Epidermal Growth Factor Receptor-Targeted Engineered Gelatin Nanovectors for Gene Delivery and Transfection in Pancreatic Cancer Cells

Padmaja Magadala and **Mansoor Amiji**
Department of Pharmaceutical Sciences, School of Pharmacy, Northeastern University,
Boston, MA 02115

ABSTRACT

Pancreatic cancer is a leading cause of cancer-death, mostly owing to lack of efficient therapeutic options. Upwards of 90% pancreatic tumors over express epidermal growth factor receptor (EGFR) and this largely influences the disease aggressiveness and poor clinical outcome in patients. Non-viral gene therapy has achieved increased attention over the last two decades. In this study type B gelatin-based nanoparticles were characterized and evaluated for gene delivery in pancreatic cancer. Further, these nanoparticles were surface-modified to actively target EGFR on the surface of Panc-1 cells.

1. INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer-related deaths in the United States (1). The poorly understood etiology, lack of early diagnostic tools, and effective treatment options of pancreatic cancer contribute to its increasing incidence and mortality rates. Epidermal growth factor receptor (EGFR) family includes EGFR (or *erb*B-1), HER-2 (or *erb*B-2 or neu), HER-3, and HER-4. Over 90% of human pancreatic cancer cases over express EGFR family members (2). Upon ligand binding, activated EGFR plays an important role in cell growth, differentiation, migration, and metastasis. Its positive signaling was found to cause increased proliferation, decreased apoptosis, enhanced tumor cell motility and angiogenesis. Also, EGFR is expressed in different non-transformed cell types in the tumor microenvironment that are involved in tumor growth and progression, including endothelial cells on the neovasculature. Targeting the EGFR receptors for therapeutic purpose has become possible with the recent introduction of chimeric and humanized monoclonal antibodies (3).

Gene therapy comprises transfer of genetic constructs intended to alter the neoplastic potential of the cancer cell. Various viral and non-viral delivery systems are currently being examined pre-clinically and clinically in different models of pancreatic cancer. In the development process of a nanoparticulate drug delivery system for *in vivo* gene therapy application, several design criteria are necessary. First, the nanovector must efficiently and stably encapsulate DNA and protect against degradation in the systemic circulation and upon cellular uptake. Second, the delivery system must be able to overcome biological barriers and accumulate at the target site and be internalized in cells by non-specific or receptor-mediated endocytosis. Third, the internalized nanovector must release plasmid DNA and allow for nuclear import and transfection. Lastly, the nanovector matrix must be degradable and non-toxic for chronic administration. Various nano-particulate systems based on natural and synthetic polymers like gelatin, chitosan, and poly(D,L-lactide-co-glycolide) (PLGA) have been extensively investigated (4).

Gelatin-based delivery systems are known for their biocompatibility and biodegradability. Over the last several years, our lab has engaged in studies of gelatin-based nanoparticulate systems for drug and gene delivery. Kaul and Amiji (5) have shown that gelatin and poly(ethylene glycol) (PEG)-modified gelatin nanoparticles are efficiently endocytosed by different types of cells and accumulate in the perinuclear region. Additionally, Kommareddy and Amiji (6) have demonstrated that, PEG-modified thiolated gelatin nanoparticles can be successfully used for *in vivo* delivery of therapeutic gene encoding for anti-angiogenesis sFlt-1 factor in an orthotopic human breast cancer xenograft model.

PEG is a biocompatible water soluble polymer that are increasingly chosen for shielding drug delivery systems, particularly for proteins, peptides and antibody fragments. Over the last two decades, PEG surface modification has become an attractive choice of drug delivery, largely due to its reduced renal clearance, reduced proteolysis and immunogenicity. Therefore, the use of PEG spacers helps in potential reduction of steric interference and long circulating nanoparticles.

For surface modification of gelatin nanoparticles for EGFR targeting, we have utilized a peptide ligand. Recently, various researchers have reported

NSTI-Nanotech 2008, www.nsti.org, ISBN 978-1-4200-8503-7 Vol. 1

the successful identification of peptide ligands by screening phage display libraries (7). In earlier gene delivery studies, EGFR-targeting-peptide-conjugated polyethylenimine (PEI) vectors were shown to be less mitogenic, but highly efficient at transfecting genes into EGFR overexpressing cells and tumor xenografts (8). EGFR-targeted gelatin-based engineered nanovectors (GENS) were synthesized by conjugating the EGFR-targeting peptide ligand via a heterobifunctional PEG spacer. Reporter plasmid DNA expressing enhanced green fluorescent protein (GFP) was encapsulated and the transfection was studied in EGFR over-expressing human pancreatic adenocarcinoma cells (Panc-1).

2. EXPERIMENTAL METHODS

2.1. Synthesis and Characterization of EGFR-Targeted Gelatin Nanoparticles

Type B gelatin (225 bloom strength) was used to make the nanoparticles (GENS) by the solvent displacement method (5). EGFP-N1 plasmid DNA, expressing GFP, was encapsulated in the nanoparticles and the surface was modified with a heterobifunctional PEG linker for covalent attachment of peptide. To enhance particle uptake by EGFR targeting, we used the 11-amino acid peptide -YHWYGYTPQNVI- (8). Mean particle size and size distribution as well as surface charge (zeta potential) values were determined for the blank and DNA-loaded nanoparticles. The spherical shape and surface morphology of the particles was confirmed by scanning electron microscopy (SEM).

The plasmid DNA loading efficiency in the control and EGFR peptide-modified gelatin nanoparticles was quantified dissolving the nanoparticles in protease-containing buffer and quantifying the released DNA using PicoGreen® dsDNA fluorescence assay. Stability of the encapsulated plasmid DNA was examined by agarose gel electrophoresis.

2.2 EGFR Expression in Panc-1 Cells

Baseline EGFR expression in human pancreatic adenocarcinoma (Panc-1) cells was analyzed by Western blot and immunocytochemistry techniques. Other pancreatic cancer (Capan-1) cell line, along with SKOV3 (human ovarian cancer) and NIH3T3 (murine fibroblast) cells were used as positive and negative controls of EGFR over-expression. Briefly, the different cells were grown in fetal bovine serum supplemented DMEM and RPMI media and lysed at 80% confluence. The cell lysate was analysed for total protein concentration using the NanoOrange® protein quantitation kit. Briefly, 50 μg of total protein was subjected to western blot analysis using SDS page gel, nitrocellulose membrane and anti-EGFR antibody, to confirm the receptor expression in Panc1 cells. Immunocytochemistry analysis was performed by incubating the cells with a primary EGFR antibody and then with secondary antibody conjugated with horse-radish peroxidase. Addition of the peroxidase substrate diaminobenzidine results in the formation of brown precipitate.

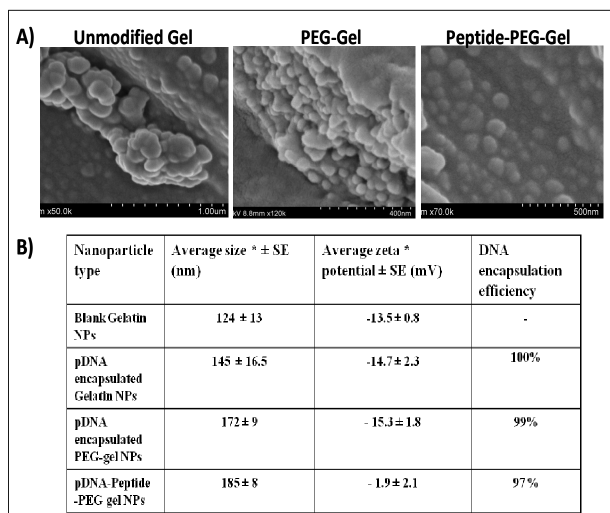
2.3 Cellular Uptake and GFP Transfection Studies

Panc1 cells, cultured in DMEM at 37°C and 5% CO_2 atmosphere, were seeded at 3 X 10^5 cells per well in 6-well cell culture plates. Cover slips were added some culture plates to facilitate microscopic analysis. Plasmid EGFP encapsulated, surface modified and functionalized gel nanoparticles were prepared as described above. Panc1 cells were incubated with control and modified nanoparticles, with final DNA concentration of 30 μg/well, in serum-free medium. At the end of 4 hours, treatments were removed, and growth medium was replaced in the culture wells following a wash with PBS. At 24, 48, 72 and 96 hour time points, transfected cells were harvested, washed and analyzed for green fluorescence intensity by fluorescence activated cell sorting (FACS). Transfected cells on cover slips were mounted on glass slides for fluorescence microscopy imaging.

3. RESULTS AND DISCUSSION

Under controlled solvent displacement, DNA-encapsulated gelatin nanoparticles of less than 200 nm in diameter were reproducibly prepared (Figure 1). These were further crosslinked with glyoxal. Further modification of the gelatin nanoparticles with bifunctional PEG and functionalization with the EGFR-targeting peptide showed that the modification process had no significant influence on particle size (<200 nm) and zeta potential. The similarity of zeta potential values to those of the blank nanoparticles indicates the encapsulation of the plasmid DNA as opposed to adsorption on the surface. The SEM images reveal the smooth surface morphology and spherical shape of these nanoparticles. The percent cell viability as a function of the polymer and EGFR-peptide concentrations was evaluated. These studies indicate that gelatin is

highly biocompatible showing 99% cell viability. However, for the EGFR-peptide, we found that the relative cell viability at the highest concentration of 200 μM was 88%. This could most likely be due to the inhibitory effect of the peptide on the cell proliferation than actual cell kill. In comparison, the positive control, poly(ethyleneimine), has shown a relative cell viability of only 40%.



Nanoparticle type	Average size * ± SE (nm)	Average zeta * potential ± SE (mV)	DNA encapsulation efficiency
Blank Gelatin NPs	124 ± 13	-13.5 ± 0.8	-
pDNA encapsulated Gelatin NPs	145 ± 16.5	-14.7 ± 2.3	100%
pDNA encapsulated PEG-gel NPs	172 ± 9	- 15.3 ± 1.8	99%
pDNA-Peptide -PEG gel NPs	185 ± 8	- 1.9 ± 2.1	97%

Figure 1. Characterization of the control and EGFR peptide-modified gelatin nanoparticles. (A) Scanning electron microscope images of unmodified, PEG-modified and peptide-modified gelatin nanoparticles and (B) size, surface charge, and percent DNA encapsulation in the control and peptide-modified gelatin nanoparticles.

An understanding of the EGFR expression pattern in different pancreatic cell lines was pertinent at this point. Past literature suggests that large variation in EGFR expression among the pancreatic adenocarcinoma cell lines was common. In fact, this is suspected to be a major challenge in the development of receptor targeting drug delivery system for pancreatic cancer treatment. Using Western blot and immunocytochemistry techniques with standard protocol (Figure 2), we found that Panc-1 cells showed increasing levels of EGFR over expression. However, in comparison, Capan-1 (another pancreatic cancer) cells showed significantly lower EGFR expression levels, which was comparable to the human ovarian cancer (SKOV3) cells. Lastly, the NIH3T3, murine fibroblast cells showed no detectable levels of EGFR over expression. This is most likely due to the efficient endocytotic sequestration and subsequent degradation of the EGF receptor in normal cells.

On the other hand, competition binding studies confirmed that over 70% of the binding and uptake mediated by the EGFR-targeted GENS could be competed, while the control nanoparticles showed no such competition. By the *in vitro* particle uptake studies, EGFR peptide-modified GENS resulted in higher uptake by the Panc-1 cells than normal murine fibroblasts (NIH3T3) due to EGFR over-expression. Additionally, we found that uptake of peptide-modified nanoparticles in the Panc-1 cells was time and dose dependent. These results confirm the specificity of the EGFR-targeting peptide in cell lines and, therefore, suggest possible receptor-mediated uptake of the functionalized nanoparticles.

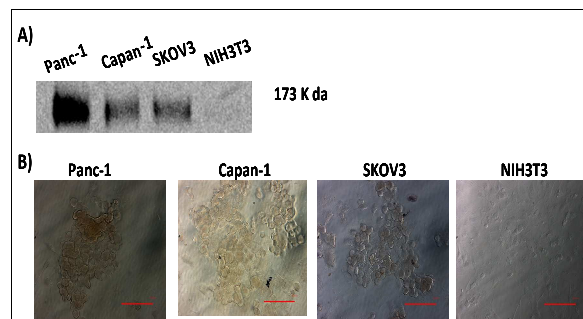


Figure 2. Epidermal growth factor receptor over-expression analysis by (A) Western blot and (B) immunocytochemistry methods in Panc-1 and Capan-1 pancreatic adenocarcinoma cell lines, SKOV3 ovarian adenocarcinoma cells, and NIH3T3 murine fibroblasts.

The DNA loading efficiency of control and peptide-modified GENS was >95%. Surface modification and functionalization of nanoparticles has insignificant effect on the encapsulation of pDNA itself. In the presence of the reducing agent glutathione, release of the encapsulated DNA was found to be 100% in five hours.

With unmodified GENS, the GFP transfection efficiency (Figure 3) in Panc-1 cells was 11%. Upon PEG-modification, the transfection efficiency increased to 30%. EGFR-targeted peptide conjugation to PEG modified nanoparticles led to further increase (~50%) in the transfection efficiency of GFP. This is predominantly attributed to the effective targeting of the functionalized nanoparticles to the EGFR receptor expressed on the tumor cell surface. In addition, the enhanced transfection levels are also due to the prolonged stability of the nanoparticles upon PEG modification. Lipofectin®-complexed plasmid DNA, used as a positive control, showed lower transfection efficiency and higher toxicity over time.

However, previous reports (9, 10) have shown that transduction levels in tumor cell lines were not significantly different from non-targeted transfection levels. Therefore, it is hypothesized that this insignificant increase may be due to the lack of external force *in vitro* to increase vector-to-cell interaction as can be seen *in vivo* post intravenous administration. This emphasizes the popular belief that a wider range of factors controling the tumor

microenvironment *in vivo* cannot be completely and successfully mimicked in the *in vitro* models.

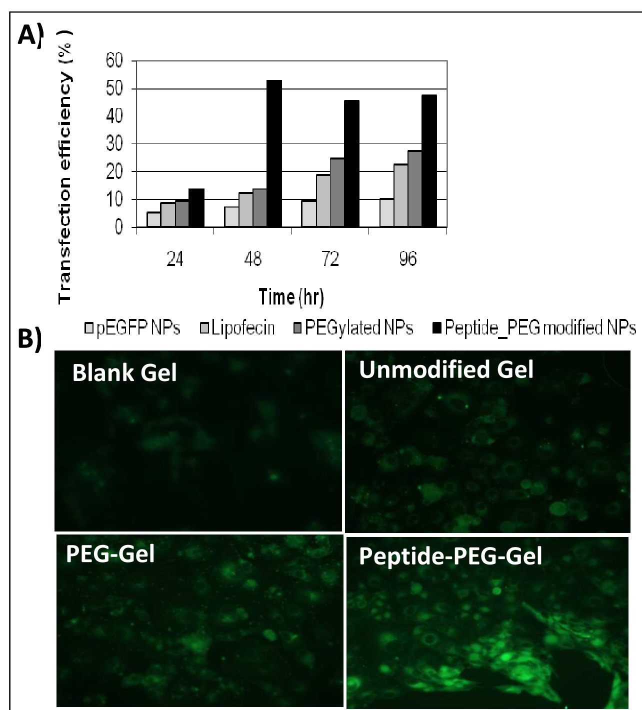


Figure 3. (A) Quantitative and (B) qualitative green fluorescent protein expression efficiency following administration of EGFP-N1 plasmid in control and EGFR peptide-modified gelatin nanoparticles in Panc-1 human pancreatic cancer cells. Quantitative and qualitative transfection was evaluated by flow cytometry and fluorescence microscopy, respectively.

4. CONCLUSIONS

The results of this study showed that type B gelatin nanoparticles, when conjugated with EGFR-targeting peptide ligand, can mediate safe and specific gene transfer to EGFR over-expressing tumor cells in vitro. Furthermore, these results demonstrate the potential to active targeting *in vivo* while achieving efficient gene therapy in pancreatic cancer.

5. ACKNOWLEDGEMENTS

Padmaja Magadala is an IGERT Fellow in Nanomedicine Science and Technology training program. This program is supported by the National Cancer Institute (NCI) and the National Science Foundation (NSF). SEM was performed at the Electron Microscopy Facility of Northeastern University (Boston, MA).

REFERENCES

1. American Cancer Society: Cancer Facts and Figures 2007. Atlanta, GA. American Cancer Society, 2007. Accessed on January 3, 2008 from http://www.cancer.org/downloads/STT/CAFF2007PWSecured.pdf
2. Woodburn, J. R. (1999). The epidermal growth factor receptor and its inhibition in cancer therapy. *Pharmacol. Ther.* **82:** 241–250.
3. Abbruzzese JL, Rosenberg A, Xiong Q, *et al.*, (2001). Phase II study of anti-epidermal growth factor receptor (EGFR) antibody cetuximab (IMC-C225) in combination with gemcitabine in patients with advanced pancreatic cancer. *Proc Annu Meet Am Soc Clin Oncol.* pp. 518.
4. Amiji, M.M. *Polymeric Gene Delivery: Principles and Applications* (2005). CRC Press, New York, NY.
5. Kaul G. and Amiji M. Cellular interactions and in vitro DNA transfection studies with poly(ethylene glycol)-modified gelatin nanoparticles. *J Pharm Sci.,* **94(1):** 184-198 (2004).
6. Kommareddy S and Amiji M. (2007). Antiangiogenic gene therapy with systemically administered sFlt-1 plasmid DNA in engineered gelatin-based nanovectors. *Cancer Gene Ther.,,* **14(5):** 488-98.
7. Brissette, R and Goldstein, NI. (2007). The use of phage display peptide libraries for basic and translational research. *Methods Mol. Biol.,* **383:** 203-13.
8. Li Z, Zhao R, Wu X, et al. (2005). Identification and characterization of a novel peptide ligand of epidermal growth factor receptor for targeted delivery of therapeutics. *FASEB Journal*, **19(14):**1978-85.