Multifunctional Polymeric Nanosystems for *EGFR* Gene Silencing

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ABSTRACT

Cetyltrimethylammonium bromide (CTAB) surface-modified poly (ethylene oxide) –modified poly (epsilon caprolactone) (PEO-PCL) nanoparticles were formulated for the simultaneous delivery of Epidermal Growth Factor Receptor (*EGFR)* and Chemotherapeutic drug (Paclitaxel) to pancreatic cancer cells (Panc-1). The multifunctional polymeric nanoparticles were capable of enhanced cell-kill response. The purpose of this study was to utilize siRNA to silence the EGFR gene thus inhibiting the host of signaling pathways required for cancer cell growth and survival initiated by activated EGFR.

Keywords: multifunctional PEO-PCL nanoparticles, EGFR, apoptosis, siRNA

1. INTRODUCTION

Adenocarcinoma of the pancreas is the fourth leading cause of cancer-related deaths in the US. It is the worst form of gastrointestinal malignancy associated with the poor prognosis and a median survival of less than one year from the time of diagnosis. The poor prognosis of pancreatic cancer is partly due to the failure to diagnose the malignancy while the tumor is resectable and the tendancy of the tumor to spread to vital organs [1]. Tumor progression is linked to the over-expression of receptors for epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF). Cytokines like transforming growth factor beta (TGF-ß), interleukins 6 and 8 (IL-6 and IL-8) and tumor necrosis factor alpha (TNF-α) are also associated with tumor progression [2].

EGFR, belonging to the ErbB family of receptors is a 170 kDa membrane spanning glycoprotein. It is composed of an extracellular ligand binding amino-terminal, a transmembrane hydrophobic region anchoring the receptor to the cell membrane, a tyrosine kinase containing cytoplasmic domain and a carboxy terminal region with the receptor regulatory motifs and essential tyrosine residues. Tumor cells require an adequate supply of oxygen, nutrients and an efficient disposal of toxic molecules for their survival [3]. Oxygen from capillaries can not diffuse beyond a distance of 150-200 μm therefore the necrotic region of the cancer is not vascularized and receives no oxygen or nutrient supply. This leads to the generation of new blood vessels from pre-existing blood vessels, angiogenesis. The expression of EGFR and its receptor correlates with angiogenesis and tumor progression [4]. When ligands like EGF, betacellulin or epiregulin bind to the EC domain of EGFR it leads to EGFR dimerization and autophosphorylation of the tyrosine residues. The phosphorylated tyrosine residues interacts with a host of signal transducers and adaptor molecules that initiate signaling pathways leading to cell proliferation, differentiation, migration, adhesion protection from apoptosis and transformation. PLC gamma-1, Akt, Src, STATs, Ras-Raf-MEK-MAPKs, Phosphatidylinositol-3 kinase (PI3K) and PAK-JNKK-JNK are the signaling pathways initiated by activated EGFR. Activated EGFR induced activation of Akt, NFκB and the PI3K pathway causes the cancer cells to escape apoptosis, leading to resistance to therapy and metastasis. EGFR is the only receptor from the ErbB family that can directly interact with c-Cb1, a ubiquitin ligase targeting EGFR to lysosomal degradation after ligand induced receptor internalization. In most cancers a deletion of the EC domain leads to ligand independent activation of the receptors [3].

RNA interference has emerged as a powerful tool for silencing genes. RNA induced silencing is the mechanism by which non-coding small endogenous RNAs down-regulate or completely suppress the expression of genes with complementary nucleotide sequences. RNAi was first described by Fire and Mello, who observed that injecting small double stranded RNA (dsRNA) in *C.elegans* resulted in post transcriptional gene silencing (PTGS) of the corresponding gene by degrading mRNA of complementary sequence. dsRNA are cleaved by Dicer, a member of the RNase III protein family into 21-23 nucleotide small interfering RNAs (siRNA). siRNA gets incorporated into a protein complex called the RNA-induced silencing complex (RISC), which preferentially retains the sense strand and guides the complex to target mRNA. RNAi has displayed tremendous potential in silencing oncogenic genes [4]. The major limitation to the clinical development of siRNA is its large molecular weight and negative charge which hinders its effective cellular uptake and intracellular delivery. The use of viral and non-viral vectors for the delivery of siRNA in vivo remains a major challenge [6].

Paclitaxel (Taxol), a diterpenoid derived from the needles and bark of *Taxus brevifolia* has a wide spectrum of activity blocking cancer cells in the late G2-mitotic phase

of the cycle by stimulating microtubule polymerization and suppressing their dynamics thereby inhibiting cancer cell replication. Paclitaxel is hydrophobic with poor solubility in water. It is solubilized in a 50:50 Cremophor EL and ethanol. Cremophor gives rise to side effects like hypersensitivity, nephrotoxicity and neurotoxicity [7]. Nanoparticles made from biodegradable polymers can overcome the above harmful allergic responses and provide controlled and targeted delivery of drug promoting higher efficacy and lower side effects this is because the drug distribution would follow the carrier rather than the physicochemical properties of the drug [7] [8].

Biodegradable, hydrophobic poly-epsilon-caprolactone (PCL) is surface modified using Pluronic®F-108 an ABA triblock copolymer consisting of polyethylene /polypropylene / polyethylene (PEO/PPO/PEO). Surface modification is the result of hydrophobic interactions between the PPO and and PCL core. The nanoparticle surface was further functionalized with cetyltrimethylammonium bromide (CTAB) which complexes with siRNA. A suitable PCL and Pluronic blend in the right proportion ensures that hydrophilic PEO side-arms extending outward from the particle surface remain in the mobile state resulting in a slow eroding, long circulating nanoparticles. Owing to the impaired reticuloendothelial/lymphatic clearance or lack of such clearance macromolecular drugs administered i.v. escape renal clearance due to their large molecular size. These drugs fail to penetrate the tight endothelial junctions of normal blood vessels but extravasate in tumor vasculature to get trapped in tumor vicinity. Due to a lack of efficient lymphatic drainage the concentration of drug in the tumor will increase several times higher than the concentration of drug in plasma. Thus these nanoparticles get concentrated preferentially in the tumor mass as a result of the enhanced permeability and retention (EPR) effect of the vasculature. The hydrophobic biodegradable polymeric nanoparticles act as a local drug depot [9].

2. EXPERIMENTAL METHODS

2.1 Nanoparticle preparation and characterization

CTAB modified-PEO-PCL nanoparticles were prepared by the solvent displacement method. These nanoparticles encapsulated 10% (w/w) PTX. The nanoparticles were formed by dissolving the drug/polymer mixture in acetone, followed by gentle dropwise addition of the polymer-drug solution to distilled water containing CTAB under rapid magnetic stirring. Following evaporation of the organic solvent, nanoparticles were collected by centrifugation, washed in distilled water, and lyophilized for storage. 1 nmole siRNA was then added to lyophilized nanoparticles re-suspended in RNase free water. Nanoparticle preparations were subsequently subjected to size and zeta-potential measurements using a Brookhaven 90Plus analyzer. A Pico green ® assay was performed to obtain siRNA loading and PTX loading was determined by UV spectroscopy.

2.2 *In-vitro* cytotoxicity studies

The human pancreatic cancer cell line Panc-1 was maintained in culture in the presence of increasing concentrations of PTX. For cytotoxicity studies Panc-1 cells with a seeding density of 8000cells/well were seeded in a 96 well plate. The panc-1 cells were subjected to dose-response studies against PTX dose ranging from 10nM to 10μM, siRNA 10pM to 100pM and PTX (100nM) combined with siRNA (100pM), delivered as free drugs in solution or delivered in PEO-PCL nanoparticles. In all studies cell growth medium was used as a negative control and polyethyleneimine (PEI) was used as a positive control and proper vehicle controls. Since PTX is a cell-cycle dependant chemotherapeutic, all treatments were carried out for a period of 6 days, allowing ample time for all cells to enter mitosis. The cell death/viability was determined by the MTS (formazan) assay measuring absorbance at 490nM.

2.3 Baseline EGFR Expression in Panc-1 Cells

Western Blot Analysis: Total protein was extracted from Pancreatic cells lines Panc-1, SW1990 and human fibroblast NIH3T3 cells and quantified using NanoOrange® assay. Proteins extracted from SW1990 and NIH3T3 were used as positive and negative control respectively. Hundred μg of denatured protein extract was diluted with loading buffer and loaded onto pre-cast 4-20% SDS-polyacrylamide gel electrophoresis (PAGE) gradient gel. After running the gel at 150 volts for 90 minutes the protein bands were transferred onto a nitrocellulose membrane where transfer was carried out at 25 volts for a period of 2 hours on ice. The transferred protein bands on the membrane were then blocked with 3% milk in Tween containing Tris buffered saline (T-BST) for 45 minutes followed by overnight incubation at 4^0C with 1:1000 dilution of primary rabbit-EGFR antibody in milk. The next morning the milk was decanted and the membrane was incubated for 1 hour with 1:2000 dilution of HRP-conjugated goat anti-rabbit secondary antibody. After 2 washes with water and a 5 minute wash with T-BST the membrane was incubated with ECL substrate for 3 minutes after which chemiluminescent bands were visualized using a Kodak imager.

Immunocytometric Studies: Cultured Panc-1 and NIH3T3 cells grown on sterile glass cover slips were fixed with 10% formalin in PBS for 10 minutes. To avoid non-specific binding of the immunoglobulins the cover slips were incubated for 30 minutes with a universal blocking solution followed by a 1:100 dilution of primary rabbit EGFR antibody. The cover slips were then incubated with

peroxidase blocking solution for 10 minutes followed by incubation with 1:200 dilution of HRP-conjugated goat anti-rabbit secondary antibody for 30 minutes. Subsequently, diaminobenzidine solution was added and allowed to incubate for 5-10 minutes for peroxide catalyzed reaction to occur resulting in the formation of brown precipitate. The cells were then dipped in Gill's hematoxylin solution and observed using a bright field microscope at 20X magnification.

3. RESULTS AND DISCUSSION

The solvent displacement technique produced reproducible CTAB modified PEO-PCL nanoparticles with a mean diameter of 231.8 ± 5.6 nm and a zeta potential of 28.4 ± 0.6 mV. PTX loaded CTAB modified PEO-PCL nanoparticles had a mean diameter of 230.0 ± 4.6 and zeta potential of 30.8 ± 0.3 mV. Lastly, siRNA + PTX loaded CTAB modified PEO-PCL nanoparticles had a mean diameter of 269.8 ± 4.0 and a zeta potential of 9.95 ± 0.8 mV. The reduction in zeta potential confirms the complexation of siRNA to the CTAB on PEO-PCL nanoparticles (Table 1). Based on the Pico green assay it was observed that 1 nmole siRNA could be loaded with an efficiency of 97% on 2 mgs nanoparticles. These nanoparticles encapsulated 10% (w/w) PTX with a loading efficiency of 91%.

Table 1. Particle size and surface charge properties of PEO-PCL nanoparticles

Nanoparticle Type	Particle Size (nm)	Zeta Potential (mV)
Blank PEO-PCL Nanoparticles	231.8 ± 5.6	28.4 ± 0.6
PTX loaded PEO-PCL Nanoparticles	230.0 ± 4.6	30.8 ± 0.3
siRNA + PTX loaded PEO-PCL Nanoparticles	269.8 ± 4.0	9.95 ± 0.8

Mean ± S.D. (n = 3)

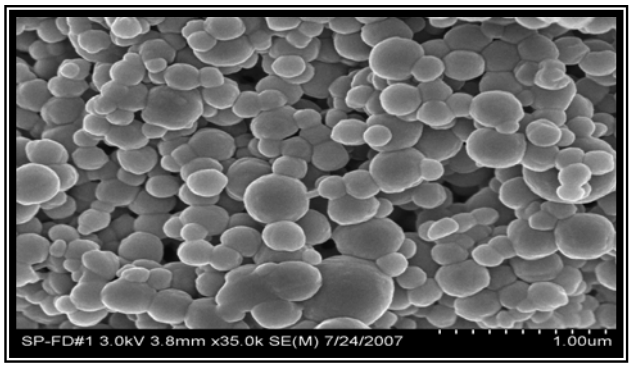


Figure 1 : *Scanning electron micrograph of CTAB-modified PEO-PCL nanoparticles for simultaneous EGFR siRNA and paclitaxel delivery in pancreatic cancer cells.*

The cytotoxicity data showed that 100 nM PTX nanoparticles reduced cell viability to 50% while the same was achieved by 300nM PTX solution. 100pM siRNA loaded nanoparticles reduced cell viability to 70% as compared to solutions where cell viability was reduced to only 80%. On the other hand, combination of siRNA (100pM) and PTX (100nM) significantly reduced cell viability to 50% when compared to only 100nM PTX loaded nanoparticles or 100pM siRNA loaded nanoparticles. Thus co-therapy is capable of enhanced cell-kill as compared to the same treatments given in solutions or same treatments given individually in nanoparticles (Figure2 and 3).

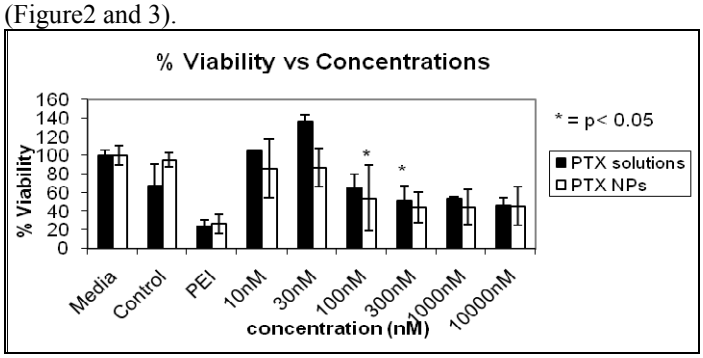


Figure 2: Cytotoxicity of PTX in solutions and Nanoparticles in Panc-1 cells. (values are expressed as mean ± S.D (n=3)

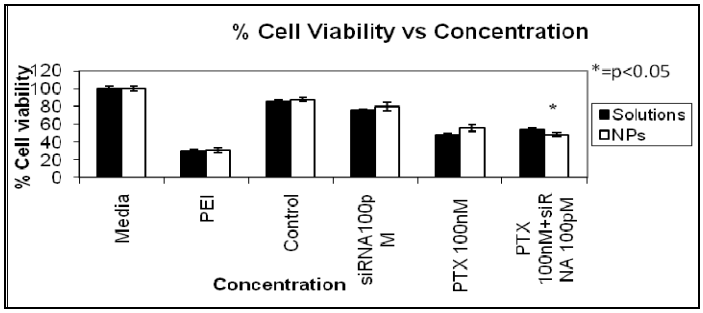


Figure 3: Cytotoxicity of EGFR silencing siRNAco-administered with PTX in aqueous solutions and nanoparticles to Panc-1 cells.
Values are expressed as mean ± S.D (n=3)

Western Blot and Immunocytochemical Analysis confirmed the overexpression *EGFR* in Panc-1 cells.

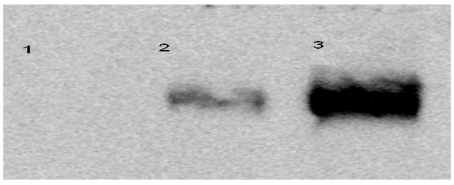
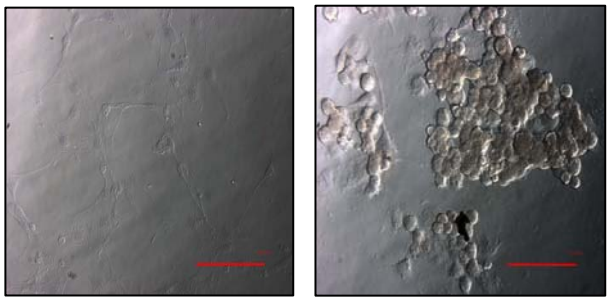


Figure 3: EGFR expression by Western Blot Analysis of Control and Panc-1 cells. Lane 1: NIH3T3, Lane 2: SW1990, and Lane 3: Panc-1 cells expressing EGFR at 175 kDa.



NIH3T3 Panc-1

Figure 4: Immunocytometric Analysis for the expression of EGFR on Panc-1 cells and NIH3T3 cells as negative control (Magnification 20X)

4. CONCLUSION

The results of this study show that multifunctional nanoparticles can be formed for simultaneous siRNA and drug delivery in cancer cells. When used for intracellular EGFR silencing and PTX delivery, the PEO-PCL nanoparticles showed enhanced efficacy in Panc-1 cells.

5. ACKNOWLEDGEMENTS

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