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BIODEGRADABLE POLYMERIC NANOPARTICLES FOR TUMOR-SELECTIVE TAMOXIFEN DELIVERY: *IN VITRO* AND *IN VIVO* STUDIES

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1. ABSTRACT

This study was performed to evaluate the *in-vitro* and *in-vivo* tumor-cellular uptake and biodistribution pattern of tamoxifen when administered intravenously as a simple solution and upon encapsulation into biodegradable, surface-modified poly(ε-caprolactone) (PCL) nanoparticles. PCL (MW ~ 15,000) nanoparticles were prepared by the solvent displacement method and characterized for particle size/charge and surface morphology (by scanning electron microscopy). We investigated the nanoparticle-surface modification potential of the hydrophilic stabilizer (Pluronic® F-68 and F-108) employed during the preparation by electron spectroscopy for chemical analysis (ESCA). Quantitative *in-vitro* cellular uptake of tritiated (^{3}H) tamoxifen in solution form and as nanoparticulate formulation was assessed in MCF-7 breast cancer cells. *In-vivo* biodistribution studies for the same formulations were carried out in Nu/Nu mice bearing MDA-MB-231 human breast carcinoma xenograft. Spherical nanoparticles having positive zeta potential (~25 mV) were obtained in the size range of 200-300 nm. Pluronics (both F-68 and F-108), the triblock copolymers of poly(ethylene oxide) (PEO) and poly(propylene oxide) induced surface hydrophilization of the nanoparticles via adsorption as evident by ESCA. Nanoparticulate formulations of tamoxifen achieved higher intracellular concentrations when exposed at therapeutic concentrations to tumor cells *in-vitro* compared to solutions. The *in-vivo* biodistribution studies carried out in nude mice bearing experimental breast tumor suggested increased tumor concentrations for the drug administered as nanoparticulate formulations besides longer retention times within tumor mass. This type of delivery system is expected to provide better therapeutic benefit by dual means: preferential concentration within the tumor mass via enhanced permeation and retention pathway, and; subsequent controlled release, thus maintaining the local drug concentration for longer periods of time to achieve maximal cell-kill.

2. INTRODUCTION

Tumor-selective delivery of anti-cancer agents at the site of action is desirable for two reasons: to maximize cell-kill effect during the tumor growth phase during which majority of the cells remain sensitive to pharmacotherapy and to protect the surrounding healthy cells from exposure to the cytotoxic agent. It is also desirable to maintain a steady infusion of the drug into the tumor interstitium to accomplish continuous extermination of the dividing cells that eventually results in tumor regression. Advances in nanobiotechnology have resulted in evolution of several novel colloidal carrier systems to achieve these multiple objectives, such as liposomes, polymeric micelles, nanoparticles, and nanoemulsions, among others [1-3].

Polymeric nanoparticles made from natural or synthetic polymers have drawn major attention due to higher stability, maneuverability for industrial manufacture, and opportunity for further surface nanoengineering [4,5]. They can be tailor-made to achieve both controlled drug release and tumor-targeting by tuning the polymer characteristics and surface chemistry [6,7]. It has been established that nanocarriers can get concentrated preferentially in the tumor mass by virtue of the enhanced permeation and retention (EPR) mechanism and once accumulated, they

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can act as local drug depot depending upon the make-up of the carrier, thus providing a source for continuous supply of encapsulated therapeutic compound into tumor mass [8,9].

3. EXPERIMENTAL METHODS

Tamoxifen base-containing PEO-PCL (Mol. wt. 14,800) nanoparticles were prepared by solvent displacement method as described by Espuelas *et al.* [10]. Briefly, 625 mg of PCL and 62.5 mg of tamoxifen was dissolved in 100 ml of acetone by mild heating and sonication. The polymer solution was introduced slowly into 200 ml of deionized distilled water containing 625 mg of Pluronic® F-68 or F108, triblock copolymers of PEO and poly(propylene oxide) (PPO), under moderate magnetic stirring. The tamoxifen-loaded nanoparticles were characterized in terms of mean size and size distribution, morphology, and surface charge. 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 nanoparticles by high resolution C_{1s} spectral analysis [11].

Estrogen receptor positive MCF-7 breast cancer cells, obtained from ATCC, were cultured in modified Eagle's minimum essential medium at 37°C in 5.0% CO_2 atmosphere. When the cells had reached 80% confluency, they were detached from the culture vessels by trypsin-EDTA treatment. For analysis of cellular uptake and distribution, the cells were grown on a glass cover slip in 6-well plates. Rhodamine-encapsulated PEO-PCL nanoparticles were prepared with 1.0% (w/w) of rhodamine for fluorescence confocal analysis of uptake and cellular distribution studies. For analysis of intracellular tamoxifen concentrations, [^{3}H]-tamoxifen-loaded nanoparticles were prepared by dissolving a known amount of the polymer, cold tamoxifen and the radiolabeled tamoxifen in acetone. The final drug concentration was 10% (w/w) and specific activity of the drug in the final formulation was 0.2 μCi/mg. Radiolabeled tamoxifen-containing PEO-PCL nanoparticles were incubated with the MCF-7 breast cancer cells (growth medium was switched to serum-free medium for trafficking studies). Periodically, the media was removed, cells were washed twice with phosphate buffered saline (pH 7.4) to remove any adhering traces of the nanoparticles particles or the drug. The MCF-7 cells were then lysed with 1.0 ml of a 0.05% (w/v) Triton® X-100 solution. The cell lysate was placed in 20-ml scintillation vials containing 10 ml of the Scintisafe® Econo 1 scintillation cocktail (Fisher Scientific, Pittsburgh, PA). The radioactivity, as counts-per-minute, was measured with a TriCarb 1600TR liquid scintillation analyzer (Packard Instrument Company, Meridian, CT). The counts-per-minute were converted into the concentration units (ng of tamoxifen per 500,000 cells) using appropriate calibration curves.

All of the animal-related experimental protocols were approved by the Institutional Animal Care and Use Committee of Northeastern University. A highly-aggressive metastatic human breast adenocarcinoma xenograft model based on MDA-MB-231 (ATCC #HTB-26) cell line has been standardized in our laboratory for the *in vivo* evaluation of the tamoxifen delivery with controls and PEO-PCL nanoparticles. The cells were maintained by standard *in vitro* propagation technique in culture flasks containing Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, sodium pyruvate and penicillin-streptomycin under standard incubating conditions of 5% CO_2 at 37°C. The *in vivo* breast cancer model was developed in female nude mice (Nu/Nu), obtained from Charles River Laboratories, (Cambridge, MA). The mice (4-6 weeks old, ~25 g) were maintained in the Division of Laboratory Animal Medicine (DLAM) animal housing facility of Northeastern University (room 21 Mugar Building). The animals housed in polycarbonate cages and had access to sterilized pellet diet and water *ad libitum.* Solid tumors were developed in the breast pad of the mice by injecting 10^6 cells (in

200 μl of serum free medium) subcutaneously. The mice were injected with test and control formulations when the tumor volume had reached 100 ± 10 mm^3.

4. RESULTS AND DISCUSSIONS

We could obtain spherical nanoparticles having a mean diameter of about 150 nm with god batch-to-batch reproducibility. The size distribution was ranging from 100 nm to 300 nm. SEM image of the nanoparticles showed distinct spherical particles with smooth surface. Tamoxifen-containing nanoparticles had a positive surface charge of 24.0 mV, due to presence of the drug on the surface. In addition, the freeze-dried PEO-modified nanoparticles were easily dispersible in aqueous medium upon hydration.

Surface modification of the nanoparticles via physical adsorption of PPO central block which leaves the flanking PEO chains extending out into the surrounding medium was ascertained with ESCA. Table 1 summarizes the results obtained with ESCA showing high-resolution -C-H- (hydrocarbon), -C-O- (ether) and –C=O- (carbonyl) peaks in the C_{1s} envelope of the control and PEO-modifed nanoparticles at their characteristic binding energies. The surface presence of the PEO chains was confirmed by an increase in the ether (-C-O-) signature of the spectra, which is indicative of the presence of ethylene oxide residues. It becomes evident that when nanoparticles are prepared in presence of Pluronics®, a PEO modification is achieved predominantly via surface adsorption. A fraction of the adsorbed surfactant is eliminated with each washing step. Pluronic® F-108 provided a better coating of PEO than F-68 and this adlayer rendered the nanoparticle surface hydrophilic and hence less prone for recognition by the RES system.

Table 1. High resolution C_{1s} peak analysis of ESCA on surfaces of the control and PEO-modified PCL nanoparticle

Formulation	Relative Peak Area (%)		
	-C-H- (285.0 eV)	-C-O- (286.4 eV)	-C=O- (288.1 eV)
Control PCL NP (no stabilizer)	67	18	13
PCL NP (0.1%, F-68, as is)	34	60	5
PCL NP (0.1%, F-68, washed once)	59	27	13
PCL NP (0.1%, F-68, washed twice)	69	18	12
PCL NP (0.1%, F-108, as is)	20	76	3
PCL NP (0.1%, F-108, washed once)	55	33	11
PCL NP (0.1%, F-108, washed twice)	61	24	14

Figure 1 provides proof for efficient uptake of the nanoparticles when presented in the vicinity of the tumor cells *in vitro*. The nanoparticles were located on periphery of the cells soon after incubation (within five minutes). After 1 hour of incubation, the PEO-PCL nanoparticle aggregates were primarily found in the endosome/lysosome complex and were seen traversing though the cytosol. After 2 hours of incubation, majority of the nanoparticle-containing vesicles were located in the perinuclear region of the cells. It is important to note that during the experimental time period, the cells remained completely viable assuring that the PEO-PCL nanoparticles did not induce any cytotoxicity. Since the estrogen receptors are located in the periphery of the nuclear membrane and in the nucleus, these nanoparticles could provide

increased therapeutic benefit by delivering apoptotic drugs in the vicinity of the intracellular target site.

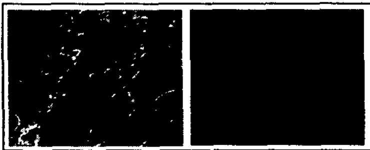

Figure 1. Differential interference contrast (DIC-left) and fluorescence (right) confocal images of rhodamine-encapsulated poly(ethylene oxide)-modified poly(ε-caprolactone) nanoparticles internalized in MCF-7 breast cancer cells after 1 hour of incubation.

Figure 2 shows the intracellular concentrations of tamoxifen in aqueous solution or nanoparticle formulation as a function of time for up to 3 hours using four different initial doses of the drug. As can be seen from each of the profile (Fig. 3-A to 3-D), the intracellular concentrations of tamoxifen increased sharply and a plateau was established within the first 30 minutes of drug administration. At lower dose of 1.9 μg per 500,000 cells, the intracellular concentration of tamoxifen from the PEO-PCL nanoparticles formulation was greater than from the drug solution. At 1 hour, for instance, the tamoxifen concentrations from the nanoparticles and solution were 460 ng/500,000 cells and 390 ng/500,000 cells, respectively. Almost 38% of the administered dose was delivered intracellularly in 1 hour in the nanoparticle formulation as compared to 30% of the dose from the solution. However, with increasing dose of the administered nanoparticles, there occurred a saturation limit for the cellular uptake. It would be possible to achieve higher drug levels within cells by increasing drug loading within nanoparticles.

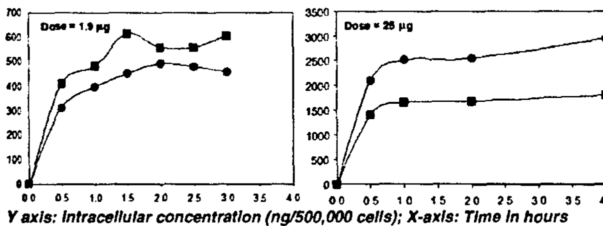

Figure 2. Intracellular concentrations of tamoxifen delivered in an aqueous solution (●) or in poly(ethylene oxide)-modified poly(ε-caprolactone) nanoparticles (■) to MCF-7 breast cancer cells as a function of time with different doses of tamoxifen.

As can be seen from Figure 3, after 1 hour and 6 hours there was a higher concentration of tamoxifen delivered per gm of tumor mass in the PEO-PCL nanoparticles as compared to control (unmodified PCL nanoparticles or aqueous solutions of the drug) after intravenous administration. The long-circulating PEO-PCL nanoparticles were able to preferentially target the payload at the tumor site.

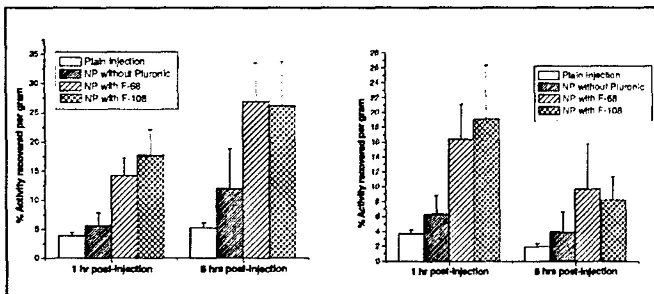

Figure 3. Tamoxifen concentration in tumor (left) and blood (right) after 1 hour and 6 hour following intravenous administration of the control and PEO-PCL nanoparticle formulations to nude mice bearing MDA-MB-231 human breast carcinoma.

Significantly higher uptake was observed for PEO-modified PCL NPs by the tumor mass compared to control (plain solution). Nanoparticles modified with F-108 adlayer showed higher

accumulation in tumor tissue – especially at 1st hour post-injection. About 60-70% of recovered dose was localized in liver which was the primary site of NP accumulation and clearance. Blood profile showed existence of NPs in systemic circulation for longer time than control (plain solution). A certain fraction of the NPs were cleared from systemic circulation with time that underwent further accumulation into tumor. F-108 offered better circulation / accumulation properties due to longer PEO chains (compared to F-68) and hence better steric stabilization.

5. CONCLUSIONS

Biodegradable polymeric nanoparticles loaded with a hydrophobic anti-cancer drug were successfully surface-modified by physical adsorption of a triblock polymer having central hydrophobic anchoring unit and hydrophilic side chains. The nanoparticles showed passive tumor-selective biodistribution with clinically exploitable circulation times. The present study provides evidence for simple means of achieving *in-situ* surface-modification to formulate a tumor-selective nanocarrier.

6. ACKNOWLEGMENTS

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