An investigation on release of DNA/ cationic ligand nano-complex for gene delivery application

A Major Project Report submitted in partial fulfillment for the award of the degree of

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Submitted by

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CERTIFICATE

This is to certify that the major project entitled

"**An investigation on release of DNA/cationic ligand nanocomplex for gene delivery application"** is being submitted in partial fulfillment for the award of degree of Master of Technology in Delhi Technological University. This work was carried out by Mansi Puri (2K11/PTE/15) under my guidance and supervision.

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CONTENTS

ABSTRACT

The present study focuses on the use of non-viral vectors for the gene delivery applications. Various non-viral vectors are available but lipids and polymers are extensively studied because of theie many advantages. In this investigation we have used G1 & G2 PAMAM dendrimer for the compaction of the DNA and the complex formed is checked by the gel condensation and the Atomic Force Microscopy (AFM imaging). Gel release assays were conducted and tranfection of the DNA with the help of dendrimer as cationic ligand is checked by transfecting the nanocpmplex in to the CHO-K1 cell line and observing the results under the fluorescent microscope.

Flow cytometry is performed to get the quantification o f the transfected cells while by fluorescent microscope helps us know about the qualitative analysis of the tranfection. With its help we are able to see very little transfection into to the by G1 PAMAM dendrimer.

1. INTRODUCTION

Successful gene therapy is still lacking an ideal vector which is safe and efficient in delivering genes to the nucleus of the target cells in vivo . Viral vectors enable high transgene expression but their clinical usage is limited due to the concerns related to safety profile and large scale production.Gene therapy as part of modern molecular medicine holds great promise for the treatment of both acute and chronic diseases and has the potential to bring a revolutionary era to cancer treatment [1,6]. It has been developed for treatment of both acquired and inherited diseases. This approach is based on the principle of correcting the basis of diseases at their origin by delivery and subsequent expression of exogenous DNA, which encodes for a missing defective gene product [2]. Appropriate gene vectors can be crucial for gene transfer. Vehicles for gene delivery which have been successfully demonstrated the delivery of exogenous genes in vivo, can be divided into two major groups: viral and non-viral vectors [3]. For the past 10 years various viral and non-viral vectors have been engineered for improved gene and drug delivery [1, 2]. Viral vectors are generally highly effective at delivering nucleic acids to a variety of cell populations, both dividing and non-dividing, yet these viral vectors are marred by significant safety concerns. Non-viral vectors are preferred for gene therapy, despite lower transfection efficiencies, and possess many customizable attributes that are desirable for tissue engineering applications [4]. Nonviral vector systems, including cationic lipids, polymers, dendrimers, and peptides, all offer potential routes for compacting DNA for systemic delivery. However, unlike viral analogues that have evolved means to overcome cellular barriers and immune defense mechanisms, nonviral gene carriers consistently exhibit significantly reduced transfection efficiency as they are hindered by numerous extra- and intracellular obstacles. However, biocompatibility and potential for large-scale production make these compounds increasingly attractive for gene therapy [5]. Cationic lipids and polymers, the most important non-viral vectors have many advantages over viral ones as they are non-immunogenic, easy to produce and not oncogenic [3].

Non-viral vectors, on the other hand, are relatively safe and easier to produce but their low gene delivery efficiency is the main bottleneck. Understanding of the detailed mechanisms and the main barriers in gene delivery both at extra- and intracellular levels and post-nuclear processes including transcription and translation of the transgene offers new perspective of the previously used vectors and designing of new proper vectors [6]. In todays time various PEG associated

PAMAM dendrimers have been used for the gene delivery applications. Also higher generation dendrimer have also been used for the same purpose [2,3].

In this investigation we study about the application of G1 PAMAMdendrimer as the non-viral vector for gene delivery.

Objectives:

- To check the compaction of DNA via dendrimer
- Transfection of the DNA into the cells using dendrimer as the non viral vector
- To study the releasing of DNA into the cells after the transfection of the nano complex.

2. REVIEW OF LITERATURE

2.1 GENE THERAPY

Gene therapy is a new therapeutic strategy that offers the promise of treating diseases via the production of therapeutic proteins within cells [1]. Gene therapy has gained significant attention over the past two decades as a potential method for treating genetic disorders such as severe combined immunodeficiency, cystic fibrosis, and Parkinson's disease, as well as an alternative method to traditional chemotherapy used in treating cancer. Gene therapy is mainly classified as:

- Germ-line gene therapy: Germ cells are modified by introduction of functional genes ordinarily integrated into their genomes. Change here is heritable i.e. passed on to later generations and now have proved to be effective theoretically incounteracting genetic disorders.
- Somatic cell gene therapy: *G*ene is introduced only in somatic cells especially of those tissues where gene expression is critical for health. Now this has emerged as the only feasible option leading to clinical trials for treatment of cancer and blood disorders. This is has further been divided on the basis of the end result of process into (i) Augmentation gene therapy and (ii) Targeted gene transfer. (I) *Augmentation gene therapy* involves introduction of functional gene in addition to defective gene endogenous to the cells i.e. the modified cells contain both defective (endogenous) as well as normal (introduced) copies of the gene. (ii) *Targeted gene transfer* involves homogenous recombination to replace endogenous gene with introduced functional gene.

Research efforts are currently focused on designing effective carrier vectors that compact and protect oligonucleotides for gene therapy: free oligonucleotides and DNA are rapidly degraded by serum nucleases in the blood when injected intravenously[3].

The characterstics of a good vector are as follows:

- Should replicate autonomously.
- Easy to isolate and purify.
- Easily introduced into host cells.
- Suitable marker genes to be present in the vector.

- Cells transformed with vector containing DNA insert (r-DNA) should be identifiable or selectable from those transformed by unaltered vectors.
- A vector should contain unique target sites for as many restriction sites as possible into which DNA insert can be integrated.

Mainly two types of vectors are used in gene therapy i.e. 1) Viral Vectors- All viruses bind to their hosts and introduce their genetic material into the host cell as part of their replication cycle. Therefore this has been recognized as a plausible strategy for gene therapy, by removing the viral DNA and using the virus as a vehicle to deliver the therapeutic DNA. A number of viruses have been used for human gene therapy, including retrovirus, adenovirus, lentivirus, herpes simplex virus, vaccinia, pox virus, and adeno-associated virus [6]. 2) Non-Viral Vectors- Non-viral methods can present certain advantages over viral methods, such as large scale production and low host immunogenicity [5, 21]. Previously, low levels of transfection and expression of the gene held non-viral methods at a disadvantage; however, recent advances in vector technology have yielded molecules and techniques that approach the transfection efficiencies of viruses. There are several methods for non-viral gene therapy, including the injection of naked DNA, electroporation, the gene gun, sonoporation, magnetofection, and the use of oligonucleotides, lipoplexes, dendrimers, and inorganic nanoparticles. Numerous researches claim that non-viral vectors (e.g. cationic surfactants, lipids, polymers, dendrimers and peptides)[4, 7] present more advantage than the viral vectors for the delivery of nucleic acid (DNA/ RNA) into specific cells for gene delivery [21]. To date, the majority of gene therapy clinical trials performed have used viral vectors for gene delivery. However, there are major practical limitations to viral gene delivery systems, including difficulties of scale-up production, lack of selectivity, inadequate infectivity in vivo, susceptibility to neutralization by serum antibodies (particularly on second and subsequent administrations) and inflammatory responses raised against successfully transduced cells. Although intense efforts are under way to overcome these problems, some are hard to address and may be insurmountable. Consequently, considerable attention is turning towards the development of nonviral systems for gene delivery, using basic molecular engineering to design vectors capable of adequate gene delivery without the baggage inherent in millions of years of evolution as a niche pathogen.

2.1.1 Types of Non-viral Vectors

Many types of synthetic vectors have been developed in recent times keeping in mind their aim of compacting DNA and protecting it throughout its journey upto cell nucleus. The various approaches include:

(I) Cationic lipids

(ii) Cationic polymers

(iii) Gold nanoparticles

(iv) Magnetic nanoparticles

(v) Quantum dots

(vi) Silica nanoparticles

(vii) Fullerenes

(viii) CNTs

(ix) Supramolecular systems

Cationic lipids: Cationic lipids are made up of a cationic head group attached by a linker to a lipid hydrophobic moiety. The positively charged head group is necessary for the binding of nucleic acid phosphate groups. All cationic lipids are therefore positively charged amphiphile systems. They can be classified into various subgroups according to their basic structural characterstics. *(1) Monovalent aliphatic lipids:* Characterized by a single amine function in their head group, e.g. N[1-(2,3- dioleyloxy) propel]-N,N,N-trimethylammonium chloride (DOTMA), 1,2-dioleyl- 3 trimethylammoniumpropane (DOTAP), N-(2-hydroxyethyl)-N,N-dimethyl 2,3-bis(tetradecycloxy-1-propanaminium bromide (DMRIE). *(2) Multivalent aliphatic lipids:* Whose polar head groups contain several amine functions such as the spermine group, e.g. dioctadecylamidoglycylspermine (DOGS). Cationic lipids present three main components: a polar head group, which allows compaction of the DNA, a lipidic chain, which allows self-association through hydrophilic interaction forming micelles or liposomes; and a linker, which interconnects the two abovementioned functional groups. Cationic lipids and DNA usually interact forming lipoplexes, which are multilayered structures where the DNA is sandwiched between the cationic lipids. The lipoplexes formed by these vectors presented strong cationic surface and great stability, resulting in strong cationic surface and great stability, resulting in strong binding affinities for cell surfaces. Used in conjunction with cholesterol to aid membrane fusion, these derivatives were seen to effectively transfecting hepatic and intra-tumoral cells both

in vitro and in vivo. They can self-assemble by cooperative hydrophobic intermolecular binding at a certain concentration, forming cationic liposomes. The lipids can then bind and compact nucleic acid by electrostatic interaction between the positively charged polar heads of the lipids and the negatively charged phosphate groups of the DNA, forming cationic lipid/DNA complexes a.k.a. lipoplexes. The nucleic acid is protected from the degradation of nucleases and is able to reach the desired site of the cells. Cationic lipids especially facilitate the transfection during the early stage of the intracellular process by condensing the DNA and binding with cell membranes. Cationic lipids offer greater ease of use and efficiency with low toxicity. However, cationic lipids become cytotoxic, usually resulting in 30-40% loss in viability beyond certain charge ratio (lipid/DNA=3:1). Taking in account the toxicity level, different vectors came into existence.

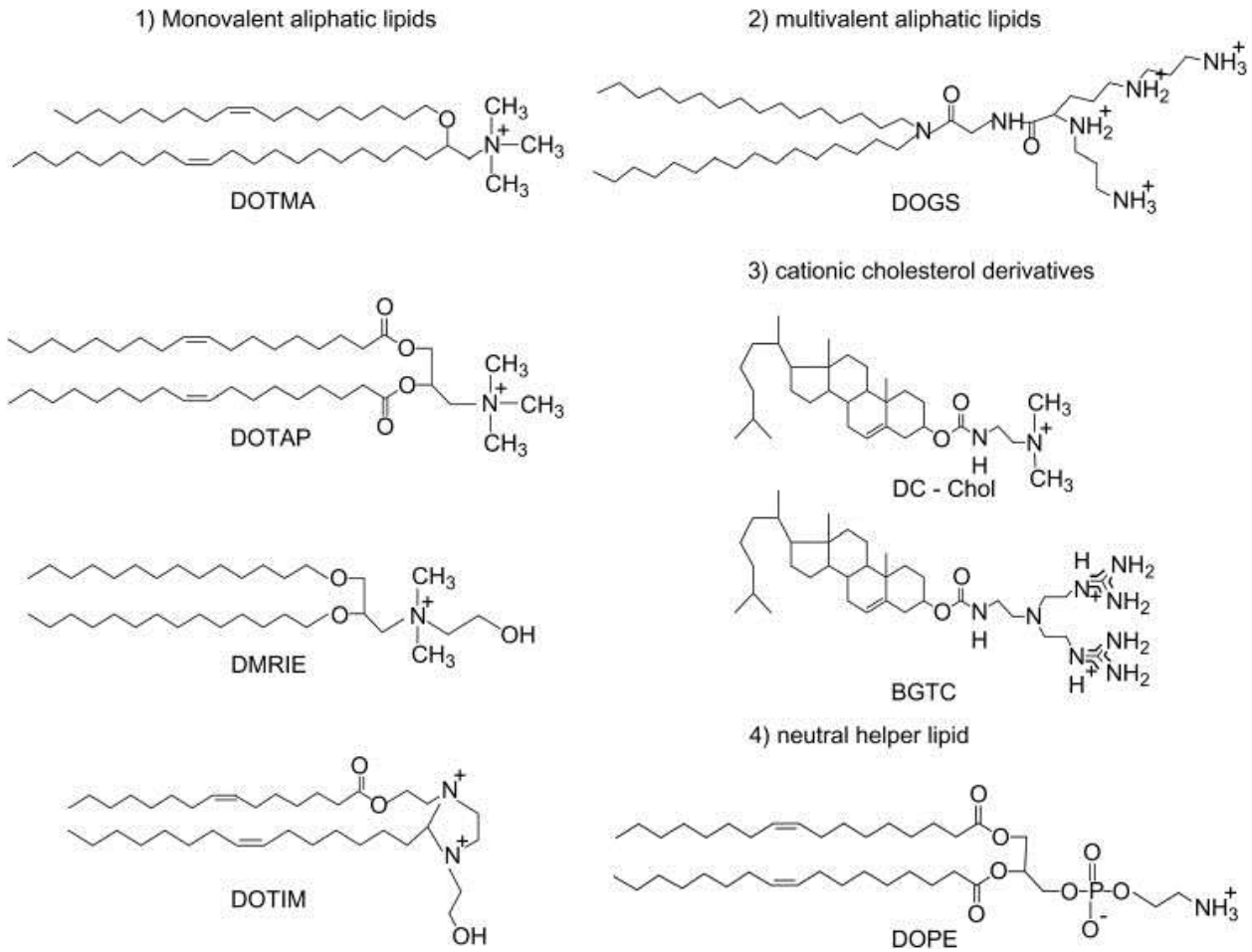


Figure 2.1: Structure of current cationic lipids used in gene therapy and the helper lipid DOPE. DOTMA: N[1-(2,3-dioleyloxy) propyl]-N,N,N-trimethylammonium chloride, DOTAP: 1,2-dioleyl-3-trimethylamonium-propane,

DMRIE: N-(2- hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy-1-propananium bromide), DOTIM: 1-[2-dioleoyloxy)ethyl]-2-oleyl-3- (2-hydroxyethyl)imidazolinium chloride, DOGS: dioctadecylamidoglycylspermine, DC-Chol: [N-(N0,N0- dimethylaminoethane)-carbamoyl]cholesterol, BGTC: bis-guanidium-tren-cholesterol, DOPE: 1, 2-dioleyl-sn-glycerol-3- phosphoethanolamine

Cationic Polymers: Cationic polymers are usually classified in two main groups: natural polymers, such as proteins and peptides, polysaccarides, and synthetic polymers, such as polyethyleneimine (PEI), dendrimers, and polyphosphoesters. They interact with the DNA through electrostatic interaction by means of amines and/ or ammonium ions. The ratio between the number of vector's amines and the number of phosphates in the pDNA is referred to as the N/P ratio, which dictates morphology and size of the complex.

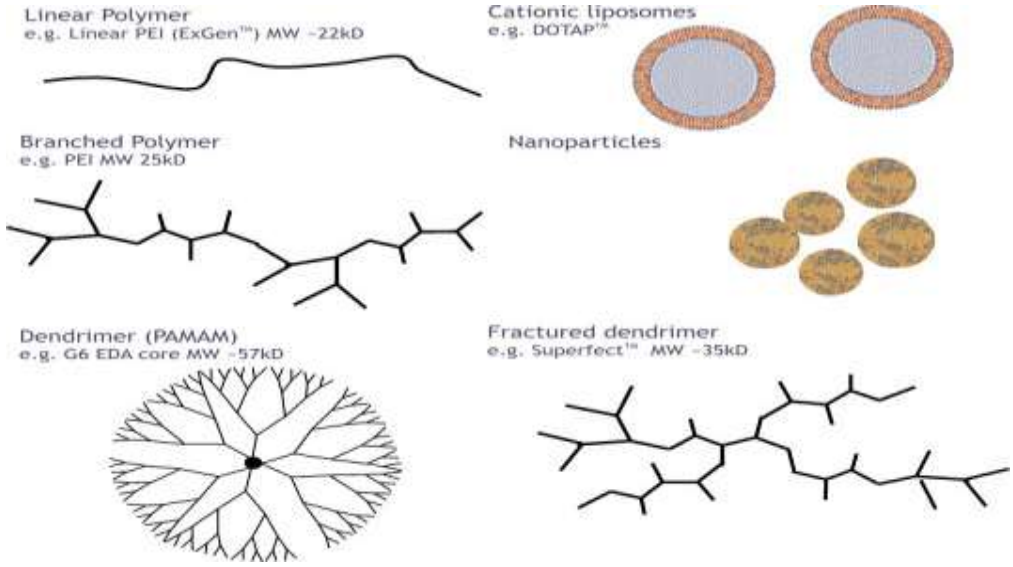


Figure 2.2 Examples of cationic polymers

Among the polycations used for gene delivery, PEI is also a very interesting candidate and another of the most extensively investigated carriers, due to its compacting skills and its buffering ability, which allow having in some cases transfection efficiencies comparable to those of viral vectors. The physicochemical properties of PEI based polyplexes and structure- function relationships were recently reviewed by Kissel. Biodegradable polymers based on poly (aminoesters) are another promising class of vectors because of their low cytotoxicity due to cleavage of the ester bonds by the plasma enzymes. Diverse poly (B-aminoester) polymers have been synthesized and tested for gene therapy. Currently, commonly used cationic polymers include poly (L-lysine) (PLL), polyethylenimines (PEI), polyamidoamine dendrimers (PAMAM), and chitosan. Among them, PEI and PAMAM are the most frequently used because

of the excellent transfection efficiency. It has been shown that (PAMAM) (< 5 nm) can selectively target tumor cells when loaded with an anticancer drug [2].

Several biological barriers must be overcome to achieve efficient nonviral gene delivery [5]. These barriers include binding to the cell surface, traversing the plasma membrane, escaping lysosomal degradation, and overcoming the nuclear envelope [1].

Functional devices can be further introduced to overcome different cellular barriers and include the use of targeting ligands to increase cellular uptake through receptor-mediated endocytosis, membrane active lipids and peptides to enhance the endosomal release, and nuclear localization signals to enhance nuclear delivery. The appropriate design of a nonviral gene vector requires a complete understanding of both the characteristics of the vectors as well as the mechanisms by which they interact with the targeted cells. Several internalization mechanisms have been proposed to explain the uptake of different synthetic vectors. The uptake mechanisms are, in general, closely linked with the intracellular trafficking and the fate of the vectors. A promising strategy for increasing the efficiency of nonviral vectors is to target certain uptake pathways that improve the intracellular fate of the particles.

2.2 Dendrimers:

Dendrimers- Dendrimers are a new class of polymeric materials. They are highly branched, monodisperse macromolecules. The structure of these materials has a great impact on their physical and chemical properties. As a result of their unique behaviour dendrimers are suitable for a wide range of biomedical and industrial applications [24]. In this investigation of our report we are using dendrimers as the DNA compacting agent therefore we will be focusing more on the dendrimers and the compaction of DNA by dendrimers. Dendrimers and hyperbranched polymers are a relatively new class of materials with unique molecular architectures and dimensions in comparison to traditional linear polymers. Polymer chemistry and technology have traditionally focused on linear polymers, which are widely in use. Linear macromolecules only occasionally contain some smaller or longer branches. In the recent past it has been found that the properties of highly branched macromolecules can be very different from conventional polymers. The structure of these materials has also a great impact on their applications. First discovered in the early 1980's by Donald Tomalia and co-workers [25], these hyperbranched molecules were called **dendrimers**. The term originates from 'dendron' meaning a tree in Greek. At the same time, Newkome's group [26] independently reported synthesis of similar

macromolecules. They called them **arborols** from the Latin word 'arbor' also meaning a tree and describes graphically the structure of this new class of macromolecules which have highly branched, three-dimensional features that resemble the architecture of a tree.

2.2.1 Structure of Dendrimer:

A typical dendrimer (Fig.2.7) consists of three main structural or architectural components [27, 28,29].

(i) An initiator core.

(ii) Interior layers (generations) composed of repeating units, radically attached to the interior core.

(iii) Exterior (terminal functionality) attached to the outermost interior generations.

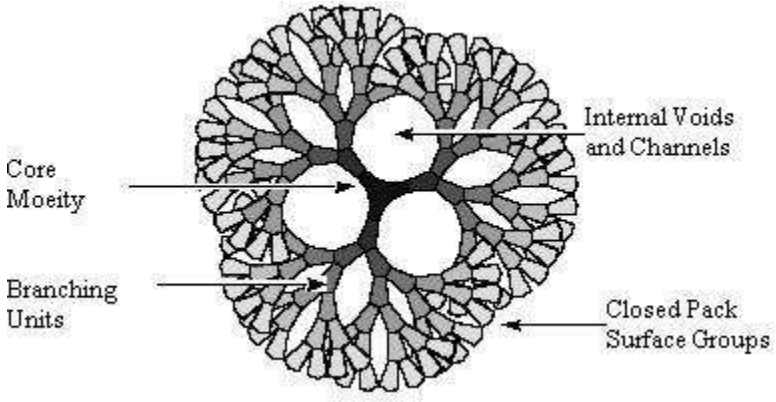


Figure2.3 Structure of Dendrimer

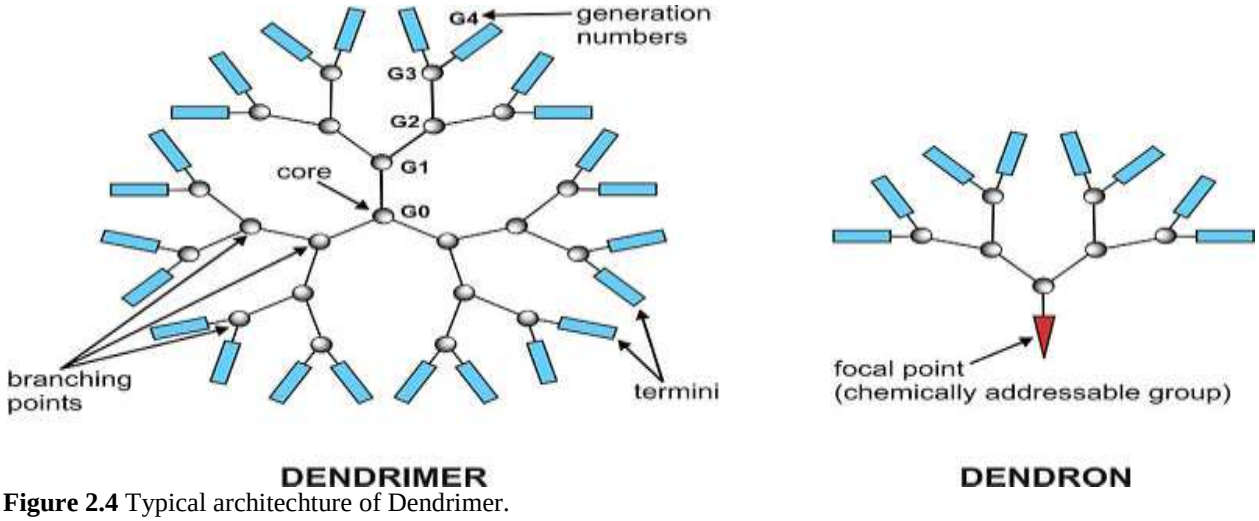


Figure 2.4 Typical architechture of Dendrimer.

The field of dendritic molecules can be roughly divided into low-molecular weight and high-molecular weight species. The first category includes dendrimers and dendrons, and the latter includes dendronized polymers, hyperbranched polymers, and the polymer brush.

2.2.1.1 Components of Dendrimer Structure [24, 27]:

2.2.1.1a) Generation- It is the hyperbranching when going from the centre of the dendrimer towards the periphery, resulting in homostructural layers between the focal points (branching points). The number of focal points when going from the core towards the dendrimer surface is the generation number. That is a dendrimer having five focal points when going from the centre to the periphery is denoted as the 5th generation dendrimer. Here, we abbreviate this term to simply a G5-dendrimer, *e.g*. a 5th generation polypropylene imine is abbreviated to a "G5-PPI-" dendrimer, The core part of the dendrimer is sometimes denoted generation "zero", or in the terminology presented here "G0". The core structure thus presents no focal points, as hydrogen substituents are not considered focal points. Intermediates during the dendrimer synthesis are sometimes denoted half-generations, a well-known example is the carboxylic acid-terminated PAMAM dendrimers.

2.2.1.1b) Shell- The dendrimer shell is the homo-structural spatial segment between the focal points, the "generation space". The "outer shell" is the space between the last outer branching point and the surface. The "inner shells" are generally referred to as the dendrimer interior.

2.2.1.1c) Pincer- In dendrimers, the outer shell consists of a varying number of pincers created by the last focal point before reaching the dendrimer surface. In PPI and PAMAM dendrimers

the number of pincers is half the number of surface groups (because in these dendrimers the chain divides into two chains in each focal point).

2.2.1.1d) End Group- It is also generally referred to as the "terminal group" or the "surface group" of the dendrimer. Dendrimers having amine end-groups are termed "amino-terminated dendrimers".

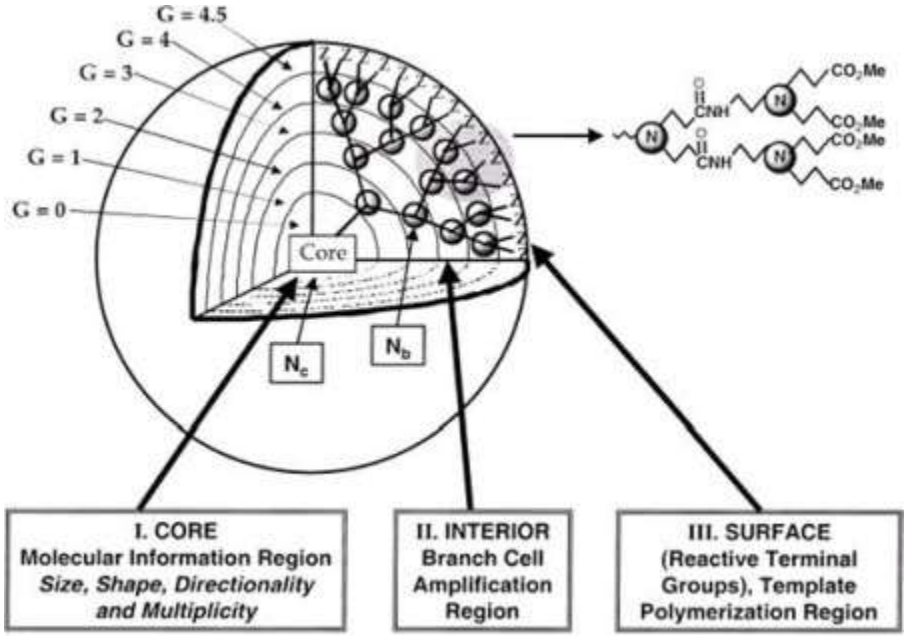


Figure2.5: Three dimensional projection of dendrimer core-shell architecture for G=4.5 PAMAM dendrimer with principal architectural components (I) core, (II) interior & (III) surface

2.2.2 Synthesis of Dendrimers:

The macromolecule dimension of dendrimer can be controlled as the synthesis involves a repetitive sequence of steps. Dendrimers are synthesized from monomers of the ABx type (xP2) through a step-growth polymerization process. There are two general synthetic approaches used to generate dendrimers: (i) divergent, and (ii) convergent [27].

(i) Divergent Method [24, 27, 33]:

In divergent method, The dendrimer is assembled from a multifunctional core, which is extended outward by a series of reactions, commonly a Michael reaction. Each step of the reaction must be driven to full completion to prevent mistakes in the dendrimer, which can cause trailing generations (some branches are shorter than the others). Such impurities can impact the

functionality and symmetry of the dendrimer, but are extremely difficult to purify out because the relative size difference between perfect and imperfect dendrimers is very small.

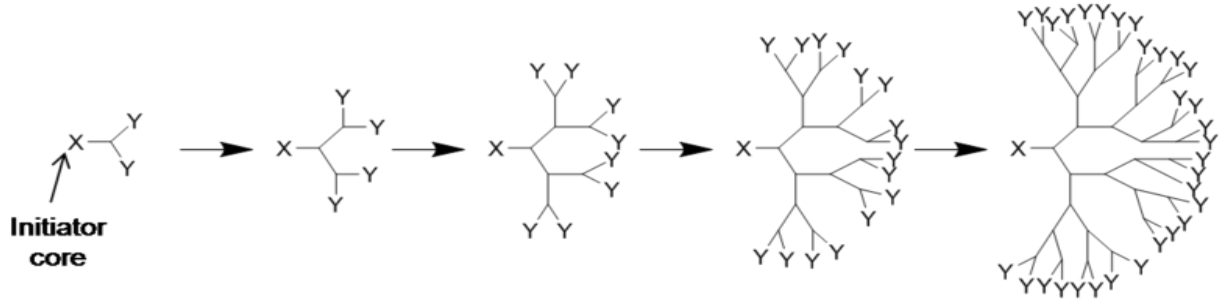


Figure 2.6 Divergent method of dendrimer synthesis

(ii) Convergent Method [33]:

Dendrimers are built from small molecules that end up at the surface of the sphere, and reactions proceed inward building inward and are eventually attached to a core. This method makes it much easier to remove impurities and shorter branches along the way, so that the final dendrimer is more monodisperse [24]. However dendrimers made this way are not as large as those made by divergent methods because crowding due to steric effects along the core is limiting.

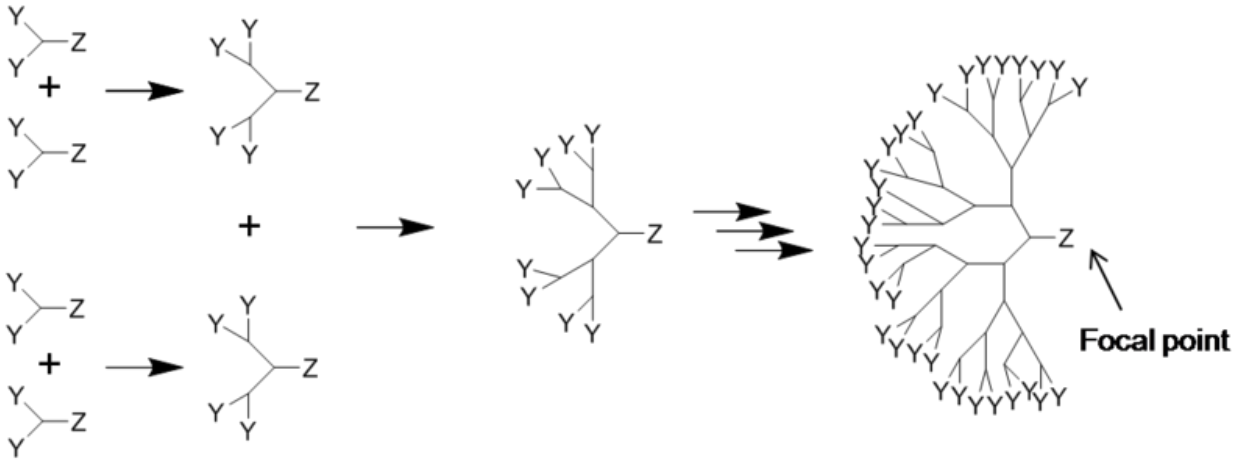


Figure 2.7 Convergent method for synthesizing dendrimers

2.2.3 Properties of dendrimers are as follows [24, 27]:

1. Dendrimers and dendrons are monodisperse and usually highly symmetric, spherical compounds [24].
2. Dendritic encapsulation of functional molecules allows for the isolation of the active site, a structure that mimics that of active sites in biomaterials.

3. Since the properties of dendrimers are dominated by the functional group, it is possible to make dendrimers water soluble, unlike most polymers, by functionalizing their outer shell with charged species or other hydrophilic groups.
4. Other controllable properties of dendrimers include toxicity, crystallinity, tecto-dendrimer formation, and chirality.

These properties of the dendrimer make them suitable vwctor for gene delivery purpose.

2.2.4 Applications of dendrimers:

There are now more than fifty families of dendrimers, each with unique properties, since the surface, interior and core can be tailored to different sorts of applications. Many potential applications of dendrimers are based on their unparalleled molecular uniformity, multifunctional surface and presence of internal cavities. These specific properties make dendrimers suitable for a variety of high technology uses including biomedical and industrial applications [24].

Applications of dendrimers typically involve conjugating other chemical species to the dendrimer surface that can function as detecting agents (such as a dye molecule), affinity ligands, targeting components, radioligands, imaging agents, or pharmaceutically active compounds. They have proved to be potentially applicable in the following fields [24, 30]:

1. Controlled Drug Delivery: A variety of molecules, such as drugs and other therapeutic agents, can be loaded both in the interior void space and on the surface of PAMAM dendrimers to control the rate of release of these agents into the body.
2. In-vitro Gene transfection: PAMAM dendrimers are used in in-vitro transfection of a broad range of cells with DNA. They form dendrimer:DNA complexes that provide reproducible high transfection efficiency and have low cytotoxicity.
3. Sensors: Due to their organized structure, ease of modification, and strong adsorption behavior to a variety of substrates, PAMAM dendrimers can be used to produce monolayers or stacked film layers, which can be used as sensors to detect hazardous chemical materials.
4. Blood Substitution
5. Synthesis of monodisperse metallic nanoparticles: eg. Silicon based dendrimer PAMAMOS.

6. In-vitro diagnostics: Dendrimer-antibody conjugates are used commercially in an immunoassay for rapid and sensitive detection of cardiac markers indicative of heart attacks.
7. Size standards/ Molecular templates: The exceptionally uniform molecular size of the various PAMAM dendrimer generations enable their use as calibration standards or precise "scaffolds" and templates to make organized thin films and stacked layers.

Poly (amidoamine), or PAMAM, is perhaps the most well known dendrimer [31]. The core of PAMAM is a diamine (commonly ethylenediamine), which is reacted with methyl acrylate, and then another ethylenediamine to make the generation-0 (G-0) PAMAM. Successive reactions create higher generations, which tend to have different properties. Lower generations can be thought of as flexible molecules with no appreciable inner regions, while medium sized (G-3 or G-4) do have internal space that is essentially separated from the outer shell of the dendrimer. Very large (G-7 and greater) dendrimers can be thought of more like solid particles with very dense surfaces due to the structure of their outer shell.

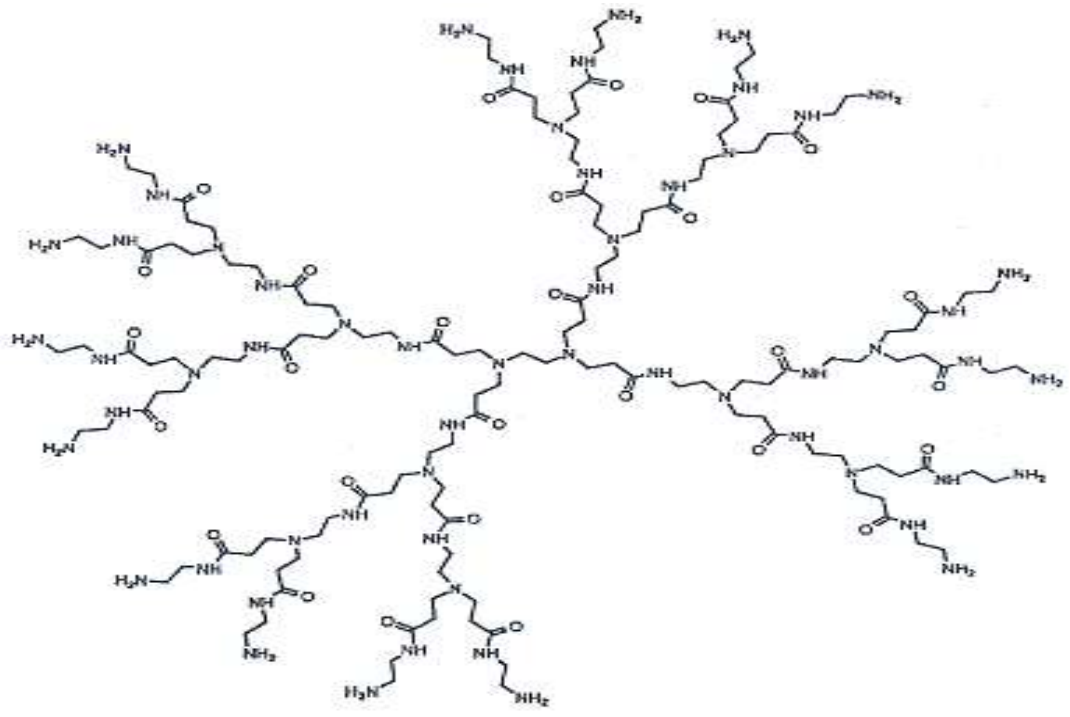


Figure 2.8 PAMAM dendrimer

The table(2.2) below shows the calculated theoretical properties of amine surface functional PAMAM dendrimers by generation:

Generation	Molecular Weight	Measured Diameter (A^0)	Surface groups
0	517	15	4
1	1,430	22	8
2	3,256	29	16
3	6,909	36	32
4	14,215	45	64
5	28,826	67	128

Table 2.2 PAMAM dendrimers properties

Polyamidoamine (PAMAM) have received most attention as potential transfection agents for gene delivery, because these macromolecules bind DNA at physiological pH [24, 31]. The binding is driven by the electrostatic interaction, and the larger the dendrimer charge, the stronger the binding affinity. As DNA wraps/ binds to the dendrimer, counter-ions originally condensed onto DNA (Na+) and the dendrimer (Cl^-) get released [34].

The aim to study PAMAM in this investigation is to examine the interaction of DNA with several dendrimers of different compositions and at physiological conditions, using constant DNA concentration and various dendrimer contents because at physiological pH, a PAMAM dendrimer is positively charged and can effectively bind negatively charged DNA [31]. Due to their binding property with DNA at physiological pH, PAMAM is majorly used for the compaction of DNA and thus very useful vector for the gene delivery. The potential of PAMAM dendrimers in controlled drug delivery has been extensively investigated and arises from the high number of arms and surface amine groups that can be utilized to immobilize drugs, enzymes, antibodies, or other bioactive agents. Such conjugates provide a high density of biological agents in a compact system. In addition, PAMAM dendrimers have shown potential as oligonucleotide and gene delivery systems, have been used to improve the solubility of sparingly soluble drugs such as piroxicam, and have increased the amount of therapeutic radionuclides delivered to cancer cells. The unique architecture of PAMAM dendrimers makes them suitable models for studying the influence of a controlled incremental increase in size, molecular weight, and number of amine surface groups on the microvascular extravasation of polymeric drug carriers [32].

The compaction of DNA using dendrimer can be studied by various techniques like Scanning Electron Microscopy (SEM), X-ray diffraction etc. Due to the progress in nanoscience and nanotechnology requires tools that enable the imaging and manipulation of matter at the atomic and molecular scale. During the last two decades or so, scanning probe-based techniques have

proven to be particularly versatile in this regard. Among the various probe-based approaches, atomic force microscopy (AFM) stands out in many ways, including the total number of citations and the breadth of possible applications, ranging from materials characterization to nanofabrication and biological studies [35]. Therefore we will be studying about Atomic force microscopy (AFM) for the analysis of compaction of DNA by PAMAM dendrimer.

2.3 STEPS INVOLVED DURING GENE DELIVERY [1,5,8,10,16,17]

Gene delivery is a potential therapeutic method for treating a wide variety of diseases [17]. An important objective of modern biology is to develop efficient methods for therapeutic gene delivery, allowing the transport of DNA or other genetic material through the cell and its release into the nucleus [18]. The first step in the preparation of the gene vehicle involves the condensation of the nucleic acid. Another crucial step in gene delivery is the dissociation of such nucleic acid based cationic complexes in the cytoplasm of the target cell. Sequential steps involved in gene transfection are as follows:

1. Gel Retardation of DNA complex
2. Gel release
3. EtBr exclusion assay
4. Uptake by cell membrane and its interation with cell membrane
5. Endosomal escape
6. Transfection into the cell line

Cationic lipids are typically used in the form of cationic liposomes. All cationic lipids possess hydrophobic groups, which may be either one or two fatty acid or alkyl moieties that are 12 to 18 carbons in length, in addition to a positively charged polar head group. The hydrophobic moieties and head groups cause the cationic lipids to assemble into bilayer vesicles (liposomes) when are dispersed in aqueous solutions. However, many cationic liposomes cannot form liposomes alone and are normally accompanied by a neutral lipid such as dioleoylphosphatidylethanolamine (DOPE) or cholesterol.

DOPE is frequently useful because it can fuse with other lipids when exposed to a low pH, such as in endosomes, which aid in the release of the associated DNA into the cytosol. Cholesterol provides structural stability, and there is evidence that it can influence targeting in vivo via

scavenger receptors. Lipoplexes are typically formed by direct mixing between cationic liposomes and DNA solutions. Positively charged liposomes bind to negatively charged phosphate molecules on the DNA backbone through electrostatic interactions. Generally, complexes are formed with a slight excess positive charge to permit them to interact with the negatively charged cell surface. The ratio between the cationic charge of the liposome and the negative charge of the DNA usually controls the size of lipoplexes. At high positive or negative charge ratios, relatively small complexes are formed, whereas large aggregates are usually formed when the net charge is close to neutrality. The cationic liposomes used are typically small (_100 nm) before adding to DNA; however, complexes formed with DNA exhibit diameters that range from as small as 200 nm to structures as large as 2 _m. The formation of lipoplexes is generally difficult to control, and different structures are produced in the same lipoplex preparation.

The proposed model for describing the interaction between cationic liposomes and DNA involves the following. First, liposomes cause a compaction of the DNA molecules and charge neutralization. Second, neutralization may induce aggregation, resulting in the formation of a heterogeneous group of multilamellar structures of different shapes and consisting of DNA sandwiched between lipid bilayers. Third, it is proposed that DNA affects the liposomes, inducing lipid mixing and rearrangement resulting in fusion of the multilamellar structures to form large DNA-lipid complexes. Usually, positively charged lipoplexes lead to more efficient gene expression by virtue of ionic interactions with the negative cell surface. In addition to the compaction and neutralization of DNA, cationic liposomes provide a protective role against extra- and intracellular nucleases. This can be attributed to the compaction and covering of DNA by the lipid bilayers. Unfortunately, the positive charge of lipoplexes makes them susceptible to interaction with negative constituents in the circulation after in vivo admini stration, which significantly limits their use in systemic administration. Although smaller-sized lipoplexes would be expected to be more efficiently internalized via endocytosis, larger lipoplexes have been reported to improve transfection activities. This may be due to the greater ability of the larger particles to sediment onto the cell surface. However, the in vivo transfection ability of larger complexes is weak, mainly because of their inability to reach the target cells because of their large size, which also renders them more susceptible to interaction with extracellular components. Cationic polymers can also condense and neutralize DNA. They do not have

hydrophobic moieties, but they can condense the DNA more efficiently resulting in smaller DNA condensed particles. Among the many cationic polymers available, the most frequently used in gene delivery are poly-L-lysine (PLL)- and PEI-based polymers. Unlike lipoplexes, polyplexes formed with PLL usually use ligands to facilitate their cellular uptake, and endosomolytic reagents are usually used to facilitate endosomal escape. PEI-based polyplexes are more efficient and do not require agents for endosomal escape. Upon mixing with DNA, electrostatic interactions occur between the cationic charge of the polymer and the negative charge of the DNA, resulting in the formation of particles, as small as 20 to 40 nm in some cases.

Cellular Binding: Unless a specific targeting ligand is incorporated in the system, the binding of lipoplexes and polyplexes to the cell surface is the result of a nonspecific ionic interaction between the positive charge of the complexes and the negative charge of the cell surface. Negatively charged cell surface constituents, such as heparan sulfate proteoglycans and integrins play a role in the cellular binding of positively charged lipoplexes, polyplexes or even cationic peptides, such as TAT. For example, in proteoglycan-deficient mutant cells, the cellular binding of lipoplexes and polyplexes is reduced. The presence of soluble heparin and heparin sulfate in the medium competitively inhibits the binding. At this point, the heparan sulfate proteoglycans may act as nonspecific receptors for cationic macromolecules, but their exact role in mediating cellular uptake is not clear. There is some evidence to show that the transmembrane proteins, syndecans, may cluster to form focal points at the plasma membrane during binding to cationic particles and this clustering induces their interaction with the actin cytoskeleton, probably resulting in the formation of tension fibers. This tension provides the energy required to engulf the particles. Similarly, a recent report suggested that the uptake of PEI polyplexes occurs through actin-mediated phagocytosis as a result of the adhesion of polyplexes to syndecan molecules followed by clustering.

Cellular Uptake: The internalization mechanism of lipoplexes is not well understood. Early reports suggested that fusion between the lipids and the plasma membrane is responsible for delivering DNA directly to the cytoso. It was suggested that the interaction between the liposomes and DNA or the cell membrane destabilize the liposomes, thus facilitating their fusion with each other and with other membranes. However, most of the following experimental evidence supports the involvement of endocytosis as a main entrance route (Fig.2.6) . For

example, the use of endocytosis inhibitors significantly reduces gene expression. Furthermore, interference of the endocytic pathway with lysosomotropic reagents such as chloroquine was found to enhance the gene expression. The strongest evidence comes from electron microscopy imaging of gold labeled DNA, which clearly shows the presence of DNA in intracellular vesicles, a typical entry via endocytosis. In general, it is currently believed that membrane fusion is important for transfection but that most of the uptake occurs through endocytosis. Membrane fusion occurs as a result of endosome acidification and is responsible for releasing the endosome contents to the cytosol. In contrast to this general belief, Almofti et al. (2003) proposed that the uptake of lipoplexes occurs by endocytosis but that membrane fusion occurs mostly (72%) at the plasma membrane level, and it is essential for endocytosis to occur. The current question is which pathway of endocytosis is responsible for uptake. The available data show diverse results. Rejman et al. (2005) reported that the uptake of lipoplexes formed between the cationic lipid DOTAP and DNA is inhibited by chlorpromazine and potassium depletion but is unaffected by filipin and genestein, suggesting that the uptake occurs solely by clathrin-mediated endocytosis. Furthermore, they have shown that particles that are internalized by CME are eventually degraded in lysosomes. Earlier, Zhou and Huang (1994) suggested that the uptake of lipopoly-Llysine lipoplexes occurs through clathrin-mediated endocytosis because the presence of the actin-depolymerizing reagent cytochalasin B increased transfection activities. Cholesterol depletion with methyl-_-cyclodextrin decreased the activities of SAINT-2-DOPE lipoplexes. This is largely indicative of nonclathrin endocytosis; however, inhibitors of caveolae such as filipin and cytochalasin D had only a slight effect on internalization. Colocalization with Tf (a CME marker) and the inhibition by potassium depletion further confirmed the involvement of CME. In contrast to these reports, Matsui et al. (1997) suggested that the uptake of LipofectACE lipoplexes occurs through phagocytosis in poorly differentiated airway epithelia cells. Similar to the uptake of large (2 _m) microspheres, which were used as markers for phagocytosis, they found that the uptake of lipoplexes was inhibited by cytochalasin B as well as by potassium depletion. The uptake of polyplexes also occurs through endocytosis, but without fusion with the cell membrane, and similarly shows some diverse results regarding specific uptake pathways. In general, it is believed that the uptake of PLL and PEI complexes occurs through CME Goncalves et al. (2004) have shown that the uptake of His-pLK polyplexes occurs through both clathrin-dependent and -independent pathways; the latter is mostly macropinocytosis, because it was

inhibited by amiloride and stimulated by phorbol esters. Furthermore, they found that macropinocytosis of the polyplexes and the recycling of DNA impaired the transfection and concluded that CME is the most productive pathway. Rejman et al. (2005) also suggested that the uptake of PEI polyplexes occurs through both clathrin-dependent and -independent pathways; however, they suggested that the latter mechanism involves the caveolae because it was inhibited by filipin and genestein. Another difference arises from their finding that CME is less productive since caveolar internalization escapes lysosomes, thus leading to efficient transfection. The diversity of results suggests that a variety of factors may affect the actual mechanism. However, in general these results collectively suggest that endosomal escape is a significant barrier to lipoplex- and polyplex-mediated transfection.

ENDOSOMAL ESCAPE: After internalization via endocytosis, the internalized molecules exist in endosomes with no access to the cytosol or the nucleus. 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. Lipoplexes containing the pH-sensitive fusiogenic lipid DOPE can release the associated DNA into the cytosol. DOPE forms a stable lipid bilayer at physiological pH _7; however, at an acidic pH 5 to 6, it undergoes a transition from a bilayer to an inverted hexagonal structure, which fuses and destabilizes the endosomal membrane, releasing its contents to the cytosol. Evidence exists to show that fusion with the endosomal membrane is essential for DOPE-containing lipoplexes. It is possible that only DNA or the lipoplex as a whole will be released to the cytosol after fusion. If lipoplexes are released, the dissociation of DNA must occur in the cytosol or even at the nuclear membrane to achieve transfection. Similar to DOPE, which has an intrinsic ability to cause endosomal release, the polycation PEI has the same ability, although through a different mechanism. This is evident by the observation that transfection with PEI polyplexes is not improved by fusiogenic peptides or chloroquine. A proton sponge hypothesis was proposed by Behr and coworkers to explain this phenomenon. This hypothesis suggests that PEI becomes more protonated at low pH as in endosomes. This protonation triggers an influx of Cl_ ions with protons leading to a water influx and finally the swelling and rupturing of the endosomes.

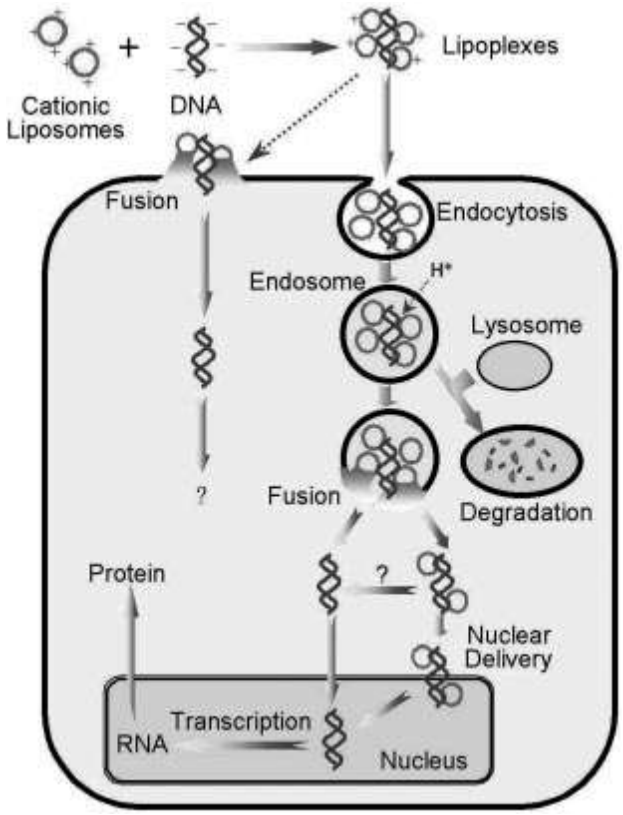


Figure 2.9 Uptake and intracellular trafficking of cationic liposome/DNA complexes (lipoplexes). Lipoplexes are mainly taken up through endocytosis. The low pH of endosomes causes fusion between the liposomal and endosomal membranes. The DNA is released to the cytosol either free or associated with the lipids and is then delivered to the nucleus for translation into protein. Fusion may also occur at the plasma membrane, thusdelivering the DNA directly to the cytosol.

NUCLEAR DELIVERY: The nuclear envelope contains nuclear pores with a passive transport limit of 70 kDa molecular mass or _10 nm diameter. This is much smaller than the size of DNA, even when condensed in lipoplexes or polyplexes. Microinjection of plasmid DNA encoding _-galactosidase into the nucleus produced a much higher gene expression than when the same plasmid was microinjected into the cytosol. This suggests that the nuclear envelope is a significant barrier against transfection. How then is the DNA is delivered to the nucleus? The most widely accepted model is that cell division is an important factor in the nuclear translocation of transgenes. During mitosis, the integrity of the nuclear membrane is transiently lost, which allows the nuclear entry of transgenes. This is the case in the in vitro transfection with dividing cells, whereas in vivo transfection usually targets differentiated nondividing cells. Therefore, the nuclear envelope cannot be neglected in in vivo situations. DNA could be detected in the nucleus in time intervals as low as 1 h after lipoplex-mediated transfection, which suggests that a different mechanism is involved in this early nuclear delivery. We have previously shown

that lipoplexes can fuse with the nuclear membrane, thus releasing DNA to the nucleus. Targeting efforts to enhance this mechanism are needed, especially for the transfection of nondividing cells. The nuclear injection of lipoplexes results in poor gene expression compared with injection of naked DNA. This finding suggests that decondensation in the nucleus is a poor process. The generally accepted model was proposed by Xu and Szoka (1996) and involves the release of DNA from lipoplexes during endosomal release, thus delivering only naked DNA to the cytosol. In contrast to cationic lipids, the microinjection of PEI polyplexes did not affect the transgene expression when the complexes are injected into the nucleus, suggesting that a rapid release of DNA from the polyplexes occurs in the nucleus, probably via an exchange with cellular DNA. Nuclear proteins require a nuclear localization signal (NLS), which contains basic amino acids and can be recognized by cytosolic factors to mediate active transport through the nuclear pore complex. During this active transport, the diameter of the nuclear pore complex is expanded to _30 nm, and this allows the delivery of nuclear proteins to the nucleus. The same approach can be used to enhance gene delivery to the nucleus. The nuclear delivery of DNA was increased by the coupling of 100 NLS peptides/kilobase pair of DNA. The amount of the NLS peptides seems to be important in delivering the gene. In addition, the peptide should be coupled to the DNA in the case of polyplexes, because coupling of the NLS to the polycation PLL did not enhance gene expression.

2.4 TECHNIQUES USED IN CHARACTERISATION OF DNA/ CATIONIC LIGAND COMPLEX

- Gel retardation- The condensation of DNA by cationic polymers can be measured by a reduction of the mobility of DNA on agarose that leads to the retention of DNA in the wells. This assay can be performed using ethidium bromide to detect DNA and may be analysed on the photo-imaging kit to quantify how much DNA is retained in the wells at different charge ratios.
- EtBr exclusion assay- The concentration of ethidium bromide used in this assay is an important parameter that can affect the condensation of DNA. If too much ethidium bromide is used it will cause stabilisation and neutralisation of the DNA structure and inhibit its condensation with cationic polymers. For this reason, and to keep the assay as physiologically relevant as possible, the concentration of ethidium bromide used was as

low as possible (400 ng/ml) given the limits of detection of the Kontron fluorimeter. This concentration of ethidium bromide was used in all fluorimetric assays to standardise the technique and allow comparison of the ability of different polymers to condense DNA.

- Circular Dichorism Measurement- Circular dichroism (CD) spectroscopy measures differences in the absorption of left-handed polarized light versus right-handed polarized light which arise due to structural asymmetry. The absence of regular structure results in zero CD intensity, while an ordered structure results in a spectrum which can contain both positive and negative signals.
- UV spectroscopy- Ultraviolet–visible spectroscopy or ultraviolet-visible spectrophotometry (UV-Vis or UV/Vis) refers to absorption spectroscopy or reflectance spectroscopy in the ultraviolet-visible spectral region. This means it uses light in the visible and adjacent (near-UV and near-infrared (NIR)) ranges. The absorption or reflectance in the visible range directly affects the perceived color of the chemicals involved. In this region of the electromagnetic spectrum, molecules undergo electronic transitions. This technique is complementary to fluorescence spectroscopy, in that fluorescence deals with transitions from the excited state to the ground state, while absorption measures transitions from the ground state to the excited state.
- UV melting- Heat denaturation of DNA, also called melting, causes the double helix structure to unwind to form single stranded DNA. When DNA in solution is heated above its melting temperature (usually more than 80 °C), the double-stranded DNA unwinds to form single-stranded DNA. The bases become unstacked and can thus absorb more light. In their native state, the bases of DNA absorb light in the 260-nm wavelength region. When the bases become unstacked, the wavelength of maximum absorbance does not change, but the amount absorbed increases by 30-40%. a double strand DNA dissociating to single strands produces a sharp cooperative transition.
- AFM imaging- **Atomic force microscopy** (AFM) or scanning force microscopy (SFM) is a very high-resolution type of scanning probe microscopy, with demonstrated resolution on the order of fractions of a nanometer, more than 1000 times better than the optical diffraction limit. The AFM consists of a cantilever with a sharp tip (probe) at its end that is used to scan the specimen surface. The cantilever is typically silicon or silicon nitride with a tip radius of curvature on the order of nanometers. When the tip is brought into

proximity of a sample surface, forces between the tip and the sample lead to a deflection of the cantilever according to Hooke's law.

2.5 Cell culture:

2.3.1 Primary Cultures

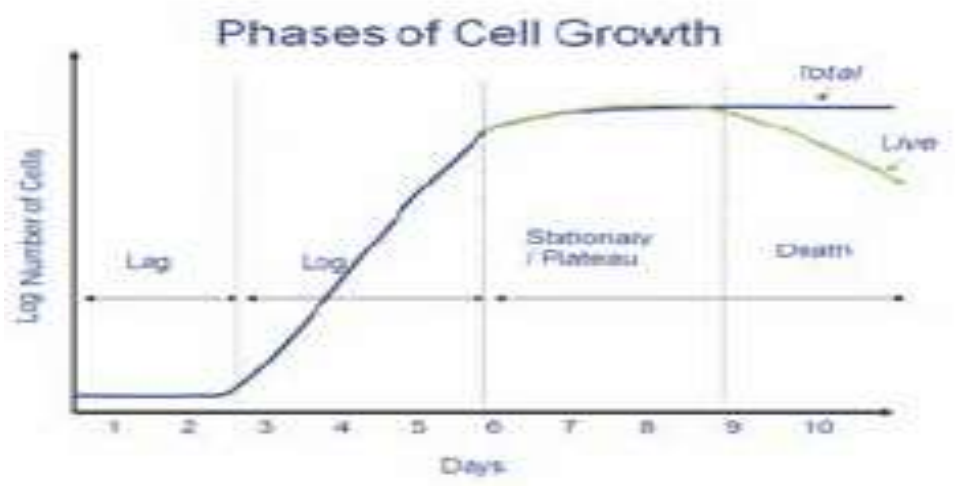
Primary cultures are derived directly from excised, normal animal tissue and cultures either as an explant culture or following dissociation into a single cell suspension by enzyme digestion. Such cultures are initially heterogeneous but later become dominated by fi broblasts. The preparation of primary cultures is labour intensive and they can be maintained *in vitro* only for a limited period of time. During their relatively limited lifespan primary cells usually retain many of the differentiated characteristics of the cell *in vivo*. **Important Note:** Primary cultures by defi nition have not been passaged, as soon as they are passaged they become a cell line and are no longer primary. 'Primary' cells sourced from most suppliers are in fact low-passage cell lines.

2.3.2 Continuous Cultures

Continuous cultures are comprised of a single cell type that can be serially propagated in culture either for a limited number of cell divisions (approximately thirty) or otherwise indefi nitely. Cell lines of a fi nite life are usually diploid and maintain some degree of differentiation. The fact that such cell lines senesce after approximately thirty cycles of division means it is essential to establish a system of Master and Working banks in order to maintain such lines for long periods.

Continuous cell lines that can be propagated indefi nitely generally have this ability because they have been transformed into tumour cells. Tumour cell lines are often derived from actual clinical tumours, but transformation may also be induced using viral oncogenes or by chemical treatments. Transformed cell lines present the advantage of almost limitless availability, but the disadvantage of having retained very little of the original *in vivo* characteristics.

A typical growth curve for cultured cells displays a sigmoid pattern of proliferation. The growth phases associated with normal cells are defined as:



2.10 Figure Growth curve

1. Lag Phase – at this stage the cells do not divide. During this period the cells adapt to the culture conditions and the length of this phase will depend upon the growth phase of the cell line at the time of subculture and
also the seeding density.

2. Logarithmic (Log) Growth Phase – cells actively proliferate and an exponential increase in cell density arises. The cell population is considered to be the most viable at this phase, therefore it is recommended to assess cellular function at this stage. Each cell line will show different cell proliferation kinetics during the log phase and it is therefore the optimal phase for determining the population doubling time.

3. Plateau (or Stationary) Phase – cellular proliferation slows down due to the cell population becoming confl uent. It is at this stage the number of cells in the active cell cycle drops to 0-10% and the cells are most susceptible to injury.

4. Decline Phase – cell death predominates in this phase and there is a reduction in the number of viable cells. Cell death is not due to the reduction in nutrient supplements but the natural path of the cellular cycle. Note: Different cell lines have different timescales for each phase, this graph is provided as a general example of a typical
growth curve.

In Vitro Age of a Cell Culture

Two terms are predominantly used to defi ne the age of a cell culture: (i) passage number - indicates the number of times the cell line has been sub-cultured and (ii) the population doubling

(pd) number - indicates the number of cell generations the cell line has undergone i.e. the number of times the cell population has doubled. The *in vitro* age of a cell culture is particularly useful to know for cell lines with a fi nite lifespan or unstable characteristics thatchange over time in continuous culture.

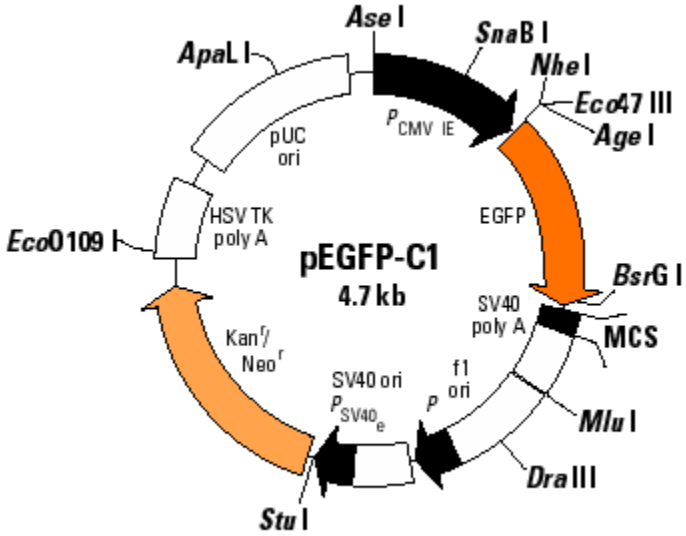
3. MATERIALS AND METHODS

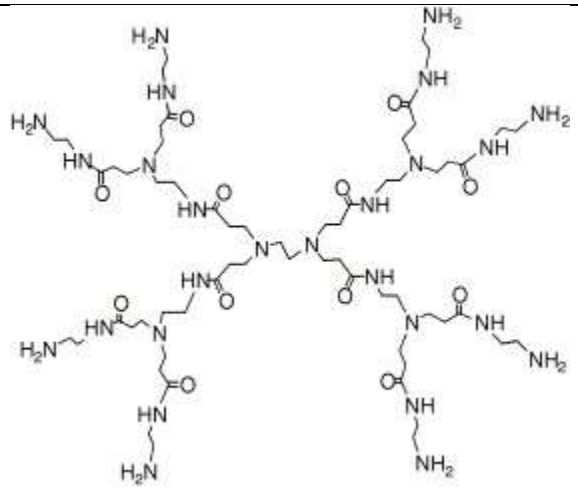
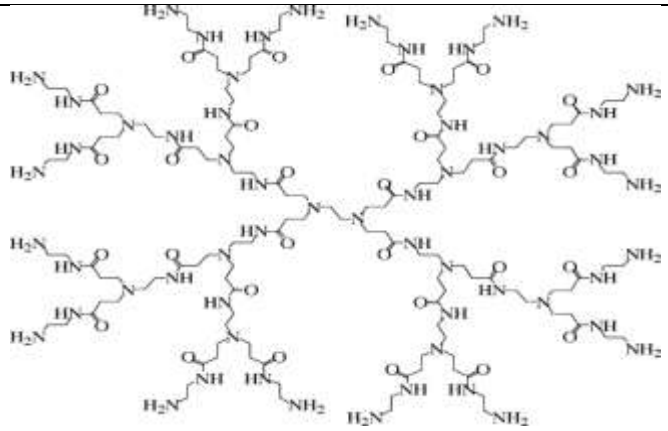
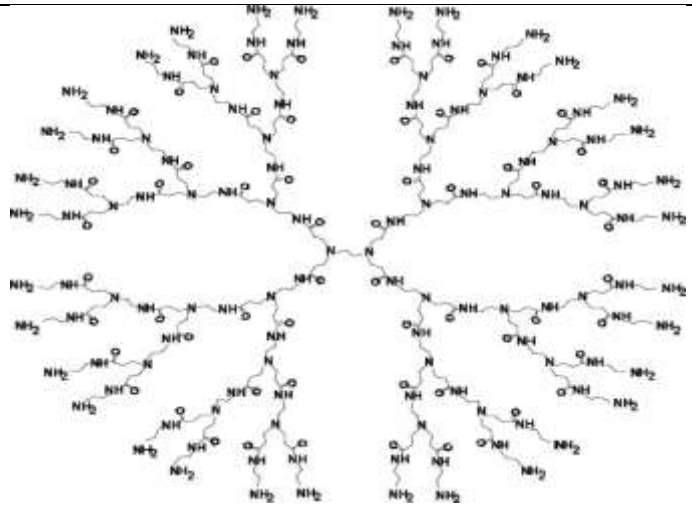
3.1 Materials

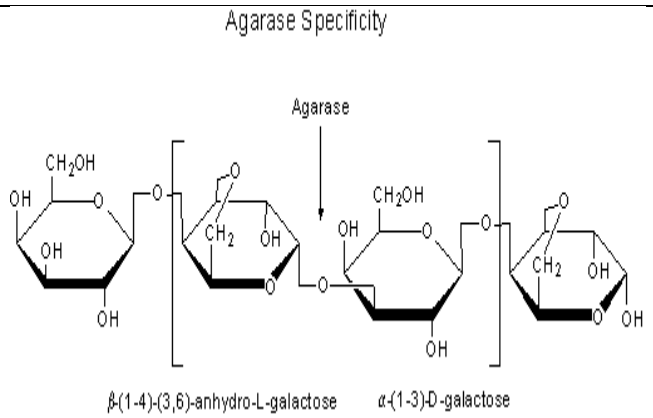
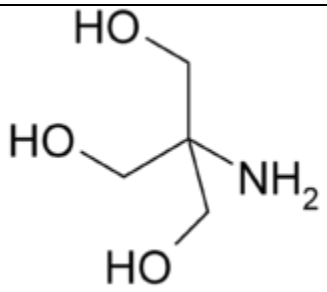
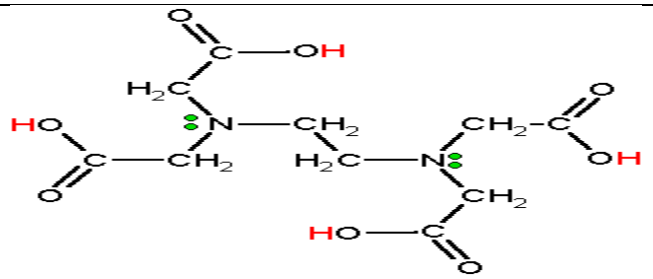
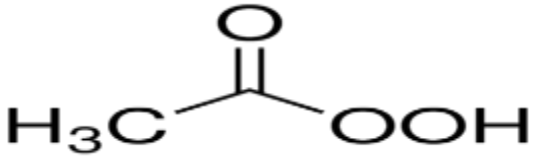
The materials required to study the study of transfection efficiency of DNA via non-viral vectors are as follows: First and foremost is the DNA sample. In this investigation Plasmid DNA is used, the compacting agent used is Dendrimer. Synthesized dendrimers are procured from Sigma-Aldrich Company.

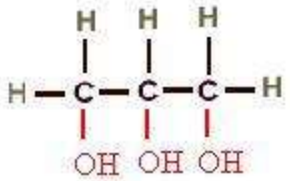
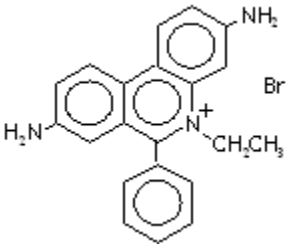
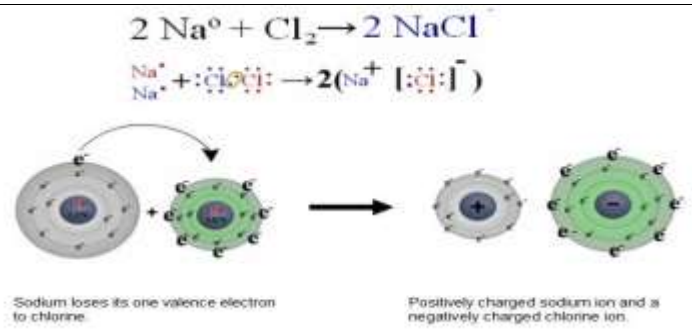
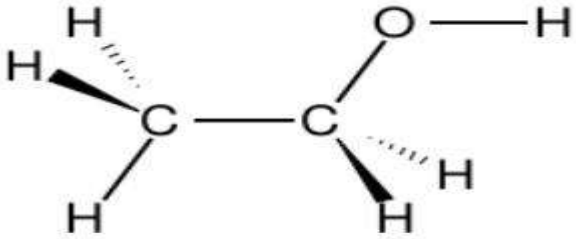
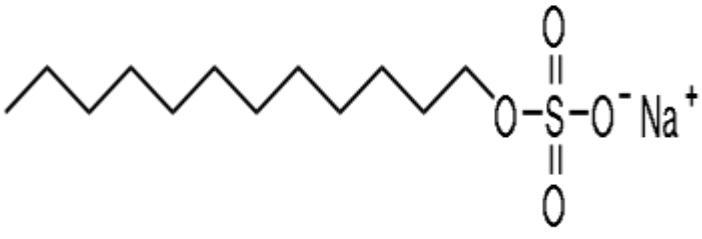
The other chemicals required for the experimentation are obtained from Sigma-Aldrich Company.

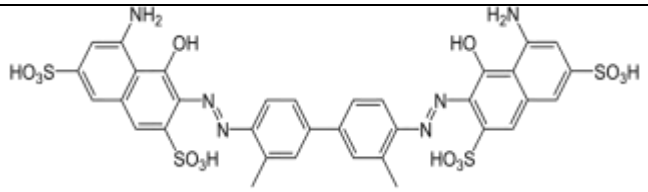
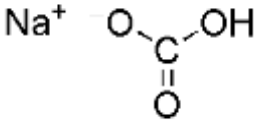
Table 3.1 List of chemicals used in the experiment along with their chemical structure:

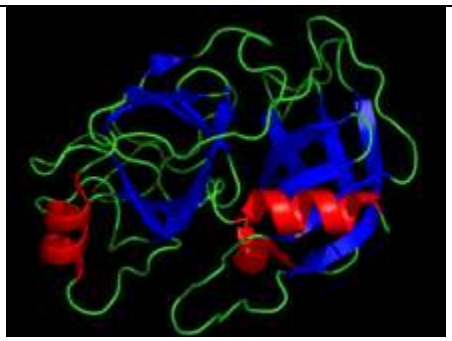
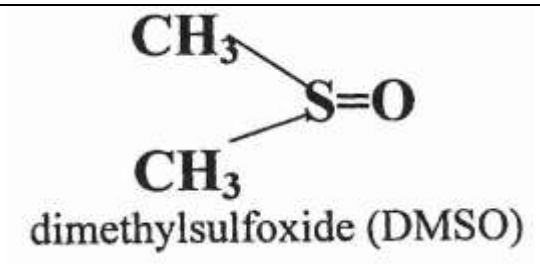
S.No.	Name of Chemicals/ Biochemicals	Chemical Structure
1.	Plasmid DNA(pEGFP-c1) Catalogue No. NA0410 Sigma Aldrich	

2.	PAMAM Dendrimer G1 Catalogue No. 412384-5G Sigma Aldrich	
3.	PAMAM Dendrimer G2 Catalogue No. 412406-5G Sigma Aldrich	
4.	PAMAM Dendrimer G3 Catalogue No. 41242-5G Sigma Aldrich	

5.	Agarose	
6.	Tris(Hydroxymethyl) aminomethane Base Catalogue No. 2044122 Sisco, Research Laboratory Pvt.Ltd.	
7.	Ethylene Diamine tetra acetic acid (EDTA)	
8.	Acetic Acid Catalogue No. TT531762 Sisco, Research Laboratories Pvt.Ltd.	

9.	Glycerol	 Glycerol (Glycerine - propane 1,2,3, triol - 3 hydroxyl (OH) functional group)
10.	Ethidium Bromide (EtBr)	
11.	Sodium Chloride	
12.	Ethyl alcohol AR 99.9% Jiang Su Huaxi International Trade	
13.	Sodium Dodecyl Sulphate	

14.	Trypan blue	
15.	Sodium Hydroxide	
16.	Milli Q	
17.	Phosphate buffer Saline	
18.	Heparin	
19.	CHO-K1 cell lines	
20.	Ham's F12 Media	
21.	Sodium Bicarbonate	
22.	FBS (Fetal bovine serum)	

23.	Trypsin	
24.	DMSO	 dimethylsulfoxide (DMSO)

3.2 Methods

Various steps are involved in the investigation of gene delivery via dendrimer. The following are the basic steps:

1. Plasmid DNA extraction
2. Gel condensation
3. Gel release assay
4. Etbr exclusion assay
5. AFM imaging
6. Cell culture
7. Transfection
8. Toxicity

3.2.1 Extraction of Plasmid DNA- The GenElute™ HP Endotoxin-Free Plasmid Maxiprep Kit offers a simple and rapid method for isolating endotoxin-free plasmid DNA from recombinant *E. coli* cultures. The kit uses a vacuum format with a filter column for the rapid clearing of the bacterial lysate and a silica column for capturing plasmid DNA.

Isolation of plasmid DNA from E.Coli is as follows:

1. Spin down $1cm^3$ of bacterial culture in a $1.5cm^3$ microcentrifuge tube for 1minute or until a mass of cells is visible.
2. Decant the supernatant into disinfectant in a waste container.
3. Add 100µl of TRIS buffer to the tube.Resuspend the cells by closing the tube. EDTA in buffer chelates Mg^{2+} and Ca^{2+} ions which are necessary for DNase action and cell membrane stability.
4. Add 20µl of SDS/NaOH solution. Mix by gently inverting the closed microcentrifuge tube. Leave it to stand for five minutes at room temperature. SDS is an ionic detergent which dissolves the phospholipids and proteins of the cell membrane. This lysis the cells, releasing the DNA. The NaOH splits both the plasmid and the chromosomal DNA into

single stranded molecules but each denatured plasmid remains stuck as two interwinded rings.

5. Add 150µl of potassium acetate and mix gently. Leave it to stand on ice for five minutes. Treatment with potassium acetate restores the pH of solution to neutral (DNA consequently renatures) Potassium acetate also precipitates the SDS, phospholipids, proteins and large DNA molecules.
6. Centirifugr tube for five minutes to spindown the cell debris and chromosomal DNA.
7. Very carefully transfer 400µl of supernatant for a clean microcentrifuge tube. The supernatant contains the plasmid DNA.
8. Add 400µl of ice cold ethanol. Mix gently.
9. Leave tube in deep freeze (-18-20°C) for 20-3-minutes, then centrifuge for 10minutes.
10. Decant the supernatant taking care not to discard the pellet of plasmid DNA.
11. Dissolve the pellet in 15µl of TE buffer. Store the plasmid solution in a freezer or run it on a gel.

3.2.2 Gel condensation (by agarose gel electrophoresis)

Agarose Gel Electrophoresis is performed in this investigation to check the conformation of the compaction of DNA via dendrimers after the AFM imaging has been done.

Protocol for Agarose Gel Electrophoresis:

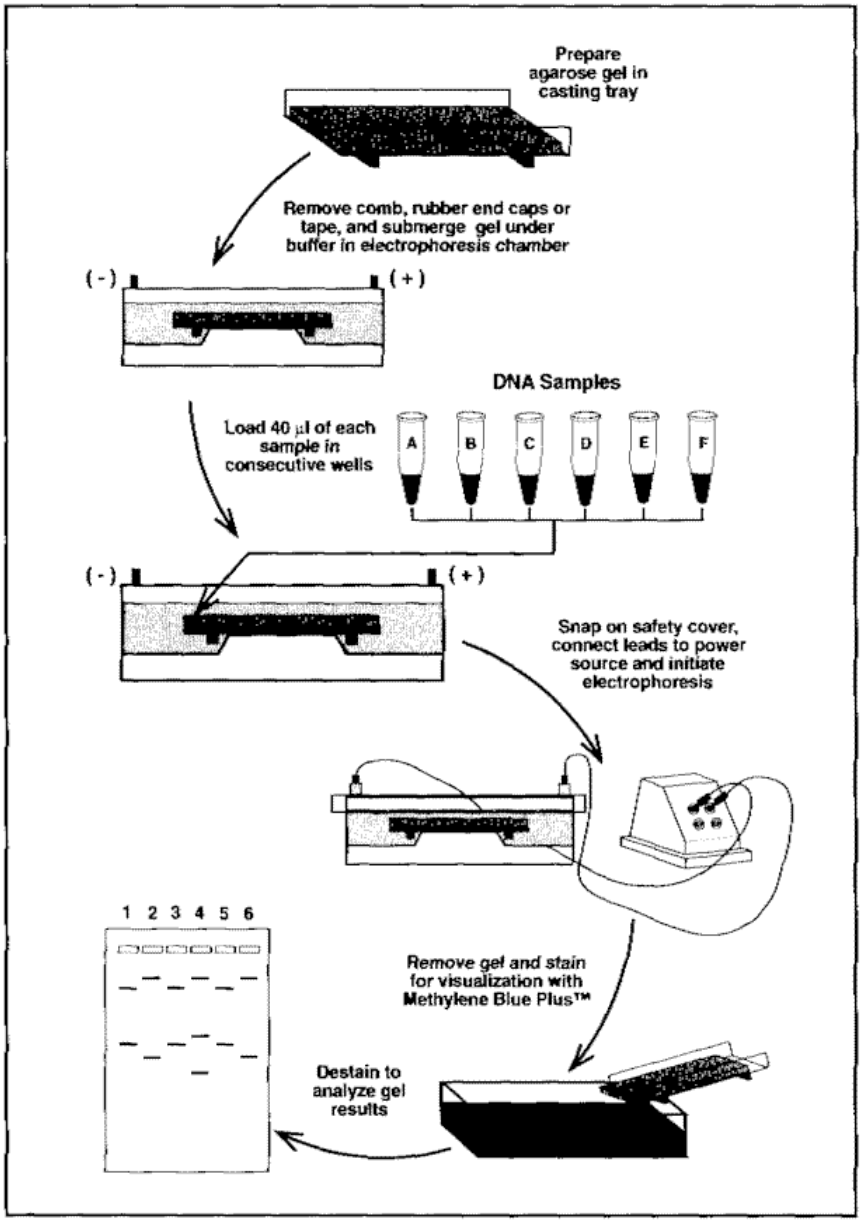
1. Prepare 1% agarose gel solution and pour the gel into the electrophoretic chamber. When the agarose has cooled to about 55°C. Insert the comb for the particular gel rig. The gel should be allowed to cool until it has set. The amount of agarose depends on the size of the gel rig. Gel should be fairly thin (approximately ¼ to ½ inch).
2. Carefully remove the comb and place the gel in gel rig with the wells closest to the cathode end.
3. Cut a piece of parafilm and place 5µl drop of glycerol loading dye onto the waxy side for each sample to be loaded.
4. Keeping the samples in ice, pipette up 5µl of sample n add one drop of loading dye to each sample. In one well, bare plasmid DNA is poured, other well contain bare

dendrimer, rest contains the different reaction mixtures of dendrimer and the DNA sample which are taken in the experiment.

5. After loading the samples, place the cover in the gel rig and run the samples towards the anode. For a small gel we set power pack at about 60ma, for large gel, we use about 120ma.
6. After completion of the process, turn off the power pack and remove the gel and place it in a stain box with 40µl of Ethidium Bromide (EtBr), 200ml of 1X TAE buffer for approximately 45minutes.
7. Visualise with UV light and photograph with a Polaroid photo documentation camera.

Pictorial depiction of gel electrophoresis is given below.

SEQUENTIAL STEPS FOR GEL ELECTROPHORESIS



3.2.3 Gel release assay

Polyplexes were prepared at Z (+/-) of 3 and incubated for 30minutes at room temperature. These were treated with increasing amount of anionic agent Heparin (H3149-100KU) in wt./wt. (heparin/peptide) ratios ranging from 0.5 to 3.0. This assay is used for analyzing stability of polyplexes against anionic challenge.

Protocol:

1. Polyplex is prepared called as mastermix at charge ratio 3 having plasmid DNA at 20ng/µl concentration and PAMAM dendrimer having concentration of 100µM.
2. Now the complex is kept for incubation for 30 minutes.
3. After the incubation period is over, add heparin (concentration 10mg/ml) into the complex and again keep it for 30 minutes.
4. After 30 minutes add loading gel onto the sample and then load it over the gel.
5. Run the gel using agarose gel electrophoresis bioanalytical technique for 45 minutes at 100 volts.
6. Now see the results using UV illuminator.

3.2.4 Ethidium bromide exclusion assay

A number of researcher have used Ethidium bromide exclusion studies using fluorescence spectroscopy as a tool for probing native DNA molecules and the degree of interaction between those and the given ligand. Similarly the binding affinity of the dendrimer with the plasmid DNA is studied using this technique. It is based on the fact that the ethidium ion shows a stricking increase in the fluorescence efficiency when it intercalated into the pDNA. The competition between the EB and dendrimer can be monitored by following decrease in intensity. Even though it does not provide information about the binding affinity or stoichiometry of binding, it is a very useful method for evaluating the binding efficiency of various ligands.

In EtBr exclusion assay, for every 6bp of DNA, we add 1 molecule of Etbr.

Protocol:

1. In 96 well plate, first of all add DNA sample, 20µl in each well.
2. This experimentation is done in duplicates therefore we use two rows and add DNA first.
3. After this, to each well 10µl of Etbr is added.
4. Now the wells are covered with foil and is shaked properly.
5. Then the plate is left undisturbed for 15-20 minutes.

6. Follwed by this, we add 20µl of the dendrimer sample in each well and again shake it properly and cover it with the foil for 15 minutes. (the 96 well plate is covered because the reaction taking place in the well is light sensitive).
7. Now take the reading using fluorescence spectroscopy.

3.2.5 AFM Imaging

In our experiment we have used Agilent 5500 SPM which is the ideal multiple user research system for Scanning Probe Microscopy (SPM) and Atomic Force Microscopy (AFM) [By Agilent Technologies]. The Agilent 5500 SPM offers features and softwares for research in material science, polymers, nanolithography and general surface characterization.

Procedure:

1. Prepare the SPM for MAC Mode imaging using one of the bare MAC levers.
   a) Insert the nose assembly into the scanner.
   b) Load the bare MAC lever into the nose assembly.
   c) Place the scanner in the SPM and connect its cable.
   d) Insert and align the detector.
   e) Prepare the sample- For the sample preparation, add 10µl of Dendrimer sample into the appendrof and after that dropwise add 10µl of Plasmid DNA sample into the same appendrof and vortex the mixture properly. Let the reaction mixture undisturbed after vortexing on to the ice bath.
   f) Now mount the sample on the mica gold sheet.
   g) Adjust the video system to focus on the cantilever.
   h) Sweep the frequency in AC tune window.
   i) Set the correct frequency (approximately 8kHz)
   j) Set the free amplitude to approximately 7nm.

2. Set the scan range to 1500nm and the scan speed to 1line/second.

3. Image the substrate.

4. Optimize the image settings:

a) Adjust the gains as higher as possible without excessive noise.

b) Tip and substrate imaging force should remain as low as possible.

5. Optimize the amplitude set point and record the imaging in digital form.

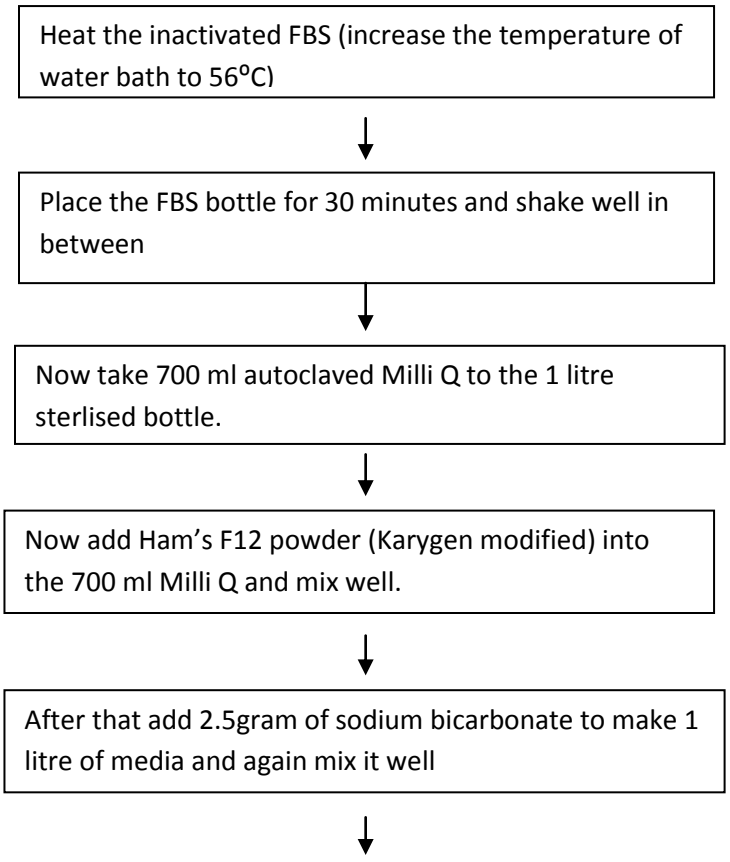
3.2.5 Cell culturing to see the transfection of dendrimer into the cell lines for gene delivery application.

Various steps involved in the culturing of cells and to see the tranfection of the nanoparticle are as follows:

1. Cell lines and reagent formulation:

   In this investigation, we are using CHO-K1 cell lines that were obtained from Sigma Aldrich. Therefore, the reagents that are used for the culturing of these cell lines are as follows-

   - Ham's F12 media
     - Media formulation



Make the volume to 900ml and add 100 ml of FBS (10%) and then filter the whole media using vacuum pump.

- 10X PBS (400ml)
  - ✓ Materials required:
    - Nacl 32gm
    - Kcl 0.8gm
    - Na_2HPO_4 5.76gm
    - KH_2PO_4 0.96gm

    Mix all these ingredients to the Milli Q and then autoclave it.

- Trypsin
  - ✓ Materials required:
    - 1X PBS (100ml)
    - 0.25% trypsin (250mg)
    - 1mM EDTA (30mg)

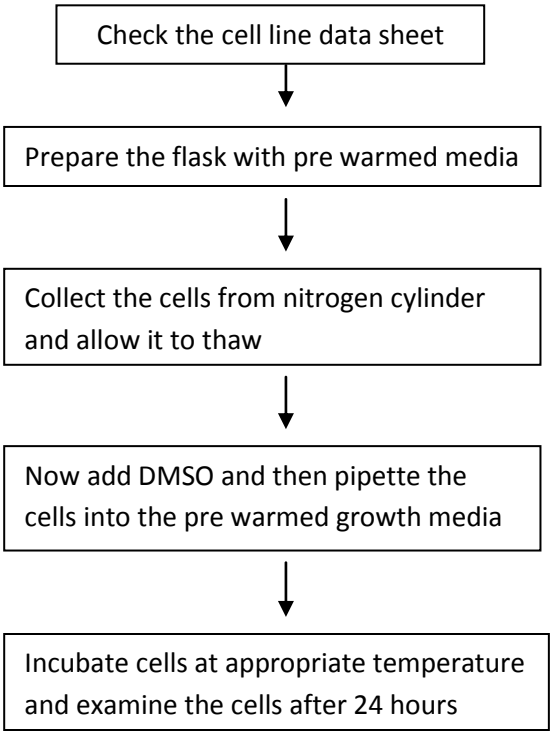
    Dissolve EDTA in PBS and then add trypsin and mix it in the Milli Q.

After all the media and reagent formulation, cell culturing can be done using all good lab practices.

2. Cell culturing:

   Various steps involved in cell culturing are as follows:

   - **Cell reviving (Resucitation of frozen cell lines)**



NOTE- Some cryoprotectant such as DMSO are toxic above 4°C therefore it is essential that cultures are thawed quickly and dilute in culture medium to minimize the toxic effects.

- **Culturing of cells**

Check the confluency of the cells under microscope in T25 flask and go for the splitting of the cells.

↓

Now pour out the old media out of the T25 flask

↓

Wash the cells with 1X PBS buffer

↓

After washing it, discard the PBS and now go for the trypsinisation of the cells using trypsin as it helps in the detachment of the adherent cells,

↓

Incubate the cells for 2 minutes at 37°C in CO_2 incubatorso that the trypsinisation can take place.

↓

Now again check the cells under the microscope to see the floating cells. After that quickly add the Ham's F12 media to the T25 flask in order to stop the action of trypsin.

↓

Thoroughly resuspend the cells into the media by pipetting it in and out. After this, collect the cells in the centrifuge tube and centrifuge the cell suspension at 600rpm for 5 minutes.

↓

After centrifugation, discard the supernatant and tap the pellet and add 2ml of the media into the centrifuge tube.

Now take two fresh T25 flasks and add 4ml of the media into it. Now take 300-400µl of cell suspension from the centriguge tube and pour it into these T25 flasks.

Leave the cell in the incubator for growth.

Note: Before making the plate for any reaction, splitting of cells should be done 3-4 times to have good growth of the cells.

- **Subculturing of the cells (passaging of the cells)**

Subculturing, also referred to as **passaging**, is the removal of the medium and transfer of cells from a previous culture into fresh growth medium, a procedure that enables the further propagation of the cell line or cell strain. The protocol is same as that of the culturing of the cells.

- **Plating of cells**

Check the confluency of the cells under microscope in T25 flask and go for the splitting of the cells.

Now pour out the old media out of the T25 flask

Wash the cells with 1X PBS buffer

After washing it, discard the PBS and now go for the trypsinisation of the cells using trypsin as it helps in the detachment of the adherent cells,

Incubate the cells for 2 minutes at $37^{o}C$ in CO_2 incubator so that the trypsinisation can take place.

Now again check the cells under the microscope to see the floating cells. After that quickly add the Ham's F12 media to the T25 flask in order to stop the action of trypsin.

Thoroughly resuspend the cells into the media by pipetting it in and out. After this, collect the cells in the centrifuge tube and centrifuge the cell suspension at 600rpm for 5 minutes

After centrifugation, discard the supernatant and tap the pellet and add 2ml of the media into the centrifuge tube.

For plating of the cells in 24 well plate, forst we have to count the cells using hemocytometer. For this we mix 10µl of cell suspension and 10µl of trypan blue dye in an appendrof. Now take the hemocytometer and add 10µl of this mix onto the hemocytometer and count the cells under the microscope.

After counting the cells, take 24 well plate and add 300µl of the media into the wells and after that add 250µl of the cell suspension and shake it well, now keep it for incubation.

NOTE: Seeding of cells into the plates depents on the generation time of the cell lines taken by us. For CHO-K1 cell lines we seed approximately 48000 of cells per well. Another important thing to be noted is that after plating, the plate should not be disturbed for 6 hours otherwise the cells will not attach to the surface of the well.

3. **Transfection of the plasmid DNA into the cell lines.**

Check the confluency of the cells under the microscope (the plate that we made).

Make polyplex having pDNA and the G1 PAMAM dendrimer and leave it for 1 hour incubation. The charge ratio taken to make the polyplex should be more than that of the gel condensation release assay.

After 1 hour incubation of the complex, take out the cell plate from the incubator and remove the media from it.

Now add 200µl of optiMEM into the wells and wash the cells and then again remove it.

Again add 300µl of optiMEM into the wells and then add 100µl of the polyplex im each well.

Leave the cells for 5 hours incubation period. After 5 hours remove the optiMEM and add 500µl of the complete media into the wells.

Now go for 24 hours incubation and then see the transfection under fluorescent microscope.

4. RESULTS & DISCUSSION

4.1 COMPACTION OF DNA VIA PAMAM DENDRIMER BY GEL CONDENSATION

The plasmid DNA that is being used in this investigation is pEGFP-c1.

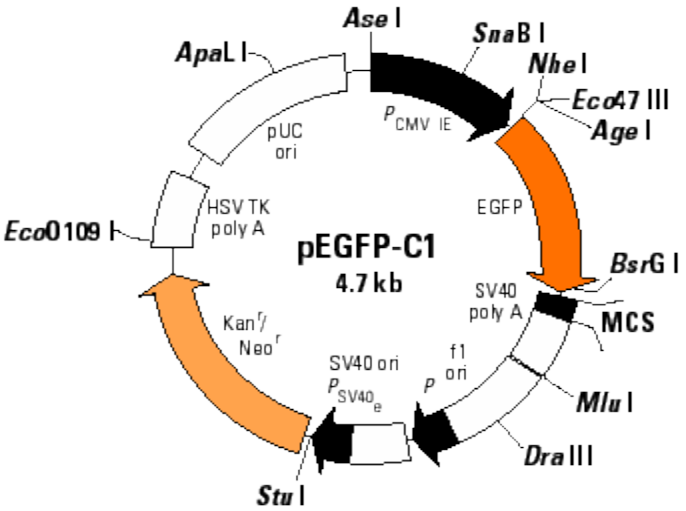


Figure: 4.1 Plasmid DNA

As we know at pH-7 (neutral pH), the phosphate groups in a phosphodiester bond are negatively charged. The pKa of phosphate groups are near 0, therefore they are negatively-charged at neutral pH (pH=7). Due to negative charge over the DNA at physiological pH, the transfection of DNA into the host cell becomes difficult as the negatively charged DNA is not able to enter through the plasma membrane easily and also there is the danger of nucleases enzyme to degrade the DNA when it enters the host cell. Therefore in order to neutalise this negative charge various polycationic species are ised such as proteins, metal ions such as magnesium, polyamines, cationic surfactants, dendrimers etc.

In our project, in order to neutralize the charge on the DNA, PAMAM dendrimers are used that are polycationic in nature. For this purpose we need to compact the DNA via dendrimer at physiological pH i.e at pH 7. Comapction of DNA is achieved when the charge over DNA is totally neutralized by the dendrimer. In order to achieve this charge ration of the complex is calculated keeping the DNA concentration to be constant.

Charge ratio(CR) is nothing but the ratio of the positive charge of the dendrimer over the negative charge of the DNA. Following is the calculation depicting the charge ratio using different concentration of dendrimer keeping concentration of Plasmid DNA constant.

❖ **Calculation of total negative charge on the plasmid DNA pEGFP-c1 sample:**

- Concentration of the plasmid DNA = 280ng/µl.
- Stock concentration of DNA that is kept constant while calculating the total negative charge on DNA sample = 20ng/µl.
- Size of the plasmid DNA =4.7kb(kilo basepairs)

  As we know that 1bp(base pair) = 620 Daltons

  Negative charge on 1bp = -2 (due to presence of two phosphate groups)

  We know, 1 Dalton = 1gram/ 1 mole.

  Therefore, 1 Mole of DNA i.e 6.023×10^{23} molecules= 620 grams

  So negative charge on 620grams of DNA = $2\times 6.023\times10^{23}$

  Therefore charge on 20ng of DNA sample= $\frac{2\times 6.023\times10^{23}\times 20\times 10^{-9}}{620}$

  $= 3.89\times10^{13}$

❖ **Calculation of total positive charge of PAMAM Dendrimer Generation 1(G1):**

- Molecular weight of G1 PAMAM dendrimer = 1430
- Stock concentration of G1 dendrimer = 100µM/ml
- Therefore amount of Dendrimer in the stock solution can be calculated by following equation→ Molar Concentration = $\frac{\text{Number of moles}}{\text{Volume of solution (litres)}}$ (1)

  Number of moles = $\frac{\text{Mass of the substance}}{\text{Molecular mass of the substance}}$ (2)

  Now with the help of above equations, we will calculate the amount of G1 PAMAM dendrimer in 100µM/ml of stock solution:

  From equation (1) & (2):

  100×10^{-6} M = $\frac{\text{x gm}}{1430} \times \frac{1000}{1\text{ml}}$ where x gm is amount of dendrimer

  X gm = 143 µg in 1000µl or

0.143 μg in 1μl of solution.

- Surface Charge group on G1 PAMAM dendrimer = 8

Therefore total positive charge on 0.143μg ofG1 dendrimer = $\frac{0.143\times10^{-6}\times 6.023\times10^{23}\times8}{1430}$

$= 4.816\times 10^{14}$

❖ **Total amount of volume to be taken for the DNA/ Dendrimer complex formation:**

- Total volume= 20μl

(10μl of DNA & 10μl of Dendrimer)

❖ **Charge ratio (CR) = Total positive charge (dendrimer)** (3)

Total negative charge (DNA)

Now, let the Charge ratio (CR) be 1,

By the following equation: $C_1V_1= CR\times C_2V_2$ (4)

(where C_1 & V_1 are the charge and volume of the dendrimer and C_2 & V_2 are the charge and volume of the plasmid DNA sample).

Therefore by applying the equation:

$4.816\times10^{14}\times V_1 = 1\times3.89\times 10^{13}\times10$

$V_1 = 0.807$μl (make this volume to 10μl by adding milliQ water into it)

By applying the same formula, volume of the dendrimer can be calculated for different charge ratios. Table 4.1 below shows different volume of dendrimers of different CR:

Charge Ratio	**Dendrimer (G1) volume (μl) 1X**	**Dendrimer (G1) volume (μl) 10X**	**PBS(μl)**	**Plasmid DNA volume (μl)**
O.1	0.0806	0.806	9.14	10
0.2	0.161	1.61	8.39	10
0.3	0.242	2.42	7.58	10
0.4	0.322	3.22	6.78	10
0.5	0.403	4.03	5.77	10
0.6	0.486	4.86	5.17	10
0.7	0.564	5.64	4.36	10
0.8	0.644	6.44	3.55	10
0.9	0.725	7.25	2.75	10

1.00	0.806	8.06	1.94	10
1.25	1.01		8.99	10
1.5	1.209		8.791	10
2.0	1.612		8.388	10
2.5	2.015		7.985	10
3.0	2.418		7.582	10
4.0	3.224		6.776	10
5.0	4.03		5.97	10

Since the charge ratios are so many therefore to run all the samples we require more than one gel.

-Total amount of G1 PAMAM dendrimer required for this experiment havinf the stock concentration of dendrimer 100μM = 1.5ml.

From this amount we make 10X and 100X solution of the dendrimer and use it for making the complex.

-Total amount of DNA required= 200μl

Stock concentration of pDNA= 246ng/μl

Required concentration= 20ng/μl, therefore amount of DNA required from the stock solution is:

$C_1V_1 = C_2V_2$ where C_1 is stock concentration of DNA

V_1 is volume of stock DNA required

C_2 is the desired concentration of the pDNA in the experiment

V_2 is the amount of DNA required in the experiment

Therefore, $246 \times V_1 = 20 \times 200$

$V_1 = 16.26\mu l$

AGAROSE GEL ELECTROPHORESIS CONFER THE NEUTRALISATION OF DNA CHARGE DURING COMPACTION BY PAMAM DENDRIMER G1:

We run the agarose gel electrophoresis to study the compaction of Plasmid DNA via PAMAM dendrimer G1 at different charge ratios. Since naked DNA is negatively charged therefore during gel electrophoresis, DNA runs from negative to positive terminal as the power is switched on.

When the total compaction of DNA takes place then the total charge on the DNA is neutralized by the positively charged PAMAM dendrimer. This can be visualized in gel electrophoresis. Following are the gel images showing the compaction of DNA by G1 PAMAM dendrimer at various charge ratios:

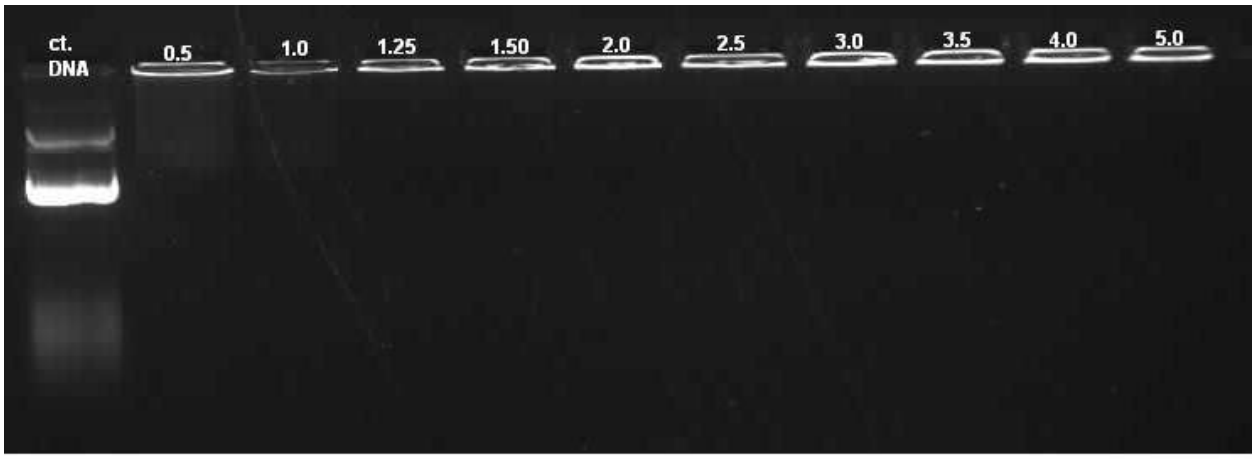


Figure 4.2 Gel condensation assay showing the compaction of pDNA by G1 PAMAM dendrimer at different charge ratios.

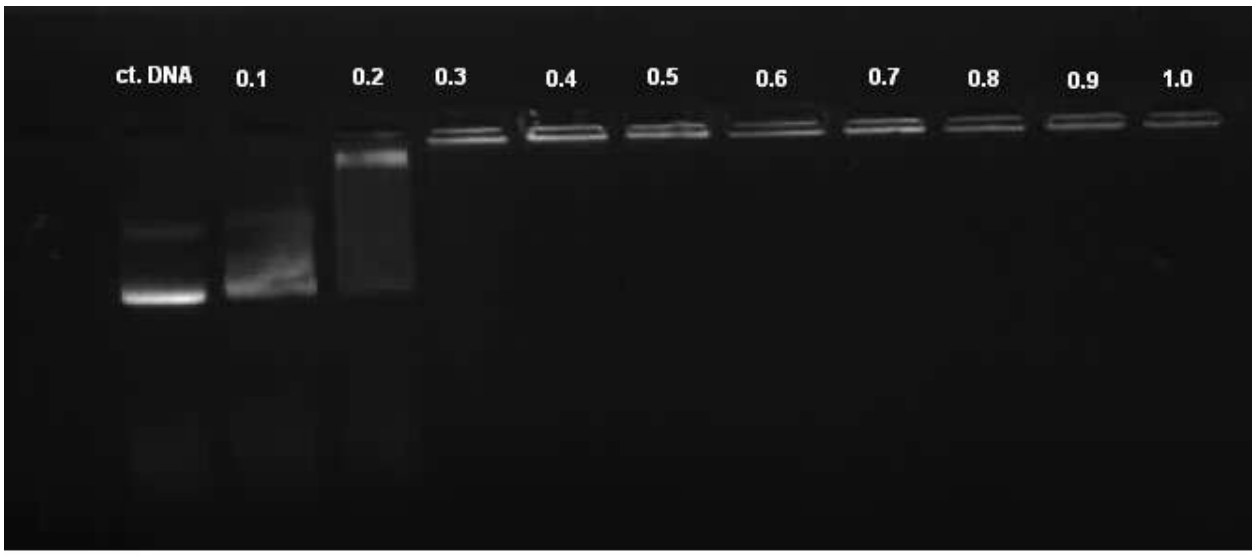


Figure 4.3: Gel condensation assay showing the compaction of pDNA by G1 PAMAM dendrimer at charge ratio 0.3 and onwards.

❖ **Calculation of total positive charge of PAMAM dendrimer Generation 2 (G2):**

- Molecular weight of PAMAM dendrimer (G2) = 3256
- Concentration of stock solution = 100μmM/ml
- Therefore amount of dendrimer in the given stock solution is calculated by using equation (1) & (2), which gives

  Xgm = 0.325μg in 1μl of solution

❖ **Total volume to be taken for the DNA/ Dendrimer complex formation:**

- Total volume = 20μl (10μl of Plasmid DNA & 10μl of the Dendrimer)
- Surface charge group on PAMAM G2 = 16

Therefore total positive charge on 0.325μg of G2 dendrimer= $\frac{0.325\times10^{-6}\times6.023\times10^{23}\times16}{3256}$

$= 9.636\times10^{14}$

- *Now by the same above formula given in equation (4) , We calculate the volume of the dendrimer required for the compaction of 10μl of Plasmid DNA at different Charge ratio.*Table 4.2 below showing the amount of Plasmid DNA and the dendrimer required along with the CR.

Charge Ratio	**Dendrimer (G2) volume (μl) 1X**	**Dendrimer (G2) volume (μl) 10X**	**Dendrimer (G2) volume (μl) 100X**	**MilliQ water volume (μl)**	**Plasmid DNA volume (μl)**
0.05	0.0201	0.201	2.01	7.99	10
0.1	0.0403	0.403	4.03	5.97	10
0.2	0.0806	0.806		9.19	10
0.3	0.121	1.21		8.79	10
0.4	0.161	1.61		8.39	10
0.5	0.201	2.01		7.99	10
0.6	0.242	2.42		7.58	10
0.7	0.282	2.82		7.18	10
0.8	0.323	3.23		6.77	10

0.9	0.363	3.63		6.37	10
1.00	0.403	4.03		5.97	10
1.25	0.504	5.04		4.96	10
1.50	0.604	6.04		3.96	10
2.00	0.806			9.194	10

Since the charge ratios are so many therefore to run all the samples we require more than one gel.

-Total amount of G2 PAMAM dendrimer required for this experiment havinf the stock concentration of dendrimer 100µM = 1.5ml.

From this amount we make 10X and 100X solution of the dendrimer and use it for making the complex.

-Total amount of DNA required= 200µl

Stock concentration of pDNA= 246ng/µl

Required concentration= 20ng/µl, therefore amount of DNA required from the stock solution is:

$C_1V_1 = C_2V_2$ where C_1 is stock concentration of DNA

V_1 is volume of stock DNA required

C_2 is the desired concentration of the pDNA in the experiment

V_2 is the amount of DNA required in the experiment

Therefore, $246 \times V_1 = 20 \times 200$

V_1= 16.26µl (Add 183.74µl of Milli Q to make up the volume to 200 µl)

AGAROSE GEL ELECTROPHORESIS CONFERS THE NEUTRALISATION OF DNA CHARGE DURING COMPACTION BY PAMAM DENDRIMER G1:

We run the agarose gel electrophoresis to study the compaction of Plasmid DNA via PAMAM dendrimer G1 at different charge ratios. Since naked DNA is negatively charged therefore during gel electrophoresis, DNA runs from negative to positive terminal as the power is switched on. When the total compaction of DNA takes place then the total charge on the DNA is neutralized by the positively charged PAMAM dendrimer. This can be visualized in gel electrophoresis.

Following are the images of the gel showing the condensation of the pDNA by G2 PAMAM dendrimer at various charge ratios:

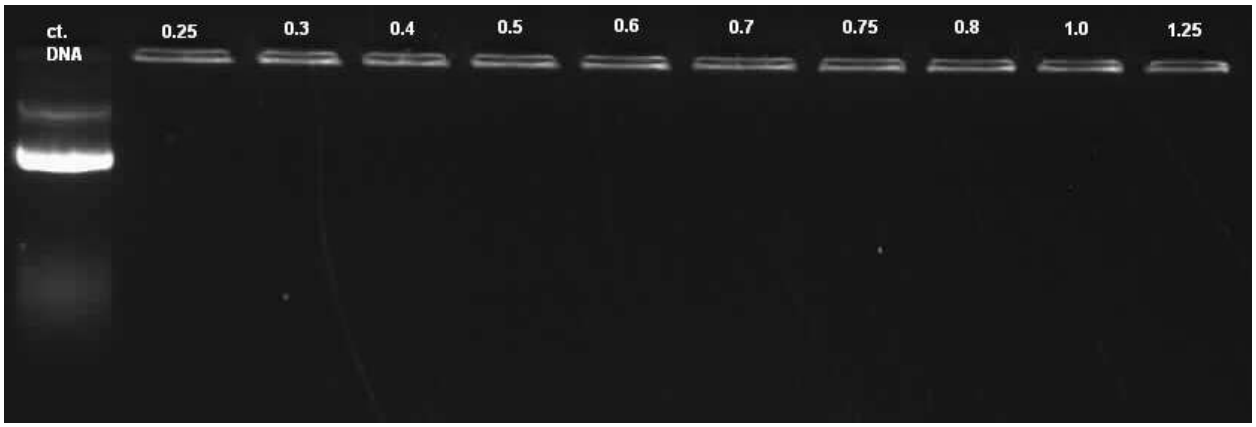


Figure 4.4: Gel condensation of pDNA by G2 PAMAM dendrimer

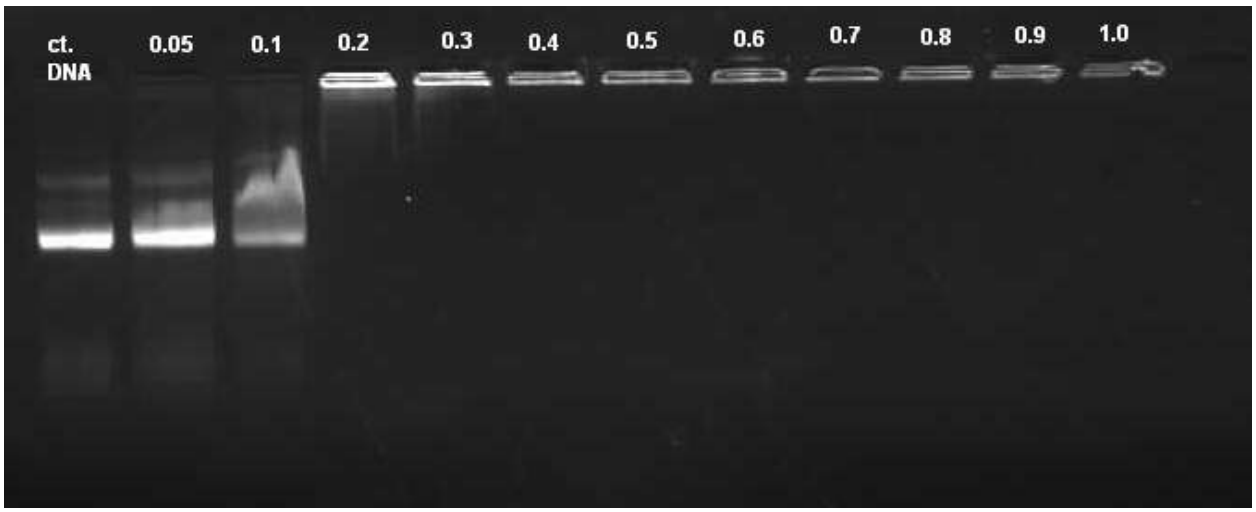


Figure 4.5: Condensation or compaction of pDNA by G2 PAMAM dendrimer at charge ratio 0.2.

4.2 Ethidium Bromide (EtBr) exclusion assay

The binding affinity of the cationic dendrimer with the plasmid DNA can be studied by Ethidium bromide exclusion assay. It is based on the fact that the ethidium ion shows striking increase in the fluorescence efficiency when it intercalated with the pDNA. The competition between the EB and the dendrimer can be monitored by following decrease in the intensity. For EtBr exclusion assay, for every 6 base pairs of DNA, we add one molecule of EtBr (Ehtidium Bromide).

Now we have to calculate the number of base pairs in 20ng/µl of the pDNA.

-Total negative charge on the plasmid DNA pEGFP-c1= 3.89×10^{13}

Size of the plasmid= 4731bp

We know, molecular weight of 1bp= 620 daltons

Therefore, molecular weight of plasmid= 620×4731= 2933220

total -2 negative charge is present = 1bp of DNA

so, 3.89×10^{13} negative charge is present on= 1.945×10^{13}bp of DNA.

For every 6 base pairs of DNA we are adding 1 molecilue of EtBr, therefore, for 1.945×10^{13}bp of DNA we are adding 3.24×10^{12} moleculesof EtBr.

The molecular weight of EtBr= 394.294gm

Or we can say that 6.023×10^{23} molecules of EtBr= 394.294gm

Therefore, molecular weight of 3.24×10^{12} moleculesof EtBr= $(394.294\times3.24\times10^{12})/ 6.023\times 10^{23}$

$= 2.12\times10^{-9}$gm or 2.12ng of Etbr/20ng of DNA

Or

2.12 ng of EtBr/ 1µl of DNA

Therefore,in 20µl of DNA we have 42.4ng of EtBr.

Stock concentration of EtBR= 1mg/ml

Stock concentration to be prepared = 4.24ng/µl

Therefore amount of EtBr required from stock solution= $C_1V_1=C_2V_2$

Where C_1 is stock concentration of EtBr

V_1 is volume of stock EtBr required

C_2 is the desired concentration of the EtBr in the experiment

V_2 is the amount of EtBr required in the experiment

Therefore V_1= (4.24× 240)/ 1000 = 1.0176µl (Add238.9µl of Milli Q to make up the volume to 240µl)

For gel condensation assay, we make 20µl of the polyplex. In EtBr exclusion assay we double the quantity of the polyplex.

For ethidium bromide exclusion assay, we run the reaction in duplex. Therefore the amount of DNA required and the dendrimer is doubled.

Table 4.3 EtBr exclusion assay for G1 PAMAM dendrimer

Charge ratio	**G1 PAMAM dendrimer volume (µl) 1X**	**G1 PAMAM dendrimer volume (µl) 10X**	**For EtBr exclusion assay volume G1 4×10X**	**PBS (µl)**	**pDNA (µl)**
0.1	0.0806	0.806	3.224	36.776	40
0.2	0.161	1.61	6.44	33.56	40
0.3	0.242	2.42	9.68	30.32	40
0.4	0.322	3.22	12.88	27.12	40
0.5	0.403	4.03	16.12	23.88	40
0.6	0.486	4.86	19.60	20.40	40
0.7	0.564	5.64	22.56	17.44	40
0.8	0.644	6.44	25.76	14.24	40
0.9	0.725	7.25	29.00	11.00	40
1.00	0.806	8.06	32.24	7.76	40

Now the amount of DNA required= 450µl

Stock concentration of pDNA= 246ng/µl

Required concentration= 20ng/µl, therefore amount of DNA required from the stock solution is:

$C_1V_1= C_2V_2$ where C_1 is stock concentration of DNA

V_1 is volume of stock DNA required

C_2 is the desired concentration of the pDNA in the experiment

V_2 is the amount of DNA required in the experiment

Therefore, $246 \times V_1 = 20 \times 450$

$V_1 =$ 36.585µl (add 413.42µl of Milli Q to make up the volume to 450µl

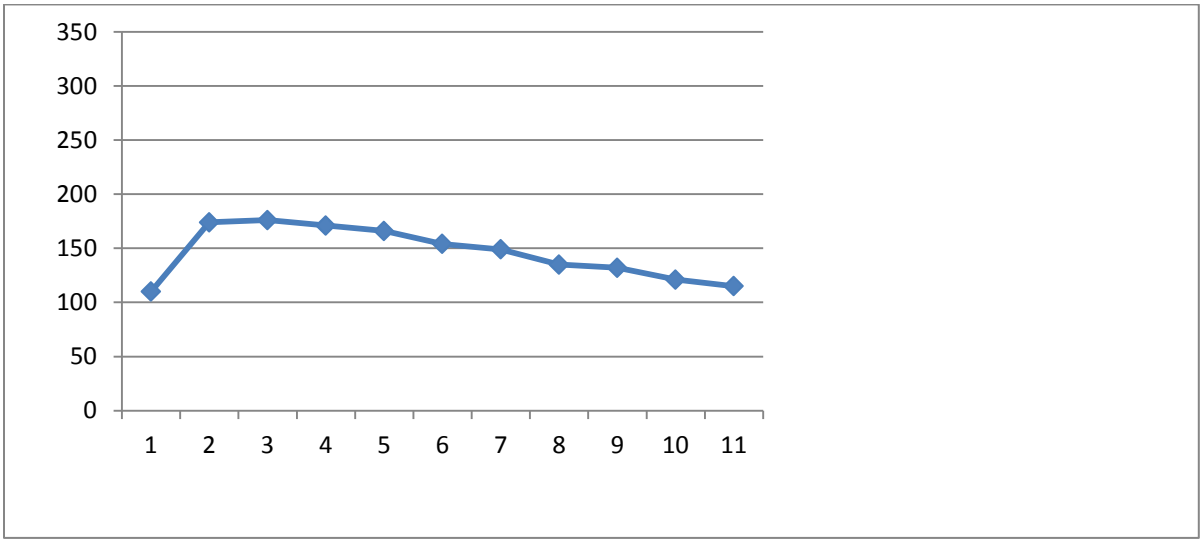


Figure 4.6: EtBr exclusion assay- graph depicting the binding of G1 PAMAM dendrimer with the DNA by showing the decrease in intensity.

Table 4.4 EtBr exclusion assay for G2 PAMAM dendrimer

Charge ratio	**G2 PAMAM dendrimer volume (µl) 1X**	**G2 PAMAM dendrimer volume (µl) 10X**	**For EtBr exclusion assay volume G2 4×10X**	**PBS (µl)**	**pDNA (µl)**
0.05	0.0201	0.201	0.804	39.19	40
0.1	0.0403	0.403	1.612	38.38	40
0.2	0.0806	0.806	3.224	36.77	40
0.3	0.121	1.21	4.84	35.16	40
0.4	0.161	1.61	6.44	33.56	40
0.5	0.201	2.01	8.04	31.96	40
0.6	0.242	2.42	9.68	30.32	40

0.7	0.282	2.82	11.28	28.72	40
0.8	0.323	3.23	12.92	27.08	40
0.9	0.363	3.63	14.52	25.48	40
1.00	0.403	4.03	16.12	23.88	40

Now the amount of DNA required= 450µl

Stock concentration of pDNA= 246ng/µl

Required concentration= 20ng/µl, therefore amount of DNA required from the stock solution is:

$C_1V_1 = C_2V_2$ where C_1 is stock concentration of DNA

V_1 is volume of stock DNA required

C_2 is the desired concentration of the pDNA in the experiment

V_2 is the amount of DNA required in the experiment

Therefore, $246 \times V_1 = 20 \times 450$

V_1= 36.585µl (add 413.42µl of Milli Q to make up the volume to 450µl

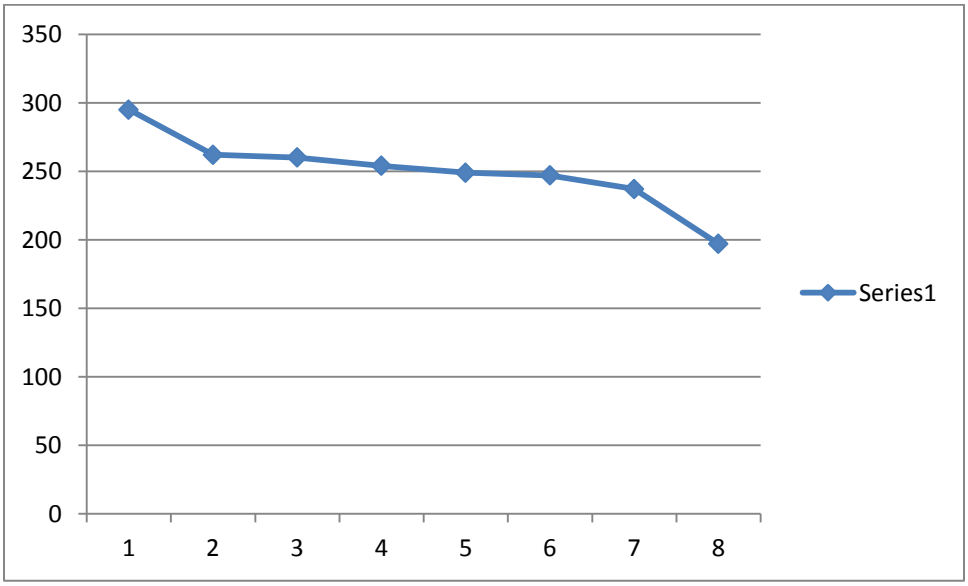


Figure 4.7: EtBr exclusion assay- graph depicting the binding of G2 PAMAM dendrimer with the DNA by showing the decrease in intensity.

4.3 Gel Release Assay

Gel release assay is basically used to study that whether our nanoparticle is able to release the DNA when it enters into our body. Polyplexes are prepared at Z (+/−) and incubated for 30 min at room temperature. These were treated with increasing amount of anionic agent heparin (H3149-100KU) in wt/wt (heparin/ peptide) ratios ranging from 0.1:1 to 3:1, incubated for a further 30 min and run on 1% agarose gel. The amount of the DNA released from the polyplexes was compared with the bare DNA band..

4.3.1 Gel release assay of G1 PAMAM dendrimer

Polyplex is prepared at charge ratio 3.

Amount of dendrimer required at Z=3 is:

$V= (3\times3.89\times10^{13}\times10)/ 4.816\times10^{14}= 2.42\mu l$ (from equation 3&4)

Stock concentration of Dendrimer= 100μM

In 100μM (1μl) of dendrimer we have, 0.143μg of the Dendrimer.

Therefore in 2.42μl of Dendrimer, we have 0.346μg of the dendrimer.

-Anionic group that is used in this release assay is heparin.

Stock concentration of Heparin= 10mg/ml

Table 4.5 Gel release assay

w/w ratio	Amount of Heparin (1X) μl	Amount of Heparin (10X) μl	Amount of Heparin (100X) μl	Milli Q μl
0.25	0.00865		0.865	4.135
0.5	0.0173		1.73	3.27
0.75	0.02595		2.595	2.41
1.00	0.0346		3.46	1.54

1.25	0.04325	0.4325		4.56
1.50	0.0519	0.519		4.481
2.0	0.0692	0.692		4.308
2.5	0.0865	0.865		4.135
3.0	0.1038	1.308		3.962

-Amount of Polyplex required= 220µl (100µl of Dendrimer G1 + 100µl of pDNA)

Stock concentration of DNA= 246ng/µl

Stock concentration of pDNA= 246ng/µl

Required concentration= 20ng/µl, therefore amount of DNA required from the stock solution is:

$C_1V_1 = C_2V_2$ where C_1 is stock concentration of DNA

V_1 is volume of stock DNA required

C_2 is the desired concentration of the pDNA in the experiment

V_2 is the amount of DNA required in the experiment

Therefore, $246 \times V_1 = 20 \times 110$

V_1= 8.94µ (add 101.06µl of Milli Q to make up the volume to 110µl)

-Amount of Dendrimer required= 26.62µl (add 83.38µl of PBS to make the volume to 110µl)

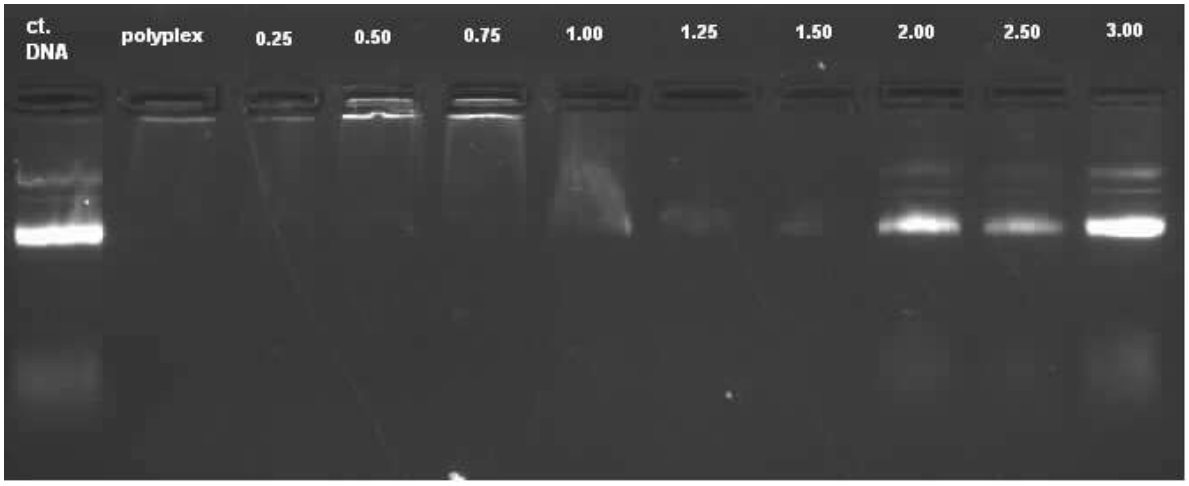


Figure 4.8 Gel release assay of G1 PAMAM dendrimer.

In the above figure we can see that the release of DNA from the dendrimer can be seen at w/w ratio 2.0. This shows the competitive binding of Heparin with the G1 PAMAM dendrimer with respect to the pDNA.

4.3.2 Gel Release Assay of G2 dendrimer

Same protocol is followed for G1 PAMAM dendrimer gel release assay as that for G1 dendrimer.

The polyplex is prepared at charge ratio=1

Stock concentration of G2 PAMAM dendrimer= 100μM

Volume of dendrimer required from the stock= $(1\times3.89\times10^{13}\times10)/9.62\times10^{14}$= 0.4038μl

So. In 1μl of G2 PAMAM dendrimer we have 0.325μg of Dendrimer

Therefore in 0.4038μl of G2 dendrimer we have 0.1312μg of Dendrimer

Stock concentration of Heparin= 10mg/ml

Now again we go for the w/w ratio of heparin & dendrimer and follow the same procedure as above.

Table 4.6 Gel Release Assay of G2

w/w ratio	Amount of Heparin (μg)	Amount of Heparin (μl) 1X	Amount of Heparin (μl) 1000X	Milli Q (μl)
0.25	0.0328	0.000328	0.328	4.672
0.50	0.0656	0.000656	0.656	4.344
0.75	0.0984	0.000984	0.984	4.016
1.00	0.1312	0.001312	1.312	3.688
1.25	0.164	0.00164	1.64	3.36
1.5	0.1968	0.001968	1.968	3.04
2.0	0.2624	0.00262	2.62	2.38
2.5	0.328	0.00328	3.28	1.72
3.0	0.3936	0.00393	3.93	1.07

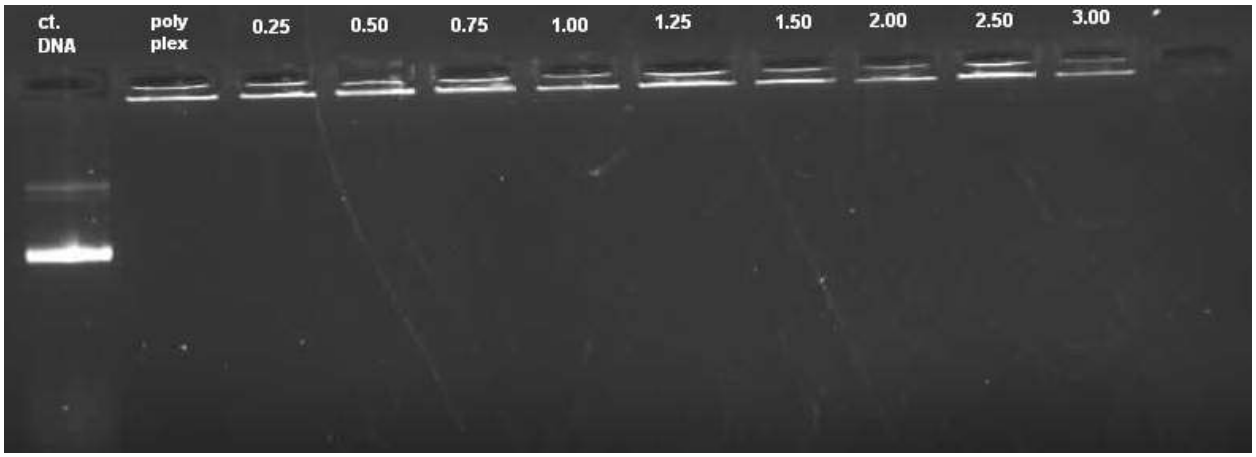


Figure 4.9: Gel release assay of G2 PAMAM Dendrimer

4.4 Atomic Force Microscopy Imaging (AFM Imaging)

In our project, in order to neutralize the charge on the DNA, PAMAM dendrimers are used that are polycationic in nature. For this purpose we need to compact the DNA via dendrimer at physiological pH i.e at pH 7. Comapction of DNA is achieved when the charge over DNA is totally neutralized by the dendrimer. In order to achieve this charge ration of the complex is calculated keeping the DNA concentration to be constant.

Charge ratio(CR) is nothing but the ratio of the positive charge of the dendrimer over the negative charge of the DNA. Following is the calculation depicting the charge ratio using different concentration of dendrimer keeping concentration of Plasmid DNA constant.

- **Calculation of total negative charge on the plasmid DNA pEGFP-c1 sample:**

- Concentration of the plasmid DNA = 280ng/µl.
- Stock concentration of DNA that is kept constant while calculating the total negative charge on DNA sample = 20ng/µl.
- Size of the plasmid DNA =4.7kb(kilo basepairs)

  As we know that 1bp(base pair) = 620 Daltons

  Negative charge on 1bp = -2 (due to presence of two phosphate groups)

  We know, 1 Dalton = 1gram/ 1 mole.

  Therefore, 1 Mole of DNA i.e 6.023×10^{23} molecules= 620 grams

  So negative charge on 620grams of DNA = $2\times 6.023\times10^{23}$

  Therefore charge on 20ng of DNA sample= $\frac{2\times 6.023\times10^{23}\times 20\times 10^{-9}}{620}$

$= 3.89\times10^{13}$

❖ **Calculation of total positive charge of PAMAM Dendrimer Generation 1(G1):**

- Molecular weight of G1 PAMAM dendrimer = 1430
- Stock concentration of G1 dendrimer = 100μM/ml
- Therefore amount of Dendrimer in the stock solution can be calculated by following equation→ Molar Concentration = $\frac{\text{Number of moles}}{\text{Volume of solution (litres)}}$ (1)

  Number of moles = $\frac{\text{Mass of the substance}}{\text{Molecular mass of the substance}}$ (2)

  Now with the help of above equations, we will calculate the amount of G1 PAMAM dendrimer in 100μM/ml of stock solution:

  From equation (1) & (2):

  100×10^{-6} M = $\frac{\text{x gm}}{1430} \times \frac{1000}{1\text{ml}}$ where x gm is amount of dendrimer

  X gm = 143 μg in 1000μl or

  0.143 μg in 1μl of solution.

- Surface Charge group on G1 PAMAM dendrimer = 8

  Therefore total positive charge on 0.143μg ofG1 dendrimer = $\frac{0.143\times10^{-6}\times 6.023\times10^{23}\times8}{1430}$

  $= 4.816\times 10^{14}$

❖ **Total amount of volume to be taken for the DNA/ Dendrimer complex formation:**

- Total volume= 20μl

  (10μl of DNA & 10μl of Dendrimer)

❖ **Charge ratio (CR) = $\frac{\text{Total positive charge (dendrimer)}}{\text{Total negative charge (DNA)}}$** (3)

Now, let the Charge ratio (CR) be 1,

By the following equation: $C_1V_1 = CR\times C_2V_2$ (4)

(where C_1 & V_1 are the charge and volume of the dendrimer and C_2 & V_2 are the charge and volume of the plasmid DNA sample).

Therefore by applying the equation:

$4.816\times10^{14}\times V_1 = 1\times3.89\times 10^{13}\times10$

V_1 = 0.807µl (make this volume to 10µl by adding milliQ water into it)

By applying the same formula, volume of the dendrimer can be calculated for different charge ratios. Table 4.1 below shows different volume of dendrimers of different CR:

Charge Ratio	**Dendrimer (G1) volume (µl)**	**Milliq water volume (µl)**	**Plasmid DNA volume (µl)**
2	1.61	8.38	10
3	2.43	7.57	10
5	4.05	5.95	10

❖ **Calculation of total positive charge of PAMAM dendrimer Generation 2 (G2):**

- Molecular weight of PAMAM dendrimer (G2) = 3256
- Concentration of stock solution = 100µmM/ml
- Therefore amount of dendrimer in the given stock solution is calculated by using equation (1) & (2), which gives

  Xgm = 0.325µg in 1µl of solution

❖ **Total volume to be taken for the DNA/ Dendrimer complex formation:**

- Total volume = 20µl (10µl of Plasmid DNA & 10µl of the Dendrimer)
- Surface charge group on PAMAM G2 = 16

Therefore total positive charge on 0.325µg of G2 dendrimer= $\frac{0.325\times10^{-6}\times6.023\times10^{23}\times16}{3256}$

$= 9.636\times10^{14}$

- *Now by the same above formula given in equation (4) , We calculate the volume of the dendrimer required for the compaction of 10µl of Plasmid DNA at different Charge ratio.*Table 4.2 below showing the amount of Plasmid DNA and the dendrimer required along with the CR.

Charge Ratio	**Dendrimer (G2) volume (µl)**	**MilliQ water volume (µl)**	**Plasmid DNA volume (µl)**
1	0.40	9.60	10
2	0.80	9.20	10
3	1.20	8.80	10

After the calculation of the charge ratio and the volume of the dendrimer required for the compaction of Plasmid DNA, AFM imaging is done to see the compaction and condensation of the DNA at different charge ratios.

- First of all, bare Plasmid DNA sample is kept for AFM imaging so that we are able to distinguish between the linear/free DNA and it's compacted form in presence of the Dendrimer.

Interpretation of the images given below: When the bare plasmid DNA is poured on the Mica sheet of the AFM sample stand, then we see that due the negative charge on both the DNA and the Mica sheet there is electrostatic repulsion, and we can see the scattered network of the free plasmid DNA in its open form (as at physiological pH, DNA is present in the linear form). Figure 4.2 & Figure 4.3 depicts the AFM imaging of the bare plasmid DNA showing the open network of the DNA without the presence of any compacting agent.

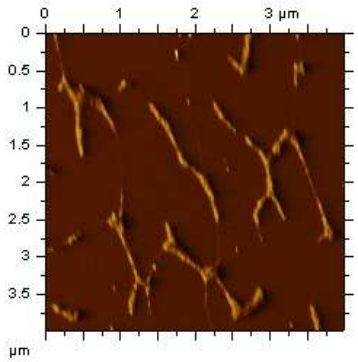


Figure 4.10 AFM image showing the free form of Plasmid DNA (control Plasmid) without the presence of any compacting agent.

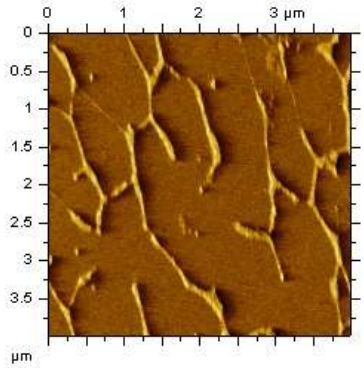


Figure 4.11 AFM image of the control plasmid without presence of any compacting agen

★After imaging the bare Plasmid DNA sample, compaction of Plasmid DNA is imaged by using the Dendrimer as the compacting agent. Now at first, reaction is prepared which includes the Plasmid DNA sample and the Dendrimer G1 at charge ratio 1 and the AFM image is recorded.

Interpretation of the images given below: When the PAMAM dendrimer G1 is used as the compacting agent for the compaction and the condensation of the Plasmid DNA at charge ratio(CR) 1 then we see that there is partial compaction of the Plasmid DNA with the Dendrimer at CR 1 as in the image we can see some compacted particles and also the free form of the plasmid DNA. This shows that at charge ratio 1, DNA's negative charge is not being completely neutralized by the dendrimer positive charge and thus we are not able to see the fully compaction of the DNA. Figure 4.4 & Figure 4.5 shows the partial compaction of the Plasmid DNA by PAMAM dendrimer G1 at CR 1.

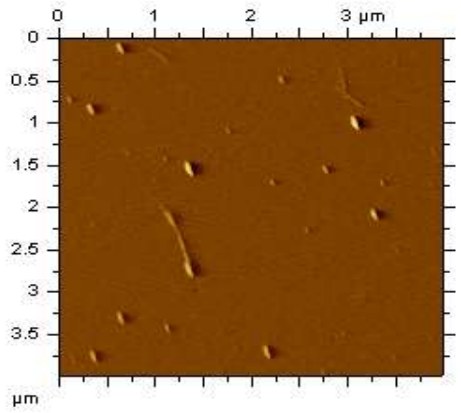


Figure 4.12 AFM image showing the complexion of the Plasmid DNA and the PAMAM dendrimer G1 at CR=1

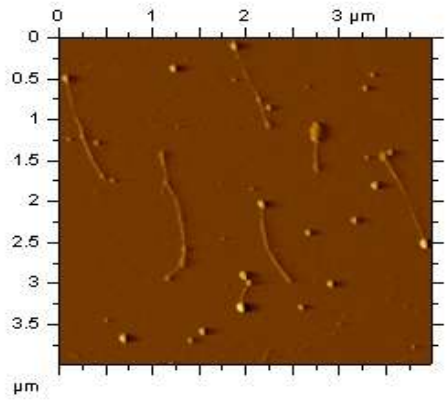


Figure 4.13 AFM image showing the complexion of the Plasmid DNA and the PAMAM dendrimer G1 at CR=1

★ Since we are not able to get the fully compacted form of the plasmid DNA by Dendrimer G1 at charge ratio 1, therefore now we use the dendrimer G1 sample at charge ratio 3 to complex the same plasmid DNA sample and see the compaction of DNA via AFM imaging.

Interpretation of the images given below: After the complexion of the plasmid DNA and the PAMAM dendrimer G1 at charge ratio 3, we see that there is the complete neutralization of the DNA negative charge by the positively charged dendrimer and thus we are able to see the total compaction of the Plasmid DNA in presence of the dendrimer at CR 3. Figure 4.6 & Figure4.7 showing the total compaction of the plasmid DNA in presence of the PAMAM dendrimer at charge ratio 3.

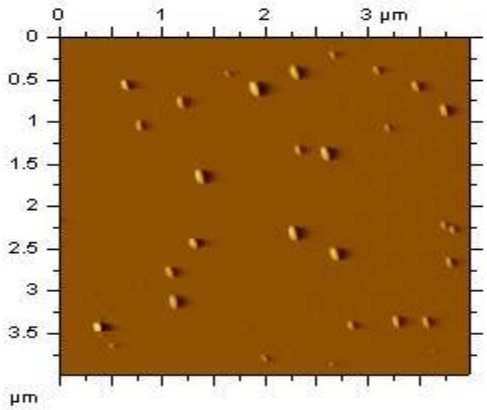


Figure 4.14 AFM image showing the compaction of plasmid DNA by PAMAM dendrimer G1 at charge ratio 3

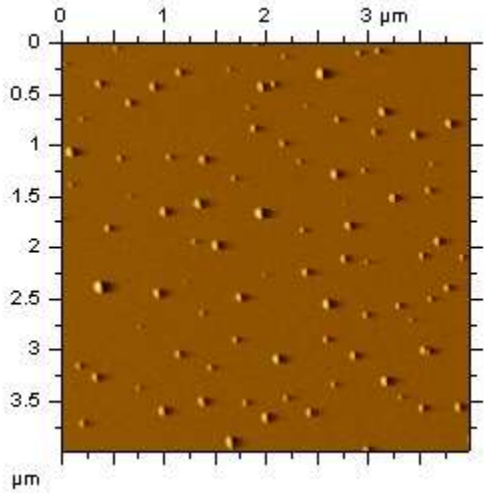


Figure 4.15 AFM image showing the compaction of plasmid DNA by PAMAM dendrimer G1 at charge ratio 3

Similarly compaction of pDNA by G2 PAMAM dendrimer is also seen under AFM at different charge ratios. At first, reaction is prepared which includes the Plasmid DNA sample and the Dendrimer G2 at charge ratio 1 and the AFM image is recorded.

Interpretation of the images given below: After the complexion of the plasmid DNA and the PAMAM dendrimer G1 at charge ratio 1, we see that there is not complete neutralization of the DNA negative charge by the positively charged dendrimer and thus we are able to see partially free DNA and some complexes of the Plasmid DNA in presence of the dendrimer at CR 1. Figure 4.16 & Figure4.17 showing the total compaction of the plasmid DNA in presence of the PAMAM dendrimer at charge ratio 1

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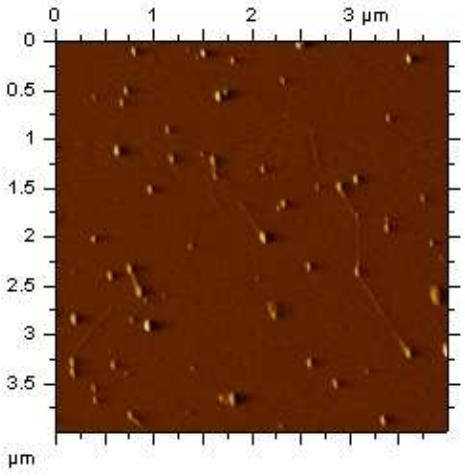


Figure 4.16: AFM imaging showing the partial compaction of the pDNA at CR 1

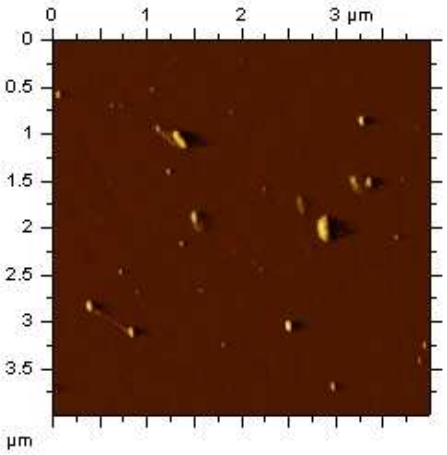


Figure 4.17: AFM imaging showing the partial compaction of the pDNA at CR 1.

Since we are not able to get the fully compacted pDNA by G2 PAMAM dendrimer at CR 1, so now we make the reaction of the dendrimer/DNA complex at CR 2 and then see it under the AFM.

Interpretation of the images given below: After the complexion of the plasmid DNA and the PAMAM dendrimer G2 at charge ratio 2, we see that there is the complete neutralization of the DNA negative charge by the positively charged dendrimer and thus we are able to see the total compaction of the Plasmid DNA in presence of the dendrimer at CR 2. Figure 4.18 & Figure4.19 showing the total compaction of the plasmid DNA in presence of the PAMAM dendrimer at charge ratio 2.

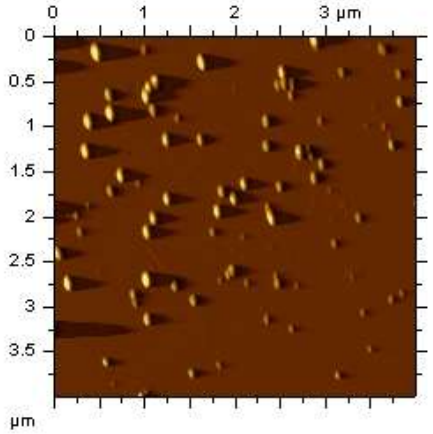


Figure 4.18: AFM imaging of G2 PAMAM dendrimer showing compaction of DNA at CR 2

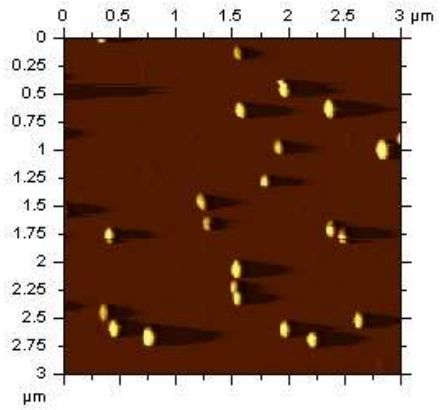


Figure 4.19: AFM imaging showing compaction of DNA by G2 PAMAM dendrimer at CR 2

4.5 Cell culturing to check the transfection of pDNA into the cells

4.5.1 Culturing of cells- CHO-K1 cells are used for this experimentation. The generation time of these cell lines are 17-24 hours. Ham's F12 media is used. T25 flask has been made and when the confluency reaches to 70-80% then we go for splitting of the cells and its plating.

Figure 4.20: T25 Flask containing cell suspension and the Ham's F12 media

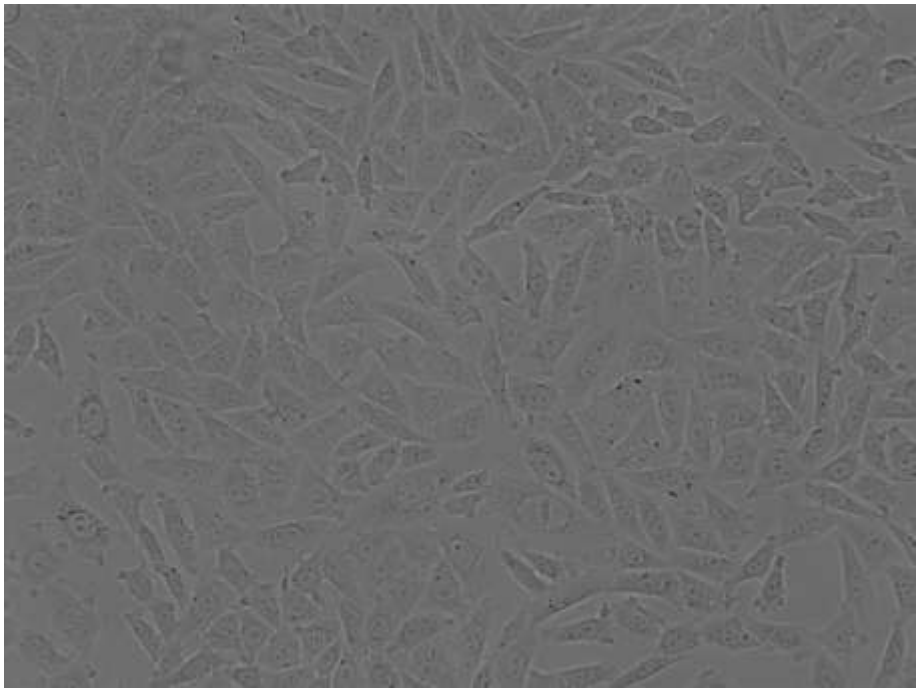


Figure 4.21: Confluent CHO-K1 cells in T25 flask

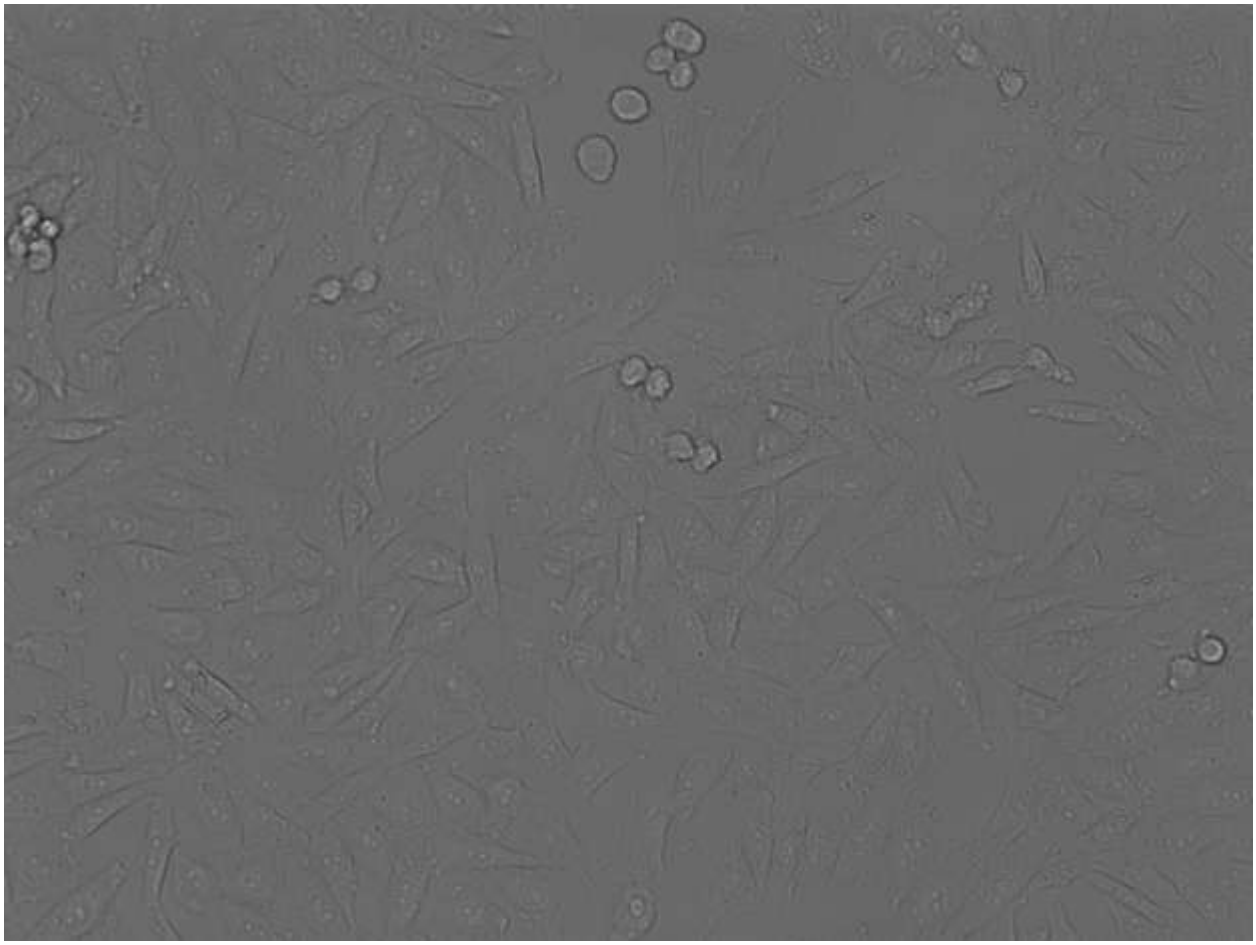
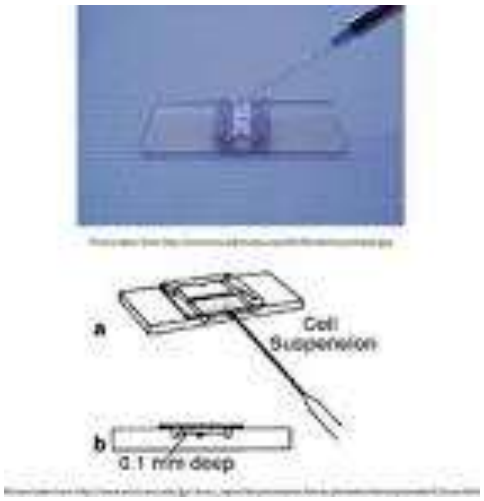


Figure 4.22: Confluent CHO-K1 cells (Image under microscope)

4.5.2 Preparation of 24 well plate

After the confluency of the cells has reached then the plating of cells is done. For that counting of the cells is done using hemocytometer.

Hemocytometers were developed for counting blood cells, but can also be used to count Chlamys. A hemocytometer has two chambers and each chamber has a microscopic grid etched on the glass surface. The chambers are overlaid with a glass coverslip that rests on pillars exactly 0.1 mm above the chamber floor. Thus, the volume of fluid above each square of the grid is known with precision.



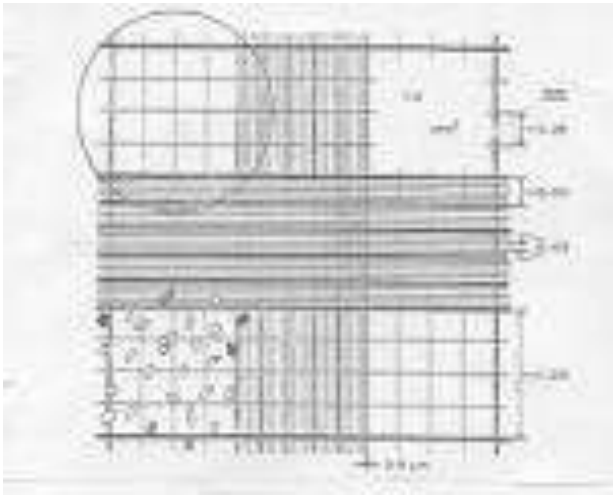


Figure 4.23 Hemocytometer

Amount of cell suspension required to seed into 24 well plate=

(No.of wells× no.of cells to be seeded/well)/ average no.of cells$\times 10^4$/well

After the confluency is reached cells are counted using hemocytometre and that comes out to be 155 in four squares of hemocytometer.

Now by applying the above formula, we get

(24×48000/well)/ (155/4) $\times 10^4$= 2.97ml or 3ml approximately

-Cell suspension to be added in each well is = 250µl

Therefore total amount needed for 24 well plate = 24× 250= 6ml

Total amount of media to be added in each well = 300µl

Therefore total amount of media required = 300× 24= 7.2ml

Now plating of the cells take place.

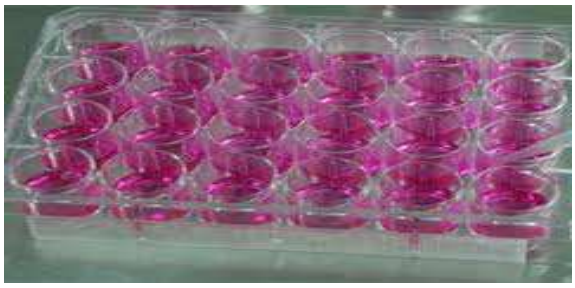
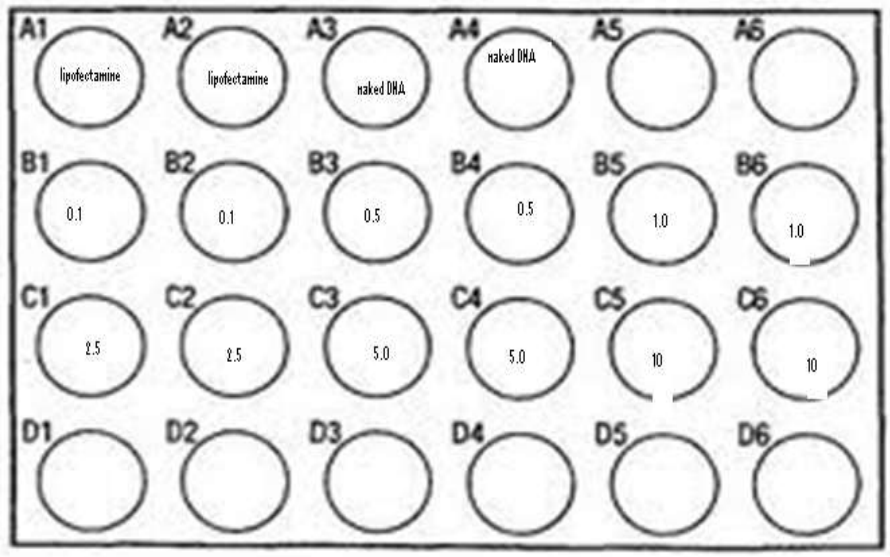


Figure 4.24: 24 well plate

4.5.3 Transfection reaction of G1 PAMAM dendrimer

As per the protocol mentioned, we follow the protocol for the transfection of the DNA into the cell lines and the results are seen under fluorescent microscope.

Polyplex were prepared at six different charge ratios 0.1, 0.5, 1.0, 2.5, 5.0 & 10.0 and were added in the wells in such manner shown below:



Following are the results of the fluorescence microscopy:

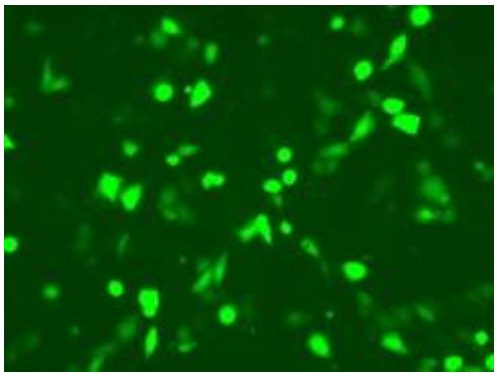


Figure 4.25: lipofectamine under fluorescent microscope

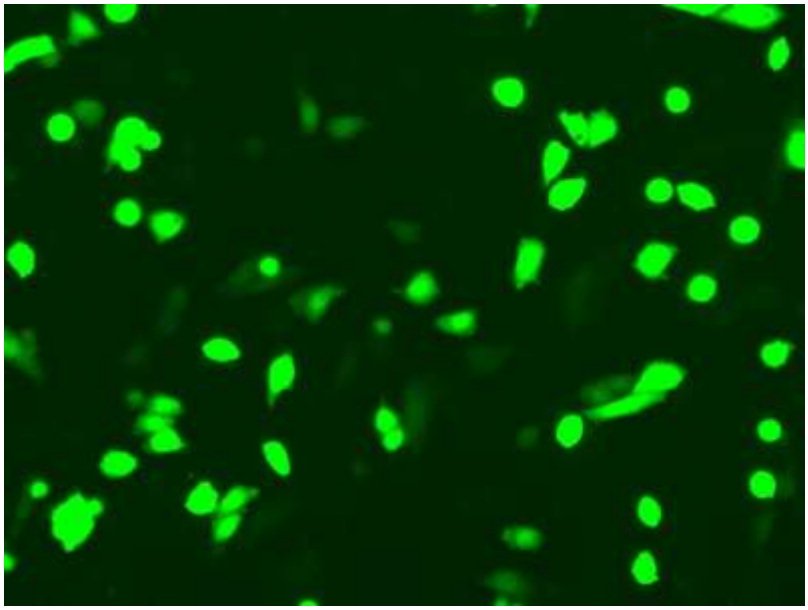


Figure 4.26: pEGFP expression in cells when lipofectamine is added

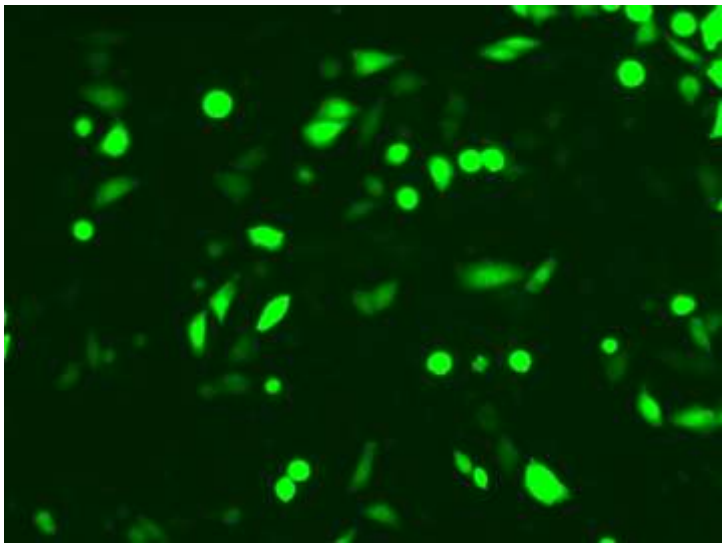


Figure 4.27: pEGFP expression in cells when lipofectamine is added

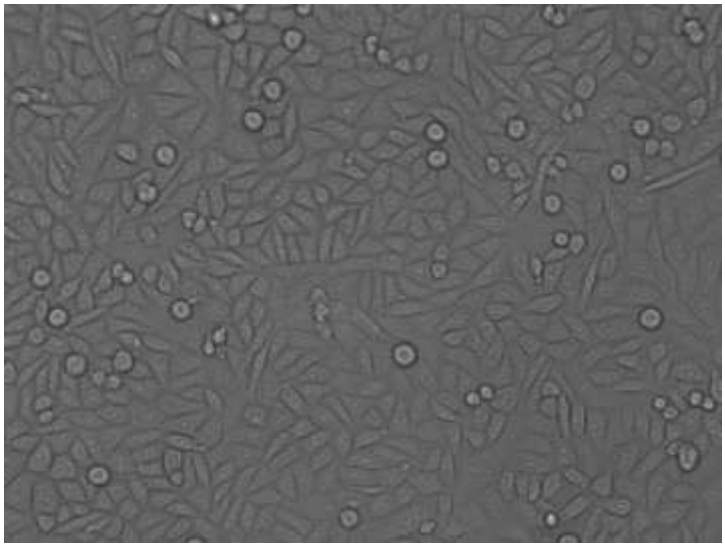


Figure 4.28: Naked DNA in the wells under microscope.

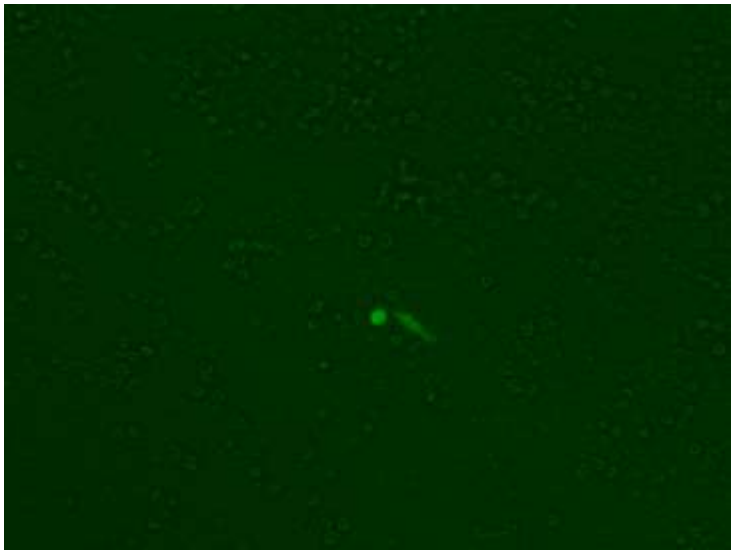


Figure 4.29: Little pEGFP expression in cells at CR 2.5

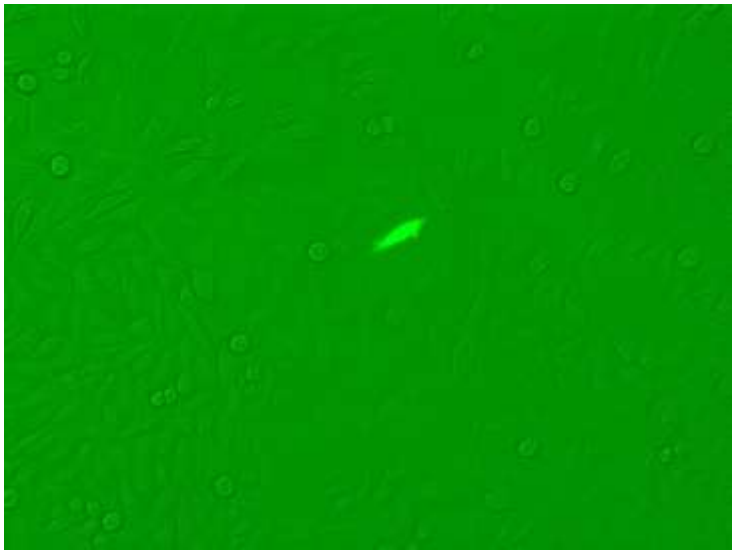


Figure 4.30: pEGFP expression in cells at CR 2.5

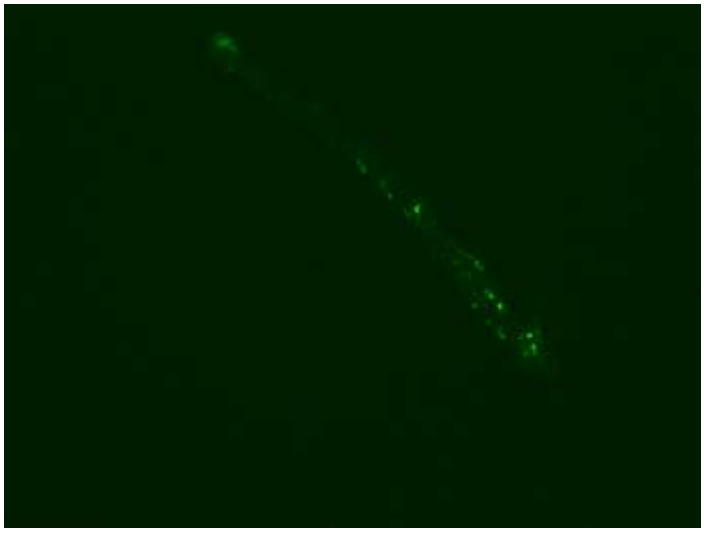


Figure 4.31: pEGFP expression at CR 5.0

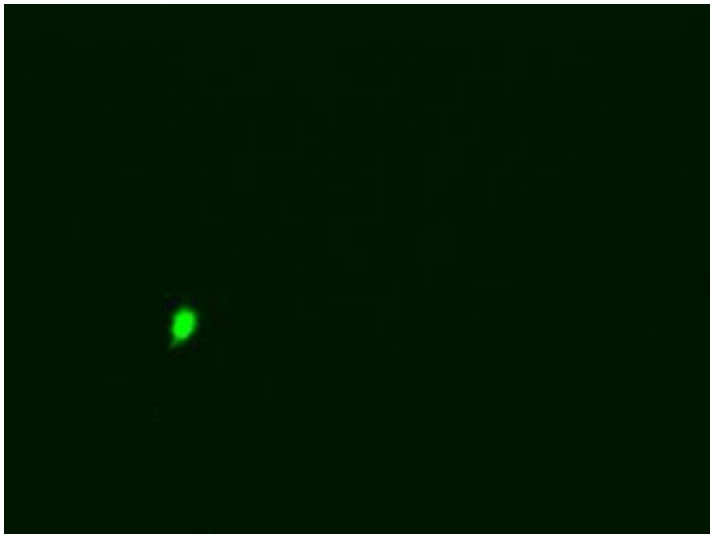


Figure 4.32: pEGFP expression at CR 5

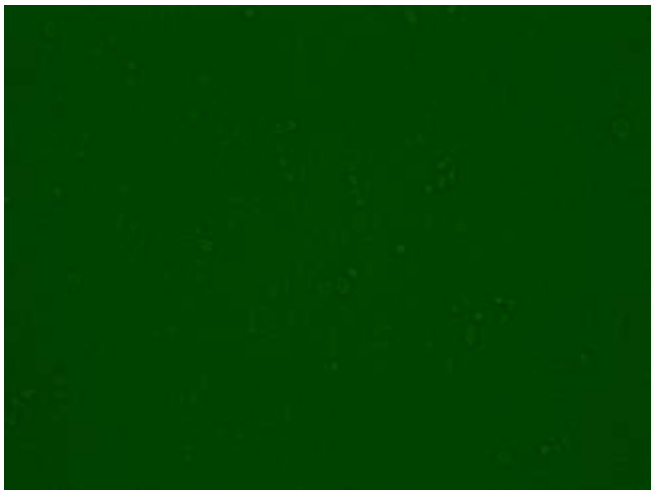


Figure 4.33: No pEGFP expression in the cells at CR 10

Interpretation of the results: From the above images it can be seen that the pEGFP expression can be seen in the lipofectamine well Very less transfection was seen at CR 5 and CR 2.5. No expression was seen at CR 10. This can be due to the following reasons:

- First, transfection efficiency of G1 PAMAM dendrimer is very low
- Or it can be possible that the complex formed of dendrimer/DNA is not able to enter into the cell via cell membrane
- It can also be the reason that the dendrimer and the DNA are so tightly bounded that after entering into the cell, dendrimer is not able to release the DNA.

To overcome these problems experimentations are still going on to get the standardized results.

5.CONCLUSION

Nonviral gene delivery systems especially cationic polymers are receiving much attention. The generation of particulate systems with a specific shape and size plays a crucial role in the development of modern gene and drug delivery systems. Dendrimers have been explored extensively in this field because their structure is well-defined and can be tailored to specific applications. Dendrimer-DNA binding is of major importance for gene delivery and transfection. Over the past decade a number of promising synthetic nonviral gene delivery systems have been developed and a profile of their potential advantages and disadvantages has emerged. The structural analysis of dendrimer-DNA binding is the first step in gene delivery and DNA condensation, which requires further investigations. In this investigation we have formed the nanocomplexes of plasmid DNA and PAMAM dendrimer at different charge ratios. The super molecular structure formed between DNA and the starburst dendrimer were studied by Atomic Force Microscopy (AFM). It was shown that while DNA- dendrimer complexation occurred via ionic interaction, DNA remained in B family structure. Studies by AFM showed that DNA condensation and particle formation were influenced by the different charge ratios of the dendrimer. AFMand TEM images provides the structural information regarding Dendrimer-DNA formulation which is important in gene delivery and DNA transfection. In order to increase the transfection efficiency of the dendrimer, we have to play with its concentration and the charge density. Also the dendrimer variants eg. Peptide conjugated, ester conjugated etc.can be taken to see the transfection of the DNA into the cells.

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