Doctor's Thesis

Regime of gene silencing: Efficient siRNA delivery into cancer cells using nanocapsules

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PREFACE

RNA interference (RNAi) is a gene regulation mechanism initiated by RNA molecules that enables sequence-specific gene silencing by promoting degradation of specific mRNAs. Molecular therapy using small interfering RNA (siRNA) has shown great therapeutic potential for diseases caused by abnormal gene overexpression or mutation. The major challenges faced in application of siRNA therapeutics include the stability and effective delivery of siRNA *in vivo*. Important progress in nanotechnology has led to the development of efficient siRNA delivery systems. Polymeric nanoparticles being biodegradable and biocompatible are used widely in the field of siRNA delivery. PLGA [poly (lactic- co-glycolic) acid] is United States Food and Drug Administration approved and possesses good physiochemical properties that are highly accessible for surface modifications. Nanoparticles synthesized from PLGA have generated increasing interest in the field of medical therapeutics and clinical trials.

This thesis entitled "**Regime of Gene Silencing: Efficient siRNA delivery using Nanocapsules**" is divided into six chapters that deals with the synthesis, characterization and biological application of siRNA encapsulated hollow PLGA nanocapsules.

In **chapter one**, we present the techniques used in siRNA delivery in RNAi therapeutics. Nanomedicine is giving rise to the advanced nano-biomedical techniques and has shown successful results in gene delivery. Gene therapy using siRNA has demonstrated therapeutic potential for diseases caused by over-expression of disease-associated genes. In this chapter, we present a brief history on the techniques used in siRNA delivery.

In **chapter two**, the theoretical details of the techniques and instrumentation to characterize the nanomaterials and its biological properties are discussed. The basic understanding and analysis of the physiochemical properties of nanomaterials are essential to acquire insight into the unique properties of nano-scale materials.

In **chapter three**, we discuss the importance of siRNA delivery in cancer cells by using different types of nanomaterials and their applications in targeted delivery. The basic understanding of gene delivery and RNAi therapeutics are discussed in this

chapter. siRNA delivery is a reliable and effective tool that can be used in the treatment of many diseases. We also discuss the application of polymeric nanoparticles in siRNA delivery.

In **chapter four**, we discuss the formulation of highly efficient and biocompatible PLGA hollow nanocapsules. Our work proposes a novel route to prepare hollow PLGA NPs (HNPs), which showed increased drug-encapsulation and release efficiency. Simple emulsion solvent evaporation technique was adopted to synthesize nano-hollow shells. We report the synthesis of PLGA hollow nanoparticles, which released the drug in controllable manner that can be used as nano vechicle to deliver gene and drug in cell organelles.

In **chapter five,** we have presented the use of hollow PLGA nano-capsules for the delivery of siRNA into the cancer cells by using highly efficient cell penetrating targeting moieties. siRNA was encapsulated in PLGA hollow nanoparticles. We found enhanced anti-proliferation and down regulation of *MYC* proto-oncogene expression in cancer cells on treatment with siRNA encapsulated functionalized PLGA nanoparticles.

The concluding remarks are presented in **chapter six**.

Contents

Chapter 1
History of Techniques used in siRNA delivery in the field of nanomedicine

Abstract

Nanomedicine is giving rise to advanced nano-biomedical techniques and producing successful results in gene delivery. The potential ability of small interfering RNA (siRNA) to silence abnormal gene over-expression created a new horizon in the field of gene therapy. The major hindrances in the application of siRNA therapeutics are safety of gene, stability and effective delivery of siRNA in *in vivo* to reach the targeted cells. In this chapter, we present a brief history on the techniques used so far in siRNA delivery. We also mentioned about the future aspects of designing nanoparticles for effective siRNA delivery.

1.1. Introduction

Nanomedicine is a rapidly developing field in science giving satisfactory and promising results in drug and gene delivery. Until now, nanoparticles (NPs) played a major role in drug delivery. They are also utilized vastly in the field of gene delivery; especially in RNAi (RNA interference) therapeutics [1]. Various types of NPs are administered and have been proved efficacious in gene delivery; however the research is still in progress.

Organic and inorganic NPs, especially those, which are cationic in nature, show enhanced results in drug and gene delivery. NPs based targeted gene therapy aims to shuttle gene to the site-specific target so as to deliver effective concentrations of therapeutic drugs [2]. Despite many cellular and tissue-level hurdles, non-viral deliveries of NP-based approaches have been developed and hold great potential to transform medicine effectively [1, 3]. NPs have played a major role in gene targeting and delivery methods, giving the results for down-regulation of certain overexpressed gene in the diseased cells. siRNA treatment in non-viral gene delivery includes the insertion of siRNA using various types of NPs [4].

siRNA are small interfering RNA segments that are 23-29 nucleotides long and are categorized in non-coding genes. These siRNAs have the ability to silence the expression of almost all genes present in eukaryotic cells hypothetically. In practice, many diseases have been treated with high success rate. Therefore, siRNA treatment is gaining attention among researchers. RNAi treatment is giving effective results however there are some barriers that siRNA faces when administered inside the bloodstream and cell [4-6].

The major drawbacks using siRNA-based gene therapies without using any vectors are (i) low cytosol uptake, (ii) degradation of gene by enzymes, nucleases and (iii) rapid clearance through kidney, spleen, liver following systemic administration [5, 6]. The negative charge of siRNA fragments prevents active intracellular delivery. Cell membrane also has a negatively charged backbone and higher molecular weight, leading to electrostatic repulsion. Therefore, in this dilemma the NPs play a critical role in transportation of siRNA [4, 7]. Nanotechnology along with gene delivery is a reliable method that results in siRNA delivery in the reduction of multiplication of cancer cells by cleaving messenger RNA (mRNA) & breaking down the transcription resulting in no protein synthesis. NPs, which are inert, cationic, relatively moderate size (ranging from 60~300 nm) and having a large surface area are used and have shown promising results in siRNA delivery [4-12].

So far NPs have been an excellent vector for gene delivery, however the major barriers faced in the complete delivery of siRNA are (i) immune clearance through the liver and spleen, (ii) permeation through the endothelium into target tissues, (iii) penetration through the tissue and renal clearance, (iv) endosomal escape in target cells, (v) diffusion through the cytoplasm and (vi) entry into the nucleus. Also, the bio distribution of NPs varies according to the route of transport. The cellular uptake of the NPs carrying siRNA is unequal, leading lower effect and thereby increase in dose of the siRNA-NPs complex becomes necessary [13, 14].

In this chapter we focused on the techniques that are used to formulate NPs for targeted delivery of siRNA. We have emphasized on the upcoming techniques used so far in fabrication of different kinds of NPs and the effect in

targeted delivery. These different techniques involved in siRNA delivery give a clear picture for inventing novel ideas for delivering the drug completely in the cells in addition to bypassing the existing barriers.

1.2. Techniques for siRNA delivery

In the past decade siRNA delivery has been researched thoroughly. Transfection, electroporation and viral gene transfer were the delivery methods used earlier for gene delivery. However, due to emergence of nanotechnology the transfection of siRNA became easy and reliable [15]. Many NPs specially carbon nanotubes, lipoplexes, polymer complexes were widely used in siRNA transfection as a delivery vectors [16, 17]. Selection of siRNA delivery method depends on three aspects namely efficiency of siRNA, toxicity and optimization of the activity of the siRNA or siRNA-nano-conjugates. siRNA treated to a certain cell population must have the potency of target-gene knockdown at a specific siRNA concentration and systemic administration. The highly efficient delivery method depends on the rate of conjugation between siRNA and nano-complexes. The efficient delivery must target and affect the targeted gene with minimum amount of siRNA. Fine-tuning of experimentation is required for acquiring the target-gene down-regulation [16, 18]. Even though drugs are directly accessible to many tissues and can reach the tissues through systemic administration with the help of delivery agents in bloodstream, overdose and side effects of drugs are still persisting.

Till now techniques like encapsulation, conjugation (chemical reaction between targeting moieties and siRNA) was studied. Recent developments in

siRNA delivery techniques include layer-by-layer assembly (LbL) assembly and PEGylation on the NPs.

1.2.1. Barriers to systemic siRNA delivery

RNAi is a biological pathway in eukaryotic cells and is considered to be the fundamental pathway that cleaves complementary mRNA in sequence-specific manner. The lengthy double stranded RNA is introduced into the cytoplasm that triggers RNAi and further cleaved into siRNA (21–23 nucleotides long) [19]. Further, the siRNA recognizes complementary mRNA and is cleaved by the Dicer complex. Practically the siRNA is directly inserted into nucleus. In practice, siRNA can be synthetically produced and then directly introduced into the nucleus of the cell. When inserted appropriately the designed siRNA targets the gene, following this RNAi machinery activates and silences that gene in the body, giving it a broader therapeutic potential than specific small-molecule drugs. Even though the siRNA are capable of knocking down the disease-causing gene including cancer, siRNA needs to overcome certain barriers *in vivo*. Safe and effective delivery of siRNA is still a hurdle. Certain reliable and efficient systems must be developed for complete delivery of siRNA. While 'naked', chemically modified siRNA have shown efficacy in certain physiological settings [20]. Some siRNA get cleared from the renal and hepatic system very easily. This is because naked siRNA is subject to degradation by enzymes, and is too large (~14 kDa) and negatively charged to cross-cellular membranes. The issue of non-toxic and effective delivery is a key challenge and serves a barrier between siRNA interference usage, technology and its therapeutic application [4, 21].

siRNA and nano-complexes need to face series of hurdles after reaching

cytoplasm and further the nucleus. Post-injection, the siRNA nano complex must navigate the circulatory system of the body while avoiding kidney filtration, uptake by phagocytes, aggregation with serum proteins, and enzymatic degradation by endogenous nucleases. Phagocytosis is one of the greatest immunological barriers found in extracellular matrix of tissues. Phagocytic cells such as macrophages and monocytes remove foreign material from body [22]. Unfortunately, phagocytes are efficient in eliminating therapeutic nano-complexes and macromolecules from the body, and therefore certain reliable techniques must be adopted to avoid opsonization, enzymatic infection when designing gene delivery vehicles [22, 23].

Efficient technique and different types of nanomaterials must be utilized for siRNA deliveries that have high loading capacity with maximum release efficiency. siRNA must be designed and also coupled with NPs in such a way that siRNA should not get cleaved in bloodstream. Also the technique must be accessible and should not affect the activity and longevity of siRNA [24-36]. Further, we have discussed about certain techniques used now a days that are studied in siRNA delivery.

1.3. Layer by layer formulation [LbL assembly]

1.3.1. Introduction

The designing and fabrication of nano carriers for the siRNA delivery has gained interest recently because of their clinical applications. Decher et al were the first group that introduced the LbL assembly method [38]. This assembly has attracted extensive attention in the field of siRNA delivery. Its

advantages in biomedical applications like easy preparation, versatility, and capability of assembling high loadings of different types of bio-structures in layers helps in formulating different types of NPs [39]. Fine control over the NP structure and robustness of the bio-moieties under provided physiological conditions enhance the activity of the vectors used. NPs used in gene delivery form ideal nano-vector. In this situation neutral moieties and positively charged biomolecules like antibodies, cell-penetrating peptides become necessary to be used in targeting application. Therefore, the LbL assembly helps to accumulate and control the size and structure that facilitates the targeted and safe delivery of the gene inside the specific cell [40-42].

Elbakry A et al [43] focused on the siRNA delivery using modified Gold NPs (AuNPs) with active and relevant siRNA molecules in LbL approach and cellular delivery. AuNPs are widely used in this type of delivery system because of its remarkable advantages like straightforward synthesis, easy surface modification, availability in different sizes and high biocompatibility with cells and tissues. Normally in LbL assembly technique the NPs are first addressed to surface modification by binding it mercaptoundecanoic acid, polyethylene glycol (PEG) and polyethyleneimine (PEI) [44-46]. Further, these surface-modified NPs are subjected to bind the subsequent layers of siRNA and polyelectrolytes with final completion of positively charged moiety around. This increase in layers can be controlled. The size of NPs could be controlled for suitable delivery of siRNA [41, 47].

To obtain a well-defined siRNA coated (layered) NPs, two oppositely charged polyelectrolytes are adsorbed on one another so as to keep the characteristics of the siRNA intact keeping siRNA safe from external forces. In

the LbL technique charge-reversible copolymers are used to improve the endosome escape capacity. Thus LbL technique improves the efficiency of transfecting the siRNA across the cell membrane [48-50]. The other main feature of LbL technique is that the film formulated is non-erodible regulatory membrane for release of siRNA to escape from the serum nucleases during delivery. These LbL assemblies show two significant approaches, namely the structural aspect and film deposition method between the interaction and driving forces during the film assembly [51, 52].

1.3.2. Structural aspect

The fabrication of multilayer on a substratum by consecutive adsorption of polymeric polyanions and polycations, polyelectrolytes controls the shape and size of functionalized NPs. It is obvious that the simplest lattice is a structure of multiple polyanion /polycation layers. This architecture built by very flexible cationic and anionic polyelectrolytes, like poly (allylamine hydrochloride) (PAH) and poly-styrenesulfonic (PSS) acid leads to the formation of one-dimensional structures along the layer. Further, if this film gets a strong hold on a substratum, then the strength of the whole moiety increases giving a structural integrity [53, 54].

More rigid ionic building blocks could be envisioned to reduce layer overlap. For example, creating a difference in the adsorption layer lipid bi-layers can be incorporated in between two anionic and cationic adsorbed moieties for the longevity of the siRNA incorporated in the NPs. In LbL assembly, the periodic laminating of organic and inorganic compounds gives a well-defined sandwiched multilayered film with distinguished super-lattices, which can be easily characterized by X-ray analysis, Zeta-potential analysis.

Size distribution is analyzed using a dynamic light scattering technique [55]. In these Nano composites, both the experimental and theoretical energy transfer efficiencies were correlated to show that the morphology of films are precisely on the molecular length scale, and that the polycation layer, once adsorbed hardly gets eroded by external forces [53, 56].

1.3.3. Polyelectrolyte assembly for deposition of film through interaction and driving forces

In a solution, there are electrostatic interactions between oppositely charged polyelectrolytes. These oppositely charged polyelectrolytes readily get adsorbed because of their specific nature as a thin film deposition. There is an enthalpic contribution between point charges on the oppositely charged polyelectrolyte chains, and entropic contribution due to conformational changes [55, 56]. These charges on the polyelectrolyte chain are surrounded by counter ions from the aqueous solution.

Usually the balance between enthalpic and entropic contribution changes with the salt concentration in the solution. The electrostatic forces are higher in high salt concentration than that of low salt concentration. This enthalpic complexion driven by entropy complex in the salt concentration depends on the nature of electrolyte used in solution [55]. Temperature also plays a great role in this film assembly. It is found that the growth of the polyelectrolyte films turns to exponential when the concentration of the supporting electrolyte is increased. Thus the step-by-step assembly in the formation of film depends on the external parameters like salt concentration, temperature and the co-solvents used in the experiment [54-56].

Although there are advantages of layer-by-layer assembly techniques, there are certain limitations that are faced in gene delivery when very nano-scaled particles are used. The major disadvantage is water insoluble substances are used that is difficult for transportation of the gene through blood capillaries. Sometimes water-soluble moieties are modified with lengthy alkyl chains (either covalently or non-covalently) to adsorb on the nanocarrier [57].

In the synthesis of functionalized NPs where LbL technique is used, the interfacial width of rough surfaces does not give any well-defined scattering length gradients. But it depends on the spread and the distance between the layers. The internal organization in the LbL technique can be decayed monotonically away from the substrate. To overcome these limitations an alternate assembly technique is needed so as to deliver gene in the targeted region. The LbL technique may give a firm assembly; however, there are many other techniques like Encapsulation, Conjugation and PEGylation techniques that are used widely for gene delivery. The mechanisms of the LbL structure have proven successful and well developed only with polymeric ions and gold NPs [58, 59].

1.3.4. Polymer particles for LbL techniques

Polymer NPs are used for siRNA delivery because of their affinity towards holding siRNA and their proper delivery in cells. Positively charged polymer NPs have the potential ability to bind and condense large nucleic acids into NPs. They serve as efficient gene delivery agents with siRNA. LbL assembled polyelectrolyte multilayers (PEMs) were engineered to tune surface properties to modulate the cell adhesion on a surface of NPs [60].

Dimitrova M et al [61] demonstrated a fabrication of cell-degradable multilayered polyelectrolyte films (MPF) with PEI-siRNA complexes targeting cell- infection. MPFs were monitored for synthesizing the layered films using Quartz Crystal Microbalance (QCM). To form this PEI-siRNA complex, positively charged layer complex, adsorption technique of layering alternative precursor films were used [61, 62]. Electrolytes namely poly-lysine and poly-glutamic acid contributed in supporting the film assembly. The film thickness of the polymer bilayers was found to be 150nm. The adsorption of PEI-siRNA increased till 10nm thickness. After the treatment to the cells the amount of siRNA released from culture medium was less for newly formed MPFs. These MPFs suggested the passive diffusion and spontaneous release of siRNA from films till the first deposited layer. These films showed efficient inhibition of nucleic acid replication using the delivered siRNA [61]. However the transfection ability of siRNA was limited.

Transfection was assessed in another framework where siRNA particularly for human PKR gene (protein kinase RNA-activated) was analyzed. A typical method to deliver small interfering RNA (siRNA) that capitalizes on a forward transfection method was adopted to increase siRNA transfection. A multilayer assembly mediated forward transfection (MFT) method separates the substrate-mediated delivery from the cell. Hence, pH responsive LbL assembled multilayer was used as the delivery platform. Micro-contact printing technique (μCP) pattern was utilized as transfection method. 25-kDa linear polyethylenimine (LPEI) was optimized as the siRNA transfection reagent through NPs. MFT efficiency ≥ 60% was achieved for LPEI-siRNA NPs in siRNA delivery system. Thus cationic polymers with siRNA complexes have

shown potential for application in the systemic assembly of siRNA layers on NPs for successful siRNA delivery. [63]

1.3.5 Other nanoparticles

AuNPs are the emerging NPs that are widely used in the nano-gene delivery systems. AuNPs being inert in nature and easy to customize, are coated with biodegradable polymers and served as the idealistic nano-carriers for siRNA delivery. Deng et al generated a nano-layered siRNA loadable LbL film onto functionalized NPs. They developed a substratum that was tailored to achieve highest loading of siRNA and other biomolecules, secured delivery, stability of siRNA in serum and tumor targeting [64]. They demonstrated the ability of AuNPs as an ideal gene carrier in MDA-MB-468 to silence GFP overexpression. This idea of creating a platform for siRNA suggests the reliability of LbL technique used in gene delivery.

Tan YF et al demonstrated the efficient delivery of siRNA using LbL NPs. The feasibility of encapsulating SPARC-siRNA using LbL NPs with poly (L-arginine) (Arg) and dextran as polyelectrolytes showed cellular binding and uptake of LbL NPs as well as siRNA delivery in FibroGRO cells. siRNA molecules were efficiently coated onto hydroxyapatite NPs [65].

Shutao Guo synthesized charge-reversal functional AuNPs using LbL assembly technique and delivered RNA (siRNA) and plasmid DNA into cancer cells. Cyanine 5-siRNA/polyethyleneimine/ cis-aconitic anhydride functionalized poly-allylamine/ polyethyleneimine/ 11-mercaptoundecanoic acid-gold NPs (cy5-siRNA/ PEI/ PAH-Cit/ PEI/ MUA-AuNP) complexes showed cytoplasmic uptake of cy5-siRNA [66]. Charge-reversal co-polymer has the property of pH-dependent shift charge nature between positive and negative.

The charge-reversal copolymers are used to improve the nucleic acid delivery efficiency by enhancing endosome escape capacity [67, 68].

Elbakry A et al therefore used LbL technique to obtain mono-dispersed AuNPs and deposited the oppositely charged polyelectrolyte thin films on the solid surfaces. AuNPs form an excellent tool for studying the size and surface properties that influence the portal entry into the cells [69]. Cystamine modified AuNPs were functionalized with hyaluronic acid (AuCM)/ siRNA/ polyethyleneimine (PEI)/ hyaluronic acid (HA) for intracellular delivery of siRNA by HA receptor mediated endocytosis. The HA in the outer layer of the AuCM/ siRNA/ PEI/ HA complex contributed to the stability and target specific gene silencing. This complex therefore, had a capacity of target specificity and ultimately gene knockdown. The layers one by one react within the cell and then the siRNA remains safe from the endosomic reactions when administered through blood circulation. Hence LbL assembly technique is widely used in siRNA delivery system and still the work is in progress with great demand and applications.

Recently, Jaganathan H et al reported the presence of tumor initiating cells (TICs) that are more lethal than the existed cancer cells [70]. These breast cancer cells are drug resistant and reappear even it is treated at initial stage. Hence the TICs were treated with siRNA that silenced Signal Tranducer and Activator of Transcription 3 (STAT 3) expression levels in MDA-MB cancer cells. They used LbL assembly technique and layered siRNA on Au tagged with poly-L-lysine. This clearly suggests the deposition of different bio-moieties on the surface of Au. Three layers were introduced on Au substratum and siRNA was delivered into the cytosol. The release of siRNA and poly-L-lysine was

measured spectrometrically at definite time intervals and accurate absorbance. Therefore in this study, LbL assembly serves as a reliable technique in delivering siRNA in TICs also. This technique could be used for siRNA delivery in cancer cells too.

1.4. Encapsulation Technique

1.4.1. Introduction

In the field of RNAi therapeutics it is known that siRNA reacts with cytoplasmic proteins and faces renal clearance inside the cell. Also, this mechanism includes first interaction with cell membrane, endosomal escape and finally complete escape from vesicle, so that siRNA gets delivered in the cytoplasm as mentioned earlier. Moreover, siRNA cannot be injected intravenously into the bloodstream as plasmatic nucleases degrade it. To overcome this consequence complete encapsulation of siRNA and its safe delivery is required [71].

Encapsulation is encasing the cargo material inside a capsule. Encapsulation of siRNA was widely adopted method using polymeric NPs, lipid NPs, liposomes, tri-block polymeric NPs and hybrid NPs were widely used giving highest encapsulation efficiency (80-90%) of siRNA. These NPs hardly interact with cytoplasmic proteins. siRNA are physically encapsulated in NPs and hence are safe. Therefore NPs face and escape endocytosis and deliver siRNA in cytosol [71, 72].

Cun D et al [73] determined the effect of PLGA (poly lactic co-glycolic acid) used during encapsulation of siRNA in synthesizing siRNA encapsulated PLGA NPs. They reported that formation of siRNA/ PLGA NPs depend on the various factors like (i) volume ratio between the inner water phase and the oil

phase, (ii) the PLGA concentration, (iii) the sonication time, (iv) the siRNA molecules, (v) charge on the molecule and the amount of acetylated bovine serum albumin (Ac-BSA) in the inner water phase that affects the encapsulation efficiency. The encapsulation efficiency finally depends upon the capacity of the molecule to get physically encapsulated in the NPs without having any effect on the activity of siRNA. Lipids substituted with PEI, which have low molecular weight, were used for siRNA delivery in MDA-MB-231 human breast cancer cell lines. The 50 to 120 fold of survivin down regulation was due to siRNA, which was hydrophobically encapsulated with modified PEI in lipid NPs. Toxicity of PEI was evaluated and found that the siRNA delivery was effective and safe [73-76].

Composite nano fibers of PLGA prepared by electro spinning were subjected to siRNA delivery. The higher integrity of encapsulated siRNA/ chitosan polyplex released siRNA to inhibit 50% of over expressed endogenous enhanced green fluorescent protein (EGFP) in H1299 cells. Hybrid NPs composed of human IgG and poloxamer 188 were efficiently used for siRNA gene delivery in A549 cells [77]. siRNA encapsulated NPs elicit immune response avoiding renal clearance and nuclease degradation that silenced the mutated KRAS gene successfully and siRNA/ NPs therapy was implemented. The important parameter that affects the encapsulation efficiency is the distribution of material that is dispersed in NPs and the siRNA distribution inside the NPs. Even the smallest volume of solidification of dispersed phase and stability of siRNA can affect the siRNA encapsulation efficiency. In order to prevent gene loss and gene migration the equal distribution of siRNA in NPs is essential. Also the optimized dispersed phase and continuous phase ratio in

the NPs determines the encapsulation efficiency and its controlled release efficiency [77, 78].

1.4.2. Polymers used for encapsulation of siRNA

Polymers are used widely in research area for siRNA delivery. Polymer encapsulated siRNA NPs or polymer-coated NPs are not only used in *in vitro*, but are successful in clinical trials too. Tagami et al [79] recently reported siRNA delivery using PEGylated cationic liposomes. They found that post-second dose injection; whole siRNA gets degraded due to the production of anti-PEG IgM [79]. Therefore, the technique of siRNA encapsulation was introduced as PEGylated lipid nanocarrier and "wrapsome" was invented. "Wrapsomes" are composed of siRNA and cationic lipofection complex in the center, which was completely enveloped with a neutral lipid bilayer and hydrophilic polymers [80]. Colombo S et al demonstrated the siRNA release kinetics of lipid-polymer hybrid NPs. Also the ratio of siRNA and PLGA used for synthesizing the NPs affects the encapsulation of siRNA. The encapsulation of siRNA depends on the surface reorganization of NPs. siRNA in its physical form gets encapsulated when the PLGA is low [81].

1.4.3. Other nanoparticles used for siRNA delivery

Nowadays magnetic NPs, including super-paramagnetic iron oxide NPs (SPIONs) and magnetic iron tetroxide particles are used as nanotheranostics for gene delivery [82]. The large surface area of SPIONs makes their functional modification feasible, enabling the conjugation of targeting molecules, siRNA, and imaging agents for enhancing siRNA delivery [83]. Z Medarova et al reported the synthesis and characterization of a new dual-purpose probe for the simultaneous non-invasive imaging and delivery of siRNAs to tumors [84]. Jing

Chen performed experiment on radioiodine labeling of human vascular endothelial growth factor (^{131}I-hVEGF) siRNA by the Bolton-Hunter method. It is simple and reliable, and improved technique to give high yield. The dual functional properties of I-hVEGF siRNA and magnetic NPs in tumor therapy increased the down regulation of VEGF. Imaging of NPs provided an attractive system where real time monitoring of gene delivery, gene therapy can be integrated. Therefore, the combined therapy of ^{131}I-hVEGF siRNA/ SPIONs might be a future treatment option against carcinoma[85]. Lu W et al [86] used near-IR (NIR) light–inducible therapy for NF-κB down regulation and coined the mode of action as "photo-thermal transfection". They attached folate receptors on hollow gold nanospheres and showed pulsatile release of siRNA using NIR irradiation [86].

1.5. PEGylation

1.5.1. Introduction

The PEGylation technique is widely adopted and preferred method of adhering highly stabilized polymers onto NPs. PEGylation simply refers to the decoration of a particle surface by the covalently grafting, adsorbing of PEG chains. PEGylation helps in maintaining the stability of cargo material inside the NPs by preventing proteolytic and enzymatic degradation [87-92]. Recent studies demonstrated the rejection ability of targeting moieties like proteins on PEGylated surfaces. It was possible to understand due to use of freeze-fracture transmission electron microscopy (TEM) [89].

Owing to the siRNA resistivity with the NPs, many of PEG-containing polymers have conjugated with NPs and proved their ability to impart specificity and integrity towards siRNA and preserve it till it is delivered. The

repeating units of PEG and polypropylene glycol contribute in the functionalization of NPs. Because of these chemical structures they are also known as polyethylene oxide (PEO) and polypropylene oxide (PPO) chains. The PEG, a PEO extension on the NPs becomes conjugated to the targeting moieties. These functionalized NPs are used for treatment of cancer cells [92-95].

Nowadays using multi-arm-PEGs, PEG-dendrimers, star-PEGs, star-PEG-co-block polymers conjugation technique have been a practice in delivery of siRNA in the cancer treatment and genetic diseases [90]. This technique actually is the process of covalent attachment of PEG-polymer chains to a NP specially inert or cationic in nature. PEGylation is basically a chemical reaction with a derivative of PEG with the target molecule and ultimately attached to a nanovector [90, 96-98]. PEGylation of the NPs improved the ability of the vector to cross the barrier like endosomal activity, lysosomal activity [99]. Multi-arm PEGs that were conjointly attached layers of PEG having a functional group at the end. One of the arms is attached to NP and other arms are free. This helps the multi-arm PEG to stabilize and the site becomes active for the adsorption of targeting molecules. These PEG-arms are resistant to serum protein giving siRNA protection from external forces and provides secured mode of delivery [100, 101].

1.5.2. Y-shaped PEGylation reagents

Functional Y-shaped PEGs have received growing interest due to some reports indicating improved biological efficacy compared to the analogous therapeutics in which a linear conjugating PEG was used. 1-(1H, 1H, 2H, 2H-perfluorodecyloxy)-3-(3, 6, 9- trioxadecyloxy)-propan-2-yl acrylate was

synthesized for the gene delivery purpose. Sometimes, conjugated three blocks namely hydrophobic, hydrophilic and a polymerizable vinyl group using epichlorohydrin as a conjugating agent are widely used. This technique is cost effective and has simple synthesizing process, hence could be utilized in large scale. Therefore the Y-shaped PEGylation reagents having specific functional groups that cannot have adverse effect on the activity of siRNA were used widely for siRNA conjugation and delivery system [102]. The macromolecular structure of the conjugating polymer has proven to be crucial for the improved properties of the corresponding bio-conjugates, compared to linear PEG, branched PEG chains [103].

1.5.3. Highly branched PEGylation reagents

Comb-shaped polymers with one specific site of attachment represent a new type of PEGylation agent. One such polymer is POLY PEG, where PEG 'teeth' are linked to a methacrylate backbone via an ester bond. POLY PEG has more degrees of freedom than linear PEG, enabling structure optimization. Conventional PEGylation agents are prepared by ring-opening polymerization of ethylene oxide, usually initiated by an alkoxide derived from an appropriate alcohol [102, 104].

1.6. Comparison of above techniques

In the past decade, it is an observation that PEGylation and encapsulation techniques are widely adopted for siRNA delivery. Even though the techniques are reliable the siRNA targeting and site-specificity is still a hurdle. Although the encapsulation of siRNA is maximum, the pulsatile release and instant release from the nano-vectors is still under research. The

encapsulation method was adopted so as to maintain the siRNA integrity and ultimately have safe delivery.

However, encapsulation of siRNA is found to be nearly 100% efficient, but delivery is still low [105]. In the method of PEGylation, the long chain of polymers had low fixable ability. There are chances that the polymer chain increases the total weight of the nanovector and thereby leading to low targeting of the cells. The PEGylation of nanovector faces, the effect of nuclease and serum degradation. Owing to large molecular weight the accumulation of the nanovector is also observed specially in *in vivo* application. The large molecular weight of the PEGylated nano vectors decreases the mobility inside the blood stream and therefore the siRNA delivery is hindered [106, 107].

The LbL technique that is the new approach in the scientific world is quite durable and reliable. It is observed that the neutral moiety as the substratum does not cause any adverse effect on the siRNA activity. As the layers can be controlled and siRNA can be released as per the necessity inside the cell, LbL technique can be used as the ideal technique. The layers generated by siRNA and targeting moieties do not react with each other due to electrostatic stabilization. The siRNA activity remains independent of the reacting activity with targeting moieties and is physically adsorbed on the nano vector [108]. Therefore LbL technique is gaining great demand in siRNA delivery field.

1.7. Summary and outlook

In this chapter, we discussed about the different techniques used widely in siRNA delivery. Of the above techniques, LbL technique is widely accepted and researchers are keenly interested in investigating the effect of siRNA

delivery and the target sites in cancer cells. A detailed review of NPs used for siRNA delivery with LbL technique is mentioned with their application.

Recent developments in biological drugs have broadened the scope of therapeutic targets against the human diseases. These emerging drugs, which include non-coding genes along with NPs, demonstrate successful gene delivery when compared to the weak activity of siRNA delivery alone. Also, it is reported that siRNA/ nano-complex delivery have shown promising results in clinical trials. Further to overcome certain barriers faced in siRNA delivery, improved synthetic carrier and chemical modification of RNA are used. Systemic delivery of siRNA is a necessary aspect so that unmodified siRNA do not tend to accumulate in the kidneys, liver, spleen like vital organs. The siRNA therapeutic relies on filtering organs of the reticulo-endothelial system. Application of siRNA delivery requires a synthetic carrier decorated with specific targeting moieties. The major advantage of siRNA nano-carriers is their ability to engineer tissue specificity. Hence, to overcome these limitations the biodegradable polymers having a highest encapsulation efficiency and pulsatile release efficiency should be employed for efficient and tissue-specific delivery. Highly advanced techniques are necessary to fully harness the power of RNA interference and increase the flexibility of RNA-based therapeutics. Engineered NPs, such as peptide nucleic acid siRNA (PNA-siRNA) chimeras, peptide-siRNA, ligand-siRNA and transferrin-decorated NPs, will continue to improve the delivery of RNA drugs. The widely adopted technique could be the LbL assembly as it involves the physical adsorption of siRNA rather any chemical activity. When nano vectors are taken into consideration, due to their size, accessibility to deliver siRNA is comparatively faster than the naked siRNA

alone. Therefore, the future prospects of RNA-based nano-carriers will require efficient technique to maximize gene potency while minimizing off target toxicity and immunogenicity. Also the proper administration technique is essential that will cure the diseases.

1.8. References

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Chapter 2

Techniques for Characterization of Nanomaterials

Abstract

This chapter deals with the principle and instrumentation available for characterization of the nanomaterials used in our research. The basic understanding and analysis of the nanomaterials like chemical composition; surface morphology, crystalline property etc. are essential to acquire insight into the unique properties of nano-scale materials compared to bulk counterparts. In addition to their application in bio-nanotechnology, nanomaterials at bio- nano interface are also analyzed at large scale. All the techniques used in this chapter are for nano-characterization and nano-application.

2.1. Introduction

Nanotechnology is an emerging interdisciplinary area that is expected to have wide range of implications in fields of science and technology like material science, electronics, optics, medicine, energy etc. Nanophasic and nanostructure materials had a great deal of impact on medicinal field and have still attracted many researchers because of their potential applications in achieving the goals of drug, gene and protein delivery. NPs are particles between 1 and 100 nm in size. In nanotechnology, a particle is defined as a small object that behaves as a whole unit with respect to its transport and properties. Particles are further classified according to diameter. Ultrafine particles are the same as NPs and between 1 and 100 nm in size. Coarse particles cover a range between 2,500 and 10,000 nm. Fine particles are sized between 100 and 2,500 nm. Hence, due to their size variation and high structure selectivity characterizing the physical and chemical properties becomes challenging. It totally depends on the material atomic-scale structure, shape, size and chemistry.

NPs characterization is necessary to establish understanding and control of NP synthesis and applications. Characterization is done by using a variety of different techniques, mainly drawn from materials science. Common techniques are transmission electron and scanning electron microscopy (TEM, SEM), atomic force microscopy (AFM), dynamic light scattering (DLS), X-ray photoelectron spectroscopy (XPS), X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), matrix-assisted laser desorption ionization with time-of-flight analyzer (MALDI-TOF), ultraviolet-visible spectroscopy,

Rutherford backscattering spectrometry (RBS), dual polarization interferometry and nuclear magnetic resonance (NMR).

The majority of these NP characterization techniques are light based. The characterization techniques become essential when the nanomaterial is functionalized or surface modified. In field of gene delivery, the NPs are functionalized using tags like monoclonal antibodies, aptamers, streptavidin or peptides. In biological applications, the surface coating should be polar to give high aqueous solubility and prevent aggregation of NPs. In serum or on the cell surface, highly charged coatings promote non-specific binding, where as polyethylene glycol linked to terminal hydroxyl or methoxy groups repel non-specific interactions. NPs can be linked to biological molecules that can act as address tags, to direct the NPs to specific sites within the body, specific organelles within the cell, or to follow specifically the movement of individual protein or RNA in living cells.

In the following sections we discuss the details about the principle, instrumentation and sample preparation of various microscopic, spectroscopic and other characterization techniques available to analyze nanomaterials.

2.2. Microscopic Techniques

2.2.1. Transmission Electron Microscopy

Transmission electron microscopy (TEM) is a microscopic characterization technique in which a beam of electrons is transmitted through specimen, interacting with the specimen as it passes. An image is formed from the interaction of the electrons transmitted through the specimen; the image is

magnified and focused onto an imaging device, such as a fluorescent screen, or detected by a sensor such as a CCD camera.

TEMs are capable of imaging at a significantly higher resolution than light microscopes. Owing to the small de Broglie wavelength of electrons it is easy for the instrument's user to examine fine details of samples. TEM forms a major analysis method in a range of scientific fields, in both physical and biological sciences. TEM finds application in materials science, semiconductor research and biological applications. At low magnifications TEM image contrast is due to absorption of electrons in the material, due to the nature and composition of the material. At higher magnifications wave interactions modulate the intensity of the image. The alternate modes of use allow for the TEM to observe modulations in chemical identity, crystal orientation, electronic structure and sample induced electron phase shift.

Instrumentation

TEM is composed of a vacuum system, an electron emission source, a series of electromagnetic lenses. Imaging devices are subsequently used to create an image from the electrons that exit the system.

- Vacuum system
- Electron gun
- Electron lens
- Specimen Stage
- Charge coupled detector (CCD)
- Data output source

Imaging

An image is formed from the electrons transmitted through the specimen, magnified and focused by an objective lens and appears on an imaging screen, a fluorescent screen in most TEM, plus a monitor, or on a layer of imaging plate, or to be detected by a sensor such as a CCD camera.

Sample preparation

In a TEM, the specimen must be of such a low density that it allows electrons to travel through the tissue. Another way to prepare specimen is to isolate it and study a solution e.g. colloidal solution of NPs. The specimen is also stained in different ways and use markers to locate specific things in the tissue. The 2-3µl of sample is dropped on the copper mesh and dried and used for imaging. As prepared samples were subjected for TEM analysis using JEM-2100FS, JEOL, Field emission electron microscope at an accelerating image of 200kV and the images were obtained.

2.2.2. Scanning Electron Microscopy

A scanning electron microscope (SEM) is an electron microscope that produces images of a sample by scanning it with a focused beam of electrons. The electrons interact with atoms in the sample, producing various signals that are detected and composition of the sample is determined. Specimens can be observed in high vacuum, in wet conditions and at a wide range of elevated temperatures.

The most common mode of detection is by secondary electrons emitted by atoms excited by the electron beam. The types of signals produced by a SEM include secondary electrons (SE), back-scattered electrons (BSE), characteristic

X-rays, cathode luminescence (CL), specimen current and transmitted electrons. Secondary electron detectors are standard equipment in all SEMs, but it is rare that a single machine would have detectors for all possible signals (Figure 2.1). Back-scattered electrons (BSE) are beam electrons that are reflected from the sample by elastic scattering. BSE are often used in analytical SEM along with the spectra made from the characteristic X-rays, because the intensity of the BSE signal is strongly related to the atomic number (Z) of the specimen. BSE images can provide information about the distribution of different elements in the sample. Characteristic X-rays are emitted when the electron beam removes an inner shell electron from the sample, causing a higher-energy electron to fill the shell and release energy. These characteristic X-rays are used to identify the composition and measure the abundance of elements in the sample.

Instrumentation

- Electron Gun
- Electron lenses
- Detectors for all signals
- Data Output devices
- Infrastructure requirement:
- Power supply
- Vacuum system
- Cooling system

Sample preparation

For conventional imaging in the SEM, specimens must be electrically conductive, at least at the surface, and electrically grounded to prevent the accumulation of electrostatic charge at the surface. Metal objects require little special preparation for SEM except for cleaning and mounting on a specimen stub. Solid samples are mounted on the sample holder using carbon tape. Liquid samples are dropped on silica substrate and then air dried for uniform layer and then subjected to analysis. SEM images are collected and observed using JEOL, JSM-7400F, Field Emission scanning electron microscope at 5kV. The operating voltage varies from 1 to 20 kV depending on the working distance.

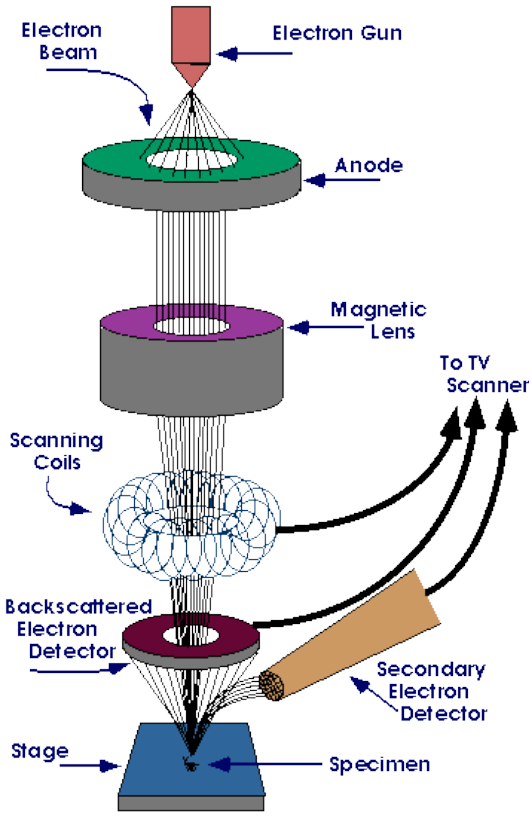

Figure 2.1 Working principle of SEM to detect the morphology of specimen

2.2.3. Confocal Microscopy

Confocal microscopy is an optical imaging technique used to increase optical resolution and contrast of a micrograph. This technique has gained popularity in the scientific and industrial communities and typical applications are in life sciences. Marvin Minsky patented the principle of confocal imaging .It aims to overcome some limitations of traditional fluorescence microscopes. All parts of the specimen in the optical path are excited at the same time and were detected by the camera. A confocal microscope uses point illumination and a pinhole in an optically conjugate plane in front of the detector to eliminate out-of- focus signal. As much of the light from sample fluorescence is blocked at the pinhole, this increased resolution is at the cost of decreased signal intensity.

As only one point in the sample is illuminated at a time, 2D or 3D imaging requires scanning over a regular raster in the specimen. The achievable thickness of the focal plane is defined mostly by the wavelength of the used light divided by the numerical aperture of the objective lens, but also by the optical properties of the specimen. Confocal laser scanning microscopes (CLSM) use a pair of mirrors one at X and other at Y axis to scan laser across the samples and determine the image across the filed pinhole and detector. The laser light source has the additional benefits of being available in a wide range of wavelength.

Instrumentation

The basic confocal microscopy consists of six components:

- Laser source

- Rotating mirrors
- Microscope
- Specimen stage
- Screen with pinhole
- Detector

Sample preparation

Confocal laser scanning micrographs of biological samples were characterized using IX81, Olympus Corporation and A1si Nikon Corporation. The Scanning unit of IX81 confocal microscope is CSU-x1, Yokogawa Electric Corporation with CCD camera iXon DU897, Andor Technology. Cells for CLSM were obtained by growing specific cell lines on 35mm glass-based confocal plate. The bright field image is obtained under normal light source while fluorescent image was obtained upon excitation and emission on the wavelengths of 405, 488 and 561 nm. To study the fluorescence of nanomaterials stains and dyes were added on cover slips and subjected for analysis.

2.2.4. Phase contrast Microscopy

The Phase contrast microscopy is widely used in life science and medical re-search. Phase contrast microscopy is an optical microscopy technique that converts phase shifts in light passing through a transparent specimen to image. Phase shifts themselves are invisible, but become visible when shown as brightness variations.

When light waves travels through a medium other than vacuum, interaction with the medium causes the wave amplitude and phase to change in a manner dependent on properties of the medium. The change in amplitude from the absorption of light creates the image on the detector. It reveals many cellular structures that are not visible with a simpler bright field microscope are clear in phase contrast microscopy. The phase contrast microscope made it possible for biologists to study living cells and how they proliferate through cell division. The basic principle to make phase changes visible in phase contrast microscopy is to separate the illuminating background light from the specimen scattered light, which make up the foreground details, and to manipulate these differently. As the technique is based on diminishment of brightness of objects, more light is needed for contrast study than for corresponding bright field view (Figure 2.2).

Instrumentation

- Light source.
- Condenser lens.
- Specimen stage
- Phase plate
- Detector

Sample preparation

For contrast analysis of biological samples, cells were grown on T25 flask on confocal plates using Leica DMi8 SIN 401683 microscope.

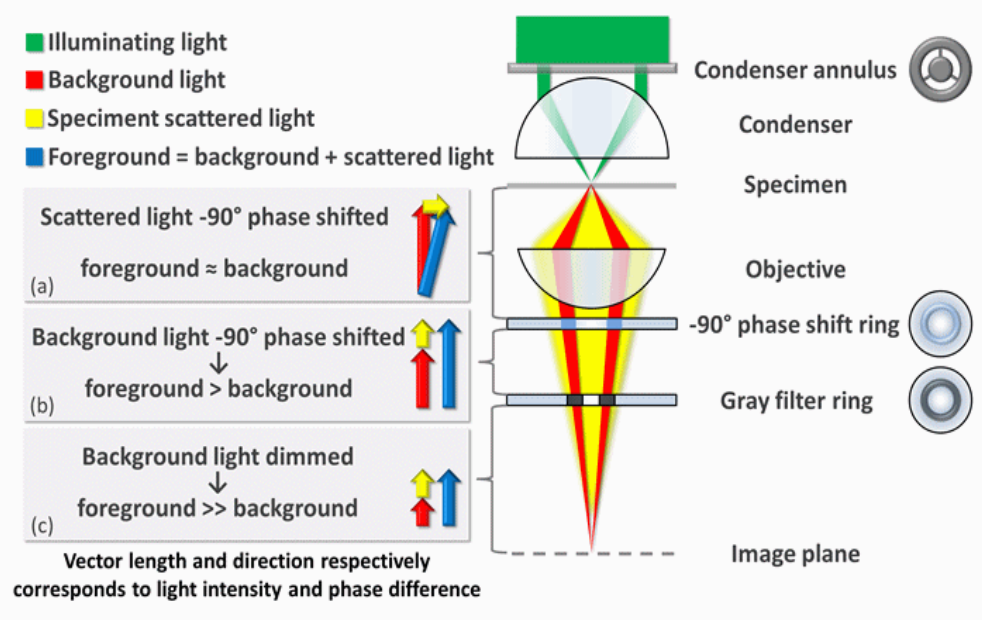

Figure 2.2: Principle of phase contrast microscopy

2.3. Spectroscopic Techniques

2.3.1. Ultraviolet Visible Spectroscopic analysis

Ultraviolet-visible spectroscopy or ultraviolet-visible spectrophotometry (UV-Vis or UV/Vis) refers to absorption spectroscopy in the ultraviolet- (near-UV and near-infrared (NIR)) ranges. The absorption in the visible range directly affects the perceived color of the chemicals involved. In the electromagnetic spectrum, molecules undergo electronic transitions. This technique is complementary to fluorescence spectroscopy, in that fluorescence deals with transitions from the excited state to the ground state, while absorption measures transitions from the ground state to the excited state. UV/Vis spectroscopy is routinely used in the quantitative determination of solutions of transition metal ions highly conjugated organic compounds, and biological macromolecules. Solutions of transition metal ions can be colored

(i.e., absorb visible light) because the electrons within the metal atoms can be excited from one electronic state to another. The color of metal ion solutions is strongly affected by the presence of other species, such as certain anions or ligands. Organic compounds, especially those with a high degree of conjugation, also absorb light in the UV or visible regions of the electromagnetic spectrum. The solvents for these determinations are often water for water-soluble compounds, or ethanol for organic-soluble compounds. The charge transfer complexes give rise to colors; the colors are often too intense to be used for quantitative measurement. The range of this spectroscopy ranges from 190-900 nm

Instrumentation

The UV-Vis spectrometer consists of four components:

- Light source-deuterium Arc lamp
- Sample holder-Cuvette
- A diffraction grating
- Detector

Sample preparation

UV-Vis spectroscopy samples are usually liquids and placed in cuvettes, a quartz cuvette with 1cm width container. The liquid samples were taken in cuvette and spectrum is read. Baseline is considered as reference. Absorption spectrum of samples was analyzed with Beckman Coulter spectrometer, DU 730 under absorbance mode.

2.3.2. X-ray Photoelectron Spectroscopy

X-ray photoelectron spectroscopy (XPS), also known as ESCA (electron spectroscopy for chemical analysis) provides both elemental and chemical state information virtually without restriction on the type of material, which can be analyzed. The sample is illuminated with X-rays - monochromatic or unfiltered aluminum (Al) Kα or magnesium (Mg) Kα and photoelectrons are emitted from the surface. The kinetic energy of these emitted electrons is characteristic of the element from which the photoelectron originated. The position and intensity of the peaks in an energy spectrum provide the desired chemical state and quantitative information.

The chemical state of an atom alters the binding energy (BE) of a photoelectron, which results in a change in the measured kinetic energy (KE). The BE is related to the measured photoelectron KE by the simple equation;

$$\mathbf{BE = h\nu - KE}$$

where,, hν is the photon X-ray energy. The chemical or bonding information of the element is derived from these chemical shifts.

In modern spectrometers the X-rays are energy filtered or monochromatized using a quartz crystal to give X-rays with very little energy spread. This monochromatic x-ray illumination of the sample enables high-energy resolution of chemical shifts and subtle bonding changes evident in the valence band. Photoelectrons may also be collected from the surface in two dimensions to generate elemental or chemical state images of the surface. This is detected on detector in form of peaks heights in spectrum. The quantitative

data obtained from peak areas and identification of chemical state can be made from exact analysis.

Instrumentation

The main components are:

- A source of X-ray
- Ultra high vacuum chamber
- An electron collection lens
- An electron collection analyzer
- Sample mount
- An electron detector
- A computer system

Sample preparation

Powder samples are added in the sample holder and it is fixed on the carbon tape. Liquid samples are dispersed on silica wafer and vacuum dried and then analyzed. XPS was performed on Kratos analytical ESCA instrument equipped with monochromatic Al Kα X-ray source with photon energy of 1487.6eV. This instrument is operated at 15mA and 12kV under vacuum conditions maintained at 10^{-9} Torr.

2.3.3. Energy Dispersive X-ray spectroscopy

Energy dispersive X-ray spectroscopy (EDS) is an analytical technique used for identification of elemental chemical composition and is usually used in conjunction with SEM or TEM. To stimulate the emission of characteristic X-

ray form a specimen, a high-energy beam of charged electrons is focused on to the sample under study.

When electron beam interacts with the sample it excites an electron in an inner shell of atom ejecting it from the shell creating a hole in the K-shell. An electron from the outer high-energy shell fills this hole and this difference in the energy between the higher and the lower energy shell is released in the form of X-ray. This emitted X-ray energy was measured by an energy dispersive spectrometer and allow measuring the elemental composition of the specimen.

Instrumentation

EDS has five major components:

- Electron source
- Specimen stage
- X-ray detector
- Pre amplifier
- Display unit

The spectrum is obtained, as plot of X-ray versus counts to determine the elemental composition of the sample. In the case of elemental mapping, characteristic X-ray intensity is measured relative to the lateral position of the sample depending on the variation in the X-ray intensity at some energy values across the surface.

Sample preparation

As the EDS analysis was carried out in conjunction with TEM or SEM the sample preparations are made according to TEM or SEM sample

preparation. EDS spectrum of samples were taken by JEM-2100, JEOL instrument.

2.4. Other Instruments

2.4.1. Zeta sizer

Zeta potential is a physical property, which is exhibited by any particle in suspension. It can be used to optimize the formulations of suspensions and emulsions. Knowledge of zeta potential can reduce the time needed to produce trial formulations. It is also an aid in predicting long term stability. Zeta potential is caused by the net electrical charge contained within the region bounded by the slipping plane, and also depends on the location of that plane. Thus, it is widely used for quantification of the magnitude of the charge.

Zeta potential is a key indicator of the stability of colloidal dispersions. The magnitude of zeta potential indicates the degree of electrostatic repulsion between adjacent, similarly charged particles in dispersion. For molecules and particles that are small enough, a high zeta potential will confer stability, i.e., the solution or dispersion will resist aggregation. When the potential is small, attractive forces may exceed this repulsion and the dispersion may break. So, colloids with high zeta potential (negative or positive) are electrically stabilized while colloids with low zeta potentials tend to coagulate or flocculate.

Instrumentation

This instrument consist of

- Laser source
- Beam splitter

- Sample holder
- Attenuator
- Detector
- Digital signal Processing

Sample Preparation

The diluted colloidal solution of nanomaterial was taken in cuvette and dip electrode was fitted in the solution. The holder was fitted in the holder and kept undisturbed for 120s to neutralize the movement of NPs in colloidal solution and measurement was carried out by using Nano- ZS Zetasizer (Malvern Instruments Ltd.) at 25 ºC.

2.4.2. Particle Size Analyzer

Dynamic light scattering (DLS), sometimes referred to as Quasi-Elastic Light Scattering (QELS), is a non-invasive, well-established technique for measuring the size and size distribution of molecules and particles typically in the submicron region, and with the latest technology lower than 1nm. The Brownian motion of particles or molecules in suspension causes laser light to be scattered at different intensities. Analysis of these intensity fluctuations yields the velocity of the Brownian motion and hence the particle size using the Stokes-Einstein relationship. The dynamic information of the particles is derived from an autocorrelation of the intensity trace recorded during the experiment. The second order autocorrelation curve is generated from the intensity trace as follows:

$$g^2(q;\tau) = \frac{\langle I(t)I(t+\tau)\rangle}{\langle I(t)\rangle^2}$$

Where g^2 (q;τ) is the autocorrelation function at a particular wave vector, delay time and intensity.

DLS is used to characterize the size of various particles including proteins, polymers, micelles, carbohydrates, and NPs. If the system is mono-disperse, the mean effective diameter of the particles can be determined. This measurement depends on the size of the particle core, the size of surface structures, particle concentration, and the type of ions in the medium. Since DLS essentially measures fluctuations in scattered light intensity due to diffusing particles, the diffusion coefficient of the particles can be determined. DLS software of commercial instruments typically displays the particle population of different diameters. If the system is mono-disperse, there should only be one population, whereas a poly-disperse system would show multiple particle populations.

Stability studies can be done conveniently using DLS. Periodical DLS measurements of a sample can show whether the particles aggregate over time by seeing whether the hydrodynamic radius of the particle increases. If particles aggregate, there will be a larger population of particles with a larger radius. Additionally, in certain DLS machines, temperature dependent stability can be analyzed by controlling the in situ temperature.

Instrumentation

This instrument consists of

- Laser source
- Sample holder
- Focusing lens

- Photon detector
- Co-relator

Sample Preparation

The diluted colloidal solution of NPs were taken in cuvette and the cuvette was fitted into the holder and kept undisturbed for 120 s to neutralize the moment of NPs in colloidal solution and the measurement was carried out by Nano-ZS Zeta-sizer (Malvern Instruments Ltd.) at 25ºC.

2.5. Molecular Biology Techniques

2.5.1. Flow cytometry (FCM)

Flow cytometry is a technology that simultaneously measures and then analyzes multiple physical characteristics of single particles, usually cells, as they flow in a fluid stream through a beam of light. The properties measured include a particle's relative size, relative granularity or internal complexity, and relative fluorescence intensity. These characteristics are determined using an optical-to-electronic coupling system that records how the cell or particle scatters incident laser light and emits fluorescence. A flow cytometer is made up of three main systems: fluidics, optics, and electronics. In the flow cytometer, particles are carried to the laser intercept in a fluid stream. Any suspended particle or cell from 0.2–150μm in size is suitable for analysis. Cells from solid tissue must be disaggregated before analysis. The portion of the fluid stream where particles are located is called the sample core. When particles pass through the laser intercept, they scatter laser light. Any fluorescent molecules present on the particle fluoresce. Appropriately positioned lenses collect the scattered and fluorescent light. A combination of beam splitters and filters steers

the scattered and fluorescent light to the appropriate detectors. The detectors produce electronic signals proportional to the optical signals striking them. List mode data are collected on each particle or event. The characteristics or parameters of each event are based on its light scattering and fluorescent properties. The data are collected and stored in the computer. This data can be analyzed to provide information about subpopulations within the sample (Figure 2.3).

Instrumentation

A flow cytometer is made up of three main systems:

- Fluidics
- Optics
- Electronics.

The fluidics system transports particles in a stream to the laser beam for interrogation. The optics system consists of lasers to illuminate the particles in the sample stream and optical filters to direct the resulting light signals to the appropriate detectors. The electronics system converts the detected light signals into electronic signals that can be processed by the computer. For some instruments equipped with a sorting feature, the electronics system is also capable of initiating sorting decisions to charge and deflect particles.

Sample Preparation

Cell suspension of NP treated cells was prepared by trypsinization and centrifugation to obtain cell pellet. This pellet was transferred to chilled PBS solution and centrifuged to get the clear cell suspension. Then this pellet was

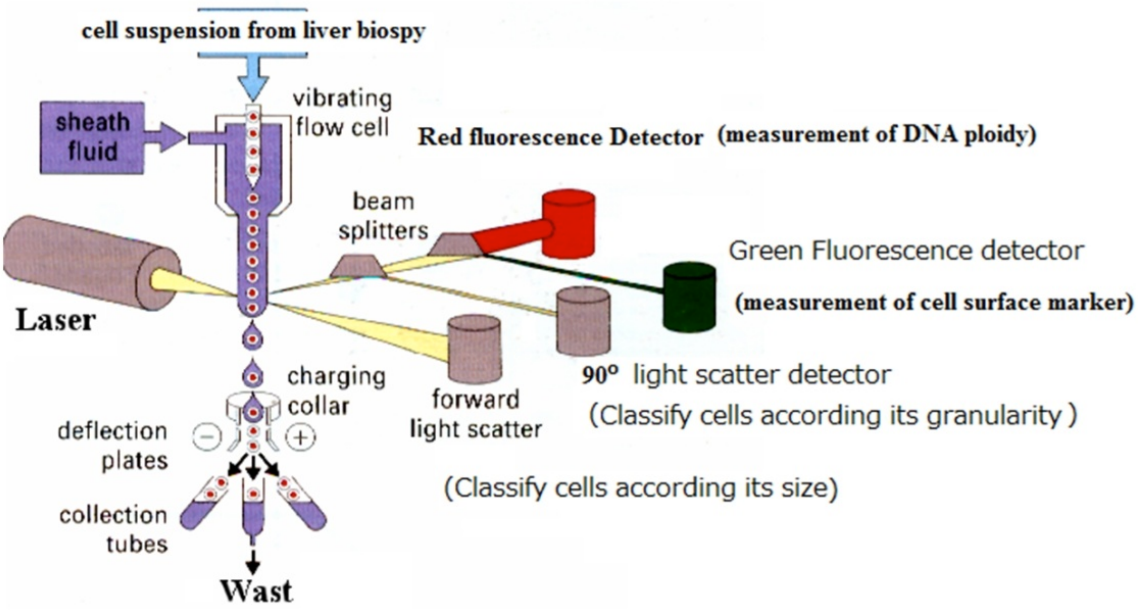

Figure 2.3. Flow cytometry working principle

incubated in buffer with respective dyes to analyze. Flow cytometry instrument used in experiment was JSAN SDM 140203.

2.5.2. Quantitative Real-Time Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

Quantitative real time polymerase chain reaction (qRT-PCR) is a major development of PCR technology that enables reliable detection and measurement of products generated during each cycle of PCR process. This technique became possible after introduction of an oligonucleotide probe, which was designed to hybridize within the target sequence. Cleavage of the probe during PCR because of the 5' nuclease activity of Taq polymerase can be used to detect amplification of the target-specific product.

Quantitative PCR is carried out in a thermal cycler with the capacity to illuminate each sample with a beam of light of a specified wavelength and

detect the fluorescence emitted by the excited fluorophore. The physicochemical properties of the nucleic acids and DNA polymerase become an advantage for running the thermal cycler.

These cycles normally consist of three stages: the first, at around 95°C, allows the separation of the nucleic acid double chain; the second, at a temperature of around 50-60°C, allows the binding of the primers with the DNA template; the third, at between 68-72°C, facilitates the polymerization carried out by the DNA polymerase. In addition, some thermal cyclers add another short temperature phase lasting only a few seconds to each cycle, with a temperature of, for example, 80°C, in order to reduce the noise caused by the presence of primer dimers when a non-specific dye is used. The temperatures and the timings used for each cycle depend on a wide variety of parameters, such as; the enzyme used to synthesize the DNA, the concentration of divalent ions and deoxynucleotides (dNTPs) in the reaction and the bonding temperature of the primers

Quantification of gene expression

qRT-PCR is used to quantify nucleic acids by two common methods: relative quantification and absolute quantification. Absolute quantification gives the exact number of target DNA molecules by comparison with DNA standards using a calibration curve. It is therefore essential that PCR of the sample and the standard have the same amplification efficiency. Relative quantification is based on internal reference genes to determine fold-differences in expression of the target gene. The quantification is expressed as the change in expression levels of mRNA interpreted as complementary DNA. Relative quantification is easier to carry out, as it does not require a calibration curve as the amount of the studied

gene is compared to the amount of a control housekeeping gene. As the units used to express the results of relative quantification are unimportant the results can be compared across a number of different qRT-PCR. A number of statistical algorithms are used to determine which gene or genes are most suitable for use under given conditions.

Instrumentation

- Thermal Controller
- Fluorometry

Sample Preparation

The tissue handling techniques are very important in determining the gene expression in the cancer cells. The cells treated with NPs are harvested from the flasks and centrifuged and the pellet is used for isolation of mRNA. The mRNA is isolated from the cells and preserved in chilled condition. Using the SYBR green method the whole mRNA expression is determined at specific cell amplification and annealing cycles. The qRT-PCR used to determine the gene expression was Applied Biosystems 7500.

2.5.3. Agarose gel electrophoresis

Agarose gel electrophoresis is used for separation of nucleic acids (DNA/ RNA) on agarose matrix. It separates nucleic acids on the basis of molecular weight. The separated molecules are read by using chemi-luminiscence principle where ethidium bromide (EtBr) is used as fluorescence dye. It is a heterocyclic molecule with phenanthridine nucleus, which shows absorbance in aqueous solution at 210 and 285 nm. It fluoresces at 605 nm due to its intercalation with DNA. The intense fluorescence is observed mainly due

to stabilization of positively charged phenyl moiety with negatively charged DNA. In agarose gel electrophoresis the electric field is applied to move charged molecules through agarose matrix. Agarose gels in the concentration of 0.7 – 2 % are commonly used in suitable electrophoresis buffers like TAE (Tris acetate) and TBE (Tris borate). DNA loading buffer consisting of Bromophenol blue, xyelene cyanol and sucrose in H_2O are commonly used for visual tracking of samples while running it on the gel.

The samples were prepared using Bromophenol blue DNA loading buffer as mentioned previously. In our experiment we used 2% agarose gel with final working concentration of 0.5μg/ml for EtBr for running the qRT-PCR products in TAE buffer. The samples were run against the DNA molecular weight marker. We ran gel electrophoresis for 1 hour at a specific voltage. Later the gels were analyzed using gel doc apparatus using BioRad GS710 analyzer.

2.6. Conclusion

Recent developments in nanotechnology and characterization techniques have created a new horizon in research and development. The active research focused on interdisciplinary sciences welcomes more characterization techniques to analyze novel particles for new applications. In future to attain commercialization and application of nano in our day today life we need economical, non-time consuming characterization techniques.

2.7. Reference

The working principle of the instruments was collected from instrument manual and respective home page of the commercial makers.

Chapter 3

Importance of Gene therapy and use of polymeric nanoparticles in siRNA delivery

Abstract

This chapter deals with the importance of polymer encapsulated gene delivery in cancer cells. siRNA delivery technique is the future of upcoming scientific and etiological studies. It is the most reliable and effective tool that can be used for treatment of many diseases. Also, nano-technology helps this gene delivery to be more successful. The application of nano-technology and gene delivery together conquers many hurdles present in curing various diseases.

3.1. Introduction

Recently, non-viral gene delivery has gained considerable attention. RNA interference (RNAi) was characterized as an essential process in gene silencing more than 10 years ago. RNAi offers targeting any pathological protein in a sequence-specific manner thereby curbing protein synthesis.

Non-viral delivery with siRNA is the method of inserting efficient non-coding RNA into host cells. There are numerous strategies for siRNA delivery developed for different cells and tissues. The strategies can be divided into two classes namely non-viral and viral delivery [1]. Non-viral techniques incorporate physical insertions, for example, electroporation, microinjection and lipofection. It can likewise incorporate the use of lipoplexes and polyplexes for siRNA insertion [2]. However, gene delivery with use of nanoparticles (NPs) has now gained attention as these methods has fewer adverse effects, but prolonged effects. In this chapter, we highlight the concepts like importance and applications of gene delivery, specially siRNA delivery and use of functionalized polymer NPs.

3.2. Importance of Gene Therapy

Gene therapy is a promising approach to treat genetic diseases without affecting existing DNA in the cell. Basically, in gene therapy the main criteria is delivering foreign genes into cells and targeting gene expression. Down regulation of targeted gene expression and gene silencing was carried out using micro RNA (miRNA) or antisense oligonucleotides. Gene therapy is useful for treating genetic, autoimmune and other acquired diseases. The first clinical trial was successful in treating adenosine deaminase deficiency in 1990. Numerous

clinical trials carried out for other congenital genetic defects such as familial hypercholesterolemia and cystic fibrosis were successful using gene therapy. Clinical trials using gene therapy performed for acquired diseases such as cancers, cardiovascular diseases and infectious diseases are still under research [3, 4]. Nowadays siRNA administration is studied widely for targeting mRNA in cells. These siRNA do not hinder and alter any other chromosomes, instead directly interact with mRNA curbing protein synthesis [5, 6].

3.3. Advantages of siRNA delivery

Compared to chemotherapeutic anti-cancer drugs, there are a lot of advantages of using siRNA. Due to the special mechanism of gene silencing, siRNA has four main advantages as a potential cancer therapeutic strategy. The first is its high degree of safety. siRNA acts on the post-translational stage of gene expression, so it does not interact with DNA and thereby avoids the mutation and teratogenic risks of gene therapy. The second advantage of siRNA is its high efficacy. In a single cancer cell, siRNA can cause dramatic suppression of gene expression with short nucleotide sequences. Compared to other small molecule drugs or antibody based drugs, the third advantage of siRNA is the unrestricted choice of target specificity determined by the principle of complementary base pairing. This strategy also benefits from rapid developments in molecular biology and whole-genome sequencing. In addition, the fourth advantage of siRNA is that it is easy to establish comprehensive nucleotide sequence databases, including human genomic databases, chimeric DNA databases and disease gene databases. This has laid a solid foundation for customizing and designing sequences of siRNA. The basic strategy of a siRNA is to treat cancer by silencing the specific cancer-promoting gene with rationally

designed siRNA. Hence it is now possible to design effective siRNA sequence targeting any disease gene according to the mRNA sequence.

However, several hurdles still exist on the road to siRNA clinical use for cancer therapy. Firstly, siRNA is unstable under physiological conditions. When siRNA travels through the blood, it is easily digested by nucleases in the serum. siRNA gets degraded in early endosomes once it is delivered inside the cytosol [7]. Once siRNA enters inside the endosomes, these early endosomes formed fuse together to form late endosomes. These endosomes have acidic environment that is not suitable for siRNA. Endosomes then are relocated to the lysosomes. They are further acidified (pH ~ 4.5) and contain various nucleases that promote the degradation of siRNA. The ideal administration route of siRNA is systemic injection, so that siRNA can reach cancer cells more efficiently. Secondly, after injection into the blood, siRNA is easily enzymatically degraded by endogenous nucleases, filtered by the kidney, taken up by phagocytes and aggregated with serum proteins [8]. Further along with the endosome and lysosome reactions, other biological barriers encountered by siRNA is, the nuclease activity in plasma and tissues [9]. To overcome these barriers many techniques are utilized namely modification of siRNA and delivery of siRNA into cytosol and nucleus using NPs. Utilizing these two approaches the research on siRNA delivery has increased and the study are still in progress. The most interesting advantage of siRNA is in targeting direct and indirect mutation inside the cell. Only 21-24 nucleotide sequence length of siRNAs along with the anti-mutant oligonucleotide located at one of four central positions within the siRNA were sufficient for targeting the mutant in

Tau [10]. Exon specific targeting is the method of silencing mRNA inside the cytosol.

Celotto AM et al have described the gene silencing in Drosophila melanogaster Down syndrome cell adhesion molecule (DSCAM) gene. This gene is endogenously expressed in Drosophila Schneider (S2) cells as mRNA isoforms. This DSCAM gene contains 95 exons having alternative exons organized into four clusters. One of these four clusters, exon 4-cluster having 12 alternative exons were targeted to destroy specific mRNA isoforms using RNAi [11]. The usage of splice isoforms also leads to targeting of genes using complementary siRNA duplexes.

Keeping these advantages of siRNA in mind there is a need for specific delivery techniques and self-targeting siRNA that can target not only the mRNA but also have multiple gene silencing capacity. Rational designing of NPs for siRNA delivery has developed successful systems. Usages of NPs were adopted as one of the reliable targeting techniques in siRNA delivery systems.

3.4. siRNA Delivery using Nanoparticles

Gene silencing mediated by small interfering RNA (siRNA) is explored as a remedial methodology for treating genetic diseases including hereditary disorders. Nonetheless, the effect of siRNA treatment is stopping protein synthesis and cleaving complementary mRNA inside the cytosol [12]. In a study, the intra-peritoneal administration of polyethyleneimine si-RNA (PEI-siRNA) complexes focused on the herceptin (HER-2) receptor in test mice brought about mRNA silencing into the tumors and reduction in tumor development [13]. Also, PEI was utilized to develop NPs bearing siRNA

focusing on vascular endothelial development element receptor-2 (VEGF) keeping in mind to target tumor neovasculature communicating integrin. The NPs with the mixture of neovasculature ligands and siRNA prompted particular siRNA delivery to tumor tissue and hindrance of tumor development through particular silencing pathway in tumor-bearing mice. Hence it was proved that siRNA delivery is one of the major aspects that could be utilized widely in gene therapy.

3.5. Criteria of siRNA delivery system for cancer therapy

To apply siRNA into cancer therapy, the in vivo delivery barriers of siRNA are the main problems to be solved. According the barriers encountered by siRNA in cancer therapy, there are several criteria that NPs have to overcome in siRNA delivery system. siRNA molecules are too large (~13 kDa) and negatively charged to diffuse across cancer cell membranes alone. The effective and non-toxic delivery of siRNA-nanocomplex is a key challenge. These functionalized NPs serve as the most significant remedy between siRNA technology and its therapeutic application [14]. To administer siRNA systemically and allow it to cross physiological barriers to reach its targeted site of action, delivery systems must be engineered to (1) provide serum stability, (2) allow immune evasion, (3) mitigate interactions with serum proteins and non-cancer cells, (4) resist renal clearance, (5) enhance vascular permeability to reach cancer tissues, (6) permit cell entry and endosome escape to enter the RNAi machinery and (7) have low toxicity [15]. To overcome these hurdles certain techniques must be adopted.

First, siRNA should be injected into blood for cancer therapy. As soon as naked RNA molecules are administered into the blood, the innate immune system is stimulated and serum nucleases immediately degrade the RNA. A common strategy to avoid these problems is to modify the siRNA backbone through chemical elements. The most frequently used strategies of chemical modification are incorporation of 2'-O- methyl groups, locked or unlocked nucleic acids, or phosphorothioate (PS) linkages [16]. Although chemical modifications can solve some problems of siRNA delivery, NPs that encapsulate siRNA are better at protecting it from degradation and immune recognition [17]. So, not only modifications of the siRNA chemical structure are needed, but also additional delivery materials are also necessary to overcome other barriers like the effect of enzymes present in blood, renal clearance etc.

There are many components in the blood that will interact with siRNA delivery in various ways. High positive charge on the surface of NPs can cause unfavorable aggregation in blood stream, but interaction between NPs and serum proteins in blood aids uptake by cancer cells. For example, many liposomal delivery systems like siRNA with lipoplexes interact with serum lipoproteins and enter hepatocytes that take up those lipoproteins. However, serum opsonin proteins can also be adsorbed on the surface of delivery NPs and tag them for uptake by the mononuclear phagocyte system (MPS) [18, 19].

After systemic administration, there are many ways by which siRNA leaves the bloodstream and passes through the liver, spleen, kidney and lungs [20]. By conjugating siRNA with synthetic materials, the size of the nanoparticle can be increased to avoid renal clearance, hepato clearance and

reserve the siRNA for alternative organ targets [21]. The NPs within the range of 100-200 nm serve as ideal vectors for siRNA delivery. Secondly, based on the enhanced permeability and retention effect (EPR effect), NPs ranging in size from 10-100 nm are passively accumulated in tumors. It is because newly formed tumor vessels are usually of abnormal architecture. Dendrimers, liposomes and inorganic hybrid particles became an alternative for overcoming this hurdle for cancer therapy [22].

Thirdly, low toxicity is the most important part of siRNA delivery systems. If siRNA delivery provokes unacceptable toxicity even after cellular or systemic level, the most efficacious siRNA delivery system will be ineffectual. Certain NPs and viral vectors, studied earlier for siRNA delivery system, can induce unacceptable levels of toxicity through the activation of immune responses [23]. Therefore, liposomes and polymers based NPs were developed to avoid any self-toxicity caused by NPs in the immune system. Biodegradable, biocompatible and low molecular mass materials were adopted for synthesis of NPs. The use of biodegradable, low molecular mass polymers containing linkages that can be cleaved inside the cell may help reduce self-cytotoxicity [24].

3.6. Potential siRNA delivery systems for cancer therapy

Although many strategies were adopted to deliver siRNA into the cytoplasm of cancer cells, non-viral delivery proved as most reliable. Till now majority of siRNA delivered in clinical trials were directly administered to pathology bearing regions to avoid the complexity of systemic delivery. This systemic delivery may be divided into different categories according to their

targets, including eye diseases, congenital, viral diseases, asthma, hypercholesterolemia, acute kidney injury, thyroxine amyloidosis, and cancer [25]. However, the excellent therapeutic potential of siRNA for cancer therapy is still under research. It is necessary to introduce systemic routes of siRNA delivery to treat most cancers.

As mentioned above, siRNA delivery system should include biocompatibility, biodegradability, and non-immunogenicity, low toxicity characteristics. Additionally, the system should protect siRNA from serum nucleases. Finally, the delivery system should provide siRNA an endosome escape ability to enter the RNAi machinery and activate RNAi pathways [26, 27]. The currently developed siRNA delivery systems for cancer therapy can be divided into four categories: chemical modifications, lipid-based nanovectors, polymer-mediated delivery systems and conjugate delivery systems.

3.7. Chemical modifications of anti-cancer siRNA

The chemical modifications in sequence of siRNA provide an ideal carrier for siRNA therapeutics; they show great potential in increasing the longevity of siRNA and hence are necessary agents in cancer therapeutic siRNA delivery systems. With rational chemical modifications, siRNA can acquire advantages such as serum stability, enzyme escape ability and RNAi machinery access. Chemical modifications can be introduced at the 5’ or 3’- terminus, backbone of sugar moiety or nucleoside base of siRNA. The most common modification site of siRNA is the 2’ position of the ribose ring, which has been proven to enhance siRNA stability by preventing degradation by endonucleases.

The basic requirement of successful modifications is enhancing siRNA serum stability without negative effects on its gene silencing activity.

3.8. Polymer nanoparticles synthesized for anti-cancer siRNA delivery system

Polymer NPs are biodegradable, biocompatible and have low molecular weight. Polymer NPs are synthesized using natural and synthetic polymers. Polymers like natural polymers and NPs synthesized using those polymers are ideal vectors for siRNA delivery. The synthetic polymers include PEI, dendrimers, cyclodextrins and PLGA etc. Natural polymers such as chitosan, cellulose, gelatin, etc. are widely used in synthesis of NPs. Synthetic polymers are easily modified with respect to surface charge and hydrophobicity. The most commonly applied polymers for siRNA delivery, focuses on (i) the physico-chemical properties of the delivery systems, (ii) successful in vivo delivery, and (iii) application and adaptability of the individual polymers in vivo.

Cyrille Boyer designed star polymers or branched polymers using dimethyl-amino-ethyl methacrylate (DMAE-MA) as a core moiety and reversible addition−fragmentation transfer polymerized (RAFT) carrier to yield uniformly dispersed siRNA conjugated NPs. These NPs were used to deliver siRNA into pancreatic cancer cells both in vitro and in vivo. The pancreatic cancer cells were treated with 100nM, Luc2 [8:1 (w/w) ratio with siRNA] of siRNA that silences firefly luciferase 2 (Luc2) gene. 50% of gene silencing was observed after 24 hours treatment. Also, siRNA was administered subcutaneously in in vivo that reduced tumor growth and observations were

taken for 48hours [28]. Polymer like PLGA with combination of different monomers, low molecular weight and linkages were used widely for synthesizing NPs. Further "nano-encapsulation technique" for siRNA encapsulation in polymer NPs is well suited for siRNA delivery. Therefore before synthesizing polymer NPs certain aspects must be considered like siRNA property, poly (lactic-co-poly glycolic acid) (PLGA) quality, final yield, manufacturing costs, personnel safety, environmental impact, and waste disposal or eco-friendly approach. Most widely adopted methods in synthesizing siRNA encapsulated PLGA NPs are emulsion solvent evaporation/extraction, salting-out, nano-precipitation, membrane, emulsification, microfluidic technology, and flow focusing technique [29]. Di-block or tri-block co-polymers, such as PLGA-poly ethylene glycol (PLGA-PEG), PLGA-PEG-PLGA, and PEG-PLGA-PEG, poly-caprolactone-PLGA (PCL-PLGA) are different types of polymers used to synthesize NPs as these NPs formed are easy to functionalize and used widely for siRNA delivery [30, 31]. Saltzman and group reported that PLGA NPs encapsulated with siRNA in the presence of spermidine lead to maximum and sustained gene silencing. Knockdown of gene expression was observed in the vaginal lumen and distal uterine region of genital parts. Penetration of NPs using biodegradable polymer was reported for first time into vaginal mucosa [32]. This proves that siRNA delivery is not only useful in treating somatic cell genetic disorders but also in germinal cell diseases.

A cationic lipid assisted polymeric NP system was fabricated with poly (ethylene glycol)-b-poly (d,l-lactide), siRNA and a cationic lipid, using a double emulsion-solvent evaporation technique. These NPs had more than 90% of

siRNA encapsulation efficiency with size distribution of 170 to 200 nm. The particles were efficient in endosomal escape and demonstrated significant down-regulation of luciferase expression in HepG2-luciferase cells which, stably express luciferase, and kinase 1 (Plk1) expression, following delivery of specific siRNAs by the NPs [33].

Biodegradable polymers that have capacity to form NPs have high capacity of encapsulating siRNA and can escape endosomal, lysosomal, nucleases degradation in in vivo conditions. Therefore polymers are reliable material that can be used for siRNA delivery in future purpose.

3.9. Conclusions and future prospects

As one of the most promising therapeutic strategy for cancer treatment, siRNA has great advantages, such as excellent safety, high efficacy, unrestricted choice of targets and specificity. To solve the delivery problems of siRNA, many delivery systems have been developed. The highly effective delivery systems are quite different in terms of structure, size and chemistry, but there are still some guidelines to be considered for optimal delivery systems. Nanoparticle delivery systems should have a particle size of about 100-200 nm, i.e. be large enough to avoid renal and hepatic filtration but small enough for immune clearance. PEG as the shielding agent has proven to be valuable in preventing non-specific interactions and avoiding immune recognition in the circulation. Chemical modifications, such as 2'-O- methyl substitutions, are necessary to reduce non-specific effects and avoid nuclease digestion. In addition, endogenous or exogenous targeting ligands are also often beneficial for siRNA uptake by cancer cells. In recent years, siRNA drug development has

experienced highs and lows. In summary, a good delivery system is the key to siRNA medical development. Once research into siRNA drug delivery systems makes a significant breakthrough, siRNA will occupy a strong position in the drug market, especially in the anti-cancer drug market.

3.10. References

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Chapter 4
Hollow Polymeric (PLGA) Nano-capsules synthesis by solvent emulsion evaporation method for enhanced drug encapsulation and release efficiency

Abstract

Nano-hollow polymer shells have captured the attention of many researchers in field of pharmaceutical and medical therapeutics. In the field of controlled drug/gene release; nano-capsules in colloidal solutions i.e., particles with hollow piths play an important role in cargo encapsulation. These NPs are synthesized using variety of procedures like emulsion polymerization, phase separation, crosslinking of micelles, inner core etching and self-assembly. Our work proposes a novel route to prepare hollow PLGA NPs (HNPs), which showed increased drug-encapsulation and release efficiency. Simple emulsion solvent evaporation technique was adopted to synthesize nano- hollow shells. The hollow characteristics of NPs were studied using SEM, TEM and Confocal microscopy analysis. The particle size was analyzed by DLS. Drug loading, encapsulation and release efficiency in *in vitro* were assessed by ultraviolet spectroscopy. The developed NPs were hollow and spherical in shape with approximately 80nm in size. The drug encapsulation efficiency is 99.4% and drug was released in controllable manner during *in vitro* analysis.

4.1. Introduction

Nanotechnology is the field of science, which is rapidly flourishing and encouraging the inventions and synthesis of various types of nanomaterials. These nanomaterials have wide range of applications in the fields of medicine, electronics and pharmaceuticals [1, 2]. Nanomaterials used for therapeutic applications faces some challenges, as they need to be biocompatible, biodegradable, cost effective and highly efficient in drug loading. Also, the NPs have to rescue themselves from the enzymatic actions and endosome reactions within the cells before they reach the specific cellular organelles for which they were designed [3]. The development in synthesis of polymeric NPs is one of the successful attempts carried out in field of gene, protein and drug delivery. The capacity of these NPs in holding and encapsulating the cargo still needs improvement [4, 5].

Until now micro- hollow particles of polymers were synthesized and used for delivery of drug in cells [6, 7]. Chiang WL et al synthesized NPs that are incorporated in polymeric micro hollow particles for pulsatile drug release [8]. Although micro-particles carry the cargo, there still remains the question of sustained release of drugs inside the cytosol [9]. Major limitation of micro-particles is that it does not have the capacity to intrude through the cytosol and reach specific targets. This limitation could be overcome by usage of highly self-modified biocompatible NPs.

The polymeric NPs replaced the micro-particles with relatively greater efficiency in encapsulating and delivery of the drug and gene [10]. However,

evidence for ‘endosomal escape’ has come either from cells treated with high concentrations of cargo above the therapeutic range or pH-sensitivity [3]. To overcome this limitation many other NPs like metal NPs [9], silica NPs [11], liposomes [12], synthesized using multi-functionalization technique and encapsulation technique were widely adopted. These NPs do show some limitation owing to its toxicity [8-14]. Use of hollow NPs is an option for overcoming these limitations and disadvantages.

Hollow NPs are said to have great efficiency for gene and drug release as compared with non-hollow NPs using targeted moiety due to their tailored porous structured, high cargo loading and encapsulating efficiency, zero order drug release kinetics and material reliability. Even though some hollow polymeric NPs were efficient, not all polymeric nanomaterials synthesized are biodegradable and biocompatible [15].

PLGA with 50:50 ratio of monomers is used in our experiment for synthesis of hollow nanoshells. This polymer has successive monomeric units (of glycolic or lactic acid), which are linked together by ester linkages yielding a linear, amorphous aliphatic polyester product, during polymerization [16]. This polymer when subjected to acidic environment showed controllable degradation. Also PLGA is still widely used in single solvent emulsion method giving highest yield of NPs wherein physical parameters like size, size distribution, morphology, surface modification and zeta potential were controlled successfully [17]. It has excellent biocompatibility and biodegradability [8].

The various methods used for synthesis of NPs include thermal annealing method, sol-gel process, crosslinking polymerization, [18] inner core

etching method [19] and solvent emulsion method. [20] Of all above, simple solvent emulsion evaporation method is cost effective method [21]. Therefore we used this method for the synthesis of hollow PLGA NPs.

Kwon S et al have reported the synthesis of PLGA Nano half shells using water/oil emulsion solvent method by adding poloxamer in organic phase, for production of nano half shells [22]. There are reports on synthesis of polystyrene colloidal solution of half shells NPs [17, 18]. Due to obvious reasons like incomplete encapsulation of drug and untimely drug release, nano half shells do not provide the support for gene and drug delivery. The sealing and fusion of the nano half shells remains a hurdle. Nano-shells prove to be more superior to nano half shells in drug and gene delivery etc.

In this work, we report the synthesis of hollow PLGA NPs, which released the drug in controllable manner and can be used as Nano vehicle to deliver gene and drug in cell organelles. The synthesis of hollow PLGA NPs by single emulsion solvent method is an economical way of synthesis with enhanced drug encapsulation and release efficiency.

4.2. Experimental

4.2.1. Materials and Methods

PLGA (50:50, Mw 17000~ 70000), Rhodamine 6G (R6G) were purchased from Sigma Aldrich, USA. Polyvinyl alcohol (PVA) and ethyl acetate were purchased from Kato Chemicals, Japan. Paclitaxel was purchased from Wako ltd. Milli-Q water was used throughout the experiment. The *in vitro* drug release was carried out at pH 7.4 and 4.2 in phosphate-buffered solution (PBS).

4.2.2. Hollow NP synthesis (HNPs)

Hollow NPs were prepared using the oil-in-water single solvent emulsion evaporation method where the organic phase is ethyl acetate and these NPs are dispersed in the PVA aqueous solution for stabilization. 50 mg of PLGA was dissolved in 1ml of ethyl acetate and was rapidly stirred on a magnetic stirrer for complete dissolution in a beaker and vortexed for one hour. Concomitantly 5% (w/v) of 2 ml of PVA aqueous solution was prepared with the help of magnetic stirrer keeping the stirrer temperature at 40 ºC to make the solution lukewarm and avoid boiling or over heating. The above vortexed PLGA solution was added drop-wise in 1 ml of aqueous PVA solution (the remaining 1ml of PVA solution was cooled down to room temperature) keeping constant vortexing. After the complete addition of PLGA solution in 1 ml of lukewarm PVA solution, 1 ml cold PVA solution was added instantly. The vortexing was continued for 5 min; even after the complete addition of cold PVA solution. After vortexing, this solution was sonicated with energy output of 40 W for 2 min to create a fine emulsion. This mixture was added to 50 ml of 0.05% (w/v) PVA solution to form a colloidal solution. The colloidal solution was stirred using magnetic stirrer for complete evaporation of organic component. The synthesized nano-shells showed good stabilization when dispersed in PVA solution and was preserved at room temperature.

4.2.3. Non-hollow Nanoparticle Preparation (non HNPs)

Non-HNPs were also prepared by simple emulsion solvent evaporation method using 5 ml of ethyl acetate as organic phase and dissolving 200 mg of PLGA in it. This solution was added to 3 % (w/v) of PVA solution and vortexed

by keeping the vortexing at maximum to obtain an emulsified solution. This solution was further dispersed in 0.01 % of 20 ml of PVA solution to increase the stability of the NPs. The whole colloidal solution was stirred in room temperature for two hours.

The above two colloidal solutions containing HNPs and non-HNPs prepared were centrifuged at 8000 rpm for 10 min to collect the NPs. These collected HNPs and non-HNPs were washed thrice using Milli-Q water; and then dispersed in water and centrifuged at 8000 rpm for 10 min. NPs were vacuum dried and further used for characterization.

4.2.4. Characterization of NPs

The shape and morphological characters of polymer NPs were analyzed by SEM. 10 mg NPs were re-suspended in 100 ml of distilled water and sonicated for 15 min. From the above solution 10 µl drop was placed on a silicon wafer and dried at room temperature to get a uniform layer of particles.

The polymer NPs were also investigated by TEM operating at 180 kV. The NPs after washing were re-suspended in equal amount of Milli-Q water and 1-2 µl were dropped on the copper grid coated with carbon film. Sample was dried in room temperature and was observed in TEM. Also, in order to confirm the mean size of the PLGA hollow and non-hollow nanoparticle, the particle size characterization based on DLS was performed using Zeta-sizer.

For confocal microscopy study; freshly prepared hollow NPs were mixed with R6G aqueous solution having dye concentration 10^{-5} M. Sufficient time was allowed to adsorb the dye molecules onto the polymer NPs to make

PLGA/R6G nano-complex. The 100 µl of this solution was pipetted and dried on a confocal plate for examining the HNPs.

4.2.5. Drug encapsulation and loading

The drug, paclitaxel was encapsulated in NPs using simple emulsion solvent evaporation technique. For the synthesis of non-HNPs and HNPs we have used 200 mg and 50 mg of PLGA respectively. The specific amount of paclitaxel ranging 10, 20, 30 upto 220 µg/ml were added into ethyl acetate, which was suitably stirred to ensure that all materials were dissolved. The synthesis of HNPs and non-HNPs were carried out, following the same above procedures mentioned. The formed oil-in-water (o/w) emulsion was gently stirred at room temperature with help of a magnetic stirrer for not less than four hours to evaporate the organic solvent. The colloidal suspension was sonicated for 3 min and then the NPs were separated at low speed centrifugation (3000 rpm, 5 min). Same procedure was carried out until the maximum amount of paclitaxel was encapsulated. The quantity of drug present is calculated according to UV-absorbance of paclitaxel present in supernatant at 227 nm [5, 23]. The calculation of determining the concentration of drug was carried out according to the procedure described earlier [5].

The percentage drug entrapped (drug encapsulation efficiency) was calculated from the amount of incorporated drug in the nano-shell using following equation:

$$\in_{DL}=\frac{D}{D+P}\times 100 \;\in_{DL}=\frac{A_{PTNP}}{A_{LPTNP}}\times 100 \qquad (1)$$

Where ;

$\in_{DL}$=Drug loading efficiency

D = Amount of drug loaded NPs

P = Amount of drug used for synthesis of drug loaded NPs

4.2.6. Drug release from NPs

Drug release study was compared between HNPs and non-HNPs in different in vitro environment keeping pH 7.4 and 4.2. Readings were observed at time interval till 72 h. Before the study freshly prepared HNPs and non-HNPs were filtered through 0.8 μm pore size Whatmann filter unit GmbH Germany. The drug encapsulated in HNPs was 150 μg/ml and 53 mg/ml in non-HNPs. After filtering the NPs were centrifuged at 3000 rpm for 10 min and vacuum dried. 25 mg of both types of NPs were collected and dispersed in 25 ml PBS solution of pH 7.4 and 4.2 separately to get 1 mg/ml of NPs. Further 1 ml aliquots of above solution were suspended and placed in orbital shaker kept a 120 rpm/min maintained at 37° C. These vials were taken out of shaker at different time intervals as mentioned above and centrifuged at 8000 rpm for 10 min. For drug release study reading of supernatant was observed at 227 nm [5, 23]. Experiment was carried out in triplicates using UV-visible spectroscopy. For calculating drug release equation 2 was used.

$$\Delta_{DR} = \frac{D_S}{D_{PTNP}} \times 100 \qquad (2)$$

Where,

Δ_{DR}= % Drug release

D_S = Amount of drug (paclitaxel) in supernatant

D_{PTNP} = Total amount of drug (paclitaxel) used for preparation of drug-encapsulated

4.3. Results and Discussion

Although PLGA NPs are not hollow they have large surface area. They prove to be having great ability to conjugate with multiple diagnostic and therapeutic agents. However, HNPs proved to be more efficient than the existing non-HNPs in drug releasing and encapsulation activity. The suggested mechanism for nano-hollow-shell formation may be explained as follows, and is presented in figure 4.1. It is reported that PLGA gets diffused to the outer water phase during emulsification after trapping water into the organic phase on account of its amphiphilic nature. Also, the hydrophilic property of PLGA may enhance the water-trapping efficiency in the organic phase, which seems to cause fast solidification at the surface and the hollow structure [22, 24]. In our case the slight modification was aeration during the procedure of emulsification. The solution was vortexed for an hour, which creates air entrapment rather than water entrapment (Figure 4.1-A) [25].

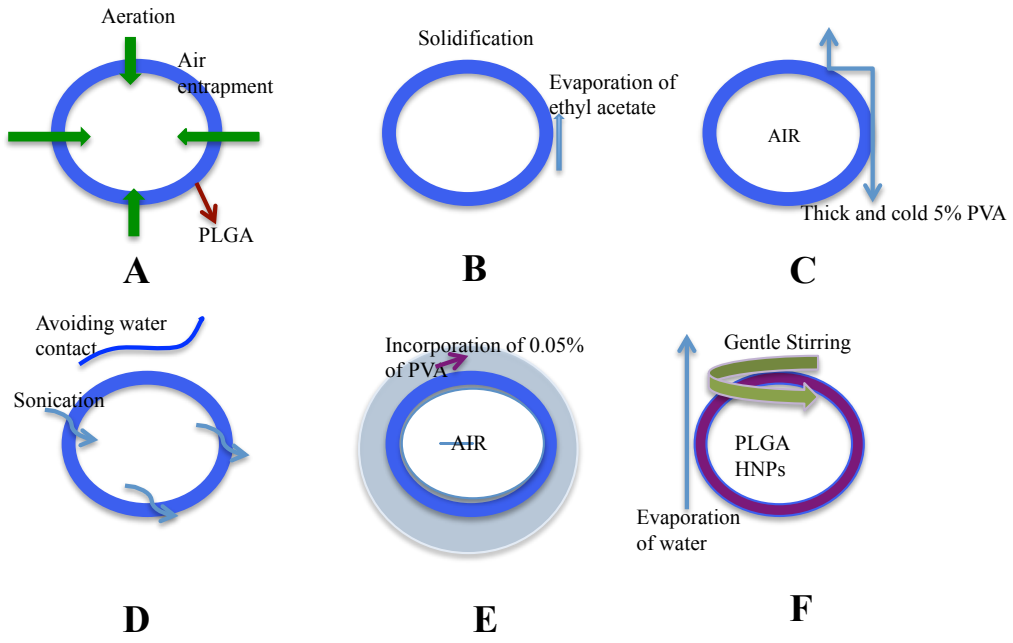


Figure 4.1. The hypothetical representation of formation of nanohollow shells

The organic phase, ethyl acetate, evaporates at the later stage of experiment (Figure 4.1-B). After vortexing, at the same instant, the surface of the emulsion solidifies quickly depending upon the air entrapment ability owing to the rapid solvent removal phase [26]. Since ethyl acetate gets evaporated and air was diffused into the inner phase through the thin solidified film, the empty space left by ethyl-acetate evaporation was replaced by thick and cold 5% PVA until the point at which the two phases are separated and an inner emulsion is created (Figure 4.1- C) with hollow piths in the NPs. Further sonication stabilizes the NPs and avoids the contact of water with NPs. (Figure 4.1-D) [27]. At this stage the incorporation of 0.05% of PVA stabilizes the NPs (Figure 4 1-E) [22]. The sonication creates more stabilization and also addition of 0.05% PVA prevents particles to burst out. Hence instead of formation of nano-half-shells; nano-hollow-shells are created (Figure 4.1-F). As the inner phase solidifies, the remaining ethyl acetate and water evaporates from outer space and nanohollow structures were generated. These HNPs were characterized using SEM, TEM and confocal microscopy.

4.3.1. Scanning electron microscopy (SEM)

As observed in figure 4.2, the NPs are spherical in shape. The size of NPs after synthesis and before filtering ranges from 30-900 nm. The surface morphology of the particles seemed to be intact with smooth surfaces and the pores were absent on surface as observed in the image. The incident electron beam melts the nanoshells and makes the nanohollow shells to shrink [22, 24-28]. Further, we successfully characterized the hollow nature of PLGA NPs using TEM.

4.3.2. Transmission Electron Microscopy (TEM)

TEM images of HNPs are shown in figure 4.3 A and B. It is observed that HNPs were mostly spherical. In each image of nanoparticle the absence of inner cavity can be easily observed. This hollow feature was confirmed by confocal study as observed in figure 4. 3 IA. The ring like structure as shown in figure 4.3 IA depicts the hollow characteristic of NPs. The NPs were stained by the dye R6G. Hence it is confirmed that the particles synthesized were hollow in nature. The size of HNPs observed in TEM ranges around 30-200 nm. Also when compared with TEM images of non-HNPs synthesized using oil/water emulsion solvent method, we were unable to see the hollow nature as shown in figure 4.4 A and B. The size of the non-hollow NPs ranged from 20-70 nm.

The mean size distribution of HNPs was further confirmed with the help of DLS. As seen in figure 4.5, it can be clearly analyzed that mean size diameter of HNPs is 79 ± 0.69 nm. The DLS graph in figure 4.5 depicts that the mean size diameter of the non-hollow NPs is 68 ± 1 nm.

The elemental composition of the material was determined using EDS and XPS. PLGA consists of carbon and oxygen as seen in figure 4.6 D. The samples containing HNPs and non-HNPs were dispersed on gold-coated silicon wafer. According to the graphs shown in figure 4.6 A, B and C, carbon and oxygen were present. Hereby it could be confirmed that there is presence of carbon and oxygen in PLGA and the HNPs prepared. There was presence of gold, as the substrate used consists of gold. Similarly from the analysis of XPS as shown in Figure 4.7, we identified carbon and oxygen on NPs. The wide spectrum obtained shows the peaks corresponding to carbon (283.5 eV), oxygen

(532 eV) and gold (82 eV). For carrying out XPS the non-HNPs, HNPS and PLGA were spread on Au coated silicon wafer.

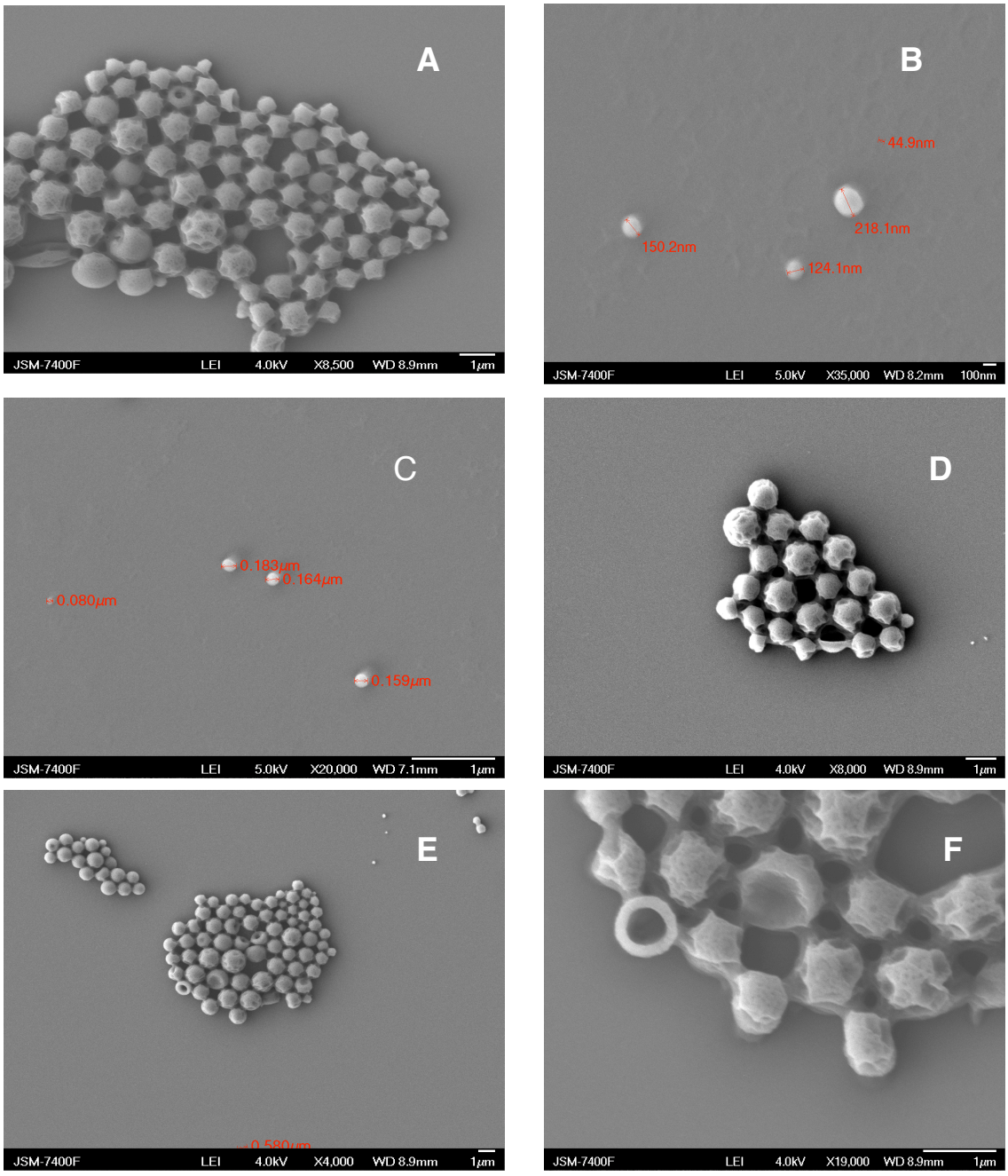


Figure 4.2 The SEM images of HNPs at various magnifications.

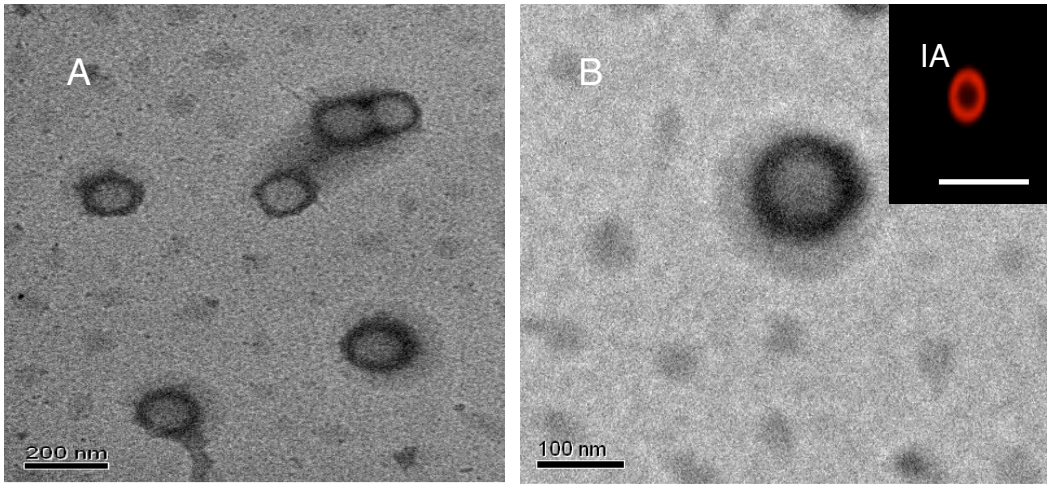


Figure 4.3 The TEM images of HNPs without inner core. Inset image IA is obtained by confocal microscopy.

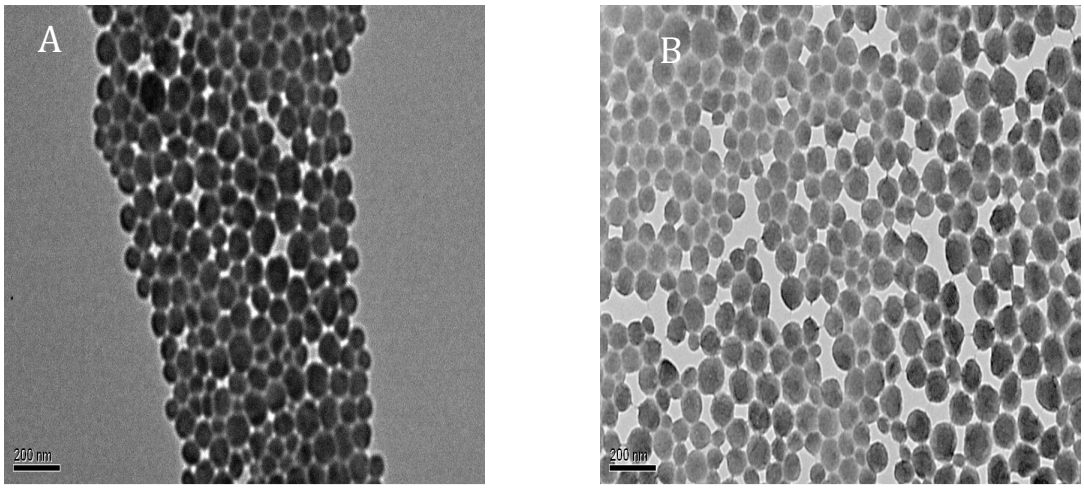


Figure 4.4 TEM images of non-hollow PLGA NPs.

According to SEM, TEM and confocal microscopic analysis it was confirmed that the NPs synthesized were hollow in nature and could be further used for drug encapsulation and release study.

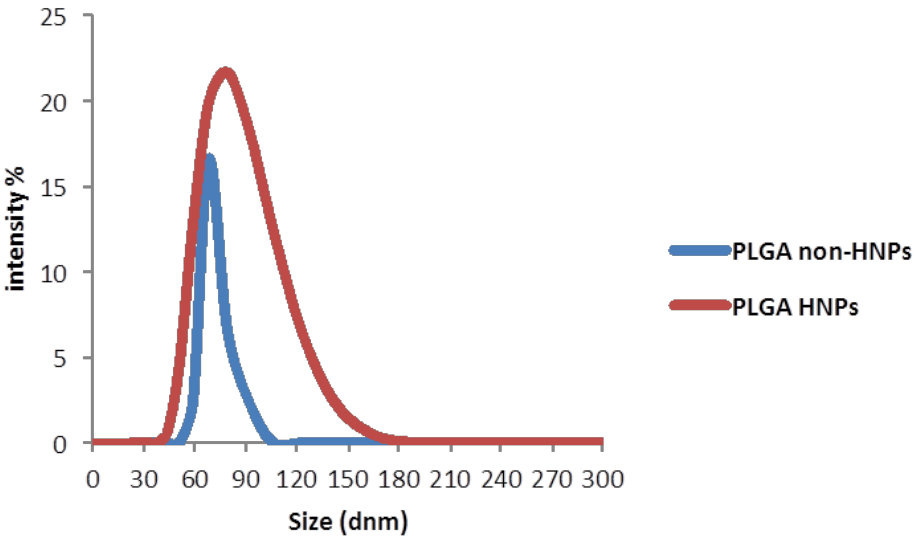


Figure 4.5 Particle size distributions of HNP and non-HNP PLGA NPs.

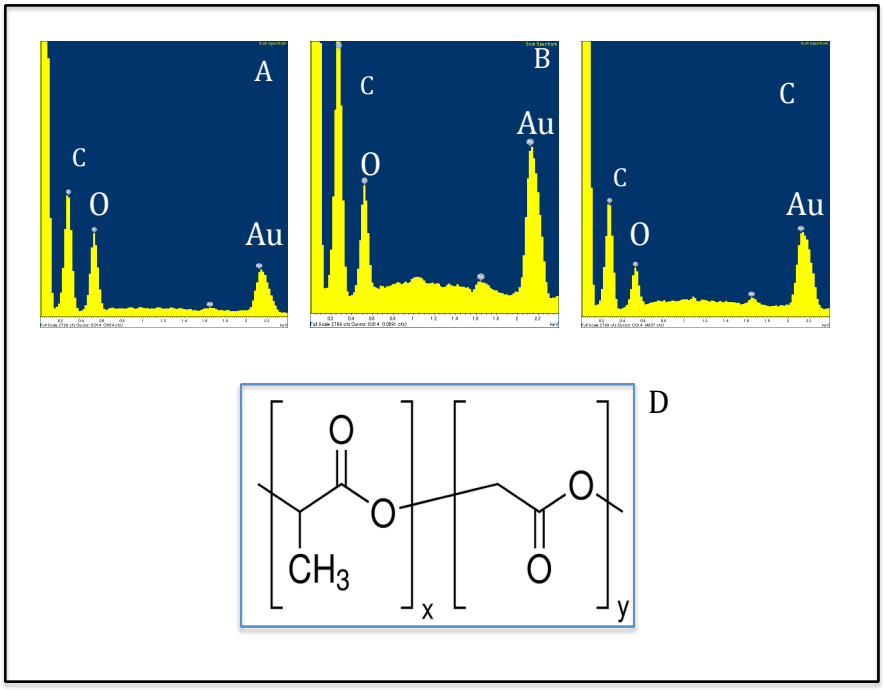


Figure 4.6 The EDS analysis graph for commercially obtained PLGA (A). (B) and (C) show the EDS of HNPs and non-HNPs with presence of oxygen and carbon.(Au is from substrate) (D) Structure of PLGA.

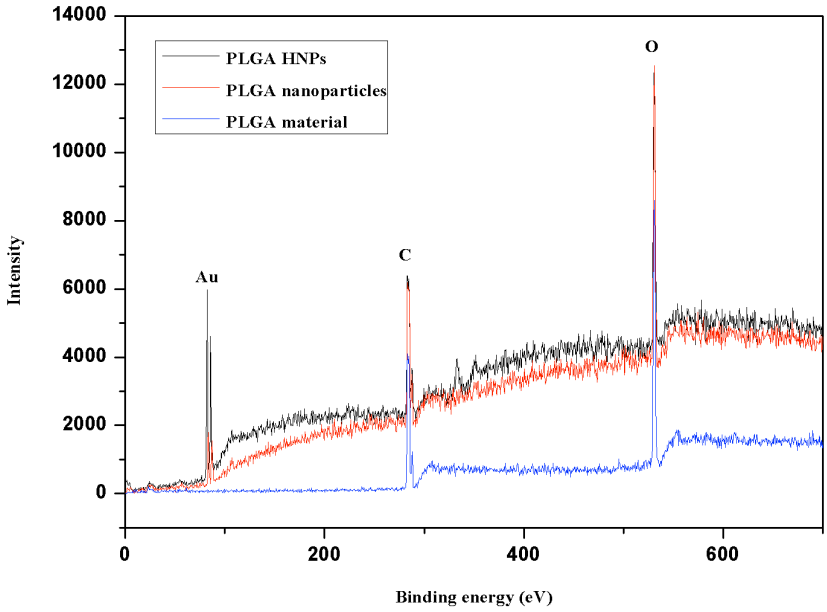


Figure 4.7 XPS spectra of NPs. XPS spectra of PLGA, PLGA HNPs and PLGA NPs and peaks showing presence of carbon and oxygen (Au from the substrate)

4.3.3. Drug encapsulation and release efficiency

The nano-shells being hollow in nature have the highest capacity to encapsulate drug compared to the non-hollow NPs. In our work we used paclitaxel as the model drug and carried on the experiment for drug encapsulation and release efficiency studies.

Paclitaxel is widely used as a mitotic inhibitor in cancer chemotherapy. Direct oral administration of paclitaxel causes elimination of drug and has many limitations in clinical application owing to its low solubility in water and pharmaceutical solvents [29, 30]. Therefore polymeric NPs like PLGA hollow NPs prove to be advantageous as ideal carriers in delivering the drug safely to targeted cells.

It is reported that the drug encapsulation efficiency of PLGA NPs synthesized through solvent emulsion method is lower as compared to any other method. The drug encapsulation efficiency reported ranges from 30 ~ 70%. [4, 5, 31]. The rate of encapsulation efficiency increases to 90% only if other co-polymers are used [31].

As per figure 4.8 we see the maximum amount of drug encapsulated in nano-shells is 150 µg/ml while 53 µg/ml of drug gets encapsulated in non-hollow PLGA NPs. Also, materials such as PLGA used for the synthesis was less in case of nano-shells that is 50mg. This implied that synthesis of HNPs utilize less material leading to greater capacity of holding the drug when compared to non-HNPs.

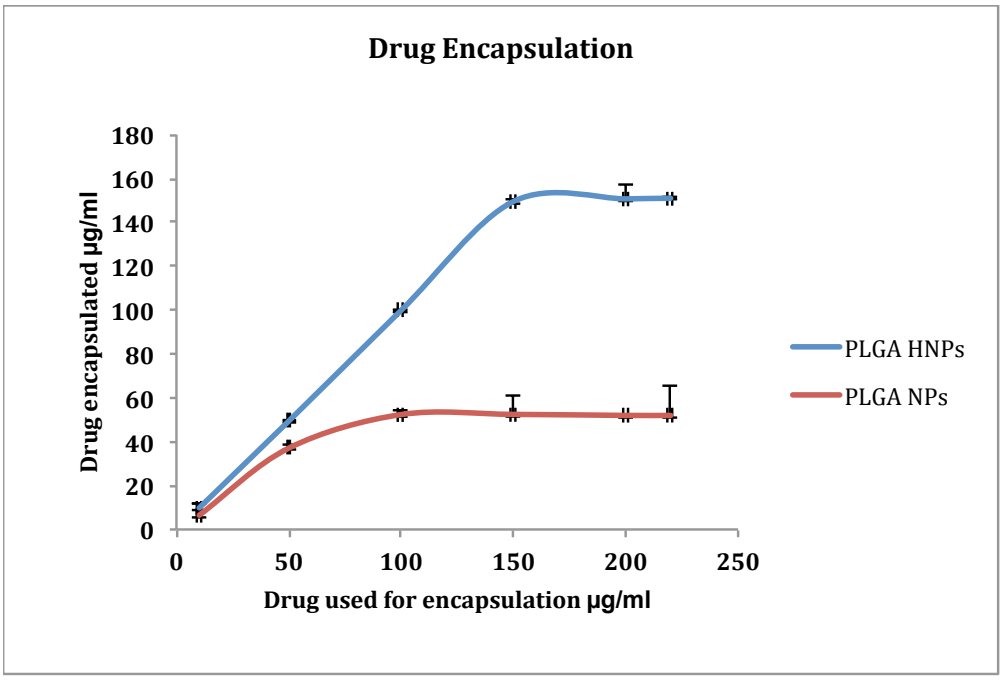


Figure 4.8: Paclitaxel encapsulation by PLGA HNPs showing the maximum encapsulation efficiency of 99%

Further, the encapsulation efficiency of the hollow NPs is 99 ± 0.4 % and non-hollow NPs is 52 ± 0.2 % calculated using equation 2. Also less amount of drug was attached over the surface of HNPs that is $1x10^{-4}$ % than in non-HNPs (20 %), which was calculated using the equation 1. Therefore we inferred that being hollow, almost all drug gets encapsulated inside the NPs leaving no drug to get attached over the NP. The efficiency of drug entrapped in the HNPs was 65 % when compared to lower efficiency in non-HNPs. Overall, this phenomenon suggested less wastage of polymer and increased drug entrapment. The hollow nature of HNPs thus secured the drug or the cargo encapsulated. This feature could be used to encapsulate the materials like oligonucleotides, proteins etc. Further we have done TEM observations for checking the difference in drug encapsulated HNPs, non-drug encapsulated HNPs and drug encapsulated non-HNPs. In figure 4.9A we can observe a shadow region in the inner core of the HNP when compared to the image in figure 4.3 A.This implied that the drug was encapsulated inside the particle. Figure 4.9 B depicts paclitaxel encapsulated PLGA non-HNPs.

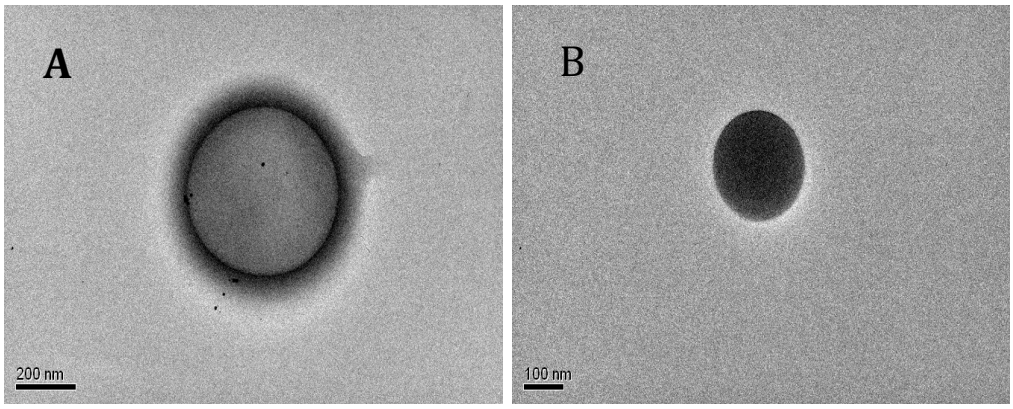


Figure 4.9: A- TEM image depicting paclitaxel encapsulated in PLGA HNPs, B- paclitaxel encapsulated in PLGA non-HNPs.

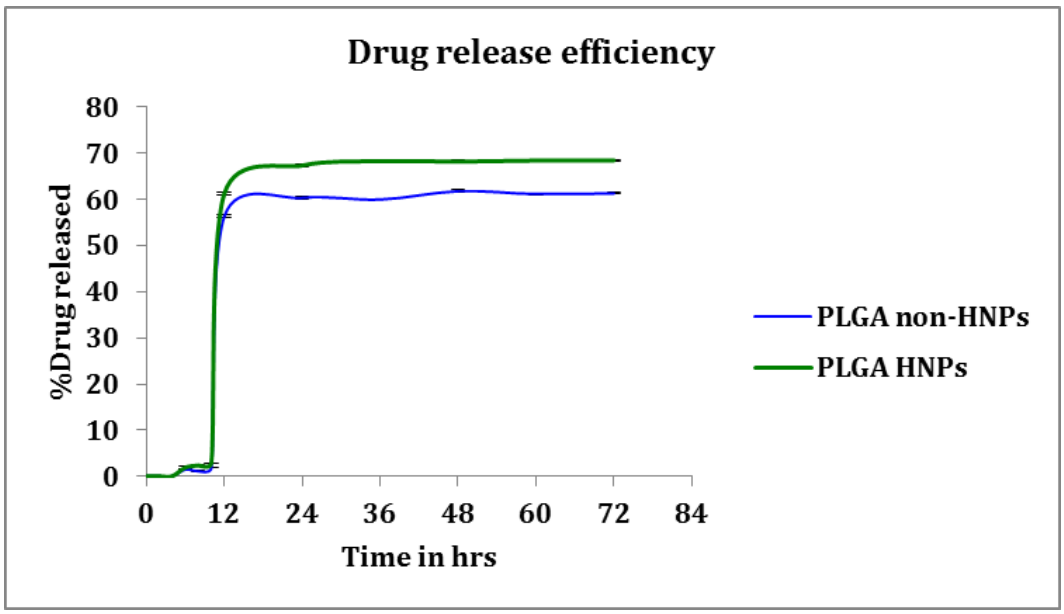


Figure 4.10: Percentage of paclitaxel released from PLGA HNPs and non-HNPs

The drug release was compared between hollow and non-HNPs as observed in figure 4.10. The drug release was observed for 72 h in PBS of pH 7.4. The drug release of the non-HNPs was 56 % at 12 h and after another 12 h it was maintained at 60 % as shown in figure 4.10. The drug release observed with HNPs was 61 % at 10 h and after another 10 h the drug release is nearly 70 %. There is 5 % increase in the drug release of HNPs when compared to non-HNPs in first few hours. Similarly a 10 % increment in the release of drug was observed till 72 h. This release was totally dependent on the bulk degradation of polymer. In non-hollow PLGA NPs, the drug encapsulated or in this case, the drug encapsulated in the polymer NPs was released with degradation of polymer [32].

In case of hollow NPs there was quick surface degradation and maximum drug release. This characteristic difference between the quantity of drug release between HNPs and non-HNPs suggested that HNPs gave quick results of drug delivery within 12 h as compared to non-HNPs.

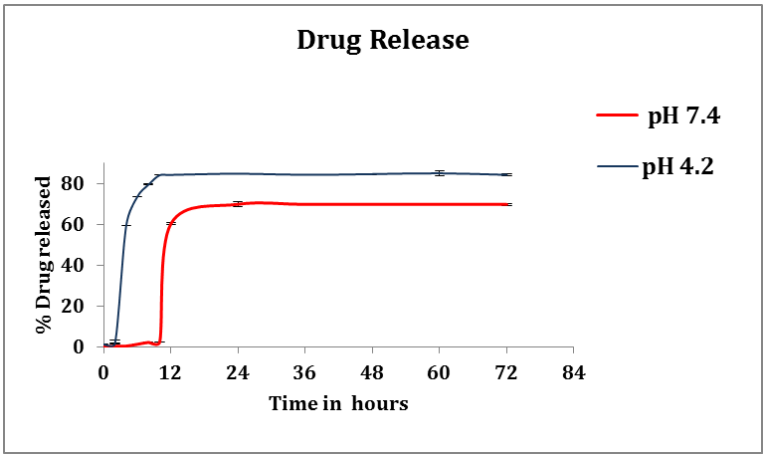
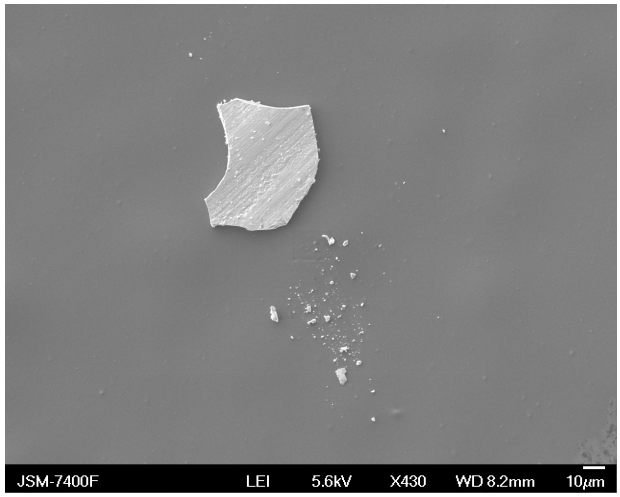


Figure 4.11: Paclitaxel released by HNPs in PBS buffer at pH 7.4 and 4.2 respectively. The drug release in pH 4.2 is greater than in pH 7.4.



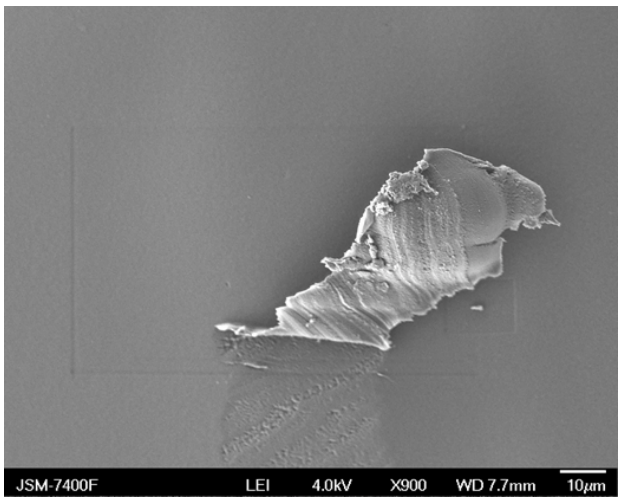


Figure 4.12: The disintegration of HNPs after 72 h.

Further, the drug release of HNPs was studied in different *in vitro* environment. The rate of drug release was determined in different pH solutions, (PBS of pH 7.4 and 4.2). The PBS of pH 4.2 was used to mimic the acidic environment found in the endosomes of cells. The amount of drug released in acidic environment is greater than in normal pH as seen in graph in figure 4.11. The percentage of drug release was 69 ± 3 % at pH 7.4 while the percentage of drug release at pH 4.2 was 84 ± 2 %. The burst release observed in acidic pH is at 4 h while at pH 7.4 was after 12 h. This implied that the matrix degradation of the NPs is fast in acidic medium and the degradation activity starts at 4 h. According to figure 4.11, we conclude that the drug release remains almost constant after 12 h. It was found that small amount of paclitaxel still remained inside the particles from the calculations using equation 2. The SEM images showed that the particles disintegrate in buffer solution after 72 h in figure 4.12. The remaining paclitaxel was released eventually as the NPs were subjected to complete degradation. It can be inferred from the above observation that as time increases, degradation of HNPs also increase giving complete release of drug to the surrounding medium [33].

4.4. Conclusion

In summary, PLGA HNPs were synthesized using simple emulsion solvent evaporation technique, which is economic, cost effective, and leads to high yield of good quality NPs. These hollow structure results from air entrapment, fast solidification, and phase separation with instant stabilization. PLGA HNPs have the capacity to hold the cargo material for long time wherein the material can remain safe. The sustained release of drug was observed. These PLGA HNPs were pH sensitive, inferring that these particles could be used for

drug or gene delivery. Also, as these hollow shells were nanostructures with large surface area, they may be used as carriers for drug and gene delivery for anticancer treatment.

4.5. Reference

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Chapter 5

Strategist PLGA Nano-capsules to deliver siRNA for Inhibition of Carcinoma and Neuroblastoma cell lines by knockdown of *MYC* proto-oncogene using CPP and PNA

Abstract

RNA interference and the therapeutic applications using small interfering RNA was discovered more than 10 years ago and currently is used in various applications including in therapeutic field. However the research in this field is still in its infancy. Many challenges like safe delivery of targeted siRNA to nucleus and cytosol of cancerous cells without compromising the activity of siRNA needs to be addressed. We have overcome this hurdle with the help of nanotechnology using PLGA hollow nanoparticles and suppressing the oncogene of *MYC* transcription factors by using anti myc-siRNAs in human cancer cell lines. siRNA was encapsulated in PLGA hollow nanoparticles. These spherical PLGA hollow nanoparticles (PLGAHNPs) of size 70 nm had high efficiency of gene release at pH 4.2 under *in vitro* conditions. Cell penetrating peptide (CPP)- Tat peptide (TAT) and peptide nucleic acid-nucleolus localizing signal (PNA-NLS) was used for siRNA delivery without affecting the therapeutic activity of siRNA. The siRNA duplex was prepared using T7 polymerase and double stranded DNA through *in vitro* transcription.

Incubation of the siRNA encapsulated PLGAHNPs functionalized with TAT and PNA-NLS (TAT-siRNA-PNA-PLGAHNPs-siRNA) with cancer cells resulted in reduced cell proliferation. A downregulation of gene expression by 90 % was observed even with low concentration of siRNA. We found complete arrest of cell division, which was mediated by downregulation of *MYC* expression.

5.1. Introduction

RNAi therapeutics is a powerful gene therapy technique for suppressing specific genes in the cells and hence has a great potential in biomedical applications including in the treatment of genetic disorders, cancer, viral infections and auto-immune diseases [1- 4]. In 1998, Andrew Fire and coworkers reported the gene silencing in the nematode *Caenorhabditis elegans* by double stranded RNA [5]. The first successful siRNA treatment of gene silencing was achieved in hepatitis C virus. Since then gene therapy opened a new horizon for treating genetic disorders with RNAi [6]. Even though use of siRNA is effective, efficient and safe *in vivo* delivery of siRNA to target tissues remains a key challenge for clinical implementation. As siRNA is negatively charged molecule, it is difficult to penetrate into the cell membrane [7]. Virus mediated nucleic acid delivery was adopted as an option [8]. Usually in viral delivery, very small pieces of DNA are introduced into the cells. However, these methods are labor-intensive and expensive. Apart from this, risk of random insertion in the genome, cytophathic effects and mutagenesis are few other issues associated with viral gene delivery [9]. Therefore, viral delivery faded gradually from bioresearch field [10]. Recently, non-viral siRNA delivery systems using NPs including cationic lipids [11], liposomes [12], cationic polymers [13], peptide - conjugates [14, 15], gold nanoparticles [16], quantum dots [17] and modified siRNA [18] are preferred. NPs made of polymers like polyethylenimine form conjugates with siRNA through electrostatic interactions. However, these cationic nanoparticles have toxicity issues [19-21]. Meanwhile inorganic nanoparticles like gold nanoparticles and quantum dots showed great potential in siRNA delivery, but researchers are still optimizing

and studying the surface chemistries and investigating their long-term toxicity issues [22, 23].

Further, for the efficient use of NPs in siRNA delivery, researchers still need to overcome certain problems related to cellular uptake, degradation and renal clearance [24]. While siRNA along with the nanoparticles were administered intravenously, they were subjected to degradation by endogenous enzymes in the blood stream. Therefore NPs should be designed in such a way that they are effective and non-toxic. Also post-injection, NP-siRNA conjugates must navigate through the circulatory system, avoiding kidney filtration, uptake by phagocytosis, aggregation with serum proteins and escape enzymatic degradation [25]. Hence, there is a need to formulate siRNA-encapsulated NPs that can target the nucleus of cancerous tissue [25, 26, 27].

Until now, biocompatible and biodegradable, PLGA NPs was used for siRNA delivery. These polymeric NPs showed enhanced encapsulation efficiency but had very low release efficiency [28]. This is because the siRNA gets entrapped in the PLGA solution during encapsulation process and its degradation takes longer time, even though PLGA is biodegradable [28]. To solve this problem we have developed PLGA hollow nanoparticles (PLGAHNPs) that could encapsulate and efficiently release siRNA. In our previous work we showed the potential ability of PLGAHNPs and non-hollow PLGAHNPs in drug encapsulation and release efficiency [29].

In this study, we developed a therapeutic approach using layer-by-layer (LBL) NPs to treat aggressive cancer. The advantages of LBL technique include ease of preparation, versatility, and capability of incorporating controlled high loadings of different types of biomolecules in layers [15]. By taking advantage

of this technique, we incorporated layers of Tat peptide and PNA-NLS on PLGAHNPs of homogenous size below 100 nm (68-70 nm). These targeting moieties layered on siRNA encapsulated PLGAHNPs enhanced the uptake of our siRNA delivery targeted cells [16].

5.2. Materials and methods

5.2.1. Materials

PLGA; (50:50, Mw 17000 ~ 70000), Rhodamine 6G (RHODAMINE), Fluorescein isothiocyanate isomer-I were purchased from Sigma Aldrich, USA. Tat peptide (TAT, 49-57) of sequence (Arg - Lys - Lys - Arg - Arg - Gln - Arg - Arg - Arg) was purchased from Sigma Aldrich. Polyvinyl alcohol (PVA) and ethyl acetate were purchased from Kanto Chemicals, Japan. Acid yellow Tartrazine was purchased from Tokyo Chemical Industry, Japan. The linear DNA templates for siRNA synthesis, primers for β2-microglobulin, c-myc and n-myc were purchased from Eurofins, Germany. Neuroblastoma IMR-32 (CCL-127), colorectal carcinoma T84 (CCL-248) and normal cortical neuron HCN-2 (CRL- 10742) were purchased from American Type Culture Collection, USA. The *in vitro* drug release of siRNA encapsulated PLGA-HNPs was carried out at pH 7.4 and 4.2. The experiment of siRNA release was carried in acidic environment (pH 4.2) to resemble the cancer tissue environment. PNA-NLS (H-TCAACGTTAGCTTCACC- PKKKRKV-NH_2) was purchased from PNA Bio, Inc. USA. Ambion siRNA construction kit, Life technology, Japan was used for the synthesis of siRNA and quatified using Ribogreen reagent (Qubit™ RNA Assay Kits, Invitrogen, Japan). For cell proliferation study MTS-based colorimetric assay (Cell titer 96 AQ One Cell Proliferation assay, Promega

Corporation's, USA) was used. Alexa Fluor® 488 annexin V/Dead Cell Apoptosis Kit with Alexa® Fluor 488 annexin and PI was used for studying apoptosis. RNeasy Mini kit (Qiagen) was used for isolation of mRNA. One Step SYBR PrimeScript PLUS RT-PCR Kit (Takara Bio Inc) was used for qRT-PCR.

5.2.2. Synthesis of PLGA Hollow nanoparticle preparation (HNPs)

Hollow nanoshells were synthesized as described in our previous paper [29], using simple solvent emulsion evaporation method. Briefly 50 mg of PLGA was dissolved in 1ml of ethyl acetate. This solution was vortexed for one hour. The above vortexed PLGA solution was emulsified in 5 % (w/v) of 2 ml of PVA solution. This solution was stabilized in 0.05 % (w/v) PVA solution to form colloidal solution containing PLGAHNPs.

5.2.3. siRNA duplex preparation

The silencer siRNA used in the experiment, corresponding to sequence 447 -449 relative to the start codon of c-myc Gene bank Accession no. AA149343 and sequence 997 - 998 of n-myc mRNA sequences Gene Bank Accession no. X03294 were synthesized after *in vitro* transcription and hybridization of the sense and antisense strands. siRNA synthesis was carried out according to the manufacturer's instruction. Briefly, the siRNA duplexes were synthesized by *in vitro* transcription with T7 RNA polymerase on linear DNA templates selecting part of mRNA reference sequences from human n-myc and c-myc. The DNA strands designed and used for synthesizing siRNA were:

1. c-myc

Sense 5'-AATAATTGGCCCAAGTCATTG-CCTGTCTC-3'

Antisense DNA 5'-AACAATGACTTGGGCCAATTA-CCTGTCTC-3'

2. n-myc

Sense DNA 5'-AAGTCAAACTCGAGGTCTGGG-CCTGTCTC-3'

Antisense DNA 5'-AACCCAGACCTCGAGTTTGAC-CCTGTCTC-3'

The above 29-mer DNA oligonucleotides (template oligonucleotides) with 21 neucleotides (nt) encoding siRNA and 8 nt complementary to the T7 Promoter Primer were synthesized. In separate reactions, the two template oligonucleotides were hybridized to a T7 Promoter Primer that is provided in the kit that contains a T7 promoter sequence. The 3' ends of the hybridized DNA oligonucleotides were extended by the Klenow fragment of DNA polymerase to create double-stranded siRNA transcription templates. The sense and antisense siRNA templates were transcribed by T7 RNA polymerase and the resulting RNA transcripts were hybridized to create dsRNA. The dsRNA consisted of 5' terminal single-stranded leader sequences, a 19 nt target specific dsRNA, and 3' terminal UUs. The leader sequences are removed by digesting the dsRNA with a single strand specific ribonuclease supplied in the kit. Overhanging UU dinucleotide remained on the siRNA and this helped in further transfection. The DNA template is removed at the same time by a deoxyribonuclease. The resulting siRNA is purified by elution method, which removes excess nucleotides, short oligomers, proteins and salts in the reaction mixture. The end product is a double-stranded 21-mer siRNA with 3' terminal uridine dimers that can effectively reduce the expression of target mRNA when transfected into mammalian cells.

Our siRNA sequences were designed according to the guidelines by Ui-Tei et.al. [30] and homology to other genes were confirmed using BLAST. The molarity and concentration of siRNA prepared were calculated according to the instructions in user manual for siRNA synthesis.

5.2.4. Preparation of siRNA encapsulated PLGA nanoparticles

siRNA encapsulated PLGA NPs were synthesized using the simple solvent emulsion evaporation method. In brief, different concentration of siRNA ranging from 100, 500, 1000, 1500, 2000 and 2500 µg/ml solubilized in Tris–EDTA buffer (10 mM Tris, 1 mM EDTA, pH 7.5, TE buffer) were mixed with 3 mg of PLGA and 60 µl of ethyl acetate in a closed vial and was emulsified to synthesize siRNA encapsulated PLGAHNPs. The NPs were collected by centrifugation at 10000 rpm for 8 min and these NPs were further washed with 5 ml of Diethylpyrocarbonate (DEPC)- treated water, vacuum dried and stored at -80 ºC till further use.

siRNA encapsulation was done according to the protocol reported by Cun et al. [31] 2 mg each of NPs containing different concentrations of siRNA was dissolved in 200 µl of chloroform and 500 µl of TE buffer. This mixture was rotated for one hour, and the phases were separated by centrifugation for 30 min at 12000 rpm at 4 ^{o}C. The supernatant was kept at room temperature for 5 min for complete evaporation of chloroform. The samples were diluted in TE buffer and concentration of siRNA was measured by Ribogreen reagent. Readings were taken using plate reader at excitation wavelength of 485 nm and emission wavelength of 520 nm. Each sample was assayed in triplicates and the following equations were used to calculate the amount of siRNA encapsulated.

$$\epsilon_{siRNA\ Ld} = \frac{W_{siRNA}}{W_{NPs}} \quad \text{....(1)}$$

Where

$\epsilon_{siRNA\ Ld}$ = siRNA loading

W_{siRNA} = The weight of siRNA in NPs

W_{NPs} = The weight of NPs

$$\epsilon_{\%\ EE} = \frac{E_{siRNA\ Encap.}}{E_{siRNA\ Used}} \times 100 \ldots\ldots \quad (2)$$

Where

$\epsilon_{\%\ EE}$ = % Encapsulation efficiency

$E_{siRNA\ Encap.}$ = actual amount of siRNA encapsulated in nanoparticles

$E_{siRNA\ Used}$ = the amount of siRNA used for encapsulation

5.2.5. Release of siRNA

The release of siRNA was determined from PLGAHNPS using UV-Vis spectrophotometer. 10 mg of siRNA encapsulated PLGAHNPs was suspended in 10 ml each of Tris-HCl buffer of pH 7.4 and 4.2. Further they were filtered through 0.45, 0.25 and 0.15 µm pores sized Whatman filters to obtain NPs ranging from 50 - 400 nm. Further, aliquots of 1 ml of the above solutions were prepared and placed on a rotator with a stirring speed of 100 rpm at 37^{o}C for 72 h. At specific time intervals (0, 2, 4, 6, 12, 24, 36, 48, 60 and 72 h) samples were centrifuged at 14000 rpm for 8 min at 4 ^{o}C. Then, the supernatant was used to estimate the amount of siRNA released from HNPs by qualitatively analyzing the concentration of siRNA at 260 nm [32, 33]. Each sample was assayed in triplicates and the release efficiency was determined.

5.2.6. Preparation of Functionalized PLGA HNPs

The synthesized siRNA encapsulated PLGAHNPs were functionalized with nucleus targeting and CPP for studying anticancer activity in two different cancer cell lines- IMR-32 and T84, and normal cell line HCN-2. 1 mg of siRNA encapsulated PLGAHNPs was functionalized with PNA-NLS and TAT peptide. Two types of functionalized NPs were prepared, a) siRNA encapsulated PLGAHNPs-coated-PNA-NLS (PAPLGAHNPs), b) TAT peptide coated over siRNA-PNA-NLS-siRNA encapsulated PLGAHNPs (TPAPLGAHNPs) The reason behind coating TAT on PAPLGAHNPs was to increase cell permeability.

PNA-coated NPs were developed by incubating 1 mg of PLGAHNPS in 1, 2.5, 5, 10, and 20 μmol/L of PNA-NLS dissolved in 10 mM Tris buffer (pH 8) for 4 h at room temperature. After incubation, PLGA-PNA NPs were collected by centrifugation at 8000 rpm for 30 min at 4 ^{o}C. They were resuspended again in fresh PNA solution and incubated on ice for an additional 3 h without rotation. These PNA-PLGA NPs were collected by centrifugation at 8000 rpm for 20 mins at 4 ^{o}C and were resuspended in fresh deionized water. These synthesized PAPLGAHNPs were rotated for 20 min prior to experiment [34]. For the preparation of PLGA-PNA-siRNA conjugates, the above prepared NPs were suspended in anti-n-myc and anti-c-myc siRNA of different concentrations (70 nM and 80 nM) with 10 mM of NaCl and 1 mM Tris buffer of pH 7.4 for 20 min at room temperature. After incubation the particles were collected by centrifugation at 5000 rpm for 10 min at 4 $^{\circ}$C [35]. Further, for preparation of TAT-siRNA-PNA-PLGAHNPs conjugates, the above prepared siRNA-PNA-PLGANPs were incubated in 1mM PBS containing TAT with concentration of

10, 50, 100 and 150 µg/ml at room temperature with rotation. The functionalized NPs were collected by centrifugation at 8000 rpm for 10 min [36].

5.2.7. Preparation of fluorescently labeled NPs

Two types of fluorescently labeled NPs were prepared for studying the cellular and nuclear uptake. As we wanted to determine the site specificity and accumulation of NPs we used PNA-NLS and TAT to target the cells. The first type of fluorescent PNA-PLGAHNPS (PAPHNPs) were prepared by conjugating RHODAMINE - PNA complex on Tartrazine- siRNA encapsulated PLGAHNPS complex. 20 µmol/L PNA-NLS was added to 5mM of RHODAMINE and incubated for 1 h. 1 mg of siRNA-PLGAHNPs of approximately 70 nm was incubated with 20 mM of Tartrazine. Both complexes were mixed in Tris buffer and incubated on ice for 7 h. Resulting PAPHNPs were collected by centrifuging the above solution at 3000 rpm for 10 min at 4 $^{\circ}$C

The second type of fluorescently labeled TAT-siRNA-PNA-PLGAHNPS (TPAPHNPs) were prepared by incubating siRNA- PAPHNPs in 0.5 mM of FITC-DMSO-TAT solution. TAT-FITC was prepared on ice by incubating TAT peptide and FITC-DMSO solutions for 20 min. These NPs were washed in deionized water to remove excess FITC and the NPs were collected and re-suspended in deionized water for further studies.

Technically, four types of siRNA encapsulated PLGAHNPs namely a) PAPLGAHNPs, b) TPAPLGAHNPs (non-fluorescent NPs), c) PAPHNPs and d) TPAPHNPs (fluorescent NPs) moieties were synthesized as represented in figure 5.1 for studying the *in vitro* effects on cancer cells. Cell proliferation

ability, mode of cell death, uptake of NPs in cancer cells and downregulation of MYC-gene was studied. These NPs were stored at -20 ^{o}C till further use.

5.2.8. Characterization of NPs

The NPs (1 mg/ml) suspended in deionized water and were characterized for their shape and size using SEM and TEM. SEM was performed at 5 kV and TEM at 180 kV. Zeta potential and DLS of PAPLGAHNPs, TPAPLGAHNPs, PAPHNPs and TPAPHNPs were analyzed.

Cell culture

Monolayer of IMR-32 and T84 were maintained within 1 – 8 passages [37, 38]. IMR-32 cells were cultured for 3 days in Eagle's Minimum Essential Medium (EMEM), with 10 % of heat inactivated fetal bovine serum (FBS) and antibiotics. The T84 cells was cultured for 5 days to get a monolayer in Dulbecco's Modified Eagle's Medium (DMEM) - Ham F12K media with 5 % serum and 10 % antibiotics. The HCN-2 cells were cultured and maintained in DMEM, with 10 % FBS and antibiotics. For studying the cell proliferation, flow cytometry and relative mRNA expression level; these cells were grown to confluency with a viability of 85 – 99 %. The viability of the cells was checked using tryphan blue assay.

5.2.9. Analysis of Cell Proliferation

IMR-32, T84 and HCN-2 cells were seeded in 96-well culture plates ($5x10^4$ cells/well) and treated with different concentration of NPs containing various concentrations of siRNA and were incubated overnight. MTS-based colorimetric assay was used for cell proliferation study, where PAPLGAHNPs

and TPAPLGAHNPs containing different concentrations of anti- c-myc and anti-n-myc siRNA (50, 70, 80 nM) were used. The absorbance was measured at 490 nm [39].

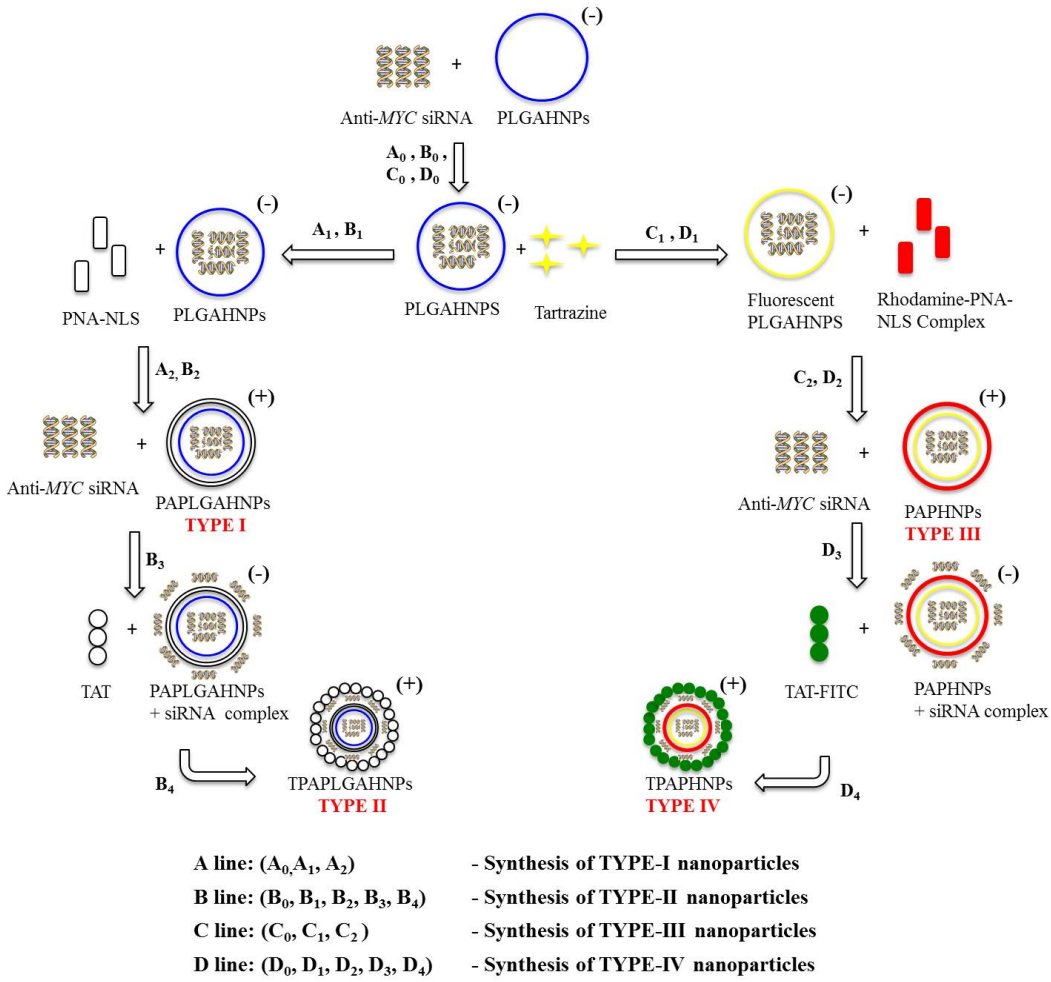


Figure 5.1: Schematic representation of synthesis of four types of anti-myc-siRNA encapsulated PLGA NPs: Type 1- PAPLGAHNPs, Type 2- TPAPLGAHNPs (non-fluorescent NPs), Type 3- PAPHNPs and Type 4- TPAPHNPs (fluorescent NPs).

5.2.10 Flow cytometry

The mode of cell death and effect of the functionalized NP on the cells were confirmed by flow cytometry. The cells were seeded at a concentration of 5 x 10^3 cells/well in 6-well plates and treated with functionalized NPs. IMR-32 cells were incubated overnight with 200 µg/ml each of TPAPLGAHNPs and PAPLGAHNPs keeping 70 nM of siRNA in PLGAHNPS. While 150 µg/ml of TPAPLGAHNPs and 250 µg/ml of PAPLGAHNPs with concentration of 80 nM of siRNA were treated with T84 cells. The HCN-2 cells were incubated with 200 µg/ml (70 nM of anti-n-myc siRNA) and 150 µg/ml (80 nM of anti-c-myc siRNA) of TPAPLGAHNPs; 200 µg/ml (70 nM of anti-n-myc siRNA) and 250 µg/ml (80 nM of anti-c-myc) of PAPLGAHNPs. The cell death was observed in both the cancer cells using live and dead cell assay kit. Flow cytometry measurements were taken at fluorescence emissions of 530 nm and 575 nm [40]. Same procedure was carried on normal cells to determine the effect of NPs.

5.2.11 Cellular uptake of nanoparticles

To investigate the site targeted delivery and accumulation of the NPs, HCN-2, IMR-32 and T84 cells were incubated with fluorescent PAPHNPs and TPAPHNPs. Two sets of each type of cell lines were plated on a 35 mm glass base confocal plate till the cells reached 70-80 % confluency. One set of each cell type was kept for 4 h uptake studies and another set for overnight observation. Hoechst 33342 nucleic acid stain was used for staining nucleus. 4 h after the treatment, one of the above sets containing control and treated cells were washed with Dulbecco's Phosphate Buffered Saline (DPBS), fixed by 4 %

formaldehyde solution in DPBS for 10 min and stained with Hoechst 33342 in DPBS for 20 min for nucleus visualization. The cells were washed twice with DPBS and images were obtained using confocal microscopy. The laser of 405, 488, 561 and 640 nm were used to accquire the images. The other set was incubated overnight for observation of effect of NPs. The fluorescence signals were quantified and efficacy of the nanoparticle's accumulation was counted using the formula [41]:

$$\frac{G}{B} = \frac{\left(\frac{Gcell}{Scell} - \frac{Gbgr}{Sbgr}\right)}{\left(\frac{Bcell}{Scell} - \frac{Bbgr}{Sbgr}\right)} \quad \ldots.(3)$$

Where

G- green fluorescence signal from FITC

B - blue signals from Hoechst

Gcell, Bcell- green and blue signal intensities in the cells

Scell - square of cell area where the fluorescent signal is measured

Sbgr- square of the background area

Gbgr, Bbgr- background green and blue signals intensities

Uptake in nucleus

Nuclear uptake of the nanoparticles was confirmed by confocal microscopy. Cells were incubated with fluorescent nanoparticles for 4 h (TAT-Anti*MYC* siRNA-PNA-NLS-PLGAHNPs). The 3D reconstruction of *in vitro* cultures was carried out to investigate the accumulation and nucleus targeting specificity of NPs that were present in and around nucleus [42].

Morphological analysis

HCN-2, IMR-32 and T84 cells were cultured for 3 days in six well plate containing PAPLGAHNPs and TPAPLGAHNPs. Phase contrast microscopy was used to check the morphological changes in cells after co- incubation with the NPs.

5.2.12 RNAi Treatment.

Investigation of gene inhibition was studied on HCN-2, IMR-32 and T-84 cells. The cells were grown to confluency and treated with 250 μg/ml (70 nM of anti-n-myc siRNA) and 150 μg/ml (80 nM of anti-c-myc siRNA) of TPAPLGAHNPs; 250 μg/ml (70 nM of anti-n-myc siRNA) and 350 μg/ml (80 nM of anti-c-myc) of PAPLGAHNPs. Cells were treated and incubated overnight for two days with NPs. The cells were then harvested to check n-myc and c-myc mRNA expression by qRT-PCR [43].

5.2.13 Relative gene expression studies using qRT-PCR

Cells were harvested and washed with DPBS. Total RNA was isolated from these cells using RNeasy Mini kit (Qiagen) and quantified by UV spectrophotometry [43]. Complementary DNA (cDNA) was prepared from 1.56 μg of total cellular RNA using oligo-dT18 primers according to the manufacturer's protocol. Relative gene expression of c-myc, n-myc mRNA was compared with that of human β2-microglobulin mRNA was determined by qRT-PCR.

Following primers were used:

c-myc forward: 5`-CTCCTCGGTGTCCGAG- GACC-3`;

c-myc reverse: 5`-GTTCGCCTCTTGACA-TTCTCC-3`;

n-myc forward: 5`-TCTGTCGGTTGCA-GTGTTG-3`;

n-myc reverse: 5`-TTCTCAAGCAGG-ATCTCCG-3`;

β2-microglobulin forward; 5`-ATCTTCAAACCTCCATGATG-3`

β2-microglobulin reverse; 5`-ACCCCCACTGAAAAAGATGA-3`

The concentration of mRNA used was 10 μM, with 1 μl primer mix (sense and antisense) in 25 μL q-PCR reaction mixture. The q-PCR was performed using One Step SYBR PrimeScript PLUS RT-PCR Kit based on the manufacturer's protocol after optimization. Inhibition of c-myc and n-myc mRNA was determined by comparison of the ratio between c-myc and n-myc and β2-microglobulin mRNA for the treated groups against the untreated group. RT-PCR products were subjected to electrophoresis on 2 % agarose gel and visualized by ethidium bromide staining. Agarose gel were photographed and intensities were determined using BioRad GS710. The standard curve obtained from PCR showed the level of mRNA concentration present in the treated and non-treated cells. We have calculated the relative mRNA expression by using the equation 4.

$$E = (10^{-1/\text{slope}} - 1) \times 100 \quad \ldots. (4)$$

5.3. Results

5.3.1. Nanoparticle Characterization

SEM, TEM and DLS were used to characterize the NPs. Figure 5.2A represents the SEM image of PLGAHNPs that were spherical in shape and hollow. In TEM image (Figure 5.2 B), we observed the absence of inner core in

the NPs. Compared to Figure 5.2 B, in figure 5.2 C we could observe the presence of light shadow region, depicting the presence of siRNA encapsulated in the hollow NPs. According to the DLS graph (figure 5.2 D) the mean diameter of siRNA encapsulated PLGAHNPs was around 70 nm after it was filtered through 0.15 µm Whatman filter.

5.3.2. siRNA encapsulation efficiency

The encapsulation efficiency of siRNA into NPs plays a significant role in "non-viral gene delivery". It is reported that the encapsulation efficiency of non-hollow PLGANPs is affected by the concentration of PLGA used to synthesize the NPs [31, 44]. We have used PLGAHNPs to encapsulate higher amount of siRNA and protect it from degradation. From figure 5.3 the amount of siRNA encapsulated was 1997 ± 0.76 µg/ml when 2000 µg/ml of siRNA was used for encapsulation. The efficiency of siRNA encapsulation was 99 ± 0.42% as calculated using equation 2.

5.3.3. siRNA release efficiency

PLGA NPs favor siRNA delivery systems because they can readily be surface-modified to enhance targeting or cellular uptake [45]. We found that PLGAHNPs show efficient encapsulation and *in vitro* release of siRNA and have the capacity to protect the siRNA from the external environment. In our experiment the PLGA HNPS were filtered through 0.45, 0.25 and 0.15 µm sized Whatman filters. As shown in figure 5.4 A we obtained NPs of mean size around 260 ± 0.49, 105 and 70 ± 0.77 nm respectively after filtering. Further from graph in figure 5.4 B and C we concluded that the maximum siRNA release is from 70 nm sized PLGAHNPS when compared to other larger

PLGAHNPS. Maximum amount of siRNA release in pH 7.4 was 1786 µg/ml, and the release efficiency was 89 ± 0.89 %. The siRNA release from other NPs was comparatively lower (78 % for 105 nm and 0.00015 % for 260 ± 0.49 nm).

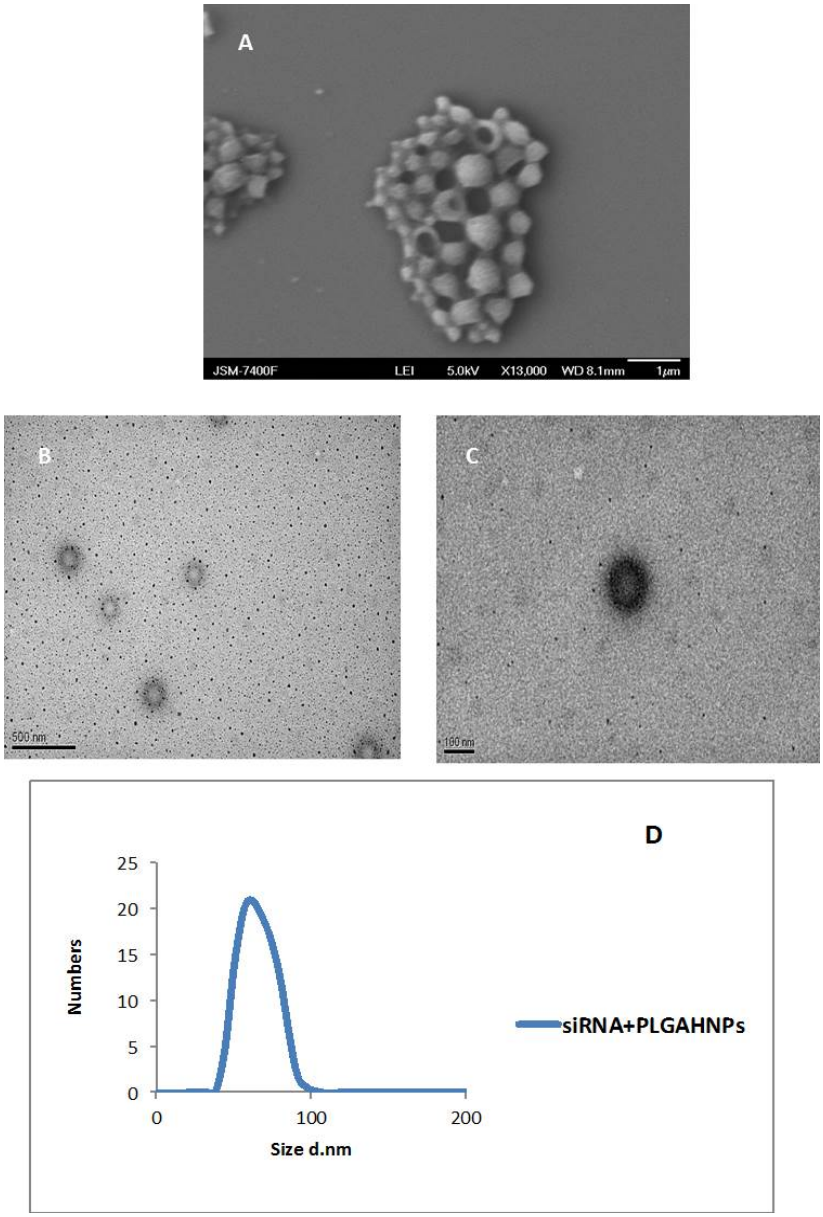


Figure 5.2 Characterization- A. SEM image, B. TEM image of PLGAHNPs and C. TEM image of siRNA encapsulated PLGAHNPs, D. DLS of siRNA encapsulated PLGAHNPs.

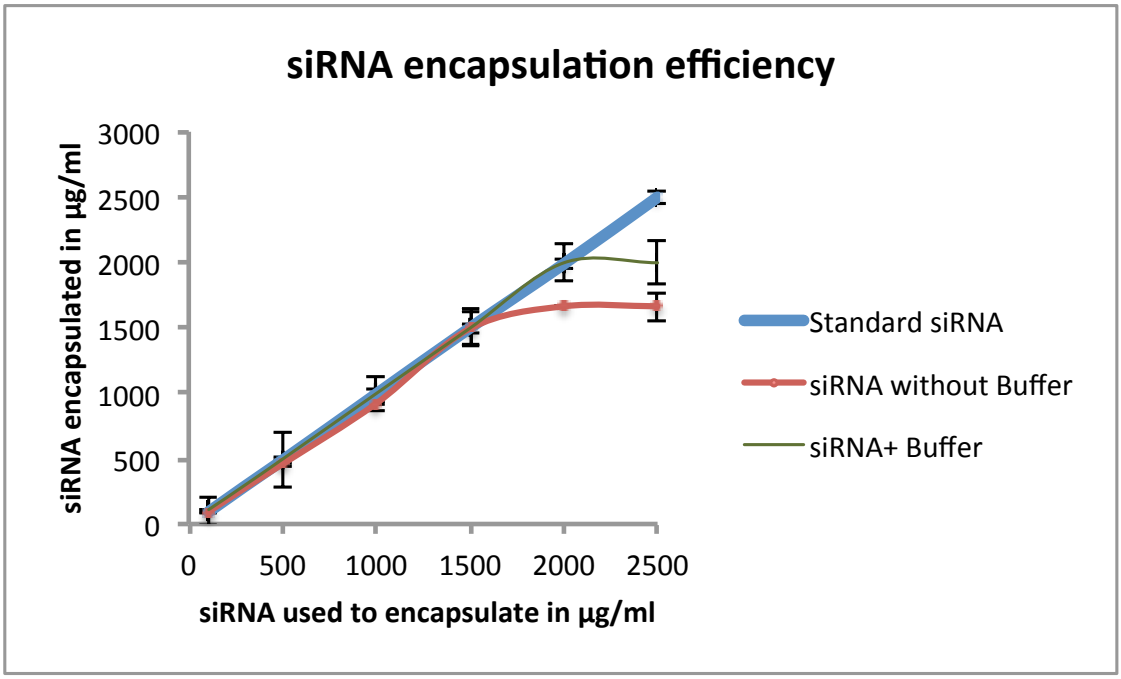


Figure 5.3. siRNA encapsulation efficiency of PLGAHNPS.

From graph in figure 5.4 C we observed that the NPs of 70 nm showed 94 % of siRNA release after 12 h in pH 4.2 and sustained release after 12 h. We performed siRNA release in pH 4.2 to mimic the acidic environment of endosomes in the cells [29, 31].

At pH 4.2 the release efficiency of 105 nm was 84 % and of 260 ± 0.49 nm was $4x10^{-4}$ %. For further studies with targeting moieties, NPs of size 70 nm were selected. The size, siRNA encapsulation and release efficiency was suitable for siRNA delivery in cancer cells. It is reported that the NPs of size ranging from 70-200 nm are best suited for *in vitro* and *in vivo* delivery [46].

5.3.4. Characterization of functionalized NPs

TAT and PNA-NLS were attached to the surface of siRNA (anti-c-myc and anti-n-myc) encapsulated PLGAHNPS by layer-by-layer assembly. After

the incubation and addition of targeting moieties, hydrodynamic size and zeta potential were analyzed. We observed that the targeting moities formed a layer on the HNPs. Figure 5.5 showed the SEM image of these functionalized NPs.

Table 5.1 summarizes surface charge, size and encapsulation efficiency of PLGAHNPS, PAPLGAHNPs, TPAPLGAHNPs, PAPHNPs and TPAPHNPs. The size of PLGAHNPS increased from 58 nm to 123 ± 0.87 nm after functionalizing with targeting moieties to form TPAPLGAHNPs. The functionalized fluroscent NPs were larger in size than non-fluroscent NPs. The surface charge of PLGA NPs without fluorescent moiety was -7 mV. After conjugation of PNA and TAT peptide, the surface charge was +12 and +29 mV respectively, suggesting the formation of peptide layer on PLGAHNPS.

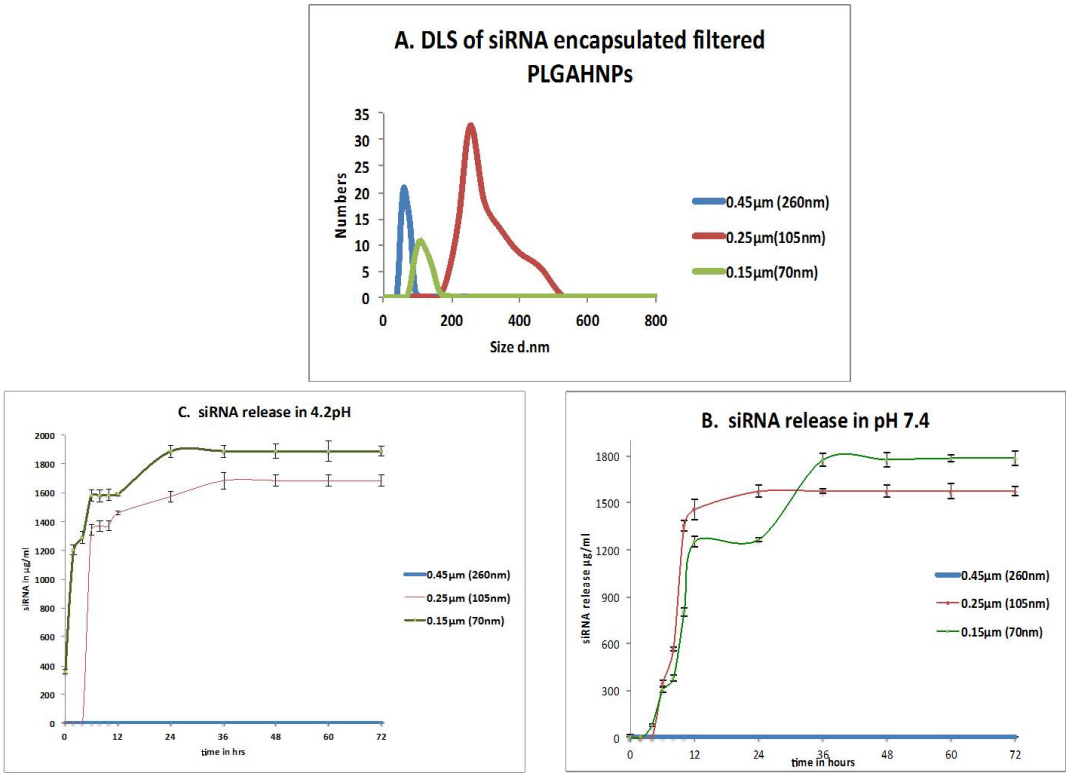


Figure 5.4 DLS of PLGAHNPS after filtering and siRNA release efficiency by PLGAHNPS at pH 7.4 and 4.2.

A layer of anti-c-myc and anti-n-myc siRNA was inserted in between two peptides. This contributed an increase in hydrodynamic size and decrease in surface charge to -5 mV after the formation of PNA layer. Although the TAT layer increased the size, the surface charge was maintained to + 29 mV creating cationic TPAPLGAHNPs, which made the penetration into the cell membrane easily [35, 47]. The targeting moieties converted the PLGAHNPs to cationic polymeric NPs, which made them ideal carriers for gene delivery [47]. Thus after the complete functionalization of PLGAHNPS, the cellular and nuclear uptake were studied in different cell lines.

5.3.5. Effect of nanoparticles on cell

The minimum inhibitory concentration (MIC) required for inhibiting cell proliferation was determined using MTS assay. Cancer cells were treated with different molar concentrations of anti-*MYC*-siRNA (anti-n-myc siRNA for IMR-32 cells and anti-c-myc siRNA for T84) ranging from 50 - 90 nM. From figure 5.6 B, D and F we can observe the effect of optimized molar concentration of anti-MYC-siRNA on IMR-32, T84 and HCN 2. 70 nM of anti-

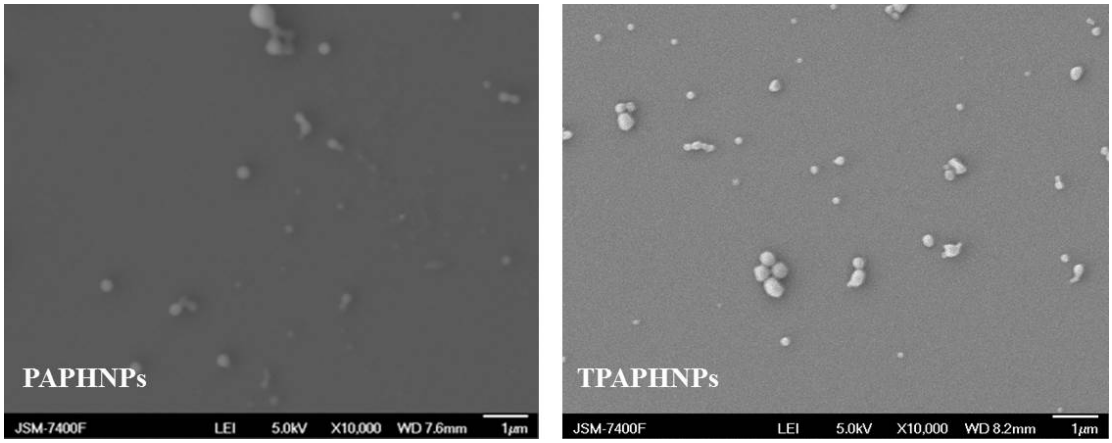


Figure 5.5 SEM images of functionalized siRNA encapsulated PLGA hollow nanoparticles

n-myc siRNA was optimized as MIC required for inhibiting IMR-32 cells and 80 nM of anti-c-myc siRNA was required to inhibit T84 cells. There was no significant effect on proliferation observed in HCN-2 cells. Further we treated IMR-32, T84 and HCN-2 cells with functionalized NPs encapsulated with optimized molar concentration of anti-MYC- siRNA ranging from 50 to 250 µg/ml. From figure 5.6 A,C and E we observe the MIC required for inhibiting IMR-32, T84 and HCN-2 cells when treated with functionalized NPs. 200 µg/ml of PAPLGAHNPs and 200 µg/ml of TPAPLGAHNPs was the MIC observed to inhibit IMR-32 cells encapsulated with anti-n-myc siRNA of 70 nM.

Nanoparticles	**Properties of NPs**				**siRNA Encapsulation efficiency (%)**
	Non- Fluorescent (Blank)		**Fluorescently Labeled**		
	Size (nm)	**Zeta potential (mV)**	**Size (nm)**	**Zeta potential (mV)**	
PLGA	**58.57**	**-7.06**	**69.06**	**-15.04**	**93.45**
PLGA+PNA-NLS	**78.82**	**16.2**	**91.28**	**12**	**96.05**
PLGA+PNA-NLS+siRNA (c-myc)	**105**	**-2.51**	**113.3**	**-5.88**	**96.00**
PLGA+PNA-NLS+siRNA (n-myc)	**115**	**-0.3**	**121**	**-1.7**	**96.00**
PLGA+PNA-NLS+siRNA+TAT (c-myc)	**121.2**	**25.3**	**122**	**29.5**	**96.00**
PLGA+PNA-NLS+siRNA+TAT (n-myc)	**123.87**	**11.5**	**124.06**	**15.7**	**96.00**

Table 5.1: Particle size, zeta potential, siRNA loading of PLGA HNPS.

Also, 250 µg/ml of PAPLGAHNPs and 150 µg/ml of TPAPLGAHNPs was the MIC required to inhibit T84 cells encapsulated with anti-c-myc siRNA of 80 nM. There was no effect on proliferation of HCN-2 when treated with 200 µg/ml of PAPLGAHNPs, 200 µg/ml of TPAPLGAHNPs encapsulated with anti-n-myc siRNA of 70 nM, 250 µg/ml of PAPLGAHNPs and 150 µg/ml of TPAPLGAHNPs encapsulated with anti-c-myc siRNA of 80 nM.

Further we performed flow cytometry experiment to determine the effect of functionalized NPs on IMR-32, T84 and HCN-2 cells (Figure 5. 7). The viability of untreated IMR-32, T84 and HCN-2 cells was 96%, 89% and 98% respectively. We observed 67.31 % and 49.12 % of cellular death in IMR-32 and T84 cells after treatment of TPAPLGAHNPs encapsulated with anti-MYC-siRNA after 4 h of incubation. Also, 9.42 % and 12.16 % of cellular death in IMR-32 and T84 cells after treatment of PAPLGAHNPs encpasulated with anti-MYC-siRNA after 4 h of incubation. Treatment of functionalized NPs did not have significant effect on HCN-2 cells (figure 5.7 B). Also, 78.24 % and 78.12 % of cellular death was observed in IMR-32 and T84 cells after overnight incubation with TPAPLGAHNPs while 46 % and 51.67 % of cellular death was observed in IMR-32 and T84 cells after overnight incubation with PAPLGAHNPs encapsulated with anti-MYC-siRNA (figure 5. 7A). There was no effect on HCN-2 cells after overnight incubation with functionalized NPs. The results are tabulated in table 5.2.

5.3.6. Cellular and nuclear uptake of PAPHNPs and TPAPHNPs

The cellular uptake of two different types of NPs (PAPHNPs and TPAPHNPs) were observed after 4 h of incubation and overnight incubation of

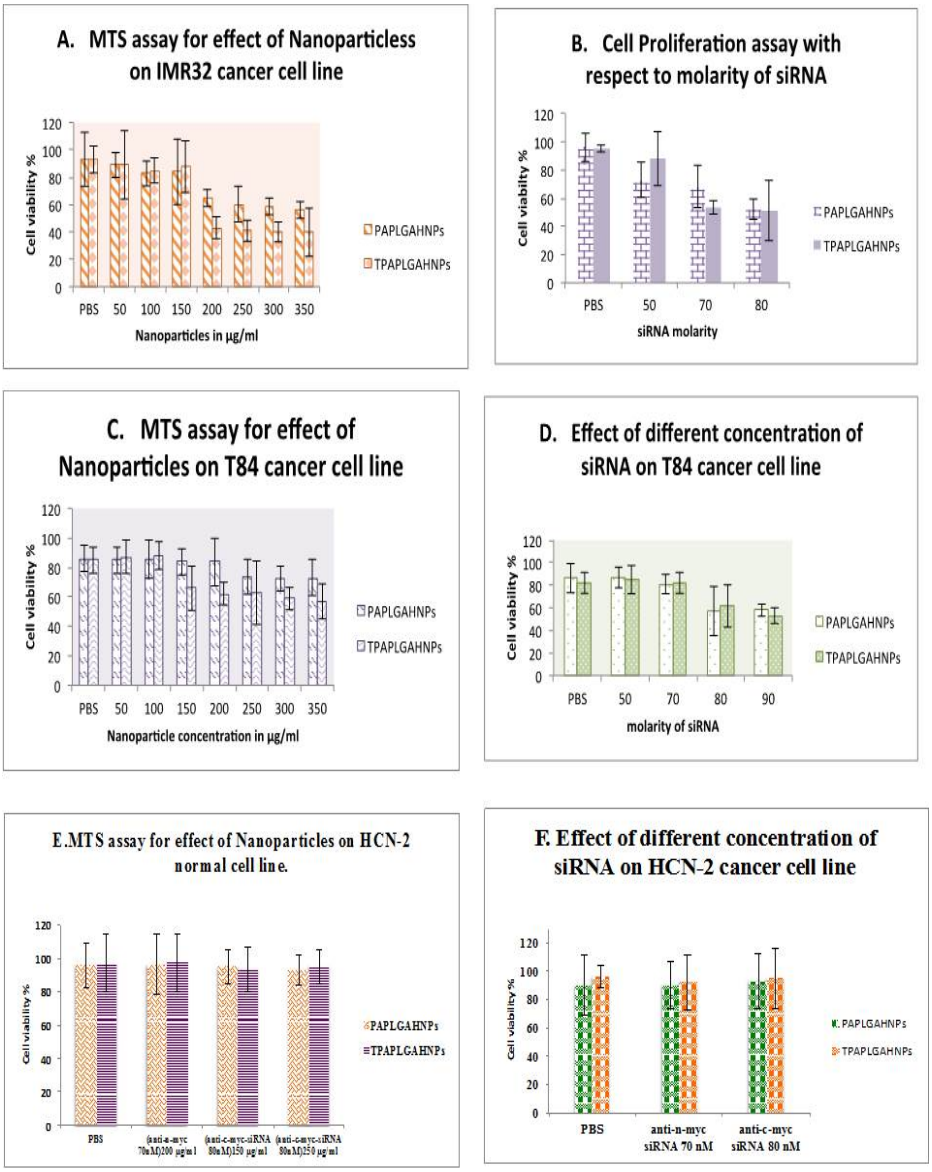


Figure 5.6 Effect of functionalized nanoparticles on cancer cell proliferation and normal cell proliferation.

NPs with cancer cells and normal cells. The 4 h as well as overnight incubation of NPs showed the presence of fluorescence in cytoplasm and nucleus as observed in figure 5.8. It is known that the successful endosomal escape is crucial for the siRNA carriers to improve siRNA silencing efficiency [24]. We have observed co-localizaion of PNA-Rhodamine and Tartrazine-PLGAHNPS in cytoplasm and nucleus of cancer cells by confocal microscopy.

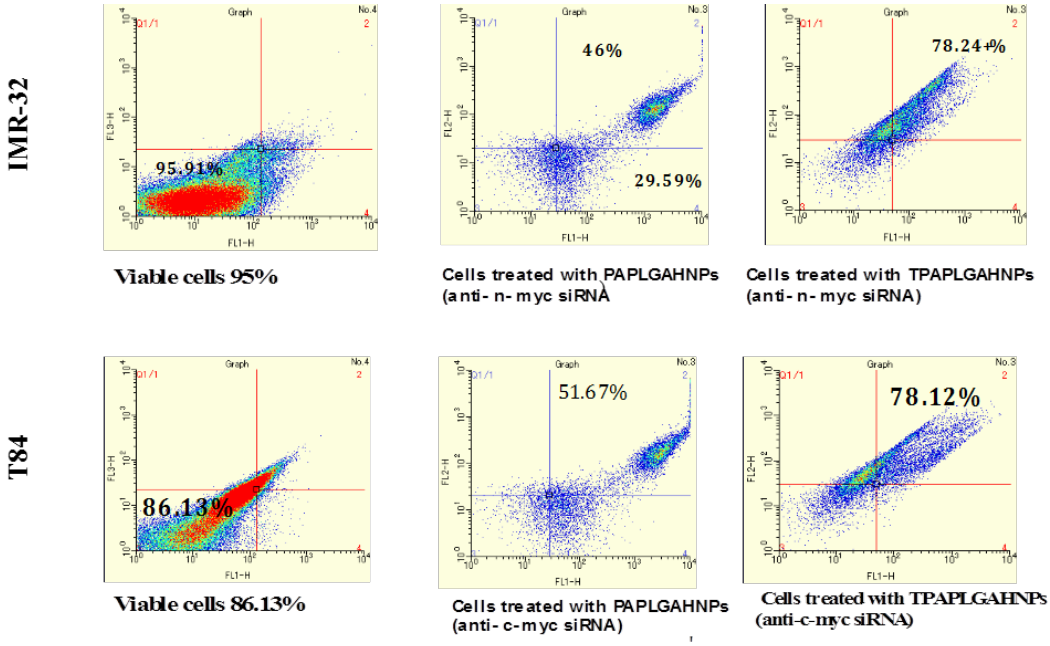


Figure 5. 7 (A)

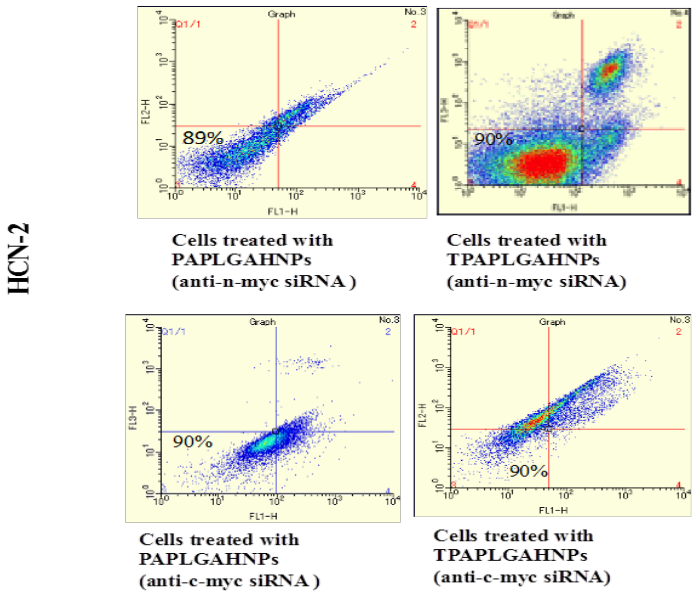


Figure 5. 7 (B)

Figure 5.7 Study of death in cancer cells after overnight treatment of functionalized NPs.

Sr. No.	Cell Lines	Cell Deaths (%)					
		TPAPLGAHNPs		PAPLGAHNPs		Untreated	
		Overnight	4 h	Overnight	4 h	Overnight	4 h
1	HCN (anti-n-myc siRNA)	10	3	11	2	9	2
2	HCN (anti-c-myc siRNA)	10	4	10	3	9	2
3	IMR-32 (anti-n-myc siRNA)	78.24	67.31	46	9.42	4.09	3
4	T-84 (anti-c-myc siRNA)	78.12	49.12	51.67	12.16	13.83	10.68

Table: 5.2. Results of flow cytometry represented in percentage cell death in cancer cells after four hours and overnight incubation with functionalized NPs.

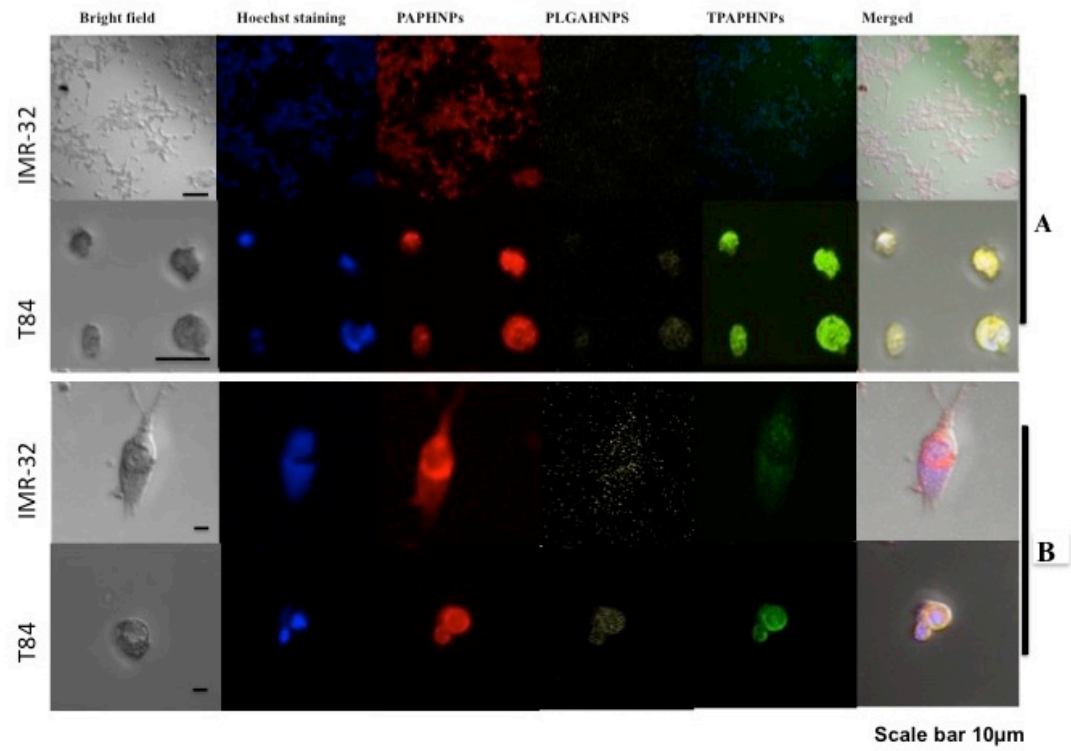
Within 4 h of coincubation with PAPHNPs and TPAPHNPs, the NPs were randomly distributed all over the cytoplasm. In figure 5.8 A we have observed co-localization indicating the presence of PNA-Rhodamine, FITC-TAT and Tartrazine-PLGAHNPS in IMR-32 and T84 cells. The single cell uptake as observed in figure 5.8 B of IMR-32 and T84 represents that NPs were present both in cytoplasm and in nucleus. In figure 5.8 C we observed morphological changes in cancer cells after overnight incubation with fluorescent NPs. In figure 5.8 D, cells treated with Tartrazine-PLGAHNPs did

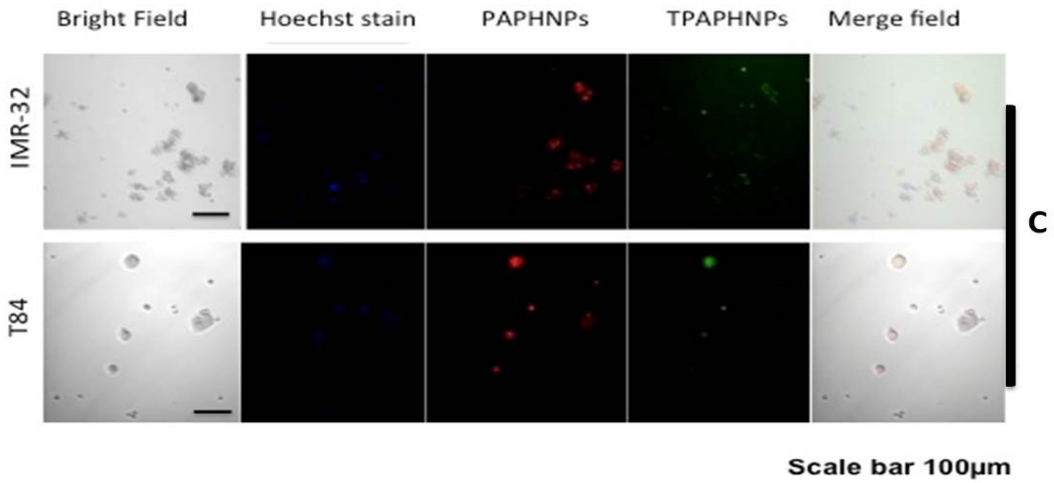
not show any cellular or nuclear uptake. Accoridng to figure 5.8 E and F cytosol and nuclear uptake of NPs was less in HCN-2 cells. These results suggest the enhanced uptake of NPs in cancer cells.

We have observed that the cells treated with TPAPHNPs showed the uptake of NPs into the nucleus. Moreover, the cells treated with PAPHNPs showed less uptake in nucleus compared to TPAPHNPs. This may be because TAT protects the NPs from external environment. Based on figure 5.8 quantification of fluorescence intensity was calculated using equation 3 and presented in figure 5.9. The fluroscence intensity of Rhodamine was less in cells treated with PAPHNPs. In IMR-32 and T84, the presence of FITC in cytosol was high, while less in nucleus. This concluded that TAT helps the PAPHNPs to cross-cellular membrane and release siRNA.

The confocal images in figure 5.8.C and the phase contrast microscopy images in figure 5.10 revealed that the cellular uptake of TPAPHNPs show morphological changes after the overnight incubation. The TPAPHNPs treated IMR-32 underwent shrinkage and were clumped. We also observed that the IMR-32 cells lost aherence and started floating. We have observed same effect in T84 cells. We observed no adverse effect of NPs on HCN-2 cells. These results clearly prove that the NPs resulted in cancer cell death. Further to confirm nuclear uptake of NPs, cells were treated with TPAPHNPs and were visualized by 3-D imaging after 4 h (Figure 5.11). In figure 5.11 A and 5.11 B we observed the nuclear uptake and colocalization of PNA-Rhodamine, TAT-FITC and Tartrazine-PLGAHNPS. The nucleus was stained with Hoechst stain. We have observed higher nuclear uptake of PNA conjugated NPs in IMR-32 and T84. The presence of PNA-Rhodamine, Tartrazine-PLGAHNPS in nucleus

determined the uptake of NPs through the nuclear membrane. The process of internalization initiates during the first 4 h. In figure 5.11 C and 5.11 D the orthogonal section of HCN-2 nucleus proved that NPs were completely absent inside the nucleus. From figure 5.11 we inferred that nuclear uptake of siRNA encapsulated PLGAHNPS were mediated by PNA and TAT.





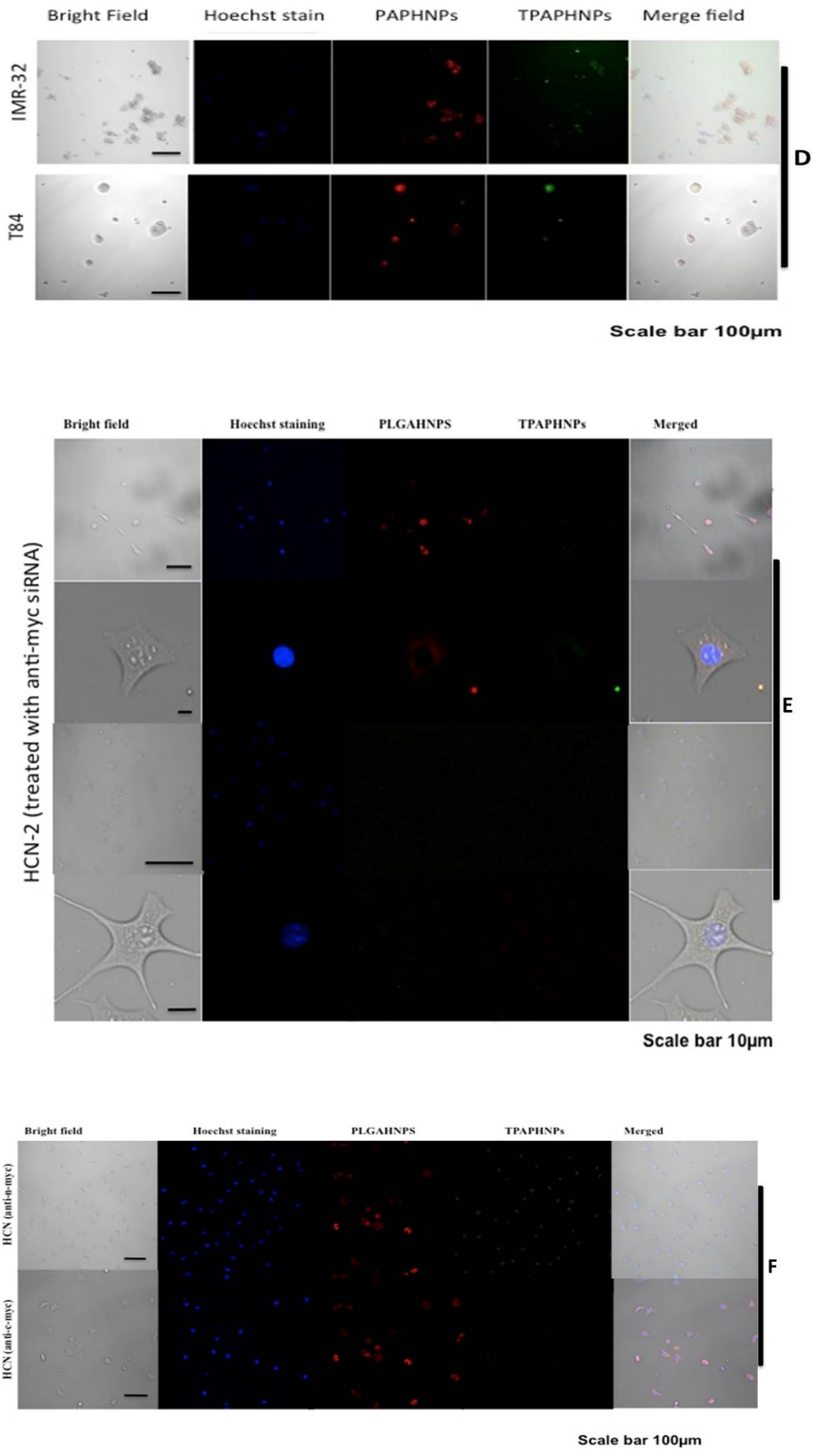


Figure 5.8 Cellular uptake of fluorescent functionalized nanoparticles to study the endosomal escape and cytoplasmic siRNA delivery.

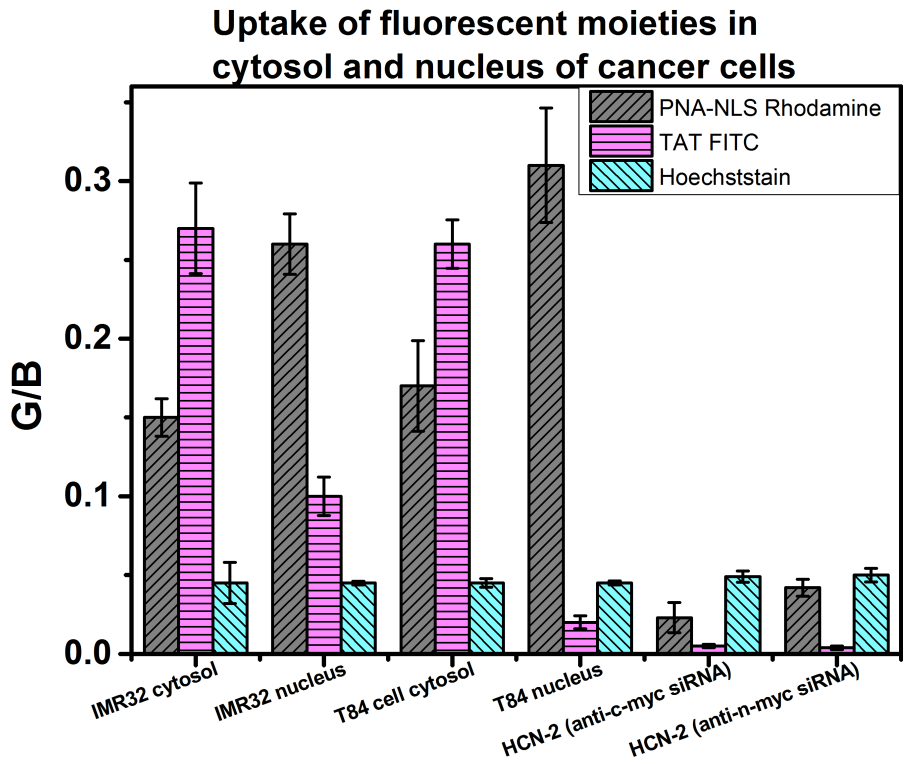


Figure 5.9. Study of fluorescent intensity of functionalized nanoparticles after nuclear and cellular uptake.

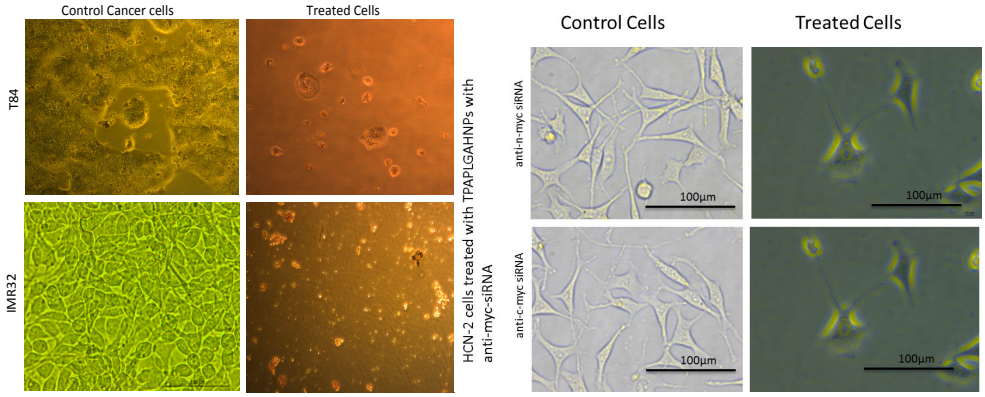
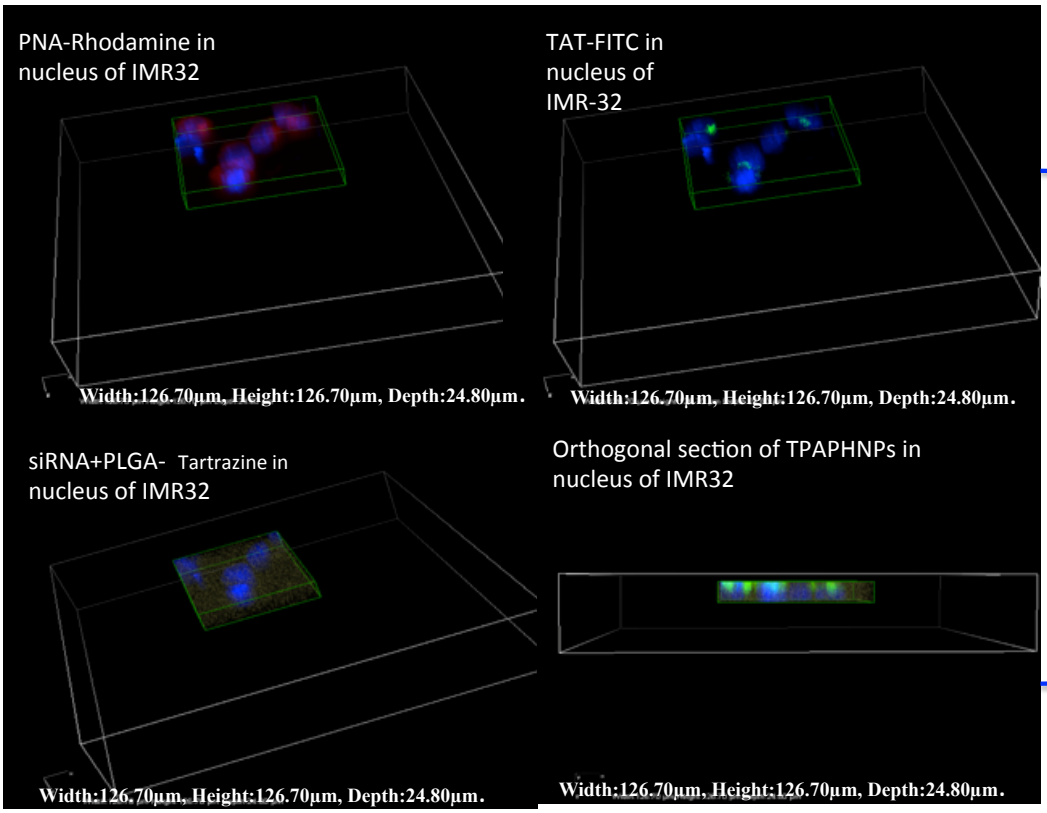
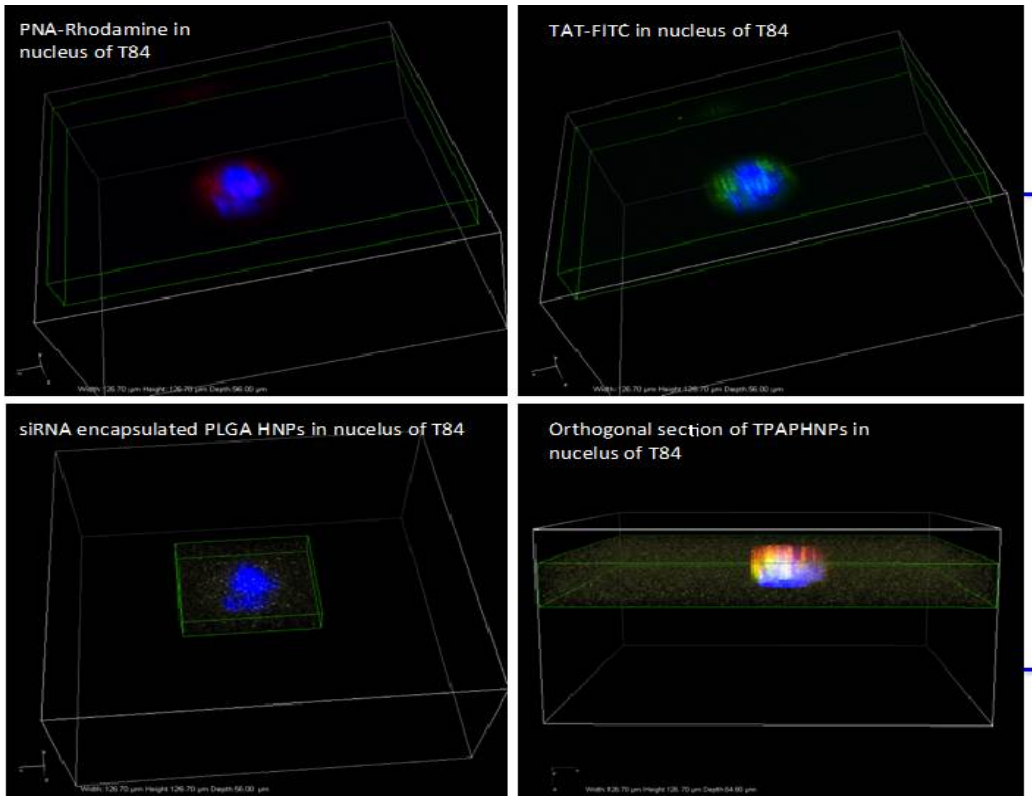


Figure 5.10 Study of Morphological changes in cancer cells. No change observed in normal cells.


PNA-Rhodamine in nucleus of IMR32
Width:126.70µm, Height:126.70µm, Depth:24.80µm.
TAT-FITC in nucleus of IMR-32
Width:126.70µm, Height:126.70µm, Depth:24.80µm.
siRNA+PLGA- Tartrazine in nucleus of IMR32
Width:126.70µm, Height:126.70µm, Depth:24.80µm.
Orthogonal section of TPAPHNPs in nucleus of IMR32
Width:126.70µm, Height:126.70µm, Depth:24.80µm.

A


PNA-Rhodamine in nucleus of T84
TAT-FITC in nucleus of T84
siRNA encapsulated PLGA HNPs in nucelus of T84
Orthogonal section of TPAPHNPs in nucelus of T84

B

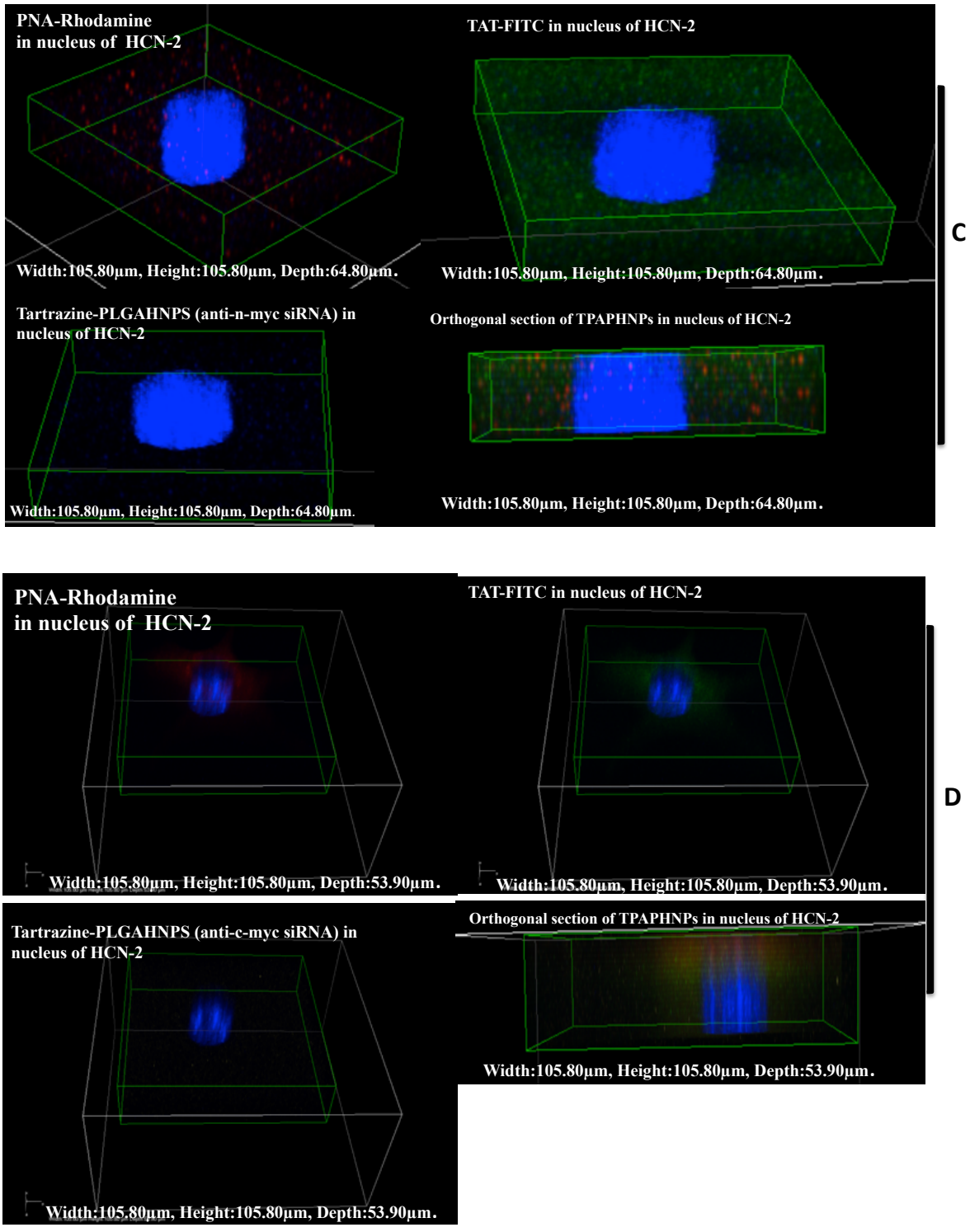


Figure 5.11. Nucleus uptake of functionalized nanoparticles after four hours of incubation.

5.3.7. Effect of NPs on gene expression

To investigate the RNAi efficiency with anti-n-myc and anti-c-myc

siRNA, IMR-32 and T84 cells were treated with PAPLGAHNPs and TPAPLGAHNPs and the relative gene expression was studied using qRT-PCR analysis. There was significant down regulation of c-myc and n-myc gene expression. To study the relative gene expression efficacy (figure 5.12), cells were incubated with different concentrations of siRNA encapsulated PAPLGAHNPs and TPAPLGAHNPs. IMR-32 cells were incubated with anti-n-myc siRNA (50 nM and 70 nM) and T84 cells were incubated with anti-c-myc siRNA (50 nM and 80 nM). We have observed a decrease in mRNA expression as observed in figure 5.12. These results suggested 1.2 fold decrease in n-myc expression and 0.6 fold decrease in c-myc mRNA expression. To further investigate the efficiency of gene silencing, qRT-PCR was performed and compared with β2-microglobulin, a housekeeping gene. HCN-2 cells were also treated with same concentration of functionalized NPs and no effect in gene regulation was observed. The relative gene expression was calculated based on equation 4. In IMR-32 cells mRNA expression was reduced from 96.7 % to 32.4 % and in T84 cells from 74 % to 31 % when treated with PAPHNPs.

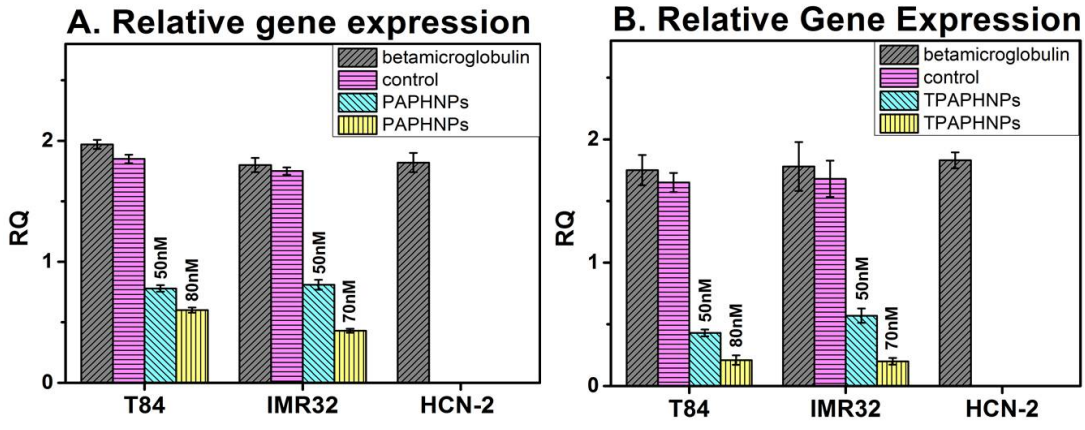


Figure 5.12: Down regulation of proto-oncogene *MYC* in cancer cells after overnight incubation with functionalized NPs.

On treatment with TPAPHNPs the gene expression in IMR-32 was reduced from 96.7 % to 5.64 % and in T84 from 74 % to 8.815 %. These results suggested gene silencing using sequence specific siRNA using PLGAHNPs as delivery vehicle.

5.4. Conclusion

In our study, PLGAHNPs were used as nano carrier for anti-myc siRNA delivery. PLGAHNPs were able to encapsulate approximately 2000 μg/ml siRNA. Targeted delivery of anti-myc siRNA encapsulated NPs was achieved using PNA-NLS and TAT. These functionalized NPs resulted in receptor-mediated endocytosis and delivered anti-myc siRNA in endolysosomal compartments. Down regulation of gene was observed due to efficient delivery of siRNA in the targeted cells. The targeted siRNA could be further applied to other disease-associated genes and could be used for *in vivo* applications. Thus, the combination of PLGA HNPs, TAT, and PNA-NLS to formulate nanocarriers provides an attractive strategy for siRNA delivery in therapeutic use. In addition, there are several ways for incorporating diagnostic and therapeutic agents into cancer cells such as protein modification, surface loading and core loading. Since the interaction between TAT, PNA-NLS and siRNA is based on surface loading, the core siRNA still can be replaced with other drugs for treatment or image-guided therapy. This system can be extended to other NPs or peptide-based delivery systems for creating safe and effective therapeutic delivery.

5.5. References

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Chapter 6

Conclusion

This chapter summarizes the complete work and major conclusions are presented in this chapter. In the first chapter, the historical background of siRNA delivery and various techniques (layer-by-layer, encapsulation, conjugation and PEGylation) that are used in RNAi therapeutics are discussed. The main advantages of these techniques are that siRNA gets delivered into the target cells without affecting any properties or stability of siRNA. We have summarized the techniques used during the synthesis, characterization and biological properties of the nanomaterials developed.

The importance of gene therapy and the significance of siRNA delivery is discussed in the third chapter. The development of nanocapsules with polymers and lipid conjugates are critical for the delivery of siRNA. To solve the delivery problems of siRNA, many delivery systems have been developed. The highly effective delivery systems are quite different in terms of structure, size and chemistry, but there are still some guidelines to be considered for optimal delivery systems. Nanoparticle delivery systems should have a particle size of about 100-200 nm, i.e. be large enough to avoid renal and hepatic filtration but small enough for immune clearance. PEG as the shielding agent has proven to be valuable in preventing non-specific interactions and avoiding immune recognition in the circulation. Chemical modifications, such as 2'-O-methyl substitutions, are necessary to reduce non-specific effects and avoid nuclease digestion. In addition, endogenous or exogenous targeting ligands are

also often beneficial for siRNA uptake by cancer cells. In recent years, siRNA drug development has experienced highs and lows. In summary, a good delivery system is the key to siRNA medical development. Once research into siRNA drug delivery systems makes a significant breakthrough, siRNA will occupy a strong position in the drug market, especially in the anti-cancer drug market.

In the fourth chapter we discuss the synthesis, characterization, enhanced drug encapsulation and release efficiency with hollow polymeric nanocapsules. These hollow nanoparticles were formed as a result of air entrapment, fast solidification, and phase separation with instant stabilization. PLGA hollow nanoparticles have the capacity to hold the cargo material for long time wherein the material can remain safe. The sustained release of drug was observed. These PLGA hollow nanoparticles were pH sensitive, inferring that these particles could be used for drug or gene delivery. Also, as these hollow shells were nanostructures with large surface area, they may be used as carriers for drug and gene delivery for anticancer treatment.

In the fifth chapter of the thesis we have described a new strategy for siRNA delivery into cancer cells using polymeric nanoparticles. PLGAHNPs were used as nano carrier for anti-myc siRNA delivery. PLGAHNPs were able to encapsulate approximately 2000 µg/ml siRNA. Targeted delivery of anti-myc siRNA encapsulated NPs was achieved using PNA-NLS and TAT. These functionalized NPs resulted in receptor-mediated endocytosis and delivered anti-myc siRNA in endolysosomal compartments. Down regulation of gene was observed due to efficient delivery of siRNA in the targeted cells. The targeted siRNA could be further applied to other disease-associated genes and could be used for *in vivo* applications. Thus, the combination of PLGA HNPs, TAT, and

PNA-NLS to formulate nanocarriers provides an attractive strategy for siRNA delivery in therapeutic use. In addition, there are several ways for incorporating diagnostic and therapeutic agents into cancer cells such as protein modification, surface loading and core loading. Since the interaction between TAT, PNA-NLS and siRNA is based on surface loading, the core siRNA still can be replaced with other drugs for treatment or image-guided therapy. This system can be extended to other NPs or peptide-based delivery systems for creating safe and effective therapeutic delivery.

Our results support the application of polymer based nano delivery system for siRNA delivery. Developments in siRNA research could bring new treatments in nanomedicine for various diseases.

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"The larger the island of knowledge, the longer the shoreline of mystery."

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Raichur Archana Mukundrao

List of Publications

1. Archana Raichur, Yoshikata Nakajima, Yutaka Nagaoka, Toru Maekawa, D. Sakthi Kumar. "Hollow polymeric (PLGA) nano-capsules synthesized using solvent emulsion evaporation method for enhanced drug encapsulation and release efficiency." Materials Research Express. IOP publication. doi:10.1088/2053- 1591/1/4/045407.

2. Archana Raichur, Yoshikata Nakajima, Yutaka Nagaoka, Toru Mizuki, Kazunori Kato, Kotaro Matsumoto, Toru Maekawa, D. Sakthi Kumar. "Strategist PLGA Nano-capsules to deliver siRNA for Inhibition of carcinoma and neuroblastoma cell lines by knockdown of MYC proto-oncogene using CPPs and PNA." Nano World Journal. United Scientific Group. doi:10.17756/nwj.2015-00.

Conferences

1. Archana Raichur, Toru Mizuki, Yutaka Nagaoka, Kazunori Kato, Toru Maekawa, D. Sakthi Kumar. "Strategist PLGA nano-capsules to deliver siRNA for inhibition of carcinoma and neuroblastoma cell lines by knockdown of proto-oncogene." The 4th International Conference on Nanotek & Expo, San Francisco, USA Dec. 2014. (Best Poster Award)

2. Archana Raichur, Toru Maekawa, D. Sakthi Kumar, Biosynthesis of "Gold nanoparticles for therapeutic pRNA/siRNA delivery in carcinoma cells using Bipartite approach." The 12th International Symposium on Bioscience and Nanotechnology, Kawagoe Campus, Toyo University, Japan.

3. Archana Raichur, Yoshikata Nakajima, Yutaka Nagaoka, Yoshida, Toru Maekawa, D. Sakthi Kumar. "Polymeric nano-hollow shells synthesized by simple solvent evaporation method." The 11th International Symposium on Bioscience and Nanotechnology, Hakusan Campus, Toyo University, Tokyo, Japan.

Abbreviations

AFM	Atomic Force Microscopy
Ac-BSA	Acetylated Bovine serum albumin
ATCC	American Type Culture Collection
AuNPs	Gold nanoparticles
CLSM	Confocal Laser Scanning Microscopy
CPPs	Cell Penetrating Peptides
DLS	Dynamic Light Scattering
DMAEMA	N, N-Dimethylaminoethyl Methacrylate
DPBS	Dulbecco's Phosphate-Buffered Saline
EDS	Energy-dispersive X-ray spectroscopy
EMEM	Eagle's Minimal Essential Medium
EPR	Enhanced permeability and retention effect
ESCA	Electron Spectroscopy for Chemical Analysis
FDA	Food and Drug Administration
FBS	Fetal bovine serum
FTIR	Fourier Transform Infrared Spectroscopy
GFP	Green Fluorescent Protein
HA	Hyaluronic Acid
HNPs	Hollow Nanoparticles
IgM	Immunoglobulin M
kDa	Kilo Daltons
KRAS	Kirsten rat sarcoma viral oncogene homolog
LbL	Layer-by-layer assembly
LPEI	Linear Polyethylenimine
MALDI-TOF	Matrix Assisted Laser Desorption/Ionization Time of- Flight Mass Spectrometry
MFT	Multilayer Mediated Forward Transfection
MPF	Multilayered polyelectrolyte films
MPS	Mononuclear phagocyte system
mRNA	messenger RNA
NIR	Near Infra Red
NMR	Nuclear Magnetic Resonance

Non-HNPs	Non-Hollow Nanoparticle
NPs	Nanoparticles
PAH	Poly (allylamine hydrochloride)
PAPHNPs	PNA-NLS-Rhodamine-siRNA-encapsulated PLGA Hollow Nanoparticles
PAPLGAHNPS	PNA-NLS- siRNA encapsulated PLGA Hollow Nanoparticles
PBS	Phosphate-buffered saline
PEG	Polyethylene glycol
PEI	Polyethylenimine
PEMs	Polyelectrolyte Multilayers
PEO	Polyethylene oxide
PKR	Protein kinase RNA-activated
PLGA	Poly (lactic-co-glycolic acid)
PLGAHNPs	PLGA Hollow Nanoparticles
PLGAHNPS	siRNA encapsulated PLGA Hollow Nanoparticles
PNA-NLS	Peptide nucleic acid- nuclear localization signal
PPO	Polypropylene oxide
PSS	Poly (styrenesulfonic acid)
PVA	Poly (vinyl alcohol)
QCM	Quartz Crystal Microbalance
QELS	Quasi-Elastic Light Scattering
RAFT	Reversible Addition−Fragmentation chain Transfer
RBS	Rutherford Backscattering Spectrometry
RNAi	RNA interference
qRT-PCR	Quantitative Real Time Reverse Transcriptase Polymerase Chain Reaction
SEM	Scanning Electron Microscopy
siRNA	Small interfering RNA
SPIONs	Super paramagnetic iron oxide nanoparticles
STAT 3	Signal Transducer and Activator of Transcription
TAT	Trans-Activator of Transcription
TEM	Transmission Electron Microscopy

TICs	Tumor Initiating Cells
TPAPLGAHNPS	TAT-PNA-NLS-siRNA encapsulated PLGA hollow nanoparticles
TPAPHNPS	TAT-FITC-PNA-Rhodamine-siRNA encapsulated PLGA Nanoparticles
VEGF	Vascular Endothelial Growth Factor
XPS	X-ray Photoelectron Spectroscopy
XRD	X-ray Diffraction