2.1 Cancer

Cancer cells share many common features with the normal host cells from which they originate, thus diminishing the chances of selective delivery of anticancer drugs to these cells. The majority of anticancer drugs acting on the dividing and proliferating cells also target normal body dividing cells as reproductive organs and germ cells, hematopoetic cells and hair follicles, leading to dose-limiting side effects like mucositis, stomatitis, alopecia and reproductive effects (Rang H.P. et al-2003). Hence, to lower overall normal cell toxicity and to increase therapeutic effects against cancer cells, targeted delivery systems for anti-tumor chemotherapeutic agents and biomolecules with selective site specific action against cancer cells are being developed.

The global market for therapeutic agents for the central nervous system (CNS) is greatly underpenetrated and would have to grow extensively in Pharmerging market as shown in Table 2.1 (Global Oncology Trend Report by IMS-2014). Pharmerging markets are: India, China, Brazil, Russia, Mexico, Turkey, Venezuela, Poland, Argentina, Saudi Arabia, Indonesia, Colombia, Thailand, Ukraine, South Africa, Egypt, Romania, Algeria, Vietnam, Pakistan, and Nigeria. Estimates are that, by 2014, more than 15% of all products on the global market will have some kind of nanotechnology incorporated into their manufacturing process (Dawson N G-2008). But, the markets for gene therapy are difficult to estimate as there is only one approved gene therapy product and it is marketed in China since 2004. Gendicine, also known as Recombinant Ad-p53 Anti-cancer Injection, was developed by Shenzhen SiBiono Gene Technologies Co. (SiBiono) Ltd. And it is the first gene therapy to be approved in China for treating squamous cell carcinoma in January 2004. The global gene therapy market is expected to grow to over $300 million by 2015, according to a new report available on companiesandmarkets.com. The gene therapy market will grow further driven by the human genome project, the increasing incidence of cancer and other critical diseases.

Table 2.1: Spending by Therapeutic Area in 2017, Pharmerging Markets
(*Top 20 Classes = 45% of total; others represent 55%)*

Therapeutic Area	Sales in 2017 (Constant exchange rates, US dollars)
Pain	$22–25 Billion
Central Nervous System	$20–23 Billion
Antibiotics	$18–21 Billion
Oncology	$17–20 Billion
Hypertension	$14–17 Billion
Diabetes	$10–12 Billion
Dermatology	$10–12 Billion
Antiulcerants	$9–11 Billion
Cholesterol	$6–8 Billion
Asthma/Chronic Obstructive Pulmonary Disease	$3–5 Billion
Anti-Epileptics	$3–5 Billion
Antivirals, excluding HIV	$3–5 Billion
Immunosuppressants	$3–5 Billion
Allergy	$3–5 Billion
Antidepressants	$3–5 Billion
Antiplatelets	$3–5 Billion
Antipsychotics	$2–3 Billion
Heparins	$1–2 Billion
Erectile dysfunction	$1–2 Billion
Immunostimulants	$1–2 Billion

Source: IMS Health Thought Leadership, September 2013, as reported in *Innovation in Cancer Care and Implications for Health Systems: Global Oncology Trend Report*, IMS Institute of Healthcare Informatics, May 2014.

Brain tumor and glioma are one of the most deadly disorders with more than 80 % mortality rate. The crude incidence of primary brain tumor in India is 3.4 per 100,000 populations for males and 1.2 per 100,000 populations for females. It represents < 1% of new cancer cases detected every year in the country. However, there has been a steady increase in the incidence of primary brain tumors over the last decade or so primarily due to higher detection rates due to more widespread availability of diagnostic imaging.

There are an estimated 141,553 people currently living with brain and other nervous system cancer in the United States. The number of new cases of brain and other nervous system cancer was 6.5 per 100,000 men and women per year. The number of deaths was 4.3 per 100,000 men and women per year. In other words, an estimated 17,200 individuals in the United States are diagnosed with malignant CNS tumors per year out of which gliomas are

the most debilitating forms and need immediate attention. Figure 2.1 shows the data related to brain and other nervous system cancer in the United States (Cancer stats website, *as accessed on Dec-2013*).

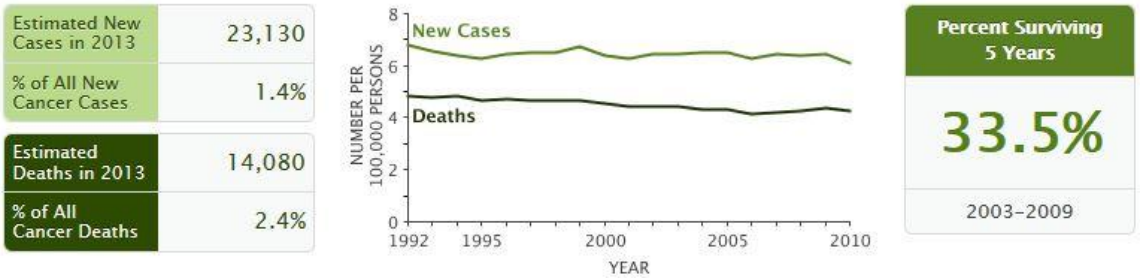


Figure 2.1: Report related to statistics for brain and other nervous system cancer in the United States provided by National Cancer Institute (*source: Surveillance, Epidemiology, and End Results Program*)

2.1.1 Brain Tumors

A brain tumor is any localized intracranial lesion that occupies space within the skull and causes a rise in intracranial pressure. Brain tumors can be classified in two broad categories i.e. benign and malignant. Benign brain tumors do not contain cancer cells and they seldom grow back and, usually, can be removed. The border or edge of a benign brain tumor can be clearly seen. Cells from benign tumors do not invade tissues around them or spread to other parts of the body. But malignant brain tumors are generally more serious and often is life threatening. Malignant tumors can be further classified as primary (the tumor originate from the brain tissue) or secondary (metastasis from others tumor elsewhere in the body). They are likely to grow rapidly and invade the surrounding healthy brain tissue. The Central Brain Tumor Registry of the United States (CBTRUS) collects the data related to brain tumors and one such data is shown below in figure 2.2.

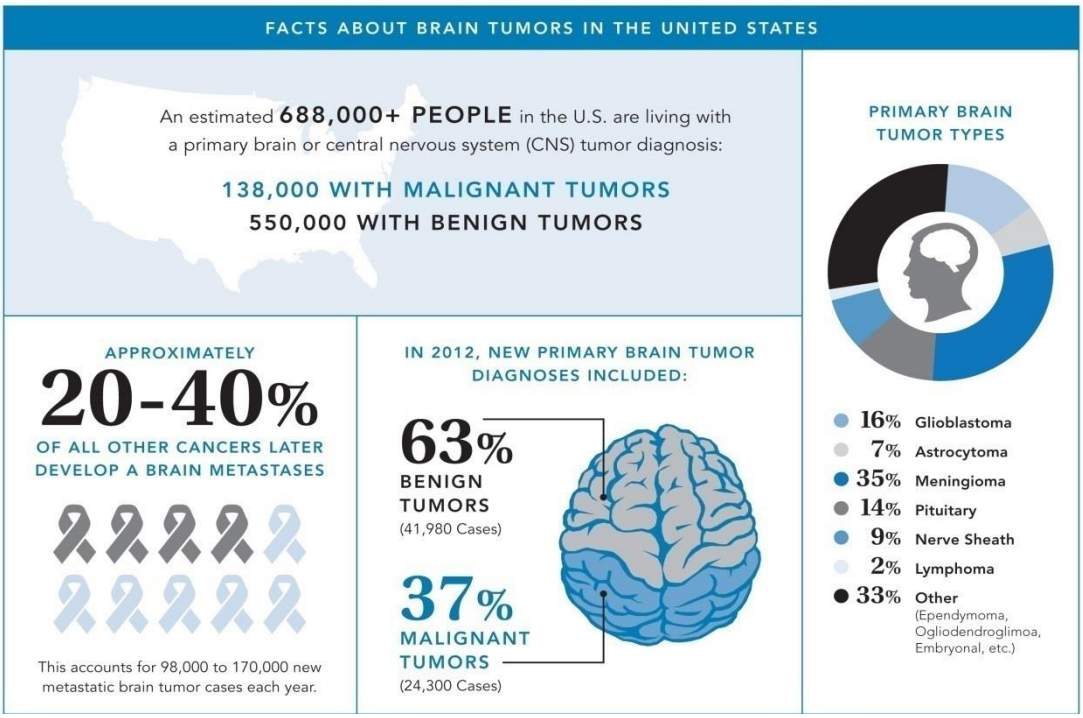


Figure 2.2: Facts about brain tumors (*Source: CBTRUS Statistical Report, 2012*)

Benign brain tumors: These types of brain tumors do not contain cancer cells and usually can be removed, and they seldom grow back.

a) The border or edge of a benign brain tumor can be clearly seen. Cells from benign tumors do not invade tissues around them or spread to other parts of the body. However, benign tumors can press on sensitive areas of the brain and cause serious health problems.

b) Unlike benign tumors in most other parts of the body, benign brain tumors are sometimes life threatening.

c) Very rarely, a benign brain tumor may become malignant.

Malignant brain tumors: These types of brain tumors contain cancer cells.

a) Malignant brain tumor is generally more serious and often is life threatening.

b) They are likely to grow rapidly and crowd or invade the surrounding healthy brain tissue.

c) Very rarely, cancer cells may break away from a malignant brain tumor and spread to other parts of the brain, to the spinal cord, or even to other parts of the body. The spread of cancer is called metastasis.

2.1.2 Types of Brain Tumors

There are many types of primary brain tumors. Primary brain tumors are named according to the type of cells or the part of the brain in which they begin. For example, most primary brain tumors begin in glial cells. This type of tumor is called a glioma. Among adults, the most common types are:

• **Astrocytoma**: The tumor arises from star-shaped glial cells called astrocytes. It can be any grade. In adults, an astrocytoma most often arises in the cerebrum.

Grade I or II astrocytoma: It may be called a low-grade glioma.

Grade III astrocytoma: It's sometimes called a high-grade or an anaplastic astrocytoma.

Grade IV astrocytoma: It may be called a glioblastoma or malignant astrocytic glioma.

• **Meningioma**: The tumor arises in the meninges. It can be grade I, II, or III. It's usually benign (grade I) and grows slowly. Oligodendroglioma: The tumor arises from cells that make the fatty substance that covers and protects nerves. It usually occurs in the cerebrum. It's most common in middle-aged adults. It can be grade II or III. Among children, the most common types are:

• **Medulloblastoma**: The tumor usually arises in the cerebellum. It's sometimes called a primitive neuroectodermal tumor. It is grade IV.

• **Ependymoma**: The tumor arises from cells that line the ventricles or the central canal of the spinal cord. It's most commonly found in children and young adults. It can be grade I, II, or III.

• **Brain stem glioma**: The tumor occurs in the lowest part of the brain. It can be a low-grade or high-grade tumor. The most common type is diffuse intrinsic pontine glioma.

Broadly, brain and CNS tumors can be classified according to the site of occurrence as well as according to the tumor histology as depicted in figure 2.3 and 2.4.

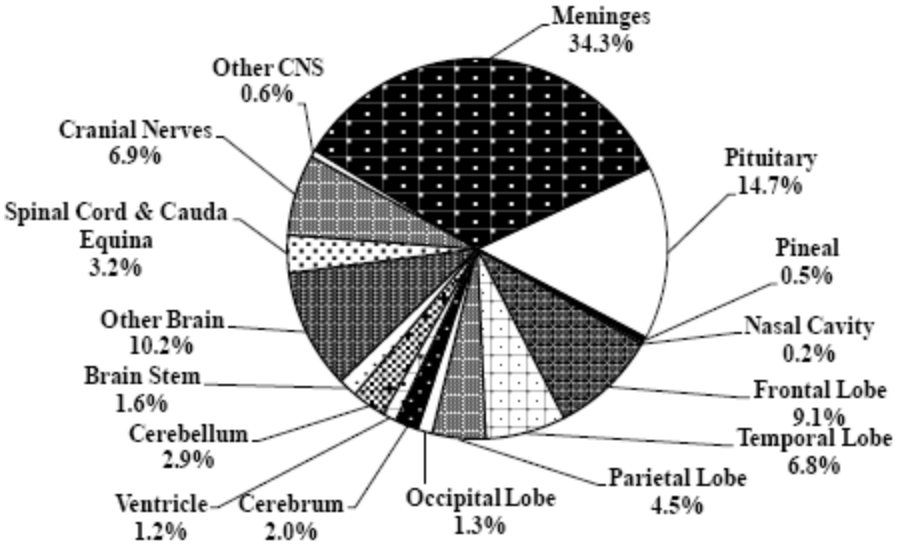


Figure 2.3: Brain and CNS tumors are disseminated according to the site of occurrence.

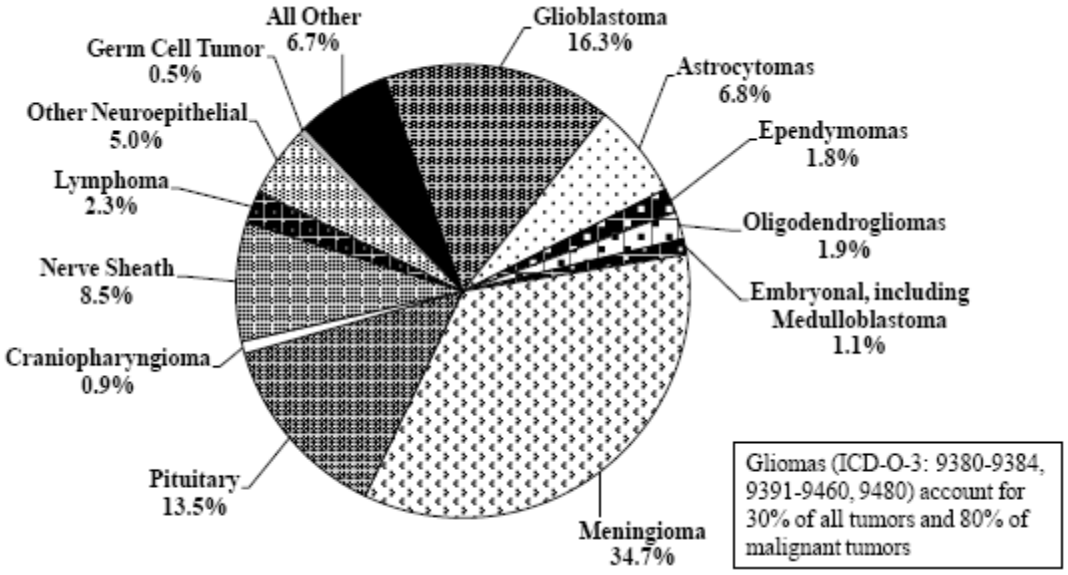


Figure 2.4: Brain and CNS tumors are disseminated according to the tumor histology.

Glioma: Gliomas are any form of tumor that arises from the supportive tissue of the brain. This tissue, called "glia," helps to keep the neurons in place and functioning well. A typical drawing of glial cells is shown below in figure 2.5.

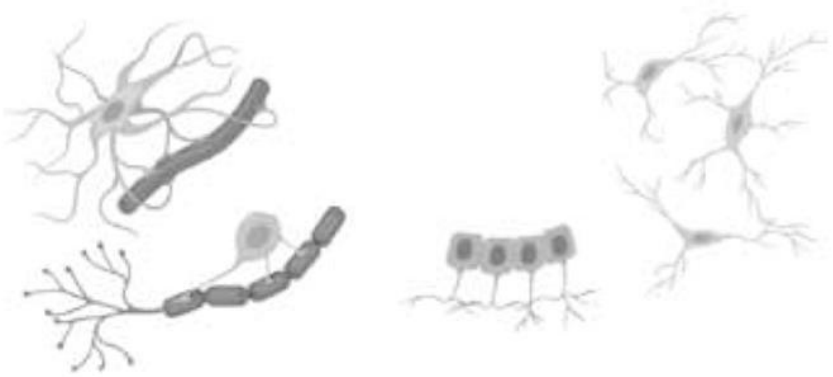


Figure 2.5: Typical drawing of glial cells.

There are three types of normal glial cells that can produce tumors. An astrocyte will produce astrocytomas (including glioblastomas), an oligodendrocyte will produce oligodendrogliomas, and ependymomas come from ependymal cells. Tumors that display a mixture of these different cells are called mixed gliomas. The location of the tumor depends on the type of cells from which it originates. A glioma is a type of tumor that starts in the brain or spine. It is called a glioma because it arises from glial cells. The prognosis for patients with high-grade gliomas is generally poor, and is especially so for older patients. Of 10,000 individuals of United States diagnosed each year with malignant gliomas, about half are alive 1 year after diagnosis, and 25% after two years. Those with anaplastic astrocytoma survive about three years (H. Kim *et al*-2013). The cure and management rate is expected to be much lower in the developing countries like India. A study conducted in the Tata Memorial Hospital provided a one-year demographic data and relevant tumor-related information on all 656 patients registered in the Neuro-oncology Clinic. Gliomas constituted 38.7% (254 cases) of CNS tumors, with high-grade gliomas comprising 151 (59.5%) and low-grade gliomas 79 (33.1%) cases (A. Munshi and R. Jalali-2009).

2.1.3 Conventional Cancer Therapy

Conventional therapies are the establishment of cancer care. Most people with cancer receive surgery, chemotherapy, radiation therapy, or other conventional therapies at some point during treatment, and many will have a combination of these treatments. Conventional therapies are constantly evolving to improve effectiveness while diminishing harmful side effects of the treatment.

Surgery: The first cancer surgery was done in 1909 by Harvey Cushing, at the Johns Hopkins Hospital, when he operated a pediatric brainstem glioma (Dmetrichuk J. M. *et al.*-2011). The surgery achieved remarkable increment of the patients' expectancy of life – increased from 3 months expected survival to 6 to 12 months. Surgery is the primary form of treatment for brain tumors in parts of the brain that can be removed without being detrimental to critical neurological functions. The extent of surgical tumor resection is the most important factor determining length of survival and hence the goal of surgery is to remove the entire tumor if possible. But a tumor is most likely to recur since in most cases not all the tumor cells are removed. Glioblastoma on average consist of 10^{11} cells, a removal of 99 % implies a residual tumor mass of 10^{9} cells. But it is hard to remove all tumor cells, especially given the infiltrative nature of the tumor. Partial removal helps to relieve symptoms by reducing pressure on the brain and it reduces the residual amount of tumor to be treated by other forms of therapy (like chemo and/or radiation therapy). Any remaining tumor may then be treated with radiotherapy and possibly chemotherapy.

Radiation therapy: Radiation therapy (RT) was introduced shortly after 1895, when Röntgen first reported the use of X-rays for diagnostic medical purposes. Radiation remained a principal treatment option of certain solid tumors. Researchers have demonstrated in a randomized trial that radiotherapy (5000 to 6000 rads to the whole brain) only improved median survival to 37.5 weeks, relative to chemotherapy only (25 weeks), and radiotherapy in combination with chemotherapy resulted in median survival of 40.5 weeks (Walker M. D. *et al.*-1978). Today the radiotherapy (RT) (2 Gy per fraction once daily, per 5 days/wk, for 6 weeks) resulting in a total cumulative radiation dose of 60 Gy) has become part of the standard of care treatment for glioblastoma multiforme (Fulton D. S. *et al*.-1992). Radiation therapy uses high-energy x-rays or other types of ionizing radiation to stop cancer cells from dividing. It is often used in addition to surgery or when surgery is not possible.

Radiation therapy variants include: Conventional radiation therapy is designed to deliver radiation to an entire region of the brain. Depending on the location and size of the tumor(s), the treatment can be either focused or whole brain radiation therapy (WBRT).The total dose of radiation over an extended period can be given as a fractionation approach. This helps to protect healthy tissue in the area of the tumor. Moreover, a 3-dimensional image of the tumor and brain region can be made in order to focus the radiation beams precisely to the tumor

such that the surrounding healthy tissue is protected. Finally, instead of X-rays, proton beam radiation therapy uses protons. Protons have ability to pass through healthy tissue without damaging it. Interstitial radiation therapy (brachytherapy) is an internal form of radiation therapy, which involves surgically implanting radioactive material directly inside the tumor. It should be noted that, no significant difference was found in the time to progression or median survival time between three-dimensional conformal radiotherapy and whole brain radiotherapy (Showalter T. N. *et al.*- 2007). A group of investigators have showed that cancer stem cells contribute to glioma radio resistance through preferential activation of the DNA damage checkpoint response and an increase in DNA repair capacity, making radiotherapy a palliative therapy (Bao S. *et al*-2006).

Chemotherapy: Chemotherapy is a drug treatment that uses one or more powerful chemicals (i.e. cytotoxic anti-neoplastic drugs or chemotherapeutic agents) as part of a standardized regimen to kill fast-growing (cancerous) cells in the body. Exposure of soldiers to nitrogen mustard gases during First World War resulted in pancytopenia. This observation triggered interest for chemotherapeutic therapy, especially in patients with hematologic malignancies. Today chemotherapy is a validated and well documented treatment option. Late seventies saw the introduction of various chemotherapeutic agents such ascarmustine (BCNU or 1,3-bis(2-chloroethyl)-l-nitrosourea), procarbazine (PCB) or dacabazine (DTIC) (Eyre H. J. *et al.,*1986). The efficacy of this treatment in conjunction with available adjuvant therapies generally increases the quality and expectancy of life of GBM patients, but only for a few months, at best (Aoki T. *et al.,* 2007).

Therefore, chemotherapy is nonstandard therapy for GBM, and only given in the context of clinical trials. However, Stupp and co-workers demonstrated that in comparison to surgery-radiotherapy without temozolomide (TMZ) the surgical resection, followed by adjuvant radiotherapy plus TMZ, improved survival in patients with GBM significantly. They concluded that the addition of TMZ to radiotherapy for newly diagnosed glioblastoma resulted in a statistically significant and clinically meaningful survival benefit with less additional toxicity (Stupp R. *et al.*-2005). TMZ is an oral alkylating agent having relatively low toxicity profile (Yung W. K. *et al.*-2000).

Chemotherapy is usually given in cycles of a treatment period which is followed by a recovery period and so on. Upon chemotherapy, some cancer cells acquire drug resistance

and survive. The surviving cell will divide and pass its drug resistance to progeny cells, thereby rendering the chemotherapeutic treatment of limited value. Hence, although chemotherapy kills most cells in a tumor, it is believed to leave tumor stem cells behind, which might be an important mechanism of resistance (Dean M *et al.*-2005).

2.1.4 Gene Therapy

Major advances in the field of genetic engineering and gene therapy can be attributed to discoveries in the 1960's of enzymes which could be used to cut and paste DNA sequences together in test-tubes. Early experiments showed that delivering engineered human genes into cells provided the instructions cells needed to make a functional protein. But it was not until 1970 that Stanfield Rogers proposed the use of "normal copy" of DNA to replace defective DNA as a treatment for inherited disease. Gene therapy works on the basic concept that the delivery of polynucleotides to the cells will alter the expression of given protein resulting in therapeutic benefit. Gene therapy involves delivering polynucleotides such as DNA, RNA, anti-sense ologonucleotides and small interfering RNA either locally or systemically. Gene therapy is currently available only in a research setting.

The U.S. Food and Drug Administration (FDA) have not yet approved any gene therapy products for sale in states (FDA website, accessed in Feb 2014). Although, commercial availability is not there in the US market, there are several products in the late stages of clinical trials (A Basarkar- 2007). Successful gene therapy is thus dependent on the development of an efficient delivery vector. The delivery of nucleic acid molecules into cells to alter physiological functions at the genetic level is a powerful approach to treat a wide range of disorders acquired (such as cancer) or inherited through a genetic disorder. However, a medicine based on a nucleic acid must be delivered to the interior of the target cell while surviving an array of biological defenses honed by evolution. The overview of the human gene therapy is shown in figure 2.6.

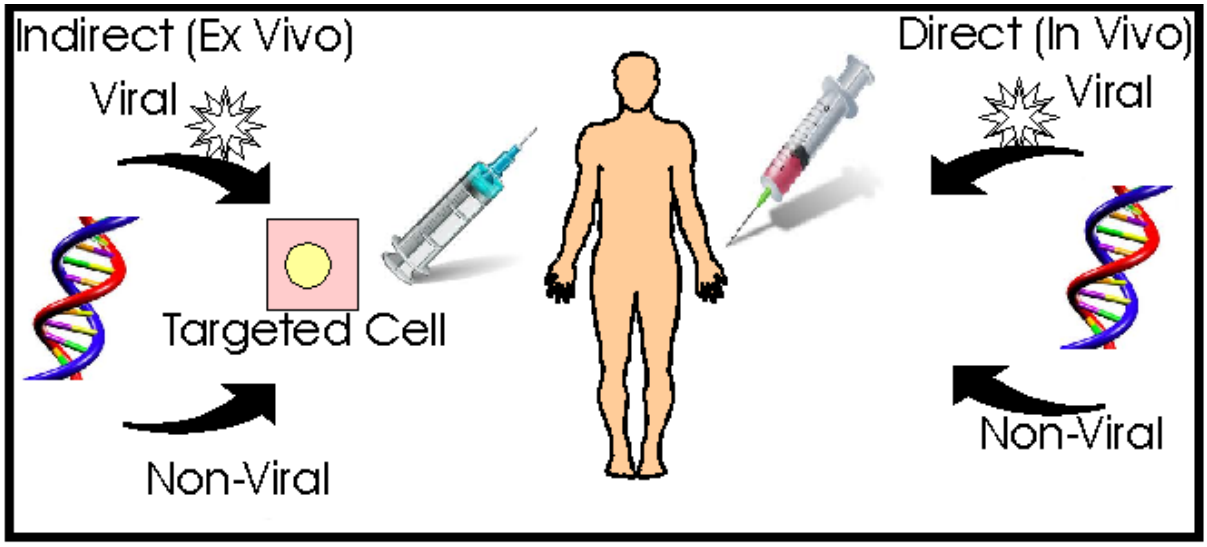


Figure 2.6: Overview of the human gene therapy

2.1.5 Tumor Suppressor Genes

The first insight into the activity of tumor suppressor genes came from somatic cell hybridization experiments, initiated by Henry Harris and his colleagues in 1969. Commonly inherited cancers and associated tumor suppressor genes with their functions are shown in table 2.2. Another tumor suppressor gene to have been identified is *p53*, which is frequently inactivated in a wide variety of human cancers, including leukemias, lymphomas, sarcomas, brain tumors, and carcinomas of many tissues, including breast, colon, and lung. In total, mutations of *p53* may play a role in up to 50% of all cancers, making it the most common target of genetic alterations in human malignancies (Cooper G. M. -2000).

The p53 gene has been mapped to chromosome 17. In the cell, p53 protein binds DNA, which in turn stimulates another gene to produce a protein called p21 that interacts with a cell division-stimulating protein (cdk2). When p21 is complexed with cdk2 the cell cannot pass through to the next stage of cell division. Mutant p53 can no longer bind DNA in an effective way, and as a consequence the p21 protein is not made available to act as the 'stop signal' for cell division. Thus cells divide uncontrollably, and form tumors. Gendicine, an adenovirus encoding tumour suppressor p53 gene, developed by SiBiono GeneTech Co., Ltd., was approved by China's state FDA for treatment of head and neck squamous cell carcinoma (A Basarkar-2007).

Table 2.2: Commonly inherited cancers and associated tumor suppressor genes (*adapted from book: Cooper G. M. -2000*).

Mutated Tumor Suppressor Gene(s)	**Gene Function(s)**	**Associated Noninherited Cancers**
RB1	Cell division, DNA replication, cell death	Many different cancers
TP53	Cell division, DNA repair, cell death	Many different cancers
CDKN2A *(*INK4A*)*	Cell division, cell death	Many different cancers
APC	Cell division, DNA damage, cell migration, cell adhesion, cell death	Most colorectal cancers
MLH1, MSH2, MSH6	DNA mismatch repair, cell cycle regulation	Colorectal, gastric, endometrial cancers
BRCA1, BRCA2	Repair of double-stranded DNA breaks, cell division, cell death	Only rare ovarian cancers
WT1, WT2	Cell division, transcriptional regulation	Wilms' tumors
NF1, NF2	RAS-mediated signal transduction, cell differentiation, cell division, developmental processes	Small numbers of colon cancers, melanomas, neuroblastoma
VHL	Cell division, cell death, cell differentiation, response to cell stress	Certain types of kidney cancer

2.1.6 Gene Therapy for Gliomas

There are very few therapeutic options available for management of malignant glioma. Gene therapy provided many interesting clinical trials about its application against brain cancer and specifically against GBM (Curtin J. F. *et al.*-2005).Cancer gene therapy is an experimental treatment that involves introduction of healthy copy of genetic material into malignant cells to treat cancer. The aim of gene therapy is to selectively kill cancer cells while leaving healthy cells unaffected. This can be achieved by introduction of a normal healthy copy of gene of interest i.e. the gene mediating cytotoxic killing of tumor cells or which encode immune system proteins enhancing anti-tumor immunity (Tai C. K. *et al.*-

2005). Gene transfer is recently being investigated as a promising approach against brain cancer (Fulci G. and Chiocca EA-2007). It is now understood that gene therapy approach fetches really encouraging results for GBM cancer treatment, but the largest limitation of gene therapy is the need to infect all tumor cells and/or evoke a bystander effect in the non-infected tumor cells. Examples include soluble cytokines, such as TNF or IL-1β, that generate an antitumor immune response that could involve the bystander effect of suicide-gene therapy.

2.2 Gene Delivery

The complexity of delivering exogenous DNA becomes apparent when we consider the properties of DNA. Plasmid DNA (which is the workhorse in majority of gene therapy protocols) is large in terms of both molecular weight and size, the phosphodiester bond is a ready substrate for degradation by nucleases (which are abundant both in serum and inside the cell) and at physiological pH, it's polyanionic nature (due to the phosphate backbone) prevents any spontaneous interaction with the negatively charged cell membrane. DNA has been shown to have a very short half-life of around 1.2 to 21 minutes, depending on topoform of DNA, within serum when given intravenously as a naked DNA (Houk B. E. *et al*-1999). This is believed to be due to both endo and exonuclease activity in the plasma. Likewise, similar degradation has been observed by investigators when plasmid DNA was delivered intramuscularly (Mumper R. J.*et al*-1996). Thus, although delivery of naked (uncomplexed) DNA has met with success under certain scenarios, the majority of approaches favor the use of gene delivery agents or "vectors" and basically, there are two types of gene carriers one is viral and other is nonviral.

2.2.1 Viral Vectors

Based on the properties of DNA mentioned above, it appears that DNA delivery would be nearly impossible to accomplish. However, such a delivery system is already present in nature. Indeed, the virus is Nature's nanoparticle that has an exceptional ability to deliver exogenous DNA into the cell nucleus (Gururaj R. *et al*-2007). A major effort in developing gene delivery systems has focused on the use of modified viruses. In this case, the virus has

to be gutted and genetically manipulated such that it cannot replicate, be infectious and does not elicit any severe immunological response.

Viruses used as gene delivery vectors include retroviruses, adeno-viruses, adeno-associated viruses, herpes simplex virus, influenza virus and hepatitis B virus. The first approved human gene therapy attempt took place in 1990 on 4 year old Ashanti DeSilva who had ADA-SCID -an inherited disease that prevents the normal development of the immune system. Ashanti DeSilva showed positive improvement without developing any complications when exposed to environment similar to her siblings. This early promise led to an increase in the number of gene therapy trials taking place in the 1990's for a variety of different inherited diseases (Website of British Society for Gene and Cell Therapy as accessed on Feb-2014). The variety of viruses has been converted to vectors to deliver genes to cells (e.g. adenoviruses, retroviruses, adeno-associated viruses and lentivirus) and it is worth mentioning that they have been implicated in 70% of gene therapy clinical trials (Waehler R. *et al*-2007). But, their use is limited because of inherent safety concerns. The use of viral vectors for gene therapy can be associated with severe inflammation and immunological problems (Verma I. M. and Somia N.-1997; Lehrman S.-1999).

Attempts at designing virus-based vectors have been very successful considering the complexity of the problem, thanks to the advances in molecular biology. Viral constructs possessing high transduction efficiencies have been developed and utilized in clinical trials. However, even presently, there are concerns regarding toxicity. These concerns have been highlighted by a couple of unfortunate incidents during clinical trials with viral based vectors (Gansbacher B.-2003 and Lehrman S.-1999). In past the case of a young boy developing leukemia after being treated using a retroviral vector brings forth another concern, which is the possibility of insertional mutagenesis. Thus, even thoughviral vectors have the excellent transduction properties the toxicity problems still plague the field (Hacein-A.S. *et al*-2003).

2.2.2 Nonviral Gene Carriers

The control over the properties of non-viral carriers is important in designing new gene carriers, although the main problem currently in polymeric carriers is overcoming their poor efficacy. Although there has been a significant progress in the understanding and applications of various nonviral gene delivery systems, the majority of nonviral approaches are still much

less efficient than viral vectors, especially for in vivo gene delivery. Therefore, some physical methods of gene delivery find interest in them all the time. Theoretically, cationic carrier molecules might complex with DNA and neutralize its electrostatic charge, thereby promoting cell-membrane–DNA interaction and increasing transfection efficiency.

An ideal nonviral gene carrier should have the following characteristics (Nguyen J. and Szoka F.C.-2012):

- its size must be below 200 nm,
- all the components have to be biocompatible,
- it must be stable enough in blood to protect the nucleic acid,
- opsonization and uptake by macrophages have to be avoided, and
- after reaching the target site, endocytosis should occur in the cell but the nucleic acid must be able to further escape the endosome and be released in the cell cytoplasm.

The research on viruses as gene carriers have given an insight into the necessary properties of a gene delivery system and that has helped researchers in the development of alternatives in the form of nonviral gene transfer systems. The research has resulted in a host of DNA carriers (both natural and synthetic) that have been designed to overcome one or several of the barriers. The use of cationic lipids and cationic polymers as gene carrier was introduced by Felgner et al. (Felgner P. L. *et al*-1987). Most of these carriers formulate DNA into discrete particles in the nano- to sub-micron size range. This size range is ideal as it permits efficient uptake into the cell via the process of endocytocis. Compaction of DNA also reduces its access to nucleases, which means that the transgene has a better chance of reaching the cell intact (Gururaj R. *et al*-2007). In addition, many of the carriers impart an excess positive charge on the surface of the particles. This promotes interaction between the particles and cell surface and thereby aids in cellular uptake. Gene delivery to tumor has been tried by different approaches as naked plasmid delivery, different colloidal carriers such as liposomes, nanoparticles, microspheres, lipoplexes, and polyplexes containing cationic lipid or polymer condensed with gene. The distinction between viral and nonviral gene carriers is shown in table 2.3.

Table 2.3: Distinct characteristics of viral and nonviral gene carriers.

Gene carriers	Viral gene carriers	Nonviral gene carriers
Features	• Relative high titre • stable gene expression due to viral genome integration into cell chromosome. • can infect non dividing cell • Total insert capacity in the virion is in the range 5-30Kb. • Transiently high level of gene.	• They aren't infectious. • Theoretically there is no limit to the size of DNA. • They are suitable for oligonucleotide delivery. • Low degree of toxicity.
Limitations	• Host immune responses, inflammatory and toxic in reactions in patient. • Random insertion of viral genome, which may possibly result in mutagenesis. • Possibly of replication competent virus formation by homologous recombination.	• Targeting isn't specific. • Low transfection efficiency. • Only transient expression. • Difficult in vivo applications. • Host immune responses, inflammatory reactions in patient, if they express chemical cell receptors on their surface, or in the presence of unmethylated CpG sequences of bacterial plasmid DNA.

Many serious disorders of the CNS are resistant to conventional small-molecule therapy but could be treated, even cured, with gene therapy. But, in current practice, delivery of the therapeutic gene to the brain requires drilling a hole in the head followed by insertion of the gene incorporated in a viral vector. However, craniotomy based delivery does not enable the expression of the therapeutic gene widely throughout the brain or even to relatively localized area such as a brain tumor, which could have a volume greater than several milliliters. However, craniotomy has to be used currently because the virus does not cross the blood–brain barrier (BBB) *in vivo*. The Blood Brain Barrier (BBB) acts as a major barrier for most hydrophilic substances including (bio-macromolecules) in reaching the brain and has been

the hurdle to overcome delivery of such therapeutic agents to brain. Therefore, brain targeting technology is need to be employed that enables the nanocontainer to traverse the BBB, a 400 miles long network in the humans, so as to distribute the therapeutic agent through-out the entire brain volume (W.M. Pardridge-2001).

Targeting of brain is very different and challenging when compared to targeting other organs. This is because of presence of the blood-brain barrier (BBB) which protects the central nervous system (CNS) from potentially harmful xenobiotics and endogenous molecules (Smith, M. W. and Gumbleton, M.-2006). The fact that >98% of candidate brain-targeting drugs have been halted mid-development due to the poor permeability of the BBB, shows the level of difficulty that the BBB poses to the pharmaceutical industry (Pardridge W. M.-2001). Another striking problem is to develop gene therapy drugs that offer the promise of an effective cure for both genetic and acquired brain diseases, since the majority of these diseases do not respond well to small molecule drugs and have no effective long-term therapy. The so called advanced gene vectors also do not cross the BBB after an i.v. administration and must be given via craniotomy or intracerebral injection, which are considered to be highly invasive and unable to deliver exogenous genes to global areas of brain (Pardridge W. M.-2002). Brain gene targeting technology, including both viral and nonviral delivery, enables widespread expression of exogenous genes throughout the CNS after an i.v. injection. But given the evident side effects of viral vectors, the goal of brain gene targeting technology is the efficient, noninvasive, and nonviral gene therapy of the brain.

The BBB does possess specific receptor-mediated transport mechanisms that potentially can be exploited as a means to target drugs to brain (Hatakeyama, H. *et al*-2004). It is known fact that nanoparticles impart accessibility to brain i.e. ability to cross BBB owing to their size and such targeting ability is deemed to be passive in nature (Kreuter J.-2001). Receptors expressed on the BBB like transferrin (Tf) receptor (Pardridge, W. M.-1999) has been used for braintargeting with different drug delivery systems like micelles (Zhang P et al-2012), dendrimers (Somani et al-2014), liposome (Lv Q et al-2013), polymerosomes (Gao H L et al-2010), nanoparticles (Wiley D. T. et al-2013) etc.

The Tf receptor is of particular interest because its expression in capillaries throughout the body is restricted to brain capillaries. The brain targeting with colloidal gene carriers has

been carried out with PEGylated immunoliposomes, which access the brain from blood via Tf receptor-mediated transcytosis and deliver exogenous genes into the brain parenchyma without damaging the BBB (Zhang, Y. *et al* -2003). Lactoferrin (Lf)-modified nanoparticles (NPs) have also been developed which demonstrated efficient expression of exogenous genes in the brain via intravenous administration. Therefore, Lf-modified NPs could be exploited as potential brain-targeting delivery systems for exogenous genes (Huang R. *et al*-2010). In addition, the nanoparticles can be modified with different ligands such as apolipoprotein E, wheat germ agglutinin and antibodies to create a smart targeted delivery system.

Overcoming the obstacles associated with nonviral vectors to improve the delivery efficiency and therapeutic effect of nucleic acids is thus an active area of current research area globally. In order to achieve this, the DNA, after being introduced into the body has to remain intact while it is carried by the circulatory system to the target tissue and the target cell, regardless of the method by which a non-viral vector is administered in vivo (e.g. by inhalation, intramuscular injection, intravascular injection, etc). While doing so, it will unavoidably come into contact with the hostile extracellular environment. Apart from activity of nucleases the activation of the immune system is also considered as an extracellular barrier. While immune activation has been most associated with viral gene delivery, some non-viral methods have been shown to induce an immune response. It was reported that intravenously injected cationic lipoplexes can induce an inflammatory response involving the release of TNFα and IFN γ into the serum (Dow S. W.*et al*-1999). The schematic representation of extracellular barriers for nonviral nanoparticulate DNA carriers is shown in figure 2.7.

Like the extracellular barriers, a gene delivery system has to overcome several intracellular barriers for successful delivery of payload (genetic material) at its workplace that is the nucleus. Therefore, a proper understanding of their uptake mechanism and their intracellular trafficking becomes necessary to lead to a rational design of efficient non-viral carriers. The schematic representation of intracellular barriers for nonviral nanoparticulate DNA carriers is shown in figure 2.8.

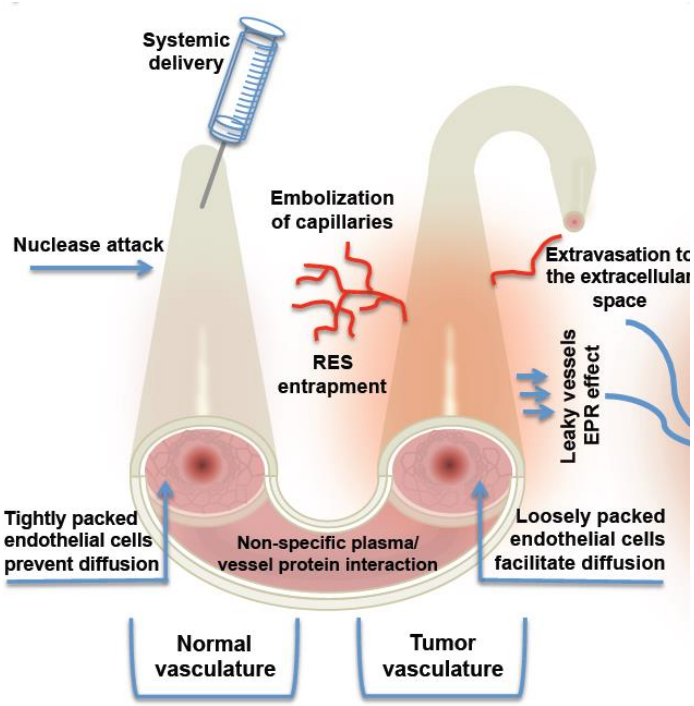


Figure 2.7: Schematic representation of extracellular barriers for nonviral nanoparticulate DNA carriers (*Source: Cian M. M. and Helen O. M.-2013*).

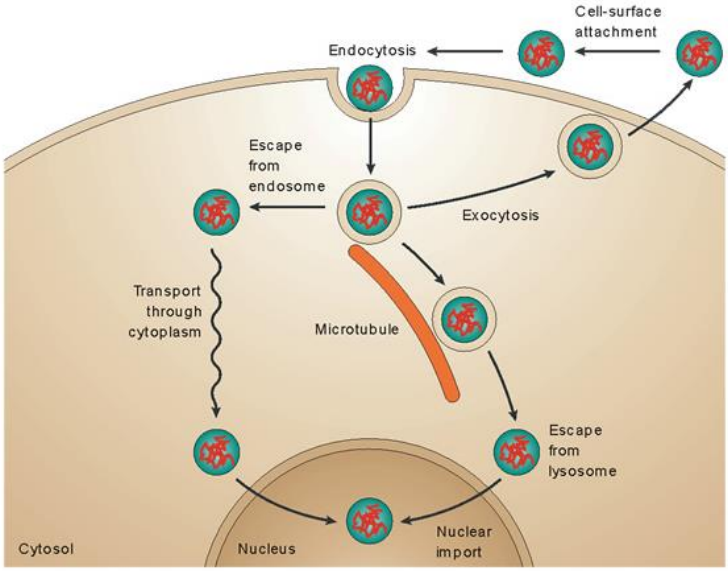


Figure 2.8: Schematic representation of intracellular barriers for nonviral nanoparticulate DNA carriers (*Source: Daniel W. P. et al-2005*).

2.2.2.1 Lipid Based Carriers

Complexation of cationic lipids with DNA was first described in 1987, and thereafter 'lipofection' was reported to be much more efficient than the earlier transfection techniques. The cationic lipids in aqueous solution forms positively charged micellar structures termed liposomes. Contemporary liposome preparations contain cationic lipids but are dependent on a neutral lipid or helper lipid to provide effective transfection. Complexes formed by self assembly of DNA with liposomes are generically known as lipoplexes. The simple mixing of diluted solutions of plasmid DNA and liposomes forms the lipoplexes (Eastman S.J. et al-1997). But, the resulting lipoplexes are generally heterogeneous in size and morphology. The heterogeneity is primarily due to the relatively large sizes of DNA and liposomes, and the multivariant nature of the interaction between the DNA and liposomes. Alternative methods involving forms of lipid assembly other than liposomes have been designed to overcome these problems. For example, direct addition of DNA solution to a dried film of cationic lipid and DOPE promotes entrapment of DNA within multilamellar liposomes. The lipoplex can alternatively be prepared by slow dialysis which procedure involves DNA condensation in mixed micelles consisting of cationic lipid and non-ionic detergent, and removal of the detergent by dialysis (Hofland H. E. *et al*-1996). At a concentration below the critical micelle concentration of single-chain cationic lipids, DNA collapses into unimolecular lipid-DNA nanoparticles that are much smaller (20-30 nm) (Liu G. et al-2003). Small particles are preferred for *in vivo* gene delivery because of their slower clearance rate in the blood and, therefore, their high probability of reaching the target cells. Conjugation to these small-sized complexes with polyethylene glycol (PEG) and targeting ligands on their surface makes it possible to construct target-specific gene carriers (Xu L. et al-2012).

It was initially suggested that several liposomes might associate with a single plasmid molecule to render it charge neutral, condensing the DNA to form a small dense lipoplex. However, electron micrograph studies have produced images of lipoplexes with a range of macromolecular structures. Immediately after complexation or at low DNA concentrations, multiple liposomes appear to bind and sandwich DNA in between them. Different complexes emerge later, which might vary depending on charge ratio, lipid formulation and mode of preparation. Condensed lipoplexes are seen with diameters of 100–200 nm, and also elongated lipoplexes, which were thought to represent DNA surrounded by a lipid uni- and/or

bilayer (Xu Y. et al-1999). Large aggregates lipoplexes were also observed, and thought to comprise numerous lipid and DNA molecules. Precisely which of these represents the most transfection-efficient fraction is not clear but it was reported that the size of lipoplex determines the nature of the entry pathway by endocytosis (Wasungu L. and Hoekstra D.-2006).

Lipoplexes are powerful systems for introducing plasmids into target cells; however, their hydrophobic and positively charged surface frequently leads to interactions with plasma proteins and other extracellular proteins, which bind nonspecifically to the lipoplexes and might inactivate them. In this regard, protein-resistant lipoplexes have been developed (Llères D. et al-2004). The effect of cell membrane- associated and extracellular-matrix associated proteoglycans on lipoplexes has been neglected for a long time. Many of these proteins are sulphated, and therefore are negatively charged, allowing them to interact with positively charged lipoplexes. It is also feasible that these proteins regulate intracellular transport and exert an effect on gene transcription/translation by a pathway that is as yet unknown. Lipoplexes are thought to be internalized by endocytosis, although fusion with the cell membrane or disruption of the cell membrane lipid bilayer have also been proposed (Parker A. L. et al-2003). Larger aggregated lipoplexes might be internalised by phagocytosis. Once the lipoplex has been internalized to the endosomal system, rapid mixing of cationic (liposome) and anionic (endosome) lipids might disturb the endosomal membrane (Farhood, H. et al-1995). The presence of the neutral lipid DOPE in the liposome is thought to promote endosomal rupture, purportedly by a mechanism involving transition from a bilayer phase to an inverted micellar structure. The vast majority of the time, the endosome will mature and fuse with lysosomes, where DNA will be degraded, and no gene expression will occur. Rarely, during lipid mixing the endosomal wall will rupture, and although most of the encapsulated DNA will remain bound to the lipid, some will manage to escape into the cytoplasm and traffic to the nucleus, culminating in gene expression. For a summary of lipoplex mediated gene delivery, the transfection of lipid mediated gene carriers after typical internalization by endocytosis is shown in figure 2.9.

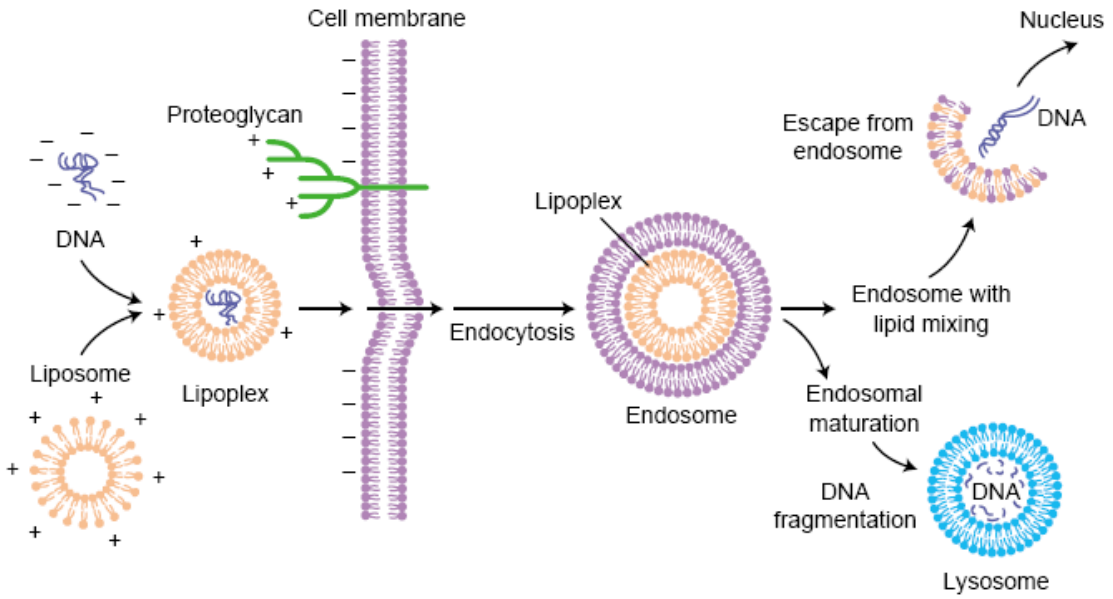


Figure 2.9: The transfection of lipid mediated gene carriers after typical internalization by endocytosis (*Source*: Parker A. L. et al-2003).

Although lipoplexes often show high levels of transgene expression following direct administration or injection into target tissues, their nonspecific membrane activity usually precludes cell-selective targeting. This leads to indiscriminate transgene expression in cells present at the site of administration. Furthermore, a major problem with the application of most nonviral systems, including lipoplexes, is their poor efficiency at transfecting nonproliferating cells. This is thought to be mainly a result of the integrity of the nuclear membrane providing a physical barrier to entry.

2.2.2.2 Polymer Based Carriers

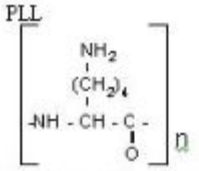
Polyelectrolyte complexes formed by self assembly of DNA with cationic polymers are referred to as polyplexes. When plasmid DNA is mixed with cationic polymers, in appropriate ratios and solvents, the resulting complexes are usually nanoparticulate (<100 nm) and surprisingly homogenous. Several cationic polymers have been evaluated for their ability to form nanoparticles with DNA, the most significant being poly-lysine (PLL) and polyethylene- imine (PEI) (Christopher M. J. et al-2008). Table 2.4 mentions various cationic polymers, copolymers both block and grafted studied as gene carriers.

Table 2.4: The various cationic polymers, copolymers both block and grafted studied as gene carriers.

Linear backbone

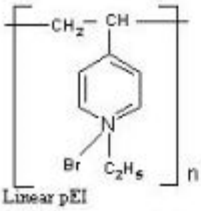
(1) DEAE- dextran [MW 500 KDa & around 40 mol DEAE/100 mol glucose

(2) PLL

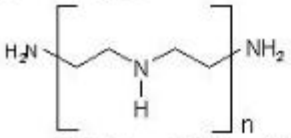


MW - 9.6 KDapKa - b/w 9 and 10

(3) pVP

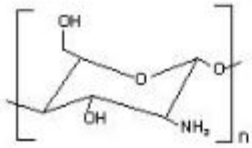


(4) Linear pEI



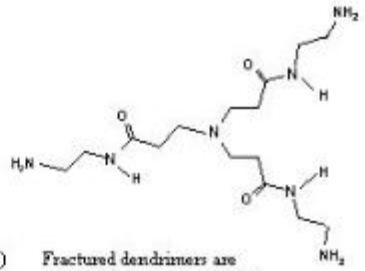
MW - between 22 KDa and 220 KDa
pKa - 5.5

(5) Chitosan



Branched backbone

(1) pAMAM dendrimer

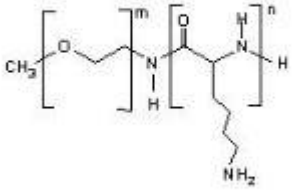


(2) Fractured dendrimers are partially degraded pAMAM Dendrimers

(3) Branched pEI

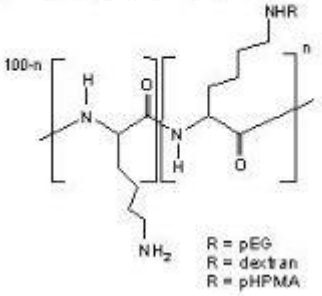
Block copolymers

(1) pEG-pLL



Grafted copolymers

(1) pLL grafted copolymers



It is clear that the properties of the complexes formed are largely determined by formulation conditions as well as polymer parameters, including molecular weight and

charge density. Although the majority of studies have involved PLL, probably the most promising complexes under development are those based on PEI, since they often exhibit remarkably high transfection activity (Park M. R. et al.-2008). The mechanism underlying the enhanced transfection rates with PEI is unclear, although it is widely stated that its ability to enhance transgene expression involves an endosomal buffering capacity. It is proposed that multiple protonatable amine groups on the polymer act as a 'proton sponge' that quenches lysosomal acidification and prevents DNA degradation (Behr J. et al-1997). Consequently, increased osmolarity of the endosome might cause rupture of the endosomal membrane and release of entrapped DNA complexes into the cytoplasm and promoting subsequent delivery to the nucleus. However, if PEI truly possesses endosomolytic properties, it is surprising that inclusion of other lysomotropic agents (e.g. chloroquine) markedly increases gene expression. One further possibility is that protonation of PEI leads to an expansion of the polymer structure, which produces physical swelling and endosomal disruption (Behr J. et al-1997).

The mechanism by which the polyplexes navigate the cellular barriers to transfection is very crucial. Therefore, experiments using microinjection of complexes directly into the cytoplasm or nucleus separate the effects of internalisation; endosomal escape and nuclear import were carried out to determine the same (Alan L. P. et al-2003). Cytoplasmic microinjection of PEI–DNA complexes appears as efficient as simple polyplex transfection, implying internalization is efficient and not the rate-limiting step. Cytoplasmic injection of DNA complexed with lipid, PLL or PEI results in 1%, 5% and up to 50% of cells exhibiting gene expression, respectively. Approximately 1% of plasmid molecules complexed with PEI and injected into the cytoplasm are calculated to reach the nucleus – greater than ten times more than the fraction for other polyplexes. Time to gene expression is also shorter, perhaps indicating cytoplasmic transport is enhanced. It has been proposed that the multiple positive charges on PEI mimic a nuclear import signal, which might also account for these findings (Pollard H. et al.-1998). In support of this, PEI complexes are less dependent on cell division to achieve gene transfection than either PLL- or lipid based complexes. Whereas nuclear injection of lipoplexes rarely results in gene expression, implying complex disassembly is not possible in the nucleus, polyplex nuclear injections do result in gene expression. Indeed, nuclear injection of PEI complexes or DNA alone result in similar rates of expression (~40–

50%). Another polymer, polyallylamine possessing primary amine groups is inefficient in transferring nucleic acids into eukaryotic cells, until it is imparted with fusogenic or lysosomotrophic effect. It was reported by Boussif O. et al that with appropriate chemical modification, namely glycolylation of the amine groups of polyallylamine, the transfection efficiency can be increased and cytotoxicity can be decreased significantly (Boussif O. et al-1999). In another research work, primary amino groups of polyallylamine (PAA, 17 kDa) were substituted with imidazolyl functions, which are presumed to enhance endosomal release, and found that enhanced gene delivery efficiency was obtained without the requirement of external lysosomotropic agents (Pathak A. et al-2007).

Similar to lipoplexes, polyplexes are cationic, rendering them prone to nonspecific interactions with plasma proteins. Complexes formed with PEI, rather than PLL, may be more susceptible to these effects. Targeting ligands can be incorporated into the polymers to mediate entry into cells by binding cognate receptors. This can lead to increased levels of uptake into receptor-positive cells, often with corresponding increases in transgene expression. Recent studies have shown that RNA can also be delivered as polyplexes, provided the molecular weight of the cationic polymer is small (Dohmen C. et al-2012). Peptides can also be incorporated into these systems to enhance selectivity and efficiency of expression.Polyplexes therefore represent a versatile strategy for gene delivery that is likely to fulfill several requirements.

2.3 Barriers and Strategies for Improved Transfection

There are several barriers encountered to a gene carrier for effective transfection of a mammalian cell. Generally, the barriers are of two types i.e. intra and extra cellular and each type encompasses barriers typical for the respective class (figure 1.10). Strategies to improve the delivery of nucleic acid therapeutics can be divided into two thrusts:

(i) Those based on carrier design to control packaging, intracellular uptake/trafficking and release of the nucleic acid cargo, and

(ii) Those based on the design of nucleic acid cargo to mediate trafficking and expression of the transgene.

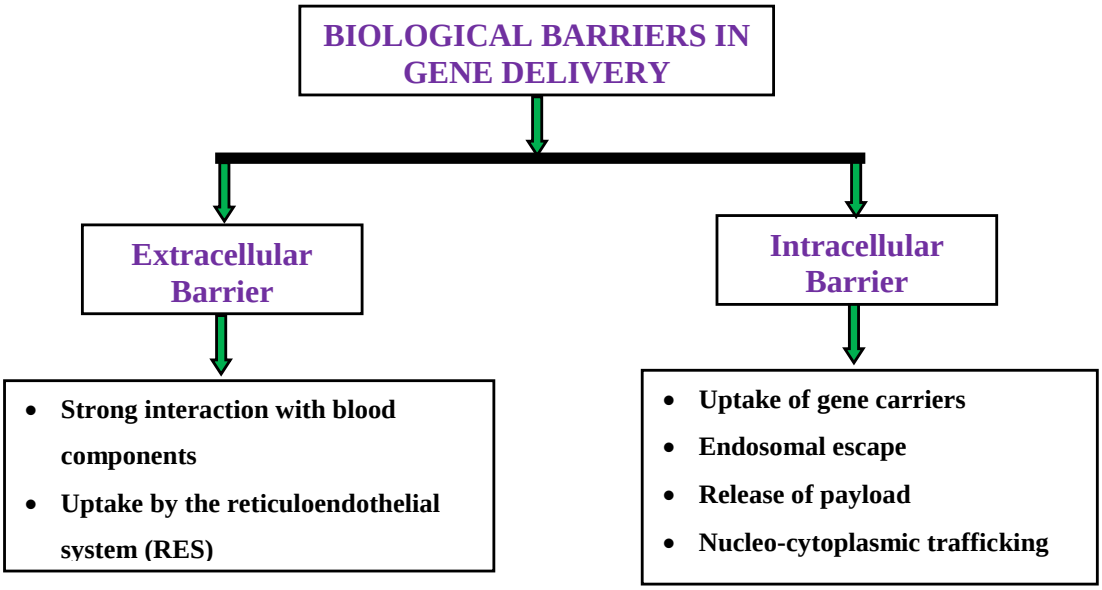


Figure 2.10: Barriers to gene transfer.

2.3.1 Extracellular barriers:

2.3.1.1 Strong interaction with blood components

Due to their highly cationic charge, the binding of plasma proteins to nonviral vectors becomes inevitable and this poses the principle challenge encountered in the blood and serum thereby causing non-specific interaction. As the proteins like albumin have negative charge, the cationic nanoplexes tend to bind them and can cause neutralization or reversion of their surface charge. Moreover, the binding of cationic complexes with serum proteins such as serum albumin hinders cellular uptake, promotes aggregation, and possibly encourages phagocytosis. Apart from non-specific interaction, the physiological salt concentration (150 mM) is also a major problem that promotes aggregation of cationic complexes, which may lead to vascular blockage. The physicochemical parameters can control the degree of particle aggregation (Ahl P.L. *et al*.-1997) in the blood as well as particle opsonization in biological fluids. Therefore, in the blood, particles and their aggregates should be small enough so that they are not removed from the circulation by simple filtration in the first capillary bed encountered. A major hurdle prohibiting the efficient uptake of pDNA is the instability of

complexes before reaching the desired cells. The charge-charge interactions can arise during delivery, leading to premature dissociation of complexes, heterologous aggregation between particles and proteins, or homologous aggregation between complexes.

There is also an evidence of accumulation or deposition of these complexes in organs such as the skin and intestine. In addition to non-specific interactions with plasma proteins, the cationic complexes also show undesirably short plasma circulation times owing to rapid hepatic uptake (Hashida M. *et al*-1996). It has been repeatedly underscored that the clearance tissue distribution of intravenously injected nanoparticulate DNA complexes are greatly influenced by their size and surface characteristics (Poznansky M. and Juliano R. L.-1984; Patel H. M.-1992). The opsonization and the activation of complement also trigger a rapid clearance of DNA-polymer complexes by the mononuclear phagocyte system (MPS). The opsonization process is the adsorption of protein entities capable of interacting with specific plasma membrane receptors on monocytes and various subsets of tissue macrophages, thus promoting particle recognition by these cells (Chonn A. et al.-1992; Moghimi S. M. and Davis S. S.-1994). Typical example of opsonic molecules include various subclasses of immunoglobulins, complement proteins like C1q and generated C3 fragments (C3b, iC3b), apolipoproteins, thrombospondin, and fibronectin, (Absolom, 1986; Patel, 1992; Serra M. V. et al.-1992; Chonn A. *et al*.-1995). Thus the process of opsonization is one of the major biological barriers for gene transfer.

The most common method of reducing these effects is decorating the periphery of the complex with hydrophilic moieties such as with polyethylene glycol(PEG).Modifying the gene carriers with PEG is the predominant method used to reduce the binding of plasma proteins to nonviral vectors and minimize clearance by the RES after intravenous administration. The biggest advantage of such modification is that the nanoparticles are not rapidly cleared from the circulation and accumulate in the tumors because of the enhanced permeability and retention effect. Therefore, it is suggested that in vivo gene delivery can be promoted by reducing salt/serum affects and protecting genetic material from nucleases.

2.3.1.2 Uptake by the reticuloendothelial system (RES)

The rapid uptake of gene carriers by the mononuclear phagocyte system (MPS) after the intravenous administration is one of the major events, which often prevents an injected

nonviral carrier system from delivering to the site of action other than the MPS tissue and organ. As one practical way to minimize the MPS uptake, the surface modification of gene carriers with polyethylene glycol (PEG) or PEG-like polymers is effective (Karinaga R. *et al*-2005). Therefore, surface modification by PEG onto gene carrier prepared through the electrostatic assembly of pDNA and polycation (i.e. polyplex) is a widely acknowledged strategy to advance their systemic application. The application of this feature has been widely used to demonstrate efficient gene expression following a single administration (Zhang Y. P. *et al*-1999).

The importance of PEG crowdedness on the polyplex surface in determining blood circulation property was demonstrated earlier however its accurate quantification has never been demonstrated. But Tockary T. A. et al reported the first successful determination of PEG crowdedness for PEGylated polyplexes (polyplex micelle) formed from PEG–poly (l-lysine) block copolymers (PEG–PLys) and plasmid DNA (pDNA). The researchers were also able to demonstrate conformation of polyplexes tethered with PEG chains through PLys segment length. In addition, the PEG crowdedness significantly correlated to blood retention profile, approving its critical role on both shape and systemic circulation property (Tockary T. A. et al-2013). Moreover, surface modification of nanocarriers with specific ligands defines a new biological identity, which assists in targeting and internalization of the nanocarriers to specific cell populations, such as cancers and disease organs. Towards this end Jing F. *et a*l demonstrated linking of transferrin (Tf) and folate (Fa) onto polyethylene glycol-phosphatidylethanolamine (PEG-PE) separately to get transferrin-PEG-PE (T-PEG-PE) and folate-PEG-PE (F-PEG-PE) ligands for the surface modification of carrier. The researchers found that the lipoplexes modified with such dual ligands displayed over 30% higher transfection efficiency than unmodified lipoplexes and single ligand modified lipoplexes in HepG2 cells (Jing F. *et al*- 2014).

2.3.2 Intracellular barriers

2.3.2.1 Charge and size of complexes

Excess cationic charge of polyplex poses several problems after systemic administration of such gene medicines and may lead to distinctive biodistribution pattern. But this distinctive biodistribution pattern can be benefitted in terms of targeting the ailment

e.g. tumor having increased vascular growth. The size of the DNA complex is one of the critical issues that can affect the transfection efficiency in a mammalian cell (Mady M. et al-2011). The electrostatic interaction leads to formation of tiny particles depending on various factors such as carrier (polymer or lipid) to DNA (N/P) ratio, concentration, ionic strength of the buffer, and kinetics of mixing etc (Lai E. et al-2001). Preparation conditions can be focused for reducing intermolecular interactions and favoring the assembly of more uniform particles. Also, parameters such as mixing order of complex components, speed of mixing, temperature, and pH are all factors that can be implemented to control aggregation (Tiia M K. et al-2013). For example, gradual dropwise polymer addition to dilute nucleic acid solution results in smaller and more uniform particle sizes. Likewise, subsequent dilution of the complexes in larger volume combined with low temperature storage can slow down rate of aggregation. Various morphological structures arise out of DNA carrier complexes which are shown in figure 2.11.

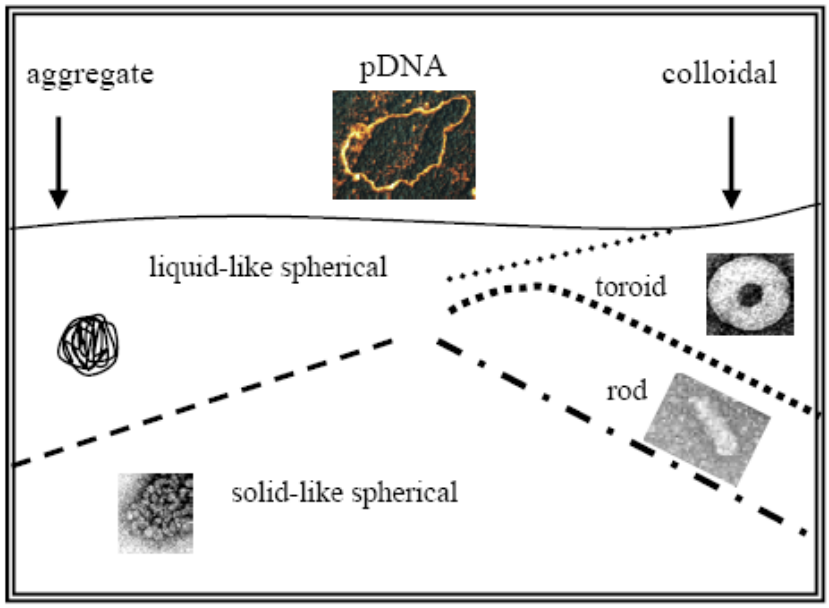


Figure 2.11: Various morphological structures arise out of DNA carrier complexes.

The nanoparticulate gene carriers can only extravasate from vascular organs from specialized sites, e.g. liver, where the endothelial linings has suitable gaps which allows particles of around 200 nm or smaller to pass. Therefore, size can become a potentially important intrinsic property of synthetic gene delivery systems that will limit organ access.

2.3.2.2 Uptake of gene carriers

The increased transfection efficiency of nonviral gene delivery vehicles may depend on how they are internalized. After administration the vectors must pass through the epithelial tissue of the blood vessels and enter the target tissue. But it is very difficult for nanoparticles with larger diameter to pass through the epithelial tissue of blood vessels and thus, it becomes important to study the cellular transport mechanism of epithelial cells. As the distance between the extracellular matrix and target cells is great, many vectors will be engulfed and cleared by macrophages after passing through the epithelial tissue of blood vessels. As an immediate next step, the vectors must attach to the cell membrane of desired cells. Therefore, it is important that the non-viral vectors should be able to identify specific cell types to avoid any nonspecific interaction/toxicity and to ensure safety. The complexes can get inside the desired cells by different processes grouped under the endocytosis as shown in figure 2.12. Different endocytosis pathways yield different intracellular fates for vectors, and this is the reason for vectors differing in their transfection efficiency in various cells (Katharina V G. et al-2006).

The addition of selective ligands such as transferrin or folate to the nonviral gene carriers was also found to be useful for internalization via endocytosis (Jing F. *et al*- 2014). A large complex of an aggregated complex of cationic is internalized with great difficulty via endocytosis, since the average diameter of endosomes is ~ 100 – 200 nm. Typical sequence of steps occurring from administration to internalization of nonviral gene carriers is demonstrated in figure 2.13.

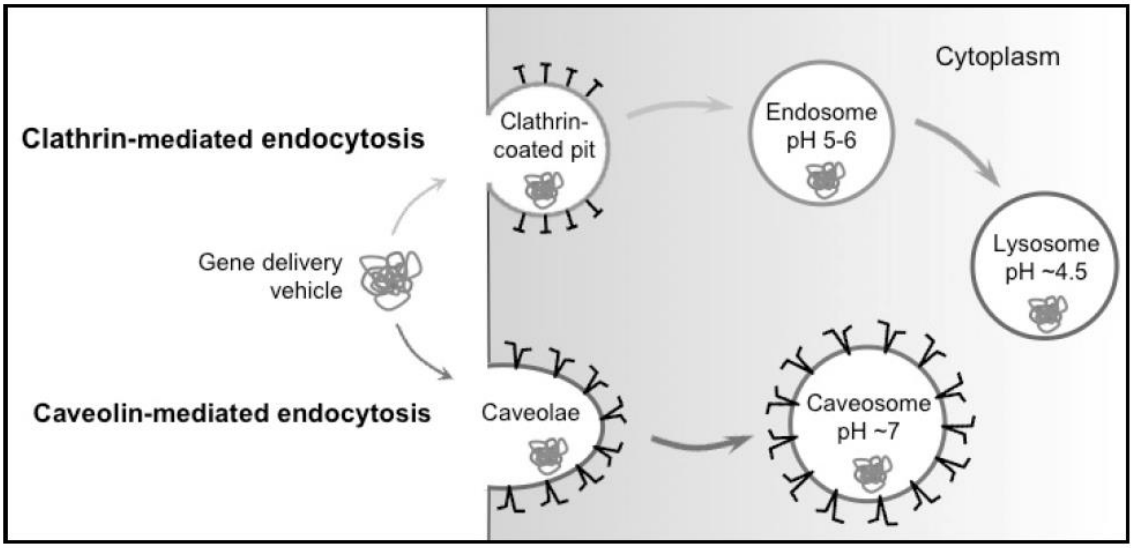


Figure 2.12: Gene delivery vehicle uptake proceeds via two main endocytic pathways with different intracellular trafficking mechanisms *(Source: C. E. Wang et al- 2010)*

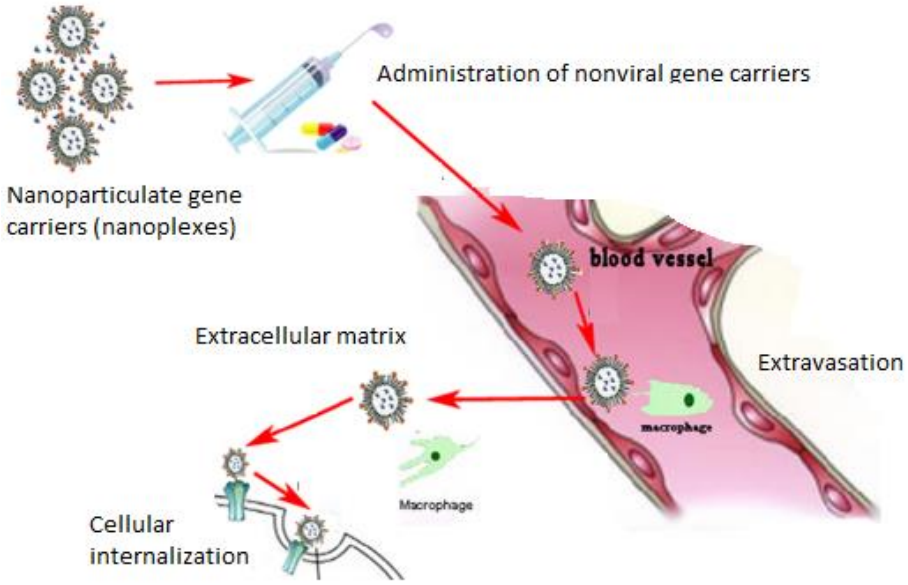


Figure 2.13: Typical sequence of steps occurring from administration to internalization of nonviral gene carriers (*adapted from: Xiang S. and Zhang X.-2013*).

Ligands can be used as a strategy to improve cellular internalization. Moreover, it can give control over the targeting ability of nonviral carriers and could avoid any non-specific interactions. For cell-specific targeting, typically utilization of receptor-mediated endocytosis through the conjugation of appropriate ligands to the carrier can also be done. Ligands such

as antibody (Chen H. *et al*-2013), transferrin & folic acid (Jing F. *et al*- 2014), RGD peptides (Gao H. *et al*-2014) and carbohydrates (Ro R. *et al*-2013) have been employed and well documented. But, there are some concerns for utilization of such ligands as these may lead to several other associated problems.

The substitution of the cationic backbone of polycation (polymer or lipid) with the help of any ligand can reduce binding affinity to anionic DNA and thereby reducing the ability of the modified carrier to compact the DNA (Patrick M. et al-2009). Also the ligand needs to be properly displayed on the surface of the particles such that the receptor can recognize and bind to the complex. The cell-specific receptors are typically presented at a lower proportion on the surface, thus, targeting to a specific subset of receptors may in effect, limit the level of nucleic acid uptake. It was estimated that at least $10^5 - 10^6$ plasmids per cell are required for transfection. If the number of plasmids taken up by the limited subset of cell-specific receptors falls below this range, then subsequent nuclear delivery and transgene expression would be reduced (Abdelatif E. et al-2005). The actual delivery efficiency of targeted carrier might be severely diminished due to the effect of the ligand on other intracellular barrier, such as endosome escape and nuclear uptake, and the ligand, particularly antibodies and peptides, can potentially be immunogenic, compromising the safety profile of a nonviral delivery system. While conceptually simple, ligand-based targeted delivery may enhance specificity at the expense of efficacy (David V. S. et al-2000).

2.3.2.3 Endosomal escape

Following the uptake and internalization of gene carriers via endocytosis, endosomal escape and nucleo-cytoplasmic trafficking are thought to be rate limiting for the transfection process. These endosomes either fuse with lysosomes for degradation or recycle their contents back to the cell surface. Therefore, escape from endosomes is essential for efficient transfection. But, methods to promote the release of complexes from endosomes largely rely on the composition of the carrier and its inherent reactive properties to disrupt of the enveloping membranes. The endosomal escape basically can be brought in by either fusogenic effect of the carrier or due to the so called “proton sponge effect”. In the proton sponge effect, endosomolysis is promoted through adsorption of H+ by amine groups found on cationic polymers. Protonation induces an inflow of ions and water into the endosome

lumen, leading to a gradual increase in osmotic pressure, swelling the vesicle, causing the membrane to destabilize and eventually. In the fusogenic effect, the carrier can undergo conformational changes upon pH drop, which triggers the molecule to adopt a conformation suitable for fusion with the lipid bilayer.

However, the inherent endosomolytic activity of the carriers can be increased by grafting additional membrane disruptive components onto the carriers to further enhance cytosolic release (Chandrashekhar C. et al-2013). Thèse functional components have been derived from viruses, bacteria, plants, mammalian (or endogenous), as well as synthetic or recombinant peptides. Attachment of these endosome-disruptive components is accomplished by either covalent linkages or through attractive interactions with the complex surface. Interestingly, there is often a minimum substitution required for inducing membrane destabilization. So, high degree of carrier modification may not be needed that may diminish the DNA binding capacity, causing the complexes to be less stable. Conjugation of functional devices could also lead to changes in the overall size of the complexes, which could in effect, redirect the uptake to a pathway that may not allow the endosomolytic component to exert its activity. Finally, the functional component should be present on the surface and not in the core of complex, which is required for their efficacy.

2.3.2.4 Release of payload

The binding efficiency of the polycation seems to be biphasic, considering its ability to bind plasmid DNA effectively so as to protect the plasmid DNA and its release intracellularly. In addition, the polymer/DNA binding helps determine high cellular uptake efficiency of the formed particles. However, it is also important to consider that the particle should not bind or encapsulate the DNA so tightly as to prevent the timely release of the DNA once in the cytoplasm. It has been shown that polymeric vector/DNA complexation significantly inhibits gene expression and that above an optimal length, overall transfection decreases as polylysine length increases (Schaffer D. V. *et al*-2000). Similarly, 25 kDa polyethylenimine(PEI) vectors are known to have higher transfection efficiency than those formed with higher molecular weight PEI, which are toxic as well (Choosakoonkriang S. *et al*-2003). The importance of release of payload is also evident from the fact that poly(*β*-amino esters) (PBAEs) are found to be superior to PEI due to their biodegradability via

hydrolytically degradable ester groups and their ability for triggered DNA release within the cell (Lynn DM, Langer R.-2000).

2.3.2.5 Nucleo-cytoplasmic trafficking

Some studies report that endosomal escape was not the rate limiting step but the limited nucleo-cytoplasmic movement might hinder the gene delivery (Marchini C. et al-2011). Movement of endosomes across the cytoplasm is facilitated by microtubules along the cytoskeleton network which extends from the plasma membrane to the microtubule organizing centre (MTOC) which is located in close vicinity to the nucleus. Movement along the MTOC appears to be bi-directional – cargo may oscillate between the perinuclear region and the cell periphery. This signifies the importance of the timing of endosome release relative to movement along the microtubules. If complexes are released far away from the nucleus, diffusive mobility of large pDNA (>2 kb) may be restricted in the crowded cytoskeleton mesh, limiting nuclear uptake. If endosome escape coincides with localization around the perinuclear region, then cycle-dependent nuclear import may be enhanced. This prompts for a method to induce endosome escape that can be spatially triggered to the vicinity of the perinuclear region. Alternatively, pDNA movement can be facilitated by signal peptides, lipids or adaptor proteins for sorting, targeting and anchoring to specific subcellular compartments. The lipid-modified polymeric carrier exhibits enhanced trafficking to the nuclear periphery, due to passive event of anchoring of the lipid group to nuclear membrane through hydrophobic interaction. Signal peptides are arguably the most widely used approach for subcellular trafficking (Hu Q. *et al*-2012). Even viruses have evolved to use specific peptide sequences to facilitate its interaction with dynein (a transport protein) to move along the cytoskeleton network, which extends into the nucleoplasm, thereby gaining entry into the nucleus. Adapting endogenous mechanisms for protein import such as conjugatingnuclear localization signal (NLS) to either the gene carrier or to the DNA vector has proven to be a viable strategy for promoting the nuclear uptake of complexes (Shi D. *et al*-2014). However, the positive effect of NLS has not been consistently demonstrated among research groups.

The problem, which is shared by conjugated carriers, is often the lack of proper spatial presentation of the ligand to its receptor. But more critically is the fact that other

cellularbarriers were not simultaneously tackled, which may inadvertently undermine the benefits of NLS. In that regard, a multifunctional gene delivery system that can incorporate all of the barrier-evading moieties in a spatially coordinated order would be ideal in overcoming multiple rate-limiting steps in the transfection pathway. Recently, the different classes of nonviral vectors (lipid, polymer or Lipopolymers etc.) appear to be converging, and the ability to combine features of different classes of nonviral vectors in a single strategy has emerged. With the strengths of several approaches working in concert, more hurdles associated with efficient nucleic acid delivery might therefore be overcome.

2.4 Cell Cycle and Gene Delivery

In many cases, nonviral particle-mediated gene delivery is highly dependent on the cell cycle status of transfected cells. Researchers carried out transfection experiment at different stages of cell cylcle to understand the effect of cell cycle stages on transfection ability of polyplexes and/or lipoplexes. In one report it was found that particle-mediated delivery with lipofection or branched PEI resulted in 2 to > 3 log-unit differences in gene expression between cells transfected in G1 and S/G2, whereas linear polyethylenimine (PEI) showed no significant difference for the same (Brunner S., et al-2002). The cell division cycle takes place in four phase, G1 (Gap1), S (Synthesis), G2 (Gap 2) and M (mitosis). G1 and G2 phases are interphases in which cells mainly grow, accumulate nutrients needed for mitosis, and monitor their size, as depicted in fig 2.14. During S phase, the chromosomal DNA, organelles and cellular contents replicate, though the ploidy of cell remains the same.

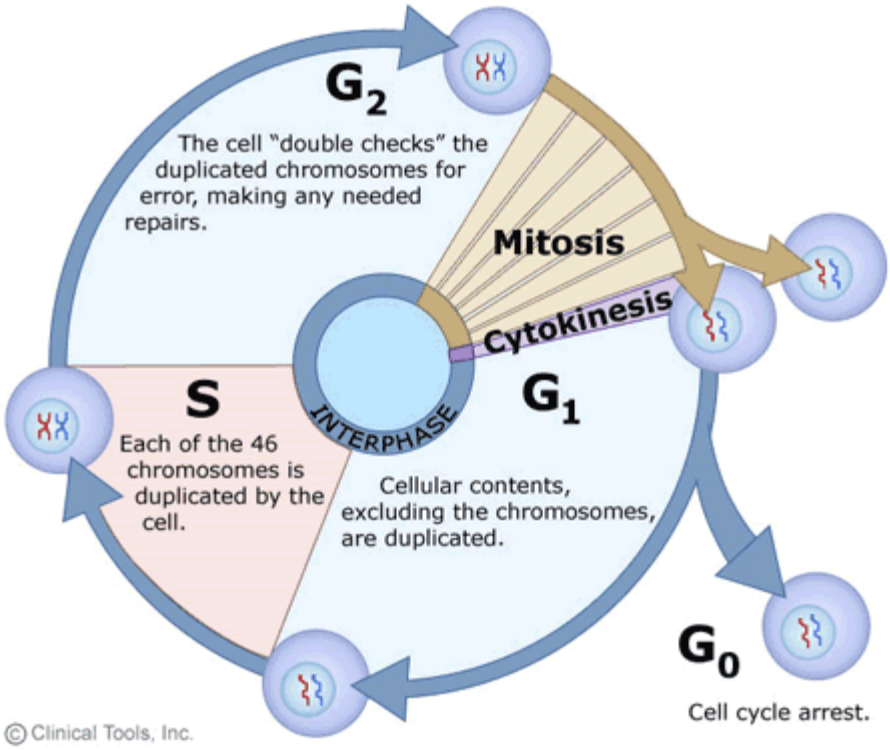


Figure 2.14: Typical cell cycle.

In G2 phase, protein synthesis occurs, involving the production of microtubules, which are required during the process of mitosis. In this phase, cells also ensure whether the newly synthesized DNA is perfect and the environment is positive to enter in last phase, mitosis, in which cell divide. Sometimes cells can pause in their progress and exit from the cell cycle to phase called quiescent G0. The transition from one cell cycle to phase to another is regulated by cyclin dependent protein kinases (Cdk) but other factor such as, cyclins, soluble growth factors, insoluble extracellular matrix molecules, mechanical force or cell distortion also contribute to the control of the cell cycle and each factor can be rate limiting step for the others. Normally, cell proliferation is tightly controlled and regulated, however when the control fails, cell can be transformed into cancerous cell. Cell volume and intracellular pH both are dependent on the the phase of cell cycle and both tend to increase toward mitosis. This may due to the proton pump activation during cell growth phase. Certain cell organelle, such as lysosomes, reacts to a change in the intracellular pH by changing their localization and shape. The nucleolus is a membrane less organelle within the nucleus and its size and morphology reflects the cell cycle phase and transcriptional activity. Cells prepare

themselves to enter mitosis by increasing the tension of the plasma membrane, decreasing the rate of endocytosis and depolymerization the microtubules. Intracellular negativity follows the charges of chromatin, being lowest at the end of mitosis, when chromosomes are in a condensed state and the charges of the DNA are partially neutralized by positively charged histones, and thereafter negativity increase until G2 phase when it is at its highest, before the condensation take places. The nuclear envelop disappear and reconstitutes at each cell division. Reassembly of an envelope is completed within 15 min after cell division. The number of nuclear pores increases throughout the cell cycle, but the rate of pore formation is at its highest soon after the cell division, decreasing continuously towards the end of the cell cycle. Finally, it is not known how these changes in cells during the cell cycle affect the nonviral carriers and their ability to deliver genes.

2.5 Cytotoxicity Concerns of Nonviral Gene Carriers

The mechanisms by which cationic polymers/lipids (polycations) induce cytotoxicity are understood poorly. There are examples reported in the literature of induced cytotoxicity of polycations, such as high molecular weight polyethylenimine and poly-l-lysine. Some investigators found that by optimizing the balance between polymer cationic density with endosomal escape moieties (such as imidazole), effective gene transfer was achieved with low cytotyoxicity (Putnam D. *et al*-2001). The cytotoxicity of cationic nanoparticles was attributed mainly due to disruption of lipid bilayers (Hong *et al.,* 2006) and induction of oxidative stress inside the cell as a result of cell-type interplay.

Fisher D. *et al* carried out an interesting comparative in vitro cytotoxicity study with different water-soluble, cationic macromolecules which have been described as gene delivery systems was performed. The investigators monitored the cytotoxicity in L929 mouse fibroblasts using the MTT assay and the release of the cytosolic enzyme lactate dehydrogenase (LDH). Both the assays yielded comparable results and allowed the following ranking of the polymers with regard to cytotoxicity: Poly (ethylenimine) = poly (L-lysine) > poly (diallyl – dimethyl - ammonium chloride) > diethyl aminoethyl – dextran > poly (vinyl pyridinium bromide) > Starburst dendrimer > cationized albumin > native albumin (Fisher D. *et al*-2003). The researchers have found that the molecular weight as well as the cationic charge density of the polycations was the key parameters for the interaction with the cell

membranes and consequently, the cell damage. On the other hand, the magnitude of the cytotoxicity of the polymers is found to be time- and concentration dependent (Fisher D. *et al*-2003). Poly (ethylenimine) induces the cell death by a necrotic cell reaction and not by apoptosis.

Hornung *et al,* described that any rupture or leakage of the endosomal or lysosomal membrane will release cathepsin B which is directly associated with the apoptosis (Hornung *et al.,* 2008). Also, the cytokine release upon PEI/nucleic acid polyplex treatment has been described by researchers (Kawakami S. *et al.,* 2006). Therefore, the in vivo use of such polycations need to be done very judiciously after taking a lot of effort to avoid the high proinflammatory effects caused by the rupture or leakage of the endosome. To form stable and protective PEI nucleic acid polyplexes, an excess of PEI polymer is needed. But after the release of nucleic acid, 60-80% PEI remains in a free form and that is mainly attributed to PEI toxicity. The high positively charged PEI molecule is able to disrupt cell membranes. Disruption of the endosome is on one hand favourable with respect to the intended cytoplasmatic delivery, but on the other hand disruption of other cell membranes (e.g., lysosomal membranes, mitochondrial membrane, plasma membrane) is not favourable as it will cause stress responses or even apoptotic or necrotic cell death. It has been shown that PEI causes apoptosis in a manner which is not specific in all kind of cells (Beyerle A. *et al.,* 2010). The area of understanding of cytotoxicity by polycations calls for an intensive effort to obtain more insights in the exact mechanisms.

2.6 Polyethylenimine as a Gene Carrier

High molecular weight polyethylenimine (PEI) has been reported to be effective for gene delivery, since plasmid DNA can be delivered to the cytoplasm via endosomes due to the proton-sponge effect of PEI (Godbey, W. T. *et al*-1999). However, high molecular weight PEI has not been clinically employed due to its non-specific cytotoxicity displayed against different cell types. On the other hand, low molecular weight PEIs are much less cytotoxic, but they are not efficient transfection agents due to limitations of DNA delivery into the cytoplasm. In an effort to improve the transfection efficiency of polymeric carriers, water-soluble lipopolymers (WSLP) were synthetized by combining lipidic components with the PEI; PEI was expected to condense the plasmid DNA and enhance endosomal release due to

its tertiary amines, while the lipid coating on plasmid DNA/polymer complexes increased the permeability of complexes through cell membranes (Han S. *et al*-2001). A versatile class of gene carriers could be designed in this way by controlling the nature of the cationic backbone, lipidic substituent and extent of substitution. Substitution of branched PEI's primary amine groups is particularly attractive since it is amenable to a variety of chemistries. Doody et al. showed that substitution of the amines generally increases the transfection efficiency with PEI, but increasing the substitution beyond ~25 mol% decreased the transfection efficiency from the optimum configuration (Doody A. M. *et al*-2006). Attempts to identify the appropriate lipid substituent suggested that steroid receptors could be beneficial in facilitating the trafficking of transfected DNA to the nucleus (Rebuffat A. *et al*-2001). Choi *et al.* conjugated dexamethasone to polyamidoamine (PAMAM) dendrimers (PAM-Dexa) and found that the transfection efficiency of PAM-Dexa conjugates was higher than that of PAMAM or PEI by one order of magnitude. In addition, more PAM-Dexa/DNA complexes were observed in the nucleus than the PAMAM/DNA complexes based on confocal microscopy studies (Choi J. S. *et al*-2006). Han S. et al. showed effectiveness of cholesterol to enhance the PEI's transfection efficiency, although no detailed studies on nuclear uptake was reported (Han S. *et al*-2001). Other lipid-modified PEIs were also observed to increase the nuclear association of polyplexes (Hsu C.Y. *et al*-2011), which was considered the main mechanism behind the improved transfection efficiency of these lipopolymers. Therefore, it was assumed that lipids with steroid nucleus in their structure may prove effective transfection reagents when conjugated with suitable hydrophilic polymers. In addition, such a hydrophobic modification was also considered beneficial in facilitating the crossing of polyplexes through the plasma membrane (Neamnark A. *et al*, 2009).

2.7 Polyallylamine as a Gene Carrier

The polyallylamine 15 kDa (PAA15**)**, a less investigated polycationic polymer**,** was also chosen to compare the effect of lipidic substitutent on transfection efficiencies. It is known fact that cationic polymers possessing primary amine groups are inefficient in transferring nucleic acids into eukaryotic cells, until they possess fusogenic or lysosomotrophic effect. PAA carries a strong positive charge, which makes it suitable to bind

and package negatively charged DNA. It is a pH-sensitive polymer, extensively used in the pharmaceutical industry. PAA also contains non-titratable primary amino groups and lacks titratable secondary and tertiary amino function, which contributes to the buffering capacity. Hence, osmotic endosomal swelling is not induced by these polymers, leading to feeble DNA-polymer complex escape (Chen D. J. et al-2005). However, due to strong polycationic character PAA causes cytotoxicity and that has severely restricted its use as a gene delivery system. In order to reduce its cytocoxicity, Pathak A. et al have modified PAA with imidazolyl functions of polyallylamine and found that its transfection efficiency was increased and cytotoxicity was decreased significantly (Pathak A. et al-2007). Also, previously, PAA was modified with hydrophilic methyl glycolates and found that its ability to mediate gene transfer into cells increased by several orders of magnitude. At the same time, it was observed that such glycolylation of the amine groups of polyallylamine decreases cytotoxicity (Boussif O. et al-1999).

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