Nano-vesicles for gene delivery

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Nano-Vesicles for Gene Delivery

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ABSTRACT

Gene therapy was considered an important modality for the potential treatment of cancer. It aimed to correct diseases through the delivery of genetic material encoding a therapeutic protein and the subsequent expression of the transgene at the appropriate sites. The hurdle for effective gene therapy hinged on the development of safe vectors that have the ability to efficiently deliver therapeutic DNA to the targeted site. In this study, the potential applications of nano-bioceramics for gene delivery were investigated. Nano-sized bio-ceramic particles including neutrally charged hydroxyapatite (HA), negatively charged silica (SiO_2) and positively charged zirconia (ZrO_2) were tested.

The ability of different chemicals to modify the surface charges of these nano-particles in enhancing transfection efficiencies was also studied. Protamine Sulfate (PS) was found to be ideal for this application. The conditions for optimal DNA binding was established using SiO_2 as the proto-type. With the SiO_2 -PS coated DNA complex, efficient transgene delivery and expression for a number of human cancer cell lines including nasopharyngeal cancer cells, hepatocellular carcinoma cells, colon cancer cells and cervical cancer cells could be achieved. Time course study revealed the stability of complex for up to 72 hours in the presence of 50% serum. This complex was subsequently employed for *in vivo* gene delivery and it had the ability to target transgene expression specifically in the spleen, following systemic delivery of the SiO_2 -PS coated DNA complex.

CHAPTER

1

INTRODUCTION

1.1 Background on gene therapy

Gene therapy provided great promise towards the advancement of treatment in genetic and acquired diseases. Previously viewed as an experimental treatment, it had gained substantial grounds in the medical arena in recent years. In June 2004, scientists at the Cancer Research Center of UK had succeeded in manipulating a gene to cause ovarian cancer cells to self destruct [1]. Shortly after the announcement, China launched Genedicine, the world's first licensed "gene therapy". Genedicine essentially comprised a virus with the insertion of the P53 gene that will serve to trigger the cancerous cells to destroy themselves when introduced into the patient [2].

The concept of gene therapy revolved around the principle of correcting the root cause of diseases at their origin by delivery and subsequent expression of exogenous DNA which encoded for a missing or defective gene product. The areas of medicine that may be most suited to gene therapy were those that have failed to respond to existing treatments, such as oncology and chronic inflammation. Cancer ranked amongst the top killer disease in developed nations. Existing primary treatment regimes included surgery, radiotherapy and chemotherapy.

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These treatments were costly and often ineffective in curing cancer. Surgery was not a viable option in tumor metastasis and in inaccessible tissues such as the brain.

Radiotherapy and chemotherapy were only applicable to cancerous cells that were receptive, and presented the disadvantage of destroying healthy as well as malignant cells. It was thus not surprising that majority of the worldwide clinical trials had been devoted towards cancer treatment.

In cancer gene therapy, it may be feasible to eliminate cancer cells without damaging normal, healthy tissues. It contained the potential to provide patient-friendly treatment regimens which were minimally invasive, affordable and easily administrated to patients. It can be deemed as a therapeutic alternative to patients whose diseases did not respond to conventional treatment regimens. It may also interact in synergistic manners with conventional treatments. For instance, it could enable a cancerous tumor to be more receptive to chemotherapy or radiation. Moreover, cancer gene therapy offered the promise of providing patients with a better life quality during and post-treatment compared to surgery, chemotherapeutic agents and radiation therapy.

Clinical trials for cancer gene therapy currently adopted several strategies which included immunotherapy, chemo protection of stem cells by insertion of drug resistance genes and the insertion of tumor suppresser genes. Although the National Institute of Health (NIH) and the Recombinant DNA Advisory Committee (RAC) had reviewed and approved more than 100 clinical protocols for gene therapy since 1999, clinical efficacy in human patients had yet to be clearly established for any gene therapy protocol.

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Majority of the clinical trials were restricted to phase 1, where the main purpose was to test on the tolerance, the activity and the safety of the gene transfer protocol, rather than an assessment of the therapeutic efficacy [5]. Further progress for gene therapy had been severely impeded by several significant roadblocks. This included shortcomings in all the current gene transfer vectors and an inadequate understanding of the biological interaction of these vectors with the host [4 and 11].

1.2 Challenges on the progress of gene therapy

The two main approaches in transferring therapeutic genes to the body included physical delivery of DNA and those mediated by vectors. The delivery of naked plasmid DNA into the tissue by physical methods resulted in limited gene expression due to the rapid degradation of naked DNA by nucleases in the serum and clearance by the mononuclear phagocyte system.

To protect the DNA from enzymatic degradation *in vivo*, vectors must be used as a mean to deliver DNA to the target cell. Vectors can be broadly segmented into viral and non-viral vectors. Viral vectors accounted for 75% of the worldwide gene therapy trials due to their ability to achieve high transfection efficiencies [4 and 5]. However, viral vectors suffered from several drawbacks particularly in terms of safety, toxicity, the elicitation of an immune response and the lack of cell-specific targeting. Virus production was also expensive and there were safety concerns involving viral manipulations. The size of delivered plasmid was also limited by the size of the viral capsid.

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Gene therapy trials came under fire with the sudden death of Jesse Gelsinger in 17th Sept 1999. Gelsinger suffered from partial ornithine transcarbamylase (OTC) deficiency, a disorder in the liver, which kept him in perpetual danger of hoarding toxic levels of ammonia in his blood. In an experiment to correct this disorder, the normal OTC gene using adeno-associated virus (AAV) was injected into his liver. Within hours, he suffered from severe immune response, slipping into coma after 24 hours. He died subsequently from multiple organ system failure. This case had propelled a hard scrutiny on the safety of virus and renewed emphasis towards the development of non-viral vectors [5, 6 and 7].

The rapid progress of nanotechnology had led to the discovery of a vast and diverse array of synthetic chemical vectors that can be designed to deliver therapeutic genes to cells without the aid of an intact virus. These were considered to be the most promising development in gene therapy [11 and 14].

Nano-particles with uniform diameters and shapes can be arranged, oriented and functionalized for specific applications [12, 21 and 62]. The transport of plasmid DNA can take place with the aid of cationic particles. One of their main advantages resided in their ability to incorporate multiple therapeutic, diagnostic and barrier-avoiding agents [11]. The targeting of these vectors might be attained by conjugating active recognition moieties to their surfaces. These vectors were also extremely attractive to the pharmaceutical industry as a result of their stability, low cost, consistent standard of production and greater safety.

Extensive researches were embarked on cationic liposomes and cationic polymers. Despite numerous literatures on the potential of these non-viral systems, their progress had been slow.

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Compared to their viral counterpart, these systems exhibited low transfection efficiency, particularly when used *in vivo*. They tended to suffer rapid clearance from the circulation as a result of their inability to bypass “first pass” organs such as liver, lung and spleen, leading to poor delivery to the disease site [24]. They were also cytotoxic, which limited administration at higher dosages. Hence, very few non-viral vectors have reached the stage of clinical trials.

1.3 Research Focus

Given the central role of vectors in gene therapy, its development represented a critical area for research. Although an enormous amount of researches had been undertaken on novel synthetic chemical vectors, the use of inorganic nano-particles as vectors had not been widely explored. Ceramic, glasses and glass ceramics represented a wide range of inorganic materials that can be synthesized at nano-sized scale.

One particular class of ceramics, known as bio-ceramics, may be suitable for use as gene delivery vectors. Bio-ceramics were an accepted group of materials by the medical community. They were commonly used to repair or replace skeletal hard connective tissues. Their success as implants had resided on their capability to achieve stable attachment to the connective tissue [12].

Nano-bioceramics were increasingly gaining attention as binding and delivery agents for drugs, genes and proteins and for vaccine production. However, researches involving this class of materials in gene therapy were still in a nascent stage. This class of biomaterials was extremely attractive from a pharmacological standpoint. They can be economically manufactured at lower temperatures, which also increased the range of possible substrates.

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Therefore, this research aimed to develop an alternate new carrier system involving nano-ceramic particulates for cancer gene therapy. The study will entail studying the effects of different surface charges of nano-bioceramic particles and the ability to modify their surface charges to enhance transfection efficiencies. Nano-Hydroxyapatite (HA), Silica nanopowder (SiO_2) and Zirconia nanopowders (ZrO_2) were selected for this study. HA, a bioactive material was used widely in dental and bone implant. SiO_2 was widely used in cosmetics and pharmaceuticals. ZrO_2 exhibited bio-inertness and was used clinically in the total hip replacement (THR) ball heads. These materials were non-toxic, possessed good biocompatibility and did not illicit immunogenic response from the body. Moreover, as synthetic chemical vectors, they can be easily produced on a large scale basis with consistent quality at low cost and were stable for long term storage.

CHAPTER

2

LITERATURE REVIEWS

2.1 Fundamentals of gene therapy

Gene therapy can be broadly defined as the transfer of genetic material into a cell to transiently or permanently alter the cellular phenotype [13]. Generally, the gene cannot be directly inserted into a person's cell. It must be delivered either by a carrier, known as a vector or through physical methods. Both viral and non-viral vectors have been employed in gene therapy. Genes are the biological units of heredity and comprised part of a deoxyribonucleic acid (DNA) molecule. Each gene carries a separate piece of information. The Human Genome project, a 13-year project completed in 2003, had identified approximately 30,000 genes in human DNA [8]. These genes carried instructions that allowed the cells to produce and regulate specific proteins such as enzymes. Changes in the sequence of the DNA molecule resulted in gene mutations. The occurrence of most diseases, including cancer had been attributed to gene mutations.

Nature of DNA molecule

DNA molecules can be single stranded or double stranded. Each strand of DNA is made of a backbone and sequences of four bases: adenine (A), guanine (G), cytosine (C) and thymine (T). The backbone consisted of five carbon sugar molecules linked together via phosphodiester bonds. This linkage gave all DNA strands a chemical polarity.

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One end terminated in a phosphate group and the other end in a sugar molecule. DNA molecules were chemically non-toxic, stable and water soluble.

It can be easily manipulated through a wide range of physical and chemical methods [16]. Individual genes or DNA fragments with desired sequences can be extracted in the form of a plasmid. Plasmids DNA were small, circular extra-chromosomal DNA molecules which can be replicated independently in a host cell. DNA can be attached to a surface via non-covalent or covalent bonds. Since DNA is highly negatively charged, it can be attached to positively charged surfaces. This non-specific and reversible attachment formed the fundamental basis in the formation of non-viral vectors-DNA complexes.

2.2 Requirements of an ideal vector

An ideal vector for gene therapy [15, 21, 24 and 25] must contain the following features:

- i) Good biocompatibility, safe and well tolerated upon systemic administration;
- ii) non-toxic and non-immunogenic;
- iii) ability to deliver intact DNA to target tissue and mediate sufficient transfection of that tissue to obtain intended therapeutic benefit;
- iv) appropriate size and pharmacokinetic attributes to ensure delivery to disease sites
- v) capacity to transport large genetic materials;
- vi) ease of modifying surface charge density, stoichiometry and hydrophobicity
- vii) stability in the blood stream; and

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- viii) stability upon manufacture so that large batches can be prepared with uniform reproducible specifications, as well as for storage purposes.

2.3 Viral Vectors

Viral vectors were the first vectors to be explored for gene therapy. Viruses have been able to efficiently infect and replicate their DNA within infected cells, due to their ability in escaping from the endosomal-lysosomal pathway. Despite being efficient gene transfer vehicles, their use in clinical applications were largely negated by safety related issues, potential for insertational mutagenesis, limited capacity to carry large gene inserts, and costly, and complicated production processes. Moreover, systemic administration of viral vectors was normally unable to target specific cells. Hence, the administration of viral vectors was often limited to local and regional tumors. Immune recognition of viral proteins with destruction of the infected cells also limited its repetitive administration.

However, viruses continued to be of interest to researchers who have placed greater emphasis in reducing their toxicity and immunogenicity.

Retrovirus

Retroviruses were RNA viruses which replicated through a DNA intermediate, where the viral DNA integrated randomly into the host genome. It had the propensity to integrate into dividing cells. It had been successful in achieving stable integration of foreign DNA into target cell and hence they were employed in majority of the clinical protocols.

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However, its usefulness had been negated by its low titers (10^7), capacity limitation on inserted genes, inactivation by serum complement, cumbersome preparation, difficulties in controlling or ensuring expression, and the potential for genetic damage due to random integration in the host genome. Another major disadvantage was their ineffectiveness in non-dividing cells [4, 43 and 52].

Adenovirus

Adenoviral vectors were based on a family of viruses that caused benign respiratory tract infections in humans. They were non-enveloped isometric particles, approximately 45-50nm in diameter, with an icosahedra surface and a DNA-containing core. This double-stranded DNA virus was capable of high-level of transduction in many cell types, regardless of the mitotic status of the cell.

The presence of intact viral genes in the recombinant virus resulted in low levels of viral gene expression, leading to cellular toxicity and cell death. The lack of an envelope made cell-specific targeting difficult, as cell-specific ligands cannot be attached to the viral particle. Other major disadvantages included limited length of gene expression, relatively high immunogenicity and the complexity of its genome. Toxic effects in a patient had been reported after administration of an adenovirus vector expressing the cystic fibrosis trans-membrane conductance regulator (CFTR) gene [4 and 43].

Adeno-associated virus (AAV)

Adeno-associated virus can be generated such that most of the viral genome was replaced with DNA encoding a potentially therapeutic gene. Hence, this virus had lesser chance of eliciting an immune response. This virus was capable of infecting both dividing and non-dividing cells, as well as hematopoietic cells.

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A major perceived strength of AAV was its ability to integrate at specific site in the infected cell genome, when the wild type virus is removed from the viral genome. However, the lack of suitable helper cell lines limited the ability to prepare large amount of uncontaminated, recombinant virus. Additionally, its capacity to carry a therapeutic gene insert was limited to only 5kb.

Herpes simplex virus

Herpes simplex virus, a large double-stranded DNA virus, had the ability to establish latent infection in the brain. Hence, it had been used in some initial applications to deliver therapeutic genes to neurons and could potentially be used to deliver therapeutic genes to some forms of brain cancer [43]. This virus had yet to be characterized due to its large genome size.

New viral vectors under development

Some of the latest research developments in viral vectors included poxvirus, vaccinia virus and baculovirus. Recombinant forms of the vaccinia virus could accommodate large inserts and were generated by homologous recombination. Avipox virus vectors could infect and express recombinant proteins in human cells without viral replication. This allowed the vector to be used for vaccination against disease. Baculovirus, an insect virus system, previously used for protein expression, was now also being considered for gene therapy application, due to its ability to express proteins at high levels. The HIV virus, a form of retrovirus, was also recently investigated as a potential vector for gene therapy [9 and 10].

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2.4 Non-viral Vectors

Non-viral gene mediated gene delivery was not as efficient as those mediated by viral vectors. However, their key advantages over viral mediated procedures included low cytotoxicity, little immunogenicity, high adaptability, capacity to carry large DNA inserts, the simplicity of use and ease of large-scale production [15, 21 and 34]. It could also circumvent safety problems associated with viral vectors, such as endogenous virus recombination, oncogenic effects and unexpected immune response [21]. These vectors may provide the best potential in the development of gene constructs that mediated episomal maintenance, replication, or integration into the host genome due to their ability to provide the necessary signals [43].

Non-viral vector systems can be categorized into two major groups [21 and 34]:

- i) Naked DNA delivery by a physical method;
- ii) Delivery mediated by a chemical carrier.

2.5 Barriers to Non viral mediated gene delivery

The ability for non-viral vectors to transfer genes was hampered by systemic and intracellular barriers as presented in Fig.1. In order to achieve an efficient expression of genes in the targeted site, the vector must be designed to overcome these barriers.

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Fig. 1: Barriers to gene delivery

2.5.1 Systemic barriers

Systemic or biological barriers will interfere with the specific transport of therapeutic genes, as it navigated through the blood compartment, serum proteins, cellular elements like erythrocytes and platelets before reaching the target site. A major limiting factor of DNA delivery using liposomes *in vivo* was its rapid blood clearance. A process known as ‘opsonization’ removed 80-90% of hydrophobic particles in the blood [14].

It was also reported that the excess positive charges of lipoplexes [31] enhanced non-specific electrostatic interactions of lipoplexes with serum proteins, cellular components and other systemic molecules. This had resulted in non-discriminatory binding of the complexes to biological surfaces and systemic molecules, leading to serious compromise in targetability and systemic stability of lipoplexes *in vivo*.

2.5.2 Intracellular Barriers

Intracellular transfection pathway for most non-viral vectors was believed to consist of the following steps:

i) Transport through the cell membrane

In the vicinity of the target cell, the plasmid complex had to penetrate the plasma membrane into the cell cytosol [23]. Membrane penetration was thought to occur predominantly by endocytosis for most cationic liposomes and polymers [24 and 76]. Endocytosis of these vectors was known to take place through clathrin-coated pit formation on the cell membrane surface [84].

Besides receptor-mediated endocytosis, cellular uptake into the cell membrane can also possibly be achieved through pinocytosis and phagocytosis [81]. Pinocytosis occurred continuously in almost all cells where the engulfed drop was relatively small. Phagocytosis, which occurred only in specialized cells such as neutrophils and macrophage, resulted in the ingestion of particulate matter from the extra cellular fluid (ECF). Some reports had also mentioned the entry of lipoplexes via direct fusion with the cellular membrane [76 and 77]. The size and composition of the vector had been attributed as influencing factors governing the internalization routes [84].

Inefficient cell-specific binding and low cellular uptake of plasmid DNA had been known as major impeding factors in non-viral mediated gene transfer [27]. The high transfection efficacy of viruses had been due to their ability to bypass endocytosis [14].

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ii) Uptake and degradation in the endo-lysosomal compartments

Low transfection efficiency of non-viral vectors may be due to intracellular degradation of DNA in the endosomes and/or lysosomes. After entering the cytoplasm, the endocytosed cationic-carrier DNA complex fused with the endosomal compartments where the pH was lowered to 6.0-5.5. Lysosomes were considered to be the end product of endocytosis. Release of DNA prior to lysosomal degradation must occur in order to achieve productive gene expression [22]. The low pH and the presence of nucleases within the endosomal/lysosomal compartments will result in the degradation of entrapped DNA and associated complexes [14].

The prevention of DNA from degradation was thus dependent on its release from the endosomal compartment prior to the fusion of the endosome with the lysosome [31]. Cationic lipids were reported to release DNA into the cytoplasm by destabilizing the endo-lysosomal membrane [84]. To aid the escape of DNA from the endosome, some vectors have incorporated fusogenic lipids or peptide which fused with the endosomal membrane [5, 84 and 85]. Chloroquine raised endosomal pH and had been used to decrease DNA degradation by inhibiting lysis.

The early escape of polyethylenimines(PEI)-DNA complex from the lysosome was believed to be the cause of their high transfection efficacy [14 and 38]. This had been attributed to the presence of a protonatable nitrogen in every third atom of PEI which rose the protonation level in the low pH environment of the lysosome compartment, leading to the influx of chloride ions and the eventual rupture of the lysosome.

However, a study embarked by W.T. Godbey et al [79] using fluorescent labeling and confocal microscopy revealed that PEI-DNA polyplex did not enter the lysosome during transfection. The study also revealed that endocytosed PEI administered with or without DNA, underwent nuclear localization in the form of ordered structures.

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iii) Intracellular trafficking of plasmid DNA to the nucleus

Nuclear trafficking was the most critical rate limiting step in the efficient delivery of the gene from the cytoplasm into the nucleus [5, 27, 28, 29, 31 and 84]. Nuclear entry of DNA molecules occurred through the nuclear pores, which was about 10nm in diameter, or during cell division. DNA must dissociate from the condensed complexes either before or after entering the nucleus. A study comparing nuclear microinjection of lipoplex and free DNA revealed that free DNA was required for transcription. Nuclear microinjection of lipoplex had resulted in minimal or no expression activity, while nuclear microinjection of free DNA resulted in gene expression in 60-70% of cell [28].

The efficiency of nuclear trafficking had been reported to be very low with one in 10,000-100,000 plasmids taken up by the cell eventually being expressed [27]. The problem was worsened with non-dividing cells, where entry into the nucleus was thought to occur solely through the nuclear pore complex. To achieve active transport to the nucleus, nucleus localizing signal (NLS) peptides was conjugated with a gene carrier or with DNA directly [31].

2.6 Naked DNA delivery

Direct injection of naked plasmid DNA into the tissue was the simplest way of administration. Naked DNA was delivered directly to the cytoplasm, bypassing endosomes and lysosomes, hence avoiding enzymatic degradation [34]. The ease of preparation and unlimited size of constructs for gene delivery made this approach attractive.

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However, the delivery of naked DNA was limited only to cells near the injected site and cannot be used for tissue targeting in systemic administration. Site of direct injection included skeletal muscle, liver, thyroid, heart muscle, urological organs, skin and tumor. Naked DNA therapies had been tested against heart disease, cancer and other disorders, but had been reported to be effective only in the liver and muscle [5].

Hickman, Malone et al [61] had shown that the direct plasmid DNA injection administered to the livers of rats and cats had resulted in luciferase activities which peaked at 24 to 48 hrs. X-gal staining of liver tissue, following injection of plasmid DNA encoding β -galactosidase, revealed that the take up of injected DNA was limited and scattered around the injection site. Histological examination revealed acute inflammatory infiltration near the injection site, which may have adversely affected the transgene expression. Despite the simplicity, reliability and cost effectiveness of this method, its use was currently limited due to low transfection efficiency, transient expression and localized area of transduction.

While gene expression can be achieved by direct intra-tissue injection of naked plasmid DNA, gene transfer via systemic administration such as intravenous injection will generally require the use of a delivery vector or vehicle. When plasmid DNA was injected slowly into the mouse via tail-vein, no enhanced gene transfer was observed *in vivo* in major organs including liver, lung, heart and spleen. This was attributed to the sensitivity of DNA to enzymatic degradation. These DNA were rapidly degraded during circulation due to the large amount of nucleases in the blood resulting in much lower levels of transfection compared to those mediated by carriers [4 and 59]. It was also suggested that the plasmid DNA may be rapidly cleared out from the blood circulation.

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Due to the low affinity of the DNA to the cell's receptor, it may also not remained in close proximity with a given cell surface receptor long enough to result in endocytosis [91].

Other physical delivery techniques including electroporation, bioballistic (gene gun), hydrodynamic injection and ultrasound have also been established to enhance the *in vivo* transfection of DNA [21 and 34].

F. Liu et al [60] had demonstrated that high level of transgene expression can be achieved by systemic administration of plasmid DNA by rapid injection of DNA in large volumes via tail-vein injection in mice. Transgene expression was observed in internal organs including the lung, spleen, heart, kidney and liver, with the highest level reported in the liver. It was postulated that the injected DNA solution was likely to accumulate in the inferior vena cava when the injection rate exceeded the cardiac output. This resulted in a buildup of hydrostatic pressure, proportional to the volume and speed of injection, in the inferior vena cava. This pressure will force the flow of DNA solution into tissue such as the liver, kidney and heart that were linked directly to the inferior vena cava. Since the liver was the largest organ in the body, it had the most direct exposure to the DNA flow. It was suggested that some of the DNA molecules were transferred to the liver during the hydrodynamic process. The pressure could also increase the permeability of liver fenestra, which would otherwise prohibit the plasmid DNA from reaching the hepatocytes.

More recently, Liu and Huang [87] have reported that the temporary clamping of the vena cava following tail vein injection of plasmid DNA in 0.1ml solution led to high gene expression in the liver. It was postulated that the clamping of the vena cava may have facilitated the egress of DNA by decreasing the local blood flow and improved the interaction of DNA with blood vessels.

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Wolff's group [91] had also demonstrated that high gene expression can be achieved in the liver and limb muscles when large volume of DNA were injected with high speed via the portal vein of liver or the artery of the limb muscles.

Their studies have suggested that naked DNA was taken up by cells *in vivo* by an active receptor-mediated process. The high pressure was thought to enhance gene expression by facilitating the egress of DNA out of the blood vessels.

2.7 DEAE-dextrane and Calcium phosphate co-precipitation (Calphos™)

Calcium phosphate precipitation and diethylaminoethyl-dextran (DEAE-dextran) were the earliest non-viral methods used in transferring exogenous genes into somatic cells [5]. Both were simple and still widely used in labs worldwide for *in vitro* transfections. However, both methods were hampered by cytotoxicity and the difficulty of applying them for *in vivo* studies [5 and 14].

Positively charged DEAE-dextran bonded to the negatively charged phosphate groups of the DNA, forming aggregates. These complexes, when applied to cells, subsequently bond to the negatively charged plasma membrane. Cellular uptake was believed to be mediated by endocytosis. DEAE-dextran had been reported to be ineffective in the presence of serum [15].

Calcium phosphate-mediated transfection, pioneered by Graham and Van der Eb [28], was based on the formation of small, insoluble calcium phosphate-DNA precipitates. The procedure required that calcium ions first bonded to the negatively charged phosphate groups of the DNA.

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When phosphate was subsequently added to the mixture, it bonded ionically to the calcium ions to form a crystalline precipitated complex. Cellular uptake was believed to occur by endocytosis, when these precipitates attached to the cell surface.

Transfection by CalphosTM had been described in several literatures as a simple, but insufficiently reproducible method [15, 45 and 73]. This was because the transfection efficiency was highly dependent on precipitation conditions such as pH, salt and DNA concentrations, the temperature and the time between precipitation and transfection techniques.

The time between precipitation and transfection had been reported to play a critical role in influencing the size of precipitates. The size of these precipitate complexes affected the transfection efficiency. It was also restricted by the amount of DNA used, as high DNA concentration would block the surface of the initially formed nuclei, thereby completely inhibiting the formation of precipitates.

2.8 Cationic Liposomes

Early experimental studies and clinical trials involving negatively charged liposomes had revealed insufficient gene transfer activity [77]. Substantial efforts had since been spent towards improving the efficiency by the development of cationic liposomes. Cationic liposomes were the most extensively investigated non-viral vectors. The most widely known cationic liposomes were 2,3-bis(oleoyl)oxipropyl-trimethylammonium chloride (DOTMA) and dioleoylphosphatidylethanolamine (DOPE), which were commercially available as transfection reagents in Lipofectin. Other cationic liposomes that had been extensively studied included DOTAP, 3- β -[N-(N'-N'-dimethyl-ethane)-carbamoyl]cholesterol (DC-chol), 3- β -[N-N'-N'-trimethyl-ethane)-carbamoyl]-cholesterol (TC-chol) and dimethyl-dioctadecyl-ammonium bromide (DDAB).

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Due to their amphiphilic nature, cationic liposomes readily and efficiently interacted with the negatively charged phosphate backbone of DNA to form lipoplexes [77]. They presented the advantages of robust manufacture, ease in handling and preparation techniques, low immunogenic response and the ability to inject large lipid-DNA complexes. These reagents can also be easily manipulated to incorporate novel biological functions, or to produce new formulations that can be screened for *in vivo* gene transfer activity [21]. The availability of a wide range of lipid molecules and the ease with which the complexes can be generated resulted in the use of cationic liposomes in majority of the clinical trials involving non-viral vectors.

Several liposomal formulations had been successfully used in gene therapy experimental models and evaluated in several clinical protocols for gene therapy in cancer and cystic fibrosis [21].

Cationic-lipid-plasmid complexes were effective as *in vitro* transfection agents, but had limited capacity for systemic applications [21 and 77]. Cationic liposomes systems lacked the ability to target specific cell types. Their success *in vivo* was limited by low transfection and lipid toxicity. Systemic and compartmental administration of cationic lipidic vectors was also found to induce the production of a number of pro-inflammatory cytokines including IFN- γ , TNF- α , IL-1 and IL-12. These cytokines can cause toxicity and inactivation of transgene expression [93]. High dosages were reported to cause death in mice [87].

Acute toxicity and silencing of transgene expression were primarily related to cationic lipid/DNA complexes. Lipid toxicity was caused by the non-natural, non-biodegradable nature of transfection lipids as well as their ability to disrupt cell membranes.

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Ester, amide and carbamate linkages had been used to tether polar and hydrophobic domains of cationic lipids to reduce toxicity [21].

The low transfection efficacy was largely associated to the aggregation of the complexes arising from their inherent instability at physiological conditions. Size and surface charge of the complexes were thought to be the most critical factor in preventing aggregation [21 and 77]. Complex formation from the addition of plasmid DNA to cationic liposomes often resulted in a heterogeneous mixture of complexes with size ranging from 100 to 1000nm in diameter. The large size and positive charge of these aggregates resulted in rapid clearance from the body. These aggregates were sieved out by the first capillary bed, with the highest level of expressions observed in first-pass organs [21 and 43].

Studies on DC-Chol:DOPE complexes had determined that the cationic liposomes were cleared from the circulation within minutes of intravenous administration. Majority of the lipid label were found to accumulate immediately in the lung, with the remainder being distributed in the spleen, heart and liver [21]. It had also been reported that repeated dosing was not effective during a refractory period of one or two weeks [87].

The positive charge of the cationic liposome system had caused it to interact with various proteins, which resulted in the lack of ability to target specific cell types. This limited the gene transductions at the site of administration [21 and 43]. S. Li et al [36 and 39] overcame this issue by increasing the charge ratio of cationic-lipid-DNA. *In vivo* efficiency of most cationic lipids including DC-Chol, TC-Chol and DDAB were found to be dependent on charge ratio.

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Condensing DNA with biocompatible polycations prior to mixing with cationic liposomes had been shown to protect DNA from DNases activities and enhance the transfection efficiencies of cationic liposomes [46 and 47]. Fl Sorgi, L. Huang, S. Li et al [20 and 39] had enhanced the transfection efficiency of cationic liposomes by employing polycations such as protamine sulfate and poly-(L-Lysine) to form LPD complexes. LPD complexes had been shown to achieve significantly higher transfection efficiency compared to the corresponding liposome-DNA complexes without the presence of polycation. The incorporation of these polycations was found to reduce the average diameter of the lipoplexes to 100nm, which allowed for more efficient cellular internalization. Systemic administration of LPD formulation had yielded a dose dependent gene expression in various tissues including heart, lung, liver, spleen and kidney with the highest level of gene expression in the lung [22].

The development of novel types of lipid molecules appeared to be saturated, and most of the current efforts had shifted towards improving the efficacy and specificity of existing lipids for *in vivo* applications [34]. Despite alterations in plasmid-liposome charge ratios or the nature of the cationic lipid, the cytotoxicity and pharmacokinetics limitations of these systems had not been overcome [21 and 24].

2.9 Cationic Polymers

Polyplexes were defined as cationic polymer-nucleic acid complexes formed by the addition of nucleic acid to cationic polymer [24]. Polylysine (PLL), polyethylenimine (PEI) and other polycations were known to interact electrostatically with plasmid DNA to form toroidal structures of 40 to 60nm in diameter. *In vitro* activity of these complexes had been found to correlate with particles that were small, stable and uniform in size and can be taken up by the cells.

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It had been suggested that the correlation between the high transfection potential and small particle size may be linked to the presence of 100nm diameter of coated pits involved in receptor-mediated endocytosis [24].

Polyplexes possessed several distinct advantages. DNA encapsulation within the polymer can protect DNA against degradation until its release into the cell cytoplasm. The injection or implantation of the polyplexes into the body can be used to target a particular cell type or tissue, and drug release from the polyplex into the tissue can be designed to occur rapidly or over an extended period of time. Polyplexes were also mechanically and thermodynamically more stable than lipopolyplexes [62].

Cationic polymers had displayed good potential *in vitro*, but were unlikely to be suitable for use *in vivo* due to their inherent cytotoxicity and rapid clearance by the reticuloendothelial system [21]. These vectors tended to attract serum proteins and blood cells when entering the body circulation, resulting in dynamic changes in their physicochemical properties [21]. Moreover, they transfected cells indiscriminately and bioactive molecules cannot be easily incorporated for cell specific delivery. The molecules would need to be covalently linked to the polymer backbone during self-assembly process.

Several novel cationic polymers such as polyamidoamine and polyethylenimines dendrimers had been investigated. These were able to mediate higher transfection efficiencies *in vitro* and *in vivo*, but were found to be cytotoxic [21]. Polyethylenimines (PEI) was the first synthetic transfection agent to be discovered, and had been extensively studied. It was a highly branched polymer, composed of two units of carbon per nitrogen. PEI was stable and mediated higher level of transfection, but toxicity was also a major drawback [24].

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To lower the toxicity, M. Thomas et al [80] had chemically modified the nitrogen atoms of PEI and had reported enhanced transfection efficacy on COS-7 cells in the presence of serum.

Chitosan was an aminopolysaccharide derived by the alkaline deacetylation of chitin. Chitin, a naturally occurring cellulose-like polymer, was found in fungal walls and the exo-skeleton of arthropods such as insects, crabs, shrimps, lobsters and other vertebrates. Chitosan had been extensively investigated for gene and drugs delivery due to its low toxicity, good biocompatibility, biodegradability and DNA complexation. However, the applications of chitosan were limited by its high molecular weight, which resulted in its low solubility in aqueous media. Chitin after deacetylation and conversion to chitosan formed very viscous solution even at low concentrations, making it difficult to be chemically modified in the neutral pH condition. Previous works [24 and 48] had utilized dextran, poly(ethylene glycol) (PEG) poly(vinyl pyrrolidone) and lactobionic acid (LA) to increase the solubility of chitosan in water. Chitosan-mediated transfection was found to be not inhibited by the presence of serum *in vitro* and was less cytotoxic compared to the other polyplex systems. However, particle aggregation and surface charge mediated serum clearance remained as key barriers for use *in vivo*.

2.10 Peptide mediated gene delivery

Naturally occurring or synthetic peptides had been utilized as gene delivery systems. A number of peptide sequences such as poly-L-lysine (PLL), protamine and tyrosine-lysine-alanine-(lysine)₈-tryptophan-lysine had been shown to bind and condense DNA [15, 20, 39 and 49]. However, the endosomal-lysosomal pathway presented a major barrier to transfection as the peptide-DNA complexes lacked the means of escaping from the endosome, following endocytosis [50].

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PLL was biodegradable, but high toxicity prevented its usage *in vivo*. The lack of amino groups was thought to prevent PLL from endo-somolysis, leading to low levels of transgene expression [24]. The addition of choloquine, a lysosomotropic agent, greatly enhanced the activity of PLL, which aided its escape from the endosome upon cell internalization.

A significant advantage of polypeptide conjugates was the possibility of coupling with cell specific ligands, allowing receptor-mediated targeting of the peptide/DNA complexes to specific cell types. J. Zeng and S. Wang [51] had developed a cationic polypeptide comprising a nerve growth factor (NGF), a member of neurotrophin family, as a targeting ligand and a 10-lysine sequence as a DNA binding moiety. When this polypeptide was conjugated with PEI, transfection was enhanced six-folds in PC12 cell line (rat pheochromocytoma) compared to COS-7 cells, which did not contain a NGF receptor.

2.11 Nano-bioceramics

Bio-ceramics referred to the class of ceramics used for repair and replacement of diseased and damaged parts of musculoskeletal systems. The bulk materials have traditionally been used for bone implant and as coatings for hard tissue metallic implants.

At nano-scale size, these materials were generally monolithic pure, possessed higher chemical reactivity and higher specific surface areas. Their small nano-scale size would also ease their transport in the blood stream.

Calcium Phosphate

Calcium phosphate (CaP) was the most ubiquitous family of bioceramics, well known for their use in biological applications [53].

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The most important properties of calcium phosphate biomaterials were their bioresorbability and bioactivity. It had been deemed as a suitable candidate for gene delivery work due to their high biocompatibility and good biodegradability.

T. Welzel et al [45] had developed spherical CaP nano-particles, with diameters ranging from 10 to 20nm, via controlled precipitation from the reaction of aqueous $\text{Ca}(\text{NO}_3)_2$ (18nM, pH 9.5) and $(\text{NH}_4)_2\text{HPO}_4$ (10.8nM, pH 9.5) at room temperature. The coating of DNA on the nano-particles resulted in the formation of a stable colloid, which did not lose their transfection ability even after 2-3 weeks of storage at 6 deg C. This complex was reported to be able to mediate transgene expression on transformed human umbilical vein endothelial cells (T-HUVEC) in the absence of serum. The lower level of transfection efficiency in the presence of serum had been postulated to the formation of larger precipitates caused by the effect of the serum.

Hydroxyapatite (HA)

Hydroxyapatite (HA) had the chemical formula of $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, Ca/P ratio of 1.67 and possessed a hexagonal structure. It was stable in body fluid, and in dry or moist air for up to 1200 deg C. HA was the most ubiquitous and well known CaP phase, studied for a number of biomedical applications, owing to its similarity to the natural bone. Due to its favorable osteo-conductive and bioactive properties, it was the most intensely studied bio-ceramic for use as scaffold materials in tissue engineering. It had also been investigated as a carrier of plasmid DNA for non-viral gene delivery [53 and 54].

Prashant K. et al [53] had developed an in-situ precipitation technique for HA by adding DNA to CaCl_2 , and then mixing it with Na_3PO_4 at pH7.5.

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NaOH was added to Na_3PO_4 precursor to modify different calcium to phosphate (Ca/P) ratio. It was reported that different Ca/P ratio of HA can alter the surface charge density of the particles due to the electrostatic interaction of Ca^{2+} , DNA and $(\text{PO}_4)^{3-}$, thus affecting the nature and kinetics of DNA binding and condensation.

S. Bisht et al [73] had also developed an in-situ precipitation technique for crystalline HA nano-particles by adding a mixture of calcium chloride, plasmid DNA and AOT solution in hexane to a mixture of AOT solution, Tris-HCl buffer, Na_2HPO_4 and plasmid DNA, followed by continuous stirring for 12 hour at 4°C. Comparable β -galactosidase activity was reported to be mediated by these HA nano-particles encapsulating pSV β gal DNA to polyfect on HeLa cell line in the absence of serum. The group had also reported the substitution of Ca^{2+} with Mg^{2+} and Mn^{2+} , which formed crystalline magnesium phosphate and amorphous manganous phosphate nano-particles respectively [53]. The transfection activity of magnesium phosphate encapsulated plasmid on HeLA cell line was found to be comparable to that of Polyfect. However, manganous phosphate was 25% less efficient compared to Polyfect. This had been attributed to the complexation reaction between manganous ion and the plasmid DNA.

HA crystalline nano-particles (40-60nm) formed by a precipitation hydrothermal technique was also recently investigated as a potential vector by S.H Zhu, B.Y Huang et al [54]. *In vitro* transfection of the HA nano-particle using EGFP-N1 pDNA on the gastric cancer SGC-7901 cell line showed transfection efficiency of about 80% compared to that of Lipofectamine TM2000. *In vivo* animal experiment showed no acute toxic adverse effect 2 weeks after tail vein injection into mice.

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Silica Nano-particles

Silica and silicon have been emerging as interesting candidate materials for injectable nano-vectors [11]. Dan Luo et al [55] reported that dense inorganic silica nano-particles with radius of 0.225 μ m, which by themselves do not deliver DNA, were able to enhance DNA transfection mediated by other commonly used transfection reagents such as SuperFect, Effectene, LipofectAmine, DMRIE-C, Lipofectin, Fugene and DEAE-dextran. The enhancement of transfection was attributed to extracellular mechanisms in which dense SiO₂ nano-particles played a role in DNA complexation. The group had further established a mathematical model based on its hypothesis that physical characteristics such as size, number or particles, complexation and medium viscosity, rather than chemical characteristics play a role in transfection enhancement [56].

C. Kneuer, M. Sameti et al [57] had reported the ability of colloidal silica nanoparticles (10-100nm) modified with various aminoalkylsilanes viz. N-(2-aminoethyl)-3-aminopropyltrimethoxysilane (AEAPS) and N-(6-aminoethyl)-amino-propyltrimethoxysilane (AHAPS) to interact with pCMV β plasmid for *in vitro* transfection. The *in vitro* transfection of COS-1 cells was observed to peak using silica nano-particles surface modified with N-(6-aminoethyl)-amino-propyltrimethoxysilane (AHAPS) and pCMV β plasmid at w/w ratio of 10 in serum-free medium and w/w ratio of 30 in 10% serum. Further addition of 100mM chloroquine was reported to enhance the transfection efficiency by 2-10 folds, reaching 30% of the efficiency achieved by a 60kDa PEI with no toxicity observed. AFM revealed a spaghetti-meatball-like structure with a mean diameter of 26nm and a zeta potential of up to +31mV.

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X. He et al [58] had investigated the use of amino-modified silica coated magnetic nanoparticles prepared by synchronous hydrolysis of tetraethoxysilane and N-(β -aminoethyl)- γ -aminopropyltriethoxysilane in water-in-oil micro-emulsion after the synthesis of aqueous magnetic ferro-fluid with the precipitation of the chloride mixture with the base. *In vitro* transfections on COS-7 cells using GFP plasmid DNA showed that the modified silica particles were able to mediate transfection activity in the absence of serum. These super-paramagnetic nano-particles may be potentially used in the filtration of successfully transfected cells under magnetic field, but more work will need to be done to establish its viability as an efficient gene carrier.

2.12 Hybrid vectors

It has been predicted that future gene therapy vectors might be a combination of both viral and non-viral transfer methods [5, 21]. Hybrid vectors could be developed by incorporating viruses or viral peptides into traditional cationic-amphiphile-based vector systems. The efficiency of synthetic vectors can also be improved by using artificial nucleic-acids carriers incorporating functional elements that mimic viruses or the use of viral coat proteins [21].

2. 13 Disease targeting

Non-viral vector targeting had been accomplished primarily through the use of molecular conjugates and protein/DNA complexes. Several ligands had been developed and used for cell-specific gene delivery.

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Specific targeting to the liver had been achieved using the glycoprotein asialoorosomucoid (ASOR) ligand to bind with the asialoglycoprotein receptor (ASGPr), which was uniquely present on hepatocytes at a high density of 500,000 receptors per cell, and retained on several human hepatoma cell lines. However, since targeting was done through a receptor that was internalized by receptor-mediated endocytosis, the fusion of the endosome with the lysosome resulted in the degradation of the DNA attached to the molecular conjugate. This limited its application *in vivo* [43].

Tissue or cell-specific promoters have also been used to generate specificity. The cytomegalovirus promoter and enhancer (CMV) had been identified to be active primarily in rapidly dividing cells [43 and 57].

The route of administration can also aid in specificity [43]. The direct injection into tumor allowed for specific transfection of cells in the site of injection. Peritumoral injection limited the administration to a neighbouring tumor. Systemic, intravenous administration provided the least specificity to tissue. *Ex vivo*, an alternative route to *in vivo* provided the greatest degree of specificity. In this approach, cells were removed from patient, transfected with the delivery system and then replaced back into the host.

2.14 Gene Therapy applications

Originally intended for treatment of hereditary genetic disorders, gene therapy has evolved in treating four major types of disease. These included single gene inherited disorders, multi-factorial disorders, cancer and infectious diseases. To date, most of the approved protocols for gene therapy involved cancer.

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2.14.1 Cancer Gene Therapy

Cancer was caused by the culmination of somatic gene alterations triggered by a multi-step process. Several strategies had been adopted in harnessing gene therapy to treat cancer.

i) Immunotherapy

The concept of immunotherapy was based on the body's natural defense system to attack cancer. A significant advantage of this approach was the ability to recruit the immune system to attack disseminated tumor cells and eliminate both local and metastatic diseases.

Passive immunotherapy aimed to increase pre-existing immune response to the cancer, while active immunotherapy stimulated the body's own immune system to fight the disease. There had been evidence that tumor regression can be achieved from manipulating the human immune response [43]. Passive immunotherapy protocols have involved the use of tumor-infiltrating lymphocytes (TILs), tumor cells or fibroblasts to express cytokine genes. Active immunotherapy focused on genetically modifying the tumor cells to increase expression of antigen presenting molecules or express tumor rejection antigens.

ii) Gene Directed Enzyme Pro-drug Therapy (GDEPT)

A substantial limitation of conventional chemotherapy and radiotherapy was the toxicity of these agents to normal tissue. This approach was based on the systemic administration of a non-toxic pro-drug following the selective transduction of tumor cells with genes that encoded enzymes capable of converting pro-drugs to cytotoxic drugs in the cancer cells. The local expression of an activating enzyme ensured that the toxicity associated with chemotherapy was reduced.

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iii) Insertion of tumour suppressor genes and oncogene suppression
Oncogenes and tumor suppressor genes have been identified in carcinogenesis. Many tumors lacked functioning tumor suppresser genes that might be able to prevent further tumor growth and promote apoptosis of the tumor cells leading to tumor regression. This strategy involved the insertion of a gene encoding a tumor suppressor protein such as P53, to restore the ability of the cells to keep the tumor in check. The inactivation of oncogenes involved the use of anti-sense ras complementary DNA or anti-sense oligonucleotides.

iv) Induction of drug resistance genes to complement existing therapies
This protocol involved the transfer of genes into normal cells to augment existing cancer treatment.

The protection of bone marrow during chemotherapy by transducing the multiple-drug resistance (MDR1) gene into marrow stem cells has been investigated [43]. Protocols involving the use of MDR1 have also been approved for treatment complementing paclitaxel (Taxol).

2.14.2 Gene Therapy in Bone Regeneration

There had been a growing need to develop artificial bones due to various clinical bone diseases such as bone infections, tumors and bone loss by trauma.

Numerous researches had been devoted to bone tissue engineering in which 3D porous scaffold was loaded with specific living cells and/or tissue-inducing factors to launch tissue regeneration or replacement. Biodegradable polymers such as poly(lactic acid) (PLA), poly(glycolic acid) (PGA) and PLGA and bio-ceramics such as hydroxyapatite (HA) and tri-calcium phosphate (TCP) had been widely used to develop porous 3D scaffolds using various fabrication techniques [70].

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These 3D scaffolds served important roles in manipulating the functions of osteoblasts and guiding the formation of new bone into desired shapes.

The ability to extract human bone morphogenetic proteins (BMP) [82], a growth factor that controlled osteogenesis, provided a huge complementary boost towards the progress of artificial bone implants. BMP carried a dual role of both mitogen and morphogen, which stimulated the multiplication of connective tissue cells and transformed connective tissue cells into osteoprogenitor cells.

BMP was a water-soluble relatively low-molecular weight protein that diffused easily in the body fluids. Hence, a carrier was necessary to contain BMP for localized effect to be achieved at the bone healing site. Bovine collagen was the most commonly used carrier and was currently used in clinical setting. However, the use of collagen restricted the use of BMP-2, which was easily squeezed out of the collagen by axial loads and under pressure.

Gene therapy thus represented a possible strategy in delivering BMP gene as a transgene to target cells. BMP genes can be mediated by viral or non-viral vector. However, the use of viruses raised concerns of safety, while the use of non-viral vectors was associated with low transduction rate and transient expression [82 and 83]. Ono et al [83] had reported that the impregnation of Superfect carrying the BMP-2 gene into the HA 3D scaffolds, was able to induce osteogenetic effects when implanted in Japanese white rabbits.

CHAPTER

3

MATERIALS AND METHODS

3.1 Nano-particles

Stoichiometric HA with calcium/phosphate (Ca/P) ratio of 1.67 was synthesized in-house using a wet chemical route according to the protocol developed by R. Kumar et al [37]. 0.28M Orthophosphoric acid (Merck) was reacted with 0.5M calcium hydroxide (Merck) according to the following equation:

The reaction occurred under steady state condition by adding H_3PO_4 drop-wise at the rate of 1.5lit/hr, with the control of a Manostate (Simon Varistalitic pump). The pH of the reaction was monitored with the HI 9017 microprocessor pH and temperature meter (Hanna Instruments). The reaction was allowed to proceed at 100°C until the pH reached 8.0. The heater was switched off and the suspension was stirred for two hours before leaving it to stand for 24 hours.

SiO_2 nano-particles were synthesized in-house using RF spray process at plate power of 50kW. This was previously described by R. Kumar in his works on RF processing of ultra-fine bio-ceramic materials [42]. The powder was made into colloidal dispersion in dd H_2O .

Zirconia nano-particles suspension was purchased from Alfa Aesar, USA.

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3.2 Surface modifying agents

Amino acids including L-Lysine (Lys), L-Histidine (His), L-Arginine (Arg) and polycations including Poly-L-Lysine (PLL) and Protamine Sulfate (PS) were used as modifying agents to modulate the surfaces of the nano-particles with net positive charges. All the above mentioned chemical agents were purchased from Sigma-Aldrich. The respective amino acids and polycations were dissolved in ddH₂O to form aqueous solutions with concentration of 10mg/ml. These solutions were sterilized using a 0.22µm Millipore filter and stored at 4°C.

3.3 Surface modification of nano-particles

Varying percentages of the various amino acids and polycations solutions were added to the respective nano-particles and allowed to stand overnight at 4 deg Celsius. The surfaces of the nano-particles were modified through the adsorption of the respective amount of amino acids and polycations.

3.4 Preparation of nano-particles-DNA complex

500ul of 10mM Tris at pH7.0 was used as a transfection buffer to suspend the respective modified nano-particles. Plasmid DNA was added to the nano-particles suspension and vortexed at half-speed setting for 3 seconds. The mixture was further incubated for 1 hour in 37 deg Celsius water-bath to allow the formation of nano-particles-DNA complexes.

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3.5 Cell Lines used for *in vitro* transfection

B16 (mice skin melanoma), CNE2 (human nasopharyngeal carcinoma), HepG2 (human liver cancer), HeLa (human cervix cancer) and 220.1 (human colon cancer) cell lines were grown in 75cm² cell flasks (BD Falcon). All cell lines were cultured in Dulbecco's modified eagle medium (DMEM, NCC in-house media) containing 0.3M glutamine (Sigma-Aldrich), 0.1M sodium pyruvate (Sigma-Aldrich), non-essential amino acids (Gibco, Invitrogen Corp.), penicillin-streptomycin (Sigma Aldrich) and 10% heat activated fetal bovine serum (Hydroclone). Cells were grown at 37°C in humidified air containing 5%CO₂ and passaged using 0.125% wt/v trypsin chelated with EDTA (NCC in-house media). All cells lines were obtained from the National Cancer Center (NCC).

3.6 Animal lines

Female Balb/c mice were purchased from the Animal Laboratory Unit of the National University of Singapore. Mice were used at 8-12 weeks of age. All mice were housed in the animal facility of the National Cancer Center according to the guidelines spelt out by the National Advisory Committee for Laboratory Animal Research (NACLAR) [95].

3.7 Reporter gene used in characterization and *in vitro* transfection studies

*p*NGVL encoding the firefly luciferase, was utilized as a reporter gene to determine the *in vitro* and *in vivo* efficiencies of the nano-ceramics particles. The plasmid was obtained from Dr. Wu XF of the Gene Vector lab at the National Cancer Center.

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3.8 Transformation of competent cells

100ul of competent cells and 5ul of plasmid DNA were mixed and incubated on ice for 15 mins. This was followed by 2 mins of incubation in 42°C hot water bath, shaken at 550 rpm, followed by a further 10 mins incubation on ice. 900ul of LB broth (NCC in-house media) was added to the competent cells/DNA mixture and shaken for 40mins in a 37°C incubator. 250ul of the competent cell mixture was scaled up by adding it to 250ml LB broth and shaken at 200rpm for 16 hours in 37°C incubator. 100mg/ml of Kanamycin (Sigma) was added to the LB broth prior to the addition of the competent cell mixture.

3.9 Extraction of plasmid DNA

Plasmid DNA was extracted using Qiagen's Maxi-kits for high plasmid copy according to the Qiagen's protocols [19]. The scaled up bacteria cells was harvested by centrifugation at 6000 x g for 15 mins at 4°C. The bacteria pellet was then re-suspended in 10ml of P1 buffer and 10ml of P2 buffer, followed by 5 mins incubation at room temperature. 10ml of pre-chilled P3 buffer was then added to the lysate and incubated on ice for 20 mins. The lysate mixture was centrifuged at 20,000 x g for 30 mins at 4°C. The Qiagen-tip 500 was equilibrated with 10ml of QBT buffer before the supernatant of the lysate was applied to the tip and allowed to enter the resin in the column by gravity flow.

The Qiagen tip was washed with 2 x 30ml QC buffer before the plasmid DNA was eluted with 15ml QF buffer. Eluted DNA was then precipitated with 10.5ml room temperature iso-propanol and centrifuged at 15,000 x g for 30 mins at 4°C. 70% ethanol was used to wash the DNA pellet which was allowed to air-dry for 10mins before dissolving it in endogen-free TE Buffer.

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The concentration and purity of the extracted plasmid DNA was then measured with the UV-spectrophotometer at wavelengths of 260nm and 280nm (Ultraspec 3000, Pharmacia Biotech).

3.10 Electron micrographs of nano-particles

The nano-particles were diluted in ddH₂O and sonicated for 30mins before depositing the suspension on a 300 mesh copper grid. The grid containing the sample was allowed to dry before loading onto the JSM-6700F FEG SEM (JEOL). Images were captured using both the scanning electron mode at 5KV and the scanning transmission electron mode at 20kV.

3.11 Resorbability of the nano-particles

10mM Tris buffers with pH values of 7.0, 6.0, 5.9, 5.5 and 5.0 were prepared. The three different nano-particles were soaked in 500ul of the respective buffers at room temperature and observed visually over a period of 1 month.

3.12 Gel Retardation Assays

1% Agarose gel was prepared by dissolving 1g of Agarose powder (Sigma Aldrich) in 100ml of 1XTris-borate EDTA (TBE) buffer (Sigma-Aldrich). 1% of Etidium Bromide (Sigma-Aldrich) was added to the gel before casting it with the comb in place. The nano-particle-DNA complexes were suspended in 6X gel loading buffer (0.25% Bromophenol blue, 0.25% Xylene cyanol FF, 15% Ficoll Type 4000, 120 mM EDTA) before loading them into the respective wells. A voltage of 120V was applied to the gel, submerged in 1XTBE for 45mins. The gel assay was visualized using Geldoc 2000 gel documentation system (Bio-Rad, Germany) equipped with a UV transilluminator.

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3.13 Measurement of Zeta Potentials

The surface charge of the nano-particles and nano-particles-DNA complexes were measured using the ZetaSizer 3000 HS (Malvern Instruments Ltd, UK). The samples were suspended in 2ml of ddH₂O and loaded into the capillary cell. The zeta potential is determined by measuring three ten-cycle runs at 1.5mA with count rates between 50KCPs and 3000KCPs.

3.14 Quantification of unbound DNA from the nano-particles

The nano-particle-DNA complex suspension was spun down at 13,000 rpm for 2 mins. The supernatant was extracted and unbound DNA were measured using the UV-spectrophotometer at wavelengths of 260nm and 280nm (Ultraspec 3000, Pharmacia Biotech).

3.15 *In vitro* transfections

Cells in their log (exponential) growth phase were seeded on six-wells plate (Nunc, USA) 24 hours before transfection. Cells viability was checked using trypan blue exclusion, and counted using a bright-line hema-cytometer (Sigma) under an inverted microscope (Nikon-TMS). Plated cells were incubated in DMEM containing glutamine and 10% Fetal Bovine Serum (FBS) at 37°C in 5% CO₂ incubator. When 70% cell confluency is reached, the culture medium was discarded and the respective nano-particles-DNA complexes were added to the cells and incubated for 10mins before adding fresh culture medium. Transfected cells were harvested after 24 hours, where luciferase and Bradford protein assays were conducted. The transfection efficiency was quantified by the luminescence units (RLU) normalized per ug of cell protein.

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All transfections were done in triplicates and repeated twice to ascertain reproducibility.

3.16 CalphosTM Transfection protocol

The Calcium Phosphate co-precipitation technique was used as a positive reference in gauging the *in vitro* transfection efficacy of the nano-particles systems. *In vitro* transfection were conducted under optimized conditions according to the Clontech's CalphosTM protocol on mammalian transfection [17]. Cells in their log (exponential) growth phase were plated on 6-wells plate in DMEM containing glutamine and 10% FBS and incubated for 24hrs in 5% CO₂ at 37°C. The culture medium was replaced with serum-free medium 1 hour prior to transfection. 3ug of plasmid DNA was added to the CaCl₂ solution and incubated for 1 hour. The plasmid/CaCl₂ mixture was then added drop wise to 2x HBS and allowed to stand for 20 minutes prior to transfection. Transfected cells were harvested after 24 hours. Luciferase and Bradford protein assays were conducted to measure transfection efficiency.

3.17 Harvesting transfected cells

Transfected cells were harvested after 24 hours. The growth medium was discarded and the cells were washed twice using 1x PBS. Cells were mechanically scrapped using cell scrappers (Nunc, USA) and spun for 10mins in 1x PBS at 2000rpm. The supernatants were discarded and the cell pellets were re-suspended in 0.25M Tris base at pH 7.8. The cells were lysed through 3 cycles of freeze-thaw and spun down for 10mins at 13000 rpm in 4 deg Celsius. The supernatants of the cell lysates were collected and stored at -20 deg C for subsequent quantifications using the luciferase and Bradford protein assays.

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3.18 Luciferase Assay

Luciferase Assay Buffer (LAB) was prepared by adding 25mM glycylglycine, pH 7.8, 15mM MgSO₄, 4mM EGTA and 15mM potassium phosphate, pH 7.8 in ddH₂O. Luciferin buffer was prepared by adding 25mM glycylglycine, pH 7.8, 15mM MgSO₄, 4mM EGTA in ddH₂O. LAB and the luciferin buffer were filtered sterile and stored at 4°C. Luciferin stock was prepared in the dark by dissolving 10mg of luciferin (Roche Diagnostic) in 25mM glycylglycine, pH 7.8 and stored at -20°C in the dark. Just prior to use, 10ul of 1M ATP and 5ul of 1M dithiothreitol (DTT) were added to 5ml of LAB. DTT and luciferin stock were added to the luciferin buffer. The cell lysate supernate (sample) was added to the luciferase assay substrate in a luminometer tube, vortexed and immediately assayed for light output with a 10s integration time in a luminometer (Lumat, EG&G Berthold). Luciferase detection signal was recorded in relative luminescence units (RLU). All reagents, with the exception of luciferin stock, were purchased from Sigma-Aldrich.

3.19 Bradford Assay

The Bradford assay provided a simple and accurate method of determining protein concentration through the binding of Coomassie Brilliant Blue G-2 dye to proteins [96]. This dye which existed in three forms viz. cationic (red), neutral (green) and anionic (blue), is converted to a stable unprotonated blue form when the dye binds to protein. Six protein standard solutions of 5ug/ml, 7.5ug/ml, 10ug/ml, 15ug/ml, 20ug/ml and 25ug/ml were prepared by performing serial dilutions with 10% of Bovine Serum Albumin (BSA) stock in 2ml ddH₂O. Each standard and unknown sample solutions were dispensed into separate clean disposable cuvettes. 1xdye reagent (Bio-rad laboratories Inc.) were added into each cuvette, vortexed and incubated at room temperature for 5 mins.

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The 1xdye (Bio-Rad Lab Inc.) was added and mixed with each of the protein concentrations and loaded onto appropriate cuvettes. The spectrophotometer (Ultraspec 3000, Pharmacia Biotech) was zeroed with a blank sample and the color absorbance measured at 595nm. A standard curve was obtained by plotting the 595nm absorbance values against their protein concentrations. The unknown sample concentration was determined from extrapolating its absorbance value using the standard curve, and multiplying by the dilution factor used.

3.20 Measurement on the concentration of modifying agent adsorped on the surfaces of the nano-particles

Varying amount of modifying agent was added to the nano-particles and allowed for adsorption. The surface modified nano-particles were suspended in ddH₂O. The protein standard solutions described in 3.19 were prepared. Each unknown and standard solutions were transferred to clean cuvettes and 1x dye was added. The amount of modifying agent adsorped on the surface of the nano-particles was determined by extrapolating its 595nm absorbance value from the standard curve.

3.21 *In vivo* gene delivery

In vivo delivery was performed by administering the nano-particles-DNA complexes in 1 ml of 10mM Tris, pH7 either intravenously through tail-vein or intraperitoneally to Balb/c mice. Mice were euthanized with carbon dioxide after 24 hrs, in accordance to NACLAR's guidelines [95]. Six vital organs including spleen, kidney, liver, lung, heart and brain were dissected from the dead animals. 1ml of 0.25M Tris, pH7.8 containing protease inhibitor (Sigma-Aldrich) was added to each of the dissected organ.

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Each organ was homogenized for 30s with the Tissue Tearor at maximal speed. An additional 1 ml of 0.25M Tris, pH7.8 containing protease inhibitor was added to each tissue homogenate and vortexed before subjecting them through 3 cycles of freeze-thaw. The lysed tissue homogenates were then centrifuged at 13,000rpm for 10min in 4 deg Celsius. The supernatants were extracted for Luciferase and Bradford assays, to quantify the luciferase activities and protein concentrations using a dilution factor of 10.

CHAPTER

4

DESIGN OF NANO-PARTICLES SYSTEM FOR GENE DELIVERY

4.1 Selection of materials

Ceramic materials specially developed for use as medical and dental implants were termed as bio-ceramics. Bio-ceramics materials were polycrystalline, non-metallic compounds that can be classified as inert, resorbable or active [12]. This implied that they can either remain unchanged, dissolved or actively participated in physiological processes. Materials that were classified as bio-ceramics included alumina (Al_2O_3), zirconia (ZrO_2), calcium phosphates, silica based glasses or glass ceramics and pyrolytic carbons. These materials were known to be non-toxic, non-carcinogenic, possessed good biocompatibility and did not illicit immune response, thus forming the basis for *in vivo* characterization.

Bio-inert zirconia (ZrO_2), bio-active silica (SiO_2) and bio-active and resorbable Hydroxyapatite (HA) nano-particles were selected for investigation as potential carriers for cancer gene therapy.

Hydroxyapatite (HA)

HA was thermodynamically the most stable among the family of calcium phosphate ceramics at physiological pH. One of its most important properties was its excellent biocompatibility, where it actively participated in bone bonding to form strong chemical bonds with the surrounding bone.

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HA had also been reported to possess high affinity towards some amino acids which could render its usefulness for functional peptides to be tagged to its surface [66]. The ability of HA particle to reflect the charged environment of the adsorbed protein molecules [67] suggested the flexibility of modifying its surface charge using amino acids and polypeptides. Past investigation had also suggested that HA could be used as a controlled release carrier of growth factors [41]. At low pH, HA had greater solubility compared to other calcium phosphates, which rendered its suitability as a digestible material [66].

Multiple techniques had been used for the preparation of HA powders. The most commonly reported approaches for synthesizing nano-sized HA included precipitation, solid state reaction, sol-gel methods, hydrothermal route, emulsion and micro-emulsion techniques, mechanochemical reaction, combination of mechanochemical and hydrothermal, and ultrasonically assisted reaction [37 and 53]. The rate of addition, pH and sintering temperature played a role in influencing the stoichiometry of Ca/P ratio and property of HA. The morphology, crystallite size, purity, stoichiometry and structure of HA also determined its bioactivity, biocompatibility, chemical and physical stability as well as its mechanical property. These properties were found to have profound impact on its *in vitro* and *in vivo* performance [37 and 53].

For this study, the wet chemical or precipitation method of synthesizing was chosen, due to its relative ease and level of reproducibility. HA with calcium/phosphate (Ca/P) ratio of 1.67 was produced by reacting 0.28M Orthophosphoric acid with 0.5M calcium hydroxide under controlled precipitation conditions at 100 deg C to yield spheroidal HA precipitates, with water as the only by-product.

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Silica (SiO_2)

SiO_2 had been widely used as a surface modification agent for tailoring the surfaces and interfacial properties of powders [14 and 65].

SiO_2 was described to play an important role in the bio-mineralization of many organisms including coral and diatoms. A study embarked by Carlisle [68] indicated the role of SiO_2 in bone calcification. Dan Luo et al [55 and 56] had reported the effective use of dense SiO_2 nano-particles to concentrate liposomes-DNA vectors complex at the surface of cell monolayers, resulting in a 8-fold increase in transfection efficiency compared to commercially available transfection reagents such as Lipofectamine.

Zirconia (ZrO_2)

Zirconia was a polymorph that occurred in three distinct forms viz. monoclinic, cubic and tetragonal. Pure zirconia possessed the form of monoclinic at room temp which was stable up to 1170°C . Above this temp, it transformed into tetragonal and then cubic phase at 2370°C . The distinct advantages of ZrO_2 ceramics over other ceramic materials were its hardness and chemical inertness. Since it had minimal interaction with the host tissues, ZrO_2 was widely used in femoral ball head in total hip replacement (THR).

C. Piconi and G. Maccauro [72] had reviewed various authors' works on the viability of Zirconia as a bio-ceramics material. Zirconia possessed superior mechanical properties compared to other ceramics including Al_2O_3 . Both zirconia and alumina were found to have lower level of cytotoxicity compared to rutile (TiO_2). No local or systemic toxic effects were reported after the implantation of zirconia ceramics into muscles and bones of different animals, as well as after the injection of powder in mice.

4.2 Physical properties of nano-particles

HA nano-particles

The electron micrograph of HA nano-particles synthesized in-house through wet chemical reaction revealed these particles to be spheroidal in shape, with an approximate diameter of 50nm (Fig. 2).

Previous XRD and FITR analyses indicated that the HA formed were crystalline in phase [37]. These HA nano-particles displayed a near neutral surface charge of +0.12mV in pH7.0. They were observed to be stable at pH7.0, partially soluble at pH values of 5.9 to 6.0 and completely soluble below pH 5.5.

Fig. 2: Electron micrograph of HA nano-particles

SiO₂ nano-particles

SiO₂ nano-particles, produced by radio frequency process at plate power of 50kW, were observed to be dense and spherical with a diameter of 30nm (Fig. 3). XRD and FITR analysis indicated the particle to be amorphous [42]. Zeta potential revealed the nano-particles to have an extremely negative surface charge of -43.4mV at pH7.0.

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These nano-particles were stable at pH7.0 but were partially resorbable at pH 6.0 to pH 5.0.

Fig. 3: Electron micrograph of SiO₂ nano-particles

ZrO₂ nano-particles

Electron micrographs in Fig. 4 revealed ZrO₂ nano-particles to be micaceous, with an estimated size of 80nm. XRD analysis revealed very broad peaks pertaining to extremely small monoclinic-zirconia particles [46]. ZrO₂ nano-particles were observed to have a very positive surface charge of +64.1mV in pH7.0. Changes in pH values from 7.0 to 5.0 did not appear to have any significant changes to the solubility of the nano-particles.

Fig. 4: Electron micrograph of ZrO₂ nano-particles

4.3 Surface Modification of nano-particles

All three nano-particles were observed to have distinctly different surface charges. HA was neutrally charged, while SiO_2 was negatively charged and ZrO_2 was positively charged. To facilitate the formation of nano-particles-DNA complex, the surfaces of the respective nano-particles needed to be modified.

Amino acids including Lysine (Lys), Histidine (His), Arginine (Arg) and polycations including Poly-L-Lysine (PLL) and Protamine Sulfate (PS) were selected as prospective surface modifying agents. The main intent was to use these agents to surround the respective nano-particles with a mono-layer coating of positive charges through adsorption. This would enable the formation of DNA complexes through electrostatic attraction of the positively charged surface modified nano-particles and the negatively charged phosphate back-bone of the DNA, as illustrated in Fig. 5.

These basic amino acids and polycations were selected based on previous studies that had shown that HA treated with a basic protein or polypeptide would produce a positive zeta potential reading, whereas an acidic protein or polypeptide would produce a negative zeta potential value [67]. Q.R Chen et al [71] had further demonstrated the effective use of the lysine and histidine copolymer to significantly enhance the transfection efficiency of cationic liposomes *in vitro* and *in vivo*. The enhanced transfection efficiency had been attributed to the ability of lysine to partially neutralize the negative charge of plasmid DNA and the buffering capacity of histidine, thus aiding the release of DNA from pre-lysosomal vesicles.

Other studies had also indicated that polycations were required in DNA condensation to neutralize the electrostatic repulsion of its phosphate

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groups [27 and 30]. The addition of polycation to the transfection media were shown to enhance cationic lipid-mediated transfection [20 and 21]. PLL and PS had been proven to be the most effective thus far, with reported enhancement in transfection of over 30-fold compared with cationic liposome-mediated transfection in various cell lines [22].

Fig. 5: Formation of nano-particles-DNA complex

4.4 Surface charges of nano-particles

Fig. 6: a) Effect of modifying agents on surface charge modulation of HA nano-particles; b) Effect of modifying agents on surface charge modulation of HA with the addition of DNA

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Fig. 6 compared the effect of various surface modifying agents on the surface charges of the HA nano-particles with and without the addition of DNA. In Fig. 6a, it was observed that the use of protamine sulfate (PS) and poly-L-lysine (PLL) as modifying agents helped to raise the zeta-potentials of the neutrally-charged HA nano-particles to the positive range. On the contrary, the use of amino acids had further lowered the surface charges of the modified HA nano-particles to the negative range. With the addition of DNA, the surface charges of the unmodified HA nano-particles and those modified with amino acids (Lys, Arg and His) became more negative (Fig. 6b) compared to those in the absence of DNA (Fig. 6a). This suggested that electrostatic attraction between the DNA and these nano-particles had occurred, resulting in the possible formation of nano-particle-DNA complexes.

Complete charge shielding was observed in HA nano-particles modified with PS (PS-HA) in the presence of DNA, thus suggesting that the bound DNA had fully condensed. The surface charges of PLL modified HA nano-particles were observed to remain in the positive range, even with the addition of DNA. The incremental addition of the modifying agents to the HA nano-particles from 1% to 3% did not appear to have significantly affected the surface charges of the nano-particles with and without the addition of DNA.

Fig. 7 provided a comparison on the effectiveness of different modifying agents in modulating the surface charges of negatively charged SiO₂ nano-particles. Unmodified SiO₂ nano-particles and those modified with amino acids (Lys, Arg and His) were observed to have negative surface charges hovering between -30 mV to -50 mV (Fig. 7a). No significant changes were observed to the surface charges of these nano-particles in the presence of DNA, which remained in the range of -30 mV to -50 mV (Fig. 7b).

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This might imply that the unmodified SiO_2 nano-particles and those modified with amino acids did not result in DNA binding due to their negatively charged surfaces.

Fig. 7: a) Effect of modifying agents on surface charge modulation of SiO_2 nano-particles; b) Effect of modifying agents on surface charge modulation of SiO_2 with the addition of DNA

Both PS and PLL were able to modulate the surface charges of the SiO_2 nano-particles towards the positive range (Fig. 7a). Complete charge neutralization was observed when DNA was added to the PS modified SiO_2 nano-particles (Fig. 7b). This suggested the formation of nano-particle-DNA complexes in which the bound DNA was fully condensed. No significant changes were observed in the surface charges of PS modified nano-particles with and without the addition of DNA when the PS content was increased from 1% to 3%. However, the incremental increase of PLL from 1% to 3% resulted in a corresponding increase in the surface charges of the modified nano-particles (Fig. 7a).

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The surface charge of SiO₂ nano-particles modified with 1%PLL was seen to dip in the negative range with the addition of DNA.

Although the surface charge of 1%PLL-SiO₂ in the presence of DNA was negative, the zeta potential value was observed to be at least 10-20mV higher compared to the unmodified nano-particles and those modified with amino acids. This suggested that partial DNA binding may have taken place, due to insufficient positive charges on the surfaces. Further addition of PLL from 2% to 3%, resulted in raising the surface charges in the positive range, which remained at the same level of +20mV (Fig. 7b).

The surface charges of unmodified and surface modified ZrO₂ nano-particles in the absence and presence of DNA were presented in Fig. 8. The use of amino acids and polycations as modifying agents were observed to lower the intrinsically high positive surface charge of the unmodified ZrO₂ nano-particles. Nevertheless, the resultant surface charges remained in the positive range (Fig. 8a). Lys and Arg were the most effective in lowering the surface charges of modified nano-particles to +15mV to +20mV, followed by PS at +30mV. The surface charges of nano-particles modified by His and PLL hovered close to that of the unmodified nano-particles.

No significant changes were observed in the surface charges of the Lys and Arg modified nano-particles in the presence and absence of DNA (Fig. 8b). This could possibly suggest that DNA binding did not take place or the amount of DNA added were insufficient in lowering the net positive surface charges. The lowering of surface charges in the unmodified and His, PLL and PS modified nano-particles in the presence of DNA indicated that DNA binding was likely to have taken place (Fig. 8b).

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Fig. 8: a) Effect of modifying agents on surface charge modulation of ZrO₂ nano-particles; b) Effect of modifying agents on surface charge modulation of ZrO₂ with the addition of DNA.

4.5 Stability of nano-particles-DNA complexes

Gel electrophoresis assays were carried out to qualitatively study the ability of the unmodified and surface modified nano-particles to bind with DNA to form stable complexes. This study was established based on the concept of separating charged species by subjecting them to a voltage gradient. Electrophoresis was performed in a gel matrix so that molecules of similar electric charge can be separated on the basis of size. The agarose gel provided mechanical support and prevented the mixing of the molecules that were being separated. Ethidium Bromide was used as a marker to identify the presence of DNA under UV light.

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Efficient complexation of DNA by the unmodified and surface modified nano-particles would lead to the immobilization of DNA and their subsequent inability to migrate towards the anode under the applied electrophoresis voltage.

The stability of DNA complexes formed by unmodified and modified HA nano-particles and DNA was analyzed in the gel retardation assay shown in Fig. 9a. The pore size of 1% agarose gel permitted only the separation of molecules with size of 500-7000kb pairs. This was small enough to allow the migration of DNA towards the anodic end of the electrophoresis plate (well 3 in Fig. 9a), but not the larger sized HA nano-particles, which were retained in the well (well 2 in Fig. 9a).

From lane 4 to lane 13, the presence of DNA marked by the florescence, was observed to smear down the lanes. This denoted that large pieces of genomic DNA were partially degraded or cut into smaller fragments, indicating the release of DNA from the respective HA-DNA complexes. Gel electrophoresis conducted on 1/50 of their respective supernatants revealed traces of DNA band (Fig. 9b). Further quantification of the freed DNA in the supernatants revealed that these nano-particles were unable to bind completely with 3 μ g of DNA (Fig. 9c). These data indicated that the HA nano-particles and those modified with amino-acids (Lys, Arg and His) had limited capacity to bind with 3 μ g of DNA and were unable to form stable complexes with DNA.

No DNA smear or band was observed from lane 14 to 19 (Fig. 9a and b). This suggested the stability of the DNA complexes formed with HA nano-particles modified with PLL and PS. Additionally, they were able to bind completely with 3 μ g of DNA (Fig. 9c).

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Fig. 9: Stability of complexes formed by HA and DNA. a) Gel Retardation Assay of complexes formed between unmodified and surface modified HA with DNA ; b) Gel Retardation assay of 1/50 supernatants from buffer containing nano-particles and DNA ; c) Quantification of unbound DNA in the supernatants of the buffers containing nano-particles and DNA. Lane 1= 1kb DNA ladder; Lane 2= HA nano-particles; Lane 3= DNA; Lane 4 = unmodified HA+ DNA ; Lane 5 to 7 = 1-3%Lys-HA+ DNA , Lane 8 to 10 = 1-3%Arg-HA+DNA; Lane 11 to 13 = 1-3%His-HA+DNA; Lane 14 to 16 = 1-3%PLL-HA+DNA and Lane 17 to 19 = 1-3%PS-HA+DNA.

The stability of DNA complexes formed by unmodified and surface modified SiO_2 nano-particles was presented in Fig.10. In Fig.10a, SiO_2 nano-particles by themselves were observed to fluoresce when exposed to UV light (well 2).

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As the size of these nano-particles was comparatively larger than DNA, they were unable to migrate towards the anodic end of the plate and remained in the well, as were reflected by the fluorescence in all the wells containing the nano-particles (wells 2 and 4 to 19). In comparison, the fluorescence band away from well 3 indicated the migration of free DNA. Single discrete DNA bands were observed from lane 4 to lane 13. The lighter intensity in these bands compared to that in lane 3 indicated that the concentration of whole genomic DNA present was less than 3 μ g.

Gel electrophoresis on 1/50 of the respective supernatants revealed similar DNA bands from lane 4 to lane 13 (Fig. 10b). Quantification of excess DNA in the supernatants revealed that unmodified SiO₂ particles and those modified with amino acids (Lys, Arg and His) were unable to bind with 3 μ g of DNA (Fig. 10c). This correlated well with the detection of discrete DNA bands in the gel electrophoresis assays (Fig. 10a and b), which further affirmed that no DNA binding can take place with unmodified SiO₂ nano-particles and those modified with amino acids (Lys, Arg and His).

No DNA smear or band was observed for DNA complexes formed with SiO₂ modified with PLL and PS (Lanes 14 to 19 in Fig. 10a). The absence of DNA bands and the inability to detect unbound DNA in their respective supernatants (Lanes 14 to 19 in Fig. 10b and Fig. 10c) affirmed the formation of stable DNA complexes.

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Fig.10: Stability of complexes formed by SiO₂ and DNA. a) Gel Retardation Assay of complexes formed by unmodified and surface modified SiO₂ and DNA; b) Gel Retardation assay of 1/50 supernatants from the buffers containing the nano-particles and DNA; c) Quantification of unbound DNA in the supernatants of the buffers containing nano-particles and DNA. Lane 1= 1kb DNA ladder; Lane 2= SiO₂ nano-particles; Lane 3= DNA; Lane 4 = unmodified SiO₂ +DNA; Lane 5 to 7 = 1-3%Lys-SiO₂+DNA, Lane 8 to 10 = 1-3%Arg-SiO₂ + DNA; Lane 11 to 13 = 1-3%His-SiO₂ + DNA; Lane 14 to 16 = 1-3%PLL-SiO₂+DNA; and Lane 17 to 19 = 1-3%PS-SiO₂+DNA.

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Fig. 11: Stability of complexes formed by unmodified and modified ZrO₂ nano-particles with DNA. a) Gel Retardation Assay of complexes formed by unmodified and surface modified ZrO₂ and DNA; b) Gel Retardation assay of 1/50 supernatants from the buffers containing nano-particles and DNA; c) Quantification of unbound DNA in the supernatants of buffers containing nano-particles and DNA. Lane 1= 1kb DNA ladder; Lane 2= ZrO₂ nano-particles; Lane 3= DNA; Lane 4 = unmodified ZrO₂+DNA; Lane 5 to 7 = 1-3%Lys-ZrO₂+DNA, Lane 8 to 10 = 1-3%Arg-ZrO₂+DNA; Lane 11 to 13 = 1-3%His-ZrO₂+DNA; Lane 14 to 16 = 1-3%PLL-ZrO₂+DNA and Lane 17 to 19 = 1-3%PS-ZrO₂+DNA.

Gel retardation assays in Fig. 11 provided an analysis on the stability of DNA complexes formed with unmodified and surface modified ZrO₂ nano-particles. Well 2 in Fig. 11a showed that ZrO₂ nano-particles by themselves were able to fluoresce without the presence of DNA.

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No DNA smear or bands were observed in the lanes containing the complexes formed by the unmodified and surface modified ZrO₂ nanoparticles with DNA (lanes 4 to 19 in Fig. 11a). Similarly, gel electrophoresis of their respective supernatants showed no traces of DNA bands (Fig. 11b) and unbound DNA was not detected in their respective supernatants (Fig. 11c). Thus, these clearly suggested that unmodified ZrO₂ and those modified with different agents were able to bind well with 3µg of DNA to form stable complexes.

4.6 Discussion

Three different types of bio-ceramics materials with differing shape, size and surface charge were investigated as potential candidates for gene therapy. The physical properties of these nano-particles, including their morphology, size, charge density and colloidal stability were considered as important parameters that will determine their success for use *in vitro* and *in vivo* [55, 56 and 76]. The size in particular, was known as one of the most crucial factor affecting gene expression efficiency. It was known to determine the extent of endocytic uptake of the nano-particle-DNA complex into the cell cytosol [20, 24, 39 and 76]. The sizes of the three selected nano-particles were below 150nm, with the smallest being SiO₂ at 30nm, followed by HA at 50nm and ZrO₂ at 80nm. These met the size requirement for endocytosis to take place in many types of the mammalian cells [24]. In addition, tissues of the reticulo-endothelial system such as the liver, spleen and bone marrow, which possessed a sinusoidal endothelial structure, would permit free passage of materials with size of 100nm and less [97].

The spherical and near spheroidal shapes of SiO₂ and HA nano-particles would ensure homogeneity with the application of surface modifying agents and provided a greater surface areas for DNA binding.

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The compactness of their shapes was also likely to promote endocytic uptake and enable ease of systemic administration via narrow catheters or syringes to the targeted site. The thin plate-like shape of ZrO_2 nano-particles tended to foster agglomeration. This, coupled with the shape structure, may result in non-uniformity of the surfaces when modifying agents were applied. Due to the sparseness of the structure, it may be difficult to control the binding of DNA. Systemic administration via narrow catheters or syringes may result in blockages or shearing of the ZrO_2 -DNA complex.

At lower pH, HA nano-particles were readily soluble while SiO_2 was partially resorbable. In contrast, ZrO_2 nano-particles were inert to changes in pH values. The bio-resorbability of the nano-particles may have implications during the intracellular trafficking of its DNA load. Differences in pH existed between the cell cytosol and the endosomal/lysosomal compartments [84 and 85]. The partial dissolution of the HA and SiO_2 nano-particles in the endosomal/lysosomal compartments may have greater ability to release the DNA cargo into the cytoplasm for transcription in the nucleus, compared with the ZrO_2 nano-particles.

The surface charge of the DNA delivery systems was known to be one of the major factors influencing the bio-distribution and transfection efficiency of non-viral vectors. At pH7.0, SiO_2 nano-particles were negatively charged at -43.4mV, ZrO_2 were highly positively charged at +64.1mV while HA were neutrally charged. Electrostatic attraction between the DNA and the nano-particles was the initial step leading to complex formation. This could only take place when the surfaces of the nano-particles were cationically charged. Hence, various chemical agents including amino acids (Lys, Arg and His) and polycations (PLL and PS) were studied for their effectiveness in modulating the surface charges of the nano-particles towards the positive range.

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The NH_2 -groups in the amino acids (Lys, Arg and His) were found to be ineffective in raising the surface charges of the neutrally charged HA and negatively charged SiO_2 nano-particles towards the positive range. The resultant negative surface charges with the addition of DNA suggested that DNA may be partially or incompletely bound to the nano-particles. However, PLL and PS were effective in modulating the surface charges of HA and SiO_2 into the positive range, a condition essential for DNA complex formation to take place.

Complete charge shielding was observed in the DNA complexes formed with HA and SiO_2 nano-particles modified with PS. The electrostatic neutralization of the DNA anionic charges can be attributed to the condensation of the DNA's poly-nucleotide structure.

DNA complexes formed with PLL modified HA and SiO_2 nano-particles bore net positive charges. Similarly, the surface charges of DNA complexes formed with unmodified and surface modified ZrO_2 nano-particles were positive. This may be favorable as high transfection efficiency were often correlated with complex bearing a net positive charge in most non-viral vectors reported [20, 22 and 76]. An excess of cationic charges could also aid colloidal stability by ensuring complete coating of the DNA to the cationically charged nano-particles resulting in the electrostatic repulsion between the particles. However, an overly excessive positive surface charge may cause the DNA complexes to interact indiscriminately with the various biological components in the body resulting in the premature dissociation of the vector [77].

Another important consideration for ensuring an efficient gene transfection resided in the stability of the nano-particle-DNA complex system.

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Upon entry into the cytosol, the nano-particle carrier would need to provide a protective environment for its DNA cargo from degradation by the nucleases, before releasing them into the nucleus. The stability of the complexes was found to be dependent on the strength of the electrostatic attraction and thus on the total charge and charge density of the carrier [98]. Similarly, the formation of stable DNA complexes with the various surface modified nano-particles was observed to have close correlations with their surface charges.

Highly negatively charged SiO_2 nano-particles and those modified with amino-acids (-30 to -50mV) did not bind with DNA to form complexes. Unmodified and amino-acid modified (Lys, Arg and His) HA nano-particles were moderately negatively charged (0 to -20mV) and bound weakly to DNA forming unstable complexes. Positively charged HA and SiO_2 nano-particles modified with PS and PLL were able to bind strongly with the negatively charged phosphate backbone of DNA to form stable DNA complexes.

Unmodified and surface modified ZrO_2 nano-particles were highly positively charged. The absence of DNA migration in the gel retardation assay, coupled with no unbound DNA detected in their supernatants, strongly suggested that stable DNA complexes have been formed. However, the sparseness of the nano-particle structure may possibly result in the non-uniform coating of DNA on the surface. Possible agglomeration may also cause DNA to be entrapped between the plate-like structures. Its non-resorbable property may lead to possible toxicity when large amount of the particles were administered *in vivo*. However, its potential as a gene delivery carrier cannot be ruled out at this stage and warranted further investigation.

Nano-Vesicles for Gene Delivery

HA and SiO₂ nano-particles modified with PS and PLL were deemed as potential carriers in gene therapy applications for their ability to form stable DNA complexes. The compactness of their size and shape encouraged endocytic uptake, provided larger DNA load bearing capacity and eased systemic administration. The positive to near neutral surface charges of their respective DNA complexes would enhanced the binding of the complexes to the negatively charged cell membrane. PS and PLL would also be able to provide a protective environment for the DNA from nuclease degradation [20].

The viability of the three nano-particles modified with the various surface agents will be further investigated *in vitro* to ascertain their suitability as carriers for gene therapy applications.

CHAPTER

5

***IN VITRO* TRANSFECTION**

The *in vitro* transfection efficacy of the DNA complexes formed with unmodified and surface modified HA, SiO₂ and ZrO₂ nano-particles systems were studied across three cancer cell lines. Investigated cell lines included CNE2 (human nasopharyngeal carcinoma), HepG2 (human liver cancer) and B16 (mouse skin melanoma). These were selected for their differing cell biology, growth kinetics and degree of transfectability.

pNGVL-luciferase encoding the firefly luciferase, was utilized as a reporter gene. pNGVL-Luciferase, when expressed in mammalian cells, will produce a molecule that can be detected. The luciferase enzyme hydrolyzed its substrate, D-luciferin, in the presence of Adenosine 5' triphosphate (ATP) and oxygen to produce photons of the emission spectra ranging from 400 to 620 nm. Luciferase in the cellular extracts can therefore be quantified through an enzymatic assay using a luminometer [18]. The luciferase activity was measured in relative light units (RLU) and normalized per ug of protein from the cellular extracts.

To form the basis for comparison, the amount of modifying agents added to each nano-particles system was kept at 2%, with a consistent DNA load of 3µg. To gauge the transfection efficacy of the various nano-particles system, the CalphosTM technique [17] was used as a positive control, while DNA alone without the presence of a carrier was used a negative control.

Nano-Vesicles for Gene Delivery

CalphosTM was chosen as a reference as it was the only inorganic particulate system with a well established transfection protocol, and was used universally as a routine laboratory practice for *in vitro* transfection. All the experiments were conducted in triplicates and repeated twice to ascertain the reproducibility.

5.1 *In vitro* transfection mediated by the unmodified and surface modified HA nano-particles

Fig. 12: Effect of modifying agents on the surface charge modulation and *in vitro* transfection efficacy of HA nano-particles. a) Surface charge modulation of HA nano-particles with and without DNA; b) Quantification of unbound DNA in the buffers containing the HA nano-particles and DNA ; c) *In vitro* transfection activities mediated by the unmodified and surface-modified HA nano-particles.

Fig. 12 investigated the effect of modifying agents on the surface charge modulation of HA nano-particles and their impact on the *in vitro* transfection efficacy.

Nano-Vesicles for Gene Delivery

In Fig. 12a, it was shown that unmodified HA nano-particles and those modified with amino acids (Lys, Arg, His) were negatively charged with the addition of DNA. Fig. 12b revealed that the unmodified nano-particles and those modified with amino acids had limited capacity in bearing the complete 3 μ g DNA load (Fig. 12b). However, HA nano-particles modified with PLL and PS (Fig. 12b) were found to bind completely with 3 μ g of DNA.

Fig. 12c showed that both unmodified and modified HA nano-particles were able to transfect HepG2 cells, and their efficacies significantly surpassed that of CalphosTM. The highest level of transfection activity in all three cell lines was observed to be mediated by the PS-HA-DNA complex. The transfection efficacy of PS-HA-DNA complex in HepG2 was 33 fold more than CalphosTM and at least 10 times more than the unmodified and other surface modified HA-DNA complex systems. PS-HA-DNA complex also displayed twice the transfection efficacy in CNE2 and B16 cells compared to CalphosTM. In spite of the ability of PLL-HA in carrying 3 μ g of DNA, it failed to deliver similar level of transfection activity as that of PS-HA-DNA complex. It was also observed that unmodified HA and those modified with amino acids were unable to mediate significant transfection activities in CNE2 and B16 cells.

5.2 *In vitro* transfection mediated by the unmodified and surface modified SiO₂ nano-particles

The effect of modifying agents on the surface charge modulation and *in vitro* transfection efficacies of SiO₂ nano-particles were studied in Fig. 13. Fig. 13a revealed that only PLL and PS modified SiO₂ nano-particles were able to form positively charged complexes with DNA.

Nano-Vesicles for Gene Delivery

These were found to bind completely with 3 μ g of DNA (Fig. 13b). In contrast, negatively charged unmodified SiO₂ nano-particles and those modified with amino acids (Lys, Arg and His) were unable to bind with DNA (Fig. 13a and 13b). This corresponded with their lack of transfection activity across the three studied cell lines (Fig. 13c).

Fig. 13: Effect of modifying agents on the surface charge modulation and *in vitro* transfection efficacies of SiO₂ nano-particles. a) Surface charge modulation of SiO₂ nano-particles with and without DNA ; b) Quantification of unbound DNA in the buffers containing the SiO₂ nano-particles and DNA ; c) *In vitro* transfection activities mediated by the unmodified and surface modified SiO₂ nano-particles.

Although PLL modified SiO₂ (PLL-SiO₂) was seen to bind with the same amount of DNA as that of PS modified SiO₂ (PS-SiO₂), its transfection activity in all three cell lines were significantly lower compared to the latter.

Nano-Vesicles for Gene Delivery

Transfection mediated by the PS-SiO₂-DNA complex was 35 folds greater in HepG2, four times higher in CNE2 and ten time more in B16 compared to those mediated by PLL-SiO₂-DNA complex. PS-SiO₂-DNA complex also demonstrated superior transfection activity compared to Calphos™, achieving transfection activities that were 30 times more in HepG2 cells and four folds greater in B16 cells.

5.3 *In vitro* transfection mediated by the unmodified and surface modified ZrO₂ nano-particles

Fig. 14: Effect of modifying agents on the surface charge modulation and *in vitro* transfection efficacies of ZrO₂ nano-particles. a) Surface charge modulation of ZrO₂ nano-particles with and without DNA; b) Quantification of unbound DNA in the buffer containing the ZrO₂ nano-particles and DNA; c) *In vitro* transfection activities mediated by the unmodified and surface modified ZrO₂ nano-particles.

Nano-Vesicles for Gene Delivery

Both unmodified and surface modified ZrO_2 nano-particles were positively charged and bound completely to $3\mu\text{g}$ of DNA (Fig. 14a and 14b). However, Fig. 14c showed that only the DNA complex formed with the PS modified ZrO_2 (PS- ZrO_2) nano-particles, was able to mediate transfection activity in the three investigated cell lines. No significant transfection activity was observed in all three cell lines by unmodified ZrO_2 and those modified by the other surface modifying agents. PS- ZrO_2 -DNA complex was able to mediate 1.4 times more transfection activity in B16 cells compared to CalphosTM, but was observed to be ten times less efficient in HepG2 and CNE2 cells.

5.4 *In vitro* transfection efficacies of the PS-HA, PS-SiO₂ and PS- ZrO_2 DNA complex systems

Fig. 15: *In vitro* transfection efficacies of PS-HA, PS-SiO₂ and PS- ZrO_2 DNA complex systems

Fig. 15 compared the *in vitro* transfection efficacies of PS-HA, PS-SiO₂ and PS- ZrO_2 DNA complex systems across three different cell lines.

Nano-Vesicles for Gene Delivery

Compared with CalphosTM and PS-ZrO₂, DNA complexes formed with PS-HA and PS-SiO₂ displayed superior transfection efficacies in HepG2, CNE2 and B16 cells. PS-HA and PS-SiO₂ DNA complexes expressed significantly higher level of transfection activity in HepG2, which were at least 30 folds higher than that exhibited by CalphosTM. PS-HA and PS-SiO₂ were observed to display comparatively similar levels of transfection activities in the three studied cell lines. PS-ZrO₂ displayed poorer level of transfection activities, and was also seen to be less efficient than CalphosTM.

5.5 Effect of 50% serum on *in vitro* transfection efficacies of the PS-HA, PS-SiO₂ and PS-ZrO₂ DNA complex systems

Fig.16: Effect of 50% serum on the *in vitro* transfection efficacies of PS-HA, PS-SiO₂ and PS-ZrO₂ DNA complexes

In vitro transfection of the PS-HA, PS-SiO₂ and PS-ZrO₂ DNA complexes were carried out in the presence of 50% serum. The presence of 50% serum appears to reduce the level of gene expression mediated by the nano-particles (Fig. 16) compared to those mediated in the absence of serum (Fig. 15).

Nano-Vesicles for Gene Delivery

However, the DNA complex of PS-SiO₂ was still able to display a significantly higher level of transfection in all three cell lines compared to PS-HA and PS-ZrO₂. This suggested greater stability of the PS-SiO₂-DNA complex in the presence of serum. The positive control, CalphosTM, was seen to perform poorly in all three cell lines in the presence of 50% serum.

5.6 Discussion

For gene expression to take place, several prerequisites must be fulfilled by the nano-particles-DNA complexes. The DNA complex must penetrate through the cell membrane into the cytosol. As the sizes of the nano-particles fell below 100nm, the intracellular uptake of their respective DNA complexes was likely to take place through endocytosis via coated pits through a non-specific clathrin-dependent process [84]. Following intracellular uptake, the nano-particles must protect its DNA load from nuclease degradation in the endosomal-lysosomal pathway, before releasing the DNA cargo into the cell nucleus for the translation and transcription of proteins. The viability of the unmodified and modified nano-particles systems for gene delivery was thus determined based on their ability to protect and transport genetic material into the cell, followed by the subsequent transgene expression in the cell nucleus.

DNA alone, without the presence of nano-particles, was unable to mediate transfection activity in any of the three investigated cell lines. This was attributed to the extended structure of hydrated DNA and its negative intrinsic charge which made it difficult to move across the nuclear membrane pore into the nucleus where transgene expression could take place. Additionally, unprotected DNA after endocytosis was rapidly degraded by the nucleases in the endosomal/lysosomal compartments.

Nano-Vesicles for Gene Delivery

Protamine Sulfate (PS) was seen to play a significant role in enhancing the transfection efficacies of the three nano-particles systems, particularly for HA and SiO₂. Due to the presence of its 21 arginine residues, PS was able to effectively modulate the surface charges of the neutrally charged HA and negatively charged SiO₂ nano-particles towards the positive range. This allowed strong electrostatic binding to take place between the cationically charged nano-particles with the negatively charged phosphate backbone of DNA. Charge neutralization of the subsequent DNA complexes indicated that the bound DNA had been effectively condensed. Previous reports on cationic liposomes and PEI-DNA complexes had also indicated their *in vitro* efficiencies when their surface charges were near to neutral [87].

It had been widely established that DNA had to be condensed into a toroidal-like structure in order for it to enter the cell cytosol [25, 26, 27, 28, 76 and 77]. Condensation of DNA resulted in the collapse of a single, long DNA chain which involved a dramatic decrease in the volume occupied by the DNA molecule [30]. This compact state of DNA would protect it from enzymatic degradation, thus allowing it to pass more easily through small openings. It has been noted that the smaller size in the condensed DNA particles would aid its *in vivo* diffusion towards target cells and the subsequent trafficking within the cell [20]. The role of polycations in DNA condensation was also documented in previous findings [20, 21, 22, 23, 27, 30, 31 and 35].

The superior transfection capability of the DNA complexes of PS-HA and PS-SiO₂ was also attributed to the nuclear localizing property of protamine. The role of protamine, a naturally occurring substance found in the sperm of salmon, was to bind and deliver DNA to the nucleus of the egg after fertilization [20].

Nano-Vesicles for Gene Delivery

This unique property of PS was also utilized by various investigators in enhancing the transfection efficacy of their gene therapy systems. Nevertheless, it was discovered that PS in the absence of a carrier was not effective in mediating high level of transfection [30, 20, 39 and 69]. However, transfection efficiency was found to be greatly enhanced when PS was added to cationic liposomes including DOTAP, DC-Chol, TC-Chol and DDAB [39]. PS enhanced liposome was also seen to provide comparable transfection enhancement as μ (Mu), which was derived from the condensed core of adenovirus [38].

PLL was seen to be effective in modulating the surface charges of HA and SiO₂ nano-particles into the positive range, resulting in complete binding of the nano-particles with DNA to form complexes. Earlier analysis on gel retardation assays (Chapter four) had shown that HA and SiO₂ nano-particles modified with PLL were able to form stable complexes with DNA. Although PLL was noted for its ability to condense DNA [20], the transfection efficacies mediated by PLL-modified HA, SiO₂ and ZrO₂ nano-particles were not significantly enhanced.

A possible explanation for the lower transfection activity was the absence of amino groups, which prevented the escape of the complexes from the endosome/lysosome compartments [24]. A previous study embarked by FL Sorgi, S Bhattacharya & L. Huang had also reported that PS displayed more superior ability than PLL in enhancing the *in vitro* transfection efficacy of DC-Chol, CLONfectin, lipofectin, DOTAP/DOPE liposomes and lipofectamine on HEK293 and CHO cells [20]. Thus, PLL could be ruled out as an effective modifying agent to enhance the *in vitro* transfection of the nano-particles. Moreover, it was not included in the list of 'generally regarded as safe (GRAS) compound and contained a wide range of molecular weights which were difficult to characterize.

Nano-Vesicles for Gene Delivery

The use of amino acids (Lys, Arg and His) as modifying agents failed to significantly enhance the transfection efficiency of HA and SiO₂ nano-particles. Lys, Arg and His were unable to modulate the surface charges of the nano-particles into the positive region. Although neutrally charged HA and moderately negatively charged amino-acids modified HA (Lys-HA, Arg-HA and His-HA) nano-particles displayed limited binding capacity to DNA, it was able to mediate transfection in HepG2 at three times the level of CalphosTM.

However, when these were compared to PS-HA, the level of transfection mediated by HA and amino acids modified HA nano-particles were 10 times more inferior. Moreover, the latter were unable to mediate transfections in CNE2 and B16 cells. The poorer level of transfections could also be attributed to the inability of unmodified and amino acids modified HA nano-particles in condensing DNA.

Negatively charged SiO₂ and amino acids modified SiO₂ (Lys, Arg and His) nano-particles did not bind with DNA to form complexes. Hence, they were unable to mediate transfection in any of the cell lines. These findings correlated well to the earlier conclusions in chapter four on surface charges and gel retardation assays.

Unmodified and modified ZrO₂ nano-particles were highly positively charged and bound well with DNA. Gel retardation assays had suggested that the DNA complexes formed were stable. With the exception of PS-ZrO₂-DNA complex, unmodified ZrO₂ and those coated with other surface modifying agents were unable to mediate transfection activity in any of the three investigated cell lines. It was highly possible that the unmodified and amino-acids (Lys, Arg and His) modified ZrO₂ nano-particles were unable to condense DNA, resulting in the degradation of the DNA by the nucleases in the endosomal/lysosomal compartments.

Nano-Vesicles for Gene Delivery

It was likely that the presence of PS coated on the ZrO_2 nanoparticles could condense DNA and thus offer additional protection to its DNA load in the endosomal/lysosomal pathways, leading to the subsequent gene expression in the nucleus.

A comparison was made on the *in vitro* transfection efficacies mediated by the DNA complexes formed by the three nano-particles modified by PS (PS-HA, PS- SiO_2 and PS- ZrO_2). DNA complexes formed by PS-HA and PS- SiO_2 displayed high level of transfection efficacies in the three investigated cell lines and were superior to that of CalphosTM and PS- ZrO_2 , even in the presence of 50% serum.

Compared to PS-HA DNA complexes and PS- SiO_2 DNA complexes, PS- ZrO_2 DNA complexes performed poorly *in vitro*. It was speculated that the insensitivity of ZrO_2 to the lower pH conditions in the endosomal/lysosomal compartments may have inhibited the release of DNA for gene expression to take place in the nucleus. This led to subsequent entrapment of the ZrO_2 -DNA complexes in the endosomal/lysosomal compartments resulting in the eventual degradation of the DNA. Agglomeration caused by the plate-like structure of the ZrO_2 nano-particles could also resulted in the formation of aggregates larger than 200nm, thus inhibiting intracellular uptake into the cytosol.

Compared to CNE2 and B16, PS-HA and PS- SiO_2 were seen to mediate significantly higher level of gene expression in HepG2. S. Li and M. Zhang [87] have drawn a correlation between the level of gene expression and the ability of the cells in taking up DNA. Cell lines that took up more DNA expressed higher level of gene expression compared to those that took up less. The authors have further concluded the importance of the role of Proteoglycans (PGs) on the cell membrane in the transfectability of the cells.

Nano-Vesicles for Gene Delivery

PGs were seen to play a functional role in the binding and entry of many viruses into cells [89]. Cells that were deficient in PGs were deemed to be more difficult to transfect. These were substantiated by previous works investigated on the role of PGs on cationic-liposomes mediated transfections [88 and 89]. Hence, the higher transgene expression in HepG2 cells may be attributed to the high density of asialoglycoprotein receptors (ASGP-R) located on the cell surface [48]. Proteolytic fragment of glypican-3, a member of the glypican family of glycosylphosphatidylinositol-anchored proteoglycans have also been detected on the surfaces of HepG2 cells [86].

The ability of different cell lines in expressing exogenous DNA may also be related to the stability of the DNA in the nucleus. Normal human fibroblasts, which were difficult to transfect, rapidly eliminated exogenous DNA from the nucleus. HeLa cells, which were easily transfected, were able to retain plasmid DNA in the nucleus for a very long time [78].

It had been reported that size of the particle was largely responsible for its margination dynamics. Particulates with size of about 100nm displayed the greatest tendency to remain distal to the endothelium. Sizes that were smaller or larger than this critical radius was more likely to marginate and deliver therapeutic action to the endothelial or parenchymal regions [23, 63 and 64]. M.A. Arangoa et al [23] had noted that the rate and extent of liposomal uptake by the liver were highly variable and dependent on the size, charge, composition, rigidity and other physicochemical properties of the particles. The proportion of administered macromolecules internalized by hepatocytes was observed to depend on their particle size and biochemical characteristics. It was possible that PS-HA and PS-SiO₂ complexes would have met the size requirement in passing the entrance of sinusoidal endothelial cells in the liver [23] and hence their superior transfection efficacy compared to CalphosTM.

Nano-Vesicles for Gene Delivery

These *in vitro* transfection results suggested the suitability of PS-HA and PS-SiO₂ DNA complexes for use in gene therapy. Their abilities to achieve *in vitro* transfection in three different cell lines aligned well with the conclusions made from the gel retardation assays and surface charges in Chapter 4. Compared to CalphosTM, PS-HA and PS-SiO₂ were more efficient in mediating gene expression. The use of PS as a modifying agent was able to effectively condense DNA and protect it from degradation by intracellular nucleases. Moreover, PS had been classified as a USP compound, approved by FDA for clinical use in counteracting the effects of heparin-induced anticoagulation after cardiac and vascular surgery [40]. Additionally, substantial transfection activity mediated by PS-HA and PS-SiO₂ in 50% serum suggested their stability in the presence of body serum and thus their viability for *in vivo* applications.

CHAPTER

6

***IN VIVO* GENE DELIVERY**

It had been shown earlier that the DNA complexes of PS-HA and PS-SiO₂ were able to mediate significant level of transfection activities in three different cell lines. Majority of the existing non-viral systems had shown great promises *in vitro*, but their use *in vivo* was limited. An ideal gene therapy system should be capable of achieving efficient and tissue-specific gene expression when directly administered *in vivo*. Hence, it would be pertinent to investigate the transfection efficacies of these nano-particles-DNA complex systems *in vivo*, in ascertaining their usefulness for gene therapy applications. This study will establish various important parameters of the nano-particle-DNA complex systems, including its bio-distribution, stability in the presence of body serum and its susceptibility to clearance from the blood circulation. All of these cannot be effectively modeled in a cell culture system.

The PS-SiO₂-DNA complex was selected as the proto-type system for *in vivo* gene delivery. It was selected based on its ability to mediate higher level of transgene expression *in vitro* in the presence of 50% serum. The PS-SiO₂-DNA complex system was optimized by varying the percentages of PS and DNA concentrations on 0.5mg of SiO₂ nano-particles. *p*NGVL-Luciferase was used as a reporter gene. *In vivo* studies were then conducted on female Balb/c mice, 6-8 weeks old, using the most optimal conditions.

Nano-Vesicles for Gene Delivery

Each group comprised 3 mice, and six vital organs viz. the spleen, kidney, lung, heart, liver and brain were assayed to analyze the bio-distribution of the transgene expressions.

6.1 Optimization of the PS-SiO₂ DNA complexes

Fig. 17: Quantification of PS concentration adsorbed on the surface of SiO₂ nano-particles

In Fig. 17, the concentration of PS coated on the surfaces of SiO₂ nano-particles was observed to increase from 0% to 2%PS, reaching a maximum adsorption level of 19ug/ml from 2%PS onwards. This suggested that the addition of PS at 2% onwards will be sufficient to adequately cover the surfaces of the SiO₂ nano-particles. This adsorption curve resembled a Langmuir curve that was also reported by T. Matsumoto et al [41] in the measurement of protein adsorption on HA particles as carriers for protein release.

Nano-Vesicles for Gene Delivery

The effect of increasing the amount of PS coated on the SiO₂ nano-particles to enhance the stability of DNA complexes, and its subsequent binding capacity to DNA were established in Fig. 18 and 19. 10µg of DNA was used to form each of the complexes.

Fig. 18: Gel retardation assays of the PS-SiO₂ DNA complexes. a) PS-SiO₂ DNA complexes in 0% serum; b) PS-SiO₂-DNA complexes in 20% serum; and c) PS-SiO₂ DNA complexes in 50% serum. Lane 1= DNA ladder; lane 2 = SiO₂ nano-particles; Lane 3= DNA alone; Lanes 4 to 12 = 0-8%PS-SiO₂-DNA complexes

Nano-Vesicles for Gene Delivery

Fig. 19: Effect of increasing PS on the binding capacity of SiO₂ nano-particles to DNA

In the absence of serum, gel assay in fig. 18a showed that the use of 2% PS as coating was sufficient to form stable complexes with 0.5mg SiO₂ nano-particles and 10μg of DNA. Smears of fluorescence bands in the presence of 20% and 50% serum may indicate the possibility of some dissociation of DNA from the complexes (fig. 18b and 18c). However, further quantification of unbound DNA in the buffer containing the complexes revealed that 10μg of DNA were completely bound to the surfaces of the nano-particles in the absence and presence of 20% and 50% serum when it was coated with 2% PS and more (fig.19). The fluorescence smears detected in the respective gel assays may be possibly contributed by the serum component rather than the presence of released DNA.

Nano-Vesicles for Gene Delivery

Fig. 20: Effect of increasing PS on the level of transgene expression mediated by the SiO₂-DNA complexes in HepG2 cells, in the presence of 50% serum

The optimal percentage of PS coating on 0.5mg SiO₂ nano-particles was further evaluated *in vitro* using HepG2 cells in the presence of 50% serum (Fig.20). 10µg of DNA was used to form the DNA complexes. The level of transgene expression was observed to increase with increasing amount of PS coated on SiO₂ nano-particles, peaking at 5%PS. Further increase of PS beyond 5% did not yield further improvement to the gene expression level.

6.2 *In vitro* transfection mediated by the optimized PS-SiO₂ DNA complexes

The *in vitro* transfection efficacy of the optimized PS-SiO₂-DNA complexes was studied in Fig. 21. Compared to CalphosTM, optimized PS-SiO₂-DNA complexes were seen to mediate higher levels of transfection in HepG2 and 220.1 cell lines in the absence of serum.

Nano-Vesicles for Gene Delivery

However, PS-SiO₂-DNA complex were able to achieve stable level of transgene expression in four out of the five studied cell lines, in the presence of 50% serum. In contrast, CalphosTM gave only marginal level of transgene expression in all the five cell lines, in the presence of 50% serum

Fig. 21: Effect of serum on the transfection efficacies of PS-SiO₂ DNA complexes and CalphosTM across five different cell lines. a) 0% serum; b) 50% serum

Nano-Vesicles for Gene Delivery

6.3 Time course studies on the optimized PS-SiO₂-DNA complexes

Fig. 22: Time course study on the stability of the 5%PS-SiO₂ DNA complexes. a) Gel retardation assays of 5%PS-SiO₂ DNA complexes. Lane 1= DNA ladder; Lane 2= 10μg of DNA, Lanes 3-6= SiO₂ DNA complexes after 6hr, 24hr, 48hr, 72hr in 0% serum, Lanes 7-10 = SiO₂ DNA complexes after 6hr, 24hr, 48hr, 72hr in 20% serum and Lanes 11-14= SiO₂ DNA complexes after 6hr, 24hr, 48hr, 72hr in 50% serum. b) Quantification of bound DNA to 5% PS-SiO₂ nano-particles.

The stability of the optimized PS-SiO₂-DNA complexes in the absence and presence of serum was investigated over a time course spanning from 6hrs to 72hrs (Fig. 22a). Gel retardation assay unveiled that the optimized complexes were stable at all time points in the absence of serum. Some partial release of DNA was observed in the presence of 20% and 50% serum. Further quantification of unbound DNA from the buffers containing the optimized PS-SiO₂-DNA complexes revealed that all 10ug of DNA were bound to 5%PS-SiO₂ nano-particles.

Nano-Vesicles for Gene Delivery

These suggested that the optimized PS-SiO₂-DNA complexes were stable for up to 72h in the absence and presence of 20% and 50% serum.

6.4 *In vivo* gene delivery

Fig. 23: Effect of different administration routes on transgene expression *in vivo*. Organs were harvested after 24 hours and assayed for luciferase activity. Each group comprised of 3 female Balb/c mice.

The ability of the optimized PS-SiO₂-DNA complexes to mediate transgene expression *in vivo* was studied across six vital organs viz. the spleen, kidney, liver, lung, heart and brain (Fig.23). The intravenous (i.v) administration of the PS-SiO₂-DNA complexes via the tail-vein of mice was limited to 1mg of nano-particles carrying 20µg of DNA in 1ml of 10mM Tris buffer. This induced very low levels of gene expressions in all the six organs assayed.

A controlled experiment involving the intravenous injection of 20µg of naked DNA using the hydrodynamic-based technique established by F. Liu et al [60] had resulted in a significantly high level of luciferase activity detected in the liver (1,578,555 RLU per µg of protein).

Nano-Vesicles for Gene Delivery

Intravenous administration of 2mg of nano-particles resulted in the immediate death of mice. However, gene expressions in all six organs were significantly enhanced with the highest expression in the spleen, when 2mg of nano-particles loaded with 40 μ g of DNA in 1ml of 10mM Tris buffer were administered intraperitoneally (i.p.) to the mice. This implied that the i.p. administration of the nano-particles-DNA complexes may be a more suitable route compared to i.v.

In vivo optimization of the PS-SiO₂-DNA complexes was thus conducted via intraperitoneal administration to the mice. Compared to the groups of mice induced with SiO₂ nano-particles alone and DNA alone, i.p. administration of PS-SiO₂-DNA complexes was seen to induce significantly higher level of transgene expression specifically in the spleen (Fig. 24). Luciferase activity in the spleen was seen to increase with increasing amount of DNA complexes, reaching an optimum level at 4mg SiO₂-80 μ g DNA. Beyond 4mg, further increase of the PS-SiO₂-DNA complexes did not appear to improve the transgene expression in the spleen.

The administration of 8mg SiO₂-160 μ g DNA led to significantly higher levels of transgene expressions in the liver and kidney, in addition to the spleen. The level of gene expression in the liver was observed to be equivalent to the level of that expressed in the spleen.

Nano-Vesicles for Gene Delivery

Fig. 24: Optimization of the PS-SiO₂ DNA complexes *in vivo*. PS-SiO₂-DNA complexes were suspended in 1ml of 10mM Tris buffer and administered intraperitoneally (i.p.) to groups of three female Balb/c mice. Organs were harvested after 24 hours and assayed for luciferase activity.

The stability of transgene expression induced by 4mg PS-SiO₂-80µg DNA complexes administered intraperitoneally was further investigated in a time course study (Fig. 25). Six hours after the transduction of the SiO₂-DNA complexes, significant transfection activity was detected in the spleen, liver and the kidney, with the highest gene expression in the spleen. Gene expressions in the three organs peaked from 6 to 12 hours and declined by half after 24 hours. Some level of luciferase activity was still detectable in the spleen after 72 hours. However, transgene expression in the liver and kidney were seen to decline to basal level after 24 hours.

Nano-Vesicles for Gene Delivery

Fig. 25: Time course study of 4mg SiO₂-80µg DNA complexes *in vivo*.

Groups of 3 female balb/c mice per time point were subjected to compartmental administration of the complexes. Organs were harvested at various time points and assayed for luciferase activities.

6.5 Discussion

Optimization of the PS-SiO₂-DNA complex system was carried out by varying the percentages of PS and the amount of DNA. The determination on the right amount of PS used to modulate the surface chemistry of SiO₂ nano-particles may be critical in enhancing the transfection efficacy. Yoram B et al [35] had suggested the existence of a threshold, whereby further increase in the amount of polycation would decrease its ability to effectively condense DNA. The addition of 2%PS to SiO₂ was seen to be sufficient in providing these nano-particles with a maximum coating of 19µg/ml of PS. This PS concentration was below the clinical recommended dosage of 50mg per 10mins and would thus be safe for use in gene delivery work [40].

Nano-Vesicles for Gene Delivery

Gel retardation assay and the quantification of unbound DNA in the buffer containing the complexes had further reaffirmed that SiO₂ nano-particles modified with 2%PS was sufficient for the formation of stable complexes with 10µg DNA in the absence and presence of up to 50% serum.

However, gene expression in HepG2 cells in the presence of 50% serum was maximized when the SiO₂ nano-particles were modified with 5%PS. This percentage of PS coated on SiO₂ was deemed as the most optimal amount in mediating 10ug of DNA for *in vitro* transfection. No significant improvement in the transfection level was observed with further increase in the percentage of PS. This trend correlated well with other research findings which reported no significant change in transfection activities beyond an optimum amount of PS [20, 22 and 39]. Thus, 5%PS-0.5mg SiO₂-10µg DNA formed a convenient base unit for the incremental determination of an optimal dosage *in vivo*.

Complex stability in serum played an important role in enabling the effective delivery of therapeutic genes *in vivo*. The degradation of the therapeutic DNA by serum nucleases represented a major obstacle in the functional delivery to the target cell. The inhibitory effect of serum on the transfection efficiency of cationic liposomes-DNA complexes had been previously reported [26, 47, 87, 92 and 99]. The binding of the liposome-DNA complexes to negatively charged serum protein was attributed to their decrease in cell association and rapid clearances from the circulation. The optimized PS-SiO₂-DNA complex system was able to achieve high level of transgene expression in four out of the five different cancer cell lines viz. HepG2, HeLa, 220.1 and CNE2 in the presence of 50% serum. In contrast, CalphosTM performed poorly in all the investigated cell lines due to its instability in the presence of 50% serum.

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Further evidence of the stability of the PS-SiO₂-DNA complexes was substantiated in the time-course study in the absence and presence of 20% and 50% serum.

The stability of the PS-SiO₂-DNA complex in 50% serum over a period of 72 hours suggested that the PS-SiO₂ nano-particles will be stable when exposed to a plethora of secreted molecules, ions and peptides when in circulation, thus protecting its DNA cargo from degradation by serum nucleases.

The route of delivery had been known to aid in the specificity of the gene to the targeted cells [43 and 87]. The limitation on the amount of PS-SiO₂-DNA complexes to be administered intravenously to the mice suggested that this may not be a feasible route of delivery. The maximum dose of 1mg SiO₂ loaded with 20µg DNA was shown to be insufficient in enhancing significant transgene expression in all the assayed organs. Further dosage increase resulted in the death of rodents. It was probable that the complexes agglomerated in the syringe to form large aggregates, which eventually clogged up the tail veins of the mice. Conversely, the intraperitoneal administration of 2mg SiO₂ loaded with 40µg of DNA had resulted in enhanced gene expression in all the six organs, with the highest level of expression in the spleen.

With compartmental administration, it was possible to increase the dosage of PS-SiO₂-DNA complexes. Gene expression was significantly higher in the spleen and increased on a dose-dependent basis, reaching a maximum level at 4mg SiO₂-80µg DNA. Beyond this dosage, there was no further improvement to the level of gene expression in the spleen. However, an equivalent level of gene expression was detected in the liver when the dosage of the complex was increased to 8mg SiO₂-160µg DNA.

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Due to a lack of studies on the pathways of the particles introduced by compartmental administration, the factors involved in the bio-circulation of the nano-particles-DNA complexes have yet to be identified. Studies drawn from the intravenous administration of cationic lipids revealed that the physicochemical properties of particles played a major role on their bio-distribution and blood clearances.

Particle size and surface charge were thought to be the most critical factors in clearances [24, 103]. These factors governed the interaction of the complexes with serum proteins, endothelium and the reticuloendothelial system.

The blood filtering role of the spleen may be responsible for the high level of gene expression mediated by the PS-SiO₂-DNA complexes. The complexes which have shown stability in the presence of serum, were speculated to be carried by the arterial blood into the venous sinuses of the splenic red pulp. These sinuses were lined by endothelium and consisted discontinuous structure of stress fibres extending underneath the basal plasma membrane, running parallel to the cellular axis. The slits formed by the stress fibres forced the flow of blood from the cords into the sinuses [94].

The nano-particles-DNA complexes might have entered through the fenestrae, which had been previously reported to permit passage of particles with size of 200nm or less [98]. The contractility of the stress fibres [94] might aid the retention of the PS-SiO₂-DNA complexes in the spleen resulting in the subsequent gene expression. However, the precise site and distribution of gene expression in different sites of the spleen has yet to be determined. Besides the red pulp, the spleen also comprised of the white pulp component. The white pulp consisted of aggregates of lymphoid tissue and was responsible for the immunological function of the spleen.

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The compartmental administration of PS-SiO₂-DNA complexes at high dosage resulted in high gene expression in the liver, in addition to the spleen. As the largest internal body organ, the liver was known to be one of the main bio-distribution organs after systemic administration [21, 60, 87 and 98]. It was postulated that the excess PS-SiO₂-DNA complexes that have not been phagocytosed by the spleen, were carried by the blood to the liver. The entry and retention of the complexes through the fenestrae on the sinusoid wall of the liver could result in the subsequent gene expression in parenchyma cells [98]. Some gene expression detected in the kidney was likely to be derived from the spleen and the liver.

Another important consideration for non-viral gene therapy was in producing sustained gene expression. Time course study following intraperitoneal administration of the PS-SiO₂-DNA at the optimized dose of 4mg SiO₂-80ug DNA revealed the peaking of transgene expression in the spleen and liver from 6 to 12 hours. After 24 hours, gene expression in the liver returned to basal level. Gene expression remained consistently higher in the spleen even after 72 hours. No significant transfection activity was observed in the rest of the harvested organs. The degradation of the complexes was likely to be the cause of the short-term expression [43]. Some percentages of the PS-SiO₂-DNA complexes were also likely to be taken up by the spleen macrophages and the liver's Kupffer cells. The liver and spleen have been reported to play a central role in the removal of foreign particles and invasive organism from the circulation, via Kupffer cells and spleen macrophages [93].

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The ability of the PS-SiO₂ system to achieve sustainable level of transgene expression targeted specifically in the spleen following systemic administration of the complex, suggested its viability for delivering therapeutic agents to correct blood disorder such as hemophilia and sickle cells anemia, as well as in immuno-modulation strategies.

CHAPTER

7

CONCLUSION

Three different types of nano-bioceramics materials with differing shape, size and surface charge were investigated as potential candidates for gene therapy applications. The DNA binding ability of these nano-particles was found to have close correlations to their surface charges. Positively charged ZrO_2 nano-particles were able to bind well with DNA, while neutrally charged HA nano-particles partially bound with DNA and negatively charged SiO_2 nano-particles did not bind with DNA.

Different chemical agents including amino acids (Lys, Arg and his) and polycations (PLL and PS) were employed in modulating the surface charges of the nano-particles in enhancing DNA binding for *in vitro* transfections. The NH_2 group of the amino acids was ineffective in raising the surface charges of HA and SiO_2 in the positive range resulting in inefficient binding of DNA. Although both PLL and PS were able to raise the surface charges of HA and SiO_2 into the positive range to form stable complexes with DNA, PS was found to be the most ideal. The positive to near neutral surface charges of their respective DNA complexes enhanced the binding of the complexes to the negatively charged cell membrane and provided a protective environment for the DNA from nuclease degradation. Moreover, PS had been classified as a USP compound, approved by FDA for clinical use in counteracting the effects of heparin-induced anticoagulation after cardiac and vascular surgery. Hence, issues with toxicity and immunogenicity will be minimal.

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In addition to surface charges, the resorbability of the nano-particles was observed to play a critical role in mediating efficient *in vitro* transfections. Despite being positively charged to form stable complexes with DNA, unmodified and surface modified ZrO₂ performed poorly *in vitro*. This can be largely attributed to its chemically inertness which may have inhibited the release of DNA in the endosomal/lysosomal compartments for gene expression to take place in the nucleus. In contrast, HA and SiO₂ which were stable at pH7.0 were partially soluble in the presence of the lower pH in the endosomal/lysosomal compartments, thus aiding the release of DNA into the cytoplasm.

Using PS-SiO₂ nano-particles as the proto-type system, the system was optimized to form complexes which were stable for up to 72hours in 50% serum. The optimized system was able to efficiently deliver and express transgene expression in various cancer cell lines including HepG2, HeLa, 220.1 and CNE2 in the presence of 50% serum. This system was subsequently employed for *in vivo* gene delivery and it had the ability to target transgene expression specifically in the spleen following systemic delivery of the PS-SiO₂-DNA complex.

Successful cancer protocols would require systemic delivery of therapeutic agents with some degree of targeting. One of the biggest challenges encountered by most non-viral vectors *in vivo* was the efficient targeting of vectors into the appropriate target cells and tissues without affecting other organ systems. Thus far, no literature has reported the targeting of genes specifically through the spleen via systemic administration. Most systems that involved the targeting of spleen employed intra-splenic injection. Given the important roles of the spleen in blood filtering and immune regulation, the PS-SiO₂ system could thus be useful for delivering therapeutic agents to correct blood disorder such as hemophilia and sickle cells anemia as well as in immuno-modulation strategies.

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7.1 Future Work

The morphology and other physico-chemical characteristics of the nano-particles including their size and charge density can strongly influence their transfection efficacy *in vitro* and *in vivo*. Future work can be embarked to correlate the shapes and sizes of the nano-particles with their transfection efficacy. Additionally, the effect of pH on the DNA nano-particle complexes can be studied in achieving the optimal stability for *in vitro* and *in vivo* transfection.

The presence of 50% serum was seen to vastly enhance the transfection efficacy of the DNA PS-SiO₂ complexes. This is currently not well understood and more work will have to be established in gaining a better understanding of the structure and stability of the serum treated nano-particles complexes with cells *in vitro*.

The DNA PS-HA and PS-SiO₂ complexes had been assumed to follow similar transfection pathways as cationic liposomes and cationic polymers. Although cationic liposomes and polymers had been extensively investigated for gene delivery, the mechanisms by which DNA was delivered into cells remained to be clearly understood. In depth studies involving the fluorescence resonance energy transfer (FRET) and confocal microscopy will be also need to be undertaken to provide better clarity on the intracellular trafficking pathway mediated by the DNA-nanoparticles systems.

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Since the bulk materials of HA and SiO₂ were commonly used for bone implant, the DNA complexes of PS-SiO₂ and PS-HA systems could also be incorporated into three-dimensional bio-ceramics scaffolds for investigation as potential platforms for gene delivery. This will complement other existing and new research efforts in skeletal tissue engineering where the success in the repair and regeneration of bone, cartilage and ligament was strongly dependent on a suitable delivery vehicle for the transfer of stem cells or genes.

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