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13. ABSTRACT (Maximum 200 Words) Current chemotherapeutic delivery systems are toxic due drug hydrophobicity. Long circulating polymeric nanoparticles (NPs) are efficient in encapsulating hydrophobic drugs and can provide high loading capacity, controlled release, and compatibility with little toxicity. The use of amphiphilic copolymers provides high solubilization and better drug stability <i>in vivo</i> . Our studies are focused on small nano carrier systems that utilize amphiphilic biodegradable polymers and interact with cellular targets. The use of PEO-Poly(β -Amino Ester) (PBAE) Nanoparticles to encapsulate paclitaxel was successful with high loading dose and provided pH dependent release and demonstrated cytotoxicity on human breast cancer cells in comparison to control nanoparticles with no encapsulated drug. Our goal is to quantitate the effects of PBAE nanoparticle system and to expand its potential to other drugs such as doxorubicin and ceramide for its efficacies in breast cancer. Our experimentation is focused on the deduction of a formulation of a combination therapy with a delivery system that is practical, reliable and reproducible.				
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Annual Report

Introduction

Cancer of the breast continues to be the most common cause of death in middle-aged women. Despite a considerable research effort and clinical trials, the response to paclitaxel (Pt) or doxorubicin (Dx) as a single agent is small with considerable toxicity and requires hospital based treatment. The documented toxicity of Pt, Dx or Ce as single agents is, in part, due to the drug delivery system utilizing alcohol, DMSO or commonly used cremophore due to the drug hydrophobicity. Our approach is based on the utilization of nanoparticles (NPs) by an efficient chemical synthesis to encapsulate chemotherapeutic drugs. The encapsulation of drug(s) in the NPs will provide high drug loading capacity, controlled drug release and good compatibility between the core-forming block and the drug. Our studies are focused on the formulation of a combination therapy with a delivery system that is practical, reliable, efficient, reproducible and overcomes harsh conditions associated with NP technology. A successful outcome of the proposed studies will provide a new avenue for future therapeutic strategies for breast cancer treatment.

Body

Our initial focus has been in the determination of the in vitro cytotoxic effects of Pt, Dx or Ce on human breast cancer cell line, MCF-7. Initially, cells were plated in a microplate with and without single drug additions at different concentrations. A 3-day cytotoxicity assay with the use of MTT dye technique demonstrated that the ED₅₀ dosage were 0.6-6 ug/ml, 10-50 ug/ml and 12.5-25 ug/ml for Pt, Dx and Ce respectively.

The second set of studies was directed to encapsulate Pt in nanoparticles optimally. To that effect, the following studies were performed:

Optimization of Nanoparticle Production:

Paclitaxel-Containing PEO-Poly(β -Amino Ester) (PBAE) Nanoparticles

Synthesis and Characterization:

PBAE are synthetic biodegradable and biocompatible pH-responsive polymers that were shown to be very effective for encapsulation and intracellular delivery of hydrophobic drugs. PBAE are synthesized by Michael's addition reaction between a primary amines or secondary diamines with diol-diacrylates. In our studies, we prepared a number of different PBAE (Figure 1) by varying the monomers to obtain structure-activity relationship.

For the purpose of illustration and use in the proposed study, PBAE was synthesized by the addition reaction of 4,4'-trimethyldipiperidine with 1,4-butanediol diacrylate. The monomers, obtained from Aldrich Chemicals (Milwaukee, WI), were independently dissolved in dehydrated methylene chloride or tetrahydrofuran. Twenty-five ml of monomer solutions were mixed and the reaction was allowed to proceed for 48 h at 50°C under stirring conditions. As the addition polymerization occurred, the viscosity of the reaction increased and the polymer was precipitated in diethyl ether. After washing with ether, the polymer was vacuum dried. Typical yield was around 85%. The PBAE was characterized by ¹H and ¹³C NMR. Organic phase gel permeation chromatography was performed to determine the molecular weight of the polymer.

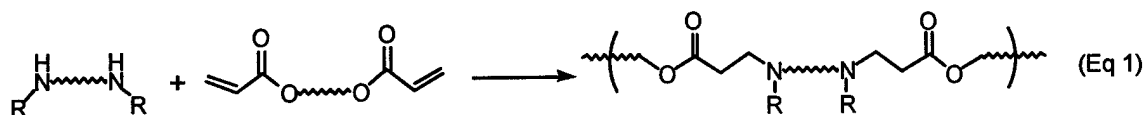


Figure 1. The chemical reaction between secondary diamines and diol-diacrylates to form poly(β -amino esters).

Preparation and Characterization of Nanoparticles:

Using polymer with a number-average molecular weight of 14,000 daltons (PDI = 2.1), PEO-modified PBAE nanoparticles (NPs) were prepared by the solvent displacement process in ethanol-water system. After a number of trials that involved changing the concentration of polymer, ethanol to water volume ratio, concentration and types of PEO-containing block copolymers, and the stirring conditions, we were able to optimize the conditions for obtaining NPs with an average diameter of 150-300 nm for efficient tumor uptake. The polymer was dissolved in dehydrated ethanol at 1.0 mg/ml concentration. Ten-ml of the polymer solution was added to 30 ml of deionized distilled water containing 0.1% (w/v) PEO/PPO/PEO triblock copolymer (Pluronic[®] F-108) under continuous stirring. [Note: Pluronic[®] F-108 is a triblock copolymer containing 122 residues of ethylene oxide and 56 residues of propylene oxide. Our studies have shown that Pluronic copolymers bind to hydrophobic surfaces through the middle PPO segment, while the two PEO chains extend into the aqueous environment for steric repulsion. In this case, the Pluronic copolymer will bind to the PBAE-NP surface during formation and permanently impart the properties of the surfactant to the surface of the nanoparticles. Singh *et al.* have used a similar approach to engineer cationic PLGA microparticles in the presence of cetyltrimethylammonium bromide (CTAB), a cationic surfactant, for the development of drug vaccine delivery system. Control NPs were prepared in the absence of Pluronic[®] F-108.] The turbid mixture was allowed to stir for an additional 2 hours to allow for the ethanol to evaporate. The hardened NP suspension was centrifuged at 10,000 rpm and the pellet was twice washed with deionized distilled water. Coulter counter was used to analyze the mean particle size and size distribution of the NP suspension. After centrifugation, the pellet was rapidly frozen in liquid nitrogen and freeze-dried. Scanning Electron Microscopy (SEM) was used to characterize the surface morphology of the freeze-dried NPs.

Coulter data, in Figure 2A below, shows NPs with a size range of 150-250 nm in diameter and a unimodal size distribution. The SEM (Figure 2B) of NPs showed distinct particles with spherical shape and smooth surface. Electron Spectroscopy for Chemical Analysis (ESCA) was performed at the National ESCA and Surface Analysis for Biomedical Problems (NESAC/BIO), University of Washington (Seattle, WA) to confirm the surface presence of PEO chains on the modified NPs by high resolution C_{1s} spectral analysis. There was a significant increase in the -C-O- (ether carbon) signal of the C_{1s} envelop in the PEO-PBAE NPs relative to control. In addition, the zeta potential of control and PEO-PBAE NPs were in the range of 15-20 mV.

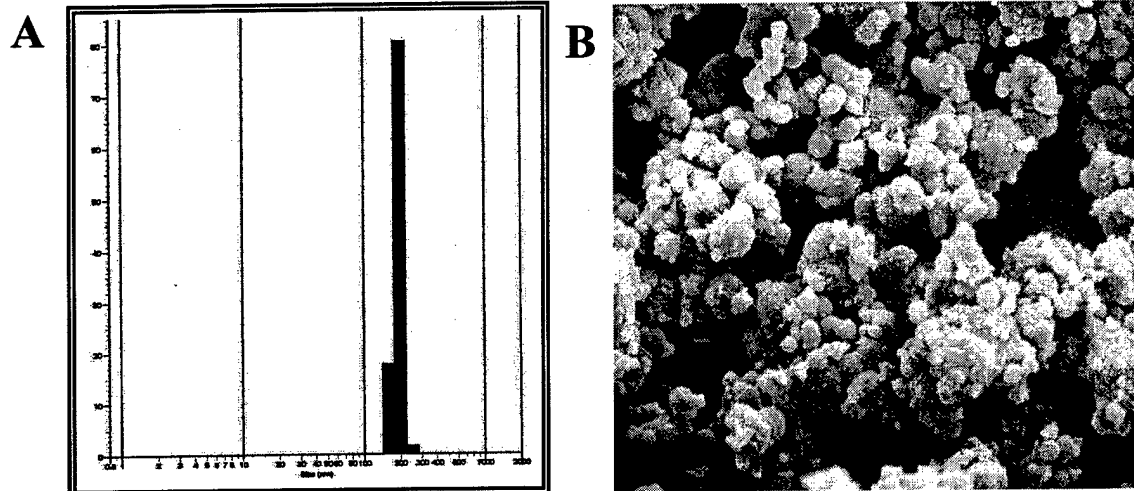


Figure 2. Particle size analysis by Coulter counter (A) and scanning electron micrograph (B) of poly(ethylene oxide)-modified poly(β -amino ester) nanoparticles prepared by solvent displacement method. For the SEM, original magnification was 17,500.

pH-Responsive Triggered Release:

PBAE microspheres and NPs display a unique pH-dependent solubility profile. They are insoluble at physiologically-relevant pH of 7.4; however, completely soluble at pH <6.5. Rhodamine- (Molecular Probes) containing PEO-PBAE-NPs were prepared as described above. A drop of the NP suspension was instilled on a glass slide and observed with a fluorescence microscope. A small volume of pH 5.1 acetate buffer was added from the upper left-hand-side of the coverslip and the rapid dissolution of the NPs at pH < 6.0 can be observed in Figure 3 below. The pH-responsive solubility of PBAE-NPs is particularly desirable for tumor-specific delivery since the pH in the vicinity of the tumor is around 6.5 as compared to 7.4 in the systemic circulation. We have observed that the release of encapsulated drug is very slow at pH 7.4, but occurs rapidly at pH of 6.5 or lower.

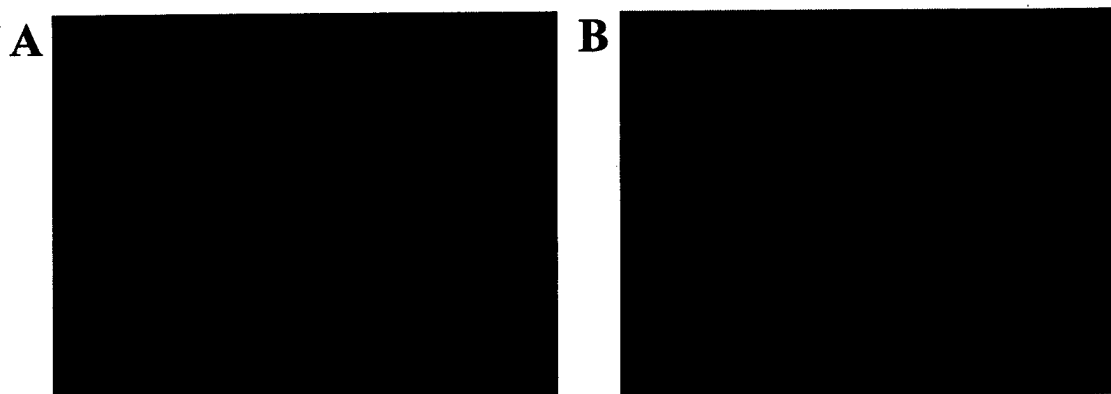


Figure 3. Fluorescence micrographs of rhodamine containing PEO-modified poly(β -amino ester) nanoparticle suspension on a glass slide (A) and after the addition of small amount of pH 5.1 acetate buffer (B). The rapid dissolution (~5 seconds) of the nanoparticles in low pH is shown by the streaks and an increase in the background rhodamine fluorescence signal.

Paclitaxel Loading and Release Studies:

Paclitaxel was added to the dehydrated ethanol solution of the PBAE at concentration ranging from 0.1% (w/w) to 1.0% (w/w) of the polymer. The solution was stirred to insure that the drug was completely dissolved. PEO-PBAE-NPs were prepared as described above by precipitating in Pluronic[®] F108-containing distilled water. Coulter analysis of the resulting suspension showed that the particle size and size distribution remained similar to those prepared in the absence of paclitaxel. Since the polymer is soluble at pH 6.5 or below, the loading studies were carried out by dissolved 10 mg on the NPs in 10 ml of 0.1 M acetate buffer (pH 5.5). The amount of paclitaxel in 10 mg of NPs was determined using a reverse-phase HPLC assay of the drug. The loading capacity and efficiency (percent) were calculated from a calibration curve of the drug in acetate buffer. As can be seen in Figure 4 below, a linear relationship was obtained between the amount of drug loaded in the nanoparticles and the amount added to the polymer solution in ethanol. In addition, more than 98% of paclitaxel added was incorporated in the nanoparticles at 1.0% (w/w) concentration.

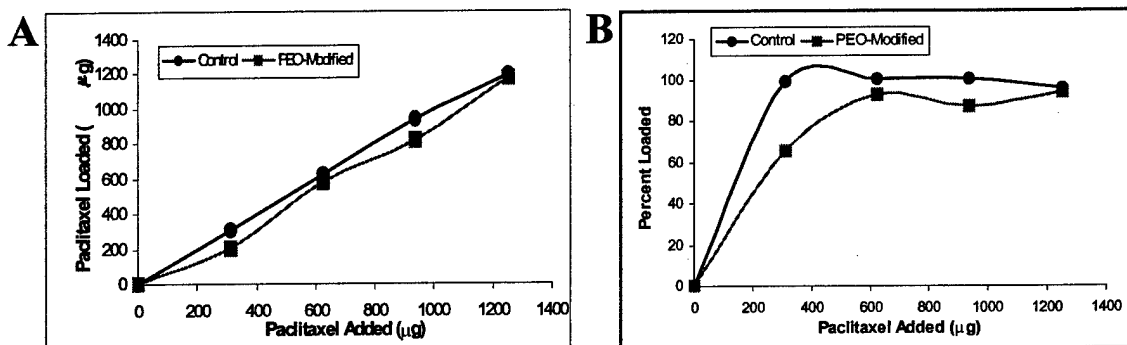


Figure 4. The capacity (A) and efficiency (B) of paclitaxel loading into the control and poly(ethylene oxide)-modified poly(β -amino ester) nanoparticles.

Since paclitaxel is insoluble in aqueous buffer, 1.0% (w/v) Tween-80, a non-ionic surfactant, was added to phosphate-buffered saline (PBS, pH 7.4) for the release studies. Tween[®]-80 enhanced the solubility of paclitaxel in to provide more physiologically relevant release medium and prevented the binding of the drug to the container surfaces. Ten-mg of paclitaxel-containing PEO-PBAE nanoparticles was added to 10 ml of Tween-containing PBS and the system was incubated at 37°C. Periodically, after centrifugation, 5 ml of the release medium was removed and 5 ml of fresh medium was added to maintain sink conditions. After filtering through a 0.22- μ m filter to remove particulates, the release medium was injected into HPLC column for analysis of the drug.

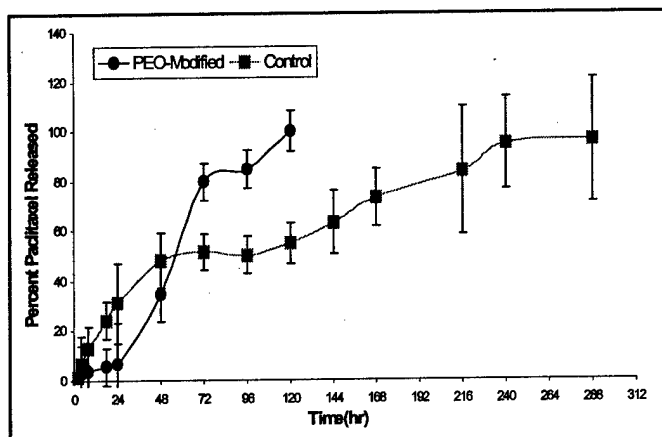


Figure 5. Paclitaxel release from the control and poly(ethylene oxide)-modified poly(β amino ester) nanoparticles at 37°C in 1.0% (w/v) Tween-containing phosphate buffered saline (pH 7.4).

Cumulative percent paclitaxel release was calculated using a calibration curve of the drug in Tween-containing PBS. Figure 5 shows the release profile of paclitaxel from the control and PEO-PBAE-NPs. PEO-PBAE-NPs release only about 10% of incorporated drug in the first 24 hours in PBS. This is consistent with our observation, which suggests that the drug was uniformly distributed in the NP matrix and diffused slowly. However, after 48-96 hours, significant paclitaxel was released from the PEO-PBAE-NPs due to degradation of the polymer at pH 7.4. Hundred-percent of the incorporated paclitaxel was released from the PEO-PBAE-NPs in 120 hours. The control (without Pluronic) NPs released the drug relatively slowly in PBS. This is probably due to the fact that the control NPs were slightly larger (~400-600 nm) as compared to the PEO-modified ones (~150-300 nm). It is very important to note that PEO-PBAE -NPs will not release significant amount of the drug in the systemic circulation for up to 24 hours. In the vicinity of the tumor, however, the NPs will rapidly release the payload due to low pH (~6.5).

The next set of experiments was directed at the examination of direct cytotoxicity of blank nanoparticles. The results initially were not encouraging as it demonstrated contamination when added to cells in a 3-day cytotoxicity assay at 37°C. This problem was then resolved by examining the chemical synthesis procedures and eventually by use of sterile filtration of diluted nanoparticle preparations. The addition of blank nanoparticles did not show any direct cytotoxicity to the cell cultures as indicated by MTT dye assay where control cells and NP blanks demonstrated no statistical differences in optical densities normalized to cellular cytotoxicities. Interestingly, Pt encapsulated NP preparations demonstrated direct cytotoxicity. At present efforts are underway to quantitate the effective concentrations of Pt released from NP and compare its ED₅₀ with non-encapsulated Pt. With the success of Pt experimentation we are encouraged and are attempting to encapsulate other agents.

Key Research Accomplishments

Studies outlined here demonstrates that Pt can be successfully encapsulated in nanoparticle system as outlines with high loading and with consistency. The results also indicated that a change in pH to acidic conditions can successfully release encapsulated Pt. Additionally, these NPs did not demonstrate any cytotoxicity *in vitro* on human cancer cells.

Reportable Outcomes

A grant has been submitted to Komen Foundation for a possible funding on the utilization of NP technology that includes chemotherapeutic drug encapsulation for a possible treatment of breast cancer in preclinical studies.

Conclusions

Our results have shown that the selection of drugs for possible treatment of breast cancer has direct cytotoxicity to human breast cancer cell lines. The encapsulation of Pt in PBAE nanoparticles is effective with high loading efficiency. In addition, a change in pH to acidic conditions triggers complete release of chemotherapeutic drug from nanoparticles in use. The expansion of the studies presented here may help encapsulate other chemotherapeutic drugs with high efficiency.

References None

Appendices None