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MAGISTRSKA NALOGA

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IZDELAVA IN VREDNOTENJE POLIMERNIH NANODELCEV Z OVALBUMINOM TER OCENA EKSPERIMENTALNEGA PROSTORA Z MATEMATIČNIM MODELOM

PREPARATION, CHARACTERIZATION AND DESIGN-SPACE ESTIMATION OF OVALBUMIN-LOADED PLGA NANOPARTICLES

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I performed the thesis research at the University of Lisbon, Faculty of Pharmacy, at the department of Pharmaceutical technology under supervision of Prof. Dr. João Pedro Martins de Almeida Lopes and home mentorship of Prof. Dr. Pegi Ahlin Grabnar. Formulations of nanoparticles were made at the Faculty of Pharmacy, University of Lisbon. The size, size distribution and zeta potential determination were performed at the Instituto Nacional de Engenharia, Tecnologia e Inovação (INETI), Lisbon, Portugal.

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Statement

I declare that I performed the experiments and wrote this thesis independently under the supervision of doc. dr. Pegi Ahlin Grabnar and Prof. Dr. João Pedro Martins de Almeida Lopes.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Breast cancer is one of the most frequently diagnosed diseases. Conventional forms of treatment demonstrate severe side effects and do not particularly target tumor cells. Thus, there is a need for new therapeutic strategies such as nanoparticle-based therapeutic vaccine, which not only target tumor cells but are also immunotherapeutic.

Poly(lactic-co-glycolic acid) nanoparticles (PLGA NPs) containing ovalbumin (OVA) as a model antigen, prepared by a modified double emulsion solvent evaporation method, were investigated for suitability as advanced drug delivery systems against this disease. To modify physicochemical characteristics of nanoparticles, variations were made in the amount of incorporated OVA, glycol-chitosan (CS) and concentration of poly(vinyl)alcohol (PVA) in the internal aqueous phase (IP). As the IP surfactant 2% (w/v) PVA was used. PVA and Pluronic® F127 (PF127) (both 0.3% (w/v)) were also tested as the external aqueous phase (EP) surfactants. Implementing the Quality-by-design approach for the development of nanoparticles ensures that quality is sustained. Average size (Z-average) and polydispersity index (PDI) were assessed by Dynamic Light Scattering. Zeta potential (ZP) was determined with Laser Doppler Velocimetry in combination with M3 Phase Analysis Light Scattering. OVA encapsulation was indirectly measured with High Pressure Liquid Chromatography and expressed as antigen encapsulation efficiency and loading capacity after calculations. Nanoparticle average size varied from 190 to 472 nm, with surface charge close to neutrality (from -5 to 5 mV) and high encapsulation efficiency (71.4% or more). For better understanding of critical process parameters and critical quality attributes, a mathematical linear modelling approach was used. Factors (% PVA, surfactant in EP, OVA, CS) and responses (Z-average, PDI, ZP, amount of encapsulated OVA) were determined. As a result, a causal predictive model showing the importance of all factors and their interactions, was established. Physicochemical properties of PLGA NPs were most affected by concentration of PVA in IP, CS and PF127 as surfactant in EP. A design-space was created by resourcing the developed models and considering target properties of the nanoparticulate system. Models were further validated by performing an additional set of experiments to confirm their prediction accuracy.

Key words: breast cancer, poly(lactic-co-glycolic acid) nanoparticles, nanoparticle-based therapeutic vaccine, quality-by-design, design-space

RAZŠIRJENI POVZETEK

Rak na dojki je ena izmed najbolj pogosto diagnosticiranih bolezni v svetu. Običajne oblike zdravljenja, kot sta kemoterapija in radioterapija, lahko povzročijo hude neželene učinke, prav tako pa ne ciljajo tumorskih celic neposredno, zato obstaja potreba po novih terapevtskih strategijah, ki bi povečale učinkovitost zdravljenja in bi pacientom izboljšale kakovost življenja. Polimerni nanodelci (ND) so se v številnih raziskavah izkazali kot učinkoviti dostavni sistemi za selektivno ciljanje. Ti dostavni sistemi lahko potencialno dostavijo bodisi antigen ali adjuvant do želene lokacije po vnaprej predvideni poti in poskrbijo za zadostno trajanje optimalnega imunskega odziva, obenem pa lahko zaščitijo učinkovino pred razgradnjo, dokler se iz dostavnega sistema ne sprosti. Uporaba biorazgradljivih ND z vgrajenim antigenom, kot so proteini in peptidi, ali DNA predstavlja napredek za kontrolirano sproščanje antigena in optimizacijo želenega imunskega odziva preko selektivnega ciljanja na antigen predstavljajoče celice (APC). Najbolj pomembna vprašanja v razvoju učinkovitih cepiv zajemajo učinkovito dostavo antigena v APC, zlasti v dendritične celice (DC) in aktivacijo APC. ND, pripravljeni iz biorazgradljivih in biokompatibilnih polimerov, kot so kopolimer mlečne in glikolne kisline (PLGA), poli (aminokisline) in polisaharidi, so se izkazali za učinkovite nosilce antigenov.

Namen predstavljenega dela je bila najprej izdelava PLGA ND, ovrednotenje le-teh in na koncu uporaba matematičnega pristopa linearnega modeliranja za boljše razumevanje kritičnih procesnih parametrov in kritičnih lastnosti kakovosti. Kot rezultat smo želeli izdelati vzročni napovedni model, ki kaže na pomembnost vseh parametrov in njihovih interakcij. Z linearnim pristopom modeliranja smo želeli določiti, (i) kateri parametri resnično vplivajo na lastnosti PLGA ND; (ii) ali so kateri od parametrov v medsebojnih interakcijah, ki so statistično značilne (sinergizem ali antagonizem); (iii) katere so najboljše nastavitve parametrov za dosego optimalnih proizvodnih pogojev; in (iv) kakšne so napovedane vrednosti lastnosti PLGA ND za dane nastavitve parametrov.

V tem raziskovalnem delu smo izdelali in ovrednotili PLGA ND z vgrajenim ovalbuminom (OVA) kot modelnim antigenom. Izdelali smo jih z modificirano metodo

dvojne emulzije z izhlapevanjem topila. PLGA ND so se razlikovali glede na količino vgrajenega OVA, glikol-hitosana (GH) in koncentracijo polivinilalkohola (PVA) v notranji vodni fazi, ter glede na uporabljeno površinsko aktivno snov (PAS) v zunanji vodni fazi. V notranji vodni fazi smo kot PAS uporabili 2 % (m/v) PVA. V zunanji vodni fazi smo kot PAS testirali PVA in Pluronic® F127 (PF127) v enakih koncentracijah.

Notranja vodna faza je bila sestavljena iz različnih koncentracij PVA in vodne raztopine GH, v kateri je bil raztopljen OVA. Nato smo jo emulgirali z organsko raztopino, ki je vsebovala polimer in organsko topilo (PLGA v diklorometanu), z uporabo ultrazvočnega procesorja. K nastali emulziji v/o smo dodali PVA in sonicirali pod enakimi pogoji. Nastalo dvojno emulzijo v/o/v smo po kapljicah dodali k zunanji vodni fazi (PVA ali PF127) in mešali na magnetnem mešalu, kar je omogočilo izhlapevanje organskega topila in tvorbo PLGA ND. PLGA ND smo nato zbrali po treh ciklih centrifugiranja, kjer smo jih sprali z ultra čisto vodo pred vsakim centrifugiranjem, da bi odstranili antigen, ki ni bil vgrajen, in odvečno količino PAS. PLGA ND smo dispergirali v fosfatnem pufru in hranili pri 4° C za nadaljnje poskuse. Za formulacije PLGA ND z vgrajenim OVA in GH smo izdelali tudi kontrolne formulacije, ki niso vsebovale OVA in GH. Vsaka formulacija je bila izdelana v triplikatu.

Pri izdelavi PLGA ND smo sledili vnaprej pripravljenemu načrtu; to je popolnemu faktorskem načrtu; ki se uporablja, ko hočemo določiti najbolj pomembne parametre v procesu; v našem primeru različne količine in koncentracije dodanih komponent. Načrt je upošteval vse možne interakcije med izbranimi parametri, kar je rezultiralo v izdelavi 32 različnih formulacij glede na vsebnost komponent. Pri načrtu in razvoju PLGA ND smo upoštevali sistematičen pristop, tako imenovan »Quality-by-design«, ki zagotavlja kakovost v razvoju.

Povprečna velikost (Z-ave) in polidisperzni indeks (PDI) izdelanih ND smo izmerili z napravo Zetasizer S, po principu dinamičnega sipanja svetlobe. Napravo Zetasizer Z smo uporabili za meritev zeta potenciala (ZP), ki smo ga določili z lasersko Dopplerjevo elektroforezo. Količino vgrajenega OVA smo posredno izmerili z visokotlačno tekočinsko kromatografijo in po izračunih izrazili kot učinkovitost vgrajevanja antigena in vsebnost v PLGA ND. Ker so PLGA ND namenjeni za subkutano ali intratumoralno aplikacijo, so bile zaželene določene lastnosti PLGA ND; Z-ave med 50 in 200 nm, absolutna vrednost ZP manjša od 5 mV, PDI nižji od 0,2; z namenom da preprečimo možno toksičnost in prezgodnjo fagocitozo ND v makrofagih v krvnem obtoku.

Povprečna velikost PLGA ND je variirala med 190 in 472 nm, delci so imeli površinski naboj blizu nevtralnosti (od -5 do 5 mV) in visoko učinkovitost vgrajevanja (71,4 % ali več). Določili smo parametre (% PVA, PAS v EP, OVA, GH) in lastnosti (Z-ave, PDI, ZP, količina vgrajenega proteina), pomembne za pripravo PLGA ND. Na fizikalno-kemijske lastnosti PLGA ND so najbolj vplivali koncentracija PVA v IP, GH in PF127 kot PAS v EP. Izdelali smo precizne modele za lastnosti PLGA ND, razen za PDI, ki na podlagi tega ni bil vključen v ustvarjanje eksperimentalnega prostora. Na podlagi izdelanih modelov smo izoblikovali eksperimentalni prostor, območje v katerem so zagotovljeni kriteriji za pripravo PLGA ND z zaželenimi lastnostmi. Modeli so bili validirani z izvajanjem dodatenega niza poskusov za potrditev njihove točnosti napovedovanja. Pomembno je omeniti, da modeli niso 100 % natančni, vendar pa nam dajejo koristne informacije o tem, kaj je bolj pomembno, če upoštevamo sistem modeliranja.

V prihodnje bi bilo smotrno opraviti več eksperimentov z večjo raznolikostjo v parametrih. Lahko bi uporabili enak pristop modeliranja z drugimi podobnimi sistemi, analizirali razlike in podobnosti. Priporočljivo bi bilo, da bi preizkusili tudi nelinearni pristop k razvoju modelov. Potrebno bi bilo dodatno raziskati model za PDI z nelinearnim pristopom, da bi videli, ali je nelinearnost razlog za visoko odstopanje.

Ključne besede: rak na dojki, nanodelci iz kopolimera mlečne in glikolne kisline, cepivo, kakovost v razvoju, eksperimentalni prostor

LIST OF ABBREVIATIONS

ADDS	Advanced drug delivery systems
ANOVA	Analysis of variance
APCs	Antigen presenting cells
BBD	Box–Bhenken design
CPP	Critical process parameters
CQA	Critical quality attributes
CS	Glycol-chitosan
CV	Cross validation
DCM	Dichloromethane
DCs	Dendritic cells
DoE	Design of experiments
DPBS	Dulbecco's phosphate buffered saline
DPMO	Defect per one million opportunities
DS	Design space
EE	Encapsulation efficiency
E.O.	Amount of encapsulated protein (ovalbumin)
EP	External aqueous phase
HPLC	High performance liquid chromatography
ICH	The International Conference on Harmonisation of technical requirements for registration of pharmaceuticals for human use

IP	Internal aqueous phase
LC	Loading capacity
MC	Monte Carlo simulations
MLR	Multiple linear regression
NPs	Nanoparticles
OVA	Ovalbumin
PBD	Plackett Burman design
PBS	Phosphate buffered saline
PDI	Polydispersity index
PF127	Pluronic® F127
PLGA	Poly(lactic-co-glycolic acid)
PLS	Partial least squares
PRESS	Predictive residual sum of squares
PVA	Poly(vinyl)alcohol
QbD	Quality-by-design
w/o	Water-in-oil
w/o/w	Water-in-oil-in water
Z-average	Average size
ZP	Zeta potential

1 INTRODUCTION

1.1 Advanced drug delivery systems

The matter of advanced drug delivery systems (ADDS) has been common in pharmaceutical exploration for some time, aiming to treat diseases with greater control of drug delivery and action over conventional formulations (1). In the last few decades, development of novel carriers for advanced drug delivery has been focused on forming personalized treatment for a wide range of highly prevalent diseases, considering ADDS evident advantages for drug adimistration (2). The main intention of using ADDS is to deliver a biologically active compound in a controlled process (releasing rate and time period), to sustain drug levels in the body within a therapeutic window, preferably directing the drug towards a specific organ or tissue (targeted drug delivery) without degradation during the whole process (2-4).

1.2 Nanotechnology

The term nanotechnology describes a multidisciplinary approach to the design, development, characterization and application of materials, structures, devices and systems through monitoring the matter at the nanoscale (5). In the context of this, the prefix nano is used when a material or a structure has at least one dimension in the range of 1-100 nm. Nevertheless, the literature about nanosystems (used as a delivery system for active ingredients) also includes a particle size of up to 1 μm. In the nano range, physical, chemical and biological properties of materials differ significantly from the properties of the origin "macro" materials, which is the starting point for their use (6).

Utilization of nanotechnology for medical use is called nanomedicine and involves the use of materials and devices in nano scale for the diagnosis, prevention and treatment of diseases (7). The formulation of active substances in the nanosystems has numerous advantages over the free active substance: the protection of active ingredients from premature degradation, to prevent premature interaction of substances with the environment, to improve the penetration of the active substance in the tissue of interest

(e.g., tumor), enable a controlled release of the active ingredient and improve the delivery of active substances in cells (8).

1.3 Nanostructures as delivery systems for active substances

Nanostructures are eminent delivery systems. The most common types are described in Table I and Figure 1. More recent forms of nanostructures, quantum dots, nanoshells, carbon nanotubes, nanogels and others are also receiving great interest from scientists all over the world (9-11).

Table I: Types of nanostructures (10-12).

Type	Description
Polymeric nanoparticles 20-400 nm	The most common and diverse in composition and functions. The active ingredient can be incorporated in the core or attached on the surface by adsorption or by covalent bonds. The surface of nanoparticles may contain a hydrophilic polymer, attached to a variety of ligands and other groups.
Polymeric micelles 20-250 nm	Amphiphilic block copolymer assembled into nanosized core/ shell structure in aqueous solution. The hydrophobic core region serves as a reservoir for hydrophobic drugs, while the hydrophilic shell region stabilizes the hydrophobic core and enables the polymer to be water-soluble.
Dendrimers 2-10 nm	Symetrically branched polymers with a large number of peripheral functional groups which facilitate fastening of ligands, fluorescent dyes or substances.
Liposomes 120-250 nm	Lipophilic bilayer, hydrophilic interior and modified surface; Four known generations of liposomes, which differ especially in the surface groups. The possibility of incorporation of hydrophilic, lipophilic or amphiphilic substances.

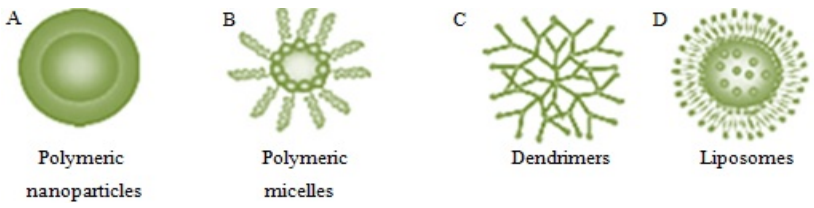

Figure 1 : Types of nanostructures A: Polymeric nanoparticles, B: Polymeric micelles, C: Dendrimers, D: Liposomes (12).

1.3.1 Polymeric nanoparticles

As already mentioned, polymeric NPs are the most common type of NPs, due to their advantages over other types. They are made of biocompatible and biodegradable polymers where the drug is entrapped, encapsulated, dissolved, or adhered to a nanoparticle matrix (13, 14). Biocompatible and biodegradable polymers are preferably used for preparation of NPs, in order to avoid toxicity and to ensure their elimination from the body (15). The selection of suitable polymers is dependent on the type of NPs that need to be produced. Important aspects to be considered are (i) required size of NPs; (ii) inherent properties of substances (hydrophility, charge and stability); (iii) surface characteristics (charge and permeability); (iv) the degree of biodegradablility, biocompatability and toxicity; (v) desired drug release profile; (vi) antigenicity of the final product; (vii) area of application among others (16, 17). Most commonly used synthetic biodegradable polymers, approved by the US Food and Drug Administration (FDA) are poly(lactic acid) (PLA), poly(D,L lactide-co-glycolide) acid (PLGA), and poly(caprolactone) (PCL) (18). Also some natural polymers are suitable for use in preparation of NPs (chitosan, albumin, gelatin, sodium alginate) (15). Polymeric NPs can be produced either by direct polymerization of monomers, from preformed polymers or ionic gelation. A schematic example of different preparation techniques for polymeric NPs is given in Figure 2 (19).

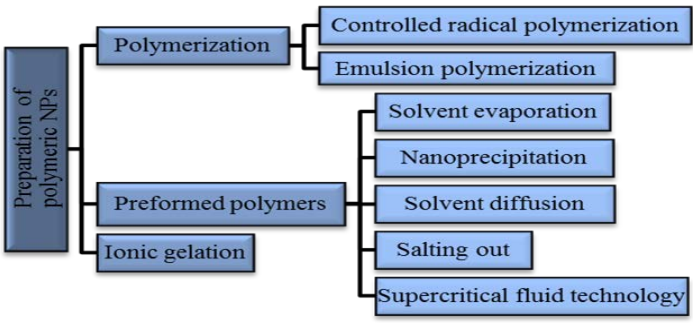

Figure 2: Schematic representation of various techniques for the preparation of polymeric nanoparticles.

Depending upon the method of preparation, nanospheres or nanocapsules can be obtained (Figure 3). Nanospheres are matrix systems in which the drug is physically and uniformly dispersed, whereas nanocapsules are systems in which the drug is enclosed in a cavity surrounded by a polymer membrane (13, 14).

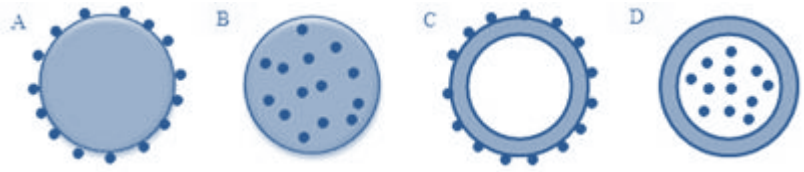

Figure 3: Nanospheres and nanocapsules. A: Nanosphere with adsorbed drug; B: Nanosphere with encapsulated drug; C: Nanocapsule with adsorbed drug; D: Nanocapsule with encapsulated drug

* Advantages of polymeric nanoparticles (15, 20, 21)

- Able of mimicking (size of pathogens)
- Increase the stability of any volatile substance
- Can carry different molecules (drugs, proteins, DNA, RNA, hydrophilic or hydrophobic compounds)
- Produced in large quantities using a wide range of methods
- Provide protection to encapsulated agents
- Show improvement over traditional routes of administration (more efficient and effective)
- Deliver a higher concentration of pharmaceutical agent to a desired location
- Lower dose needed (reduction of toxicity)
- Ability to modify drug release (sustained release)
- Passive targeting
- Active targeting (surface functionalization can target specific cell receptors)

* Disadvantages of polymeric nanoparticles (15, 20, 21)

- Particle aggregation (small size vs. large surface area)
- Stability of dosage forms
- Costly formulation
- Highly sophisticated techology
- Required skills to manufacture

1.3.1.1 Modified double emulsion solvent evaporation method

The double emulsion w/o/w solvent evaporation method was developed to encapsulate hydrophilic drugs, such as proteins, peptides and nucleic acids. It is a modification of the solvent evaporation technique, which is applicable for encapsulation of hydrophobic drugs (22). Organic solvents are used in the solvent evaporation method to dissolve the polymer which is also used as the solvent for the hydrophobic drug. The drug dissolved or dispersed in polymer solution is next emulsified in an aqueous solution containing a surfactant or emulsifying agent to form oil in water (o/w) emulsion. After a stable emulsion is formed, the organic solvent is evaporated either by continuous stirring or by reducing the pressure. Preferably, for attaining the small uniform sized particle size, high-speed homogenizer or ultrasonication may be applied to the process (10). Modifications introduced into the solvent evaporation technique in this work are presented in Figure 4.

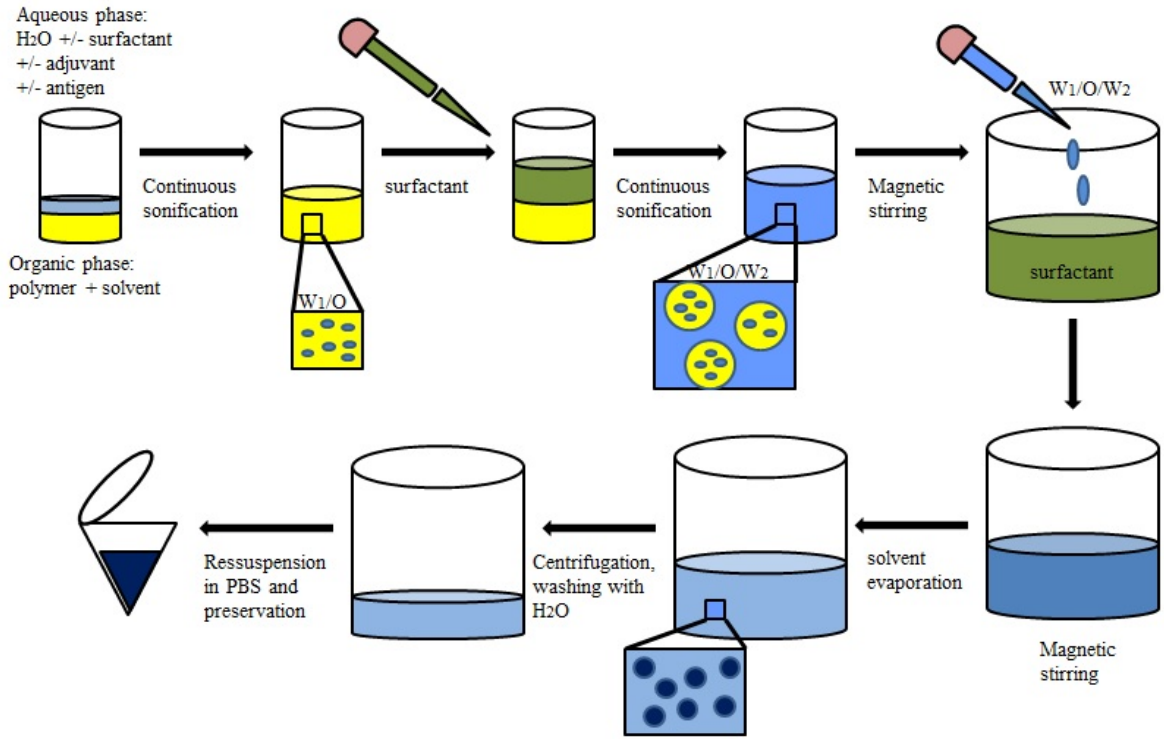

Figure 4: Schematic description of modified double emulsion sovent evaporation method. (adapted and altered from 23).

In this work, organic phase consisted from PLGA (polymer) dissolved in dichloromethane (solvent). Aqueous phase consisted from different concentration of glycol-chitosan (adjuvant) and ovalbumin (model antigen). As surfactants poly(vinyl)alcohol and

Pluronic® F127 were tested. Chemical structures of the compounds used are shown in Figure5.

Poly(lactic-co-glycolic acid) (PLGA)

PLGA is prepared from D,L-poly lactide (D,L-PLA) and poly glycolide (PGA), which are amorphous in nature. PLGA is approved by FDA for drug delivery use, thus being commonly used and is already commercialized for a variety of drug delivery systems. PLGA co-polymer undergoes degradation in an aqueous environment (hydrolytic degradation or biodegradation) through cleavage of its backbone ester linkages, dividing it into its original monomers; lactic acid and glycolic acid. These degradation products are physiologically eliminated and therefore cause a minimal toxicity related to PLGA. It has good mechanical properties, low immunogenicity and toxicity, excellent biocompatibility and predictable biodegradation kinetics (24).

Dichloromethane (DCM)

Dichloromethane is a volatile, colourless liquid, with a mildly sweet, not unpleasant odour. It can dissolve a lot of organic compounds but is immiscible with water. In the lab, it is used as a solvent. Too long exposure can be fatal, with symptoms such as respiratory depression and narcosis (25).

Ovalbumin (OVA)

Ovalbumin is a glycoprotein that comprises 60-65% of the total proteins of avian egg white. It consists of 385 amino acid residues, has four cysteine residues and a single cystine disulfide bridge. It belongs to the serpin family although it lacks any protease inhibitory activity. In pharmaceutical technology it is commonly used as model antigen/vaccine (26).

Glycol-chitosan (CS)

Chitosan is a natural product made from crab shell. It is a derivative of natural chitin, polysaccharide of β(1-4) linked D-glucosamine and N-acetyl-D-glucosamine. Commercially available preparations differ in molecular weight, degree of deacetylation and purity. Chitosan is nontoxic, biocompatible, biodegradable and has good

mucoadhesive properties due to its positive charge, hence acts both as an adjuvant and matrix for delivery systems. It also has antibacterial properties. The presence of the amino group alows its water solubility. In the preparation of nanoparticles, it is used to increase the viscosity and drug encapsulation (27, 28).

Pluronic® F127 (PF127)

PF127 is a polymer of polyoxyethylene (PEO) and polyoxypropylene (PPO) with two 96-unit hydrophilic PEO chains surrounding one 69-unit hydrophobic PPO chain. Since it shows low toxicity and immunogenicity, this non-ionic bifunctional triblock copolymer is widely used as surfactant for the production of polymeric NPs (29).

Poly(vinyl)alcohol (PVA)

PVA is a water-soluble polymeric non-ionic surfactant, known to form PLGA NPs with uniform size distribution. It is produced by polimerization of vinyl acetate. Chemical properties depend on the degree of hydrolysis or the content of acetate groups (30).

Dulbecco's phosphate buffered saline (DPBS)

Phosphate buffered saline (PBS) is a balanced salt solution, containing sodium phosphate, sodium chloride and in some formulations potassium chloride and potassium phosphate. Its main function is to maintain pH and osmotic balance as well as to provide cells with water and essential inorganic ions. Renato Dulbecco developed one of the early formulas of PBS (DPBS). The pH value of PBS is set to be within the range of 7 to 7.6, maintaining a constant pH. Frequently, it is used as a washing buffer in tissue culture and protein chemistry, as well as being used in the preservation of products, to prevent any changes during storage (31).

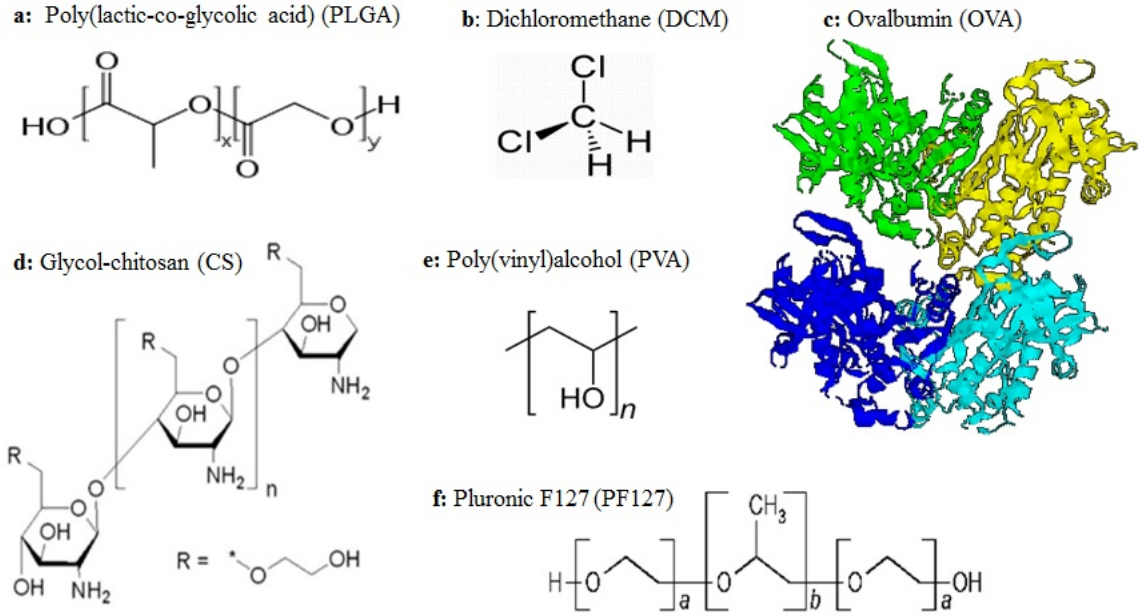

Figure 5: Chemical structures. a: Poly(lactic-co-glycolic) acid (adapted from 32), b: Dichloromethane (adapted from 33), c: Ovalbumin (adapted from 34), d: Glycol-chitosan (adapted from 35), e: Poly(vinyl)alchohol (adapted from 36), f: Pluronic F127 (a=96, b=69) (adapted from 37)

1.3.2 Nano-particulate PLGA therapeutic cancer vaccine

Currently used methods in breast cancer therapy do not particularly target tumor cells, which can lead to severe adverse effects. Thus, there is a desire for new treatment strategies (33).

Essential components of an adequate vaccine are: antigen, adjuvant, and delivery system. Antigens are molecules (proteins, peptides, lipids, etc.) recognized by the immune system that can induce an adaptive immune response. An adjuvant is a substance able to provoke the innate immune system. A delivery system is defined as a platform that assures optimal delivery of antigen and adjuvant for the efficient activation of both innate and adaptive immune systems (37).

The aim of the therapeutic cancer vaccines is to reduce the immunosupression caused by tumor cells. Nanomedicine-based systems have appeared effective in recognition of tumor associated antigens (TAAs), as well as in capturing and presentation by antigen presenting cells (APCs). This leads to an extensive, specific and long-lasting immune response, preventing removal of antigens from circulation. The type of PLGA NPs with loaded

antigen (e.g., ovalbumin), is being developed to deliver breast cancer antigens to dendritic cells (DCs) and enhance their recognition by T cells in the tumor microenvironment. They are biodegradable, biocompatible, and have low toxicity (38, 39). Natural polymers have also been used to prepare nanoparticle adjuvants, such as chitosan. Chitosan-based NPs have been extensively studied due to their biocompatibility, biodegradability, nontoxic nature and their ability to be easily converted into desired shapes and sizes (40). The use of NPs as a form of a vaccine enhances antigen stability and immunogenicity, and enables targeted delivery and slow release. Many of nanoparticle vaccines varying in their properties have been approved for human use. Still, a lack of understanding regarding the in vivo behavior of nanoparticles remains.

Liu et al. (41) reported a nanoparticle-based multi-adjuvant whole cell tumor vaccine for cancer immunotherapy. PLGA NPs were tested as a carrier of whole cell tumor vaccine. They proved a sufficient inhibition of tumor growth and metastasis, as well as prevention of recurrence.

1.4 Quality-by-Design

To assure pharmaceutical quality in development or manufacturing process, a concept called Quality-by-design (QbD) has been applied within pharmaceutical companies in the last few years. It consists of systematic methods to guarantee the quality of a finished product and all components and processes involved in the production. QbD provides insights throughout the development process, so any quality problem can be efficiently analyzed and its cause quickly identified. QbD approach is based on concepts from the International Conference on Harmonisation of technical requirements for registration of pharmaceuticals for human use (ICH) Q8, Q9 and Q10 guidelines, principles of which have been introduced to the pharmaceutical industry by FDA. Companies can acquire several benefits for development and manufacturing if properly implementing QbD, such as: (i) more efficient consumption of time and costs; (ii) simpler meeting submission guidelines and expectations; (iii) reducing time for approval; (iv) quicker response to any manufacturing deviation (42-46).

Throughout process development, raw materials (drug substance and excipients), process parameters (equipment, batch size, operating conditions, environmental conditions) and

quality attributes (physical, chemical or microbiological typical features of components) are investigated. The purpose is to ascertain the critical quality attributes (CQA), critical process parameters (CPP) and also to establish any possible relations between them. CQA are physical, chemical, biological or microbiological properties or characteristics that must be controlled directly or indirectly to ensure the quality of the product (e.g., particle size). CPP are process inputs that have a direct and significant influence on critical quality attributes when they are varied within regular operational range (e.g., sonication conditions) (42, 47).

1.4.1 Design of experiments

Experiments are comonly used for solving problems in many areas of daily life or within specific scientific areas. Simplified, an experiment is an observation which leads to particular information about a studied object (49). The concept of Design of Experiments (DoE) selects a diverse and representative set of experiments in which all factors are independent of each other despite being varied simultaneously. With the design each experiment is performed and then conclusions about the studied object are gathered. The intent is to optimize a process or system, hence minimizing the number of performed experiments and lowering costs. With DoE the most informative combination of factors is chosen, thus DoE represents an effective and economical solution (49).

With DoE the combined influence of all factors is evaluated, therefore it is possible to obtain more precise data about the studied process or system (49, 50). There are two types of variables when we perform experiments using the concept of DoE: responses and factors (Figure 6). Responses give us information about the studied system and factors are used to manipulate them (49, 50).

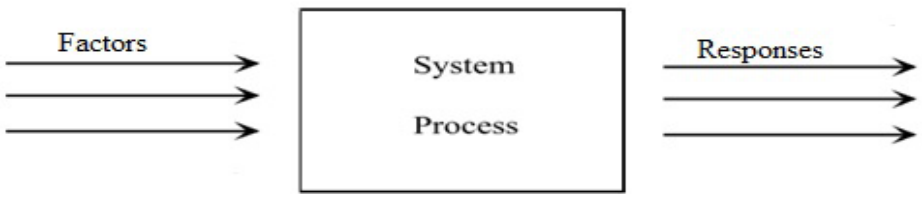

Figure 6: Factors and responses. The determined responses describe the characteristics of the process or system. The characteristics can be altered for a desired response profile if the most influential factors are changed (adapted from 50).

1.4.2 Design space

According to the ICH guidelines, the design-space (DS) is the region where all specifications are fulfilled at a specified risk level. The ICH draft guidance: Q8(R2) has outlined QbD principles for pharmaceutical development where the concept of DS was presented. ICH Q8 defines DS as “the multidimensional combination and interaction of input variables (e.g. material attributes) that have been demonstrated to provide assurance of quality” (44). The aspects defining the DS are the response specifications, prediction models and the accepted risk level. Since each response may have a unique model describing the interaction between the investigated factors and the corresponding response, the resulting DS region will be limited from different directions. This can result in a DS volume that is irregular in its shape. A resolution is then to divide the experimental region into smaller sections and estimate the probability of fulfilling the specifications in each section (Figure 7). If the experimental region is divided into sub regions, the predictions from the models can be used to estimate how well the specifications are fulfilled in each sub region. With different simulations and DoE models we can estimate the risk of getting predictions that are outside the limits in each investigated section. The risk of getting predictions outside the limits is estimated as Defect Per one Million Opportunities (DPMO). With this strategy any type of DS region can be described and it will allow the flexibility to use a variety of precision estimates for the factors that are present in the models.

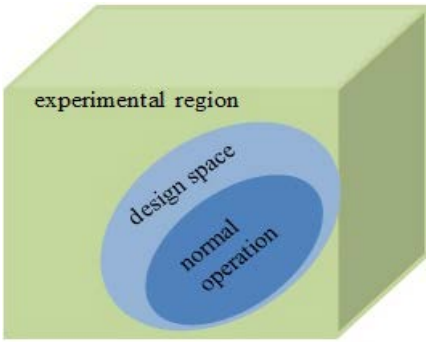

Figure 7: The design space (DS). The DS is a region within the investigated area spanned by the experimental design. This investigated area is often designated experimental region, as in the picture here, or knowledge space (adapted from 50).

The range of normal operation is defined as the upper and/or lower limits for the critical raw material attributes and process parameters. Within these limits, materials are controlled during the process in order to assure reproducibility. The operating range should be within the DS. When it is smaller than the DS, the process is considered robust.

Conventional techniques used for process control, include a combination of mathematical and knowledge-based models. Testing during a process is very important for monitoring and controlling. When any in-process testing result does not meet predefined limits, the batch is discarded and the cause of the failure is identified and fixed if possible. When applying the QbD approach, any inadequacy in the process steps is recognized during the design phase (42, 50).

1.5 Modelling

The basic idea of modelling is to alter all significant factors at the same time, over a set of prepared experiments and then combine the results by means of a mathematical model. The model is then used for interpretation, optimization and further predictions.

Objectives of modelling and experimental design include detecting factors that have a real impact on the responses, detecting factors that have significant interactions (synergies or antagonisms), choosing the best parameters of the factors for the best performance of a system, and establishing the predicted values of the responses for given parameters of the factors.

An important factor is a factor that causes consequential changes (effects) in the response when it varies. We need to differentiate between qualitative and quantitative factors. The values of a qualitative factor have only distinct values, while quantitative factor have a given range on a continuous scale. On the other hand, response is the general condition of a studied system during the change of the factors and can differe in nature (e.g., numerical, cathegorical). In the screening stage simple models (linear or linear with interactions) are used. The data collected during experiments defined by the experimental design is used to estimate the model coefficients. The model represents the relationship between the response Y and the factors X_1, X_2, etc. Multiple linear regression (MLR) and Partial least squares (PLS) regression are the preferred methods to estimate model coefficients (50).

The use of computers to analyze chemical data has grown dramatically in the last twenty years, partly due to recent advances in "hardware" and "software". As a result a causal predictive model is obtained. It shows the importance of all factors and their interactions. Models can then be summarized as informative plots. It is important to know that a model is an approximation. It is very useful for understanding important mechanisms and for manipulating units in the process according to a desired outcome (50).

Linear regression: A regression equation (or function) is linear when it is linear in its parameters. The aim of linear regression is to adjust the values of the parameter and constant to find the line that best predicts Y (response) from X (factors). A linear equation consists of the results for each term:

Response = constant + parameter * factor + ... + parameter * factor + residual

$$Y = b_o + b_1X_1 + b_2X_2 + ... + b_kX_k + e \qquad \text{(equation } 1\text{)}$$

The equation must be linear in its parameters but it is possible to transform the factor variables in ways that produce curvature. For instance, you can include a squared variable or use log and inverse functional forms that are linear in the parameters to produce different types of curves (variable transformation) (50-53).

Nonlinear regression**:** With this regression any model can be fit to the data. Compared to a linear model, non-linear models can take many different forms (Figure 8). If the equation does not fit for a linear equation (equation *1)*, it is nonlinear. These functions can have more than one parameter per a factor variable. A computational approach that can be explained using calculus and matrix algebra needs to be used. The method usually requires initial estimated values for each parameter (50-53).

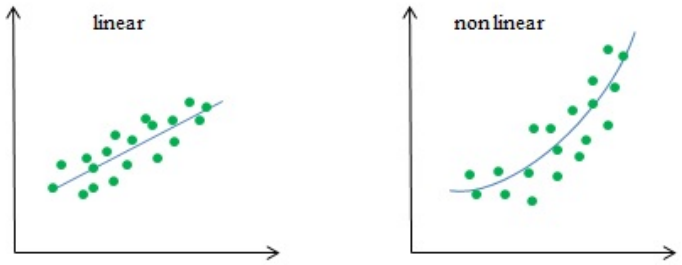

Figure 8: Graphic representation of linear and nonlinear function.

1.6 Previous studies

There are several research papers on implementing the concepts of DoE and QbD approach into the development of nanoparticulate systems.

Punna et al. (54) developed solid lipid NPs for oral delivery of raloxifene, in order to improve drug's oral bioavailability, using DoE to optimize the manufacturing process of solid lipid NPs. Since many variables were involved in the process, a single design could not be sufficient. Therefore, they followed a hybrid-design approach: Plackett Burman design (PBD) for initial screening, followed by Box–Bhenken design (BBD), a subtype of response surface methodology (RSM) design for process optimisation. Significant processing conditions for development of these solid lipid NPs were identified and optimized, presenting a good correlation between actual and predicted values.

Yerlikaya et al. (55) implemented QbD approach into development and evaluation of paclitaxel NPs. CQA were determined to be Z-average, ZP and EE. Furthermore, the Ishikawa diagram was used to determine potential risk factors, where eight potential risk factors were identified and then further evaluated with experimental designs. PBD design was used for further screening and finally BBD design for optimisation of NPs.

In the study by Soema et al. (56), the effect of lipid composition on the physicochemical characteristics and adjuvanticity of liposomes was investigated. DoE approach was used for developing peptide-containing liposomes containing various lipids and different peptide concentrations. The acquired data of characteristics were fitted with regression models. These models were applied to predict a lipid composition that resulted in a liposome with a target zeta potential. To investigate the effect of the liposome composition on liposome size, zeta potential and liposome-induced DC maturation, a linear mixture model was selected with MODDE 10 (Umetrics) software.

These studies, including this thesis work, proved that the DoE and QbD approach are useful for understanding formulation and process parameters and are applicable for the development and optimisation of complex drug delivery systems.

2 OBJECTIVES

The aim of the presented thesis is to formulate, characterise and model modified drug delivery systems based on poly(lactic-co-glycolic acid) (PLGA) nanoparticles (NPs) containing ovalbumin (OVA) as the model antigen prepared by a modified double emulsion solvent evaporation method previously established at the host laboratory, and will be intended for use as a nanoparticle-based therapeutic vaccine for breast cancer immunotherapy. Taking advantage of the developed models, a quality-by-design approach will be implemented for the manufacturing of these systems.

To modify physicochemical characteristics of NPs, different excepients will be tested in various proportions. Variations will be made in amount of incorporated OVA, glycol-chitosan (CS) and concentration of poly(vinyl)alcohol (PVA) in the internal aqueous phase (IP). PVA and Pluronic® F127 (PF127) will also be tested as external aqueous phase (EP) surfactants. The PLGA NPs size (Z-average), zeta potential (ZP), polydispersity index (PDI), antigen encapsulation efficiency (EE) and loading capacity (LC) will be evaluated.

The NPs are intended for subcutaneous or intratumoral administration, therefore the following specific properties are desirable:

- Z-average between 50 and 200 nm
- ZP under 5 mV (positive or negative)
- PDI lower than 0.2;

Furthermore, we will model data for the process interpretation following a linear modelling approach. As a result, we want to establish a causal predictive model.

We would like to determine (i) which factors have a real impact on the responses; (ii) which factors have interactions that are statistically significant (synergies or antagonisms); (iii) what are the best factor parameters to achieve optimal manufacturing conditions for best performance; and (iv) what are the predicted values of the responses for the given factor parameters. Finally, design-space will be established and additional validation experiments performed.

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Equipment

1. Analytical balance VWR LA314, VWR International, LLC (USA)
2. Vortex mixer 230V, Labnet, Labnet Internacional (USA)
3. Ultrasonic processor SonifierVibracell VC 375, Sonics & Materials (INC. DANBURY, CT. USA)
4. Centrifuge Beckman Coulter, lnc, Avanti® J-E Centrifuge JA-20 (USA)
5. Zetasizer Nano S, Malvern Instruments (Worcestershire, UK)
6. Zetasizer Nano Z, Malvern Instruments (Worcestershire, UK)
7. Folded Capillary cell (DTS1060), Malvern Instruments (Worcestershire, UK)
8. Cell ZEN0112, Malvern Instruments (Worcestershire, UK)
9. Beckman System Gold High Performance Liquid Chromatograph: UV-vis Detector (Beckman 166), Beckman 126 solvent module, Midas autosampler
10. Shodex PROTEIN KW-803 series column (8.0 mm ID x 300 mm, 5 µm particle size, 300 Å pore size)
11. Eppendorf® tubes 1.5 mL, 2 mL, 15 mL, 50 mL
12. Nalgene® centifuge tubes
13. Laboratorial material: spatules; beakers; glass vials; gratuated cylinder; micropipettes 5000 µL, 1000 µL, 200 µL, 20 µL, 10 µL; magnetic stirrer plate; Al foil; heaters

3.1.2 Chemicals

1. Dichloromethane (DCM), Merck (Darmstadt, Germany)
2. Poly(lactic-co-glycolic acid) (PLGA), Resomer® RG 502 (lactide:glycolide 50:50, Mw 7,000-17,000 g/mol), Boehringer Ingelheim GmbH (Ingelheim, Germany)
3. Glycol-chitosan (CS) , G7753, ≥60% (titration), crystalline, Sigma-Aldrich (St. Louis, MO, USA)

4. Ovalbumin (OVA), A5503, albumin from chicken egg white (molecular weight (Mw) 45,000 Da), Sigma-Aldrich (St. Louis, MO, USA)
5. Poly(vinyl) alcohol (PVA), Mw 13,000~23,000 Da, 87-89% hydrolyzed, Sigma-Aldrich (St. Louis, MO, USA)
6. Pluronic® F127 (PF127), P2443, Mw 12,600 Da, Sigma-Aldrich (St. Louis, MO, USA)
7. Millipore Milli-Q ultrapure water (resistivity ≥ 15.0 MΩcm)
8. Phosphate buffered saline (PBS), 0.01 M pH 7.4, Sigma-Aldrich (St. Louis, MO, USA)
9. Calcium chloride ($CaCl_2$), Sigma-Aldrich (St. Louis, MO, USA)
10. Magnesium chloride ($MgCl_2$), Sigma-Aldrich (St. Louis, MO, USA)

3.1.3 Prepared solutions

1. Dulbecco's PBS (DPBS) (0.01 M pH 7.4, [+] 0.9 mM $CaCl_2$, 0.45 mM $MgCl_2$)
2. Mobile phase for HPLC (50 mM Sodium phosphate buffer (pH 7.0) + 0.3 M NaCl)
3. Standard stock solution of OVA (0.125 mg/mL)

3.2 Methods

3.2.1 Preparation of nanoparticles

PLGA NPs were prepared using a double emulsion (w/o/w) solvent evaporation method as explained before (Figure 4). The internal aqueous phase (IP) consisted of 25 µL of 2% (w/v) PVA, 6% (w/v) PVA, 10% (w/v) PVA or 14% (w/v) PVA and 25 µL of CS aqueous solution (2 mg/mL), in which OVA (125 µg) was dissolved. This IP was then emulsified with an organic solution (10 mg PLGA dissolved in 200 µL DCM), under continuous sonication for 15 s, at 70 W, using an ultrasonic processor (SonifierVibracell VC 375, Sonics & Materials (INC. DANBURY, CT. USA)). Single w/o emulsion was formed. After, 2% (w/v) PVA solution (surfactant in IP) was added to the single w/o emulsion and sonicated under the same conditions. The obtained double w/o/w emulsion was added dropwise to the external aqueous phase (EP) (20 mL 0.3% (w/v) PVA or 20 mL 0.3%

(w/v) PF127) and magnetically stirred at 37 °C for 1h, enabling DCM evaporation and NPs formation. The details of formulations are shown in Table III. PLGA NPs were collected through three centrifugations (22,000 × g, 40 min, 4 °C) using Centrifuge Beckman Coulter (lnc, Avanti® J-E Centrifuge JA-20, USA) and washed with millipore ultrapure water before each centrifugation to remove the excess of non-encapsulated antigen and surfactant, for formulations with or without CS, respectively. NPs were then dispersed in DPBS and kept at 4 ºC (Eppendorf® tubes 2 mL) for following experiments. After each centrifugation, supernatants were stored and kept at 4 ºC, or frozen at -20 ºC (Eppendorf® tubes 2 mL, 15 mL) until analysis. For each OVA-loaded NPs and CS containing NPs, plain NPs were made. Each formulation was made in triplicate or more.

3.2.1.1 Full factorial (Mixed) design

In the early stages of an investigation, several screening designs can be used to find which factors are significant and if their ranges need to be modified. When choosing full factorial designs it is possible to work with interaction models, i.e. all interactions possible. They can be created at 2 or more factor levels, comprising all the possible combinations of the factor levels (e.g., for p factors at 2 levels one needs N =2p runs). Full factorial designs are orthogonal (balanced), hence the estimated effect of a factor is independent of the effects of all other factors. Full factorial designs with factors of a different number of levels are called Full factorial mixed designs (50, 53). Experimental design matrix of performed experiments (for NPs with or without loaded OVA) is shown in Table II. All together 32 experiments were designed.

Table II: Experimental design matrix. Coding in italics refers to combinations of factors A, B and C; with numbers 0, 1, 2, 3 implying the level of factor.

Factor A (surfactant in EP)		**Factor B (CS in IP (µg))**	**Factor C (PVA in IP (%))**
0 (PVA)	**1** (PF127)		
000	***100***	**0** (0 µg)	**0** (2% PVA)
001	***101***	**0** (0 µg)	**1** (6% PVA)
002	***102***	**0** (0 µg)	**2** (10% PVA)
003	***103***	**0** (0 µg)	**3** (14% PVA)
010	***110***	**1** (25 µg)	**0** (2% PVA)
011	***111***	**1** (25 µg)	**1** (6% PVA)
012	***112***	**1** (25 µg)	**2** (10% PVA)
013	***113***	**1** (25 µg)	**3** (14% PVA)

Table III: Details of prepared formulations.

Formu-lation	**IP**	**surfactant in IP**	**surfactant in EP**	**Formu-lation**	**IP**	**surfactant in IP**	**surfactant in EP**
F1b	2% PVA, H2O	2% PVA	0.3% PVA	F9b	2% PVA, H2O	2% PVA	0.3% PF127
F1o	2% PVA, OVA	2% PVA	0.3% PVA	F9o	2% PVA, OVA	2% PVA	0.3% PF127
F2bC	2% PVA, CS	2% PVA	0.3% PVA	F10bC	2% PVA, CS	2% PVA	0.3% PF127
F2oC	2% PVA, CS, OVA	2% PVA	0.3% PVA	F10oC	2% PVA, CS, OVA	2% PVA	0.3% PF127
F3b	6% PVA, H2O	2% PVA	0.3% PVA	F11b	6% PVA, H2O	2% PVA	0.3% PF127
F3o	6% PVA, OVA	2% PVA	0.3% PVA	F11o	6% PVA, OVA	2% PVA	0.3% PF127
F4bC	6% PVA, CS	2% PVA	0.3% PVA	F12bC	6% PVA, CS	2% PVA	0.3% PF127
F4oC	6% PVA, CS, OVA	2% PVA	0.3% PVA	F12oC	6% PVA, CS, OVA	2% PVA	0.3% PF127
F5b	10% PVA, H2O	2% PVA	0.3% PVA	F13b	10% PVA, H2O	2% PVA	0.3% PF127
F5o	10% PVA, OVA	2% PVA	0.3% PVA	F13o	10% PVA, OVA	2% PVA	0.3% PF127
F6bC	10% PVA, CS	2% PVA	0.3% PVA	F14bC	10% PVA, CS	2% PVA	0.3% PF127
F6oC	10% PVA, CS, OVA	2% PVA	0.3% PVA	F14oC	10% PVA, CS, OVA	2% PVA	0.3% PF127
F7b	14% PVA, H2O	2% PVA	0.3% PVA	F15b	14% PVA, H2O	2% PVA	0.3% PF127
F7o	14% PVA, OVA	2% PVA	0.3% PVA	F15o	14% PVA, OVA	2% PVA	0.3% PF127
F8bC	14% PVA, CS	2% PVA	0.3% PVA	F16bC	14% PVA, CS	2% PVA	0.3% PF127
F8oC	14% PVA, CS, OVA	2% PVA	0.3% PVA	F16oC	14% PVA, CS, OVA	2% PVA	0.3% PF127

3.2.2 Physicochemical characterization of nanoparticles

3.2.2.1 Size and polydispersity index

Mean size (Z-average) and polydispersity index (PDI) of NPs were determined by Dynamic Light Scattering (DLS) using Zetasizer Nano S (Malvern Instruments, Worcestershire, UK). 50 µL of each batch of NP suspension (20 mg/mL) plain or loaded, was suspended in 950 µL DPBS in Eppendorf® tubes (1.5 mL). The diluted suspension was primarily introduced into a cell (Cell ZEN0112, Malvern Instruments, Worcestershire, UK) to evaluate the Brownian motion of NPs based on laser light scattering, measuring size and PDI. Working conditions and measurments were always maintained constant to obtain comparable results. Each diluted suspension was measured in triplicates, where each size and PDI data corresponds to 10 measurments.

3.2.2.2 Zeta potential

After size and PDI analysis, the same suspension was inserted into an electrode specific cell (Folded Capillary cell (DTS1060), Malvern Instruments, Worcestershire, UK) for electrophoretic mobility. Surface charge of NPs was inferred from the determination of zeta potential (ZP), assessed by Laser Doppler Velocimetry (LDV) in combination with M3 Phase Analysis Light Scattering (M3-PALS), using Zetasizer Nano Z (Malvern Instruments, Worcestershire, UK), at 25 ºC. Working conditions and measurments were always maintained constant, due to dependance of ZP on pH and ionic strength of the dispersant. Each diluted suspension was measured in triplicates where each ZP measurment represents 100 readings.

3.2.3 Ovalbumin loading analysis

Supernatants were collected in triplicates from each centrifugation, performed for each batch of NPs. Before each determination, supernatants were defrosted at room temperature.

A calibration curve, using OVA aqueous solution (obtained from stock solution of 1000 µg/mL) and first supernatants recovered after centrifugation of plain NPs, in the concentration a range of 0–10 µg/mL was prepared (Table IV).

Table IV: Solutions for preparing the calibration curve.

Solutions	Final concentration (µg/mL)	Volume of OVA solution with first supernatant of plain NPs (50µg/mL) (µL)	Volume of first supernatants of plain NPs (µL)
S0	0	0	1000
S1	0.5	10	990
S2	2	40	960
S3	4	80	920
S4	5	100	900
S5	10	200	800

The amount of encapsulated protein OVA was quantified indirectly by High Performance Liquid Chromatography (HPLC) using a Beckman System Gold High performance Liquid Chromatograph (UV-vis Detector (Beckman 166), Beckman 126 solvent module, Midas autosampler). Samples (20 µL; supernatants) were injected in the column (Shodex PROTEIN KW-803 series column (8.0 mm ID x 300 mm, 5 µm particle size, 300 Å pore size)) and eluted with buffer (50mM Sodium phosphate buffer (pH 7.0) + 0.3M NaCl), at room temperature (run time: 20 min, flow rate: 1 mL/min). OVA elution from each supernatant was monitored following absorption at 220 nm.

The entrapment efficiency (EE,% (w/w)) is the encapsulated protein in the polymeric matrix of NPs over the initial amount of protein (Equation *2*) expressed in%.

$$EE\ (\%) = \frac{amount\ of\ encapsulated\ protein}{initial\ amount\ of\ protein} \times 100 \qquad \text{(Equation 2)}$$

The loading capacity (LC, µg/mg) is defined as the protein amount (µg) per mg of NPs (Equation *3*).

$$\text{LC (µg/mg)} = \frac{amount\ of\ encapsulated\ protein}{total\ amount\ of\ polymers} \qquad \text{(Equation 3)}$$

The amount of encapsulated OVA was calculated using the standard calibration curve and equations, as mentioned above.

3.2.4 Modelling

To construct mathematical models, explaining NPs size, PDI, ZP and OVA loading analysis, the software Modde version 10.1 (Umetrics, Sweden) was used. The software uses the principles of DoE to analyze processes or products and helps the user to get valuable information from the raw data. With different analyzing and visualization tools, the user gets help to understand complex processes and has the opportunity to improve the processes by performing suggested experimental designs. With a user-friendly design wizard and easy visualization methods, the evaluation of raw data and the corresponding decision making is made simple.

In order to enter the data in this computer program, factors (% PVA, surfactant in EP, OVA, glycol chitosan) and responses (Z-average, PDI, ZP, amount of encapsulated OVA) were determined. Figure 9 illustrates the specified factors and responses entered in the software for the construction of models and Table V describes the characteristics of factors and responses.

Figure 9: Specified factors and responses.

Table V: Characteristics of factors and responses.

Factors	**Description**
% PVA	The percentage of PVA used in IP. Values varied within 2%, 6%, 10% and 14%. Factor type is multilevel.
s. EP	The type of the surfactant used in EP. PF 127 or PVA (both 0.3%) were used. Factor type is qualitative.
OVA	OVA used in preparation of NPs. Factor type is quantitative and constant, considering only NPs with encapsulated OVA were included in modelling.
CS	CS used in preparation of NPs. Factor type is multilevel, since CS was or was not used in preparation of NPs.
Responses	**Description**
Z-ave	Results from measuring size of NPs were recalculated into average values (from triplicates or more of each formualtion).
PDI	Results from measuring PDI of NPs were recalculated into average values (from triplicates or more of each formulation).
ZP	Results from measuring ZP of NPs were recalculated into average values (from triplicates or more of each formulation).
E.O.	Results obtained from HPLC measurments, recalculated into average values. Transformed into quantity (µg) of encapsulated OVA in NPs (triplicates or more) for each formulation.

After determination of the data illustrated in Figure 9 in Modde, results (data collected from measurments described above) for responses were inserted. The study design consisted of twenty runs (including replicates) with zero centre points. The experiment was designed and analyzed using Modde. Work then proceeded to the fitting, optimization and validation of models for Z-average, PDI, ZP and amount of encapsulated OVA. The models were fitted with the PLS algorithm.

3.2.4.1 Partial least squares (PLS) regression

PLS regression is appropriate when one wants to fit a model that can represent the variation of all the responses to the variation of the factors (dealing with many responses simultaneously, taking their covariances into account) and when the number of degrees of freedom do not allow the application of MLR. An overview of how factors affect the responses is the target. It finds a linear regression model by projecting the predicted variables and the observable variables to a new space.

PLS finds the relationship between a matrix Y (response variables) and a matrix X (factor variables) expressed as:

$$Y = XB+E \qquad \text{(equation } 4\text{)}$$

The PLS model consists of a simultaneous projection of the Y and X spaces on a low dimensional hyper plane with new coordinates T (summarizing X) and U (summarizing Y), and then relating U to T. This kind of analysis is desirable to approximate X and Y well and to maximize the correlation between X and Y in the projected space (between U and T). Loadings are the weights with which each variable contributes to formation of the main components (P and Q) (Figure 10).

$$X = TP^t + E \qquad \text{(Equation } 5\text{)}$$

$$Y = UQ^t + E \qquad \text{(Equation } 6\text{)}$$

$$U = bT \qquad \text{(Equation } 7\text{)}$$

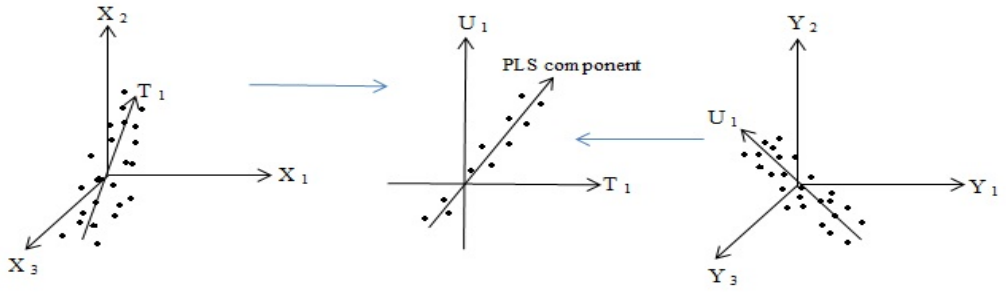

Figure 10: A geometric representation of PLS regression.

The number of significant PLS components (the dimensionality), is determined by cross-validation (CV), where the predictive residual sum of squares (PRESS) is computed for each model dimension. Modde selects automatically the number of PLS dimensions that give the smallest PRESS. PRESS is then re-expressed as

$$Q^2 = (1 - PRESS/SS_Y) \quad \text{(Equation } \textit{8}\text{)}$$

where SS_Y is the sum of Y's squares (49).

Since PLS regression was the adopted method, the number of latent variables should be optimized. This optimization was done automatically by the software Modde using the cross-validation type leave-one-out (50).

3.2.4.2 Analysis

To examine the model fit, different approaches are considered. First, the summary of fit plot displays accuracy of a model with four columns: (i) R^2 shows the model fit. A model with R^2 of 0.5 is a model with rather low significance; (ii) Q^2 estimates future predictions precision. Roughly, it should be greater than 0.1 for a significant model and greater than 0.5 for a good model. Q^2 is the best and most sensitive indicator; (iii) Model validity is a test of diverse model problems. A value less than 0.25 for model validity indicates statistically significant model problems, such as the presence of outliers, an incorrect model, or a variable transformation problem. A low value here may also indicate that a term, such as an interaction or square is missing. When the pure error is very small (replicates almost identical), the model validity can be low even though the model is good

and complete; (iv) Reproducibility explains the variation of the replicates compared to overall variability. The value should be greater than 0.5 (50).

After, diagnostics are made with a number of diagnostic plots, for instance residual plots to find outliers and analysis of variance (ANOVA) to review the lack of fit, etc. To interpret the influence of terms on the model, the coefficients and effect plots and lists can be used.

If any outliers are discovered or we want to remove or add a term to the model we can refine the model.

3.2.4.3 Predictions

When one is satisfied with the model, one can use the model to make further predictions and find the area (design space) with the optimal conditions. Then, one can validate the predicted values by performing a new set of the experiments, to confirm that predictions are reliable.

4 RESULTS AND DISCUSSION

4.1 Characterization of PLGA nanoparticles

In the nanoparticle formulation process, the organic phase was composed of 10 mg PLGA powder dissolved in 200 µL of DCM. Differences in IP between NPs were related to PVA concentration (2% (w/v) PVA, 6% (w/v) PVA, 10% (w/v) PVA or 14% (w/v) PVA solutions were used), aditions of CS (0% ($w_{CS}/w_{polymer}$) or 0.25% ($w_{CS}/w_{polymer}$) were added) and additions of OVA (0 µg or 125 µg was added). As a surfactant in IP, 400 µL of 2% (w/v) PVA solution was used in all formulations. Formulations also varied depending on the surfactant used in the EP, namely 20 mL of 0.3% (w/v) PVA or 20 mL of 0.3% (w/v) PF127 solutions were used. For all formulations the same production process was used. Multiple emulsions w/o/w were formulated.

We can divide results into two groups: plain NPs and NPs with encapsulated OVA. Furthermore, each group can be divided in terms of the concentration of PVA used in IP, incorporation of CS into IP and the type of surfactant used in EP (PVA or PF127). Plain NPs were used as a control, hence their results will not be considered for modelling since our interest is OVA loaded NPs, also containing CS.

Changes in formulations were made with the purpose of reducing toxicity and meeting the desired requirements as close as possible. We want Z-average of NPs to be between 50 and 200 nm, ZP under 5 mV (positive or negative) and PDI lower than 0.2, so NPs would be suitable for vaccine use. Results of measured Z-average, PDI, ZP, EE and LC are shown in Table VI. Statistical analysis of results and models will be performed with Modde. Note that all statistical tests in this work were performed to a significance level of 0.05.

Table VI: Z-average, PDI, ZP, EE and LC (Mean ± SD; n ≥3). Details of prepared formulations were described in Table III.

Formulation	Z-average (nm)	PDI	ZP (mV)	EE (%)	LC (µg/mg)	Formulation	Z-average (nm)	PDI	ZP (mV)	EE (%)	LC (µg/mg)
F1b	258 ± 5	0.094 ± 0.019	-1.77 ± 0.36			F9b	419 ± 33	0.580 ± 0.070	-1.74 ± 0.21		
F1o	317 ± 3	0.177 ± 0.003	-3.56 ± 0.39	80.68	10.09	F9o	403 ± 6	0.391 ± 0.047	-5.75 ± 0.84	82.55	10.32
F2bC	310 ± 7	0.184 ± 0.017	0.92 ± 0.96			F10bC	379 ± 6	0.447 ± 0.010	-0.19 ± 0.63		
F2oC	359 ± 8	0.208 ± 0.022	0.64 ± 0.51	81.93	10.24	F10oC	444 ± 11	0.304 ± 0.053	-1.01 ± 0.63	80.97	10.12
F3b	223 ± 6	0.291 ± 0.033	-1.34 ± 0,35			F11b	444 ± 19	0.501 ± 0.018	-1.16 ± 0.23		
F3o	208 ± 5	0.187 ± 0.019	-1.95 ± 0.65	81.44	10.18	F11o	311 ± 3	0.334 ± 0.048	-2.27 ± 0.34	71.24	8.9
F4bC	388 ± 11	0.462 ± 0.045	-0.19 ± 0.19			F12bC	299 ± 5	0.38 ± 0.005	-0.31 ± 0.81		
F4oC	295 ± 1	0.309 ± 0.034	-0.84 ± 0.11	88.94	11.12	F12oC	420 ± 17	0.461 ± 0.033	-0.71 ± 0.57	81.88	10.24
F5b	197 ± 6	0.189 ± 0.016	-1.45 ± 0.27			F13b	245 ± 12	0.28 ± 0.018	-1.39 ± 0.39		
F5o	210 ± 3	0.217 ± 0.019	-1.79 ± 0.19	79.5	9.94	F13o	233 ± 4	0.219 ± 0.021	-2.14 ± 0.35	74.81	9.35
F6bC	232 ± 9	0.252 ± 0.029	-0.083 ± 0.34			F14bC	243 ± 7	0.3 ± 0.012	-1.05 ± 0.31		
F6oC	201 ± 3	0.137 ± 0.013	-1.59 ± 0.71	85.95	10.74	F14oC	420 ± 14	0.5 ± 0.013	-0.70 ± 0.58	76.64	9.58
F7b	190 ± 5	0.1 ± 0.022	-2.0 ± 0.50			F15b	472 ± 43	0.558 ± 0.011	-1.27 ± 0.25		
F7o	191 ± 6	0.129 ± 0.027	-2.07 ± 0.71	76.66	9.58	F15o	219 ± 4	0.293 ± 0.013	-2.13 ± 0.41	84.91	10.61
F8bC	304 ± 8	0.405 ± 0.002	0.26 ± 0.45			F16bC	234 ± 5	0.256 ± 0.024	-0.32 ± 0.98		
F8oC	270 ± 6	0.324 ± 0.005	-0.65 ± 0.28	83.6	10.45	F16oC	429 ± 11	0.38 ± 0.019	-0.48 ± 1.20	82.51	10.31

4.1.1 Particle size

Size is an important aspect of nanoparticulate systems. It has a significant influence on the physicochemical properties, degradation of NPs *in vivo*, NPs intracellular uptake, toxicity and efficacy. Particle size also has an impact on drug loading, drug release and stability of particles. In this study, Z-average was measured by dynamic light scattering (photon correlation spectroscopy) with Zetasizer Nano S. Each sample was measured in triplicate. Results are shown in Table VI.

Different concentrations (2, 6, 10 and 14% w/v) of PVA in IP were used in order to assess its effect on NPs size. Higher concentrations of PVA have increased emulsion stability in general and thus the size of both plain and antigen-loaded NPs (both without CS) mostly decreased when higher concentrations of PVA in IP were used. However, in the case of plain NPs with PF127 used in EP, this trend is not visible. We can assume, since PF127 is a bigger molecule (12,5 kDa) than PVA, it was the former that affected the size of NPs. The viscosity of IP increases as the concentration of PVA increases. This decreases the impact of shear stress which is the most important independent variable liable for size reduction, leading to system instability in consideration of the small volume of this phase. Therefore at 14% PVA in IP we have noticed that NPs (F15b) were the biggest due to an increased viscosity, the size of PF127 molecule and the absence of additional stabilization of OVA. Another interesting observation arises from OVA loaded NPs (without CS) that are smaller compared to plain controls (F3o, F9o F11o, F13o), which could be due to the additional stabilization of emulsion by OVA. We can also see that all NPs (plain, loaded, with or without CS), except F4bc and F8bc, with PF127 in EP have higher Z-average values. When CS was added to plain or OVA loaded NPs, we expected higher Z-average of NPs, since it represents an addition of a big molecule (82 kDa). In the case of PVA used in EP, NPs Z-average did increase, except in F6oc (10% PVA in IP) where Z-average was lower. This could impact the formulation process regarding our desire for OVA and CS containing NPs with an appropriate size range. The opposite results with plain NPs occured with PF127 in EP when CS was added Z-average decreased. However, Z-average of OVA loaded NPs, when CS was added, also increased. The best result, considering we want OVA and CS included, was obtained by F6oc. In addition, the low standard deviation (SD)

obtained for the Z-Average (with PVA in EP) implies that the method used for NPs preparation is reproducible.

4.1.1.1 Modelling

As mentioned before, only OVA loaded NPs (with or without CS) were analyzed. In Figure 11, we can see the coefficients and effects plots for Z-average. In the coefficients plot we can assess the significance of model terms (factors). A significant model term is one with a large distance from y=0 as well as having an uncertainty level that does not extend across y=0 (50). During the modelling process non-significant terms can be excluded in order to obtain the best model. The most significant factors that explain the Z-average variability are surfactants in EP (PVA and PF127), CS and% (w/v) of PVA in IP, which means they have the biggest influence on NPs size. Next, in the effects plot, we can see how the significant factors are influencing Z-average. The effect represents the change in the response values when one factor varies over its range and all other factors are kept in the same proportion as in the reference formulation (50). Concentration of PVA in IP has a negative effect on Z-average, meaning that Z-average values are decreasing while% (w/v) of PVA in IP is increasing. On the other hand, positive effects are visible for PF127 as the surfactant in EP and CS, which implies that NPs were bigger, when PF127 was used or CS was added for NPs loaded with OVA, comparing to NPs only loaded with OVA (without CS and PF127 in EP).

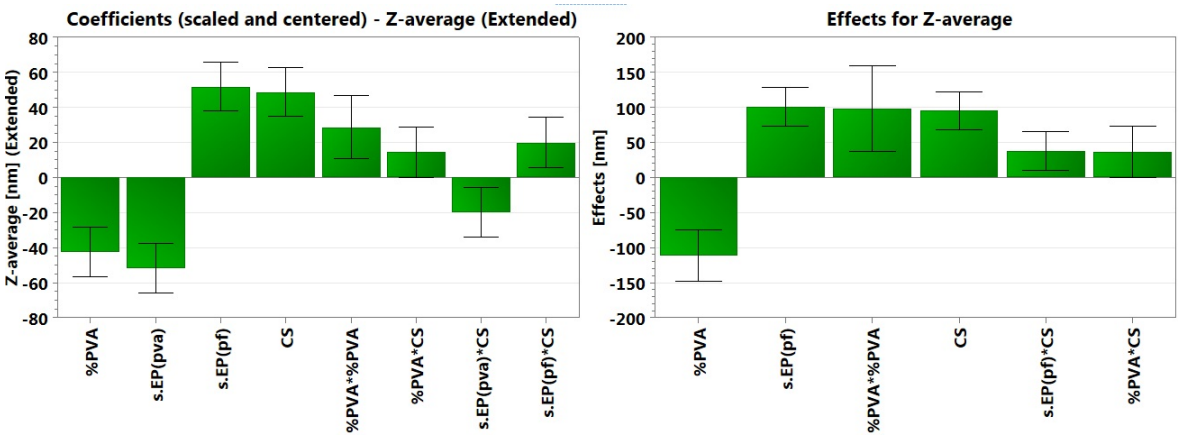

Figure 11: Coefficients and effects plot for Z-average.

We can also see if there are any factor interactions. With interaction plots (Figure 12) the predicted values of the response, when one factor varies from its lowest to its highest level, are plotted for both levels of each factor and all remaining factors in the design are set on their average (50).

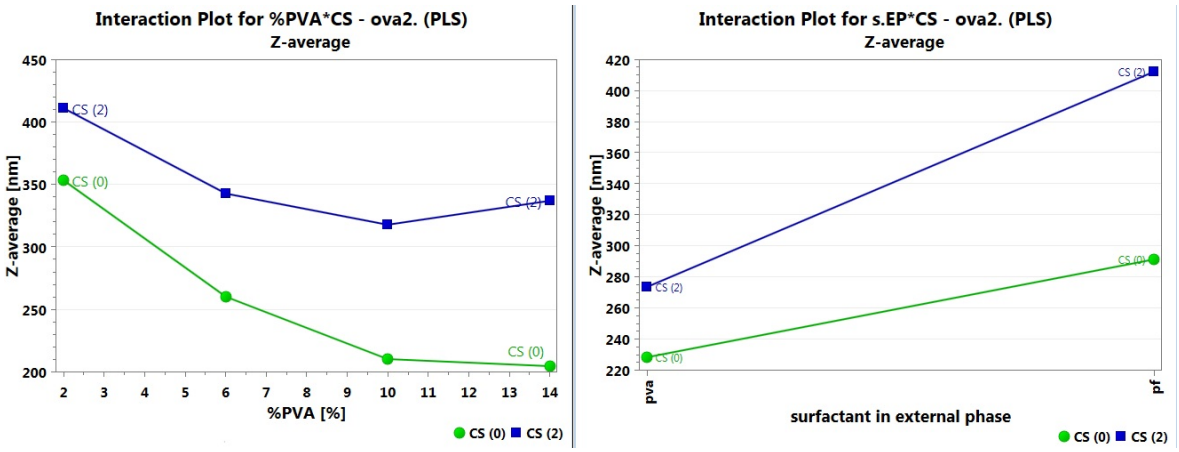

Figure 12: Interaction plots for Z-average. Interaction plot on the left represents interaction between % (w/v) of PVA in IP with CS. Interaction plot on the right represents interaction between surfactant used in EP with CS. Blue line stands for CS being used in preparation of NPs and green line means there is no CS in formulations.

We can see from the interaction plot on the left of Fig. 12, that Z-average decreased when the% (w/v) of PVA increased and no CS was added. When CS was added, the trend remained the same, except with 14% (w/v) PVA, which can be explained by the increased viscosity of IP, making the system less stable (as explained before). Regarding the interaction plot on the right, it is visible that particle size increases drasticly when PF127 and CS are combined. As previously stated, Z-average is lower for PVA in EP (with or without CS).

After analysis, adjustments, refining and alterations of model terms, the final model was established. Summary of fit provides a summary of the basic model statistics, presented visually. For every response there are 4 columns displayed: R^2, Q^2, model validity and reproducibility (as explained in **3.2.4.3 Analysis**). The summary of fit plot for Z-average is displayed in Figure 13. The R^2 column is over 0.9 which indicates a good model fit with high significance. The Q^2 column being greater than 0.5 implies a good model, thereby providing good estimations of future predictions. Model validity with values over 0.25

shows there is no diverse model problems, such as the presence of outliers, an incorrect model, or a transformation problem. Where the reproducibility column is greater than 0.5 we can conclude that the variation of the replicates compared to the overall variability is not significant, hence results for Z-average are repeatable.

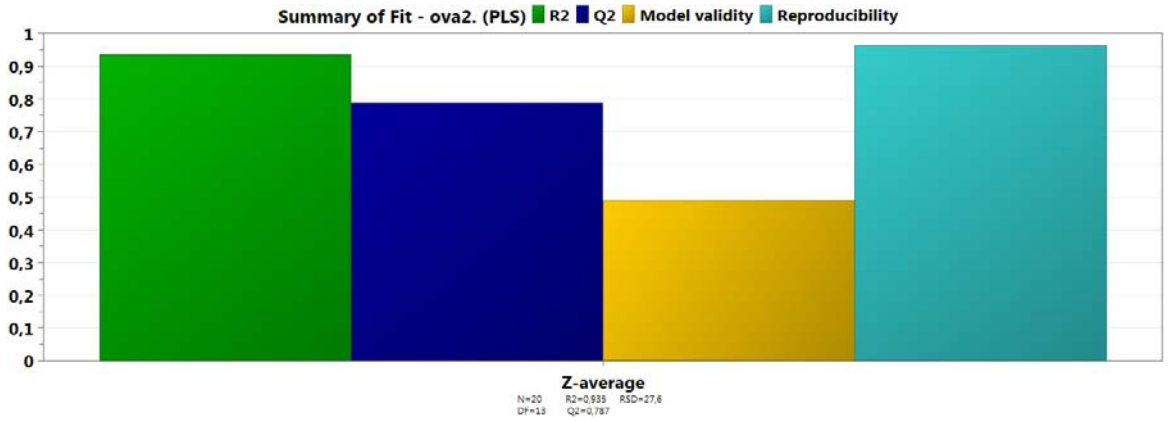

Figure 13: Summary of fit for Z-average.

The obtained model for Z-average is significant ($p < 0.05$). Also, the lack of fit test is not significant, hence statistically the model has no lack-of-fit ($p < 0.05$), meaning that replicate errors are similar to model errors (Table VII).

Table VII: : The analysis of variance (ANOVA) plot for Z-average, PDI, ZP and E.O. The regression is significant when $p < 0.05$. The lack-of-fit test shows no significance when $p < 0.05$.

ANOVA	**Z-average**	**PDI**	**ZP**	**E.O.**
regression	p = 0.000	p = 0.000	p = 0.000	p = 0.017
lack-of-fit	p = 0.132	p = 0.108	p = 0.063	p = 0.087

Testing model predictions is an important step in modelling. A scatter plot of observed vs. predicted values is one of the most common alternatives to evaluate model predictions.
A plot with points lying on a straight line indicates that predictions are similar to the experiment values. From the observed vs. predicted plot for Z-average we can confirm a good agreement between model predictions and experimental data (Figure 14).

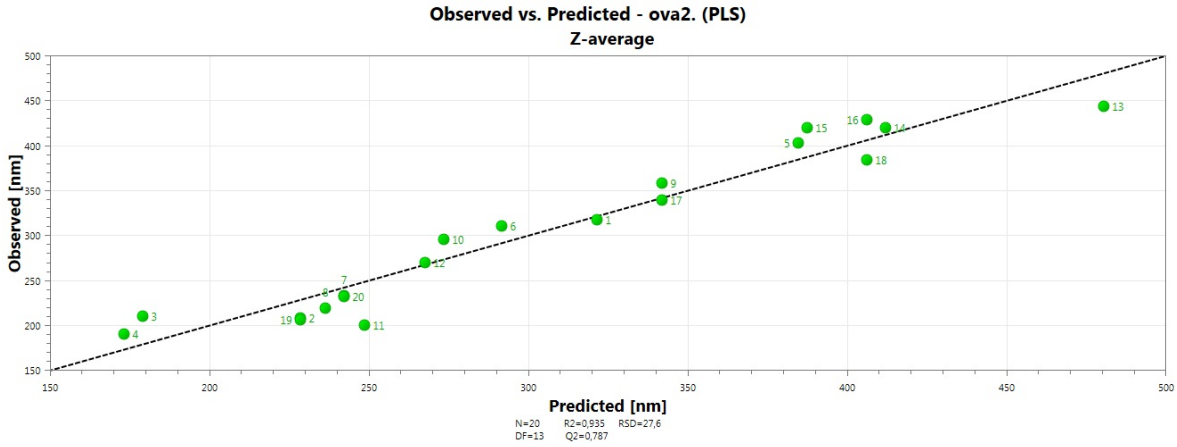

Figure 14: Observed vs. predicted plot for Z-average.

4.1.2 Size distribution

Size distribution is an equaly important aspect of nanoparticulate systems. It also has an influence on physicochemical properties, degradation of NPs *in vivo*, NPs intracellular uptake, toxicity and efficacy. Size distribution in this study is expressed with PDI, which was measured using dynamic light scattering (photon correlation spectroscopy) with Zetasizer Nano S. Each sample was measured in triplicate.

PDI is the measure of the distribution of particle size in a sample. If NPs are mostly the same size, the sample is called uniform (monodisperse). If NPs are very different in size in the sample, it is non-uniform (polydisperse). In our study we want samples to be monodisperse.

Results for measured PDI are displayed in Table VI. Comparing plain and OVA loaded NPs (both without CS) with PVA in EP we notice that PDI slightly increases, apparently as a consequence of the increased size of NPs, which can be explained by the fact that the bigger particles are, the wider the size distribution. In similar formulations containing PF127 in the EP, we see an opposite trend. PDI lowered but also the size of NPs is smaller, so we can explain this with the same logic as above. When CS was added, PDI increased for almost all formulations. This can also be explained by the bigger size of these NPs, when adding CS. The best result, considering we want OVA and CS included, was obtained by F6oc.

4.1.2.1 Modelling

In Figure 15, we can see coefficients and effect plots (definitions described previously in the section **4.1.1.1 Modelling**) for PDI. Significant factors in the PDI model are surfactant in EP (PVA or PF127) and CS. Next, in the effects plot we can see that both PF127 and CS have a positive effect on PDI, meaning that when these two factors were present in the formulation, PDI is expected to increase.

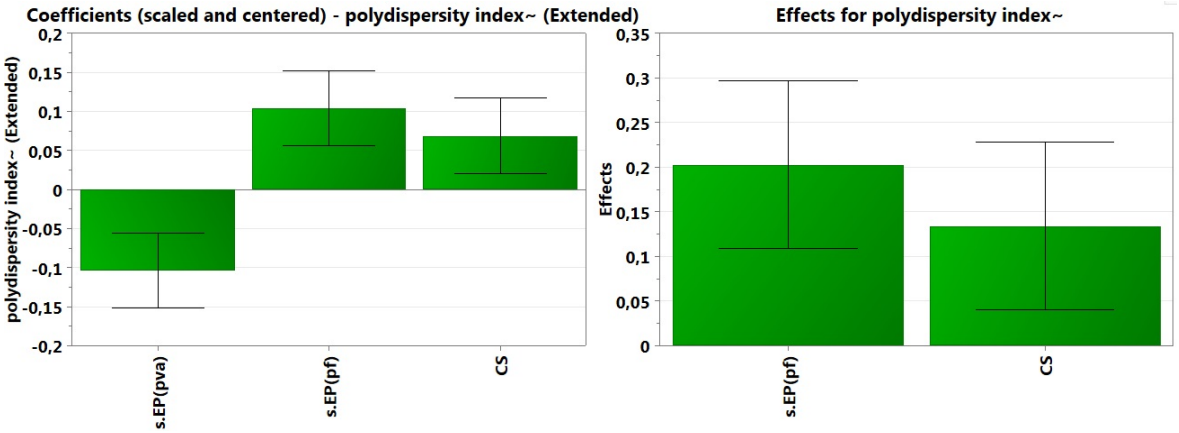

Figure 15: Coefficients and effects plot for PDI.

For the PDI model there are no observed interactions between model terms. This is due to few significant factors.

Summary of fit for PDI is displayed in Figure 16. R^2 is greater than 0.5 which indicates a good model fit with high significance. Q^2 being greater than 0.5 implies a good model, thereby a good estimation of future predictions. Model validity with value over 0.25 shows there is no diverse model problems, such as the presence of outliers, an incorrect model, or a transformation problem. With reproducibility higher than 0.5 we can conclude that the variation of the replicates compared to overall residuals variability is not significant, hence results for PDI are repeatable.

Obtained model for PDI shows a statistically significant regression ($p \leq 0.001$). Also the lack-of-fit test shows no statistical significance ($p = 0.108$), meaning that replicate differences are similar to model residuals (Table VII).

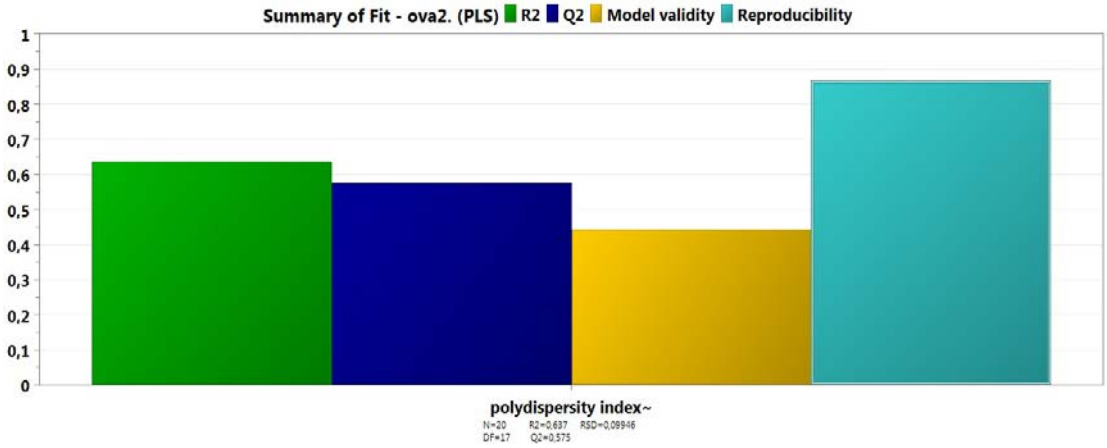

Figure 16: Summary of fit plot PDI.

The scatter plot of observed vs. predicted values for PDI does not show all points close to a straight line, which would usually indicate a bad model (Figure 17). We can see points in four lines. This happened as a result of not enough variabilty in significant factors. We had 2 significant factors (surfactant in EP and CS), both at two levels (PVA or PF127 and with or without CS), which could lead only to four predictions on the observed vs. predicted plot for PDI.

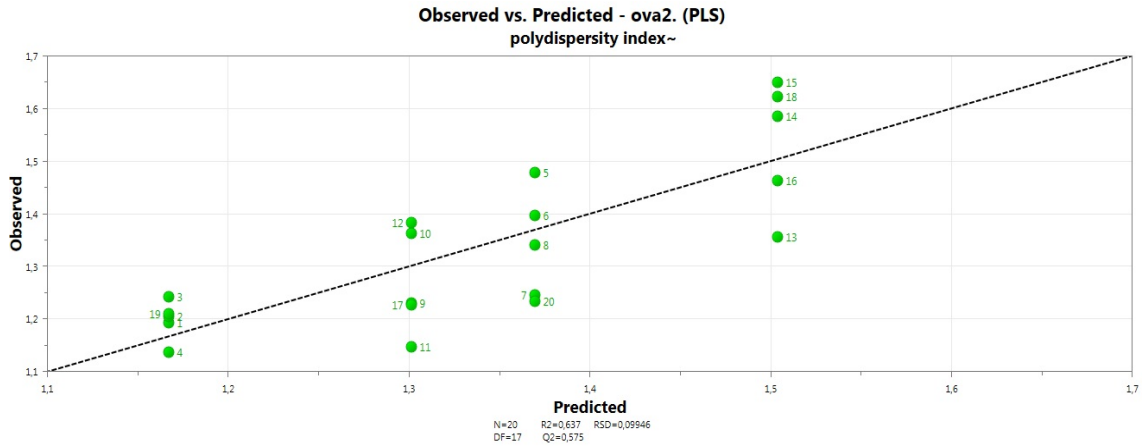

Figure 17: Observed vs. predicted plot for PDI.

4.1.3 Zeta potential

The liquid layer surrounding the particle occurs in two parts; an inner area (Stern layer), where the ions are strongly bound and an outer (diffuse) area, where they are less stably associated. Inside the diffuse layer there is a notional boundary within which the ions and particles form a stable entity (57). When a particle moves, ions within the boundary move with it. The ions outside the boundary stay with the bulk dispersant. The potential at this boundary (surface of hydrodynamic shear) is named the zeta potential (ZP). The extent of the zeta potential gives an implication of the potential stability of the colloidal system. If the particles in the suspension have an abundant positive or negative zeta potential then they will tend to repulse each other and there will be no tendency for the particles to flocculate. However, if the particles have low zeta potential values there will be no force to prevent the particles coming together and flocculating (57).

In this study ZP was measured with Zetasizer Nano Z, assessed by Laser Doppler Velocimetry (LDV) in combination with M3 Phase Analysis Light Scattering (M3-PALS). Each sample was measured in triplicates.

Results of measured ZP of formulations are presented in Table VI. The results show slightly negative surface charge mostly, but still close to neutrality, with ZP values ranging from -5.75 to 0.90 mV. By comparing plain NPs and OVA loaded NPs (without CS) we can observe that ZP of NPs with OVA is slightly more negative (for either PVA or PF127 used in EP), owing to the negative charge of the protein. All formulations with CS have more positive ZP than plain controls, due to the positive charge of CS. Also formulations with loaded OVA and CS have less negative ZP then formulations with only OVA (for either PVA or PF127 used in EP), meaning that more OVA was encapsulated, hence a less negative charge of NPs. Except F9o, all formulations showed desired ZP, close to neutrality (between -5 mV and 5 mV).

4.1.3.1 Modelling

Determining significant factors for ZP model was different compared to previous models. From the coefficents plot (Figure 18) we can observe that between significant factors there are also interactions. Interaction between% (w/v) of PVA in IP and surfactant

in EP (PVA or PF127) and interaction between% (w/v) of PVA and CS seem to be significant.

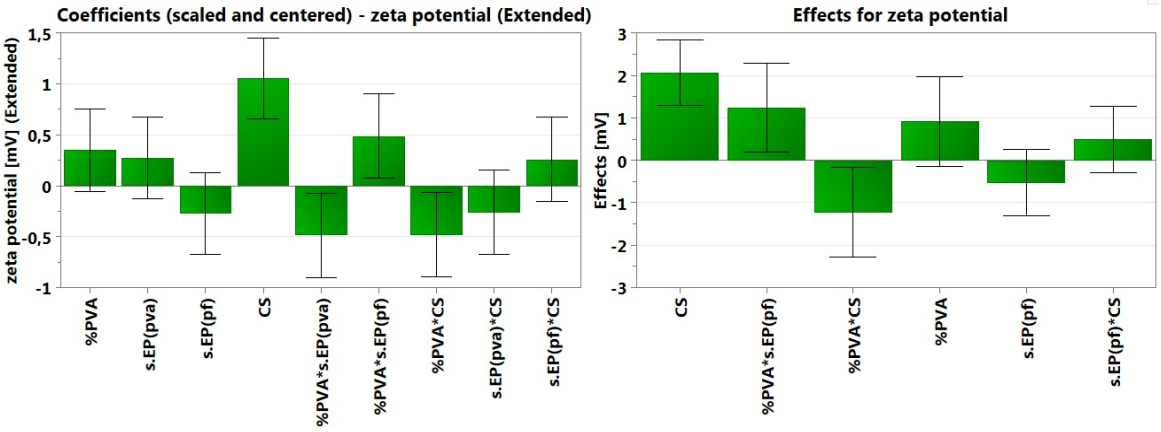

Figure 18: Coefficients and effects plot for ZP.

CS alone is the most significant factor for ZP, as can be seen on the effects plot for ZP (Figure 18) it has the biggest positive effect. As mentioned above, when CS was added, surface charge of NPs became more positive, due to the positive charge of CS. In both plots we can detect factors that are not statistically significant (they do not show a large distance from y=0 as well as having an uncertainty level that does extend across y=0). Sometimes when refining model terms it is not possible to exclude all of the non-significant terms in order to obtain a better model, that is why they stay included in plots.

As previously seen, there are interactions between model terms in ZP model. We can see from the interaction plot on the left (Figure 19) that ZP became more positive when% (w/v) of PVA in IP increased (with PVA or PF127 in EP). When CS was added, ZP did not change regardless of the changing% (w/v) of PVA or surfactant in the external phase (interaction plots in the middle and on the right). For NPs with CS, ZP changed to more positive values when increasing the% (w/v) of PVA in IP and to more negative when PF127 in EP was used.

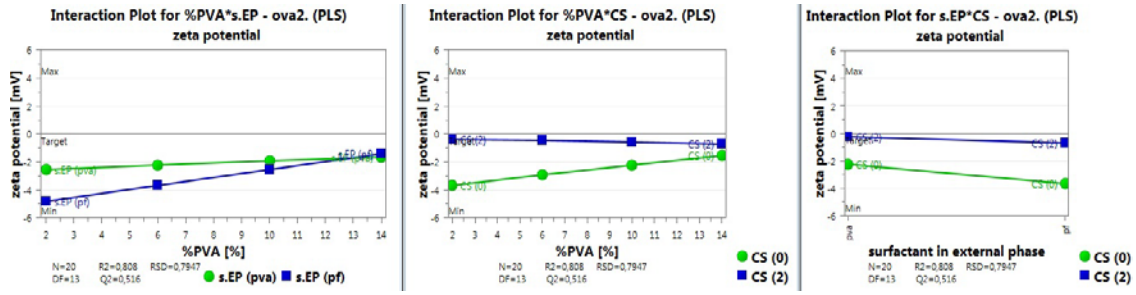

Figure 19: Interaction plots for ZP. Interaction plot on the left represents interaction between % (w/v) of PVA in IP and surfactant used in EP. Interaction plot in the middle represents interaction between % (w/v) of PVA in IP with CS. Interaction plot on the right represents interaction between surfactant used in EP with CS.

Summary of fit for ZP is displayed in Figure 20. R^2 is 0.8 which indicates a good model fit with high significance. Q^2 is slightly over 0.5, which implies a good model, thereby good estimation of future predictions. Model validity with a value over 0.25 shows that there are no diverse model problems, such as the presence of outliers, an incorrect model, or a transformation problem. With reproducibility greater than 0.5 we can conclude that the variation of the replicates compared to the overall variability is not significant, meaning the results for ZP are repeatable.

Obtained model for ZP has a significant probability for the regression ($p \leq 0.001$), meaning that model is statistically valid. Also the lack-of-fit test is not significant, hence statistically the model has no lack of fit ($p = 0.063$), meaning that replicate differences are similar to model residuals (Table VII).

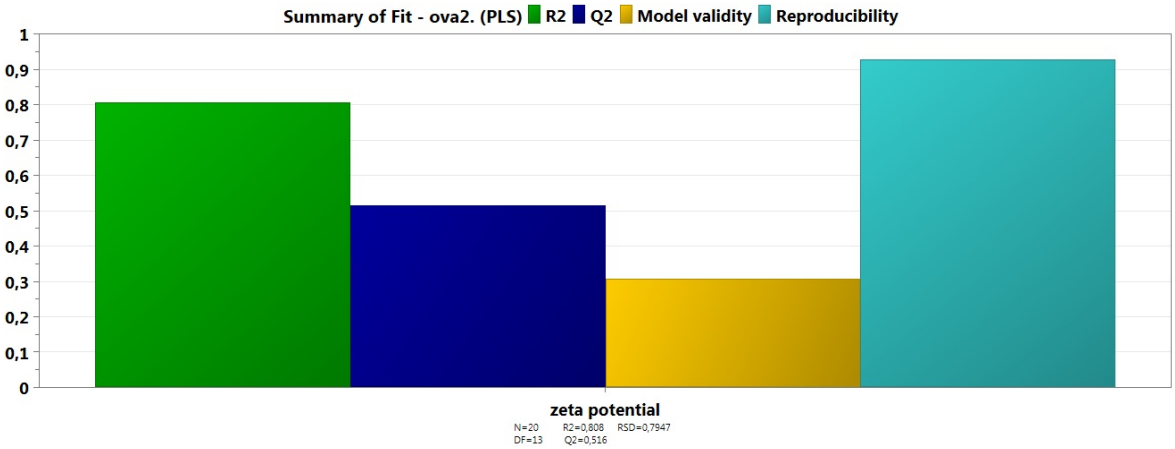

Figure 20: Summary of fit for ZP.

From the observed vs. predicted plot for ZP we can identify some deviations (low accuracy when compared with the Z-average model), still predictions follow the expected trend (Figure 21).

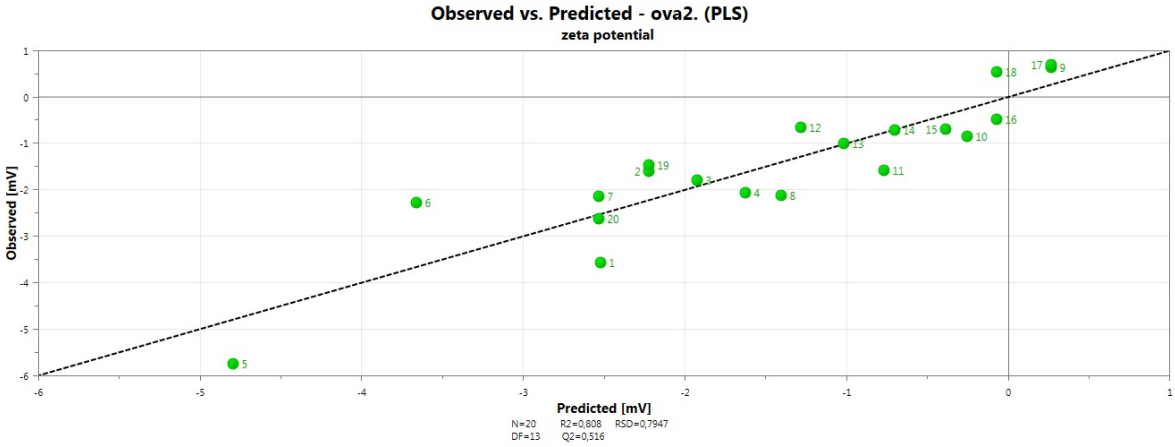

Figure 21: Observed vs. predicted plot for ZP.

4.1.4 Ovalbumin encapsulation efficiency and loading capacity

The ability of PLGA NPs to incorporate model antigen OVA was determined as previously described (**3.2.3 Ovalbumin loading analysis**). The EE represents the percentage of entrapped OVA to the initial OVA and LC represent the quantity of the loaded OVA to the overall particles, respectively. They are mostly affected by development conditions, copolymer compositions, particle size and characterization techniques. Results of the calculated EE and LC for all formulations are shown in Table VI.

The amount of OVA that was not incorporated into NPs was determined by quantifying the OVA in particle supernatants. The amount of loaded OVA was calculated by the difference betweent the total amount of OVA used during formulation and the mass of OVA that was not incorporated into the NPs (Equation *2*, Equation *3*), this is the amount that was detected in supernatants (collected during the washing and centrifugation steps of NPs).

When PVA was used in the EP we could see that EE was higher with incorporated CS. CS polymer is able to protect biomolecules during the particle formulation process and also throughout the release in physiologic media. The higher viscosity that is expected for IP after CS dissolutiom can decrease the interaction between proteins and the interface of droplets of the primary (w/o) emulsion, endorsing the maintenance of their structure and

activity, as well as enhancing NPs LC by preventing protein leakage to the external aqueous phase. The highest EE and LC is shown for 6% PVA in IP. With PF127 in the EP we can notice lower EE and LC values compared to when PVA is in the EP, except for F1o and F15o. Highest EE and LC are observed for 6% and 10% PVA in IP.

Overall, we can conclude that we managed to formulate NPs with high EE and LC values, with the best results obtained by F4oc and F6oc.

HPLC analysis also showed that the excess of surfactant in EP is efficiently removed from the surface of NPs during the washing and centrifugation procedures (data not shown).

4.1.4.1 Modelling

When modelling we only used EE results for establishing the model, leaving out the results of LC, because of its lack of predictive capacity. To be able to model LC we would probably need a more complex modelling structure (a non-linear model) or else more information from the process (other factors) to help explain LC's variability. Also, we converted percentage values of EE into quantitave values of µg (naming it »amount of encapsulated protein« (E.O.)).

From the coefficients plot (Figure 22) we can see that only CS is a significant factor. However, the type of surfactant used in the EP had an influence on the amount of encapsulated OVA (E.O.). CS has a positive effect on E.O., thus increasing E.O. when incorporated into NPs (Figure 22) and PF127 has a negative effect. This could imply that the surfactant PF127 allowed a leakage of OVA to the external phase. This is probably due to its lower ability to decrease the free energy at the specific emulsion droplets interface.

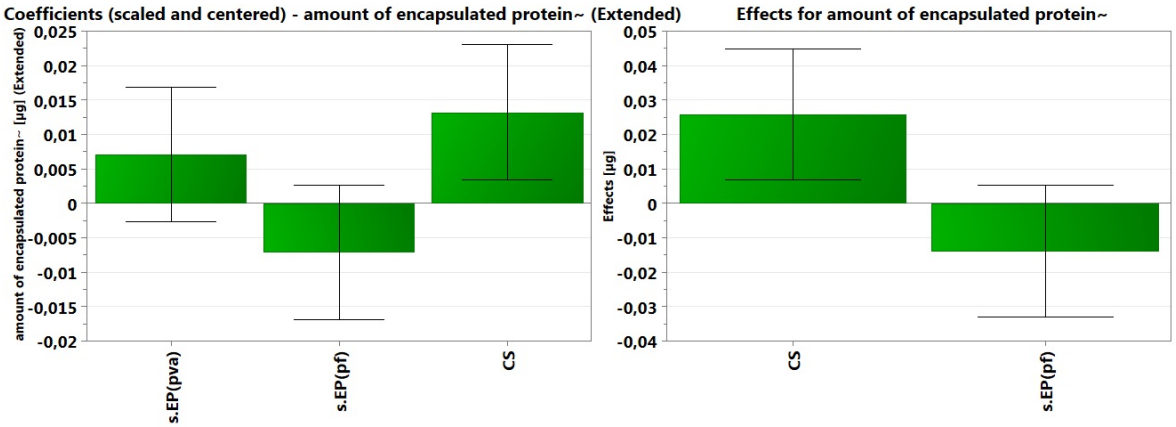

Figure 22: Coefficients and effects plot for E.O.

For E.O. model there are no observed interactions between model terms. This is due to a lack of significant factors.

The summary of fit for E.O. shows that the performance in terms of accuracy is lower when compared to the previous models (Figure 23).

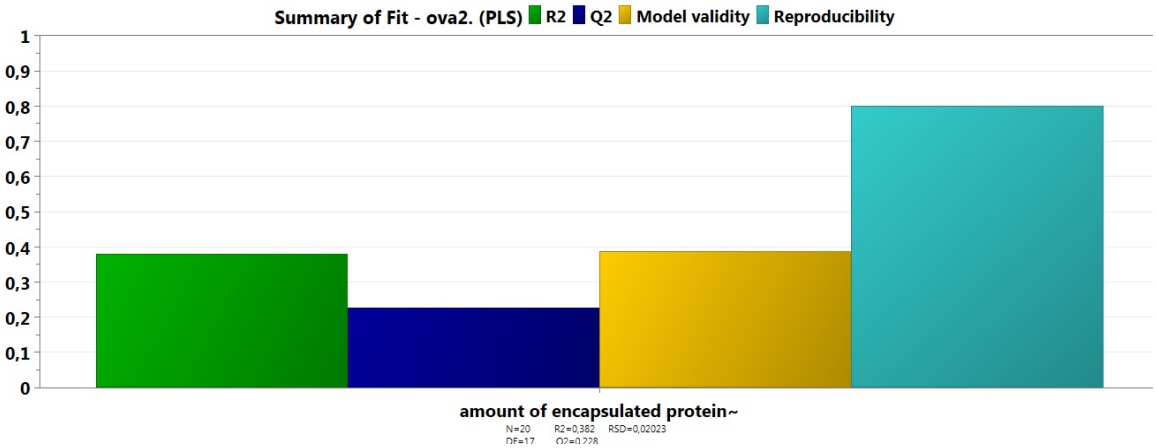

Figure 23: Summary of fit for E.O.

The observed lower R^2 is confirmed with a Q^2 which is only greater than 0.1. Model validity being greater than 0.25 and reproducibility being greater than 0.5 are on the other hand good characteristics of this model.

From the ANOVA we can see that the obtained model for E.O.is statistically valid ($p = 0.017$). Also there is no lack-of-fit ($p < 0.05$), meaning that replicate differences are comparable to model residuals (Table VII).

The scatter plot of observed vs. predicted values for E.O. (Figure 24) shows similar disturbance as the plot for PDI. We do not see points close to the straight line. This happened as a result of not enough variabilty in the significant factors to explain E.O.. We had 2 significant factors (surfactant in EP and CS), both at two levels (PVA or PF127 and with or without CS), which could lead only to four predictions on the observed vs. predicted plot for PDI.

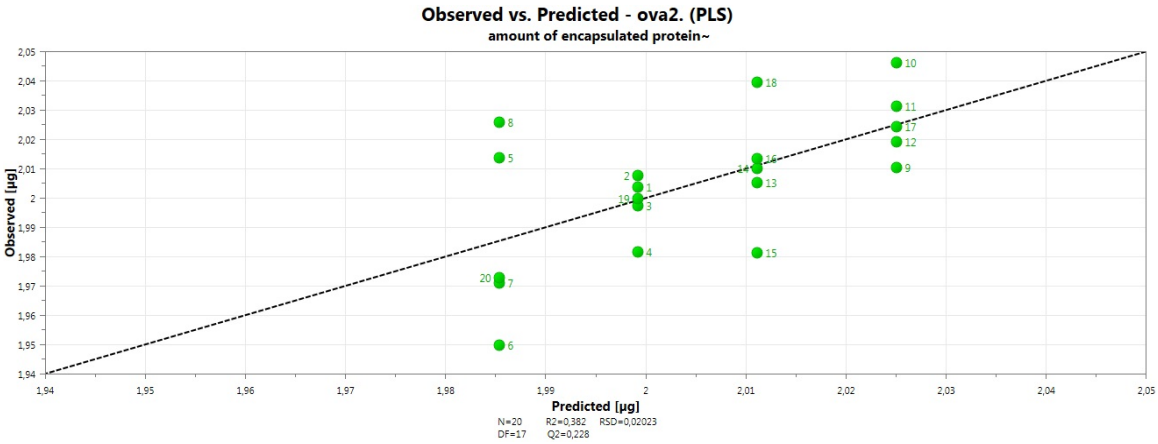

Figure 24: Observed vs. predicted plot for E.O.

4.2 Models optimisation

The very first result obtained for the models for all responses is represented in Figure 25. These models were simply calibrated using the factors terms and interactions without any optimisation. They represent thus, the starting point for the model's optimisation procedure.

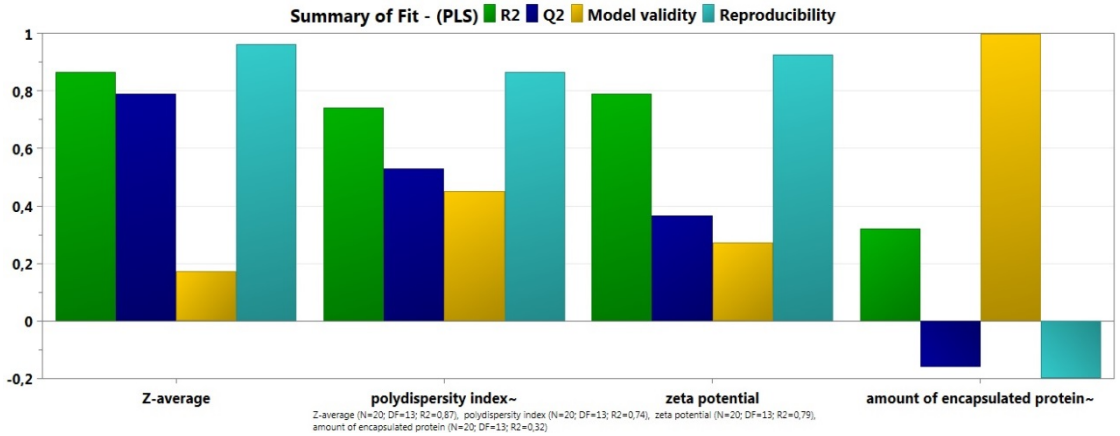

Figure 25: Summary of fit for Z-average, PDI, ZP and E.O. before optimisation.

Figure 26 shows the same models after the optimisation process. If we compare the initial and optimised models (Figures 25 and 26), the difference is very relevant. Especially for Z-average and E.O. where we can claim that models were improved significantly.

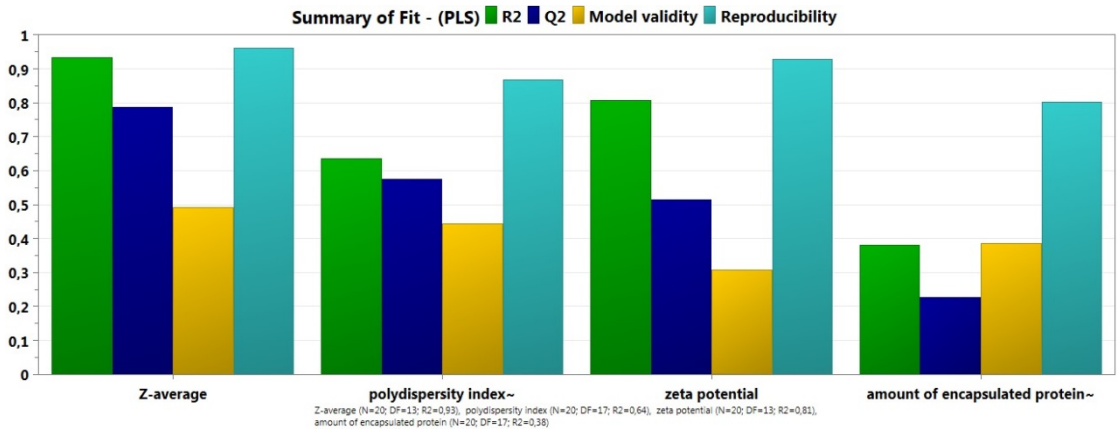

Figure 26: Summary of fit for Z-average, PDI, ZP and E.O. after optimisation.

We can describe the relationship between the response variables and the predictors (or factors) mathematically on a matrix format (Figure 27). Values are taken from the scaled and centered coefficients list available in Modde. It allows an unfied perspective of all developed models.

$$Y = XB$$

	offset	%PVA	s.EP(pva)	s.EP(pf)	gCS	%PVA*%PVA	%PVA*gCS	s.EP(pva)*gCS	s.EP(pf)*gCS	%PVA*s.EP(pva)	%PVA*s.EP(pf)	
Z-average =	277,3	-42,6	-51,7	+51,7	+48,7	+28,7	+14,5	-19,8	+19,8	0	0	Offset
PDI	1,33	0	-0,1	+0,1	+0,07	0	0	0	0	0	0	%PVA
ZP	-1,55	+0,4	+0,3	-0,3	+1,05	0	-0,48	-0,26	+0,26	-0,48	+0,48	s.EP(pva)
E.O.	2	0	+0,007	-0,007	+0,01	0	0	0	0	0	0	s.EP(pf)
												gCS
												%PVA*%PVA
												%PVA*gCS
												s.EP(pva)*gCS
												s.EP(pf)*gCS
												%PVA*s.EP(pva)
												%PVA*s.EP(pf)

Figure 27: Models structure and coefficients in matricial format.

4.3 Formulation optimisation

To take advantage of the developed models, we used the software optimizer feature to estimate a formulation (factors) given to a specific set of values for the responses (desired target for the NP). This search procedure is based on the simple algorithm that attempts to estimate the most appropriate combination of factors to produce a desired response. Of course, it might happen that multiple possibilities exist and it is our duty to select the most appropriate solution. Considering the desired quality target for the product, the optimiser was run and yielded the following expected values for the factors: 12.1% (w/v) of PVA in IP; (ii) 0.075% ($w_{CS}/w_{polymer}$) CS; (iii) 0.3% (w/v) of PVA in the EP.

4.4 Design-space estimation

Before estimating the DS for this manufacturing process, it is necessary to set the desired range for the response values (target quality) to obtain the most appropriate combination of factors. Our target limits were as mentioned before: Z-average between 50 and 200 nm, ZP under 5 mV (positive or negative), PDI lower than 0.2 and for E.O. we decided on a minimum of 80 µg. After running the algorithm for the DS estimation, we checked at DPMO's of all the responses (considering a DPMO limit of 1000). The value of DPMO for PDI was very high (208000), implying that the PDI model is yielding estimations that do not provide a product within the expected PDI range. On the other hand, DPMO for all other responses was 0, which proves optimal for the definition of the DS and final quality of the product. For that reason, we decided that the PDI response will not be included in the definition of the DS. A possible reason for DPMO being so high could be the possible non-linear behaviour of the PDI in relation to the factors, which would explain why we could not improve the value of DPMO. The established DS shows the estimated volume in the investigated region where we can expect that all specifications are fulfilled at a specific risk level (risk assesment). Each response may have a unique model describing the connection between the investigated factors and the corresponding response.

Figure 28 shows the obtained DS established from our study. We were not able to obtain a product within specification when PF127 in EP is used. On the other hand, DS for formulations with PVA in EP shows an acceptable quality. The green and yellow colors

represent the area within the defined limit for DPMO. The optimal values for the factors for the desired product as estimated by the optimiser can be seen in the figure's left panel (12.1% (w/v) PVA, 0.6 mg/mL CS).

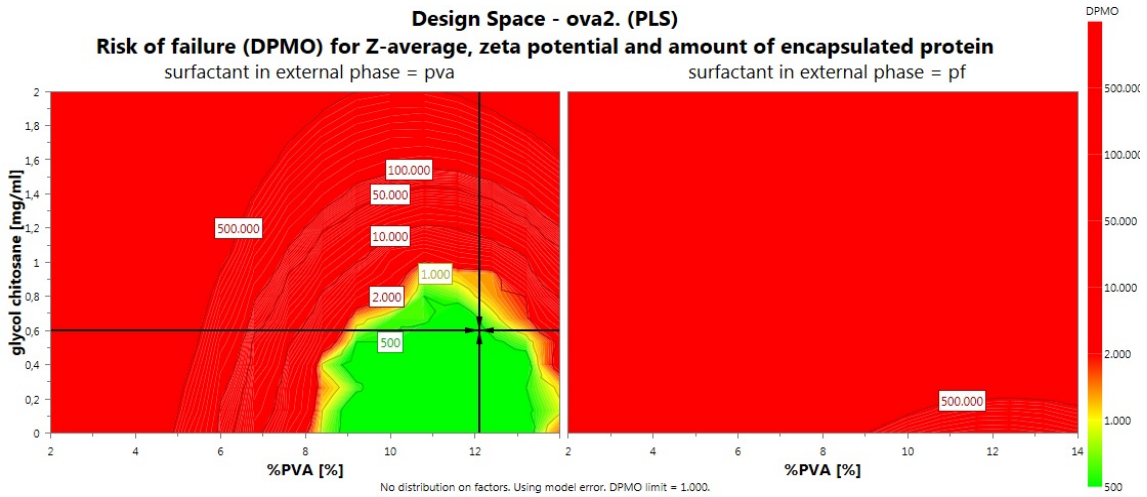

Figure 28: Design space for PVA in the EP and for PF127 in the EP.

4.5 Validation experiments

Model validation is an important aspect of the modelling procedure. We need to validate the model's predictions with a few additional experiments, to see if these predictions are accurate enough for the production of NPs to rely on the model. Validation relies on testing the optimised models for formulations not included in the models' development. There are many ways to perform this process. One possibility is to split the available formulations in two sets, and use one to develop the models and then use the remaining to test the models. Testing is based on comparing the models predictions for these validation samples with the experimental data for the responses. In our case, this strategy was not adequate since the samples were made according to a DoE meaning that if we leave some samples to test the models, it will mean that they are expected to extrapolate. This is because DoE samples are estimated to be as independent as possible. Therefore the strategy here was to make a couple of new formulations within the boundaries of the factors used in the DoE. The best formulation was defined for: (i) 12.1% (w/v) of PVA in the IP; (ii) 0.075% ($w_{CS}/w_{polymer}$) CS; (iii) 0.3% (w/v) of PVA in the EP. For our validation experiment we chose two points

from the established DS (Table VIII). Because of easier and more accurate preparation of NPs (specially by weighing) we decided to prepare NPs with 12% (w/v) of PVA in IP and 0.125% ($w_{CS}/w_{polymer}$) CS or without CS. The choice was also based on DPMO. Formulation F1 has a low DPMO value while F2 has a DPMO value over the limit. This was chosen in order to see if there are large differences between observed and predicted results, considering that the DPMO is at its limit for the F2 formulation.

Table VIII: Details of formulations for the validation experiments.

F	IP	Surfactant IP	Surfactant EP	DPMO
F1	12% PVA, H2O, OVA (1.25% ($w_{OVA}/w_{polymer}$))	2% PVA	0.3% PVA	50
F2	12% PVA, CS (0.125% ($w_{CS}/w_{polymer}$)) , OVA (1.25% ($w_{OVA}/w_{polymer}$))	2% PVA	0.3% PVA	3244

Results of the validation experiment are shown in Table IX. From the model predicted values we can notice that the SD is quite large, giving a higher possibility of observed values to be in the predicted range. Observed values showed more narow deviation. All observed results had values in the predicted ranges, confirming the models validity. With the F2 formulation, selected for DPMO on the boundary, we can see that residual errors are mainly higher than with F1, meaning that DPMO is an indicator that should be considered.

Table IX: Results for the validation experiments.

F1	observed	predicted	prediction error	prediction error (%)
Z-average (nm)	175 ± 2	170.7 ± 34	4.3	2.5
PDI	0.08 ± 0.014	0.154 ± 0.072	-0.074	48
ZP (mV)	-2.29 ± 0.77	-2.05 ± 0.98	-0.24	11.7
E.O. (µg)	96.06	99.93 ± 3.81	-3.87	3.8
F2				
Z-average (nm)	190 ± 4	211.66 ± 24.16	-21.66	10.1
PDI	0.134 ± 0.013	0.211 ± 0.056	-0.077	36.5
ZP (mV)	-0.41 ± 1.62	-1.39 ± 0.82	0.98	70.5
E.O. (µg)	99.27	102.91± 3.11	-3.64	3.5

From the results of the validation experiments we can conclude that the developed models were yielding accurate predictions. It confirms that models could be used to assist the development of further formulations.

5 CONCLUSIONS

In this thesis, PLGA NPs incorporating model antigen OVA were investigated with the ultimate goal of developing mathematical models for relevant attributes. Different excipients have been tested in various proportions. Variations were made in the amount of incorporated OVA, CS and PVA in IP. PVA and PF127 were also tested as EP surfactants. Different concentrations of PVA in IP were used to change the viscosity of IP, 2% (w/v) PVA was used as a surfactant in IP to build the w/o emulsion and then the 0.3% (w/v) PVA or 0.3% (w/v) PF127 were used as surfactants in EP for the w/o/w emulsion. The produced NPs with appropriate physicochemical characteristics are intended for subcutaneous or intratumoral administration. We were able to obtain proper NPs acording to our target: Z-average between 50 and 200 nm; ZP under 5 mV (positive or negative); PDI lower than 0.2. All these formulations have 0.3% (w/v) PVA as a surfactant in EP. If we take in consideration that NPs should contain OVA as the model antigen and CS for higher EE, only F6oc formulation is applicable (Z-average: 201 ± 3 nm; PDI: 0.137 ± 0.013; ZP: -1.59 ± 0.71 mV; EE: 85.95%; LC: 10.74 µg/mg). With HPLC analysis we confirmed that the excess PVA as surfactant in EP was efficiently removed from the surface of NPs during the washing and centrifugation procedure.

Transforming data into information was achieved with a linear modelling approach using the software Modde. It enabled us to estimate the importance and influence of terms of particles properties. Z-average was most affected by% (w/v) of PVA in IP, CS and PF127 as surfactant in EP. PDI was most affected by CS and PF127 as surfactant in EP. ZP and EE were most affected by CS.

We were able to establish accurate models for responses. It is important to mention that models are never 100% right but they give us useful information about what is more important considering the system we are modelling. A DS was created, from which admissible predictions were made and then confirmed with additional validation experiments. We also confirmed that DPMO is an indicator that should be considered. Our validation experiments showed that our models could be used to assist the development of further formulations.

For future work, we should perform more experiments with more variations in factors. We could also use the same modelling approach with other similar systems, to analyse differences and similarities. It would be recommended to test a non-linear approach to develop the model. Particularly, the PDI model should be additionally investigated with a non-linear approach to see if the non-linearity was the reason for the very high DPMO when trying to create the DS.

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