

Nanocarriers as non-viral vectors: Potential in cancer gene therapy

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Introduction

Cancer is one of the giant killers across the world and the number of cancer related deaths is rising every year. Although perpetual efforts are going on to improvise current chemotherapeutic and radiotherapeutic regimens for cancer, the survival rates vary widely both between tumor types and between individual patients. Furthermore, there are no effective treatments for advanced stage cancers and metastatic cancers. In the terminal stages of cancer, patients usually suffer from extensive tumor nodules characterized with multitude of genetic aberrations. This results in development of resistance to standard chemotherapeutic agents. Additionally, it is well known that chemotherapeutic agents have several side effects due to lack of specificity which severely compromises the quality of patient's life. Hence, researchers have always been in the search of therapeutic options with greater target specificity.

Interestingly, with increasing understanding of molecular pathogenesis of cancer and also genetic aberrations in the cancer progression, the concept of genetic manipulation or cancer gene therapy has emerged as an alternative strategy for cancer treatment. Gene therapy, which can be defined as transfer of genetic material into cells in order to elicit a therapeutic effect against disease, is currently regarded as a potential and promising therapeutic strategy for cancer with special emphasis on treatment of metastatic cancers and refractory solid tumors. The priority of cancer gene therapy is very high, as nearly two-thirds of all current clinical gene therapy trials are directed against cancer.

Deep insight into the genetic changes occurring in cancer cells has provided opportunities to identify, repair or destroy those cells as desired. Hence, the therapeutic approaches employing cancer gene therapy have diverse mechanisms or effects e.g. 1) rectifying errors in oncogenes (mutations and rearrangements), tumor suppressor genes (correcting inactivating mutations) or DNA

pathway repair genes (inactivating mutations), 2) inhibition of neoangiogenesis, 3) activation of cytokine or immunostimulatory responses and 4) induction of apoptosis. The modalities which have been explored for cancer gene therapy include plasmid DNA (pDNA) or synthetic nucleic acids such as antisense oligonucleotides, small interfering RNA (siRNA), or other double stranded RNAs like poly inosine-cytosine (pIC) [1]. The various types of nucleic acids elicit different effects at the molecular genetic level. Generally, pDNA vectors are used for intranuclear delivery to replace or to substitute a specific genetic function in the target cell resulting in a 'gain of gene function' whereas synthetic antisense oligonucleotides or siRNA are employed for 'loss of gene function' resulting in reduced expression of endogenous genes in a sequence specific manner.

Although emergence of cancer gene therapy has unraveled the opportunities for target specific therapy with lesser side effects, the efficient delivery of therapeutic genes to a target site is a major challenge. An ideal gene delivery system should offer [1,2]

- Protection of transgene (therapeutic gene) molecules against enzymatic and nonenzymatic degradation.
- Enhanced cellular uptake, i.e. transfer through biological membranes
- Targeted delivery of transgene to the tumor site
- Escape from endosomes / lysosomes within the cell
- Correct intracellular localization, i.e. in the cytoplasm
- Release of transgene from its formulation at the site of action
- Favourable pharmacokinetic properties of the formulation e.g. with regard to serum half- life and biodistribution.
- Minimal or zero toxicity to other organs and blood components

Considering the challenges associated with delivery of transgenes, researchers have been working on numerous approaches. Initially, approaches such as electroporation and microinjection were proposed to facilitate cellular delivery of genes [3]. However, these approaches are possible only in vitro. The approaches such as hydrodynamic injection were also proposed for systemic delivery of therapeutic genes. However, this approach can not be employed in humans. Furthermore, this approach cannot improve stability of transgene in the plasma [1-3]. Because viruses are naturally adept at infecting target cells and transferring genetic material, they were a logical choice for the delivery of transgenes in the initial phases. However, despite efficient transgene transfection potential of viruses, their utility in clinic is severely limited due to several reasons such as immunogenicity of viral proteins, risk of oncogenesis, insertional mutagenesis, dose limiting hepatotoxicity and inadvertent creation of infectious viral particles [1-3]. Hence, there has been paradigm shift towards exploration of non-viral approaches for cancer gene therapy.

Nanocarriers have emerged as powerful and efficient non-viral vectors for cancer gene therapy. Various types of nanocarriers have been evaluated for the effective cancer gene therapy. The nanocarriers fulfill almost all the aforementioned requirements of the ideal gene delivery system and they are devoid of disadvantages associated with the viral vectors such as immunogenicity and potential carcinogenicity. The several nanoarchitectures that have been explored for cancer gene therapy are discussed below

Cationic Liposomes

The non-viral vector mediated delivery took a great leap when Felgner et al first reported that a double chain monovalent quaternary ammonium lipid, N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride, effectively binds and delivers DNA to cultured cells [3].

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Since then there has been extensive research to develop various cationic lipids with different structures for the improved delivery of various therapeutic genes. Although some cationic lipids alone exhibit good transfection activity, they are often formulated with a noncharged phospholipid or cholesterol as a helper lipid to form cationic liposomes. It was observed that cationic liposomes spontaneously interact with DNA solution upon mixing to yield small quasi-stable particles called lipoplexes [3-6]. The process involves an initial rapid association of polycationic liposomes and polyanionic DNA through electrostatic interaction, followed by a slower lipid rearrangement process. This process is called as DNA condensation and it was observed that DNA in lipoplexes is well protected from nuclease degradation. Lipoplexes are able to trigger cellular uptake and the helper lipids such as DOPE facilitate the release of DNA (or transgene) from the intracellular vesicles before reaching destructive lysosomal compartments [3-6].

After the initial success with lipoplexes, researchers focused on development of various cationic lipids. Among various cationic lipids that have been developed, DOTAP (Dioleoyl 3-Trimethylammonium-Propane) has emerged as the most preferred cationic lipid because of its ease of use and robust transfection efficiency. Nabel and coworkers first reported the intratumoral injection of plasmid DNA complexed to cationic lipids to elicit antitumor responses in mice; a similar approach was evaluated and found safe in a pilot study of humans with melanoma. Intratumoral administration of lipoplexes containing genes encoding cytokines (e.g., IL-2, TNF-) or suicide genes (e.g., Pseudomonas exotoxin A) resulted in therapeutic benefit. Seki et al. showed that intratumoral transfection of therapeutic genes could be enhanced by the inclusion of transferrin in the lipoplex [4]. Ito and coworkers have established that DOTAP-Cholesterol liposomes can successfully deliver FSU1 gene, a tumor suppressor gene for lung cancers. It was observed that intravenous injections of DOTAP:Chol-FUS1 lipoplex into mice bearing experimental A549 lung metastasis demonstrated significant ($P < 0.001$) decrease in the number of metastatic tumor nodules as compared to control [7]. Furthermore, lung tumor-bearing animals when treated with DOTAP:Chol-FUS1 lipoplex demonstrated prolonged survival (median survival time: 80 days, $P < 0.01$) compared to control animals. Ramesh et al. showed that the systemic application of cationic liposomes loaded with either p53 or FHIT genes resulted in transgene expression in 25% of cells in primary tumors and 10% in disseminated tumors [4].

Although there are few examples on systemic administration of lipoplexes for gene delivery, by and large, it has been observed that lipoplexes can form aggregates in the presence of serum proteins, limiting potential transfection sites to "first-pass" organs such as the liver, spleen, and lungs. In another approach to stabilize cationic liposomes for systemic application, PEG has been incorporated, resulting in longer circulation times and reduced accumulation in first-pass organs. PEG-stabilized cationic liposomes resulted in higher reporter gene expression at the tumor site (Lewis lung tumor in the mouse flank) [4]. The utility of ligand targeted liposomes in cancer gene therapy has also been established. Prof. Chang and coworkers at Georgetown University, NY, USA have explored potential of targeted cancer gene therapy in several types of cancer. Prof. Chang and coworkers employed cationic immunolipoplex containing tumor suppressor p53 gene and a ligand that targets transferrin receptor. The potential of this immunolipoplex has been evaluated in various types of cancer such as head and neck squamous cell carcinoma xenografts and breast cancer. The cationic immunolipoplex demonstrated great therapeutic potential in treatment of various cancers on systemic administration. This cationic immunolipoplex has entered Phase 1 clinical trial in the year 2005. The potential of cationic liposomes containing various other targeting ligands such as antibodies for human estrogenic receptor (anti-HER2), human epidermal growth factor receptor (anti-EGFR) and RGD peptide has also been evaluated for the cancer gene therapy.

Researchers have also developed lipoplexes containing DNA sequences encoding HLA-B7 and 2 microglobulin, which together form a major histocompatibility complex, or MHC, class I. This lipoplex formulation is commercially available as Allovectin-7® and is indicated in the treatment of metastatic melanoma. It is believed that injection of Allovectin-7® directly into tumor lesions directs an immune response against metastatic tumors through several mechanisms. The product is currently in Phase 3 trial. The utility of cationic liposomes containing γ -interferon gene has been evaluated to treat patients suffering from glioblastoma in Japan [5]. In short, cationic liposomes have demonstrated a very good potential in cancer gene therapy.

Polymeric nanocarriers (Polyplexes)

Polymers used as non-viral vectors to enhance gene expression can be divided into two categories based on

biodegradability. Various cationized non-biodegradable polymers have been evaluated with regard to their success of delivering DNA into cells, resulting in improved gene expression. These include linear cationized polymers of poly(ethyleneimine) and poly-L-lysine. Others are poly(N-ethyl-4-vinylpyridinium bromide), poly(dimethylaminoethyl methacrylate), chitosan, and dimethylaminodextran, or cationic polymers branched poly(ethyleneimine) [3-6]. Generally, because DNA is a large and negatively charged molecule, it has difficulty attaching to the negatively charged cell membrane for internalization. It is well recognized that cationized polymers readily form complexes with negatively charged DNA through electrostatic interactions. This condenses the DNA and creates a positive net electric charge under appropriate conditions. This facilitates cell attachment and subsequent internalization by means of endocytosis. The major advantage of using polyplexes is that they can be easily conjugated to targeting agents; these include proteins (e.g., transferrin, epidermal growth factor, antibodies) and small molecules (e.g., folate, galactose). Of the cationic nonbiodegradable polymers tested, PEI has the highest in vitro transfection efficiency, which is believed to be because of its intrinsic ability to facilitate endosomal release. One proposed mechanism for the endosomolytic activity of PEI is the so-called proton sponge hypothesis that an osmotic imbalance is caused on endosomal acidification, resulting in the breakup of the endosome. However, PEI has also been shown to be toxic to the cells [3-6].

Various in vivo studies have shown that a near-neutral surface of the polyplexes is essential to minimize nonspecific interactions in the blood, allowing greater circulation time for the vector to reach its target. To reduce the surface charge, hydrophilic agents have been attached to the polyplex surface to provide so-called steric stabilization. Shielding agents investigated include polyethylene glycol (PEG), hydroxypropyl methacrylic acid, and the serum protein transferrin. Shielding by PEG not only improves circulation times, but also reduces toxicity, increases solubility, and provides stability for freezing-thawing. Of the shielding agents, most work has been done with PEG, with several different strategies of PEGylation developed. PEG can be covalently attached after polyplex formation (postPEGylation) [4].

Wagner and coworkers first demonstrated tumor-targeted gene expression in distant tumors when PEG-shielded polyplexes of PEI (800 kDa) targeted with transferrin were injected into the tail vein of mice bearing subcutaneous Neuro-2A neuroblastoma tumors [4].

Luciferase reporter gene expression was enhanced from 100- to 1000-fold in the tumors compared to the other major organs. This specific gene expression could also be achieved with transferrin-shielded polyplexes with lower molecular weight (22 and 25 kDa) PEIs as gene carriers. This work demonstrated that transferrin could be used as an alternative shielding agent, and that the lower molecular weight PEIs, which have lower toxicity, were effective as gene carriers. With these encouraging results, the transferrin-targeted/shielded PEI polyplexes were used to apply the therapeutic gene TNF- systemically to mice. Gene expression of TNF- was localized within the tumors in three different tumor models, resulting in pronounced hemorrhagic tumor necrosis and inhibition of tumor growth, with complete tumor regressions observed in the MethA model. No systemic TNF-related toxicity was observed [4].

Biodegradable polymers have been used to achieve controlled-release of DNA, thus enhancing and prolonging gene expression. Controlled-release technology increases and prolongs the concentration of DNA around an injection site. Several reports describe the controlled-release of DNA from the matrixes of various biodegradable polymers, including

poly(D,L-lactic acid-coglycolic acid), poly(lactic acid)-poly (ethylene glycol), poly(2-aminoethyl propylene phosphate), silk-elastin like polymer, atelocollagen, and gelatin [5]. It has been observed that biodegradable polymers like PLGA have ability to undergo rapid endosomal escape upon cellular uptake and release the encapsulated cargo such as plasmid DNA or gene in cytoplasm. Labhasetwar and coworkers evaluated the antiproliferative activity of wild-type (wt) p53 gene-loaded nanoparticles in a breast cancer cell line. Cells transfected with wt-p53 DNA-loaded nanoparticles demonstrated a sustained and significantly greater antiproliferative effect than those with naked wt-p53 DNA or wt-p53 DNA complexed with a commercially available transfecting agent (Lipofectamine). Cells transfected with wt-p53 DNA-loaded nanoparticles demonstrated sustained p53 mRNA levels compared to cells which were transfected with naked wt-p53 DNA or the wt-p53 DNA-Lipofectamine complex, thus explaining the sustained antiproliferative activity of nanoparticles. This clearly demonstrates the potential of PLGA nanoparticles in cancer gene therapy [8].

Researchers have also evaluated potential of various cationic derivatives natural polymers such as pullulan and dextran in cancer gene therapy [5]. Lim et al. described a new biodegradable

polymer of amino esters that has transfection efficiencies in mammalian cells similar to 25-kDa PEI but with minimal cytotoxicity [4]. The polymer consists of a branched network of amino esters with synthesis based on that of the branched 25-kDa PEI. The high activity was attributed to its ability to mediate endosome acidification efficiently. This new polymer fulfills the two main requirements for a gene carrier-efficient transfection and low toxicity-and should be a useful tool in cancer gene therapy in the future.

Dendrimers [9]

Dendrimers are branched, nanometer-sized, spherical polymeric structures that, depending on the monomers, surface and internal modifications, can be engineered to carry out various functions. Dendrimers are being explored for various applications including gene delivery. Although various polymers can be employed to yield dendritic structures, polyamidoamine (PAMAM) and polypropylenimine (PPI) dendrimers have been predominantly used for the gene therapy. Biophysical characterization tools have shown that dendrimers, when complexed with DNA, are capable of forming spontaneously in solution a supramolecular assembly that possesses all the features required to diffuse in experimental tumors through the enhanced permeability and retention effect.

PAMAM dendrimers alone or conjugated to various targeting ligands such as antibodies, luteinizing hormone releasing hormone (LHRH), biotin and transferrin have been evaluated for cancer gene therapy. PPI dendrimers have gained a lot of interest in recent years as it has been observed that the intravenous injection of PPI dendrimers and gene complexes can lead to liver targeted gene expression. Liver, where large numbers of proteins are produced, is an attractive candidate for gene therapy. Dufes et al. applied different generations of PPI dendrimers as transfection agents and found that target gene efficiently expressed in the liver rather than other organs after intravenous administration via the tail vein [9]. In another study, Dufes and coworkers further evaluated efficacy of modified PPI dendrimers containing

tumor necrosis factor alpha (TNFalpha) gene in the treatment of epidermoid carcinoma, cervix carcinoma, and colorectal adenocarcinoma on systemic administration. Specifically, the systemic injection of dendrimer nanoparticles containing a TNFalpha expression plasmid regulated by telomerase gene promoters (hTR and hTERT) led to transgene expression, regression of remote xenograft murine tumors, and long-term survival of up to

100% of the animals [9]. Interestingly, these dendrimers also exhibited plasmid-independent antitumor activity, ranging from pronounced growth retardation to complete tumor regression. This indicated that dendrimers may have intrinsic anticancer activity. Although dendrimers have emerged as an attractive approach for gene delivery, extensive investigation from toxicological aspects is necessary.

Lipid-Polymer Hybrid Nanocarriers [3-6,10]

The reported lipid-polymer hybrid systems include DNA precondensed with polycations, then coated with cationic liposomes, anionic liposomes, or amphiphilic polymers with or without helper lipids. These hybrid nanocarriers are usually referred to as LPD. Linear poly-L-lysine, protamine, histone, and several synthetic polypeptides have been used as the DNA condensation component; the polyplexes formed are then coated with a lipid layer. DNA is better protected in these lipid-wrapping polyplexes. The 3-part system appears to be more efficient in transfection than lipid-DNA complexes in vitro and is equally active in vivo. LPD have been shown to be efficient than lipoplexes and polyplexes. The improved transfection efficiency has been attributed to the smaller particle size, greater uptake due to presence of lipids and provision of better protection against nucleases.

The LPD containing DOTAP and cholesterol as lipids and protamine as polymer have been explored in various types of cancers by Prof. Huang and his research group. It has been observed that i.v. administration of DOTAP: Chol LPD carrying tumor suppressor genes Rb or E1A in cancer animal models resulted in apoptosis induction, tumor size reduction and life span increase in the treated animals. As expected, antitumoral synergistic effects were obtained when E1A LPD treatment was combined with standard chemotherapeutic agents such as paclitaxel. LPD modified with various targeting ligands such as PEG-folate, PEG-PE-anisamide and asialofetuin have been explored for targeted cancer gene therapy. It has been observed that LPD coated with the liver targeting ligand asialofetuin which significantly increased uptake of the encapsulated DNA in HepG2, liver cancer cells. LPD composed of DC-Chol/DOPE was also tested in clinical settings when two children with Canavan disease (a fatal CNS disease characterized by spongy degradation of cerebral white matter) were treated with ASPA gene via intracerebral application; both subjects showed some clinical improvements.

Miscellaneous examples of nanocarriers

Potential of inorganic nanomaterials in cancer gene therapy has also been explored. Liu et al., fabricated calcium phosphate nanoparticles for the gene delivery [11]. Preliminary studies indicated that calcium phosphate nanoparticles could condense DNA and the complex did not exhibit inherent cytotoxicity unlike standard transfection reagents such as Lipofectamine or PEI. The potential of calcium phosphate nanoparticles in the delivery of a suicide gene (yCDglyTK) for the treatment of nasopharyngeal carcinoma was evaluated. It was observed that calcium phosphate nanoparticles could successfully deliver the gene in vitro in CNE-2 cells. Furthermore, intra-tumoral injection of nanoparticle-gene complex showed significant anti-cancer activity [11]. Recently, researchers have developed a series of well defined polycationic amphiphilic cyclodextrins (paCDs) capable of complexing and compacting DNA into homogeneous nanoparticles of size 70 nm. These cationic cyclodextrins resemble both cationic polymers and cationic lipids and would have advantages of lipopolyplexes. One representative cationic cyclodextrin derivative is shown in figure 1. The cationic cyclodextrin derivative shown in the figure 1 was found to give superior transfection as compared to standard polycation PEI in BNL-CL2 and COS-7 cell lines whereas the cytotoxicity was lesser as compared to PEI.

These cationic cyclodextrins have great potential in cancer gene therapy [12]

Conclusion

Nanocarriers have demonstrated a great potential as non viral vectors for the delivery of various genes targeted at various cancers and have already reached human clinical trials. The commercialization of nanocarrier based vector for cancer gene therapy is not far from the sight.

References

1. Russ V, Wagner E. Cell and tissue targeting of nucleic acids for cancer gene therapy. *Pharm Res.* 2007, 24:1047-57.
2. Pathak A, Patnaik S, Gupta KC. Recent trends in non-viral vector-mediated gene delivery. *Biotechnol J.* 2009, 4:1559-72.
3. Gao X, Kim KS, Liu D. Nonviral Gene Delivery: What We Know and What Is Next? *The AAPS Journal* 2007, 9:Article 9.
4. Walker GF, Wagner E. Nonviral Vector Systems for Cancer Gene Therapy. In: Curiel DT, Douglas JT. (Ed). *Cancer Gene Therapy.* Humana Press Inc., Totowa, NJ, 2005, pp.367-378
5. Kaneda Y, Tabata Y. Non-viral vectors for cancer therapy. *Cancer Sci.* 2006, 97:348-54.
6. El-Aneed A. An overview of current delivery systems in cancer gene therapy.

J Control Release. 2004, 94:1-14.

7. Ito I, Ji L, Tanaka F, Saito Y, Gopalan B, Branch CD, Xu K, Atkinson EN, Bekele BN, Stephens LC, Minna JD, Roth JA, Ramesh R. Liposomal vector mediated delivery of the 3pFUS1 gene - demonstrates potent antitumor activity against human lung cancer in vivo. *Cancer Gene Ther.* 2004, 11:733-9.
8. Prabha S, Labhasetwar V. Nanoparticle-mediated wild-type p53 gene delivery results in sustained antiproliferative activity in breast cancer cells. *Mol Pharm.* 2004, 1:211-219.
9. Dufès C, Uchegbu IF, Schätzlein AG. Dendrimers in gene delivery. *Adv Drug Deliv Rev.* 2005, 57:2177-2202.
10. Tan Y, Whitmore M, Li S, Frederik P, Huang L. LPD nanoparticles-novel nonviral vector for efficient gene delivery. *Methods Mol Med.* 2002, 69:73-81.
11. Liu T, Tang A, Zhang G, Chen Y, Zhang J, Peng S, Cai Z. Calcium phosphate nanoparticles as a novel nonviral vector for efficient transfection of DNA in cancer gene therapy. *Cancer Biother Radiopharm.* 2005, 20:141-9.
12. Díaz-Moscoso A, Vercauteren D, Rejman J, Benito JM, Ortiz Mellet C, De Smedt SC, Fernández JM. Insights in cellular uptake mechanisms of pDNA-polycationic amphiphilic cyclodextrin nanoparticles (CDplexes). *J Control Release.* 2010, In press.

Regulatory Update

WHO drafts guidelines on starting material quality

The WHO has published its views on the production of starting materials in draft guidance which details recommendations to ensure product quality while keeping costs down.

Focusing primarily on specified starting materials, those used as, or in the production of, active pharmaceutical ingredients (API), the document aims to offer a global approach to defining quality.

The World Health Organization (WHO) believes it is important quality standards are achieved without overburdening companies with regulations. This would drive up prices and could lead to people using cheaper counterfeit or substandard drugs, according to the guidance.

Consequently, the document states it is unhelpful to uniformly apply good manufacturing practices (GMP) to specified starting materials. Instead, the WHO recommends manufacturers decide what level of quality is required based on the guidance or risk assessments if appropriate.

The guidance document details quality levels for some materials and states that for others a risk assessment, considering factors such as the number and nature of synthesis steps, may be suitable.

Quality control

When establishing quality control levels certain factors, such as impurity profile and isomers, are critical.

Consequently, standards should be designed to detect isomeric or other impurities which are potentially reactive and could impact on the final product.

The WHO recommends that this threat is discussed and controlled with suitably validated methods if appropriate. Furthermore, acceptance criteria should be established based on the fate of impurities in the starting material following normal synthesis or process.

Comments on the document can be submitted until May 1 and those received by April 23 could be discussed at the consultation on paediatrics and generic guideline development.