ABSTRACT

This chapter describes the motivation for the present work, the rationale for the choice of the targeting ligand, target gene and design of the nano-carriers. The research questions addressed in the present work have been listed based on which the overall objective has been defined. The specific aims designed to achieve the overall objective and the underlying hypothesis has also been elaborated.
2.1 Introduction

The advent of nanotechnology has introduced a new facet towards treatment of diseases. In the past decade, nanotechnology has made giant strides in treatment of various cancers and several nano-delivery systems have reached different phases of clinical trials [1]. Use of nano-dimensional structures that can entrap therapeutic moieties has not only ensured their sustained release, but these carriers can also be surface-modified to impart target-specific delivery of their cargo [2]. These nano-delivery systems can reduce the adverse effects arising from ‘off-targeting’ of the therapeutic moiety while improving its treatment efficacy by enhancing its stability and cellular uptake [3]. Gene silencing strategies have especially benefitted from the emergence of nano-carriers. The inherent instability of si-RNA and off-targeting can be greatly overcome through the use of appropriate targeted nano-carrier. However, the selection of an appropriate targeting ligand is the prime requisite for achieving site-specific delivery of the gene.

2.2 Choice of targeting moiety

Cancer cells exhibit cell surface molecules that are over-expressed only in cancerous tissue at all times unlike normal cells [4]. These cell surface markers can be targeted for internalization of the nanoparticles in to the cancer cell through receptor-mediated endocytosis [5]. Many such cell markers have been employed as targets to home in to cancer cells. These include folic acid receptors that are over-expressed in many forms of cancer [6-11], prostate specific membrane antigen (PSMA), which is over-expressed in prostate cancers [12-16], epidermal growth factor receptor that is abundantly expressed on the surface of epithelial cancers [17-19], luteinizing hormone receptor that is targeted for delivery of drugs in to prostate and ovarian cancers [20-21], transferrin receptor that is highly expressed
in brain [22-25] and integrins, which are cell surface receptors over-expressed in many forms of cancer [26-30]. One of the potential hurdles in development of a targeting strategy is that normal cells also express these cell surface markers, albeit in smaller numbers, and hence some amount of the carrier with its cargo can internalize in to normal cells. This can have serious implications in gene therapy, as the therapeutic gene will interfere with the normal cellular functions, thereby transforming in to a toxic entity [31-35]. Hence, the identification of superior targeting moieties for the treatment of cancers still continues. It is also desirable to identify a target that is expressed in a wide range of cancers so that any targeting strategy developed can be employed as a platform technology to treat many types of cancer. In this context, use of epithelial cell adhesion molecule as a target for site-specific delivery in to epithelial cancers has emerged as a promising strategy. The choice of anti-EpCAM as the targeting ligand is based on the fact that EpCAM is over-expressed in cancer cells when compared to normal cells [36-37]. Another interesting aspect of EpCAM is that it is localized in the baso-lateral side in normal cells and hence is not accessible to the antibody. But in cancer cells, the EpCAM is localized in the apical side making it more accessible to the antibodies [39]. Thus, the difference in expression levels as well as localization between cancerous and normal cells makes EpCAM an attractive target for site-specific delivery of drugs and genes. Figure 2.1 depicts a cartoon showing the different localization of EpCAM in normal and cancer cells.

Several studies have employed EpCAM antibodies as a targeting moiety to home in to EpCAM-expressing cancers and deliver an anticancer agent. Poly(lactide-co-glycolide) nanoparticles conjugated with EpCAM antibody were reported to deliver nutlin-3a, an anti-
Figure 2.1: Expression of EpCAM in normal and cancer cells.

Cancer drug to retinoblastoma, leukemia and colon cancer cells [39]. A recent study had reported EpCAM antibody tagged gold-poly(ethyleneimine) nanoparticles encapsulated with EpCAM si-RNA exhibited increased cell uptake and significant down-regulation of the EpCAM expression levels in Y79 RB cell line underlying the importance of targeting for effective treatment of cancers [40]. A humanized single chain antibody fragment exhibiting specific binding affinity to EpCAM was conjugated to liposomes for delivery of the anti-cancer agent doxorubicin to breast cancer and ovarian cancer cells [41]. Similarly, an designed ankyrin-repeat protein (DARPin) that exhibited specificity towards EpCAM was employed to deliver si-RNA against bcl-2 in breast cancer cells [42-43]. A novel epithelial cell adhesion molecule (EpCAM) aptamer conjugated with Alexa-fluor 594 (AF594) fluorescent probe tagged alkyne (alkyne-DIBO) was developed for the imaging of EpCAM expressing cancer cell lines MCF7, MDA-MB-453, Weri-RB1 and PC3 [44]. This suggests that EpCAM as a targeting moiety could ensure cancer-specific delivery of the therapeutic molecule and hence was chosen for the present work.
2.3 Choice of target gene

Recently, several investigations on EpCAM have also revealed its oncogenic potential. It has been demonstrated that the extracellular domain of EpCAM undergoes proteolytic cleavage in cancer cells. It is believed that this fragment could be involved in signaling pathways leading to proliferation of the cancer cells [45]. Similarly, an intra-cellular oncogenic domain has been identified in EpCAM (Ep-ICD) that has been implicated in nuclear signaling leading to uncontrolled proliferation of the cells [46]. EpCAM levels have been found to be up-regulated both at the gene level and protein level in many types of cancers including breast cancer [4], hepatic cancer [47-48], ovarian cancer [49], colorectal cancer [50], prostate cancer [51] and retinoblastoma [52]. Another study has revealed that EpCAM levels influence the JNK/AP-1 signaling pathway, which may be responsible for the invasive nature of EpCAM-expressing cancer cells [53]. Similar correlations between cancer invasiveness and EpCAM expression levels have also been reported in urothelial carcinoma of the bladder [54]. In another independent work, it has been found that silencing EpCAM gene in lung cancer cells reduced the metastatic nature of the cancer thereby indicating a strong relation between EpCAM expression and invasiveness of a tumor [55]. EpCAM suppression in lung carcinoma has also been found to retard the proliferation and clonogenic nature of the cancer cells without inducing cell cycle arrest. The same study also revealed that EpCAM silencing induced significantly high levels of apoptosis in cancer cells while the magnitude of apoptosis in normal bronchial epithelial cells was considerably lower [56]. Retinoblastoma, a type of ocular cancer that affects children, has been found to over-express EpCAM [52]. Silencing of EpCAM gene in Y79 retinoblastoma cells has been found to down-regulate mitogen-activated protein kinase (MAPK) and suppress p53-mediated pathways. Similarly,
studies using different breast cancer cell lines have also revealed the therapeutic potential of EpCAM silencing by reducing the cell proliferation, migration and invasion [57-58]. It has been reported that suppression of EpCAM expression in prostate cancer cells reduced the expression of androgen receptor and prostate specific antigen – two key markers of prostate cancer [59]. Silencing of EpCAM has shown to up-regulate apoptosis genes and down-regulate genes responsible for cell proliferation and tumor invasion. Thus, silencing EpCAM appears to be a good strategy to mitigate a wide range of cancers [52, 57, 58, 60]. However, since it is known that administration of free si-RNA against EpCAM will not be effective \textit{in vivo} as in the case of all gene therapy applications, use of a gene delivery vehicle can realize the therapeutic potential of this important gene target. Surprisingly, a scan of the literature reveals that no significant efforts have been carried out to develop a nanocarrier-mediated silencing of this gene for cancer therapy. Hence, this work aims to address this lacuna through the development of nanocarriers to deliver EpCAM si-RNA specifically to epithelial cancer cells.

\textbf{2.4 Choice of carrier system}

The previous section has elaborated on the various challenges in designing gene delivery systems and the wide range of delivery systems that have been employed for gene delivery. Figure 2.2 depicts some of the carrier systems that have been reported for delivery of si-RNA to cancer cells.
Cationic polymers such as chitosan [61], poly(ethylene imine) [62], polyamidoamine (PAMAM) dendrimers [63-64], poly(L-lysine) [65] have been reported for gene delivery applications. In addition, a wide range of cationic liposomes [1], mesoporous silica [66] and poly(lactide-co-glycolide) [67-68] have been investigated for delivery of oligonucleotides. Liposomal systems are extensively investigated nano-dimensional carriers due to their ease of formation through self-assembly and amenability to surface modification for imparting specific functionalities and surface charge [3, 69]. In addition, liposomal systems have demonstrated good cell internalization [3, 69]. However, incorporation of cationic lipids has been found to elicit immune response and cause cytotoxic effects [70] and hence attempts to mask the cationic charge or substitute the cationic lipids with non-cationic lipids are underway. But replacement of the cationic components may compromise the encapsulation efficiency of the liposomes. Hence, this work envisages the development of an effective non-cationic liposomal carrier for gene delivery with good therapeutic potential. Two types of liposomal carriers were investigated in this work to explore their potential for gene silencing.
applications. Liposomal carriers formed using non-cationic lipid components like egg phosphatidyl choline (egg PC or EPC) that serves as the primary vesicle-forming lipid, dioleoyl phosphatidyl ethanolamine (DOPE) that can serve as helper lipid enabling cell internalization and endosomal escape through its fusogenic properties, cholesterol (Chol) that can impart stability to the liposome and finally distearoyl phosphatidyl ethanolamine-poly(ethylene glycol) (DSPE-PEG), which contains PEG chains at one end thereby facilitating the formation of a liposome with PEGylated surface. Different combinations of these lipids would be employed to identify the composition that displays maximum encapsulation efficiency of si-RNA. The second system to be investigated is a liposomal carrier that encapsulates a lipoplex formed between a cationic polymer [poly(L-lysine)] and si-RNA. Poly-L-lysine (PLL) with its cationic charges can condense si-RNA with high efficiency through electrostatic interactions and can therefore protect the si-RNA from nuclease degradation [71]. However, its use as a carrier is limited by its high immunogenicity [72]. Therefore, the present work envisions encapsulation of the PLL-si-RNA hybrid in to liposomes. This strategy that has never been explored earlier, attempts to reduce the immunogenic nature of the PLL-si-RNA lipoplex while retaining its desirable high complexation property through its encapsulation in liposomes. The non-cationic liposomal composition optimized in the first case will be used for the formation of the hybrid liposomes. Both liposomal systems will also be surface modified through covalent linking of the EpCAM antibody to understand the influence of the antibody on the silencing efficacy of the systems. In vitro and in vivo experiments will be designed to determine the efficacy of the nano-carriers developed in the present study.
2.5 Scope and objective of the present work

In the light of the current scenario, it is evident that the field of gene therapy remains wide open for the development of a nanocarrier with less immune response and superior targeting efficiency. The present work focuses on the development of novel liposomal carriers for the delivery of EpCAM si-RNA against EpCAM expressing cancer cells. The efficacy of the delivery systems will be evaluated both in vitro and in vivo. The nanocarrier is expected to improve the stability of the si-RNA and enable selective transfection of the oligonucleotide to EpCAM-expressing cells in an effort to reduce the expression of EpCAM in epithelial cancers thereby enabling tumor regression.

The present work attempts to address the following questions:

- Can nanocarrier-mediated targeted delivery of EpCAM si-RNA contribute to better tumor regression?
- Can non-cationic carriers be effective in gene silencing?
- Can lipoplexes formed between a cationic polymer and si-RNA be encapsulated in a nano-carrier and can such hybrid systems exhibit superior gene silencing effect over conventional nano-carriers?

2.6 Objective and Scope of work

The overall objective of the work is “To develop a novel stealth nanoimmunoliposome to deliver si-RNA against EpCAM expressing cancer cells”

To achieve this objective, the work is divided into three specific aims.
Specific Aim 1: To synthesize and characterize PEGylated liposomes encapsulating si-RNA linked with anti-EpCAM

Hypothesis: We believe the encapsulation of si-RNA in to liposomes and linking with EpCAM antibody will impart stability in circulation due to the presence of poly(ethylene glycol) chains and reduce off-target effects due to target-specific internalization.

Specific Aim 2: Evaluate the efficacy of si-RNA uptake and stability using in vitro techniques

Hypothesis: The antibody-mediated cell internalization coupled with the presence of the helper lipid in the liposomes is expected to improve the silencing efficiency of the nano-carriers in vitro.

Specific Aim 3: Evaluate the in vivo efficacy of the si-RNA delivery system using animal models.

Hypothesis: We believe that target-specific delivery of EpCAM si-RNA will reduce the tumor size and control the metastasis.
The overall plan for the present work is depicted pictorially in Figure 2.3.

Figure 2.3: Schematic representation of work plan
2.8 References


