การพัฒนาอนุภาคนาโนไคโทซานสำหรับการส่งพลาสมิดดีเอ็นเอเข้าสู่เซลล์แบบจำเพาะ

นายกมล จึงอภิโสดม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตร์มหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2553

DEVELOPMENT OF CHITOSAN NANOPARTICLES FOR CELL TYPE SPECIFIC PLASMID DNA DELIVERY

Mr Kamon Jungapisodom

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biotechnology
Faculty of Science
Chulalongkorn University
Academic Year 2010

Thesis Title	DEVELOPMENT OF CHITOSAN NANOPARTICLES FOR CELL TYPE SPECIFIC PLASMID DNA DELIVERY
By	Mr. Kamon Jungapisodom
Field of Study	Biotechnology
Thesis Advisor	Associate Professor Tanapat Palaga, Ph.D.
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กมล จึงอภิโสดม : การพัฒนาอนุภาคนาโนไคโทซานสำหรับการส่งพลาสมิดดีเอ็นเอเข้าสู่เซลล์แบบจำเพาะ. (DEVELOPMENT OF CHITOSAN NANOPARTICLES FOR CELL TYPE SPECIFIC PLASMID DNA DELIVERY) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ธนาภัทร ปาลกะ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ.ดร.ศุภศร วนิชเวชารุ่งเรือง, 86 หน้า.

ไคโทซานถือเป็นทางเลือกที่ดีสำหรับการขนส่งยีนเนื่องจากมีความเข้ากันได้กับเนื้อเยื่อในร่างกาย, ย่อยสลายได้และมีความเป็นพิษต่อเซลล์ต่ำ ไคโทซานเป็นพอลิเมอร์ประจุบวกสามารถรวมตัวกับพลาสมิดเป็นสารประกอบเชิงซ้อนแบบโพลีอิเล็กโทรไลต์ได้อนุภาคนาโนไคโทซานสามารถนำส่งพลาสมิดดีเอ็นเอ (CNP-DNA complex) เข้าสู่เซลล์ผ่านทางเอ็นโดโซมเข้าสู่นิวเคลียสต่อไปได้ การศึกษานี้ได้เตรียมอนุภาคนาโนจากไคโทซาน (CNP-DNA complex) โดยใช้วิธีโคอะเซอเวชันเชิงซ้อนและสร้างอนุภาค CNP-DNA complex ที่สามารถขนส่งพลาสมิดดีเอ็นเอเข้าสู่เซลล์ได้ ประสิทธิภาพในทรานสเฟกชันพลาสมิดที่มีส่วนเรืองแสง (pMAXGFP) ของ CNP-DNA complex ที่เตรียมที่อัตราส่วนพอลิเมอร์ต่อพลาสมิด (N/P) 6:1 ในเซลล์ตัวอ่อนในไตของมนุษย์ 293T มีค่าเท่ากับ 55% สำหรับเซลล์แมคโครฟาจที่มาจากหนู อนุภาค CNP-DNA complex ให้ประสิทธิภาพในการทรานสเฟกชันสูงสุดเท่ากับ 3.5% FugeneHD ให้ค่าอยู่ที่ 19.3% ขนาดอนุภาค CNP อยู่ในช่วง 350-650 นาโนเมตรและประจุพื้นผิวอยู่ในช่วง 6-11 mV ในขณะที่ภาพถ่ายจากกล้องจุลทรรศน์อิเล็กตรอนแบบส่องกราดจะให้ขนาดอนุภาคมีค่าประมาณ 125 nm นอกจากนี้ได้นำไคโทซานมาทำการติดสเตรปตาวิดิน โดยอนุภาค streptavidin CNP-DNA complex มีขนาด 290 นาโนเมตร และมีประจุพื้นผิว -31 mV โดยขนาดอนุภาค streptavidin CNP-DNA complex มีค่าประมาณ 134 และ 350 นาโนเมตรเมื่อดูด้วยกล้องจุลทรรศน์อิเล็กตรอนแบบส่องกราดและกล้องจุลทรรศน์อิเล็กตรอนแบบส่องผ่าน อย่างไรก็ตามในการทรานสเฟกชันสำหรับอนุภาค streptavidin CNP-DNA complex ที่มีแอนติบอดี้ (F4/80) ติดอยู่ พบว่าอนุภาคที่ได้สามารถขนส่งพาสมิดดีเอ็นเอเข้าสู่เซลล์แมคโครฟาจที่มาจากหนูได้ในระดับที่เห็นได้ชัด

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต.....

ปีการศึกษา 2553 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

5072204023 : MAJOR BIOTECHNOLOGY
KEYWORDS: CHITOSAN, STREPTAVIDIN HYDAZIDED CNPS, GENE THERAPY, NON-VIRAL VECTOR, COMPLEX COACERVATION PROCESS

KAMON JUNGAPISODOM : DEVELOPMENT OF CHITOSAN NANOPARTICLES FOR CELL TYPE SPECIFIC PLASMID DNA DELIVERY. ADVISOR : ASSO. PROF. TANAPAT PALAGA, PH.D., CO-ADVISOR : ASSO. PROF. SUPASON WANICHWECHARUNGRUANG, PH.D., 86 pp.

Chitosan is considered to be a good candidate for gene delivery since it is biocompatible, biodegradable and exhibits low toxicity against cells. Chitosans are cationic polymers with component of amino group and can self-assemble with plasmid into polyelectrolyte complexes. The chitosan-DNA nanoparticles (CNP-DNA complex) enter cells by endocytosis and are released from endosome into the nucleus, leading to transcription and translation of genes. The aim of this study was to prepare CNP-DNA complex using a complex coacervation process and to construct a cell-type specific DNA delivery system. The transfection efficiency of CNP-DNA complex with an polymer/DNA (N/P) ratios of 6:1 for human embryonic kidney cell line, 293T, was 55%. For murine macrophage cell line, Raw 264.7, the N/P ratio of CNP-DNA complex showed the highest transfection efficicey of 3.5%, in comparison to commercial transfection reagent, FUGENE HD, which yielded around 19.3% of transfection. The CNP-DNA complex particle size was within the range of 350–650 nm with a surface charge ranging from +6 to +11 mV at various N/P ratios, whereas the particle size of CNP-DNA complex was approximately of 125 nm as measured by scanning electron microscope. To modify chitosan with strepavidin, the chitosan was reacted with sodium *meta*-periodate and streptavidin hydrazide. The streptavidin CNP-DNA complex have a particle size of 290 nm with a surface charge ranging from -31 mV. The streptavidin CNP-DNA complex particle size was approximately of 134 nm and 350 nm by scanning electron microscope and transmission electron microscopy. Nevertheless the streptavidin CNP-DNA complex which was bound to F4/80 biotin, could mediate the expression of green fluorescent protein in RAW 264.7 cells at a noticeable level.

Field of Study : Biotechnology Student's Signature .....

Academic Year : 2010 Advisor's Signature .....

Co-advisor's Signature .....

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my advisor, Associate Professor Dr. Tanapat Palaga, Ph.D. and Associate Professor Supason Wanichweacharungruang, Ph.D. for the excellent instructions, guidance and support throughout this thesis.

My gratitude is also extended to Associate Professor Suthep Thaniyavarn, Ph.D., Associate Professor Sirirat Rengpipat, Ph.D. and Nuttapun Supaka, Ph.D. for serving as thesis committee.

I wish to thank Associate Professor Dr. Supason Wanichweacharungruang for chitosan 30 kDa from Core squid, FTIR and NMR. I would like to thank Scientific and technological research equipment centre of Chula university for SEM and TEM. I wish to thank Nanotec for Nanosizer. I also thank Noppadol Sa-Ard-Iam for his help on FACS analysis.

I would like to thank Department of Microbiology, Faculty of science, Chulalongkorn University for providing some research facilities.

I would to thank all of my 403 laboratory members for their assistant and friendship.

Finally, I would like to express my deepest gratitude to my parents for their love, understanding and encouragement extended throughout my study.

CONTENTS

Page

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF SCHEME

ABBREVIATIONS

1.	%	Percentage
2.	°C	Degree Celsius
3.	µg	Microgram
4.	µm	Micrometer
5.	µM	Micromolar
6.	/	Per
7.	:	Ratio
8.	×	Fold
9.	A	Absorbance
10.	Ab	Antibody
11.	APC	Antigen Presenting Cell
12.	bp	Base pair
13.	cm-1	A Reciprocal Wavelength
14.	CNP	Chitosan Nanoparticles
15.	CO_2	Carbon dioxide
16.	CTL	Cytotoxic T Cells
17.	DD	Degree of Deacetylation
18.	DNA	Deoxyribonucleic acid
19.	DP	Degree of Polymerization
20.	g (centrifugation speed)	Gravity
21.	GlcN	Glucosamine Hydrochloride
22.	GlcNAc	N-acetyl-D-glucosamine
23.	hr	Hour
24.	kDa	Kilo Dalton
25.	mA	Milliampere

26.	mAbs	Monoclonal Antibodies
27.	mg	Milligram
28.	MHC	Major Histocompatibility Complex
29.	ml	Milliliter
30.	mM	Millimolar
31.	mϕ	Macrophage
32.	MW	Molecular Weight
33.	nm	Nanometer
34.	NMR	Nuclear Magnetic Resonance
35.	No.	Number
36.	OD	Optical density
37.	PdI	The polydispersity index
38.	ppm	Part Per Million
39.	psi	Pound per square inch
40.	PBS	Phosphate buffer saline
41.	PEI	Polyethylenimine
42.	pmaxGFP	Plasmid maxGFP
43.	RNA	Ribonucleic acid
44.	rpm	Round per minute
45.	SEM	Scanning Electron Microscopy
46.	TAE	Tris-acetate-EDTA
47.	TEM	Transmission Electron Microscopy
48.	TPP	Thepolyanion Tripolyphosphate
49.	U	Unit
50.	v	Volume
51.	w	Weight
52.	α	Alpha

53.	β	Beta
54.	γ	Gamma
55.	θ	Scattering Angle
56.	δ	Delta

CHAPTER I

BACKGROUND

Nanotechnology

Nanotechnology is the understanding and control of matter at dimensions of roughly 1–100 nanometers, where unique phenomena enable novel applications. Nanotechnology is a multi-disciplinary field that is expected to lead to breakthroughs in molecular biology, diagnostics, and therapeutics. The developments in nanotechnology have considerably increased the interest in platform for the delivery of drugs and plasmid DNA into cells. Delivery of plasmid DNA containing genes of interest into mammalian cells is an important process requiring high efficiency and specificity. The technology of DNA delivery into cells have been increasingly applied in clinical research such as gene therapy and DNA vaccines. Gene therapy has attracted considerable attentions and interest because it is expected to treat devastating inherited genetic diseases, prevent, correct and modulate genetic and acquired diseases by introducing genes coding for therapeutic proteins (Kim et al., 2007). DNA vaccine is a novel type of vaccine using DNA as immunogen. Because of its stability and low cost for production, it is expected to facilitate protective immunization in the developing world (Khatri et al., 2008).

Gene delivery

There are two types of vectors currently in use for gene delivery: viral vector and non-viral vector. There are several types of viral vectors, such as adenovirus, retrovirus, adeno-associated virus, herpes simplex. virus and lentivirus (Jiang et al., 2006). Viral vectors are highly

effective in gene delivery and high specificity for the target cells, but they have potentials to have dangerous side effects from the stimulation of the immune response and from the viral vector itself (Zheng et al., 2009). The non-viral vector is often used in the form of lipid-based vector and cationic polymer. This type of vector is safe with little to no side effects, low immunogenicity, low toxicity to cells. However, it was difficult to increase the amount of vector to be used and the efficacy and specificity in introducing DNA into cells is relative low (Mansouri et al., 2006).

Chitosan

Chitosan is a linear cationic polysaccharide composed of β (1-4) linked D-glucosamine, partly containing N-acetylglucosamine. Two methods, namely chemical and enzymatic, are known for preparation chitosan and their oligomers from chitin, with different degrees of deacetylation, polymerization, and molecular weight (MW). Chitosan is consider to be a good candidate as vector for gene delivery since it is biocompatible, biodegradable and had low toxicity. It is cationic polymers with amino component and can self-assemble with plasmid into polyelectrolyte complexes. The chitosan-DNA nanoparticles enter cells by endocytosis and is released from endosome into the nucleus, leading to transcription and translation of genes.

Previous studies have been carried out on chitosan-DNA nanoparticles as gene delivery system. Delivery of plasmids into various cell types such as human embryonic kidney cells (HEK293), human lung carcinoma cells (A549), B16 melanoma cells, COS-1, CHO-K1 and HeLa cells were achieved by chitosan-DNA nanoparticle (Li et al., 2003; Corsi et al., 2003). In addition, studied on synthesis, characterization and transfection efficiency of chitosan-DNA nanoparticles as gene carriers have been reported (Mao et al., 2001). Bozkir et al. have reported on the effects of molecular structure of chitosan on formulation in chitosan-DNA nanoparticles (Bozkir and Saka., 2004). Using the complex coacervation technique, the nanoparticles as non-

viral gene deliverly vehicles for either DNA or siRNA have been succesfully used (Yuan et al., 2009). The transfection ability of chitosan was found to be a cell type dependent and the efficiency was affected by various factors such as the presence or absence of serum, media pH and the molecular weight of chitosan (Koping-Hoggard et al., 2001; Ishii et al., 2001). In General, transfection efficiency of chitosan-DNA nanoparticles was generally low, compared with that of the viral vectors (Lee et al., 2007). Thus, chitosan-DNA nanoparticles modified to increase transfection efficiency. For instance, it was reported that galactosylated 6-amino-6-deoxychitosan as a DNA carrier was used for an *in vitro* gene delivery to HepG2 cells (Satoh et al., 2007). Furthermore, it has been reported that receptor mediated gene delivery was achieved by folate conjugated N-trimethylchitosan *in vitro*. weF reports, however, on the delivery plasmids DNA into cells in a specific group of immune cells. Developing the techniques to delivery plasmids DNA into immune cells is useful for application such as DNA vaccines and gene therapy. For DNA vaccine, the main target cells are antigen presenting cell (APC) such as dendritic cells and macrophages that function mainly to present antigens to T lyphocytes) Khatri et al., 2008).

The aim of this study is to prepare chitosan–DNA nanoparticles using a complex coacervation process and modified chitosan so that it can be used to specifically deliver plasmid DNA into macrophages.

Objectives

The purposes of this research is to investigate the development of chitosan nanoparticles for cell type specific plasmid DNA delivery.

CHAPTER II

LITERATURE REVIEWS

2.1 Nanotechnology and its application

Nanotechnology, is the understanding and control of matter at dimensions of roughly 1–100 nanometers, where unique phenomena enable novel applications. Two main approaches are used in nanotechnology. In the "bottom-up" approach, materials and devices are built from molecular components which assemble themselves chemically by principles of molecular recognition. In the "top-down" approach, nano-objects are constructed from larger entities without atomic-level control. Nanotchnology has been increasingly used for manufacturing diverse industrial items such as cosmetics or clothes and for infinite applications in electronics, aerospace and computer industry. In addition, nanotechnology is being used to refine discovery of biomarkers, molecular diagnostics, and drug discovery and drug delivery, which could be applicable to management of these patients.(Soloviev, 2007) Nanoparticle research is currently an area of intense scientific research, due to a wide variety of potential applications in biomedical, optical, and electronic fields. Nanoparticles are of great scientific interest as they are effectively a bridge between materials and atomic or molecular structures. Nanoparticles often have unexpected visible properties because they are small enough to confine their electrons and produce quantum effects. Nanoparticles are able to enter the body through the skin, lungs or intestinal tract, depositing in several organs and may cause adverse biological reactions by modifying the physiochemical properties of living matter at the nano level (Oberdorster et al., 2005).

2.1.1 Different classes of nanoparticles

Several varieties of nanoparticles are available: different polymeric and metal nanoparticles, liposomes, micelles, quantum dots, dendrimers, microcapsules, cells, cell ghosts, lipoproteins, and many different nanoassemblies. Nanoparticle for *in vivo* use are expected to be easy and reasonably cheap to prepare, biodegradable, have small particle size, possess high loading capacity, demonstrate prolonged circulation, and, ideally, specifically or non-specifically accumulate in required pathological sites in the body. (Gref et al., 1994) Here, different classes of nanoparticles offen used for DNA and active compound carrier.

Liposomes are nanoparticles comprising lipid bilayer membranes surrounding an aqueous interior. The amphilic molecules used for the preparation of these particles have similarities with biological membranes and have been used as carrier for improving the efficacy and safety of different drugs (Hofheinz et al., 2005). Usually, liposomes are classified into three categories on the basis of their size and lamellarity (number of bilayers): small unilamellar vesicles or oligo-lamellar, large unilamellar vesicles and multilamellar vesicles. The active compound can be located either in the aqueous spaces, if it is water-soluble, or in the lipid membrane, if it is lipid-soluble. (Figure 2.1)

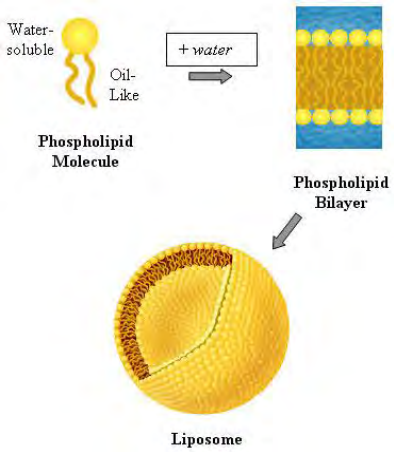


Figure 2.1 Structure of Liposomes. The interior of liposomes was filled with water and therefore, molecules which were soluble in water could be encapsulated in the interior of the liposome. (http://www.nanolifenutra.com/; 2/9/2010)

Emulsions comprise oil in water-type mixtures that are stabilized with surfactants to maintain size and shape. The lipophilic material can be dissolved in a water organic solvent that is emulsified in an aqueous phase. Like liposomes, emulsions have been used in delivery for improving the efficacy and safety of diverse compounds (Sarker et al., 2005). (Figure 2.2)

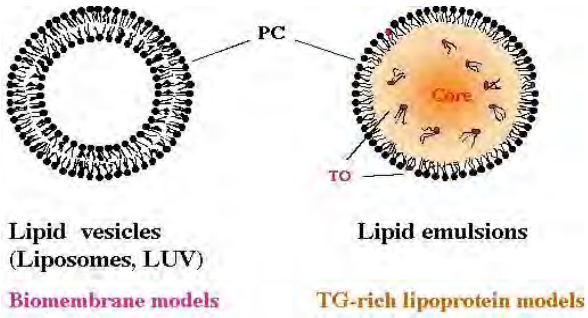


Figure 2.2 Schematic representation of lipid emulsions and liposomes. Lipid emulsion is used as a model compound of a plasma lipoprotein particle and it is applied to the drug delivery system (http://www.pharm.kyoto-u.ac.jp/seizai/research/research-e.html; 2/9/2010).

Ceramic nanoparticles are inorganic systems with porous characteristics that have recently emerged as drug vehicles (Cherian et al., 2000). These vehicles are biocompatible ceramic nanoparticles such as silica, titania and alumina that can be used in cancer therapy. However, one of the main concerns is that these particles are non-biodegradable, as they can accumulate in the body, thus causing undesirable effects. (Figure 2.3)

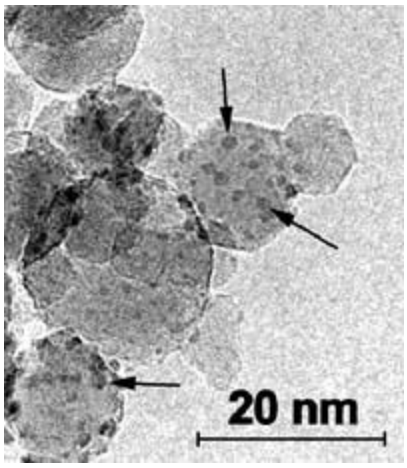


Figure 2.3 Structure of ceramic nanoparticles. Flame made Pt/TiO_2. Pt particles d< 3nm are indicated by arrows. (http://www.ptl.ethz.ch/research/res_top_metals; 2/9/2010)

Metallic particles such as iron oxide nanoparticles (15–60 nm) generally comprise a class of superparamagnetic agents that can be coated with dextran, phospholipids or other compounds to inhibit aggregation and enhance stability. The particles are used as passive or active targeting agents (Gupta and Gupta, 2005). (Figure 2.4)

Figure 2.4 The assembly of metallic particles. The Iron/steel and ferrous steel alloys are the most widely used standard methods of measurement for oil filters.

(http://www.prccusa.com/turbomag%20reviews.html; 15/10/2010)

Gold shell nanoparticles, other metal-based agents, are a novel category of spherical nanoparticles consisting of a dielectric core covered by a thin metallic shell, which is typically gold. These particles possess highly favourable optical and chemical properties for biomedical imaging and therapeutic applications (Hirsch et al., 2006). (Figure 2.5)

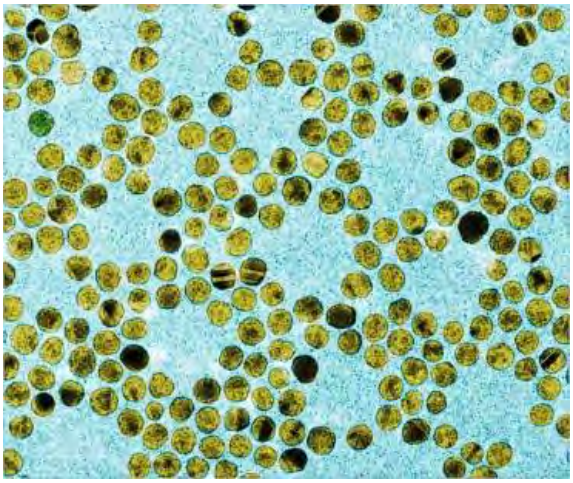


Figure 2.5 Gold nanoparticles has tiny spheres of gold just a litte billionths of a meter in diameter. They have become useful tools in modern medicine.
(http://web.mit.edu/newsoffice/2010/sticky-nanoparticles-0611.html; 22/9/2010)

Carbon nanomaterials include fullerenes and nanotubes. Fullerenes are novel carbon allotrope with a polygonal structure made up exclusively by 60 carbon atoms. (Figure 2.6) These nanoparticles are characterized by having numerous points of attachment whose surfaces also can be functionalized for tissue binding (Bosi et al., 2003). Nanotubes have been one of the most extensively used types of nanoparticles because of their high electrical conductivity and excellent

strength. Functionalized carbon nanotubes are emerging as novel components in nanoformulations for the delivery of therapeutic molecules (Pagona and Tagmatarchis, 2006)

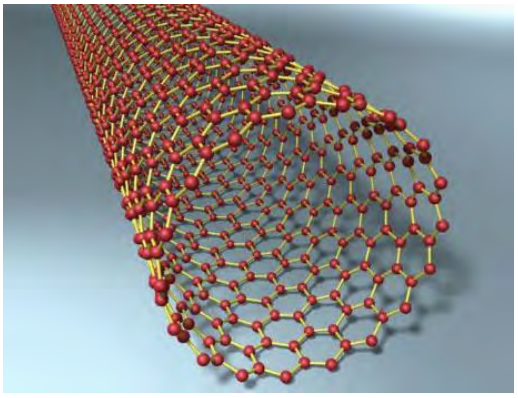


Figure 2.6 Carbon nanomaterials has ability to superconduct adds to their many intriguing electrical and physical characteristics. (http://thedakepage.blogspot.com/2010/06/british-report-laments-lack-of-exposure.html; 12/9/2010)

Quantum dots are nanoparticles made of semiconductor materials with fluorescent properties. A quantum dot has a discrete quantized energy spectrum. The corresponding wave functions are spatially localized within the quantum dot, but extend over many periods of the crystal lattice. (Figure 2.7) For self-assembled quantum dots are typically between 10 and 50 nm in size. Biological applications, quantum dots must be covered with other materials allowing dispersion and preventing leaking of the toxic heavy metals (Weng and Ren, 2006).

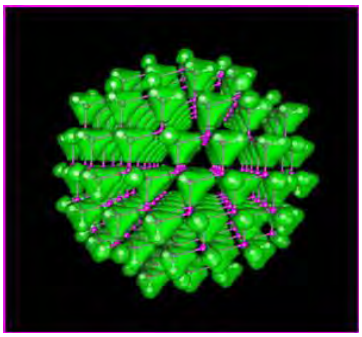


Figure 2.7 Quantum Dots with atomic numbers in the hundreds of thousands have good fluorescence properties, and lighting that can be change color.
(http://almonz.blogspot.com/2010/01/quantum-dots.html; 2/9/2010)

Polymers such as polysaccharide chitosan nanoparticles have been used for some time as drug delivery systems (Agnihotri et al., 2004). Recently, water-soluble polymer hybrid constructs have been developed. These are polymer–protein conjugates or polymer–drug conjugates. Polymer conjugation to proteins reduces immunogenicity, prolongs plasma half-life and enhances protein stability. Polymer drug conjugation promotes tumour targeting through the enhanced permeability and retention effect and, at the cellular level following endocytic capture, allows lysosomotropic drug delivery (Lee, 2006). (Figure 2.8)

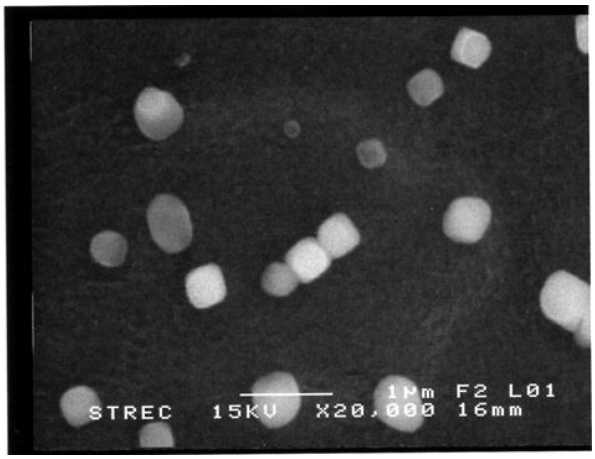


Figure 2.8 Polymers of chitosan nanoparticles on SEM. Chitosan nanoparticles have been spherical and in size from 100 to 200 nm.

2.2 Chitosan

Chitosan, a copolymer of d-glucosamine and N-acetyl-d-glucosamine with b-(164) linkage, is obtained by alkaline or enzymatic deacetylation of chitin and is an abundant polymeric product in nature. (Figure 2.9) Chitosan was first discovered by Rouget in 1859 when he heated chitin to the boiling point in a concentrated KOH solution (Dunn et al., 1997). With unique structures, they possess high biological and mechanical properties as they are biorenewable, biodegradable, and biofunctional (Hirano et al., 2000). Two methods, namely chemical and enzymatic, are known for preparation chitosan and their oligomers, with different degrees of deacetylation, polymerization, and molecular weight (MW). Meanwhile, chitin and chitosan oligomers can be prepared through microbiological and fungal treatments (enzymatic preparation). Chitosan is soluble in dilute acidic solutions and highly viscous when dissolved in, formic acid/water, acetic acid/water, lactic acid/water and glutamic acid/water.

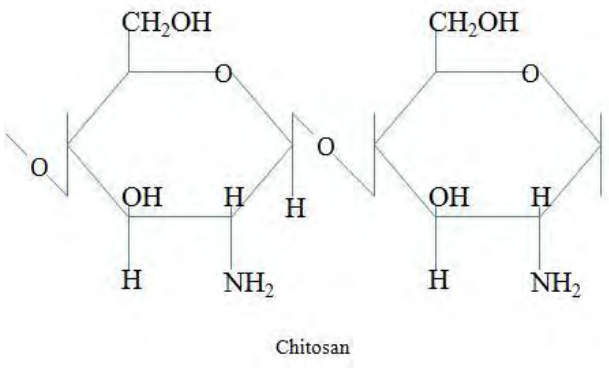


Figure 2.9 The chemical structures of chitosan has a copolymer of glucosamine and N-acetyl-d-glucosamine linked together by β (1, 4) glycosidic bonds.

Chitosan has many useful applications in different fields, mainly because of the presence of amino groups at the C2 position, and because of the primary and secondary hydroxyl groups at the C3 and C6 positions (Furusaki et al., 1996; Kurita, 1986). Unlike most polysaccharides, the presence of positively charged amino groups repeatedly placed along the chitosan polymer chain allows the molecule to bind to negatively charged surfaces via ionic or hydrogen bonding (Muzzarelli, 1973; Rha, 1984; Shahidi, 1995). The term chitosan is favored when the nitrogen content of the molecule is higher than 7% by weight (Muzzarelli, 1985) and the degree of deacetylation (DD) is more than 70% (Li et al., 1992). Chitosan are weak bases and the usual neutralization reactions of basic compounds. The non-bonding pair of electrons on the primary amino group of the glucosamine unit accepts a proton, and thus becomes positively charged (Winterowd and Sandford, 1995). Chitosan are labile to acid- or alkaline-assisted degradation. Under acidic or basic conditions, acetic acid can be freed as N-acetyl groups at the C2 positions of N-acetyl glucosamine units are released, leaving behind primary amine groups. In addition, the presence of the primary amino groups in chitosan presents further potentials for modification of the molecule such as N-acylation, N-alkylation, and N-alkylidenation. Acidic conditions also cause some degree of depolymerization as degradation of the β-glycosidic bonds occurs. The solubility characteristics of chitosan are governed mostly by the extent of N-acetylation, the

distribution of acetyl groups, the pH, and the ionic strength (Anthonsen et al., 1993). The amino group in chitosan has a pKa value of 6.2 to 7.0, which makes chitosan a polyelectrolyte at low pH values (Claesson and Ninham, 1992).

2.2.1 Preparation of chitosan

Two hydrolytic methods were reported for action of chitin and chitosan. These are acid hydrolysis (chemical treatment) and enzymatic hydrolysis (Shahidi et al., 1999).

Chemical treatment; The normal procedure for preparation of chitin from crustacean shells includes the use of NaOH, HCl, and decoloring agents to remove the remaining proteins, calcium, and color, respectively. The chitin that is produced can then be deacetylated with sodium hydroxide to produce chitosan (Tsai et al., 2002). Jaworska and Konieczna (2001) reported that chitosan can be prepared via chemical means using concentrated hydroxides (40–50%) at high temperatures (100–130 ^{o}C). The resulting chitosans from chemical method are diffent from that enzymatic methods are different with respect to their degree of deacetylation (DD), distribution of acetyl groups, chain length, and conformational structure of chitin and chitosan molecules. These factors together will affect the characteristics of chitin and chitosan.

2.2.1.1 Degree of deacetylation and molecular weight of chitosan

DD is highly important because of affects physical properties of chitosan. The DD of chitosan is affected by the concentration of alkali, temperature, reaction time, previous treatment of chitin, particle size, and chitin concentration. Many techniques are used to evaluate the average degree of acetylation of chitosan, such as infrared, solid-state nuclear magnetic resonance (NMR), ultraviolet spectrometry and potentiometric titration, ^{1}H liquidstate NMR, and elemental

analysis (Heux et al., 2000), as well as C solid-state NMR and elemental analysis. These techniques do not require solubilization of the polymer. DD has no effect on the acid binding properties of chitosan (Scheruhn et al., 1999). Chitosans have a relatively high DD and strongly enhance fibroblast proliferation, whereas chitosans with lower levels of deacetylation show less activity. The MW and polymer chain length were of little importance (Howling et al., 2001).

The MW of chitosan have an important effect on its activity. Chitosan preparations with a MW of 5–50 kDa had the ability to reduce serum cholesterol levels in rats (Ikeda et al., 1993). Oh et al. (2001) reported that chitosan with an MW of 12,000 Da (DD, 87%) was most effective against *L. fructivorans*, and chitosan with an MW of 32,500 Da (DD, 80%) was most effective against *L. plantarum*. From these results, it is clear that there is a relationship between the type of microorganism and antimicrobial activity of different MW chitosans. (Li et al., 1992).

2.2.1.2 Depolymerization

Chitosan, like other polysaccharides, is influenced by several degradation mechanisms, including oxidative-reductive free radical depolymerization, and acid-, alkaline-, and enzymatic-catalyzed hydrolysis (Holme et al., 2001). Degradation of polysaccharides usually occurs via cleavage of glycosidic bonds; it is very important to control the depolymerization of chitosan to maintain other properties such as viscosity, solubility, and biological activity. There reports also found that the degradation rate of chitosan increased by acid hydrolysis with increasing temperature and degree of acetylation (Holme et al., 2001).

Enzymatic hydrolysis; The enzymatic hydrolysis of chitin and chitosan may occur because of the action of chitinases, chitosanases, lysozymes, and cellulases (Shahidi et al., 1999). The products of chitin hydrolysis are of high degree of polymerization (DP). Tsigos et al. (2000)

reported the necessity for pretreatment (alkali treatment) of crystalline chitin before adding the enzyme to increase the rate of deacetylation to produce new polymers with new physical and chemical characteristics. The compounds are easily soluble if produced with different distribution of N-deacetylated residues. A synthetic procedure for chitin with N-acetyl-d-glucosamine and chitosan derivatives with d-glucosamine branches has been reported (Kurita et al., 2000). These resulting nonnatural branched chitin and chitosan have extra amino sugars in branches that render them much improved properties in comparison with linear ones, such as the affinity for solvents and hygroscopicity.

2.2.2 Applications of chitosan

2.2.2.1 Medical applications

2.2.2.1.1 Wound-healing agent

Chitosan have been tested to have both material and biological properties that might be beneficial to enhance wound repair. Howling et al. (2001) found that chitosan polymers interact with and modulate the migration behavior of neutrophils and macrophages, modifying subsequent repair process such as fibroplastic and epithelialization. It was reported that chitosan have both stimulatory and inhibitory effects on proliferation of human dermal fibroblasts and keratinocytes (Howling et al., 2001). They also have an enhancing effect on the survival function of osteoblasts and chondrocytes (Lahiji et al., 2000). It was reported that the wound recovering material composed of polyelectrolytic complexes of chitosan and sulfonated chitosan that speeded up wound healing and resulting a good-looking skin surface (Lahiji et al., 2000). Other studies have examined the effect of chitin and chitosan samples with different deacetylation levels and polymer chain length on the proliferation of human dermal fibroblasts *in vitro* (Howling et al.,

2001). It was found that chitosans with a high DD strongly motivated fibroblast proliferation; meanwhile, samples with lower degrees of deacetylation showed less activity.

2.2.2.1.2 Dietary applications

Chitosan may be considered as a dietary supplement for reducing body weight in humans. Industrial production of chitosan tablets (Muzzarelli et al., 2000) and chitosan dietary fibers (Hughes, 2002) has been conducted. Furthermore, Schiller et al. (2001) reported that a rapidly soluble chitosan (LipoSan Ultra that has a higher density and solubility than chitosan itself) facilitated weight loss and reduced body fat. This effect was due to the fact that this chitosan was able to prevent dietary fat absorption in overweight and mildly obese individuals that consumed a high-fat diet. Dietary fibers have many health advantages such as lowering low-density-lipoprotein (LDL) cholesterol levels and hence reduce heart disease and lower the risk of colon cancer. Moreover, dietary fibers are involved in weight loss. The fat-absorbing mechanism of chitosan has been explained by Hughes (2002); the chitosan with its positively charged amino groups ($-NH^{+}_{3}$) is attracted to the anionic carboxyl groups of fatty acids and bile acids forming films passing through the digestive system undigested (Hughes, 2002; Ylitalo et al., 2002). Chitosans have also been used to prevent body weight increase in animals (Hughes, 2002). Meanwhile, negative results were recorded regarding chitosan effectiveness in this field (Hughes, 2002).

2.2.2.1.3 Antitumor activity

Suzuki (1996) reported that chitin and chitosan oligomers act as inhibitors of growth up tumor cells via their immunoenhancing effects. Suzuki et al. (1985) found that chitin oligomers from (GlcNAc)4 to (GlcNAc)7 have strong attracting responses to peritoneal exudate cells in

BALB/c mice. However, chitooligosaccharides from (GlcN)2 to (GlcN)6 did not exhibit such an effect. With regard to hexamers, both (GlcNAc)6 and (GlcN)6 were reported to process growth inhibitory effects against allogenic and syngeneic mouse systems (Suzuki et al., 1986a). These results indicated that the effect was not by direct cytocidal action on tumor cells but was host mediated.

2.2.2.2 Food applications of chitosan

Chitosan derivatives offer a wide range of applications including bioconversion for the production of value-added food products, preservation of foods from microbial spoilage, formation of biodegradable films, recovery of waste material from food processing discards, purification of water, and clarification and deacidification of fruit juices (Shahidi et al., 1999) (**Table I**).

TABLE I

FOOD APPLICATIONS OF CHITOSAN

Area of application	**Examples**
Antimicrobial agent	Bactericidal Fungicidal Measure of mold contamination in agricultural commodities
Edible film	Controlled moisture transfer between food and th surrounding environment Controlled release of antimicrobial substances Controlled release of antioxidants Controlled release of nutrients, flavors, and drugs Reduction of oxygen partial pressure Controlled rate of respiration Temperature control C ntrolled enzymatic browning in fruits Reverse osmosis membranes

Area of application	Examples
Food additive	Clarification and deacidification of fruit juices Natural flavor extender Texture adjusting agent Emulsifying agent Food mimetic Thickening a d stabilizing agent Color st bilization
Nutrition	Dietary fiber Hypocholesterolemic agent Livestock and fish feed additive Reduction of lipid absorption Production of single cell protein Antigastritis agent
Water treatment	Recovery of metal ions, pesticides, phenols, and PCB Removal of dyes, radioisotopes
Agriculture	Seed and fruit covering Fertilizer Fungicide
Cosmetics	Skin and hair products
Biomedical and Pharmaceutical Materials	Artificial skin Surgical structures Contact len Treating major burns Blood ialysis membranes Artificial blood vesicles
Others	Enzyme immobilization Encapsulation of nutraceuticals Chromatography Analytical reagent Synthetic fiber Chitosan-coated paper Manufacturing material for fiber Film an sponges

Table 1 Show food applications of chitosan

2.2.2.2.1 Antimicrobial activity

Chitosan has activity against bacteria, yeast, and fungi. The exact mechanism of antimicrobial action of chitin and chitosan and their derivatives remains illusive, but different mechanisms have been proposed (Shahidi et al., 1999). Chitosan is considered to be a soluble chelating agent and activator due to the presence of a positive charge on the C-2 of its glucosamine monomer at pH values less than 6. A leakage of proteineous and intercellular components occurs due to the interaction between the positively charged chitosan molecules and the negatively charged microbial cell membranes. This is affected by the MW of chitosan (Tsai et al., 2002). The DD increased, its antimicrobial effect on bacteria increased, even to a greater extent than that on fungi. Furthermore, chitosan can enter the nuclei of a microorganism and bind with DNA. This binding inhibits mRNA and protein synthesis (Hadwiger et al., 1985; Sudharashan et al., 1992).

2.2.2.3 Preservation of foods

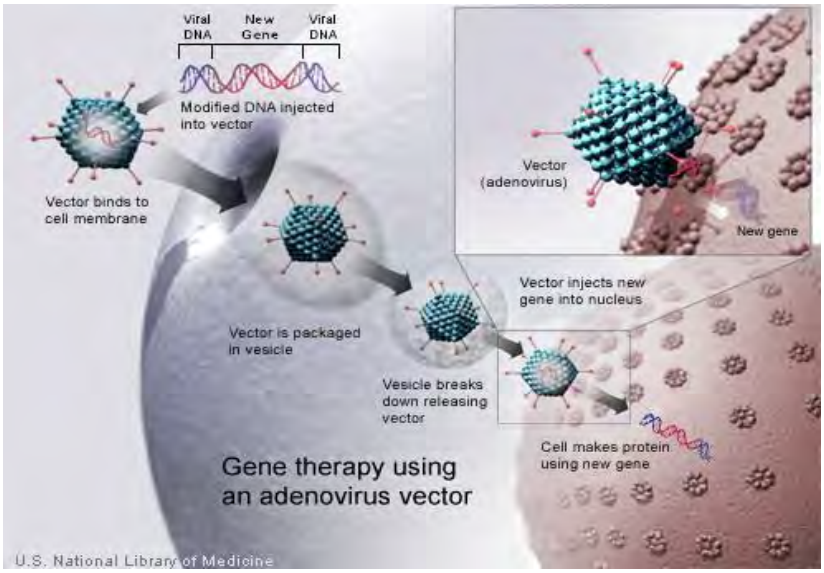
Chitosan can be used for food preservation by inhibiting the growth of spoilage microorganisms in mayonnaise (Oh et al., 2001). Four species of food spoilage microorganisms were treated by chitosans to examine their effects on microbial activity (Oh et al., 2001). These were *Lactobacillus plantarum*, *Lactobacillus fructivorans*, *Serratia liquefaciens*, and *Zygosacchaomyces bailii*. Chitosan had a microbiocidal effect as the number of cells grown was clearly reduced. It has been found that after an extended phase, some strains recovered and started to grow. As the concentration of chitosan increased, the activities of these strains increased. It was noticed that chitosan-50 had the most efficiency against *L. fructivorans*; meanwhile, the inhibition of *L. plantarum* growth was mostly by chitosan-55 and no difference was found among the chitosans against *S. liquefaciens* and *Z. bailii*.

2.3 Gene delivery

Gene delivery is the process of introducing foreign DNA into host cells. Gene delivery is, for example, one of the steps necessary for gene therapy and the genetic modification of crops. In most gene therapy studies, a wildtype gene is inserted into the genome to replace an "abnormal," disease-causing gene (Oligino et al., 2000). A carrier molecule called a vector must be used to deliver the therapeutic gene to target cells. Currently, the most common vector is viral vector that has been genetically altered to carry human DNA. Viruses have evolved a way of encapsulating and delivering their genes to human cells in a pathogenic manner. Therefore, it is possible to manipulate the virus genome to remove disease-causing genes and insert therapeutic genes. There are many different methods of gene delivery developed for a various types of cells and tissues, from bacterial to mammalian. Generally, the methods can be divided into two categories, viral and non-viral approaches.

Viruses are obligate intra-cellular parasites, designed through the course of evolution to infect cells, often with great specificity to a particular cell type. They are highly efficient at transfecting their own DNA into the host cell, which is expressed to produced new viral particles. (Figures 2.10) By replacing genes that are needed for the replication phase of their life cycle with foreign genes of interest, the recombinant viral vectors can transduce the cell type it would normally infect (Humrich et al., 2003). To produce such recombinant viral vectors the non-essential genes are provided in trans, either integrated into the genome of the packaging cell line or on a plasmid. As viruses have evolved as parasites, they all elicit a host immune system response to some extent. Though a number of viruses have been developed, interest has centred on four types; retroviruses (including lentiviruses), adenoviruses, adeno-associated viruses & herpes simplex virus type 1. The acute immune response, immunogenicity, and insertion mutagenesis uncovered in gene therapy clinical trials have raised serious safety concerns about

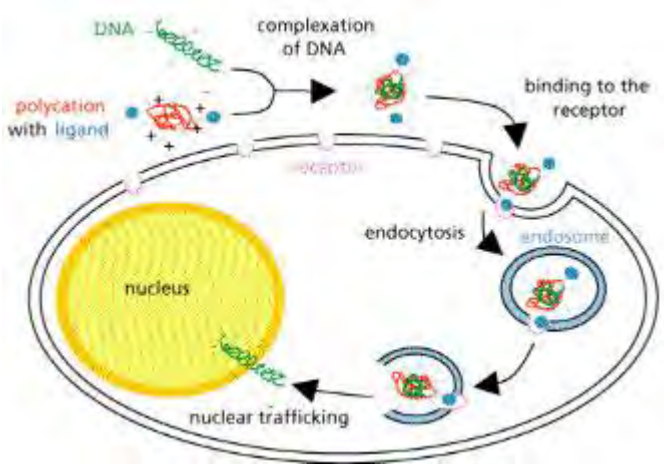
some commonly used viral vectors. The limitation in the size of the transgene that recombinant viruses can carry and issues related to the production of viral vectors present additional practical challenges (Gao et al., 2007). Viral vectors all induce an immunological response to some degree & may have safety risks (such as insertional mutagenesis & toxicity problems).



Figures 2.10 A new gene is injected into an adenovirus vector, which is ugsed to introduce the modified DNA into a human cell. If the treatment is successful, the new gene will make a functional protein. (http://ghr.nlm.nih.gov/handbook/therapy?show=all; 2/9/2010)

Besides virus-mediated gene-delivery systems, there are several non-viral options for gene delivery. The simplest method is the direct introduction of therapeutic DNA into target cells. This approach is limited in its application because it can be used only with certain tissues and requires large amounts of DNA. Nonviral gene delivery have also been explored using physical (carrier-free gene delivery) and chemical approaches (synthetic vector-based gene delivery). Physical approaches, including needle injection, electroporation, gene gun, ultrasound, and

hydrodynamic delivery, employ a physical force that permeates the cell membrane and facilitates intracellular gene transfer (Mao et al., 2001). The chemical approaches use synthetic or naturally occurring compounds as carriers to deliver the transgene into cells. Although significant progress has been made in the basic science and applications of various non-viral gene delivery systems, the majority of nonviral approaches are still much less efficient than viral vectors, especially for *in vivo* gene delivery.



Figures 2.11 A non-viral vector bind DNA into the nucleus, the simplest vectors include cationic-polymer-DNA complexes, also known as polyplexes; can be used to deliver DNA into cells. Polyplexes are positively charged complexes of cationic polymers with anionic DNA. Use of cationic materials help condense the negatively charged DNA and reduce its susceptibility to nucleases. (http://www.expresspharmaonline.com/20080115/research02.shtml; 2/9/2010)

Gene delivery by chemical methods as strategy for non-viral gene delivery formulate DNA into condensed particles by using cationic lipids or cationic polymers. The DNA-containing

particles are subsequently taken up by cells via endocytosis, macropinocytosis, or phagocytosis in the form of intracellular vesicles, from which a small fraction of the DNA is released into the cytoplasm and migrates into the nucleus. Synthetic and naturally occurring cationic polymers constitute another category of DNA carriers that have been used widely for gene delivery. A significant number of cationic polymers in linear configuration have been explored as carriers for in vitro and *in vivo* gene delivery (Oster et al., 2006). These include polyethylenimine (PEI), polyamidoamine and polypropylamine dendrimers, polyallylamine, cationic dextran, chitosan, cationic proteins (polylysine, protamine, and histones), and cationic peptides. Although cationic polymers share the function of condensing DNA into small particles and facilitating cellular uptake via endocytosis through charge-charge interaction with anionic sites on cell surfaces, their transfection activity and toxicity differently (Zhang et al., 2004). For PEI- mediated transfection, DNA-to-PEI ratios, the molecular weight and configuration of PEI, the concentration of DNA and polymer, and the ionic strength of the solvent for preparation are all important factors that determine the physical properties of the DNA/PEI complexes (polyplexes) and their transfection activity. Treatment of low – molecular weight PEI with several bifunctional cross-linking reagents generates PEI oligomers that are transfectionally active. Cross-linking of small PEI with a biodegradable bond such as a disulfide or ester bond resulted in oligomers that were as active as PEI 25k but significantly less toxic to cells.

2.4 DNA Vaccine

Genetic DNA immunization is a novel technique used to efficiently stimulate humoral and cellular immune responses to protein antigens. The direct injection of genetic material into a living host causes a small amount of cells to produce the introduced gene products. This inappropriate gene expression within the host has important immunological consequences, resulting in the specific immune activation of the host against the gene delivered antigen. DNA

vaccine is a novel type of vaccine using DNA as immunogen (Toda et al., 2002). Because of its stability and low cost for production, it is expected to facilitate protective immunization in the developing world. (Figure 2.12)

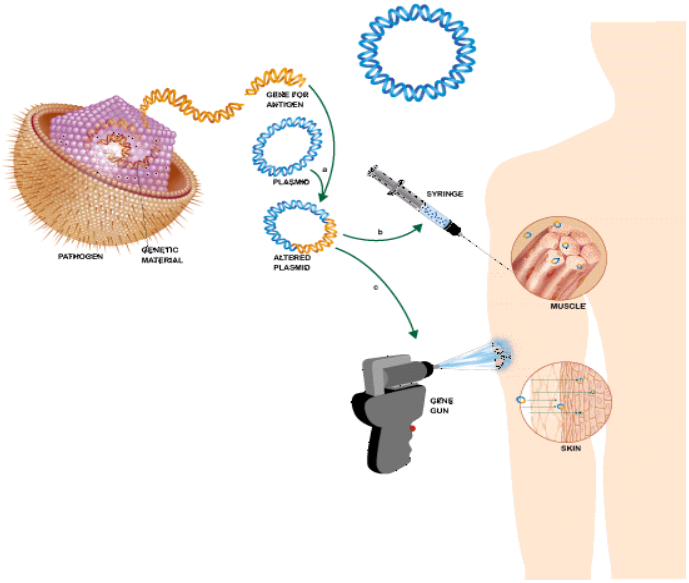


Figure 2.12 Principles of DNA Vaccine is one or more genes from a disease-causing agent with known antigenic properties and splicing those genes into plasmids (which are closed rings of self replicating DNA.) The rings are then delivered into small groups of cells, often by injection into muscle cells or by propulsion into the skin via a so-called gene gun. (Scientific American: Feature Article: Genetic Vaccines; 1999)

Mechanisms of DNA vaccine using a plasmid vector expressing the protein of interest under the control of an appropriate promoter is injected into the skin or muscle of the the host. After uptake of the plasmid, the protein is produced endogenously and intracellularly processed into small antigenic peptides by the host proteases. (Figure 2.13) The peptides then enter the lumen of the endoplasmic reticulum by membrane-associated transporters. In the endoplasmic reticulum, peptides bind to MHC class I molecules. These peptides are presented on the cell

surface in the context of the MHC class I. Subsequent CD8+ cytotoxic T cells (CTL) are stimulated and they evoke cell-mediated immunity. CTLs inhibit viruses through both cytolysis of infected cells and noncytolysis mechanisms such as cytokine production (Encke et al, 1999).

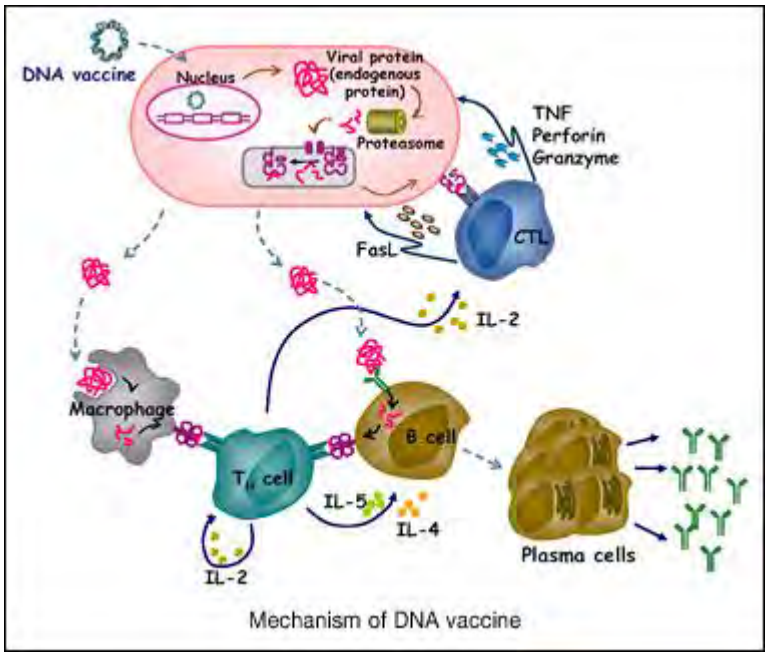


Figure 2.13 Mechanisms of DNA vaccine by 1) the development of an antigen presenting cell-specific vector system; 2) the manipulation of the envelope gene to either change its cellular location or to increase its immunogenicity and 3) the evaluation of several co-stimulatory molecules as potential DNA vaccine adjutants.
(http://www.mogam.re.kr/eng/page/im01.asp; 2/9/2010)

2.5 Macrophage

Macrophages are white blood cells within tissues, produced by the differentiation of monocytes. Monocytes and macrophages are phagocytes, acting in both innate immunity as well as to help adaptive immunity of vertebrate animals (Plowden et a., 2004). They move by action of

amoeboid movement. Macrophages are a highly heterogeneous population of cells and specialized subpopulations of macrophages occupy distinct anatomical compartments in the body. The development of monoclonal antibodies (mAbs) against macrophages has not only been useful for defining these cells but has also created the opportunity to identify molecules expressed specifically bymacrophages and their subsets.

2.5.1 Function of macrophage

2.5.1.1 Role in innate immunity

Macrophages are versatile cells that play many roles. As scavengers, they rid the body of worn-out cells and other debris. They are foremost among the cells that "present" antigen, a crucial role in initiating an immune response. As secretory cells, monocytes and macrophages are vital to the regulation of immune responses and the development of inflammation; they produce a wide array of powerful chemical substances including enzymes, complement proteins, and regulatory factors. At the same time, they carry receptors for lymphokines that allow them to be "activated" into single-minded pursuit of microbes and tumour cells. (Mantovani et al., 2005 and Edwards et al., 2006).

After digesting a pathogen, a macrophage will present the antigen of the pathogen to the corresponding helper T cell. The presentation is done by integrating it into the cell membrane and displaying it attached to an MHC class II molecule, indicating to other white blood cells that the macrophage is not a pathogen, despite having antigens on its surface (Ladanyi et al., 2000).

Eventually, the antigen presentation results in the production of antibodies that attach to the antigens of pathogens, making them easier for macrophages to adhere to with their cell membrane and phagocytose. In some cases, pathogens are very resistant to adhesion by the macrophages.

The antigen presentation on the surface of infected macrophages (in the context of MHC class II) in a lymph node stimulates TH1 (type 1 helper T cells) to proliferate (mainly due to IL-12 secretion from the macrophage). When a B-cell in the lymph node recognizes the same unprocessed surface antigen on the bacterium with its surface bound antibody, the antigen is endocytosed and processed. The processed antigen is then presented in MHCII on the surface of the B-cell. TH1 receptor that has proliferated recognizes the antigen-MHCII complex and causes the B-cell to produce antibodies that help opsonisation of the antigen so that the bacteria can be better cleared by phagocytes. The macrophage does not generate a response specific for an antigen, but attacks the cells present in the local area in which it was activated. (Singh et al., 2000)

2.6 Streptavidin

Streptavidin is a 52,800 dalton tetrameric protein purified from the bacterium *Streptomyces avidinii*. (Figure 2.14) Streptavidin is a tetrameric protein which binds very tightly to the small molecule, biotin. The biological function of many proteins is triggered and modulated by the binding of ligands. For this reason, an understanding of the mechanism of protein-ligand interactions is essential for a detailed knowledge of protein function at the molecular level. Ligand binding, in most cases, involves the formation of noncovalent bonds at specific interacting surfaces between the protein and the ligand. (Gonzalez et al., 1997) The binding constant for this interaction is very high and has made the streptavidin/biotin system the focus of a number of

studies aimed at determining what particular intermolecular interactions give rise to the tight binding.

Figure 2.14 Monomeric streptavidin (ribbon diagram) with bound biotin (spheres). (Weber et al;. 1997)

The numerous crystal structures of the streptavidin-biotin complex have shed light on the origins of the remarkable affinity. Firstly, there is high shape complementarity between the binding pocket and biotin. Secondly, there is an extensive network of hydrogen bonds formed to biotin when in the binding site.

2.6.1 F4/80

F4/80 is a 160 kD glycoprotein. It is characterized as a member of the epidermal growth factor (EGF)-transmembrane 7 (TM7) family. F4/80, also known as EMR1 or Ly71, has been widely used as a murine macrophage marker, which is expressed on majority of tissue macrophages including peritoneal macrophages, macrophages in lung, gut, thymus and red pulp of spleen (but not on the macrophages located in T cell areas of the spleen, lymph node and Peyer's patch), Kuffer cells, Langerhans cells, bone marrow stromal cells. F4/80 has also been

shown to be expressed by subset of dendritic cells. The biological ligand of F4/80 has not been identified, but it has been reported that F4/80 is required for induction of CD8 T cells-mediated peripheral tolerance (Nakmura et al., 2009). Furthermore, low levels of F4/80 are found on monocytes and certain subsets of mature macrophages, including those found in the T-cell areas of the secondary lymphoid tissues, whereas the marginal zone macrophages and metallophilic macrophages of the splenic marginal zone appear F4/80-negative. The F4/80 molecule is expressed selectively on subpopulations of myeloid cells, including macrophages and DCs. (van der Berg et al., 2005) (Figure 2.15)

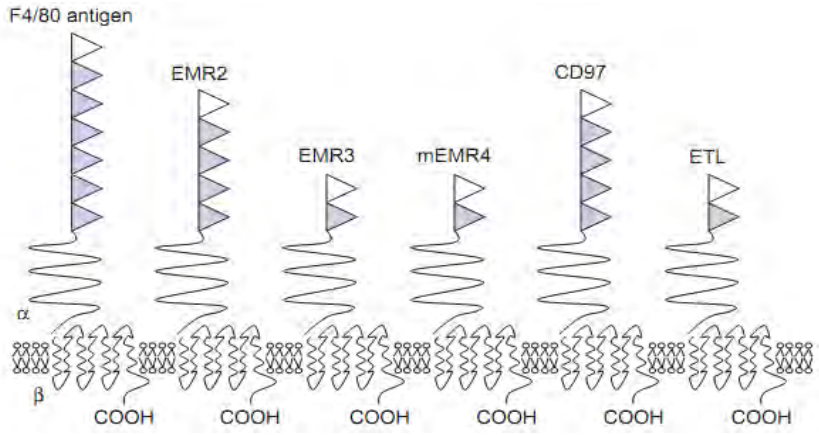


Figure 2.15 Structures of the mouse F4/80 molecule and other members of the EGF-TM7 family.

Although, the previous has studied on synthesis, characterization and transfection efficiency of chitosan-DNA nanoparticles as gene carriers have been reported (Mao et al., 2001). Bozkir et al. have reported on the effects of chitosan molecular structure on formulation in chitosan-DNA nanoparticles (Bozkir and Saka, 2004). Using the complex coacervation technique, the nanoparticles as non-viral gene deliverly vehicles for either DNA or siRNA has been

succesfully used (Yuan et al., 2009). The transfection ability of chitosan was found to be a cell type dependent and the efficiency was affected by various factors such as the presence or absence of serum, media pH and the molecular weight of chitosan (Koping-Hoggard et al., 2001; Ishii et al., 2001). However, transfection efficiency of chitosan-DNA nanoparticles was generally low compared with that of the viral vectors (Lee et al., 2007). Thus, chitosan-DNA nanoparticles has been modified to increase transfection efficiency. For instance, it was reported that galactosylated 6-amino-6-deoxychitosan as a DNA carrier was used for an *in vitro* gene delivery to HepG2 cells (Satoh et al., 2007). The fast expanding research of the useful physicochemical and biological properties of chitosan has led to the recognition of the cationic polysaccharide as a promising natural polymer for drug delivery, especially for therapeutic proteins and genes. By using thepolyanion tripolyphosphate (TPP) as the coacervation crosslink agent to form chitosan–BSA–TPP nanoparticles, which can be conditioned to exert control over protein encapsulation efficiency and subsequent release profile (Quan and Tao, 2008). This study investigated the delivery system of plasmids DNA into cells in a specific group of immune cells by modified chitosan. Chitosan is modified to conjugate with streptavidin are this modified chitosan as used to bind to anti-F4/80 with direct the particle specifically to macrophages.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Equipments

1.	-20˚C Freezer model MDF-U332	Sanyo, Japan
2.	37˚C Incubator	Memmert, Germany
3.	4˚C Refrigerator	Mitsubishi Electric, Japan
4.	5% CO_2 Incubator model 311	Thermo Electron Corperation, USA
5.	500 ml Bottle Top Filter w/33 mm Neck 0.2 μm	Hycon, Germany
6.	-70˚C Deep Freezer model ULT1780	Forma Scientific, USA
7.	Autoclave model MLS 3020	Sanyo, Japan
8.	Balance	Metler Toledo, Switzerland
9.	Bench-Top Centrifuge model Stratagene	Profuge, USA
10.	Centrifuge : Rotafix 32	Hettich, Germany
11.	Centrifuge Tube 15 and 50 ml	Corning Incorperation, USA
12.	Cryotube	Corning Incorperation, USA
13.	Cuvette 0.4 cm	BioRad, USA
14.	Dynamic Light Scattering (Nanosizer)	National Nanotechnology Center (NANOTEC), Thailand
15.	Eppendorf Thermomixer	Eppendorf, Germany
16.	Flow Cytometry (FACS)	

17.	Fluorescence Microscope	Nikon, Japan
18.	Fourier Transform Infrared Spectroscopy (FT-IR)	Nicolet Instruments Technologies, Inc., WI,USA
19.	Gel Documentation and Quantity One 4.4.1	BioRad, USA
20.	Haemocytometer	Boeco, Germany
21.	Heat Block : Thermomixer Compact	Eppendorf, Germany
22.	Hot Air Oven model D06063	Memmert, Germany
23.	Inverted Microscope	Olympus, USA
24.	Inverted Fluorescence Microscope	Olympus, USA
25.	JEM-2100 Transmission Electron Microscope (TEM)	Scientific and Technological Research Equipment Centre, Thailand
26.	JSM-6400 Scanning Electron Microscope (SEM)	Scientific and Technological Research Equipment Centre, Thailand
27.	Laminar Flow Cabinet model H1	Lab Survice LTD part, Thailand
28.	Liquid Nitrogen Tank 34 HC Taylor Wharton Cryogenic	Harsco Corperation, USA
29.	Magnetic Stirrer	Clifton, USA
30.	Mass spectrometry (MS)	
31.	Microcentrifuge Tube 1.5 ml	Axygen Scientific, USA
32.	Micropipette P2, P20, P100 and P1000	Gilson, France
33.	Microplate Reader : Elx 800	Bio-Tek instrument, Canada
34.	Mini-rotator Bio RS-24	Biosan, Latvia
35.	Nuclear magnetic resonance (NMR)	Variance Company, USA

36.	PCR Tube 200 µl	Corning Incorperation, USA
37.	Petri Dish	Hycon, Germany
38.	pH-meter model S20-K	Metler Toledo, Switzerland
39.	Pipette Aid	Drummond, USA
40.	Refrigerated Centrifuge model 1920	Kubota, Japan
41.	RNase-free Tip 2, 10, 100 and 1000 µl	Corning Incorperation, USA
42.	Spectrophotometer model Lambda 25	Perkin-Elmer, USA
43.	Syringe 1 ml	Nipro, Japan
44.	Syringe Fliter 0.20 µm	Corning Incorperation, USA
45.	Tissue Culture Plate 12, 24 and 96 well	Nunc™, Denmark
46.	Ultra-Pure Water Purification System	Elga, England
47.	Vortex mixer model G560E	Scientific Industries, USA
48.	Water bath	Memmert, Germany

3.1.2 Chemicals, Antibodies and Kits

1.	1 kbp DNA Ladder	Fermentas, Canada
2.	Absolute ethanol	Merck, Germany
3.	Absolute methanol	Merck, Germany
4.	Acetic acid	Merck, Germany
5.	Agar	
6.	Agarose Gel	Research Organics, USA
7.	Ammonium persulfate	Bio Basic Inc., Canada
8.	Bactotryptone	Becton, Dickinson and company, France

9.	Bovine Serum Albumin (BSA)	Sigma Aldrich, USA
10.	Biotin anti-mouse F4/80	Biolegend, USA
11.	Bromphenol blue	Sigma Aldrich, USA
12.	Chloroform	Lab-Scan, Ireland
13.	DEPC (diethylpyrocarbonate)	Sigma Aldrich, USA
14.	Dimethylsulfoxide (DMSO)	Sigma Aldrich, USA
15.	Disodium hydrogen phosphate (Na_2HPO_4)	Merck, Germany
16.	DMEM	Hyclone, England
17.	Ethanol 95%	Merck, Germany
18.	Ethanol 70%	
19.	EDTA (ethylenediaminetetraacetic acid)	Merck, Germany
20.	*Escherichia coli* DH5α	Max Plank Institute of Infection Biology, Germany
21.	Ethidium Bromide	Sigma Aldrich, USA
22.	Fetal Bovine Serum (FBS)	Hyclone, England
23.	FuGeneHD Reagent	Roche, Germany
24.	Glycerol	Cario ERBA, France
25.	HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)	Hyclone, England
26.	HPLC grade water	Merck, Germany
27.	Hydrochloric acid (HCl)	Merck, Germany
28.	Hydrogen peroxide	Merck, Germany
29.	Kanamycin	General Drugs House Co., Ltd., Thailand
30.	Isopropanol	Merck, Germany

31.	Opti-MEM	Invitrogen, USA
32.	Paraformaldehyde	Sigma Aldrich, USA
33.	Penicillin	General Drugs House Co., Ltd., Thailand
34.	Plasmid pmax GFP	Amaxa, USA
35.	Potassium chloride (KCl)	Merck, Germany
36.	Potassium di-hidrogen phosphate (KH_2PO_4)	Merck, Germany
37.	QIA prep spin midiprep kit	Qiagen, Germany
38.	Restriction Endonuclease *Kpn* I	Roche, Germany
39	Sodium acetate	Merck, Germany
40.	Sodium azide	Merck, Germany
41.	Sodium chloride (NaCl)	Merck, Germany
42.	Sodium hydrogen carbonate ($NaHCO_3$)	Sigma Aldrich, USA
43.	Sodium *meta*-Periodate	Pierce Biotechnology, USA
44.	Sodium nitrite	Carlo Erba
45.	Sodium pyruvate	Hyclone, England
46.	Sodium sulfate (Na_2SO_4)	Merck, Germany
47.	Spectra/Por Dialysis Tubing	Spectrum Laboratories, USA
48.	Spectra/Por Float-A-Lyzer G2	Spectrum Laboratories, USA
49.	Streptavidin Hydrazide	Pierce Biotechnology, USA
50.	Streptomycin	General Drugs House Co., Ltd., Thailand
51.	Sulfanilamide	BDH Chemicals Ltd., England
52.	Trypan Blue 0.5% w/v	Biochrom AG, Germany

53.	Trypsin 0.25% in 1 mM EDTA	Invitrogen, USA
54.	Yeast Extract	Bio Springer, France

3.2 Cell line and media

Human embryonic kidney cell line 293T cell (ATCC No.CRL - 11268) and murine macrophage cell line RAW 264.7 (ATCC No. TIB - 71) were maintained in DMEM media supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 0.4 mg/ml streptomycin, 1% sodium pyruvate and 1% HEPES. Cells were cultured in a humidified atmosphere containing 5% CO_2 at 37 ^oC.

3.3 Cell culture and treatment

3.3.1 Cell culture

RAW 264.7 was detached from a tissue culture dish by repetitive pipetting in 1X PBS (Appendix). Single cell suspension was centrifuged at 1,000 rpm for 5 min. After centrifugation, 1X PBS layer was discarded and complete DMEM media were added. Cell viability was assessed by trypan blue dye exclusion method using a haemocytometer. The viable cell number was calculated according to the following formula. 293T cell was detached from a tissue culture dish by treatment with trypsin/EDTA. Cells were incubated in a humidified atmosphere containing 5% CO_2 at 37 ^oC for 2 min and complete media were added at the end of treatment. Single cell suspension was centrifuged at 1,000 rpm for 5 min. After centrifugation, trysin was discarded and DMEM complete media was added. Cell viability was assessed by trypan blue dye exclusion

method using a haemocytometer. The viable cell number was calculated with the same formula as RAW 264.7.

Viable cell number (cell/ml) = number of counted cell in 16-large squares $\times 2 \times 10^4$

After calculation, cells were diluted to appropriate cell concentration and plated in tissue culture plates for further experiments.

3.3.2 Cell storage

Cell frozen media were prepared by adding 10% DMSO (v/v) to complete DMEM media. After collecting cells by centrifugation, cells were resuspended in 1 ml cold frozen media and stored in cryotubes. The frozen cells were immediately stored in -80˚C refrigerator overnight and moved for long term storage in liquid nitrogen the next day.

3.3.3 Thawing cell from stock

Frozen cells in cryogenic vials in liquid nitrogen were immediately thawed in 37˚C water bath. Cell suspensions were added to 9 ml serum-free media and centrifuged at 1000 rpm for 5 min. Freezing media in serum-free media was removed and DMEM complete media was added. Cells in complete media were plated in a tissue culture dish for experiments.

Figure 3.1 Plasmid maps of pmaxGFP used in this study. The pmaxGFP had green fluorescent protein (GFP) derived from *Pontelina sp* and the control of cytomegalovirus (CMV) promoter. (Amaxa news #3, 2000)

3.4 Plasmid preparation

3.4.1 Transformation of plasmid into *E.coli* competent cells by heat-shock technique

Competent *E. coli* DH5α from -80˚C was thawed on ice. Next, 50 µl of the competent cell was added to the microcentrifuge tube containing 2 µl of plasmid. The reaction was incubated on ice for 30 min. The reaction was heated in 42˚C water bath for 90 sec and quickly chilled to 4˚C for 2 min. After incubation, total volume of competent cells and plasmid were transferred to 2 ml LB broth (Appendix A) and incubated at room temperature for 1 hr in shaker incubator. After incubated, bacterial culture (50 µl) was spread on LB agar plate containing 30

µg/ml kanamycin. The plate was incubated at 37˚C for 24 hr. The colonies was observed and used for experiments.

3.4.2 Plasmid isolation

For pmaxGFP, an *E. coli* colony was picked in order to culture overnight in 2 ml LB broth containing 30 mg/ml kanamycin (Appendix) at 37˚C. 100 ml of LB medium were inoculated with 200 µl of starter culture at 37˚C for starter cultured. After that, the culture (100 ml) was sterilely poured to microcentrifuge tube, and cell was harvested by centrifugation at 6,000 g for 15 min. The supernatant was than discarded and plasmids were extracted using QIAGEN prep spin midiprep kit, according to manufacturer's instructions. The obtained plasmid was resuspended in sterile HPLC grade water and stored at -20˚C until use.

3.4.3 Quantitation of plasmid

Plasmid stored at -20˚C was diluted in deionized water at 50 fold dilution. The diluted plasmid was subjected to measure the optical density measurement at 260 and 280 nm using spectrophotometry. An OD_{260} of 1.0 corresponds to a concentration of 50 µg/ml plasmid DNA. Therefore, the concentration of plasmid was calculated in µg/ml by using the following equation.

Plasmid (µg/ml) = $OD_{260} \times 50 \times$ dilution factor

The purity of plasmid was evaluated from a ratio of OD_{260}/ OD_{280}. The ratio of appropriately purified plasmid was 1.8-2.0.

3.5 Preparation of chitosan-pmaxGFP nanoparticles (CNP-DNA complex)

Chitosan (1g, MW 30,000 Da, Seafresh Laboratory, Thailand) was dissolved in1% acetic acid (50 ml) with gentle heating. After getting a clear solution, the pH of the solution was adjusted to 5.5 with 1N sodium hydroxide. The solution was diluted to 0.02% of chitosan (w/v) [stock solution: 200 µg/ml] using 5 mM of sodium acetate buffer (pH 5.5) and the obtained solution was passed through a 0.22 µm filter. The pmaxGFP (stock solution: 200 µg/ml) used for nanoparticle formation was at the concentration 20 µg/ml in 25 mM sodium sulfate. Nanoparticles were prepared by a complex coacervation technique as previously described (Mao et al, 2001). Brifely, after heating to 55°C, equal volume of the chitosan and the pmaxGFP solutions were mixed at various N/P ratios (the ratios of moles of the amine groups of cationic polymers to those of the phosphate ones of DNA, Table 3.1) to produce a series of chitosan/DNA complexes of various N/P ratios. The mixture was immediately vortexed at maximum speed for 1 min. The obtained suspension was kept at 4^{o}C until being used for the transfection experiments without further modification.

N/P ratio	Final conc. Chitosan (µg/ml)	Final conc. DNA (µg/ml)	Volume of 200 ug/ml chitosan solution (µl)	5 mM sodium acetate buffer (µl)	Volume of 200 ug/ml pMAXGFP solution (µl)
12:1	100	20	100	0	100
6:1	50	20	50	50	100
3:1	25	20	25	75	100
0.6:1	12.5	20	12.5	87.5	100

Chitosan (µl) + 5 mM sodium acetate buffer (µl)

pMAXGFP (µl) + 25 mM NA_2SO_4 (µl)

↓ Incubate, 55 °C 5 min

Mix and vortex 1 min

stock pMAXGFP 200 µg/ml (µl)	25 mM NA_2SO_4 (µl)
40	60

Table 3.1 Prepare nanoparticles method by a complex coacervation technique at various N/P ratios.

3.6 Agarose gel electrophoresis

CNP – DNA complex was evaluated by agarose gel electrophoresis. The CNP-DNA complex were prepared at N/P ratios of 0.6, 3, 6, and 12 by varying the concentration of chitosans. The nanoparticles and the naked plasmid were loaded on to a 2% agarose gel (Appendix) in 0.5X TAE buffer, with the same amount of DNA. The samples were separate at 100V for 80 min and the gel was stained with ethidium bromide and photographed using a Gel documentation system (Biorad)

3.7 Cell transfection

293T cell line was harvested by trypsin/EDTA treatment and seeded, 24h prior to transfection, in 24-well plates at a density of 5×10^4 cells/well in 0.5 ml DMEM with 10% FBS. When cells reached the 50–70% confluency, CNP-DNA complex (50 µl) was added to the media at differrent N/P ratio. Fugene HD transfection reagent was used as the positive control according to the manufacturer's procedures. Following an incubation of 48h and 72h, cells were observed under an inverted fluorescent microscope and subjected to FACS analysis.

RAW 264.7 cell line was harvested by incubating cells in cold 1xPBS (Phosphate buffered saline) and seeded, 24h prior to transfection, on 24-well plates at a density of 50,000 cells/well in 0.5 ml DMEM with 10% FBS. Similar procedures as has been described for 293T cell line were repeated.

3.8 Determination of transfection efficiency

Transfection efficiency was analyzed by observation under fluorescent microscope and FACS analysis. For observation under an inverted fluorescence microscope (Olympus, Model: DP71- SET, Department of Microbiology, Chulalongkorn University), cells were observed in three random fileds and total cells in bright feild with GFP were counted. For FACS analysis, cells were resuspended and fix with 4% paraformaldehyde (Appendix) and subjected to FACS analysis (Faculty of Dentistry, Chulalongkorn University).

3.9 Transmission electron microscopy (TEM) and scanning electron microscopy (SEM)

To examine the morphology of the CNP-DNA complex, 20µl of nanoparticle suspension was placed on a copper grid, the excess liquid was removed with a piece of filter paper, and the grid was air dried. Samples were visualized using JEM-2100 transmission electron microscope at a 80kV – 100kV operation (Scientific and Technological Research Equipment Centre, Chulalongkorn University, Bangkok). For SEM analysis, 20µl of nanoparticle suspension was air dried and coated with gold at the voltage of ??? under vacuum. Samples were visualized using JSM-6400 scanning electron microscope (Scientific and Technological Research Equipment Centre, Chulalongkorn University, Bangkok).

3.10 Determination of particle size and zeta potential

Particle size and zeta potential of the freshly prepared CNP-DNA complex were determined using Dynamic Light Scattering (Nanosizer) (National Nanotechnology Center (NANOTEC), National Science and Technology Development Agency, Thailand).

3.11 Chitosan oxidation and conjugation to streptavidin hydrazide

One ml of the chitosan stock solution (1% chitosan in 1% acetic acid) was dissolved in 1 ml of 0.1 M sodium acetate buffer, pH 5.5 (Appendix). Then, 1 ml of cold 20 mM sodium *meta*-periodate solution (Appendix) was added to the chitosan solution (1 ml) and the mixture was mixed and left in the dark for 30 minutes at 4^{o}C. The obtained mixture was dialyzed against PBS using cellulose membrane with the MW. cut-off of 12-14 KDa to remove excess periodate and exchange the buffer to PBS buffer. Then, the mixture (1 ml) was added with 20 µl of streptavidin hydrazide (diluted $1/10^{4}$ times from stock of 1.66 mg/ml) and mixed for 2 hours at room temperature. The sample was dialyzed against water using cellulose membrane with MW. cut-off of 100 KDa. The dialyzed sample was then freeze-dried and the dry sample was subjected to nuclear magnetic resonance (NMR), fourier transform infrared spectroscopy (FT-IR) and mass spectrometry (MS) analysis.

3.12 Cytotoxicity test

Cytotoxicity test were performed at 72 h after transfection using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich). RAW 264.7 cells were seeded on 96-well plates at a density of 10,000 cells/well in 100 µl complete DMEM. After 24 h of incubation, chitosan, CNP-DNA complex and streptavidin CNP-DNA complex suspensions (50 µl) were then added at various concentrations. Cell viability was analyzed using MTT after a further 48 h by measuring absorbance at 570 nm. The viability of non-treated control cells was arbitrarily defined as 100%.

$$\text{The percentage of cell viability (\%)} = \frac{\text{OD of treated cells} \times 100}{\text{OD of control untreated cells}}$$

3.13 Transfection efficiency of streptavidin CNP-DNA complex in RAW 264.7 cell used F4/80 antibody

Streptavidin CNP-DNA complex was used to transfect RAW 264.7 cell line by adding a specific biotinyled antibody to streptavidin-chitosan. Streptavidin CNP-DNA complex were prepared by using the optimum conditions obtained as described above. Using specific biotinyled antibodies against specific markers on the cell surface of macrophage (anti-F4/80 Ab). Used 1 µg/ml and 2 µg/ml of F4/80 biotinyled antibody (stock solution 0.5 mg/ml) were added to streptavidin CNP-DNA complex and incubated 30 min at RT. RAW 264.7 cell line were grown in complete DMEM at 37 ◦C. Cells were seeded into 24-well plates at a density of 5×10^4 cells per well and were incubated for 24h to achieve approximately 60–80% confluency. Streptavidin CNP-DNA complex antibodies were added to each well and then incubated with RAW 264.7 cells in a humidified atmosphere containing 5% CO_2 at 37 ^oC. Following an incubation at 48h and 72h, cells were observed under an inversted fluorescent microscope. Fugene HD transfect reagent was used as the positive control according to the manufacturer's procedures and macrophage cell line by cell skin cancer with no F4/80 antigen negative control cells.

CHAPTER IV

RESULTS

4.1 Characterization of chitosan - pmaxGFP nanoparticles

Based on the preparation methods described by Mao et. al., we synthesized the chitosan - pmaxGFP nanoparticles (CNP-DNA complex) through the complex coacervation of the chitosan and pmaxGFP at various N/P ratios. The particle size and zeta potential of the CNP-DNA complex are shown in Figure 1. CNP-DNA complex prepared with chitosan of MW 30 kDa, showed a particle size as follow:

N/P ratio	Particle size (nm)	PdI	Zeta potential (mV)
12:1	432.7	0.408	8.47
6:1	348.6	0.304	11.6
3:1	236	0.571	5.67
0.6:1	293.1	0.547	9.26

Table 4.1 Particle size and zeta potential of nanoparticles freshly synthesized (pH 5.5) with chitosan and pMAXGFP at various N/P ratios as measured by light scattering.

As measured by light scattering technique (scattering angle, $\theta = 173$). The CNP-DNA complex in sodium acetate buffer, sodium sulfate and distilled water. SEM and TEM revealed the spherical morphology of the obtained complex (Figure 2) with the size of the dry CNPs as follow:

N/P ratio	Paticle size (nm) on SEM	Paticle size (nm) on TEM
6:1	125	60

Table 4.2 Particle size of nanoparticles with chitosan and pMAXGFP at N/P ratios 6:1 by SEM and TEM.

The difference between the size from DLS and SEM/TEM was probably from the swelling of the particles in water.

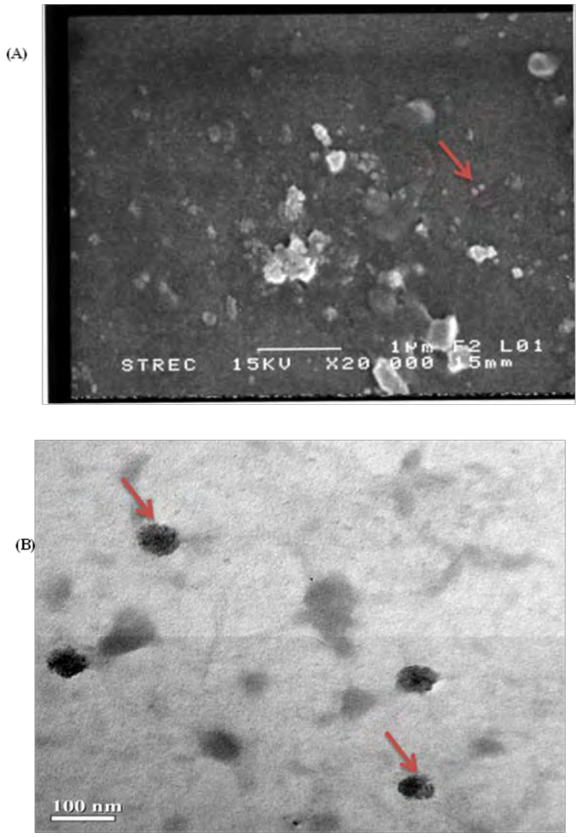


Figure 4.1 (A) SEM images of chitosan-pMAXGFP nanoparticles at N/P ratio of 6:1, scale bar = 1 μm (red arrow points); (B) TEM images of chitosan-pMAXGFP nanoparticles at N/P ratio of 6:1, scale bar = 100 nm. (red arrow points)

4.2 Complexation of plasmid pMAXGFP DNA with CNP-DNA complex

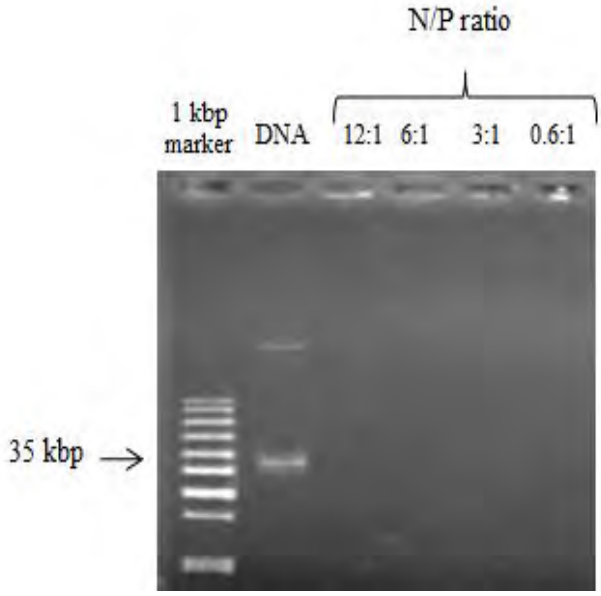
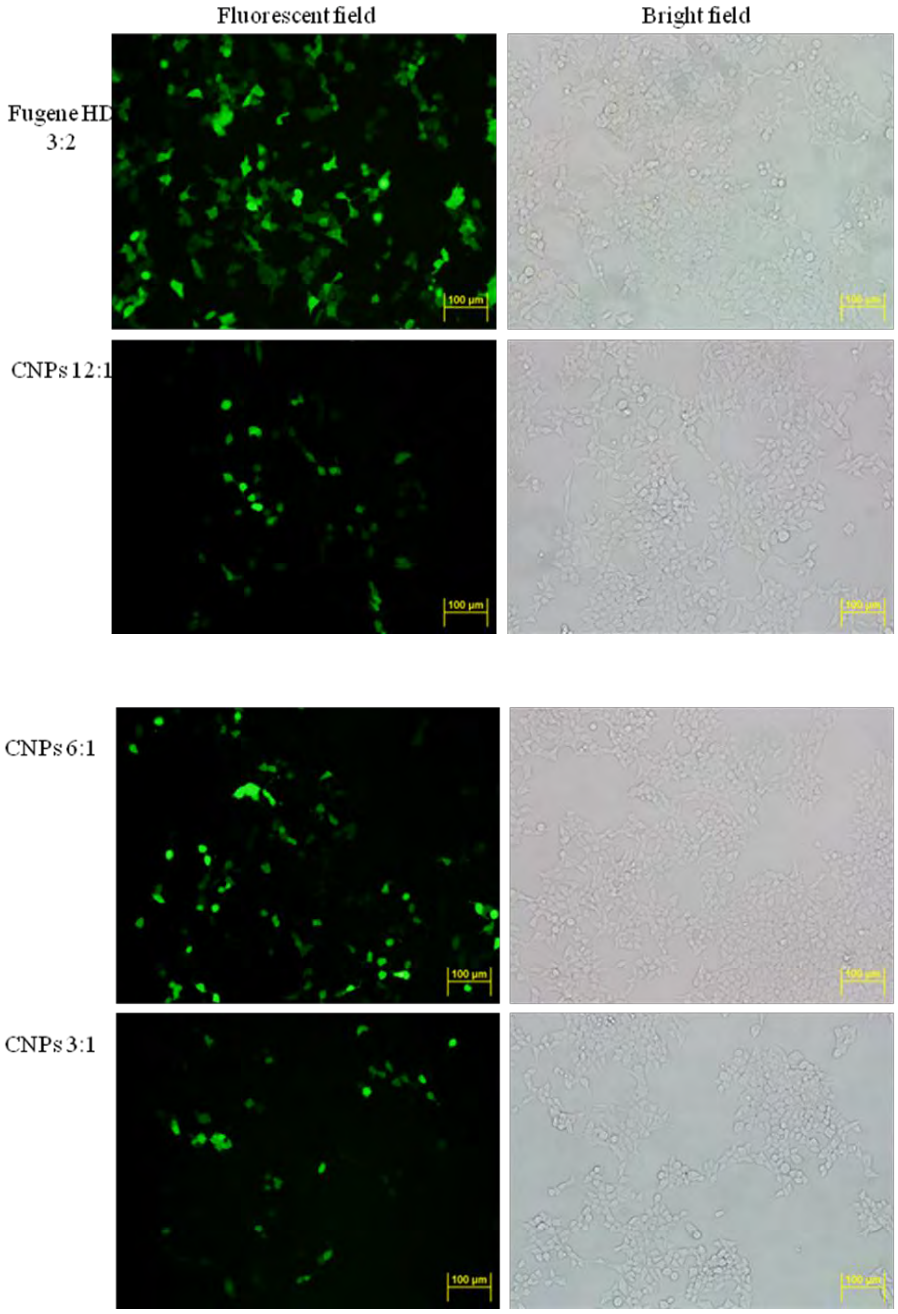


Figure 4.2 Complexation of pMAXGFP plasmid was indirectly examined by agarose gel electrophoresis. CNP-DNA complex at various N/P ratios and control naked plasmid pMAXGFP DNA were analyzed on 2% agarose gel. DNA was visualized by ethidium bromide staining.

To indirectly examine the complexation of plasmid pMAXGFP DNA with CNP, CNP-DNA complex pMAXGFP plasmid complexes prepared at various N/P ratio were subjected to agarose gel eletrophoresis (Figure 3). Naked plasmid pMAXGFP was clearly seen on this gel, whereas the complexed plasmid DNA was not visible in all N/P ratios tested, suggesting that CNP could complex with plasmid DNA.

4.3 Transfection efficiency of CNP-DNA complex



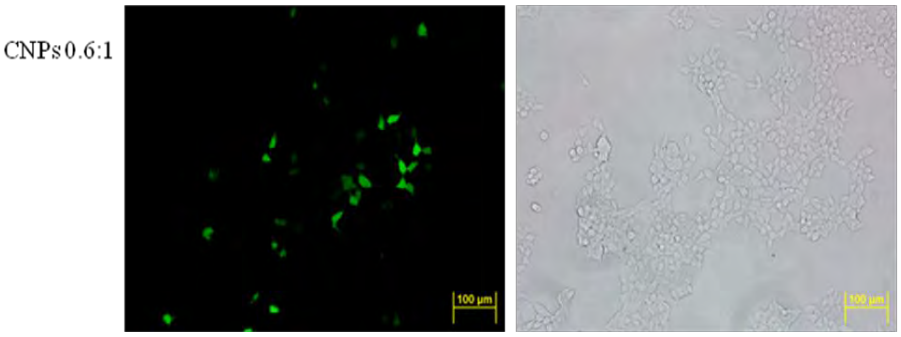


Figure 4.3 Transfection efficiency of CNP-DNA complex in 293T cell. Cells were analyzed 72 h after transfection by fluorescent microscope (left) and bright field microscope (right). FuGene HD was used as positive control.

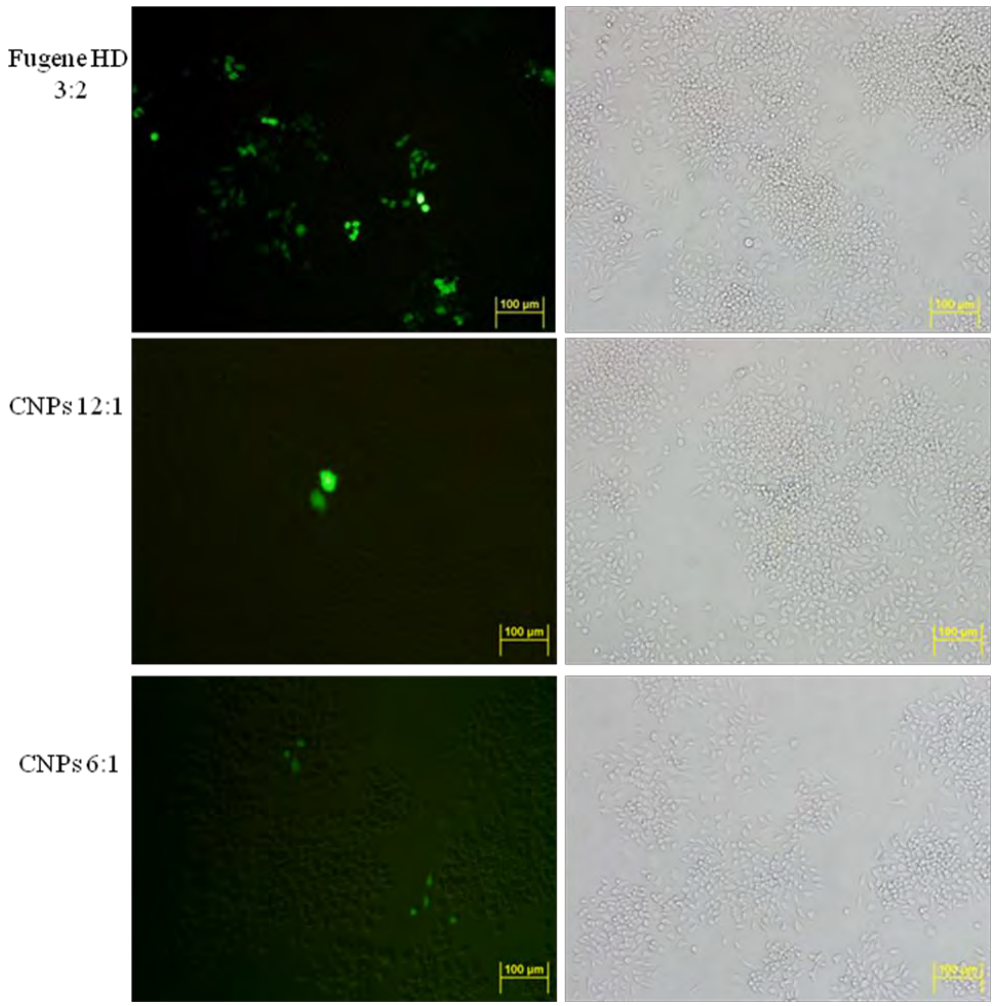
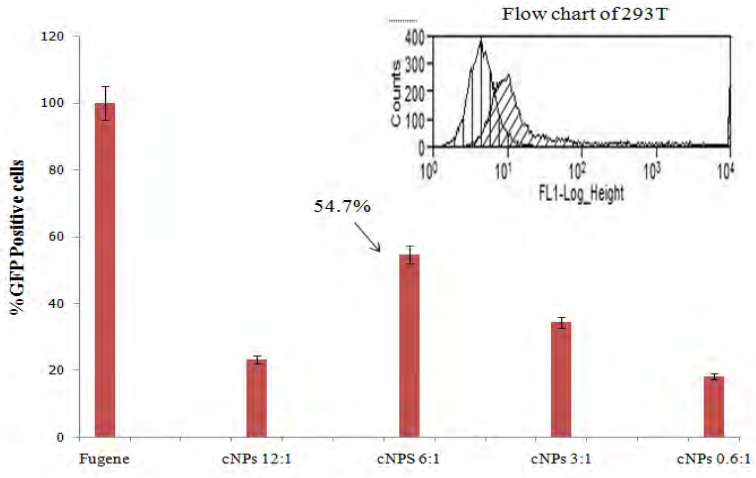


Figure 4.4 Transfection efficiency of CNP-DNA complex in RAW 264.7 cell line. Cells were analyzed 72 h after transfection by fluorescent microscope (left) and bright field microscope (right). FuGene HD was used as positive control.

When transfection efficiency was evaluated by varying N/P ratios of CNP-DNA complex and observing for GFP positive cells. The results showed that all CNP-DNA complex mediated expression of green fluorescent protein in 293T cells with varying degrees (Fig.4 and 6) The transfection efficiencies of CNP-DNA complex prepared at N/P ratio of 12:1, 6:1, 3:1 and 0.6:1 were 23.2%,54.7%, 34.4%, and 18.3%, respectively. Therefore, CNP-DNA complex at N/P ratio

of 6:1 which gave the highest transfection efficiency was chosen for further study (Fig.6.). As for Raw 264.7 cells, CNP-DNA complex and commercial transfection reagents showed only modest transfection efficiency (Figure 5 and 6). The highest transfection efficiency of the CNP-DNA complex was achived at 3.3% using CNP-DNA complex with N/P ratio of 6:1 (Figure 6). Taken together, the CNP-DNA complex with N/P ratios of 6:1 showed the highest transfection effcicency both in 293T cells and RAW 264.7 cells. Previous studies have demonstrated that the gene delivery potential of chitosan in mammalian cells depend on several factors such as cell type, pH of transfection medium, molecular weight of chitosan and plasmid dosage (Mao et al., 2001, Sato et al., 2001 and Ishii et al., 2001) In addition, percentage of transfection efficiency in HEK293 cells transfected *in vitro* using complexes made with chitosans was measured. Cells were incubated for 48 h in 24-well culture plates with complexes at different N/P ratios of 12:1 and 6:1 with fixed 2.5 mg of pMAXGFP plasmid per well and fixed media pH of 7.1. The N/P ratio of 6:1 resulted in undetectable transfected cells by flow cytometry. It was reported that the CNP-DNA complex with the N/P ratio of 5:1 resulted in 8% of transfection in macrophages. (Lavertu et al;. 2006)



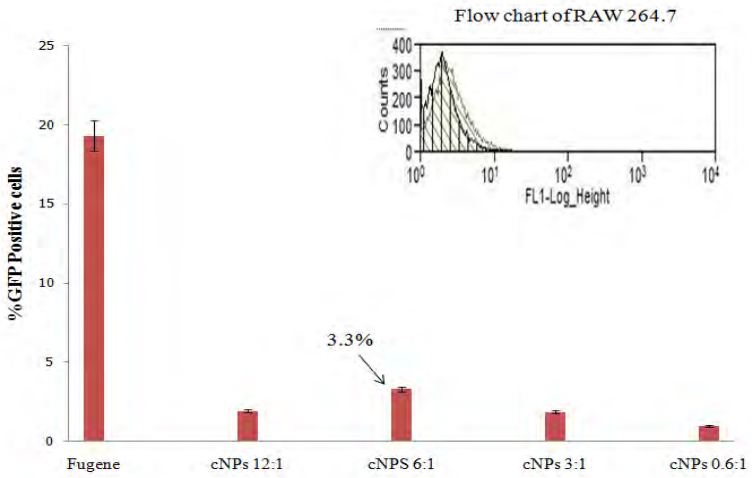
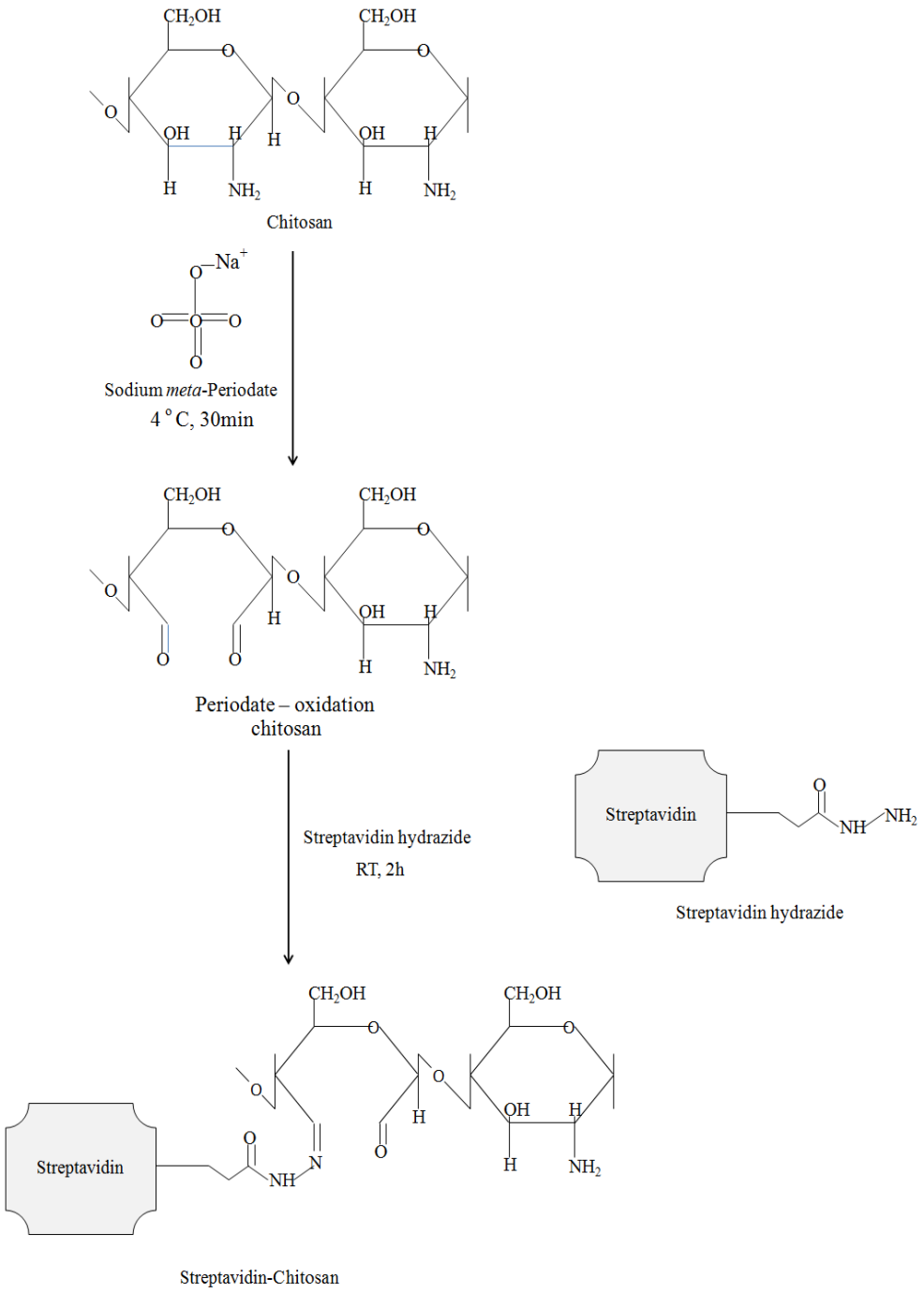


Figure 4.5 GFP expressed in a 293T cell line (top) and Raw 264.7 cell line (bottom) transfected with CNP-DNA complex at difference N/P ratio of FuGene HD was measured by FACS analysis. Small histograms in the upper right conner represented the histogram obtained from FACS analysis.

4.4 Conjugating and characterization of streptavidin hydrazide to chitosan



Scheme 4.1 Synthesis of chitosan, periodate-oxidation chitosan and streptavidin-chitosan.

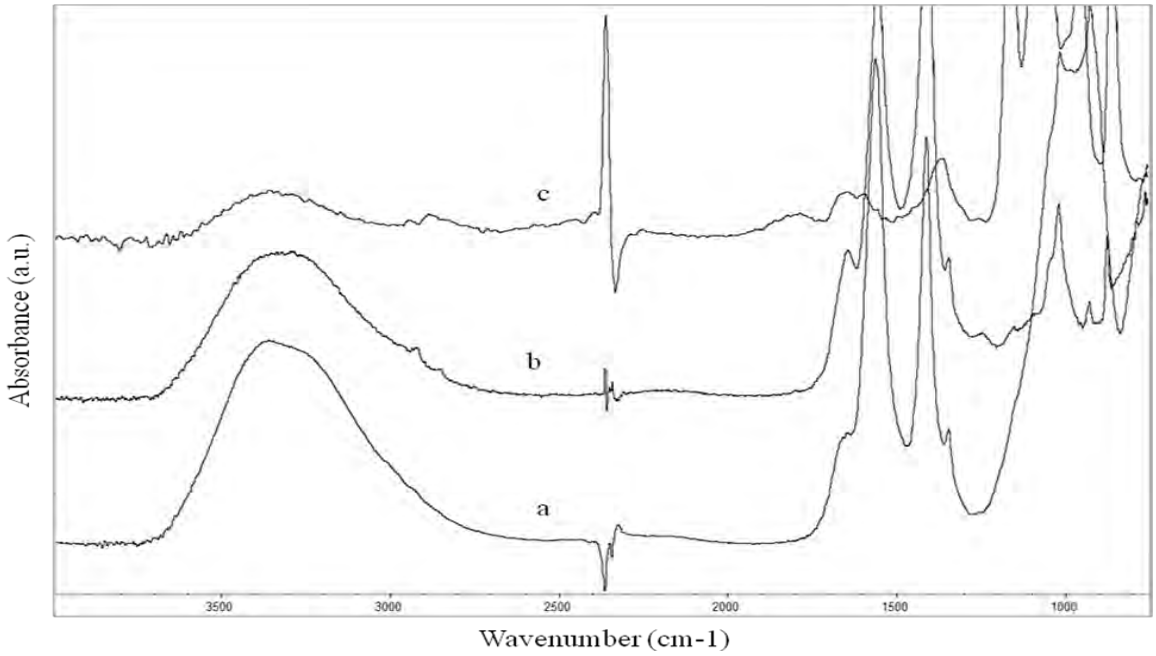


Figure 4.6 Representative FTIR spectrometer of chitosan (a), chitosan oxidation with sodium *meta*-periodate (b) and streptavidin-chitosan (c).

The streptavidin-chitosan was synthesized by the reaction between streptavidin hydrazide and chitosan. First the chitosan was activated by oxidation with sodium *meta*–periodate. The chitosan-streptavidin was then obtained from the reaction between the oxidized chitosan and the streptavidin hydrazide (Scheme 4.1). The product was confirmed by FTIR (Figure 7). The Figure 7b showed the characteristics of the oxidized chitosan with aldehyde group (C=O stretching at 1643 wavenumber (cm-1) and C-H stretching at 2925 wavenumber (cm-1). The disappeared of peak at 2894 wavenumber (cm^{-1}) (aldehyde) indicated the reaction of aldehyde with amino groups of the streptavidin protein. The conjugated products also showed prominent peaks at 2900-2883 wavenumber (cm-1) indicating more C-H stretching from streptavidin proteins. Streptavidin-chitosan (Figure 7c) showed carbonyl stretching at 1778 wavenumber (cm-1) from the protein with reduction of carbonyl of aldehyde at 1651 wavenumber (cm-1). It was likely that the amine

groups ($-NH_2$) of the streptavidin protein reacted with the aldehyde groups in the periodate chitosan.

The composition of the synthesized copolymer was analyzed by ^{1}H-NMR (Figure 8). The chemical shifts are referenced to the residual solvent peak of monodeuterated water (HDO/1% acetic acid, $\delta = 4.75$ ppm). The streptavidin-chitosan was characterized by ^{1}H-NMR spectroscopy as a solution of 10 mg in 0.5 mL D_2O/1% acetic acid. Chitosan normally (Figure 8a) showed resonances at $\delta = 1.859$ ppm for H of acetyl groups, $\delta = 2.593$ ppm for H2 of glucosamine, GlcN, $\delta = 3.776 – 3.191$ ppm for N-acetylglucosamine, GlcNAc, H3, H4, H5 and H6 of GlcNAc and GlcN and $\delta = 4.361$ for H1 of GlcNAc and GlcN. Figure 8b showed all the mentioned resonances with additional peaks at $\delta = 8.080 – 7.701$ ppm for aromatic amino acid residues of streptavidin protein.

Complexation of the streptavidin-chitosan was confirmed by mass spectrometry (MALDI-TOF). The result showed peak at molecular weight of 10920 – 10930 m/z (Figure 9) which corresponded to the conjugation of 2 chitosan (Mw of 30,000 Da each) and 1 streptavidin molecule (Mw of 52,800 Da). Therefore, it was concluded that the streptavidin-chitosan complex was successfully synthesized.

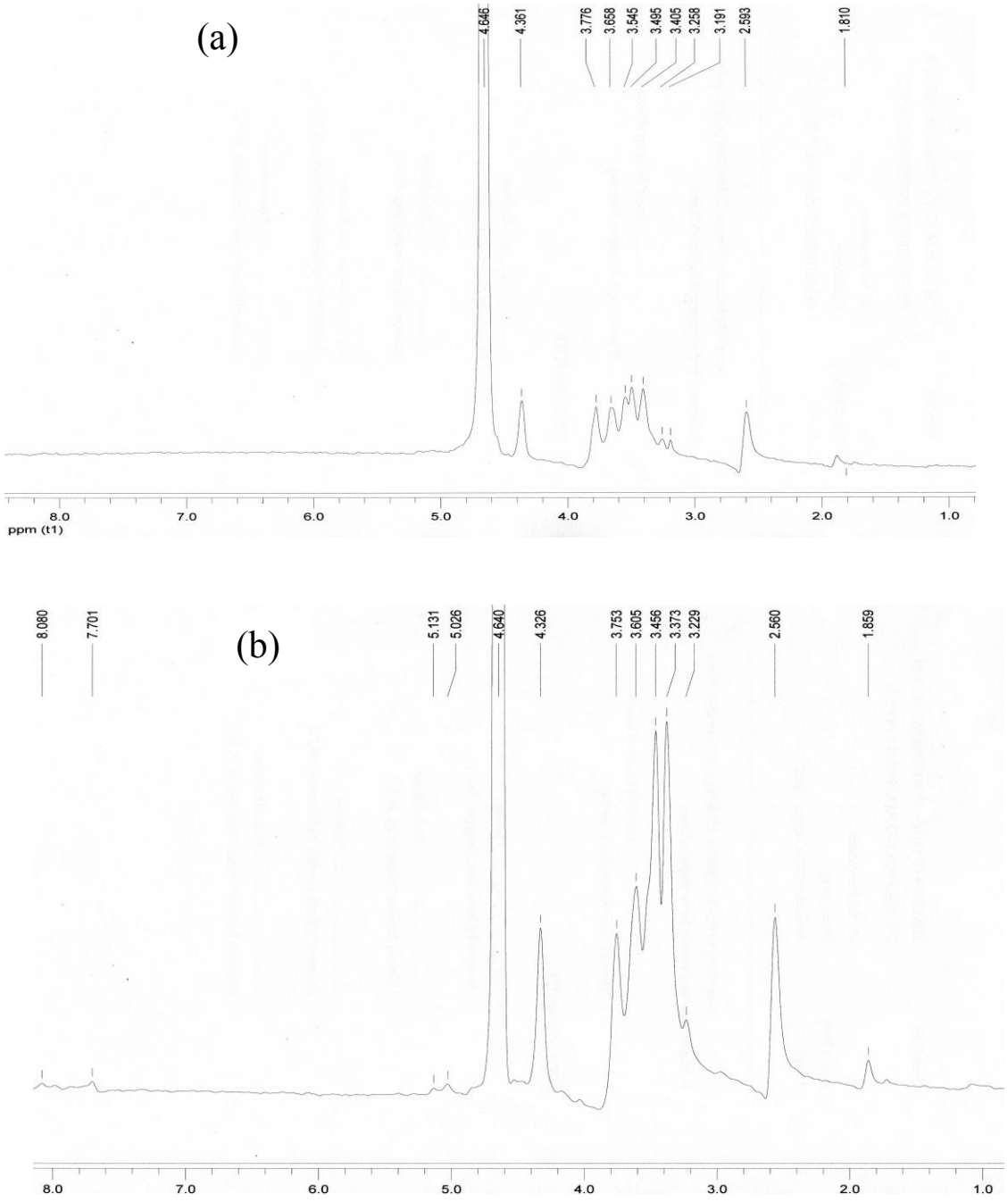


Figure 4.7 Representative ^{1}H-NMR spectra of chitosan (a) and streptavidin-chitosan (b). streptavidin-chitosan indicated the proton peak of aromatic amino acid residue of streptavidin (δ = 8.080 –7.701 ppm).

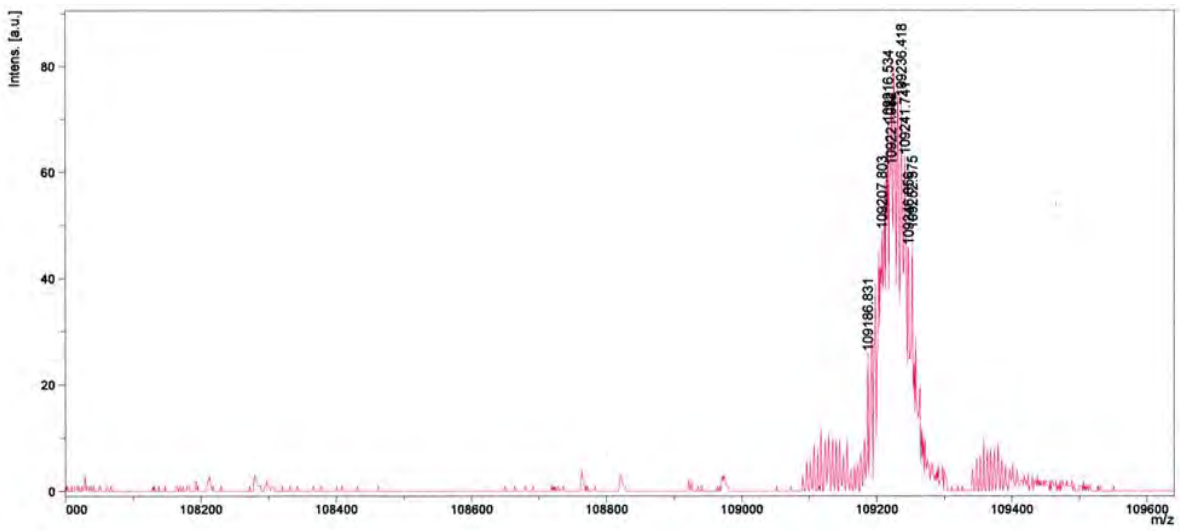


Figure 4.8 Representative Mass spectra of streptavidin-chitosan. The peak indicated molecular weight of streptavidin-chitosan (10920 – 10930 m/z).

Streptavidin-chitosan (1 mg/ml) and pMAXGFP plasmid (200 ug/ml) was a complex coacervated at the weight ratio between chitosan: DNA of 5:1. The streptavidin CNP-DNA complex possessed hydrodynamic size of 290 nm (PDI = 0.322) as measured by light scattering technique (scattering angle, θ = 173) with a surface charge of -31 mV. Streptavidin CNP-DNA complex dry particle size was the range of 134 nm and 350 nm as measured by TEM and SEM (Figure 10, Figure 11(B)). The difference between the dry and water suspended particles was probably from the swelling of the chitosan in water. The streptavidin CNP-DNA complex were subjected to agarose gel electrophoresis and the resulted clearly revealed that DNA was complexed tightly with the chitosan particles (Figure 13). Streptavidin CNP-DNA complex was more stable than CNP-DNA complex and did not aggregate easily (agreed with the highly negative zeta potential). It was speculated that interactions between chitosan and DNA were ion–ion interactions (Tang and Szoka., 1997).

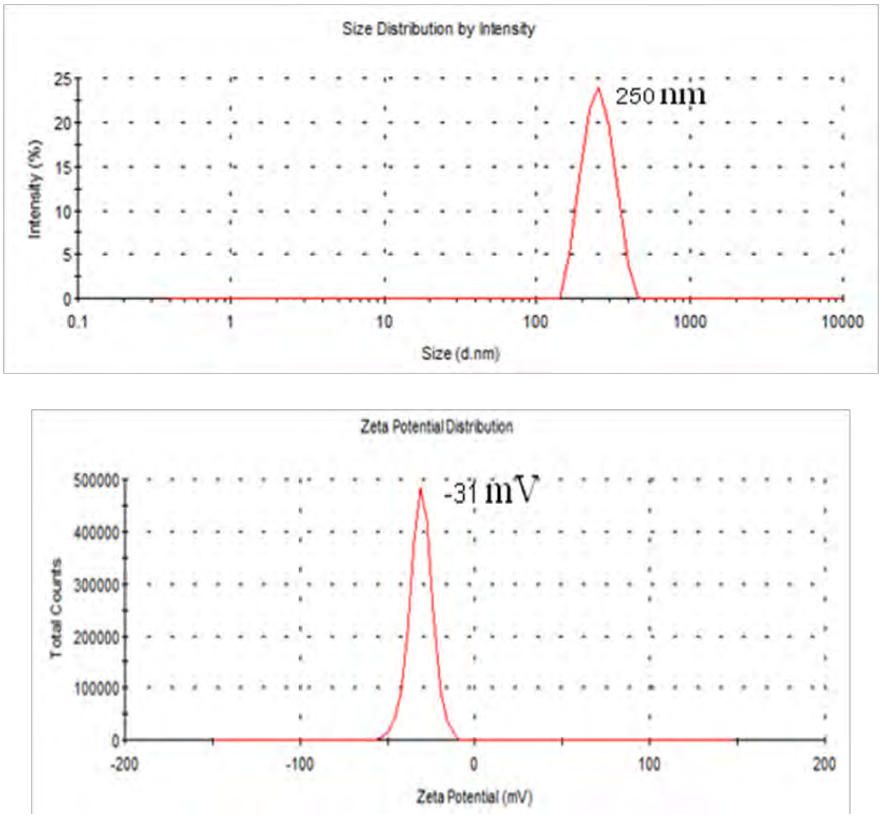


Figure 4.9 Particle sizes and zeta potentials of streptavidin CNP-DNA complex at concentration of 5:1 (streptavidin-chitosan:DNA) by Nanosizer.

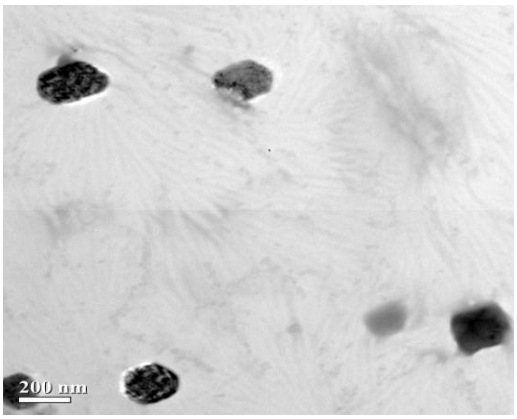


Figure 4.10 Representative TEM images of streptavidin CNP-DNA complex at concentration (w/w = chitosan /DNA) of 5:1; scale bar = 200 nm.

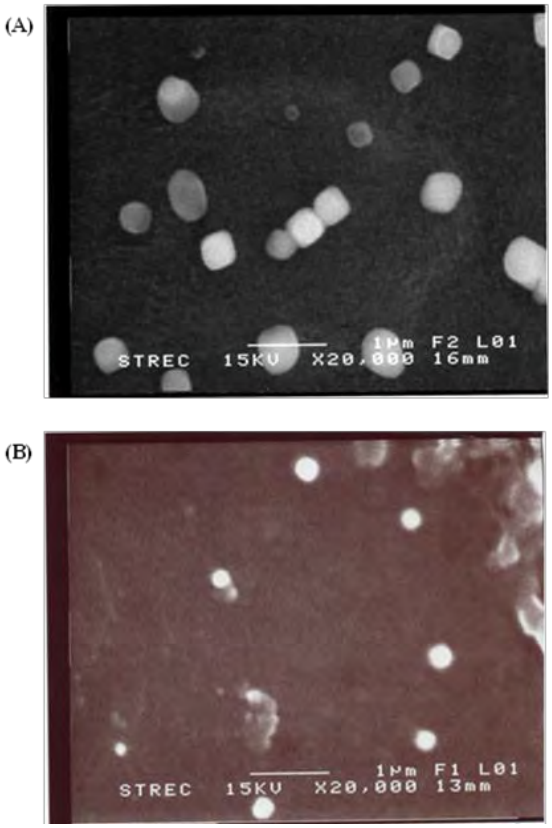


Figure 4.11 (A) SEM images of streptavidin-chitosan, scale bar = 1 µm; (B) SEM images of streptavidin CNP-DNA complex at [chitosan:DNA] 5:1, scale bar = 1 µm.

4.5 Cytotoxicity of chitosan, CNP-DNA complex and Streptavidin CNP-DNA complex

Polycations are considered to be cytotoxic because cationic macromolecules could interact with cell membranes, extracellular matrix proteins and blood components leading to side effect (Kircheis et al., 2001). The cytotoxicity of polycation was greatly influenced by polymer structures (Fischer et al., 2003). In order to investigate the cytotoxicity of chitosan, CNP-DNA complex and Streptavidin CNP-DNA complex on RAW 264.7 cell line, the MTT assay was used. As shown in Figure 12, chitosan and CNP-DNA complex 6:1 showed very low toxicity at tested concentrations when compared with streptavidin CNP-DNA complex [chitosan:DNA] 5:1. Although chitosan has been reported to have a low toxicity, reviewed by Illum, a few investigators, including us, have observed a dose-dependent toxicity of chitosan at high doses *in vitro*. The CNP-DNA complex involved a dose-dependent toxicity after transfected into cell line.

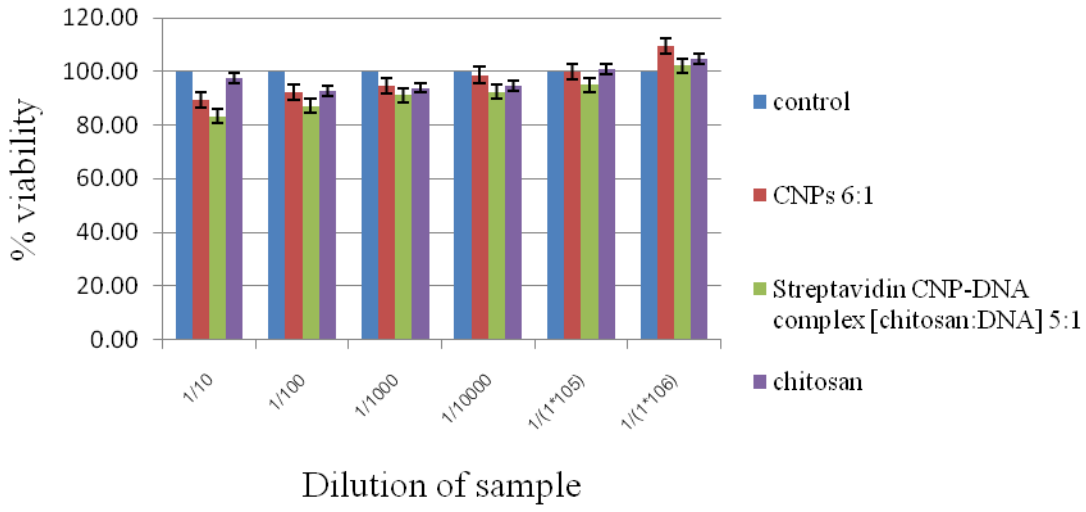


Figure 4.12 Cytotoxicity of empty RAW 264.7, chitosan, CNP-DNA complex 6:1 and streptavidin CNP-DNA complex [chitosan:DNA] 5:1 by MTT assay.

4.6 Transfection efficiency of streptavidin CNP-DNA complex to the macrophage cell line-specific

Streptavidin-chitosan and pMAXGFP plasmid was prepared by a complex coacervation technique as previously described using the N/P ratio of 6:1. But when streptavidin-chitosan and pMAXGFP plasmid was prepared a complex, which resulted that it can not form complex. Therefore, using the experimental preparation of chitosan–DNA nanoparticles (Fei Li et al;. 2009) by changing the incubate time of 5 minutes to 20 minutes. Resulted that show Fig. 13, (1) streptavidin CNP-DNA complex [chitosan:DNA] 5:1 prepared incubated at 55 ° C 20 min; (2) streptavidin CNP-DNA complex [chitosan:DNA] 5:1 prepared incubated at 55 ° C 5 min; (3) streptavidin CNP-DNA complex [chitosan:DNA] 0.5:1 prepared incubated at 55 ° C 20 min and (4) streptavidin CNP-DNA complex [chitosan:DNA] 0.5:1 prepared incubated at 55 ° C 5 min. Sample (2), sample (3) and sample (4) showed the retarded DNA migration (Fig. 13), while sample (1) showed stronger condensation ability with retarded DNA migration (Fig. 13).

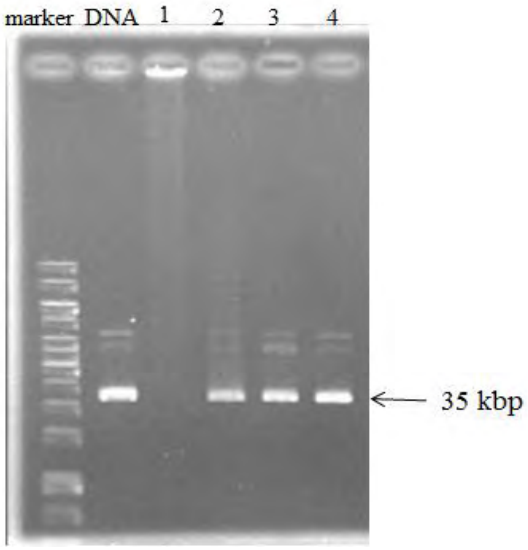


Figure 4.13 Agarose gel electrophoresis of streptavidin CNP-DNA complex. Lane 1: 1 kb marker; Lane 2: naked DNA control; Lanes 3 – 6: streptavidin CNP-DNA complex prepared incubated at 55 ° C using different time by a complex coacervation technique, respectively.

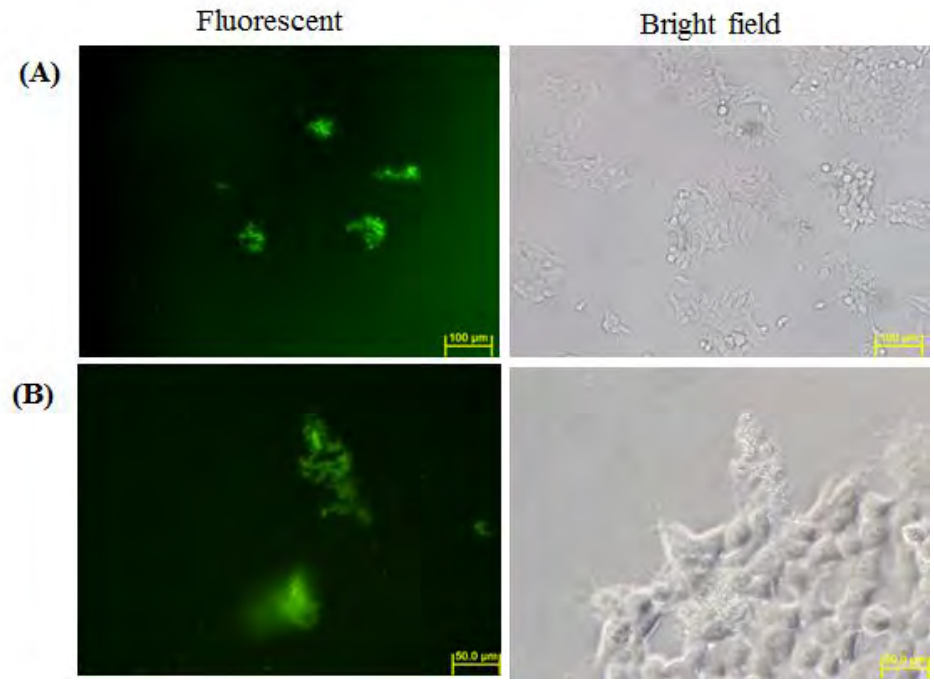


Figure 4.14 Streptavidin CNP-DNA complex [chitosan:DNA] 5:1 were transfected in 293T cell line. Cells were analyzed 72 h after transfection by fluorescent microscope and bright field microscope at magnification 20X (A) and 40X (B).

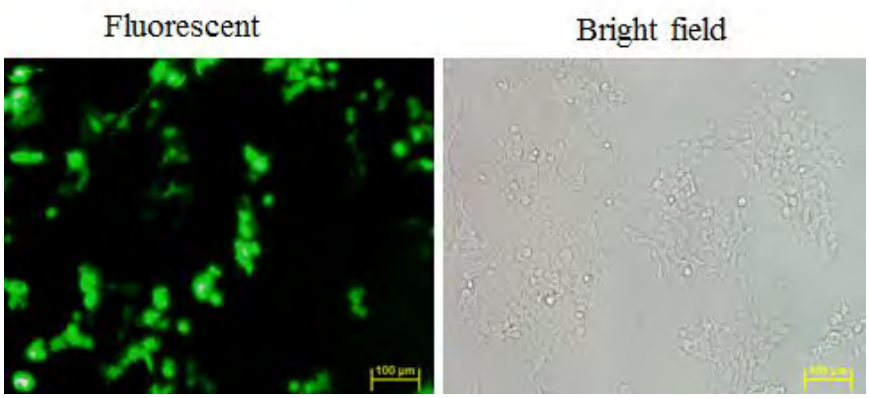


Figure 4.15 Test plasmid DNA at temperature 55 ° C incubation; 20 minutes. Fugene HD were transfected in 293T cell line. Cells were analyzed 72 h after transfection by fluorescent microscope (left) and bright field microscope (right).

When transfection efficiency was evaluated by streptavidin CNP-DNA complex [chitosan:DNA] 5:1 and observing for GFP positive cells. The results showed that streptavidin CNP-DNA complex [chitosan:DNA] 5:1 mediated expression of green fluorescent protein in 293T cells at 72 h (Fig. 14) after modified chitosan with sodium *meta*-periodate and streptavidin hydrazide. Figure.15 was showed test plasmid DNA at temperature 55 ° C incubation; 20 minutes by using fugene HD were transfected in 293T cell line at 72 h**.** This result implies that incubate DNA at 55 ° C did not break DNA plasmid to be transfection into cells.

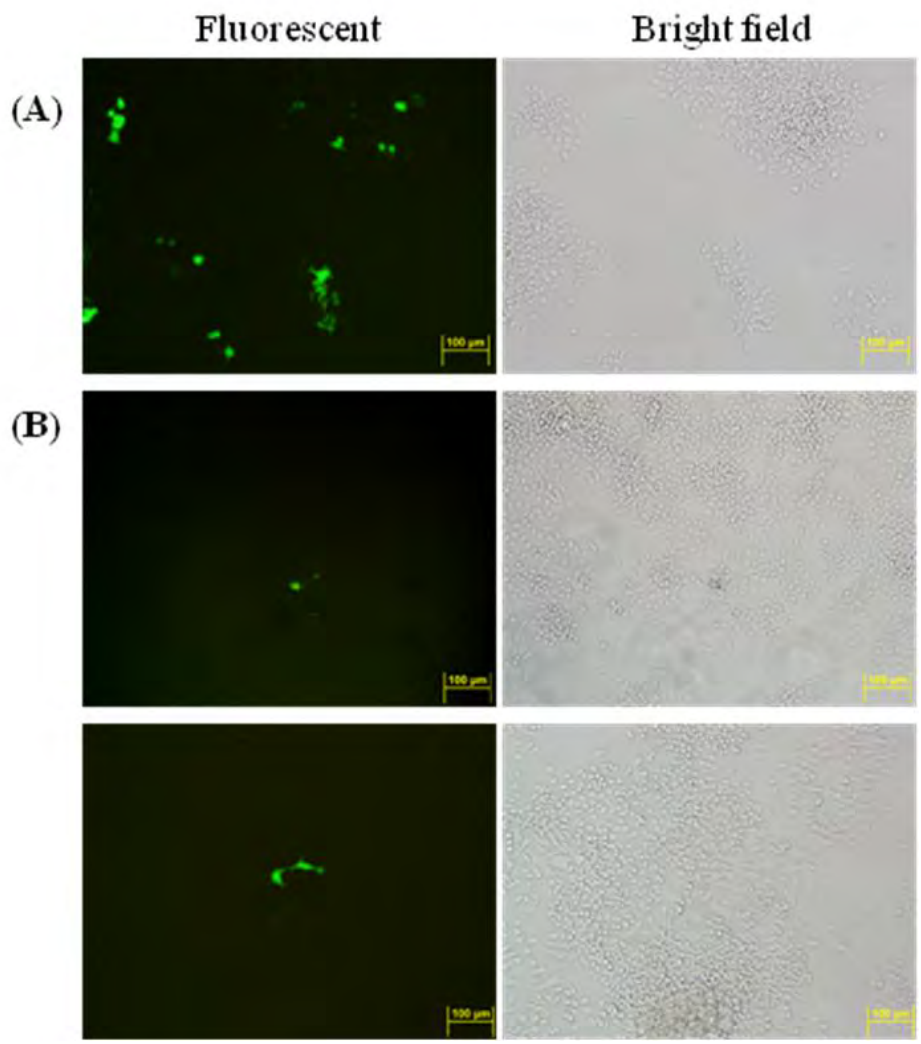


Figure 4.16 Streptavidin CNP-DNA complex [chitosan:DNA] 5:1 binding to F4/80 biotin (B) were transfected in RAW264.7 cell line. Cells were analyzed 72 h after transfection by fluorescent microscope and bright field microscope. (A) Fugene HD was positive control.

When transfection efficiency was evaluated by streptavidin CNP-DNA complex [chitosan:DNA] 5:1 binding to F4/80 biotin (1 µg and 2 µg) and observing for GFP positive cells. The results showed that can be expression a few of green fluorescent protein in RAW 264.7 cells (Fig. 16 (B)). That were not a difference with used CNP-DNA complex transfection in RAW 264.7. gene delivery. Possibly, streptavidin CNP-DNA complex has a low transfected because the

transfection efficiency may depend on several factors, such as the chemical structure of polycations, cell type, nanoparticle size and composition, interactions with cells (Sania et al., 2006). One of the primary causes of poor gene delivery is the inefficient release from endosomes into the cytoplasm (Kim et al., 2005) and negative charges of CNP-DNA complex involved endocytosis. To improve transfection efficiency, several derivatives of chitosan have been designed, based on reactions with free amino groups. Park et al. prepared modified galactosylated chitosan (GC)/DNA complexes, and these systems could efficiently transfect liver cells, expressing asialoglycoprotein receptors, which specifically recognize the galactose ligands on chitosan, although the transfection was still low.

CHAPTER V

CONCLUSIONS

1. Here in this work we have successfully prepared a new streptavidin CNP-DNA complex

2. The chitosan and CNP-DNA complex showed very low toxicity when compared with streptavidin CNP-DNA complex when tested with RAW264.7 cell line.

3. The streptavidin CNP-DNA complex could mediate expression of green fluorescent protein in 293T cells at 72 h. However, the complex failed to mediate the expression of green fluorescent protein in RAW 284.7 cells at 72 h. Nevertheless the streptavidin CNP-DNA complex which was bound to F4/80 biotin, could mediate the expression of green fluorescent protein in RAW 264.7 cells at a noticeable level.

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APPENDIX

APPENDIX

1. Complete DMEM 100 ml

DMEM	90%
FBS	10%
Penicillin	100 U/ml
Streptomycin	0.4 mg/ml
Sodium pyruvate	1%
HEPES	1%

2. Freezing media 10 ml

CompleteDMEM	90%
DMSO	10%

3. Penicillin and streptomycin

Streptomycin was prepared at final concentration 50 mg/ml, and penicillin was prepared at final concentration 10^6 U/ml by diluting in strile deionized water. The solutions were filtered by using 0.22 µm syringe filter and then aliquoted and kept at -20 ^{o}C.

4. FBS inactivation

Before using FBS, FBS must be inactivated at 56˚C for 30 minutes using water bath.

5. 50×TAE buffer for agarose gel electrophoresis 200 ml

Trisma base	48.4 g
Glacial acetic acid	11.42 ml
0.5 M EDTA	20 ml

Adjusted pH to 8.0 and volume to 200 ml using deionized water. Autoclaved at 121˚C and pressure 15 psi for 15 minutes

5.1 2% agarose gel preparation

Agarose gel	2%
1×TAE	20 ml

5.2 running buffer for agarose gel electrophoresis

50×TAE was diluted to final concentration 0.5× in 400 ml of deionized water.

6. Lauria-Bertani (LB) broth and agar plate 1000 ml

6.1 LB broth preparation

Bacto tryptone	10 g
Yeast extracts	5 g
NaCl	10 g

Adjusted pH to 7.4 and volume to 1000 ml using deionized water

6.2 LB agar preparation

After LB broth preparation, 1.5% of agar was added to the broth. Next, the LB broth and agar were autoclaved at 121˚C and pressure 15 psi for 15 minutes.

6.3 LB agar containing 30 µg/ml kanamycin

After sterilization, the LB agar was warmed to approximately 50˚C using water bath. Next, 30 µg/ml kanamycin was added to the warmed agar, and the agar was mixed well and poured plate.

7. Bacterial glycerol stock

An inoculum preparation, a bacterial colony on agar plate was picked and cultured in 2 ml of LB broth overnight. Then, bacterial culture (250 µl) was taken to 25 ml of LB broth overnight. The bacterial culture (400 µl) was transferred to a new microcentrifuge tube, and glycerol was added to 20% final concentration. The aliquots were kept at –80˚C. To measure the CFU of stock culture, the aliquot was diluted and determined the CFU by spread plate.

8. 4% Paraformaldehyde

Paraformaldehyde (4g) was dissolved in 100 ml of PBS. Then, 1 N NaOH was added for 2 – 3 drops. The solution was heated at 65°C in a chemical hood. Then, the solution was cooled to room temperature and, the pH was adjusted to 7.4.

9. 1% Acetic acid

1% acetic acid would be prepared by adding 10 mL of glacial acetic acid to a 1000 mL flask half filled with water; mixing, then diluting to 1000 mL.

10. 5 mM of sodium acetate buffer (pH 5.5)

Sodium acetate	0.41 g
Glacial acetic acid	300 µl
deionized water	1000 µl

Mix the two solution and check pH by 1M NaOH or 1M glacial acetic acid. Autoclave at 121 ^{o} C and pressure 15 psi for 15 min.

11. 1xPBS pH 7.4

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g
Deionized water	1000 ml

Autoclave at 121 ^{o} C and pressure 15 psi for 15 min.

12. 25 mM sodium sulfate

Sodium sulfate	5.33 g
Deionized water	1000 ml

Autoclave at 121 ^{o} C and pressure 15 psi for 15 min.

13. 20 mM Sodium *meta*-periodate 1 ml.

Sodium *meta*-periodate	4.3 mg
0.1 M sodium acetate buffer,pH 5.5	1 ml.

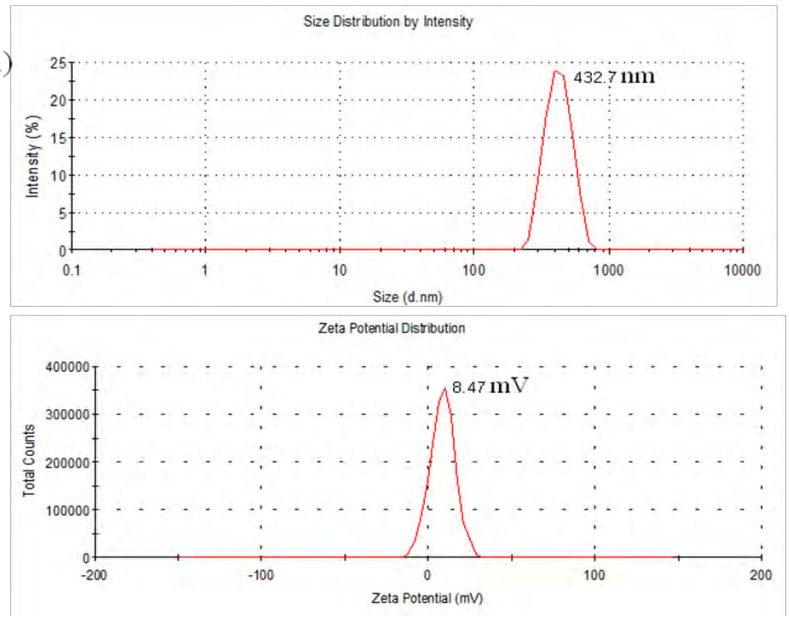
Keep solution on ice and protect it from light.

14. 0.1 M sodium acetate buffer, pH 5.5 (oxidation buffer)

Sodium acetate	0.82 g
Glacial acetic acid	60 µl
deionized water	100 µl

Mix the two solution and check pH by 1M NaOH or 1M glacial acetic acid. Autoclave at 121 ^{o} C and pressure 15 psi for 15 min.

(a)


Size Distribution by Intensity
Intensity (%)
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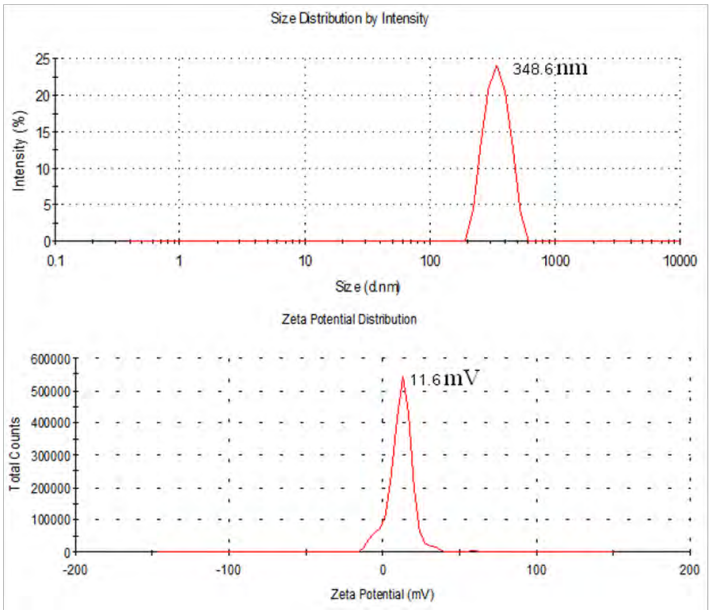
20
15
10
5
0
0.1
1
10
100
1000
10000
Size (d.nm)
432.7 nm
Zeta Potential Distribution
Total Counts
400000
300000
200000
100000
0
-200
-100
0
100
200
Zeta Potential (mV)
8.47 mV

(b)


Size Distribution by Intensity
Intensity (%)
25
20
15
10
5
0
0.1
1
10
100
1000
10000
Size (d.nm)
348.6 nm
Zeta Potential Distribution
Total Counts
600000
500000
400000
300000
200000
100000
0
-200
-100
0
100
200
Zeta Potential (mV)
11.6 mV

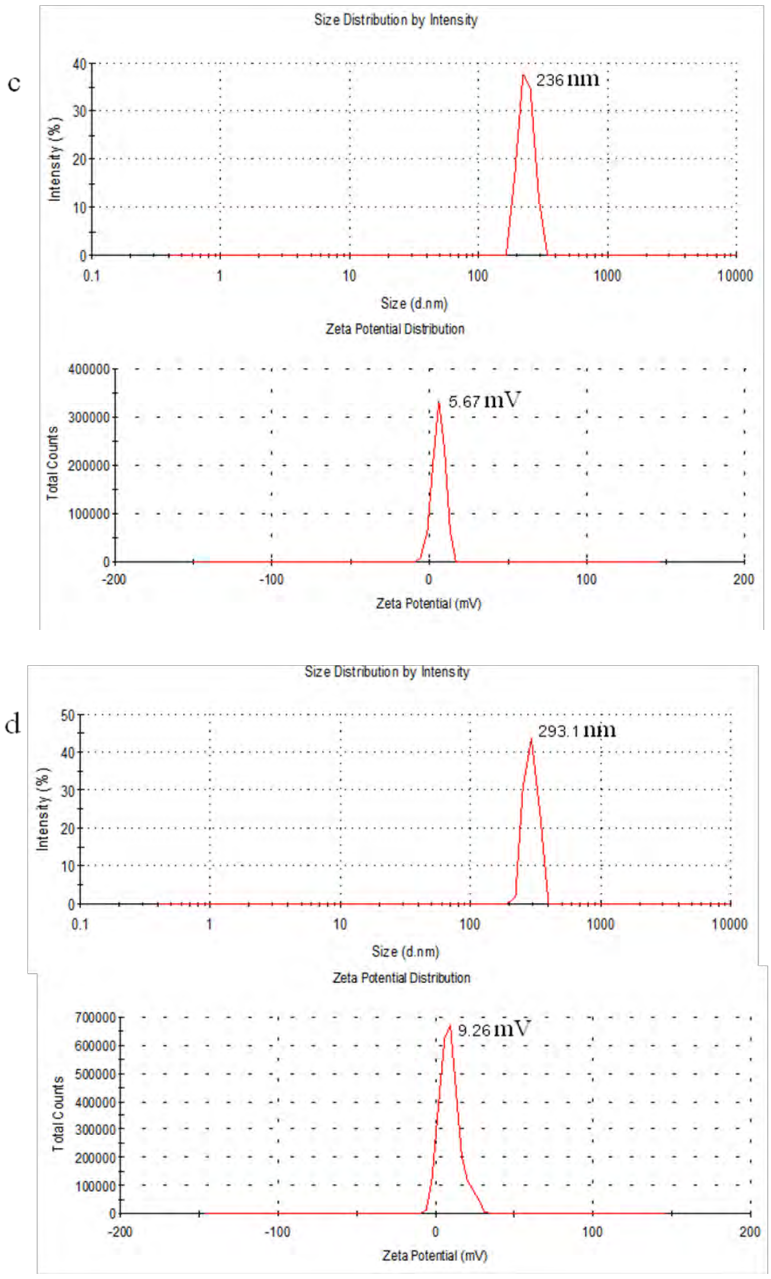


Figure A.1 Particle size and zeta potential of nanoparticles freshly synthesized (pH 5.5) with chitosan and pMAXGFP of N/P ratio 0.6:1 (d), 3:1 (c), 6:1 (b) and 12:1 (a), respectively, molecular weight at 30 kDa.

Biography

Kamon Jungapisodom was born in Nakornratchasima, Thailand on September 16, 1984. After graduate with the second honor of bachelor degree of science from the Department of Biotechnology, Faculty of Science at Rangsit University in 2006, he enrolled in the Master degree of Science in Biotechnology at Chulalongkorn University in 2007.

Academic presentation

In 2010, I made the oral presentation in the topic of development of chitosan nanoparticles for cell type specific plasmid DNA delivery. In the presentation of the 3rd Technology and Innovation for Sustainable Development International Conference at Nongkhai, Thailand.