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Chitosan/TPP Nanoparticles as a Gene Delivery Agent For Tumor Suppressant P53

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Gaojun Liu

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Abstract of the Thesis

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In the last decade, non-viral polymeric vectors have become more attractive than their viral counterparts due to their nontoxicity and good biocompatibility. However, one of the major drawbacks is the low transfection efficiency when compared to viruses. In this work, a naturally cationic polysaccharide, chitosan, was cross-linked with negatively charged tripolyphosphate (TPP) to synthesize chitosan/TPP nanoparticles (CNPs) for delivery of plasmid DNA (pDNA). Particle size and zeta potential were characterized for CNPs with chitosan-TPP mass ratios of 4:1 and 6:1 (w/w) using benchtop dynamic light scattering. And both potentiometric titration method and FTIR spectrometer were applied to measure the degree of deacetylation of chitosan. Release kinetics of a model protein (bovine serum albumin, BSA) showed a steady release that reached 7% after 6 days. Besides that, we also assessed the in vitro transfection efficiency of the CNP-pDNA system using fluorescence microscopy, as well as the effect of tumor suppressant p53. Later the release kinetics and encapsulation efficiency of plasmid DNA bound to the CNPs will be investigated. Additionally, we will try to improve the gene transfection efficiency in both MC3T3-E1 and osteosarcoma cells by applying Sonicator 740 therapeutic ultrasound.

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Acknowledgments

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Introduction

In 1972, the idea of gene therapy was firstly raised by Theodore Friedmann and Richard Roblin. At that time, more than 1500 human diseases were known to be genetically determined, and new cases were coming into being every year. Worryingly, some genetic diseases are rare. For example, in the United States one per 18,000 live births will come into the world with phenylketonuria, while cystic fibrosis of the pancreas occurs about once in every 2500 live births [1]. The increasing number of genetic diseases has become a difficult and ethical problem.

Over the past thirty years, large numbers of scientists have been attracted to study gene therapy. One remaining challenge they are currently facing is how to deliver enough amount of DNA to specific target cells. Nowadays, DNA molecules can be delivered by either direct injection or gene vectors, which can be divided into two groups, viral vectors and non-viral vectors. Both of them are quickly developed, because they are biologically safe, and have abilities to transfer larger numbers of genes at low costs than other physical means, such as particle-mediated gene transfer, electroporation-mediated gene delivery, direct injection of plasmid DNA, etc [2]. Owing to the limitations of viral carriers, such as toxicity, lack of biocompatibility, non-viral vectors have become more attractive in the studies of gene delivery in the past decade. However, the low transfection efficiency is one of the most significant drawbacks when non-viral vectors are studied in the field of gene therapy [3].

In order to improve the transfection efficiency of non-viral vectors, recent research trials have been done to control the release rate of DNA. Biodegradable hydrogels (e.g. cationized gelatin, poly-L-lysine in collagen) [4] came into people's sights, and some physical means were used to aid the transfection of DNA [5].

The key to designing a novel gene vector is to take the critical properties biocompatibility, biodegradability and transfection efficiency into consideration. Polymer-based gene vector have promising future due to their easy fabrication and biocompatibility.

1. Review of The Literature

1.1 Gene Therapy

Gene therapy is a type of technique for removing, replacing, or correcting a defective gene and inserting an intrinsic healing one to deal with the gene determined diseases [6]. Actually, hundreds of human diseases are determined by genes, and genetic diseases have become a significant medical problem around the world.

Human genetic diseases are usually treated by dietary therapy and drug therapy. Dietary therapy refers to practical applications of nutrition as a preventative or corrective treatment of disease, while drug therapy is used to help impede or reduce the accumulation of undesired and possible harmful metabolites [1]. Both of these therapies have some limitations for disease treatment. In order to break the limits, gene therapy has been successfully developed, which has potential application in the replacement of dysfunctional gene and treatment of acquired disease. In 1972, gene therapy was firstly mentioned by Theodore Friedmann and Richard Roblin. They held a view that research for the development of techniques for gene therapy should continue and some human genetic diseases would be ameliorated by gene therapy in a few years [1].

The first gene therapy case took place in United States on September 14, 1990. A four-year-old girl, with an Immune System deficiency, accepted the gene therapy, and the effects lasted a short period of time, but successful.[7]

In 2000s, large numbers of scientists were attracted to devote themselves into gene therapy. Block and graft copolymers [8] came into our sights and were studied to prepare polyelectrolyte complexes with DNA intended for targeted delivery of genes in vivo [9]. In addition, vital properties of vectors for DNA transfection have been taken into consideration, when they were designed.

1.2 Vectors in Gene Therapy

"Vectors" in gene therapy are defined as a kind of carrier for allowing a therapeutic gene to be delivered specifically to the target cell in a particular sequence and timing [3]. A proper vector should have the following properties:

a) Biocompatibility

"Biocompatibility refers to the ability of a biomaterial to perform its desired function with respect to a medical therapy, without eliciting any undesirable local or systemic effects in the recipient or beneficiary of that therapy, but generating the most appropriate beneficial cellular or tissue response in that specific situation, and optimizing the clinically relevant performance of that therapy." The concept was concluded and re-defined by Williams in 2008. [10]

b) Biodegradability

The biomaterials designed for gene delivery should perform their functions before or during the delivery process by which they were degraded and eliminated from the body, which means the degradation rate makes a vital effect to the gene delivery. [10]

1.2.1 Viral Carriers

For the viral system, the vectors of retrovirus, herpes simplex virus, lentivirus, adenovirus, and adeno-associated virus (AAV) have been used as potentially efficient gene carriers (Table.1), although there still remain some drawbacks to be resolved for the clinical applications. For example, AAV has a low capacity of DNA, and herpes simplex virus has cytotoxicity issues and has short-lived transgene expression, although it has better DNA capacity than AAV. [2] In 1999, G. Duisit et al. prepared three different E1- deleted replication-defective adenoviral vectors, Moloney murine leukemia virus (Mo-MuLV) Gap-Pol core particle proteins, gibbon ape

leukemia virus (GALV) envelope glycoproteins, and anMuLV-derived retroviral vector. All of them showed high efficiency. [11]

Vector	Packaging capacity (kb)	Host range	Clinical Trial	Features
AAV	Low < 4	Broad; infects both non-dividing and dividing cells	+	Slow expression onset; genome integration (±); long-term expression; inefficient production
Adenovirus	Medium < 7.5	Broad; low transduction of neurons	+	Transient expression; strong immunogenicity
Alphaviruses	Medium < 7.5	Broad; neuron and glial cell-specific strains	+	Transient but extreme expression levels; low immunogenicity
Lentivirus	Medium 8	Broad, dividing and nondividing cells	−	Genome integration; long-term expression; safety concerns; low titers; inefficient production
Retrovirus	Medium 8	Restricted; dividing cells only	+	Genome integration; long-term expression
Herpes simplex virus	High > 30	Broad; neurons; stem cells, muscle cells	−	Latent infection; long-term expression; low toxicity mutations

Table 1 Viral vectors used for gene delivery [2]

1.2.2 Non-viral Carriers

Both naked plasmid DNA and plasmid DNA complex with cationized carriers, are safe and have no limitation in the molecular size of DNA applied. However, the low transfection efficiency in vitro and in vivo is one major drawback for the therapeutic applications.

There are at least four categories by which the research approach of non-viral vectors design can be classified. The first group includes cationic polymers which are poly (L-lysine), polyethylenimine (PEI), chitosan, poly (amidoamine) dendrimers, and polymer micelles. The second group are cationic liposomes, which are made up of positively charged lipids, like dioctadecylamido- glycylspermine (DDGS, Transfectam^{TM}), N-[1-(2,3dioleyloxy)propyl]-n,n,n-trimethylammoniumchoride (DOTMA). Lipfectamine^{TM} is known as a commercially product for gene transfection. The third approach deals with several trials, which can enhance the gene expression based on biological phenomena, e.g. receptor-mediated internalization and endosomal escape. For example, the plasmid DNA and poly(L-lysine) can be released from the endocytotoc pathway quickly to accelerate DNA entry into the cytoplasm when a peptide derived from vitus

coats was used. And the last type is the conventional phosphate method. Darbre and Lee have co-precipitated of plasmid DNA with calcium phosphate to cells for transfection. [12]

1.3 Polymer-based Non-viral Carriers

Polymer-based gene carriers can be divided into three types in terms of the apparent molecular size. The carriers with different size have different character and can be used in various cases. For example, nano-sized gene carriers are used for transit gene expression in vitro and in vivo targeting of gene expression in most cases, while micro-sized gene carriers are mainly used for the controlled release of gene in the organ, tissue or in the inside of cells. Figure 1 shows the classification of non-viral gene carriers in terms of the carrier size and the characteristics of these vectors.

In addition, a variety of polymers have been used for the study of gene delivery. Table 2 summarizes some studies about the controlled release of plasmid DNA with different biodegradable biomaterials. [3]

1) Nano-sized gene carriers

In vitro

DNA · Ligand
(DNA-carrier complex)

(a) Enhancement of gene internalization (cell-specific gene internalization)

(b) Efficient intracellular trafficking (endosomal escape, transport to the nucleus)

Endosome   Nucleus

In vivo

(c) Blood circulation for long time

(d) Gene delivery using EPR effect and gene targeting delivery

2) Micro-sized gene carriers

In vitro   DNA

Micro-sized carrier

(a) Micro-sized gene carriers taken up by phagocyttic cells

(b) Controlled release of gene inside the cells

In vivo   Tissue or organ

Carriers injectable

(c) Controlled release of DNA in the tissue and organ site to be transfected to enhance the level of gene expression as well as prolong the expression duration

3) Macro-sized gene carriers

In vitro

DNA

Macro-sized carrier (carrier matrix)

(a) DNAs are immobilized in the carrier matrix

(b) Cells to be transfected are applied on the carrier matix

(c) Controlled release of DNA by the carrier of matrix

In vivo   Tissue or organ

Carrier matrix

(d) Controlled release of DNA in the tissue and organ site to be transfected to enhance the level of gene expression as well as prolong the expression duration

Figure 1 Classification of non-viral gene carriers in terms of the carrier size[6]

7

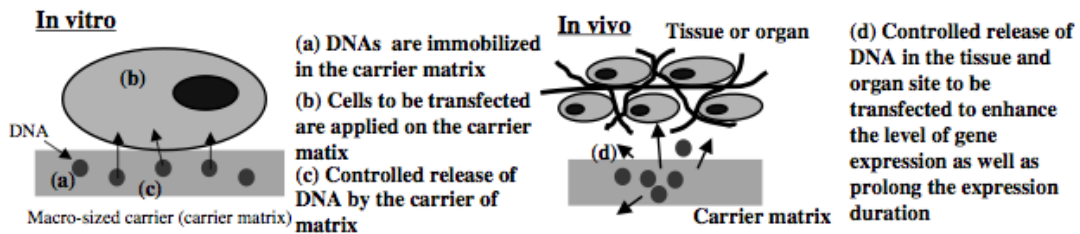| Carrier material	Plasmid DNA	Biological function	References
Poly(D,L-lactic acid-co-glycolic acid) (PLGA)	β-Galactosidase	Delivery intact and functional plasmid DNA at controlled rates. The ability to create porous polymer scaffolds capable of controlled release rates may provide a means to enhance and regulate gene transfer within a developing tissue, which will increase their utility in tissue engineering.	Murphy et al.[13]
Polymethacrylic acid (PMA) and Polyethylene glycol (PEG), Hydroxypropylmethylcellulose-carbopol		The in situ gelling systems can be considered as a valuable controlled-delivery system for plasmid DNA in their role to provide protection from DNase degradation.	Ismail et al.[14]
Poly(lactic acid)-poly(ethylene glycol) (PLA-PEG)	Luciferase	Release plasmid DNA from nanoparticles in controlled manner.	Perez et al.[15]
Poly(2-aminoethyl propylene phosphate)	β-Galactosidase	Enhanced β-Galactosidase expression in anterior tibialis muscle in mice, as compared with naked DNA solution injections.	Wang et al.[16]
Poly(β-(4-aminobutyl)-L-glycolic acid) (PAGA)	β-Galactosidase	The complexes showed about 2-fold higher transfection efficiency than DNA complexes of poly-L-lysine (PLL) which is the most commonly used poly-cation for gene delivery.	Lim et al.[17]
Poly(ethylene-co-vinyl acetate) (EVAc)	β-Galactosidase	The EVAc disks are efficient and convenient vehicles for delivering DNA to the vaginal tract and providing long-term local immunity.	Shen et al.[18]
Denatured collagen-PLGA	β-Galactosidase	Increase the level of gene expression because of integrin-related mechanisms and associated changes in the arterial smooth muscle cell actin cytoskeleton.	Perlstein et al.[19]
Gelatin	β-Galactosidase	Plasmid DNA release period can be regulated only by changing the hydrogel degradability.	Fukunaka et al.[20]

Table 2 Research reports on the release of plasmid DNA with polymer-based vectors

1.3.1 Chitosan

Chitosan, poly [β-(1,4)-amino-2-deoxy-β-D-glucose], a natural nontoxic biopolymer, has attracted a lot of attention due to its antimicrobial and antifungal activity. This biomaterial is derived by the deacetylation of chitin, and can also be found naturally in some fungi. But the commercial chitosan is obtained from chitin, one of the abundant polymers in nature. [21]

Compared to chitosan powder itself, chitosan nanoparticles have much smaller size so that the nanoparticles show the unique characteristics such as lower molecular weight, magnetism, high surface area, and higher solubility in water.

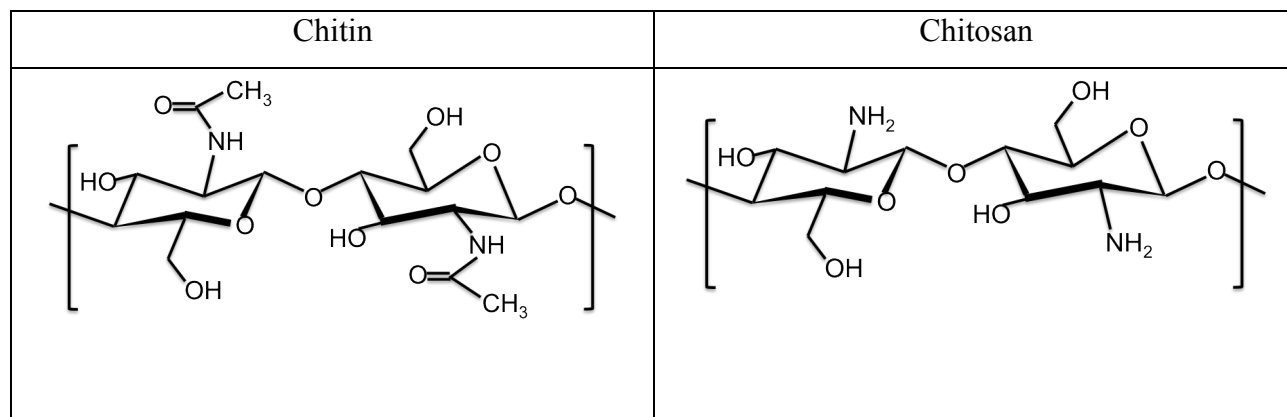| Chitin	Chitosan

Figure 2 The molecular structures of chitin and chitosan

1.3.1.1 Chitosan in Gene Delivery

Chitosan is a polymer that is able to open tight junctions and allow the paracellular transport of molecules across mucosal delivery of vaccines. Chitosan microparticles and nanoparticles loaded with DNA plasmids were reported to induce protective immune responses in mice.

Chitosan is considered as a good candidate of gene delivery vector due to its positive charge, which is opposite to that of DNA. So chitosan can bind DNA well and protect it from nuclease degradation. Mao and Boy et al. used a complex coacervation process to prepare chitosan-DNA nanoparticles in 2001. And the electrophoretic mobility analysis showed this kind of vector could protect the plasmid DNA from degradation, and they found the transfection efficiency of this nanoparticles is cell-type dependent. The gene expression levels in HEK293 (human embryonic kidney 293 cells) and IB-3-1 cells (an immortalized human bronchial epithelial cell line bearing the Δ508 mutation) were apparently higher than the levels in 9HTEo (a human tracheal epithelial cell line) and HeLa (human cervix adenocarcinoma) cells. [22]

In 2011, Gaspar et al. used inotropic gelation technique to prepare chitosan-TPP nanoparticles as a kind of gene delivery vector. They found that both the degree of deacetylation of chitosan and the chitosan-TPP ratio were critical to nanoparticle size, encapsulation efficiency, and release of DNA. Nanoparticles with high degree of deacetylation and high chitosan-TPP ratios had higher encapsulation than other samples. [23]

1.3.2 Gelatin

Gelatin, a natural, biocompatible and nontoxic macromolecule [24], is an irreversibly hydrolyzed form of collagen. In general, gelatin can be obtained by the hydrolysis of collagen derived from skin, white connective tissue, and bones of animals. Why is gelatin widely used in pharmaceutical, medical, and cosmetic products? It is owing to the fact that gelatin is natural, biodegradable,biocompatible in physiological environments, edible, water permeable, and insoluble in cold water, but completely soluble in hot water.

The most outstanding advantage of gelatin is its low toxicity, which makes it become a promising non-viral vector for gene delivery, compared to those polymer-based non-viral vectors with non-biodegradability and cell toxicity. [24]

In 1972, gelatin was used to synthesize gelatin-albumin nanoparticles as a kind of carriers for pharmaceutical applications. With more than thirty years' development, gelatin, cationic polymer, has been often used with poly-anions materials to synthesize nanoparticles for gene delivery. In 2011, G. K. Zorzi et al. designed hybrid nanoparticles, composed of gelatin and dextran sulfate, for ocular gene therapy. This kind of nanoparticles was reported to protect pDNA from degradation for at least 60 min, while naked DNA was degraded in 5 min. Although the transfection efficiency of the biomaterial was lower than the Lipofectamine control, the stability and nontoxicity made this kind of nanoparticles a promising gene vector in the ocular surface. [25]

1.4 Ultrasound in Gene Delivery

Nowadays, ultrasound (US) irradiation has been widely used in clinical medicine, not only during the operative and diagnostic procedures, but also intherapeutic process. US is composed of a propagating pressure wave or sound wave, which can help transfer mechanical energy into various body tissues. [12] And the frequency, duty cycle and time will make an effect to the absorbance of energy. US can also make positive effects on bone growth, which can enhance bone fracture healing [26] , and drug release [27].

Now, US is also used to improve the transfection efficiency of plasmid DNA in vitro and in vivo. Hosseinkhani et al. prepared three different types of complexes of plasmid DNA with gelatin derivatives of aminization, and examined the effects of US on transfection. [28] In

addition, in 2009, S. J. Yang et al. successfully synthesized chitosan-alginate nanoparticles, used as carriers of the pAcGFP1-C1 plasmid, and applied US to enhance the gene transfection. The results showed US could enhance the transgene product expression in both HeLa cells and 293T cells. [29]

2. Objective

Because it is very attractive in clinical application that the foreign materials can eventually be removed from the body, with the intention that no second surgery will be needed. Two naturally derived polymers were used in this study: chitosan and gelatin.

In this work, a novel non-viral gene vector based on a polymer phase consisting of a naturally derived component, chitosan, and its polyanioniccrosslinker, tripolyphosphate (TPP), was investigated. Different characterization techniques were used to measure the materials properties of chitosan nanoparticles, and to determine their suitability of non-viral gene carriers with high gene transfection.

First, ionic gelation method is used to synthesize chitosan nanoparticles (CNPs), composed of positively charged chitosan and negatively charged tripolyphosphate (TPP), with two mass ratios of chitosan/TPP (4:1 and 6:1). This process is simple, mild, and does not require any other chemical cross-link reagents. Then dynamic light scattering (DLS) was used to indicate the size and zeta potential of two groups of CNPs, and help us choose a mass ratio for the synthesis of CNP-pDNA.

Secondly, before CNP-pDNA was prepared, the degree of deacetylation of chitosan was investigated by two methods, which was the theory evidence why CNP-pDNA can keep positive charge and then go through cell membranes easily.

Thirdly, a model drug protein, bovine serum albumin (BSA), was loaded into chitosan nanoparticles to examine the rate of release from the CNPs. And at the same time, Sonicator 740 therapeutic ultrasound device was applied to stimulate the release of BSA.

Afterwards, GFP-p53 and pcDNA3-flag-p53 were encapsulated into chitosan nanoparticles to investigate gene transfection in MC3T3-E1 cells.

3. Materials And Methods

3.1 Materials

Chitosan (low molecular weight, deacetylation degree 75%-85%), sodium tripolyphosphate (technical grade, 85%), D-(+)-trehalose dehydrate (premium, ≥98.5%), glycerol (BioReagent, suitable for cell culture, suitable for insect cell culture, ≈99% (GC)), and Triton X-100 (laboratory grade) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium Hydroxide (reagent grade, ≈98.8%) and acetic acid (≈99.9%) were obtained from J. T. Baker. MC3T3-E1 subclone 4 and subclone 24 (mouse calvaria-derived cells) were purchased from ATCC (Manassas, VA, USA). Alexa Fluor® 546 Phalloidin, Lipofectamine™2000 and cell culture reagents were obtained from Invitrogen (Carlsbad, CA, USA). Plasmid was extracted from *Escherichiacoli (E. coli)* culture using a Qiagen Mini Prep kit (Valencia, CA, USA).

3.2 Methods

3.2.1 Synthesis of Chitosan/TPP Nanoparticles

In this paper, chitosan/TPP nanoparticles were prepared by the ionic gelation method. A chitosan solution (0.5% in 1% acetic acid) and a TPP solution (0.25%) were prepared separately. A few drops of NaOH (10mol/L) were added into chitosan solution to adjust the PH between 4.6 and 4.8. Afterwards, a certain volume of TPP solution was added to CS solution drop by drop, under stirring, at room temperature. Then 5% trehalose or 10% glycerol was used to prevent aggregation of nanoparticles. The formulated nanoparticles were centrifuged at 2700 rpm for 30 min, rinsed with DI water three times [30]. The nanoparticles were kept in freezer at -80°C overnight and then freeze-dried.

3.2.2 Characterization of CNPs

3.2.2.1 Nanoparticle size and zeta potential

Particle size of our chitosan nanoparticles was measured by dynamic light scattering (DLS). Chitosan nanoparticles were suspended in PBS to form a 5mg/ml solution, followed by 5 min ultra sonication at room temperature. Size measurements were taken in a Malvern Zetasizer Nano instrument. And all the measurements were performed in triplicate.

To measure the zeta potential, chitosan nanoparticles were prepared as previous described for particle size, but DI water was used as the solvent. Zeta potential was also investigated by Malvern Zetasizer Nano, but with a zeta dip cell. All the measurements were performed in triplicate and about 100 measurements for each sample.

3.2.2.2 Degree of deacetylation of chitosan

The deacetylation of chitosan was investigated by three analytical methods: potentiometric titration method and FTIR spectrometer [31].

When potentiometric titration method was used, 0.2 g of chitosan powder was weighted with an accuracy of ±0.001 g, and then dissolved in 20 mL of HCl solution (0.1mol/L). The resulting solution was titrated with a 0.1mol/L NaOH solution. Briefly, the standard NaOH solution was added into chitosan solution stepwise, and 0.2 mL was added each step. Then the volumes of NaOH used for titration and the pHs of the solution after each step were recorded. The titration curve was plotted by using Origin 8.0 software, and the points of the maxima of the first derivative, which is related to the equivalence points of the excess HCl (V_1) and amino groups (V_2) can be found from the titration curve.

By using the following equation, the degree of deacetylation was calculated:

$$DD = \frac{203.2}{42.0 + \frac{1000m}{c_{NaOH}(V_2 - V_1)}} \times 100,$$

where m is the mass of chitosan in the solution, c_{NaOH} is the concentration of the standard NaOH

solution, V_2-V_1 is the volume of the NaOH solution used in titration of the amino group, 203.2 is

the molecular weight of the acetylated monomeric unit of polysaccharide, 42.0 is the difference

between the molecular weights of the acetylated and deacetylated monomeric units, 1000 is the

factor of conversion of milliliters to liters, and 100 is the factor of conversion of degree of

deacetylation to percentage.

The degree of deacetylation of chitosan can also be measured by FTIR spectroscopy. The

absorption bands at 2878 cm^{-1} and 1655 cm^{-1} represented the stretching vibrations of the C-H

bond and the bending vibrations of the amino bond, respectively. Then after some image

processing, the optical density can be calculated, and the DDA was obtained by using the optical

density.

3.2.3 Encapsulation of BSA and release kinetics

A model drug, bovine serum albumin (BSA), was first used to examine the rate of release

from the CNPs. 50 mg of chitosan nanoparticles was suspended into 2 mL of PBS in a 15 mL

Falcon tube, which was left on a rotating agitator for an hour at room temperature. Then a certain

volume of BSA solution (30 mg/mL) was added in the nanoparticles solution. After that, in order

to keep the total volume of 3 mL, PBS was added in the Falcon tube. The system was left on the

rotating agitator for 24 hours for adsorption of protein. The following table shows the three

different drug-loading concentrations analyzed in my study.

	BSA Concentrations		
	0 mg/mL (A, B)	5 mg/mL (C, D)	2.5 mg/mL (E, F)
PBS (mL)	2	2	2
Chitosan nanoparticles (g)	0.05	0.05	0.05
30 mg/mL BSA solution (mL)	0	0.5	0.25
PBS (mL)	1	0.5	0.75
Total volume (mL)	3	3	3

Table 3 BSA loading concentrations

The release samples were collected every day for 7 days. Every day, all the samples were homogenized by using vortex agitator, then 0.5 mL of the mix was taken out and put into a siliconized tube. Another 0.5 mL of PBS was added to each tube to keep the total volume 3 mL. Additionally, ultrasound was applied to sample A, C, and E for 15 min everyday, at a frequency of 1 MHz, a duty cycle of 20% at 2.0 W/cm^2. The siliconized tubes for each day were micro-centrifuged at 13,000 rpm for 5 min, and only the supernatant was collected in new siliconized tubes, which were left in freezer until samples for all time-points had been collected. Six days later, the released protein was analyzed by BCA test.

3.2.4 Bicinchoninic acid protein assay

Bicinchoninic acid protein assay (BCA) is a common and efficient way to detect the total quantitation of protein. This method is according to the well-known reduction of Cu^{2+} to Cu^+ by protein in an alkaline medium, and the cuprous cation (Cu^+) can be detected with high sensitivity, by using a unique reagent containing bicinchoninic acid. [32]

During the BCA assay, diluted BSA standards were prepared firstly.Six different concentrations of BSA (0 µg/mL, 0.5 µg/mL, 5 µg/mL, 10 µg/mL, 20 µg/mL, and 40 µg/mL) were made by diluting the standard BSA (1 mg/mL). Then BCA working reagent (WR) should be prepared. The ratio of Reagent A: Reagent B: Reagent C is 25:25:1, and the volume of WR was calculated according to the amount of samples.

Afterwards, 0.1 mL of each sample was transferred to a 96-well plate, and 0.1 mL of WR was added. After the plate was incubated at 60 °C for an hour, the absorbance was measured by Microplate reader at 562 nm. If the purple color is too dark to be read, each sample ought to be dilute with DI at 1:3 (sample:DI).

3.2.5 Plasmid production and purification

The plasmids GFP-p53 and pcDNA3-FLAG-p53 were amplified in cell cultures of *E.coli* DH5alpha. The bacterial culture grew for about 16 hours in a 37°C shaking incubator. After the bacterial growth became visible on the second day, all the cells were centrifuged (>9,000 rpm, 3 min) to a pellet, and then recovered by the Qiagen Plasmid Mini Kit according to the supplier's protocol.

3.2.6 Preparation of CNP-pDNA system

When CNP-pDNA system was prepared, the pDNA was added to TPP solution firstly to make sure that negatively charged pDNA has the same opportunity to combine with positively charged chitosan as TPP. Then both the chitosan solution (5 mg/mL) and TPP-pDNA solution were filtrated to prevent clustering by using 0.2 µm filter. After that, a certain volume of TPP-pDNA solution was dropped into 1 mL of chitosan solution to get a cloudy mix, and the whole

mix was homogenized by vortex agitator. In order to lower the pH of the CNP-pDNA system and sterilize the nanoparticles for later use, nanoparticles were rinsed with 70% ethanol and DI water.

3.2.7 Protection and release of encapsulated pDNA

CNP-pDNAsolution was incubated with α-modified minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS) for 1 h at 37°C. The release of pDNA was incubated with lysozyme solution (10 mg/mL) and visualizedon 1% agarose gel stained with ethidium bromide.

3.2.8 Cell culture and *in vitro*gene transfection

MC3T3-E1 subclone 4 and subclone 24 (murine osteoblasts) were cultured in α-modified minimum essential medium (α-MEM), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin streptomycin (Pen-Strep), at 37°C in a humidified atmosphere of 5% CO_2. The medium was changed every two days until cells were confluent.

In order to obtain ideal gene transfection, cells were seeded in 24 well-plates with a density of 3×10^4 cells/well in 1 mL of α-MEM supplemented with 10% FBS and 1% pen-strep, and cultured overnight before transfection experiments. The medium was replaced with 500 μL of fresh medium, supplemented only with 10% FBS, containing different amount of CNP-pDNA, CNPs, pure pDNA, or Lipofectamine™ 2000-pDNA complex. It took more than 12 hours for the cells to uptake nanoparticles, pure plasmids, or Lipfectamine™ 2000-pDNA complex.

3.2.9 Immunofluorescence

Fluorescence images were taken after in vitro gene transfection experiments. Briefly, all the cells were rinsed with PBS twice, and then fixed with 3.7% formaldehyde. After permeabilized by 0.4% Triton X-100 (in PBS) for 8 min and rinsed again with PBS for two times, 1% Alexa Fluor® 546 Phalloidin (in PBS) was used to dye the cytoskeleton of the cells. Afterwards, all the samples were washed with PBS. Both GFP fluorescence and AF 546 fluorescence were investigated with the inverted fluorescence microscope.

4. Results

4.1 Physical Characterization of CNPs

When we prepared chitosan nanoparticles, the ratio of chitosan/TPP was set at 6:1 and 4:1 to check whether a high concentration of polyanion could improve the formation of nanoparticles. In fact, aggregation of particles may happen, if the polyanion concentration is too high. However, a low concentration of TPP may lead to a failure in synthesis of chitosan-TPP particles.

4.1.1 Particle Size

The average size and size distribution of two batches of nanoparticles suspension (chitosan:TPP=6:1 or 4:1, freeze-dried) were shown in the following Figures 3 and 4. Figure 3 shows a batch of chitosan nanoparticles with a chitosan/TPP ratio of 6:1, and the z-average size was 165.7 nm, with nearly 100% numbers of particles around 128.9 nm. On the other hand, when the chitosan/TPP ratio was changed to 4:1, the z-average size became larger, 183.3 nm.

Results

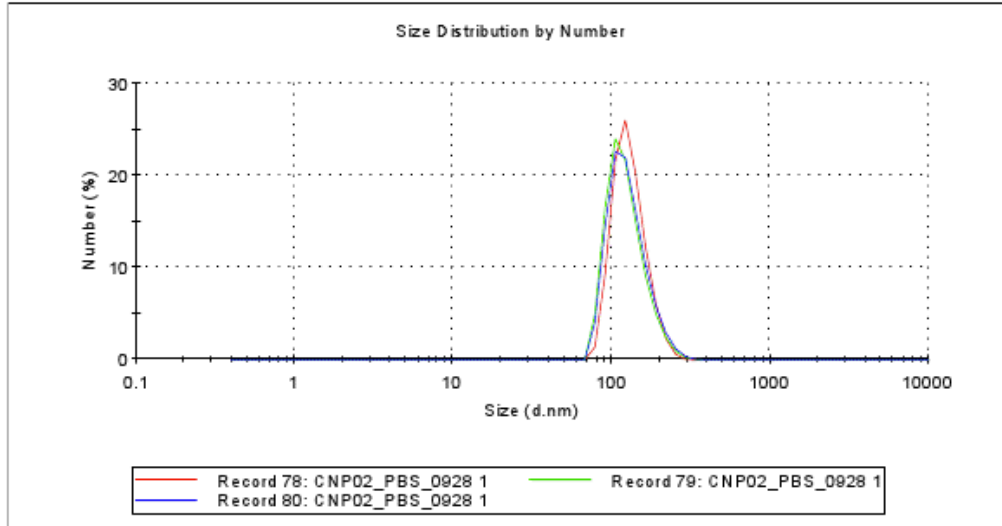|	Size (d.nm):	% Number	Width (d.nm...
Z-Average (d.nm): 165.7	Peak 1: 128.9	100.0	36.80
PdI: 0.166	Peak 2: 0.000	0.0	0.000
Intercept: 0.909	Peak 3: 0.000	0.0	0.000
Result quality : Good			

Figure 3 The size distribution by number of chitosan nanoparticles (chitosan:TPP=6:1)

Results

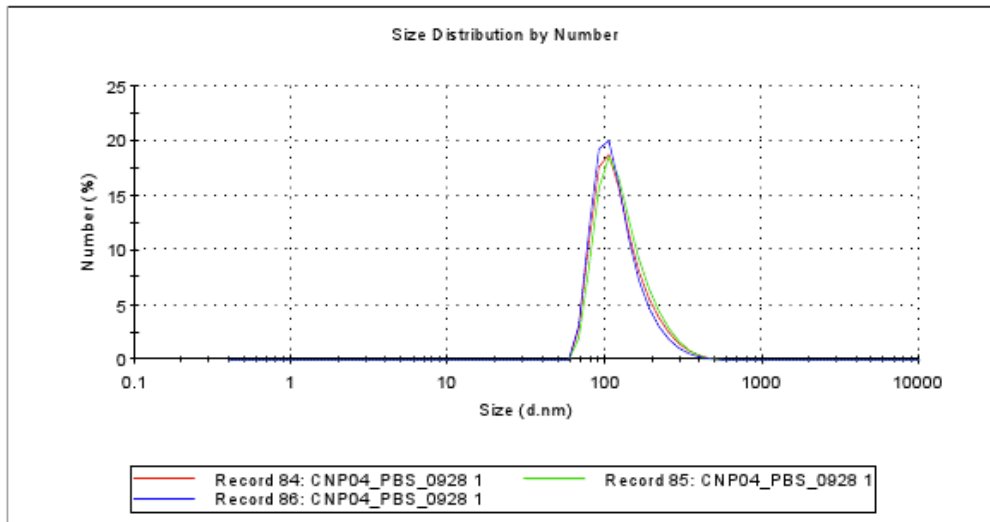|	Size (d.nm):	% Number	Width (d.nm...
Z-Average (d.nm): 183.3	Peak 1: 124.5	100.0	50.09
PdI: 0.189	Peak 2: 0.000	0.0	0.000
Intercept: 0.924	Peak 3: 0.000	0.0	0.000
Result quality : Good			

Figure 4 The size distribution by number of chitosan nanoparticles (chitosan:TPP=4:1)

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4.1.2 Zeta Potential

Zeta potential represents the surface charge of the nanoparticles, which is related to the particle stability in suspension. Besides, it can also make affect the uptake of particles by the cells, owing to the negatively charged cell membrane.

Figure 5 shows that the batch of nanoparticles with chitosan/TPP ratio of 6:1 had a positive charge about 27.1 mV, while another batch (chitosan/TPP=4:1) showed a positive charge of 28.6 mV in Figure 6.
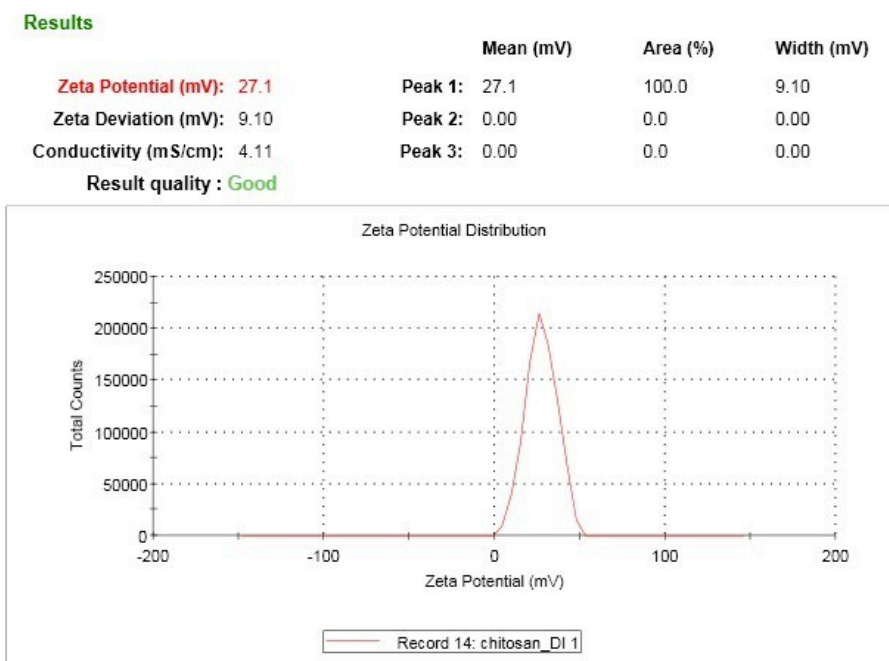

Figure 5 Zeta potential distribution of chitosan nanoparticles (chitosan: TPP=6:1)
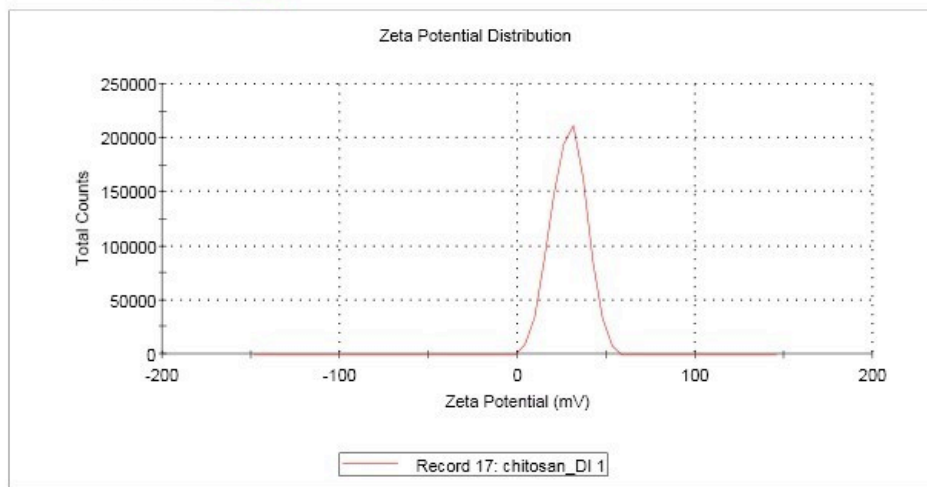
Figure 6 Zeta potential distribution of chitosan nanoparticles (chitosan: TPP=4:1)

4.1.3 Degree of Deacetylation of Chitosan

The degree of deacetylation of chitosan was measured by two different methods. The Figure 7 shows the potentiometric titration curve of chitosan and the first derivative curve drawn by Origin 8.0 software. According to the two peaks of the first derivative curve and the equation:

$$DD = \frac{203.2}{42.0 + \frac{1000m}{c_{NaOH}(V_2 - V_1)}} \times 100$$

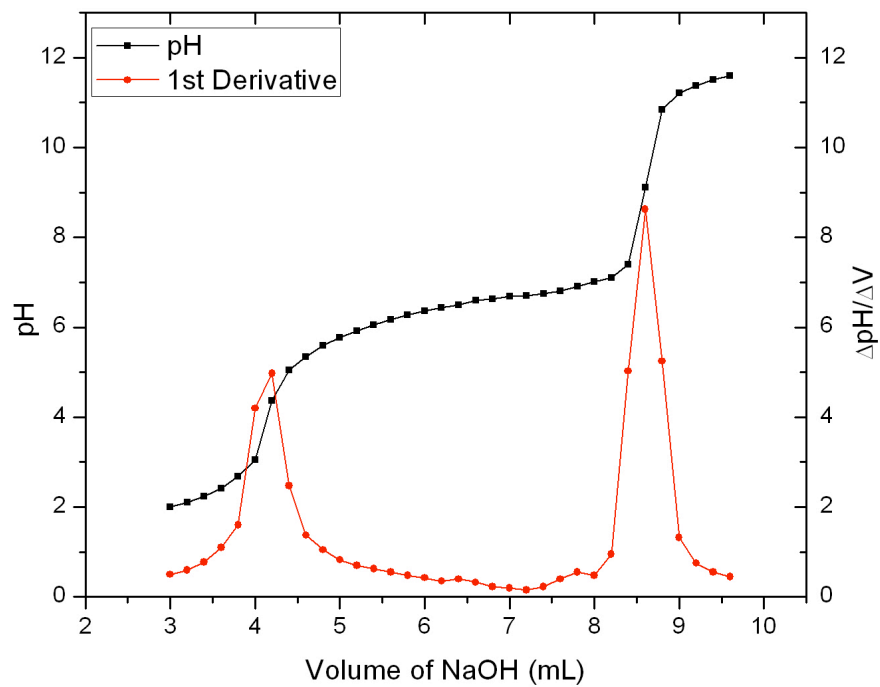The deacetylation of chitosan was calculated around 74%.

Figure 7 Potentiometric titration curve of chitosan and first derivative curve for the determination of degree of deacetylation of chitosan

According to the second method, FTIR spectroscopy, the absorption spectrum of a chitosan sample is shown in the following Figure 8. The absorption bands at 2878 cm^{-1} and 1655 cm^{-1} represented the stretching vibrations of the C-H bond and the bending vibrations of the amino bond, respectively. After some image processing, the optical density was calculated as follows:

$$A_{2878} = \log(AC/AB) = \log(110/106) = 0.01609$$

$$A_{1655} = \log(DF/DE) = \log(104/101) = 0.01271$$

The degree of deacetylation was calculated from the following regression equation:

$$A_{1655}/A_{2827} = -0.04529DD + 4.4214, r^2 = 0.9946$$
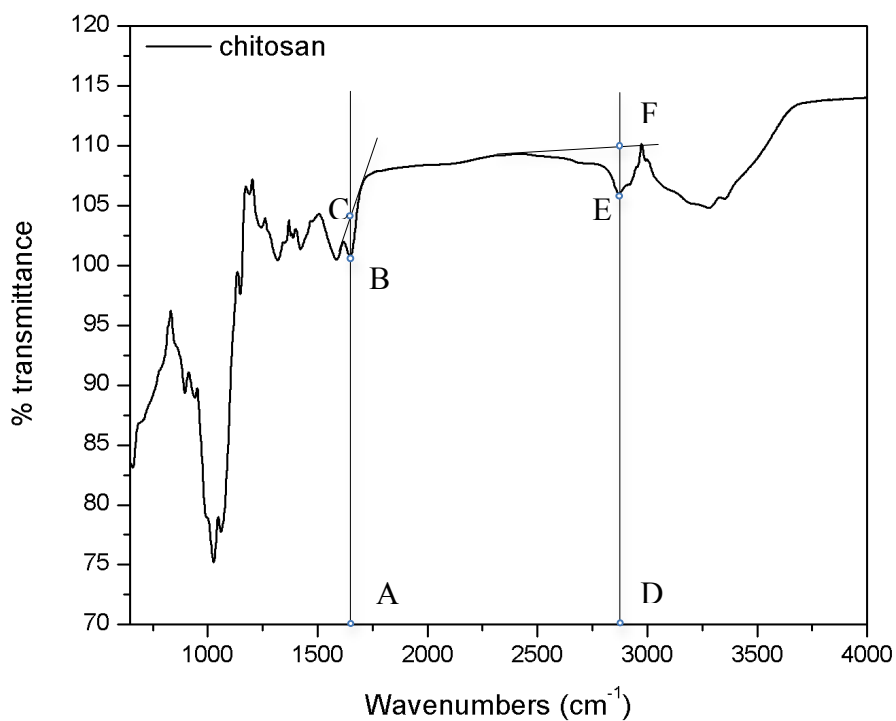
$$DD \approx 80\%$$

Figure 8 FTIR absorbance spectrum of a chitosan sample. Lines and points illustrate the graphical processing of the data for degree of deacetylation of chitosan

4.2 BSA Release From CNPs

We investigated the BSA release from chitosan nanoparticles with and without ultrasound, and with two different initial BSA concentrations (2.5 mg/mL and 5.0 mg/mL). Figure 9shows nearly 8% of BSA was released from chitosan nanoparticles, containing 7.5 mg of BSA initially, with and without the help of ultrasound, while the nanoparticles encapsulated with 15 mg BSA just released about 4% of protein with ultrasonic stimulation.

On the other hand, as a kind of protein carrier, chitosan nanoparticle showed a slow release rate, which proved it can be considered as a good drug carrier.
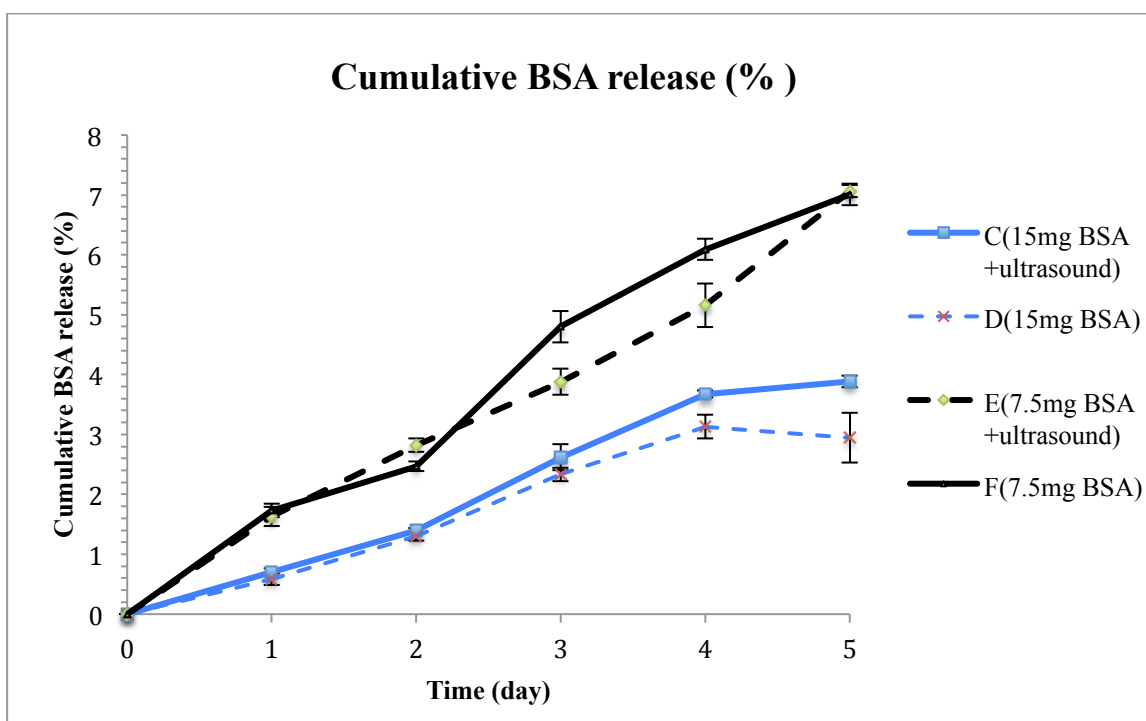

Figure 9 Effect of ultrasound and loading BSA concentration on BSA release profile from the CNP-BSA.

4.3 Plasmid DNA integrity analysis

From the agarose gel image (Figure 10), no pDNA in the nanocapsules was degraded after incubated with α-MEM supplemented with 10% FBS or lysozyme solution (10 mg/mL). And the

bright band in the wells of the gel (lane 5, 6, 7, and 9) indicated pDNA was really encapsulated
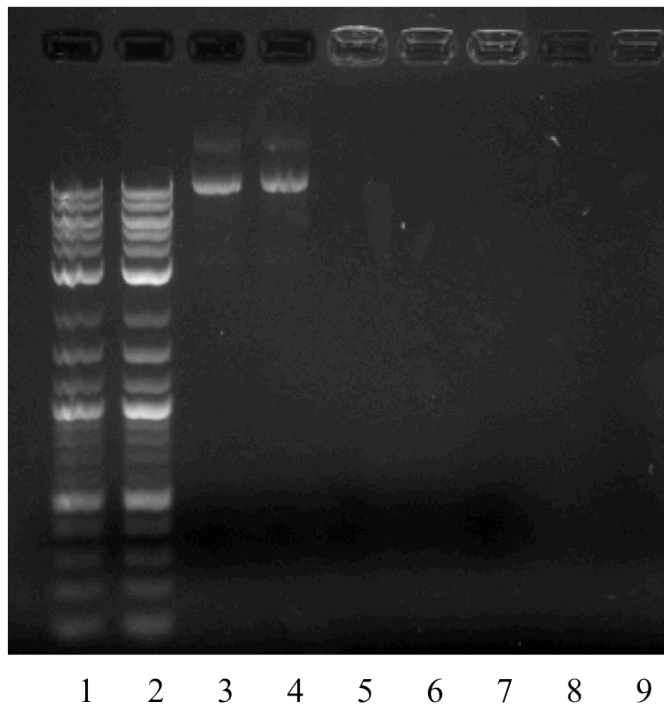
in our chitosan/TPP nanoparticles.

Figure 10 Agarose gel electrophoresis of CNPs protection of pDNA. Lane 1: molecular weight marker; lane 2: molecular weight marker; lane 3: 200 ng pDNA; lane 4: 200 ng pDNA; lane 5: CNP-pDNA; lane 6: CNP-pDNA; lane 7: CNP-pDNA incubated with α-MEM supplemented with 10% of FBS; lane 8: CNPs; lane 9: CNP-pDNA incubated with lysozyme.

4.4 *In vitro* Gene Transfection

Figure 11 shows the transfection efficiency of CNP-pDNA in both MC3T3-E1 subclone 4

and subclone 24 after 4 h, 8 h, and 24 h. Cells were fed with 50 μLof CNP-pDNA nanoparticles,

75 μL of CNP-pDNA, pure chitosan nanoparticles and naked plasmid, separately. And both the

two kinds of cells were allowed to uptake particles and plasmids for 6 hours or 24 hours.

After 4 hours of incubation, all the cells showed slightly green fluorescence, except the

ones uptook pure plasmids and control samples. Both subclone 4 and subclone 24 were detected

nearly 50% and 80% cellular uptake (%) respectively with 24 hours of incubation. However,

pure chitosan nanoparticles also gave some fluorescence, 40% for subclone 4 and 30% for subclone 24, which indicated the inaccuracy of the transfection efficiencies of chitosan/TPP nanoparticles loaded with GFP-p53.
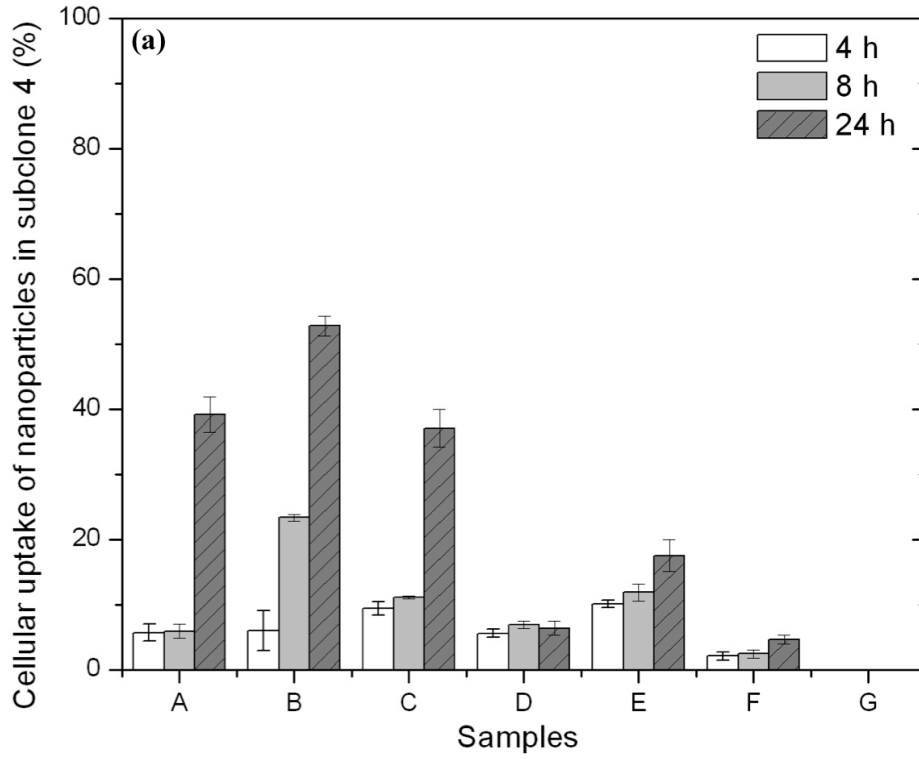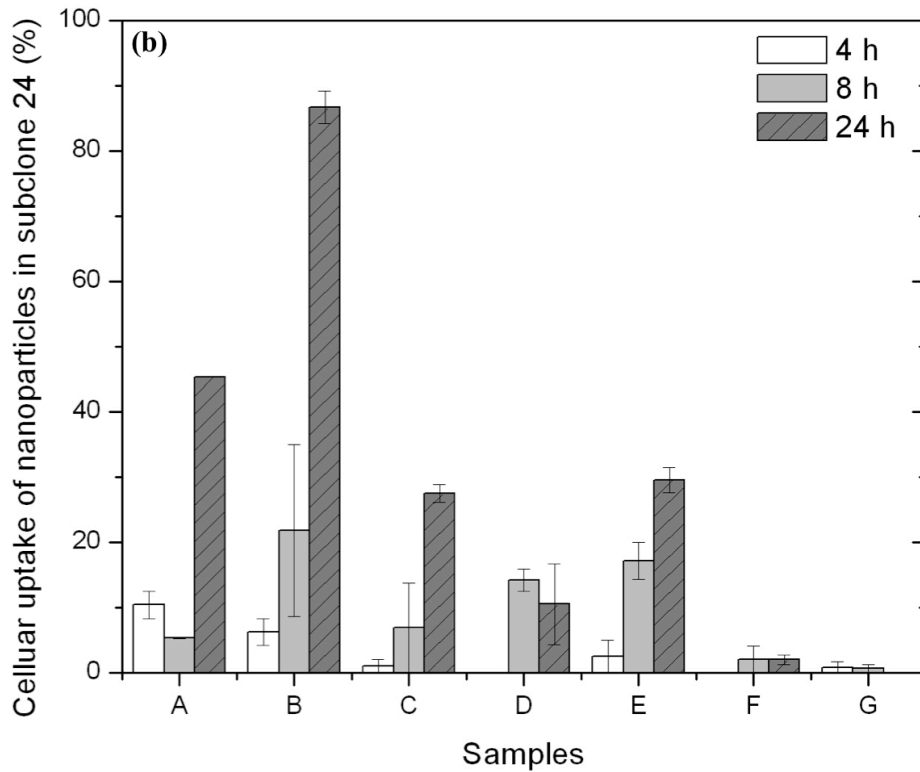
Figure 11 Effect of pDNA concentration and incubation time on uptake of nanoparticles by MC3T3-E1 subclone 4 (a) and subclone 24 (b). The concentrations of nanoparticles per 30,000 cells were (A, D) 50 µL of CNP-pDNA (corresponding to 1.1 µg of pDNA), (B, E) 75 µL of CNP-pDNA, and (C, F) 50 µL of CNPs only. For samples A, B, and C, medium was replaced after 24 hours of incubation, while in samples E, F, and G, medium was replaced after 6 hours. Sample (G) was cells in medium only.

4.5 Fluorescence microscopy

Figure 12 shows six fluorescence images of MC3T3-E1 (subclone 4) transfected for 24 hours with nanoparticles. The green dots in the images present the pDNA loaded nanoparticles or pure chitosan/TPP nanoparticles. Most of the green dots were located in the cells. Moreover, from figure (c) we found the cells shrank, due to the presence of 75 µL of CNP-GFP-p53 in the medium, and the cell density of sample (f) was a little higher than other five samples, which were encapsulated with CNP-pDNA or CNPs.
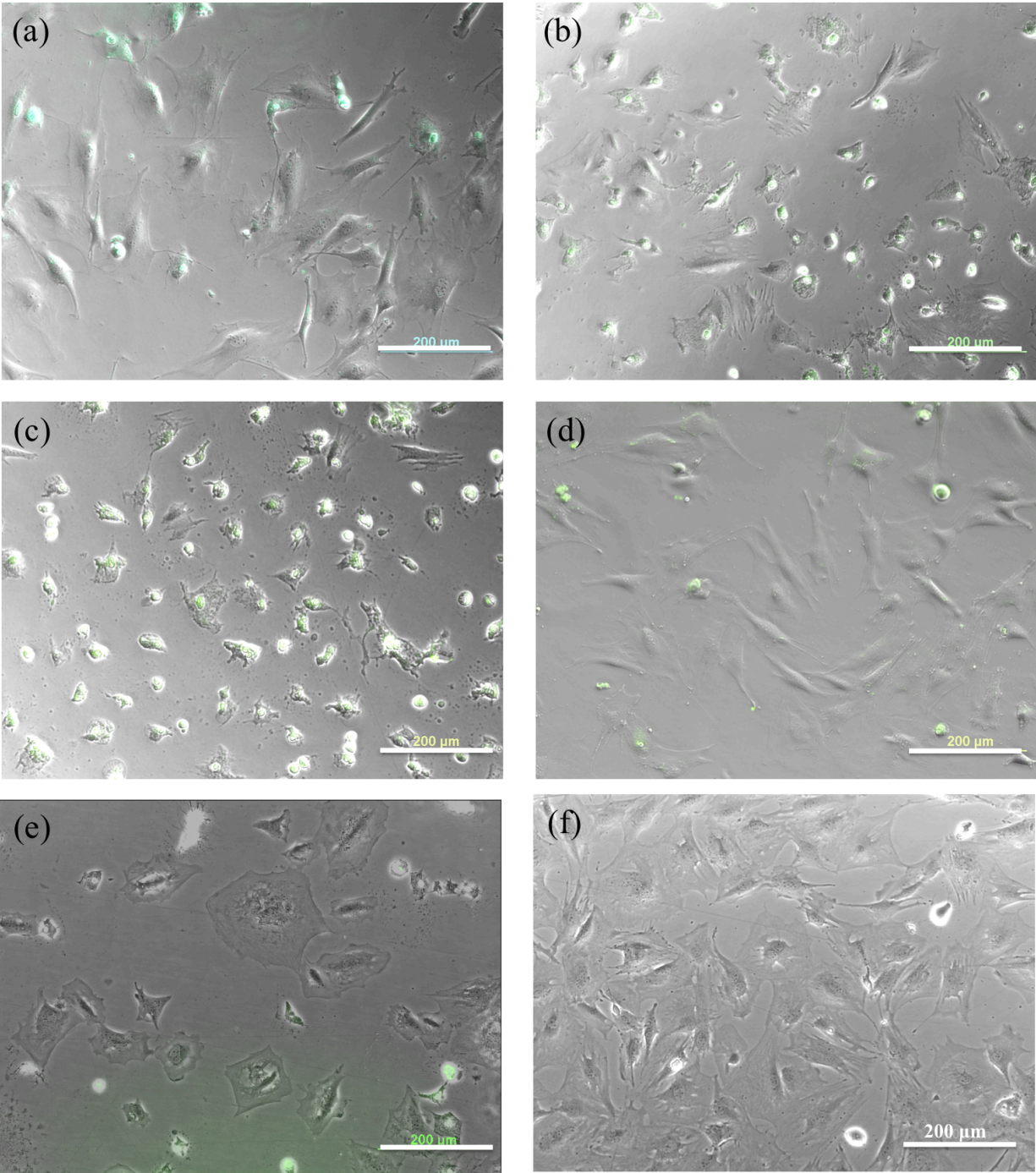
Figure 12 Fluorescence images of MC3T3-E1 (subclone 4) transfected for 24 hours with nanoparticles, (a) 25 μL of CNP-GFP-p53, (b) 50 μL of CNP-GFP-p53, (c) 75 μL of CNP-GFP-p53, (d) 0.6 μg of GFP-p53, and (f) 50 μL of CNPs. Sample (f) is control sample of cells in medium only.

In an attempt to evaluate the cell spreading areas, immunofluorescence micrographs were took after 48 hours of incubation. From the micrographs (a)-(d), the cell density of sample (c), which was incubated with Lipofectamine™-pDNA complex, was the lowest among the four samples. Apart from this, the cell spreading areas increased a little, after the cells were transfected with CNP-pDNA, for example, the cell average area of sample (b) reached nearly $3000\ \mu m^2$.
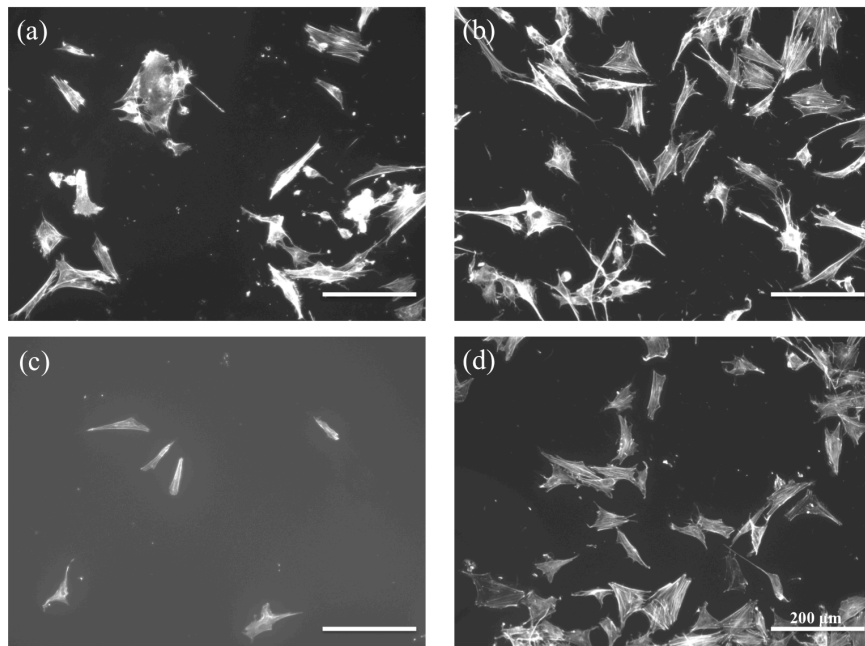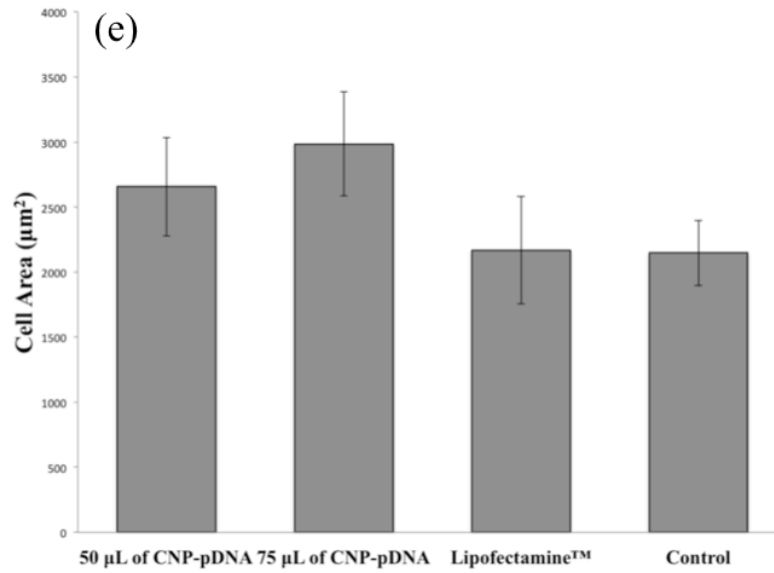
Figure 13 Immunofluorescence micrographs and cell areas after 48 hours transfection with (a) 50 µL of CNP-pDNA (corresponding to 1.1 µg of pDNA), (b) 75 µL of CNP-pDNA, (c) 2.0 µL of Lipofectamine™ loaded with 1.1 µg of pDNA. Control samples (d) were cells in medium only. Cell spreading areas were calculated from fluorescence micrographs in (a)-(d).

5. Discussion

5.1 Particle Size and Zeta Potential

Both particle size and zeta potential are two significant parameters for gene carrier. Nanoparticles (~100 nm) are known to be able to enhance arterial uptake compared to large particles (~300 nm) in an ex vivo canine carotid artery model. And particles positively charged are easier to cross over cell membranes than neutral or negatively charged.

In my studies, CNPs with a chitosan/TPP ratio of 4:1 were slightly larger (183.3 nm) than CNPs with a ratio of 6:1 (165.7 nm). This is probably due to the higher concentration of TPP in 4:1 nanoparticles. TPP, a kind of polyanion, is considered as a cross-linker in the synthesis of chitosan nanoparticles. The increasing of the amount of TPP will help attract more positively charged chitosan, which may lead to the enlargement of the nanoparticle size and higher positive charge.

5.2 Degree of Deacetylation of Chitosan

The degree of deacetylation of chitosan provides a theoretical basis of why chitosan nanoparticles and CNP-pDNA can express positive charges.

When the chitosan/TPP ratio was 6:1, we found the positive charge from chitosan is nearly twice more than negative charge obtained from TPP. Assuming 0.5 g of chitosan was used, when we prepared chitosan nanoparticles, the chitosan powder may provide 0.0023 mol of positive charge, according to the following equation (1).

$$\frac{0.5\ g \times DD}{161\ g/mol} \times 1 = \frac{0.5\ g \times 74\%}{161\ g/mol} \times 1 \approx 0.0023\ mol \quad (1)$$

DD is the degree of deacetylation of chitosan, 161 g/mol is the monomeric unit of chitosan, and 1 represents each unit of chitosan can give 1 positive charge (Figure 13).

Meanwhile, TPP solution can give nearly 0.0011 mol of negative charge, according to equation (2).

$$\frac{33.3 \; ml \times 0.25\% \; (g/ml)}{367.91 \; g/mol} \times 5 = 0.0011 \; mol \quad (2)$$

In equation (2) 33.3 ml is the volume of TPP solution used for synthesis of CNPs, 0.25% (w/v) is the concentration of our TPP solution, 367.91 g/mol is the molecular weight of sodium tripolyphosphate, and 5 represents every tripolyphosphate ion can give 5 electrons (Figure 14).

So when TPP was used as a cross-linker between chitosan, a linear polymer, chitosan/TPP particles can be obtained with different sizes.

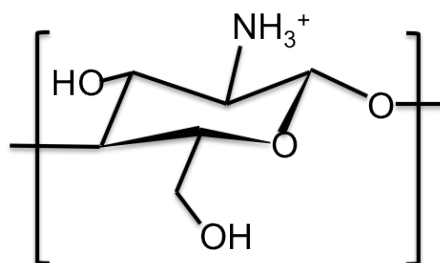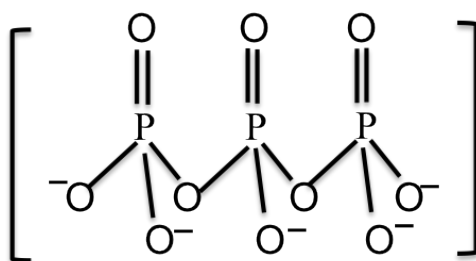Figure 14 Molecular structure of a unit of chitosan

Figure 15 Molecular structure of a TPP

5.3 Nanocapsule protection and in vitro uptake of nanoparticles

In order to evaluate whether CNP-pDNA are stable enough to protect genetic materials in extracellular environment, we incubated CNP-pDNA in α-modified minimum essential medium (α-MEM), supplemented with 10% fetal bovine serum. Then the result of agarose gel

electrophoresis showed no pDNA was degraded under the presence of serum, which can degrade naked pDNA. This suggested our CNP-pDNA is a kind of suitable delivery agents for gene delivery applications *in vitro* and *in vivo*.

Both naked pDNA and CNP-pDNA were applied to transfect MC3T3-E1 cells. (Figure 11) The negative charge of naked pDNA hindered the delivery into cells, while CNP-pDNA showed a higher cellular uptake in subclone 4 cells after 24 hours of incubation. It was due to CNPs, positively charged, helped encapsulate naked DNA and eliminate the barriers (negatively charged cell membranes) for delivery of genetic materials to a certain degree.

Compared with Lipofectamine™, our CNP-pDNA can be treated as a non-viral gene carrier without toxicity. A low cell density was found, when cells were incubated with Lipofectamine™-pDNA complex for 48 hours (Figure 12). Although good transfection efficiency is a key property for gene carriers, nontoxicity cannot be neglected in the synthesis of non-viral gene delivery agent. CNP-pDNA showed good compatibility in our study, and can be left in tissues for gene delivery, owing to its nontoxicity and biodegradability.

6. Conclusions

In this study, we successfully synthesized chitosan/TPP nanoparticles by using ionic gelation method. We tried two different chitosan/TPP mass ratios, 6:1 and 4:1. Then, particle sizes and zeta potentials of two groups of nanoparticles were investigated by DLS. Owing to the large size of the cotton-shaped particles (4:1), we decided to keep the chitosan/TPP mass ratio of 6:1 in the following experiments. In order to detect the stability of this novel gene carrier, we used bovine serum albumin as a model drug protein to examine the effect of loading density and ultrasound stimulation on BSA releases. The release profile showed that the more BSA was loaded, the less amount of BSA was released. And the ultrasound stimulation did improve the release, especially when the loading density was high.

After that, we tried to combine tumor suppressor p53 gene with chitosan/TPP nanoparticles. The agarose gel electrophoresis of the nanocapsule proved that chitosan/TPP nanoparticles could protect pDNA from degradation. Then, we transfected murine pre-osteoblasts with CNP-GFP-p53. After 24 hours of incubation, the cellular uptakes of nanoparticles reached nearly 50% in both subclone 4 and subclone 24 cells. However, chitosan/TPP nanoparticles themselves also showed fluorescence, so we cannot determine the transfection efficiencies accurately from the green fluorescence images. In addition, we calculated the cell spreading area, and the cells showed a little higher cell areas after they uptook CNP-pDNA, while the commercial gene vector Lipofectamine™ decreased the areas.

Chitosan based CNP-pDNA system obtained good protection property and high transfection efficiency in this study. Besides that, this kind of gene carrier is a stable protein carrier with stimulus-responsive property. So this study may help provide an alternative for non-viral gene delivery agents in the future.

7. Future work

In the future, encapsulation efficiency of CNPs will be studied by using UV-Vis, and we will try some new methods to prevent the aggregation of CNPs, although glycerol has helped a lot. Additionally, we will continue to study the effect of the CNP-pDNA on cell growth and motility of MC3T3-E1 pre-osteoblast cells. Further, we will try to apply Sonicator 740 therapeutic ultrasound to improve the transfection of CNP-pDNA *in vitro* in MC3T3-E1 and osteosarcoma cells.

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