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Surfactant–Polymer Nanoparticles Overcome P-Glycoprotein-Mediated Drug Efflux

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Abstract: Nanoparticles enhance the therapeutic efficacy of an encapsulated drug by increasing and sustaining the delivery of the drug inside the cell. We have previously demonstrated that Aerosol OT (AOT)–alginate nanoparticles, a novel formulation developed recently in our laboratory, significantly enhance the therapeutic efficacy of encapsulated drugs like doxorubicin in drug-sensitive tumor cells. The purpose of this study is to evaluate the drug delivery potential of AOT–alginate nanoparticles in drug-resistant cells overexpressing the drug efflux transporter, P-glycoprotein (P-gp). AOT–alginate nanoparticles were formulated using an emulsion–cross-linking process. Rhodamine 123 and doxorubicin were used as model P-gp substrates. Cytotoxicity of nanoparticle-encapsulated doxorubicin and kinetics of nanoparticle-mediated cellular drug delivery were evaluated in both drug-sensitive and -resistant cell lines. AOT–alginate nanoparticles enhanced the cytotoxicity of doxorubicin significantly in drug-resistant cells. The enhancement in cytotoxicity with nanoparticles was sustained over a period of 10 days. Uptake studies with rhodamine-loaded nanoparticles indicated that nanoparticles significantly increased the level of drug accumulation in resistant cells at nanoparticle doses higher than 200 μg/mL. Blank nanoparticles also improved rhodamine accumulation in drug-resistant cells in a dose-dependent manner. Nanoparticle-mediated enhancement in rhodamine accumulation was not because of membrane permeabilization. Fluorescence microscopy studies demonstrated that nanoparticle-encapsulated doxorubicin was predominantly localized in the perinuclear vesicles and to a lesser extent in the nucleus, whereas free doxorubicin accumulated mainly in peripheral endocytic vesicles. Inhibition of P-gp-mediated rhodamine efflux with AOT–alginate nanoparticles was confirmed in primary brain microvessel endothelial cells. In conclusion, an AOT–alginate nanoparticle system enhanced the cellular delivery and therapeutic efficacy of P-gp substrates in P-gp-overexpressing cells.

Keywords: Sustained release; drug efflux; surfactants; polymer; cytotoxicity; multidrug resistance

Introduction

Development of simultaneous resistance to multiple drugs, termed multidrug resistance (MDR), is a frequent phenomenon in cancer cells.[1] The significance of this problem is highlighted by the estimations that up to 500 000 new cases of cancer each year will eventually exhibit a drug-resistant

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phenotype.[2] Overexpression of drug transporters, stress-response proteins, antiapoptotic factors, or other cellular proteins in tumor cells results in the development of MDR. Overexpression of P-glycoprotein (P-gp), a membrane-bound efflux pump and a product of the *ABCB1* (*MDR1*) gene, is a key factor contributing to MDR.[3]

Expression of P-gp leads to energy-dependent drug efflux and a reduction in intracellular drug concentration. While the exact mechanism by which P-gp interacts with its substrate is not fully understood, it is thought that binding of a substrate to the high-affinity binding site results in ATP hydrolysis, causing a conformational change that shifts the substrate to a lower-affinity binding site and then releases it into the extracellular space.[4] Tumor cells that overexpress P-gp do not accumulate therapeutically effective concentrations of the drug and are, therefore, resistant to the drug's cytotoxicity.

A number of studies demonstrate that P-gp-mediated drug efflux and MDR could be potentially overcome by the use of specific delivery systems. Studies indicate nanoparticles formulated using specific polymers like polyalkylcyanoacrylates can overcome P-gp-mediated drug efflux in cancer cells.[5,6] Similarly, cetyl alcohol/polysorbate-based nanoparticles have also been effective in overcoming P-gp-mediated efflux of paclitaxel in tumor cells.[7] Micellar delivery systems formulated using Pluronics inhibit P-gp-mediated drug efflux in both tumor cells and brain capillary endothelial cells.[8] Other studies demonstrate that conjugation of specific ligands such as transferrin[9] or incorporation of lysosomotropic functional groups[10] can improve the efficacy of nanoparticle-encapsulated drug accumulation in drug-resistant cells. More recently, a novel lipid–polymer nanocapsule formulation was also shown to successfully overcome MDR.[11] Incorporation of a P-gp inhibitor along with the drug in nanoparticles could enable a stronger anticancer activity in drug-resistant cancer cells.[12]

We have recently reported the fabrication of a novel surfactant–polymer nanoparticle system for efficient encapsulation and sustained release of water-soluble drugs.[13] These nanoparticles are formulated using dioctylsodium sulfosuccinate [Aerosol OT (AOT)] and sodium alginate. AOT is an anionic surfactant that is approved by the U.S. Food and Drug Administration as oral, topical, and intramuscular excipient. While intravenous administration of AOT could cause toxicity at high doses, previous studies have shown that AOT is eliminated completely through renal mechanisms and does not accumulate even after repeated dosing.[14] Sodium alginate is a naturally occurring polysaccharide polymer that has been extensively investigated for drug delivery and tissue engineering applications.[15,16] We have shown that AOT–alginate nanoparticles can sustain the release of water-soluble drugs such as doxorubicin and verapamil over a period of 4 weeks.[13] Importantly, AOT–alginate nanoparticles can significantly enhance and sustain the cellular delivery of water-soluble molecules such as doxorubicin, resulting in enhanced therapeutic efficacy in

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drug-sensitive tumor cells.[17] In this report, we demonstrate that AOT–alginate nanoparticles overcome P-gp-mediated drug efflux and drug resistance in P-gp-overexpressing cells without the use of additional P-gp inhibitors.

Materials and Methods

Materials. Rhodamine 123, doxorubicin, sodium alginate, polyvinyl alcohol, and calcium chloride were purchased from Sigma-Aldrich (St. Louis, MO). AOT, methanol, and methylene chloride were purchased from Fisher Scientific (Chicago, IL).

Methods. Nanoparticle Formulation and Characterization. Nanoparticles were formulated by a previously reported emulsification–cross-linking process.[18] In brief, an aqueous solution of sodium alginate [1.0% (w/v), 1 mL] and drug (5 mg) was emulsified into an AOT solution in methylene chloride [5% (w/v), 2 mL] using sonication over an ice bath. The primary emulsion was further emulsified into 15 mL of a 2% (w/v) aqueous PVA solution by sonication for 1 min to form a water-in-oil-in-water emulsion. Five milliliters of an aqueous calcium chloride solution [60% (w/v)] was added to the emulsion described above with stirring. The emulsion was stirred overnight to evaporate methylene chloride. Nanoparticles formed were recovered by ultracentrifugation (Beckman, Palo Alto, CA) at 145000*g*, washed two times with distilled water to remove unentrapped drug, resuspended in water, and lyophilized. Drug loading in nanoparticles was assessed by extracting 5 mg of nanoparticles with 5 mL of methanol for 30 min and analyzing the methanol extract for drug content. Doxorubicin and rhodamine concentrations were determined by HPLC (see below). Drug loading was represented as percent (w/w) and defined as the amount of drug encapsulated in 100 mg of nanoparticles. Particle size and ζ potential were determined using the Brookhaven 90Plus ζ potential equipment fitted with particle sizing software (Brookhaven Instruments, Holtsville, NY). Nanoparticles (0.1 mg) were dispersed in 1 mL of distilled water by sonication and were subjected to both particle size and ζ potential analysis.

HPLC Determination of Doxorubicin and Rhodamine. A Beckman Coulter HPLC system connected to Linear Fluor LC 305 fluorescence detector (Altech) and a C-18 column (Beckman Ultrasphere, octadecylsilane, 4.6 mm × 250 mm) was used. For doxorubicin, a 70:30 acetonitrile/water (adjusted to pH 3 with glacial acetic acid) mixture was used as the mobile phase at a flow rate of 1 mL/min. For rhodamine, a 50:20:30 acetonitrile/sodium acetate (adjusted to pH 4 with glacial acetic acid)/tetrabutylammonium bromide mixture was used as the mobile phase at a flow rate of 1 mL/min. Detection wavelengths were 505 and 550 nm for doxorubicin and 490 and 526 nm for rhodamine. Retention times were 7 and 3.2 min for doxorubicin and rhodamine, respectively.

Cell Culture. Human breast cancer cells (MCF-7) and RPMI-1640 medium were obtained from American Type Culture Collection (ATCC, Manassas, VA). NCI-ADR/RES (previously known as MCF-7/ADR) cells were obtained from the National Cancer Institute. Both cell lines were passaged in T-75 tissue culture flasks in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum. Bovine brain microvessel endothelial cells (BBMECs) were isolated from the gray matter of fresh bovine cerebral cortices using enzymatic digestion and centrifugal separation methods as described previously.[19] Freshly isolated cells were used to establish primary cultures of BBMEC. For the primary cultures, BBMEC were seeded (50 000 cells/cm^2) on collagen-coated, fibronectin-treated, 24-well polystyrene tissue culture plates (2 cm^2/well). The culture media consisted of 45% minimum essential medium, 45% Ham's F-12 nutrient mix, 10 mM HEPES, 13 mM sodium bicarbonate, 50 μg/mL gentamicin, 10% equine serum, 2.5 μg/mL amphotericin B, and 100 μg/mL heparin. The BBMECs were cultured in a humidified 37 °C incubator with 5% CO_2, with medium replacement occurring every other day until the monolayers reached confluence (approximately 10–14 days).

Cytotoxicity Studies. NCI-ADR/RES or MCF-7 cells were seeded in 96-well plates at a seeding density of 5000–10000 cells per well per 0.1 mL of medium and allowed to attach overnight. Following attachment, cells were treated with doxorubicin in solution or doxorubicin in nanoparticles. Untreated cells and empty nanoparticles were used as controls. The medium was replaced every alternate day, and no further dose of doxorubicin or nanoparticles was added. Cytotoxicity was determined over a period of 10 days using a commercially available MTS assay (Promega). Results were analyzed by using an ANOVA. Differences were considered significant at $P < 0.05$.

Uptake Studies. Nanoparticles containing rhodamine 123 were used for the study to avoid the complications of doxorubicin-induced cytotoxicity while evaluating drug accumulation. All the studies were performed at 37 °C unless specified. Cells were seeded in a 24-well plate at a density of 50 000 cells/well and allowed to attach overnight. Following attachment, cells were treated with rhodamine in solution or encapsulated in nanoparticles. To determine the effect of the dose of nanoparticles on rhodamine uptake, cells were treated with various doses (25–300 μg/mL) of nanoparticles containing rhodamine for 2 h. To determine the effect of ATP depletion on nanoparticle uptake, cells were preincubated with growth medium containing 0.1% (w/v) sodium azide and 50 mM deoxyglucose for 1 h and then

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incubated with a nanoparticle suspension (100 μg/mL) containing 0.1% (w/v) sodium azide and 50 mM deoxyglucose for 2 h. To study the effect of inhibition of active processes on cellular uptake of nanoparticles, cells were preincubated at 4 °C for 1 h and then treated with the nanoparticle suspension (100 μg/mL) at 4 °C for 2 h. To determine the effect of blank nanoparticles on rhodamine uptake, cells were treated with a mixture of blank nanoparticles (0, 30, or 300 μg/mL) and rhodamine in solution. To determine the effect of blank nanoparticles on fluorescein uptake, cells were treated with a mixture of blank nanoparticles (30 or 300 μg/mL) and fluorescein in solution.

At the end of the treatment period, cells were washed three times with cold PBS and then lysed using 100 μL of cell culture lysis reagent (CCLR; Promega). The protein content of the cell lysates was determined using the Pierce (Rockford, IL) BCA protein assay. Cell lysates were then mixed with 300 μL of methanol and incubated at 37 °C for 6 h at 100 rpm. Samples were centrifuged at 14 000 rpm for 10 min at 4 °C. The concentration of rhodamine in the methanolic extract was determined by HPLC as described before. Data are expressed as rhodamine accumulation normalized to total cell protein.

Fluorescence Microscopy. The uptake and intracellular distribution of doxorubicin in NCI-ADR/RES cells were determined qualitatively using fluorescence microscopy. Cells (5×10^5) were seeded on coverslips placed in 35 mm dishes. The following day, medium was replaced with fresh medium containing 2.5 μg/mL doxorubicin in solution or in nanoparticles. At 2 h post-treatment, cells were rinsed with drug-free medium and incubated with 75 nM Lysotracker Green (Invitrogen) for 30 min. Cells were then washed and counterstained with DAPI (4′,6-diamidino-2-phenylindole, Invitrogen). Images were captured with a BX60 Olympus fluorescence microscope. Images captured using red, blue, and green filters were overlaid to determine localization and association of doxorubicin-associated red fluorescence in the nucleus and endolysosomes, respectively.

Results

AOT–Alginate Nanoparticles Loaded with Doxorubicin or Rhodamine. Nanoparticles used in the study were essentially similar to those reported in our earlier publication.[17] Both rhodamine-loaded nanoparticles and doxorubicin-loaded nanoparticles were in a similar size range (500–700 nm) and had similar polydispersity indices (~0.28). The ζ potential of nanoparticles containing doxorubicin or rhodamine was around −13 to −14 mV. It is expected that the ζ potential reported for these formulations will be marginally stable. Drug loading was 4.6% (w/w) and 3.8% (w/w) for rhodamine and doxorubicin, respectively. The suspension stability of nanoparticles was unaffected by lyophilization, salt, or the presence of serum.

Enhanced and Sustained Cytotoxicity in MDR Cells. The cytotoxicity of nanoparticle-encapsulated doxorubicin was evaluated in vitro. Drug-sensitive MCF-7 cells demonstrated dose-dependent cytotoxicity to doxorubicin in solution, whereas concentrations of >50 μg/mL were required to induce cytotoxicity in the drug-resistant NCI-ADR/RES cells (Figure 1A,B). Addition of verapamil, a P-gp inhibitor, reversed the resistance to doxorubicin in NCI-ADR/RES cells (Figure 2). Nanoparticles enhanced the cytotoxicity of doxorubicin significantly in both drug-sensitive and drug-resistant cells. Nanoparticle-mediated enhancement of cytotoxicity observed in the drug-resistant cells was sustained during the 10 days of the study [$P < 0.05$ for all the days that were tested (Figure 2)]. There was no additional benefit of combining verapamil with doxorubicin in nanoparticles. Blank nanoparticles had no effect on cell survival, indicating that blank nanoparticles were not toxic to cells in the dose range that was tested.

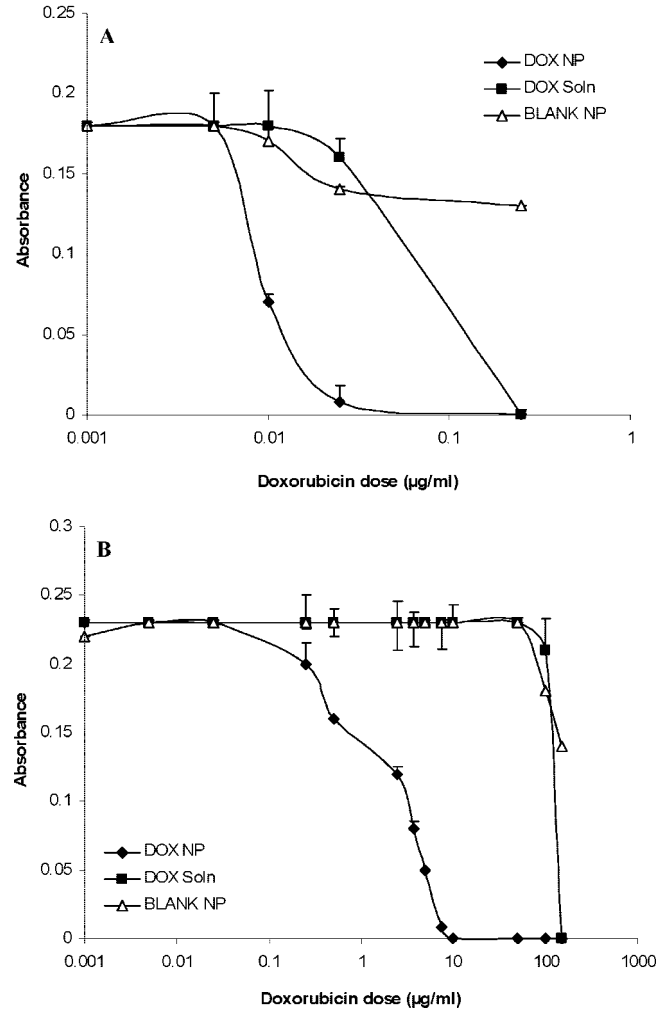

Figure 1. Enhanced cytotoxicity with doxorubicin nanoparticles in (A) MCF-7 cells and (B) NCI-ADR/RES cells. Cells were treated with blank nanoparticles (BLANK NP), doxorubicin in solution (DOX Soln), or doxorubicin in nanoparticles (DOX NP). Results are expressed as means ± the standard error from three independent experiments, each performed in duplicate.

Kinetics of Accumulation of Rhodamine in Resistant and Sensitive Cells. To determine the efficacy of cellular drug delivery with AOT–alginate nanoparticles, we compared

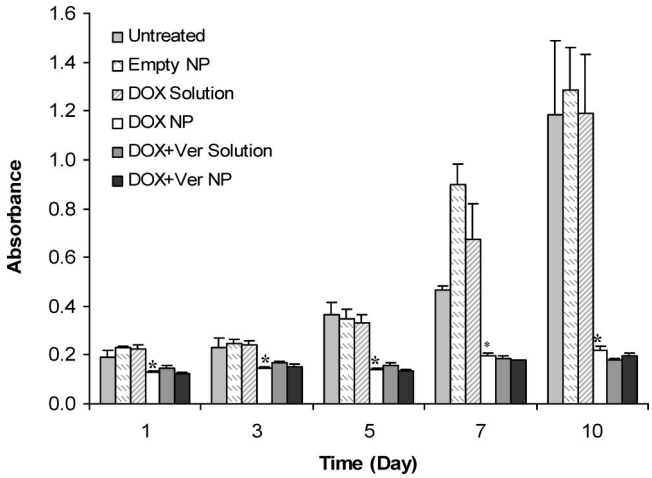

Figure 2. Sustained cytotoxicity with doxorubicin nanoparticles in NCI-ADR/RES cells. Cells were incubated with doxorubicin in solution (0.4 μg/mL), doxorubicin and verapamil (23.0 μg/mL) in solution (DOX+Ver Solution), doxorubicin in nanoparticles (equivalent to 0.4 μg/mL doxorubicin), or doxorubicin and verapamil in nanoparticles (DOX+Ver NP; equivalent to 0.4 μg/mL doxorubicin and 23.0 μg/mL verapamil). An asterisk indicates a P of <0.05 vs untreated cells ($n = 6$).

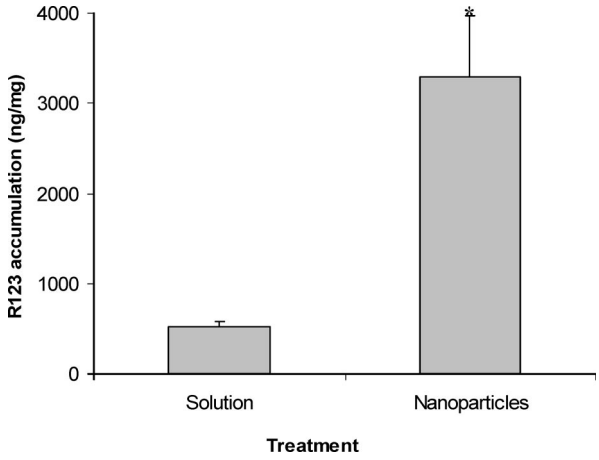

Figure 3. Cellular accumulation of rhodamine 123 (R123) in NCI-ADR/RES cells ($n = 4$). The asterisk indicates a P of <0.05 (t test).

cellular accumulation of rhodamine, a P-gp substrate, following treatment with an equivalent dose of rhodamine in solution and in nanoparticles. As one can see in Figure 3, treatment with rhodamine in nanoparticles resulted in a significantly higher level of accumulation of rhodamine than treatment with rhodamine in solution ($P < 0.05$). To determine the kinetics of drug accumulation with nanoparticles, we evaluated the cellular accumulation of rhodamine following treatment with different doses of nanoparticles containing rhodamine. As one can see in Figure 4A, in drug-sensitive MCF-7 cells, the level of accumulation of rhodamine increased in proportion to the nanoparticle dose. However, in drug-resistant NCI-ADR/RES cells, the level of rhodamine accumulation was low and nonlinear at nanoparticle doses

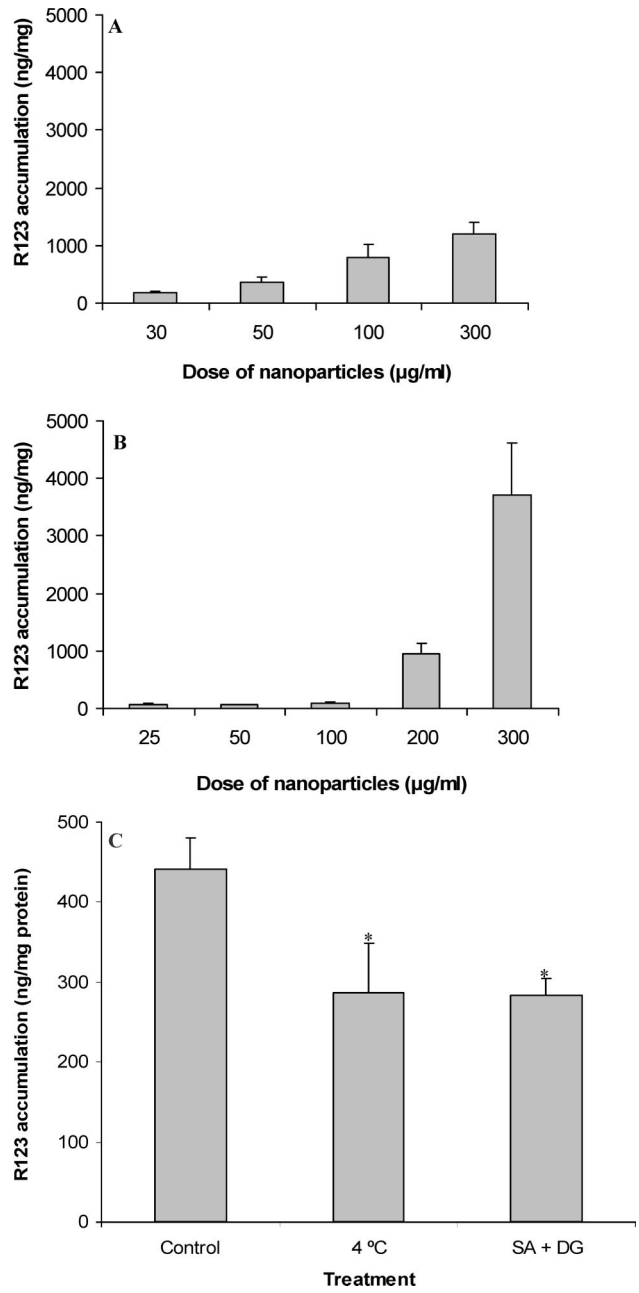

Figure 4. Effect of nanoparticle dose on rhodamine 123 (R123) accumulation in (A) MCF-7 cells and (B) NCI-ADR/RES cells. Cells were incubated with various doses of nanoparticles containing rhodamine for 2 h ($n = 4$). (C) Energy dependence of nanoparticle uptake in NCI-ADR/RES cells. Data are means $\pm$ the standard deviation ($n = 4$). An asterisk indicates a P of <0.05 compared to control (nanoparticle treatment at 37 °C and in the absence of inhibitors) (t test).

of less than 100 μg/mL (Figure 4B). At doses above 200 μg/mL, nanoparticles significantly enhanced cellular accumulation of rhodamine. To determine the mechanism of uptake of nanoparticles into cells, we evaluated the energy dependence of nanoparticle uptake in cells. Decreasing the rate of endocytosis by incubating cells at 4 °C or with metabolic inhibitors resulted in an ~40% reduction in the

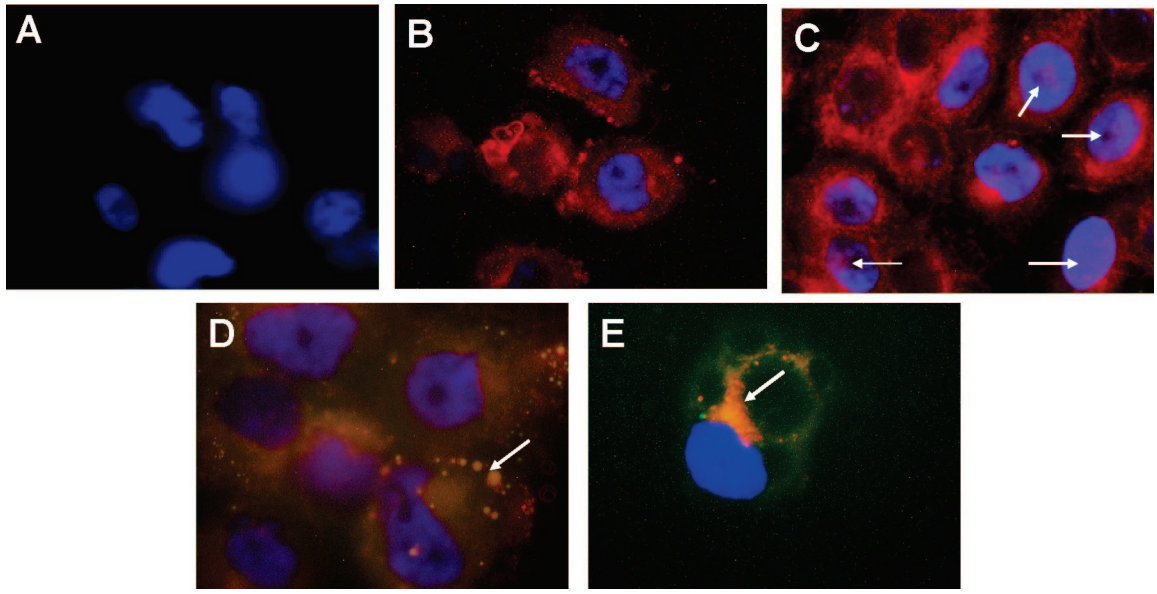

Figure 5. Intracellular distribution of doxorubicin. NCI-ADR/RES cells were treated with blank nanoparticles (A), doxorubicin in solution (B and D), or doxorubicin in nanoparticles (C and E) for 2 h. Cells were rinsed, counterstained with DAPI, and imaged by fluorescence microscopy (A–C). The magnification is 40×. In panels D and E, cells were also incubated with 75 nM Lysotracker Green for 30 min at 37 °C before being imaged. The magnification is 100×. Note that free doxorubicin is present near the cell surface (arrow in panel D) and is localized in endocytic vesicles. In the case of nanoparticles, a majority of doxorubicin is endocytosed and is present inside the cells rather than at the cell surface, extending all the way to the nucleus (arrows in panels C and E).

rate of cellular uptake of nanoparticles (Figure 4C). The energy dependence of nanoparticle uptake, along with dose and time dependence, suggests that cells internalize AOT–alginate nanoparticles through an endocytic process.

Intracellular Distribution of Doxorubicin. To determine whether encapsulation of doxorubicin in nanoparticles affected its trafficking inside drug-resistant cells, we evaluated the intracellular distribution of free and nanoparticle-encapsulated doxorubicin in NCI-ADR/RES cells that were stained for nucleus and endolysosomes. Free doxorubicin demonstrated a diffuse distribution within the cells, with a significant fraction appearing in vesicles located near the cell membrane (Figure 5B,D). Those vesicles stained positively with Lysotracker Green (Figure 5D), indicating that they were endolysosomal in nature. A significant proportion of nanoparticle-encapsulated doxorubicin also appeared to be present in endolysosomal vesicles (Figure 5C,E); however, these vesicles were concentrated at the peri-nuclear region rather than at the cell periphery. Further, doxorubicin was also present in the nuclei of cells treated with nanoparticle-encapsulated doxorubicin (Figure 5C). No or insignificant doxorubicin fluorescence was observed in nuclei of cells treated with doxorubicin in solution (Figure 5B).

Effect of Blank Nanoparticles on Rhodamine and Fluorescein Uptake. To determine whether blank nanoparticles had any effect on drug efflux, we studied the accumulation of rhodamine in drug-resistant cells in the presence and absence of blank nanoparticles. As one can see in Figure 6A, blank nanoparticles significantly enhanced rhodamine accumulation in drug-resistant cells at a nanoparticle dose of 300 μg/mL ($P < 0.05$) but not at a dose of 30 μg/mL. To determine whether nanoparticle-mediated enhancement in cellular uptake was nonspecific, we evaluated the effect of blank nanoparticles on the cellular accumulation of fluorescein sodium in drug-resistant cells. As one can see in Figure 6B, irrespective of the nanoparticle dose, blank nanoparticles did not affect the cellular accumulation of fluorescein.

Effect of Nanoparticles on the Uptake of Rhodamine in BBMECs. Cellular accumulation of rhodamine in the presence and absence of P-gp inhibitors was studied in primary BBMECs. As one can see in Figure 7, treatment with rhodamine in nanoparticles resulted in a >12-fold increase in the level of accumulation of rhodamine in BBMECs. Similar to that seen in drug-resistant tumor cells, blank nanoparticles significantly enhanced rhodamine accumulation in BBMECs. The enhancement observed with nanoparticles was much greater than that observed with P-gp inhibitors verapamil and GF 120918. Co-encapsulation of verapamil, a P-gp inhibitor, along with rhodamine in nanoparticles did not influence the effect of nanoparticles on rhodamine accumulation.

Discussion

Overexpression of P-gp results in active efflux of the anticancer agent from tumor cells. Whether P-gp extracts its substrate from the cytoplasm or from within the membrane ("vacuum cleaner" hypothesis) is not clear, but evidence suggests that substrates diffuse from the lipid bilayer into

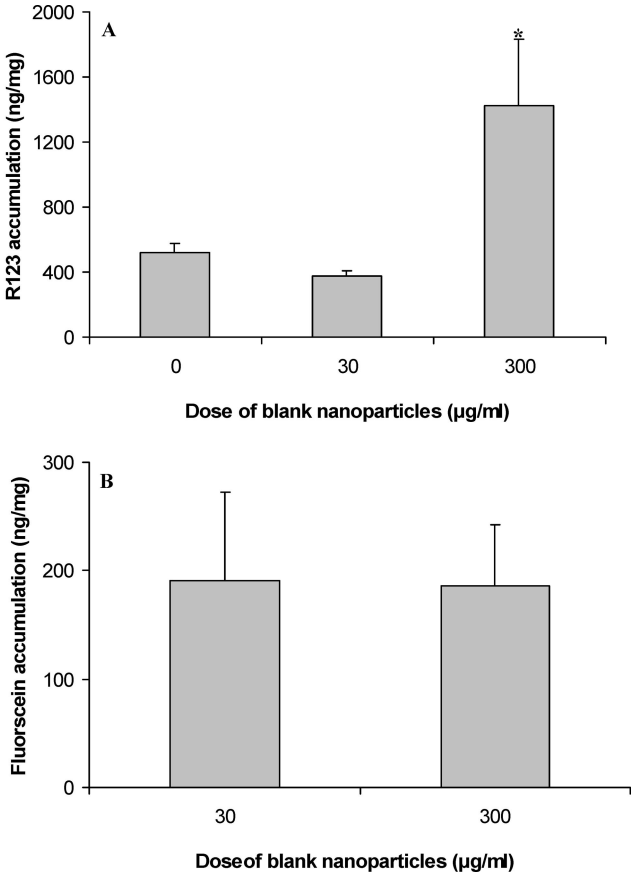

Figure 6. Effect of blank nanoparticles on the accumulation of (A) rhodamine 123 (R123) and (B) fluorescein in NCI-ADR/RES cells. Cells were incubated with a mixture of blank nanoparticles and rhodamine or fluorescein in solution for 2 h ($n = 4$). The asterisk indicates a P of <0.05 (t test).

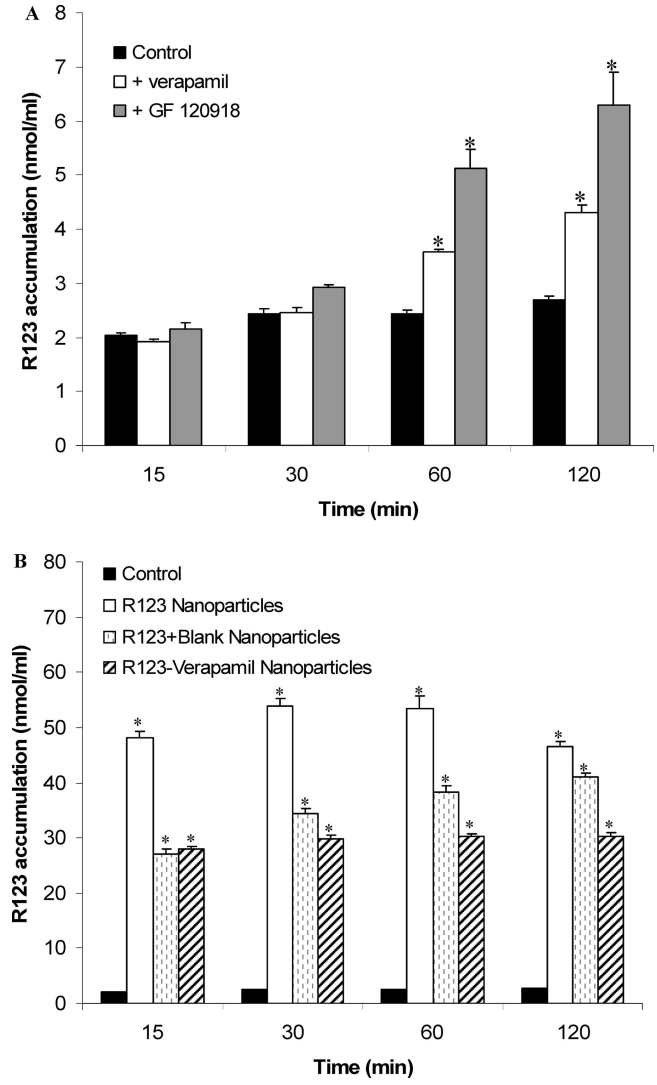

Figure 7. Cellular accumulation of rhodamine 123 (R123) in BBMEC monolayers. (A) Cellular accumulation of rhodamine in BBMEC monolayers in the absence and presence of P-gp inhibitors verapamil and GF120918. (B) Cellular accumulation of rhodamine in BBMEC monolayers exposed to either blank nanoparticles, nanoparticles containing rhodamine, or nanoparticles containing rhodamine and verapamil. Cells were exposed to the 300 μg/mL equivalent dose of nanoparticles. Control group consisted of cells that were exposed to R123 alone. Values represent the mean $\pm$ standard error of four monolayers per treatment group. An asterisk indicates a P of <0.05 (t test).

the drug-binding pocket of P-gp located in a hydrophobic environment.[20,21]

The objective of this study was to determine whether doxorubicin, a P-gp substrate, encapsulated in AOT–alginate nanoparticles was susceptible to P-gp-mediated drug efflux. Cytotoxicity studies in P-gp-overexpressing tumor cells demonstrated that nanoparticles loaded with doxorubicin alone were as effective as nanoparticles containing both doxorubicin and verapamil, suggesting that AOT–alginate nanoparticles can overcome P-gp-mediated drug resistance. However, this effect was dose-dependent; enhanced cytotoxicity was observed with a 300 μg/mL dose of nanoparticles but not with a 30 μg/mL dose (data not shown). Sustained cytotoxicity observed with nanoparticle-encapsulated doxorubicin correlates well with the sustained release properties of AOT–alginate nanoparticles. We have previously shown that AOT–alginate nanoparticles sustain the release of encapsulated doxorubicin over 15 days.[13] Further, we have shown that doxorubicin-loaded nanoparticles resulted in sustained cytotoxicity in drug-sensitive MCF-7 cells over 10 days of the study.[17] Thus, the duration of cytotoxicity observed in drug-resistant cells in this study is similar to that observed in drug-sensitive cells in our previous study.

We have previously shown that an increased level of cellular drug accumulation following treatment with AOT–alginate nanoparticles contributes to the enhanced therapeutic efficacy of a nanoparticle-encapsulated drug in drug-sensitive

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cells as well.[17] To evaluate whether AOT–alginate nanoparticles increase the level of drug accumulation in drug-resistant cells, we assessed the cellular accumulation of rhodamine, another model P-gp substrate, in NCI/ADR-RES cells. We observed that cells treated with nanoparticle-encapsulated rhodamine demonstrated higher levels of accumulation of rhodamine than those treated with a rhodamine solution. To further understand the dose effect observed in cytotoxicity studies, we determined the dose response in cellular accumulation of rhodamine in both drug-sensitive and -resistant cells. In drug-sensitive cells, nanoparticles demonstrated a near-linear dose–response relationship. A similar dose–response relationship has been observed for other nanoparticle systems and in other cell types that do not overexpress P-gp.[22] However, in drug-resistant cells, an inflection was observed in the dose–response curve, with significant drug accumulation observed only at doses higher than 200 μg/mL. This is consistent with the observations that nanoparticles enhanced doxorubicin cytotoxicity in P-gp-overexpressing cells at a 300 μg/mL dose but not at a 30 μg/mL dose.

Previous studies have shown that certain excipients such as Pluronics and polyethylene glycol can inhibit P-gp-mediated drug efflux.[23,24] To determine whether AOT–alginate nanoparticle formulation has a similar activity, we investigated the effect of blank nanoparticles on the cellular accumulation of rhodamine. Because the reversal of drug efflux appeared to be dependent on nanoparticle dose, we used two doses in the study. Consistent with the previous finding, enhancement in cellular accumulation was observed with the 300 μg/mL blank nanoparticle dose and not the 30 μg/mL dose.

One possible mechanism by which nanoparticles could enhance cellular accumulation of P-gp substrates is through permeabilization of the cell membrane. This is especially a concern, because surfactants are known to create pores in cellular membranes, [25] and nanoparticles used in this study contain anionic surfactant AOT. If the increased level of cellular accumulation observed with nanoparticles in this study were attributable to a permeabilized cell membrane, then it would be expected that similar enhancements would be seen in cells without P-gp overexpression and with drugs that are not P-gp substrates and that nanoparticles would cause toxicity. Blank nanoparticles, at the 300 μg/mL dose, did not enhance the accumulation of rhodamine in the non-P-gp-expressing MCF-7 cells (data not shown). Similarly, blank nanoparticles did not enhance the accumulation of fluorescein sodium, a non-P-gp substrate, in P-gp-overexpressing cells. Further, blank nanoparticles did not cause a significant toxicity in NCI/ADR-RES cells at the 300 μg/mL dose. The energy dependence of nanoparticle accumulation in cells suggests the involvement of endocytosis in nanoparticle uptake. Taken together, these results provide compelling evidence that the effects of the nanoparticles on drug or probe accumulation are not due to nonspecific effects on membrane permeabilization.

To further understand the mechanism of efficacy of nanoparticle-encapsulated doxorubicin, we studied the intracellular trafficking of doxorubicin. Doxorubicin causes cytotoxicity in tumor cells through several mechanisms; however, intercalation with genomic DNA in the nucleus and topoisomerase inhibition are considered primary events in doxorubicin-induced cytotoxicity. Thus, nucleus is the chief site of action for doxorubicin. Interestingly, cells treated with nanoparticle-encapsulated doxorubicin were found to accumulate doxorubicin in the nucleus, whereas cells treated with a doxorubicin solution did not. Enhanced nuclear delivery of doxorubicin by AOT–alginate nanoparticles could have contributed to the enhanced cytotoxicity observed with nanoparticle-encapsulated doxorubicin.

Enhanced nuclear accumulation of doxorubicin could be explained on the basis of the increased level of cellular accumulation of doxorubicin due to inhibition of P-gp-mediated drug efflux. The fact that blank nanoparticles also enable an increased level of cellular accumulation of free doxorubicin supports this hypothesis. The molecular mechanism by which AOT–alginate nanoparticles overcome P-gp-mediated drug efflux has not been established in this report. Also, it is not clear why the P-gp inhibition activity is seen only beyond a certain dose. Lack of activity in drug-sensitive cells and with non-P-gp substrates suggests that nonspecific membrane permeabilization is not involved. Different mechanisms of action have been proposed for other delivery systems that have exhibited activity in P-gp-overexpressing cells. Ion pair formation between doxorubicin and one of the polymer degradation products (cyanoacrylic acid), allowing shielding of doxorubicin's positive charge, has been suggested for polyalkylcyanoacrylate nanoparticles.[26] Ion pair formation and adsorption onto the cell surface have been suggested to promote an increased rate of diffusion of doxorubicin across the cell membrane, resulting in enhanced cell accumulation of doxorubicin. Recently, a lipid–polymer hybrid nanoparticle system formulated using anionic polymer dextran sulfate was shown to overcome P-gp-mediated efflux of doxorubicin.[11] It was shown that doxorubicin physically

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associated with nanoparticles bypasses the membrane-associated P-gp when delivered as nanoparticles, and in this form, the drug was better retained within the P-gp-overexpressing cells than the free drug. Transient inhibition of P-gp-associated ATPase, and the consequent depletion of the cellular ATP reserve, has been established as the mechanism by which Pluronics overcome P-gp-mediated drug efflux.[23] One could speculate that, like polyalkylcyanoacrylate nanoparticles, AOT–alginate nanoparticles shield the positive charge of doxorubicin and rhodamine and protect the drugs from efflux by P-gp. This is supported by the fact that drug-loaded AOT–alginate nanoparticles have a net negative charge. It is also pertinent to point out that in a previous study,[25] certain nonionic surfactants were shown also to specifically inhibit P-gp-mediated drug efflux, without inducing membrane permeabilization or cytotoxicity. While the mechanism of P-gp inhibition was not reported in that study, it can be expected that charge shielding was not involved because the surfactants used were nonionic. In addition, it is possible that the dose of the substrate delivered to the cell with nanoparticles was higher than the maximum capacity of the efflux pump, resulting in saturation. This could explain the enhanced efficacy observed at higher doses of nanoparticles.

To determine whether the drug efflux reversal activity of AOT–alginate nanoparticles is observed in other P-gp-overexpressing cells, we determined the effect of AOT–alginate nanoparticles on rhodamine accumulation in BBMEC monolayers. The BBMECs are primary cells isolated from brain microvessels that form the blood–brain barrier. BBMECs overexpress P-gp, which contributes to the decreased rate of brain transport of P-gp substrates across the blood–brain barrier.[27] Similar to that observed in tumor cells, nanoparticles loaded with rhodamine, or a mixture of blank nanoparticles and rhodamine solution, demonstrated a significant enhancement of rhodamine accumulation in BBMECs. Nanoparticle-mediated enhancement of the level of cellular accumulation was much greater than that observed with low-molecular weight P-gp inhibitors. This study confirms that AOT–alginate nanoparticles can overcome P-gp-mediated drug efflux not only in tumor cells but also in normal, nontransformed cells.

In addition to P-gp inhibition, another significant advantage of AOT–alginate nanoparticles is the fact that following encapsulation of weakly basic drugs, nanoparticles have a net negative charge, which stabilizes nanoparticles in buffer and in medium containing serum. This is an advantage over other nanoparticle delivery systems such as polycyanoacrylate nanoparticles that become cationic following encapsulation of weakly basic drugs like doxorubicin.[28] We speculate that due to the presence of an excess of highly electronegative sulfosuccinate groups from AOT and carboxyl groups from alginate in nanoparticles, loading of cationic drugs does not alter the ζ potential of nanoparticles. The ability to sustain doxorubicin-induced cytotoxicity over a period of 10 days is another important advantage of AOT–alginate nanoparticles over other delivery systems.

Conclusions

Encapsulation of doxorubicin in AOT–alginate nanoparticles resulted in a significant and sustained enhancement of doxorubicin-induced cytotoxicity in drug-resistant tumor cells. Increased therapeutic efficacy of nanoparticle-encapsulated drug was associated with an increase in the level of cellular and nuclear drug accumulation. An increase in the level of cellular accumulation was observed even with a mixture of blank nanoparticles and rhodamine solution. Enhancement of cellular accumulation of rhodamine in drug-resistant cells was not caused by membrane permeabilization. Finally, inhibition of P-gp-mediated drug efflux with AOT–alginate nanoparticles was not limited to drug-resistant tumor cells but was also observed in primary cells overexpressing P-gp.

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