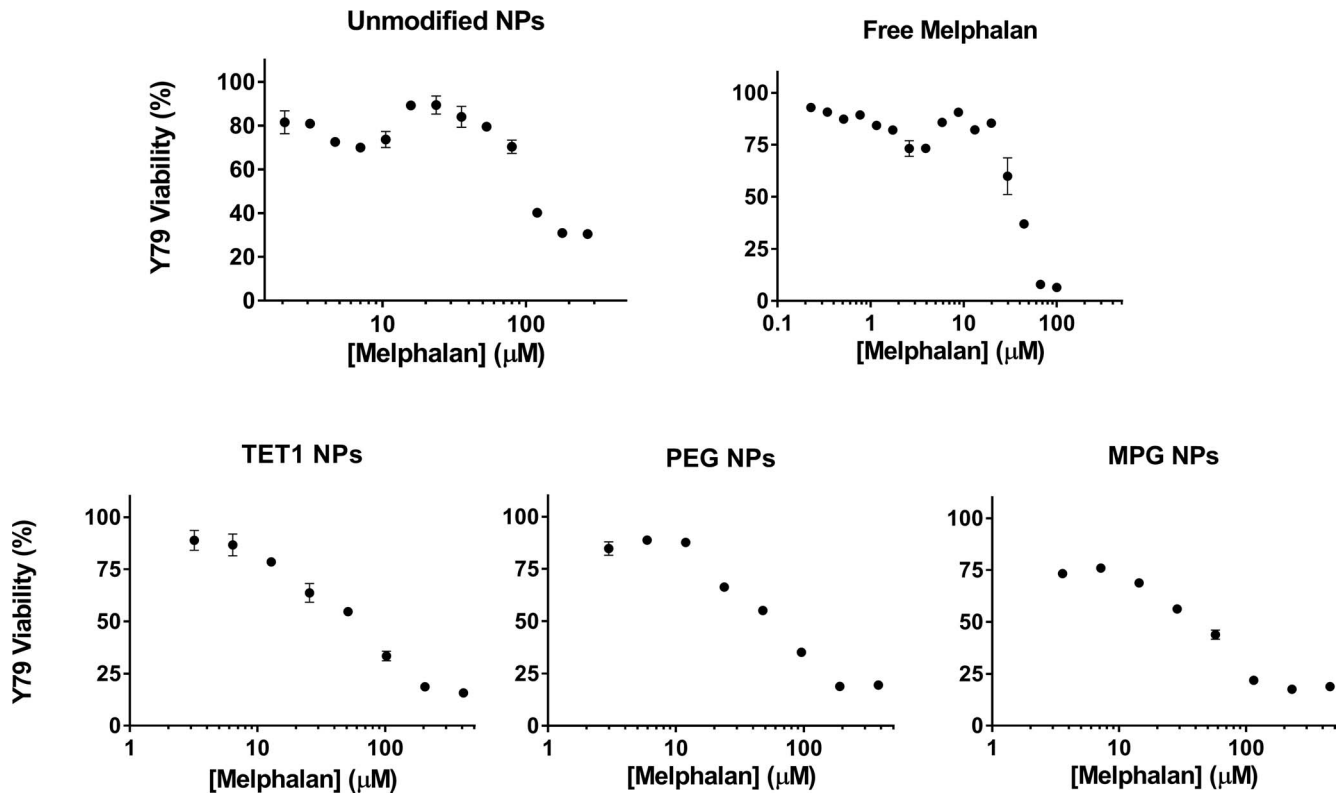


FIGURE 6. The cytotoxicity of surface-modified melphalan NPs, formulated with the 1 mg/mL saturation process, in Y79 retinoblastoma cells, as a function of melphalan dose. Cells were treated with varying concentrations of unmodified or surface-modified melphalan NPs for 24 hours, and cellular viability was compared to Y79 cells treated with free melphalan.

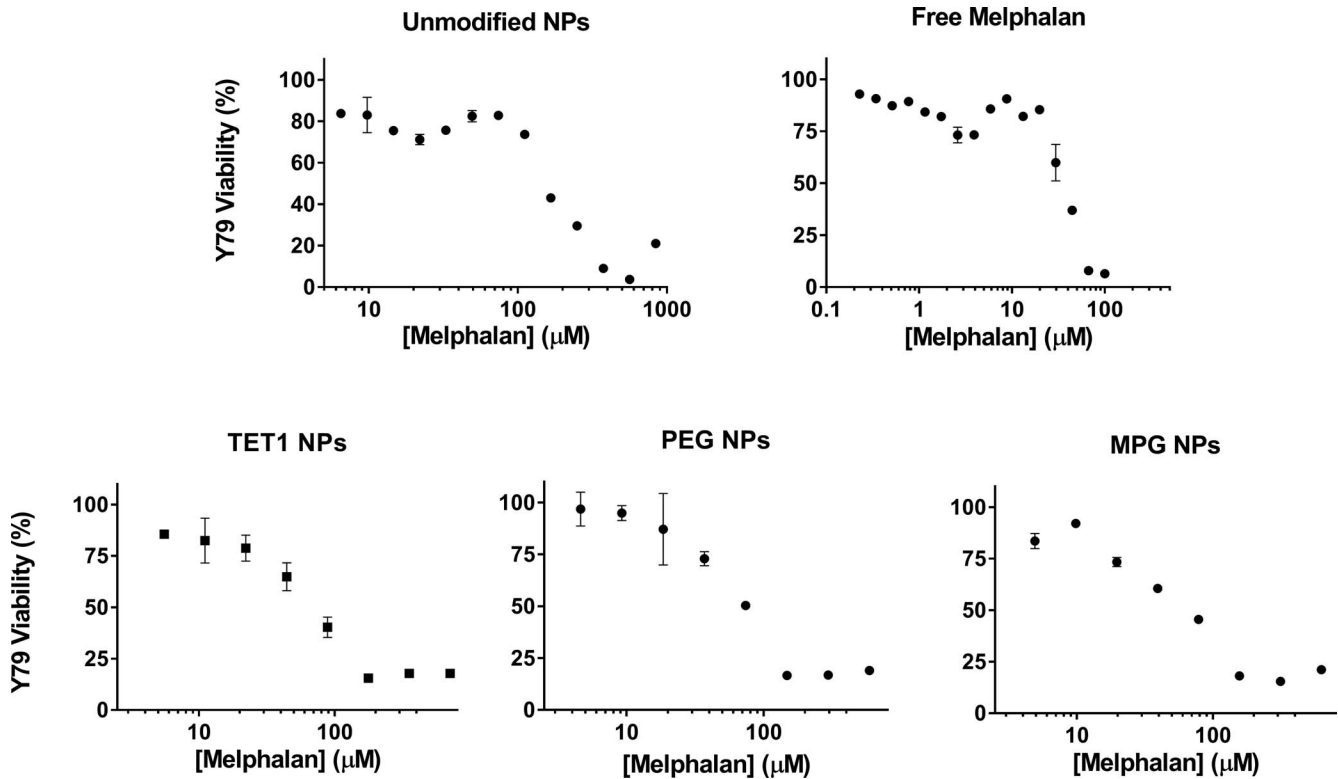


FIGURE 7. The cytotoxicity of surface-modified melphalan NPs, formulated with the 10 mg/mL saturation process, in Y79 retinoblastoma cells, as a function of melphalan dose. Cells were treated with varying concentrations of unmodified or surface-modified melphalan NPs for 24 hours, and cellular viability was compared to Y79 cells treated with free melphalan.

DISCUSSION

Many challenges face the ocular delivery of chemotherapeutic agents, including adverse side effects, an inability to treat avascular tumor seeds, poor bioavailability, and a need for multiple and frequent administrations. The chemotherapeutic melphalan, which is frequently used to treat retinoblastoma, faces additional challenges due to its poor aqueous solubility and rapid hydrolysis, forming two less efficacious byproducts. As an alternative to free drug administration, polymeric NPs present a potential platform to improve the ocular delivery of chemotherapeutic agents such as melphalan, by maintaining drug stability, reducing the need for frequent administration, targeting diseased tissues, and prolonging therapeutic effect.

PLGA is of particular interest for ocular drug delivery due to its biocompatibility, degradation profile, and clinically ap-

proved applications. To date, a variety of studies have investigated the effect of encapsulating active agents for ocular delivery to increase bioavailability and to improve NP transport in the vitreous space.^{6,13} Previously, PLGA NPs have been used to deliver active agents such as flurbiprofen to the anterior segment of the eye.^{22,23} Flurbiprofen-loaded NPs demonstrated enhanced anti-inflammatory effect over traditional eye drops in a rabbit ocular inflammation model, suggesting that the NPs increase the ocular residence time and bioavailability of flurbiprofen. In another study, polymeric NPs encapsulating pilocarpine to treat glaucoma demonstrated a prolonged mitotic effect by 40% relative to the traditional treatment of free pilocarpine.²⁴

In comparison, the treatment of ocular neoplasms at the posterior segment of the eye can present additional drug delivery challenges, such as specifically targeting and navigat-

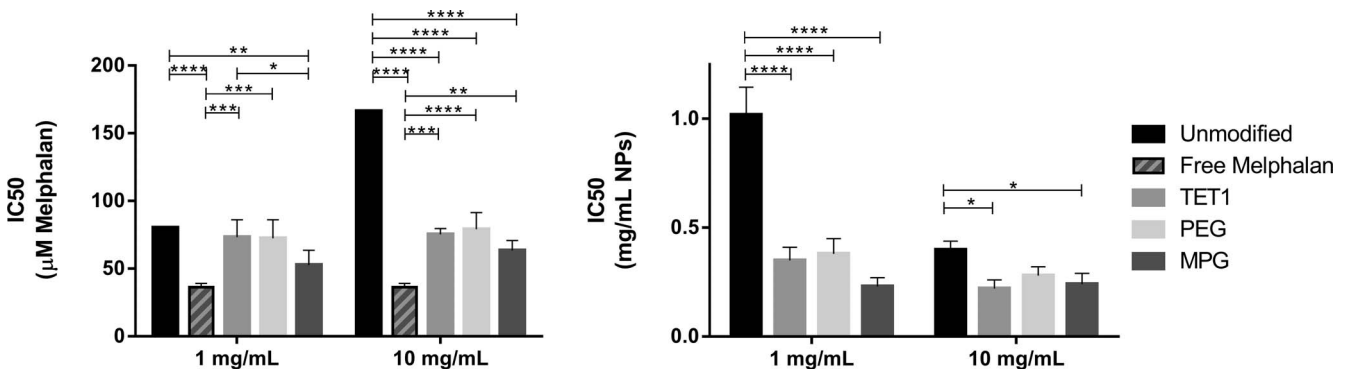


FIGURE 8. The IC₅₀ values of surface-modified 1 and 10 mg/mL saturated melphalan NPs as a function melphalan and NP concentrations. IC₅₀ values are shown as the mean ± SD with statistical significance. **P* ≤ 0.05, ***P* ≤ 0.01, ****P* ≤ 0.001, *****P* ≤ 0.0001.

TABLE 2. The Cytotoxicity of Surface-Modified Melphalan NPs, Expressed in Terms of IC₅₀, Formulated With the 1 and 10 mg/mL Saturation Processes, as a Function of NP Dose and Melphalan Concentration

	1 mg/mL				10 mg/mL			
	Unmodified	MPG	PEG	TET1	Unmodified	MPG	PEG	TET1
Y79 IC ₅₀ , μM	80.2 ± 0.5	52.6 ± 11	72.4 ± 13.8	73.3 ± 12.8	166.3 ± 0.3	63.4 ± 7.4	79.1 ± 12.3	75.3 ± 4.2
Y79 IC ₅₀ NP, mg/mL	1.02 ± 0.13	0.23 ± 0.04	0.38 ± 0.07	0.35 ± 0.06	0.39 ± 0.04	0.24 ± 0.05	0.28 ± 0.04	0.22 ± 0.04

ing the tumor microenvironment. To overcome these delivery challenges, NPs can be surface-modified with ligands to promote tumor cell specificity or uptake. Doxorubicin-loaded chitosan NPs that were conjugated to folic acid²⁵ demonstrated elevated levels of cellular uptake, relative to unmodified NPs and free drug. In a similar study in Y79 retinoblastoma cells, PLGA NPs encapsulating doxorubicin were linked to folate; it was observed that these modified NPs exhibited improved cell uptake and were more efficacious than unmodified NPs and free drug alone (Gupta J, et al. *IOVS* 2009;50:ARVO E-Abstract 5976). Additionally, studies have evaluated the impact of PEGylation on NP intravitreal diffusivity and drug delivery. To enhance NP diffusivity, PLGA NPs coated with poly(vinyl) alcohol and polystyrene NPs functionalized with PEG were assessed *ex vivo* in bovine vitreous. Surface functionalization of both NP platforms was observed to significantly improve NP diffusion within the vitreous and resulted in rapid penetration through the vitreous space.¹³ Together these studies suggest the impact of surface modification on drug delivery and efficacy when treating complex pathophysiologies at the posterior segment of the eye.

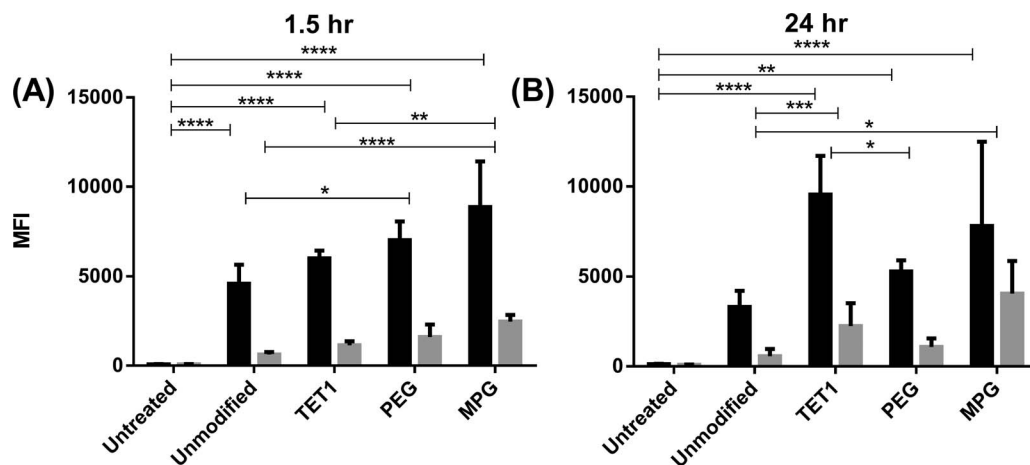
However, one of the challenges facing polymeric delivery platforms is the often observed burst release of active agent.²⁶⁻²⁸ Using our conventional NP synthesis protocol, we observed that melphalan was poorly loaded into PLGA NPs, likely resulting from melphalan diffusion from the NPs during the synthesis process. Similar studies have observed these trends with small hydrophilic molecules.²⁹

To help overcome the poor drug loading, and hence, inadequate release of melphalan, we employed an adapted synthesis technique in which the PVA solution used for NP hardening was saturated with melphalan (either 1 or 10 mg/mL) to prevent unwanted diffusion from the NPs (Fig. 1). As can be seen from Figure 3 and Table 1, the amount of drug associated (adsorbed to the NP surface or encapsulated within)

dramatically increased using the 1 and 10 mg/mL saturation methods by approximately 5- and 85-fold, respectively, which may be partially attributed to drug adsorption to the NP surface. Moreover, release studies demonstrated that NP formulations saturated with 1 and 10 mg/mL melphalan released 33 ± 1.1 and 286 ± 3.5 μg melphalan/mg NP, respectively, whereas unsaturated NPs released only 2.6 ± 0.3 μg melphalan/mg NP after 24 hours, attributed to the low melphalan loading within NPs.

After it was confirmed that the adapted fabrication process provided increased drug loading, surface-modified NPs were synthesized using this process. In addition to achieving similar high drug loading as with unmodified NPs, melphalan release from surface-modified NPs was dependent on the concentration used to “saturate” the PVA solution during fabrication, with the 10 mg/mL NPs releasing more melphalan than the 1 mg/mL formulations. While we acknowledge that some of the drug may be surface adsorbed, the saturation process offers a method to prevent unwanted melphalan loss during synthesis, thereby improving overall loading.

In addition to achieving high, localized drug concentrations, a serious challenge facing the conventional intravitreal administration of melphalan is the poor aqueous solubility, in which melphalan precipitates from solution after ~30 minutes. Under these conditions, where practical preparation involves melphalan solutions prepared by the pharmacy followed by immediate administration in the operating room, melphalan can rapidly precipitate, preventing the physician from administering the melphalan. In addition, melphalan may be rapidly hydrolyzed to less efficacious byproducts, when administered freely in buffered solution. Currently, the only way to overcome these challenges is by rapidly preparing and administering melphalan, which is inconvenient for clinicians and potentially reduces the efficacy of melphalan as it degrades.

**FIGURE 9.** The total cell association (*black*) and internalization (*gray*) of surface-modified C6 NPs with Y79 cells after (A) 1.5 hours and (B) 24 hours. Quantification of association and binding was assessed with flow cytometry and values are shown as the mean ± SD with statistical significance. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.

Given these challenges, frequent serial injections are often performed to maintain a therapeutically efficacious dose of melphalan within the vitreous space. However, each repeated injection, increases the risk of extraocular tumor dissemination and other adverse effects. The NPs used in this study present an advantageous technology that may maintain efficacious levels of active melphalan within the vitreous and thus reduce the need for frequent intravitreal administration, by binding to retinoblastoma cells and delivering their cargo directly, and avoiding the degradation associated with free melphalan administered in aqueous solution. Yet while NPs may decrease the need for repeated intravitreal injections, the presence of increased drug in the eye may increase the potential for drug toxicity. Although melphalan demonstrates *in vitro* safety at the concentrations tested, additional studies are needed to explore the potential of off-target effects *in vivo*.

In addition to maintaining stability of the encapsulants, this ability of delivery vehicles to enhance cellular association may significantly impact the therapeutic efficacy of the active agent, particularly *in vivo*. Cell-NP interactions can be enhanced with the use of surface modifications, to obtain increased NP localization at the surface or within target cells. In this work, TET1 and MPG NPs provided a significant increase in retinoblastoma cell association after 24 hours, and moreover, MPG NPs enhanced NP internalization by as much as 5-fold, relative to unmodified, TET1, and PEG-modified NPs. In contrast, cell association decreased for unmodified and PEG NPs. We believe that some of these NPs may have been internalized or recycled during this time frame as we have observed in other work.¹⁵

In parallel with these observations, we wanted to understand how surface modification and cell association correlates with efficacy in Y79 retinoblastoma cells. MPG NPs were the most efficacious formulation for NPs formulated using the low (1 mg/mL) saturated process, demonstrating similar efficacy to free melphalan. However, as a function of the NP concentration, all surface-modified NPs were more efficacious than unmodified NPs. For NPs fabricated with a more saturated melphalan solution (10 mg/mL), all surface-modified NPs were more efficacious than unmodified NPs but did not achieve efficacy similar to free melphalan. As a function of NP dose, the TET1 and MPG groups improved efficacy relative to unmodified NPs. These results in combination with the increased association and internalization observed in flow cytometry suggest that surface-modified NPs, and more specifically, TET1 or MPG NPs, may provide a platform that can more readily associate with Y79 cells. While not examined here, these modifications will likely have an additional benefit in future *in vivo* tissue transport as we have observed in References 16 and 17.

Based on these data, we postulate that the surface-modified NP formulations may significantly improve retinoblastoma treatment when administered *in vivo*. The physiologic conditions of the vitreous space create a much more complex environment through which the NPs must navigate to provide efficacious therapy. Having NPs surface-modified with ligands—such as MPG or TET1—may provide a way to overcome the challenges of *in vivo* delivery to enhance melphalan efficacy. Given the current clinical need for an efficacious, stable platform to deliver melphalan to treat retinoblastoma, the surface-modified NP vehicles presented here exhibit preliminary potential to meet this need.

In addition to the benefit these NPs may have in future transport studies, the technology presented also has direct implications as it may reduce the burden faced during the clinical administration of melphalan. The NP technology presented here provides a vehicle in which melphalan may be protected from aqueous solution during administration, and immediately after injection. Long-term, we envision this may

result in a more stable delivery method, provide ease-of-administration, and minimize side effects, with the potential to reduce the number of surgical procedures.

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