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Poly lactide-based Paclitaxel-loaded Nanoparticles Fabricated by Dispersion Polymerization: Characterization, Evaluation in Cancer Cell Lines, and Preliminary Biodistribution Studies

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Abstract

The macromonomer method was used to prepare crosslinked, paclitaxel-loaded polylactide-polyethylene glycol (stealth) nanoparticles using free-radical dispersion polymerization. The method can facilitate the attachment of other molecules to the nanoparticle surface to make it multifunctional. Proton NMR and FT-IR spectra confirm the synthesis of polylactide macromonomer and crosslinking agent. Formation of stealth nanoparticles was confirmed by scanning and transmission electron microscopy. The drug release isotherm of paclitaxel-loaded nanoparticles shows that the encapsulated drug is released over 7 days. *In vitro* cytotoxicity assay in selected breast and ovarian cancer cell lines reveal that the blank nanoparticle is biocompatible compared to medium-only treated controls. In addition, the paclitaxel-loaded nanoparticles exhibit similar cytotoxicity compared to paclitaxel in solution. Confocal microscopy reveals that the nanoparticles are internalized by MCF-7 breast cancer cells within one hour. Preliminary biodistribution studies also show nanoparticle accumulation in tumour xenograft model. The nanoparticles are suitable for the controlled delivery of bioactive agents.

Keywords

Macromonomer; Dispersion polymerization; Drug Release; Cytotoxicity; Intracellular Localization; Cell culture; Stealth Nanoparticle

BACKGROUND

Lack of selective toxicity has been identified as one of the main problems of conventional cancer chemotherapy resulting in untoward effects to normal tissues.^{1,2} The chemotherapeutic agents currently in use do not differentiate between healthy and cancerous cells giving rise to systemic toxicity and adverse effects which limit the maximum dose that can be administered.³ Successful chemotherapy therefore requires a “magic bullet” that

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selectively kills cancer cells while leaving normal issues intact. While the attainment of this goal has not been met, advances in nanotechnology offers great potential to bring the concept to the clinics. One of such advances is the development of stealth polymeric biodegradable nanoparticles for drug delivery with the capacity for site-specific drug delivery and controlled drug release. Using this approach, high concentrations of chemotherapeutic agents can be selectively delivered to the tumor sites without affecting healthy cells thereby increasing the efficiency of the administered dose (the amount of drug per dose is minimized).^{4,5} Paclitaxel is among the most active, FDA approved drugs for cases of advanced breast, non-small-cell lung and ovarian carcinomas.⁶⁻⁸ Due to its hydrophobic nature, the initial commercial formulation for clinical use involves the solubilization of paclitaxel with polyoxyethylated castor oil, a nonionic surfactant called Cremophor® EL and ethanol to enhance drug solubility and thereby permit parenteral administration.⁶⁻⁹ However, since the amount of Cremophor® EL per administration in Taxol® is high⁷, its toxicological and pharmacological characteristics become of utmost importance leading to well-reported adverse effects and other surfactant-related problems.^{6,8,9}

To circumvent the problems associated with Cremophor®-solubilized paclitaxel, several research groups have reported the preparation of Cremophor-free paclitaxel delivery systems.¹⁰⁻¹⁴ Abraxane® is an example of a FDA-approved, paclitaxel-loaded nanotechnological intervention for cancer chemotherapy.^{8,9} It has been reported that preferential intratumoral accumulation of Abraxane® is due to the enhanced permeability and retention (EPR) effect and by a natural specific receptor-mediated transcytosis facilitated by albumin.^{6,7} However, the tumor response is only about 30-35%¹⁵. Furthermore, the nanoparticle system is not actively targeted to tumors. Thus, the advantages associated with the nanoparticle system and the low tumor response underscores the importance of developing suitable nanoparticle alternatives for the delivery of hydrophobic paclitaxel and other chemotherapeutic agents.

Nanoparticles of biodegradable polymers are among the best delivery systems for achieving sustained and targeted delivery of chemotherapeutic agents.^{4,16} They degrade under physiological conditions into harmless byproducts and the drug can either be entrapped or encapsulated in the polymer matrix. Poly(lactide) (PLA) is one of the few biodegradable polymers approved for human use by the Food and Drug Administration (FDA) that has reached the clinical application stage.¹⁷ However, due to the lack of reactive functional groups along the PLA chain, free radical polymerization reactions for nanoparticle synthesis are not feasible.¹⁸ Thus, nanoparticle fabrication with poly(lactide) is limited to the dispersion of preformed polymers which makes it difficult to covalently attach targeting moieties to the surface of the particle and to introduce crosslinked networks.

We report here the use of the macromonomer approach to prepare crosslinked, drug-loaded PLA-PEG (stealth) nanoparticles with the capacity for drug targeting using redox free-radical dispersion polymerization at room temperature. The nanoparticles have also been characterized. In these studies, paclitaxel was used as a model drug. *In vitro* cytotoxicity testing in breast and ovarian cancer cell lines was conducted using the CellTiter-Glo® luminescent cell viability assay, and compared with that of free paclitaxel at the same drug

concentration. The *in vitro* intracellular localization of rhodamine-123 loaded nanoparticles was studied by confocal laser scanning microscopy in MCF7 breast cancer cells. Preliminary biodistribution studies in a mouse tumor model were also carried out.

MATERIALS AND METHODS

Materials

L(-) lactide (Polysciences Inc.) was recrystallized from toluene before use. 2-Hydroxyethyl methacrylate (HEMA) (Aldrich, 97%) was dried over molecular sieves (4Å) for 24 hours and distilled under negative pressure before use. Toluene (Acros, 99%) and pyridine (Sigma, 99%) were refluxed over calcium hydride for one hour and distilled prior to use. Methacryloyl chloride (Aldrich, 97%) was distilled prior to use. Stannous octoate (Sigma, 95%), phosphorous pentoxide (Aldrich, 97%), hydroxylamine hydrochloride reagentplus® (Sigma, 99%), chloroform (Certified A.C.S, Fisher Scientific), hydrochloric acid (Aldrich, 37%) were used as received. All other solvents were used as received. Paclitaxel from *Taxus brevifolia* was obtained from Sigma-Aldrich (St. Louis, MO, USA). All cell media, heat-inactivated fetal bovine serum (FBS), trypsin-EDTA, penicillin-streptomycin were obtained from Invitrogen (Carlsbad, CA, USA). Hoechst® 33342, CellMask™ deep red plasma membrane stain kit, Rhodamine-123 and Bodipy® 665/676 were obtained from Invitrogen. Phosphate-buffered saline (PBS) was from Mediatech (Manassas, VA, USA) and the CellTiter-Glo® Luminescent Cell Viability Assay Kit was obtained from Promega (Madison, WI, USA).

Methods

Synthesis and Characterization of P(LLA-HEMA) Macromonomer and Crosslinking Agent N, O-Dimethacryloylhydroxylamine (MANHOMA)—The macromonomer used for nanoparticle fabrication was synthesized by modification of a reported method.^{19,20} The crosslinker (MANHOMA) was synthesized based on literature reports with a slight modification.^{20,21} The details of the synthesis of both monomers and the methods of characterization are presented in the supplementary material section.

Preparation of Crosslinked Stealth Paclitaxel-Loaded Nanospheres by Dispersion Polymerization Using Redox Initiator System—The fabrication, characterization and optimization of the blank nanoparticles were as previously reported.²⁰ For fabrication of drug-loaded nanoparticles, paclitaxel (model hydrophobic anticancer drug), P(LLA-HEMA) macromonomer (0.24 mmol), crosslinker (MANHOMA) (0.016 mmol), and PEG-MMA macromonomer (0.252 mmol) were dissolved in dioxane to form a clear homogenous solution. The solution was added to a Dioxane : DMSO : Water (12:5:2.5) solvent system. 0.196 mmol of N-phenyldiethanolamine (NPDEA) and 0.196 mmol of benzoyl peroxide (BPO) were injected into the reaction mixture at 10 min and 20 min respectively through a rubber closure under continuous flushing with nitrogen gas and with continuous stirring at 400 rpm. The overall polymerization time was 24 hours. The resulting particles were recovered by centrifugation. Fluorescent Bodipy® 665/676 and rhodamine-labeled nanoparticles were also prepared according to the procedure described above. Paclitaxel was replaced with Bodipy® 665/676 and rhodamine-123 in the synthesis

of fluorescent nanoparticles. Rhodamine 123- and Bodipy-labeled nanoparticles were prepared by loading 5 mg of the fluorescent dyes in the nanoparticle formulation.

Characterization of Drug-Loaded Nanoparticle Formulation—Particle size and size distribution of nanoparticles were determined by dynamic light scattering (DLS) using Zetasizer Nano-ZS (Malvern Instruments, USA) as described previously.²⁰ Briefly, 10 mg of freeze-dried particles was dispersed in 5 mL of filtered distilled water using a probe sonicator (Vibra-Cell; Model VC 750, Sonics and Materials, Inc, Newton, CT). The sonicated suspension was filtered through an Acrodisc syringe filter with a 5 μ m Versapor membrane (Pall Corporation). Particle size was determined at 25 °C. For determination of zeta potential, 1.5mL of the resulting suspension for particle size analysis was diluted with 2mL of filtered distilled water and mixed by vortexing. Zeta potential was determined at 25 °C using Zetasizer Nano-ZS (Malvern Instruments, USA). In both cases, the mean of three measurements was recorded.

Electron Microscopic Techniques

(a)Transmission Electron Microscopy (Structure): Lyophilized nanoparticles were suspended in 100% ethanol for 30 minutes, and re-suspended in Spurr's embedding medium. The samples were allowed to settle in the tips of BEEM capsules that were incubated for three days at 60 °C. Sections of light gold interference color (approximately ninety nanometers thickness) were cut with a diamond knife using a Leica ultramicrotome. The sections were placed on 200 mesh copper grids, lightly stained with uranyl acetate, and observed at 30-200 X magnification under a Zeiss Libra 120 electron microscope.

(b) Scanning Electron Microscopy (Surface morphology): The surface morphology of the nanoparticles was evaluated using scanning electron microscopy (SEM) (FEI Quanta 200F environmental scanning electron microscope). With this equipment, coating with a metal (e.g. gold) is not necessary and this confers the advantage of imaging accurate morphological features. To evaluate surface morphology, different dilutions of nanoparticle suspension in distilled water were placed on a carbon tape affixed to a specimen stub and dried *in vacuo*. The samples were then viewed using a scanning electron microscope under high vacuum at 10 KV and a working distance of 10 mm. Images were taken at different sample magnifications.

Drug Loading and Release Profile

Drug loading (DL) (the weight percent of paclitaxel in the nanoparticle formulation) was determined by dissolving 5mg of paclitaxel-loaded nanoparticles (A_{NP}) in 2 mL of acetonitrile. The solution was then filtered through a 0.45 μ m syringe filter and the amount of paclitaxel dissolved in the solution (A_{PT}) was quantified using a validated method by high performance liquid chromatography (HPLC) using a HP series 1100 HPLC equipped with a Zorbax 300SB-C18 column kept at 37°C. The mobile phase for HPLC studies was 60:40 {acetonitrile: 12.5 mmol ammonium phosphate buffer (pH-4.5)} at a flow rate of 1 mL/min. Quantitation of paclitaxel was done using Diode Array Detector (DAD) at wavelength 227nm.

The percent drug loading was calculated from the equation below²²:

$$DL = \frac{(A_{PIS}) \times 100\%}{(A_{NP})}$$

Encapsulation efficiency (EE) (percentage of paclitaxel that is encapsulated out of the total used for nanoparticle preparation) was determined by quantifying the amount of paclitaxel in the washings (A_{wash}) by HPLC and assuming that the rest of the drug used for nanoparticle preparation (A_{prep}) had been encapsulated. A known volume of the washings (3 mL) was extracted twice with 3 mL quantities of 1-octanol. To 500 μ L of the combined octanol layer was added 4.5 mL of acetonitrile and filtered through a 0.45 μ m syringe filter. The solution was injected into a HPLC as described above and the amount of paclitaxel in the total volume of washing was determined. The EE was determined from the equation below:

$$EE = \frac{(A_{prep}) - (A_{wash}) \times 100\%}{(A_{prep})}$$

To determine the release profile, a known weight of paclitaxel-loaded nanospheres was dispersed in 10 mL of freshly prepared phosphate buffered saline (PBS) in a 15 mL Eppendorf tube with a screw cap. Due to the poor solubility of paclitaxel in PBS, 3 mL of 1-octanol was added to continuously extract the released drug and therefore maintain sink conditions. The tube was clamped to a Labquake® shaker capable of 360° rotation maintained at 37 °C in a laboratory oven. At different time intervals, the octanol layer was completely removed and replaced with a fresh 3 mL of 1-octanol. The removed octanol layer was analyzed by HPLC using the calibration curve to determine the amount of paclitaxel released as shown in Figure 2.

Cell Cultures

The human breast cancer cell lines, MCF7, MDA-MB-231 and BT-474 were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). The MCF7 and MDA-MB-231 cell lines were maintained in Dulbecco's Modified Eagles Medium (DMEM) and BT474 in RPMI 1640 supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 100U/mL penicillin G and 100 μ g/mL of streptomycin sulfate. The ovarian cancer cell line SK-OV-3 was cultured in DMEM-F/12 medium under the same conditions. The cells were maintained as a monolayer in an incubator at 37 °C in a humid atmosphere with 5% CO₂.

Cytotoxicity of Paclitaxel-Loaded Nanoparticles

Cultured cells (MCF7, MDA-MB-231 and SK-OV-3) were seeded in 96-well plates at a seeding density of 6000 cells/well/0.1mL medium and allowed to attach for 24 hours. Cells were then treated with 100 μ L of culture medium containing paclitaxel-loaded nanoparticles or paclitaxel in solution (To prepare the control paclitaxel solution, the required amount of

paclitaxel was dissolved in 0.5 mL of DMSO and 9.5 mL of medium to make 10 mL of stock solution). Serial dilutions from this stock solution were then done in cell-specific media for the different cell lines. To allow direct comparison, the amount of paclitaxel-loaded nanoparticles containing the same amount of paclitaxel as the paclitaxel in solution was used. The concentrations tested range from 2.5 nM to 120 nM. Control cells were treated with culture medium only, culture medium with 0.05% DMSO and culture medium containing blank nanoparticles at the highest concentration tested. Cell viability was assessed at 48-, 72- and 96 hours post treatment using the CellTiter®glo luminescent cell viability assay (Promega Corporation, Madison, WI, USA). Briefly, at the predetermined time intervals the treatment was washed off and fresh medium added. An amount of the CellTiter®-Glo reagent equal to the amount of medium was added. The plate was mixed by shaking on an orbital shaker for 2 min to induce cell lysis and luminescence was measured after 10 min using the FLUOstar OPTIMA plate reader (BMG LABTECH). Results are presented as percent viability normalized to controls and represent the mean $\pm$ S.D. of 4 replicates per concentration tested. The results were analyzed using Student's T-test with the aid of SPSS® statistical software. Differences were considered significant at $p < 0.05$.

In vitro Intracellular Localization of Nanoparticle

MCF7 cells were seeded in glass-bottom microwell dishes (MatTek Corp., MA, USA) at a seeding density of 6×10^5 cells/dish/1.5mL medium and allowed to attach for 24 h. After 24 h, medium was replaced with suspension of rhodamine-123 loaded nanoparticles in medium (1.71mg/mL) and incubated at 37 °C for additional 1, 6 and 24 hours. Nuclei and cell membranes were counterstained with Hoechst® 33342 (5 μ g/mL) and CellMask™ deep red plasma membrane stain (4 μ g/mL) respectively. Directly before imaging, cells were washed three times with PBS to remove nanoparticles that were not internalized and were observed with a confocal laser scanning microscope (CLSM 510, Carl Zeiss, GmbH) by using a 60 $\times$ 1.3 NA Plan-Apochromat oil immersion objective and a multitrack configuration. The Hoechst® 33342, rhodamine-123-loaded nanoparticles and CellMask™ deep red plasma membrane stain signals were collected by using BP 385–470nm filter, 505 – 550nm filter and LP 650nm filter after excitation with the 364, 488, and 633 nm laser lines, respectively. Images (512 $\times$ 512 pixels) were acquired with line average of four by using the Zeiss AIM software.

Tumor Model

All animal studies were conducted in accordance with the principles and procedures outlined in the *Guide for the Care and Use of Laboratory Animals* (National Academy Press, 1996) and were approved by the Institutional Animal Care and Use Committee of the National Institutes of Health. A method earlier reported by the Capala group was followed.²³ Briefly, estrogen pellets (1.72 mg; Innovative Research of America) were implanted 48 h before tumor cell inoculation and remained in place until the end of the study. BT474 breast cancer cells (5.5×10^6) suspended in Matrigel (BD Bioscience) were implanted subcutaneously in the shoulder region of female athymic nude mice (5- to 7-week old). Tumors (100-250 mg) developed after 3-5 weeks.

Preliminary Biodistribution Studies of fluorescent, Bodipy®-loaded Nanoparticles in Mice

Biodistribution of fluorescent nanoparticles was evaluated by optical imaging. For biodistribution studies, 125 μ L of the Bodipy®-loaded nanoparticle suspension in PBS (100 mg/kg) was administered by intravenous injection through the tail vein into mice. Control mice were injected with saline. At predetermined time intervals, the mice were anesthetized using isoflurane/O₂ (1.5%-5% v/v) and fluorescent images were acquired. The mice were later sacrificed by cervical dislocation and selected tissues (heart, lung, small intestine, liver, kidney, spleen, muscle and the tumor) were removed for imaging *ex vivo*. The fluorescent images were acquired at 24 h, 48 h, 72 h, and 150 h after the administration of Bodipy®-labeled nanoparticles with an IVIS-100 imager (Xenogen Co., Alameda, CA) using an excitation passband of 615-665 nm and an emission passband of 695-770 nm.

RESULTS

Preparation and characterization of P(LLA-HEMA) macromonomer and crosslinking agent (MANHOMA)

We slightly modified the reported method of synthesis of P(LLA-HEMA) macromonomer and MANHOMA used for the synthesis of nanoparticles. P(LLA-HEMA) macromonomer was synthesized by ring opening polymerization of L-lactide using 2-hydroxyethyl methacrylate as initiator in the presence of stannous octoate as catalyst. The macromonomer was characterized by ¹H NMR and FT-IR. The molecular weight was determined by GPC and also by ¹H NMR. MANHOMA was also synthesized based on literature reports and characterized by ¹H NMR, FT-IR and melting point determination. Results show that the spectra obtained are consistent with earlier reported data for the synthesis of the macromonomer¹⁹ and MANHOMA.^{20, 21} All the ¹H NMR, FT-IR and GPC data are presented in the supporting information (S1-S5).

Preparation and Characterization of Nanoparticles

Nanoparticles were synthesized by free radical dispersion polymerization using different amounts of macromonomer, initiators, crosslinking agent and stabilizer in a dioxane/DMSO/water solvent system (Scheme 1). The hydrodynamic particle size was measured by the dynamic light scattering (DLS) technique. We selected from optimized formulations earlier reported²⁰ for this work. The optimized formulation yielding the smallest mean particle size (244.2 ± 4.2 nm) was selected for drug loading and other *in vitro* applications. The surface charge of the selected optimized formulation dispersed in filtered distilled water was negative with a mean zeta potential of -17.7 ± 0.6 mV. The mean values for particle size and zeta potential after drug loading are 230.8 ± 4.7 nm and -27.4 ± 0.6 mV respectively. Nanoparticle fabrication was confirmed by scanning electron microscopy (SEM)²⁰. SEM images of the drug-loaded nanoparticle formulation revealed smooth spherical particles (Figure 1A); while Figure 1B shows the stealth characteristic of the nanoparticles (lactide core with a corona of PEG).

Release Profile and Loading of Paclitaxel

The drug loading of the optimized formulation was determined by HPLC to be 0.25 % w/w while the encapsulation efficiency was determined to be 31.9%. A validated HPLC method was developed for the quantitation of paclitaxel. The *in vitro* release of paclitaxel from nanoparticles was determined in phosphate buffered saline (PBS) (pH 7.4; IS 0.16) in triplicate. To continuously extract released paclitaxel from the PBS and maintain sink conditions, a layer of 1-octanol was placed on top of the PBS layer.²⁴⁻²⁶ It has been reported that the solubility of paclitaxel in octanol (>5mg/ml) is 5000 times that in PBS such that the drug will readily partition into it.²⁴ At various time intervals, the octanol layer was removed and replaced with fresh octanol. The octanol layer was then assayed for paclitaxel by HPLC after a 1:6 dilution with acetonitrile.

Figure 2 shows the *in vitro* release isotherm of paclitaxel from nanoparticles using the calibration curve for paclitaxel (supplemental information S7). The release is biphasic with an initial rapid release followed by a slower rate of release for an extended duration of time. About 40% of the encapsulated drug was released at 24 hours while complete drug release was observed at 7 days.

Cytotoxicity of Paclitaxel-Loaded Nanoparticles

The cell viability assay shows that paclitaxel was released from the nanoparticles under study conditions and that both paclitaxel-loaded nanoparticles and the control paclitaxel solution inhibited cell growth in the cell lines used for the assay to relatively similar extents (Figure 3). This result is also similar to what was observed for 48 h and 96 h (data not shown). Furthermore, MCF-7 appears to be the most sensitive to paclitaxel while SK-OV-3 appears to be most resistant. A particularly interesting finding is that the viability of the cells reduces as paclitaxel concentration increases but at a particular concentration above 40nM, the observed viability levels off and no appreciable increase in cell killing was observed. Similar observations were made for 48 hours to 96 hours for the 3 cell lines (Fig. 4A, 4B and 4C). Another observation worthy of note is the effect of exposure time on cell viability. Increased cytotoxicity was observed with the MCF-7 and MDA-MB-231 cell lines as the duration of treatment increased from 48 hours to 96 hours (Fig. 4A and 4B).

Nanoparticle Uptake Studies

Cellular uptake was determined by confocal laser scanning microscopy (CLSM). Results show that uptake of discrete particles was observed in 1 hour and intense uptake at 6 hours. Figure 5 (A, B and C) shows the internalization of the nanoparticles in MCF-7 cells following exposure to the particles for 1, 6 and 24 hours respectively. The images show rhodamine-123-loaded (green color) nanoparticles surrounded the nucleus (stained blue) and bound by the plasma membrane (red color). The images clearly show discrete nanoparticles within the cell membrane boundary. To further prove that the nanoparticles are taken up by the cell and not located on or adhering to the cell surface, Z-stacks (which are images of planes at various depths within the cell) confirm that the particles are within the cell (supplementary information S7).

Preliminary Nanoparticle Biodistribution Studies

Preliminary biodistribution studies in the tumor xenograft model show nanoparticle accumulation in tumor from 48 h post injection up to 150 h. *Ex vivo* imaging analysis of those tumors show fluorescence compared with tumors from control mice which do not fluoresce (Figure 6A).

Relatively high accumulation of particles was observed in the lungs at the early time point (< 48 h) suggesting that the bulk of the nanoparticles were trapped there (Data not shown). Tumor uptake of the nanoparticles was also clearly visualized at 48 and 72 hours after nanoparticle injection. Figure 6B shows a representative image of mouse tumor model injected with fluorescent nanoparticles.

DISCUSSION

The fabrication of poly(lactide) for nanoparticles has been largely limited to the use of preformed polymer. The macromonomer approach and free radical dispersion polymerization allow the preparation of covalently crosslinked paclitaxel-loaded nanoparticles without the use of surfactants. Nanoparticle preparation without the use of surfactants is an obvious advantage of the dispersion polymerization technique. Functionalization of polylactide to make it amenable to free radical dispersion polymerization is an important step in our approach. The use of redox initiators also allows the fabrication of nanoparticles at room temperature which is important when loading thermolabile drugs like proteins.

Following fabrication, characterization and optimization of PLA-based nanoparticles²⁰; the formulation that gave the smallest particle size was selected and loaded with paclitaxel as a model drug. Figure 1A shows that smooth spherical nanoparticles were obtained. The particle size of the optimized formulation is 244nm and the zeta potential is -17.7mV. The values for particle size and zeta potential after drug loading are 230.8 ± 4.7 nm and -27.4 ± 0.6 mV respectively. Various research groups have reported that a high surface charge as characterized by the zeta potential is important for the stability of nanoparticle suspension and that a low zeta potential value suggests colloidal instability and may lead to aggregation.^{22,27} The mechanism for improved colloidal stability has been ascribed to higher Coulombic repulsion forces at high zeta potential values which overcome the Van der Waals attraction between them.^{22,28}

The *in vitro* release isotherm of paclitaxel shows that approximately 40% of the encapsulated drug was released at 24 hours while complete drug release was observed at 7 days. The burst effect can be ascribed to the presence of drug embedded on the surface of the nanoparticles while the second phase is due to the hydrolysis of the crosslinked PLA core.^{29,30} This release profile may be appropriate for paclitaxel chemotherapy since too fast release of the drug payload may lead to drug loss before reaching the biophase while slow release may yield suboptimal concentrations at the biophase thus encouraging drug resistance in cells.³¹

To investigate the translational potential of the nanoparticle formulation, the *in vitro* cytotoxicity of paclitaxel-loaded nanoparticle formulation was investigated and compared to free paclitaxel in solution using the CellTiter®glo luminescent cell viability assay. In this assay, the number of viable cells in culture is determined by quantification of the ATP present which is an index of the presence of metabolically active cells.^{32,33} It involves adding the cellTiter-Glo reagent (which includes a lysis buffer, luciferin substrate and luciferase enzyme) to cells which in a single step generate a measurable luminescent signal proportional to the amount of ATP present in cells. Non-viable cells do not synthesize ATP and also contain endogenous ATPases that rapidly deplete existing ATP.³⁴ For data analysis, the measured luminescence from paclitaxel-loaded nanoparticles treated cells were normalized to the luminescence from cells with no treatment (medium only) which is set at 100%. Cytotoxicity was determined in breast cancer (MCF-7 and MDA-MB-231) and ovarian cancer (SK-OV-3) cell lines (Figure 4 A, B and C).

The characteristics of the cell lines used in this study are well known. The breast cancer cell lines MCF-7 and MDA-MB-231 are estrogen-dependent and estrogen-independent respectively and do not overexpress the HER2 receptor.^{35,36} On the other hand, the ovarian cancer SK-OV-3 cells strongly overexpress HER2 and are not estrogen responsive.³⁶ The overexpression of the HER 2 receptor has been shown to correlate to paclitaxel resistance^{37,38} and resistance to other therapeutic interventions leading to poor outcomes.³⁹ In addition an important factor which has been shown to mediate resistance to paclitaxel is P-glycoprotein.⁴⁰ P-glycoprotein acts as a drug efflux pump contributing to resistance to its substrates such as paclitaxel. SK-OV-3 ovarian cancer cells have also been reported to overexpress P-glycoproteins (P-gp)⁴¹ and these factors may contribute to the lower sensitivity of SK-OV-3 cells compared to the other two cell lines tested.

For use as a drug carrier, nanoparticles must be biocompatible and biodegradable showing no toxic effects *in vitro* or *in vivo*.⁴² Blank nanoparticles (synthesized without loading paclitaxel) showed no toxicity to cells at the highest concentration of drug-loaded nanoparticles tested for the longest duration of exposure tested (96 hours) (Figure 4 A-C). Statistical analysis comparing viability of cells treated with blank nanoparticles to control cells (medium only) using the T test show no significant difference in viability at 5% level of significance ($p = 0.212$ and 0.068) for SK-OV-3 and MDA-MB-231 cells respectively. The significance observed with MCF-7 cells and ($p = 0.049$) is due to the greater viability of the cells in the presence of blank nanoparticles compared to control cells (Figure 4 A). The data show that the nanoparticles are biocompatible and that the cytotoxicity observed with paclitaxel-loaded nanoparticles is due to the encapsulated paclitaxel. These data suggest that they are suitable as a carrier for controlled delivery of drugs and that the paclitaxel-loaded particles can serve as an alternative to Taxol® without the attendant adverse effects.

Our results show that at higher paclitaxel concentrations (above 40 nM) there is no decrease in cell viability. Also, we observe an increase in cytotoxicity with increase in time of cell exposure to paclitaxel from 48 h to 96 h (Figures 4, A-C). The cytotoxicity data at 24 hours was not considered because only about 40% drug release would have occurred from nanoparticles. It has earlier been reported that cytotoxicity increased in all cell lines tested with increase in time of exposure to paclitaxel and that no additional cytotoxicity was

observed at paclitaxel concentrations greater than 50 nM.⁴³ The authors concluded that paclitaxel concentrations above 50 nM may not lead to increased tumor response and that extended exposure to paclitaxel is likely to yield greater tumor response. This information is a good justification for a nanoparticle formulation of paclitaxel that is capable of releasing the encapsulated drug for extended periods of time at the tumor site. Other groups have shown that the cytotoxic activity of the taxanes increased with time of exposure to cells.⁴⁴ It has also been reported that the upregulation of the p53 protein by paclitaxel stimulates another gene to produce a protein (p21) that interacts with a cell division-stimulating protein (cdk2).⁴⁵ When p21 is complexed with cdk2 the cells cannot go to the next phase of cell division causing G1/G2 arrest and not mitotic arrest thereby protecting against paclitaxel cytotoxicity due to prevention of mitotic arrest. This reasoning agrees with the observation that if there is a G1 block, then cells would be unable to enter the M phase where paclitaxel exerts its effect.⁴³ Our results are consistent and agree with the above observations.

Reports have shown that drug-loaded nanoparticles can exhibit cytotoxicity by two main pathways: (i) by adsorbing to the cell membrane and releasing the drug which leads to the generation of a concentration gradient that would favor cellular drug influx by passive diffusion, and (ii) by uptake into the cell by endocytosis leading to drug release in the interior of the cells.^{25,46} In the latter case, the nanoparticles are retained in the cell cytoplasm for a prolonged period, acting as intracellular drug depots by slowly releasing the encapsulated drug. This condition leads to an increase in therapeutic efficacy for drugs such as paclitaxel that have the cytoplasm as their site of action by a sustained drug effect.^{40,47} To determine which of these two mechanisms is predominant and responsible for the observed cytotoxicity, cellular uptake was determined by confocal laser scanning microscopy. Our results show that the latter mechanism was predominant as uptake of discrete particles was observed in 1 hour and intense uptake at 6 hours (Figure 5, A and B). At 24 hours, the interior of the cell showed diffuse fluorescence which might have resulted from release of the fluorescent dye from the nanoparticles (Figure 5, C). The question therefore arises – why is there no difference in viability between the paclitaxel nanoparticle formulation and the paclitaxel solution despite the obvious reported advantage of cellular particle internalization? The answer may be one of two factors or a combination of both and may be explained as follows: (a) Paclitaxel is a hydrophobic and membrane-permeable drug that is expected to preferentially partition into the lipophilic cell membrane when present in a hydrophilic media such as culture medium used for *in vitro* studies. This preferential partitioning creates a concentration gradient which favors diffusion of the drug into the cell. As such, intracellular paclitaxel concentrations are expected to be high which may mask the cellular uptake advantage of the nanoparticle paclitaxel formulation when cells are continually exposed to treatment. (b) At 96 hours, not all the paclitaxel encapsulated in nanoparticles has been released; consequently, the paclitaxel solution formulation technically has a higher drug concentration available to the cells. Thus the similar cytotoxicity at 96 hours for paclitaxel solution and paclitaxel-loaded nanoparticles shows an advantage of the nanoparticle formulation. From the foregoing, our results suggest that as a result of the hydrophobic nature of the drug, the *in vitro* cell culture model may not be discriminatory enough to reveal any advantages the nanoparticle paclitaxel formulation may have over the control paclitaxel solution, as the cells' exposure to paclitaxel in solution is

not limited and no targeting is involved as is the case *in vivo*. Consequently, *in vivo* tumor models could be more useful to show the advantages of the nanoparticle formulation in inhibiting tumor growth.²⁵

There have been other reports showing no difference in cytotoxicity between different paclitaxel-loaded nanoparticle formulations and paclitaxel solutions within the time frame for our studies.^{25,47,48} Our data are consistent with these reports and suggest that the *in vivo* model may be a better discriminatory model to compare the effects of paclitaxel-loaded nanoparticles and paclitaxel solution.

To ascertain that the nanoparticles preferentially accumulate in tumors and as a first step towards evaluating the *in vivo* efficacy of drug loaded nanoparticles, biodistribution studies were carried out using nanoparticles loaded with a near infrared fluorophore (NIRF), Bodipy®. Near infrared fluorophore molecules such as Cy5.5 have been used for studying the biodistribution of nanoparticles.⁴⁹ Data from *ex vivo* imaging reveal that at 24 h, the nanoparticles were largely sequestered in the lungs and cells of the reticuloendothelial system such as the spleen and liver as well as the kidneys (data not shown). This is consistent with literature reports.⁴⁹ However, at 48 h, whole organ imaging shows fluorescence in the tumor suggesting that some accumulation of nanoparticles in the tumor had taken place. At 72 h, increased accumulation had taken place such that fluorescence was observed in the tumor region during whole animal imaging (Figure 6). Accumulation in tumor could be adduced to the enhanced permeability and retention effect which allows extravasation and accumulation of the stealth nanoparticles (Figure 1B) in the tumor region.⁵⁰ Rapid vascularization to establish growing tumor blood supply due to the release of proangiogenic factors by tumor cells leads to leaky and defective blood vessels that have incomplete endothelial linings and basement membranes.³ This hyperpermeability allows the passage of macromolecules and drug delivery systems through the blood vessels that supply tumors leading to the entrapment and accumulation of the drug carriers for prolonged periods as a result of deficient or impaired lymphatic drainage in the tumor bed.^{3,22} This phenomenon is defined as enhanced permeability and retention (EPR) effect⁵¹ and is the primary mechanism for the passive targeting of stealth nanoparticles to tumors. Other researchers have observed that the prospects of passive targeting are enhanced if the circulation time of the delivery vehicle is prolonged.⁵ Thus, the “stealth” characteristic of the nanoparticles developed in this work (Figure 1) enables the nanoparticles to avoid the cells of the reticuloendothelial system (RES) (due to PEG on the surface) to achieve prolonged circulation times essential for nanoparticle drug delivery by passive targeting.

The data suggest that the nanoparticle formulation shows promise to selectively accumulate in tumor and require further investigation of its *in vivo* anticancer efficacy.

CONCLUSION

The macromonomer method has been used to successfully prepare crosslinked, paclitaxel-loaded PLA-PEG (stealth) nanoparticles with the capacity for drug targeting using redox free-radical polymerization at room temperature. The nanoparticles have also been characterized. *In vitro* cytotoxicity testing in breast and ovarian cancer cell lines revealed

that the nanoparticle formulation compared to free paclitaxel at the same drug concentration exhibited similar cytotoxicity for the duration of the study. The cellular uptake of rhodamine-123 loaded nanoparticles show that the nanoparticles are internalized by MCF-7 breast cancer cells within one hour. Biodistribution studies have shown that the nanoparticles accumulate in tumor.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
(A): SEM image of paclitaxel-loaded nanoparticles prepared by *in situ* dispersion polymerization. (B): TEM image of paclitaxel-loaded nanoparticles prepared by *in situ* dispersion polymerization.

Figure 2.
Cumulative amount released vs time of paclitaxel-loaded nanoparticles using the selected optimized formulation. Standard deviation (0.36-7.73%; n = 3)

Figure 3.

Cytotoxicity of free (Pac. Solution) and paclitaxel-loaded nanoparticles (NP) to MCF-7, MDA-MB-231 and SK-OV-3 cancer cells (72h treatment) at different concentrations. Controls represent blank nanoparticles and 0.05% DMSO in medium. Data represent mean $\pm$ S.D. (n=4)

Figure 4.

Effect of treatment duration on cytotoxicity of paclitaxel-loaded nanoparticles on (A) MCF-7 cells (B) MDA-MB-231 cells (C) SK-OV-3 cells. Data represent mean $\pm$ S.D. (n=4). Error bars represent percent standard deviation.

Figure 5.

Internalization of nanoparticles by MCF-7 cells (A) 1 hour; (B) 6 hours and (C) 24 hours after incubation with fluorescent particles. Lower left quadrant shows cell membrane staining only; upper left quadrant shows fluorescent nanoparticles only; upper right quadrant shows nuclei staining only while the lower right quadrant shows overlay of all the quadrants.

Figure 6.

(A) *Ex vivo* images of tumors from bodipy®-loaded nanoparticle injected mice at 72 h (right) and tumor from control animal (left) at 72 h (B) Representative image showing accumulation of fluorescent Bodipy® dye-loaded nanoparticles in tumor at 72 hour post injection

Scheme 1.

Synthesis and structure of stealth crosslinked P(LLA-HEMA) nanoparticle