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DEVELOPMENT AND CHARACTERISATION OF CHITOSAN-PLASMID DNA NANOPARTICLES

Master of Science Thesis

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and M.Sc. (Eng.) Elina Talvitie
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ABSTRACT

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Chitosan has been explored as a non-viral vector for gene therapy due to favourable characteristics such as good biocompatibility and biodegradability. The aim of this thesis was to prepare and characterise chitosan-plasmid DNA (chitosan-pDNA) nanoparticles, using 17 different types of chitosan, in order to evaluate which chitosan yields the most favourable nanoparticle characteristics in terms of size and zeta potential. Also size stability and correlations between chitosan properties such as molecular weight (M_w) and degree of deacetylation (DD), and particle properties were assessed in this study. Both basic chitosan and chitosan salts and derivatives were tested.

The particles were prepared by complex coacervation. Size and zeta potential of particles were determined by dynamic light scattering (DLS) and Laser Doppler velocimetry, respectively. Integration of pDNA with chitosan was assessed by agarose gel electrophoresis and morphology of prominent samples was observed using field emission gun scanning electron microscope (FEG-SEM). With a fixed chitosan to pDNA weight ratio 4:1 most chitosan types, excluding carboxymethylchitosan and chitosan oligosaccharide, integrated with pDNA to form nanoscale particles. The size of the remaining chitosans ranged from approximately 100 nm to 180 nm and the zeta potential was relatively stable ranging from +20 to +30 mV. Smallest particles were achieved with chitosan 85/25 (101.2 ± 0.9 nm) and chitosan hydrochloride (100.3 ± 1.3 nm). Statistical analysis indicated that for most samples particle size increased significantly during the first week and then remained relatively stable.

No clear correlations between chitosan and nanoparticle properties were detected. However, it appears that high DD and high M_w chitosans yield more stable complexes indicated by higher zeta potentials. According to FEG-SEM images particles were spherical and their size ranged from 20-200 nm. Smaller particles appeared to have a more even structure. In many cases, however, the particle surface was not smooth suggesting possible aggregation. Although chitosan 85/25 and chitosan hydrochloride yielded good results in terms of size, it was noted by other researchers in the same project that functionalisation of the particles failed due to sedimentation. When the weight ratio was changed, functionalisation properties improved. Therefore, future research should focus on exploring the effects of different chitosan:pDNA weight ratios on particle parameters and also functionalisation possibilities should be considered.

TIIVISTELMÄ

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Kitosaania pidetään kudosyhteensopivuutensa ja biohajoavuutensa ansiosta lupaavana biomateriaalina geeniterapeuttisiin sovelluksiin. Tämän työn tavoitteena oli valmistaa sekä karakterisoida kitosaani-pDNA-nanopartikkeleita käyttäen 17:ta erilaista kitosaanityyppiä. Näin pyrittiin selvittämään, onko eri kitosaanityyppien välillä suuria eroja ja mikä kitosaani tuottaa parhaimmat tulokset koskien nanopartikkelien kokoa ja zetapotentiaalia. Myös korrelaatiota nanopartikkeliominaisuuksien, kuten koon, sekä kitosaaniominaisuuksien, kuten molekyylipainon (M_w) ja deasetylaatioasteen (DD), välillä selvitettiin. Lisäksi tutkittiin partikkelikoon pysyvyyttä kahden viikon seurantajaksolla.

Työssä tutkittiin sekä peruskitosaaneja että kitosaanisuoloja ja -johdannaisia. Partikkelit valmistettiin kompleksikoaservaatiomenetelmällä. Partikkelikoko mitattiin perustuen dynaamiseen valosirontaan ja zetapotentiaali määritettiin Laser Doppler -virtaustekniikan avulla. Plasmidi-DNA:n yhdentyminen kitosaaniin todettiin agaroosigeelielektroforeesilla, ja lupaavimmat näytteet kuvattiin kenttäpyyhkäisy-elektronimikroskoopilla (FEG-SEM). Kitosaani-pDNA massasuhteella 4:1 suurin osa kitosaaneista muodosti nanopartikkeleita, poikkeuksena karboksyylimetyylikitosaani ja kitosaani-oligosakkaridi. Partikkelien koko vaihteli välillä 100-180 nm. Pienimmät partikkelit saatiin aikaiseksi kitosaani 85/25:llä ja kitosaanikloridilla (CH HCl). Koot keskihajontoineen olivat 101.2 ± 0.9 nm ja 100.3 ± 1.3 nm. Stabiiliutta mittaava zetapotentiaali oli yleisesti ottaen vakaa (20-30 mV). Useimpien näytteiden partikkelikoko muuttui tilastollisesti merkittävästi ensimmäisen viikon aikana, jonka jälkeen koko ei enää muuttunut merkittävästi.

Selkeitä korrelaatioita kitosaanin ja partikkelien ominaisuuksien välillä ei ollut nähtävissä. Kitosaanit, joilla oli suurempi M_w ja DD, johtivat kuitenkin vakaampiin komplekseihin, joiden zetapotentiaali oli korkeampi. FEG-SEM-kuvien perusteella partikkelit olivat pallomaisia ja niiden koko vaihteli 20 ja 200 nm:n välillä. Pienimmillä partikkeleilla oli tasaisempi pintarakenne, kun taas usean isomman partikkelin pinta oli raemainen. On mahdollista, että partikkelit aggregoituivat. Vaikka 85/25 ja CH HCl -kitosaaneilla saatiin aikaiseksi parhaimmat tulokset koskien partikkelikokoa, ne sedimentoituvat jatkokäsiteltäessä. Kun kitosaani-pDNA massasuhdetta muutettiin, tulokset paranivat. Näin ollen jatkotutkimuksissa tulisi selvittää eri massasuhteiden vaikutus partikkeliominaisuuksiin, sekä myös pohtia funktionalisointimahdollisuuksia.

PREFACE

This Master of Science thesis was conducted at the Department of Biomedical Engineering at Tampere University of Technology as a part of EU project "NanoEar".

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ABBREVIATIONS AND DEFINITIONS

α	Polymorphic form of chitin
β	Polymorphic form of chitin
γ	Polymorphic form of chitin
η	Viscosity
AFM	Atomic force microscopy
AIDS	Acquired immune deficiency syndrome
Atoh-1	gene regulating the differentiation of cochlear hair cells and supporting cells from their precursor cells during embryogenesis
CH	Chitosan
CH_3COOH	Acetic acid
CH_3COONa	Sodium acetate
CMC	Carboxymethylchitosan
COS	Chitosan oligosaccharide
Da	Dalton. A unit of mass used to express atomic and molecular masses. One Dalton, also known as an atomic mass unit (u), is one twelfth of the mass of an isolated carbon-12 atom at rest in its ground state. In biochemistry, Da is sometimes used as a unit for molar mass, where 1Da = 1g/mol, even if it is really a unit for molecular mass (1Da = 1.660 538 782(83) $\times 10^{-27}$ kg)
DA	Degree of acetylation
DD	Degree of deacetylation
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid. A nucleic acid that contains the genetic material needed for the development of all living organisms and some viruses. DNA is composed of a double

	stranded helix structure, in which the polymer chain backbone consists of phosphate groups and sugars attached to bases.
F_A	Fraction of acetylation
GAG	Glycosaminoglycan
HEK293	Human embryonic kidney cell
HeLa	Immortal cell line derived from cervical cancer cells taken from Henrietta Lacks
HCl	Hydrogen chloride
HMWC	High molecular weight chitosan
IMT	Institute of Medical Technology (University of Tampere)
IR	Infrared
kbp	kilo base pair
KNOB	Protein developed by erythrocyte membrane infected by the human malaria parasite Plasmodium falciparum
LDV	Laser Doppler Velocimetry
LD_{50}	Lethal dose. A dose at which 50% of subjects die.
LMWC	Low molecular weight chitosan
Math-1	See Atoh-1
M_n	Number average molecular weight
M_v	Viscosity average molecular weight
M_w	Weight average molecular weight
N	Normality. The number of gram equivalent weight of a solute per litre of its solution. The definition of normality depends on the exact reaction intended.
NaOH	Sodium hydroxide
Na_2SO_4	Sodium sulphate
NIBS	Non-invasive back scattering

NMR	Nuclear magnetic resonance
N/P	Polymer nitrogen (N) per DNA phosphate (P) ratio
PCS	Photon correlation spectroscopy
PCR	Polymer chain reaction
PDI	Polydispersity index
pDNA	Plasmid DNA. Extra-chromosomal DNA capable of independent replication.
PEI	Poly(ethylene)imine
PEG	Polyethyleneglycol
pK_a	Acid dissociation constant
QELS	Quasi-elastic light scattering
siRNA	Small interfering RNA, also known as short interfering RNA and silencing interfering RNA. A class of double-stranded ribonucleic acid 20-25 nucleotides in length. siRNA is involved in the RNA interference pathway, where it interferes with the expression of a specific gene
Std	Standard deviation
RNA	Ribonucleic acid. A single-stranded polymer structure whose backbone consists of a nitrogenous base attached to a sugar (ribose) and phosphate. RNA participates in protein synthesis and gene expression
TAE	Tris Acetate EDTA
TBE	Tris Borate EDTA
TE	Tissue engineering
UV	Ultraviolet
WHO	World Health Organization
w/v	Weight to volume ratio
Å	Ångström. A unit of length equal to 1×10^{-10}m

Anion	A negative ion
Anode	A positively charged electrode
Anticoagulant	A substance that prevents blood from clotting
Antitumoral	A substance that counteracts or prevents the development of tumours
Biocompatibility	The ability of a material to perform with an appropriate host response in a specific application
Biodegradability	The ability of a chemical substance to be broken down by living organisms
Cathode	A negatively charged electrode
Cation	A positive ion
Chlorhexidine	A chemical antiseptic that kills both gram-positive and gram-negative microbes
Cytocompatibility	The compatibility of a biomaterial at the cellular level
Deacetylation	The removal of an acetyl group from a chemical structure
Electrical double layer	An electrical layer that surrounds an object in a liquid. The first layer of ions is in contact with the particle surface and it is called the Stern layer. The second layer is in contact with the Stern layer and it is more diffuse.
Endocytosis	The process by which cells absorb molecules from outside the cell by engulfing it with their cell membrane
ex vivo	Experiment taking place in or on a tissue in an artificial environment, not necessarily in a culture, outside an organism
Glycosaminoglycan	A long unbranched polysaccharide that has nitrogen in one of its sugars
Hemocompatibility	The compatibility of a biomaterial, when it is in contact with blood

Hydrophilic	A molecule or part of a molecule that is water-soluble due to charged-polarization and hydrogen-bonding
Hydrophobic	Non-polar molecules, which are usually insoluble in water
in vitro	Experiment taking place in a controlled environment outside an organism
in vivo	Experiment taking place in a whole, living organism
Isoelectric point	The pH at which a molecule or surface carries no net charge
Luciferase	A class of oxidative enzymes that act as catalysts in bioluminescence
Lysosome	A spherical cell organelle that contains digestive enzymes
Mark-Houwink equation	An equation determining the relation between viscosity and molecular weight
Mucopolysaccharide	See glycosaminoglycan
Plasmid	An extra chromosomal DNA molecule, capable of replicating independently of the chromosomal DNA
Schiff base	A functional group that consists of a carbon-nitrogen double bond with the nitrogen connected to an aryl or alkyl group
Siver sulfadiazine	Topical antibacterial used to treat burns. It prevents the growth of bacteria and yeast on damaged skin
Stern layer	The first layer of the electric double layer
Weak acid	An acid that does not fully dissociate in an aqueous solution
Weak base	A base that does not fully dissociate in an aqueous solution

1. INTRODUCTION

This thesis was conducted at the Department of Biomedical Engineering (TUT) as part of an EU project called "NanoEar". The aim of the project is to develop multifunctional nanoparticles in order to treat hearing disorders. At the moment approximately 250 million people worldwide suffer from hearing loss and deafness. It is estimated that this number will increase to 900 million by 2050. Hearing loss or other sensory impairment usually leads to a lower quality of life, which is why it is important to find means to treat the disorder. Currently, hearing aids such as cochlear implants are used in the treatment of inner ear damage. However, new technologies involving nanomedicine are emerging. NanoEar is an early stage project exploring the possibilities of nanotechnology in inner ear treatment. Nanosize particles interact on the cell level, which permits lower drug doses and better targetability. Other important factors related to nanoparticles are biodegradability, traceability *in vivo* and controlled drug release. [82]

A key gene known as Atoh 1 or Math 1 has been identified as one of the main genes regulating the differentiation of cochlear hair cells and supporting cells from their precursor cells during embryogenesis [82]. Cochlear hair cells have an important role in hearing as mechanical oscillations of the hair cells stimulate sensory neurons of the cochlear nerve to transmit electrical signals to the brain that is ultimately responsible for the hearing sensation [34, p.299-306 and 342]. Thus, the encapsulation of nucleotides encoding Atoh 1 in nanostructures could prove useful for the treatment of inner ear disorders [82]. Chitosan is one of the most promising biomaterials examined in NanoEar and it has been widely studied as a candidate for non-viral gene delivery due to favourable characteristics such as biocompatibility and non-toxicity. This study is concerned with the preparation and characterisation of chitosan-pDNA nanoparticles using different types of chitosans. Also the effect of different material properties on particle parameters is explored.

The thesis consists of two parts: a literature review and an experimental study. Theory related to chitosan, gene therapy, nanoparticles, and characterisation techniques is reviewed in Chapters 1-5. Chapter 2 discusses the history, chemistry, synthesis, properties, and applications of chitosan as a biomaterial. Also the synthesis of chitosan salts and derivatives is discussed in detail. Chapter 3 provides an insight to gene therapy, which is the field of interest for chitosan-pDNA nanoparticles. Chapter 4 describes nanoparticle preparation methods with a specific focus on complex coacervation, which is the method used in this study. Also particle parameters and properties such as size, zeta potential, and transfection efficiency are described and discussed in this section. Chapter 5 introduces various characterisation techniques,

including dynamic light scattering (DLS) and Laser Doppler velocimetry, for chitosan-pDNA nanoparticles. Also agarose gel electrophoresis and field emission gun scanning electron microscopy are explained. The experimental part (Chapters 6-7) of the thesis aims to provide an insight to the practical work concerning this study. Chapter 6 elaborates the materials, devices and methods that were used in particle preparation. In Chapter 7 results are presented and discussed. The results focus mainly on particle size, size stability, zeta potential, and correlations between chitosan properties and particle parameters. Finally, conclusions are drawn in Chapter 8.

2. CHITOSAN

Chitosan is the second most abundant natural polysaccharide on Earth. Due to favourable characteristics such as good biocompatibility, biodegradability, antimicrobial activity and chelating abilities, chitosan is used in various fields and numerous applications.

2.1. History and nomenclature of chitin and chitosan

To better comprehend the following discussion concerning chitin and chitosan, it is necessary to briefly introduce the history and nomenclature of these two polysaccharides. Chitosan is the partially N-deacetylated derivative of chitin which, after cellulose, is the second most abundant biopolymer on Earth [100]. Chitin occurs as a structural mucopolysaccharide in a wide range of species. For example, chitin can be found in the exoskeletons of arthropods such as crustaceans, as well as in the endoskeletons of cephalopods. In addition, chitin is also present in algae and in the cell walls of most fungi. Chitosan, on the other hand, is much less abundant in nature and so far it has only been found in the cell walls of certain fungal species. [19; 44; 111]

Chitin was first discovered by French scientist Henri Braconnot during his studies on mushroom in 1811. He initially named chitin *fungine*. [19] Later, in 1823, the term *chitin* was proposed by Odier, who found the same compound in the cuticles of insects. The term "chitin" is derived from the Greek word "chiton" signifying nail coat. [102] Chitosan, on the other hand, was discovered by C. Rouget in 1859 when he treated chitin in boiling and concentrated potassium hydroxide. Rouget named this structure "modified chitin". This chitin-derivative was renamed *chitosan* by F. Hoppe-Seyler in 1894. [19; 119] In 1934, the production of chitosan, as well as the preparation of chitosan films and fibres was patented [23, p.336-337]. The modern research of chitin and chitosan can be considered to have begun in the early 1970s, when it was realised that chitin constitutes a significant waste in sea food related processes. Consequently, governments even donated grants in order to valorise this substance. [19] Currently chitin is mainly used as the raw material for chitosan, whose application fields include agriculture, cosmetics, dietetics, pharmacology, gene therapy, and biomaterials, just to name a few [19; 102]. Some useful properties of chitosan are the ability to chelate harmful metal ions from waste water, to form water clarifying or filtrating membranes, as well as to act as an agent in controlled release [23, p.336-337].

Despite the colourful nomenclature history of the above-mentioned polysaccharides, the terms chitin and chitosan persist in scientific language of today [19]. Even so, the distinction between chitin and chitosan remains vague, as the only differentiating

feature in their chemical structure appears to be the degree of deacetylation (DD, see Section 2.2) [19]. Some sources state that when the DD of chitin is more than 50%, the polymer in question is, in fact, chitosan, while a DD less than 50% suggests that the material is chitin [99]. In addition, some sources propose that also the solubility of the material has to be taken into account for nomenclature purposes. For instance, if the substance is soluble in dilute acids, it is usually considered as chitosan. [99; 111] In some rare cases, when the degree of acetylation (DA) is 100%, such as in some marine diatoms, the term *chitan* has also been proposed [38; 44].

2.2. Chemical structure of chitin and chitosan

Chitin and chitosan can be considered to belong to the family of glycosaminoglycans (GAGs); a subcategory of polysaccharides. Among the latter, it appears that GAGs are the only polysaccharides that express bioactivity, which make them especially interesting when considering medical applications. [19] The relatively high nitrogen content (6.9 wt%) of the chitin structure also makes it useful for chelation [38].

Chitin is a cationic, linear and high molecular weight polysaccharide that consists of β(1-4)-linked 2-acetamido-2-deoxy-D-glucose units [23, p.336-337; 102; 111]. This unit is often referred to as N-acetyl-D-glucosamine [19; 102]. However, chitin may be slightly N-deacetylated depending on its isolation process and therefore some randomly distributed D-glucosamine units may remain in its structure. *Chitosan* is considered as an N-deacetylated form of chitin and it is composed of randomly distributed β(1-4)-linked 2-acetamido-2-deoxy-β-D-glucopyranose and 2-amino-2-deoxy-β-D-glucopyranose units. [111] The units in both chitin and chitosan are linked together by glycosidic bonds [38]. The chemical structures of chitin and chitosan are presented in Figure 2.1. The degree of deacetylation (DD) is one way of defining whether the structure in question is chitin or chitosan. A majority of D-glucosamine units in the material suggests that the substance is chitosan [44].

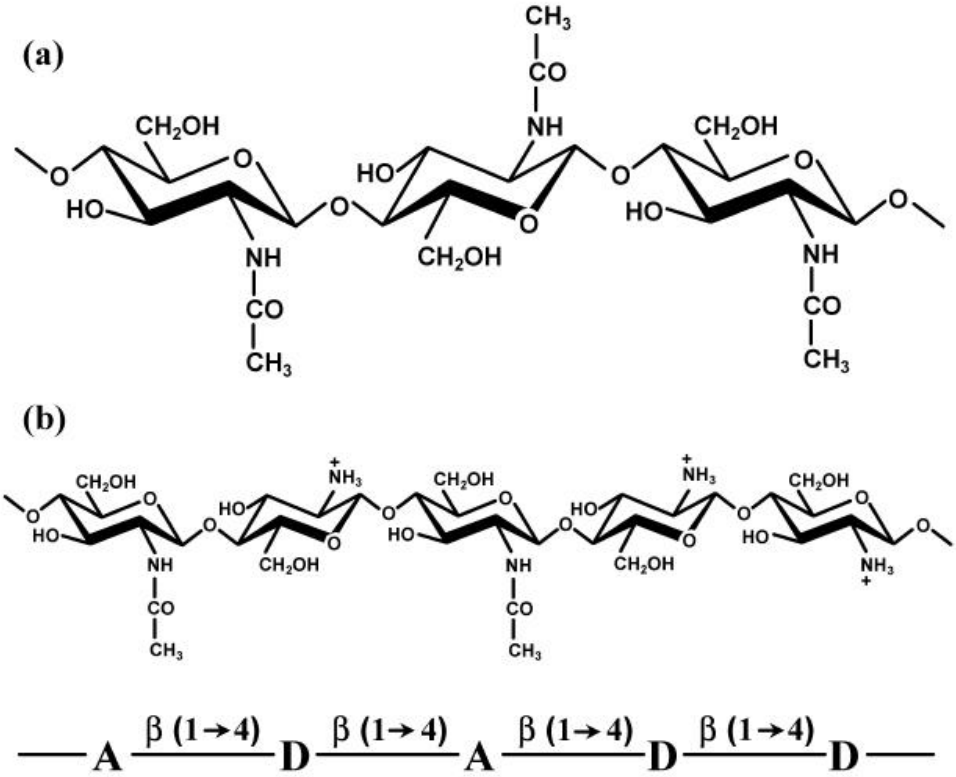


Figure 2.1. *a) Chemical structure of chitin and b) protonated chitosan. "A" represents the acetylated unit of chitin and "D" the deacetylated unit. [111]*

Chitin and chitosan exist in three different polymorphic forms, namely α-, β- and γ-chitin [5]. These structures form sheets or stacks of essentially linear chitin chains running in certain directions, with each individual chain undergoing a full twist every 10.1-10.5 Å [119]. α-chitin, present in arthropods, fungi or cysts of *Entamoea*, is the most common allomorph and its antiparallel chain alignment allows it to form strong interactions with other molecules. These interactions are due to strong intra- and intersheet hydrogen bonding in α-chitin. The α-structure is also commonly used for preparing chitosan. [51] The structure of γ-chitin, found in cocoon fibers of the *Ptinus* beetle and in the stomach of *Loligo* squid, resembles that of α-chitin, but with every third stack having the opposite direction to the two previous stacks. It has also been suggested that the γ-chitin is a distorted form of α- or β-chitin instead of a true third polymorphic form. [5] β-chitin, derived from the pen of the *Loligo* squid, on the other hand, is characterised by the parallel arrangement of chitin molecules that causes weak intermolecular forces [23, p.336-337; 40]. The β-conformation also exhibits a higher reactivity and higher affinity for solvents compared to α-chitin [40]. β-chitin can readily and irreversibly be transformed to α-chitin via steam annealing in the swollen state without losing its fibrous orientation, although shrinkage may occur in longitudinal direction [38]. Due to the different crystalline structure of β-chitin, it proposes an

attractive alternative to α-chitin and studies related to its chemistry are steadily progressing [51].

It should be mentioned that chitosan has three reactive functional groups. These include a primary amino group at the C-2 position, as well as a primary and secondary hydroxyl group at the C-3 and C-6 positions, respectively [64; 93]. The repeating unit of chitosan and its functional groups are illustrated in Figure 2.2.

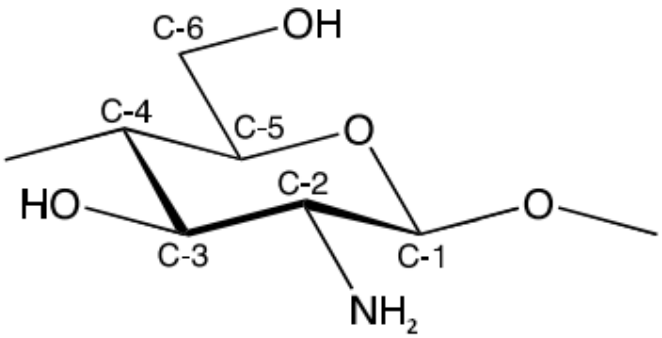


Figure 2.2. *Repeating structural unit of chitosan showing the functional groups at positions C-2 (primary amino group), C-3 (primary hydroxyl group) and C-6 (secondary hydroxyl group). Modified from [51].*

In contrast to chitosan, chitin has an acetamido group at the C-2 position. This acetamido group can be deacetylated to an amino group in order to produce chitosan. It should be noted that the chemical structure of cellulose resembles that of chitin, with the only difference being that there is a hydroxyl group at the C-2 position of cellulose instead of an acetamido group. [102] The main function of chitin, chitosan and cellulose is to provide structural support in nature. Due to the many functional groups in chitin and chitosan, the polysaccharides can readily be modified. For example, some chemical reactions related to chitosan modification are acylation, deacetylation, alkylation, hydrolysis of the main chain, Schiff base formation, reductive alkylation, O-carboxymethylation, N-carboxyalkylation, silylation, and graft copolymerization. [38; 52; 52].

2.3. Synthesis of chitin and chitosan

The main source for commercially available chitosan is chitin which, as mentioned in Section 2.1, occurs widely in the lower plant and animal kingdoms. The most common source for chitin and chitosan syntheses are arthropod shells of marine crustaceans like crabs and shrimps, that are readily available as waste from the seafood processing industry. The shells contain approximately 15-40% of α-chitin. Other major constituents

of shells are proteins (20-40%) and calcium carbonate ($CaCO_3$) (20-50%). In addition, also pigments and other metal salts are present in the shells. [51]

2.3.1. Synthesis of chitin

Chitin is insoluble in most common solvents and therefore the solvent extraction method is excluded in the isolation of chitin [51]. Two hydrolytic methods have been proposed to prepare chitin and chitosan, namely acid hydrolysis (chemical treatment) and enzymatic hydrolysis [102]. Even if not soluble in common solvents, chitin is quite stable in mild acidic and basic conditions. Chemical chitin isolation essentially involves three steps: decalcification, deproteinisation and decolorisation of crustacean shells. [102; 111] After these steps, chitin is obtained as the residue after the decomposition of the other constituents of the shells [51].

More specifically, the chemical synthesis of chitin involves treating crustacean shells with dilute hydrochloric acid (HCl) at room temperature in order to remove calcium carbonate and metal salts. Carbonate ions react with protons to form bicarbonate (HCO_3^-) and carbonic acid (H_2CO_3) that are liberated from the solution as carbon dioxide (CO_2). [51; 111] Deproteinisation is achieved by alkali treatment at high temperatures. Decalcified shells are ground and then heated at about 100°C in 1-2 mol/L sodium hydroxide (NaOH). This step should remove the proteins and possible pigments from the shells. In mild conditions proteases can be used to replace the alkali treatment. [51] To ensure the removal of organic substances, the HCl and NaOH treatments may be repeated a few times. The order of decalcification and demineralisation may also be reversed [111]. Some chitin producers use an organic solvent to remove pigments and fat from chitin. Upon drying, the alkali treatment results in a flake-like chitin with off-to-white colour. [51] A schematic representation of chemical chitin and chitosan synthesis is illustrated in Figure 2.3.

It should be noted that the cost of shellfish waste and therefore also chitin, is dependent on the production region, collection costs and other costs. Uncertainties relating to chitin production include the quantity of available waste, the waste composition, as well as the selling price and market volume for end use. [38]

2.3.2. Synthesis of chitosan

Although chitosan can be extracted from certain fungi, it is currently obtained mainly by the N-deacetylation of chitin, as this method is presently more convenient and less costly [51]. Chitosan can also be reacetylated from highly deacetylated chitosans [111].

The N-deacetylation of chitin is based on using highly concentrated aqueous or alcoholic alkali, normally sodium or potassium hydroxide (40-50%), at temperatures of 100-150°C for periods of 1-5 h under heterogeneous conditions [5; 51; 98; 102]. This treatment results in approximately 70% deacetylated chitosan [99]. Repeating the procedure increases the degree of deacetylation to a certain extent. However, the molecular weight of the chitosan decreases with repeated alkali treatment leading to a

decrease in chain length. To overcome this problem, the use of sodium thiophenolate has been suggested. Sodium thiophenolate protects the polymer chains from degradation while simultaneously allowing the catalysis of the reaction. [19] To minimise the amount of NaOH used, also the mixing of chitin with NaOH powder (weight ratio 1.5) by extrusion at 180°C has been proposed. This method results in highly deacetylated and soluble chitosan with only one half of the NaOH needed for the aqueous treatment. [98] Figure 2.3 demonstrates the production process of chitosan, whereby the acetamido group of chitin is deacetylated to an amino group due to alkali treatment in the last stage of the process.

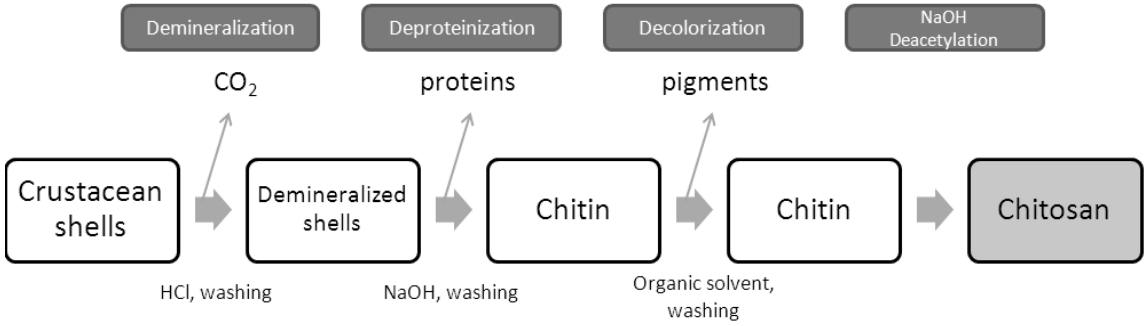


Figure 2.3. *Production process of chitin and chitosan. Modified from [112] and [99].*

Chitin and chitosan can also be synthesised by enzymatic hydrolysis. Examples of applicable enzymes include chitinases, chitosanases, lyzosomes and/or cellulases. [102; 121] Enzymatic synthesis usually leads to low molecular weight chitosans (LMWC), or chitosan oligosaccharides (COS), that are readily soluble in water due to shorter chain lengths and free amino groups in the D-glucosamine units. This is considered as an advantage to insoluble chitosan, as it is believed that solubility and low viscosity of the COS will increase the use of chitosan in food and biomedical applications. [46]

Enzymatic hydrolysis of chitin or chitosan is generally preferable to chemical hydrolysis associated with toxic compounds, environmental pollution and low production yields. However, the high cost of enzymatic hydrolysis restricts its use in COS production, although reuse of hydrolytic enzymes can decrease the production costs. Chitosan oligosaccharide synthesis can also be carried out using, for example, oxidative degradation and ultrasonic degradation. [46] Also physical depolymerisation using gamma radiation can be performed [5]. The resulting physical forms after chitosan synthesis and processing usually include hydrogels, solutions, and solid state forms. Solid forms of chitosan include, for example, powders, flakes, particles, films, and fibres [19].

2.4. Synthesis of chitosan salts and derivatives

Although unmodified chitosan has many favourable characteristics such as good biocompatibility and low toxicity, its use is still limited by its low solubility at physiological pH 7.4. This restricts the use of chitosan in nasal or oral delivery systems. Moreover, the rapid adsorption of water and high swelling limit the use of chitosan in controlled release systems. [95] Therefore, alternatives such as chitosan derivatives have been developed. Chitosan can be chemically modified due to the functional groups in its structure (see Section 2.2). This modification often results in chitosan derivatives that aim to have improved properties over native chitosan. The modified material may, for example, exhibit a better drug loading capacity and sustained release than the basic chitosan. Also the mechanical strength, stimuli sensitivity and solubility can be affected. [95] A concrete example of a chitosan derivative is thiolated chitosan, which possesses better mucoadhesive properties than basic chitosan as a result of the formation of disulfide bonds with subdomains of mucus glycoproteins [91]. Chitosan can also be graft-polymerised to produce different chitosan derivatives. Some examples of chitosan derivatives are acetylated chitosan, carboxymethylchitosan, thiolated chitosan, chitosan oligosaccharides (COS), chitosan glutamate, chitosan acetate, chitosan lactate, chitosan hydrochloride, cyclodextrin-linked chitosan, and so on. [95]

Despite the common use of chitosan salts and derivatives, little comprehensive literature exists in this field. Some attempts to gather information about various chitosan salts and derivatives and their preparation methods have recently been made by Prabaharan. [95] Nevertheless, numerous chitosan derivatives are uncovered in this review. Some information about specific chitosan derivatives can be found in articles, though these mainly focus on an application of the material and not the material itself. In this thesis, chitosan derivatives such as chitosan acetate, chitosan lactate, chitosan hydrochloride, carboxymethylchitosan, chitosan oligosaccharide and chitosan glutamate are used.

2.4.1. Carboxymethylchitosan

Carboxymethylchitosans (CMCs) are a family of water-soluble chitosans which form semipermeable films and membranes [31]. There are various methods for preparing CMCs. In N-carboxyalkylation, an acidic group is introduced to the amino group of the polymer backbone. Kurita synthesised N-carboxymethylchitosan by adding glyoxylic acid ($C_2H_2O_3$) to a chitosan solution. After that sodium cyanoborohydride was used to reduce the Schiff base intermediate. Finally, N-carboxymethyl chitosan was formed. [12; 50] This method is illustrated in Figure 2.4.

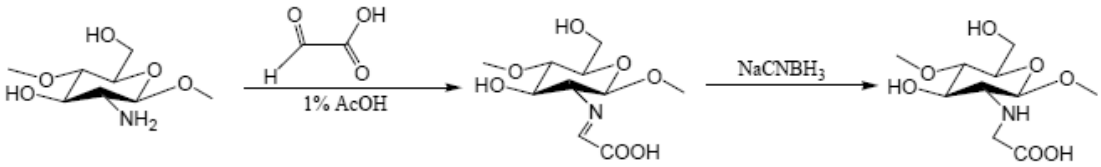


Figure 2.4. *Synthesis of N-carboxymethyl chitosan (CMC). [12]*

Also other preparation methods for carboxymethylchitosan have been described and patented. [31; 41; 108; 117] For example, O-carboxymethylchitosan can be prepared by pretreating chitin powder with 50% NaOH solution for 12h at -18°C, and then reacting it with monochloroacetic acid in isopropyl alcohol using a condensation reaction. The synthesis of O-carboxymethylchitosan is illustrated in Figure 2.5.

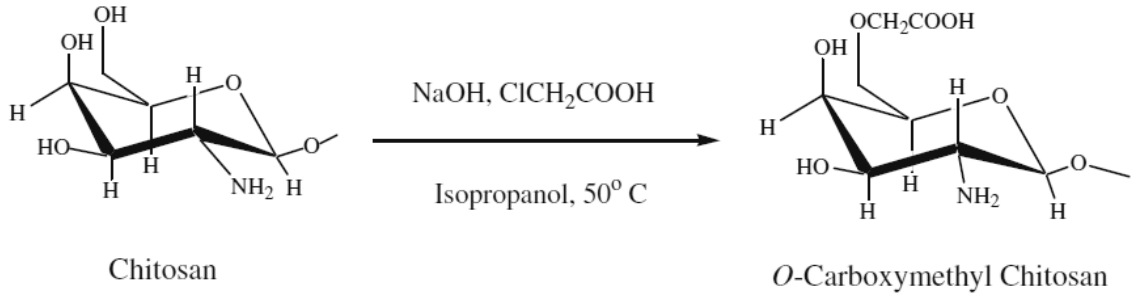


Figure 2.5. *Synthesis of O-carboxymethylchitosan. [41]*

The water-solubility of the resulting CMC is greatly dependent on preparation conditions, especially temperature. It seems that higher preparation temperature leads to more insoluble CMCs at neutral pH. [41] Recently, Jayakumar et al. have written an extensive review concerning novel carboxymethylchitosan derivatives and their applications. The nomenclature of carboxymethylchitosans greatly depends on which functional group has been altered during preparation. Modifying the amino or hydroxyl group results in N-carboxymethyl or O-carboxymethyl chitosan, respectively. It is also possible to modify both groups to obtain N,O-carboxymethylchitosan. These CMCs can be further modified to include different functional groups. [41]

2.4.2. Chitosan acetate and chitosan lactate

Chitosan acetate can be prepared by linking chitosan and acetic acid by electrovalent bonding resulting in a salt whose chemical structure is displayed in Figure 2.6a [58]. Due to protonation of amine groups ($-NH_2$), the derivative is water-soluble. Chitosan acetate is also more stable than chitosan dissolved in acetic acid and also manages to retain the original chitosan structure and antibacterial activity [58]. In addition to electrovalent bonding, chitosan acetate has also been prepared by spray-drying. In this method chitosan powder is dissolved in a small amount of distilled water containing acetic acid. Finally, the chitosan acidic solution is sprayed using a spray dryer, and the obtained powder is collected and stored in a dessicator containing dry silica gel. [88]

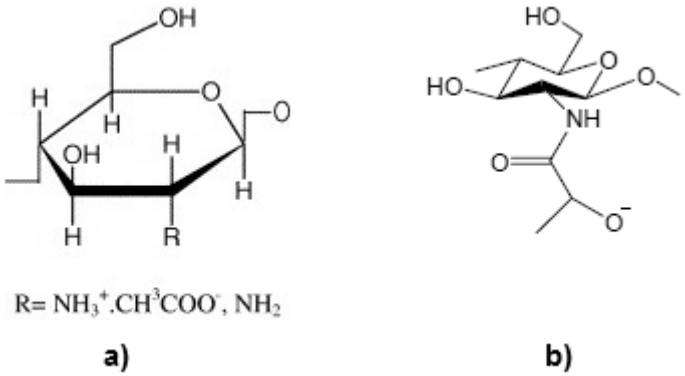


Figure 2.6. *a) Chemical structure of chitosan acetate [58] and b) chitosan lactate [12].*

Chitosan lactate can be prepared by spray-drying or by adding the minimum amount of lactic acid that is capable of dissolving chitosan in an aqueous chitosan and propranolol hydrochloride solution [11; 115]. The structure of chitosan lactate is represented in Figure 2.6b. Unfortunately, there is a lack of information about chitosan lactate as a material. Therefore, more studies and literature concerning the synthesis and properties of chitosan lactate are needed.

2.4.3. Chitosan oligosaccharide

Chitosan oligosaccharides (COS) are low-molecular-weight chitosans that are usually water-soluble. In the case of chitosan bases, the solution viscosity is usually quite high, which makes it difficult to prepare high concentration chitosan solutions that can be advantageously used in, for example, pharmaceuticals. As a result, chitosan oligosaccharides that allow high concentration solutions with small viscosities have been prepared. [122]

There are a number of preparation methods for chitosan oligosaccharides. To mention a few, COS can be fabricated by adding chitosan to water containing a monobasic acid, such as hydrochloric or lactic acid, in order to prepare an intimate chitosan-water mixture that can be treated further with cellulase. Alternatively, the glycoside bonds of chitin can be split to reduce the molecular weight as chitin is deacetylated. Chitin glycoside linkages can also be cleaved by a dilute acid before deacetylation. These two methods include preparation in severe conditions, which may adversely influence chitosan properties. It is also difficult to prepare chitosans with an arbitrarily reduced molecular weight. COS can also be prepared by introducing chitosan to chlorine gas or treating it with aqueous peroxide solution of an aqueous peroboric acid solution. Naturally, using a toxic gas is undesirable especially in biomedical applications. The peroxide treatment, on the other hand, results in a molecular weight of 10 000 g/mol at the lowest, and can therefore not be dissolved in water in neutral conditions. [122]

A microwave-based preparation technique for LMW chitosan oligosaccharides has recently been patented. This method involves adding an acid solvent containing an electrolyte to chitosan and performing the reaction at 480-800 W for 3-12 minutes. After irradiation the solution is cooled down to room temperature and neutralised with 1-10 M NaOH or KOH to obtain a yellow floc. Thereafter, precipitation, filtering and dessiccation is performed to the solution and finally chitosan oligosaccharides are obtained. [57] Finally, also enzymatic hydrolysis and other synthetisation techniques for COS mentioned in Section 2.3.2 are applicable.

2.4.4. Chitosan hydrochloride

Chitosan hydrochloride (chitosan HCl) can be chemically modified from chitosan by preparing an aqueous chitosan suspension to pH 4.7 with 1N HCl. Next, the solution is filtrated to remove traces of undissolved material and the resulting solution is evaporated and a new water-soluble chitosan is formed. [18; 95]

2.5. Physicochemical properties of chitosan

The physicochemical properties of chitosan vary greatly depending on the type of chitosan, the production process, the natural source and other factors. Important chemical characteristics that influence the properties and hence the applications of chitosan, include the degree of deacetylation (DD), molecular weight, polydispersity, solubility, and charge. [5; 19; 111] These properties are discussed in more detail below.

2.5.1. Degree of deacetylation

The degree of deacetylation (DD) of chitosan is more than 50%, which means that the majority of the chitosan monomers are in the deacetylated form and possess an amino group at the C-2 position instead of an acetamido group (see Section 2.2) [44]. For commercial chitosans the DD is usually 70-95%. For medical applications the appropriate DD is 70-100%. [5] The DD is an important determinant of, for instance, the biocompatibility and solubility of chitosan [99]. It appears that a higher DD is characteristic of a higher biocompatibility [35]. Therefore it is important to choose chitosans with an appropriate DD for biomedical purposes. The DD can be determined using, for example, IR spectroscopy, gel permeation chromatography, UV spectrophotometry, ^{1}H-NMR spectroscopy, ^{13}C solid state NMR, acid-base titration methods, and near-infrared spectroscopy. It has been proposed that for gene delivery purposes the DD should be $\leq$ 80%, while for drug delivery systems the DD should be higher. [5]

2.5.2. Molecular weight, viscosity and polydispersity

The molecular weight (M_w) of chitosan ranges from 5 to 2,000 kDa [32; 114]. In some chitosan derivatives such as chitosan oligosaccharides, the M_w can be as low as 1.5 kDa

[33]. Due to this extensive range of molecular weights, chitosans can be categorised into low molecular weight chitosans (LMWC, 5-20 kDa) [60; 124], medium molecular weight chitosans (MMWC, ~100 kDa) and high molecular weight chitosans (HMWC > 300 kDa) [14]. It has been suggested that the LMWCs express higher bioactivity than MMWCs and HMWCs [14].

Like most polysaccharides, chitosan is polydisperse with respect to its molecular weight. Therefore, the "molecular weight" of a chitosan sample is the average over a whole distribution of molecular weights. This average can be calculated using the number average molecular weight (M_n) and the weight average molecular weight (M_w). The formulae for calculating these averages are given below in Equations 1 and 2. [111]

$$M_n = \frac{\sum_i N_i M_i}{\sum_i N_i} \tag{1}$$

$$M_w = \frac{\sum_i w_i M_i}{\sum_i w_i} = \frac{\sum_i N_i M_i^2}{\sum_i N_i M_i} \tag{2}$$

N_i is the number of molecules and w_i is the weight of molecules with a specific molecular weight M_i.

The molecular weight can be determined using light scattering, viscosimetry or chromatography [28, p.6-7; 111]. The viscosity average molecular weight (M_v) for chitosan can be determined using the Mark-Houwink equation:

$$[\eta] = K M_v^a \tag{3}$$

where K and a are constants for a given polymer-solvent system [38]. As can be seen from Equation 3, the Mark-Houwink equation describes the relationship between viscosity and molecular weight. The viscosity of chitosan solutions depends on factors such as degree of deacetylation, molecular weight, temperature, pH, ionic strength, and concentration. For instance, higher temperatures will generally lead to lower viscosities. [96]

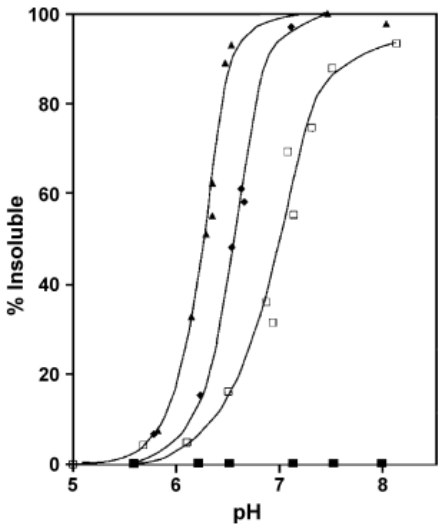
It could be argued that the molecular weight of chitosan depends on the degree of deacetylation. A higher DD would therefore imply a smaller M_w as the acetamido groups are replaced by amino groups of lower molecular weight. However, this relationship between M_w and DD is not very predictable. [49] For gene delivery purposes, it has been suggested that the molecular weight should be low (around 10 kDa) [5]. For drug delivery systems, on the other hand, it has been stated that HMWCs are desirable. [5]

The polymer dispersity of chitosan can be assessed using polydispersity (PD). The polydispersity refers to the width of the molecular weight distribution and it is calculated by dividing the weight average molecular weight by the number average molecular weight (M_w/M_n) [68]. In a polydisperse population $M_w > M_n$, while in a monodisperse molecule the number and weight average molecular weights are equal. Polydispersity near 2 is characteristic to a polymer that has been subjected to random depolymerisation, while PD below 2 indicates that fractionation has occurred during the production process. PD over 2 suggests a wider distribution and perhaps even a mixing

of products of different molecular weights to obtain a sample of certain average molecular weight. [111] In the literature chitosan has a PD of 1.4-7 depending on DD and M_w [54]. In dynamic light scattering (see Section 5.1) the concept of polydispersity is used to give an idea about the width of particle size distribution [68]. A polydispersity index (PdI) with a maximum of 1 is often used. In the literature, chitosan nanoparticle PDIs vary from 0.027-0.6 [63; 66].

2.5.3. Solubility and charge

Chitosan contains amino groups with a pKa value of 6.2-7, and it is considered as a strong base [99; 111]. Chitosan is soluble in dilute acidic solutions at a pH below 6. Acetic acid, formic acid, and lactic acid can be used to dissolve chitosan. Chitosan is also soluble in some dilute inorganic acids such as hydrochloric acid. Chitosan can also form water-soluble salts, such as chitosan acetate or chitosan lactate, with some aqueous inorganic or organic acids. [32; 93] The solubility of chitosan generally increases as the pH decreases. This is because at a low pH the amino groups of chitosan get protonated resulting in a water-soluble cationic polyelectrolyte. [19; 93; 111] In these conditions the surface charge of chitosan nanoparticles is positive, allowing it to interact with negatively charged surfaces [32]. However, if the pH is more than 6, the amines in chitosan become deprotonated and lose their charge, resulting in a neutral insoluble polymer. Also precipitation or gelation may occur. The soluble-insoluble transition of chitosan occurs at the pKa value when pH is 6-6.5. [111] Figure 2.7 illustrates the relationship between solubility and pH of chitosan in water. At the physiological pH 7.4 chitosan has low solubility in aqueous solutions. To overcome this problem, many soluble chitosan derivatives have been developed by modifying the reactive functional groups of chitosan (see Sections 2.2 and 2.4) or by depolymerising the chitosan. [5; 100]



Solubility vs. pH curves of chitosans. ▲ F_A = 0.01, ◆ F_A = 0.17, □ F_A = 0.37, ■ F_A = 0.60.

Figure 2.7. *Chitosan water-solubility vs. pH. F_A is the fraction of acetylated units. Modified from [112].*

Because the pKa of the amino groups depends on the degree of deacetylation, also the solubility of chitosan depends on this parameter as well as the method of deacetylation used. Other important factors affecting the solubility of chitosan are, for example, temperature, ionic strength, alkali concentration, time of deacetylation, and particle size. [19; 93; 111] As a result, it can be stated that the solubility of chitosan is quite difficult to control as it depends on so many parameters [93].

2.6. Biological properties of chitosan

Properties such as biocompatibility, non-toxicity and biodegradability make chitosan an interesting material for biomedical and pharmaceutical applications [5; 19]. Also hypocholesterolemic, antimicrobial, antitumor and antiviral activity has been associated with chitosan [5]. The following discussion elaborates some of the before-mentioned properties in more detail.

2.6.1. Biocompatibility and toxicity

Chitosan is generally considered biocompatible both *in vitro* and *in vivo*. This property is somewhat attributable to factors such as the natural source, molecular weight, DD and especially the preparation method [5]. The lethal dose (LD_{50}) for oral administration of chitosan has been reported to be 16 g/kg body weight in rabbits [39] and 10 g/kg body weight in mice [5; 19]. The cytocompatibility of chitosan has been reported *in vitro* with epithelial and myocardial cells. In addition, chitosan is cytocompatible with fibroblasts, chondrocytes, hepatocytes, and keratinocytes. Although the DD does not affect the cytocompatibility of chitosan on fibroblasts and keratinocytes, it does affect the cell adhesion *in vitro.* [13] In general, cell adhesion increases with increasing DD [19].

Chitosan is haemocompatible and it has been reported as a coagulating factor both *in vivo* and *in vitro*. *In vitro* chitosan maintains its coagulating properties even in severe anticoagulating conditions and in the presence of abnormal activity by platelets. In mice the LD_{50} of directly injected chitosan is 10 mg/day/kg body weight. However, high doses (200 mg/kg) have been reported to lead to hemorrhagic pneumonia in dogs. [19] The haemostatic properties of chitosan are due to the positive charge that interacts with negatively charged erythrocytes [5]. As a whole, chitosan can be regarded as non-toxic when administered in appropriate amounts.

2.6.2. Biodegradability

Chitosan can be hydrolyzed by enzymes such as chitosanase, glucosaminidase, chitobiase and N-acetyl-glucosaminidase [19]. In mammals, various proteases, including lysozyme and pepsin, are capable of degrading chitosan in appropriate pH, temperature and ionic strength [5; 19; 119]. The degradation leads to non-toxic chitosan oligosaccharides of different lengths that can be utilized in glycosaminoglycans and glycoproteins. Alternatively, these degradation products may contribute to metabolic pathways or simply be excreted. [5] Therefore, chitosan can be considered to be both biodegradable and bioresorbable *in vivo*.

The rate of degradation of chitosan is very important because it affects the biocompatibility of the material. For instance, scaffold degradation should be compatible with the rate of new tissue formation or be adequate for the controlled release of bioactive molecules. A very fast degradation will lead to an accumulation of amino sugars and cause an inflammatory response. Chitosan with low DD induce acute inflammatory responses, while high DD chitosans induce a minimal response as they degrade more slowly. [5; 19; 119] Also factors such as viscosity, molecular weight and crystallinity affect the biodegradability of chitosan. [5; 19] The rate of biodegradation can be assessed using for example viscosimetry and NMR spectrometry. [111]

2.6.3. Antimicrobial activity

Chitosan possesses antimicriobial activity towards a number of microorganisms such as bacteria, yeast, and fungi [5]. The antimicrobial activity of chitosan may be due to the interaction of the positively charged chitosan with anionic groups of bacterial cell surface. It has been suggested that this interaction creates an impermeable layer around the cell, which inhibits the transportation of essential solutes to the cell. Another antimicrobial mechanism is related to the inhibition of RNA and protein synthesis by permeation of chitosan into the cell nucleus. This mechanism is related to low molecular weight chitosans. Chitosan may also act as a chelating agent rendering trace elements, metals, and essential nutrients from the microorganism. [5; 30] Factors affecting the antibacterial activity of chitosan are DD, molecular weight, pH of the medium, and concentration in solution [61].

2.7. Medical applications of chitosan

Chitosan is diversely used in a number of fields such as agriculture, cosmetics, food industry, waste water treatment, pharmaceutics, dietetics, and biomedicine. Because this thesis concerns gene therapy and chitosan-DNA nanoparticles, only biomedical applications are discussed in more detail. Nevertheless, Table 2.1 gives an idea about the applications of chitosan in various fields.

Table 2.1. *Applications of chitosan. Modified from [28, p.10] and [30].*

(Bio)medical	Cosmetics	Water treatment	Pulp & paper	Food industry
gene delivery controlled drug release tissue engineering blood cholesterol control contact lenses oral hygiene wound dressing bandages, sponges anticoagulant antitumor	nail polish moisturiser creams bath lotions toothpaste make-up powder	flocculant chelating filtration	surface treatment carbon free copy paper photographic paper	preservation dietary supplements antioxidant prebiotics

As can be observed from Table 2.1, the main applications of chitosan in the biomedical field are related to gene delivery, wound healing, and tissue engineering. These are elaborated further in the following discussion with a specific focus on gene and drug delivery.

2.7.1. Drug and gene delivery systems

In drug delivery, the drug is usually combined with a polymeric material which protects the drug before its therapeutic action as well as delivers the drug to the body. The polymeric material should be biocompatible, non-toxic, biodegradable, stable, and sterilisable. Chitosan fulfills these requirements and is therefore an important drug delivery vehicle that enhances controlled release, drug absorption and bioadhesion. Chitosan can be delivered via parenteral, oral, nasal, transdermal, and ocular routes. Drug delivery systems of chitosan include nanoparticles, microparticles, hydrogels, solutions, films, tablets and so on. The applied system depends on the delivery route. For example, for oral delivery tablets, films or capsules may be used, while solutions and microspheres may be applied for the parenteral route. [5; 44]

The ability of chitosan to form drug and gene delivery systems is due to its cationic charge that interacts with negatively charged polyanions, such as DNA, in order to form polyelectrolyte complexes. Important properties affecting the drug delivery of chitosan are DD, M_w, and purity. Chitosans with a lower M_w have shorter chain lengths and are therefore more soluble in water at pH 7, which is important in, for example, cosmetics.

However, the relation of DD and M_w to chitosan properties is not very clear and their role depends very much on the application. Table 2.2 summarises the effect of DD and M_w on microspheres. [5]

Table 2.2. *Effect of physicochemical properties of chitosan on microsphere properties. Modified from [5].*

Physicochemical property	Properties of microsphere
DD ↑	size ↓
	loading capacity ↓
	surface roughness ↓
	swelling ↓
Mw ↑	sphericity ↑
	morphology homogeneity ↑
	release rate ↓

Relating to gene therapy, chitosan acts as a non-viral vector incorporating pDNA. Compared to viral vectors chitosan offers many advantages as it is not prone to oncogenic effects, immunological reactions or endogenous recombination. The preparation of chitosan-pDNA complexes is also cheaper than the production of viral vectors. The transfection efficiency of chitosan-pDNA complexes is highly dependent on the molecular weight. For instance, high M_w chitosans result in very stable complexes, but the transfection efficiency of these complexes is low. Therefore, LMWCs and chitosan oligomers have been applied in gene delivery. It appears that an appropriate balance has to be achieved between the extracellular DNA protection (better with high M_w) versus efficient intracellular unpackaging (better with low M_w). Also the effect of DD on gene transfection of chitosan-DNA nanoparticles has been studied and it appears that a very high DD (over 80%) releases DNA quite slowly. Hence, a DD below 80% may facilitate the release of DNA by lowering charge density, accelerating the degradation rate and increasing the steric hindrance in complexing with DNA. [5]

2.7.2. Wound dressing / healing

Chitosan activates immunocytes and inflammatory cells such as macrophages and fibroblasts, thereby contributing to wound healing. Chitosan also enhances the wound healing process by stimulating fibroblast production by affecting the fibroblast growth factor (FGF). Chitosan oligo- and monomers are used in wound healing and chitosan has also been combined with other materials, such as collagen and glycosaminoglycans, to improve its wound healing properties. In addition, antimicrobial agents such as silver sulfadiazine and chlorhexidine, have been added to chitosan. [5; 19; 44]

2.7.3. Tissue engineering

Tissue engineering (TE) is defined by Williams as the “creation (or formation) of new tissue for the therapeutic reconstruction of the human body, by the deliberate and

controlled stimulation of selected target cells through a systematic combination of molecular and mechanical signals". Classically, a material such as a scaffold or matrix can be used to support, reinforce or organise the regenerating structure. [118] Chitosan scaffolds are promising materials for tissue engineering applications because of their controlled biodegradability, biocompatibility, low immunogenicity, and porous structure. Other advantages of chitosan for TE include facile processing of the material into various shapes and forms as well as the maintenance of the structure at pH 7 once formed. Some TE applications of chitosan include nerve tubes, film sponges and hydrogels. [44]

3. GENE THERAPY

Gene therapy is based on treating inherited or acquired diseases by replacing or substituting defective or missing genes, altering the regulation of genes, or by silencing undesired gene expression. [75; 118] In detail, gene delivery refers to incorporating genetic material, such as DNA or RNA, directly or indirectly to specific cells, tissues or organs of a patient in order to start encoding desired proteins. Gene therapy is currently already applied in treating diseases such as AIDS, cystic fibrosis and cancer. [42]

3.1. Gene delivery

There are a number of methods used in gene therapy. These can roughly be divided into viral and non-viral methods. [126] Viral gene delivery is concerned with delivering genetic material via viral vectors such as adenovirus, adenoassociated virus, herpes simplex virus, or retrovirus. Non-viral methods can be categorised further into physical and chemical delivery systems. Non-viral physical systems include the delivery of naked DNA via needle/jet microinjection, hydrodynamic gene transfer, electroporation, gene gun, ultrasound, or sonoporation. Non-viral chemical gene delivery, on the other hand, relies on the delivery of genetic material via chemical carriers such as cationic lipids, cationic polymers, or inorganic nanoparticles. [3; 87; 123; 126]. Figure 3.1 summarizes the basic delivery methods of gene therapy as they are described in literature.

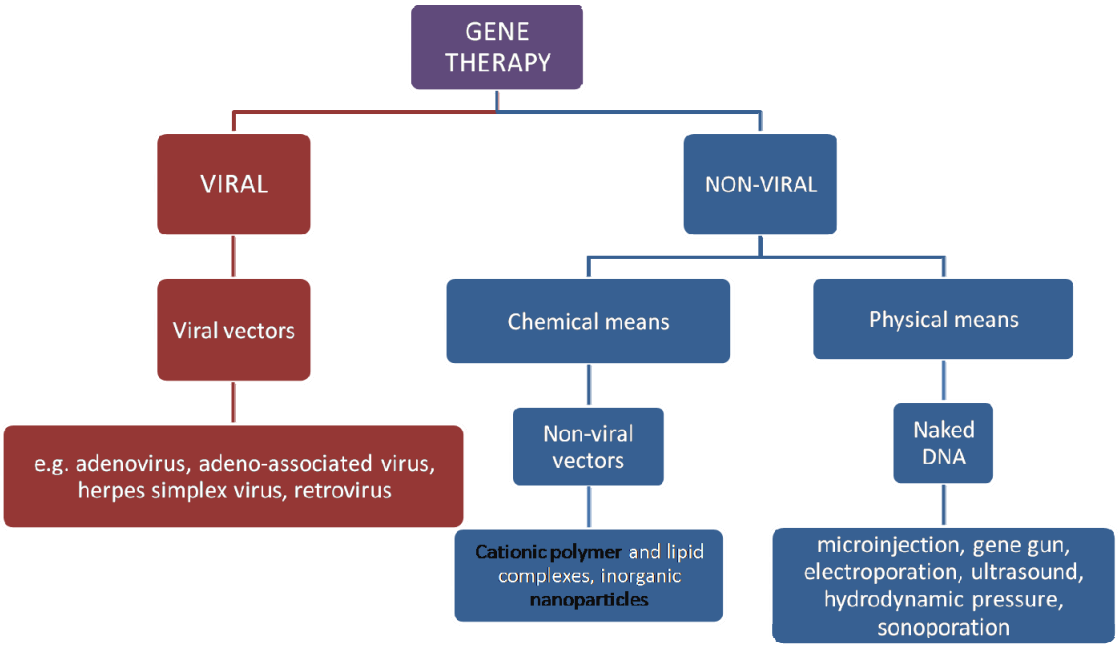


Figure 3.1. *Basic delivery methods of gene therapy. Figure based on information in [3; 72; 87; 123; 126].*

Despite the method used, numerous requirements have to be met when administering genetic material to patients. First, the gene delivery system should not be toxic or initiate immune responses. Second, the system should be able to protect the genetic material from nuclease degradation and other macromolecule disposal processes until it reaches the target site. Usually the target is the nucleus of a specific cell, which means that the system should be small enough for cell internalisation. Third, it should be possible for cells to take up the delivery system by endocytosis, pinocytosis or phagocytosis. Alternatively, the system can pass through transient holes created by physical means. Either way, it is important that the system does not transform into digestive lysosomes from the endosome. To prevent this, peptides, lipid components or osmotic pressure can be used to rupture the endosome. [3; 72] Finally, the system should be affordable, biocompatible, and biodegradable. At present, repeated administration is also a desired quality in gene therapy. [3]

Given the extensive list of requirements for gene delivery, it is not surprising that there are several obstacles related to this application. Some of these obstacles are biological barriers that can roughly be divided into biological extra- and intracellular barriers. [3; 92] Extra-cellular obstacles include anatomical features such as the epithelium, extra cellular matrix (ECM), and endothelial cell linings that directly prohibit the entry of macromolecules to target cells. Intracellular obstacles can be classified as enzymatic degradation, encapsulation in endosomes, inefficient cellular uptake, and failure of drug-polymer dissociation. [7; 75; 92]

3.2. Vectors

As described earlier, there are currently two main transport channels for exogenous DNA delivery to cells: viral and non-viral vectors. Basically, a non-viral vector is formed by the incorporation of DNA to a vector material, such as chitosan. The vector material may contain functional groups for environmental interactions including the promotion of cell binding or the limitation of serum component interaction. Also intracellular trafficking agents may be introduced. These serve to ease endosomal escape or facilitate nuclear pore trafficking. The vector is introduced to the body by a certain delivery method, such as injection. When the vector reaches the target cell, it is taken up by endocytosis and an endosome containing the vector is formed. Thereafter, endosomal escape occurs and the vector material is released into the cytoplasm of the cell. Some of the functional groups may dissociate at this point if they have already fulfilled their purpose. Finally, the vector containing the genetic material is transported to the nuclear membrane of the cell. The genetic material is released to the nucleus, where the desired proteins will be encoded. [3; 17; 72] The basic principle for the delivery of a non-viral vector into a cell nucleus is illustrated in Figure 3.2.

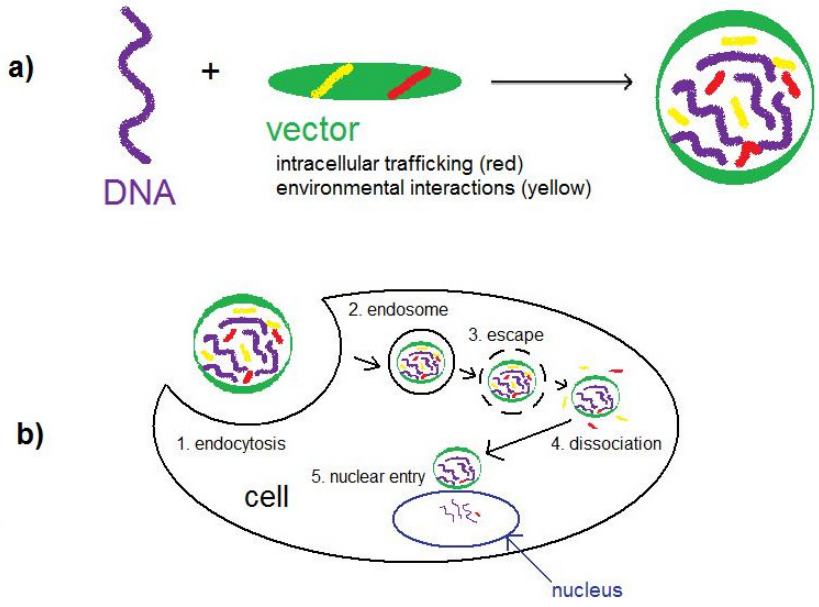


Figure 3.2. *Vector path in gene delivery. a) DNA is incorporated into a vector backbone (green) that may contain functional groups for environmental interactions (yellow) and/or intracellular trafficking (red). The intracellular functional groups enhance nuclear accumulation by facilitating endosomal escape or nuclear pore trafficking. Environmental functional groups may promote specific cell binding or limit serum component interaction. b) The vector enters the cell by endocytosis (1) and an endosome is formed (2). The vector contents then escape from the endosome in a process called endosomal escape (3). Most molecular components are dissociated from the vector (4) before it enters the nucleus (5) and releases the genetic material to be encoded. Modified from [3; 17; 72].*

Each vector type and vector subclass beholds its own specific characteristics. Viral vectors have been praised for high transfection efficiency *in vivo* and immunisation. However, there are several drawbacks such as toxicity, immunogenicity and inflammatory response, related to the use of viral vectors in gene delivery. Also insertional mutagenesis can occur. The latter refers to a situation in which the ectopic chromosomal fusion of viral DNA disturbs the expression of a tumour-suppressor gene or activates oncogenes, resulting in malignant transformation of cells. [75] Although some trials in gene therapy have been executed successfully [27; 94], unfortunately at least 2 patient deaths related to viral gene therapy have been reported [22; 97]. Therefore, it is clear that the use of viral vectors pose a safety concern in gene therapy, and it is utterly important to modify viral vectors in such a way that risks are minimised or preferably eliminated. Alternatively, other means to treat patients via gene therapy, such as the use of non-viral vectors, can be applied.

Non-viral vectors usually consist of cationic polymers (polyplexes) or cationic lipids (lipoplexes). The cationic charge of these molecules enables the electrostatic interaction with negatively charged DNA. As a result, polymer-DNA complexes are formed. Although non-viral vectors possess lower transfection efficiencies than viral vectors, they are generally less toxic and immunogenic than their viral counterparts. Other advantages of non-viral vectors include ease of synthesis and the possibility for repeatable administration with minimal host immune response. Large scale production is also easier compared to viral vectors and due to the larger size, more DNA can be packaged to non-viral carriers. [3; 72; 75]

The administration of naked DNA can also be considered as non-viral gene delivery. However, its use is limited only to tissues that are appropriate for direct injection such as skin and muscle. The use of naked DNA is also related to low transfection efficiency, as it is exposed to nuclease degradation. Hence, naked DNA cannot be administered systematically. [3; 72] Table 3.1 summarises the advantages and drawbacks of viral, non-viral and naked DNA gene delivery systems.

Table 3.1. *Advantages and drawbacks of some delivery systems in gene therapy. Adapted from [42].*

DELIVERY SYSTEM	ADVANTAGES	DISADVANTAGES
Viral vectors	• high transfection rate • rapid transcription.	• toxic • immune and inflammatory effects • viral wild-type mutations • potential oncogenic effects • insertional mutagenesis • safety issues • only small sequences of DNA can be inserted • poor target-specificity • low capacity to incorporate foreign DNA sequences to genome.
Non-viral vectors	• repeated administration • minimal host immune response • targetable • stable in storage • easy and cheap to produce in large quantities • low immunogenicity • protect DNA from nuclease degradation.	• toxicity (cationic lipids) • relatively low transfection efficiency (cationic lipids) • relatively low efficiency (polymers).
Naked DNA	• can be used for tissues that are suitable for direct administration, such as skin and muscle.	• unsuitable for systemic administration • low expression.

As can be seen from the table, the advantages related to non-viral vectors outweigh those of viral vectors by far. However, low transfection efficiencies still pose a challenge for the consistent use of non-viral vectors.

3.2.1. Chitosan as a non-viral vector

Mumper et al. were the first to report the use of chitosan as a gene delivery system in 1995 [20; 75; 81]. Since then, numerous authors have reported studies on chitosan as a non-viral vector for DNA [2; 7; 20; 37; 42; 43; 72; 74; 75; 127]. The reasons underlying the popularity of chitosan as a gene delivery system are attributable to its favourable characteristics such as biocompatibility, biodegradability, low immunogenicity, and reduced cytotoxicity compared to other cationic polymers. In addition, chitosan is relatively cheap and readily available. [7] Most commonly, chitosan has been complexed to DNA by ionic interactions between the negatively charged backbone of DNA and the positively charged amine groups of chitosan. This interaction results in a

stable complex, in which the chitosan protects the DNA from nuclease degradation. [75] Figure 3.3 illustrates the formation of chitosan-DNA nanoparticles through electrostatic interaction. Moreover, DNA can also be encapsulated in chitosan nanoparticles via traditional nanoparticle preparation methods (section 4.1) or it can be adsorbed on the particle surface through non-electrostatic secondary interactions such as hydrogen bonding. [75]

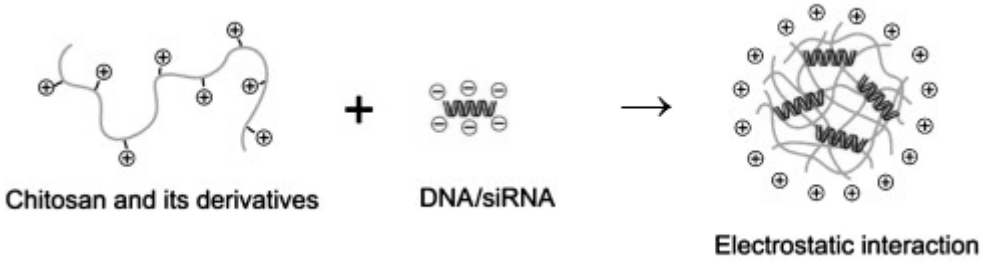


Figure 3.3. *Chitosan-DNA nanoparticles are formed through the electrostatic interaction between negatively charged phosphate groups in DNA and positively charged amine groups in chitosan. Modified from [75].*

Despite its many favourable characteristics, the low transfection efficiency of chitosan still remains a problem [75; 110]. This is due to the relatively low stability and buffering capacity of chitosan. Nevertheless, technical progress has allowed chemical modification of chitosan whereby its transfection efficiency can be improved without harming useful properties such as biocompatibility and biodegradability. [110]

4. NANOPARTICLES

Nanomedicine is concerned with applying nanotechnology to the treatment, diagnosis, monitoring, and control of biological systems. Nanotechnology, in turn, relates to creating objects, such as nanoparticles, that have at least one dimension of the order of 100 nanometres or less. One nanometre (nm) can be expressed as the billionth of one meter, 1 x 10^{-9} m. [1, p.22] Nanoparticles are solid colloidal particles that include both nanospheres and nanocapsules [79; 113]. Figure 4.1 illustrates the nature of these particles. The main difference between nanocapsules and nanospheres is that nanocapsules are reservoir particles in which a solid shell encapsulates a liquid phase, while nanospheres are particles whose entire mass is solid. Typically, the nanospheres are spherical in shape and their size ranges from tenths of nanometres to a few hundred nanometres. [113]

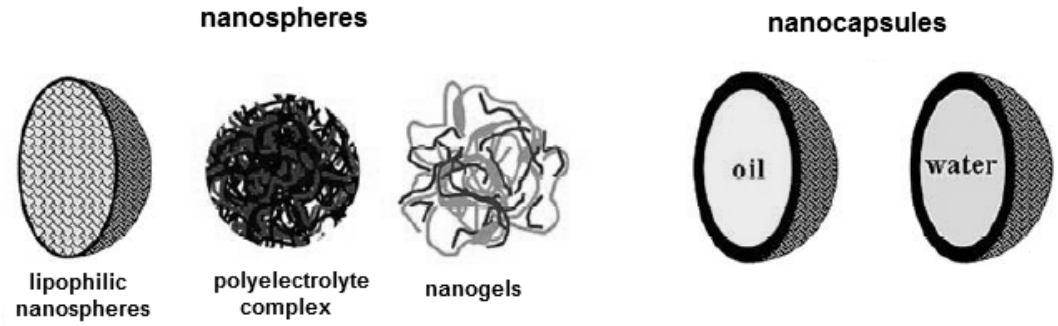


Figure 4.1. *The nature of nanoparticles including different nanospheres and nanocapsules. Modified from [113].*

Nanoparticles possess a vast number of favourable characteristics, which make them applicable for drug delivery. Nanoparticles designed for drug or gene delivery are capable of entrapping the desired drug or gene in the particle or adsorbing the drug on the particle surface. As a result, the drug is protected from instant enzymatic degradation in a biological medium.

The subcellular size of nanoparticles allows higher intracellular uptake than other particle systems. In addition, targetability and functionality of nanoparticles can be improved by attaching, for example, ligands, polysaccharides or functional groups to the particle. [79; 113] Table 4.1 below summarises common materials used in nanoparticulate carrier systems. As can be seen from the table, chitosan is one of the natural polymers used in drug delivery, in addition to alginate, gelatin and albumin.

Table 4.1. *Common materials for nanoparticles in drug delivery. [113]*

Material	Full name	Abbreviation or Commercial names*
Synthetic homopolymers	Poly(lactide)	PLA
	Poly(lactide-co-glycolide)	PLGA
	Poly(epsilon-caprolactone)	PCL
	Poly(isobutylcyanoacrylate)	PICBA
	Poly(isohexylcyanoacrylate)	PIHCA
	Poly(n-butylcyanoacrylate)	PBCA
	Poly(acrylate) and poly(methacrylate)	Eudragit*
Natural polymers	Chitosan	
	Alginate	
	Gelatin	
	Albumin	
Copolymers	Poly(lactide)-poly(ethylene glycol)	PLA-PEG
	Poly(lactide-co-glycolide)-poly(ethylene glycol)	PLGA-PEG
	Poly(epsilon-caprolactone)-poly(ethylene glycol)	PCL-PEG
	Poly(hexadecylcyanoacrylate-co-poly(ethylene glycol) cyanoacrylate)	Poly(HDCA-PEGCA)
Colloid stabilisers	Dextran	F68
	Pluronic F68	PVA
	Poly(vinyl alcohol)	
	Copolymers (see above)	
	Tween® 20 or Tween® 80	

Nanoparticle properties may vary depending on the preparation process, particle size, zeta potential, pH, or morphology. Therefore, it is important to control these parameters to attain desired properties. [79] In addition to chitosan, other common polycations for the preparation of polyplexes include poly(ethylenimine) (PEI), poly(lysine) (PLL) and poly(ornithine). The resulting internal nanoparticle structure prepared by polyelectrolyte complexation can be described as a gel like internal structure where the complexed polyelectrolyte chains are swollen by water molecules. [113]

4.1. Preparation of chitosan-DNA nanoparticles

Various methods, such as complex coacervation, ionic (or ionotropic) gelation, chemical cross-linking, as well as solvent evaporation have been used to prepare chitosan micro- and nanoparticles [2; 75; 76; 89; 104; 113]. Preparation methods for chitosan nanoparticles are summarised in Figure 4.2.

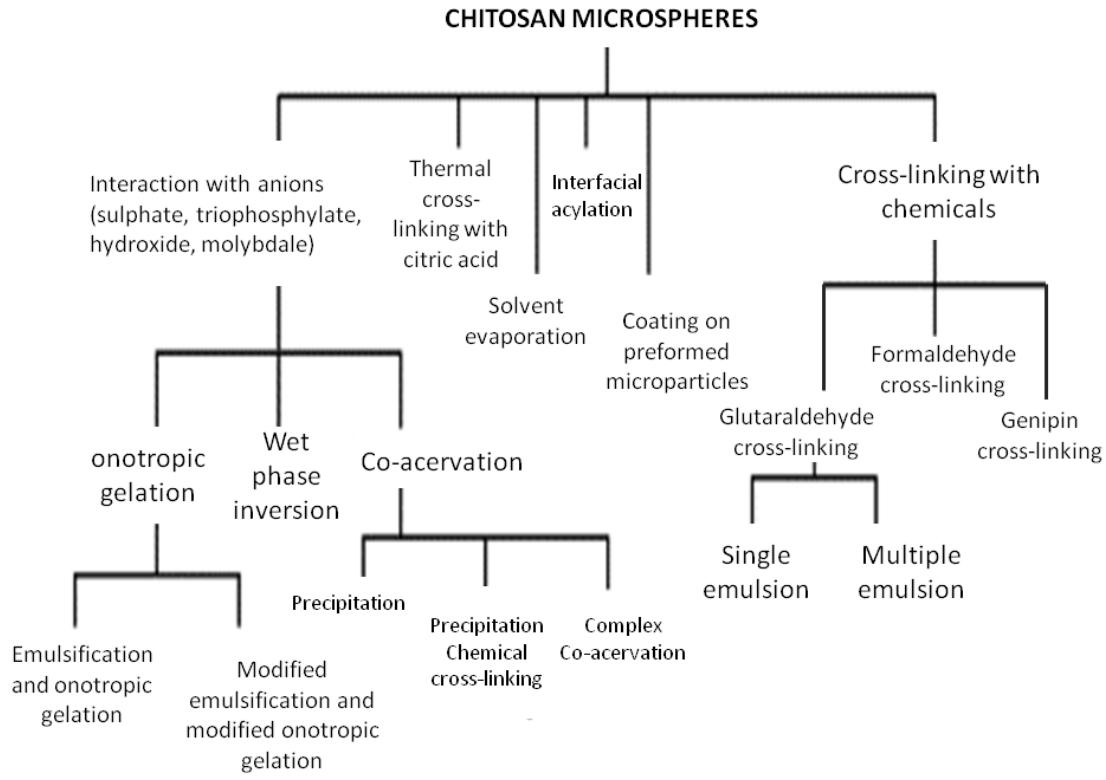


Figure 4.2. *Fabrication techniques for chitosan particles. [104]*

Micro- or nanoparticle preparation methods can also be used to prepare polymeric drug or gene delivery devices. Vauthier and Bouchemal have recently reviewed preparation techniques for polymeric drug loaded nanoparticles. Also strategies for attaining controlled release in vivo are explained in this review. In addition, further processing techniques such as sterilisation, lyophilisation and purification are discussed. [113] Many nanoparticle preparation techniques include two steps: preparation of an emulsified system and formation of nanoparticles. Particle formation is often due to precipitation or gelation of the polymer. It is also possible that the particles form already in the emulsified system or by another method requiring only one preparation step. For example, the self assembly of macromolecules to form polyelectrolyte complexes from polymer solutions can be regarded as a one step process. [113]

In the case of active agent loaded chitosan nanoparticles, the most common preparation methods include complex coacervation, emulsion droplet coalescence, ionic gelation, and a reverse micellar method [2]. Table 4.2 summarises the preparation techniques for chitosan nanoparticles involving specific drugs or components.

Table 4.2. *Preparation methods of nanoparticles for delivery of different drugs and components. Modified from [2].*

System type	Preparation method	Drug / component
Nanoparticles	emulsion-droplet coalescence	gadopentetic acid
	coacervation/precipitation	DNA, doxorubicin
	ionic gelation	insulin, ricin, bovine serum albumin, cyclosporin A
	reverse micellar method	doxorubicin

Ultimately, the selected preparation method depends on desired qualities such a specific particle size, chemical stability of the active agent, degradation kinetics, as well as the stability and residual toxicity of the final product. As can be seen from Table 4.2, complex coacervation is used to prepare chitosan-DNA nanoparticles. Recently, an osmosis-based preparation technique for chitosan-DNA nanoparticles has also been introduced and patented. [2; 76] Because complex coacervation is the method of choice for this thesis, only this technique will be elaborated in the following discussion.

4.1.1. Complex coacervation

In complex coacervation, an aqueous polymeric solution is separated into two immiscible liquid phases: a dense, polymer-rich coacervate phase and a dilute equilibrium phase [25]. The basic principle of complex coacervation in chitosan nanoparticle preparation is that chitosan is insoluble in alkaline medium, but precipitates when it comes into contact with alkaline solution such as NaOH, NaOH-methanol or ethanediamine. As a result, particles are formed by blowing chitosan solution into alkali solution using a compressed air nozzle. This results in the formation of coacervate droplets. The coacervation process is illustrated in Figure 4.3. The size of the droplets can be controlled by using specific air pressure or spray nozzle diameters. Finally droplets are filtered and purified. A cross-linking agent can be used to harden the particles and to facilitate controlled drug release. [2]

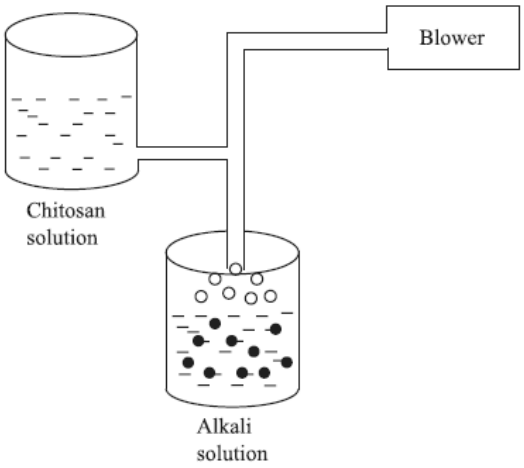


Figure 4.3. *Coacervation using an air nozzle. [2]*

Coacervation can also be accomplished by adding sodium sulphate or citrate dropwise to an aqueous acidic chitosan solution under stirring and ultrasonication for 30 minutes [2]. The function of sodium sulphate (Na_2SO_4) is to act as a coacervate agent that facilitates the phase separation process of the chitosan solution. However, addition of a non-ionic surfactant will stimulate particle formation with the sulphate itself. [53] Before addition of sodium sulphate, acetic acid is usually added to dissolve chitosan into an aqueous solution. Also sodium acetate solution is added to act as a buffer. In the case of water-soluble chitosans addition of acetic acid can naturally be omitted. [74]

Mao et al. have described in detail the application of complex coacervation for the preparation of chitosan-DNA nanoparticles. This method includes heating a chitosan solution (0.02% w/v in 5 mM CH_3COONa buffer, pH 5.5) and a DNA solution (100 µg/ml in 5~50 mM Na_2SO_4 solution) to 50-55°C separately. An equal amount, typically 500 µl of each solution is mixed and vortexed quickly for about 30 s to yield uniform chitosan-DNA nanoparticles. [74] The formation of the particles is based on electrostatic interactions between the positively charged chitosan and the negatively charged DNA backbone [75].

4.2. Properties of chitosan-DNA nanoparticles

This section highlights the properties of different chitosan-DNA nanoparticles prepared by complex coacervation. Special attention is paid to factors affecting the size and zeta potential of the particles. Also biological properties of nanoparticles will be discussed in brief.

4.2.1. Size

In the literature, chitosan-DNA nanoparticle size generally varies from 50 to 500 nm [74; 76; 81]. However, it should be kept in mind that the variation in preparation techniques may affect the size. [75] Polyelectrolyte complexation usually results in sizes of tenths of nanometres to a few hundred nanometres [113]. Mumper et al. were the first to report the use of chitosan as a gene delivery system in 1995. They concluded that the particle size ranging from 150-500 nm was dependent on the molecular weight of the chitosan (108-540 kDa). Buffer composition or sugar presence did not affect the size. [81] Mao et al. have recently made an extensive literature review concerning the different factors affecting chitosan-DNA nanoparticle properties. Research suggests that a decrease in molecular weight will also lead to a decrease in particle size. [75] For example, Huang et al. found that mean particle size decreased from 181 to 155 nm as M_w was reduced from 213 to 48 kDa [37]. However, decreasing the molecular weight further caused an increase in particle size and chitosans with a M_w of 10 and 17 kDa yielded nanoparticles of size 289 and 269 nm, respectively. [37] On the contrary, Maclaughlin et al. discovered that particle size decreases while molecular weight is decreased, even if low molecular weight chitosans are used [65]. Figure 4.4 illustrates

the correlation between molecular weight, degree of deacetylation, particle size and zeta potential.

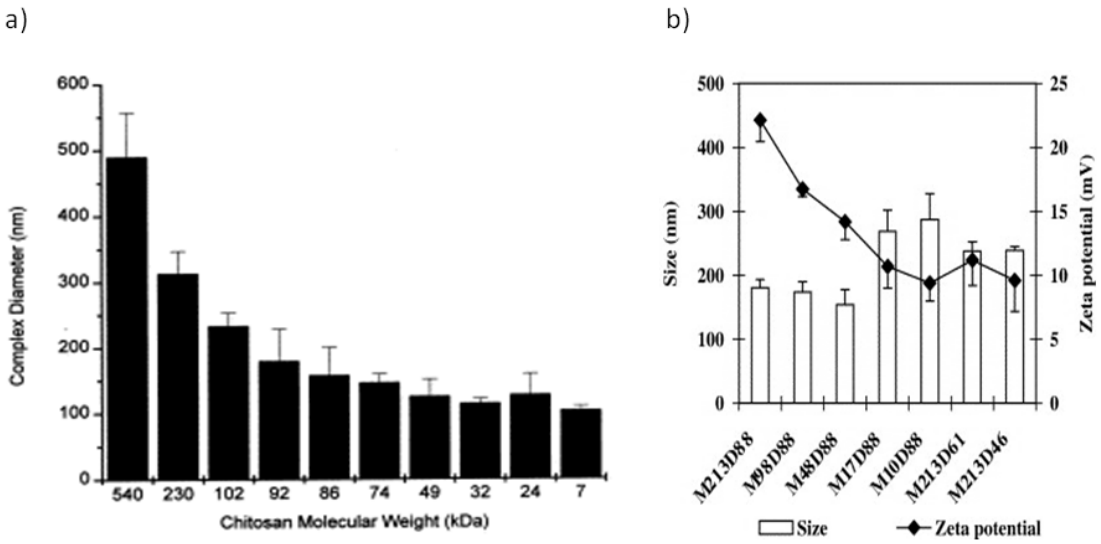


Figure 4.4. *a) Effect of molecular weight on particle size and b) effect of molecular weight and degree of deacetylation on the size and zeta potential of chitosan-DNA nanoparticles. On the x-axis, M and D signify the molecular weight and degree of deacetylation, respectively. Modified from [65] and [37].*

In addition to the molecular weight, also chitosan:pDNA charge ratio appears to be an important parameter affecting the nanoparticle size [7]. Chitosan:pDNA ratio is commonly expressed as the ratio between polymer nitrogen (N) per DNA phosphate (P) or N/P. [7; 20] It has been reported that at an N/P ratio of 2 and a zeta potential of 0 mV particles will aggregate [20]. Mao et al. also found that the particle size was dependent on the N/P ratio and reported particle sizes of 150-250 nm with N/P ratios of 3-8 in a complex coacervation process, where a chitosan concentration of 100 µg/ml was used. An N/P ratio of 1 yielded large aggregates. Nevertheless, an N/P ratio below 0.75 or above 2 produced submicron range particles. The M_w and DD of the chitosan used in this study was 390 kDa and 83.5%, respectively. The size of the plasmid, or the sodium sulphate concentration had no effect on the particle size. [74] Moreover, Mansouri et al. have found that the size of chitosan-DNA nanoparticles prepared by complex coacervation is indirectly proportional to the charge ratio, while the zeta potential is directly proportional to the charge ratio [71]. As a whole, it could be stated that preparation method, molecular weight, and charge ratio are important factors affecting the size of chitosan-DNA nanoparticles. However, the exact methods of action of these factors remain unclear, and there is a lot of controversy in literature. [20; 37; 65; 71; 74] Currently there is also a lack of research addressing the effect of the degree of deacetylation on particle size.

4.2.2. Zeta potential

Zeta potential determines whether particles in a liquid are likely to flocculate, and it can be used as one factor, in addition to size, pH, and conductivity, to assess the stability of particles. Zeta potential is an important parameter in a variety of fields such as medicine, pharmaceuticals, water treatment, and brewing. [69] In the case of chitosan nanoparticles, the zeta potential can be used to evaluate suspension stability and adhesion of particles to biological systems [55].

The zeta potential results from the electric charges related to particles in a liquid. A positively charged sample will attract negative ions from the surrounding liquid. Ions close to the sample surface will bind to it more strongly than ions that are further away. This first layer of tightly bound ions on the particle surface forms the Stern layer, while the more distant ions form a diffuse layer that is composed of loosely attached positively and negatively charged ions surrounding the particle. There is a notional boundary within the diffuse layer that separates ions that will move along with the particle as it moves in the liquid from those around the boundary that will stay where they are. This boundary is referred to as the slipping plane or shear plane. The charge at the slipping plane is extremely sensitive to the concentration and types of ions in the solution. The zeta potential is, in fact, the electrical potential that exists at the slipping plane. [103] Figure 4.5 is a schematic illustration of the zeta potential of a particle. As can be seen, the potential decreases as the distance from the particle surface increases.

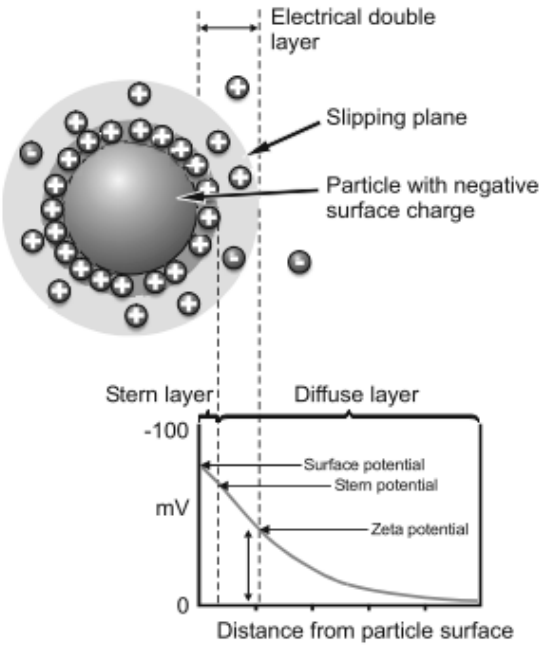


Figure 4.5. *Relation between particle surface charge and zeta potential. [67, p.1602]*

Particles with a high zeta potential of the same charge will repel each other. In general, a potential less than -30 mV and more than +30 mV is considered high. This is also the dividing line between stable and unstable suspensions. That is, particles with a

zeta potential of less than -30 mV or more than +30 mV are considered stable, whereas particles possessing a zeta potential from -30 mV to +30 mV are considered unstable. For the latter there is no force preventing molecules from coming together and flocculating. [67, p.1602]

Like chitosan, chitosan nanoparticles also possess a positive surface charge. This is explained by residual protonated amino groups of chitosan that do not take part in neutralisation with negatively charged DNA. It has been speculated that a higher degree of deacetylation would lead to higher zeta potentials as more protonated amino groups are available. Also pH has a crucial effect on zeta potential and, in general, zeta potential is higher when pH is less than 6 and chitosan is in its protonated form. Moreover, also the type of chitosan used may affect the zeta potential. Although N-acyl chitosan particles initially have a lower zeta potential than unmodified chitosan, they also undergo less reduction of zeta potential when increasing the pH closer to physiological conditions. [55]

In the literature, the zeta potential of chitosan-DNA nanoparticles varies between 0 and 40 mV at a pH of 5.5. The majority of the studies present a zeta potential of approximately 20 mV. [37; 55; 74; 80]. Therefore, the majority of zeta potentials presented in the literature suggest that particles actually are theoretically unstable. Nevertheless, the dividing line of stability at -30 or +30 mV is not always very clear [67, p.1602]. Particles possessing a zeta potential of about 20-25 mV may therefore sometimes be considered relatively stable [55]. It should be noted that some studies do not present which calculation parameters were used while measuring the zeta potential. There is also controversy among the parameters that are explained. For example, in the study by Moreira et al., a Smoluchowski approximation was used in zeta potential calculation, whereas Klausner et al. used a Huckel approximation. [48; 80] This gives rise to error, as using a Smoluchowski approximation instead of a Huckel approximation yields smaller values for the zeta potential (see Section 5.2.1). However, articles usually agree on a measurement temperature of 25°C and a measurement angle of 90° or 173° [48; 54; 62; 74].

Huang et al. have studied the effect of M_w and DD on the on the transfection efficiencies of chitosan vectors. In their study, the correlation between the zeta potential, cellular uptake, and transfection efficiency of chitosan-DNA nanoparticles was demonstrated. It appears that electrostatic interactions with the cell membrane mediate cellular uptake and improve transfection efficiency. The highest zeta potential (23 mV) was achieved with a chitosan possessing a DD of 88% and an M_w of 213 kDa. This result is illustrated in Figure 4.3b. From the figure it could also be concluded that the zeta potential decreases when M_w decreases at a constant DD of 88%. [37]

Klausner et al. have recently examined chitosan-DNA nanoparticles based on NOVAFECT ultrapure chitosan oligomers. It was found that oligomeric chitosan-DNA nanoparticles possessed a strong positive surface charge and had a zeta potential of 44.1 ± 3.5 mV. The size of the particles was small, $\leq$ 98.2±4.4 nm. They also demonstrated good transfection efficiency in vitro. It should be noted, however, that the

Huckel approximation yielding higher zeta potential was used in this study. More consistency and justification for the use of certain parameters is evidently needed in scientific research to make studies more comparable. [48] Table 4.3 summarises some results of chitosan-DNA nanoparticles prepared by complex coacervation.

Table 4.3. *Size and zeta potential of chitosan-DNA nanoparticles.*

Chitosan M_w (kDa)	**Chitosan DD (%)**	**N/P**	**Size (nm)**	**Zeta potential (mV)**	**pH**	**Reference**
-	87	> 3	260	16	5.5	[80]
390	83.5	3-6	100-250	12-18	<6	[74]
60	90	10	167.4	8.6	5.5	[24]
-	-	10	498.6	0.8	7.4	[24]
150	-	1 > 1 7	>300 -> 120	~0 - 15	-	[71]
varies	-	-	≤ 98	44.1*	-	[48]
-	-	-	148-179.5	12.10-14.6	-	[120]
150 400 600	-	-	< 100	-	5.5	[15]
9 12 65 114 170 173	95 77 78 84 84 54	50 50 50 50 50 50	3500 500 200 200 200 200	10-20 (increased slightly with higher M_w, except 173-54, due to low DD)	5.5	[62]**
chitosan lactate	87				-	[115]
20		2,4,8,**12**,24	246.7 ± 6.6	28.2 ± 0.30		
45		2,4,8,**12**,24	300.0 ± 3.7	30.3 ± 1.10		
200		2,4,8,**12**,24	481.1 ± 12.9	35.7 ± 0.80		
460		2,4,8,**12**,24	538.9 ± 15.0	35.4 ± 0.10		

**Huckel approximation was used*

*** In this study, chitosan-siRNA nanoparticles were prepared*

- Information not available

As can be seen from the table above, the pH has an important role in the determination of size and zeta potential. Increasing the pH from 5.5 to the physiological pH 7.4 may result in drastic increases in size and reduction of particle stability (see [24]). It also appears that the size and zeta potential of chitosan lactate increases with increasing M_w with a constant N/P ratio of 12. Another important observation from the table is that data concerning specific studies is relatively poor, which makes it difficult to compare results.

Although not much extensive literature exists on the effect of different material properties on particle parameters, some studies concerning this have been reported in

[116]. Weecharangsan et al. prepared chitosan derivatives (chitosan hydrochloride, chitosan acetate, chitosan lactate, chitosan aspartate and chitosan glutamate) with different molecular weights and N/P ratios and evaluated the effect of these parameters on chitosan-pDNA nanoparticle size and zeta potential. The DD of the chitosan was 87% and pH of the solution was 6.5. It was found that complete complexes formed in all chitosans at N/P ratios above 2 in all chitosans of M_w 45 kDa. Particle size increased with increasing charge ratio from 1 to 2 and then decreased to constant in the range of 101-299 nm when N/P was above 2. At N/P 2, the zeta potential was approximately neutral but above 2 the zeta potential ranged from 15 to 28 mV. Weecharangsan et al. also found that higher M_w chitosans yielded higher particle size and zeta potential than LMWCs. Among the chitosan derivatives, the size ranks were the following at N/P 2 where size was greatest: chitosan glutamate (856 nm) < chitosan aspartate (1276 nm) < chitosan acetate (1390 nm) < chitosan lactate 2553) < chitosan hydrochloride (3446 nm). [116]

4.2.3. Long-term stability of nanoparticle size and zeta potential

The stability of physicochemical particle properties, such as size, has been extensively studied *in vitro*. Degradation rate and drug release rate affect the particle size *in vivo* and hence these factors also have an important role in the biocompatibility and toxicity of the nanoparticles. Also transfection efficiencies have been explored vastly. [6; 8; 37; 101] However, there is a lack of information about the long-term stability of chitosan based polyplexes *ex vivo* [101]. Nevertheless, it is equally important to consider stability in these conditions as preparation processes may last a long time, particles may not be directly used *in vivo* and in the long term, off-the-shelf nanoparticles may be desired.

Romøren et al. assessed the long-term transfection efficiencies and the stability of chitosan-based polyplexes at different temperatures in an acetate buffer at pH 5.5. It was found that storage at 4°C for one year did not result in any major changes in particle properties. During storage at 25°C there were minor changes in the physicochemical characteristics and the transfection efficiency was reduced. At storage of 45°C, major differences occurred in particle properties after a short time. More specifically, particle size remained stable over the period of 1 year during storage at 4°C independent of the type of chitosan used. However, particle size was affected by storage at 25°C and this change was chitosan-type dependent. For instance, chitosan particles with lowest M_n (6.6 kDa, DD 83%) increased by 40% in size from an initial size of 148±26 nm, while HMWCs increased by 3-14% At storage of 45°C, a massive increase in particle size was observed independent of the chitosan used. [101]

The zeta potential of the particles stored at 4ºC and 25°C over a period of one year decreased by approximately 5 mV, as can be seen from Table 4.4. The particles stored at 45°C, however, experienced dramatic changes. [101]

Table 4.4. *Effect of temperature on chitosan-DNA nanoparticle properties during a period of 1 year. Chitosan types SC113 (M_n 6.6 kDa, F_A 0.17), SC211 (M_n 90 kDa, F_A 0.32), SC214 (M_n 160 kDa, F_A 0.15), SC312 (M_n 160 kDa, F_A 0.15). [101]*

	Zeta potential (mV)								
	4°C			25°C			45°C		
Chitosan/DNA	0 w	22 w	52 w	0 w	22 w	52 w	0 w	22 w	52 w
SC113									
PP	15.9 ± 2.6	17.8 ± 1.1	11.7 ± 1.9	15.9 ± 2.6	17.5 ± 0.9	14.8 ± 2.4	15.9 ± 2.6	−6.1 ± 0.7	ND
Sol	15.9 ± 2.6	19.7 ± 3.0	12.1 ± 1.3	15.9 ± 2.6	16.1 ± 2.2	15.2 ± 1.4	15.9 ± 2.6	−27.1 ± 0.3	ND
SC211									
PP	21.3 ± 1.8	19.6 ± 0.5	14.3 ± 3.4	21.3 ± 1.8	19.3 ± 0.7	13.7 ± 2.9	21.3 ± 1.8	1.6 ± 1.8	ND
Sol	21.3 ± 1.8	19.4 ± 0.9	10.9 ± 3.5	21.3 ± 1.8	18.3 ± 1.8	10.3 ± 1.8	21.3 ± 1.8	0.8 ± 4.8	ND
SC214									
PP	23.5 ± 4.5	22.9 ± 1.3	16.7 ± 3.4	23.5 ± 4.5	21.7 ± 0.2	17.9 ± 0.6	23.5 ± 4.5	−0.5 ± 0.3	ND
Sol	23.5 ± 4.5	22.1 ± 0.7	13.6 ± 3.0	23.5 ± 4.5	22.2 ± 0.3	14.3 ± 3.4	23.5 ± 4.5	1.6 ± 0.8	ND
SC312									
PP	22.4 ± 1.9	23.3 ± 1.3	17.5 ± 2.8	22.4 ± 1.9	21.3 ± 1.3	17.9 ± 3.2	22.4 ± 1.9	−4.1 ± 5.7	ND
Sol	22.4 ± 1.9	22.7 ± 1.1	18.5 ± 1.4	22.4 ± 1.9	23.1 ± 0.8	14.3 ± 3.1	22.4 ± 1.9	8.9 ± 3.43	ND
DNA									
Sol	22.4 ± 1.9	23.5 ± 1.3	23.5 ± 1.7	22.4 ± 1.9	22.9 ± 1.0	22.9 ± 0.3	22.4 ± 1.9	22.1 ± 1	ND

w, weeks; ND, not determined; PP, chitosan aged in polyplexes; sol, chitosan/DNA aged in solution.
The results are expressed as means ± SD (n = 3).

In a study by Bozkir and Saka it was found that particle size and zeta potential of chitosan-DNA nanoparticles prepared by complex coacervation does not change significantly over a period of 3 months at pH 5.5. However, after lyophilisation with a cryoprotective agent, differences in particle sizes were observed.[8] Vauthier and Bouchemal have proposed the storage of nanoparticles as solids, as the storage as suspensions may result in premature polymer degradation, microbiological contamination, loss of drug activity, or physicochemical instability due to particle sedimentation or aggregation. The transformation from a liquid system to solids can be achieved through freeze-drying or spray-drying processes. However, the applicability of these processes to polyelectrolyte complexes is not addressed. [113]

4.2.4. DNA binding

One important aspect in the preparation of chitosan-DNA nanoparticles is the binding capacity of DNA. Studies have shown that DNA binding is dependent on the degree of deacetylation and molecular weight. For example, high molecular weight chitosans bind more DNA than medium or low molecular weight chitosans. If the molecular weight remains constant, a higher degree of deacetylation will lead to better binding than lower deacetylation. [36; 45] This is most likely due to the fact that a higher DD will result in increased positive charge, which enables a higher DNA binding [75]. Huang et al. have found that at N/P ratio of 6, more than 90% of the DNA is condensated regardless of the DD or M_w. [37] For low molecular weight chitosans of a similar DD, the charge ratio must be increased to completely bind the DNA [45].

4.2.5. Morphology of chitosan-pDNA nanoparticles

The morphology of chitosan-DNA nanoparticles has been assessed by using scanning electron microscopy (SEM), transmission electron microscopy (TEM) and atomic force microscopy (AFM). [15; 45; 47; 74; 77] TEM allows the visualization of DNA inside chitosan complexes, while AFM gives a better idea of the size and morphology of the particles [15]. Because of the high resolution and ability to yield 3-dimensional images, SEM has also been used to evaluate the size and surface morphology of chitosan-DNA nanoparticles. However, images differ somewhat depending on the type of SEM and the preparation method of the nanoparticles. Figure 4.6 illustrates some SEM images. A more detailed description of the function of SEM is presented in Section 5.4.

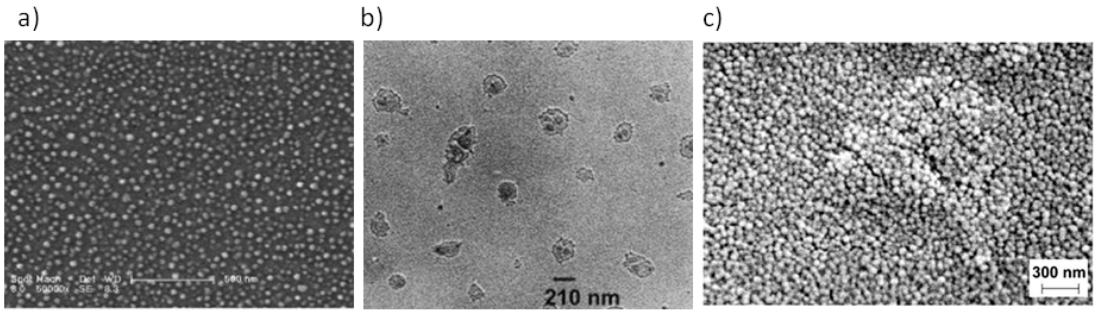


Figure 4.6. *a) Chitosan-pDNA (pC1-PDGF) nanoparticles prepared by complex coacervation and characterized by SEM. Nanoparticles were about 30-40 nm in diameter and of spherical shape. Modified from [59]. b) Chitosan-pDNA nanoparticles prepared by complex coacervation. Modified from [74]. c) Chitosan-pDNA nanoparticles prepared by an osmosis-based method. Average size 45 (±10) nm. Modified from [76].*

As can be seen from Figure 4.6, particles are generally of spherical shape. Corsi et al. prepared chitosan-DNA nanoparticles by complex coacervation and then characterized the particles with TEM and AFM. Their results also indicate that particles are of spherical shape and have a diameter of less than 100 nm. Particle size is essential, as it has been shown that polycation-DNA complexes mainly enter cells via endo- or pinocytosis. For this, it is crucial that the diameter is less than 100 nm. [15] Also Kim et al. have found that chitosan-DNA nanoparticles are of spherical shape and have compacted structure. The size of the particles depended on the charge ratio of galactosylated chitosan to DNA, and it was observed that the size decreased while the ratio increased.[47] In a study by Kiang et al. particles were of spherical shape, and the size and morphology was not significantly affected by different DDs and charge ratios. [45]

4.2.6. Biological properties

Biological properties of chitosan-DNA nanoparticles are usually described in terms of transfection efficiency and cytotoxicity *in vitro* or *in vivo*. Nanoparticles intended for this use are often slightly modified by, for instance, altering pH or adding appropriate ligands to enhance cell specific targeting. [2; 15; 20; 37; 71; 74; 107] Mao et al. found that transfection of chitosan-DNA nanoparticles is cell-type dependent. In this study LipofectAMINE™ was used as a control and it was found that transferrin conjugated chitosans yielded a 4-fold increase in transfection efficiency in HEK293 and HeLa cells, while KNOB protein conjugated particles were able to improve the gene expression level in HeLa cells 130-fold. On the other hand, the initial clearance of PEG conjugated chitosan nanoparticles in mice was slower than that of unmodified chitosans, despite the fact that they did not aggregate upon lyophilisation [74]. Also Corsi et al. used LipofectAMINE™ as a control to study transfection efficiencies of unmodified chitosan-DNA nanoparticles on human osteosarcoma cells (MG63), mesenchymal stem cells (MSC) and human embryonic kidney cells (HEK293). It was observed that while the transfection efficiency of HEK293 was superior to that of MSC and MG63, it did not surpass that of LipofectAMINE™. Cell viability studies by Corsi et al. also confirmed the non-toxicity of chitosan. [15; 74] The studies conducted by Mao et al. and Corsi et al. demonstrate that surface modification may, indeed, have a crucial role impacting the transfection efficiency of chitosan-DNA nanoparticles.

As mentioned earlier, the most important factors affecting transfection efficiency are the size and zeta potential. These were discussed in more detail in the previous sections. However, it should be stressed out that generally small size, high surface charge, and complex stability facilitate endocytosis and protect the complex in endosomal-lysosomal conditions before cargo release resulting in higher transfection efficiency. [62]

In addition to transfection efficiency and cytotoxicity, also other biological factors such as gene expression and biocompatibility have to be considered in the administration of chitosan-DNA nanoparticles. Dai et al. compared the gene expression of PEI-DNA and chitosan-DNA nanoparticles *in vivo* in rat liver, and it was demonstrated that chitosan-DNA nanoparticles achieved a 17-fold higher luciferase expression than PEI-DNA nanoparticles. [16] Furthermore, Liu et al. studied the cytotoxicity and gene expression of chitosan-siRNA nanoparticles on the human lung cancer cell line (H1299) that expresses enhanced green fluorescent protein (EGFP). The results obtained in the study indicate that chitosan-siRNA nanoparticles prepared from higher M_w and DD chitosan show higher gene silencing efficiencies (45-65%) than low M_w and low DD chitosan-siRNA nanoparticles. However, the positive control (transfection reagent *Trans*IT-TKO), showed an EGFP knockdown of 85%. The cytotoxicity test demonstrated that chitosan formulations showed a reduction of 20-40% in cell viability compared to the control. The study also suggests that higher N/P ratios increase EGFP knockdown. [62]

Although plenty of research has been made on transfection efficiency, cell uptake and cytotoxicity of chitosan-DNA nanoparticles, information concerning gene expression is still incomplete. More knowledge is needed about the degree and impact of the desired gene expression *in vivo*.

5. CHARACTERISATION OF CHITOSAN-pDNA NANOPARTICLES

There are a number of characterisation techniques for chitosan-DNA nanoparticles. In the literature, dynamic light scattering and laser Doppler velocimetry have been extensively used to evaluate particle size and zeta potential, respectively [48; 54; 74]. Incorporation of pDNA to the chitosan nanoparticles has been characterised by electrophoresis mobility testing in agarose gel containing [43; 48; 74]. The morphology of chitosan-DNA nanoparticles has been examined, among other techniques, using scanning electron microscopy (SEM) [15; 74; 77].

5.1. Determining particle size – DLS and FEG-SEM

There are a number of characterisation techniques for measuring, for instance, the size of micro- and nanoparticles. Figure 5.1 specifies the appropriate characterisation techniques for different particle size ranges.

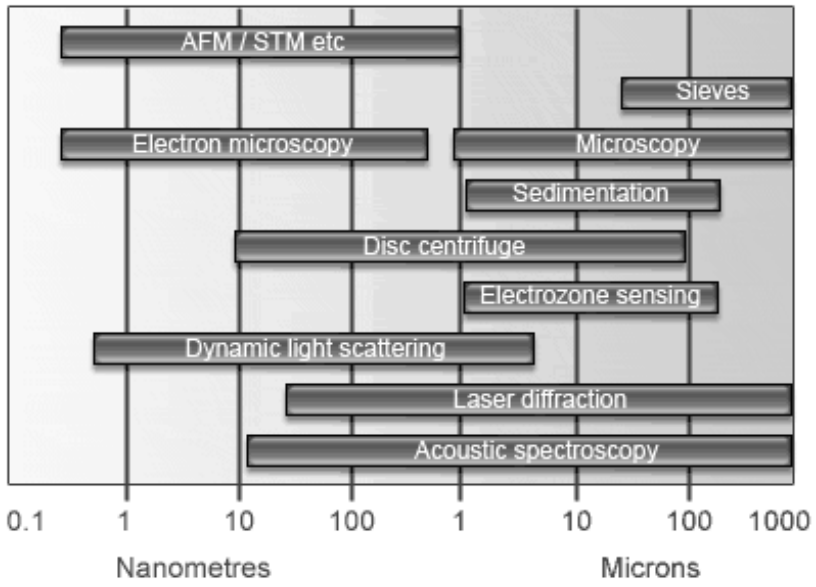


Figure 5.1. *Characterisation techniques for nanoparticles. [70]*

As can be seen from the figure, only a few techniques are suitable for the accurate characterisation of particles less than 10 nm in size. These include dynamic light scattering (DLS), electron microscopy and atomic force microscopy (AFM). DLS is the only method which allows particle size measurement in a fast, routine manner without

the need of a trained technician. However, electron microscopy and AFM provide more information about particle morphology and shape. [70]

5.1.1. Dynamic light scattering

Dynamic light scattering (DLS), also known as photon correlation spectroscopy (PCS) or quasi-elastic light scattering (QELS), is a technique for measuring the size of small particles and molecules in the submicron range. DLS is based on measuring the Brownian motion of particles and relating this to their size. The measurement is achieved by laser illumination of the samples and by analysing the intensity fluctuations of scattered light. In general, particles will scatter light at a certain angle when they are illuminated. This scattered light can be observed by placing a detector close to the particles, as in Figure 5.2. Light of the same phase will act constructively and form bright patches on the screen, while mutually destructive phase additions will appear dark. [67, p.1401-1402]

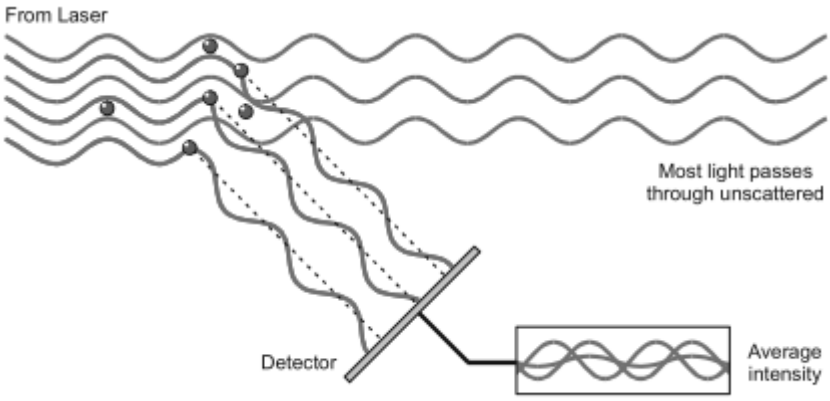


Figure 5.2. *Scattered light passed on to a detector. [67, p.1402]*

After the detector has collected a sufficient amount of data, a correlator is used to measure the degree of similarity between two intensity signals over a short period of time, usually in the order of nano- or microseconds. If the signal intensity at a time (t) is compared to itself then there is perfect correlation as the signals are identical. Perfect correlation is reported as 1 and no correlation is reported as 0. [67, p.1403]

In Brownian motion particles move due to the random collision with the molecules of the liquid that surrounds them. Small particles move faster than large particles. The Brownian motion of particles is related to their size via the Stokes-Einstein equation:

$$D = \frac{kT}{6\pi\eta R_H} \tag{4}$$

where D is the diffusion coefficient, k is the Boltzmann constant, T is absolute temperature, η is solvent viscosity and R_H is the hydrodynamic radius. According to the equation, the diffusion coefficient is related to the size of the diffusing object. If the other parameters are known, the hydrodynamic radius and the diameter of the particle

can be calculated. The hydrodynamic radius is the effective size of the molecule. [67, p.1307]

5.1.2. DLS equipment and operation

DLS equipment generally comprises six distinctive parts. First, a laser acts as a light source to illuminate the particle samples within a cell. Most of the light passes through the sample, but some is scattered from the particles. A detector is used to measure the intensity of the scattered light. The detector can only measure the intensity successfully in a certain intensity range. Therefore, an attenuator is used to reduce the intensity of the laser, and hence the intensity of scattered light, if too much light is detected. In contrast, the amount of scattered light must be increased for particles that do not scatter very much light. These include very small particles or samples of low concentration. In this case, the attenuator should allow more laser light through to the sample. After the scattered light has been processed by the detector, the scattering intensity signal is passed on to a digital signal processor – a correlator. The function of the correlator is to compare the scattering intensity at successive time intervals in order to derive the rate at which the intensity is varying. Finally, the correlator information is sent to a computer where a specific software analyses the retrieved data and determines the particle size. [67, p.1406-1407] A schematic presentation of the DLS equipment and process can be seen in Figure 5.3. The same measurement equipment can generally be used to determine both particle size and zeta potential. However, the cell type and software parameters for each are different.

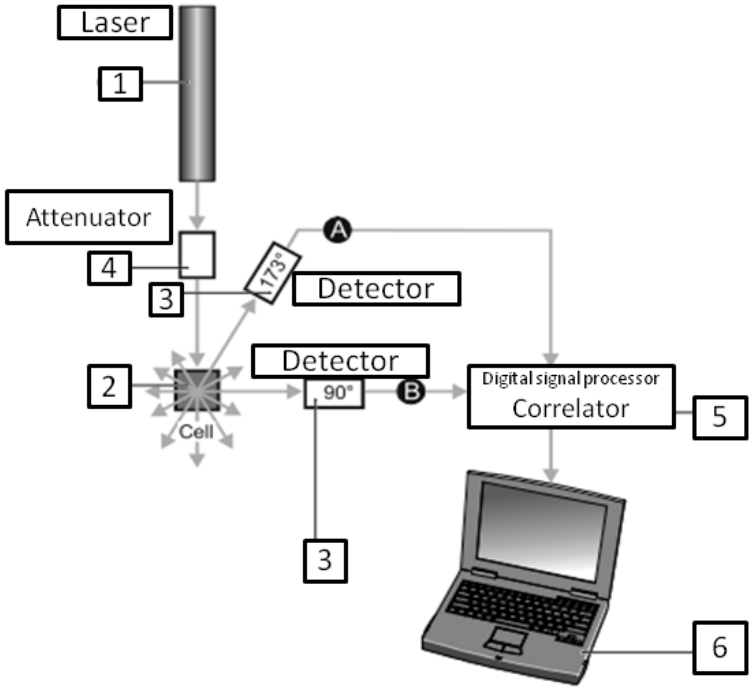


Figure 5.3. *DLS equipment. 1) laser, 2) cell, 3) detector, 4) "attenuator", 5) correlator , 6) computer. Modified from [67, p.1406].*

In some DLS equipment, such as Malvern Instruments Zetasizer Nano ZS, the scattering information is measured at an angle of 173°. The method is known as backscatter detection and it is based on a technology called NIBS (Non-Invasive-Back-Scatter). Advantages of this method include reduction in multiple scattering, elimination of large particle contaminants such as dust, and measurement of higher sample concentrations. [67, p.1407]

5.1.3. Field emission gun electron microscope

The scanning electron microscope (SEM) is, in addition to the transmission electron microscope (TEM), one of the two main types of electron microscopes. As the names indicate, TEM is based on transmitting an electron beam through a thin sample in order to obtain an image, while SEM relies on sweeping an electron beam across a specimen, and collecting the secondary or back-scattered electrons by an anode connected to a cathode-ray-tube. In contrast to TEM, SEM provides a more 3D-like image and is also suitable for the observation of thicker samples. Nevertheless, in both TEM and SEM, the function of electron microscopes is based on producing a magnified image of a sample by illuminating a specimen with a beam of electrons. The narrow electron beam is achieved by the objective and the eyepiece lenses that act as magnetic fields bringing the electrons to a focus. [26, p.838-839; 125, p.1502-1503] In sample scanning, an electron beam is typically emitted from a thermionic electron gun fitted with a tungsten filament cathode. The high melting point and low vapour pressure account for the use of tungsten in SEM. Also other types of electron emitters are used. For instance, in field emission SEM, a field emission gun (FEG) is used. The field emission gun may be a cold-cathode type fitted with tungsten single crystal emitters, or it can be fitted with zirconium oxide. Compared to conventional thermionic emitters, the field emission gun produces an electron beam that is smaller in diameter and more coherent. In addition, the current density or brightness is up to three orders of magnitude. As a result, the resolution, reliability and signal-to-noise ratio is significantly better in FEG-SEM than in conventional SEM emitters.

The emitted electron beam typically has an energy ranging between 0,5 keV and 40 keV. After electron emission, the initial beam is focused by condenser lenses to about 0.4 nm to 5 nm in diameter in the microscope column. The beam then passes scanning coils, which deflect the beam over a rectangular are over the sample surface. The energy exchange between the electron beam and sample surface results in scattered and secondary electrons, as well as electromagnetic radiation, which are detected by specific detectors connected to the microscope. The resulting image is obtained by using detectors that are sensitive to the particular emission method in question. The detector signal then controls the brightness and contrast of the cathode ray tube (CRT) that acts as the image screen. [26, p.838-839; 125, p.1502-1503] Nowadays, the SEM is usually connected to a computer, which allows observation on a computer screen and saving the image on a computer hard disc. [34, p.134] A schematic illustration of the scanning electron microscope is presented in Figure 5.4.

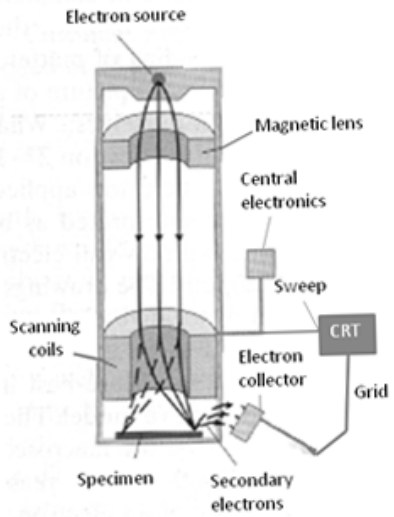


Figure 5.4. *Scanning electron microscope. Scanning coils move an electron beam across the sample surface. Secondary electrons are collected and modulate the intensity in the CRT to produce a picture. Modified from [26, p.838].*

In secondary electron emission, the image is formed due to differences in the surface topography, which results in differences in contrast. When the secondary electron hits the scintillator material, a light signal is formed and it is optically guided to the light multiplier tube. Signals are collected, amplified and passed to a cathode ray tube, where the image is formed. Back-scattered electrons are a result of the atomic number of the atoms in the sample surface. In general, heavier elements will cause lighter areas in the SEM image than lighter elements. When back-scattered electrons migrate to the semiconductor detector, they form a current that is conducted to amplifiers and the image is formed on a cathode ray tube. [34, p.154]

The resolution of the SEM depends on the brightness and the electron beam size on the sample surface [34, p.154]. Theoretically, the resolution can be as small as the wavelength of an accelerated electron. For example, an electron accelerated by a voltage on the order of 10^5 V has a wavelength on the order of 0.004 nm. [26, p.838] In general, the higher the voltage the better the resolution, as demonstrated in Equations 5 and 6:

$$\lambda = \frac{h}{mv} \tag{5}$$

$$v = \sqrt{\frac{2eU}{m}} \tag{6}$$

where λ is the wavelength of an electron, h is Planck's constant, m is the mass of an electron, v is the velocity of an electron, e is the charge of an electron and U is the acceleration voltage. In practice, aberrations in the magnetic lenses limit the maximum resolution of the scanning electron microscope to 1-10 nm. The use of high voltages is limited with biological samples, as the generated heat may cause damage to the specimens. [26, p.836-839; 34, p.154-155] Moreover, biological material is often coated

by carbon or with an adequate metal in order to increase conductivity of the specimen and thus improve image quality. [34, p.154]

5.2. Determining zeta potential- Laser Doppler Velocimetry

Zeta potential is calculated by Laser Doppler Velocimetry (LDV). When an electric field is applied to an electrolyte, charged particles move to the electrode of opposite charge. However, the viscosity of the solution opposes this movement. When a balance is achieved between the opposing forces, the particles move with constant velocity, referred to as electrophoretic mobility, and Henry's equation (Equation 7) can be applied to calculate the zeta potential:

$$U_E = \frac{2\varepsilon z f(ka)}{3\eta} \tag{7}$$

where U_E is the electrophoretic mobility, z is the zeta potential, ε is the dielectric constant, η is viscosity and *f(ka)* is the Henry function. There are two values commonly used to approximate the Henry function. In aqueous media *f(ka)* is normally 1.5. This value is also known as the Smoluchowski approximation and it is suitable for particles larger than 0.2 microns dispersed in electrolytes containing more that 10^{-3} molar salt and high ionic strengths. For non-aqueous solutions or for small particles in a low dielectric media, f(ka) 1 is used and it is referred to as the Huckel approximation. Both the Smoluchowski and the Huckel approximation have been used in studies concerning chitosan-DNA nanoparticles. [48; 80]

As can be seen from Henry's equation, the electrophoretic mobility has to be known in order to calculate the zeta potential (*z*). Laser Doppler Velocimetry is used to measure the velocity of tiny particles within a fluid. This includes scattering light at an angle of 17° and combining it with a reference beam. Once again a fluctuating intensity signal is produced. This signal is proportional to the velocity of the particles and hence the electrophoretic mobility can be calculated with the help of a digital signal processor. When the electrophoretic mobility is known, the zeta potential can be calculated according to Equation 7. [67, p.1604-1605] Considering Equation 7, it could be noted that the zeta potential is directly proportional to the viscosity and materials with higher viscosities should therefore be expected to have higher zeta potentials. A schematic representation of the measurement of electrophoretic mobility is given in Figure 5.5.

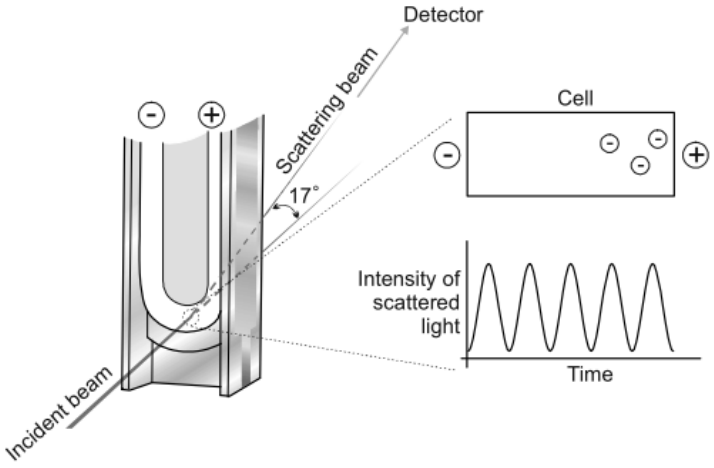


Figure 5.5. *Principles of LDV in measuring the zeta potential. [67, p.1606]*

The most important factor affecting the zeta potential is pH. For example, adding alkali to a solution with a negative charge will cause the particles to acquire more negative charge, whereas adding acid may neutralise or build up a positive charge of the solution. [67, p.1602-1603] This effect is visualised in Figure 5.6.

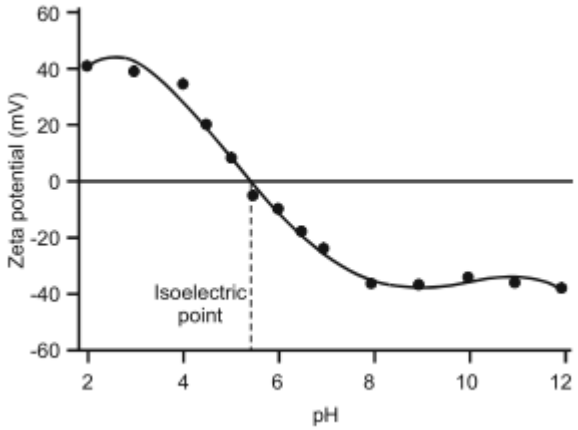


Figure 5.6. *A typical plot of zeta potential versus pH. [67, p.1603]*

As can be seen from Figure 5.6, the zeta potential versus pH curve will be positive at low pH and lower or negative at high pH. The point where the curve passes zero zeta potential is called the isoelectric point. At this point the colloidal system is least stable. [67, p.1603] In literature, zeta potential is commonly measured at a temperature of 25°C and at a scattering angle of 90° or 173°, depending on the measuring device [48; 71; 74; 80].

5.3. DNA incorporation - gel electrophoresis

Electrophoresis can be used in combination with other methods like LDV, as described in the previous section. However, it can also be used to determine the size, molecular weight, isoelectric point or biological activity of different compounds. Although

electrophoresis can be conducted using different media such as gel or paper, in literature agarose gel electrophoresis has successfully been applied to assess the incorporation of DNA into chitosan nanoparticles. [15; 71; 74] Hence, agarose gel electrophoresis is also used in this particular study to evaluate the success of DNA and chitosan nanoparticle complexation.

5.3.1. Physical principles of gel electrophoresis – sample mobility

Electrophoretic methods are based on the migration of charged ions or molecules towards an anode or cathode in an electric field. Positively charged molecules will migrate towards the negative cathode, whereas the positive anode attracts negatively charged anions. [34, p.162] This process is referred to as electrophoresis, and it is illustrated in Figure 5.7 [73, p.48]. The higher the charge of the particle and the applied voltage, the faster the compounds will move in the electric field. In addition, smaller particles will move through the field medium faster than larger molecules, especially if a restrictive medium is applied to act as a molecular sieve. If particles are of the same size, they can be differentiated according to their charge. If particles possess the same charge, they can be differentiated according to size. [73, p.49 and 56]

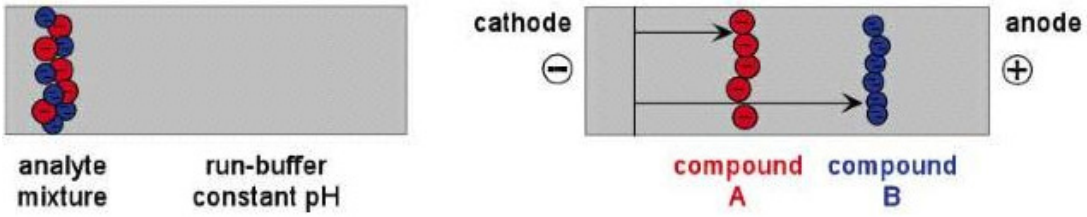


Figure 5.7. *The basic principle of electrophoresis. Positively charged particles migrate towards the negatively charged cathode and negatively charged ions migrate towards the anode. [73, p.48]*

In electrophoresis molecule samples are prepared accordingly and then loaded to wells in a non-conductive matrix such as agarose or polyacrylamide gel. The medium, often covered by a conductive aqueous buffer, is then connected to a power supply by electrodes and an electric field is applied. Typically voltages from 200 to 500 V and currents from 100 µA to 400 mA are used. The pH is usually chosen so that most analytes will be negatively charged and migrate toward the anode. In the case of chitosan-DNA nanoparticles, a voltage of 80-100 V and a current of 400 mA are chosen on a 0.8% agarose gel, and the gel is run for about an hour. [45] Figure 5.8 illustrates a typical gel electrophoresis setup.

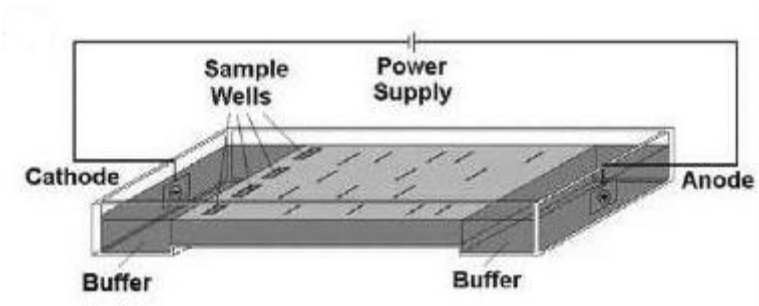


Figure 5.8. *Gel electrophoresis setup. The gel containing sample wells is placed in a buffer container, which is attached to a DC power supply with electrodes. [73, p.58]*

An electrophoresis apparatus can be considered as a simple DC circuit composed of one voltage source connected to three resistors in series, namely two electrode buffer chambers and a medium such as gel. Because most of the resistance is provided by the gel slab that is both longer in length and has a smaller cross-section than the electrode chambers, it is usually sufficient to consider the gel as the only resistor connected to the power supply. The electrical current of the circuit consists of both sample molecules and buffer ions of which the latter represent the majority of the current. As a voltage is applied to the system, cations migrate towards the cathode and anions as well as negatively charged sample molecules move towards the anode. [83]

As illustrated in Figure 5.8, the matrix material and conductive buffer are connected to a power supply. Two principles of electricity can be applied to gel electrophoresis: Ohm's law and the power equation. [34, p.163; 83] Ohm's law can be expressed as

$$V = RI \tag{8}$$

where V is the voltage between the cathode and anode, I is the current passing the electric field and R is the resistance of the field. It should be noted that although the above equation is often referred to as Ohm's law, the actual content of Ohm's law expresses the direct proportionality of V to I only when R is constant. [125, p.951] The power equation, on the other hand, is

$$P = VI \text{ or } P = I^2R \text{ or } P = \frac{V^2}{R} \tag{9}$$

where P is power. The power equation is a variation of Ohm's law and it illustrates how a small change in current can introduce large changes in the expenditure of power. [125, p.970]

The applied electric field generates the movement of particles and allows separation of molecules due to differences in electrophoretic mobility [73, p.49-50]. The electrophoretic mobility describes the ratio between the migration velocity and the electric field strength and can be expressed with equation 10:

$$\mu_{ep} = \frac{v_{ep}}{E} = \frac{q}{6\pi\eta r} \tag{10}$$

assuming a constant electric field in which the electric and frictional force are equal. Also electroosmotic force (EOF) and Joule heating affect particle separation. However,

these will not be elaborated in the scope of this study. For more information about EOF and Joule heating, see reference Manz et al. [73, p.49-50]

5.3.2. Agarose gel electrophoresis

In gel electrophoresis, the medium through which the sample molecules migrate commonly consists of an agarose or polyacrylamide (PA) gel [73, p.56]. The purpose of the porous matrix material is to provide greater resistance for larger molecules, reduce convection currents and inhibit sample diffusion [83]. In addition, samples can be fixed on the solid matrix and be detected in post-electrophoretic analysis. Agarose gel electrophoresis can be used to, for example, view, separate, purify, or isolate DNA fragments [29]. It can also be used for polymerase chain reactions (PCR) [10, p.154].

Agarose is a natural, linear polysaccharide derived from seaweed and it is composed of repeating agarobiose units. Agarobiose consists of alternating units of 1,3-linked b-D-galactopyranose and 1,4-linked 3,6-anhydro-a-L-galactopyranose. [9, p.91] Typically the pore size of agarose gel is bigger than that of polyacrylamide gel. Agarose gel is therefore more suitable for the separation of bigger nucleic acid molecules (> 400 base pairs). Agarose gel electrophoresis has often been used in the characterisation of chitosan-DNA nanoparticles. [15; 71; 74] Agarose gel is also faster to prepare and safer to handle than PA gel. [73, p.59]

To prepare agarose gel, agarose powder is mixed with a buffer such as Tris Borate EDTA (TBE) or Tris Acetate EDTA (TAE). Commonly, concentrations of 0.7 – 2 % are used. Smaller concentrations will show better resolution for bigger DNA fragments (5-10 kbp) and higher concentrations will provide better separation of smaller DNA fragments (0.2-1 kbp). [84] Typically, a 0.8% agarose gel has been applied for chitosan-DNA nanoparticles [71; 74]. Usually ethidium bromide or an alternative DNA gel stain is added to the gel for visualisation of DNA. [71] Ethidium bromide intercalates between the bases of DNA and due to its fluorescent nature it absorbs UV light. [56] More detailed information about gel and sample preparation is readily available [85].

When gel electrophoresis is complete, the gel slab is usually removed from the buffer container and further studies are conducted. For example, a UV illuminator can be used to visualise sample movement on the gel [105]. Normally DNA marker and plasmid DNA should “run” on the gel due to the negatively charged ions in their structure. However, if a sample does not have a negative net charge, it will not migrate on the gel. This should be the case with chitosan-DNA nanoparticles. [20; 74]

5.4. Statistical analysis

The statistical significance of chitosan-pDNA nanoparticle properties has been assessed by both non-parametric tests, such as the Mann-Whitney U test, and parametric tests like the Student’s t-test. [4; 90]

The Mann-Whitney U test is used to compare two population means from the same population. As the test is non-parametric, it does not make any assumptions of the

sample distribution. Instead, the Mann-Whitney U test assumes that samples are randomly drawn from the population, samples are mutually independent and the measurement scale is ordinal. The Mann-Whitney U test is calculated by Equation 11. [106]

$$U = n_1 n_2 + \frac{n_2 (n_2 + 1)}{2} - \sum_{i=n_1+1}^{n_2} R_i \tag{11}$$

where n_1 is sample size one, n_2 is sample size two and R is the rank of the sample size. U is the value of the Mann-Whitney U test. [106]

Also the Student's t-test is used to compare the means of two samples. [78, p.118] This parametric test assumes a normal distribution of the samples. When samples sizes and variances are expected to be equal, Equation 12 is used to calculate the value of t:

$$t = \frac{\bar{X}_1 - \bar{X}_2}{S \cdot \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}} \tag{12}$$

$$S^2 = \frac{(n_1 - 1)s_1^2 - (n_2 - 1)s_2^2}{n_1 + n_2 - 2} \tag{13}$$

where X_1 is the mean of sample one, X_2 is the mean of sample two, n is the number of participants in each sample, and s_1 and s_2 are the standard deviations of the samples. S^2 is an estimate of the common variance of the samples and it can be calculated using Equation 13. When measuring significance, the degrees of freedom can be calculated as (2n – 2). When t has been calculated and the degrees of freedom have been determined, the statistical significance can be evaluated by using a t distribution table. A null hypothesis assuming that there is no difference in the sample means is usually applied when using the t-test. Hence, if the calculated value of t exceeds the value given in the table, the null hypothesis is rejected. In this case, the sample means are considered significantly different at the chosen level of probability. [21, p.366; 78, p.118-122; 86]

6. MATERIALS AND METHODS

6.1. Materials for chitosan-pDNA nanoparticle preparation

Chitosans were purchased from Heppe Medical Chitosan (Germany) and FMC BioPolymer/Novamatrix (Norway). The chitosans and chitosan salts and derivatives used in this study, as well as some of their properties, are presented in Table 6.1.

Table 6.1. *Chitosans used for the preparation of chitosan-DNA nanoparticles. Chitosans written in bold are water-soluble chitosan salts and derivatives.*

Nr	Chitosan	Sample name	DD (%)	M_w (kDa)	Manufacturer (country)
1	Chitoceuticals Chitosan 75/5	75/25	76.5	25	Heppe Medical Chitosan (Germany)
2	Chitoceuticals Chitosan 85/5	85/25	84.3	25	
3	Chitoceuticals Chitosan 95/5	95/25	94.5	25	
4	Chitoceuticals Chitosan 75/50	75/150	74.1	150	
5	Chitoceuticals Chitosan 85/50	85/150	83.7	150	
6	Chitoceuticals Chitosan 95/50	95/150	92.8	150	
7	Chitoceuticals Chitosan 75/200	75/300	74.9	300	
8	Chitoceuticals Chitosan 85/200	85/300	87.3	300	
9	Chitoceuticals Chitosan 95/200	95/300	94.3	300	
10	Chitosan base (PROTASAN UP B 80/20)	80/250	86	250	FMC BioPolymer/NovaMatrix (Norway)
11	Chitosan base (PROTASAN UP B 90/500)	90/460	91	460	FMC BioPolymer/NovaMatrix (Norway)
12	**Chitosan acetate**	**CH ace (85/100)**	83.6	100	Heppe Medical Chitosan (Germany)
13	**Chitosan lactate**	**CH lac (95/400)**	93.4	400	
14	**Chitosan hydrochloride**	**CH HCl (95/40)**	91.7	40	
15	**Chitosan oligosaccharide**	**CH oligo (85/1.5)**	83.1	1,5	
16	**Carboxymethylchitosan**	**CMCH (95/150)**	94.4	150	
17	**Chitosan glutamate (PROTASAN UP G 113)**	**CH glut (86/)**	86	50-200	FMC BioPolymer/NovaMatrix (Norway)

The specific molecular weight of chitosan glutamate was not specified and hence it is omitted from the sample name. According to the manufacturer, the molecular weight should be from 50 to 200 kDa. In addition to chitosan, chemical reagents and physical

equipment were used in combination with chitosan to form chitosan-pDNA nanoparticles. These are presented in Table 6.2.

Table 6.2. *Additional materials in chitosan-DNA nanoparticle preparation.*

Reagent (chemical formula)	**Manufacturer/Supplier (city, country)**
Sodium sulphate (Na_2SO_4)	Merck (Darmstadt, Germany)
Sodium acetate (CH_3COONa)	Merck (Darmstadt, Germany)
Acetic acid (CH_3COOH)	J.T. Baker (Deventer, Holland)
Sodium hydroxide (NaOH)	J.T. Baker (Deventer, Holland)
Hydrochloric acid (HCl)	Sigma-Aldrich (Helsinki, Finland)
Plasmid DNA expressing GFP (green fluorescent protein) (500 ng/µl)	University of Tampere (Tampere, Finland)
Equipment	**Manufacturer/Supplier (city, country)**
Magnetic stirrers: Schott Ikamag® REO	 Schott Geräte GmBh (Mainz, Germany) Ika® Werke GmBh & Co. (Staufen, Germany)
pH meter: Mettler Toledo 225	Mettler Toledo (Greifensee, Switzerland)
0.2 µm syringe filters	PALL Life Sciences (Washington, U.S.A.)
5 ml syringes	BD DiscardIt™ II (U.S.A.)

Other essential materials included regular laboratory equipment.

6.2. Equipment in nanoparticle characterisation and gel electrophoresis

After particle preparation, another set of materials was used to characterise the particles. Reagents and devices are presented in Table 6.3.

Table 6.3. *Reagents and devices in chitosan-pDNA nanoparticle characterisation.*

Reagent	**Manufacturer/Supplier (city, country)**
Multipurpose agarose	Bioline (London, U.K.)
10,000x SYBR® Safe DNA gel stain	Invitrogen, part of Life Technologies™ (U.S.A.)
10 x TBE (Tris-borate-EDTA buffer) 6x DNA Loading Dye #0611 GeneRuler™ 1kb DNA Ladder	Fermentas Life Sciences (Canada)
Equipment	**Material/Supplier (city, country)**
Zetasizer Nano ZS	Malvern Instruments (Worcestershire, U.K.)
UV-transparent disposable cuvettes	Sarstedt (Nümbrecht, Germany)
Sub-cell GT Cell, Mini-sub cell GT Cell	Bio-Rad Laboratories (California, U.S.A.)
Electrophoresis Power Supply – EPS 300	Pharmacia Biotech (Uppsala, Sweden)
UV illuminator/CCD camera: Molecular imager Chemidoc™ XRS System	Bio-Rad Laboratories (California, U.S.A.)
Slide-A-Lyzer® 10K Dialysis Cassettes	Thermo Scientific (Rockford, U.S.A.)
Zeiss ULTRAplus FEG-SEM	Carl Zeiss SMT AG (Germany)

Other essential materials included regular laboratory equipment.

6.3. Preparation of chitosan-pDNA nanoparticles by complex coacervation

Each nanoparticle batch was prepared in two stages on two consecutive days. The first day involved weighing materials and preparing solutions. The second day, solution pH was adjusted and plasmid DNA was added to the chitosan solutions in order to form chitosan-pDNA nanoparticles. Below is a more detailed description of the nanoparticle preparation process.

6.3.1. Preparation of solutions

Briefly, a 200 µg/ml chitosan solution was prepared by weighing 8 mg of the desired chitosan and mixing it with 20ml of distilled water and 20 ml of 100 mM sodium acetate (CH_3COONa) solution. However, if the chitosan was non-soluble in water, 114 µl of acetic acid (CH_3COOH) was pipetted in the water solution in order to dissolve the chitosan, and the solution was left to mix on a magnetic stirrer for about an hour before the addition of sodium acetate solution. The basic principle of the first stage of particle preparation is illustrated in Figure 6.1.

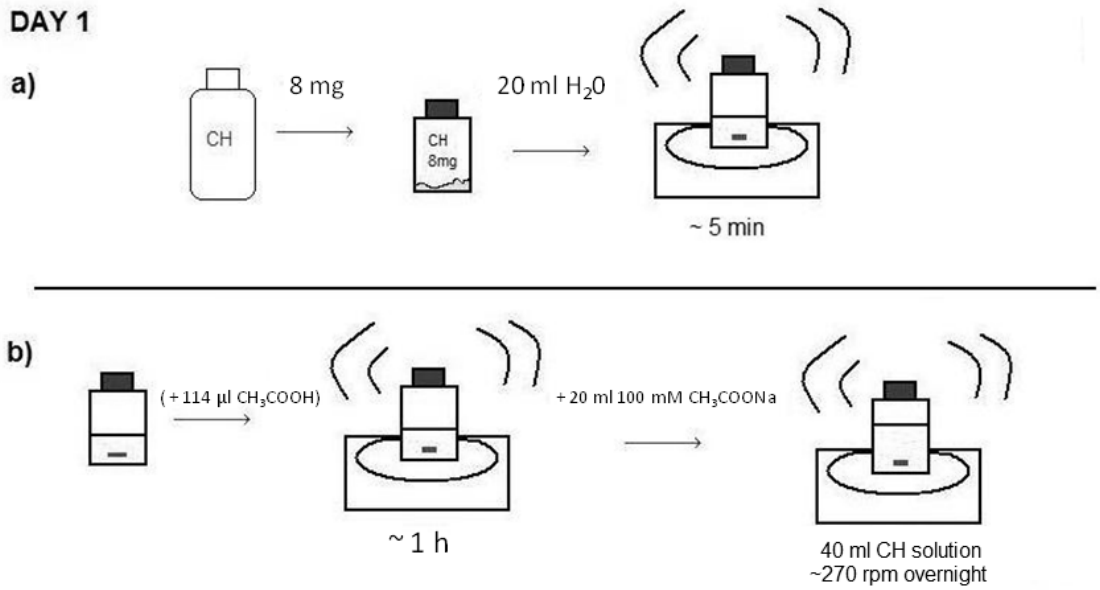


Figure 6.1. *Preparation of solutions – stage 1 a) Chitosan (CH) is weighed and inserted to a glass bottle. Distilled water is added and the solution is placed on a magnetic stirrer b) Acetic acid is added, if necessary, and the solution is placed back on the magnetic stirrer for ~1h. 100 mM of sodium acetate is added and the solution is left to mix at ~270 rpm overnight.*

In detail, 8 mg of chitosan was weighed and inserted into a 50 ml glass bottle. 20 ml of distilled water was added to the bottle using a 10 ml automatic pipette. A magnetic rod was placed into the bottle, the glass bottle was closed with a cap, and the mixture was placed in a magnetic stirrer (~350 rpm) for about 5-10 minutes. Thereafter, if needed, 114 µl of acetic acid was added using a pipette and the solution was left to mix on the magnetic stirrer for about an hour. Finally, 20 ml of 100 mM sodium acetate solution was added with a 10 ml pipette and the resulting solution was mixed on the magnetic stirrer (~270 rpm) overnight at room temperature. If there were more chitosan samples to be prepared than one, the steps described above were applied to each sample.

100 mM CH_3COONa solution was prepared whilst the chitosan-acetic acid solution was mixing on the magnetic stirrer. First, 574 mg of CH_3COONa was weighed in a 100 ml glass bottle. Thereafter, 70 ml of distilled water was added using a 10 ml pipette. Finally, a magnetic rod was placed in the bottle, the bottle was closed with a cap and the solution was mixed on a magnetic stirrer for about an hour before it was added to the chitosan solution.

Also sodium sulphate solution (5 mM Na_2SO_4) was prepared on the first day of nanoparticle preparation. For this, 28.4 mg of Na_2SO_4 was measured in a weighing boat and then poured into a 50 ml glass bottle. The weighing was executed simultaneously with chitosan weighing. The desired amount of distilled water, in this case 40 ml, was added to the glass bottle using a 10 ml pipette. A magnetic rod was placed in the bottle, the bottle was closed with a cap, and then placed on a magnetic stirrer for a couple of hours. Finally, the solution was removed from the stirrer and left to stay at room temperature overnight. Table 6.4 summarises the amount of reagents used for each solution.

Table 6.4. *Reagents used in solution preparation.*

Reagent	200 µg/ml chitosan solution	100 mM CH_3COONa solution	5 mM Na_2SO_4 solution
Chitosan	8 mg	-	-
Distilled water	20 ml	70 ml	40 ml
Acetic acid	(114 µl)	-	-
100 mM CH_3COONa	20 ml	-	-
CH_3COONa	-	574 mg	-
Na_2SO_4	-	-	28.4 mg

In addition to preparing solutions, the desired number of test tubes was cleaned and left to dry for the next day.

6.3.2. Adjusting pH and formation of chitosan-pDNA nanoparticles

On the second day of chitosan nanoparticle preparation, the 5 mM Na_2SO_4 solution prepared on the previous day was first placed back on the magnetic stirrer. Then, half of the chitosan solution was poured into another glass bottle and the pH of the remaining solution (~20 ml) was adjusted to approximately 5.5 with 10 M NaOH or with 1 M HCl,

depending on the initial pH. Also the pH of the 5 mM Na_2SO_4 solution was checked, but not adjusted. The pH meter was calibrated before use and the electrode of the meter was rinsed and dried both before use and between switching samples.

Next, plasmid DNA (pDNA) was taken from the freezer to melt. The aim was to prepare a 1.2 ml sample of chitosan-pDNA nanoparticle solution with a chitosan:pDNA weight ratio of 4:1. Simply put, this was achieved by mixing 600 µl of chitosan solution with 600 µl of pDNA-Na_2SO_4 solution. In detail, pH-adjusted chitosan solution was filtered to a plastic test tube using a 0.2 µm syringe filter and a 5 ml syringe. The function of filtering was to remove insoluble chitosan and impurities from the solutions. Also 5 mM Na_2SO_4 solution was filtered to a separate test tube. Then, 600 µl of the filtered chitosan solution was extracted and pipetted to another test tube. The volume of extracted filtered 5 mM Na_2SO_4 solution to another test tube was dependent on the volume of plasmid DNA which, in turn, depended on the concentration of the plasmid (500 ng/µl). For a CH:pDNA weight ratio of 4:1, 60 µl of pDNA was needed. Therefore, to achieve a Na_2SO_4-pDNA solution of 600 µl, 60 µl of plasmid pDNA was pipetted to 540 µl of 5 mM Na_2SO_4 solution. Table 6.5 summarises the amounts of reagents used for preparing a chitosan-pDNA nanoparticles with a weight ratio of 4:1 from a 200 µg/ml chitosan solution.

Table 6.5. *Amounts of reagents used for chitosan-DNA nanoparticles.*

Reagent	**200 µg/ml chitosan solution**
Volume of chitosan solution	600 µl
Mass of chitosan	120 µg
Volume of (5 mM Na_2SO_4-pDNA)	600 µl
Concentration of pDNA	0.5 µg/µl
Volume of pDNA	60 µl
Mass of pDNA	30 µg
Volume of 5 mM Na_2SO_4 solution	540 µl
Total sample volume	**1.2ml**

After pipetting filtered chitosan and (Na_2SO_4- pDNA) solutions (à 600 µl), test tubes were placed in a water bath in a 1000 ml decanter containing a magnetic rod. The decanter was covered with aluminium foil to protect samples from light. Finally, the bath was heated to 55°C while simultaneously stirring the bath on a magnetic stirrer (300 rpm). When the temperature reached 55°C, the decanter was removed from the heater and magnetic stirrer.

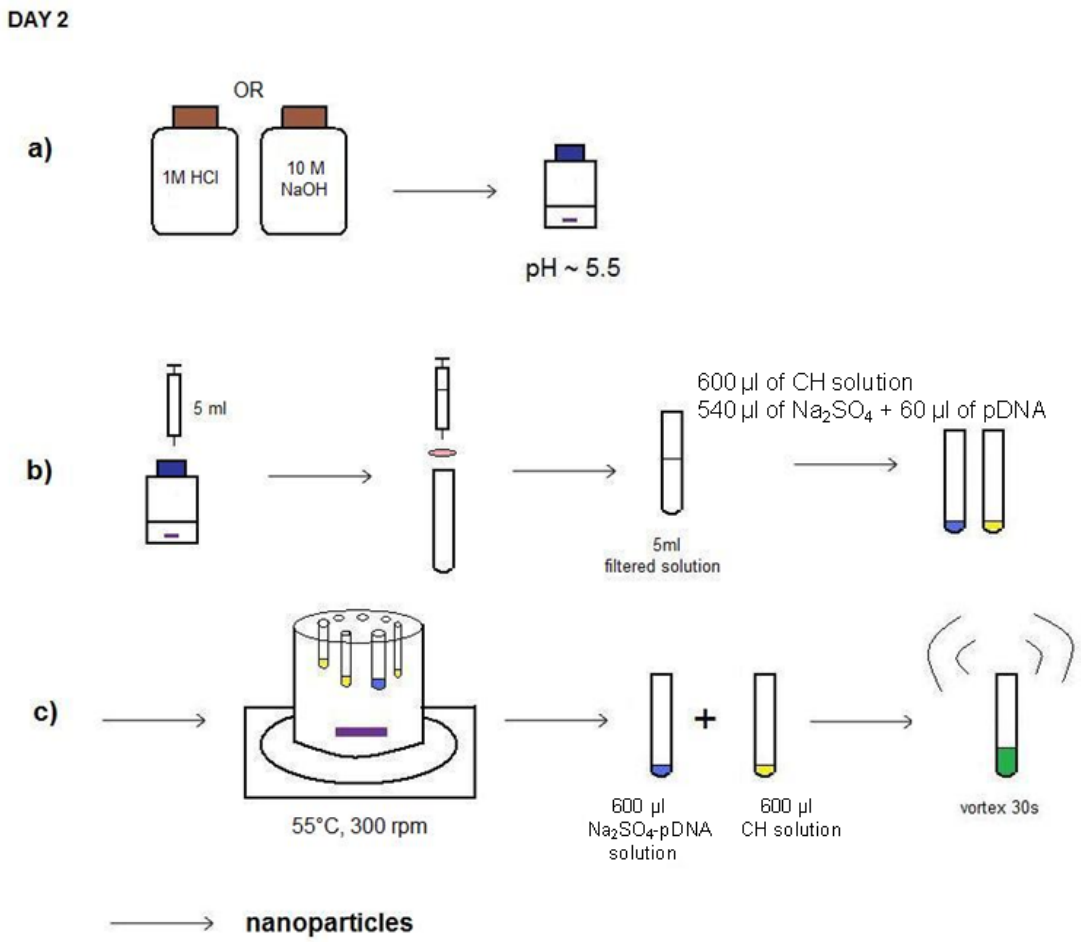


Figure 6.2. *Preparation of chitosan-pDNA nanoparticles - stage 2 a) The pH of the chitosan (CH) solution was adjusted to 5.5 by 10M NaOH or 1M HCl. b) The chitosan and 5mM Na_2SO_4 solutions were filtered and the appropriate amount of each solution was pipetted to a separate test tube. c) The solutions were placed in a water bath on a magnetic stirrer and they were heated to 55°C at 300 rpm. Test tubes were removed and the Na_2SO_4-pDNA solution was pipetted to the CH solution. The sample was vortexed for 30 s to yield nanoparticles. The yellow sample represents CH solution and the blue sample Na_2SO_4-pDNA solution for illustrative reasons. In reality, the solutions were clear.*

After the heating, 600 µl of Na_2SO_4-pDNA solution was pipetted up and down to 600 µl of the chitosan solution. Finally, chitosan-pDNA nanoparticles were formed by vortexing the mixed solution for 30 seconds. The sample was left to stand in room temperature for 30-60 minutes before further investigations. Figure 6.2 summarises the steps of chitosan-pDNA nanoparticle preparation.

6.4. Size and zeta potential measurement

Particle size and zeta potential was determined by dynamic light scattering and laser Doppler velocimetry, respectively. Measurements were performed approximately 1−4

hours after particle preparation using Zetasizer Nano ZS (Malvern Instruments, United Kingdom).

Samples were quickly vortexed before measurements. For size determination, a 100 µl sample of the chitosan-pDNA solution was pipetted in a cuvette that was inserted in the measuring device. Appropriate parameters, presented in Table 6.6, were set on the device software and the measurement was initiated. The procedure described above was repeated three times for each chitosan sample and for each 100 µl sample 3 subruns were performed. Thus, 9 values were obtained for each sample. After initial size measurements, particles were stored still at 4°C. The stability of particle size was evaluated on week 1 and week 2 after particle preparation. The same measurements settings were used during these experiments.

Table 6.6. *Software parameters used in size and zeta potential measurements.*

Parameter	Specifications Size	Specifications Zeta potential
Measurement type	Size	Zeta potential
Material	Chitosan RI 1.33, absorption 0.00	Chitosan RI 1.33, absorption 0.00
Dispersant	Water, temperature 25°C, viscosity 0.8872, RI 1.33	Water, temperature 25°C, viscosity 0.8872, RI 1.33
Temperature	25°C Equilibration time 60 s	25°C Equilibration time 60 s
Cell/cuvette	ZEN0117 disposable low volume cuvette (100 µl)	DTS1060C clear disposable zeta cell
Measurement	Angle 173° Backscatter (NIBS default) Measurement duration: automatic Number of measurements: 3 Delay between measurements: 0	Number of measurements: 3
General	-	Smoluchowski f(ka) = 1.5

In zeta potential measurements, approximately 750 µl of chitosan sample was pipetted into an electrode cuvette. The cuvette was cleaned with ethanol and water between switching samples. The zeta potential was measured by inserting the cuvette containing the desired sample into the Zetasizer and setting the right parameters on the device software. Table 6.6 above presents the parameters used in zeta potential determination. Only one measurement with three subruns was performed on each sample, resulting in 3 values for the zeta potential.

6.5. Agarose gel electrophoresis

Agarose gel electrophoresis was performed on the chitosan-pDNA solutions the day after size and zeta potential determinations. A 0.8% agarose gel was prepared by weighing the agarose in a decanter, adding 1xTBE and heating the mixture up in a microwave oven on full power. The solution was mixed every now and then to ensure

mixing of the agarose powder to the TBE solution. When the solution was clear, it was removed from the microwave and it was cooled down to approximately 65°C before adding SYBR® Safe DNA gel stain. Finally, the mixture was poured on a gel plate and the sample well comb was inserted on the gel. The gel was left to stiffen for about an hour, while preparing the samples. Table 6.7 presents the amounts of reagents used for a big and small gel plate. The size of the gel was dependent on the available equipment.

Table 6.7. *Reagents used for agarose gel.*

Reagent	0.8% small gel	0.8% big gel
Multipurpose agarose	0.48 g	1.2 g
1 x TBE	60 ml	150 ml
SYBR® Safe DNA gel stain	6 µl	15 µl

Gel samples were prepared by mixing 20 µl of chitosan-pDNA solution with 4 µl of loading dye (6 x DNA Loading Dye, Fermentas Life Sciences). DNA ladder was used as a reference and it was prepared by mixing 6 µl of sterilised water, 1.5 µl of loading dye and 1.5 µl of DNA ladder (GeneRuler™ 1kb, Fermentas Life Sciences). Plasmid DNA was used as the control and the pDNA sample was prepared by pipetting 2 µl of plasmid and 0.4 µl of loading dye. Finally, gel samples were vortexed and spinned quickly to ensure the uniformity of the solutions.

When the gel had stiffened, the well comb was removed from the gel and the possible tapes surrounding the gel plate were removed. The plate containing the gel was then placed to a gel electrophoresis apparatus and 1xTBE was poured into the apparatus until the gel was covered. Gel samples were loaded in gel wells in the following order: DNA ladder, plasmid, chitosan-pDNA samples. The gel apparatus was connected to a power supply and the gel was run at 80 V and 400 mA for approximately an hour. Table 6.8 demonstrates the amount of reagents used for each gel sample and the amount of the gel sample loaded on the gel.

Table 6.8. *Preparation of samples for agarose gel electrophoresis.*

Reagent	DNA ladder GeneRuler™ 1kb	Plasmid	Chitosan-pDNA solution
Sample	1.5 µl	2 µl	20 µl
6x DNA loading dye	1.5 µl	0.4 µl	4 µl
sterilised water	6 µl	-	-
amount of sample on gel	9 µl	2.4 µl	20 µl

When the gel electrophoresis was complete, the gel slab was removed from the plate, and it was inspected and visualised under UV light using molecular imager Chemidoc XRS System and appropriate software (Quantity One) in order to detect migration of the samples.

6.6. Particle characterisation using FEG-SEM

Based on obtained results shown in Appendix 1, three samples were chosen for morphological studies. These included chitosan 85/25, chitosan HCl and chitosan 80/250. The last was used as a reference, while the previous were chosen based on the promising nanoparticle size. Samples were prepared according to instructions given in section 6.3. A small amount of the sample was separated for size measurement comparison. Then, samples were dialysed in order to remove salts from interfering with the microscope image. First, a small amount of sample was injected to a dialysis cassette (Thermo Scientific). Then, a buoy was attached to the cassette and the sample was placed on a magnetic stirrer in a sterilised water bath. The dialysis fluid was renewed after a few hours and finally the system was left on a magnetic stirrer overnight. On the next day, the sample was removed from the cassette using a syringe. Size measurements were performed on non-dialysed and dialysed samples. Figure 6.3 illustrates the dialysis procedure.

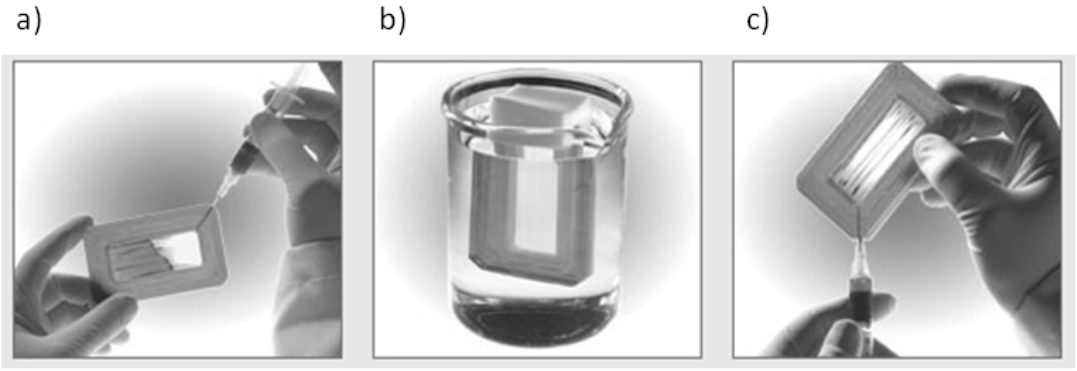


Figure 6.3. *Dialysis of chitosan-pDNA solution. a) The sample is injected to the cassette by inserting the syringe needle through the gasket via one of the corner points in the cassette. b) A float buoy is attached to the cassette and dialysis is performed. c) The sample is withdrawn by inserting a needle in one of the unused corners of the cassette. [109]*

The remaining dialysed samples were prepared for FEG-SEM imaging. Basically, a small amount of the chitosan-pDNA solution was injected on a TEM grid and the sample was left to dry for a few days. A parallel sample was prepared for carbon coating. When the samples had dried, they were observed under FEG-SEM using a voltage of 5 kV and an aperture of 20. Images were obtained with a 50 000x magnification.

7. RESULTS AND DISCUSSION

7.1. Particle size, zeta potential and PDI

7.1.1. Particle size

Particle size was measured using DLS. Figure 7.1 illustrates the mean particle diameter of the formed chitosan-pDNA nanoparticles using different chitosans. Numerical details concerning size and deviations in size are presented in Appendix 1.

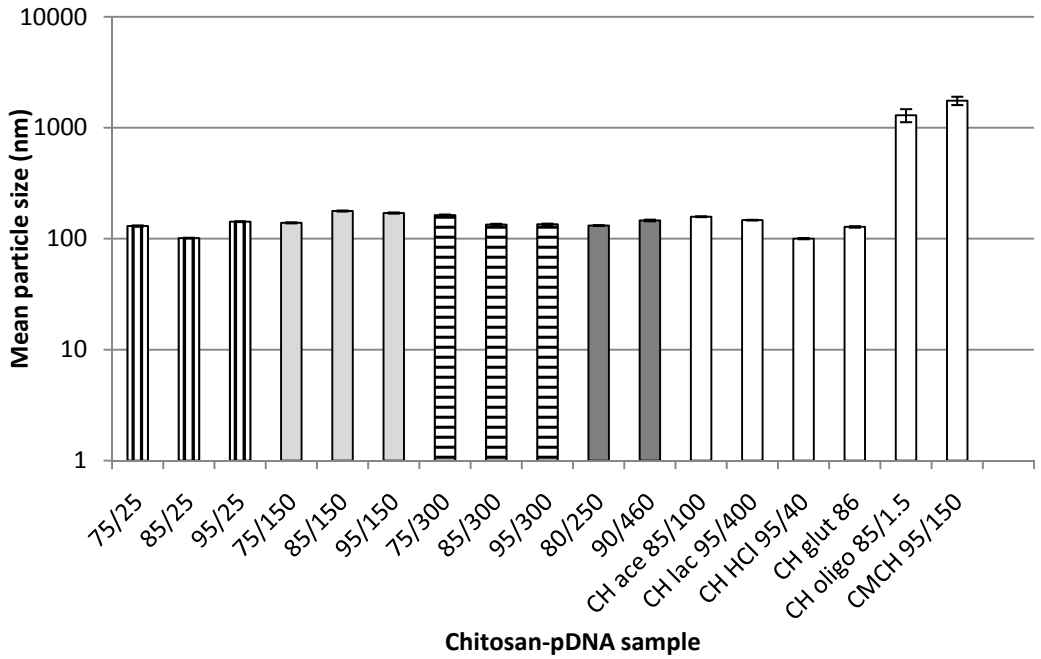


Figure 7.1. *Particle size of chitosan-pDNA nanoparticles measured by DLS on a logarithmic scale. Chitosan base samples with a M_w of 25 kDa, 50 kDa and 300 kDa are illustrated by vertical lines, light gray and horizontal lines, respectively. Other bases are coloured in dark gray, and chitosan salts and derivatives are plain. Sample names are given in the form DD/M_w. Sample specifications can be found in Appendix 1.*

Particle size generally ranged from 100 to 180 nm. Smallest particles were obtained using chitosan hydrochloride (CH HCl) and chitosan 85/25. The average size of these particles was 100.3 nm (std 1.3 nm) and 101.9 nm (std 0.9 nm), respectively. However, two of the tested chitosans did not yield nanoparticles at all. These included chitosan oligosaccharide (CH oligo) and carboxymethylchitosan (CMCH). This might be explained by the film-forming capacity of CMCH and the very small molecular weight

(1.5 kDa) of chitosan oligosaccharide. At least at this solution concentration, it appears that the chitosan oligomers were not able to make complexes with pDNA, which may be due to, for instance, short chain lengths. According to theory, chitosan oligosaccharide can be used to prepare high concentration solutions with low viscosity [122]. Therefore, it is highly likely that the used preparation parameters were not optimal. Particle formation of CMCH, on the other hand, was perhaps hindered by carboxymethylation of chitosan and reduction of amino groups.

Particle size distributions were observed to evaluate the possible aggregation of samples and also the reliability of the results. Figure 7.2 shows the intensity distributions for samples 85/25 and CH HCl. The intensity and volume distributions for all nanoparticles over a period of 2 weeks are presented in Appendix 2. As can be seen from the graphs, the intensity distributions are generally more stable and normally distributed than their respective volume distributions. Therefore, it is likely that different size complexes exist. This may be due to particle aggregation. CH oligo and CMCH did not yield stable distribution curves, which is why these results should be regarded with some scepticism.

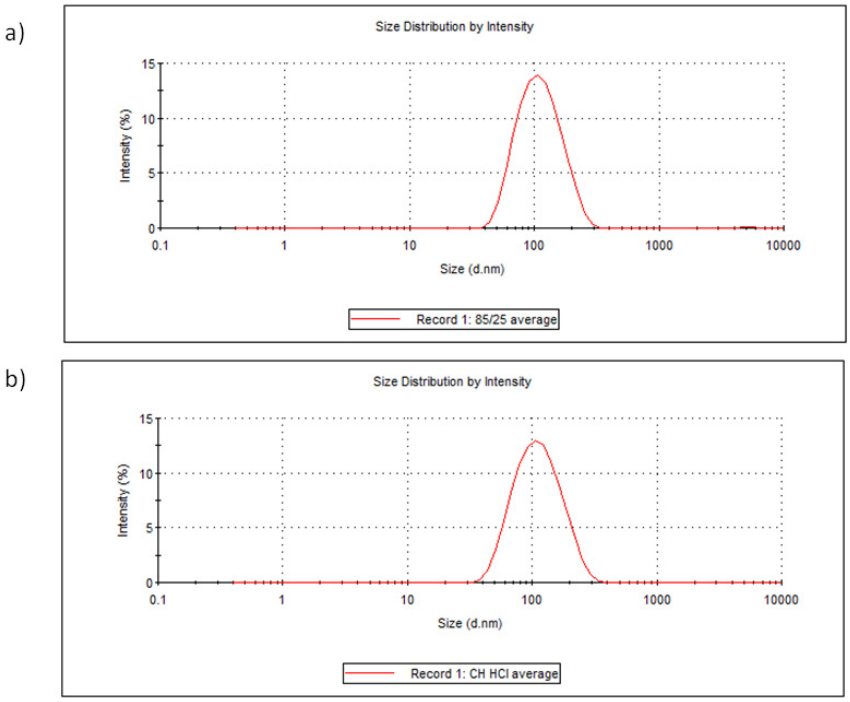


Figure 7.2. *Size distributions of a) chitosan 85/25 and b) chitosan hydrochloride (CH HCl).*

The effect of pH on particle formation was assessed for chitosan acetate and chitosan lactate. Trials were made with both pH-adjusted (~5.5) and non-adjusted sample solutions. Table 7.1 presents the effect of pH on size, size stability, and zeta potential of nanoparticles.

Table 7.1. *Effect of pH on particle properties.*

Chitosan	pH	Size (nm)	StD_0	Size week 1	Std_1	Size week 2	Std_2	$Zeta_0$ (mV)	Std zeta
Chitosan acetate	5.6	158	1.3	168.4	2.3	170.5	1.9	26	1.9
	6.7	202.4	3.1	231.3	42.9	201.7	4.1	11	0.8
Chitosan lactate	5.6	147	1.3	158.6	2.1	158.2	1.7	28	0.7
	6.7	1352	960.3	-	-	-	-	10.5	1.2

As can be observed from Table 7.1, non-pH-adjusted chitosans yielded larger particles with a significantly smaller zeta potential compared to the pH-adjusted chitosan solution. Especially chitosan lactate appeared to be affected by the initial pH 6.7 and nanoscale particles were not achieved. Although non-pH-adjusted chitosan acetate formed approximately 40 nm bigger particles than the pH-adjusted version, the particles were still in nanoscale (~200 nm) and managed to retain a somewhat stable size during a period of 3 weeks.

7.1.2. Particle size stability

Follow-up size measurements were performed at week 1 and week 2 after particle preparation. The nanoparticle solutions were also observed for any changes in clarity due to aggregation. Appendix 3 shows the size development of nanoparticles prepared by different chitosans during a period of 2 weeks. As can be seen from the graphs, there is a slight increasing trend in size. However, by pure observation it is questionable whether this increase is significant as the results generally appear to fall within the standard deviation range. Therefore, the Mann-Whitney U test and the Student's t-test (two-tailed, $p < 0.05$) were applied to assess the statistical significance of size stability using equations 11 and 12 provided in Section 5.4. Values of $p < 0.05$ were considered significant.

Appendix 4 summarises the statistical significance of size development during a period of 2 weeks. From the tables it can be concluded that the parametric and non-parametric tests generally support each other, with the exception of chitosan lactate whose size development is considered significant from week 0 to 1 by the Mann-Whitney U test and non-significant by the Student's t-test. Nevertheless, the statistical analysis shows that the particle size generally increased significantly from week 0 to week 1. The increase in size from week 0 to week 2 was also statistically significant for nearly all samples. The size development from week 1 to week 2, on the other hand, was not as significant, and only 8 out of 17 samples experienced a statistically significant increase in size. Therefore, it could be argued that the particle size stabilises after one week of preparation.

The greatest numerical change in size was achieved by the low molecular weight (25 kDa) chitosans with higher DD (85 and 95). The size development of these chitosans is presented in Figure 7.3.

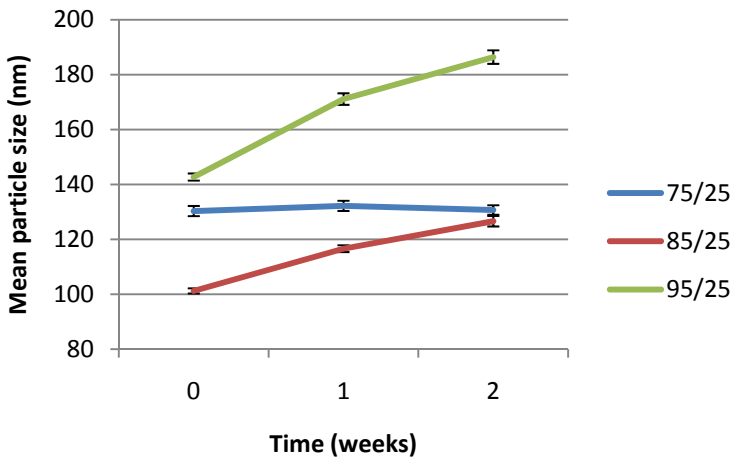


Figure 7.3. *Development of particle size in relation to DD over a period of 2 weeks. It appears that higher DD with low M_w seems to have a strong impact on particle size stability.*

As can be noted from Figure 7.3, the size of sample 95/25 increased by approximately 45 nm and the size of sample 85/25 by about 20 nm. From this it can be concluded that low molecular weight chitosan bases with a high DD are more unstable concerning size stability. It should also be noted that CH HCl (95/40) increased in size with approximately 10 nm during a period of 2 weeks. As a whole, this is quite a small change and it is also less than the size change for low M_w and high DD chitosan bases. This may be due to the slightly higher molecular weight of CH HCl or to the chemical nature of chitosan hydrochloride.

In general, the size of chitosan-pDNA nanoparticles first increased significantly during the first week after preparation and then remained relatively stable for the next week. Although size development was statistically significant especially during the first follow-up week, it may be argued that this statistical significance is not so important, as numerically only small changes in particle size were noted. In addition, many of the obtained values for the follow-up period lie within the standard deviations of measurements. No changes in solution clarity could be detected by the human eye.

7.1.3. Zeta potential

Zeta potential generally varied from approximately +20 mV to +30mV, which is in accordance with values presented in the literature. Figure 7.4 illustrates the zeta potentials of different nanoparticles.

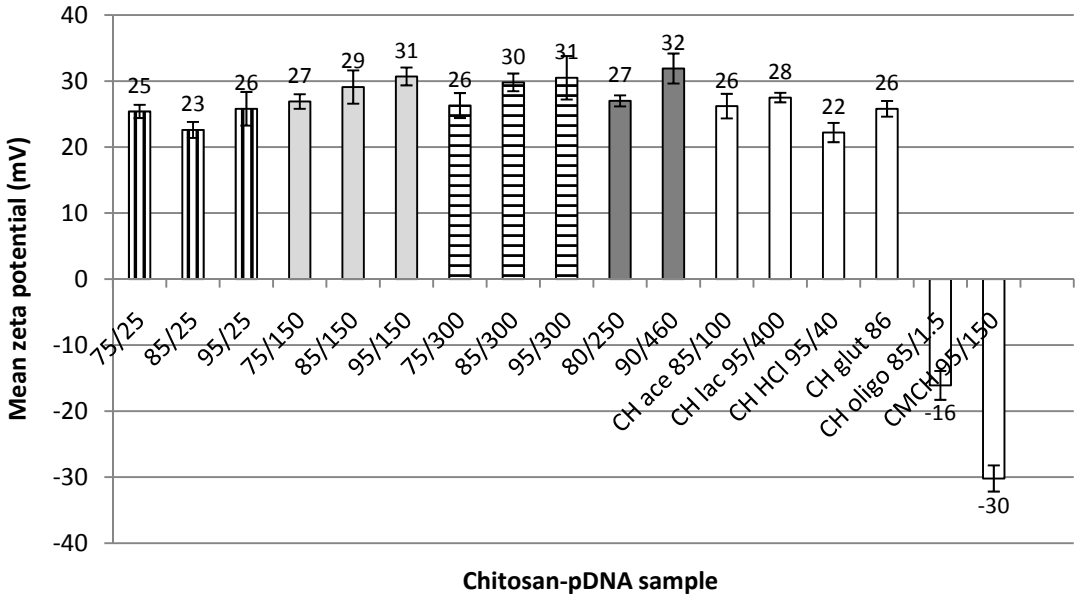


Figure 7.4. *Zeta potential of chitosan-pDNA nanoparticles.*

Greatest zeta potentials were obtained with high DD high M_w chitosan base samples (90/460, 95/300, 85/300, 95/150, 85/150). Zeta potentials of chitosan salts and derivatives were comparable to those of low DD and low M_w chitosan base samples. A slight correlation was observed between DD and zeta potential for chitosan base with M_w 150 and 300 kDa. Zeta potential appeared to increase with increased DD as M_w remained constant. However, measurements do express some deviation, which makes drawing conclusions complicated. In many cases, the particle concentration was also too low for reliable zeta potential measurements. Nevertheless, it is likely that high DD enables a higher amount of protonated amino groups in the chitosan thereby making the complex more stable. Naturally, a high M_w also provides a greater range for deacetylated units. This is in accordance with theory [37]. It should also be noted that in addition to DD and M_w, also pH has an important effect on zeta potential. In this study, the zeta potential was assessed only at pH 5.5.

Like in most of the other measurements, the values of CMCH and CH oligo differed from the other samples. In this case, the values were negative. Zeta potential of CMCH and CH oligo was about -30 mV and -15 mV, respectively. Therefore, zeta potentials obtained for these samples indicate instability. As a whole, however, zeta potentials of chitosan samples were relatively stable and in accordance with previously presented values.

7.1.4. PDI

The polydispersity index of the chitosan nanoparticles was generally in accordance with values presented in the literature. Theoretical values range from 0.027 to 0.6. In this

study, values from 0.14 to 0.25 were obtained, excluding the values for CMCH and CH oligo, which were unreliable. Figure 7.5 presents the polydispersity indices for different samples.

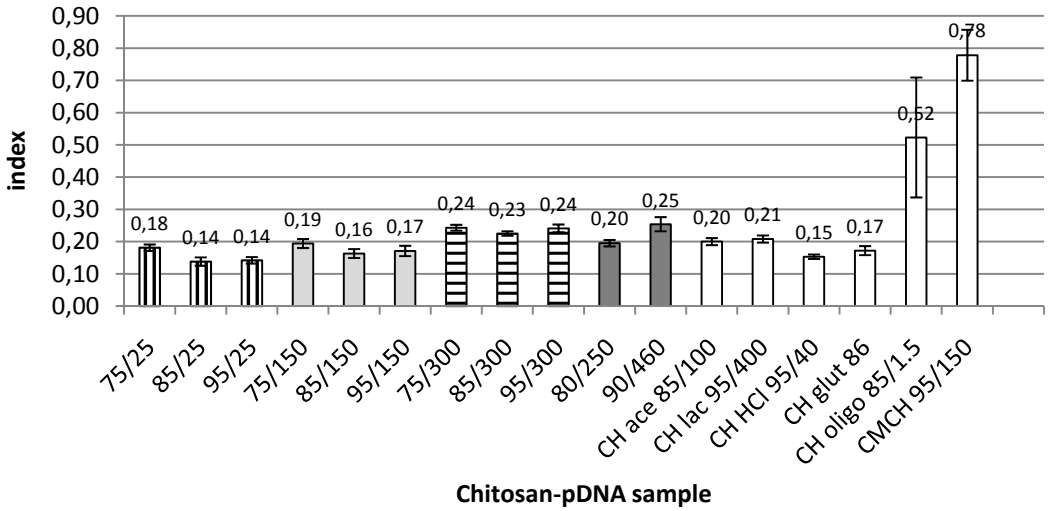


Figure 7.5. *Polydispersity index of chitosan-pDNA nanoparticles.*

Most values were located between 0.14 and 0.25. Comparing the PDI with size volume distributions, it seems that the smaller the PDI, the less polydisperse the sample.

7.1.5. Correlations: size and M_w, size and DD, size and ZP

It was attempted to evaluate whether some correlation existed between different chitosan properties and chitosan-pDNA nanoparticle parameters. Unfortunately, no clear correlations were detected. Considering chitosan bases, it appears that with a DD of 75, the particle size increases with increasing M_w. Samples with a DD of 85 and 95, on the other hand, first increased in size when M_w was increased from 25 kDa to 150 kDa. However, when the M_w was further increased to 300 kDa, the particle size decreased. This suggests that for high DD chitosans, either a low or high M_w is more suitable to yield small nanoparticles than medium molecular weight chitosan. In the case of DD 85, the smallest M_w produced the smallest particles, while the high molecular weight chitosan of DD 95 produced slightly smaller particles than the respective LMW chitosan. Figure 7.6 demonstrates the particle size and zeta potential as a function of M_w with constant DD.

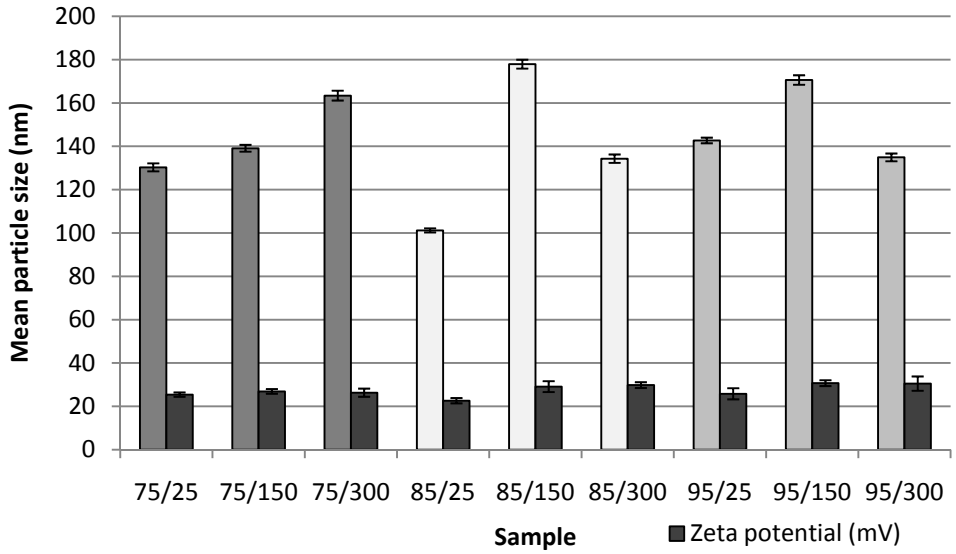


***Figure 7.6**. Particle size and zeta potential as a function of M_w with constant DD.*

Interestingly, considering the chitosan bases with respect to the DD with constant M_w, it appears that the size of HMWC will decrease as a function of increased DD. With LMWC and MMWC this relationship was not evident. Figure 7.7 illustrates the particle size and zeta potential as a function of DD with constant M_w. In the literature, it has been presented that particle size decreases with decreasing M_w or possibly increases with very small M_w if the DD remains constant. Therefore, the results of this study are in accordance with literature. In addition, they demonstrate that high DD and high M_w chitosans can yield smaller particles than high DD medium molecular weight chitosans.

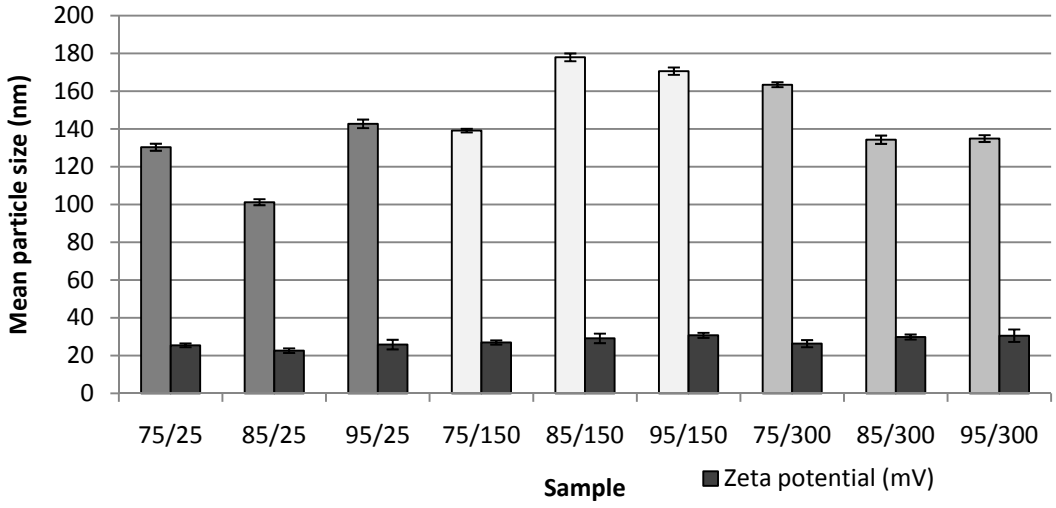


***Figure 7.7.** Particle size and zeta potential as a function of DD with constant M_w.*

Zeta potential did not directly correspond to particle size, but it was mentioned earlier that high DD and high M_w chitosans produced the most stable complexes.

7.2. Agarose gel electrophoresis

Agarose gel electrophoresis illustrated that pDNA coupled with most chitosans, excluding carboxymethylchitosan (CMCH) and chitosan oligosaccharide (CH oligo) that migrated on the gel (see Figure 7.8).

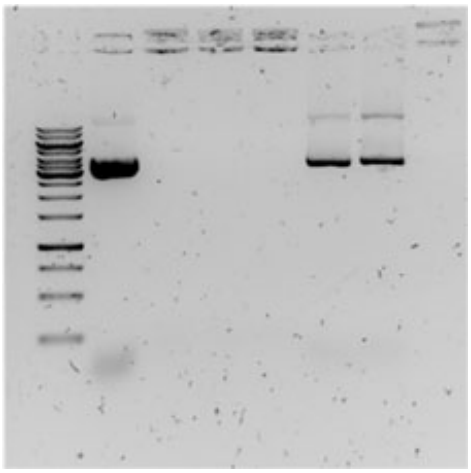


Figure 7.8. *Agarose gel electrophoresis image. From left to right: DNA ladder, plasmid, 75/50, 85/50, 95/50, CMCH, CH oligo and CH HCl.*

A more extensive gel electrophoresis image collage is given in Appendix 5. Possible explanations for the failure of chitosan oligosaccharide and carboxymethylchitosan to integrate plasmid DNA include the inability to form particles due to, for instance, low molecular weight (CH oligo) and the lack of an appropriate amount of amino groups (CMCH). Based on the gel electrophoresis images, it is likely that the pDNA resided free in the solution. CH oligo and CMCH –pDNA nanoparticles could be prepared with different chitosan:pDNA weight ratios in order to assess whether this would yield nanoparticles.

7.3. FEG-SEM

FEG-SEM images were obtained for chitosan 85/25-, 80/250- and chitosan hydrochloride pDNA nanoparticles. Carbon-coated samples produced better quality images. Some images are shown in Figure 7.9.

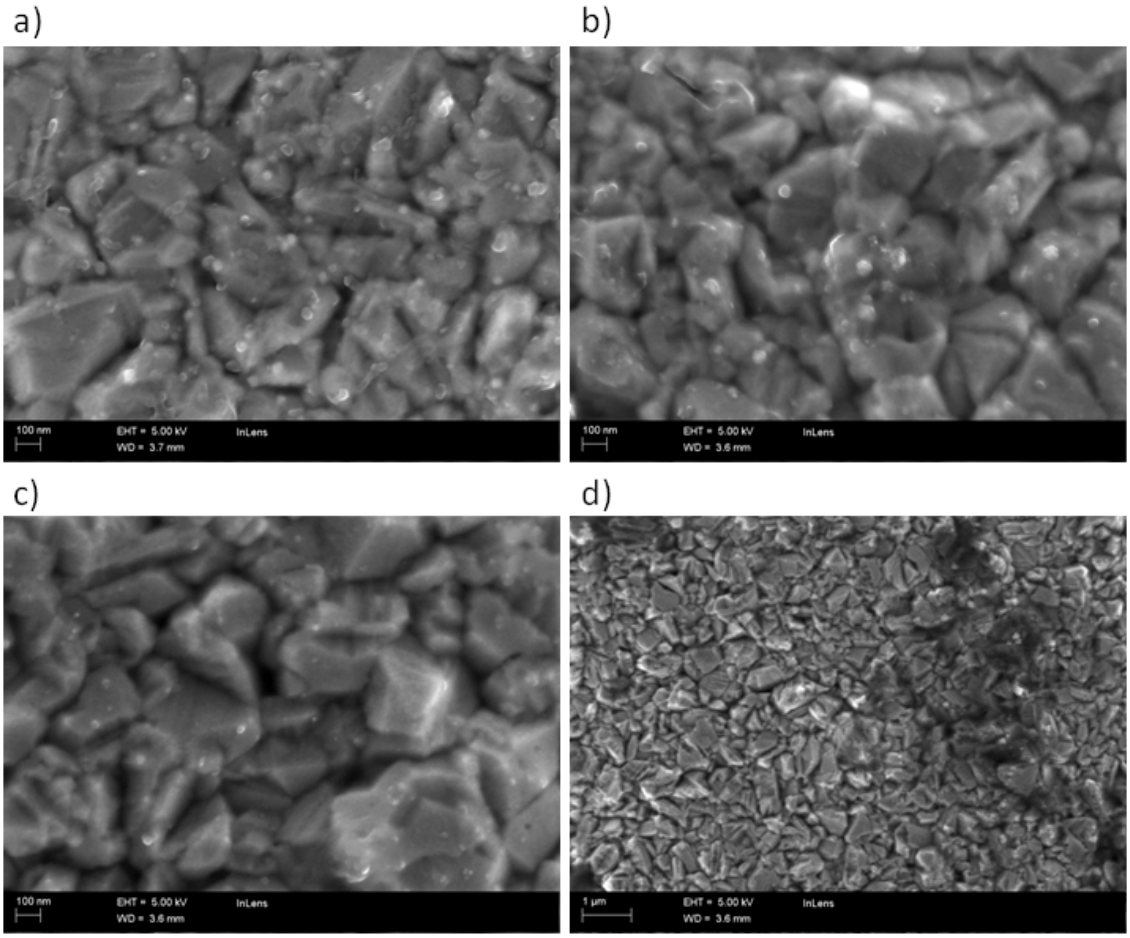


Figure 7.9. *FEG-SEM images of chitosan-pDNA nanoparticles. Nanoparticles were prepares using a) CH HCl b) 85/25 and c and d) 80/250. Magnifications are 50 000x (a-c) and 100x (d).*

As can be seen from Figure 7.9a, chitosan HCl yielded relatively uniform and numerous nanoparticles. Some particles appeared sphere-like, while some resembled flakes. Also 85/25 produced sphere-like particles, though these were not as evenly spread and numerous as CH HCl. Some of the particles in the 100 nm range of 85/25 seemed slightly raisin-like and not as spherical (see Figure 7.9b). This could be explained by aggregation of smaller particles. Another possible explanation may be the hydrogel nature of chitosan-pDNA nanoparticles. In a solution, the shape may be more spherical due to swelling. However, the latter is subject to contradiction as previous studies [43; 59; 74] have demonstrated spherical structures for chitosan-pDNA nanoparticles. Nevertheless, this explanation could be verified by observing the particles by AFM, which allows imaging in aqueous conditions and thus, the real environment of the particles.

The particle yield of the reference chitosan, 80/250, was surprisingly low compared to previous studies using the same parameters (Figure 7.9c). The reference particles also appeared to produce raisin-like structures. Nevertheless, while the particle size was quite small (~20 nm) the particles were relatively spherical. In the case of bigger

particles (100-200 nm), the raisin-like structures persisted, which suggests aggregation. The raisin-like structures of sample 80/250 and 85/25 are shown in Figure 7.10.

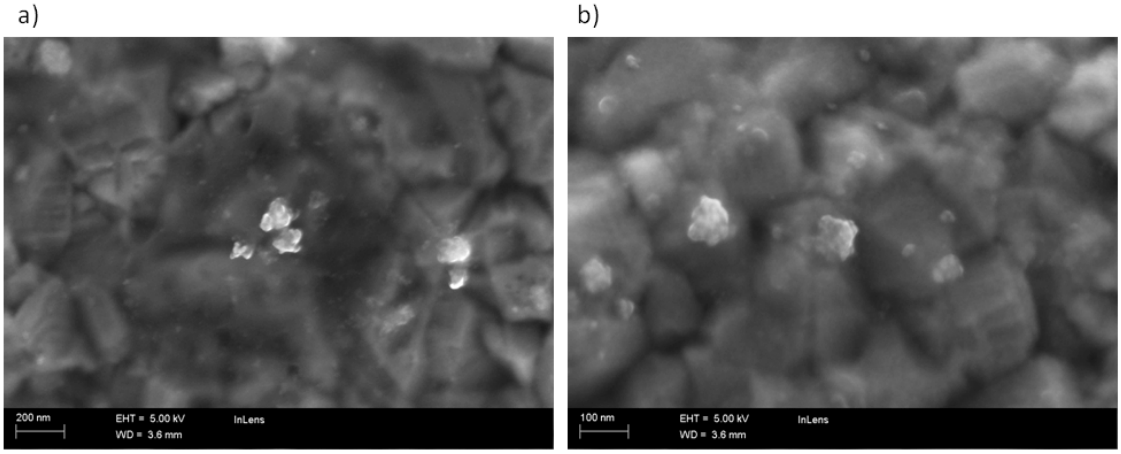


Figure 7.10. *a) Chitosan-pDNA nanoparticles prepared using chitosan 80/250 and b) 85/25.*

Although particles were not as spherical and uniformly distributed as desired, FEG-SEM images supported the nanoscale size measurements of DLS. In fact, particles were even smaller than in DLS measurements, probably due to drying. Some possible sources for error in FEG-SEM imaging may include the arbitrary image spot on the grid, disturbances in carbon coating and technical problems with the FEG-SEM. Indeed, there were some issues with the focus of the FEG-SEM, and it was suggested that perhaps there is a disturbance in the microscope column. However, changing the aperture from 30 to 20 made imaging possible, though close-ups did not succeed.

8. CONCLUSIONS

The objective of this study was to prepare and characterise chitosan-pDNA nanoparticles using 17 different types of chitosan in order to assess which chitosan yields the most favourable nanoparticle characteristics in terms of size, size stability and zeta potential. Also possible correlations between chitosan properties like DD, M_w, and particle parameters were explored. Both chitosan bases and water-soluble chitosan salts and derivatives were tested.

Chitosan-pDNA nanoparticles were successfully prepared by complex coacervation with a fixed chitosan to pDNA weight ratio of 4:1. Only two chitosans, including chitosan oligosaccharide and carboxymethylchitosan, did not form nanoparticles at this ratio. The remaining chitosans, however, formed particles with sizes ranging from 100 to 180 nm. Also the zeta potential of these chitosans was relatively stable and ranged from +20 to +30 mV. Particle size and zeta potential was assessed by dynamic light scattering and laser Doppler velocimetry, respectively. Integration of pDNA to the particles was evaluated using gel electrophoresis and finally the morphology of the most prominent samples, including chitosan base 85/25 and chitosan hydrochloride, was observed under FEG-SEM. The combination of the mentioned characterisation techniques appeared successful as results supported each other. For example, CH oligo and CMCH that did not form particles according to DLS expectedly migrated during gel electrophoresis, unlike other samples. Moreover, the zeta potential of CH oligo and CMCH was negative, which differed from other samples. The underlying reasons for the unsuccessful results for CH oligo and CMCH may be explained by the chemical nature of these materials. For instance, the molecular weight of CH oligo at the given preparation parameters may simply be too low to produce nanoparticles. Moreover, the chemical properties of CMCH may significantly affect particle formation. For example, N,O-carboxymethylchitosan has a reduced amount of protonated amino groups due to N-carboxymethylation. Particle formation based on electrostatic interaction may therefore be hindered.

Unfortunately, no clear correlations between chitosan properties and chitosan-pDNA nanoparticle properties were found. However, it would seem that a higher DD and M_w lead to more stable particles, as the zeta potential is increased due to an elevated number of protonated amino groups. It appears that for low DD (75%) chitosans, an increase in M_w leads to an increase in particle size. For higher DDs, this relationship was not evident. In fact, particle size first increased when the M_w was increased from 25 kDa to 150 kDa. Thereafter, particle size decreased when the M_w was further increased to 300 kDa with a constant DD. Size stability experienced a slight increasing

trend over a period of 2 weeks. Statistical analysis indicated that for most samples particle size increased significantly during the first week and then remained relatively stable. Low M_w chitosan bases with high DD (85/25 and 95/25) seemed to experience the greatest increase in size ranging from 20-40 nm. Therefore, these chitosans should be used with care in cases where size stability is of utmost importance.

FEG-SEM was a useful tool for particle visualisation. Unfortunately, some technical problems were encountered during the use of SEM and also an inexplicable black membrane covered the sample grid. Nevertheless, particles were detected despite the fact that close-ups could not be obtained due to a lower aperture. According to FEG-SEM, particle size ranged from approximately 20 to 200 nm. Smaller particles appeared more uniform and spherical in size than bigger complexes that appeared raisin-like. This suggests that some aggregation is present. Alternatively, the particles may possess a hydrogel nature which diminishes when the sample is dried, resulting in "shrunken" complexes. AFM, that allows observation of particles in aqueous media, may be a useful imaging technique to assess the latter, as it better reflects the true environment of nanoparticles. Chitosan hydrochloride appeared to produce more numerous and uniform particles than chitosan base 85/25. Therefore, the choice between a chitosan base and a chitosan derivative seems insignificant, though it should be verified that the chosen derivative or salt actually yields nanoparticles at a given chitosan to pDNA weight ratio.

As a whole, most samples (15/17) yielded acceptable results in terms of size, size stability and zeta potential. Only chitosan oligosaccharide and carboxymethylchitosan displayed unsatisfactory results at the given preparation parameters. As the chemical nature of chitosan and especially chitosan derivatives may vary significantly, it is perhaps somewhat naive to believe that the best results would be obtained at a fixed chitosan to pDNA ratio for all samples. Therefore, future experiments should focus on exploring the effects of different chitosan:pDNA weight ratios on particle parameters. Also the possibility to functionalise the prepared samples should be explored. Although chitosan 85/25 and chitosan HCl produced the smallest particles at the given weight ratio, it was observed by other researchers in the same project that functionalisation of these particles was unachievable due to sedimentation. However, when the weight ratio was changed, functionalisation properties were improved. Only after careful consideration of all parameters should the most prominent samples be tested *in vitro* and finally *in vivo*. Also other factors such as cost, time, manufacturability and sterilisation properties should be considered. In addition, different preparation techniques, such as a recently introduced osmosis-based method, could be explored.

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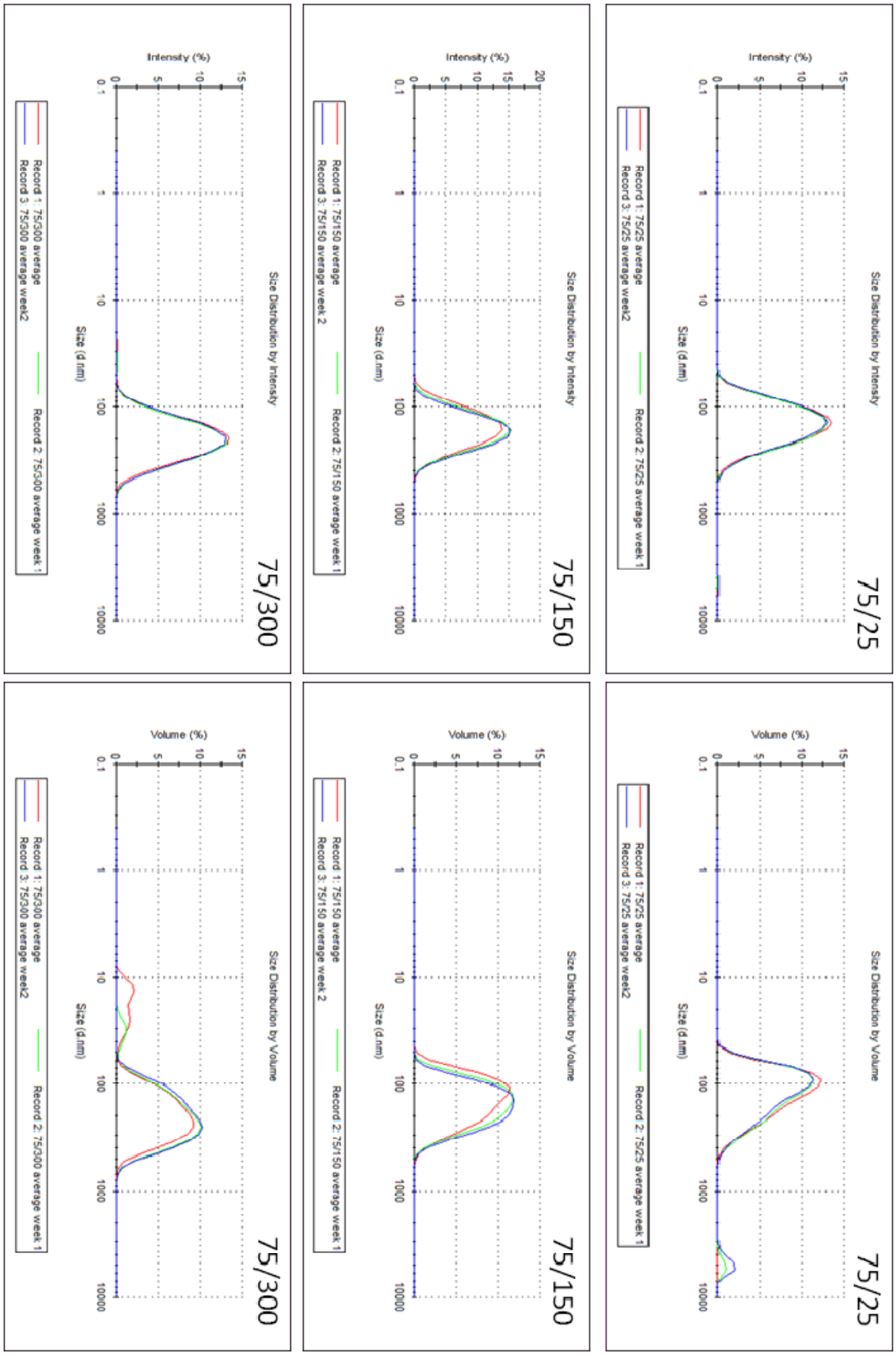
APPENDIX 1: Results and chitosan parameters

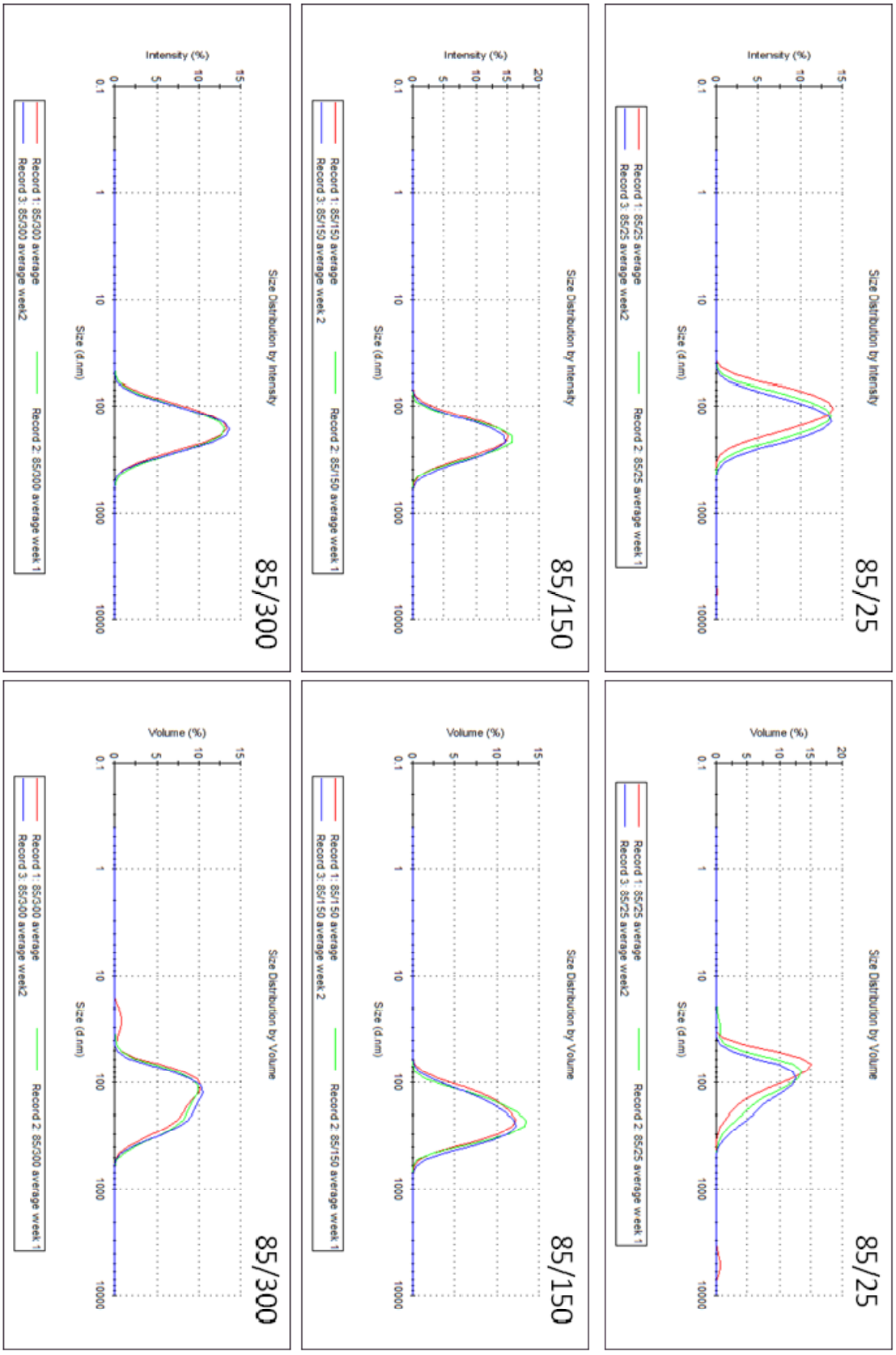
Nr	Chitosan	Sample name	Mw (kDa)	η (mPas)	DD%	H_2O-solub	pH	Size (nm)	StD0	Size 1 week	StD1	Size 2wee	StD2	Zeta 0	StD zeta	DNA incor	PDI	StDpdi
1	Chitoceuticals Chitosan 75/5	75/25	25	7	76.5	N	5.51	130	1.8	132.2	1.9	130.7	1.7	25	1.0	Y	0.18	0.01
2	Chitoceuticals Chitosan 85/5	85/25	25	5	84.3	N	5.51	101	0.9	116.6	1.2	126.6	1.9	23	1.2	Y	0.14	0.013
3	Chitoceuticals Chitosan 95/5	95/25	25	7	94.5	N	5.51	143	1.3	171.1	2.1	186.4	2.5	26	2.6	Y	0.14	0.01
4	Chitoceuticals Chitosan 75/50	75/150	150	41	74.1	N	5.46	139	1.6	144.6	1.9	149.2	3.4	27	1.1	Y	0.19	0.014
5	Chitoceuticals Chitosan 85/50	85/150	150	36	83.7	N	5.53	178	2.0	186.1	2.7	187.9	3.1	29	2.5	Y	0.16	0.014
6	Chitoceuticals Chitosan 95/50	95/150	150	45	92.8	N	5.48	171	2.2	177	2.6	179.3	2.6	31	1.4	Y	0.17	0.016
7	Chitoceuticals Chitosan 75/200	75/300	300	300	74.9	N	5.5	163	2.3	168.8	2.4	167.8	2.3	26	1.9	Y	0.24	0.009
8	Chitoceuticals Chitosan 85/200	85/300	300	180	87.3	N	5.47	134	1.9	139.5	1.6	141	2.4	30	1.3	Y	0.23	0.007
9	Chitoceuticals Chitosan 95/200	95/300	300	330	94.3	N	5.47	135	1.8	143.2	2.9	146.5	2.1	31	3.3	Y	0.24	0.012
10	Chitosan base (PROTASAN UP B 80/20)	80/250	250	48	86	N	5.47	132	1.2	139.2	2.8	138.6	1.7	27	0.8	Y	0.20	0.01
11	Chitosan base (PROTASAN UP B 90/500)	90/460	460	548	91	N	5.49	146	2.5	153.6	1.3	157.2	3.9	32	2.3	Y	0.25	0.022
12	Chitosan acetate	CH ace 85/100	100	45	83.6	Y	5.55	158	1.3	168.4	2.3	170.5	1.9	26	1.9	Y	0.20	0.011
13	Chitosan lactate	CH lac 95/400	400	220	93.4	Y	5.55	147	1.3	158.6	2.1	158.2	1.7	28	0.7	Y	0.21	0.011
14	Chitosan hydrochloride	CH HCl 95/40	40	7	91.7	Y	5.52	100	1.3	107	1.8	111.2	1.9	22	1.5	Y	0.15	0.007
15	Chitosan glutamate (PROTASAN UP G 113)	CH glut 86	50-200	16	86	Y	5.51	128	2.1	131.3	2.0	132.3	1.9	26	1.2	Y	0.17	0.014
16	Chitosan oligosaccharide	CH oligo 85/1.5	1.5	1	83.1	Y	5.52	1298	174.5	891.8	338.8	345.8	193.4	-16	2.2	N	0.52	0.186
17	Carboxymethyl-chitosan	CMCH 95/150	150	45	94.4	Y	5.52	1755	151.0	1000	105.9	1543	149.8	-30	2.0	N	0.78	0.079

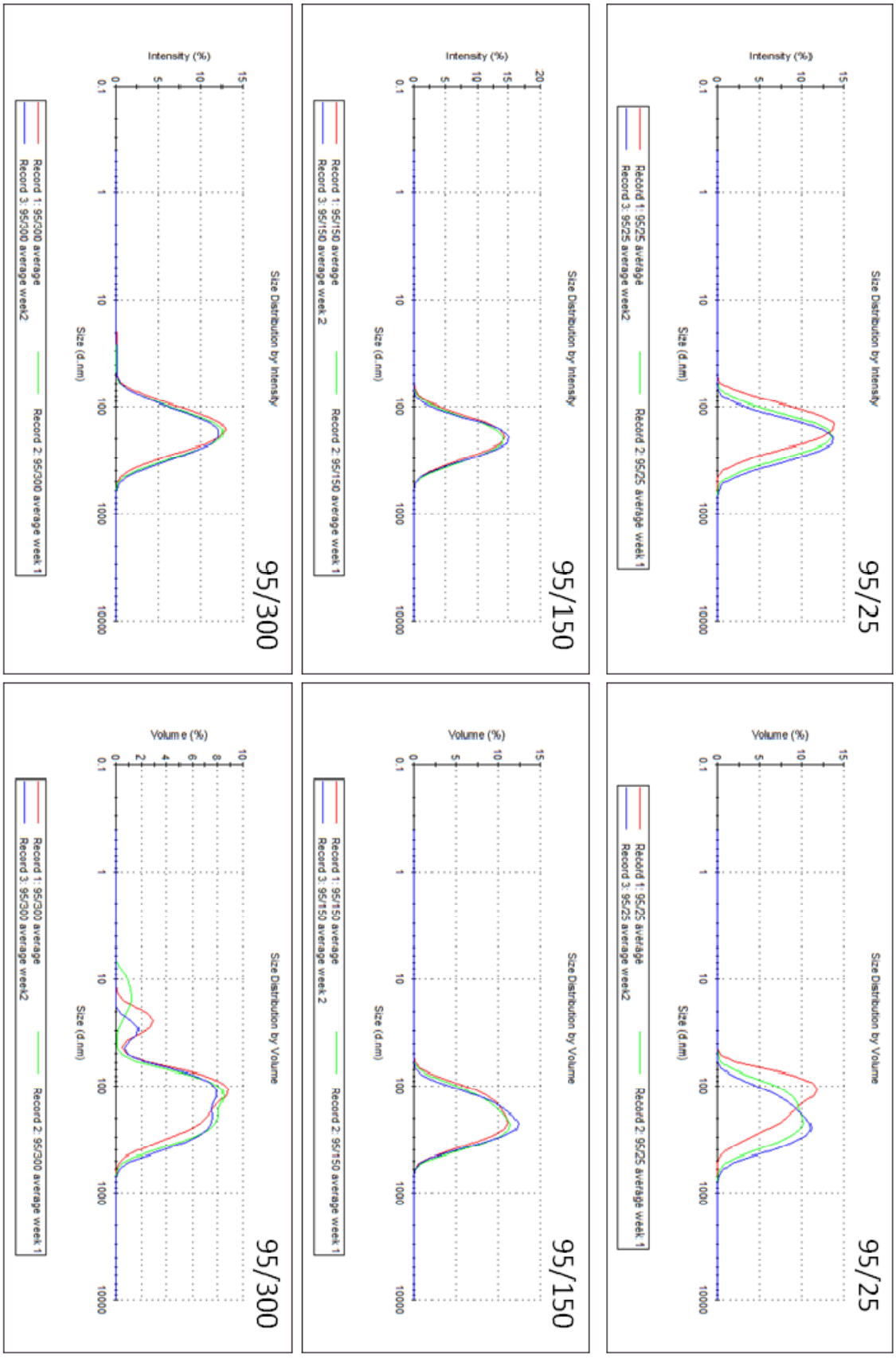
pH adjusted with 1M HCl

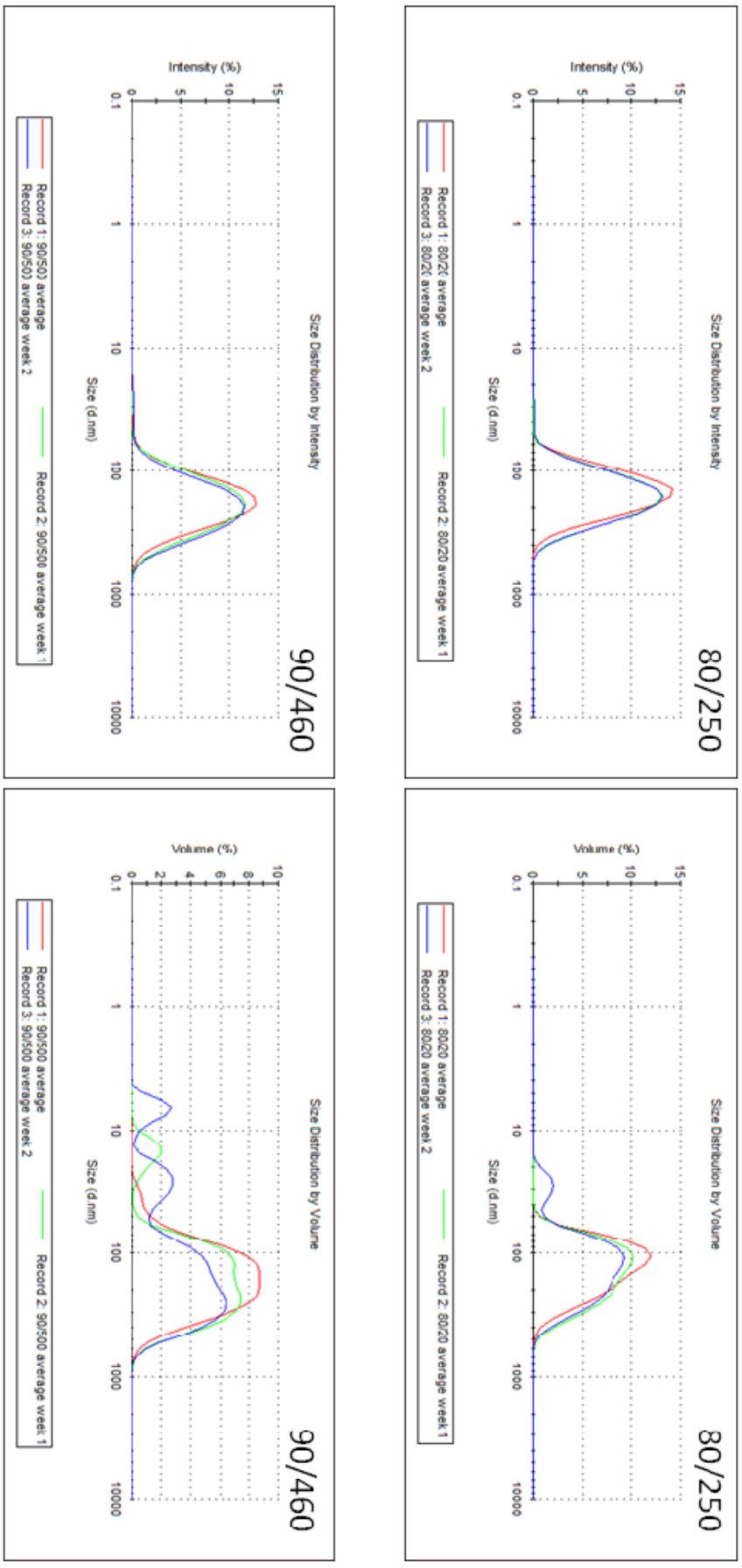
pH adjusted with 10M NaOH

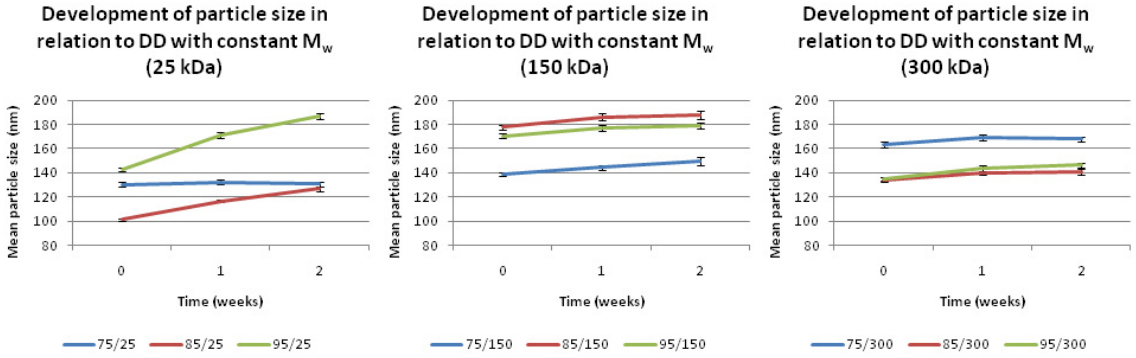
smallest size values

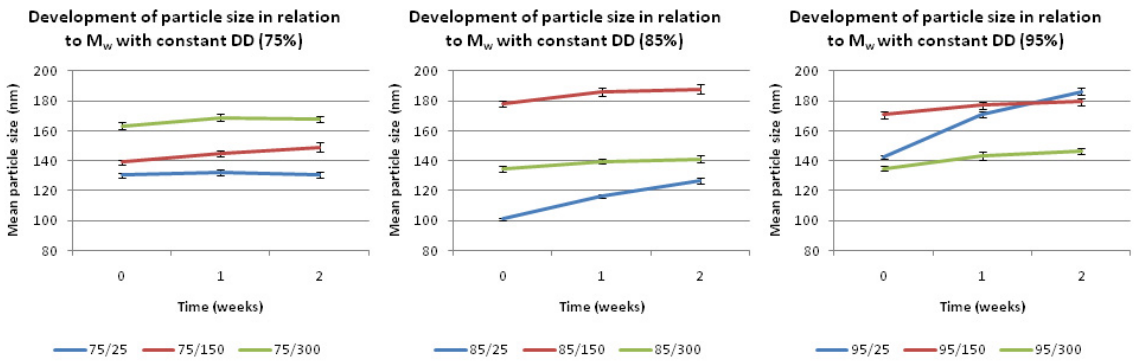

Size Distribution by Intensity
75/25
Intensity (%)
Size (d.nm)
Record 1: 75/25 average
Record 3: 75/25 average week2
Record 2: 75/25 average week 1
Size Distribution by Intensity
75/150
Intensity (%)
Size (d.nm)
Record 1: 75/150 average
Record 3: 75/150 average week 2
Record 2: 75/150 average week 1
Size Distribution by Intensity
75/300
Intensity (%)
Size (d.nm)
Record 1: 75/300 average
Record 3: 75/300 average week2
Record 2: 75/300 average week 1
Size Distribution by Volume
75/25
Volume (%)
Size (d.nm)
Record 1: 75/25 average
Record 3: 75/25 average week2
Record 2: 75/25 average week 1
Size Distribution by Volume
75/150
Volume (%)
Size (d.nm)
Record 1: 75/150 average
Record 3: 75/150 average week 2
Record 2: 75/150 average week 1
Size Distribution by Volume
75/300
Volume (%)
Size (d.nm)
Record 1: 75/300 average
Record 3: 75/300 average week2
Record 2: 75/300 average week 1

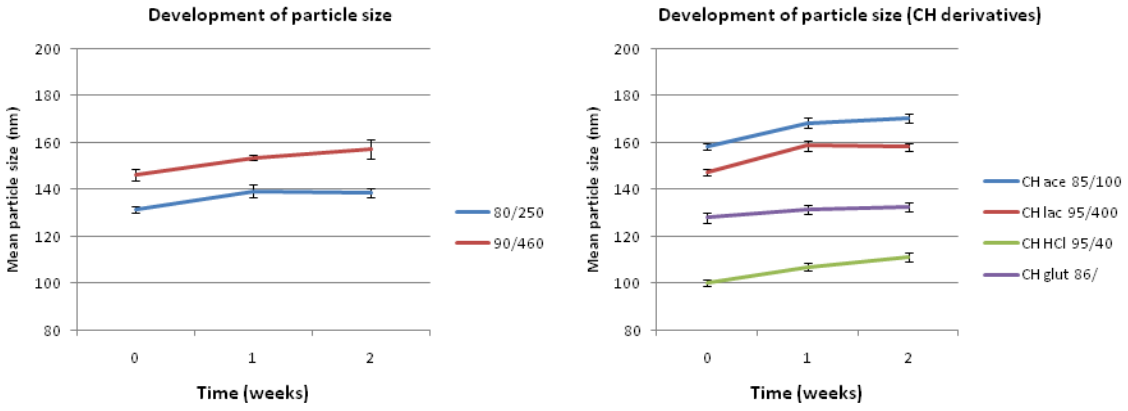

Size Distribution by Intensity
85/25
Intensity (%)
Size (d.nm)
Record 1: 85/25 average
Record 3: 85/25 average week2
Record 2: 85/25 average week 1
Size Distribution by Intensity
85/150
Intensity (%)
Size (d.nm)
Record 1: 85/150 average
Record 3: 85/150 average week 2
Record 2: 85/150 average week 1
Size Distribution by Intensity
85/300
Intensity (%)
Size (d.nm)
Record 1: 85/300 average
Record 3: 85/300 average week2
Record 2: 85/300 average week 1
Size Distribution by Volume
85/25
Volume (%)
Size (d.nm)
Record 1: 85/25 average
Record 3: 85/25 average week2
Record 2: 85/25 average week 1
Size Distribution by Volume
85/150
Volume (%)
Size (d.nm)
Record 1: 85/150 average
Record 3: 85/150 average week 2
Record 2: 85/150 average week 1
Size Distribution by Volume
85/300
Volume (%)
Size (d.nm)
Record 1: 85/300 average
Record 3: 85/300 average week2
Record 2: 85/300 average week 1


Size Distribution by Intensity
95/25
Intensity (%)
Size (d.nm)
Record 1: 95/25 average
Record 3: 95/25 average week2
Record 2: 95/25 average week 1
Size Distribution by Intensity
95/150
Intensity (%)
Size (d.nm)
Record 1: 95/150 average
Record 3: 95/150 average week 2
Record 2: 95/150 average week 1
Size Distribution by Intensity
95/300
Intensity (%)
Size (d.nm)
Record 1: 95/300 average
Record 3: 95/300 average week2
Record 2: 95/300 average week 1
Size Distribution by Volume
95/25
Volume (%)
Size (d.nm)
Record 1: 95/25 average
Record 3: 95/25 average week2
Record 2: 95/25 average week 1
Size Distribution by Volume
95/150
Volume (%)
Size (d.nm)
Record 1: 95/150 average
Record 3: 95/150 average week 2
Record 2: 95/150 average week 1
Size Distribution by Volume
95/300
Volume (%)
Size (d.nm)
Record 1: 95/300 average
Record 3: 95/300 average week2
Record 2: 95/300 average week 1


Size Distribution by Intensity
80/250
Intensity (%)
Size (d.nm)
Record 1: 80/20 average
Record 3: 80/20 average week 2
Record 2: 80/20 average week 1
Size Distribution by Intensity
90/460
Intensity (%)
Size (d.nm)
Record 1: 90/500 average
Record 3: 90/500 average week 2
Record 2: 90/500 average week 1
Size Distribution by Volume
80/250
Volume (%)
Size (d.nm)
Record 1: 80/20 average
Record 3: 80/20 average week 2
Record 2: 80/20 average week 1
Size Distribution by Volume
90/460
Volume (%)
Size (d.nm)
Record 1: 90/500 average
Record 3: 90/500 average week 2
Record 2: 90/500 average week 1


Development of particle size in relation to DD with constant M_w (25 kDa)
Development of particle size in relation to DD with constant M_w (150 kDa)
Development of particle size in relation to DD with constant M_w (300 kDa)
Mean particle size (nm)
Time (weeks)
75/25
85/25
95/25
75/150
85/150
95/150
75/300
85/300
95/300


Development of particle size in relation to M_w with constant DD (75%)
Development of particle size in relation to M_w with constant DD (85%)
Development of particle size in relation to M_w with constant DD (95%)
Mean particle size (nm)
Time (weeks)
75/25
75/150
75/300
85/25
85/150
85/300
95/25
95/150
95/300


Development of particle size
Development of particle size (CH derivatives)
Mean particle size (nm)
Time (weeks)
80/250
90/460
CH ace 85/100
CH lac 95/400
CH HCl 95/40
CH glut 86/

Mann-Whitney U, p < 0.05 is significant				**Student's t-test, p < 0.05, df 16, two-tailed 95%, t = 2.120**		
Sample	Week 0-1	Week 1-2	Week 0-2	Week 0-1	Week 1-2	Week 0-2
80/250	S	NS	S	S	NS	S
90/460	S	S	S	S	S	S
CH glut	S	NS	S	S	NS	S
75/25	S	NS	NS	S	NS	NS
85/25	S	S	S	S	S	S
95/25	S	S	S	S	S	S
75/300	S	NS	S	S	NS	S
85/300	S	NS	S	S	NS	S
95/300	S	S	S	S	S	S
75/150	S	S	S	S	S	S
85/150	S	NS	S	S	NS	S
95/150	S	NS	S	S	NS	S
CMCH	S	S	S	S	S	S
CH oligo	S	S	S	S	S	S
CH HCl	S	S	S	S	S	S
CH ace	S	NS	S	S	NS	S
CH ace pH 6.7	NS	NS	NS	NS	NS	NS
CH lac	S	NS	S	NS	NS	S

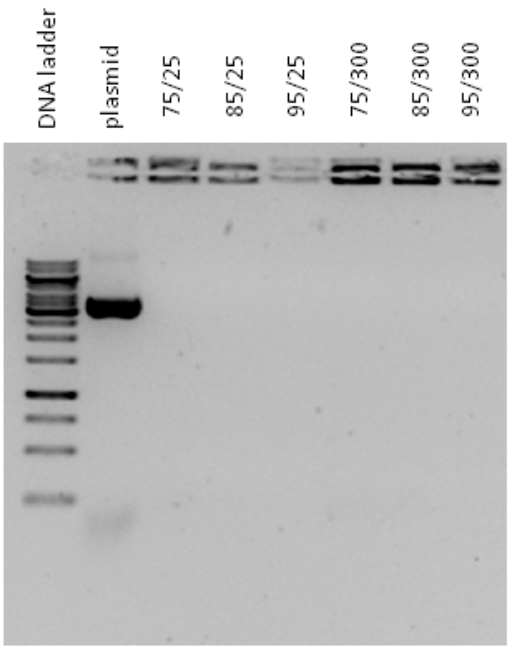
Student's t-test values, p < 0.05, df 16, two-tailed 95%, t = 2.120			
Sample	Week 0-1*	Week 1-2*	Week 0-2*
80/250	-7.602	0.552	-10.017
90/460	-7.768	-2.618	-7.056
CH glut	-3.519	-1.085	-4.634
75/25	-2.180	1.776	-0.474
85/25	-29.586	-13.126	-35.696
95/25	-34.577	-14.191	-47.205
75/300	-4.918	0.905	-4.072
85/300	-6.267	-1.564	-6.498
95/300	-7.328	-2.789	-12.748
75/150	-6.585	-3.534	-8.106
85/150	-7.189	-1.297	-8.029
95/150	23.682	46.476	46.833
CMCH	12.281	-8.880	2.990
CH oligo	3.198	4.199	10.966
CH HCl	-9.086	-4.815	-14.208
CH ace	-11.583	-2.102	-16.179
CH ace pH 6.7	-2.015	2.060	0.409
CH lac	-1.855	1.842	-15.525

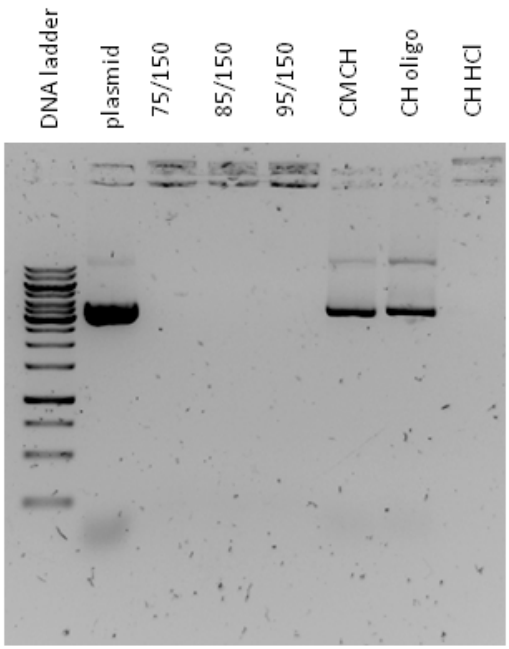
*absolute values were used when assessing significance

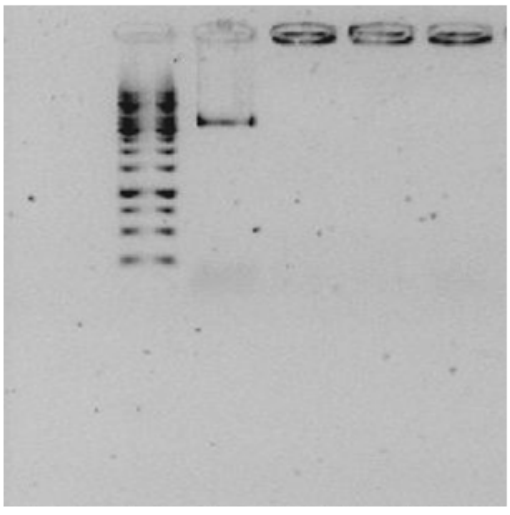
S = significant change

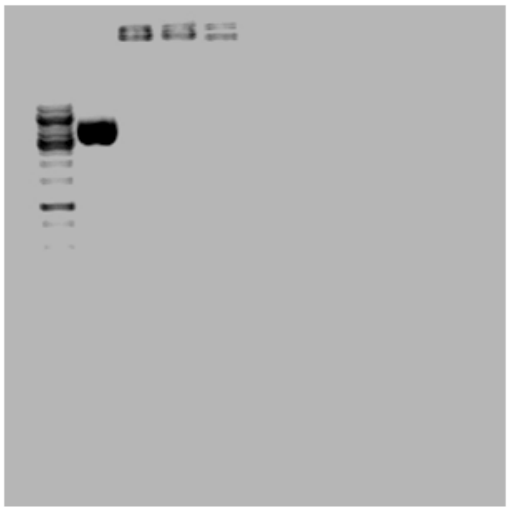
NS = non-significant

df = degrees of freedom


DNA ladder
plasmid
75/25
85/25
95/25
75/300
85/300
95/300


DNA ladder
plasmid
75/150
85/150
95/150
CMCH
CH oligo
CH HCl


DNA ladder
plasmid
80/250
90/460
CH glut


DNA ladder
plasmid
CH ace
CH ace pH 6.7
CH lac