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***In Vitro* Activity of Paclitaxel-Loaded Polymeric Expansile Nanoparticles in Breast Cancer Cells**

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Abstract

Through a series of *in vitro* studies, the essential steps for intracellular drug delivery of paclitaxel using a pH-responsive nanoparticle system have been investigated in breast cancer cells. We successfully encapsulated paclitaxel within polymeric expansile nanoparticles (Pax-eNPs) at 5% loading via a miniemulsion polymerization procedure. Fluorescently tagged eNPs were readily taken up by MDA-MB-231 breast cancer cells grown in culture as confirmed by confocal microscopy and flow cytometry. The ability of the encapsulated paclitaxel to reach the cytoplasm was also observed using confocal microscopy and fluorescently labeled paclitaxel. Pax-eNPs were shown to be efficacious against three *in vitro* human breast adenocarcinoma cell lines (MDA-MB-231, MCF-7 and SK-BR-3) as well as cells isolated from the pleural effusions of two different breast cancer patients. Lastly, macropinocytosis was identified as the major cellular pathway responsible for eNP uptake, as confirmed using temperature-sensitive metabolic reduction, pharmacologic inhibitors, and fluid-phase marker co-localization.

Keywords

drug delivery; nanoparticle; paclitaxel; breast cancer; cellular uptake; endocytosis

Introduction

Nanoparticle drug delivery systems are a promising technology platform to overcome potential drug inadequacies and provide improved specificity, solubility, stability, and appropriate pharmacokinetics.^{1–9} One field of medicine where drug delivery systems can have a significant influence is cancer therapy. Breast cancer is the most common malignancy in women and ranks second in cancer-related deaths, impacting the lives and families of nearly 200,000 US women and 1.2 million women worldwide.¹⁰ Most patients undergo surgery (i.e., lumpectomy, mastectomy, radical mastectomy), as well as treatment with adjuvant therapies such as radiation or systemic therapy, including biologic therapy, chemotherapy and/or hormone therapy in order to prevent recurrence or for treatment of advanced tumors not amenable to resection. Several factors influence the effectiveness of

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Conflict of Interest

The authors do not have a conflict of interest.

Supporting Information Available

HPLC trace of paclitaxel-loaded nanoparticles, breast cancer cell line *in vitro* viability, and inhibitor cytotoxicity data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

chemotherapy in breast cancer, specifically tumor size, extent of lymph node involvement, the presence or absence of different cell receptors, and the method of delivery.

Due to their potential ability to improve the delivery of chemotherapeutic agents (e.g., methotrexate, doxorubicin, paclitaxel) to cancer cells while maintaining antitumor efficacy and minimizing toxicity to healthy tissues, researchers are investigating nanoparticle (NP) formulations of several anticancer drugs.^{5,11–13} The use of nanoparticles in breast cancer therapy has the potential to allow simultaneous tumor targeting and drug delivery.¹⁴ Nanoparticle formulations of several common breast cancer chemotherapeutic agents, including nanoparticle albumin bound (*nab*)-paclitaxel, Abraxane,^{15,16} poly(L-glutamylglutamate)-conjugated paclitaxel,¹⁷ liposomal doxorubicin and PEG-ylated liposomal doxorubicin,^{18,19} and doxorubicin-loaded, folate-conjugated polymeric micelles²⁰ have demonstrated improved efficacy and fewer toxic side effects compared with their conventional formulations.

A particular clinical problem with the use of paclitaxel delivered in its clinical formulation of Cremophor/ethanol (C/E) is the minimal amount of paclitaxel that reaches the diseased tissue(s). In order to maximize intra-tumoral drug delivery, paclitaxel-loaded expansile nanoparticles (Pax-eNPs) were designed to release paclitaxel locally in the presence of a mildly acidic pH, as occurs within the tumor cell endosome.²¹ We have recently reported the *in vivo* efficacy and superiority of Pax-eNPs compared to Pax-C/E against human triple negative breast cancer in both microscopic and established murine breast cancer models.²² Specifically *in vivo* growth rates of the primary tumor following Pax-eNP treatment in the microscopic prevention model were significantly slower than the equivalent dose of Pax-C/E administered locally or the 3-fold higher Pax-C/E dose given systemically. Moreover, only 25% of Pax-eNP treated mice had clinical evidence of tumor compared to 67% and 92% of the mice following treatment with local or systemic Pax-C/E, respectively. In the established model, the Pax-eNP treated mice exhibited smaller tumors and significantly slower tumor growth than all other treatment groups. The average tumor doubling time in Pax-eNP treated mice was about 18 days compared to 7 days with locally or systemically administered Pax-C/E. In addition, migration of Pax-eNP to axillary lymph nodes resulted in higher intranodal paclitaxel concentrations (9.2 fold greater) and a significantly lower incidence of lymph node metastases (2.5 fold less) compared to the Pax-C/E treatment group. These results provide motivation to further study this NP drug delivery system by ascertaining the mechanism of cellular uptake of eNPs, and evaluating efficacy against other breast cancer cell lines. Therefore, in this study we have investigated the cellular uptake, paclitaxel delivery, and *in vitro* cytotoxic activity of Pax-eNPs against three different established human breast adenocarcinoma cell lines (MDA-MB-231, MCF-7 and SK-BR-3) as well as non-immortalized cancer cells isolated from the pleural effusions of two breast cancer patients.

Materials and Methods

General procedures and materials

All chemicals were purchased from Sigma-Aldrich or Lancaster Synthesis and used without further purification. MDA-MB-231 breast adenocarcinoma cells (ATCC) were cultured in Leibovitz's L-15 media supplemented with 10% fetal bovine serum (FBS), 3.5 g of glucose/L and 2.5 mL of penicillin-streptomycin/L (complete growth media) at 37 °C and atmospheric air. Cell viability studies were performed using a CellTiter 96® AQueous One Solution tetrazolium-based assay (Promega) where the absorbance was measured at 492 nm via a Beckman Coulter AD 340 96-well plate reader. In this MTS assay, the measured absorbance is proportional to the total metabolic activity of the cells in the well, giving a relative measure of the viable cell population in each well.

Synthesis of paclitaxel-loaded nanoparticles

Nanoparticles were prepared using a miniemulsion polymerization method.²¹ First, 50 mg of monomer (5-methyl-2-(2,4,6-trimethoxyphenyl)-[1,3]-5-dioxanylmethyl methacrylate) and 0.5 mg of crosslinker (1,4-*O*-methacryloylhydroquinone) were dissolved in 0.5 mL of CH₂Cl₂. For the encapsulation of paclitaxel as 5% Pax-eNPs, 2.5 mg of the drug was dissolved in the CH₂Cl₂ along with the monomer and crosslinker. This organic solution was added to 2 mL of a 10 mM aqueous buffer solution containing sodium dodecyl sulfate (SDS). By changing the amount of SDS the size of eNPs can be varied, and for these experiments we selected eNPs in the 50 nm range by using 5 – 10 mg of SDS. This mixture was sonicated for 10 min (1 s pulses with a 2 s delay) with 20 W of power under an argon blanket to create the miniemulsion. Using a base-catalyzed reaction following sonication, 20 μ L of a 200 mM aqueous ammonium peroxydisulfate (APS) solution and 2 μ L of *N,N,N',N'*-tetramethylethylenediamine (TEMED) were added to the emulsion and stirred under an argon blanket for 2 h. Following polymerization, the suspension was stirred overnight while open to the atmosphere to allow for evaporation of the remaining solvent. The resulting polymeric nanoparticles were dialyzed against 5 mM pH 8.0 phosphate buffer over two days to remove excess surfactant, salts and non-encapsulated drug. When synthesizing rhodamine-labeled nanoparticles, 0.05 mg of PolyFluor™ 570 (methacryloxyethyl tricarbonyl rhodamine B; Polysciences, Inc.) was dissolved in the CH₂Cl₂ along with the monomer and crosslinker before addition of this organic phase to the aqueous phase. The size of the nanoparticles was determined by DLS and SEM. By DLS the NP were on average $\approx$ 100 nm in diameter. SEM analysis revealed 100 nm as well as many smaller nm eNPs, and the average diameter and standard deviation was determined to be 32 ± 23 nm via sizing 200+ particles in a viewing field (Figure 1).

Measurement of paclitaxel encapsulation efficiency

A 300 μ L sample of nanoparticles was lyophilized and 1 mg of the dried nanoparticles was weighed on a microbalance. The sample of nanoparticles was dissolved in 200 μ L of CH₂Cl₂ and mixed with 0.5 mL of 1:1 acetonitrile/water. The dichloromethane was evaporated while stirring under a stream of nitrogen, and the precipitated polymer was removed by centrifuging the samples at 5000 $\times$ g for 10 min. The concentration of paclitaxel in the remaining acetonitrile/water phase was determined by high performance liquid chromatography (HPLC). HPLC detection of paclitaxel was performed using a 20 min linear gradient from 60:40 to 35:65 water:acetonitrile at a flow rate of 1.0 mL/min on a MetaChem 5 Taxsil 250 $\times$ 4.6 mm column and Varian ProStar HPLC with detection at 210–218 nm. This process also confirmed the ability to synthesize Pax-eNPs without degradation of the drug as evidenced by the observation of a single paclitaxel peak on all HPLC traces.

Confocal laser microscopy

MDA-MB-231 cancer cells were cultured in 6-well plates containing 2 mL of complete growth medium. Before seeding the cells onto the 6-well plates, a sterile 22 $\times$ 22 mm glass coverslip was placed on the bottom of each well. After 2 days of incubation, cells were treated for 8 hours with eNPs loaded with paclitaxel Oregon Green® 488 conjugate (Invitrogen) (PaxOG-eNPs) vs. media only as a control. At the end of the treatment period, the media was aspirated from the wells and the coverslips were moved to new wells in a 6-well plate. The coverslips were rinsed with phosphate buffered saline (PBS) (2 mL $\times$ 1) and incubated for 20 min with 2 mL of 3.7% neutral buffered formalin to fix the cells. The coverslips were washed with PBS (2 mL $\times$ 1) and PBS containing calcium and magnesium (complete PBS; 2 mL $\times$ 1) and then incubated with 50 μ g/mL concanavalin A-Alexa Fluor 647 conjugate (Invitrogen) in PBS (1 mL) for 5 min in order to identify the cell membrane. Lastly, the coverslips were washed with PBS (2 mL $\times$ 1) and complete PBS (2 mL $\times$ 1)

before being mounted on glass microscope slides for imaging. The fixed and stained cells were imaged on an Olympus FluoView FV1000 scanning confocal laser microscope using 488 nm and 633 nm lasers to detect paclitaxel Oregon Green® 488 and concanavalin A-Alexa Fluor 647 independently.

Quantitative measurement of nanoparticle uptake by flow cytometry

Flow cytometry was used to quantify the cellular uptake of rhodamine-labeled eNPs in MDA-MB-231 human breast adenocarcinoma cells. Cells at 80,000 cells/mL were cultured in 6-well plates containing 2 mL of complete growth media per well. After allowing the cells to adhere and grow for 24 hours, the media was removed from the wells and replaced with 2 mL of media containing 50 µg/mL rhodamine-eNPs (polymer concentration). The cells were incubated with the nanoparticles for 2, 4, 8, 12 or 24 hours at either 37 °C or 4 °C. Control experiments not containing nanoparticles were also performed. At the end of the incubation time, the media was removed and the cells were washed 3X with 2 mL PBS before harvesting by trypsin treatment. The cell suspensions were centrifuged at $250 \times g$ for 5 minutes before being resuspended in 1 mL PBS and fixed in 3.7% paraformaldehyde for 15 minutes. After fixation, all samples were washed twice with cold FACS buffer (PBS with 1% BSA) by centrifugation at $250 \times g$ for 5 minutes and resuspended in FACS buffer at 1×10^6 cells/mL. Cell-associated nanoparticle content was immediately assayed by flow cytometry using a Beckman-Coulter (Dako) MoFlo™ high-speed cell analyzer/sorter.

Confirmation of late-endosome localization of Rho-eNP

To determine if the rhodamine-labeled eNPs were observed in the late endosome, a second imaging experiment was performed. The cells were seeded at 20,000 cells in glass bottom dishes (35 mm diameter with coverslip No. 1.5, MatTek Corporation, Ashland, MA) with complete L-15 media for 24 hours at 37 °C in 5% CO₂. Cells were incubated with Rho-eNP for another 24 hours. The dishes were washed twice with phenol-red-free culture media, and twice with HBSS. Cells were stained with Hoechst 33342 (Life Technologies, Carlsbad, CA) and Alexa Fluor® 633 conjugated wheat germ agglutinin (WGA 633, Life Technologies, Carlsbad, CA) for identification of the nucleus and cell membranes respectively. LysoTracker® Green DND-26 (Life Technologies, Carlsbad, CA) was added to achieve final concentration of 50 nmol/mL in the culture media at 15 minutes prior confocal imaging. Confocal microscope images were obtained with Zeiss LSM 510 inverted confocal laser scanning microscope with Plan-Apochromat 10×/0.45 or C-Apochromat 40×1.2 W corr lens (Carl Zeiss Microscopy, Thornwood, NY). Z-stack images were obtained for the analysis of co-localization. Velocity 6.0.1 software (PerkinElmer Inc., Waltham, MA) was utilized to quantify co-localization with calculation of the co-efficient parameter “MX”, which indicates percentage of Rho-eNP in late endosome. Two to 3 representative fields were selected in each culture dishes for the co-localization analysis.

In vitro cell viability

Nanoparticle cytotoxicity was evaluated using MDA-MB-231, MCF-7 and SK-BR-3 human breast adenocarcinoma cell lines in culture. Cells were plated at 30,000 cells/mL in 96-well plates containing 100 µL of complete growth media at 37 °C. The culture media and gas exchange conditions used for the cell lines were Leibovitz's L-15 medium in atmospheric air (MDA-MB-231), EMEM in 5% CO₂ (MCF-7) and McCoy's 5A medium in 5% CO₂ (SK-BR-3). After culture for 24 h, the media was removed from the wells and replaced with complete growth media containing a known concentration of 1) drug-free nanoparticles, 2) paclitaxel-loaded nanoparticles, or 3) free paclitaxel (diluted from a stock solution of 1 mg/mL paclitaxel in DMSO) without nanoparticles. Media alone controls were also included. After 7 d of exposure, the media was removed from the wells and replaced with 100 µL of

fresh media, followed by the addition of 20 μ L of CellTiter 96® AQueous One Solution (Promega) in order to assess viability using a standard MTS assay.

Pleural effusion *in vitro* cell viability

Pleural fluid was collected, under an approved IRB protocol through Dana Farber Cancer Institute at the time of therapeutic thoracentesis performed in two patients with known malignant effusion due to breast cancer as determined on previous cytopathologic characterization. Tumor cells were isolated with centrifugation and Ficoll gradient separation, washed and seeded in culture flasks in serum-free culture media overnight. A one-minute selective trypsinization was performed to remove remaining stromal cells, and serum-positive culture media was added. Cells were grown to near-confluence, trypsinized and used for *in vitro* viability assays. Cells were plated at 30,000 cells/mL in 96-well plates containing 100 μ L of complete growth media at 37 °C and 5% CO₂. After culture for 24 h, the media was removed from the wells and replaced with media alone or media containing a known concentration of drug-free or paclitaxel-loaded nanoparticles, or free paclitaxel. After 7 d of exposure, the media was removed and an MTS *in vitro* cell viability assay as described above was performed.

Determination of endocytosis inhibitor concentration

To determine non-toxic doses of the pharmacological endocytosis inhibitors, viability assays were performed. MDA-MB-231 breast cancer cells were initially plated in 96 wells at a concentration of 30,000 cells/mL in complete L-15 media. After allowing the cells to adhere and grow for 24 hours, the media was removed from the wells and replaced with 2 mL media containing a range of inhibitor concentrations. Cells were incubated with the inhibitors for 4 hours at 37 °C. Controls not containing inhibitors were also performed. At the end of the incubation time, the media was removed from the wells and the cells were washed 3X with 2 mL PBS before fresh culture media was added. After 48 h, the media was removed from the wells and replaced with 100 μ L of fresh media, followed by the addition of 20 μ L of CellTiter 96® AQueous One Solution (Promega) in order to assess viability using a standard MTS assay. The highest inhibitor concentration, which was not itself cytotoxic, was chosen for all subsequent inhibitor assays.

Determination of uptake pathway via endocytotic inhibition

For cellular uptake experiments, MDA-MB-231 breast cancer cells were plated in 6-well plates at 30,000 cells/mL in complete L-15 media (2 mL) and allowed to adhere for 24 hours. Cells were pre-incubated in the presence of the inhibitors for 1 hour before exposure to a 50 μ g/mL (polymer concentration) suspension of rhodamine-labeled eNPs in full growth media for an additional 4 hours, during which time an inhibitor was also present. The pharmacological inhibitors were used at the following concentrations: sodium azide 0.01%, 2-deoxyglucose 20 mM, chlorpromazine 10 μ M, hexamethylene amiloride 20 μ M, wortmannin 10 μ M, genistein 50 μ M, methyl- β -cyclodextrin 5 mM. Following nanoparticle exposure, the media was removed and all wells were washed 3X with 2 mL PBS before harvesting by trypsin treatment. Cell suspensions were centrifuged at 250 $\times$ g for 5 minutes before being resuspended in 1 mL PBS and fixed in 10% paraformaldehyde for 15 minutes. After fixation, all samples were washed with cold FACS buffer (PBS with 1% BSA) by centrifugation at 250 $\times$ g for 5 minutes and resuspended in FACS buffer (1 $\times$ 10⁶ cells/mL). Cell-associated nanoparticle content was immediately assayed by flow cytometry using a Beckman-Coulter (Dako) MoFlo™ high-speed cell analyzer.

Confirmation of uptake pathway via endocytosis marker co-localization

Laser scanning confocal microscopy was used to directly visualize the co-localization of the expansile nanoparticles with an endocytotic fluid phase marker. MDA-MB-231 cells were plated in complete L-15 media at 25,000 cells/mL in collagen-coated glass bottom 35mm dishes (MatTek, Ashland MA) and allowed to adhere for 24 hours at 37 °C in 5% CO₂. Cells were treated with rhodamine-labeled eNPs (50 µg/mL polymer concentration) in the presence of the endocytosis marker Cascade Blue® hydrazide (5 µM). Following nanoparticle/Cascade Blue® exposure for 4 hours, the media was removed and all wells were washed 2X with 2 mL PBS before fixation in 3.7% formalin for 15 minutes. Cells were imaged using an Olympus FluoView FV1000 laser scanning confocal microscope in sequential mode to eliminate emission cross-talk between the various dyes.

Results and Discussion

Paclitaxel is used clinically in the treatment of breast cancer but suffers from poor aqueous solubility. We therefore selected paclitaxel for use in our expansile nanoparticle delivery system for this study. A mild miniemulsion polymerization process allowed us to successfully encapsulate paclitaxel without degradation, as determined by HPLC, following our published procedure.²¹ Specifically, a base-catalyzed free-radical polymerization method was employed. Paclitaxel was encapsulated at a loading level of 5% (wt/wt monomer) by dissolving the drug in the organic phase along with the monomer and crosslinker prior to emulsification. The encapsulation efficiency was found to be $98.3 \pm 3.4\%$ (mean $\pm$ SD). These nanoparticles of 20–100 nm were designed to undergo expansion in a pH-dependent fashion with release of the encapsulated drug at pH 5, as found within the cellular endosome, thereby achieving intracellular drug release (Figure 1).^{21,23,24}

We first investigated the ability of Pax-eNPs to enter tumor cells and reach the late endosomal compartments where their pH-triggered release of paclitaxel would be activated. In this confocal microscopy experiment, the triple negative human breast cancer cells, MDA-MB-231, were treated with rhodamine-labeled eNPs and then stained with Hoechst 33342, Alexa Fluor® 633 conjugated wheat germ agglutinin, and LysoTracker® Green DND-26 for identification of the nucleus, cell membrane, and late endosome respectively (Figure 2). The red fluorescent signal from the eNPs is clearly observed within the cells confirming uptake. Furthermore, the green fluorescent signal from the LysoTracker and the red fluorescent signal from the eNPs were observed from the same position for a number of cytosol locations. The average co-localization efficiency was determined to be $52\% \pm 5\%$ (mean $\pm$ SEM) indicating that 52% of the Rho-eNPs were located in the late endosome. In a second confocal microscopy study, eNPs were loaded with a fluorescently labeled paclitaxel Oregon Green® 488 conjugate (PaxOG; Invitrogen) and exposed to the MDA-MB-231 cells. Following incubation for 8 hours with the nanoparticles, the MDA-MB-231 cells were washed, fixed, and stained with concanavalin A-Alexa Fluor 647 to highlight the cell membrane (Figure 3). As can be seen in the composite confocal laser microscopy image on the bottom right of Figure 3, the extracellular membrane is distinctly seen in red, while the fluorescent drug (pseudo-colored green) is distributed evenly throughout the cytoplasm, indicating successful delivery of paclitaxel to the cytoplasm.

Flow cytometry was subsequently employed to quantify eNP cellular uptake. After exposing MDA-MB-231 human breast cancer cells to nanoparticles covalently labeled with rhodamine for 2, 4, 8, 12 or 24 hours, the cells were analyzed. As shown in Figure 4, the number of cells demonstrating eNP uptake progressively increased as a function of incubation time with $36.20 \pm 0.9\%$ of cells experiencing particle uptake within 24 hours at 37 °C. In contrast, when cells were incubated with eNP at 4 °C to decrease metabolic activity, cellular uptake was significantly reduced to <1% at all time points ($P < 0.001$). The

decrease in intracellular eNP detected at low temperature indicates that the cellular uptake of the expansile nanoparticles is an energy-dependent process. We also evaluated the uptake of eNP in human mammary epithelial cells, MCF-10A, as a control cell line. Similar uptake was observed under these *in vitro* conditions between the MCF-10A and MDA-MB-231 lines (see SI).

Given that the eNPs are taken up by breast cancer cells and that paclitaxel can be delivered intracellularly using the eNPs, we evaluated the dose-dependent anti-tumor activity of paclitaxel-loaded expansile nanoparticles (Pax-eNPs) *in vitro* using three human breast cancer cell lines that differ in terms of the key clinical determinants of estrogen receptor and Her-2/neu expression: MDA-MB-231, MCF-7 and SK-BR-3. After 7 days of exposure to Pax-eNPs, unloaded-eNPs, or paclitaxel alone, the cell viability was determined using a colorimetric MTS assay. Unloaded (drug-free) nanoparticles failed to exhibit cytotoxicity against any of the cell lines at the concentrations tested (0.01 to 100 $\mu\text{g/mL}$ polymer concentration), indicating that the nanoparticles themselves were not toxic to the cells. Furthermore, as shown in Figure 5, Pax-eNPs (5% wt paclitaxel/wt polymer) resulted in significant cytotoxicity against each of the cell lines tested. The cytotoxicity profiles resembled that of free paclitaxel with the calculated IC_{50} of Pax-eNP being ~ 10 , 7.5 and 5 ng/mL of paclitaxel for the MDA-MB-231, MCF-7, and SK-BR-3 cell lines, respectively, compared to 2.5–5 ng/mL for free paclitaxel demonstrating that paclitaxel is released from the eNP and is effective (individual data graphs are shown in SI). The IC_{50} obtained with the MDA-MB-231 agreed with our previous result obtained with a MDA-MB-231-Luc cell line.²² A similar increase in the IC_{50} compared to paclitaxel has been observed with other cell lines (A549) and attributed to the expanded eNP acting as reservoir for the drug thereby, in fact, limiting the immediate bioavailability of the drug.²⁵ Thus, the performance of Pax-eNPs was not significantly better than paclitaxel alone in this *in vitro* mode, and, furthermore, Pax-eNP and Pax were both cytotoxic to MCF-10A cells, as this cell line is sensitive to paclitaxel (see SI).²⁶ Despite this lack of *in vitro* superiority of the nanoparticle delivery system, we observed a significant improvement in paclitaxel performance when delivered using eNPs in three separate *in vivo* MDA-MB-231-Luc tumor models (i.e., microscopic disease, established disease, and lymph node metastases).²² The superior performance *in vivo* may stem from the ability of eNPs to maintain high concentrations of the drug within tumor tissue for prolonged periods of time, which may also be the reason eNPs perform poorly compared to free drug in *in vitro* assays. These results speak to some of the limitations in performing only *in vitro* assessments to characterize a NP drug delivery system, where drug and NP are not subjected to biodistribution, clearance, or degradation and are confined to the culture well with the tumor cells for as long as the assay is performed.

We next evaluated the cytotoxicity of Pax-eNPs against cultured cells obtained from the pleural effusions of two different breast cancer patients. Tumor cell samples were collected at the time of a therapeutic thoracentesis performed in patients with known malignant effusions due to breast cancer as determined on previous cytopathologic characterization. Patient 1 had a poorly differentiated invasive ductal carcinoma that was estrogen receptor, progesterone receptor and HER2 negative, and had failed prior treatment that included Adriamycin, cytoxan, paclitaxel, gemcitabine, and cisplatin. Patient 2 had a moderately differentiated invasive ductal carcinoma that was estrogen receptor and progesterone receptor positive, and had previously failed treatment regimens that included letrozole, zoledronic acid, capecitabine, tamoxifen, protein-bound paclitaxel, doxorubicin, and fulvestrant.

Similar to the *in vitro* viability assays with the established cell culture lines, fresh tumor cells from malignant pleural effusion were exposed to unloaded-eNPs, 5% Pax-eNPs,

paclitaxel alone, or were left untreated for direct viability comparison. After 7 days of exposure, cell viability was determined using the MTS assay; the results of exposure of cells from Patient 1 and 2 to the various treatment groups are displayed in Figure 6. For both patient samples, the Pax-eNPs had a cytotoxicity profile similar to the profile of free paclitaxel, but with a slight rightward shift. The calculated Pax-eNP IC₅₀ values was ≈ 9 ng/mL. This demonstrates that the Pax-eNPs are able to deliver encapsulated drug and effectively inhibit *in vitro* proliferation of breast cancer cells directly harvested from patients.

Having established the *in vitro* efficacy of Pax-eNPs, we next investigated the mechanism of eNP cellular uptake. Based on previous reports, size, charge, shape, material composition, and surface chemistry are key parameters that determine the mechanism and rate of cellular uptake of nanomaterials.^{27–29} We determined the mechanism of eNP uptake by using several different types of endocytotic inhibitors to block various cellular uptake pathways followed by flow cytometry quantification. Generally, endocytosis can be divided into two broad categories: phagocytosis (the uptake of large particles) and pinocytosis (the uptake of fluids and solutes). Given the small size of eNPs, pinocytosis is most relevant to these cells and has multiple forms depending on the cell origin and function. The most current classification system for pinocytosis is based on the proteins involved in the different endocytotic pathways and is divided into clathrin-dependent or -mediated endocytosis and clathrin-independent endocytosis.^{27,30,31} The clathrin-independent pathways are further classified as 1) caveolae-mediated endocytosis, 2) macropinocytosis, and 3) clathrin- and caveolae-independent endocytosis.³⁰

Clathrin-mediated endocytosis is the “classical route” of cellular entry, which is present and inherently active in all mammalian cells.^{30,31} Mechanistically, it involves the engulfment of receptors associated with their ligands in a coated pit.³² Caveolae-mediated endocytosis relies on the presence of the hairpin-like membrane protein, caveolin-1, which is necessary for the biogenesis of the caveolae.^{31,33} Macropinocytosis is a special case of clathrin- and caveolae- independent endocytosis, which is initiated by the transient activation of receptor tyrosine kinases.^{34,35} The receptor activation triggers formation of membrane ruffles that protrude to engulf the surrounding fluid in the extracellular milieu, forming large (0.5 – 10 μ m) intracellular vacuoles termed macropinosomes.^{35,36} The final pinocytosis classification, clathrin- and caveolae- independent endocytosis, includes those pathways not usually classified by the above criteria.

Exclusion of specific endocytotic mechanisms is a distinct and powerful technique to elucidate endocytosis. This can be achieved by using various pharmacologic inhibitors of endocytosis that include chemical or biological agents.^{29,34,37–40} This method offers several advantages over other techniques, such as genetic manipulation, since exposure to pharmacologic inhibitors affect all cells during exposure. This results in homogenous cell responses and less need for compensatory mechanisms, since the cells are usually exposed to inhibitors for only a short time period.³⁷ One limitation with the use of chemical inhibitors is that they are rarely selective and often disturb multiple endocytosis pathways.³⁷ Therefore, it is essential to utilize a combination of such inhibitors to validate the inhibitory mechanism and to establish effective inhibitor concentration in particular cell types.

The optimal inhibitor concentrations (as defined by a maximum dose while maintaining at least 75% cell viability compared to non-treated cells) were identified via cell proliferation assays. Based on this criterion, the pharmacological inhibitors were used at the following concentrations: sodium azide 0.01%, 2-deoxyglucose 20 mM, chlorpromazine 10 μ M, hexamethylene amiloride 20 μ M, wortmannin 10 μ M, genistein 50 μ M, methyl- β -cyclodextrin 5 mM (See Figure S5 in the SI). For the cellular uptake experiments, cells were

pre-incubated in the presence of the inhibitors for 1 hour before exposure to a suspension of rhodamine-labeled eNPs in media for 4 hours, during which time the inhibitors were also present. Following nanoparticle exposure, the media was removed and the cells were washed, fixed, and analyzed by flow cytometry. The cellular uptake data were expressed as the percentage of uptake relative to cells that had not been inhibited (positive uptake control).

Consistent with the eNP uptake studies performed at 4 °C, the results in Figure 7 suggest that endocytosis is the dominant pathway of cellular uptake, since treatment with $\text{NaN}_3/2$ -deoxyglucose almost completely diminished nanoparticle uptake (>95% decrease in uptake). Significant inhibition of eNP uptake (~80% inhibition) was also observed in the presence of wortmannin, which blocks the action of phosphoinositide 3-kinases (PI3Ks). PI3K is required for spontaneous surface ruffling, an integral part of macropinocytosis; suggesting that eNPs enter the cell through macropinocytosis. Since loss of PI3K function is known to block internalization of ligands needed for the clathrin- and caveolae-mediated pathways,⁴¹ confirmation of macropinocytosis involvement was obtained by the use of a more selective ion channel inhibitor, hexamethylene amiloride.³⁷ Similar to wortmannin, treatment with this compound afforded a ~60% inhibition on nanoparticle uptake, thus confirming a role for macropinocytosis in eNP uptake. Similarly, uptake was reduced by ~65% when cells were treated with methyl- β -cyclodextrin, which inhibits cholesterol-dependent endocytosis by extracting cholesterol from the plasma membrane. Cholesterol is involved in membrane ruffling and actin reorganization, among other endocytotic functions,⁴² such that inhibition of eNP uptake by methyl- β -cyclodextrin is also indicative of macropinocytosis.^{42–44} In contrast, no reduction in nanoparticle uptake was observed when inhibiting specific caveolae-mediated and clathrin-mediated pathways with either genistein or chlorpromazine, respectively, suggesting that these pathways do not play a significant role. Therefore, the pharmacological interference data presented above support macropinocytosis as a major pathway responsible for uptake of the eNPs.

Finally, laser scanning confocal microscopy was used to directly visualize the intracellular trafficking of the expansile nanoparticles based on the above results. The MDA-MB-231 cells were treated with rhodamine-labeled eNPs in the presence of the endocytosis marker Cascade Blue® hydrazide, a small-molecule fluid-phase uptake marker used for macropinocytosis visualization. When cells were co-incubated with nanoparticles and Cascade Blue®, co-localization of the rhodamine-eNPs and Cascade Blue® was observed (Figure 8). In addition, the co-localization results obtained with the LysoTracker® Green DND-26 and rhodamine-labeled eNPs discussed earlier (Figure 3) showed that the eNPs localize to the late endosome. Both of these results support the conclusion that eNPs are actively brought into tumor cells via the macropinocytotic pathway.

Conclusions

The findings from these studies provide important information on this delivery system while also highlighting some of the advantages and limitations of using *in vitro* experiments to predict *in vivo* performance. First, eNPs are internalized in MDA-MB-231 breast cancer cells primarily via the macropinocytosis pathway as confirmed by temperature-sensitive metabolic reduction, pharmacologic inhibitors, and fluid-phase and endosomal marker co-localization. Once internalized, eNPs enter the late endosome where the locally acidic environment results in particle expansion and liberation of their encapsulated paclitaxel payload allowing it to reach the cytoplasm. This escape of paclitaxel from the endosome to the cytoplasm is crucial to the successful performance of this delivery system because paclitaxel acts by stabilizing microtubules in the cytoplasm and, thereby, preventing cell division and inducing apoptosis. Pax-eNPs release sufficient drug to induce cytotoxicity

with an IC₅₀ value of 10 ng/mL for MDA-MB-231 breast cancer cells. The Pax-eNP formulation was active against two other human breast cancer cell lines, which possess different estrogen receptor and Her-2/neu expression levels, as well as tumor samples harvested from two patients who had undergone prior treatment with paclitaxel. It is important to note that in all of these *in vitro* toxicity assays, the Pax-eNPs did not perform better than Pax alone. In these *in vitro* assays drug is unable to diffuse away from the local cellular environment, as happens rapidly *in vivo*, and therefore sufficient time is provided for the Pax-eNPs or Pax to interact with the cell and lead to apoptosis. Nevertheless, *in vitro* experiments are generally easier and more rapidly accomplished than *in vivo* studies and allow greater knowledge of and control over experimental parameters thereby affording a method to screen delivery system performance and pathways of uptake (rate, pathway, cellular localization). *In vitro* studies do not substitute for *in vivo* experiments and the results obtained *in vitro* may, or may not, be good indicators of *in vivo* performance. As in the case with this NP system, Pax-eNPs perform significantly better than paclitaxel alone in the *in vivo* studies but not the *in vitro* cytotoxicity experiments.

These results combined with our recently published *in vivo* studies²² provide a detailed account of the activity and performance of Pax-eNPs against human breast cancer. Additionally, Pax-eNPs have shown *in vivo* efficacy in several other murine tumor models including non-small cell lung cancer,^{21,25,45} mesothelioma,^{46,47} and ovarian,⁴⁸ indicating broad anticancer applicability. Given these results, future experiments must focus on development of scalable eNP synthesis, identification of suitable storage conditions, pharmacokinetic studies of encapsulated radiolabeled-paclitaxel, and biodistribution studies of the eNP in a large animal model in order to move this technology closer to the clinic.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. (top) The chemical structure of the polymeric expansile nanoparticles (eNPs) and the polymer cleavage reaction at pH 5 lead to a swollen, expanded hydrogel state. The nanoparticles (light blue spheres; initially ≈ 50 nm diameter) are taken up into cells where they undergo a hydrophobic to hydrophilic transition, swell several hundred-fold in volume and release drug (yellow spheres). Not to scale. (bottom) Scanning electron micrograph of the synthesized eNPs (scale bar = 200 nm) and histogram of particle size.

Figure 2.

Localization study with Rho-eNPs after 24 hours incubation with cells using various fluorescent markers. (**top left**) Blue - Hoechst DNA stain; (**top right**) Green - LysoTracker stain; (**bottom left**) Red - rhodamine-labeled eNPs; (**bottom right**): White - WGA-633 cell membrane stain; (**far right**) merged image displaying the fluorescence overlay of merged image from all four channels.

Figure 3. Confocal laser microscopy images of MDA-MB-231 breast cancer cells incubated for 8 hours with PaxOG-eNPs. **(top left)** Intracellular Oregon Green® 488 Taxol signal detected using 488 nm laser. **(top right)** Concanavalin A-Alexa Fluor 647 signal detected using 633 nm laser and marking the plasma membrane in red. **(bottom left)** Differential interference contrast image. **(bottom right)** Composite image showing intracellular delivery of the Oregon Green® 488 Taxol to the cell cytoplasm. Scale bar = 10 μ m.

Figure 4. Uptake of rhodamine-eNPs in MDA-MB-231 breast adenocarcinoma cells determined via flow cytometry as a function of time and temperature. The % cells with eNP uptake at 4 °C is minimal (<1%) at all time points. Data displayed as mean $\pm$ SD (n = 3).

Figure 5.

Established breast cancer cell line viability relative to untreated controls following treatment for 7 days with varying concentrations of 5% Pax-eNPs. The same trends were observed when treating cells with free paclitaxel (see additional data in SI). In contrast, unloaded eNPs were not toxic (data shown is for MDA-MB-231 and was similar for the other two cell lines). Data are displayed as the mean $\pm$ SD ($n = 3$).

Figure 6.

Relative viability of tumor cells harvested from the malignant pleural effusion from breast cancer Patient 1 (**top**) and Patient 2 (**bottom**) treated with 5% Pax-eNP, unloaded eNP, or paclitaxel alone for 7 days. Data are displayed as the mean $\pm$ SE (n = 3).

Figure 7. Relative cell uptake of rhodamine-labeled eNPs in the presence of inhibitors as determined by flow cytometry. Results are expressed as percentage of uptake compared to positive Rho-eNP control without inhibitors. Data displayed as mean $\pm$ SD ($n = 3$).

Figure 8.

Uptake of rhodamine-labeled eNPs after 4 hours of co-incubation in the presence of the fluid-phase marker Cascade Blue® hydrazide. Red: rhodamine-labeled eNPs; Blue: Cascade Blue® hydrazide. White arrows indicate representative examples of co-localization (purple). Note: pseudo-color was used to enhance contrast and facilitate observation and the bottom image displays fluorescence overlay of the differential interference contrast image.