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Dendrimer Polycation for Delivery of Genes

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Genetic diseases are either monogenic, which involve defect of single gene and are an easy target for gene therapy or polygenic, which involve a number of defective genes and are poor targets for gene therapy. Earlier gene therapy used viral vectors but the advantages of non-viral system are being fully exploited. This review focuses on cationic polymers especially dendrimers as a delivery system for DNA as ligand targeting. Where possible the relative efficiency of different cationic polymeric delivery systems is compared. It also aims to focus on the immense potential of dendrimer as carrier for gene.

Gene therapy is a new approach for the treatment of inherited and acquired diseases. It generally requires a delivery system to transfer a gene sequence into target cells to achieve therapeutic benefits for the recipients[1,2]. Gene therapy thus may be an effective tool in the treatment of chronic diseases like diabetes[3]. Out of the two potential strategies for gene therapy, the *ex vivo* and the *in vivo*, the *ex vivo* strategy allows a wider range for therapy. In this approach, cells would be extracted from patients, transfected with a new gene, expanded in culture and reimplanted in the patient. In the *in vivo* approach, the gene could be treated more like any other pharmaceutical agent and administered directly to the patient. In this option, the delivery system would have to avoid the body's defense mechanism, reach the cell to be treated and transfect it *in situ*. While the former approach is less admissible scientifically, the latter is less compliable to the patient and hospital scientific support staff. The genetics and the molecular biology are relatively well understood for many genetic diseases and the knowledge is increasing rapidly. The progress is dependent upon delivering a large, highly ionized and easily detachable piece of DNA into the nucleus compartment of appropriate cells in the body. Ideally, the piece of DNA should be delivered only to the relevant cells and prevented from further spreading to other tissues.

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In delivery aspects of gene therapy, the prime consideration is the physio-chemical properties of DNA that will be similar for all. Positively charged histone proteins and polyamines condense DNA to within the restricted volume of nucleus that is half full in expression of DNA. Even the condensed volume of DNA is too large to pass unaided across the membrane that separates the compartments of the cell and the inside of the cell from its environment, which necessitates the entry of DNA by another route. Experiments on uptake of oligonucleotides into cells, have shown that even methyl phosphate oligonucleotides up to 15mer long that have been chemically modified to be more hydrophobic than the naturally occurring phosphodiester, still do not cross the membrane by passive diffusion at a significant rate[4].

Cellular transport mechanisms like pinocytosis[5] and receptor-mediated transport[6] are responsible for the uptake of macromolecules in the cells. In pinocytosis, a portion of the membrane invaginates and pinches off a vesicle inside the cell. The vesicle even can enclose the suspended ma-

terial or attach to the membrane that invaginates which is a degradative process since most of the biological materials are susceptible to the breakdown by the collection of enzymes in the cytosomal compartment. In receptor-mediated breakdown, the ligand attaches to the receptor that is associated to a coated pit[6]. A coated pit is a specialized area of the membrane containing a particular protein called clathrin[7]. The binding of the ligand to the receptor at the cell surface and clustering in the coated pit leads to the subsequent internalization of the receptor ligand complex via clathrin-coated vesicular intermediate (clathrin-dependent pathway) which enters into the cytosol. However, the receptor along with the bound ligand can also be internalized without coating of the clathrin proteins (clathrin-independent pathway).

One of the widely investigated clathrin-independent pathways has been demonstrated with caveolae which invaginates of plasma membrane. The major difference in receptor disposition from the clathrin-coated pits is that they do not separate from the plasma membrane while unloading the cargo, which is called as potocytosis. Non-clathrin-coated pathways[8-10], which is independent of clathrin-coated on caveolae pathway or both, can attribute to fluid phase pinocytosis. This results when the perturbations are formed from cultured cells which collapse back onto the cell surface and trap extracellular fluid in large vesicles called macropinosomes and the process is found to be stimulated by epithelial growth factor.

For all these routes of uptake, the size of particles for uptake is likely to be critical which leads to the need of an early recovery from the lysosomal or endosomal compartment in order to avoid enzymatic degradation and to achieve successful targeting. An efficient mechanism of DNA transport across the membrane is essential for an effective gene therapy and also the DNA must be condensed to a sufficiently small size to be taken up into the cell.

Interaction of polycations with DNA:

Condensation of DNA is achieved by three compounds. First is neutral compounds such as polyethylene glycol[11-13], polyvinylpyrrolidone[13] and second is anionic compounds such as polyacrylates[13]. Third are cationic compounds, such as polyamines[14-16], polyaminoacids[17-21], histone fraction[22] and protamines[23]. These are most efficient and useful condensing agents. The effect of different cationic polymeric materials as condensing agents and transfection enhancer are considered first.

Transfection of polycations with DNA:

The properties of various cationic polymers are reviewed in terms of physiochemical interaction where possible and related to their ability to transfect cells. Description of binding between polymer and DNA is almost nonexistent to the point that even DNA-polycation ratios are poorly defined.

Poly 4-vinyl pyridine:

Transfection efficiency of this polycation has been reported by Kabanov *et al.* in both prokaryotic[24] and eukaryotic systems[25]. In earlier study, DNA was complexed with either poly-(N-ethyl-4-vinyl pyridinium) bromide (PVP) or PVP modified with 3% N-cetyl-4-vinyl pyridinium unit (PVP-C). Increase in binding of PEC to bacterial membrane as compared to plasmid increases transfection. Further studies on binding of PVP and PVP-C to liposomes as a model of cell membranes showed that PVP-DNA complexes did not interact with negatively charged liposomes surfaces, but that PVP-C-DNA complexes did[26]. The effect of pluronic block copolymers on uptake and transfection efficiency of PVP-DNA complex was also investigated[27]. Pluronic block copolymers containing both hydrophobic polypropylene oxide segments and hydrophilic polyethylene oxide segment have been used either to make micellar system for drug delivery[28], or to coat hydrophobic particles thereby avoiding cellular uptake of particles by macrophages[29]. Incubation in the presence of 0.1% pluronic copolymer increases the uptake and transfection efficiency of complexes in to NIH3T3 cells[27].

Chitosan:

Chitosans are natural basic polysaccharides obtained in various molecular weights from partial deacetylation of chitin (chitin is based on a repeating N-acetyl-D-glucosamine unit). It is nontoxic and of low immunogenicity[30]. Interactions with DNA have shown that chitosan trimers and larger oligomers bind strongly to DNA or RNA. Binding of chitosan oligomers to DNA results in stabilizing the helix, which leads to a sharp rise in Tm for DNA[31]. DNA chitosan complexes completely incorporate all DNA when mixed at a 1:1 ratio, or at excess with chitosan. TEM studies show that condensation of DNA forms toroids, the particle size dependent on the Mw of the chitosan. Transfection of C_2C_{12} myoblasts using a CMV-β gal plasmid was also reported[32]. Chitosan nanospheres have also been prepared by coacervation technique at 55° in sodium sulfate solution[33]. Chitosan has been substituted with deoxycholic acid to in-

crease hydrophobicity of polysaccharides[34].

Synthetic cationic sugar polymer:

Transfection has also been reported using synthetic polyamino polymer with glucose backbone[35]. Optimum condition for *in vitro* transfection of HT 1080 human sarcoma cells using pCLuc4 plasmid driven by CMV promoter are, however, found to be at polymer:DNA weight ratio of 10. Transfection efficiency of the polymer was assessed on a further 10 cell lines, and in all but one case reported superior to lipofectamine. In addition, the polymer is unaffected by the presence of up to 8% serum in the medium, whereas transfection of lipofectamine is seriously affected by as little as 2% serum. *In vivo* experiments were carried out on D54 MG glioma cell xenografts implanted intracerebrally in adult B17SCID mice injection of Lac plasmid complexed with polymer resulted in comparable expression to a recombinant adenoviral vector, while control polymer and cationic lipid preparations resulted in detectable expression.

Poly [(2-dimethyl amino) ethyl methacrylate]:

The transfection efficiency of poly (2-((dimethyl amino) ethyl methacrylate) (PDAEMA) was investigated by forming polymer: DNA complexes in growth medium[36]. Particle size of PDEMA-DNA complexes varied markedly with polymer: DNA ratio. The smallest with diameter of 200 nm, appeared at weight ratio of 2 and above. The particles were of narrow polydispersity and greater stability at weight ratios where good transfection was achieved. If particles were made in presence of serum, or serum added after manufacture, particle size increased to 600 nm, although presence of serum did not affect transfection. Transfection efficiency of PDAEMA-DNA complexes was compared with other transfection inducing agents[37]. These complexes were found twice as active as DEAE-dextran complexes and 8-fold more active than PLL-DNA complexes in the presence of chloroquine.

Dendrimers:

In general, dendrimers are globular, highly branched, fractal like polymers of "well-defined" three-dimensional shape, size and molecular weight that are constructed via iterative or stepwise procedures. The distinction between dendrimers and hyperbranched polymers is that the hyperbranched polymers are prepared via a one-step or single-pot process and possess greater molecular weight distributions or polydispersities. The term dendrimer, coined by Tomalia *et al.*[39] from the Greek term dendron for tree-like, is synonymous with both arborol[40] founded in the Latin term arbor for tree and cascade referring to successive stages, which also defines the nomenclature family name[41]. Dendrimers can be tailored to possess uniform or discrete functionality and posses tunable internal packing densities, void volume, surface moieties, molecular mass, solvent-dependent size and branching dimensions. Two conceptually different synthetic approaches for the construction of higher generation dendrimer exist: the convergent approach and the divergent approach. Both approaches consist of a repetition of reaction steps, each repetition step for the creation of an additional generation. Polyamidoamine (PAMAM) dendrimers are a class of dendrimers in which an amine starting material is repeatedly substituted at its amino termini to provide a branched structure. At each round of synthesis, another layer of branched chain is added and termed a "generation" e.g. a dendrimer with 3 layers is a generation 3 dendrimer[42]. Because each layer should be fully substituted, these polymers should be well defined and chemically very homogeneous. They also have high cationic charge density of primary amine groups on the surface of the polymer. Because of their high charge density and unique well-defined properties, they have been proposed as DNA delivery agents.

Effect of charge and generation:

Haensler and Szoka[43] were first to report the advantages of these novel polymers for gene delivery. In their studies a range of dendrimer generations were investigated against different charge ratio of polymer to DNA. Optimal transfection of pCLuc4 plasmid into CV-I fibroblast cells takes place at charge ratio of 6 or 10 with generation-6 dendrimers. The most marked change in transfection efficiency is seen going from generation-4 dendrimers to generation 5 dendrimers and this change may relate to changes in the physical shape of dendrimers. Transfection of optimal complex increased by two orders of magnitude over 2-6 μg DNA per $5x10^5$ cells. Optimal transfection efficiency with dendrimers is almost three orders of magnitude greater than that seen with polylysine, but is variable among different cells lines. Inclusion of chloroquine in the medium was reported to have no effect on transfection efficiency and inclusion of 10% serum resulted only in having activity. These results suggest that the lower pKa of the dendrimer cause them to act as proton sponges, and to help to deliver complexes from the lysosomal compartment. Dendrimer did exhibit some toxicity to cells as shown by decrease in protein recovery of transfection cells. Reports of cytotoxicity tests using MTT show that toxicity of dendrimer is enhanced on complexation with DNA, but is less toxic than polylysine

even when complexed.

Effect of size and generation:

Mumper *et al.*[32] reported that a generation-5 dendrimer condensed DNA and that as charge ratio increased from I to 4 particle size decreased from >500 nm to about 100 nm. Also, it was accompanied by increased transfection efficiency of a CMV β-gal plasmid in C_2C_{12} myoblasts. In a more comprehensive study, Kukowskalatallo *et al.*[44] demonstrated that complexes with DNA could be formed with dendrimers of generation-3 or higher over sodium chloride concentration of 50 mM-1.5 M. and pH range of 5.0-9.8. Transfection studies were carried out on 18 different cell lines using pRSV Luc, pCMV Luc. pRSVβ-gal or pCMVβ-gal expression plasmids. Transfection occurs with dendrimers from generation-5 to generation-10, transfection efficiency increases as a ratio of dendrimer to DNA increases upto 50. Transfection efficiency also increases by a number of additions to the transfection medium, including chloroquine (50 μM) and DEAE-dextran (0.5 μM) although dimethylsulfoxide has no effect. In the presence of DEAE-dextran, transfection efficiency increases with dendrimer generation is optimum at generation-9 to generation-10. On most of the cell lines tested, dendrimer in the presence of DEAE-dextran was more effective than lipofectamine, lipofection, or TFX-50. However, more efficient long-term transfection of upto 1×10^{-3} per cell is seen in the absence of DEAE dextran. DNA G10 dendrimer complexes were shown by TEM to produce large aggregate of >200 nm diameter, which were not seen in the presence of DEAE-dextran. This may account for increased transfection efficiency using this additional component. Dendrimers at concentrations used in transfection experiments were of negligible toxicity[45].

Gonez *et al.*[46] have reported that mutant genomic cystic fibrosis (CF) transmembrane conductance regulator (CFTR) sequences can be corrected in transformed CF airway epithelial cell lines by targeted replacement with small fragments of DNA with wild type sequence. To determine if the observed genotype modification following small fragment homologous placement (SFHR) was limited to transformed CF cell lines, further studies were carried out in both transformed and non-transformed primary normal airway epithelial cells. The endogenous genotype of these normal cell lines was modified following liposome or dendrimer transfection using DNA fragments with Delta F508 CFTR sequence (488 rt, complementary single strands) designed to also contain a unique restriction enzyme cleavage site (Xho I). Replacement at the appropriate genomic locus by exogenous DeltaF508 CFTR DNA and mRNA-derived cDNA as well as Xho I digestion of the PCR products. These studies show that SFHR occurs in both transformed and non-transformed primary human airway epithelial cells and indicate that single base substitution (the silent mutation giving rise to the Xho I site) and deletion or insertion of at least three consecutive bases can be achieved in both normal and CF epithelial cells. Furthermore, these studies reiterate the potential of SFHR as a strategy for a number of gene targeting application, such as site specific mutagenesis, development of transgenic animals, development of isogenic cell lines and for gene therapy.

Effect of heat and molecular weight:

Tang *et al.*[47] have reported transfection of cultured cells using complexes between DNA and spherical cationic polyamidoamine polymers (starburst dendrimers) that consist of primary amines on the surface and tertiary amines in the interior. The transfection activity of the dendrimers is dramatically enhanced (>50 fold) by heat treatment in a variety of solvolytic solvents, e.g., water or butanol. Such treatment induces significant degradation of the dendrimer at the amide linkage, resulting in a heterodisperse population of compounds with molecular weights ranging from the very low (<1500 Da) to several tens of kilodaltons. The compound facilitating transfection is the high molecular weight component of the degraded product and is denoted as a "fractured" dendrimer. Transfection activity is related both to the initial size of the dendrimer and its degree of degradation. Fractured dendrimers exhibit an increased apparent volume change as measured by an increase in the reduced viscosity upon protonation of the terminal amines as pH is reduced from 10.5 to 7.2, where as intact dendrimers do not. Dendrimers with defective branching have been synthesized and also have improved transfection activity compared to that of the intact dendrimers. For a series of heat treated dendrimers we observe a correlation between transfection activity and the degree of flexibility, computed with a random cleavage simulation of the degradation process. We suggest that the increased transfection after the heating process is principally due to the increase in flexibility that enables the fractured dendrimer to be compact when complexed with DNA and swell when released from DNA.

Effect of activating agents:

Plank *et al.*[48] have examined the complement activating properties of synthetic cationic molecules and their complexes with DNA. Commonly used gene delivery vehicles include complexes of DNA with polylysine of various chain

lengths, transferrin-polylysine, a fifth-generation poly (amidoamine) PAMAM dendrimer, ethyleneimine and several cationic lipids (DOTAP, DC-Chol/DOPE, DOGS/DOPE, and DOTMA/DOPE). These agents activate the complement system to varying extents. Strong complement activation is seen with long chain polylysines, the dendrimer, poly (ethyleneimine) and DOGS (half-maximal at about 2-micron amine content in the assay used). Compared to these compounds, the other cationic lipids (in liposome formulations) are weak activators of the complement system (half maximal approximately 50-100 micron positive charge in assay) complement activation by polylysine is strongly dependent on the chain length. Short chain oligolysines are comparable to cationic lipids in their activation of complement. Incubation of these compounds with DNA to form complexes reduces complement activation in virtually all cases, the degree of complement activation by DNA complexes is strongly dependent on the ratio of polycation and DNA (expressed as the charge ratio) for polylysine, dendrimer, poly (ethyleneimine) and DOGS. To a lesser degree, charge ratio also influences complement activation by monovalent cationic lipid DNA complexes. For polylysine DNA complexes, modifying the surface of preformed DNA complexes with polyethyleneglycol (half-maximal approximately 20-micron amine content) can considerably reduce complement activation. The data suggests that, by appropriate formulation of DNA complexes, complement activation can be minimized or even avoided. These findings should facilitate the search for DNA complex formulations appropriate for reproducible intravenous gene delivery.

Effectiveness in plasmid mediated gene transfer:

Chin *et al.*[49] have reported that starburst dendrimer, a structurally defined, spherical macromolecule composed of repeating polyamidoamine subunits, was investigated to augment plasmid-mediated gene transfer efficiency in a murine cardiac transplantation model. The grafts were directly injected with naked pCH110, a plasmid encoding beta-galactosidase (beta-Gal) or pCH110 dendrimer complex, and reporter gene expression determined by X-Gal staining. The grafts injected with pCH110 dendrimer demonstrated widespread and extended beta-Gal expression in both myocytes and the grafts infiltrating cells from 7 to 28 d, compared to the grafts injected with naked pCH110 that expressed beta-Gal only in myocytes for less than 14 d. p-AlphaMHC-cIL-10 as plasmid encoding viral interleukin-10 (vIL-10) under the control of alpha-myosin heavy chain promoter, was able to prolong allograft survival from 13.9±-0.9 d to 21.4±2.3 d ($p<0.005$). When dendrimer G5DEA was used with p alphaMHC-vIL-10, 60 fold less DNA resulted in significant prolongation of graft survival to 38.6±4.7 d ($p<0.0005$). The dose of DNA, the charge ratio of DNA to dendrimer, and the size generation of the dendrimers were all determined to be critical variables for prolongation of allograft survival in this model system. Thus, the use of the starburst dendrimer dramatically increased the efficiency of plasmid mediated gene transfer and expression. Production of immunosuppressive cytokines at higher amounts for longer periods of time in a greater expanse of tissue enhanced the immunosuppressive effect and prolonged graft survival furthers.

Transfer of artificial chromosome into primary cell line:

Artificial chromosome expression system[50] are non-integrating, non-viral deliverable gene expression systems which function like natural chromosomes. The technology of artificial chromosome expression system has immense potential since it overcomes many of the limitations of other gene delivery and expression system including small carrying capacity, random integration, low level and short term expression and viral vector related safety. Unlike natural chromosomes that contain thousands of genes that make protein vital to human life, ACES contain only those genes required for the expression of the desired therapeutic response. Moreover, the ability to engineer and fast *in vitro* delivery expression and stability enhances the probabilities that ACES will perform to specification once inserted into the *ex vivo* target cells. Jong *et al.*[50] have evaluated the commercially available transfection reagents for this ability to mediate the *in vitro* satellite DNA and a pay load including a marker gene (hygromycin B) and a reporter gene (Lac Z). Delivery was measured within 24 h after transfection using a rapid screening technique to quantify the transfer of ACES labeled with a thymidine analog. The results demonstrate that the artificial chromosome can be successfully transferred to primary target cells.

Dendrosomes:

Sarbolouki *et al.*[51] have developed a novel family of non-viral vehicles that forms hyperbranched spherical entities called dendrosomes. They are readily synthesized, highly stable (nearly four years at ambient conditions), nontoxic, inexpensive and extremely convenient to handle and use. Their reports of transfection studies on human cell culture and vaccination of mice against hepatitis B are highly encouraging.

CONCLUSIONS

It is quite clear from the above that a variety of polycations are capable of condensing DNA into small particles, especially dendrimers, due to very precise structure. The condensing agents may change the conformation of DNA and even may remain unchanged. The condition under which these complexes form can vary, resulting in a wide range of possible size and properties of particles. Physiochemical characterization and other studies strongly suggest that polymers may be considerably better for transfection. The relative toxicity of different polymers appears to be the key issue.

Abbreviations:

ACES : Artificial chromosome expression system
Da : Daltons
DEAE : Diethylamino ethyl
Mn : Number average molecular weight
MW : Molecular weight
Mw : Weight average molecular weight
PDAEMA : Poly (2-dimethyl amino) ethyl methacrylate
PEC : (Inter) poly electrolyte complexes.
PLL : Poly-L-lysine
SCID : Severe combined immuno deficiency
TEM : Transmission electron microscopy.
Tm : Transition melting temperature

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