PROPOSAL I

A Nonviral Gene Transfer Agent Based on N-(carboxymethyl)-*trans*-4-hydroxy-L-proline

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Gene therapy, the treatment or prevention of disease by gene transfer, is regarded by many as a potential revolution in medicine[1]. The means of effecting gene transfer, through a vehicle known as a vector, has been a field of research with many variations, each with their advantages and disadvantages. These vectors are categorized in two sections: (I) viral vectors: retroviruses, adenoviruses, adeno-associated and other viral vectors; (II) nonviral vectors: liposomal gene delivery, liposome / polycation / DNA (LPD) complexes, direct injection of naked DNA, peptide mediated gene delivery, and polymer vectors. The focus of this proposal in on a hyperbranched polymer vector based on N-(carboxymethyl)-*trans*-4-hydroxyl-L-proline (Figure 1).

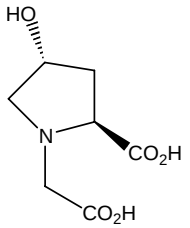


Figure 1. N-(carboxymethyl)-*trans*-4-hydroxyl-L-proline.

Viral vectors take advantage of millions of years of evolution for efficient transfer of viral nucleic acids, but our bodies have correspondingly evolved an efficient immune system to forestall infection[2]. Although viruses are efficient in transferring DNA, the amount they can carry is limited. Other issues limiting their broad use are: restricted targeting of specific cell types, production and packaging problems, recombination, and high cost. In addition, the evocation of an immune system response, as well as the toxicity of viral systems, restrain their routine use in basic research laboratories. In order to make viral vectors more useful, harmful genes have to be removed. In effect the viral vectors are de-evolved.

On the other hand, nonviral vectors are evolving to gain the elegant functions and

complexities of viruses[3]. Many different types of nonviral vectors are being designed to form a complex with DNA, transport into cells, and then transfer the nucleic acids into cells *in situ*. The expected result is to enhance or decrease the expression of a formulated transgene[4]. Nonviral vectors are more amenable to engineering to overcome immune and other toxic effects as well as tackle production challenges. Of the many forms of nonviral vectors that are designed, those with the ability to effect gene transfer *in vivo* will prove to be the best selection. It would be a remarkable achievement for a nonviral system to reach the level of *in vivo* viral-mediated gene expression.

The formation of a complex with DNA, followed by transport into cells, and the transfer of DNA are the three levels at which most DNA delivery systems operate. They are also known as DNA condensation and complexation, endocytosis, and nuclear targeting/entry, respectively. Supramolecular complexes are formed when DNA is mixed with multivalent cations such as spermine, cationic lipids, or polylysine. Previous work has shown that DNA in these complexes condenses into toroids or rods when about 90% of its charge is neutralized by counterions[5]. This step is sometimes referred to as the "packaging" step, and also serves as extracellular protection for the DNA.

Endocytosis is a multi-step process involving binding, internalization, formation of endosomes, fusion with lysosomes, and lysis[6]. Degradation of the supramolecular complex, both the DNA and the vector, is the usual result from the very low pH (4-5) and enzymes within the endosomes and lysosomes. DNA that has survived both endocytotic processing and nucleases within the cytoplasm must then dissociate from the condensed complexes either before or after entering the nucleus.

Once DNA had entered the nucleus, through nuclear pores or during cell division,

its transfection efficiency depends upon the construction of the gene expression system, which has been covered in a recent review[7]. The low efficiency of DNA delivery from outside the cell to inside the nucleus is a natural consequence of this multi-step process. As a result, the number of DNA molecules decreases at each step of the journey to the nucleus.

There are three major barriers to DNA delivery: low uptake across the plasma membrane, inadequate release of DNA molecules with limited stability, and lack of nuclear targeting[8]. To overcome these obstacles work has been done mostly in the form of uptake-enhancing chemicals, as well as extracellular and intracellular protection of DNA, while the understanding of nuclear targeting is still in its infancy.

The use of uptake-enhancing chemicals is based on complex formation between positively charged chemicals and negatively charged DNA molecules. It is arguably the easiest, most versatile, most effective, and most desirable of the DNA delivery methods, and was demonstrated more than 30 years ago[9]. The earliest chemical methods were introduced in the late 1950s and used high salt concentration with polycationic proteins to enhance nucleic acid entry into the cell[10]. Over a 10-year period starting in 1965, a variety of other chemicals were introduced, including 2-(diethylamino)ether (DEAE)-dextran[11] and calcium phosphate[12], which interact to form DEAE-dextran-DNA and calcium phosphate-DNA complexes, respectively. After the complexes are deposited onto cells, they are brought in via endocytosis. The problem with these methods though is that while they work well *in vitro*, the opposite in true *in vivo*. Besides having issues with reproducibility, they are cytotoxic when given *in vivo*.

Lipid-based systems are the most commonly used methods of DNA delivery and

have been used in human clinical trials. Lipid-based vectors have several deficiencies that include: lack of targeting, poorly understood structure of DNA-lipid complexes, and variations that arise during fabrication. As with earlier methods, lipid-based systems have still shown toxicity upon systemic administration[13].

In several instances, direct administration of DNA, either through intramuscular injection[14] or inhalation[15], was expressed in tissue cells. Although the success of naked DNA is limited, the very fact that it worked questions the role of vectors in human gene therapy and mechanisms by which they can aid or enhance gene transfer.

The use of protein-based methods of DNA delivery is increasing in popularity due in some part as a result of the versatility offered by chemical modification. The cationic peptide poly-L-lysine (PLL) can condense DNA for more efficient uptake[16], yet there are still problems with toxicity.

Along with improving the efficiency of DNA uptake by cells, it is also very important to protect DNA from both extracellular and intracellular degradation along its trek into the cell and then the nucleus. Extracellular protection of DNA is found in complex formation with various polymers and lipids, as mentioned above. When delivered systemically DNA is cleared from the blood through a process known as opsonization. As a defense mechanism, it removes 80-90% of hydrophobic particles in blood, and limits the use of artificial lipids for DNA delivery. A recent review describes the biodistribution of various nonviral gene delivery systems, including comparison of potential delivery routes, stability in blood circulation, and extravasation into tissues[17].

DNA must be provided intracellular protection in order to survive the endocytotic pathway. By comparing three different cationic lipid compounds, Ouhabi et al.[18] noticed

that the efficiency of DNA delivery is correlated not only with uptake, but also with destabilization and escape from endosomes. Branched cationic polymers, such as PEI or starburst dendrimers promote early release of DNA[19,20,21] from endosomes, thus decreasing the amount of degradation that occurs.

After getting into the cell, DNA must be released from its supramolecular complex before transcription can proceed. The rate at which the DNA is released or "unpackaged" from the complex influences the efficiency of gene expression. The rate of decomplexation goes hand in hand with the stability of the complex. Recent work by Schaffer, Lauffenburger, et al.[22] demonstrates the importance of this concept. The greatest gene expression occurs at intermediate stability, because stable complexes restrict DNA transcription and unstable complexes permit rapid DNA degradation.

In order for transcription to occur, DNA must survive the cytoplasm, and get into the nucleus. While nuclear targeting is not well understood at this point, it has been seen that certain synthetic polymers, such as PEI, but not cationic lipids, protect DNA in the cytoplasm and are known to promote entry into the nucleus[23]. In general though, nonviral systems are much less efficient than viral systems.

PEI and dendrimers contain secondary and tertiary amines that apparently serve to buffer the endosomal compartment, resulting in osmotic lysis. Midoux and Monsigny have extended this concept by conjugated His to the side chain of polylysine resulting in a polypeptide that buffers endosomes and enhances gene expression[24].

The number of Lys residues in a DNA condensing peptide can influence the particle size, stability, and gene transfer efficiency of the peptide DNA condensates. A significant problem with all of these methods, though, is that the simple mixing of

DNA and cationic lipids or polymers produces several species of particles that aggregate in physiologic solutions, with deleterious consequences for gene delivery. Aggregation is potentiated by serum proteins that coat polycation (polyethylenimine or polylysine) complexes.

Other factors that influence the efficiency of cellular DNA delivery have been discussed recently in reviews[25,26,27,28] and include: optimization of DNA condensation, size of DNA complexes, route of administration, biodistribution, bioavailability, cell and tissue targeting, and cytotoxicity.

The best nonviral vector for DNA delivery should form a complex with DNA easily. It should be made of nontoxic and biocompatible materials. It should protect DNA outside and within the target cells. It should be capable of bypassing or escaping from endocytotic pathways. The vector should also release the DNA from the complex efficiently. The DNA should be delivered to most the intended target cells, leading to total transfection. With the above, the best nonviral DNA delivery system should include efficient nuclear targeting, along with high, persistent and adjustable therapeutic levels or proteins.

The focus of this proposal in on a hyperbranched polymer vector based on N-(carboxymethyl)-*trans*-4-hydroxyl-L-proline, Figure 1. One of the main reasons for this choice is because the molecule it is based on 4-hydroxy-L-proline, an important structural constituent of the fibrous protein collagen, the most abundant protein in mammals. Collagen occurs in all multi-cellular animals and is the most abundant protein of vertebrates. Collagen occurs in virtually every tissue. It is an extra-cellular protein that is organized into insoluble fibers of great tensile strength. It is the major stress bearing

component of connective tissues such as bone, teeth, cartilage, tendon, ligament, and the fibrous matrices of skin and blood vessels[29].

Also, N-(carboxymethyl)-*trans*-4-hydroxyl-L-proline contains a tertiary amine that can serve to as a buffer, and allow for early escape in the endocytotic pathway. PEI and dendrimers that contain secondary and tertiary amines that apparently serve to buffer the endosomal compartment, resulting in osmotic lysis[30]. Thus, a polymer made from N-(carboxymethyl)-*trans*-4-hydroxyl-L-proline should be able to protect DNA as it enters the cell.

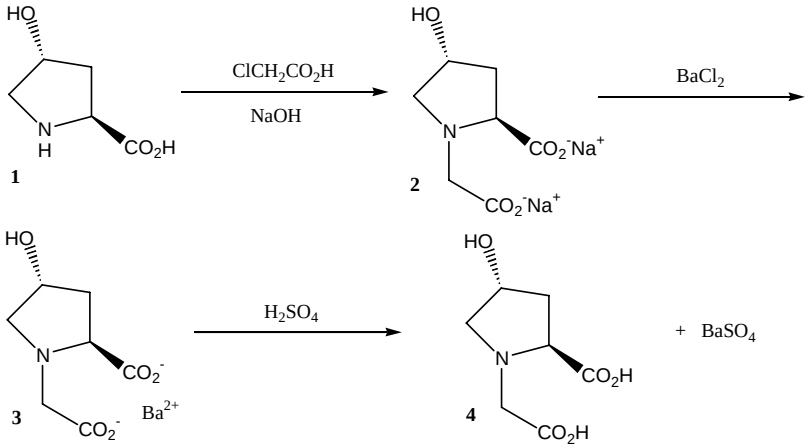
There are several requirements that a non-viral polymeric gene transfer agent must possess. They are as follows: be minimally toxic; efficiently transfect DNA; biodegradable ester backbone linkages; tertiary amine groups in the interior; and primary amine groups in the exterior. High transfection efficiency of the transfer agents must also be balanced with minimal toxicity.

Ester group inserted as biodegradable functionality, which serves to reduce cytotoxicity and for an eventual accelerated release of DNA from complexes by the action of esterases.[31]

The size and homogeneity of carrier molecules play a critical role in the success of a nonviral gene delivery system. These two parameters impact the toxicity, anti-genicity, and the ability to systematically optimize gene delivery carriers for in vivo applications.

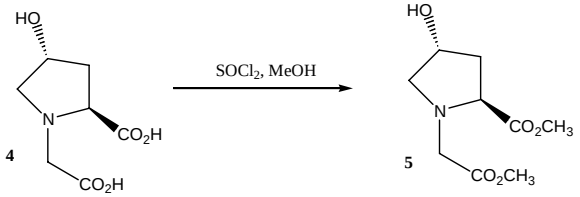
In addition to stabilizing peptide DNA condensates, the delivery system needs to facilitate the escape from lysosomal trafficking and delivery of DNA to the cytosol.

The synthesis of the proposed monomer is shown in Scheme 1. The common amino acid, 4-hydroxy-L-proline (**1**), is N-alkylated using chloroacetic acid and sodium hydroxide in aqueous media. The diacid (**2**) is isolated from the starting material by precipitation from solution as the barium salt (**3**). The barium salt (**3**) is subsequently hydrolyzed with sulfuric acid to give N-(carboxymethyl)-*trans*-4-hydroxyl-L-proline (**4**).



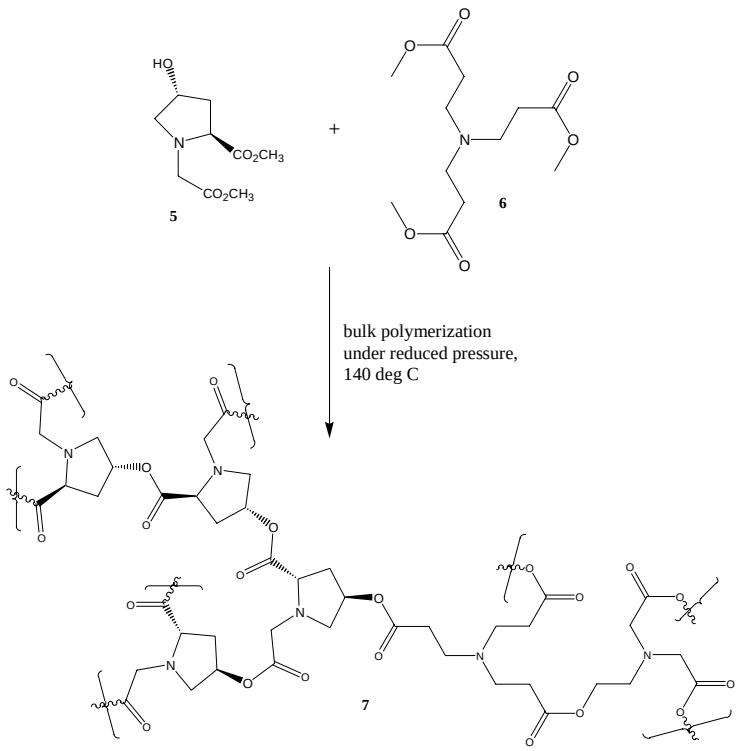
Scheme 1. Synthesis of N-(carboxymethyl)-*trans*-4-hydroxyl-L-proline.

In Scheme 2, the diacid (**4**) is converted into the dimethyl ester (**5**) by reaction with thionyl chloride and methanol,[32] in order for the monomer to be soluble in organic solvents.



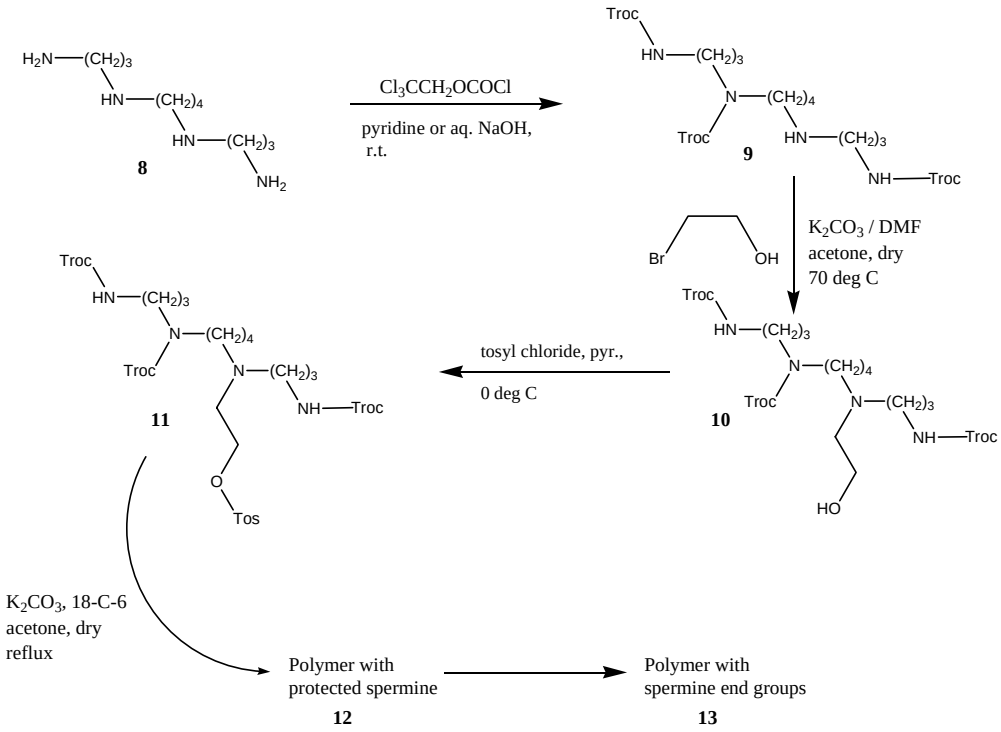
Scheme 2. Synthesis of N-(carboxymethyl)-trans-4-hydroxy-L-proline dimethyl ester.

The polymerization (Scheme 3) is carried out in bulk in the presence of ammonia core starburst PAMAM dendrimer -0.5 generation as a core moiety (monomer/core ratio, 200/1) and $Al(OiPr)_3$ as a catalyst (1 mol %). The core moiety is added to decrease polydispersity and prevent the formation of cross-linked or excessively high molecular weight polymers.[33]



Scheme 3. Polymerization of N-(carboxymethyl)-trans-4-hydroxy-L-proline dimethyl ester.

Terminal amine groups are added to the hyperbranched polymer via coupling to a modified spermine, a molecule that is known for its ability to cross the cellular membrane using specific polyamine transporters that a common to most mammalian cells.[34] In Scheme 4, three of the amines in spermine (**8**) are protected with trichloroethyl (Troc) groups. The remaining amine of compound **9** is coupled to 2-bromoethanol with potassium carbonate in DMF. The hydroxyl group of compound **10** is then tosylated with p-toluenesulfonyl chloride in pyridine in an ice bath. The protected and alkylated spermine (**11**) is then coupled to the polymer with potassium carbonate and 18-crown-6 in refluxing anhydrous acetone. The Troc groups are then removed using zinc, THF, and water, with a pH between 5.5 and 7.2, leaving the target macromolecule (**13, Figure 2**) The polymer is then purified and characterized by size exclusion chromatography.



Scheme 4. Spermine modification and addition to polymer.

Following ^{1}H and ^{13}C NMR characterization of the end groups and the degree of branching, the toxicity of the polymer should be measured using an MTT assay. MTT is 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, which is a pale yellow color until it is cleaved by living cells, yielding a dark blue product. MTT is a common test for measuring cell proliferation and cytotoxicity. The polymer's transfection efficiency should also be measured and compared to other polymers that are known to transfect DNA into cells.

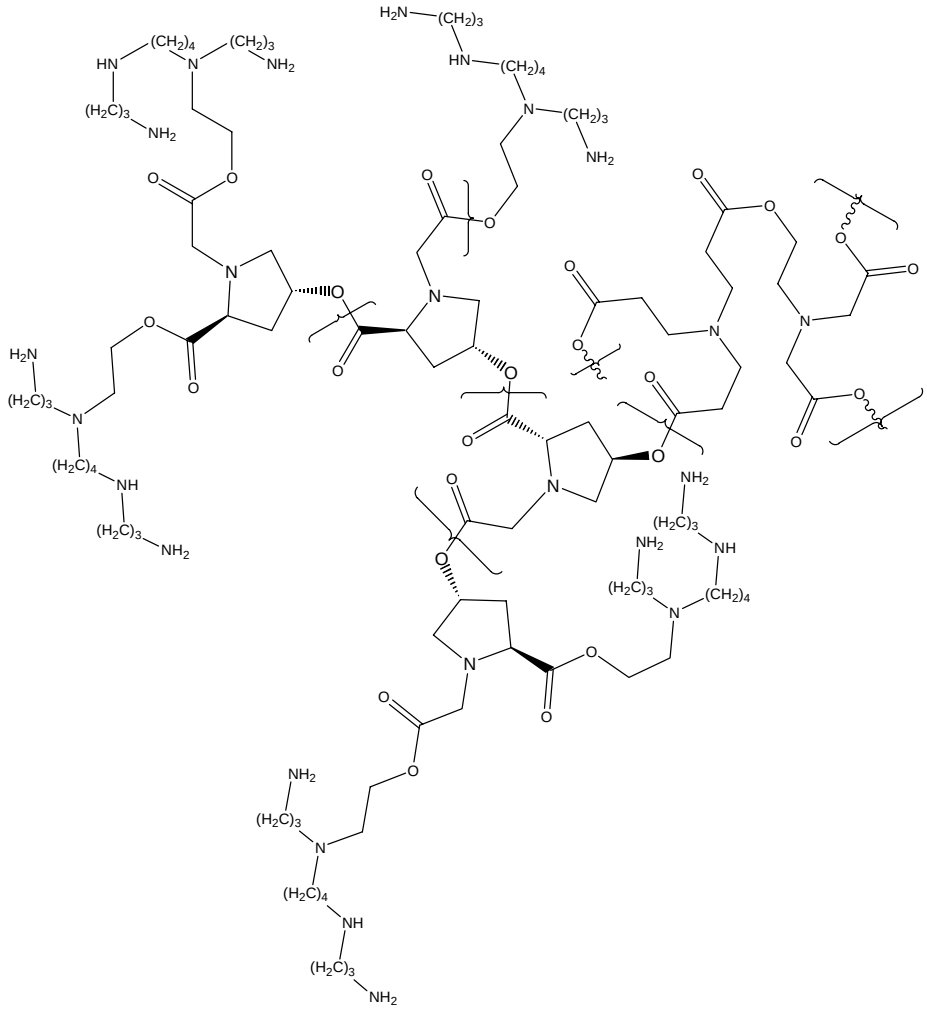


Figure 2. Structure of hyperbranched polymer based on N-(carboxymethyl)-trans-4-hydroxy-L-proline, spermine, and PAMAM.

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