Characterization of Chitosan Nanocomplexes for the Delivery of Double Stranded MicroRNA to Glioblastoma Cells

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The candidate confirms that the work submitted is his own, and that appropriate credit has been given where reference has been made to the work of others.

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Abstract

Nanoscale drug delivery systems has gained significant traction, particularly for rare diseases still lacking a curative treatment such as glioblastoma. To complete the treating system of glioblastoma employing chitosan (CS) as a potential non-viral gene delivery vehicle, the present study aimed to assess the non-viral delivery of microRNA (miRNA) to glioblastoma cells (U251 cell line) and hence, its potential to inhibit the intracellular synthesis of proteins, while exerting a cytotoxic effect in this cell line. To this end a research, chitosan (molecular weight (Mw): 25 Kg/mol, degree of acetylation (DA): 29%) was used to form polyelectrolyte complexes with double-stranded miRNA (ds-miRNA) of $[-NH_3^+]/[-PO_4^-]$ molar charge ratio (N/P = 20), and their transfection efficiency was assessed in vitro. The particle size distribution, ζ potential, stability and electrophoretic mobility of CS-miRNA complexes of varying N/P ratios were characterized. The transfection efficiency was evaluated from the cytotoxicity U251 cells using the MTT assay. A linear dose-response dependence was established and the cell viability upon treating the cells with CS-ds-miRNA complexes (200μM) for 72 hours was reduced up to 55.35%, thus indicating a targeted cytotoxic effect. Neither ds-miRNA nor chitosan applied in free form exerted a cytotoxic effect. The results demonstrate that chitosan (DA of 29%) has the ability to serve as a non-viral gene delivery vector for miRNA in glioblastoma cells. The in vitro proof of concept gained may serve as the basis for future animal and pre-clinical studies, in the roadmap for potential translation to clinical use in cancer therapy.

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Chapter 1

Introduction

Recently, technology and medical science have experienced both great development and innovation, resulting in the improvement of nanomedicine. Such a multidisciplinary focus is of enormous importance in the study of human diseases, such as hemophilia, cystic fibrosis or malignant tumor, among others. In the domains of nanoscience and nanotechnology, human body defense system is based on cells that perform their life cycle functions partially by genetic programming and responding to molecular signals generated within the cell at the electrical, molecular, macromolecular and supramolecular levels (Santos-Carballal et al., 2018). Extensive studies on nanomedicine have been focused on searching for fatal disease treatments through gene delivery.

1.1 Background and related research

In 1972, the authors brought the idea of gene therapy. However, they gave cautions before they could start the study on this. (Hamilton 1972)

The first attempt was not successful, as there was a gene therapy's conducted on 10 July 1980 by Martin Cline. Cline made an argument on how one of his patient's genes got seen to be active six months later, though the data was not published, and even if he could have it correct, it was not probable that it would generate any therapeutic benefits against beta-thalassemia (Fletcher, 1983).

Having done comprehensive research using animal models in1980s and 1989, bacterial genes tag some trials on humans. It is a demonstration of gene therapy. It was done and was a success and thus accepted.

In 1993, there was the start of a somatic treatment, which brought some permanent genetic progress. This was done to cure harmful brain tumors by employing the recombined DNA use in transfecting some genes as it brings a sensitivity of the tumor cells to a drug that would consequently cause the tumor cell to die (Curtin., et al 2005).

There is also translating the polymers to the proteins that consequently cause interference with the expression of genes being targeted or cause a correction in gene mutation. The most commonly employed form is that a mutated gene is replaced by the DNA that encodes a functional, therapeutic gene. The vectors are used for packaging the polymer, which harbors the molecules inside cells.

Initially, there occurred failures in clinical trials, which led to their cancellation. Clinical trials have done since 2006 gave researchers renewed hope despite 2014 still being a method largely experimented on. It included the retinal Leber's congenital amaurosis disease treatment and chloride mix, Parkinson's disease, severe combined immunodeficiency (SCID), hemophilia, chronic lymphocytic leukemia, multiple myeloma acute lymphocytic leukemia, adrenoleukodystrophy, and X-linked SCID. This was between 2013 and April 2014 that there was an extensive investment of the US companies in the field. In 2013, Gendicine was approved in China as it treated a variety of cancers. In 2011, there was also approval by Neovasculgen in Russia as a peripheral artery disease treatment. In 2012 Glybera, was developed as the first treatment of a rare inherited disorder and a lipoprotein lipase deficiency. Due to the advancement in genetic engineering done on the cells, bacteria, and animals, the scientists have to make the knowledge much more applicable in medicine. Researchers employed methods to do the replacement or disruption genes that are defective. Researchers had to consider the single-gene defects disease, for instance, hemophilia, cystic fibrosis, hemophilia, muscular dystrophy, thalassemia, and sickle cell anemia.

Glybera has a therapeutic effect against one of the diseases caused by an abnormality in lipoprotein lipase. Usually, DNA is transfected, interacts with the damaged cells, and penetrates the cell, and its effect is to either disrupt or express a protein. A variety of delivery methods have also been used extensively. The first technique was the incorporation of DNA into the virus to transfect the DNA into the chromosome. An exploration of naked DNA has also been used in the process of developing the vaccine.

Usually, the efforts aim to administer a gene that brings about the expression of the desired protein. DNA editing is performed using methods, for example, the zinc finger nucleases and also the CRISPR. Incorporation of genes is done into chromosomes using the vector. This leads to the silencing and replacing of genes in the chromosome. Editing of genes gives insight into the treatment of hereditary diseases, viral diseases, and cancer by causing changes in humans' genome. (Feinberg, 2007).

The central nervous system's diseases present a noticeable worry to society, and the present drug and biological-based therapeutic ways have demonstrated not much to mitigate it. The use of genes in treating is an appropriate solution to combat than available because they can change gene expression and retain the normal function. (Saad et al., 2008) The breadth of dealing with diseases at the molecular level has widened over the years with the arrival of genome editing and ways of altering the gene expression. As a result, impressive results have been obtained. As we move to an era where gene therapy will hopefully provide an optimal solution to uncovering disease treatment, such information is available for CNS-associated gene delivery. This study provides an analysis of major problems and results from last two decades and an evaluation of the future opportunities for brain gene delivery.

Additionally, it provides possible concerns to major worries, such as ways to ease the introduction of therapeutic molecules into the brain since there is great permeability in the brain. (Muldoon et al., 2013)

1.2 Gene therapy and delivery

Main purpose of gene therapy is to carry out the introducing of normal genes or therapeutic genes into human targeted cells in a certain way to correct gene defects or play a therapeutic role so as to achieve diseases treatment. According to Sharma et al., (2010), Gene therapy is seen as a field in medicine that employs the therapeutic delivery of nucleic acids into the parent cell to treat or prevent disease or a disorder. The first trial to modify DNA in humans was done by Martin Cline in 1980, and as the first successful nuclear gene transferrin, the humans got approved by the Health national institute, which got done in May in 1980.

French Anderson was the first scientist to conduct the first therapeutic gene transfer and the first direct insertion of human DNA into the nuclear genome in 1990. It is anticipated that this will lead to a breakthrough in curing many genetic disorders and even treat them over time. A perfect example is using an adenovirus as a vector. The adenovirus will possibly insert a new gene into the cell, consequently making a functional protein in the host to treat the disease. In 1989 and December 2018, more than 2900 clinical trials were done, with more than half of them in the first phase (Inana et al., 2009).

According to Sum et al., (2014), gene therapy is a method that employs exogenous nucleic acids, that are vital therapeutic agents that can be adequately employed in correcting defective genes. According to those authors, exogenous nucleic acids are more important and efficient than the conventional means of treatment since they act against diseases, such as Parkinson’s disease, at the molecular level. Gene therapy relies on the breakthrough of the development of efficient delivery systems that release nucleic acids to the targeted cells. Thus, it is of utmost importance the development of appropriate delivery systems.

The gene delivery can be performed by using both non-viral and viral systems. Due to their higher transfection capacity, viral gene delivery vectors

have gained much more popularity than non-viral ones (Yin et al., 2014). Moreover, safety concerns, this is the possibility of the system becoming toxic inducing cancers or causing immunological reactions, also affects the choice of the delivery vector. Thus, non-viral vectors have been used because they are structurally flexible, safe, and can introduce a wide range of genetic materials into the cells. Three major barriers have to be overcome when reaching the target cells.

1. Nucleic acids have to pass the cell membrane,

2. Nucleic acid molecules have to be protected and effectively released,

3. Nucleic acids have to pass the nuclear membrane.

According to Perez-Martinez et al., (2011), the main problem for non-viral gene delivery systems is that the DNA has to pass across the nuclear membrane to the nucleus for being transcribed. It has been considered a limiting step, especially for gene transfection. Thus, developing a highly efficient gene delivery system with minimum toxicity is the major problem in the study of non-viral gene therapy.

Cationic polymers, such as polyethylene, chitosan, and poly-L-lysine, are widely used for developing non-viral gene delivery systems (Rai et al., 2019). Because of the multivalent-functionalized surface amino groups and the interaction of nucleic acid and polycation structure result in compact structures.

According to Schmidt-Wolf et al., (2003), dendritic polymers are famous for their clearly-defined 3-dimensional molecular structure that has an index with some low polydispersity that is controlled at the functionalities surface, which is based on the stepwise synthesis through the utilization of the divergent and that of the convergent method. Polyamidoamine (PAMAM) dendrimers also get developed towards promising the delivery of the non-viral gene vehicles. There can also be the creation of the PAMAM-DNA complex as this will be

between the plasmid DNA and PAMAM dendrimers, which can get created as there can also be a successful transfected to the host cells. However, utilization of PAMAM dendrimers suffers two major setbacks: as there is their naturally occurring cytotoxicity and low transfection efficiency. Great strides have been made towards improving the gene efficiency and decreasing the cytotoxicity of the dendrimers at the same time. In enhancing the efficiency of gene delivery and its specificity in reducing PAMAM dendrimers' cytotoxicity, PEGylation, acetylation, alkylation, and peptide-conjugation are the modifications that are greatly utilized to enable this (Kong and Shi, 2017).

Despite gene delivery providing future insights for the discovery of treatment alternatives for various diseases like cancer, genetic disorders, and certain viral infections, the method still poses risks. It is still being worked on to bring about complete acceptance until it becomes much effective. Gene therapy is now being examined exclusively for diseases that have no therapeutic methods against them. The target cells of the patient receive exogenous genetic particles that induce the expression of the proteins. Gene therapy aims to provide the recipient's somatic cells with genetic information, which produces a therapeutic protein to treat genetic disorders. Comprehensive knowledge for the interaction that is between the targeted cell and that of gene delivery system has to be derived from creating a successful design of the gene delivery system, which constitutes three different components, 1) the system can regulate the gene's function that is contained in the target cell, 2) it is a gene expressing a specific therapeutic protein, 3) not forgetting the gene delivery system which has a regulation of the transfection of gene expression plasmid to a targeted location in the body. It's required in the success of gene delivery. The system should have the genetic material introduced into the host cell to remain unstabilized. As a gene also, in 1994, there was the usage of the vaccines as a viral vector especially in the means for the protection of the chimpanzees against Hepatitis B. Besides, there was

a reporting of the non-viral gene delivery system as it was on the change of the revealing cellular phenotype using the DNA exposures in the body.

Cells introduced by exogenous genes are then allowed to produce required therapeutic substances, mainly specific protein. The whole process of gene delivery can be summarized as 3 steps:

1. The in vitro fabrication of a stable vector-genes complex, vector of which is usually positively charged cationic liposomes, cell penetrating peptides (CPPs) and deactivated virus coat. These vectors can be divided into two classes: viral vectors and non-viral vectors depending on whether a deactivated virus coat is used. Viral vectors in gene delivery is generally more efficient, but there is a risk of triggering allergic reactions due to incompletely deactivated viral coat. Non-viral vectors have a relatively low risk of allergenic reaction, but are also relatively less efficient at delivery.

2. For the complexes to work in cells, complexes need to be delivered to a certain cell. In the viral gene delivery system, the foreign gene is assembled on the virus through gene recombination technology, so that the recombinant virus can infect the target cell. Or cationic macromolecules can be used in the non-viral gene delivery system to package the exogenous gene and then introduced into the target cell via endocytosis.

3. The exogenous genes then will be released into the cell and escapes from lysosome, and eventually enter into the nucleus, where genes participate in the protein synthesis process. Foreign genes can correct the abnormal sequence of the defective gene and repair the defective gene precisely in situ without any other changes in the genome. Or by introducing foreign genes to make them express normal products, so as to compensate for the functions of defective genes, or specifically interference the translation or transcription of certain proteins to suppress the expression of certain abnormal genes, namely transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS). The whole process of gene delivery involves many

practical operations and human physiological activities, in which many factors will affect the efficiency of gene transfer, for example, the molecular weight of complex, the effective residence time of the foreign gene in vivo, the gene release rate of complex in cells, the type of genes carried by vector, and the targeting ability of complex.

1.3 Gene delivery system development

There has been a review for the viral gene delivery systems, which are for the viral-based vectors based on RNA, DNA, and the oncolytic viral vectors. It also applies to the gene delivery system, variety of the cationic biochemical polymers, for example, polysaccharide, polyethylene (PEI), and Poly (L-lysine) (PLL) derivatives (Al-Dujaili et al., 2011).

1.3.1 Viral vectors in gene delivery

Gene delivery systems employ viruses as vehicles to introduce the DNA into host cells. According to Santos-Carballal et al., (2018), viral gene delivery vectors have gained much more popularity than non-viral ones because they are more efficient. This technique is important because it uses viruses to have their own genetic material to get replicated. The efficiency's contribution is by its structure, which prevents it from being degraded through the liposome of DNA. Viruses used as gene delivery vehicles include retrovirus, adenovirus, adeno-associated viruses, and herpes simplex viruses. Germ line gene delivery systems and somatic gene delivery systems are the two categories of gene delivery systems. Germ line gene delivery systems possibly have great potential, but they are not ethically considered for this. Practically, the use of human gene delivery systems is restricted to somatic cell alteration. Somatic gene delivery systems involve in vivo and vitro. In vivo delivery systems, genetic materials, the genetic materials transfer directly

into the target tissue. In vitro gene delivery, the genetic material is implanted into host bone marrow, cultivated, and changed in vitro. The method is not adequately advanced now, but it is anticipated to be of great use in the future. (Arbab et al., 2004).

Viral vectors based on DNA for gene delivery systems

Gene delivery systems using viruses as delivery vehicles are often long-lasting and incorporate into the genomes. (Robbins et al., 1998). Virus vectors based on DNA are lentiviruses, poxvirus, adenovirus, adeno-associated virus, retrovirus, human foamy virus, and herpes virus. The DNA-base delivery systems use plasmids that provide exogenous genes for gene therapy. Even though the DNA based gene delivery systems are still in development stages, the class of materials has been studied to yield promising candidate of gene delivery system for gene therapy in a variety of diseases such as cancer, AIDS, neurological disorders such as Parkinson's disease, and Alzheimer's disease, and cardiovascular disorders. (Liu et al., 2011)

Viral vectors based on RNA for gene delivery systems

As by Robbins et al. (1998), the direct transcription of the viral vectors based on the RNA gene delivery can transcribe the RNA. However, the delivery of RNA-based gene is not permanent as it is often transient. The human foamy virus, the lentiviral vectors, and the oncolytic viral vectors are used. Also, the complex system is seen to give RNA dependent polymerase complexes and with the negative RNA strands which are based on the gene delivery systems which have been done using some lentiviruses as the HIV vectors victims who are undergoing transplantation for lymphoma caused by HIV.

Oncolytic viral vectors for gene delivery systems

There is a development of the Oncolytic viruses (OVs) as they are for the therapeutic effect against cancer type diseases (Lawler et al., 2017). There has been a discussion in the effects of the many changes towards the evaluation of their infectivity, antitumor immunity, and treatment safety for the association of OVs and tumor cells. The utmost goal is to create a virus that has the ability to multiply effectively within the host, special target, and destroy tumor cells.

Adenovirus-mediated decorin expression causes cancer cell destruction via activation of p53 and apoptosis of mitochondrion. Oncolytic adenovirus expressing interleukin-23 and p35 activates interferon-gamma and tumor necrotic factor-alpha-Co producing T cell mediator antitumor immunity. A report shows that cytokine immune-gene therapy is one of the plans for cancer treatment (Lasek et al., 2014).

1.3.2 Non-viral vectors in gene delivery

Recently, it has been proposed for the non-viral vectors to be used in gene delivery systems. It has been established that non-viral vectors have minimal chances of causing reacting in the immunity of biologically acceptable materials. These systems utilize the cell membrane to transfect the genetic material into the target cells. Physical techniques are used to introduce the genetic material. The physical means includes the needle, in which sound waves can be imposed to enhance the effect. These techniques cause the cell membrane to become more permeable to the nanoparticles that carry the exogenous genes. In non-viral gene delivery, complexes are formed with the nucleic acid and will be introduced into the target cell via endocytosis (Santos-Carballal et al., 2015). The exogenous genes then will be released into cytoplasm under changes in the intracellular pH environment, and

eventually participate in the protein synthesis process. Different effects can be produced depending on the introduced gene. By introducing foreign genes to trigger transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS), it can compensate for the functions of defective genes, or specifically interference the translation or transcription of certain proteins to suppress the expression of certain abnormal genes.

1.4 Present study

1.4.1 Chitosan

Chitosan (CS) is a linear aminopolysaccharide comprised by D-glucoamine and N-acetyl-D-glucosamine (Figure 1.1) and it is obtained by deacetylation of chitin. Chitin occurs in a a wide range of natural sources, for example, the shells of crustaceans, like shrimp and crabs, exoskeleton of coleoptera and diptera insects, fungal cell walls and in the cell wall of some microalgal species. The annual yield of chitin in the biosphere can reach 10 billion tons, making chitin one of the most abundant biopolymers and an inexhaustible natural renewable resource.

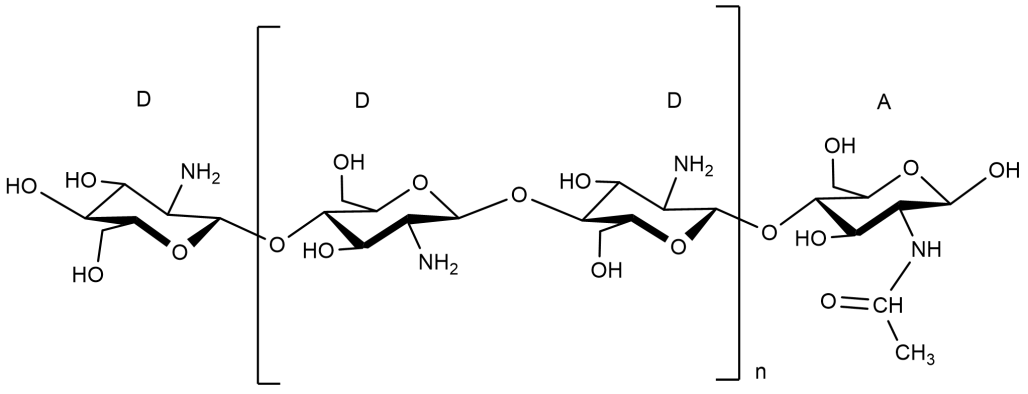

Figure 1.1 Rrepresenative chitosan chemical structure showing D-glucosamine (D) and N-acetyl-D-glucosamine (A) residues. The degree of polymerization (i.e. the total number of residues) is given by 2n+2. The degree of acetylation (DA) is given by the molar fraction of A residues as ([A]/([A]+[D])x100)

There are a large number of fields in which chitosan has been used. Chitosan molecule contains positively charged protic amino group, which is one of the few natural positively charged biopolymers. The protonated amino group has a strong adsorption effect on negatively charged substances and remove "garbage" of human body. It can also combine with the anion components on cell wall of bacteria, thus impeding the synthesis of cell wall and inhibiting the growth of microorganisms. In addition, seen from the molecular structure of chitosan, chitosan molecular structure is similar to the composition of amino acids and glucose in the human body, and it is also similar to human collagen tissue structure. This similarity gives chitosan excellent biomedical properties, namely, non-toxic, no irritancy to human body, can be absorbed by lysozyme in the body break, and have a good compatibility to human tissue at the cellular level. Chitosan chain contains a large number of hydrophilic groups, such as hydroxyl group and amino group, which gives it good water absorption and moisture absorption properties. In food processing, chitosan can be used not only as an outer film but also as a thickener, adhesives and so on. All the advantages mentioned above fit well with chitosan as a non-viral gene delivery vector, various researched have been conducted to explore the possibility of chitosan as gene delivery vector.

Chitosan as gene delivery vector

For gene delivery, variety systems delivering nucleic acid rely on the polymers positively charged. But these polymers have toxicity concerns due to their non-biodegradability. Cationic polymers that are degraded biologically usually form complexes, with transfection being much longer than dendrimers and PEI. (Sewbalas, 2010) To obtain a desirable transgene expression, modifying it with the site being targeted or introducing an alternative compound to condense the DNA, such as lipid with a negative charge

construction, is necessary. Besides, toxicity is exhibited by some polymers, which are degraded biologically. Due to this limitation, we have decided to study the polymers that are biologically degradable and cationic and exhibit high transfection efficiency. It's preferred that these polymers should possess well-defined chemical properties such as; (1) It should be able to condense the nucleic acid to facilitate an even tissue distribution adequately, which is followed by endocytosis; (2) endosmotic features that facilitate the escape of the nucleic acid from the endosome; (3) Biocompatibility; (4) simple synthesis and purification that makes it easy for large scale production; (5) functional groups that allow modification to allow simpler binding with the target molecule either extracellularly or intracellularly.

One of the main features that chitosan convenes is that it is a biocompatible and biodegradable natural cationic biopolymer capable of forming stable nanocomplexes with nucleic acids. All these properties make chitosan an adequate alternative in non-viral gene delivery applications.

The polymer met desired interest is chitosan, which is a linear polysaccharide and biodegradable. It has a random distribution of beta1-4 bound N-acetylglucosamine and D-glucosamine and is thought to be considerably nontoxic after being orally administered to humans. It has therefore received acceptance as the food additive and has been incorporated into the wound healing product. Therefore, there need to be distinct clarity of the effectiveness of chitosan-based delivery models (Sondhi et al., 2017). According to Nassif *et al*., (2002), chitosan inherently possesses a positive charge and has been discovered to provide another solution as systems that do not utilize viruses to carry genes. It has been found out that there occurs an association between the structure and the properties of chitosan-nucleic acid complexes in vitro. Additionally, the safety of using chitosan has been done on mice, and it has been established that it prevents the DNA from being degraded (Issa and M.M., 2006).

Chitosan used as nucleic acid delivering non-viral gene delivery systems were much studied to give additional insight into the both in vivo and vitro gene delivery. Since the start of early studies to do DNA delivery, chitosan has been proven to be excellent in intellectually delivering some nucleic acids as it creates transgenic response, causing either some increase in some protein expression or its decrease (Buschmann et al., 2013). This is explained as a result of several steps that involve nucleic acid condensation, shielding it from being degraded, not disrupting their normal state, cellular internalization, release from end lysosome, releasing and initiating transfection to DNA to the nucleus or RNA to the sequence in which the RNA is silenced. Due to many and sophisticated procedures involving transfecting genes to the host cell, limited understanding of the role of the structure of chitosan make-up on each procedure leads to the final limited transfection level of efficiency. Employing the whole of chitosan's sample and biophysical and biological methods is important in providing vital knowledge and finding out the utmost chitosan for transfecting a particular gene. Features such as the type of cell and route through which delivery is done also play important roles. In addition, The current change shown by the recent discovery of the technology of CRISPR-Cas9 gets seen as a transformation in a short period. It is anticipated the important role it will play in preventing and curing many diseases such as hereditary diseases (Fellmann et al., 2017).

According to the research carried out by Kievit et al., (2009), Chitosan is positively charged. Because of this, it can form DNA complexes obtained through a variety of ways and can give protection to the nucleic acid. Mao et al., (2010). said how two ways are seen in obtaining some nanoparticles of the nucleic acids of the chitosans, which are highlighted in the study: starting from a simple complexation which consists of a depolymerized chitosan and also some different salts that are with plasmid and also the ionic gelation that has some absorption of the plasmid which is in the nanoparticles or through

the encapsulation of the plasmid the nanoparticles as this helps in finding out the loading capacity of the chitosan nanoparticles that has plasmid. Also, electrophoretic mobility gets carried out as this is on the agarose gel. In addition, the nanoparticles are seen to have some characteristics that are exhibited by their morphology, their size, and their surface charge with the use of transmission electron microscope (TEM), atomic force microscope (AFM), laser diffraction, and also the methods of dynamic light scattering. The polyplexes obtained get seen to be spherical as they also have a nanometric size with a ζ potential, which has some positive results between 37 and 48 mV. It's through agarose gel electrophoresis that the positive results have been obtained for all cases that have been studied: A concentration is also between 20 and 30 micrograms per milliliters of the chitosan salts, which is required for the remaining studied as the loading efficiency of 100 percent is seen not to occur until there is a concentration that is equivalent to 100 micrograms per milliliters (Mao et al., 2010).

1.4.2 Micro RNA

Micro RNA (miRNA) is the most widely studied type of non-coding RNA, which was first discovered in nematode *C. elegans* in 1993 (Lee et al., 1993). According to Andaloussi *et al*., (2007) micro RNAs belong to a class of small RNAs, which cause modulation of gene expression. Lately, they are abnormally expressed in several cancer types. The role played by miRNA found in the human kidney is done by analyzing how the 245-micro RNAs are expressed in cancers of the kidney and bladder. More than 2300 true human miRNAs have been documented (Alles et al., 2019).

Here, RNA was used to hybridize small RNA profiling that was being developed in the laboratory. The micro-RNA has tags that corresponded to the human and mouse miRNA genes. Four different human micro-RNAs were found to have caused increased expression of the bladder's cancer

compared to the normal kidney. They were also found to have caused a significant upregulation of blood cancers compared to the normal mucosa of the bladder. In conclusion, these results reveal that different miRNAs are downregulated in renal cancers of the blood, suggesting the role of the genes in the processes that promote the development and propagation of several cancer types.

The location of these genes can be observed through citing the cause of different manners of miRNAs expression in the cancers development compared with the normal cells in genomic regions associated with cancers, by epigenetic ways, and by altering the machinery micro-RNA is processed.

RNA interference silencing, a method to silence the genes in humans by using RNA can be achieved by employing miRNA (Mittal and V., 2004). It is clearly established that these modulators have a role in disrupting the expression of the genes targeted. The small interfering RNA (siRNA) and short hairpin RNA (shRNA) cause the gene-targeted to be silenced in cancer cells. A study of an enzyme called luciferase, which causes apoptosis, was done. It was revealed that the inducement of apoptosis in transfected cells was initiated by the silencing of surviving expression selectively.

MiRNA in cancer

MiRNA miR-145 has been found as a potential tumor suppressor gene in breast cancer cells (Götte et al., 2010) and has been used in the development of chitosan-miRNA complexes for the delivery to breast cancer cells (see next section).

Recent studies conducted at Leeds Institute of Medical Research (LIMR) at St. James's Hospital, undertook a high-throughput screening of a series of miRNAs with cytotoxic activity against glioblastoma cells and uncovered miRNA-1300 (miR-1300) as the most potent cytotoxic and robust candidate.

A striking binucleated phenotype was observed in miR-1300 transfected cells due to cytokinesis failure followed by apoptosis. This was also observed in two stem-like patient-derived cultures.

The physiological role of miRNA 1300 as a regulator of endomitosis in megakaryocyte differentiation where blockade of cytokinesis is an essential step. In glioblastoma cells, where miR-1300 is normally not expressed, the oncogene Epithelial Cell Transforming 2 (ECT2) was validated as a direct key target (Boissinot et al., 2020). This study highlighted the potential of miR-1300 as a novel RNA gen with translatable potential for clinical application. The importance of the development of delivery strategies in this regards, was also mentioned.

1.4.3 Chitosan-RNA complexes

Chitosan-RNA complexes are extensively used in non-viral gene delivery system because they exhibit several distinct features:

1. It is polycationic inherently, therefore it can form complexes with miRNA;

2. It is biodegradable.

3. The stability of its chemical structure

The process in which chitosan plays a crucial role ensures the utmost silencing of the genes with the effect of CS-RNA complexes. The following are novel medical cases that patients were treated or prevented using this combination for gene delivery system:

1. Promoting the regeneration of nerves and local nanotherapeutics,

2. Efficient delivery of siRNA to the brain to act against Alzheimer's disease,

3. Self-crosslinking nanoparticles to carry polymerized siRNAs to target cancer treatment by inducing therapeutic effects against tumors,

4. Treatment of multidrug resistance for the tumor treatment,

5. Improve structural stability of siRNA for a prolonged therapeutic efficacy.

Chitosan-miRNA-145 nanocomplexes have been developed and found to improve the delivery of miRNA to breast cancer cell MCF-7 (Santos-Carballal et al., 2015). The greatest transfection efficiency, measured in terms of the downregulation of the target gene, was observed for complexes of chitosan with DA 29%, when compared with a series of DA between 1.6% and 49% (Mw: 18 - 26 kDa) (Santos-Carballal et al., 2015). This was consistent with preceding studies that addressed the role of the molar mass and degree of acetylation of chitosan and revealed that the most successful in vitro knockdown rates were obtained with chitosan (DA: 28%, Mw: 10 kDa). (Alameh et al., 2018).

1.4.4 The U251 cell line (Glioblastoma cells)

Glioblastoma is the most occurring and malignant tumor that exhibits extensive heterogenicity at cellular and molecular levels. Glioblastoma stem cell discovery helped in tumor studies' paradigm shift (Pollard et al., 2009). This study focuses on utilizing U251 glioblastoma cells to evaluate phenotype of these cells when cultured under varied culture states. Previous study revealed that U251 cells showed unique growth patterns and had the capacity to regenerate themselves. Remarkably, the glioblastoma cells exhibit reversible adaptive plasticity in response to their environment, leading to its heterogeneous nature of glioblastoma and the changes in responses to current therapies. Thus, there is a need to increase the understanding of U251 to make the therapies against this malignant brain tumor much more effective.

Glioblastoma is a malignant type of tumor that occurs in the brain. It exhibits signs including headaches, personality changes, nausea, and signs are relative to that of stroke patients may be unconscious when symptoms go to

the worst. The progression to this cancer can either occur from a normal cell or a benign tumor. Treatment is by chemotherapy or radiation therapy.

Upon studying tumor vasculature, Glioblastoma is characterized by abnormal vessels, of which the morphology was functionality disrupted. The high permeability and limited perfusion of the vasculature causing disrupted blood flow within the tumor and results in augmented hypoxia, consequentially assisting cancer progression. Joshi *et al*., (2011) studied glioblastoma causes numerous changes in genes responsible for ion channels. By augmenting these ion channels, these malignant cells are thought to facilitate an elevated transit of ion through the cell membrane, consequently augmenting water entry via the osmotic process and facilitating Glioblastoma cells in varying cellar volume extremely fast.

Unfortunately, there is no preventive measure against glioblastoma for now, recent studies have to focus on the treatment of glioblastoma.Treatment of glioblastoma is not easy, several inhibiting factors are listed as following:

1. The tumor cells exhibit resistance to available medicine,

2. Susceptibility of the brain to be damaged by the available therapy,

3. The capacity of the brain to regenerate itself is so minimal,

4. The blood-brain barrier inhibits the administration of the therapeutic molecules into the brain.

Studies done have shown that miRNA is the main regulator of the signaling in the gliomas. MiRNA in clusters regulated epigenetic pathways in the disease. These researched have found the need to utilize gene therapy methodologies since, with the limitations highlighted above, it provided a greater insight to cure glioblastoma. Early clinical trials have been done using animal models, and as of 2017, gene therapy was explored as a method to treat glioblastoma with promising results.

The first treatment method involves surgery. An advantage of surgery is

excision of tissue for pathological diagnosis, reduced symptoms related to an effect of a greater extend, and eliminating the disease before its recurrence. Following surgery, radiotherapy follows as the next treatment for people with glioblastoma. Usually, temozolomide will be used. Glioblastoma tumors have a limitation as they exhibit hypoxia, which is extremely resistant to radiotherapy. Thirdly, chemotherapy, though used to treat glioblastoma, shows little benefit against this tumor.

In addition, the cannabinoids are used efficiently to reduce nausea and vomiting caused by chemotherapy and to stimulate appetite, and reduce the sense of anguish or the actual pain. It has been actually demonstrated that they can inhibit growth and angiogenesis in malignant gliomas. Their ability to attack neoplastic stem cells of glioblastoma, which induces differential maturity of cells and simultaneously inhibiting tumorigenesis.

1.5 Research methods

1.5.1 Synthesis of Chitosan-double stranded miRNA complexes

At a physiological pH, below about 6.5, the amino groups in chitosan molecular are able to be protonated and positively charged. Meanwhile ribonucleotides in nucleic acids (miRNA in this case) provide negatively charged phosphate groups, able to spontaneously bind to chitosan forming a complex in suspension. This self-assembled system is the main mechanism for the formation of complexes in this case.

Depending on the charge ratio between the protonated amines from the chitosan and the negatively charged phosphate groups from nucleic acid, namely nitrogen to phosphorus ratio (N/P ratio), the complexes will also show different performance in gene transfection.

According to the result of the research conducted by Matulis et al., they used isothermal titration calorimetry (ITC) to study the compaction of DNA by

multivalent cations. Authors summarize the whole process in the following two stages:

1. The cation binds to nucleic acid through non-specific electrostatic forces, resulting in neutralization of the charges of the nucleotides, which leading to a decrease in charge repulsion and therefore an increase in the flexibility of the chains,

2. After reaching a critical ligand concentration, DNA-DNA interactions occur and they self-assembled, which is an entropically driven process.

1.5.2 Electrophoresis retardation assay

Electrophoresis is defined as the movement of dispersed particles in response to a fluid under the influence of a spatially uniform electric field (Carter et al., 1978) studied two types of electrophoresis: cataphoresis and anaphoretic, which use positively and negatively charged particle respectively. It is a technique for separating the components of a mixture based on their size and charges, macromolecules moving in the direction of the electrode opposite to their charges under the action of an electric field. Electrophoresis is conducted by the presence of a charged interface and the surrounding fluid. This provides the basis for the analytical methods employed in chemistry for separation according to size, charge, or binding affinity. Electrophoresis is extensively applied in separating DNA, RNA, and protein analysis.

Usually, the method uses a negatively charged molecule, so the proteins move towards a positive charge. Once the electric field is applied, nucleic acids with the phosphate group is driven towards the anode (positive pole) by the negative charge on the group. And the molecular weight and charge amount of the nucleic acids would determine the migration speed.

Native electrophoresis

The polyacrylamide gel electrophoresis (PAGE) is seen to be under the native conditions as it is also well established and a versatile method that helps in the probing of the acid conformation and the interaction of the nucleic-acid protein. The native PAGE on the other hand has been seen to be used in measuring RNA as its fluids the equilibria and the kinetics that are under a wide variety of some conditions. This method gets seen to have some advantages like the adaptability, the ease seen in the radiolabeling RNA, a direct analysis of the conformational heterogenicity hat is within a sample. The native page is also useful for resolving the ligand-induced structural changes.

Denaturants are not required in native PAGE, and because of this, small unit interactions in the multimeric proteins usually remain, and data is generated on the 3D structure. Furthermore, other proteins have their enzymatic activity conserved after a resolution by this technique. Therefore, it may be facilitated to actively purify proteins.

In the case of studying binding affinity of chitosan to nucleic acids, native electrophoresis is essential to be involved instead of conducting electrophoresis with denaturing compound, such as sodium dodecyl sulphate (SDS) and 2-mercaptoethanol or dithiotreitol (DTT). Advantages of utilizing native electrophoresis can be included as, firstly, unnecessary degradation of RNA and chitosan induced by reagents should be avoided. Secondly, electrophoresis can intuitively show that macromolecules mobility would be retarded to a considerable extent once the chitosan-RNA complexes formed, which is relative to the binding ability of chitosan.

In the end, the migration status of CS-miRNA complexes and CS-comp miRNA complexes will be recorded and discussed. Image of the electrophoresis study show the binding affinity of chitosan to RNA.The stronger the binding affinity, the shorter the migration distance of complex in

electrophoresis. Meanwhile, compared with the control group with pure RNA, similar migration distance of the experimental group indicates weaker binding affinity. A higher N/P ratio of complex would be expected to have more positive charges and therefore would have a stronger binding affinity to nucleic acids, which would also have a stronger stability and inhibition effect on migration. The expected result of electrophoresis is that at a certain N/P ratio, when the electrophoresis was paused, the sample stays at a higher position than other groups at other ratios or cannot be migrated at all.

1.5.3. Particle size and ζ potential measurement

Since nanoparticles are complex systems, various parameters have to be determined to understand their behavior and state comprehensively. Also, the size of distribution is the relevance (Hassellov et al., 2008)

A variety of analytical methods can be harnessed to obtain information about nanoparticle size. Below are the lists of techniques that are used to provide information on nanoparticle size:

1.Dynamic light scattering

2.Disc centrifugation

3.Nanoparticle tracking analysis

4.Tunable Resistive Pulse Sensing

5.Atomic force microscopy

6.Electron microscopy

Additionally, other features are crucial in characterization, as the concentration in solution, shape, surface charge, and chemical composition. Here, we shall focus our study on dynamic light scattering.

Dynamic light scattering (DLS)

There is a need to characterize nanoparticles in solution before assessing toxicity in vitro. Particle size, particle distribution, particle morphology, particle composition, surface area, surface chemistry, and ζ potential in solution are factors to be accurately assessed to determine the behavior of complexes in vitro transfection.

A determination of the particle's size and ζ potential is done by the measurement of the light intensity caused by the scattering of light from a suspension or a solution. Nanoparticles size is usually analyzed using this method.

Dynamic light scattering (DLS) is the most common and practical set of techniques mainly used to observe physical properties of wet nanoparticles based on time fluctuation of nanoparticles and its Brownian motion in suspension. In researches, DLS could be applied in particle size, size distribution and ζ potential measurement of nanoparticles including whether metals, metal oxides or carbon-based materials. Compare to those methods such as electron microscopy, DLS is not a technique working on dry materials but giving a better understand on changes to their characteristics when in an solution or suspension. Under a dry condition, it is impossible to show particle size, size distribution and aggregating status of nanoparticles in liquid dispersion by visual analysis techniques. Drying sample can cause degeneration and physical aggregation which would interfere with the measurement of sample nature.

In the development of anti-HIV activity medicine, DLS was used to reveal the effect of a long-chain alkyl group in sulfated alkyl oligosaccharides. Furthermore, researches in the food shows DLS can be applicable to measurement on food ingredients. And in colloidal suspensions it is important to accurately quantify the physical properties of nanoparticles. In order to evaluate the performance of apparatus and provide a new insight into the

mechanism of the scale inhibition, a novel dynamic light scattering special technique is used to study the bulk supersaturated gypsum aqueous solutions during the induction period. Lastly, during the process of fabricating vaccine, Dynamic Light Scattering was validated to be used as a quality control technique in the pilot production plant.

In present study, suspension of CS-ds miRNA complexes were the object of study. Due to the characteristics of chitosan and ds miRNA, their particle size and size distribution cannot be measured under dry condition, otherwise the complex will denatured and break down. In suspension, a certain ratio of chitosan to RNA can form relatively stable complexes by charge interaction. Furthermore, for the reason that chitosan and ds miRNA have definite charged characteristics in suspension, DLS technique can also be used to measure ζ potential.

Particle size measurement

The reason why DLS is applicable to particles in dispersion is that in principle DLS directly measures hydrodynamic quantities, usually translational and/or rotational diffusion coefficients, which can be converted to quantitative size and shape by a set of subsequent analysis and calculations. In measurement, geometry characteristics of nanoparticles is not directly observed and measured. Instead, apparatus monitors the time fluctuation of light intensity scattered by nanoparticles dispersion, and the decay rate of the time autocorrelation function of these intensity fluctuations is used to directly measure the particle translational diffusion coefficient, which is in turn related to the particle hydrodynamic radius. In the calculation, the hydrodynamic radius of a particle can essentially be equivalent to its geometric particle radius for spherical particles. Those above all eventually allow the apparatus to observe and calculate the size and size distribution of particles in dispersion.

ζ potential measurement

ζ potential shows the positive and negative polarity of charge on a particle surface in liquid dispersion. Because of several behaviors of particles in dispersed system, like ionisation, ion adsorption, and ion dissolution, the particles have a certain electric charges on its surface. And in a dispersed system, the ionic environment around the particle surface is also affected by the electrical properties of charges on surface to form a electrical polarized region, which is described as a double layer model - the stern layer of ions firmly attached adjacent to the particle surface, and the diffuse layer further away from the particle surface.

Particle ζ potential's significance is that the value obtained has a relationship to the stability of the colloidal dispersions. It shows the magnitude in which the adjacent similarly charged particles repel each other in a dispersion. There is a high ζ potential for the molecules that are very small and will provide stability. This will make the solution or dispersion to resist aggregation. Low potential makes attraction go beyond repulsion, and the dispersion breaks evaluate the size and distribution profile of small particles in suspension or polymer in solution. On the other hand, an investigation of complex fluid behavior as concentrated polymer solutions can be done.

In present study, chitosan molecule is positively charged and micro RNA 1300 molecule has 20 negative charges. The complex of the two will have different electrical properties depending on the proportion of chitosan and RNA they are made of.

In order to measure ζ potential, a small quantity of liquid dispersion is injected into a cell containing two electrodes that are used to create an induced electric field. Once the electric field is applied, particles move towards either the anode or cathode of the electric field depending on the positive or negative charge they carry. Whether they are positively charged or negatively charged can be determined by the direction in which polar they are moving.

And ζ potential of the particles is one of the factors affecting particle motion which is measured via the same apparatus as particle size measurement but within an applied electric field. The stability of dispersion is then determined by the useful information obtained from ζ potential measurement.

1.5.4 Cell experiments

MTT cytotoxicity assay

This is a colorimetric assay that determines the extent to which yellow3-(4,5-dimethylthiaol)-2,5-diphenyl tetrazolium bromide (MTT) is reduced. Adding MTT into media, then the MTT enters the cells, passes into the cells, and reaches the mitochondria, where it is reduced to an insoluble, colored (dark purple) product of formazan.

The cells are then made soluble with an organic solvent like Isopropanol or DMSO, and release solubilized formazan. Solubilized formazan is a reagent and can be measured using a spectrometer. The reduction of MTT can only occur in metabolically active cells. Therefore, the level at which the cells are viably active determines the cell viability and the quantity of formazan formed in cell.

The cytotoxicity of the test is based on the reduction of MTT reagent to form formazan to enable the evaluation of succinate dehydrogenase enzymatic activity and then lead to the determination of vitality of the chitosan. Later, the quantity of formazan formed is determined by measuring in the spectrometer, which will be presented as absorbance of cell suspension.

In vitro transfection of chitosan-RNA complexes on glioblastoma cells

Small interfering RNA carried by chitosan in complexes can inhibit life process of glioblastoma cell. Furnari et al., (2015) studied and realized the

role of epidermal growth factor receptor (EGFR) in glioblastoma. Scientists have been able to silence or knockdown EGFR by using small interfering ribonucleic acid (siRNA).

Lately, the interfering strategy has been directed at micro RNA, which is aimed at reducing mRNA expression. This process can be enabled by introducing miRNA having double-stranded RNA of 21-23 nucleotides. Incorporation of this miRNA into the RNA-induced silencing complex (RISC). Subsequently, the sense strand of the RISC complex is removed while the anti-sense strand will be remaining in the RISC complex and inducing the RNA enzymatic machinery, which reduces mRNA expression and protein synthesis.

1.6 Aim of the project

RNA based therapeutics hold great a potential in treating various types of cancer, which are currently difficulty to treat. Yet, this approach has presently two major limitations: 1) it requires the delivery of functional nucleic acids into the cell cytoplasm, 2) their delivery must be limited to diseased cells. Recent advances in gene delivery, may enable to overcome such limitations. Previous studies by our group, have demonstrated the feasibility of chitosan to deliver miRNA to MCF-7 breast cancer cells. Independent research at Leeds Institute of Medical Research (LIMR) at St. James's Hospital have also shown that miRNA 1300 targets glioblastoma U251 cells and induces a cytotoxic effect and a binucleated phenotype.

Given this preceding background, the aim of the present thesis is to investigate the biophysical characteristics (including average particle size and ζ potential of complexes, retardation ability of chitosan to miRNA) and potential of CS-miRNA nanocomplexes to improve the delivery of miRNA to glioblastoma U251 cells to serve as in vitro proof of concept of the feasibility of this approach.

The work is divided in the following parts:

1. Develop chitosan-miRNA (CS-miRNA) complex, chitosan-complementary miRNA (CS-comp-miRNA) complex, and chitosan-double stranded miRNA (CS-ds-miRNA) complex at varying N/P ratios (0.1, 0.5, 1, 10 and 20) and characterize their particle size, size distribution, ζ potential, electrophoretic mobility, and stability in dispersion system,
2. Evaluate the delivery and transfection efficiency of the CS-miRNA-1300 nanocomplexes in U251 glioblastoma cells by determining the cell viability using the MTT assay.

Chapter 2

Methodologies

2.1 Materials

1. Chitosan (CS) Luki HDP30 (Degree of acetylation (DA): 29%), synthesized in the researching group.

2. MicroRNA (miRNA) 1300 (5'UUG AGA AGG AGG CUG CUG3', negative charges: 20, Mw: 5857g/mol) and its complementary (5' CAG CAG CCU CCU UCU CAA 3'), purchased from Biomers GmbH (Germany).

3. DNase/RNase free water (10977-035, gibco).

4. 14% polyacrylamide precast gels (43269.01, Generon).

5. GeneRuler ultra low range DNA ladder (SM1213, Thermo Fisher Scientific (Life Technologies)).

6. RNA loading dye (R0641, VWR International).

7. SYBR gold nucleic acid gel Stain (10358492, Fisher Scientific Ltd).

8. Tris-Acetate-EDTA buffer (TAE buffer) (2116-500, Cambridge Bioscience).

9. Media (DMEM (1X) + GlutaMAX™-1, Dulbecco's Modified Eagle Medium, [+] 4.5g/L D-Glucose, [+] Pyruvate. 31966-021, gibco).

10. Minimal media (DMEM (1X), Dulbecco's Modified Eagle Medium, [+] 4.5g/L D-Glucose, [-] Glutamine, [-] Pyruvate. 31053-028, gibco).

11. Fetal bovine serum (FBS) (F9665-500ML, SIGMA).

12. Penicillin/Streptomycin (DE17-603E, LONZA).

13. Non-essential amid acid (NEAA) (11140-035, gibco).

14. L-Glutamine (17-605E, LONZA).

15. Sodium pyruvate (11360-070, gibco).

16. Phosphate buffer solution (PBS) (D8537-500ML, SIGMA).

17. Trypsin-EDTA 10X solution (BE02-007E, LONZA).

18.OPTI-MEM (1X) (Reduced serum medium, [+] HEPES, [+] 2.4g/L Sodium Bicarbonate, [+] L-Glutamine, 31985-062, gibco).

19. Lipofectamine RNAiMAX reagent (13778-030, invitrogen).

20. Thiazolyl Blue Tetrazolium Bromide (MTT) (M2128-IG, SIGMA).

2.2 Synthesis of Chitosan-double stranded miRNA complexes

Complexes were composed of chitosan (CS) and double stranded miRNA (ds-miRNA) (hybridized with miRNA 1300 and its complementary).

In practice, the sequence of adding RNA first and then chitosan should be followed when fabricating CS-ds miRNA complexes. As the standard of a finished suspension, final suspension should be clear and transparent with good fluidity. According to the different proportions of chitosan and RNA in suspension, the experimental groups were divided into five groups by fives N/P ratios of 0.1, 0.5, 1, 10, and 20.

2.2 Native electrophoresis retardation assay

The miRNA binding ability of chitosan at different ratios was determined by native electrophoresis retardation assay. 14% polyacrylamide precast gels and SYBR gold was facilitated to label nucleic acids. Include all the reagents used such as the buffer used, the gene ruler, the loading buffer, and so on.

In total, 5 samples of CS-ds miRNA complexes were prepared, with 2 lanes of gene ruler and pure double stranded miRNA as control. In practice, samples were loaded into the gel wells in Tris-Acetate-EDTA buffer (TAE buffer) as shown in table 2.1.

Table 2.1. Samples loading of native electrophoresis retardation assay

Well	1	2	3	4	5	6	7	8
Sample	Gene ruler	Pure RNA	Complex ratio 0.1	Complex ratio 0.5	Complex ratio 1	Complex ratio 10	Complex ratio 20	Gene ruler

5 µL of complexes were loaded in each lane. Electrophoresis was performed at 120 V, 37 ^{o}C for 2 hours running. After electrophoresis, gel was fixed with 10 % acetic acid, 40 % methanol and 50 % water at room temperature for one hour. Then, SYBR gold nucleic acid gel Stain was used for miRNA dying. For that purpose, SYBR gold was diluted to one tenth in Tris-EDTA buffer prior its application to the gel, and the staining was allowed at room temperature for one hour. Then, the gel was washed with milliQ water and then the presence of nucleic acids was revealed by using ultraviolet light by using a ChemiDocTM (MP imaging system (170-8280) by Bio-Rad).

2.3 Particle size and ζ potential measurement

In present study, CS-miRNA complexes, CS-comp-miRNA complexes, and CS-ds-miRNA complexes at five N/P ratios (0.1, 0.5, 1, 10 and 20) were studied at room temperature to determine which ratio was the most suitable one for gene delivery and whether there are commonalities and connections between different complexes consisted of chitosan with different RNAs. Results of particle size and ζ potential were measured by Zetasizer Ultra (ZSU5700, Malvern Panalytical Limited, UK).

2.4 Cell experiments

U251 cell line (glioblastoma cells) was cultured in the incubator at 37 ^{o}C, 5% CO_2, and 95% humidity ratio (HR). Density of cells in culturing flask was within the range of 1000 cells/cm^2 - 4000 cells/cm^2 in subculturing to prevent

cells overgrowth. Complete media was changed and cells were passaged once a week.

Ten groups were finally included in MTT cytotoxicity assay as follows:

1) No additives (positive control of growth), 2) DNase/RNase free water,

3) Pure ds miRNA (100nM), 4) Chitosan (in the same concentration as that in the 2X complex),

5) Chitosan-RNA complex 1X, 6) Chitosan-RNA complex 0.5X,

7) Chitosan-RNA complex 2X, 8) Triton X100 (negative control of growth),

9) Lipofectamine, 10) Lipofectamine-RNA complex.

Cells were seeded on the plate with 200 µL complete media, a density of $1x10^4$ cells per well and incubated at 37 ^{0}C, 5% CO_2 and 95% HR for 24 hours to ensure the cells have attached to the plate and the growing environment was stable. Then ten groups of 10 µL particles were added, the cells were incubated under previous conditions for 24 hours then replace the media by 200 µL complete media. MTT assays were performed and absorbance was measured after 48 and 72 hours of incubation. The media was removed and replaced by 100 µL minimal media and 25 µL MTT solution (5 mg/mL in PBS). The cells were incubated under previous conditions for another 3.5 hours to allow the formation of a purple formazan salt before the medium was removed and 100 µL DMSO was added to each well in order to dissolve the formazan. The absorbance was measured 10 minutes later. After obtaining the absorbance (at 570nm, and at 690nm as reference) of the MTT assay results, the following formula was used to calculate the cell metabolic activity under the current treatment conditions:

$$\text{Cell metabolic activity } (\%) = \frac{\text{Abs}_{\text{sample}} - \text{Abs}_{\text{Triton}}}{\text{Abs}_{\text{no additives}} - \text{Abs}_{\text{Triton}}} \times 100 \qquad \text{E}$$

Chapter 3

Result and analysis

3.1. Particle size and ζ potential

Physicochemical characterization of the nanocomplexes carried out by DLS is shown below plotted with Origin Pro 9.1. Namely, particle size distribution by intensity, particle size distribution by number, correlation function of the measurements, polydispersity index (PDI), average particle size, phase plot, ζ potential distribution and average ζ potential of CS-miRNA complexes, CS-comp-miRNA complexes, and CS-ds-miRNA complexes at N/P (charge) ratio 0.1, 0.5, 1, 10 and 20.

These results indicated whether three different RNAs and chitosan could form stable nanocomplexes with relatively uniform particle size in the suspension, and determined at which ratio the subsequent cell experiments can be proceed with at the best condition and efficiency. And these results will eventually be used for analysis in combination with electrophoresis, MTT assay, and in vitro transfection on U251 cell line.

3.1.1 Chitosan-miRNA (CS-miRNA) complexes

Figure 3.1, represents particle size distribution by intensity, particle size distribution by number, and the correlation functions of the measurements of CS-miRNA complexes in DLS experiment at N/P ratio equals to 0.1, 0.5, 1, 10 and 20.

According to the particle size distribution by intensity (A of Figure 3.1) of CS-miRNA complexes at ratio 0.1, 0.5, 1, 10, and 20 mainly concentrated in the range between 30 - 1040 nm, 20 - 2990 nm, 20 - 2000 nm, 20 - 570 nm, and 10 - 570 nm, respectively, indicating a higher N/P ratio resulted in a smaller range of size distribution. Meanwhile, the distribution by number (B of

Figure 3.1) of the same nanocomplexes were found in the ranges between 5 - 270 nm, 5 - 50 nm, 15 - 80 nm, 4 - 68 nm, and 4 - 93 nm, for the nanocomplexes at N/P ratio 0..1, 0.5, 1, 10 and 20, respectively. And peaks of the same nanocomplexes were found in 13.06/34.56 nm, 17.66 nm, 37.56 nm, 13.06/32.3 nm, 11.23/37.56 nm,respectively. It can be roughly deduced that the integral area of the curve indicating the particle size of complexes decreased first then increased (more precise results need to be calculated by the equipment, which were shown in B of Figure 3.2).

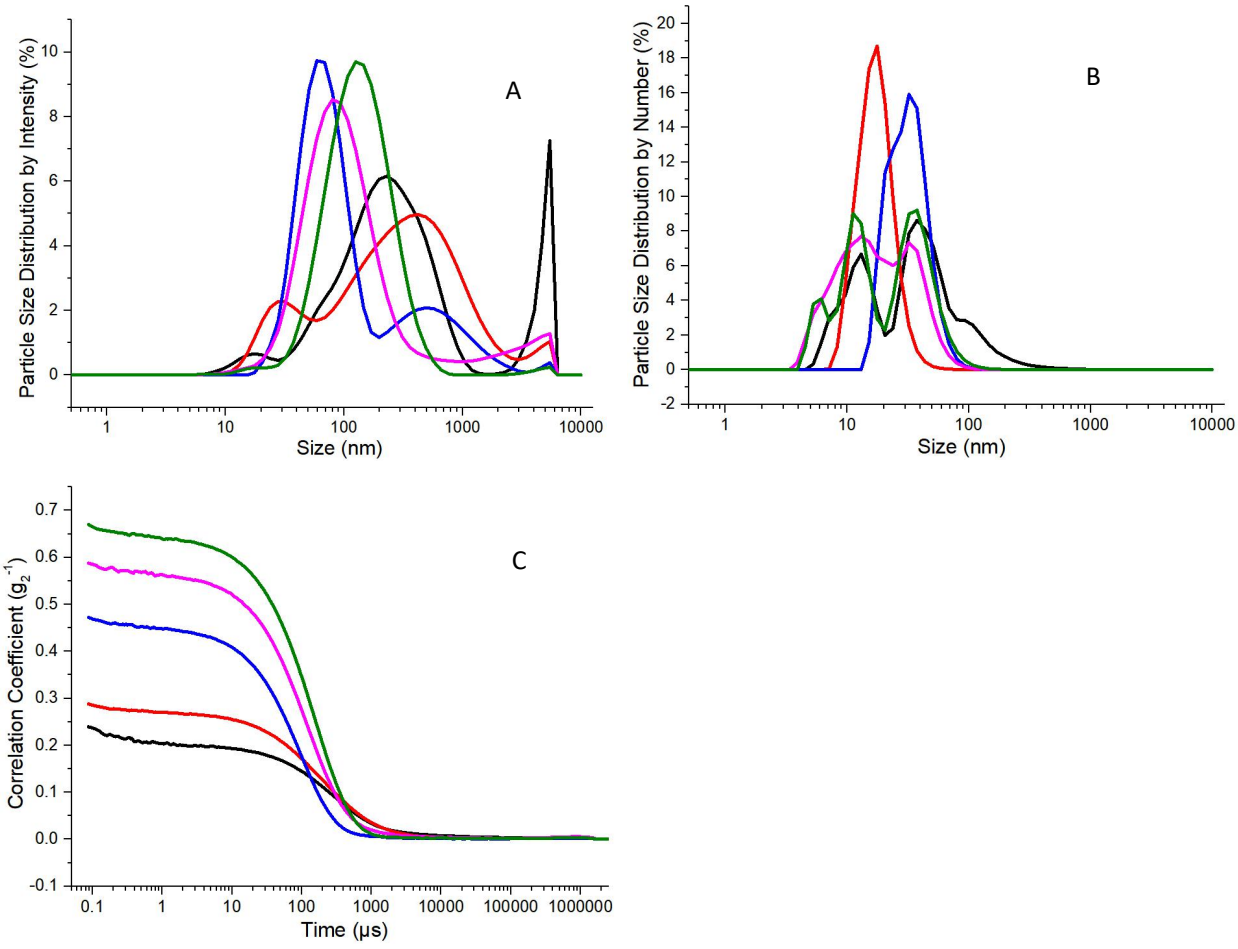

Figure 3.1. Particle size distribution by intensity (A), particle size distribution by number (B) and correlation functions (C) of CS-miRNA complexes at charge ratio 0.1 (black), 0.5 (red) 1 (blue), 10 (pink) and 20 (green)

It is important to point that according to the correlation function (C of Figure 3.1), detectable particles in the suspension increased as the increasing of N/P ratio. In the cases of N/P ratios higher than 1, intercept of complexes were over 0.6 indicating better result than those at ratio lower than 1 in which noisy signals were recorded.

Figure 3.2 show polydispersity index (PDI) and average particle size of CS-miRNA complexes at N/P ratios 0.1, 0.5, 1, 10 and 20. According to the PDI recorded (A of Figure 3.2), at N/P ratio equal or below 1, the interaction between chitosan and miRNA resulted in poor reproducible nanocomplexes with high PDI which involves the polydispersity of the particles in the suspension. However, nanocomplexes prepared at N/P 10 and 20, error bars of which were smaller and the values were in the range of 0.2 - 0.4, revealing the monodispersity of the nanocomplexes suspensions. According to the results, in nanocomplexes prepared at N/P ratio over 0.5, the higher the N/P ratio, the lower the PDI. According to the average particle size results (B of Figure 3.2), the minimum size recorded was found for nanocomplexes prepared at N/P ratio equal to 1, namely 82.214 nm. Reduction or increment of N/P ratio resulted in an increment of particle size.

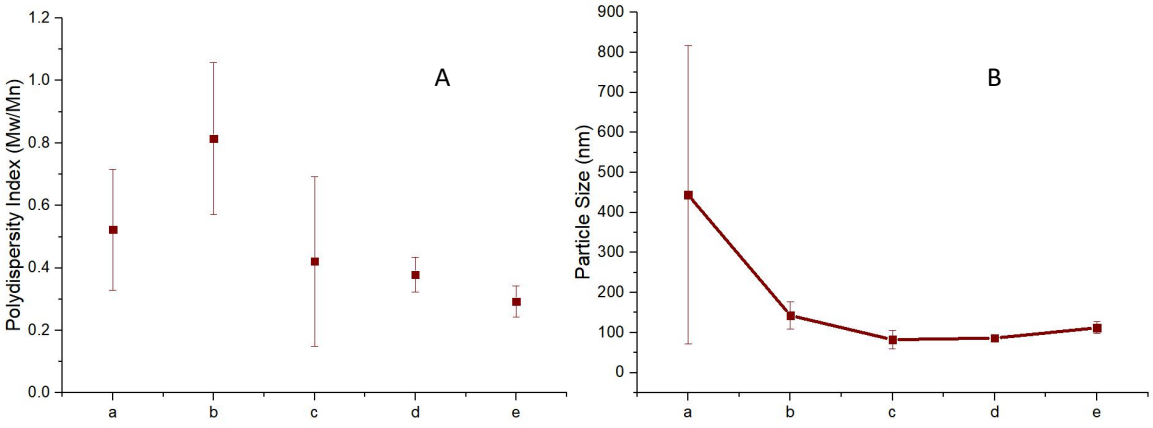

Figure 3.2. Polydispersity index (PDI) (A) and average particle size (B) of CS-miRNA complexes at ratio a) ratio 0.1, b) ratio 0.5, c) ratio 1, d) ratio 10 and e) ratio 20

According to the phase plot of CS-miRNA complex in suspension (A of Figure 3.3), nanocomplexes prepared at N/P ratios 0.1 and 0.5 were negatively charged, whereas those prepared at N/P ratios 10 and 20 were positively charged. Of particular interest here are those nanocomplexes prepared at N/P ratio equal to 1 that show ζ potential close to 0 mV as expected due to the balance between positive and negative charges in nanocomplexes. Thus, the reversion in the net charge of the nanocomplexes is around N/P ratio equal to 1.

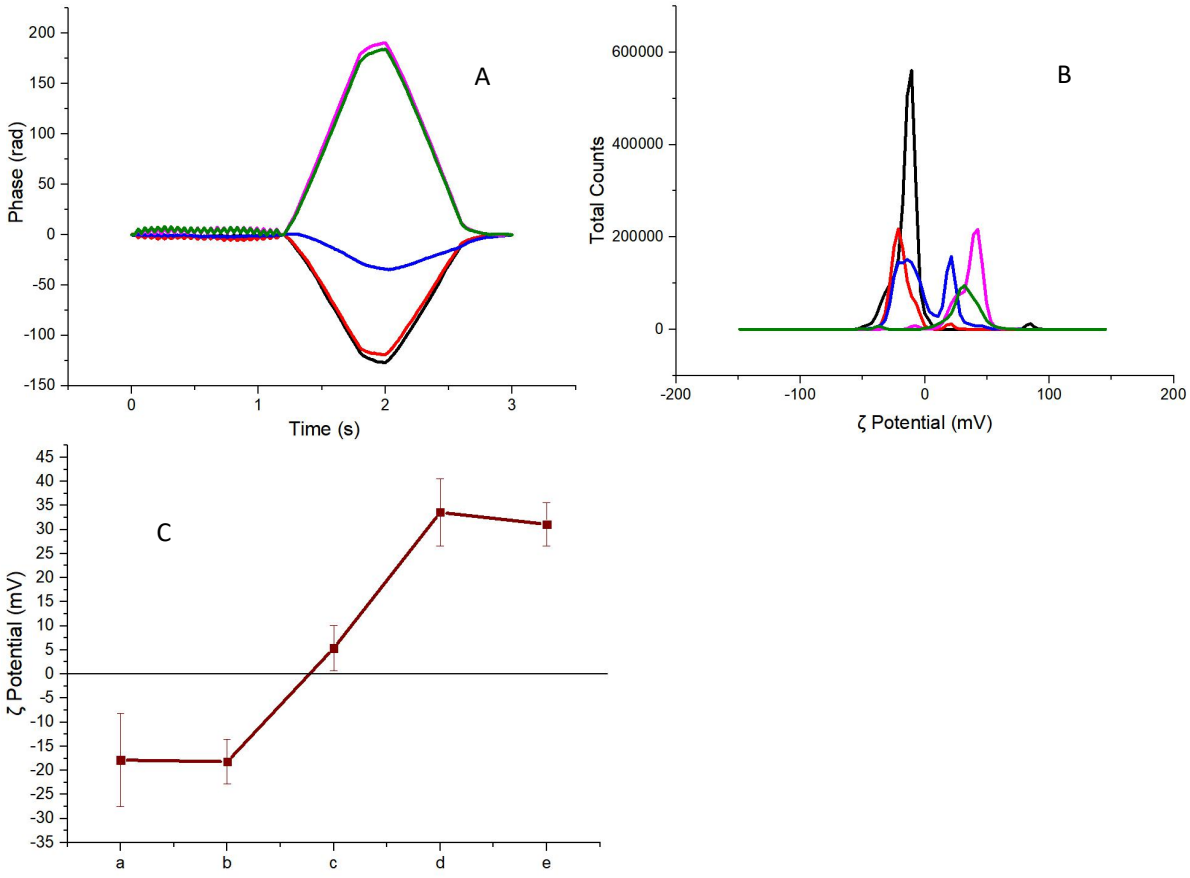

Figure 3.3. Phase plot (A), ζ potential distribution (B) and average ζ potential (C) of CS-miRNA complexes at N/P ratio a) 0.1 (black), b) 0.5 (red), c) 1 (blue), d) 10 (pink) and e) 20 (green)

Increasing the N/P ratio resulted in an increase of ζ potential (B and C of Figure 3.3, Table 3.1). The increment was not seen in all range of N/P ratios

studied. In the range of N/P from 0.1 to 0.5, average ζ potential was barely changed. Explanation for these results is that at low N/P ratios, total amount of negatively charged phosphate groups on nucleic acid exceeded that of positively charged amino group on chitosan molecules. In the N/P ratios 10 and over, the ζ potential remain unchanged when reach a value around +30 mV. The most probable explanation is that the positive charges that chitosan carried were limited, and it was unable to overload the ζ potential of the complex go beyond chitosan's own ζ potential.

Table 3.1. Average ζ potential of CS-miRNA complexes

N/P ratio	ζ potential (mV)
0.1	-17.86
0.5	-18.20
1	+5.37
10	+33.58
20	+31.09

In brief, with the addition of chitosan into suspension, positively charged cationic macromolecules interacted with negatively charged nucleic acid molecules forming CS-RNA complexes. At low N/P ratios, negatively charged phosphate groups over protonated amino groups and negatively charged nanocomplexes were obtained. Increasing chitosan, resulted in positive charged nanocomplexes.

In summary, particle size distribution was broader for CS-miRNA nanocomplexes at low N/P ratio equal to or below 1. The balance charges of nanocomplexes prepared at N/P ratio below 1 means that the charges from chitosan were less abundant than the charges from phosphate groups in miRNA. Moreover, the negative ζ potential of such nanocomplexes indicated

that the miRNA was exposed on their surface. It is important to note that the amount of miRNA was kept constant in all the nanocomplexes formulations, therefore, the changes in N/P ratio was achieved by the addition of chitosan. In those CS-miRNA nanocomplexes with N/P ratio below 1, the amount of chitosan added was low enough to not produce a change in the ζ potential of the miRNA. Although it is important to point that there was enough amount of chitosan to obtain nanocomplexes with uniform particle size distribution as shown from the DLS analysis. Also, of great importance is the fact that the system had so little mass in it that the nanocomplexes were difficult to detect in the system, as seen from the correlation function. When increasing the amount of chitosan in the system to create CS-miRNA nanocomplexes at N/P ratio equal to 1, the charges were balanced, as can be seen from the ζ potential value. And higher N/P ratio in the system resulted in a better detection of the nanocomplexes as seen from the correlation function. In this condition the particle size distribution was still broad, with two populations in the distribution by intensity. The most plausible explanation for that result is that the lack of electrostatic repulsive forces on the surface of the nanocomplexes did not avoid the presence of aggregation of complexes. The increase of chitosan in the system to prepare CS-miRNA nanocomplexes at N/P ratio equal to and above 10 resulted in nanocomplexes more detectable in the system and seen from their correlation function. Those nanocomplexes showed a strong positive net charge that could be responsible for the presence of nanocomplexes of lower size and with a narrow distribution. Together, these results indicate that chitosan and miRNA interacted at all the ratios prepared in this experimental work and that the higher the content of chitosan, the more the compact the nanocomplexes obtained.

3.1.2 Chitosan-complementary miRNA (CS-comp-miRNA) complexes

Particle size distribution by intensity of CS-comp-miRNA complexes (A of

Figure 3.4) at N/P ratio 0.1, 0.5, 1, 10, and 20 were found in the range between 24 - 3477 nm, 16 - 1900 nm, 27 - 3477 nm, 33 - 660 nm, and 50 - 768 nm, respectively. And the particle size distribution by number of CS-comp-miRNA complexes at the same ratios were found in the range between 0 - 80 nm, 12 - 80 nm, 21 - 93 nm, 24 - 125 nm, and 28 - 230 nm, respectively. Peaks of the particle size distribution by number of CS-comp-miRNA complexes at the same ratios were found in 0.638/5.279/20.53 nm, 20.53 nm, 32.3 nm, 37.56 nm, 59.07 nm (B of Figure 3.4). Regarding the correlation function of the measurements, detectable particles in the suspension increased as increasing N/P ratio (C of Figure 3.4). Low parameters of complexes at low ratio in B and C indicated that there were low amount of detectable particles formed in the suspension.

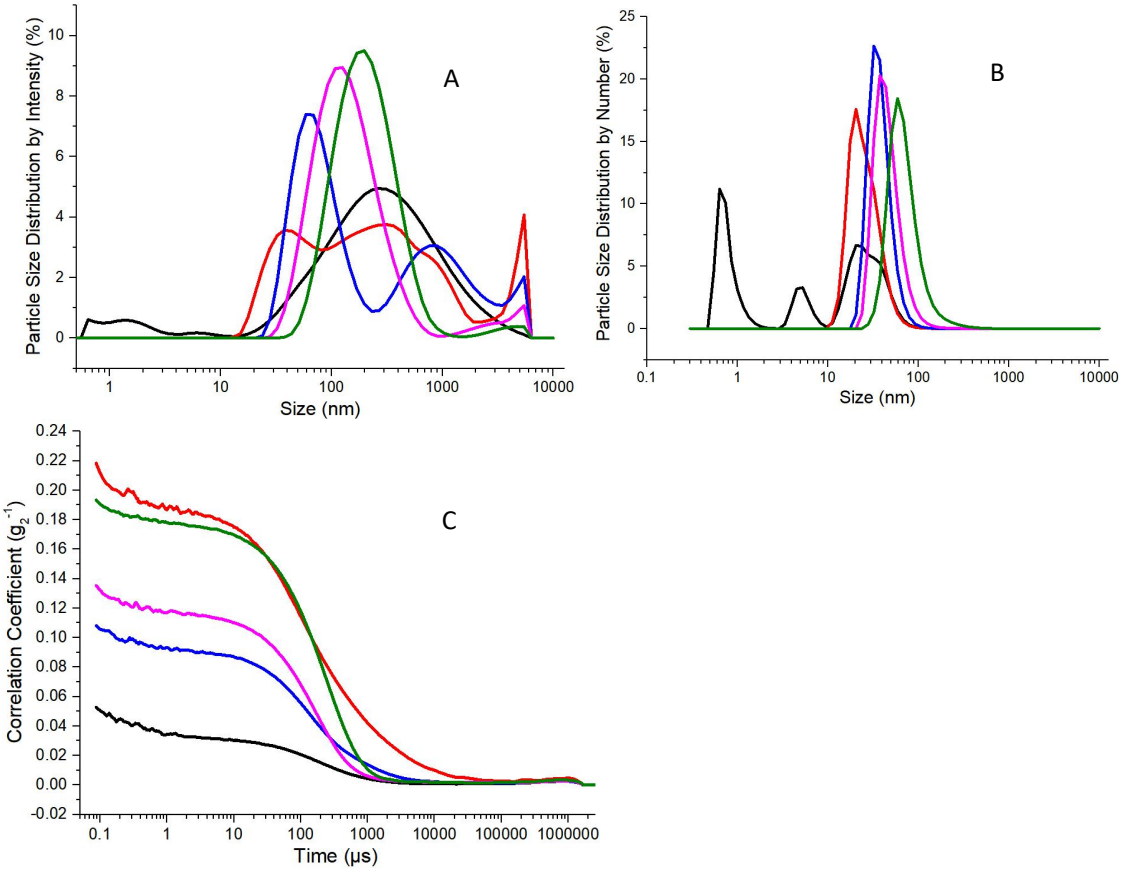

Figure 3.4. Particle size distribution by intensity (A), particle size distribution by number (B) and correlation functions (C) of CS-comp-miRNA complexes at charge ratio 0.1 (black), 0.5 (red) 1 (blue), 10 (pink) and 20 (green)

PDI results from the analysis of CS-comp-miRNA nanocomplexes (A of Figure 3.5) were in line with the results obtained for CS-miRNA nanocomplexes. This is chitosan and comp-miRNA nanocomplexes at N/P ratio equal to or below 1 were not reproducible, as can be seen by the great standard deviation of the averages. Again, increasing N/P ratio of CS-comp-miRNA nanocomplexes resulted in decreasing the standard deviation of the averages and increasing the reproducibility of the measurements. The PDI values of CS-comp-miRNA nanocomplexes prepared at N/P ratios equal to or higher than 10 showed the lowest value, namely 0.3, indicating that these systems were monodisperse. However, the systems prepared at N/P ratio 0.1, 0.5 and 1 showed PDI value over 0.4, indicating polydisperse nature of the samples. Indeed, the lower the N/P ratio, the higher the PDI value (Figure 3.5).

According to the average particle size (B of Figure 3.5), the minimum average size was recorded for CS-comp-miRNA nanocomplexes prepared at N/P ratio 1 that showed an average hydrodynamic diameter of 119.23 nm. As seen in the case of CS-miRNA nanocomplexes, the average size of the nanocomplexes incremented when decreasing or increasing the N/P ratio.

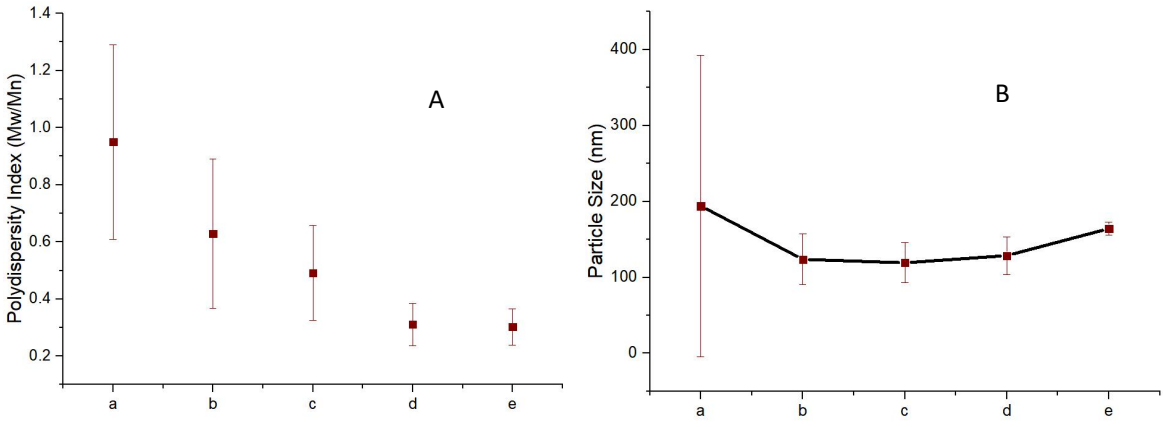

Figure 3.5. Polydispersity index (PDI) (A) and average particle size (B) of CS-miRNA complexes at ratio a) ratio 0.1, b) ratio 0.5, c) ratio 1, d) ratio 10 and e) ratio 20

Figure 3.6 listed below were electric phase diagram, ζ potential distribution, and average ζ potential results of CS-comp-miRNA complexes in DLS experiment at five different N/P ratios, a) ratio 0.1, b) ratio 0.5, c) ratio 1, d) ratio 10 and e) ratio 20.

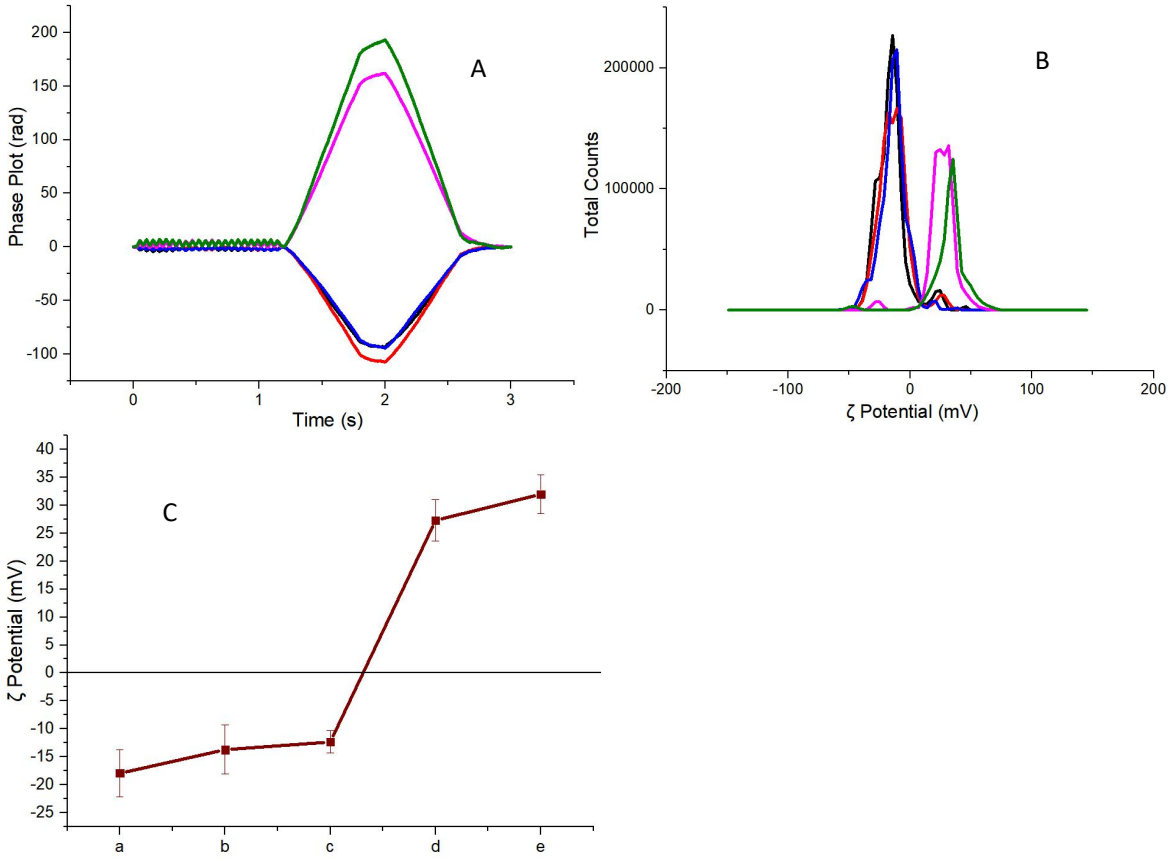

Figure 3.6. Phase plot (A), ζ potential distribution (B) and average ζ potential (C) of CS-comp-miRNA complexes at N/P ratio a) 0.1 (black), b) 0.5 (red), c) 1 (blue), d) 10 (pink) and e) 20 (green)

CS-comp-miRNA complexes in suspension were negatively charged at ratio 0.1, 0.5 and 1 (Figure 3.6). When N/P was increased to 10 and above, the nanocomplexes were positively charged. Unlike CS-miRNA nanocomplexes, CS-comp-miRNA nanocomplexes showed a monodisperse ζ potential distribution, even at N/P ratio 1, the population of nanocomplexes were found in the negative part of the x-axis instead than being found around 0 mV (C of Figure 3.6).

The ζ potential of CS-comp-miRNA nanocomplexes showed that the reversion in the net charge occurred at any point between N/P ratio 1 and 10 (Table 3.2). As in the case of CS-miRNA nanocomplexes, CS-comp-miRNA at N/P ratio 0.1 the ζ potential was -18 mV, and the N/P ratio 20 was +32 mV. Nevertheless, the other ζ potential values were not the same as the case of nanocomplexes prepared with chitosan and miRNA, indicating that the complementary miRNA and the miRNA interacted differently with chitosan.

Table 3.2. Average ζ potential of CS-comp-miRNA complexes

N/P ratio	ζ potential (mV)
0.1	-17.95
0.5	-13.74
1	-12.34
10	+27.31
20	+31.99

3.1.3 Chitosan-double stranded miRNA (CS-ds-miRNA) complexes

Particle size distribution by intensity of CS-ds-miRNA complexes at ratio 0.1, 0.5, 1, 10, and 20 were found in the range between 28 - 1209 nm, 20 - 5468 nm, 21 - 4702 nm, 11 - 488 nm, and 24 - 568 nm, respectively (A of Figure 3.7). The particle size distribution by number of the same nanocomplexes were found in the range between 9 - 108 nm, 8 - 80 nm, 10 - 69 nm, 7 - 51 nm, and 7 - 60 nm, respectively. And peaks of the particle size distribution by number of the same nanocomplexes were found in 0.638/37.56 nm, 20.53 nm, 27.77 nm, 17.66 nm, 11.23/23.88 nm, respectively. (B of Figure 3.7). According to the correlation functions of the measurements of CS-ds-miRNA nanocomplexes, increasing the N/P ratio resulted in increasing the intercept

and, thus, increase the sensitivity of the measurements (C of Figure 3.7), reaching 0.65 in the case of the N/P ratio equal to or higher than 10.

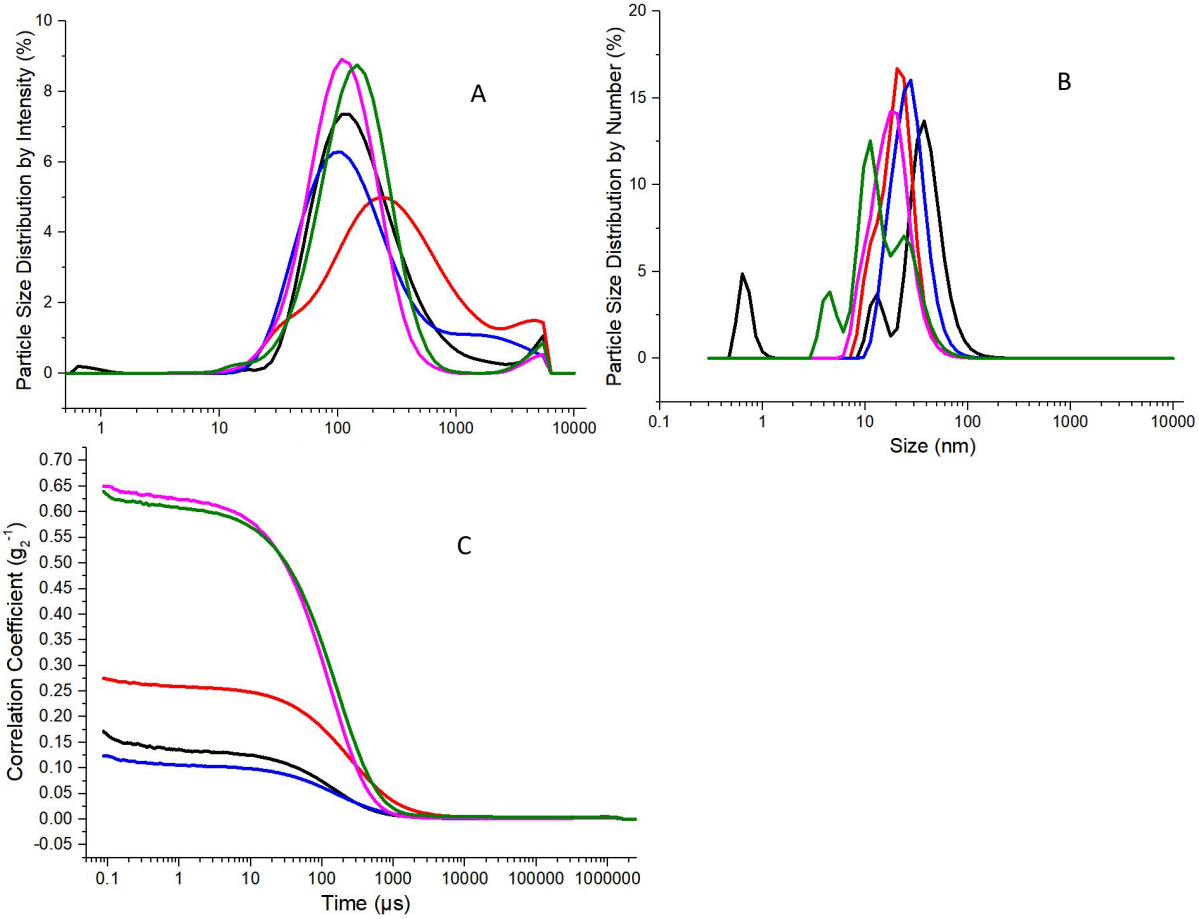

Figure 3.7. Particle size distribution by intensity (A), particle size distribution by number (B) and correlation functions (C) of CS-ds-miRNA complexes at charge ratio 0.1 (black), 0.5 (red) 1 (blue), 10 (pink) and 20 (green)

The PDI values recorded from the measurements of CS-ds-miRNA nanocomplexes (A of Figure 3.8) were similar to the results of CS-miRNA nanocomplexes (A of Figure 3.2). This is, nanocomplexes prepared at N/P ratio equal to or below 1 showed poor reproducibility as per their standard deviation. The reproducibility was increased at N/P ratio higher than 1. Again, the PDI value was in the range 0.2 to 0.3 in the case of CS-ds-miRNA nanocomplexes prepared at N/P ratio equally to 10 and 20, respectively, indicating monodispersity of these systems. On the other side, the PDI value was higher than 0.4 for the nanocomplexes prepared at N/P ratio below 10,

which revealed the polydispersity of the systems.

Regarding the average particle size (B of Figure 3.8) of the CS-ds-miRNA nanocomplexes, the average size reached its minimum for the nanocomplexes prepared at N/P ratio 10, that showed a hydrodynamic diameter equal to 97.31 nm. As in the cases of both CS-miRNA nanocomplexes and CS-comp-miRNA nanocomplexes, the size of CS-ds-miRNA nanocomplexes increased when either decreasing or increasing the N/P ratio.

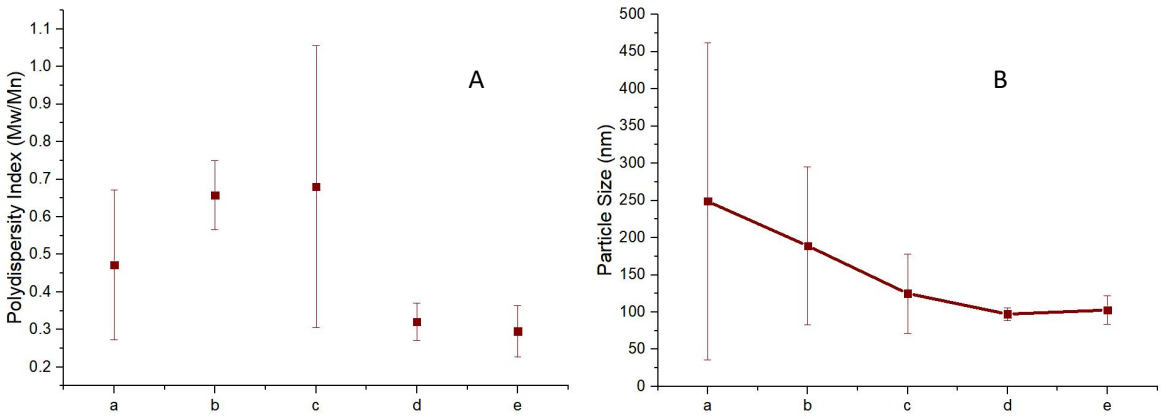

Figure 3.8. Polydispersity index (PDI) (A) and average particle size (B) of CS-ds-miRNA complexes at ratio a) ratio 0.1, b) ratio 0.5, c) ratio 1, d) ratio 10 and e) ratio 20

Figure 3.9 below were electric phase diagram, ζ potential distribution, and average ζ potential results of CS-ds-miRNA complexes in DLS experiment at five different N/P ratios, a) ratio 0.1, b) ratio 0.5, c) ratio 1, d) ratio 10 and e) ratio 20.

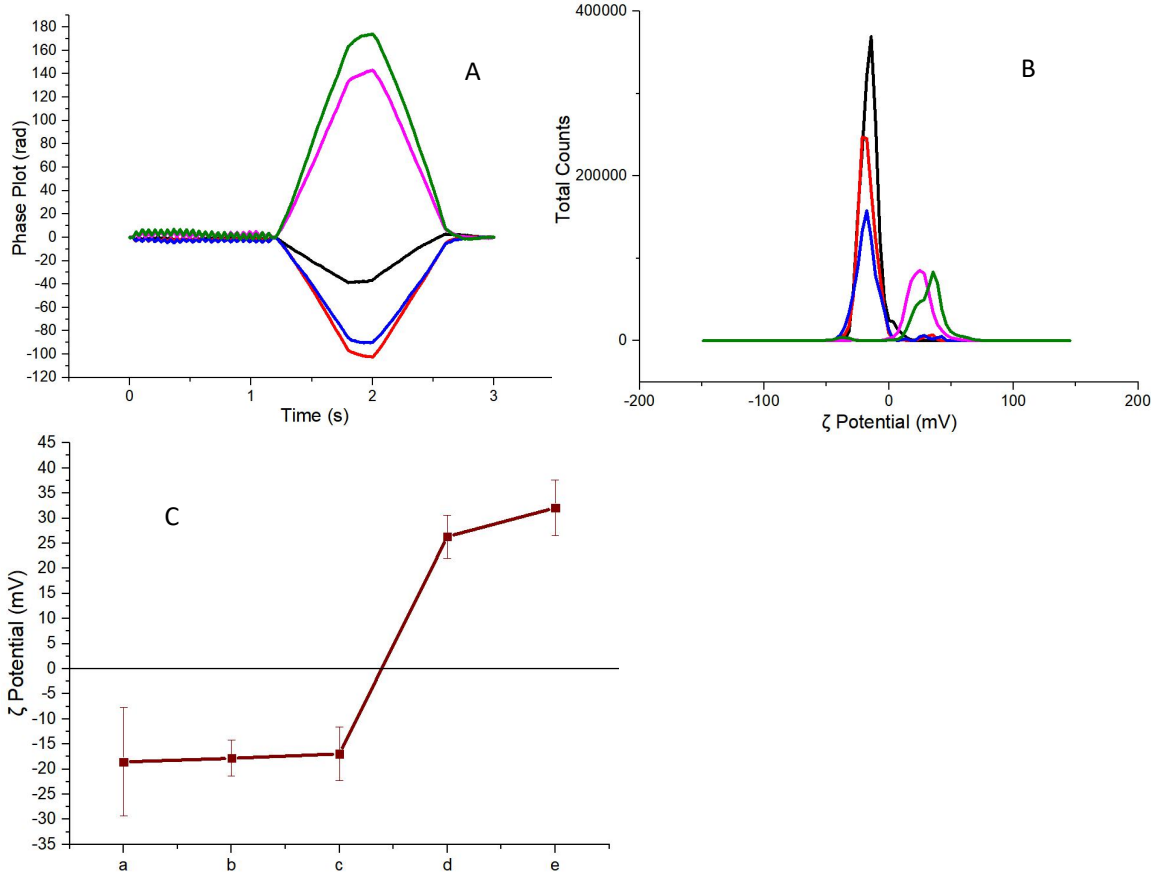

Figure 3.9. Phase plot (A), ζ potential distribution (B) and average ζ potential (C) of CS-ds-miRNA complexes at N/P ratio a) 0.1 (black), b) 0.5 (red), c) 1 (blue), d) 10 (pink) and e) 20 (green)

CS-ds-miRNA nanocomplexes in suspension were negatively charged at N/P ratios 0.1, 0.5 and 1, according to the phase plot (A of Figure 3.9). However, when increasing the N/P ratio up to 10 and above, the nanocomplexes showed a positive net charge. The amount of ds-miRNA was constant in all the nanocomplexes. Thus, the increase in N/P ratio was achieved by the addition of chitosan. The increment of chitosan in the formulation resulted in the complete reversion of the net charge of the nanocomplexes as can be seen from the ζ potential distribution plot (B of Figure 3.9), which showed the nanocomplexes populations in the negative or positive part of the x-axis but none of them on the neutral net charge region. The average values of the ζ potential of CS-ds-miRNA nanocomplexes are included in Table 3.3. As in

previous cases, ζ potential value increased as the N/P ratio, at which the nanocomplexes were prepared, increased. The ζ potential at N/P ratio 0.1 was -19 mV and +32 mV at ratio 20, which corresponded to the ζ potential of ds-miRNA and chitosan, respectively. These results were the same than found in previous cases. The reversion of the ζ potential occurred at N/P ratio between 1 and 10, as seen previously for N/P nanocomplexes composed of CS and comp-miRNA.

Table 3.3. Average ζ potential of CS-ds-miRNA complexes

N/P ratio	ζ potential (mV)
0.1	-18.55
0.5	-17.83
1	-16.94
10	+26.32
20	+32.05

3.2 Native electrophoresis retardation assay

To prove the effective interaction between chitosan and ds-miRNA to result in nanocomplexes, gel retardation assay was performed. For that purpose, given its importance to the nucleic acids, the electrophoresis was performed under native conditions. The success in the gel retardaiton assay was proved by the presence of the ds-miRNA close to the 35 base pairs in the gene ruler (Figure 3.10). The CS-ds-miRNA nanocomplexes at N/P ratio 0.1, 0.5 and 1 appeared as bands in the same region were ds-miRNA appeared. In the cases of nanocomplexes prepared at N/P ratio 10 and 20, smeared bands were observed. Indeed, the higher the N/P ratio was, the longer the smeared band appeared (Figure 3.10).

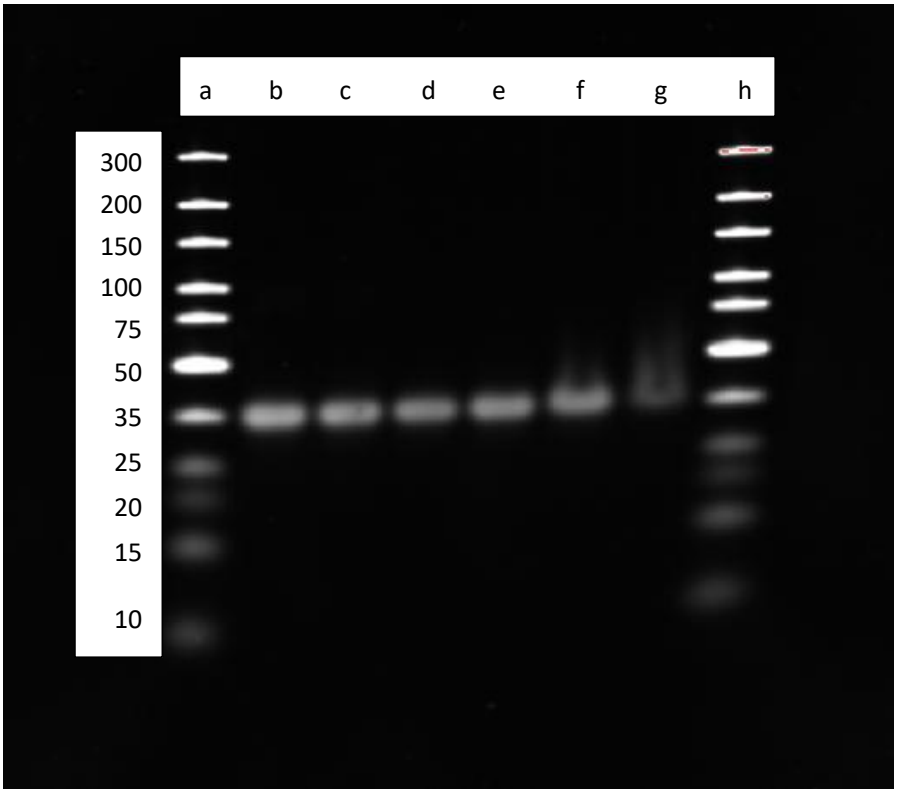

Figure 3.10. Gel retardation assay of CS-ds-miRNA nanocomplexes at different N/P charge ratios. The content of the lanes were pure ds-miRNA (b), CS-ds-miRNA nanocomplexes at N/P ratio 0.1 (c), 0.5 (d), 1 (e), 10 (f) and 20 (g). As control of size, Gene ruler (a, h) was included in the experiment

Migration of ds-miRNA occurred due to its negative charge. Naked nucleic acid can run through the gel and show a specific band. Nanocomplexes prepared at low N/P ratios did not show a different aspect compared to ds-miRNA. In fact, these negatively charged nanocomplexes showed similar electrophoretic mobility as ds-miRNA under electric field. However, nanocomplexes prepared at N/P ratio above 1, showed different electrophoretic mobility as their ζ potential were different. The presence of smeared bands, that appeared in lanes containing nanocomplexes prepared at N/P ratio equal to 10 and 20, evidenced that the electrophoretic mobility of ds-miRNA was retarded due to the interaction with chitosan.

Eventually, considering the biophysical characterization of CS-ds-miRNA nanocomplexes, those CS-ds-miRNA nanocomplexes at ratio 20 were chosen as carriers for treating glioblastoma cells in subsequent cell experiments.

3.3 Evaluation of cytotoxicity

In glioblastoma cells (U251 cell line), MTT assay was conducted to evaluate the cytotoxicity effect of chitosan and ds-miRNA. Dilution control due to the application of the treatment was included. This condition was named as DNase/RNase free water. Negative control of metabolic activity was performed by the addition of triton X-100 to the culture media. And positive control of metabolic activity was consisted on the growth of U251 cells with no additives to the culture media. The MTT assay was performed after 48 hours and 72 hours from the application of the treatments to the cells (Figure 3.11). The determination of the cytotoxic activity was calculated as in equation E (page 33) and the results were included in Table 3.4. Neither ds-miRNA nor chitosan affected the metabolic activity of glioblastoma cells. The metabolic activity was around 95 – 97 %, in both cases for the 72 h period (Table 3.4).

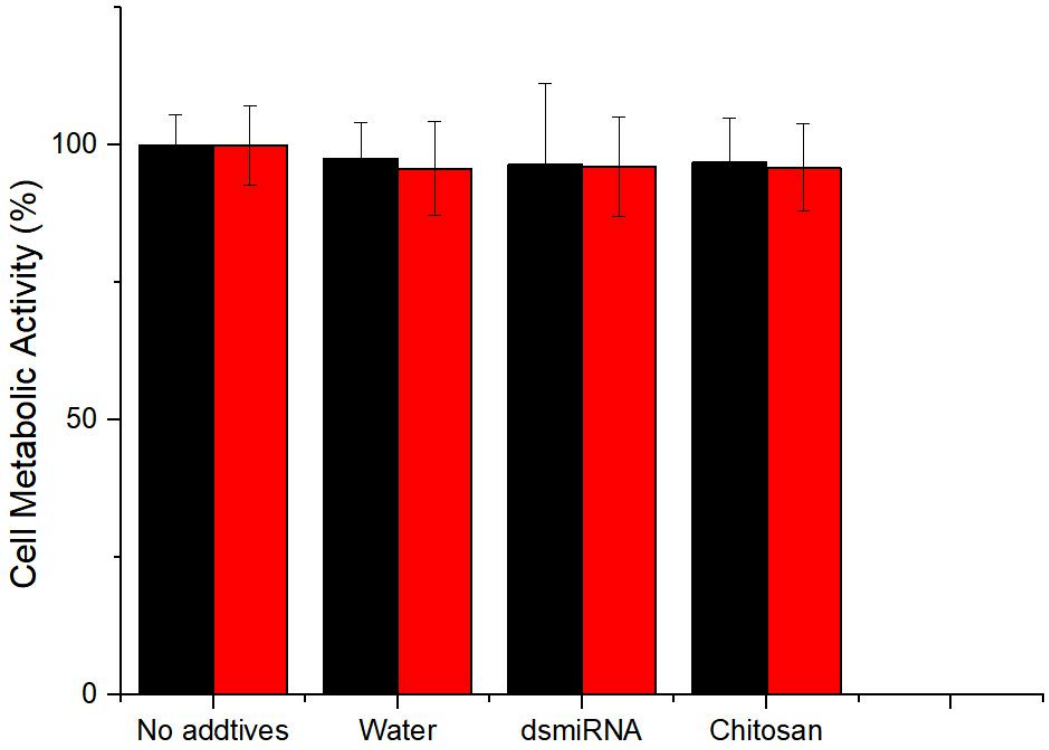

Figure 3.11. Results of cell metabolic activity determined by MTT assay on glioblastoma cells treated under different conditions (no additives, DNase/RNase free water, ds-miRNA, chitosan). Measurements were carried out after 48 hours (black) and 72 hours (red) from the application of treatment

Table 3.4. Metabolic activity of glioblastoma cells by MTT cytotoxicity assay after being treated under different conditions (no additives, DNase/RNase free water, ds-miRNA, chitosan)

	No additives	**DNase/RNase free water**	**Ds-miRNA**	**Chitosan**
48h (%)	99.99	97.54	96.45	96.99
72h (%)	100.00	95.77	96.12	95.97

3.4 In vitro transfection of CS-ds-miRNA complexes on glioblastoma cells

The cytotoxic effect of the treatment of the cells with the gene delivery system developed, CS-ds-miRNA nanocomplexes at N/P ratio equal to 20, on glioblastoma cells was carried out by MTT assay. The cell metabolic activity was calculated by using the equation E (page 33) in methodologies.

Lipofectamine was confirmed as an effective gene delivery carrier, that effectively delivered ds-miRNA (5 nM) within the cell, which impaired in a significant way the metabolic cell activity ($p < 0.001$) (Figure 3.12). Also lipofectamine itself impaired cell metabolic activity after 72 hours of treatment ($p = 0.016$).

Cell metabolic activity of glioblastoma cells indicated an inhibitory effect of the CS-ds-miRNA nanocomplexes. The proposed concentration for the treatment with ds-miRNA was 100 nM. The cell metabolic activity of three different dilutions 0.5X, 1X and 2X, considering X as the proposed concentration, was 82.74%, 78.79% and 68.46%, respectively, after 48h of treatment application. And it was 65.17%, 62.13%, 55.35%, respectively, after 72h of treatment application. The statistics analysis, to what extent α was considered as 0.05, revealed that all treatments with CS-ds-miRNA

resulted in a significant decrease in the metabolic activity ($p < 0.001$) when compared to the control of metabolic activity. Results from Figure 3.12 and Table 3.5 indicate that after treatment with CS-ds-miRNA nanocomplexes 2X for 72 hours of incubation, cell metabolic activity dropped to around 50% indicating a good inhibitory ability of this nanosystem on glioblastoma cells. Results revealed a linear dependence between the effect on the metabolic activity of the cells and the CS-ds-miRNA nanocomplexes concentration in culture media at different times studied, this is 48 hours and 72 hours (Figure 3.13). It was indicated that the more nucleic acids that successfully delivered into cytoplasm and escape from lysosome, the greater the chance of intervening in the process of cell division and protein synthesis over time.

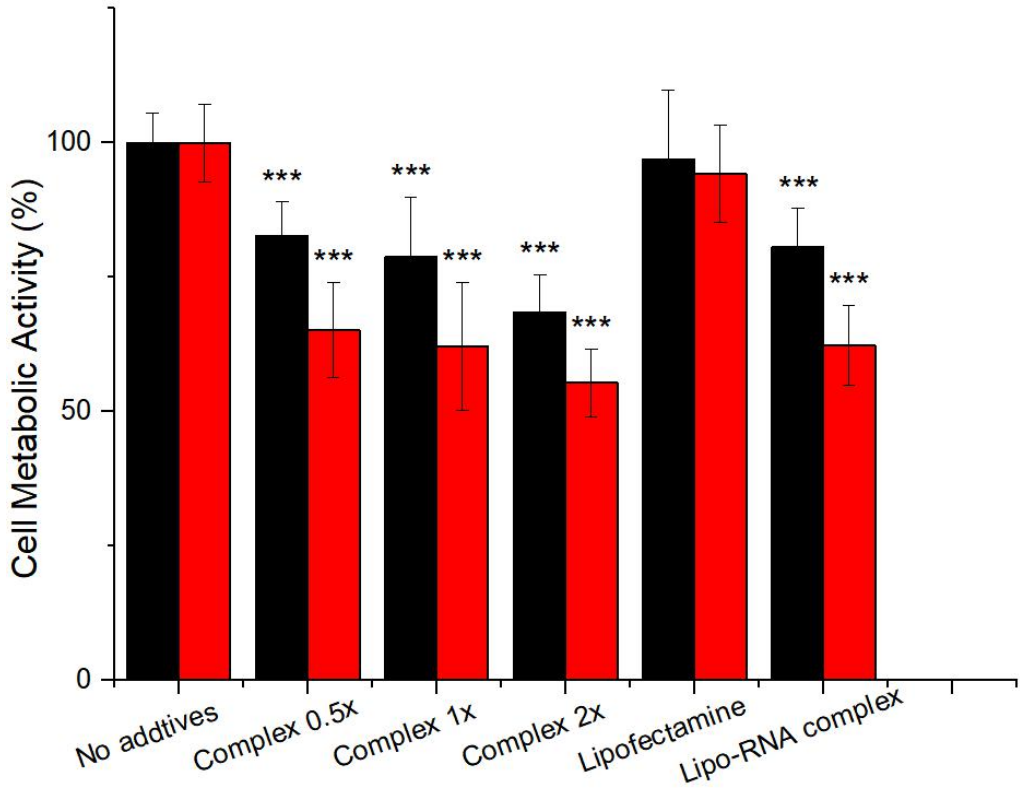

Figure 3.12. Cell metabolic activity of glioblastoma cells by MTT assay after cells were treated by CS-ds-miRNA complexes at N/P ratio 20 applied at 0.5X, 1X and 2X proposed doses of 100 nM ds-miRNA. Positive control release consisted on the treatment of cells by ds-miRNA delivered by lipofectamine. The lipofectamine itself was included as a control. Measurements were carried out after 48 hours (black) and 72 hours (red) from the application of treatment

Table 3.5. Cytotoxicity of complexes measured by MTT assay. Glioblastoma cells were treated by CS-ds-miRNA nanocomplexes at N/P ratio equal to 20 and at 0.5X, 1X, and 2X proposed doses (X= 100 nM), by lipofectamine, and Lipofectamine-ds-miRNA (5 nM)

	No additives	**Complexes 0.5X**	**Complexes 1X**	**Complexes 2X**	**Lipofectamine**	**Lipo-RNA complexes**
48h (%)	99.99	82.74	78.79	68.46	96.91	80.58
72h (%)	100.00	65.17	62.13	55.35	94.29	62.39

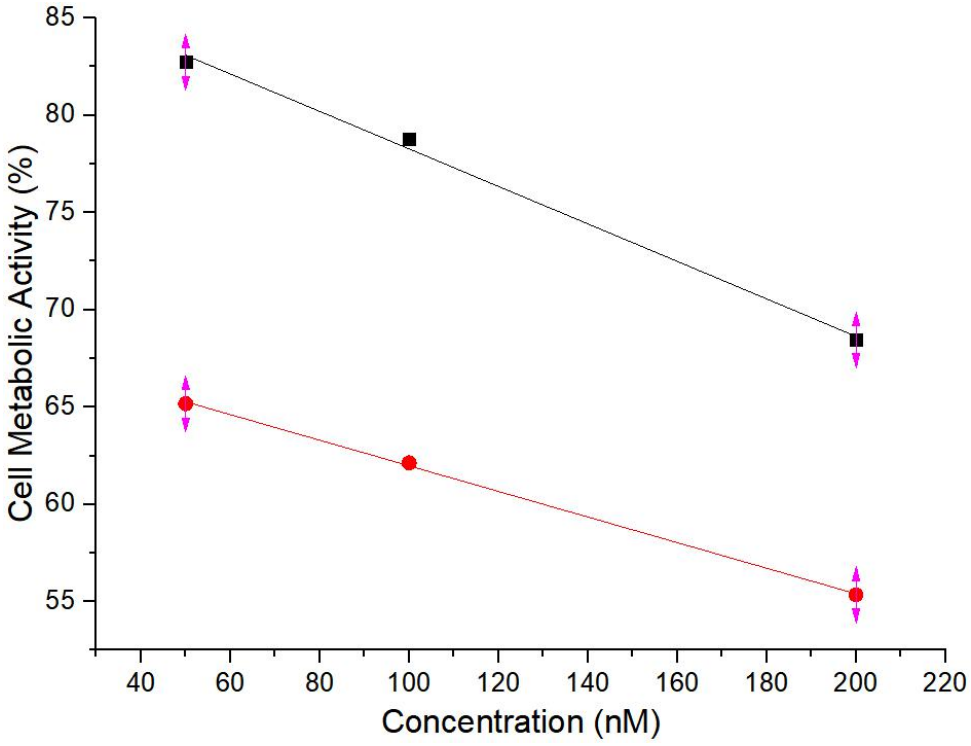

Figure 3.13. Linear fit for results of MTT assay on cell metabolic activity of samples treated by CS-ds-miRNA nanocomplexes at N/P ratio 20 after 48 hours (black) (Fitting line: $y = -0.09636x + 87.905$, $R^2 = 0.99224$) and 72 hours (red) (Fitting line $y = -0.0658x + 68.56$, $R^2 = 0.99862$) of incubation

Chapter 4

Conclusion

So far, chitosan has been widely used in many scientific research studies and life applications for decades which stated that chitosan has a promising future in various areas. Chitosan in gene delivery is thought to be able to condense nucleic acid via electrostatic self-assembly and successfully delivers it into cytoplasm, protecting it from degrading by lysosome in the intracellular environment. Inspect from all these researches, miRNA has been proved having RNA interference silencing effect on U251 cell line (glioblastoma cells), inhibiting synthesis of proteins intracellular.

This study presented the success of a non-viral gene delivery based on chitosan for the treatment of glioblastoma cells (U251 cell line). Nanocomplexes in this study were prepared with chitosan (DA: 29 %). Such nanocomplexes were used to load a double stranded miRNA for the treatment of glioblastoma cells.

From the results in this work, it can be concluded that chitosan successfully combined with miRNA, complementary miRNA and double stranded miRNA by the interaction between protonated amino groups on chitosan and negatively charged phosphate group on nucleic acid.

The dynamic light scattering experiments revealed that there is a N/P ratio that showed a minimal average particle size and either increasing or decreasing the N/P ratio, the size of the particles increased. Regarding ζ potential results, complexes were negatively charged when N/P ratio was lower than 1, meaning amount of phosphate group in suspension was exposed to the surface of the nanostructures. ζ potential reached zero at a N/P ratio between 1 and 10. And ζ potential of nanocomplexes at N/P ratio equal to 20 reached ζ potential of the chitosan, this is around +33 mV.

Electrophoresis retardation assay revealed a band in the lanes containing CS-ds-miRNA at N/P ratios 0.1, 0.5 and 1 but smeared bands in the cases of CS-ds-miRNA nanocomplexes at N/P ratios 10 and 20, demonstrating the interaction between chitosan and ds-miRNA.

Cytotoxicity analyses by MTT assay indicated that chitosan and ds-miRNA alone did not impaired the metabolic activity of glioblastoma cells, whereas CS-ds-miRNA nanocomplexes at N/P ratio equal to 20 successfully impaired the cell metabolic activity. It was found a linear dependence between the nanocomplexes concentration and the metabolic activity impairment.

It summary, it can be concluded that chitosan (DA: 29 %) can be facilitated as a non-viral gene delivery vector for loading and releasing ds-miRNA, by the preparation of CS-ds-miRNA nanocomplexes at N/P ratio equal to 20. And that the use of the cited nanocomplexes can be used for the treatments against glioblastoma cells.

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