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Chitosan and Its Modifications: Are They Possible Vehicles for Gene Therapy?

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1. Introduction

In gene therapy, the most important step is how to effectively deliver the therapeutic gene to the target cells or organ. At present, there are two methods, which are those using a viral and a non-viral vector system. The most common viral vectors that have been used include retroviruses, herpes simplex viruses, lentiviruses, adenoviruses and adeno-associated viruses (Oligino et al., 2000). The advantages of most viral vectors are high transfection efficiency and fast transcription of the foreign material inserted in the viral genome. However, a number of disadvantages have also been described, such as poor target-specificity, low capacity to incorporate foreign deoxyribonucleic acid (DNA) sequence to their genome (Mansouri et al., 2004), toxic and inflammatory effects, wild-type mutations, potential oncogenic effects (Lee et al., 1998), and, in particular, unwanted immune responses. In clinical trials of gene replacement therapy using viral vectors, significant adverse effects have been reported, including a fatal inflammatory response associated with adenoviral vector (Raper et al., 2003), and the development of acute leukaemia in recipients of ex-vivo, adenoviral vector-transduced hematopoietic cells (Woods et al., 2006). Intravenous adenoviral vector has also resulted in high liver toxicity due to uptake by hepatocytes or Kupffer cells of the liver reticular endothelial system, immediately following systemic administration.

2. Non-viral vectors for gene delivery

Due to the limitations and disadvantages of using viral vectors, there has been an ongoing search for an efficient safe vector for gene therapy, which has lead to the development of non-viral gene therapy. They have some advantages over viral methods, including simple large scale production and relatively low host immunogenicity. Previously, low levels of transfection and expression of the gene limited the usefulness of non-viral methods. However, recent advances in vector technology have been useful in yielding molecules and techniques with transfection efficiencies approaching or surpassing those of viral vectors. Table 1 provides examples of the main non-viral methods of gene delivery.

Non-viral method	Examples
Direct methods	Injection of naked DNA
Physical methods	Electroporation
	Gene gun
	Sonoporation
	Magnetofection
Chemical methods	Oligonucleotides
	Lipoplexes and polyplexes
	Dendrimers

Table 1. Examples of non-viral methods of gene delivery

Most non-viral vectors have no limitation in DNA size for packaging and they have the possibility of modification with ligands for tissue- or cell-specific targeting with low commercial cost and high reproducibility. Among these carriers, cationic lipids (lipoplexes) and cationic polymers (polycations) are primarily used, especially in *in vitro* gene transfection. Lipoplexes can form micelles or liposomes, which are multilayered structures, where the DNA is sandwiched between the cationic lipids. The lipoplexes present some problems due to their low physiological stability, reproducibility, and their toxicity of polar and hydrophobic moiety containing structure. *In vivo*, the intravenous administration of cationic lipid/DNA complexes presented significant problems, as these reagents can be quite toxic. On the other hand, polycations are more stable than lipoplexes and can protect DNA against nuclease degradation (Gao & Huang, 1996). Their structures also show more variability and versatility, including the possibility of incorporation of target-specific cellular receptors. Thus, modifications to these polymers, such as molecular weight (Mw), geometry (linear verses branched) and ligand attachment, can be easily undertaken successfully (Kim et al., 2007; Pack et al., 2005). Furthermore, they can compact DNA molecules to a relatively small particle size. However, the efficiency of gene delivery by both complexes is still relatively low, when compared to viral vectors. Polyethylenimine (PEI) is a cationic polymer that has been used for non-viral gene transfection for some time, but due to its toxicity and the variable results, it has not been widely accepted.

3. Chitosan as a non-viral carrier

At present, chitosan is the most prominent of the non-viral carriers being investigated. The biomaterial, chitosan, has interested many researchers around the world, particularly in relation to its ability to be a gene delivery vehicle or the ability to modify this biopolymer for the gene delivery vehicle. This is because of its properties of biodegradability, biocompatibility, and low toxicity, and because it can be modified for increasing transfection efficiency, as well as for targeting gene delivery development.

3.1 Molecular structure of chitosan

Chitosan (poly[β-(1-4)-2-amino-2-deoxy-D-glucopyranose]) is a deacetylation product of chitin (see Figure 1), a high Mw natural polymer found in the shells of marine crustaceans, such as shrimps (see Figure 2), as well as various insects, the internal structures of other invertebrates, and in the cell walls of fungi. It also provides an avenue for recycling of marine shellfish waste, which can now be “mined” for chitin and chitosan (Hayes et al., 2008).

Fig. 1. Chemical structure of Chitin

Fig. 2. Commercial chitosan is derived from the shells of shrimp and other sea crustaceans, including the Alaskan pink shrimp, pictured here (US National Oceanic and Atmospheric Administration, 2011)

Deacetylation of chitin can be performed by boiling chitin from crab or shrimp shells in sodium hydroxide after decolourisation with potassium permanganate (Van Der Lubben et al., 2001). Chitosan is a co-polymer of glucosamine and *N*-acetyl-D-glucosamine. When the number of *N*-acetylglucosamine units exceeds 50%, the biopolymer is called chitin; the term 'chitosan' is used to describe an *N*-acetyl-glucosamine unit content less than 50%. The chemical structure of chitosan is given in Figure 3.

Fig. 3. Chemical structure of chitosan. It is a linear polysaccharide composed of randomly distributed β -(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit)

3.2 Low toxicity of chitosan

Chitosan has low toxicity with an LD₅₀ (lethal dose for 50% of test population) level in the same dose as sugar or salt (Arai, 1968). Toxicity tests reported the LD₅₀ of chitosan in mice exceeded 16 g/kg. The molecular mass has minimal effect on cell viability, while the degree of deacetylation (DDA) of the polymer has greater effect on its toxicity (Richardson et al., 1999). DDA also affects the solubility, hydrophobicity and its ability to interact electrostatically with polyanions by affecting the number of protonatable amine groups of chitosan. Chitosan nanoparticles with lower DDA showed lower toxicity *in vitro* (Huang et al., 2004).

3.3 Applications of chitosan

Chitosan is a biodegradable polymer used in various industrial, biomedical and pharmaceutical applications due to its biocompatibility and the slow release of active molecules. Table 2 summarizes some of these broader applications. The novel properties of chitosan make it a versatile biomaterial for cell therapy, tissue engineering and gene therapy (Sui et al., 2006). Chitosan has a positive charge and hydrophilic character at an acidic pH. It is a continuum of primary aliphatic amine that can be protonated by acids; the pKa of the chitosan amine groups being around 6.3-6.5 (Kumar et al., 2004). The cationic amino groups on the C2 position of the repeating glucopyranose units of chitosan can interact electrostatically with the anionic groups (usually carboxylic acid groups) of other polyions to form polyelectrolyte complexes (Hamman, 2010). Many different polyanions from a natural origin (e.g. alginate, chondrotin sulfate or dextran sulphate) or from a synthetic orgin [e.g. poly(acrylic acid), polyphosphoric acid, or poly(L-lactide)] have been used to form polyelectrolyte complexes with chitosan, in order to provide the required physicochemical properties for design of specific drug delivery system, as well as specific target gene delivery (J. H. Park et al., 2010).

Usage	Examples of applications
Agricultural and horticultural	Natural biocontrol and elicitor
Water process engineering	Part of filtration process
Biomedical and pharmaceutical	Hemostatic agents
	Wound healing
	Tissue engineering
	Drug delivery
	Gene therapy

Table 2. Some applications of chitosan

4. Chitosan and gene delivery

Chitosan has been broadly studied as a promising non-viral vector for gene delivery (Bowman & Leong, 2006). This cationic polysaccharide can bind DNA between the positive charges of its amino groups and the negative charges of the phosphate groups of the DNA backbone in order to form nano- or microparticles. The interaction between chitosan and nucleic acids is electrostatic. The charge interaction is sufficiently strong that chitosan-DNA or small interfering ribonucleic acid (SiRNA) complex does not dissociate until it has entered the cell. Moreover, chitosan also protected nucleic acids from enzymatic degradation before entering the nucleus.

4.1 Transfection efficiency of chitosan

Tong et al. (2009) describes seven steps that should be overcome before the expression of exogenous DNA. They are complexation, *in vivo* administration, endocytosis, escape from endolysosome, release of DNA, trafficking through cytoplasm and finally importation of DNA into the nucleus. The transfection efficiency of chitosan itself is; however, relatively low, when compared to lipoplex or other methods. But this aminopolysaccharide can be modified for ease of DNA delivery, as well as for target gene delivery, which currently attracts many researchers to use chitosan and its modifications for gene delivery. Chitosan can be modified by ligand conjugation, such as transferrin-, folate- (folate and transferrin are over expressed in cancer cells), mannose- (target dendritic cells in tumor) and galactose (target Kupffer cells of the liver) conjugated chitosan, which can improve transfection efficiency of the targeted cells via receptor-mediated endocytosis (Duceppe & Tabrizian, 2010; Mao et al., 2010).

4.2 Factors affecting transfection efficiency

There are many factors that affect transfection efficiency. These include Mw, DDA, DNA complexes' charge ratio, pH and particle sizes, as well as the type of cell lines used.

4.2.1 Molecular weight (Mw)

High Mw chitosan can bind DNA tightly, which is due to the high number of positive charge of amino groups, but binding DNA tightly may give low transfection efficiency, due to not releasing the DNA to the nucleus after endocytosis to the cell. The Mw of chitosan also influences the size of the chitosan-DNA complexes, as the higher sizes of chitosan-DNA complexes can affect the cellular uptake. These factors lead to transfection efficiency (see review of Mao et al., 2010).

If the N/P ratio, which is the molar ratio between the amino groups of chitosan and the phosphate groups of DNA, was fixed, then the higher the Mw, the larger the chitosan-DNA complexes diameter (MacLaughlin et al., 1998). However, there have been differing conclusions proffered between the Mw of chitosan and transfection efficiency. Some studies have reported of high transfection efficiency with high Mw chitosan (Huang et al., 2005; Kiang et al., 2004; MacLaughlin et al., 1998). Other studies have reported that low Mw chitosan has better transfection efficiency (Koping-Hoggard et al., 2004; Lavertu et al., 2006; Supaprutsakul et al., 2010).

MacLaughlin et al. (1998) synthesised depolymerised chitosan oligomers with a Mw from 7 - 92, but the transfection efficiency was much lower than at the higher Mw of 102 and 230 kDa, respectively, and being about 1000 times lower in transfection efficiency compared to

lipofectamine™. Haung et al. (2005) also found a decreased A549 cellular uptake with the decreasing Mw or DDA of chitosan and a N/P ratio of 6 was used in that study. But the study of Supaprutsakul et al. (2010) revealed much higher transfection efficiency with the depolymerised chitosan at Mw ~16 kDa (or Mn ~ 6.5). This may be because of the different chitosan/DNA ratio, as the previous study used low N/P ratio, while Supaprutsakul et al. (2010) used chitosan/plasmid at an N/P ratio of about 56:1, which meant that a much higher amount of chitosan was used for the lower Mw. This was consistent with the study of Romøren et al. (2003), who found that low Mw chitosan was beneficial at the higher charge ratio of the complexes.

4.2.2 pH and degree of deacetylation (DDA)

The study of Lavertu et al. (2006) also found that the low Mw chitosan, which had a numeric average Mw (Mn) of about 10 and 80% DDA at N/P ratio 10:1, gave higher transfection efficiency at the same level as lipofectamine™ at pH 6.5. However, the very low Mw (1.9-7.7 kDa) chitosans with high DDA were found to form aggregates easily, even at very low charge ratios (Morris et al., 2008), and this might lower the transfection. However, the depolymerised LW chitosan in this study had only 54% DDA, which may reduce the problem of particles aggregation and, after cells uptake the chitosan-DNA nanoparticles, the DNA may be released from the nanoparticles more easily, as DNA binding efficacy was reduced as DDA was decreased (Kiang et al., 2004). Hence, many factors may have to be considered for improving transfection efficiency of chitosan, not only the ligand binding, but also the method of binding or conjugation, the size and morphology of the particles, the aggregation of the complexes, and especially the chitosan itself, as Mw, DDA and charge ratio, which may have to be adjusted.

4.2.3 Cell line dependency

Another factor, which may affect transfection efficiency of chitosan, is cell line dependency. Higher mitotic cell lines, such as cancer cells, usually have higher transfection efficiencies than lower proliferative rates of the cell line. This may be related to differences in cell physiology affecting the internalisation mechanism and subsequent internal trafficking of the vectors (Douglas et al., 2008). It has also been found that dividing cells have higher transfection ability compared to quiescent cells (Brunner et al., 2000) and higher levels of gene expression have been observed just before or during mitosis (Mortimer et al., 1999). This may explain why the immortalised cell line, with higher mitotic activity, has higher transfection ability than normal or primary cell lines. However, this factor requires further investigation.

4.3 Improving transfection efficiency

There have also been some attempts to modify the chemical structure of chitosan to improve transfection efficiency, which have involved hydrophilic and hydrophobic modifications. The main purpose of hydrophilic modification of chitosan is to increase solubility and reduce sensitivity of chitosan-DNA complexes to pH, as well as reduce the chitosan-DNA complexes aggregation, which may improve transfection efficiency. The hydrophilic chitosan modification includes quaternised chitosan (Thanou et al., 2002), PEGylated (covalent attachment of polyethylene glycol polymer chains to another molecule) chitosan (Jiang et al., 2006) and low Mw soluble chitosan (Ercelen et al., 2006). Interestingly, Brannon-Peppas & Blanchette (2004) found that particles with more hydrophobic surfaces were also preferentially taken up by the liver, followed by the spleen and the lungs.

Hydrophobic modifications of chitosan have been performed in many studies. The main objectives of these modifications were increasing transfection efficiency by modulating complex interactions with cells, especially in the complexes' adsorption on the cell surfaces and cell uptake (Kurisawa et al., 2000). Some hydrophobic units also help in the dissociation between the chitosan DNA complexes to release DNA to enter the nucleus after cellular uptake, as well as protecting it from enzymatic degradation and facilitating intra cellular pDNA (plasmid DNA) association, which can enhance transfection efficiency. These hydrophobic modifications included deoxycholic chitosan, N-alkylated chitosan, thiolated chitosan and hybrid chitosan (Mao et al., 2010). The combination of hydrophilic and hydrophobic modification of chitosan structure has been another interesting area that looks highly promising for the development of high transfection efficiency in a non-viral vector, using chitosan as a core structure.

5. Chitosan and gene therapy

One of the significant applications of chitosan is in its application to gene therapy. It has a number of benefits. It has non-toxic, biodegradable and biocompatible with high cationic charge potential; protects DNA from degradation by nucleases; and has high yield transfection efficiency (Sui et al., 2006). Genetic material (DNA and RNA) has been explored for use as a treatment of genetic abnormalities or deficiencies, which is described as gene therapy. Gene therapy functions by transferring healthy genetic material or nucleic acid constructs, such as ribozymes, antisense molecules, decoy oligodeoxy nucleotides (ODNs), DNazymes and siRNA, into diseased cells in an attempt to achieve a therapeutic effect that results in restoration of protein production, which was absent or deficient due to the pre-existing genetic disorder (Tan et al., 2009). But using small nucleic acid, such as DNazymes and siRNA, has some limitations, since they are rapidly degraded in plasma and cellular cytoplasm and cannot passively diffuse through cellular membrane, which is due to the strong anionic charge of the phosphate backbone and the consequent electrostatic repulsion from the anionic cell membrane surface as well as limited size of cellular entrance. So, these small nucleic acids encapsulated with chitosan nanoparticles can reduce the limitations of these small nucleic acids.

5.1 Use of chitosan nanoparticles

The development of chitosan and its modification for non-viral gene delivery is also a target for gene therapy. This is because chitosan nanoparticles have a low toxicity and are taken up by endosomes allowing the DNA or nucleic acid to overcome the permeability barrier posed by epithelium and also to protect against enzymatic degradation. There are some studies that have attempted to use chitosan for cancer therapy. Chitosan itself was able to demonstrate growth inhibitory effects on cancer cells and has apoptosis effect on bladder tumour cells via caspase-3 activation (Tan et al., 2009). The various manufacturing processes for chitosan nano-/micro- particles/spheres (nanofabrication) has been described elsewhere (Masotti et al., 2009).

5.2 Use of siRNA loaded chitosan nanoparticles

In current developments in chitosan for gene therapy, there is an attempt to develop siRNA loaded chitosan nanoparticles to silence the target gene. This method can silence the gene by means of RNA interfering (RNAi). SiRNAs, usually containing 20-25 base pairs (see Figure

4), assemble into endoribonuclease containing complexes known as RNA-induced silencing complexes (RISCs).

Fig. 4. Schematic representation of a siRNA molecule. SiRNA have a well-defined structure: a short (usually 21-nucleotide-long) double-strand of RNA with 2-nucleotide 3’ overhangs on either end (Alper, 2006).

The siRNA strands guide the RISCs to complementary RNA molecules leading to cleavage and destroy the target RNA (Manjunath & Dykxhoorn, 2010). The mechanism of RNAi is described in Figure 5.

Fig. 5. Mechanism of RNAi (Hood, 2004). dsRNA=double stranded RNA; shRNA=small hairpin RNA (sequence of RNA that can be used to silence gene expression via RNA interference); mRNA=messenger RNA

This system is of interest to numerous researchers, as well as many pharmaceutical companies, since the efficient siRNA delivery system will have clinical therapeutic impact in gene therapy (Mao et al., 2010; Park et al., 2010; Rudzinski & Aminabhavi, 2010). However, further investigations are needed, especially *in vivo* experiments/clinical trials.

6. Limitations in the use of chitosan

There are some limitations in the use of chitosan for non-viral gene therapy. Firstly, there is a lack of knowledge of the pharmacokinetics of chitosan-nucleic acid complexes during uptake inside the body. When chitosan-DNA nanoparticles enter the body, they were quickly removed from the blood and deposited on different organs. Administration of larger nanoparticles results in a substantial increase of the particles in the lung with a subsequent decrease in the liver, indicating a strong dependence of the tissue distribution on particle size (Liu, 2007). However, more information on this topic is required. Secondly, there is a need for more studies in animals, including clinical trials. Most of the studies in the past few years about chitosan and gene therapy continue to use an *in vitro* model; however, more studies have been performed using mouse model, as summarised in Table 3.

Route of transmission	Form of chitosan complexes	Target organ/ Disease	Study design	Reference
Utero gene transfer (injection in amniotic sacs)	Chitosan-DNA (reporter gene)	Expected route for fetal gene therapy	Mouse (murine)	Yang et al., 2008; Jang et al., in press
Local gene delivery via endovascular stent	Chitosan-DNA coated with dodecylated in endovascular stent	Expected route for diseased blood vessel wall	Mouse	Zhu et al., 2010
Local: inhalation	Chitosan-DNA (interferon-beta gene) complexes powder	Lung cancer	Mouse	Okamoto et al., 2010
Local: inhalation	Spray -freeze dry chitosan-DNA	Expected route for pulmonary gene therapy	Mouse	Mohri et al., 2010
Local: localized hydrogel by intra-tumoural injection	Chitosan-SiRNA	Expected route for multiple localized disease	Mouse models of melanoma and breast cancers	Han et al., 2010
Local: intra-tumoural injection	Chitosan-SiRNA design to down regulate RXFP1 expression	Prostate cancer	Mouse: xenograft model	Feng et al., 2010

Table 3. Summary of current animal experiments using chitosan as non-viral for gene therapy

Lastly, the route of transmission and target gene delivery are the major factors which contribute to the success in gene therapy, which still requires further investigation. Table 4 summarises some attempts to modify the chitosan-nucleic acid complexes for target gene therapy.

Modification	Chitosan complexes	Conclusion	Study design	Reference
Folate mediated targeting induced by conjugating poly(ethylene glycol)-folate (PEG-FA) with arginine modified chitosan	PEG-FA-chitosan-DNA	The transfection efficiency was higher than PEI when transfected in KB cell line, which over expressed the folate receptor (FR) in presence of 10% foetal bovine serum (FBS).	<i>In vitro</i> (KB cell line)	Morris & Sharma, 2010
Arg-Gly-Asp (RGD) peptide-labelled chitosan nanoparticle (RGD-CH-NP) as a novel tumour targeted delivery system for short interfering RNA (siRNA).	RGD-CH – SiRNA nanoparticles	RGD-CH-NP is a novel and highly selective delivery system for siRNA with the potential for broad applications in human disease.	Orthotopic mouse models of ovarian carcinoma	Han et al., 2010
Antisense oligodeoxynucleotides (asODN), using folic acid (FA) conjugated hydroxypropyl-chitosan	FA-Chitosan-asODN nanoparticles	Targeted antisense agent would be a potential approach to overcome tumour drug resistance.	<i>In vitro</i> (KB cell line)	Wang et al., 2010
Tumour- of adenoviral complexes targeting of Adenovirus (Ad)/chitosan-PEG-FA nanocomplexes formed by electrosining	Ad/chitosan-PEG-FA nano-complexes	Transduction efficiency of Ad/chitosan-PEG-FA was 57% higher than Ad/chitosan. This system aims for development of systemic administration of the vectors to target lesion.	<i>In vitro</i> (KB cell line)	Park et al., 2010

Table 4. Some modifications of chitosan for target gene therapy

7. Summary

An efficient gene delivery system is very important for gene therapy. Currently, the most efficient of these systems is a viral vector, which usually yields a transfection efficiency of more than 90%. However, by using a viral vector for gene therapy, there is a concern about the host versus vector immunological response, mutation and oncogenic effects; hence the need to develop a non-viral vector. There are many non-viral vectors; the high efficient one is cationic lipid, which gives high transfection efficiency, especially in tissue culture or *in vitro* conditions. *In vivo*, the intravenous administration of cationic lipid/DNA complexes presented significant problems, as these reagents can be quite toxic. PEI is another non-viral transfection material that has been used for some time, but due to its toxicity and the variable results, it has not been widely accepted.

Chitosan (poly[β -(1-4)-2-amino-2-deoxy-D-glucopyranose]), a nontoxic biodegradable biopolymer, has been broadly studied as a promising non-viral vector for gene delivery. This cationic polysaccharide has been produced by partial deacetylation of chitin, a naturally polymer from crustacean shells. However, the transfection efficiency of chitosan itself is not efficient enough and depends on many factors such as Mw, DDA, DNA complexes charge ratio, pH and particle sizes, as well as the type of cells. There have been many attempts to modify chitosan in order to improve transfection efficiency. Some studies have revealed that low Mw chitosan, especially the product of oxidative depolymerisation from higher Mw chitosan with NaNO_2 , had low cytotoxicity and improved solubility properties, as well as having potential for gene delivery both *in vitro* and *in vivo*. However, some studies have reported decreased transfection efficiency with lower Mw chitosan.

There have been other attempts modifying the chemical structure of chitosan. These have included introducing a hydrophilic group, such as coupling dextran, as well as incorporating poly (vinyl pyrrolidone) into the galactosylated chitosan, which can reduce the aggregation of particles and increase transfection efficiency. Some studies have also using hydrophobic modification of chitosan, such as deoxycholic acid-modified chitosan, in order to increase transfection efficiency through enhancement of complex interaction with cells and cellular uptake of the particles. Chitosan can be modified by conjugation of chitosan-DNA complexes with ligands to target specific cell surface receptors, but these attempts have had variable results. Many factors may have to be considered for improving transfection efficiency of chitosan, not just ligand binding, but also the method of binding or conjugation, the size and morphology of the particles, the aggregation of the complexes, and especially the chitosan itself, as Mw, DDA and charge ratio may have to be adjusted.

The design criteria of the effective vector for non-viral gene therapy should also consider cost-effectiveness in synthesis and purification steps, serum stability and efficient packaging of large amount of the vector-nucleic acid complexes. Moreover, the route of administration of this vector to the target cells or tumour lesion, high transfection efficiency, specific target gene delivery should also be considered. Once the complexes enter the target cells, they have to escape from enzyme degradation. The complexes then release the therapeutic gene/ nucleic acid to the target organelle, such as DNA, which has to enter the nucleus, while siRNA functions in cytoplasm. This release has to occur without too many difficulties, which means that the bonding between the vectors and nucleic acid should not be too strong. Most importantly, these non-viral vectors have to be

safe enough for the patient, including being non toxic to the host body, non-immunogenic and non pathogenic. Chitosan is now one of the candidate biomaterials for selection as an effective non-viral vector for gene therapy, especially as it is safe, cheap and easy to modify.

8. Acknowledgment

The authors wish to acknowledge the support of the World Safety Organization Collaborating Centre within the Anton Breinl Centre for Public Health and Tropical Medicine, James Cook University, Townsville, Queensland, Australia, for their kind support of this project.

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Non-Viral Gene Therapy

Edited by Prof. Xubo Yuan

ISBN 978-953-307-538-9

Hard cover, 696 pages

Publisher InTech

Published online 07, November, 2011

Published in print edition November, 2011

This book focuses on recent advancement of gene delivery systems research. With the multidisciplinary contribution in gene delivery, the book covers several aspects in the gene therapy development: various gene delivery systems, methods to enhance delivery, materials with modification and multifunction for the tumor or tissue targeting. This book will help molecular biologists gain a basic knowledge of gene delivery vehicles, while drug delivery scientist will better understand DNA, molecular biology, and DNA manipulation.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Ureporu Kedjarune-Leggat and Peter A. Leggat (2011). Chitosan and Its Modifications: Are They Possible Vehicles for Gene Therapy?, Non-Viral Gene Therapy, Prof. Xubo Yuan (Ed.), ISBN: 978-953-307-538-9, InTech, Available from: <http://www.intechopen.com/books/non-viral-gene-therapy/chitosan-and-its-modifications-are-they-possible-vehicles-for-gene-therapy->

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