Synthesis and Evaluation of Modified Polydopamine Nanoparticles for Biomedical Applications

To be submitted as the Major Project under the partial fulfillment of the requirement for the degree of

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In

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Submitted by

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(2K15/IBT/09)

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CERTIFICATE

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This is to certify that **Miss Prachi Nagar**, a student of Delhi Technological University, pursuing Master of Technology in Industrial Biotechnology has successfully completed the training and the project work entitled "**Synthesis and Evaluation of Modified Polydopamine Nanoparticles for Biomedical Applications**" from January, 2017 – June, 2017 under my supervision.

She has duly submitted her project report to the **CSIR-Institute of Genomics and Integrative Biology.**

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DECLARATION

I hereby declare that the project work entitled **"Synthesis and Evaluation of Modified Polydopamine Nanoparticles for Biomedical Applications"** is a record of an original work done by me at CSIR-IGIB, Delhi under the knowledge and guidance of **Dr. Kriti Bhandari, Assistant Professor**, Department of Biotechnology, Delhi Technological University. This project work has been submitted in the partial fulfillment of the requirement for the award of the degree of Master of Technology in Industrial Biotechnology. The results reported here are true to the best of my knowledge.

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LIST OF ABBREVIATIONS

µg/ml: microgram per milliliter

µg: microgram

µl: microliter

µm: micrometer

^{1}H-NMR: Proton Nuclear Magnetic Resonance Microscopy

A.U. /mg: Arbitrary units per milligram

Abs: absorbance

B.cereus: Bacillus cereus

bPEI: branched polyethylenimine

BSA: Bovine serum albumin

CHO: Chinese Hamster Ovary cells

D2-methanol: Methyl-d3 alcohol-d4

D_2O: Deuterium Oxide

DA.HCl: Dopamine Hydrochloride

Da: Dalton

DLS: Dynamic Light Scattering

DMEM: Dulbecco's Modified Eagle's medium

DNA: Deoxyribonucleic acid

DNase: Deoxyribonuclease

E.coli: Escherichia coli

EDTA: Ethylenediaminetetraacetic acid

EGFP: Enhanced Green Fluorescent Protein

EMSA: Electrophoretic mobility shift assay

EtBr: Ethidium Bromide

FBS: Fetal Bovine Serum

FTIR: Fourier Transform Infrared spectroscopy

g/mol: gram per mole

Gram +ve: Gram positive

Gram –ve: Gram negative

h: hour

HCl: hydrogen chloride

HEK 293: Human Embryo Kidney 293 cell line

HEPA: High Efficiency Particulate Air

IGIB: Institute of Genomics and Integrative Biology

K.pneumoniae: Klebsiella pneumoniae

KBr: potassium bromide

kDa: Kilo Dalton

kV: kilo volt

L: liter

LDH: Lactate dehydrogenase

M.W.: molecular weight

M: molar

MCF-7: Michigan Cancer Foundation-7

MCT: micro centrifuge tube

MeOH: methanol

mg/ml: milligram per milliliter

MHz: Mega hertz

min: minute

ml: milliliter

mM: milli molar

MTCC: Microbial Type Culture Collection

MTT: 3-(4, 5-Dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium

N: normailty

NaCl: sodium chloride

NaOH: sodium hydroxide

NCCS: National Centre for Cell Science

ng/µl: nanogram per microliter

nm: Nanometer

NMR: Nuclear Magnetic Resonance

NPs: Nanoparticles

O.D.: optical density

^{o}C: degree Celsius

P.aeruginosa: Pseudomonas aeruginosa

PBS: Phosphate buffer saline

PDA: Polydopamine

pDNA: plasmid deoxyribonucleic acid

PMS: Phenazine Methosulphate

ppm: parts per million

S.aureus: Staphylococcus aureus

S.D.: standard deviation

SDS: sodium dodecyl sulphate

TAE: Tris Acetate EDTA buffer

TEM: Transmission Electron Microscopy

TNBS: 2,4,6- trinitrobenzene sulfonic acid

U.A.: Uranyl acetate

U/mg: activity unit per milligram

USIC: University Science Instrumentation Centre

w/w: weight/weight

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Chapter 1

Abstract

ABSTRACT

Polymeric nanoparticles have provided for an answer to many biomedical queries and diagnosis and treatment of the diseases can be carried out with their help. In this study, polydopamine was used as the parent polymer which was modified to fulfill two aims of the project, i.e. gene delivery as well as antimicrobial activity. For gene therapy, efficient delivery of DNA to cells is the primary concern. An efficient carrier must have a good stability, transfection efficiency and minimal toxicity. One such example of polymeric vector is branched polyethylenimine (bPEI). It is regarded by the scientists as the "gold standard for gene delivery" but suffers a major drawback of high cytotoxicity. Here, we have attempted to develop nanoparticles by choosing a mussel-derived polymer, polydopamine (PDA) cross-linked with polyethylenimine in such a way that the toxic nature of bPEI is suppressed by the conversion of free primary amine groups to secondary and tertiary amines. Keeping the amount of PDA fixed, varying amounts of bPEI (10 kDa and 1.8 kDa) are linked. We observed a trend in hydrodynamic size with changing ratio of PEI onto PDA ranging from 160-300 nm and zeta potential from +12-30 mV in the developed two series – 1) PDA_1:$^{10}bPEI_{0.5}$, PDA_1:$^{10}bPEI_1$, PDA_1:$^{10}bPEI_2$ and 2) PDA_1:$^{1.8}bPEI_{0.5}$, PDA_1:$^{1.8}bPEI_1$, PDA_1:$^{1.8}bPEI_2$. A visible trend in the DNA condensation ability and buffering capacity is also reported. Further, we report that pDNA complexes of PDA-PEI nanoparticles are non-toxic to mammalian cells and exhibit higher transfection than the native PEI. Further for antimicrobial study, polydopamine was modified by using three antibiotic aminoglycosides which were-gentamicin sulfate, kanamycin sulfate and neomycin sulfate hydrate. Their antibacterial activity against some strains like *S.aureus, K.pneumoniae, P.aeruginosa and B.cereus* was tested and evaluated by zone inhibition method. To sum up, the developed nanoparticles can be effectively used for further in vivo applications.

Keywords: Nanocarriers, polydopamine, polyethylenimine, plasmid DNA, transfection, cytotoxicity, antibiotic, aminoglycosides, antimicrobial activity

Chapter 2

Introduction

INTRODUCTION

Nanobiotechnology is the branch of science that aims to combine the technology in biological and nanoscale realm i.e. biology and nanotechnology. Nanotechnology basically deals with entities on a nanoscale range i.e. 1-100nm. Advancement and research in this field has led to development of many approaches such as nano-devices, nano-carriers for drug and other therapeutic agents, different diagnostic techniques, treatment approaches etc. A number of applications are coming up daily such as in medical and clinical fields, textile industries, food industries, electrical and electronics industries in devising nanochips etc.

Research and development involving the use of nanoparticles for the diagnosis and treatment of a number of debilitating and serious diseases is taking the biomedical world by storm. An array of applications in the biomedical field is fulfilled by the use of nanomaterials. These biomedical applications may include use of nanobiosensors, targeted drug delivery for diseases like cancer, pathogenic detection, controlled drug delivery, MRI imaging, nanomedicines, gene therapy for genetic diseases, antibacterial coatings etc. [1, 2] The utmost requirements for nanomaterials to be used within body or in the devices which come in contact with body is that they should be biocompatible, biodegradable, longer bioavailability, ability to escape endosomal attack and other immune defenses and minimum toxicity. All these properties are highly desirable when the aim is to send nanoparticles within the body and targeted to a particular diseased organ.

A variety of materials are being widely used to make such nanoparticles. These include metals like gold, silver, platinum etc. and synthetic polymers like chitosan, polyglycolic acid, polylactide etc. and natural polymers such peptides, polysaccharides etc. Biological macromolecules such peptides, polysaccharides, lipids etc. are also being used to form biocompatible nanoparticles. Owing to advantages like safety, biodegradability, easy elimination from body and biocompatibility, organic polymers are preferred over inorganic polymers for use in nanoparticles and nanocomposites having different biological applications.

The key dependency of gene therapy is on successful delivery of nucleic acids into the cells. Efficient delivery counts on safe and stable carrier. The viral gene delivery method has limitations such as insertional mutagenesis, acute immune response and restriction on the size of the transgene which have brought this method under scrutiny. Hence, much focus in research is being given on the non-viral methods that have several advantages such as flexibility in the size of the transgene, biocompatibility, less immunogenicity, safe handling and control on the molecular composition of the particle/vector prepared. Therefore, a variety of non-viral vectors ranging from liposomes to polymeric nanoparticles are being employed for the purpose of gene delivery [3, 4]. Among these cationic lipids and polymers are being majorly exploited for their virtue to form nano-complexes with nucleic acids [5].

Another major cause of concern is the bacterial infections that are so frequent and contagious among people. Nosocomial infections that spread from places like hospitals and clinics due to contact with medical devices, dressings, other surfaces coming in contact with body of patients, affect the patients with low immunity. The causative agents for such nosocomial infections are mostly bacteria. Hence, work is being done in the direction to device antimicrobial materials and coatings that may be used to make medical implants, syringes, prosthetics etc. that come in body contact. These surfaces will inhibit the growth of microorganisms. This will prevent the spread of infections and diseases.

One such organic polymer that is being widely worked upon is polydopamine, which is a biomimetic polymer resembling naturally occurring melanin and some mussel adhesive proteins. Much research work has been done on the synthesis and characterization of PDA nanomaterials like nanoshells, nanocapsules, nanoparticles etc. [6-9]. Dopamine is an important neurotransmitter of mammalian nervous systems. Dopamine hydrochloride has the property to undergo self-polymerization under oxidative and alkaline conditions and gives rise to a polymer commonly known as polydopamine. Such alkaline conditions in the reaction mixture can be achieved by using reagents like ammonia water, tris buffer, sodium hydroxide etc. Under such reaction conditions, self-polymerization of polydopamine monomers starts. Change in color is observed from colorless to pale brown

and finally to dark brown as the reaction proceeds. Methods involving enzymatic polymerization and electropolymerization have also been reported and some of these are also being used on industrial scale. Studies have been done showing that variation of pH affects the size of the PDA nanoparticles so formed [10-12]. This polydopamine has different functional groups such as the free amino groups, catechol groups and hydroxyl groups that can be used to functionalize its surface and attach other entities/molecules such as aminoglycosides or other polymers by chemical bond formation [13-16].

Complexes of polydopamine with other polymers or entities may be used for the purpose of gene therapy by developing them into efficient non-viral vectors for gene and drug delivery. Nanoparticle, made up of polydopamine, have been reported to be loaded and also coated with anti-cancer drugs and other antibiotics and delivered them with minimum toxicity to the cells in-vitro.[17-19] Polydopamine linked with branched polyethylenimine has been developed as fluorescent nanoprobe and its biocompatibility and uptake has been previously evaluated on mammalian cell line (A549) [20].

In this study, an effort was made to develop a chemically modified compound by using dopamine hydrochloride derived polymer, polydopamine as the native compound and this was further modified with polyethylenimine. An attempt was made to develop non-viral vectors for gene delivery by linking polymer polydopamine with polyethylenimine of molecular weights 10kDa and 1.8kDa by varying the amount of PEI. These would combine through different free functional groups in a Schiff's base reaction and Michael addition reaction mechanisms. The free amine groups on the surface give positive charge to the nanoparticles formed. Due to positive charge, these will readily take up the negatively charged DNA and form complexes. These nanoparticle/pDNA complexes can be used to transfect cells and eventually deliver the cells with our gene of interest and then assessed for their efficiency in gene delivery.

Furthermore, polydopamine was modified by conjugating them with antibiotic aminoglycosides. The three antibiotic aminoglycosides which were used are gentamicin sulfate, kanamycin sulfate and neomycin sulfate hydrate. The conjugation takes place again because of the presence of amine and hydroxyl groups on the surface of aminoglycosides.by the catecholamine reaction, rearrangement of atoms will take place

and the desired conjugates will be formed. Experiments were done to find if these conjugates possess any antibacterial activity against some of the strains like *S.aureus, K.pneumoniae, P.aeruginosa and B.cereus* and this was tested and evaluated by zone inhibition methods. Hence, these developed compounds and the nanoparticles may be further considered for use in *in-vivo* experiments and study.

Chapter 3

Objectives

OBJECTIVES

The project was carried in the direction of fulfilling the following objectives:

1. Synthesis and characterization of Polydopamine nanoparticles
2. Functionalization of PDA NPs with branched polyethylenimine (10kDa and 1.8kDa) for gene delivery
3. Preparation of PDA-aminoglycosides conjugates and to determine their antibacterial activity

The antibiotic aminoglycosides that were used are:

Kanamycin sulfate, Gentamicin sulfate and Neomycin sulfate hydrate

Chapter 4

Review of Literature

REVIEW OF LITERATURE

Much research work has been done on the synthesis and characterization of PDA nanomaterials like nanoshells, nanocapsules, nanoparticles etc. [21, 22] The surface of PDA possesses functional groups like $–NH_2$ and –OH which are fairly active towards reaction. This facilitates a convenient method for synthesizing the different nanostructures. Alkaline conditions are required for the self-polymerization of dopamine into polydopamine nanoparticles. Such alkaline conditions in the reaction mixture can be achieved by using reagents like ammonia water, tris buffer, sodium hydroxide etc. PDA nanoparticles have also been prepared by using a mixture of ethanol and water, the pH of which is adjusted in the alkaline range. However, the particle sizes obtained by using former reagents yield smaller nanoparticles (size range 25-50nm in diameter) while the latter solution of ethanol and water may lead to formation of slightly larger nanoparticles (size range 50-100nm in diameter) [23]. Under alkaline reaction conditions, self-polymerization of polydopamine monomers starts due to the reaction between the amine and hydroxyl groups present. The reaction proceeds with the catecholamine mechanism and rearrangement takes place of dopamine to lead to the formation of polydopamine. Change in color is observed from colorless to pale brown and finally to dark brown as the reaction proceeds. Methods involving enzymatic polymerization and electropolymerization have also been reported and some of these are also being used on industrial scale. Studies show that variation of pH affects the size of the PDA nanoparticles so formed. [24, 25]

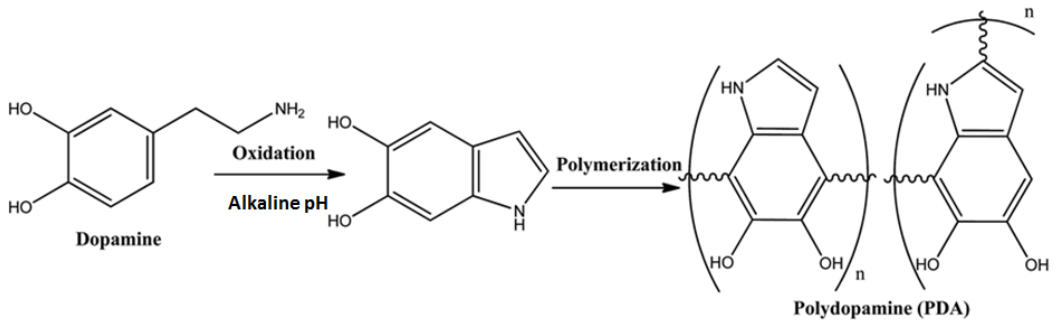


Figure 1: Schematic representation for self-polymerization of dopamine monomers into polydopamine.

Polydopamine has the advantage of having properties like biocompatibility and availability of functional groups that may be exploited in biosensors and other biodevices. Their surfaces have also been functionalized to immobilize the different molecules and agents by the formation of different covalent bonds. Metal nanocomposites with iron (Fe), copper (Cu), platinum (Pt), hydrogels, graphene based nanocomposites are some of the other examples. These have been proposed to have different applications and importance such as electrochemical sensing, pH sensing etc. [26, 27] Nanomaterials and coatings made of polydopamine have been found to have various properties like adhesive properties, optical properties, paramagnetism, chelating activity, electrical conductivity, biocompatibility, biodegradation etc. making it an advanced material that offers a scope to be used in a number of applications and research fields. PDA nanomaterials formed have also been used as template materials to link other molecules or form other nanostructures on them by using materials like Fe_3O_4, MnO_2, Ag etc. [28, 29] Different works have been reported on the linking of PDA with polyethylenimine. These complexes have been used for gene delivery. Various modifications of these have been carried out as well. Various tests have also been done to test their biocompatibility and cytotoxicity on cell lines like HeLa, HEK293 and A549 etc. [30]

Functionalization of PDA NPs using cationic polymers for gene delivery:

Cationic polymers can be used to functionalize the surface of polydopamine nanoparticles. The polymer/polydopamine complex hence formed has the ability of DNA condensation, complexation. Positively charged polymer/polydopamine complex tends to bind the negatively charged DNA and binds this complex into a compact particle. This can now be used to transfer the desired plasmid DNA into the target cells. Polyplexes formed by condensation are able to safely deliver the genetic material since the genetic material is now protected against the different nucleases present within the cell. Because the size of the particle being small, this can easily be taken up by the cell through natural processes like endocytosis, pinocytosis etc.

Gene delivery has also been carried out using the nanoparticles synthesized by these methods. Experiments show that nanoparticles made or coated with polydopamine pose minimal toxicity to the cells in-vitro. For studying polymer based gene deliveries, polyethylenimine has been considered as "the gold standard" due to the high transfection efficiencies. Polydopamine and polyethylenimine can undergo self-polymerization. Branched polyethylenimine thus helps in making the study of gene delivery easier by improving transfection into the cells. Work has been done that show that the molecular weight of polyethylenimine greatly affects the transfection efficiency. Polyethylenimine has positively charged surface due to the presence of amine groups. This forms a polyplex with the negatively charged DNA in the aqueous environment which has to cross the cell membrane barriers to get delivered within the cells. Depending on the molecular weights of the polyethylenimine, the size of these polyplexes formed may be less or more intact, the surface charge may be less or more which would clearly affect the transfection. Hence, for this purpose polyethylenimine with various molecular weights like 25kDa, 2kDa and 5kDa have been tested to get the adequate combination of high efficacy and low toxicity during transfection. [31-35]

pDNA used for transfection studies

pDNA isolated from the DH5α strain of *E.coli* is commonly employed for the transfection studies. This strain used of *E.coli* is non-pathogenic and is widely used for research purposes in the molecular biology and cell culture laboratories. D. Hanahan carried out multiple mutations on this strain to make it suitable for high-efficiency transformation and cloning purposes.

pEGFP which stands for plasmid Enhanced Green Fluorescent Protein was used as the reporter gene to ascertain that the pDNA was delivered safely within the mammalian cells. Using GFP as a reporter gene for studying cells is a noninvasive technique and it can be easily illuminated with blue light for the visualization purpose. The fluorescence produced helps in determining the transfection efficiency.

Lipofectamine as a transfection agent

Lipofectamine is an important transfection agent. It is commonly used in molecular and cellular biology works. Lipofectamine contains cationic lipid subunits which form liposomes and can easily form complexes with the negatively charged plasmid DNA. These complexes can now cross the cell membrane and lipofectamine will thus help the genetic material to penetrate within the cell and finally inside the nucleus.

Mammalian cell lines used in transfection

Transfection is the process of insertion of a genetic material (RNA, DNA) into the mammalian cell. Often this genetic material would code for a particular protein expression. The process of transfection incorporates this genetic material within the cell's genome and thus helps to achieve this protein expression or other desired function by the use of the cell's own machinery. No single method or reagent can be effective in transfecting all the mammalian cell lines. However, over the years, many methods and reagents have been developed that can transfect a wide variety of cell lines. Some of the most commonly used mammalian cell lines are HEK 293 (Human Embryonic Kidney) cells, CHO (Chinese Hamster Ovary) cells, HeLa cells, MCF-7 (Michigan Cancer Foundation-7) cell line etc.

HEK 293 (Human Embryonic Kidney) cell line:

HEK 293 is a mammalian cell line which is derived basically from the human embryonic kidney cells that have been grown and maintained in tissue culture. HEK 293 cells are widely used in research work involving the study of cell lines and the expression of certain protein in them. HEK 293 cells are easy to maintain and grow, and also show higher transfection as compared to other cell lines. Transfection efficiency for HEK 293 cells has even been reported to be more for PEI-mediated gene delivery.

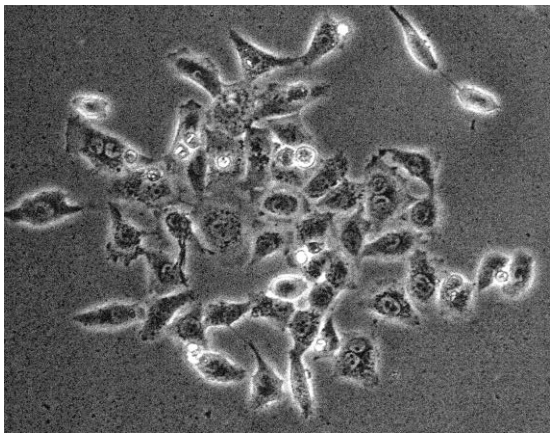


Figure 2: HEK293 cell line (Source: Google images)

Researches involving modifications in the PDA have also been done to study their probable action against microorganisms. Various surface coatings like antibacterial biofilms have also been prepared by linking PDA with other polymers and materials. [36, 37] PDA coatings on silk fibers to be made into antimicrobial fabric have also been developed. [38] Dopamine is basically a natural compound with different modifications and roles such as a neurotransmitter, adhesive proteins in marine organisms and a pigment of melanin. This makes polydopamine very much biocompatible and causes minimum toxicity. At the same time, it has the ability to escape acute responses of the body's immune system. Nanoparticles like those of silica, platinum, gold and silver have also been coated with polydopamine to enhance their biocompatibility factor. Research studies have been done on PDA NPs loaded with drugs like rifampicin, doxorubicin, amptothecin and camptothecin, carvacrol (natural agent) and checked for their ability to deliver them with minimum toxicity to the cells in-vitro. PDA NPs loaded with carvacrol have been shown to possess antibacterial activity against *Pseudomonas* and *Salmonella* spp. [39-41]

Functionalization of PDA NPs using aminoglycosides for antimicrobial activity:

Aminoglycosides are the different natural and semisynthetic compounds that are rich in amino (-NH_2) groups linked with the glycoside residues. Some of the aminoglycosides have an antibiotic action as well. The examples of such antibiotic aminoglycosides are as follows: kanamycin, gentamicin, neomycin, tobramycin, amikacin etc. These antibiotic aminoglycosides may be used to functionalize the surface of PDA NPs and then used for the formation of antimicrobial coatings and compounds. Studies have shown such PDA-aminoglycoside conjugates to have more antimicrobial action as compared to aminoglycosides or PDA alone. These aminoglycosides combine with the PDA via catecholamine reaction method.

However, the antibacterial activity of native polydopamine nanoparticles has only been reported for the PDA nanoparticles synthesized by the facile shaking method against *E.coli* and *P.aeruginosa*. No activity for native PDA nanoparticles and coatings prepared by any other method has been reported so far. [42, 43] Silver coated SiO_2/PDA nanoparticles have been tested for their antibacterial activity against *Vibrio natrigiens* (Gram negative) and *Bacillus subtilis* (Gram positive). The mechanism of action of these nanoparticles against *V.natrigiens* was the release of silver ions from the surface and the formation and release of reactive oxygen species within the cytoplasm due to the activity of silica and polydopamine. The mechanism of action against gram positive bacteria was due to the disruption of cell wall by the silver ions. [44] Hence, as can be seen there is a difference in the manner an antibacterial agent acts against the bacteria based on whether they are Gram positive or Gram negative.

In 1884, Christian Gram gave a method of differentially staining bacteria depending on the structural differences of their cell walls. Based on whether or not the bacteria will retain crystal violet dye or not, they can be categorized into two groups, namely: Gram positive bacteria and Gram negative bacteria. Bacteria belonging to these two major groups show characteristic differences in their nature, susceptibility and resistance to heat, stress, antibiotics etc. These two classes can be explained as under:

Gram-positive bacteria: These retain the crystal violet stain even after the washing with acetone or alcohol, and appear violet or purple under the light microscope. They have a thick cell wall (20-30nm) with no outer membrane present, thick peptidoglycan layer, teichoic acids absent and with lipid content very low. Few pathogenic bacteria belong to this group, and primarily produce exotoxins. These are more susceptible to antibiotics. Examples: *Staphylococcus aureus, Bacillus cereus, Streptomyces, Clostridium, Lactobacillus, Streptococcus* etc.

Staphylococcus aureus

Staphylococcus aureus is a gram positive bacterial strain (dia. 0.5-1.5µm). These are facultative anaerobes and require organic source of nitrogen to grow. The pathogenic strains of *staphylococcus* are *S. aureus* (pathogenic to humans) and *S. hyicus* (pathogenic to animals). The ability that *S.aureus* can bind to the various biological molecules, extracellular matrix and plasma proteins such as fibrinogen etc.; enables bacteria to widely grow on the medical devices and instruments and this leads to nosocomial infections. Infection with *S.aureus* may cause skin infections, food poisoning, metal-biomaterial, bone-joint and soft tissues infections. Excessive using of antibiotics and other agents has led to the development of multi drug resistant strains of *Staphylococcus*.

Bacillus cereus

Bacillus cereus is a gram positive bacterium having a rod-shaped morphology. They are nearly 1µm wide and 5-10µm in length. Their arrangement may be in chains or in single. It is a facultative aerobic bacterium and bears spores that are heat resistant. *Bacillus cereus* secretes lethal toxins and enterotoxins that have diarrheal and emetic effect. Diarrheal and emetic infection due to bacillus cereus may lead to symptoms like food poisoning, abdominal cramps, vomiting, nausea etc.

Gram-negative bacteria: These get decolorized after washing with acetone or alcohol, and take up the counter stain (Safranin or Fuchsine) and appear red or pink under the light microscope. They have a thin cell wall (8-12nm) with a membrane present, thin

peptidoglycan layer, teichoic acids present with high lipid content (20-30%). Most pathogenic bacteria belong to this group, and primarily produce endotoxins. These are more resistant to antibiotics. Examples: *Klebsiella pneumoniae, Pseudomonas aeruginosa, Enterobacter, Vibrio, E.coli, Salmonella* etc.

Klebsiella pneumoniae

Klebsiella pneumoniae is a gram negative bacterium. It is an opportunistic pathogenic bacterium that may lead to diseases and infections such as soft tissues, pneumonia and urinary tract infections. *Klebsiella* is a member of intestinal flora of human beings. People suffering from cirrhosis, biliary tract disorders, diabetes mellitus and alcoholism may have an impaired immune defense and may be at increased risk of infection. *K. pneumoniae* are mostly responsible for the nosocomial infections and hospital acquired infections. These are non-motile in nature. *Klebsiella* are resistant to a wide variety of aminoglycosides, cephalosporins and antibiotics like ampicillin and amoxicillin.

Pseudomonas aeruginosa

Pseudomonas aeruginosa is a gram negative, non-spore forming bacterium. It can be commonly found in soil and water. It has capsule of polysaccharide, and can grow in temperature conditions of up to 42ºC, with an optimum growth temperature of 37ºC. It leads to different infections and diseases such as respiratory tract infections, urinary tract infections, wounds with blue green pus, eye and ear infections etc.

E.coli

E.coli commonly inhabits our digestive tracts and may serve useful functions like Vitamin k and Vitamin B production. However, infections due to some harmful strains like *E.coli O157:H7* may lead to diarrhoea, urinary tract infections, kidney failure, anemia etc.

Evaluating activity of an antibacterial agent

Whenever an antibacterial agent is tested against bacterial strains in laboratories, there are a number of methods which can be used to find their antibacterial activity in quantitative terms. For an antibacterial agent, there is always a parameter called as Minimum Inhibitory Concentration which is "the minimal amount of an antimicrobial agent required to hinder the growth of a microorganism which is kept under incubation with the media for about an overnight".

A number of methods that can be used to evaluate the antibacterial action are agar well diffusion method, agar disk diffusion method, broth dilution method; antibacterial gradient method, ATP bioluminescence assay etc. and more have been reported in different research studies. [45]

Hence, all these studies which have been previously done provide an insight that by modifying the native polydopamine with different ligands, molecules and linkers, an altogether new compound may be developed with novel and useful properties that can be exploited for a number of biomedical applications.

Chapter 5

Materials, Buffers and Instruments

MATERIALS

SOURCE	NAME OF REAGENTS
Alfa Aesar , A Johnson Matthey Company (Great Britain)	Neomycin Sulfate
Armar Chemicals (Switzerland)	Deuterium oxide, D_2O
Bio Basic Inc.	Ethylenediaminetetraacetic acid (EDTA)
Bio Rad Inc., (USA)	Bradford Reagent
Gibco (Carlsbad, CA)	Fetal Bovine Serum
HiMedia Laboratories Pvt. Ltd. (India)	Agar Agarose Orange G
Invitrogen (USA)	Lipofectamine 2000
Polysciences Inc. (PA)	Branched Polyethylenimine (10kDa)
Sigma Aldrich Chemical Co. (USA)	Branched Polyethylenimine (1.8kDa) Dopamine Hydrochloride Ethidium bromide Heparin Iodonitrotetrazolium chloride Kanamycin Sulfate Lithium lactate DMEM (Dulbecco's Modified Eagle Medium) MTT (Thiazolyl Blue Tetrazolium Bromide) NAD sodium salt Phenazine Methosulphate (PMS) Tris base
Sisco Research Laboratories Pvt. Ltd. (India)	Gentamicin Sulfate

Spectrum Laboratories Pvt. Ltd. (USA)	Spectra/Por dialysis membrane (MWCO 3.5kDa)
HEK 293 cell line	NCCS
E.coli, Pseudomonas aeruginosa, Klebsiella pneumonia, Bacillus cereus, Staphylococcus aureus	MTCC

BUFFERS

Tris Acetate EDTA (TAE) buffer:

This buffer solution is used in agarose gel electrophoresis, and it helps in the separation of nucleic acids like DNA and RNA. It comprises of tris acetate buffer (~pH 8.0), and EDTA, which would sequester all the divalent cations.

For 1L of 50X TAE buffer-

- 242g tris base (2-amino-2-hydroxymethyl-propane-1,3-diol) (2 mole)
- 57.1 ml glacial acetic acid (1 mole)
- 100 ml 0.5M Na_2EDTA (pH 8.0)

Add water to make up the volume to 1L.

Phosphate buffer saline (PBS) (10X) (pH 7.4, 1L)

In 800ml of distilled water dissolve

- 8g of NaCl
- 0.2g of KCl
- 1.44g of Na_2HPO_4
- 0.24g of KH_2PO_4

Add water to make up the volume to 1L.

Buffer helps in maintaining a constant pH. The osmolarity and concentration of solution is similar to that of the human body (isotonic), and thus it is non-toxic to cells. Therefore, in tissue culture, cells are washed with PBS.

Cell lysis buffer (pH 7.2, 50ml)

- 60.5mg Tris (for 10mM concentration)
- 250mg SDS (for 0.5% SDS)
- 18.6mg EDTA (for 1mM)
- 30ml MilliQ water

Add water to make up the volume to 50ml.

MTT solution

MTT is reduced to formazan in living cells, giving purple colour. It is made by dissolving 10mg MTT in 10ml media i.e. 1mg/ml of solution is prepared.

MTT Lysis buffer (for two 96 well plates)

- 24ml isopropanol
- 136µl conc. HCl
- 1.25ml SDS (10%)

INSTRUMENTS

1. Zetasizer Nano-ZS

The Zetasizer Nano-ZS is an instrument used for analyzing the particle and molecular size, detection of aggregates and measurement of small or dilute samples, and samples with very low or high concentrations using dynamic light scattering. The ZS comprises of a zeta potential analyzer which makes use of electrophoretic light scattering for particles. Parameters measured are size of particle and zeta potential at high and low concentrations. The particle and surface charge of the samples were measured on Zetasizer Nano-ZS (Malvern Instruments, UK) which uses a 4 mW He-Ne laser (633 nm wavelength).

Figure 3: Zetasizer Nano-ZS for DLS (Source: IGIB lab)

2. Gel Documentation System

A Gel Doc instrument is incorporated with an ultraviolet (UV) light transilluminator, a hood to prevent outside light and a camera to take image. After the Electrophoretic proccess, agarose gels were visualized with the gel Doc System (G: box UV illuminator) (Syngene, USA), fitted with camera resolution range of 0.3-1.2 megapixels with Gene snap software tool.

Figure 4: Gel Documentation System (Source: IGIB lab)

3. Elisa Plate Reader

μQuant MCX200 microplate reader (Biotek, USA) operated with Gene5 software with high powered xenon flash lamp and tunable monochromator was used to calculate cell viability. The 540 nm and 590 nm wavelength was set to measure the amount of formazan formed after using MTT and for Bradford assay respectively.

Figure 5: Elisa Plate Reader (Source: IGIB lab)

4. Lyophilizer

The samples were lyophilized in a Hetovac VR-1. The speed vac comprises a vacuum pump connected to a centrifuged chamber in which the samples were placed. In this process, samples were dried by lowering the pressure so that vacuum was created in the chamber. Samples were dried by the process of sublimation.

Figure 6: Lyophilizer (Source: IGIB lab)

5. Transmission Electron Microscopy

Characterization by TEM was done on Tecnai G2 30U-twin microscope (operating voltage 200 kV). Transmission Electron Microscopy uses a coherent beam of high speed electrons travelling through vacuum that travel through the specimen, and these transmitted electrons are detected and further used to produce the image of the specimen.. The wavelength of electrons is much smaller than that of light; hence the resolution obtained for the TEM images is many times the order of resolution as obtained by the light microscopy. TEM can be used to view internal structures and crystal lattices of compounds, their shapes, sizes and hence enables to even get images of nanoparticles and nanomaterial with very high precision.

Figure 7: Transmission Electron Microscope (Source: Google images)

6. Nuclear Magnetic Resonance Spectroscopy

Characterization by NMR is done to get information about the physic-chemical properties of atoms and molecules contained in an environment. The technique utilizes the fact that that every nuclei possess own spin and when placed in an outer magnetic field, energy transitions take place from lower to higher orbitals and returning to lower energy state. These energy transitions are dependent on the chemical environment of atoms and molecules and can thus be used to determine the chemical groups present within the sample.^{1}H-NMR, ^{13}C-NMR and ^{15}N-NMR are most commonly used. NMR spectroscopy is widely used in applications such as chemical research and development, biological and biochemical study, food industries, pharmaceutical production, agrochemical development, polymer industry. This technique helps in characterization by elucidation of structure, chemical composition, formulation, raw materials fingerprinting, mixture analysis, determination of sample purity etc.

Figure 8: NMR spectrometer (Source: USIC, Delhi University)

7. Fourier Transform Infrared Spectroscopy

Fourier transform infrared spectroscopy is the analytical technique used to identify the different chemical and molecular structures and components. When IR is passed through the sample, some of the part is absorbed while the rest is transmitted, which is detected as the signal and that spectrum is the molecular 'fingerprint' of the sample. The different uses of FTIR involve identification of organic and inorganic compounds (solids or liquids), identification of polymers and polymer blends, knowledge of trace organic contaminants, qualitative & quantitative FTIR analysis, analysis of thin films, coatings coupling agents etc.

Figure 9: FTIR spectrometer (Source: USIC, Delhi University)

8. Laminar Air Flow Hood

Laminar air flows serve the aim of maintaining a working area devoid of any contaminants. Sterile working area must be maintained in medical and research laboratories while carrying out specialized work, hence laminar flow cabinets are used. HEPA filters are used to keep the working environment and products free from any undesired particles and contamination. Air passes from a filtration system and over the work area according to the laminar flows process.

9. Incubator

An incubator is a closed chamber aimed at providing controlled conditions for the growth and maintenance of different cell lines and microorganisms. Temperature control, humidity control and atmosphere control are provided. The incubator used for maintaining cell line had the controlled parameters of 37ºC and 5% CO_2 atmosphere. The incubator used for microbial growth and maintenance was set at 37ºC.

Chapter 6

Part A

PDA/bPEI for Gene Delivery

PART A

PDA/bPEI FOR GENE DELIVERY

Methods

Synthesis of bPEI crosslinked polydopamine nanoparticles (PDA-bPEI NPs)

Polydopamine particles were synthesized as described in the literature previously. [46] In an alkaline water-ethanol solution, dopamine polymerizes via oxidation. To a solution of ammonium hydroxide (0.5 ml), ethanol (10 ml) and de-ionized water (22.5 ml), dopamine was added in dropwise manner at a concentration of 50 mg/ml in de-ionized water. The reaction was allowed to proceed for 30 h at constant stirring. In vacuum, the reaction mixture was concentrated to approximately one fifth of the total volume and then transferred to dialysis bag of 3.5 kDa cut off limit. The dialysis process was carried out for 24 h with intermittent change of water at least thrice. The final solution was then lyophilized in speed vacuum to get solidified product. Polyethylenimine (10 kDa and 1.8 kDa) were linked then to the synthesized polydopamine in ratios of 0.5:1, 1:1 and 2:1. To sum up, appropriate amounts of PDA and bPEI were taken and dissolved in de-ionized water and were left on mixing for 24 h. The reaction mixture was then transferred to dialysis bag of 3.5 kDa cut off limit. The dialysis process was carried out for 24 h with intermittent change of water at least thrice. The final solution was then lyophilized in speed vacuum to get solidified product.

Preparation of pDNA complexes of PDA-bPEI nanoparticles

PDA-bPEI/pDNA complexes were formed by mixing appropriate volumes of aqueous solutions of PDA-bPEI nanoparticles (1mg/ml) separately with a set amount of pDNA (1 µl of 300 ng/µl) to attain various w/w ratios. For various biophysical studies, the final volume was made up to 20 µl with de-ionized water and for *in vitro* experiments, 5 µl of 20% dextrose was added. Then the final volume of this solution was made upto 20µl with de-ionized water. The resulting samples were vortexed gently and kept under incubation for 30 minutes at room temperature before the experimental use in each case.

TNBS assay

To determine the extent of reaction undergone by primary amines of polymer, 2,4,6-trinitrobenzene sulfonic acid (TNBS) assay was carried out as reported previously[47]. Aqueous solutions of modified polydopamine samples (50 µL, 1 mg/ml) were mixed with an aqueous solution of 4% $NaHCO_3$ (50 µL) and a solution of TNBS (0.1%, 50 µL, 1mg/ml in water) was added to each. The reaction mixture was kept under incubation for 2 h at 40°C. Following incubation, aqueous solutions of 10% SDS (50 µL) and dil. HCl (1 N, 25 µL) were added. Absorbance was recorded at 335 nm against the buffer without the sample. A standard curve was drawn using native polymer to calculate the extent of substitution of the polymer.

Physical characterization of PDA-bPEI nanoparticles and their pDNA complexes

The hydrodynamic diameter and surface charge measurement were carried out by Dynamic Light Scattering (DLS) instrument. At the exhibited w/w ratio of the highest transfection, bPEI/pDNA and PDA-bPEI/pDNA complexes were prepared by mixing appropriate amount. The final volume was made up with de-ionized water after addition of corresponding volume of 20% dextrose. In addition, respective complexes were prepared in the presence of 10% FBS as well. The complexes were then incubated for 30 minutes duration at room temperature, before taking measurements. The measurements were performed by carrying out 30 runs in triplicate for each hydrodynamic diameter and zeta potential. The data analysis was performed by presenting the measured size as the average value from the mentioned runs. The average values for zeta potential are expressed in mV. The DLS measurements were also made with 1mg/ml concentration of PEI and PDA-PEI.

Transmission electron microscopy analysis of PDA-PEI nanoparticles and their pDNA complexes

By using Tecnai G2 30U-twin transmission electron microscope, the size and morphology of PDA-bPEI/pDNA complexes was determined. Carbon coated copper

grids for TEM were loaded with the samples prepared in de-ionized water. It was stained with 1% uranyl acetate and dried well before analyzing at the accelerating voltage of 200 kV.

Nuclear Magnetic Resonance Spectroscopy

The synthesized compounds were analysed by ^{1}H-NMR by dissolving in D_2O (heavy water) with the help of ^{1}H-NMR Bruker Spectrospin spectrometer, 400 MHz and the structure was thus elucidated. The chemical shifts (δ) are presented in ppm.

Fourier Transform Infrared Spectroscopy

FTIR spectra were recorded on a single beam Perkin Elmer Spectrum (BX Series), USA at room temperature in the range of 4000-400 cm^{-1} with a resolution of 4 cm^{-1} and 100 scan per samples. The samples were prepared by KBr disk method. The presence and absence of functional groups were used to elucidate the structure of compounds.

Electrophoretic Mobility Shift Assay (EMSA) of PDA-PEI/pDNA complexes

To determine the amount of synthesized nanoparticles binding with negatively charged pDNA in order to retard its mobility completely, agarose gel electrophoresis was performed. PDA-bPEI/pDNA complexes were made for different w/w ratio (0.2, 0.5, 0.7, 1, 2, 3 and 5) with fixed amount of 1µl pDNA (300 ng/µl) and kept for incubation for 30 min at room temperature. The prepared complexes were mixed with 2µl Orange G dye (10X), electrophoresed (100V, 1h) in 0.8% agarose gel containing EtBr (2µl/100 ml gel) in 1x TAE buffer and the resulting bands were visualized in Gel Doc System (i.e. UV transilluminator). The reference standard contained a solution of only pDNA (20µl in volume).

Ethidium Bromide Quenching Assay

Ethidium bromide (EtBr) intercalates into DNA and this intercalation leads to a 10 fold increase in the fluorescence intensity of the dye. When a nanoparticle binds to DNA it displaces EtBr from the DNA and this would result in a drop in fluorescence intensity. This decrease in fluorescence can be used to measure of DNA condensation. The assay was performed in opaque 96-well plates where 4.22 ml EtBr (10 ng/ml) and 20 ml plasmid DNA (20 ng/ml) i.e., one EtBr molecule per 6 base pairs of DNA, was put in each well and left for incubation at room temperature for 5 minutes in dark. 20 ml of nanoparticle solution at increasing w/w ratio was added and again incubated for 30 minutes in dark. Fluorescence was measured in microplate reader (Infinite 200 Pro, Tecan Inc., Switzerland) equipped with 535nm excitation and 595nm emission filters. The fluorescence of the DNA-EtBr complex thus formed was considered as the maximum, i.e. 100%. The relative percentage of fluorescence intensity was calculated at increasing charge ratios. The result was plotted as a percentage of maximum.

Buffering capacity of PDA-bPEI nanoparticles

An acid – base titration method described earlier [48], was carried out to demonstrate the resistance of PDA-bPEI nanoparticles against acidification. 3 mg of PDA-bPEI were dissolved in an aqueous solution of 0.1 N NaCl (30 ml). The pH was then adjusted to 10 by addition of 20 µl of 0.1 N NaOH solution subsequently. This solution was then titrated against 0.1 N HCl by addition of 20 µl each time until the pH was 3. The pH values were recorded after each addition. A titration curve was drawn between pH values corresponding to the volume of 0.1 N HCl.

DNA release studies

To address the unpacking of DNA from the stable complexes, DNA release studies were conducted. PDA-bPEI/pDNA complexes were prepared at the w/w where best transfection was exhibited. Following a 30 minutes incubation, heparin, an anionic polysaccharide is added to the mix in increasing concentration to compete with pDNA. After a 30 min incubation interval, the samples were mixed with 2µl Orange G dye and

electrophoresed (100 V, 1 h) in a 0.8% agarose gel containing EtBr. The resulting bands were visualized on a UV transilluminator. The pDNA released from the complexes was quantified densitometrically using Gene Tool Software from Syngene.

In vitro transfection studies

HEK cells were seeded using DMEM high glucose media with 10% FBS in 96- well plate to assess the transfection efficiency of PDA-bPEI nanoparticles by carrying out delivery of pDNA. Seeding density was 10^4 cells/well. The cells were incubated at a temperature of 37°C for 24 h in a humidified environment in CO_2 incubator. Transfection was carried out when the cells attained 70-75% confluency. The media was aspirated and cells were washed with 1x PBS once (50µl in each well). bPEI/pDNA and PDA-bPEI/pDNA complexes were prepared at different w/w ratio in DMEM. Lipofectamine/pDNA complex was also prepared according to the manufacturer's protocol so as to carry out a comparative analysis. These complexes were first incubated for 30 minutes at room temperature and later added on to the cells gently. The plate was kept in the incubator at 37°C in a humidified CO_2 incubator. After time interval of 3 h, the transfection mixture was aspirated out, followed by addition of fresh complete media (DMEM containing 10% FBS; 100 µL/well) to the cells in each well. Cells were again incubated at 37°C in CO_2 incubator for 45 h. GFP reporter gene expression was observed under Nikon Eclipse TE 2000-S inverted fluorescence microscope equipped with an excitation filter (450-490nm)

Quantification of GFP expression using nanodrop

HEK 293 cells were seeded at the initial cell density of 10^4 cells/well in a 96 well-plate and *in vitro* transfection was carried out as described above. Post 48 hours of transfection, expression of GFP was estimated quantitatively using Nanodrop ND-3000 spectrofluorometer. The cells were washed with 1X PBS and then were treated with cell lysis buffer (50 µL/well). The cells were then incubated at 37°C for 45 minutes and then were centrifuged. 2 µL of the soup was placed at the reading arm of Nanodrop from each well at least five times for statistically significant data. To calibrate the spectrofluorometer, 1X PBS was used as blank. Further, in order to estimate the total

protein content, Bradford's reagent with bovine serum albumin (BSA) as the standard was used and it was quantitatively read at 590 nm using ELISA plate reader. GFP fluorescence was then expressed as arbitrary units (A.U.) / mg of protein.

Cell Viability Assay

A. MTT colorimetric assay

Cytotoxicity of PDA-bPEI/pDNA, bPEI10/pDNA, bPEI1.8/pDNA and Lipofectamine/pDNA complexes was assessed on HEK 293 cells by MTT colorimetric assay. The assay is based on the reduction of tetrazolium group (yellow color) into the formazan crystals (dark purple) due to the action of mitochondrial succinate dehydrogenase in live cells. After 48 h of transfection, media was aspirated and MTT reagent (100 µL, 1 mg/ml) mixed in DMEM was added to the cells. The plate was kept in a humidified CO_2 incubator for 2 h at 37°C. After incubation, the supernatant was removed and the formazan crystals were dissolved in 100 µL of isopropanol containing SDS (0.5%) and HCl (0.06M). Intensity of the color was recorded spectrophotometrically on an ELISA plate reader at 570 nm. The untreated cells were considered as control with 100% viability. Cells, devoid of MTT, were taken as blank for calibrating the spectrophotometer to zero absorbance. Experiment was repeated in triplicate. The percent cell viability was evaluated by the formula:

$$\text{Cell viability (\%)} = A_{transfected} / A_{control} \times 100$$

B. LDH Assay

HEK 293 cells were seeded at the initial cell density of 10^4 cells/well in a 96 well-plate and *in vitro* transfection was carried out as described above. In addition, to a similar cell density only nanoparticles were added at different concentrations from 1mg/ml stock concentration. Assay was carried out after 48 hours of transfection and 24 hours of sample treatment. Cytotoxicity of the samples was evaluated on the principle of cytosolic LDH release from disintegrated cells [49]. Briefly, 50 µL each of 200mM Tris (pH 8), 50mM Lithium Lactate and a cocktail of PMS, INT and NAD was added to the wells in a 96-well ELISA plate. To the mix, 50 µL of samples were

added into corresponding wells from the treated plates. The plate was incubated in dark for 5 minutes and then read at 490 nm spectrophotometrically on an ELISA plate reader. Untreated lysed cells were considered as control with 100% LDH release. Experiment was repeated in triplicate. The percent LDH release was determined using the formula:

LDH Release (%) = $A_{treated}$ / $A_{control}$ x 100

Enzymatic Protection assay

The capability of modified nanoparticles to provide a protection to bound pDNA against nucleases was evaluated by treating the complex with DNase I [50]. Native pDNA and particle/pDNA complex (prepared at the best working w/w ratio) were incubated with or without DNase I (1 µL, 1U/µL in a buffer consisting of 100 mM Tris, 25 mM $MgCl_2$ and 5 mM $CaCl_2$) for varying time intervals at ambient temperature. Samples without DNase I were taken in PBS buffer alone and used as controls. After a stated time interval, the reaction was quenched by the addition of EDTA (100 mM, 5 µL) for 10 minutes followed by incubation at 80°C for 10 minutes in a dry bath to degrade DNase I. After this, the samples were treated with heparin (10 µL, 1 U/ µL) for 2 h and the released pDNA was loaded onto 0.8% agarose gel, electrophoresed at 100 V for 1 h, stained with EtBr. The result was visualized in a Gel Documentation System.

RESULTS AND DISCUSSION

The primary goal of the present study was the development of an efficient system for the delivery of DNA into mammalian cells with minimal cytotoxicity, high uptake and internalization and higher gene expression as compared to the commercially available transfecting agent.

Synthesis of polydopamine was done from commercially available dopamine hydrochloride under alkaline conditions. It is formed by covalent oxidative self-polymerization reaction as suggested in literature [51], wherein oxidized and cyclized dopamine monomers were covalently linked via aryl – aryl linkages. Synthesis of bPEI crosslinked polydopamine nanoparticles was achieved by reaction via amine groups (1° and 2°) of bPEI. In bPEI, the 1° amines are more exposed while the 2° amines reside majorly in the polymer backbone. Thus, despite of 2° amines being stronger nucleophiles, crosslinking is achieved predominantly via 1° amines. Depending upon the arrangement and orientation of the functional groups in the polymeric structure, the amine groups react either with the hydroxyl group on the polydopamine. The schematic representation could be shown in the diagram below describing the crosslinking reaction.

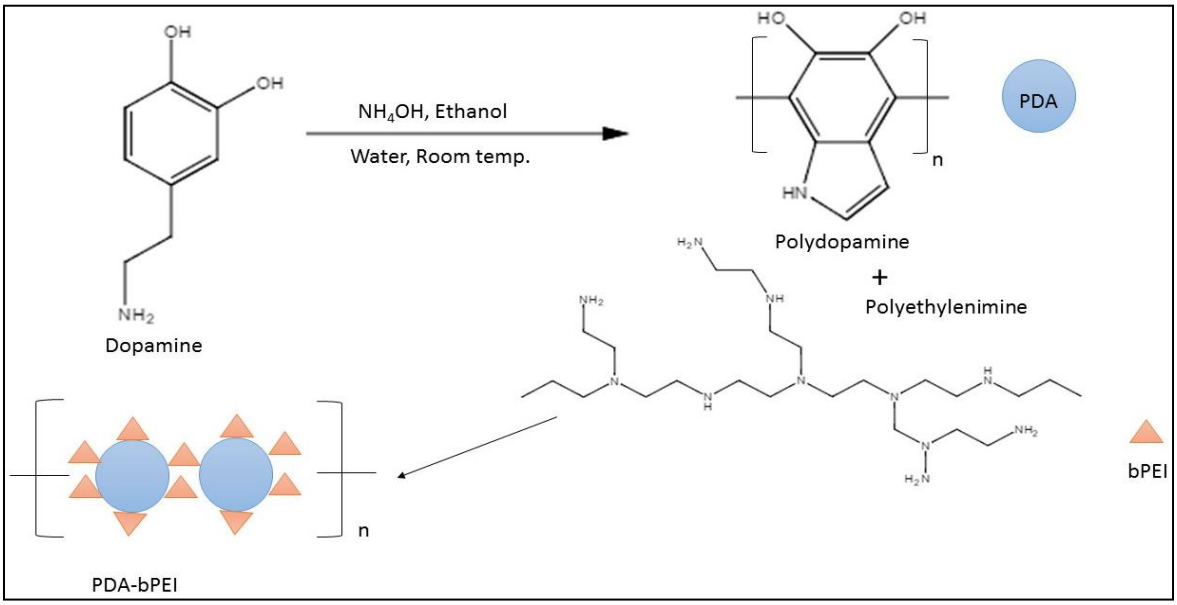


Figure 10: Schematic representation of synthesis of Polydopamine (PDA) – branched Polyethylenimine (bPEI) cross-linked particles

By varying the amounts of bPEI, three series were prepared and the yield of nanoparticles was calculated in terms of percentage after complete drying of nanoparticles by using the following formula:

$$\% \text{ yield} = \frac{\text{Weight of dried nanoparticles}}{\text{Total weight of polymer taken}} * 100$$

Table 1: Percentage yield of modified particles

Modification Ratio ↓	bPEI Molecular Weight →	10 kDa	1.8 kDa
$PDA_1 : bPEI_{0.5}$		84.47	91.27
$PDA_1 : bPEI_1$		77.25	82.35
$PDA_1 : bPEI_2$		87.67	88.67

Theoretically, 33%, 50% and 66% substitution was attempted respectively for the ratio 1:0.5, 1:1 and 1:2 of PDA:bPEI. Percentage substitution was calculated from TNBS assay as described above. After the work up, the percent crosslinking in the nanoparticles varied from ~35 – 51% of the attempted crosslinking.

Table 2: Percentage Substitution from TNBS Assay

Attempted Substitution	Substitution from TNBS Assay	
	With 10 kDa bPEI	With 1.8 kDa bPEI
33%	11.45	12.93
50%	16.35	16.65
66%	21.85	21.85

Native bPEI, lacking any defined structure, had an average hydrodynamic diameter ~313 nm. On crosslinking of varied amounts of bPEI with PDA the average hydrodynamic diameter of nanoparticles was found to be in the range of 141-171 nm. As the amount of crosslinker increased, the average size of particles increased slightly. On interaction with negatively charged pDNA, a further increase in the overall size of the PDA-bPEI particles was observed which could be due to swelling of the complexes to more extent.

Although, with the increasing amount of crosslinking, the average size of particles was found to be decreasing, this suggests more compactness in the structures. When measured in the presence of 10% FBS the size reduced drastically to the range of ~ 25-46 nm primarily because of the interaction with serum proteins.

In the zeta potential measurements, slightly increase in the values is observed with pDNA complexes than in native solutions. This could possibly because of swelling of nanoparticle/pDNA complex and hence the exposure of charge which might was previously buried in core. There has been a decrease in zeta potential values of PDA-bPEI/pDNA complexes from bPEI/pDNA complex. This change in surface charge could be due to rearrangement of polymers into nanoparticles, and because of this, the ratio of primary, secondary and tertiary amines might change and some of the charge might also be buried inside the core. In presence of 10% FBS, the polarity gets reversed due to adsorption of serum proteins on positively charged complexes and hence negative values of zeta potential are observed.

Table 3(a) : DLS measurements values for PDA-^{10}bPEI at different ratios, without pDNA, complexed with pDNA with and without 10%FBS.

Sample	Average Particle Size (d.nm ± S.D.)			Average Zeta Potential (mV ± S.D.)		
	NPs (1mg/ml)	NP/pDNA complex in water	NP/pDNA complex in 10% FBS	NPs (1mg/ml)	NP/pDNA complex in water	NP/pDNA complex in 10% FBS
PDA_1-$^{10}bPEI_{0.5}$	142.4 ± 0.8	199.1 ± 1.85	38.25 ± 0.43	23.2 ± 0.1	25.5 ± 0.611	-13.4 ± 0.24
PDA_1-$^{10}bPEI_1$	161.3 ± 6.19	193.9 ± 3.1	33.25 ± 0.1	24.8 ± 0.265	25.45 ± 2.13	-12.2 ± 1.41
PDA_1-$^{10}bPEI_2$	165.2 ± 6.24	173.1 ± 14.7	29.52 ± 1.45	29.3 ± 0.85	29.957 ± 0.733	-5.79 ± 1.82

Table 3(b): DLS measurements values for PDA-$^{1.8}$bPEI at different ratios, without pDNA, complexed with pDNA with and without 10%FBS.

Sample	Average Particle Size (d.nm ± S.D.)			Average Zeta Potential (mV ± S.D.)		
	NPs (1mg/ml)	NP/pDNA complex in water	NP/pDNA complex in 10% FBS	NPs (1mg/ml)	NP/pDNA complex in water	NP/pDNA complex in 10% FBS
PDA_1-$^{1.8}bPEI_{0.5}$	140.5 ± 6.08	191.7 ± 5.52	42.87 ± 1.346	23.8 ± 0.153	24.3 ± 4.38	-12.6 ± 0.755
PDA_1-$^{1.8}bPEI_1$	149.3 ± 3.16	186.9 ± 4.04	38.22 ± 2.019	25.1 ± 0.416	25.3 ± 0.46	-11.4 ± 2.87
PDA_1-$^{1.8}bPEI_2$	151.6 ± 3.64	174.6 ± 2.40	33.49 ± 8.384	33.2 ± 2.70	31.30 ± 0.409	-10.8 ± 2.44

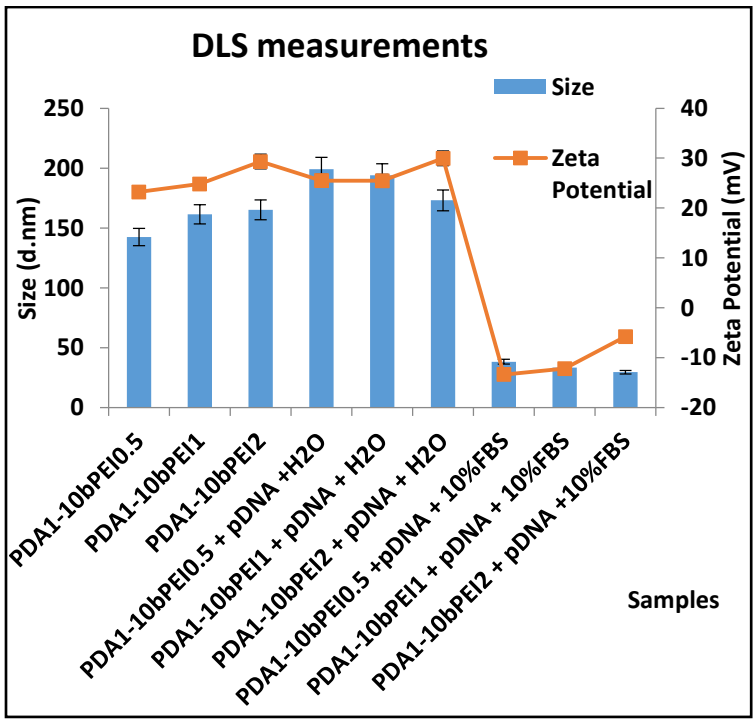


Figure 11(a): Size and Zeta Potential values from DLS measurements for PDA-^{10}bPEI at different ratios, without pDNA, complexed with pDNA with and without 10%FBS.

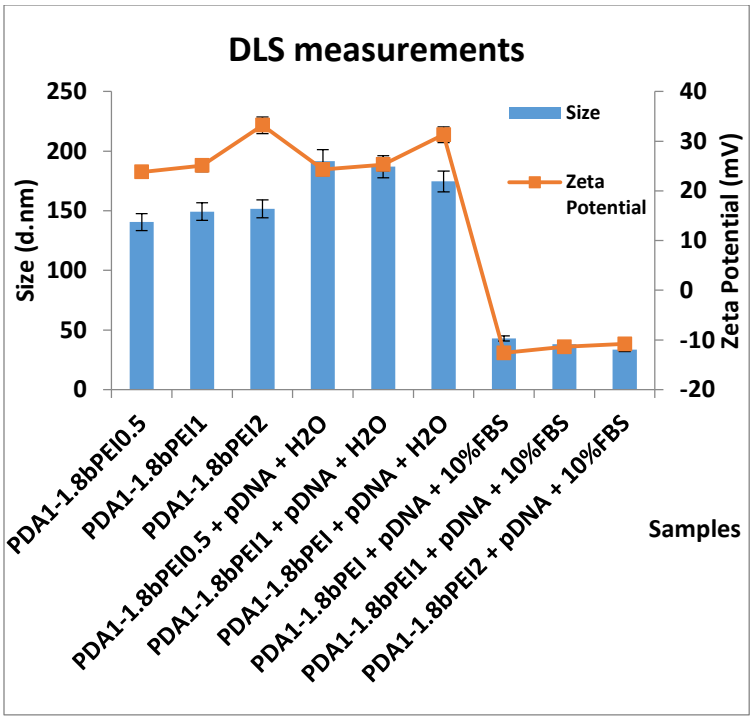
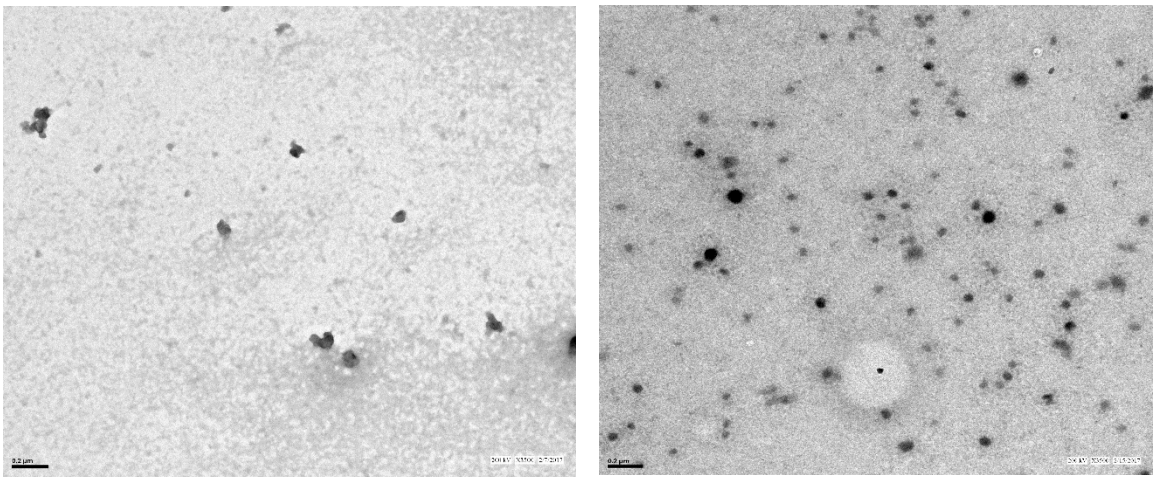


Figure 11(b): Size and Zeta Potential values from DLS measurements for PDA-$^{1.8}$bPEI at different ratios, without pDNA, complexed with pDNA with and without 10%FBS.

On examining the size and morphology of PDA-bPEI/pDNA complexes, it was found that the particles were spherically shaped with size in the range of ~60-80 nm. The size revealed from TEM is smaller than that obtained from DLS. This could be explained on the basis of difference in working principle of the two techniques.

In DLS, the size of the particle that is measured is actually the hydrodynamic diameter. This includes the actual size of the particle in medium as well as the size of the solvation layers formed round the particle's surface due to electrostatic interactions. DLS also measures the intensity-based size distribution which accounts for aggregated particles. On the other hand, TEM measures the actual size since the particle size is in a dry state. TEM provides for the number-based size distribution of the sample. Such a significant difference in size from DLS and TEM has been reported in literature previously for various particles [52-55].



PDA-bPEI PDA-bPEI/pDNA

Figure 12: TEM images of PDA-bPEI and PDA-bPEI/pDNA particles. Size of complexes were in the range of ~60-80 nm, images having scale bar of 0.2 µm.

The particles were characterized by ^{1}H-NMR spectroscopy with D_2O as solvent. An NMR spectrum in heavy water helps in the elucidation of structure of self-assembled polymeric micelles. The following results were obtained that show characteristic peaks indicating particular side chain groups within the compounds. As can be seen, characteristic peak at 2.6 ppm is obtained in the NMR spectrum of native polydopamine polymer which could be of hydroxylic groups that are exposed upon polymerization of dopamine molecules. Other peaks of lower intensity are also obtained in the region of 1-5ppm indicating the presence of amine groups. Strong peaks in the region of 1-5 ppm are obtained in the NMR spectra of all other complexes of aminoglycosides and branched polyethylenimine with polydopamine indicating presence of free amine groups on the surfaces of micellar nanoparticles so formed in the water.

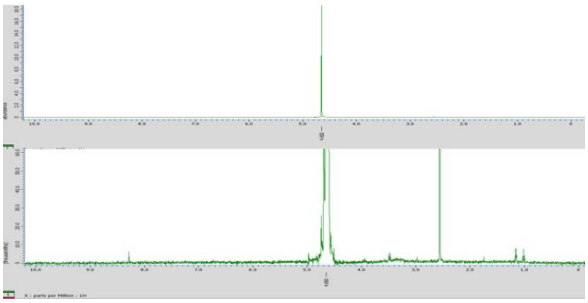


Fig. 13(a): NMR spectra of native PDA

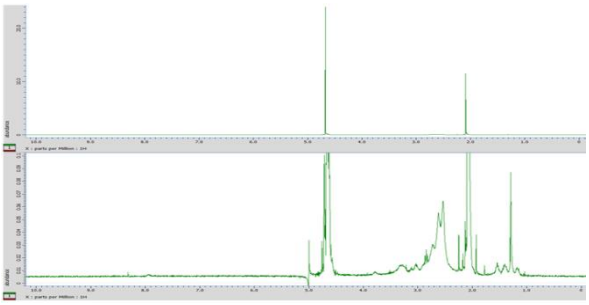


Fig.13(b): NMR spectra of PDA_1-$^{10}bPEI_{0.5}$

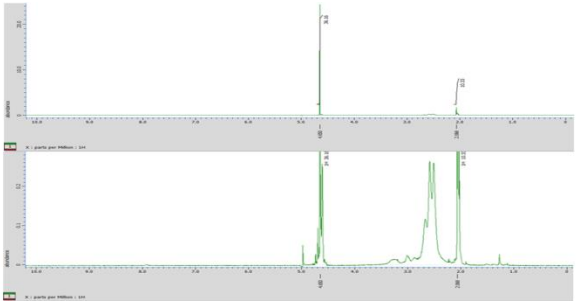


Fig.13(c): NMR spectra of PDA_1-$^{10}bPEI_1$

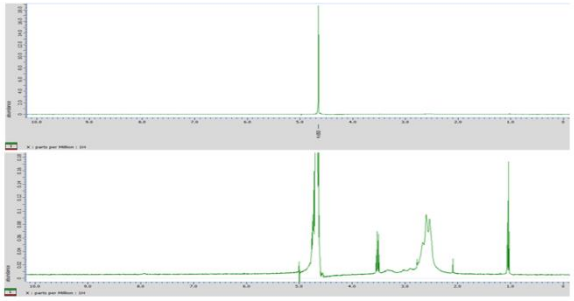


Fig.13(d): NMR spectra of PDA_1-$^{10}bPEI_2$

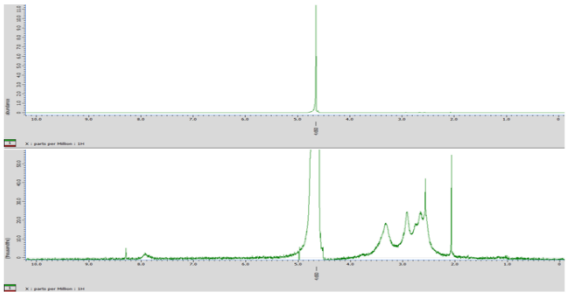


Fig. 13 (e): NMR spectra of PDA_1-$^{1.8}bPEI_{0.5}$

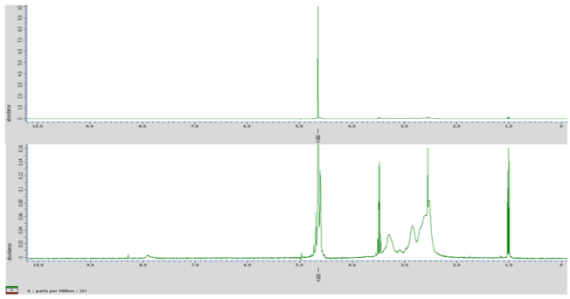


Fig. 13 (f): NMR spectra of PDA_1-$^{1.8}bPEI_1$

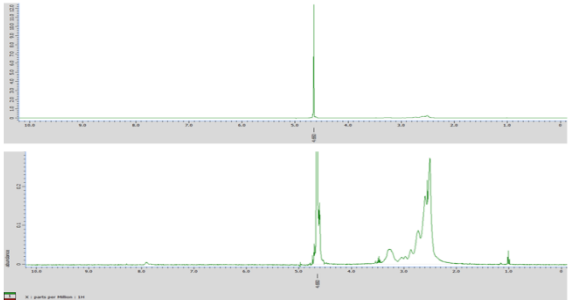


Fig. 13 (g): NMR spectra of PDA_1-$^{1.8}bPEI_2$

FTIR spectroscopy was done to characterize PDA-bPEI particles. There is a visible trend in the percentage transmittance with varying ratios of bPEI onto PDA.

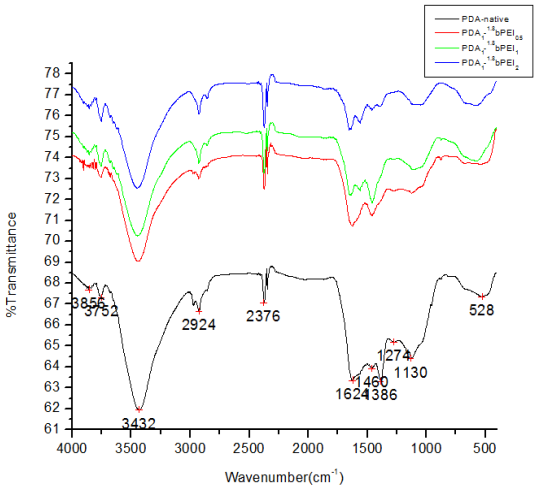


Fig. 14(a): FTIR spectra for PDA-$^{1.8}$bPEI

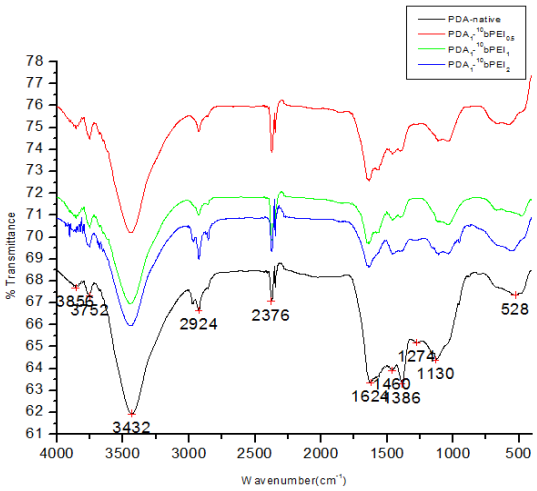
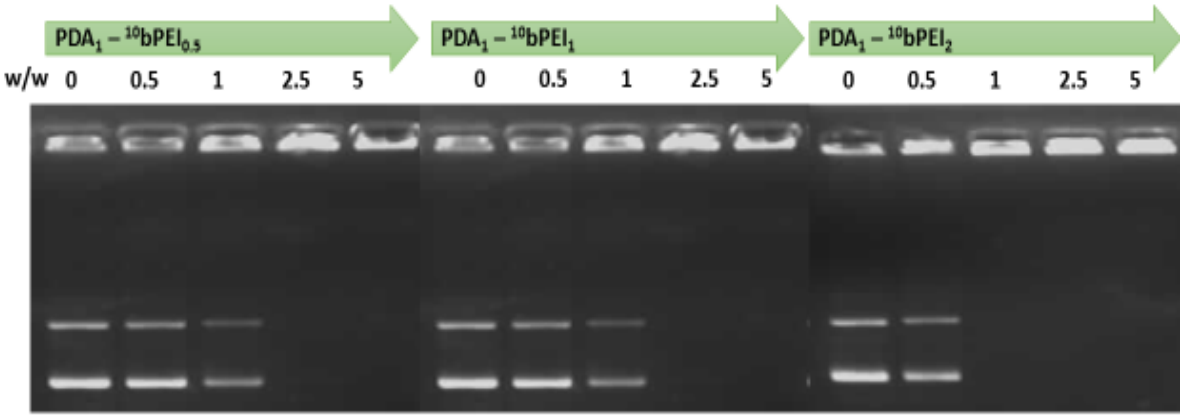


Fig. 14(b): FTIR spectra for PDA-^{10}bPEI

To determine the DNA binding efficacy of the synthesized nanoparticles, agarose gel electrophoresis was performed. The mobility of fixed amount of pDNA (300 ng/µL) complexed with varied amounts of bPEI and PDA-bPEI particles was compared with that of free pDNA under same conditions. It was found that with increasing crosslinking pDNA mobility was retarded at lower w/w ratio with 2.5, 2.5, 1 with 10 kDa and 1.8 kDa modifications for PDA_1:$bPEI_{0.5}$, PDA_1:$bPEI_1$ and PDA_1:$bPEI_2$ respectively. This could be inferred along with the increasing surface charge on increasing crosslinking.



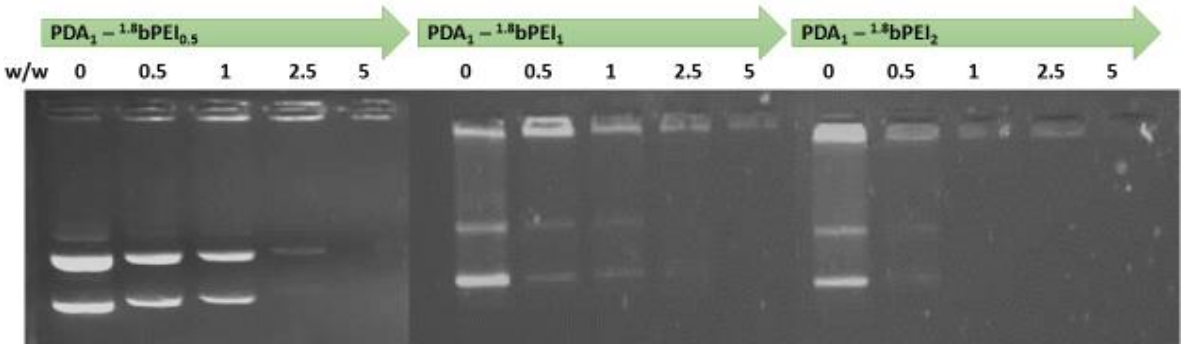


Figure 15: Electrophoretic mobility shift assay for PDA_1:$^{10}bPEI_{0.5}$, PDA_1:$^{10}bPEI_1$, PDA_1:$^{10}bPEI_2$; PDA_1:$^{1.8}bPEI_{0.5}$, PDA_1:$^{1.8}bPEI_1$, PDA_1:$^{1.8}bPEI_2$ and bPEI at various w/w ratios.

The ability of the modified nanoparticles to condense DNA was monitored by EtBr condensation assay. DNA intercalated with EtBr shows a significant fluorescence by increasing the fluorescence intensity of dye. This intensity is reduced if the DNA gets complexed with some other entity. Hence, the stronger the binding of nanoparticle with DNA, the lower is the fluorescence intensity. It is well observed that with increasing w/w ratio of nanoparticle to pDNA, the fluorescence intensity decreases significantly in all the variations with different ratios.

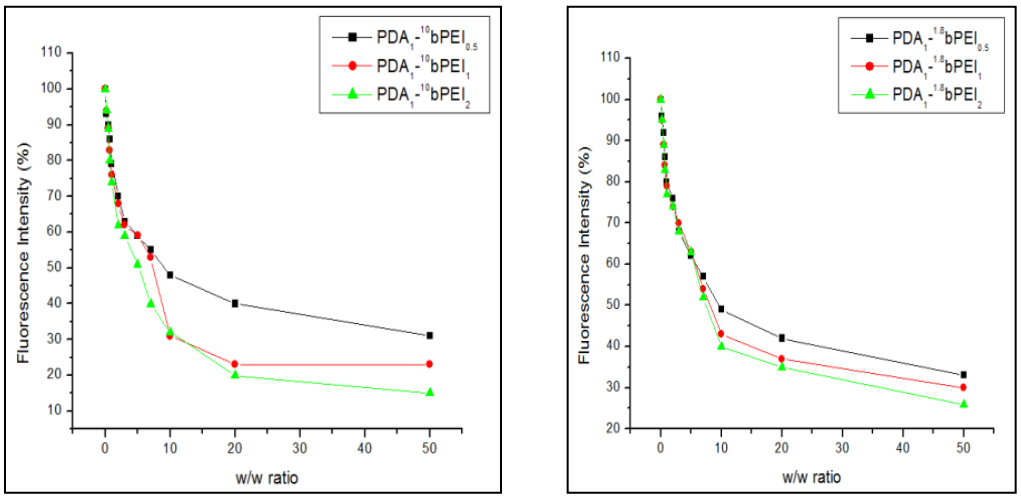


Figure 16: DNA condensation assay via EtBr quenching by different w/w ratios of PDA_1:$^{10}bPEI_{0.5}$, PDA_1:$^{10}bPEI_1$, PDA_1:$^{10}bPEI_2$; PDA_1:$^{1.8}bPEI_{0.5}$ and PDA_1:$^{1.8}bPEI_1$, PDA_1:$^{1.8}bPEI_2$

For cationic polymers, "proton-sponge hypothesis" has been proposed which describes the early endosomal escape of complexes and thereby preventing the lysosomal internalization. With the presence of primary, secondary and tertiary amines and high charge density, bPEI is capable to inhibit the endosomal enzymes, causing its swelling followed by rupturing. [56-58] When crosslinked with PDA at varied amounts, buffering capacity was checked by performing standard acid- bas titration. Buffering capacity is decreased for modifications PDA-^{10}bPEI and PDA-$^{1.8}$bPEI as compared to native ^{10}bPEI and $^{1.8}$bPEI, although they have shown better gene expression. Results like these have been reported previously in the literature where superior transfection has been observed even with decreased buffering capacity [55, 59].

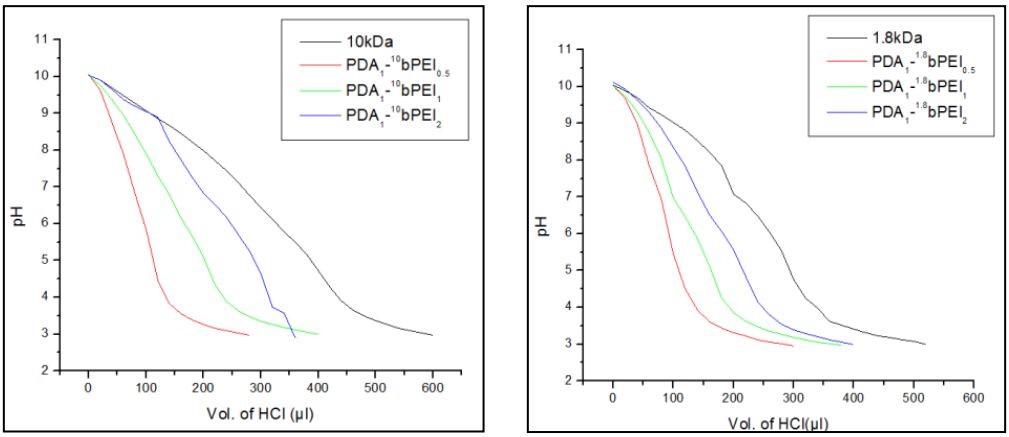


Figure 17: Titration profiles of PDA$_1$:^{10}bPEI$_{0.5}$, PDA$_1$:^{10}bPEI$_1$, PDA$_1$:^{10}bPEI$_2$, ^{10}bPEI and PDA$_1$:$^{1.8}$bPEI$_{0.5}$ and PDA$_1$:$^{1.8}$bPEI$_1$, PDA$_1$:$^{1.8}$bPEI$_2$, $^{1.8}$bPEI$_2$.

PDA-bPEI nanoparticles were evaluated for their ability to carry pDNA inside the HEK cells for efficient gene expression. After 48h of transfection, GFP gene expression was assessed by observing the cells under inverted fluorescence microscope. Figure 8, shows transfection results. After quantification, it was observed that PDA-bPEI/pDNA complexes exhibited higher transfection efficiency than respective native bPEI. The best transfection was achieved at 15 w/w ratio for PDA$_1$-^{10}bPEI$_{0.5}$ and 10 w/w ratio for PDA$_1$-^{10}bPEI$_1$ and PDA$_1$-^{10}bPEI$_2$ each. For PDA-1.8bPEI complexes, transfection was achieved to much lower level at 15 w/w ratio for PDA$_1$-$^{1.8}$bPEI$_{0.5}$ and 7.5 w/w ratio for PDA$_1$-$^{1.8}$bPEI$_1$ and PDA$_1$-$^{1.8}$bPEI$_2$ each. The transfection efficacy varied with w/w ratio. It

increased gradually until a particular w/w ratio (best w/w ratio of highest transfection) and then decreased.

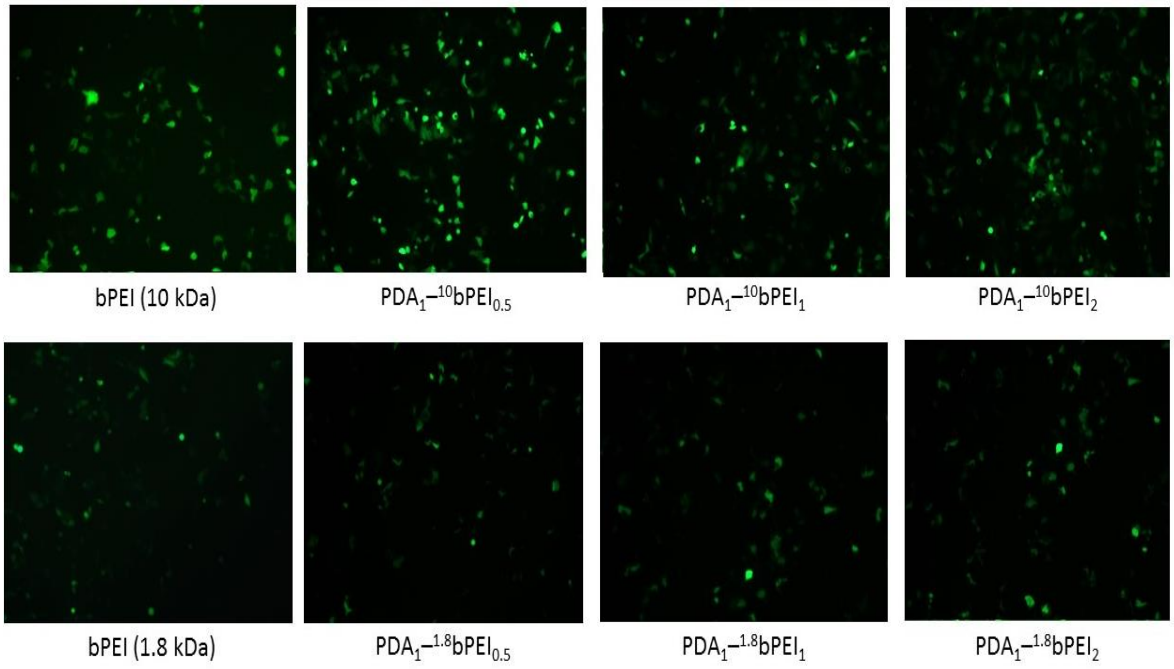
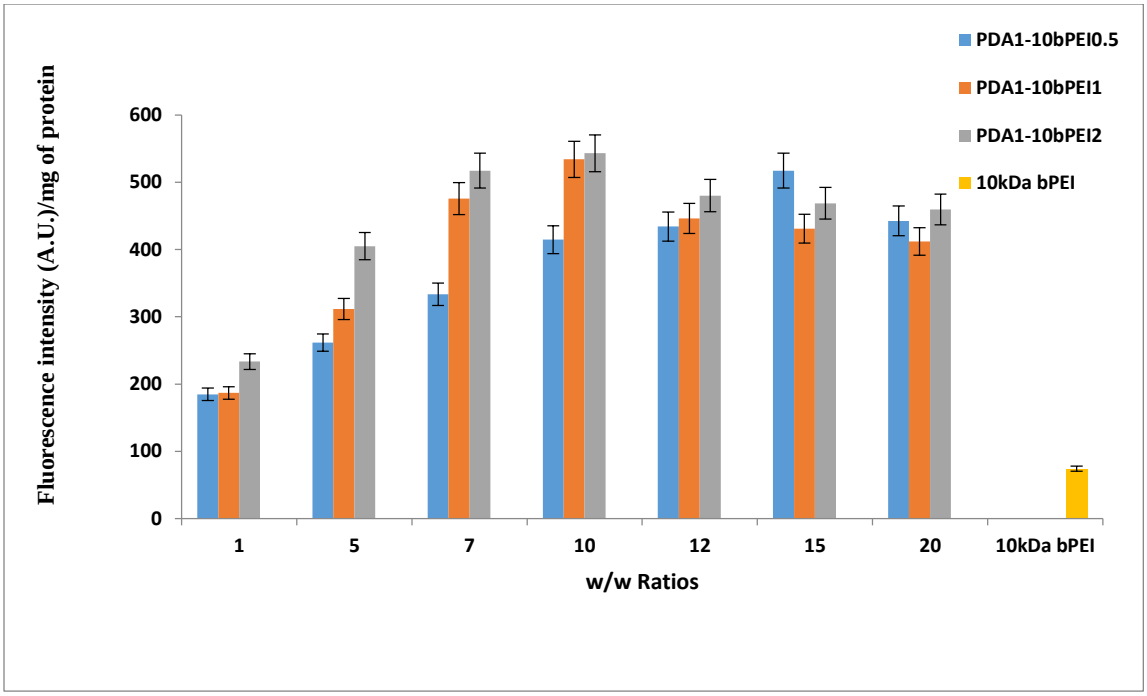


Figure 18: Fluorescence microscopic images of GFP gene expression in HEK cells at their best w/w ratios



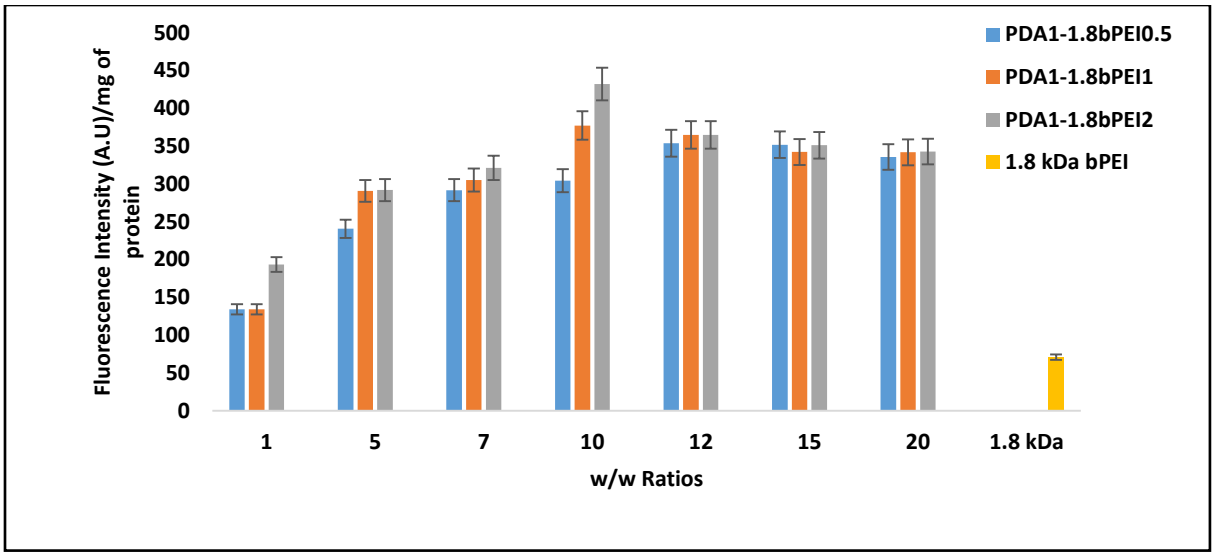


Figure 19: Gene expression quantification by Nanodrop in HEK cells for PDA-^{10}bPEI and PDA-$^{1.8}$bPEI modifications at different w/w ratios.

PDA-$_{10}$bPEI/pDNA complexes showed higher transfection efficiency than PDA-$_{1.8}$bPEI/pDNA complexes. All the complexes showed higher transfection than the native bPEIs.

To deliver nucleic acids efficiently, their unpacking from the carriers is vital. This corresponds with the transfection efficiency of the carriers [60]. The nanocarrier/pDNA complex should be stable enough to overcome the cellular barriers but must be amenable in such a way that the pDNA is localized into the nucleus. To estimate the rigidity of the nanocarrier/pDNA complex, heparin release assay is done. Due to highly anionic nature heparin competes with the complexed DNA and depending on the strength of nanocarrier/pDNA complex, releases pDNA at a particular amount. This was analyzed by gel electrophoresis and further by densitometry. The modifications on PDA by bPEI have a better percentage release at the same amount of heparin than the native bPEI. This is in complementation with the observed transfection results.

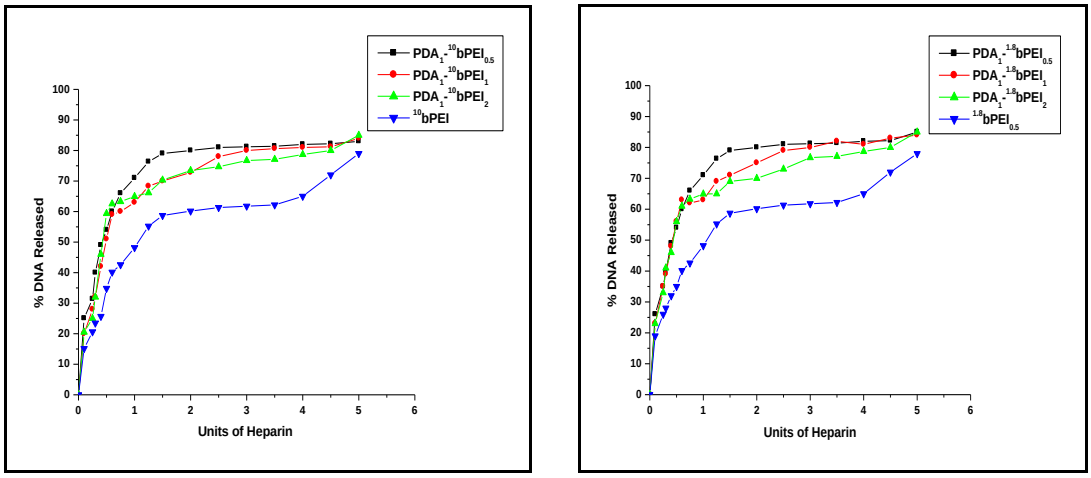
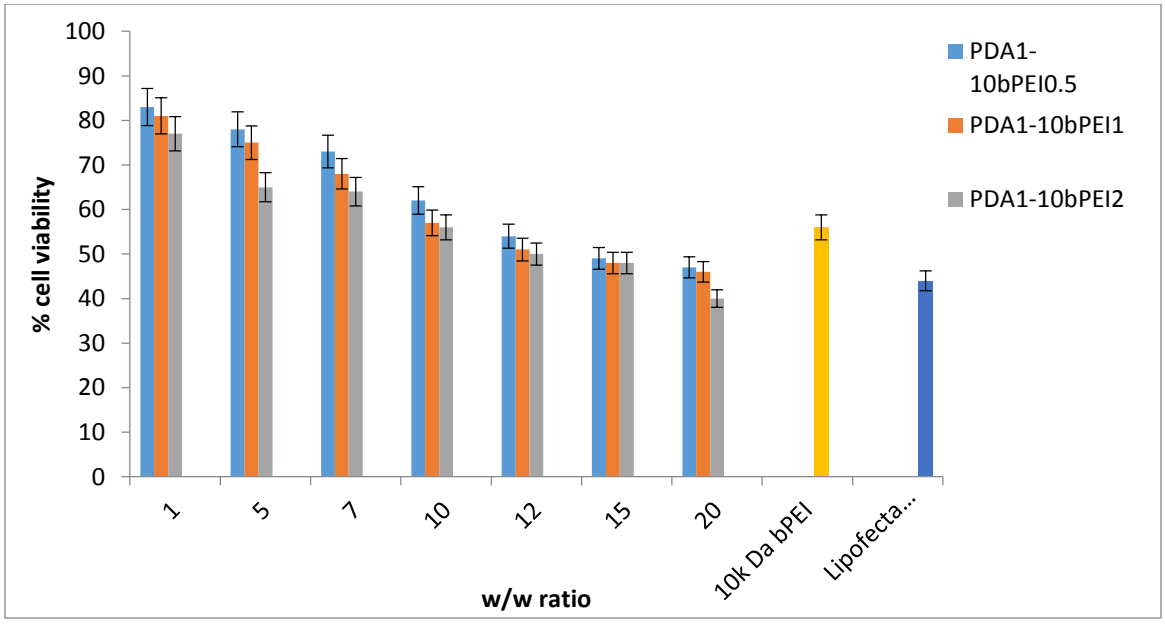


Figure 20: DNA release assay of pDNA complexes of PDA-bPEI nanoparticles and bPEI using heparin (anionic polymer) at their respective best working w/w ratios.

Next to assess the non-toxicity of nanoparticle/pDNA complexes on HEK cells; a range of w/w ratios relevant to transfection was taken. The cell viability came out to be significantly higher for the PDA-bPEI formulations as compared to native bPEI and the commercial transfecting agent Lipofectamine. This might be due to the conversion of toxicity causing primary amines to secondary and then tertiary amines. Such formulations reduce the number of free amines that are responsible for toxicity in cells [61].



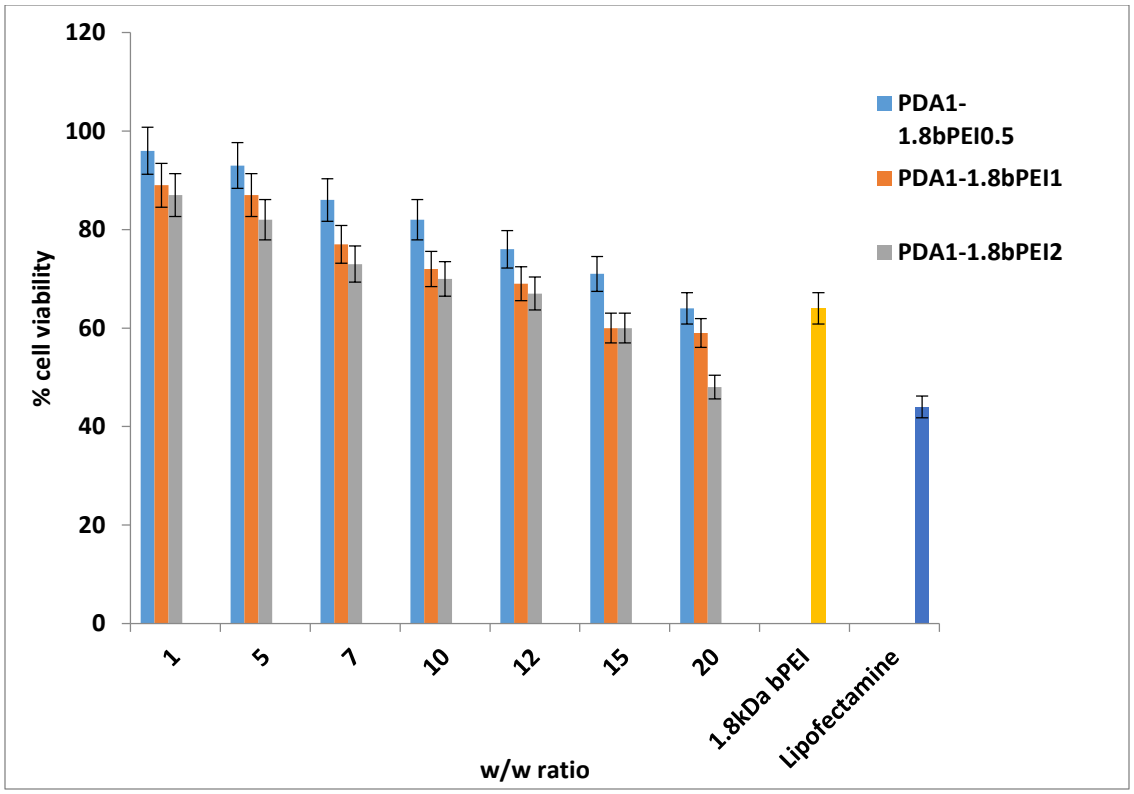


Figure 21: Percentage cell viability assessment using MTT Assay for different modifications

Release of lactate dehydrogenase was monitored in case of cell toxicity caused by PDA-bPEI nanoparticles at different w/w ratios with pDNA after transfection. Results from figure show that LDH is released at an increasing percentage with increasing w/w ratio in each case when compared with the maximum release (100%) by cell lysis buffer. At the best transfection, w/w ratio the percentage LDH is significantly lesser than that of native bPEI and commercial transfecting agent Lipofectamine. This is in agreement with the MTT data obtained and thus signifies that there is no significant cell death caused by proposed nanoparticles at their best transfection ratios.

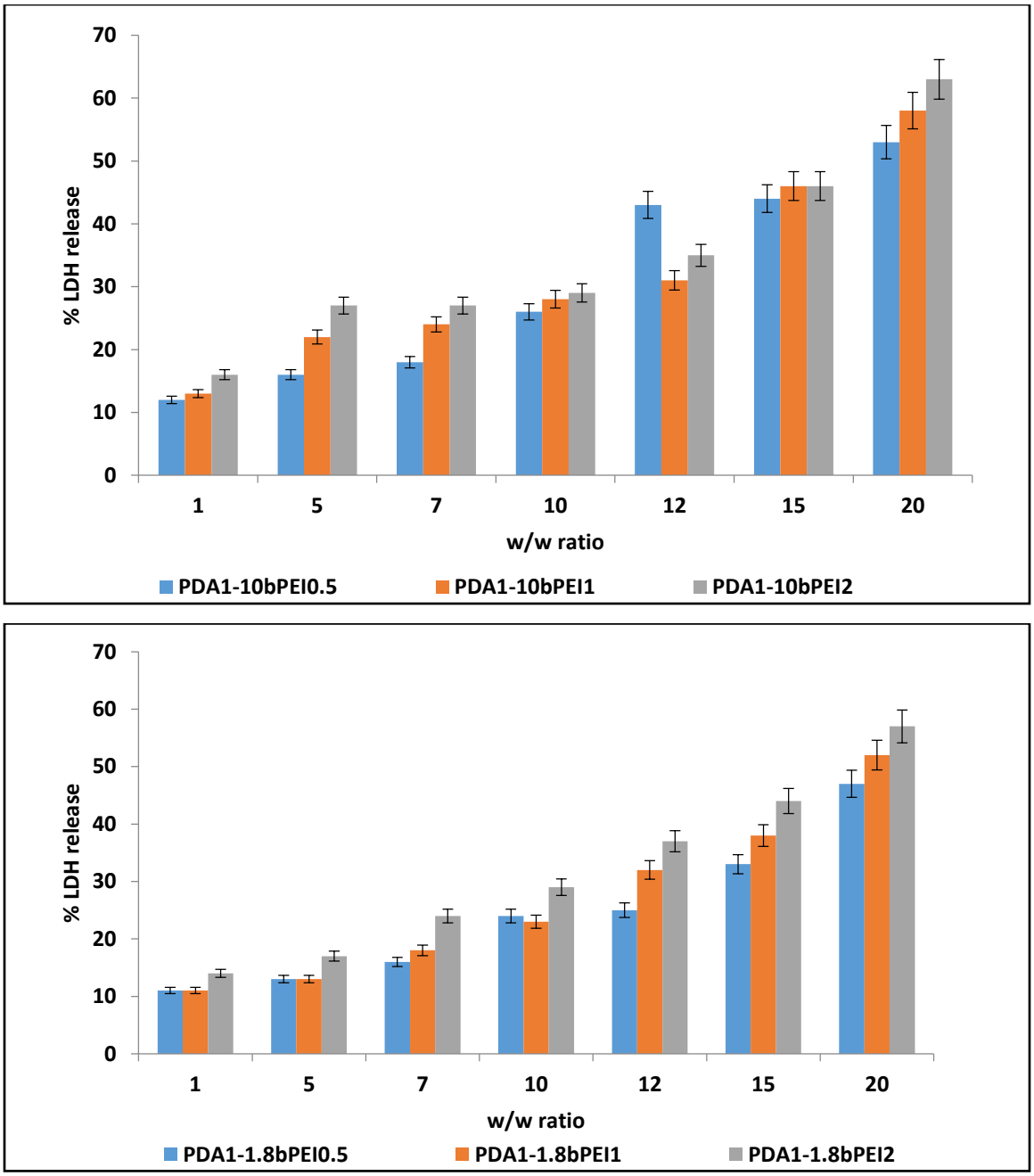


Figure 22: Percentage LDH Release w.r.t to 100% lysed cells showing non-toxic nature of different modifications for PDA-bPEI particles complexed with pDNA

Protection of pDNA from degradation against nucleases is a pre-requisite for its successful delivery into the cells. Therefore, the potential of PDA-bPEI particles to protect the pDNA from such a degradation is examined via DNase I protection assay. At

the best working formulations, PDA-bPEI/pDNA complexes were incubated with DNase I for different time intervals. Only pDNA was found to be digested by DNase I within 0.25h whereas PDA-bPEI/pDNA complex resisted any such degradation even after 2h of the incubation with DNase I. The results demonstrate that PDA-bPEI can protect pDNA from nucleases present in cellular environment, therefore making it suitable carrier for gene delivery.

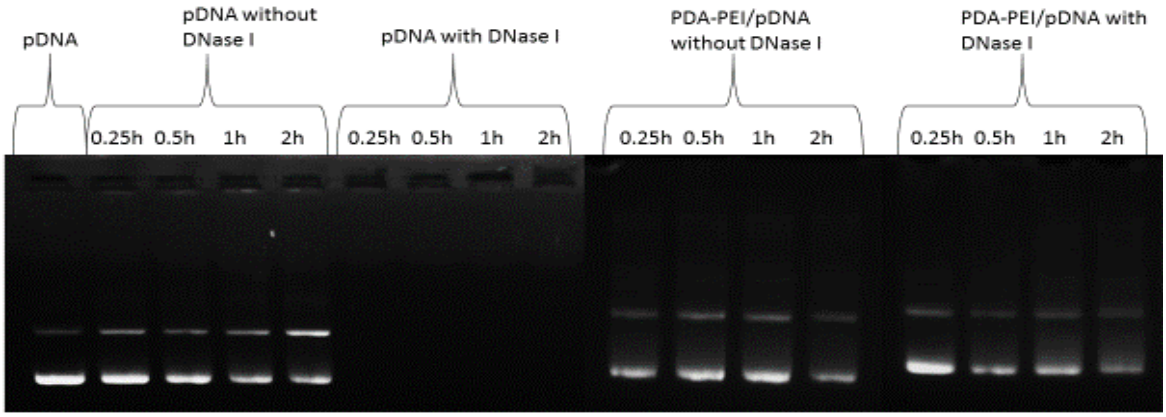


Figure 23: DNase I protection assay carried at various time intervals (0.25h. 0.5h, 1h, 2h) for PDA-bPEI/pDNA complex at the best transfecting w/w ratio.

CONCLUSION

We have reported the development of nanoparticle as gene vehicle by linking varying amounts of different molecular weight polyethylenimine onto polydopamine. The nanoparticles thus formed were characterized by the different physico-chemical techniques and were evaluated biologically for gene transfection. The developed nanoparticles with higher molecular weight PEI showed higher transfection efficiency than that of the particles linked to lower molecular weight PEI, native PEI and lipofectamine. In addition to this, the most prominent feature of the developed strategy is the minimal toxicity of the developed particles. The nanoparticles developed from the mentioned protocols also have shown to protect the DNA from nuclease activity. The results from the strategies adopted are evident to establish the synthesized nanoparticles for efficient gene delivery and hence, could be further explored for gene therapy by developing advanced *in vivo* protocols.

Chapter 6

Part B

PDA-Aminoglycosides Conjugates for Antimicrobial Activity

PART B

PDA-AMINOGLYCOSIDES CONJUGATES FOR ANTIMICROBIAL ACTIVITY

Methods

Synthesis of PDA-Aminoglycosides Conjugates

The aminoglycosides antibiotics that were used were Gentamicin sulfate, neomycin sulfate hydrate and kanamycin sulfate. 60mg each of these were taken along with 120 mg of PDA in each of the three MCTs. These were mixed in 1ml of 100mM Tris buffer (pH 8.5). The reaction mixture was kept under incubation on a thermomixer for 4 days under the conditions of 50ºC and 200rpm. Later, these were subjected to dialysis by using the dialysis membrane tubes of 1kDa cut off limit for 24 hours so that the unreacted molecules may diffuse out into the surrounding dialysis medium. The dialysed product was then lyophilized and the PDA-aminoglycosides were obtained.

Nuclear Magnetic Resonance Spectroscopy

The synthesized compounds were analysed by ^{1}H-NMR by dissolving in D_2O (heavy water) with the help of ^{1}H-NMR Bruker Spectrospin spectrometer, 400 MHz and the structure was thus elucidated. The chemical shifts (δ) are presented in ppm.

Fourier Transform Infrared Spectroscopy

FTIR spectra were recorded on a single beam Perkin Elmer Spectrum (BX Series), USA at room temperature in the range of 4000-400 cm^{-1} with a resolution of 4 cm^{-1} and 100 scan per samples. The samples were prepared by KBr disk method. The presence and absence of functional groups were used to elucidate the structure of compounds.

Microbiological Assay (Agar Disk Diffusion Method)

Microbiological assay was done by *Karby beaur* single disc susceptibility test. [62, 63] In disc diffusion method we used sterile disc procured from HiMedia with thickness 3mm and diameter 6 mm. Firstly, different bacterial strains i.e. (Gram +ve) *Staphylococcus aureus*, *Bacillus cereus*, (Gram –ve) *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* were inoculated using nutrient broth in separate centrifuge tubes and allowed them to shake in shaking incubator at 37ºC for at least 3- 4hours. The absorbance was measured at 600nm to check growth of respective bacterial strain. Secondly, particular bacterial strain was spread on nutrient agar plates aseptically simultaneously, all the polydopamine conjugated aminoglycosides (PDA-Neomycin, PDA-Gentamicin, PDA-Kanamycin) at different concentrations (24µg per disc, 50µg per disc, 100µg per disc) were impregnated on sterile disc placed along with native polydopamine nanoparticle (10µg per disc) on nutrient agar plates prior seeded with target test organisms i. and then kept in an incubator for 37ºC for 24 hours. Gentamicin sulphate an aminoglycoside used as positive control at concentration of (10µg per disk) and negative control as water. The results were recorded by measuring diameter of zone inhibited by respective sample on respective test organisms.

RESULTS AND DISCUSSION

Synthesis of PDA-aminoglycosides conjugates involves the reaction between the exposed 1º amine groups and the hydroxyl groups on the surface of the aminoglycosides as well as on PDA nanoparticles which allow the crosslinking to take place and hence the conjugates of PDA-gentamicin sulfate, PDA-kanamycin sulfate and PDA neomycin sulfate hydrate were formed.

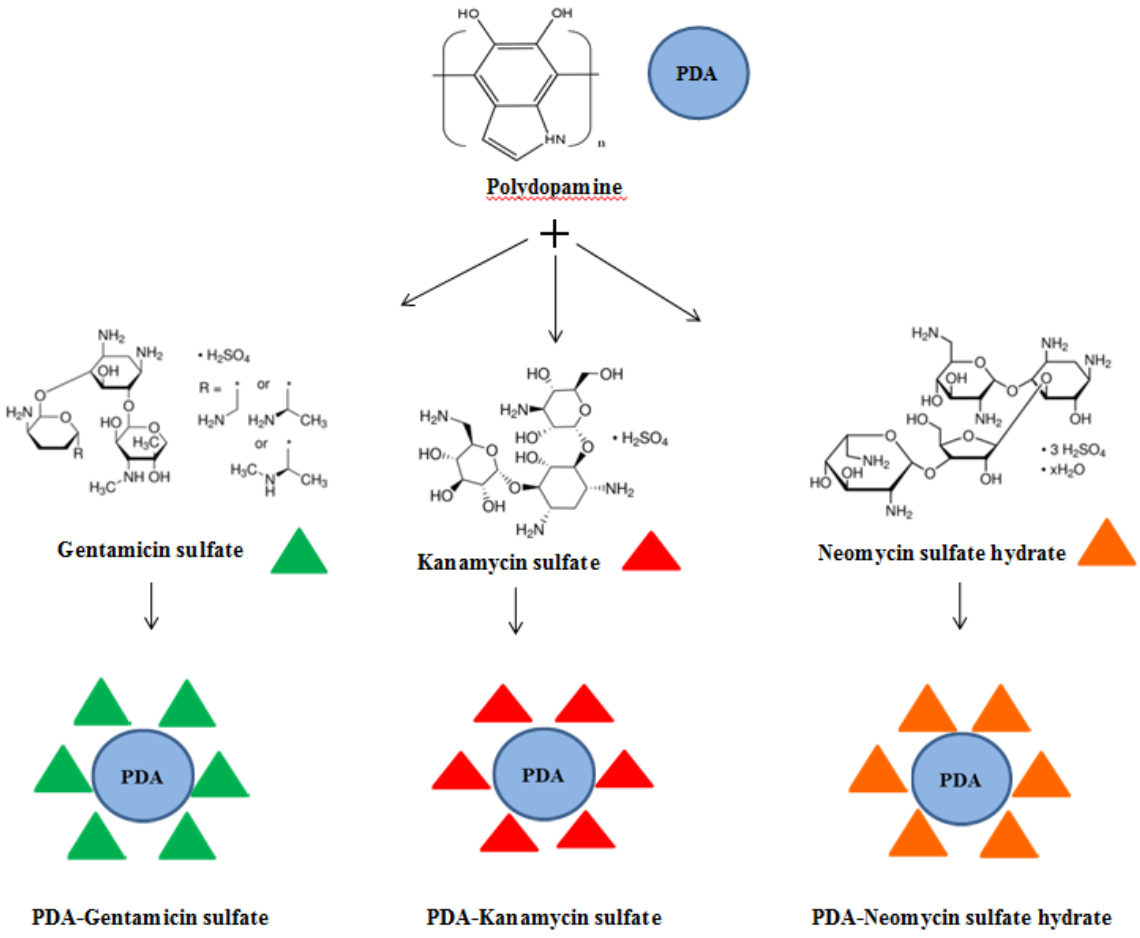


Figure 24: Schematic representation of formation of PDA-aminoglycosides conjugates.

FTIR characterization of the PDA-aminoglycosides conjugates:

FTIR characterization was done to obtain the information regarding the functional groups, bonds involved etc. in the compound.

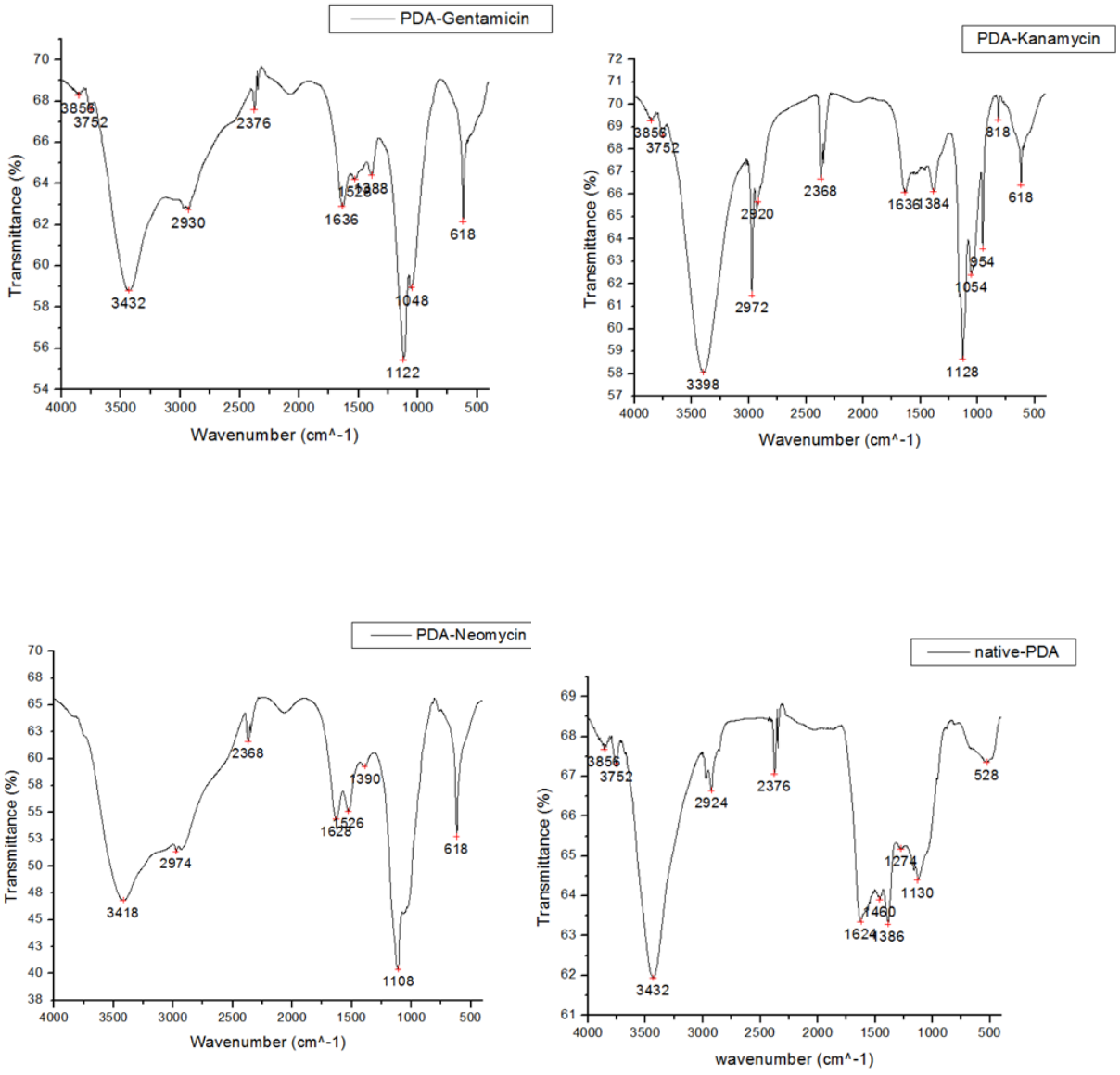


Figure 25: Graphs showing FTIR spectra for PDA-gentamicin, PDA kanamycin and PDA-neomycin conjugates and native-PDA.

NMR characterization for PDA-conjugates:

NMR characterization for the conjugates was done in D2O which helped in elucidating the micelle structure of the particles in water. The particles were characterized by ^{1}H-NMR spectroscopy with D_2O as solvent. The following results were obtained that show

characteristic peaks indicating particular side chain groups within the compounds. As can be seen, characteristic peak near 2.6 ppm is obtained in the NMR spectrum of native polydopamine polymer which could be of hydroxylic groups that are exposed upon polymerization of dopamine molecules. Strong peaks in the region of 1-5 ppm are obtained in the NMR spectra of all other complexes of aminoglycosides and branched polyethylenimine with polydopamine indicating presence of free amine groups on the surfaces of micellar nanoparticles so formed in the water.

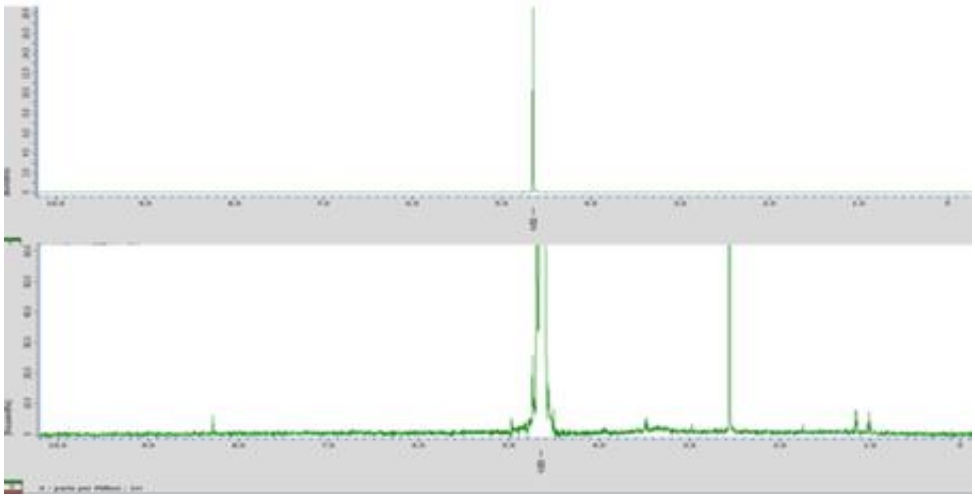


Figure 26(a): NMR spectrum for PDA

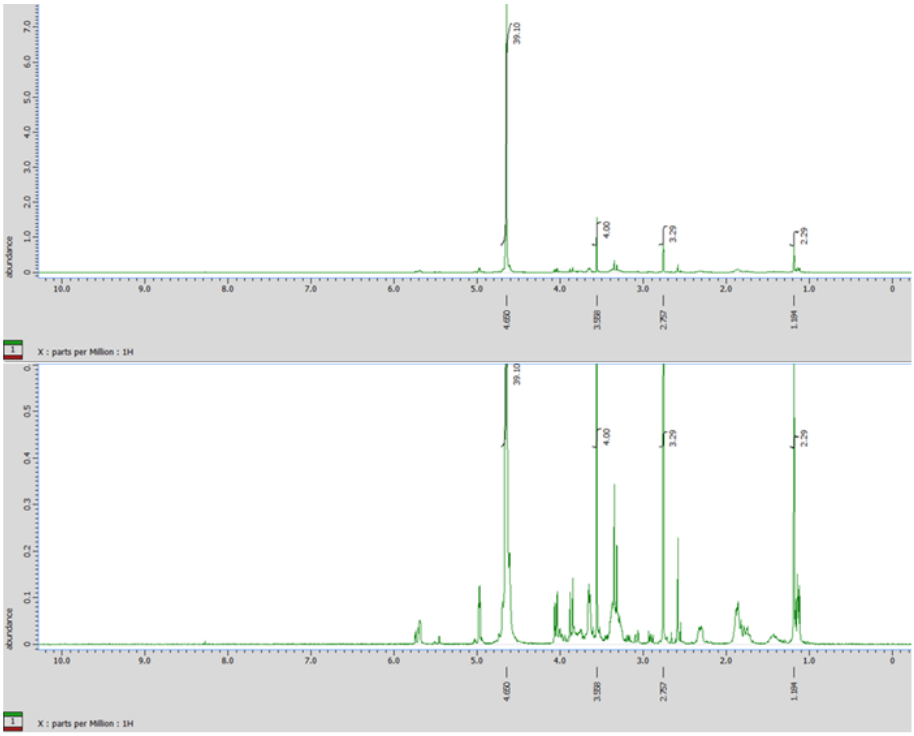


Figure 26(b): NMR spectrum for PDA-Gentamicin conjugate

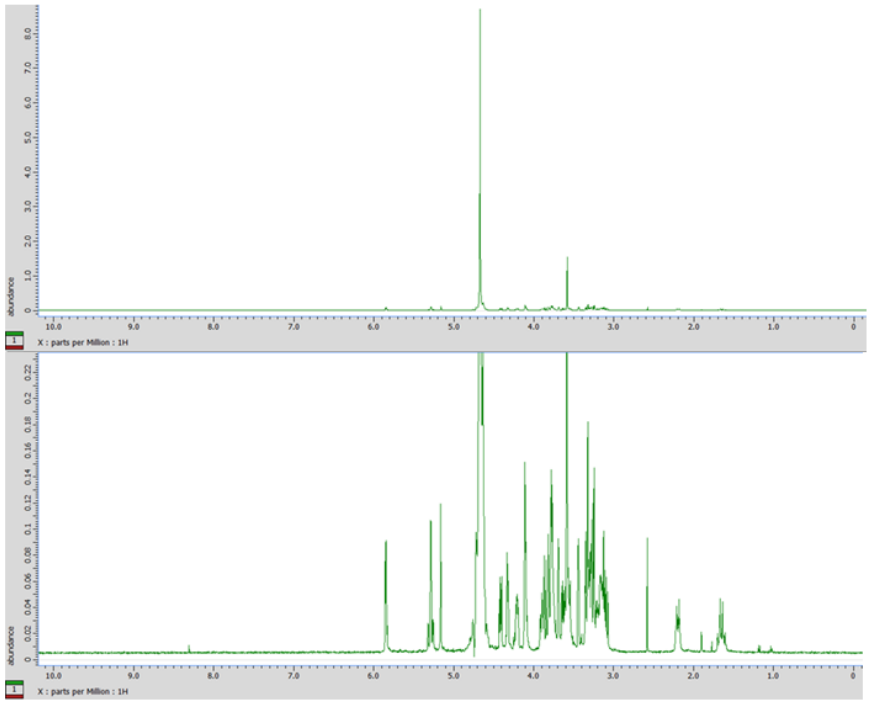


Figure 26(c): NMR spectrum for PDA-Kanamycin conjugate

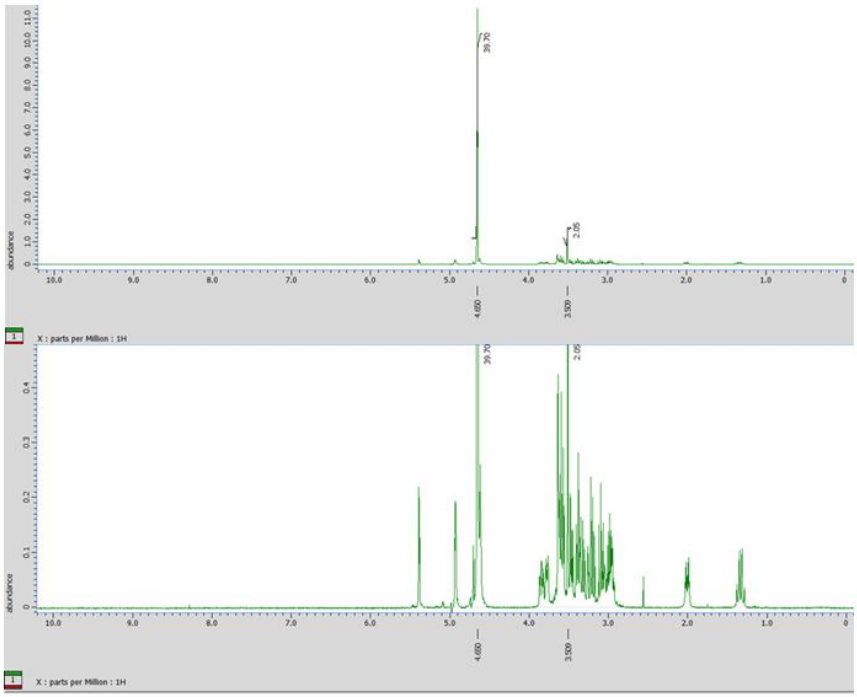


Figure 26(d): NMR spectrum for PDA-Neomycin conjugate

Microbiological assay for Zone of Inhibition by Agar Disk Diffusion Method:

In above experiment, zone inhibited by polydopamine nanoparticle was found to be nil but some literature have shown that polydopamine nanoparticles reduces the population of Escherichia coli (Gram –ve) to be extent by facile shaking method. On the other hand, polydopamine conjugated aminoglycosides found to be higher zone of inhibition when compared to positive control i.e. gentamycin. Conjugated neomycin and kanamycin elicit better antibacterial activity in every test organism in comparison to gentamycin conjugation and native aminoglycoside (positive control). The polydopamine (PDA) nanoparticle activity is contradictory, but in present susceptibility test, PDA conjugated aminoglycoside having synergistic bactericidal effects comparatively native PDA nanoparticles. For identifying reason behind the synergism of PDA with aminoglycoside further studies need to be done including Minimum Inhibitory Concentration, hemolytic activity, cell viability test.

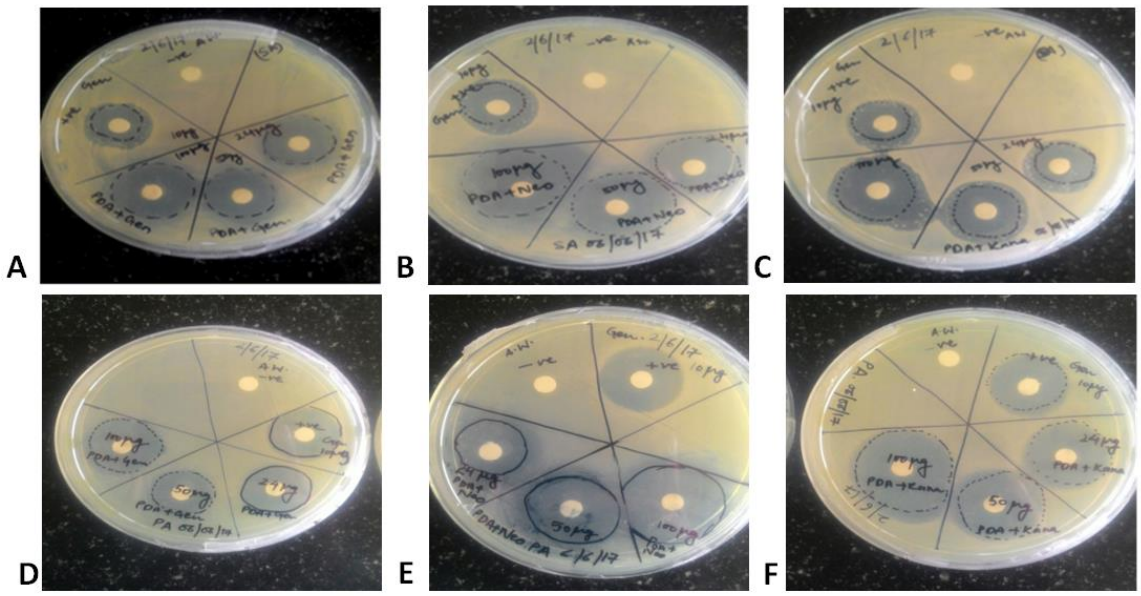


Figure 27(A,B,C): PDA with gentamicin , neomycin, kanamycin at different concentrations on *S.aureus* (Gram +ve) respectively

Figure 27(D,E,F): PDA with gentamicin, neomycin, kanamycin at different concentrations on *P. aeruginosa* (Gram -ve) respectively

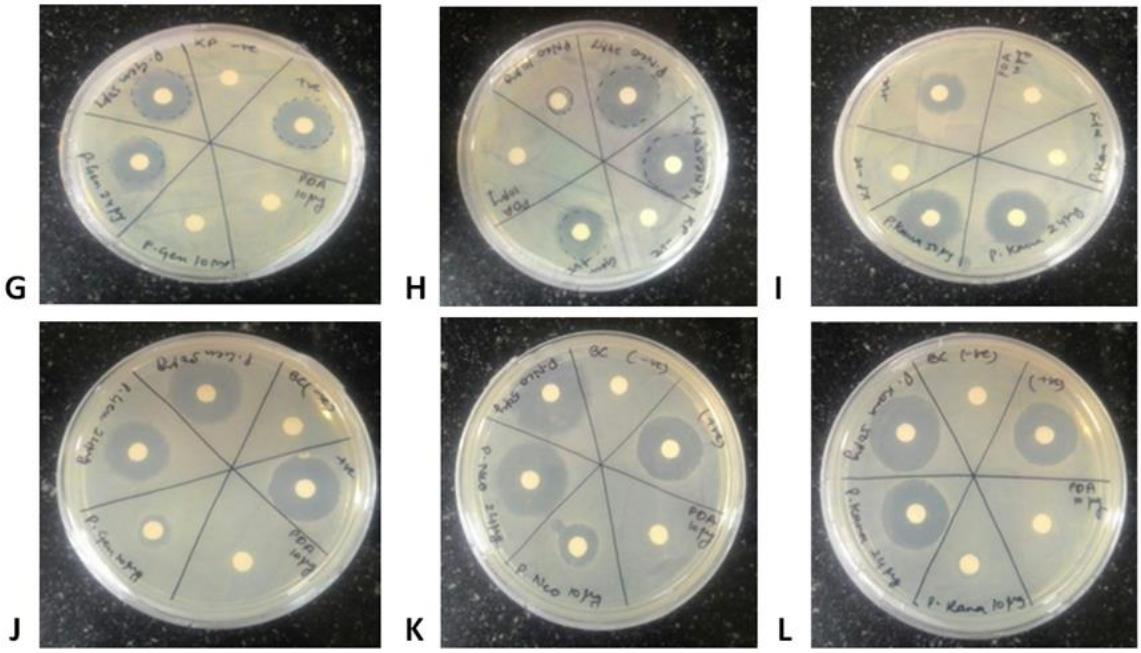


Figure 27(G,H,I): PDA with gentamicin, neomycin, kanamycin at different concentrations on *K. pneumoniae* (Gram -ve) respectively

Figure 27(J,K,L): PDA with gentamicin, neomycin, kanamycin at different concentrations on *B. cereus* (Gram -ve) respectively

Table 4: Diameters (in mm.) for zone of inhibition of PDA-aminoglycosides at different concentration of samples for the different bacterial strains

Bacterial Strain	**Concentration Of Sample**			**+ve Control (Gentamicin)**	**Polydopamine**
	10µg	**24µg**	**50µg**	**10µg**	**10µg**
Gram –ve bacteria	**Zone of inhibition (dia. In mm.)**				
Pseudomonas aeruginosa					
PDA-Neomycin	11	18	21	20	NIL
PDA-Gentamicin	10	17	19	20	NIL
PDA-Kanamycin	<10	21	24	20	NIL
Klebsiella pneumoniae					
PDA-Neomycin	<10	20	21	16	NIL
PDA-Gentamicin	NIL	17	18	16	NIL
PDA-Kanamycin	NIL	19	20	16	NIL
Gram +ve bacteria					
Staphylococcus aureus					
PDA-Neomycin	11	21	23	18	NIL
PDA-Gentamicin	10	20	22	18	NIL
PDA-Kanamycin	<10	18	20	18	NIL
Bacillus cereus					
PDA-Neomycin	12	22	24	20	NIL
PDA-Gentamicin	10	17	19	20	NIL
PDA-Kanamycin	<10	19	22	20	NIL

CONCLUSION

The PDA conjugates with Neomycin sulfate, Gentamicin sulfate and Kanamycin sulphate showed better antibacterial against all the bacterial strains (both Gram –ve and Gram +ve) in comparison to the native aminoglycoside Gentamicin sulfate ("gold standard") used as the positive control and also as compared to the native PDA nanoparticles. Hence based on these preliminary results, we can say that these conjugates may be developed further to be used in preparing antibacterial composites, surfaces etc. However, to validate these results other important tests including Minimum Inhibitory Concentration determination, cell viability test and hemolytic test would be needed to be done.

Chapter 7

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