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GELATINE NANOPARTICLES AS IMMUNOMODULATORY DRUG DELIVERY SYSTEM

ADVANCED PRODUCTION PROCESSES AND CLINICAL TRIALS

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(Katharina Geh)

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Für meine Familie

In Liebe und Dankbarkeit

"All our dreams can come true,

if we have the courage to pursue them."

Walt Disney (1901 – 1966)

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CHAPTER I

GENERAL INTRODUCTION

1 NANOPARTICLES AS DRUG DELIVERY SYSTEM

Since the 1970s nanoparticles are increasingly researched as drug delivery systems. One reason is that they have several advantages, such as being able to target different organs, e.g. the lymphatic system, the brain, the lung, the liver and the spleen, as well as tumours. Furthermore, nanoparticles are capable to carry various drugs, including hydrophilic and lipophilic molecules, proteins, nucleic acids, vaccines and other biological macromolecules. This specific delivery enables that therapeutic effects can be improved at the intended target site and systemic toxic side effects can be reduced [1]. Besides this, nanoparticles can protect the drug from (bio)degradation and consequently increase its bioavailability [2]. Both characteristics may allow a dose reduction. Other important advantage of nanoparticles is their ability to create a controlled and sustained release of the drug, as well as an enhanced cellular uptake [3-5]. All these points show why there is a strong research interest in nanoparticulate drug delivery systems.

2 MATERIALS FOR NANOPARTICLE PREPARATION

2.1 SYNTHETIC AND NON-PROTEINEOUS BASE MATERIALS

A variety of different starting materials is available to prepare nanoparticles for pharmaceutical purposes. Generally, these materials should be biocompatible and at least partly biodegradable. Nanoparticles may be prepared from synthetic polymers, such as polyethylenimine (PEI), poly(lactic-co-glycolic) acid (PLGA), or natural polymers, such as polysaccharides or lipids [3, 6-8].

The polymer PEI was demonstrated to be a potential non-viral gene delivery system *in vitro* and *in vivo.* Here, nucleic acids were attached to the cationic polymer by electrostatic interactions [9, 10]. However, due to a rather high toxicity of the material combined with a strong complement activation, the dosing is limited [11, 12]. Certainly, this could successfully be overcome by structural modifications of the polymer, such as PEGylation or introduction of negatively charged residues [12-14].

Nonetheless, due to the lack of biodegradability and subsequent accumulation of the polymer when multiple administered, further biodegradable derivatives need to be developed [15].

On the other hand, PLGA is a widely used starting material for drug delivery systems as it is biocompatible, biodegradable and approved by the US Food and Drug Administration (FDA). It solely consists of acids, which are part of the human metabolism, lactic acid and glycolic acid. Furthermore, by changing the ratio of the particular components or the molecular weight, its physical properties, such as mechanical strength, swelling behaviour or degradation time frame can be controlled. Therefore, it is mostly researched for controlled and sustained delivery of small molecules, proteins or nucleic acids [16, 17]. Due to tuneable particle sizes, PLGA based nanoparticles can be used to target different parts of the immune system, such as antigen presenting cells (APCs) or the lymph nodes. In combination with a prolonged release, a more effective immune response can be initiated when antigens are applied via PLGA nanoparticles [16]. Nevertheless, when PLGA derivatives are degraded, acidic components are released resulting in a microclimate pH drop [18, 19]. This may affect sensitive active pharmaceutical ingredients, such as proteins or nucleic acids.

Thirdly, lipids are established materials to prepare nanoparticulate carries for drug delivery as they are biodegradable and non-toxic. This class includes, inter alia, solid lipid nanoparticles (SLNs) and liposomes. SLNs are composed of lipids that are solid at room and body temperature and offer several advantages: great physical stability, controlled or sustained drug release, protection of the drug from degradation and good physiological tolerability [8]. Their main disadvantage is a possible burst drug release due to polymorphic transitions of the lipids during storage [8]. However, this could be circumvented by optimal storage conditions, lipid composition and addition of emulsifiers [20]. Besides small molecules, SLNs are also used to carry macromolecules, such as proteins or nucleic acids [21-23]. However, there is still few research regarding vaccination or immunotherapy using SLNs. On the other hand, liposomes demonstrate a lipidic nanoparticulate drug delivery system

intensively utilised to target the immune system [24]. They offer the possibility to incorporate various types of antigens and adjuvants, either into their aqueous core or into the phospholipid bilayer. In addition, attachment of the payload onto the particle's surface is possible. Furthermore, due to modifiable features, such as particle size, size distribution, lipid composition or charge, different types of immune reaction can be stimulated [25]. The excellent potential of liposomes to acitvate the immune system is proved by two marketed vaccines (Epaxal®, hepatitis A vaccine and Inflexal® V, influenza vaccine) and one cancer vaccine (Stimuvax®), which is tested in a phase III clinical trial [26]. Moreover, liposomes showed good clinical effects in delivering plasmid DNA to treat allergic diseases, such as canine atopic dermatitis, and are able to enhance the immunotherapeutic efficacy of cytosine phosphate guanine oligonucleotides (CpG ODNs) in the treatment of cancer and infectious diseases [27, 28]. However, to prepare effective lipoplexes (liposomes carrying nucleic acids) cationic lipids are often required, which are known to be cytotoxic *in vitro* and *in vivo* [29]. Furthermore, they are less stable against biological and physiological stresses compared to polymeric nanoparticles [30].

2.2 PROTEINS AS BIODEGRADABLE BASE MATERIALS FOR NANOPARTICLES

Proteins are intensely studied for the preparation of nanoparticles. Proteins consist of different amino acids and therefore, many moieties are available for chemical modification (covalent or non-covalent) in the matrix or on the particle surface. Altering the particle surface allows attaching bioactives and/or targeted delivery [31]. Due to their biodegradability, the accumulation of proteins is unlikely to occur and degradation products are usually non-toxic [32].

Considering multiple dose administrations of protein nanoparticles, possible immunogenicity associated with the protein particles should be kept in mind. However, there are mechanisms to metabolise natural proteins. Rapid enzymatic degradation is expected to decrease the chance of triggering an immune response [31]. The long parenteral use of gelatine and albumin nanoparticles

support this statement. Particles based on human serum albumin (HSA) have been thoroughly researched and their characteristics are well-established [3]. The first nanoparticulate product licensed for the use in humans is based on HSA (Abraxane®) and was marketed in 2005 [3]. Many *in vitro* and *in vivo* studies showed that albumin nanoparticles have a high drug loading capacity for a variety of active agents (hydrophilic, hydrophobic, proteins, oligonucleotides) [30]. Furthermore, they are both biodegradable and biocompatible and capable of crossing the blood brain barrier [33]. Although, albumin nanoparticles are promising drug carriers and successfully tested in delivering interferon γ (IFN-γ) to macrophages, there is only few research on targeting the immune system [34, 35].
Particles based on recombinant silk protein have been developed as promising drug delivery systems due to their biocompatibility, slow biodegradability, mechanical properties, controllable morphology and structure [36]. Further advantage is that silk nanoparticles can be prepared by desolvation of the protein without the need of organic solvents [37-39]. Moreover, they show a constant drug release and promising results as vaccine carriers [40, 41]. However, the recombinant production causes high prices for the starting material.

Besides these different synthetic and natural starting materials, this work will concentrate on nanoparticles prepared of gelatine.

2.3 WHY GELATINE NANOPARTICLES?

Gelatine is a natural polymer obtained from collagen mainly by acidic (Type A, from porcine skin, isoelectric point (IEP) pH 9.0) or alkaline (Type B, from bovine ossein and skin, IEP pH 5.0) denaturation [42]. Abundant natural sources are an advantage over some other proteins and lead to low prices. Besides, gelatine is available from recombinant origin (recombinant human gelatine, rHG) [43]. The latter overcomes the problem of impurities and inhomogeneity of molar mass [43], as well as the risk of immunogenicity of proteins from non-human sources [42].

Gelatine has a long history of use in medicine due to its biodegradability, biocompatibility, low immunogenicity and high physiological tolerance [4]. The FDA classifies gelatine as "Generally Recognised as Safe" (GRAS) in the record of safety for food supplement [42]. Gelatine derivatives are intravenously applied as e.g. plasma expander (Gelafundin®, Gelafusal®) since the 1950s without serious adverse effects [44, 45]. Another successful medicinal use of gelatine is the application as patches for vascular seal (Gelsoft®, Gelseal®) [46, 47].

Further benefit of gelatine as starting material for nanoparticles is its variety of functional groups. This allows different possibilities of surface modification [48, 49], cross-linking [42, 50, 51] and marker coupling [52, 53]. In addition, targeting-ligands [54, 55] as well as various types of drugs [56-58] may be coupled. Mainly, the amino acid lysine, providing a primary amino group, is very useful for all these modifications.

Altogether, these characteristics make gelatine nanoparticles (GNPs) a promising carrier system for drug delivery. This is supported by the emerging interest in gelatine nanoparticles as drug delivery system displayed in an increasing number of publications over the last 20 years (Figure I-1).

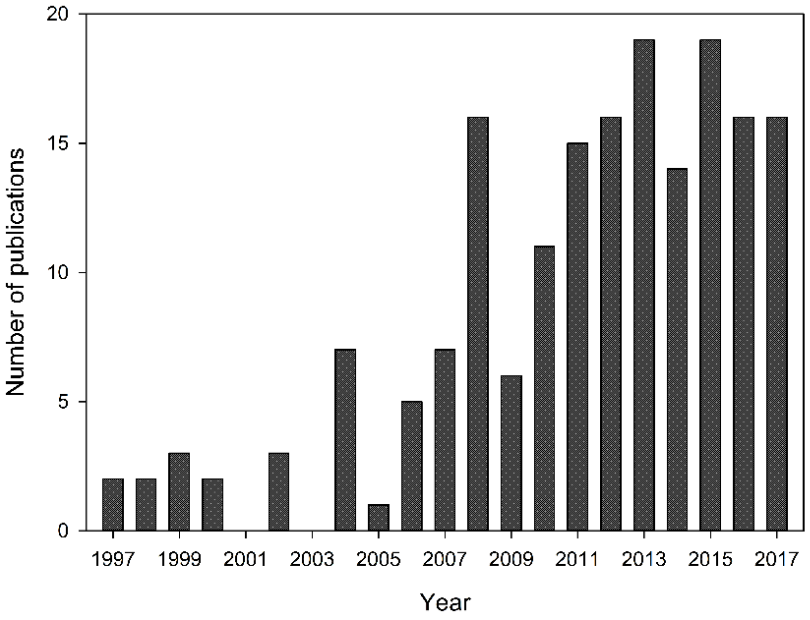

Figure I-1 Number of publications per year regarding gelatine nanoparticles. (Source: Pubmed; search criteria: “gelatine nanoparticles” or “gelatin nanoparticles”).

3 Oligodeoxynucleotide-Loaded Gelatine Nanoparticles as Approach in Immunomodulatory Therapy

3.1 CpG Oligodeoxynucleotides as Potential Therapeutic Option in Allergic Diseases

The prevalence of allergic diseases, such as atopic dermatitis, is steadily rising, in humans as well as in domestic animals [27, 59, 60]. A prominent example in veterinary medicine is canine atopic dermatitis (CAD), a chronic relapsing inflammatory and pruritic allergic skin disease similar to human neurodermatitis. This multifactorial disease results from a complex interaction between genetic and environmental factors and involves a disrupted skin barrier, flare factors, allergic sensitisation and cutaneous inflammation. Furthermore, CAD is associated with IgE antibodies most commonly directed against environmental allergens, such as house dust mites and pollen [61, 62]. The acute reaction is characterised by an increase of Th2-derived cytokines, such as IL (Interleukine)-4, IL-5, which are involved in activation and degranulation of granulocytes as well as immunoglobulin isotype switching to pro-allergic IgE. Furthermore, IgE-coated mast cells degranulate and release histamine and proteases when IgEs are crosslinked by antigen. Proteins released from granula induce acute and delayed dermal inflammation [61, 63]. The acute inflammation is characterised by hyperpermeability of vasculature, whereas the delayed inflammation is related to tissue damage caused by pro-inflammatory cells. This acute allergic reaction is followed by a chronic phase of CAD, which shows Th1-dominant cellular inflammation marked by cytokines, such as pro-inflammatory tumour necrosis factor α (TNF-α) and INF-γ, which activate macrophages [63]. Characteristic acute clinical signs are pruritus, erythrema, oedema or excoriations (Figure I-2) [64]. In the chronic phase, symptoms such as self-induced alopezia, hyperpigmentation and/or lichenification may additionally develop [61]. Furthermore, due to pruritus and subsequent scratching skin lesions increase. This is often followed by secondary infections with Malassezia yeasts or staphylococci, which exacerbate inflammatory reactions [63].

Allergen-specific immunotherapy (ASIT) is the only therapeutic approach, which is able to prevent the development of symptoms and modify long-term course of CAD [61]. However, for successful treatment, ASIT has to be performed up to a year and in some cases life-long therapy is necessary. Despite all efforts, the success rate of ASIT may only be between 50-70% [27].

Other available treatment options aim to control the symptoms rather than the origin of the disease. This includes reduction of the allergen burden, anti-inflammatory glucocorticoids or immunosuppressive drugs, such as ciclosporin or tacrolimus [63].

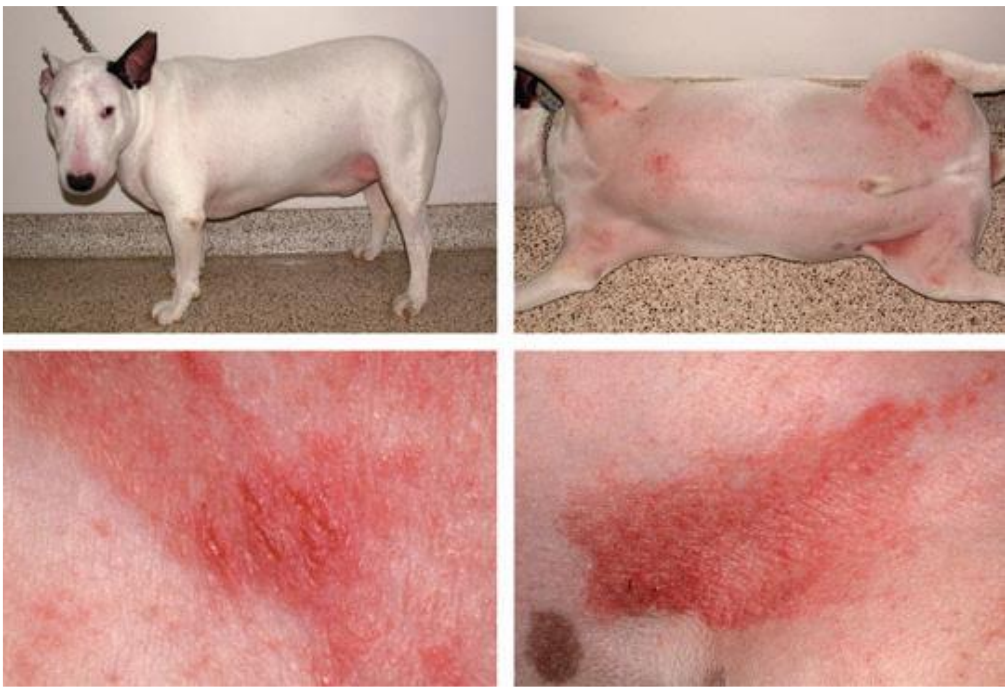

Figure I-2 Clinical signs of acute flare of canine atopic dermatitis including erythrema, oedema and excoriations taken from [61].

A causal therapy approach would include unmethylated cytosine phosphate guanosine oligodeoxynucleotides (CpG ODNs) that are recognised by the innate immune system via Toll-like receptor (TLR) 9 [65]. The activation cascade following CpG ODN recognition is displayed in Figure I-3. Pro-inflammatory cytokines, such as INF-α and β, TNF-α or IL-6 are secreted and cellular non-specific defence mechanisms are induced. This includes the activation of natural killer cells, as well

as differentiation of Th1 effector cells. On the other hand, a humoral immune reaction is initiated. This leads to the suppression of allergy-associated IgE secretion together with an isotype switch from IgE to IgG [65]. Thus, less allergic reactions such as mast cell degranulation can be induced by antigen-binding IgE [65].

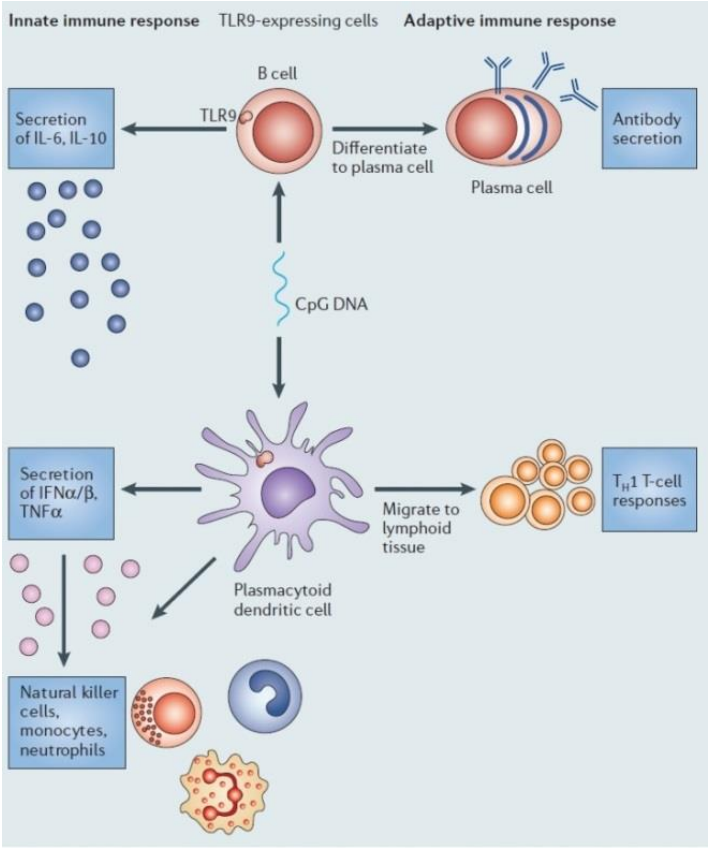

Figure I-3 Representation of the cascade initiated by CpG-mediated TLR 9 activation taken from [65].

Furthermore, IL-10 releasing regulatory T (Treg) cells are involved in the cascade initiated by TLR 9 activation by CpG ODNs [66]. IL-10 is a beneficial agent in the pathophysiology of atopic diseases by modulating mechanisms associated with allergies. For instance, IL-10 inhibits the pro-allergic IgG to IgE switch as well as the activation of mast cells and dendritic cells. In this way, the production of pro-inflammatory cytokines, such as TNF-α and IL-6 is reduced [66]. Thus, the pro-inflammatory Th1 shift can be controlled, too. Recent studies proved the activation of Treg and further release of IL-10 as promising therapeutic option in allergies [66, 67].

In summary, CpG ODNs lead to a shift from a Th2-dependent pro-allergic immune response to a Th1-mediated immune response. In the treatment of atopic diseases, such as canine atopic dermatitis, this redirection of the immune responses from Th2 to Th1 is a very promising approach.

3.2 GELATINE NANOPARTICLES AS DELIVERY SYSTEM FOR CPG ODNS

When immunomodulating nucleic acids should be applied *in vivo*, the most important technical aspect is to protect them from enzymatic degradation through DNases. Therefore, DNAse-resistant synthetic CpG ODNs have been developed. This resistance could be achieved by the partial or complete substitution of oxygen of the phosphodiester backbone by sulphur, which results in a stable phosphorothioate (PTO) backbone [68]. A further approach is the application of nanoparticulate drug delivery systems, such as GNPs [68].

So far, only a few groups investigated the capability of nanoparticles to prevent DNase-dependent degradation of CpG ODNs [69]. For instance, a study by Zorzi et al. investigated the DNase resistance of plasmid DNA when it was incorporated into GNPs [70]. The authors showed stability against DNase I of the GNP-DNA system for at least one hour, whereas free DNA was degraded immediately. Moreover, there is a lack of studies probing the DNase protection of electrostatically bound CpG ODNs onto the surface of GNPs. Nevertheless, various successful *in vitro* and *in vivo* studies support the assumption that GNPs are able to protect DNA when it is attached to their surface [71-79].
Additionally, through co-delivery of CpG ODNs with GNPs the cellular uptake may be enhanced and an interaction of CpG ODNs with the intracellular target TLR 9 is more likely. Due to their sizes between 150 nm and 350 nm, which is similar to those of microorganisms, ODN-loaded GNPs are predominantly phagocytised by APCs [5, 80].

In a previous murine *in vitro* and *in vivo* study, GNPs have proven to enhance the uptake and the immunostimulatory effects of CpG ODNs [71]. In the same study, CpG-GNPs were successfully evaluated to induce production of proinflammatory cytokines in human primary plasmacytoid dendritic cells and B cells [71]. The authors concluded that GNPs are biodegradable and well tolerated drug delivery systems for CpG ODNs and strongly increase activation of the immune system. A follow up *in vivo* study in a murine melanoma model confirmed these conclusions and showed that CpG-GNPs are superior in activating an antitumoral immune response compared to free CpG ODNs [72]. Furthermore, GNPs were able to prevent a CpG-mediated destruction of lymphoid follicles [72].
A further *in vitro* study dealing with the investigation of CpG-GNPs in the treatment of allergy-derived canine atopic dermatitis demonstrated a significant stronger increase in IL-10 production compared to free CpG ODNs [75]. Consequently, GNPs again showed their potential to protect nucleic acids from degradation and to enhance cellular uptake.

Moreover, CpG-GNPs have a long history in the experimental treatment of recurrent airway obstruction (RAO) in horses, an allergic disease similar to human asthma. A first *in vitro* study found the optimal CpG ODN sequence to induce the desired immune responses, IL-4 downregulation as well as IL-10 and IFN-γ upregulation in equine bronchoalveolar lavage (BAL) cells [74]. Furthermore, the advantage of delivering CpG ODNs via GNPs was demonstrated by higher cell viabilities [74]. A second study showed that CpG-GNPs can be efficiently nebulised and retained their immunostimulatory effects in equine BAL cells [81].
These *in vitro* studies paved the way for several *in vivo* studies in RAO-affected horses and a formulation patent [76-78, 82]. Firstly, five successive inhalations of CpG-GNPs led to a significant induction of IL-10 release and a partial remission of the clinical signs [76]. This was followed by a double-blinded, placebo-controlled, prospective, randomized clinical trial, which showed an potent and prolonged clinical effect [78]. This included a decrease in respiratory effort, nasal discharge,

tracheal secretion and an increase in arterial oxygen pressure. Furthermore, the effect of a co-administration of the relevant allergens was investigated [77]. This study revealed that a co-application of the specific allergen is not relevant to initiate an appropriate immunomodulatory effect and to improve clinical parameters [77]. Currently, the results of a fourth clinical trial are evaluated. This investigation combined a dose-response study and a comparison to the standard therapy inhalative glucocorticoids to inhaled CpG-GNPs [83].

Besides, different *in vitro* and *in vivo* studies showed that GNPs are also capable to carry, protect and efficiently deliver other types of nucleic acids, such as plasmid DNA, RNA oligonucleotides, NF-κB inhibiting decoy oligodeoxynucleotides or double stranded DNA and RNA oligonucleotides [49, 73, 84, 85].

These positive attributes as well as the previously mentioned biodegradability, biocompatibility and physiological tolerance of gelatine make GNPs very attractive delivery systems for CpG ODNs.

4 AIM OF THE THESIS

This thesis is based on long successful research and development of GNPs in the field of treating allergic diseases and aimed to achieve a further step into commercialisation of CpG ODN-loaded GNPs.

The work focusses on the preparation of gelatine nanoparticles with the aim to optimise the production process and provide methods for scale-up. For this, a straightforward one-step desolvation method was introduced to replace the common, but delicate two-step desolvation process. A commercially available gelatine type should be found that enables to perform the already described one-step desolvation without the need of customised gelatine and subsequent large-scale production of GNPs. Additionally, with regards to future application in humans, suitable non-toxic cross-linkers are investigated to substitute the standardly used glutaraldehyde **(Chapter II)**.

Furthermore, bearing a future commercial implementation and wide medicinal use in mind, this project aimed to develop a storage stable ready-to-use formulation. In order to achieve this, freeze-dried ODN-loaded GNPs were further developed, and new lyophilisation approaches were investigated, such as controlled nucleation prior to freeze-drying or novel amino acid containing formulation compositions. Furthermore, MALDI MS was examined as a versatile tool to evaluate ODN integrity **(Chapter III)**.

A further requirement for commercialisation and clinical use is an approach to sterilise the final drug product. Therefore, this project addressed the goal to establish suitable sterilisation processes for GNPs. For plain GNPs, this work researched steam sterilisation as an easy and suitable method. On the other hand, gamma irradiation was studied as promising sterilisation process for lyophilised ODN-loaded GNPs **(Chapter IV)**.

In addition, this work concentrates for the first time on *in vivo* effects of ODN-loaded GNPs in the treatment of canine atopic dermatitis. A preliminary study was examined to provide the basis for further clinical studies. This study was carried out in cooperation with the small animal clinic of the Ludwig-Maximillians-Universität München **(Chapter V)**.

Further aim of this project, but not explicitly described in this thesis, was to supply different clinical studies in recurrent airway obstruction (RAO) affected horses with CpG-loaded GNPs. The first study dealt with the question if a co-application of CpG-GNPs and specific allergens would further increase the efficacy of the treatment [77]. The main outcome of this investigation was that additive allergens are not necessary to initiate an efficient improvement of RAO by CpG-GNPs. The second study supplied during this project focussed on the determination of a dose response relationship and the comparison of CpG-GNP treatment with the standard inhalative glucocorticoid therapy [83]. The results are currently under evaluation. Both studies were carried out at the equine clinic of the Ludwig-Maximillians-Universität München. Lastly, lyophilised CpG-GNPs were provided for a future clinical trial in racehorses suffering from a mild form of asthma, so called inflammatory airway disease (IAD). This study will be conducted at the equine clinic of the Freie Universität Berlin.

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CHAPTER II

OPTIMISATION OF ONE-STEP DESOLVATION AND SCALE-UP OF GELATINE NANOPARTICLE PRODUCTION

Parts of the following chapter have been published in *Journal of Microencapsulation*:

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ABSTRACT

Gelatine nanoparticles (GNPs) are biodegradable and biocompatible drug delivery systems with excellent clinical performances. A two-step desolvation is commonly used for their preparation, although this methodology has several shortcomings: lack of reproducibility, small scales and low yields. A straightforward and more consistent GNP preparation approach is presented with focus on the development of a one-step desolvation with the use of a commercially available gelatine type. Controlled stirring conditions and ultrafiltration are used to achieve large-scale production of nanoparticles of up to 2.6 g per batch. Particle size distributions are conserved and comparable to those determined for two-step desolvation on small scale. Moreover, further approaches are investigated to scale GNP production: an increasing contact area between gelatine solution and acetone during common desolvation process, as well as the alternative preparation method nanoprecipitation. Additionally, a range of cross-linking agents is examined for their effectiveness in stabilising GNPs as an alternative to glutaraldehyde. Glyceraldehyde demonstrated outstanding properties, which led to high colloidal stability. This approach optimises the manufacturing process and the scale-up of the production capacity, providing a clear potential for future applications.

KEYWORDS

Gelatine nanoparticles, one-step desolvation, scale-up, cross-linking, AF4

1 INTRODUCTION

Over the past few decades, the frequency of allergic diseases, such as asthma, has been steadily increasing in the human population. Today, it is estimated that 300 million people suffer from asthma worldwide, furthermore by 2025, an additional 100 million people will be affected [1]. Evidence also indicates that there are an increasing number of domestic animals, which is afflicted with allergic pulmonary disorders. For example, recurrent airway obstruction (RAO) is currently the most common airway disease in horses [2]. RAO shares many similarities with human asthma and is described as a genetically predisposed allergic immune response to inhaled environmental allergens [3]. The allergic response leads to the development of major clinical signs, such as bronchoconstriction, mucus hypersecretion and inflammation of the lower airways [2, 4].

Conventional therapies include corticosteroids or β_2-sympathomimetics. However, these therapeutics only aim to improve symptoms instead of treating the underlying disease mechanism. Thus, there is a strong need for novel causal treatment options. Cytosine phosphate guanosine oligodeoxynucleotides (CpG ODNs) have been identified to redirect the immune response from the pro-allergic Th2 pathway to the pro-inflammatory Th1 via the toll-like receptor (TLR) 9 stimulation [5]. The most important aspects of applying immunomodulatory ODNs *in vivo* are their protection from enzymatic degradation by DNase and their delivery into cells. Both can be achieved by using nanoparticles as delivery systems [6, 7]. Several nanoparticles have provided promising results as carrier systems for CpG ODNs, such as mesoporous silica nanoparticles (MSN) [8], protamine nanoparticles ("proticles") [9] or gelatine nanoparticles [10-13]. MSN could successfully prevent CpG ODNs from degradation and enhance cellular uptake [8]. However, *in vitro* studies using MSN revealed complications, such as formation of reactive oxygen species or inhibition of cellular respiration [14]. "Proticles" loaded with CpG ODNs showed the ability to prevent an allergen-induced Th2 immune response in mice [9]. Nevertheless, protamine may induce severe side effects, such as histamine release or anaphylactic reactions [15].

On the other hand, gelatine is one of the most popular biopolymers and widely used in pharmaceutical and medical applications due to its biodegradability, biocompatibility and its physiological tolerance. Its unfunctionalised amine groups allow surface modifications that enable loading of CpG ODNs via electrostatic interactions. All of these features make GNPs a very attractive delivery system for CpG ODNs.

An aerosol formulation of cationised GNPs loaded with CpG ODNs (CpG-GNPs) has previously been developed to improve the immunotherapy of RAO, and was recently applied successfully in several *in vivo* studies [10-12, 16]. The inhalation of CpG-GNPs led to a significant improvement of clinical parameters, such as respiratory effort, nasal discharge or tracheal secretion in comparison to a placebo [12]. However, the co-application of allergens did not further increase the efficacy of this treatment [11, 17].

Besides RAO in horses, CpG-GNPs also showed the first positive results in the treatment of allergy-derived canine atopic dermatitis [18, 19]. All these studies indicate that CpG-GNPs are very effective for the treatment of allergic diseases and provide a promising and innovative strategy beyond the conventional symptomatic therapies.

The most common preparation method for GNPs is two-step desolvation [20]. In principle, stretched gelatine molecules change their conformation into coiled structures due to the controlled addition of acetone to a gelatine solution followed by the stabilisation of GNPs with a chemical cross-linker. During the first desolvation step, the high molecular weight (HMW) fraction of gelatine is separated from the low molecular weight (LMW) fraction by precipitation. In the second desolvation step, GNPs are formed. This separation is necessary due to the heterogeneous molecular mass distribution of gelatine. Monodisperse GNPs can only be formed from the HMW fraction. Without discarding the LMW fraction, the desolvation method would lead to the formation of large nanoparticles in a wide size range, which are prone to aggregation [20, 21]. The lab-scale preparation of these

nanoparticles has become a standard method, although it is susceptible to several issues: low particle yields, lack of reproducibility of the first desolvation step, and difficult process scale-up.

Due to the exceptional clinical results of GNPs as carrier systems in the treatment of asthmatic horses, GNPs are no longer only a research tool [10, 12]. The present work provides an improved and more reproducible process that enables the transfer from the conventional bench lab methodology to the large-scale production of GNPs. This novel approach is based on preliminary studies by our group led by C. Coester using a non-commercial, customised high molecular weight gelatine type A [22-24], which allowed to neglect the first irreproducible desolvation step. A previous study by Ofokansi and co-workers [25] demonstrated how the commercially available gelatine type B 225 bloom could be used in a one-step desolvation. However, this procedure involved a complex series of incubation steps and a strong effect of pH on particle size was reported. The current study was performed to establish a more robust and straightforward one-step desolvation for monodisperse GNPs from a commercially available gelatine type A 300 bloom as well as gelatine type B 300 bloom.

In addition to the gelatine quality, the process conditions during desolvation are crucial parameters for nanoparticle formation [22]. A higher gelatine concentration promotes higher inter-molecular interactions and co-aggregation of gelatine during desolvation. As a result larger nanoparticles are formed [22]. The pH value strongly influences the net charge of gelatine. If the pH of the gelatine solution is similar to the isoelectric point (IEP), the overall net charge is insufficient and particle aggregation most likely occurs [22, 26]. However, the further away the pH value is from the IEP, the more sufficiently charged the particles are and higher intermolecular electrostatic repulsion forces prevent aggregation, but the particle size and yield decrease. If the pH is too far away from IEP, the net charge is too strong to allow desolvation and nanoparticle formation. Moreover, the solvent used for desolvation has an influence on particle characteristics. Commonly used solvents are acetone and ethanol, where acetone is the preferred desolvation agent due to

smaller particle sizes and lower PDI values [26]. Azarmi et al. could show that GNPs prepared with ethanol showed particles, which were 100 – 150 nm larger in size than GNPs prepared with acetone [26].

Two-step desolvation has become the standard preparation process for gelatine nanoparticles, but a reliable scale-up method has not yet been established [27]. It is known from human serum albumin (HSA) nanoparticles that a higher stirring efficiency during desolvation enabled large-scale preparation without a negative influence on particle size or size distribution [28]. With a paddle stirrer, a homogeneous distribution of the HSA molecules could be ensured, which was not achieved sufficiently with a stirring bar due to reduced stirring efficiency in higher volumes and irregular hydrodynamics. This principle was transferrable to GNP preparation by one-step desolvation. Furthermore, the purification process could be enhanced by ultrafiltration.

Aside from pH value and stirring efficiency, the contact area between gelatine solution and desolvation agent was defined as an important process parameter [29]. The GNP formation mainly occurs at the surface of the gelatine solution where the desolvation agent gets in contact with the gelatine molecules and causes interfacial turbulences [30]. By enlarging this area, GNP output should increase, too. The present study was conducted to evaluate if expanding this area via spreading the tubes, which are used to add acetone, over the whole gelatine solution or using a dual syringe pump system could efficiently raise GNP yield.

A completely different concept to optimise and scale GNP preparation is via nanoprecipitation [31]. For this approach two miscible solvents are required. Gelatine should be soluble in one of them (typically water) and insoluble in the other liquid (“non-solvent”, typically ethanol). The aqueous gelatine solution is slowly poured into the “non-solvent” phase containing a stabiliser, such as poloxamer 407 [31]. Due to the miscibility of the liquids a violent diffusion is observed, which causes the torn of small solvent droplets from the interface. The stabilising agent rapidly preserves these droplets until the solvent is completely spread and protein coagulation occurs [30]. Khan and Schneider have stated that

nanoprecipitation is an advantageous, rapid and easy method that enables the preparation of nanoparticles (approximately 200 - 250 nm) with unimodal size distribution [31]. The following study was performed to investigate the feasibility of nanoprecipitation for GNP scale-up.

Micro- and nanoparticles are commonly prepared through glutaraldehyde cross-linking of gelatine [32, 33]. Although glutaraldehyde is well established as a cross-linker, it represents a potential risk to humans and can cause irritations and inflammations at low concentrations [34, 35]. It is therefore essential to remove any unconsumed glutaraldehyde after particle preparation. As these systems could potentially be used for treating human diseases, more suitable and safer cross-linking agents have to be identified. Alternative cross-linking methods for GNP preparation such as genipin [36], transglutaminase [37] or glyceraldehyde [38] have previously been investigated. Nonetheless, none of these has successfully substituted glutaraldehyde as the standard cross-linking agent. In this study, we addressed whether particle stabilisation with genipin or glyceraldehyde could generate GNPs with properties comparable to those stabilised with glutaraldehyde.

Due to the increasing biological application of CpG-loaded GNPs, this study aimed to simplify the desolvation manufacturing process in order to improve reproducibility, as well as the rate of yield. A screening of factors, such as gelatine type, concentration, pH value and contact area was performed on small scales. Furthermore, nanoprecipitation was examined as a different GNP preparation method. To evaluate the effect of alternative cross-linking agents, we studied incubation time, cross-linking degree and colloidal stability.

2 MATERIALS AND METHODS

2.1 MATERIALS

Gelatine type A 300 bloom and gelatine type B 300 bloom were obtained from Gelita AG (Eberbach, Germany). Acetone was supplied by Fisher Chemicals (Loughborough, UK). Gelatine type A 175 bloom, type A 100 bloom, type B 75 bloom, glutaraldehyde (25% solution), glyceraldehyde, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), (2-Aminoethyl) trimethylammonium chloride hydrochloride (Cholamine), 2,4,6-Trinitrobenzenesulfonic acid (TNBS) and poloxamer 407 were purchased from Sigma (Taufkirchen, Germany). Genipin was acquired from Wako Chemicals GmbH (Neuss, Germany). Highly purified water (HPW), which was produced by a PURELAB Plus device (conductivity < 0.055 µS/cm, Elga Labwater, Celle, Germany), was used in all experiments.

2.2 PREPARATION OF GELATINE NANOPARTICLES

2.2.1 OPTIMISATION OF GELATINE NANOPARTICLE PREPARATION

Gelatine nanoparticles were prepared either by two-step desolvation [20] or one-step desolvation [24], as a modification of the common two-step desolvation method. In brief, an amount of 750 mg gelatine type A 300 bloom was dissolved in a volume of 25 mL of HPW under constant stirring at 50°C. The pH was adjusted to a value below the isoelectric point (IEP pH 8 – 9). In case of gelatine type B 300 bloom the pH was adjusted to a value above the isoelectric point (IEP pH 4.5 – 5.0). Acetone was then added drop-wise to the gelatine solution in order to initiate desolvation and nanoparticle formation. With respect to particle stability, a volume of 175 µL glutaraldehyde solution was added to cross-link GNPs. The dispersion was stirred overnight and purified by two-fold centrifugation (20000*g for 15 min; Sigma Laborzentrifugen, Osterode, Germany).

Varied gelatine concentrations [2.0%, 3.0%, 4.0% and 5.0% (w/v)] were investigated as well as different pH values between 2.5 - 3.0 and 6.0 – 8.0 for gelatine type A and B, respectively, at a fixed initial gelatine concentration [3.0% (w/v)].

With the aim to scale up the one-step desolvation process, the five-fold amount (3.75 g) of gelatine type A 300 bloom was used and GNP preparation was performed as mentioned above.

2.2.2 Cationisation of Gelatine Nanoparticles

Cationisation of GNPs was performed according to the standard protocol [39]. In brief, GNP dispersion was diluted with HPW (1-2 mg/mL) and pH was adjusted to a value between 4.5 and 5.0. Then, 50 mg of each 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and (2-Aminoethyl) trimethylammonium chloride hydrochloride (Cholamine) were added. The reaction mixture was incubated for 30 min and purified by two-fold centrifugation (16000*g for 15 min; Sigma Laborzentrifugen, Osterode, Germany).

2.2.3 Gelatine Nanoparticle Purification by Ultrafiltration

The GNP dispersion was purified via ultrafiltration using a solvent resistant stirred cell (Millipore S.A.S., Molsheim, France) with an ultrafiltration disc of regenerated cellulose and a molecular weight cut-off of 100’000 kDa (Millipore S.A.S., Molsheim, France). To ensure purification from acetone and residual glutaraldehyde, the filtration was repeated three times.

2.2.4 Alternative Approaches to Increase Particle Yield

Increasing the Contact Area Between Gelatine and Acetone

Gelatine nanoparticles were prepared using the standard two-step desolvation method [20]. As a modification, the way of adding acetone to induce desolvation, was changed. During the second desolvation step the contact area between gelatine

and acetone was increased by positioning the two tubes in opposite position. In a second experiment, a peristaltic pump using six tubes (MINIPLUS 3, Gilson Inc., Middleton, USA) was utilised. During the acetone addition, the tubes were placed over the whole area of the gelatine solution.

DUAL SYRINGE PUMP SYSTEM

The standard two step desolvation method [20] was modified to adopt the process to the dual syringe pump 100 DX system (Teledyne Isco, Lincoln, USA) used for e.g. spider silk particle production [40]. The first desolvation step was performed in the standard manner, which is necessary to separate the LMW fraction of gelatine. The dual syringe pump system was used to perform the second desolvation step. After discarding the LMW fraction, the gelatine sediment was redispersed in a volume of 25 mL of HPW and pH adjusted (pH 2.5 - 3.0). Subsequently, the gelatine solution was filled into one of the two syringes (max. filling volume 100 mL). The other syringe was filled with 100 mL acetone and both syringes were connected via a T-shaped mixing element. Both liquids were mixed with a flow rate ratio of 1:3 (gelatine solution: acetone). Afterwards, GNPs were cross-linked by glutaraldehyde (25%).

NANOPRECIPITATION

Nanoprecipitation was performed as described by Khan and Schneider [31]. Gelatine of different type and bloom number was dissolved in highly purified water under stirring and heating (50°C). Afterwards, the gelatine solution was added dropwise to an ethanol solution 95% (v/v) that contained poloxamer 407 as a stabiliser. Subsequently, formed GNPs were cross-linked by the addition of glutaraldehyde (25%). The exact compositions of the solutions are presented in Table II-1.

Table II-1 Different formulation compositions for preparation of GNPs by nanoprecipitation.

Formulation Composition	Gelatine type/ Bloom number	Gelatine conc. [mg/mL]	Ratio gelatine solution: ethanol	Conc. Poloxamer 407 [%] (w/v)
A	A / 175 bloom	25	1:10	7
B	A / 175 bloom	25	1:10	10
C	A / 100 bloom	25	1:10	7
D	A / 100 bloom	25	1:10	10
E	B / 75 bloom	25	1:10	7
F	B / 75 bloom	25	1:10	10
G	B / 75 bloom	20	1:10	10

2.2.5 Evaluation of Alternative Cross-Linking Agents

Plain GNPs were prepared by one-step desolvation according to the aforementioned protocol without subsequent cross-linking by glutaraldehyde. To stabilise GNPs, either glyceraldehyde or genipin were added. Different pH conditions as well as cross-linking agent concentrations were evaluated (Table II-2). After incubation, GNPs were purified by two-fold centrifugation and redispersed in HPW. GNPs were stored at 4°C and colloidal stability was tested by measuring particle size and PDI values over a period of 35 days.

Table II-2 Concentrations and pH conditions of alternative cross-linking agents. (*referred to volume of gelatine solution).

Gelatine	Cross-linking agent	pH value	Conc. cross-linking agent [mg/mL] *	Incubation time [h]
Type A	Glyceraldehyde	2.5-3	8-20	20-65
Type A	Genipin	2.5-4.5	10-30	24-48
Type B	Glyceraldehyde	6-7	10-30	19
Type B	Genipin	6	10-30	19

Scaled batches using glyceraldehyde as cross-linking agent were performed following the standard procedure of one-step desolvation with the five-fold amount of gelatine (3.75 g) and purification by ultrafiltration.

2.3 Characterisation of Gelatine Bulk Material by Asymmetric Flow Field-Flow Fractionation (AF4)

Characterisation of gelatine bulk material was performed by asymmetric field flow-field fractionation (AF4). Gelatine type A 300 bloom and gelatine type B 300 bloom were analysed. Control samples were standard gelatine type A 175 bloom, the sediment, which is obtained by the first desolvation step during two-step desolvation, as well as customised gelatine (VP413-2) that possessed less than 20% (w/w) peptides < 65 kDa. Measurements were conducted with a Wyatt Eclipse 2 system (Wyatt Technology, Dernbach Germany) combined with an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, USA) equipped with UV and RI detection and a Wyatt Dawn Eos multi-angle laser light scattering (MALS) detector. The refractive index increment dn/dc was set to 0.174 mL/g and the second virial coefficient was set to 0. The channel height was 350 µm and a regenerated cellulose membrane with 10 kDa molecular weight cut-off was applied. Phosphate buffer (2M $Na_2HPO_4 * 2\ H_2O$) pH 6.0 was chosen as running buffer. According to Schultes et al. [41] channel flow was set to 1.0 mL/min and a cross flow of 0.05 mL/min was applied. The complete measurement period was 20 minutes.

2.4 Characterisation of Gelatine Nanoparticles

2.4.1 Particle Size and Zeta Potential Measurements

Particle size and polydispersity index (PDI) were determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). Zeta potential measurements were carried out by electrophoretic light scattering with the Zetasizer Nano ZS.

2.4.2 Particle Concentration

The particle concentration was obtained via gravimetric determination using a UMX2 ultra-microbalance (Mettler Toledo, Greifensee, Switzerland).

2.4.3 Determination of Cross-Linking Degree

Cross-linking degree of GNPs was determined by TNBS assay. Briefly, an aliquot of the GNP dispersion was diluted with HPW to a certain concentration (1 mg GNPs in total volume of 250 µL). A volume of 0.25 mL of 0.05% TNBS (v/v) (Sigma Aldrich Chemie GmbH, Steinheim, Germany) and 0.25 mL of 4% $NaHCO_3$ (w/v) (pH 8.5, Sigma Aldrich Chemie GmbH, Steinheim, Germany) were added. The samples were incubated in a Thermomixer (Eppendorf, Hamburg, Germany) for 2 hours under constant shaking (500 rpm) at 40°C. A volume of 750 µL of HCl 6M was then added to each sample, which were further incubated for 90 min at 60°C under constant shaking at 500 rpm. Subsequently, specimens were diluted with HPW for photometric determination of the reaction product at 349 nm (Agilent 8453 UV-visible sprectrophotometer, Agilent Technologies, Santa Clara, CA, USA). Blank samples of gelatine (≙ 0% cross-linking) and control samples of gelatine (≙ 100% cross-linking) were prepared. The control samples were treated as the specimens except that HCl was added prior to the TNBS solution to avoid the reaction between TNBS and free amino groups of gelatine. Cross-linking degree was determined by the following equation:

$$CL\ [\%] = \left(1 - \frac{A(sample) - A(blank)}{A(control) - A(blank)}\right) * 100\%$$

2.4.4 Scanning Electron Microscopy (SEM)

Gelatine nanoparticles were freeze dried according to the protocol of Zillies et al. [42] and immobilised on an aluminium sample grid. Samples were carbon sputtered under vacuum and analysed by a Helios NanoLab G3 UC scanning electron microscope (FEI, Hillsboro, Oregon, USA) at 2.0 kV and a working distance of 4.0 – 4.2 mm.

2.5 STATISTICAL EVALUATION

Data were analysed for difference in particle yields between standard and scaled batches using a paired t-test performed by SigmaPlot 12.5 (Systat Software Inc., Erkrath, Germany).

3 RESULTS

The objective of the present study was to optimise the manufacturing procedure for gelatine-based nanoparticles with the main focus on method robustness and overall particle yield. Here we present an enhanced and scalable gelatine nanoparticle preparation process using a commercially available gelatine in combination with a paddle stirring system (Figure II-1).

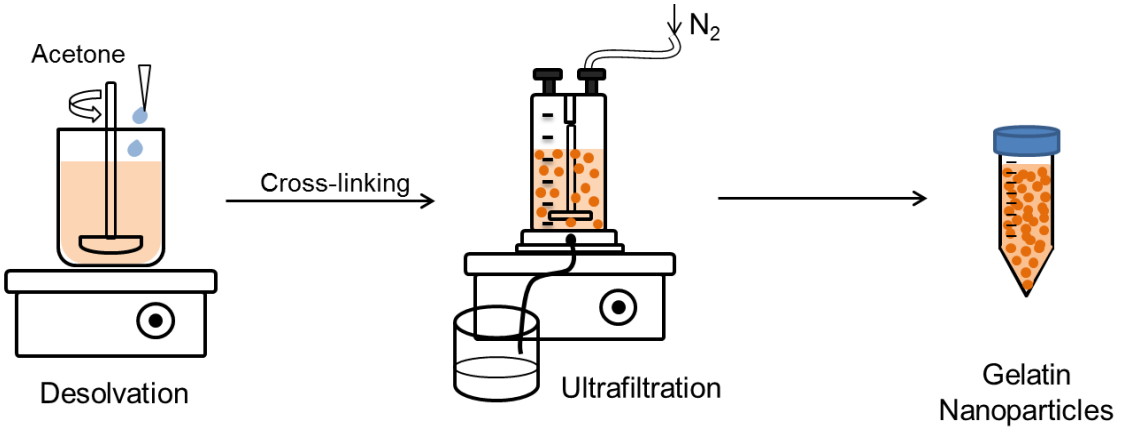

Figure II-1 Schematic representation of the optimised gelatine nanoparticle preparation process and scale-up.

3.1 PREPARATION OF GELATINE NANOPARTICLES BY ONE-STEP DESOLVATION

3.1.1 EFFECT OF GELATINE CONCENTRATION AND PH VALUE

TYPE A 300 BLOOM

As reported in a previous study [22], the initial gelatine concentration and the pH value during desolvation with acetone are crucial parameters for nanoparticle formation. Here, we screened various gelatine concentrations and a range of pH values in order to define optimal conditions required for particle formation during one-step desolvation using gelatine type A.

The different gelatine amounts and their effect on particle size and yields are shown in Figure II-2. The particles obtained had diameters between 150 – 300 nm, with a uniform size distribution (PDI < 0.15). The gelatine concentration affected the

particle size, whereby a higher input led to an increase in measured diameter. An initial increase in yield was observed with increasing gelatine concentrations; however, the percentage decreased with 4% and 5% of gelatine. In comparison to the two-step desolvation (yield ca. 1.5%), all batches showed higher particle yields.

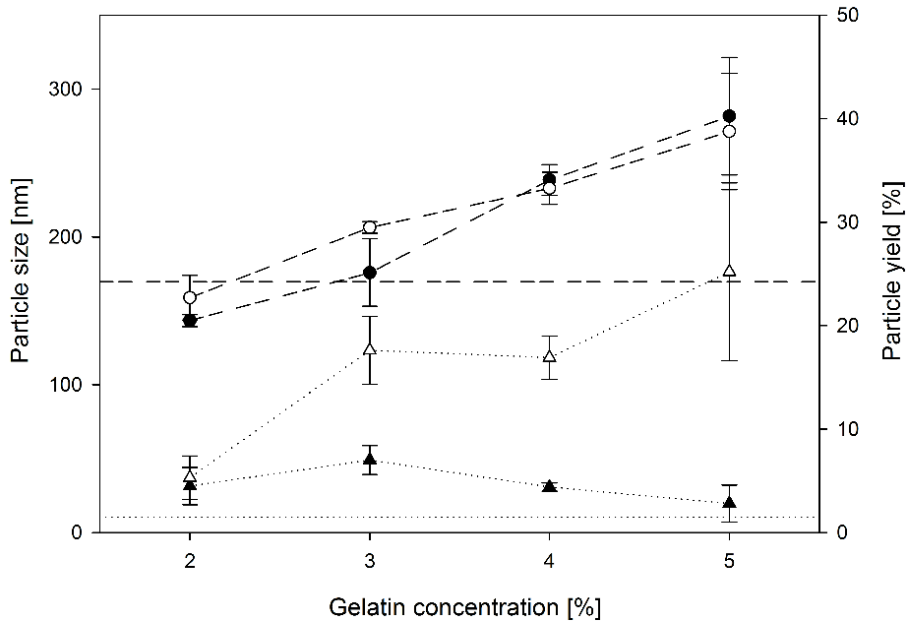

Figure II-2 Particle characteristics of GNPs prepared by one-step desolvation. Effect of concentrations on size and yield for gelatine type A 300 bloom (particle size: black dots, relative particle yield: black triangles) and type B 300 bloom (particle size: white dots, relative particle yield: white triangles) compared to two-step desolvation (particle size: dashed line, relative particle yield: dotted line). Data is presented as mean ± SD (n=3).

To ensure that particles were formed with the pH conditions used for two-step desolvation, a range of pH 2.5-3.0 was tested during the desolvation process. At any value investigated, particles were obtained, which met the required criteria based on the results of common two-step desolvation (Figure II-3). This includes particle sizes between 150 – 200 nm and PDI values below 0.2. Thus, by using a gelatine type with 300 bloom, successful one-step desolvation can be performed without the initial drawback of a broad size distribution.

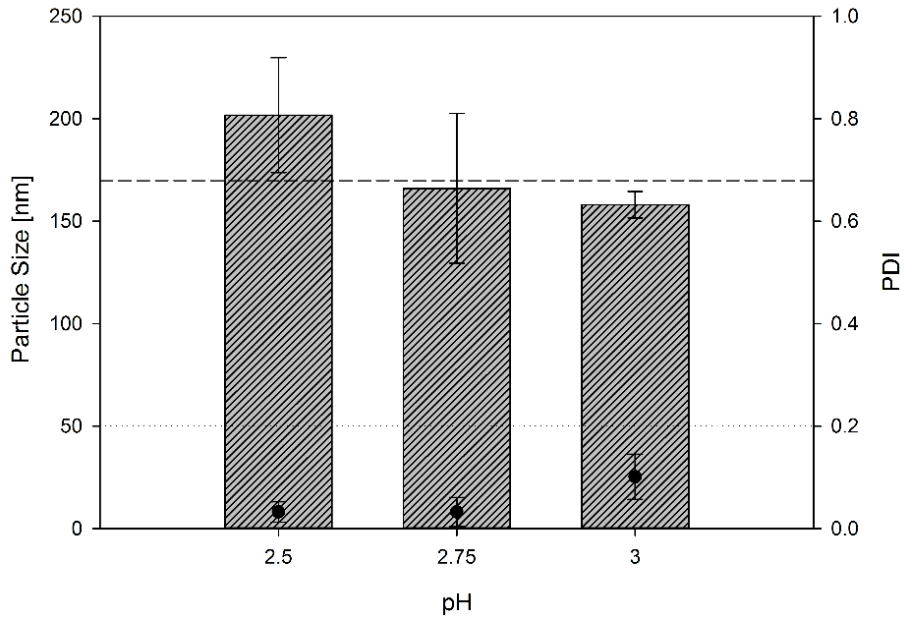

Figure II-3 Particle sizes (bars) and PDI values (dots) of GNPs prepared by one-step desolvation of gelatine type A 300 bloom at different pH values compared to two-step desolvation (particle size: dashed line, upper limit PDI value: dotted line). Data is represented as mean ± SD (n=3):

TYPE B 300 BLOOM

In the interest of producing GNPs with alternative particle characteristics, such as a negative surface charge, the one-step desolvation process was adapted to gelatine type B 300 bloom. Gelatine type B has an IEP of 4.7 – 5.6 [43] and thus leads to the formation of negatively charged particles at pH value between 6.0 – 8.0. Again, different initial gelatine concentrations [2-5% (w/v)] and pH values beyond the IEP were evaluated (pH 4.7 – 5.6). Similar to gelatine type A 300 bloom, the particle diameter became larger with increased initial gelatine amount (Figure II-2). No effect was observed on the homogeneity of the samples and all GNP batches showed uniform size distribution (PDI < 0.15). In contrast to gelatine type A 300 bloom, higher initial concentrations of type B resulted in higher particle yields.

For further experiments, a gelatine concentration of 3% was chosen for both gelatine types due to acceptable particle yields combined with adequate particle size and PDI value.

The evaluation of different pH values during desolvation showed smaller particle sizes as well as decreasing particle yields with increasing pH (Figure II-4). The further away the pH value was from the IEP, the higher the net charge of the gelatine molecules was. This results in stronger intermolecular electrostatic repulsion forces, which hinder the inter-molecular co-aggregation and thus particles with smaller diameter are formed.

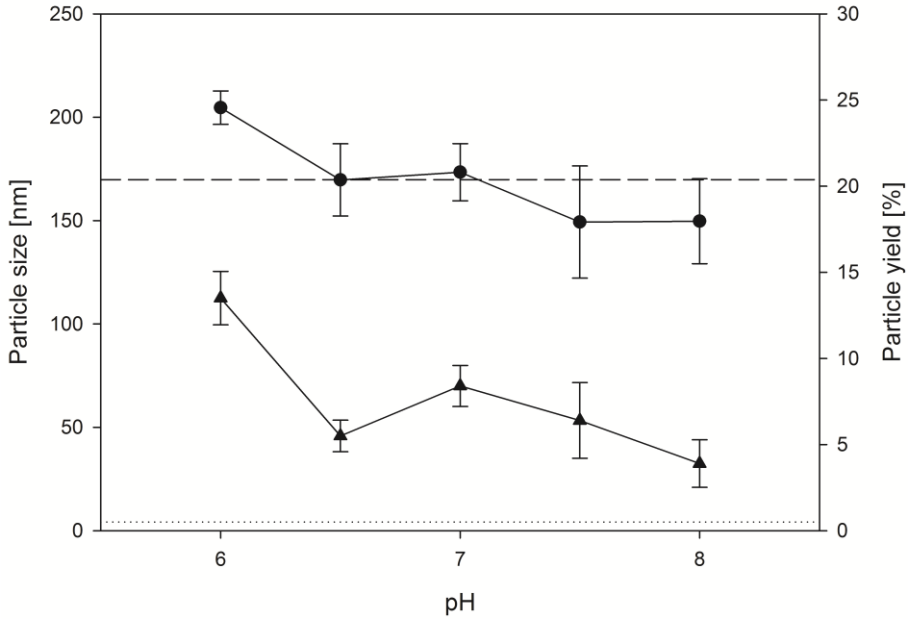

Figure II-4 Particle characteristics of GNPs prepared by one-step desolvation. Particle size (dots) and relative particle yield (triangles) of GNPs prepared at different pH values by one-step desolvation from gelatine type B 300 bloom compared to two-step desolvation (particle size: dashed line, relative particle yield: dotted line). Data is presented as mean ± SD (n=3).

3.1.2 Mean Molecular Weight of Gelatine Base Material

The different types of gelatine starting material were analysed by AF4/MALS to determine their molecular weight distributions. The samples suitable for one-step desolvation (A 300 bloom and B 300 bloom) were compared to the standard gelatine type A 175 bloom, its sediment, as well as customised gelatine VP413-2 with a reduced LMW fraction. The aim of this study was to identify a range of molecular weights where GNP preparation by one-step desolvation is possible. Compared to

the customised gelatine batch VP413-2 (ca. 700 kDa), the standard gelatine A 175 bloom (ca. 300 kDa) showed a lower mean molecular weight (Figure II-5). The molecular weight of the sediment was found to be around 400 – 500 kDa and the distribution of the gelatine qualities used for the one-step approach were comparable to that of the sediment. This demonstrated that a slight shift to a higher mean molecular weight was sufficient to enable one-step desolvation instead of two-step desolvation.

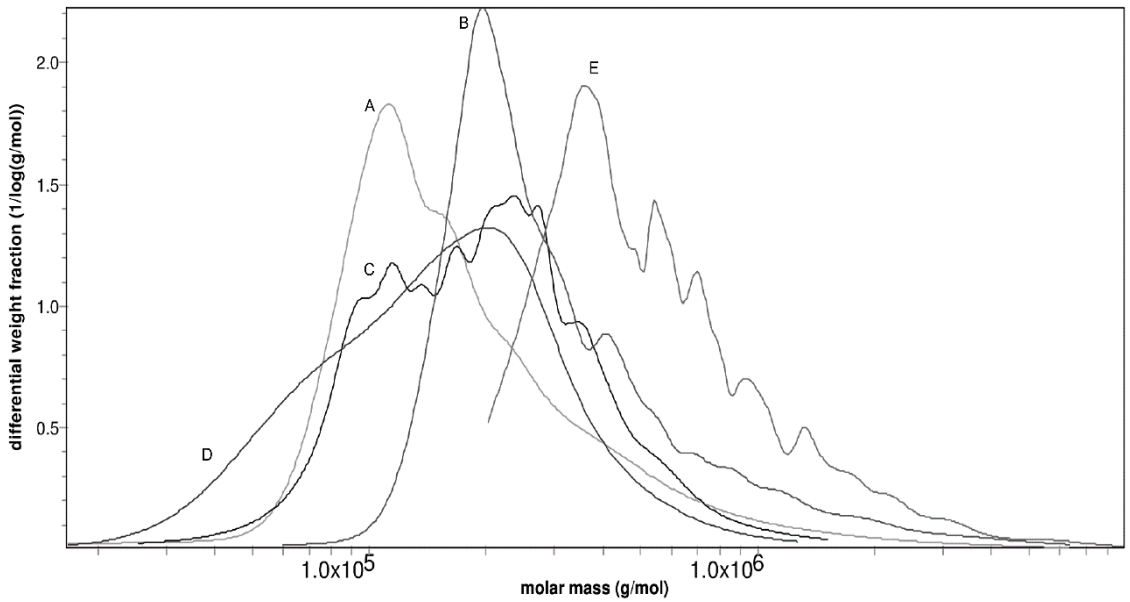

Figure II-5 Differences in molecular weight distribution of the various gelatine types and the sediment: Gelatine type A 175 bloom (A), sediment of gelatine type A 175 bloom after first desolvation (B), gelatine type A 300 bloom (C), gelatine type B 300 bloom (D), customised gelatine VP413–2 (E).

3.2 SCALE-UP OF DESOLVATION PROCESS

In addition to the optimisation of the GNP preparation process, scale-up was a central focus of this work. Attempts to maximize the production capacity of the two-step desolvation have met major obstacles, such as reduced efficiency of the process. We were able to successfully enlarge the overall yield of the GNP preparation process by combining the one-step desolvation method with a paddle stirrer system that provided a tailored mixing intensity and thus more control over the mixing

efficiency (Figure II-6). Application of a five-fold initial gelatine amount (type A 300 bloom) yielded 388.6 ± 53.3 mg per batch as opposed to 50-60 mg achieved with the standard batch size for one-step desolvation. Measured particle diameters were 185.2 ± 32.6 nm (PDI 0.070 ± 0,050) and therefore met the requirements.

To increase the particle yield further and also to lower the particle loss during purification by centrifugation, ultrafiltration was performed. The purification of GNP dispersions using an ultrafiltration cell enabled the preparation of GNPs with a particle size of 120.4 nm ± 5.0 nm with a homogeneous size distribution (PDI 0.076 ± 0.014). Highly concentrated GNP dispersions were achieved with a particle yield of 69 – 83%, referring to 587.5 mg ± 58.4 mg GNPs (standard batch size). The combination of the scaled batch size and purification with the ultrafiltration cell significantly increased the yield to 2627 mg ± 163.8 mg, corresponding to ca. 70% (Figure II-6, $p < 0.001$). Taken together, with a 130-fold overall particle gain compared to two-step desolvation, this advanced methodology provides GNPs in high availability with reproducible product quality.

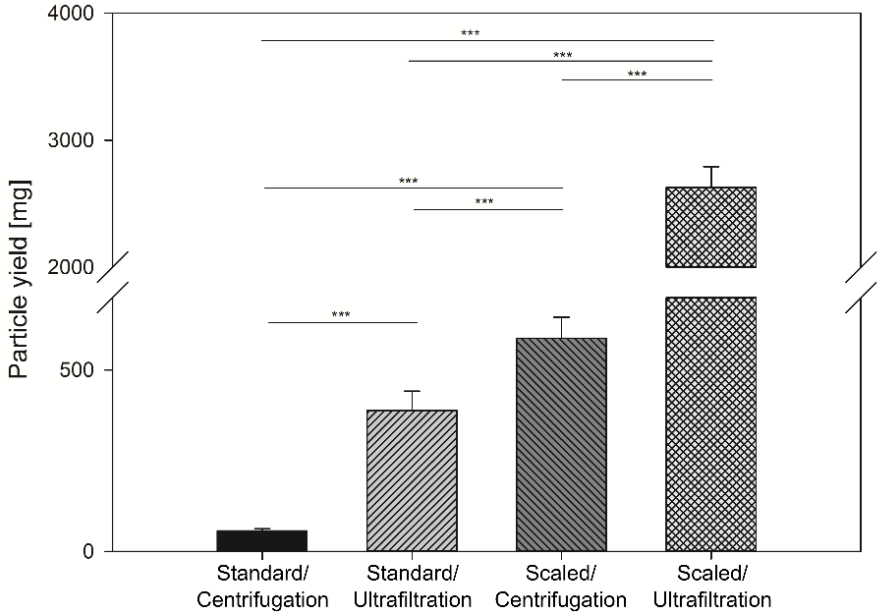

Figure II-6 Particle yields of GNPs prepared by one-step desolvation from gelatine type A 300 bloom. Comparison of standard batch size and purification by centrifugation (black bar), scaled batch size and purification by centrifugation (light grey striped bar), standard batch size and purification by ultrafiltration (dark grey striped bar) and scaled batch size and purification by ultrafiltration (light grey chequered bar). Data is presented as mean + SD (n=3). *** $p < 0.001$.

3.3 SURFACE PROPERTIES OF GNPS

The overall surface charge of gelatine nanoparticles prepared by two- or one-step desolvation, different preparation process and types of gelatine (A 300 bloom and B 300 bloom) were investigated using electrophoretic light scattering (Figure II-7). These measurements enabled comparison of the surface properties of the different GNP batches from gelatine type A prepared by two-step or one-step desolvation. Through cationisation, the zeta potential of the particles can be increased by at least 5 mV. Interestingly, the scaled one-step desolvation batches (gelatine type A 300 bloom) showed the highest zeta potential before and after cationisation. In contrast, GNPs from gelatine type B 300 bloom showed negative surface charge due to the pH value beyond the IEP during particle formation. Nevertheless, the standard cationisation process generated a permanent positive surface charge on GNPs from

gelatine type B, which is comparable to the zeta potential of cationised GNPs from gelatine type A.

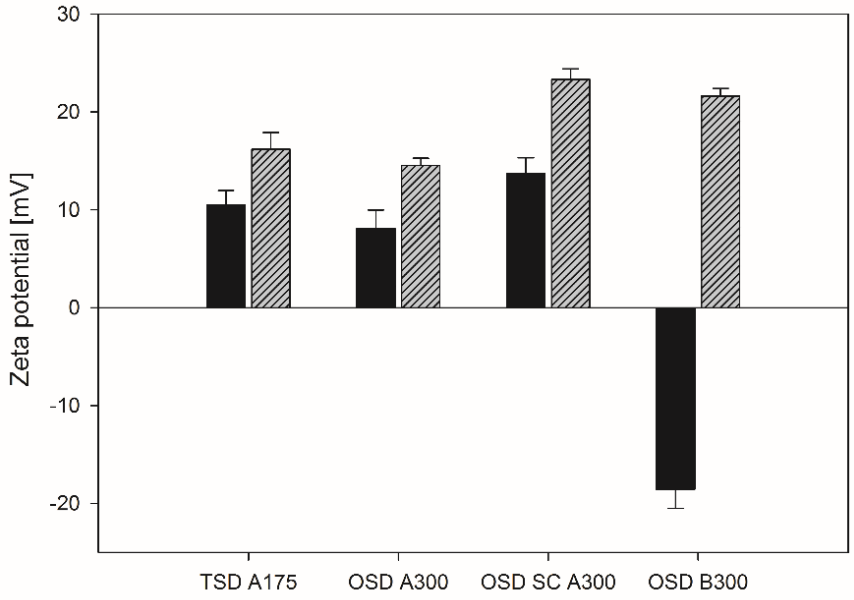

Figure II-7 Zeta potential of non-cationised (black bars) and cationised (grey striped bars) GNPs prepared by two-step desolvation from gelatine type A 175 bloom (TSD A175), one-step desolvation from gelatine type A 300 bloom (OSD A300), large scale one-step desolvation from gelatine type A 300 bloom (OSD SC A300) and one-step desolvation from gelatine type B 300 bloom (OSD B300). Data is presented as mean ± SD (n=3).

3.4 Alternative Approaches to Increase Particle Yield in Two-Step Desolvation

3.4.1 Increasing the Contact Area Between Gelatine and Acetone

Besides the importance of gelatine quality and pH value, it was stated that the area where acetone is added to the gelatine solution during the second desolvation step is crucial [29]. For that reason, two different approaches were conducted to increase the contact area between acetone and gelatine solution: Firstly, the two tubes for acetone addition were placed in the opposite position or even six tubes were used and evenly distributed above the gelatine solution area. Secondly, the contact area between the two solutions was maximized by using a dual syringe pump system for the second desolvation step. In this technique, the two liquids are filled into two

identical syringes, which are connected via a T-shaped mixing element. The precipitation of the nanoparticles occurs in this mixing element.
Results of these experiments are shown in Figure II-8. Keeping apart the tubes for acetone addition had no negative influence on particle characteristics, as particle sizes and PDI values did not differ from GNPs prepared by the standard method (188.5 ± 25.9 nm vs. 172.2 ± 19.0 nm and 0.077 ± 0.024 vs. 0.079 ± 0.043). However, there was no beneficial effect on particle yield (1.3 ± 0.27% vs. 1.3 ± 0.35%). By using six tubes and thus further enlarging the contact area, no trend to higher particle yields (1.4 ± 0.50%) could be found. Nevertheless, this further expansion of contact area correlated with increasing particle sizes (207.8 ± 61.2 nm) and PDI values (0.143 ± 0.05), plus broadening of standard deviations. Based on these results, it can be assumed that the increase of the acetone contact area by a higher number of tubes would be not an option for escalation of GNP yields in common two-step desolvation.

The maximisation of the contact area between gelatine solution and acetone by using the dual syringe pump system led to 50% higher particle output (2.2 ± 0.52%). Furthermore, this method seems to be appropriate for the preparation of GNPs with smaller particle sizes (150.1 ± 12.7 nm). Nonetheless, by making use of this benefit in production quantity, one needs to accept a reduction in monodispersity of the GNPs (PDI 0.143 ± 0.075) compared to the standard method by using a peristaltic pump for acetone addition.

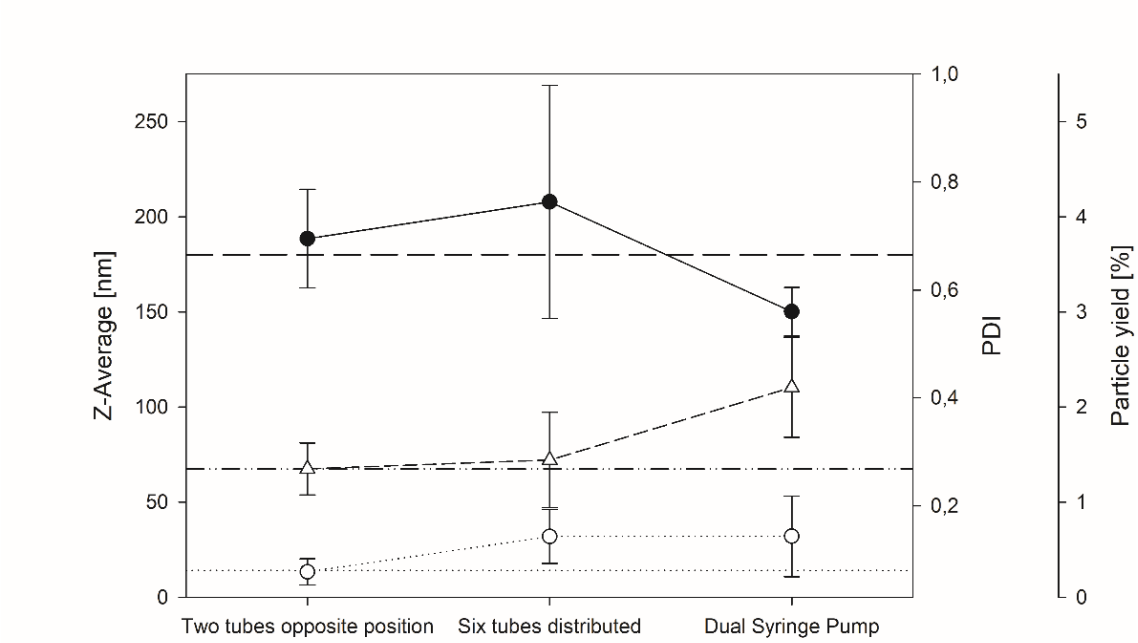

Figure II-8 Particle characteristics of GNPs prepared by two-step desolvation with either two acetone tubes in opposite position, six tubes evenly distributed or using a dual syringe pump (particle size: black dots, PDI value: white dots, relative particle yield: white triangles) compared to standard two-step desolvation (particle size: dashed line, PDI value dotted line, relative particle yield: dashed/dotted line). Data is presented as mean ± SD (n=3).

3.4.2 NANOPRECIPITATION

As a completely different approach, nanoprecipitation was investigated as option for scaling GNP preparation. Different types of gelatine, gelatine concentrations and stabiliser concentrations were screened in order to find appropriate conditions.
Results are displayed in Figure II-9. Interestingly, original conditions of gelatine type B 75 and 7-10% stabiliser as used by Khan and Schneider [31] led to very large and inhomogeneous GNPs with particle sizes between 443.4 ± 43.4 nm and 649.0 ± 226.8 nm and PDI values between 0.424 ± 0.032 and 0.672 ± 0.155 (formulation compositions E-G). By using gelatine type A with a similar bloom number (100 bloom), hardly any change in particle size could be achieved (358 ± 87.4 nm and 443.8 ± 8.76 nm), but PDI values decreased to values between 0.298 ± 0.097 and 0.330 ± 0.035 (formulation compositions C-D). The best results were achieved with the standard two-step desolvation gelatine type A 175

bloom (formulation compositions A-B). Particle sizes ranged between 293.0 ± 12.9 nm and 328.7 ± 36.9 nm with corresponding PDI values of 0.272 ± 0.049 and 0.355 ± 0.085. Nonetheless, GNP characteristics were still far from those prepared by two-step desolvation (see reference lines in Figure II-9). However, nanoprecipitation enabled much higher particle yields than two-step desolvation. Relative outcomes varied between 13.4 ± 6.5% and 33.9 ± 10.4%.

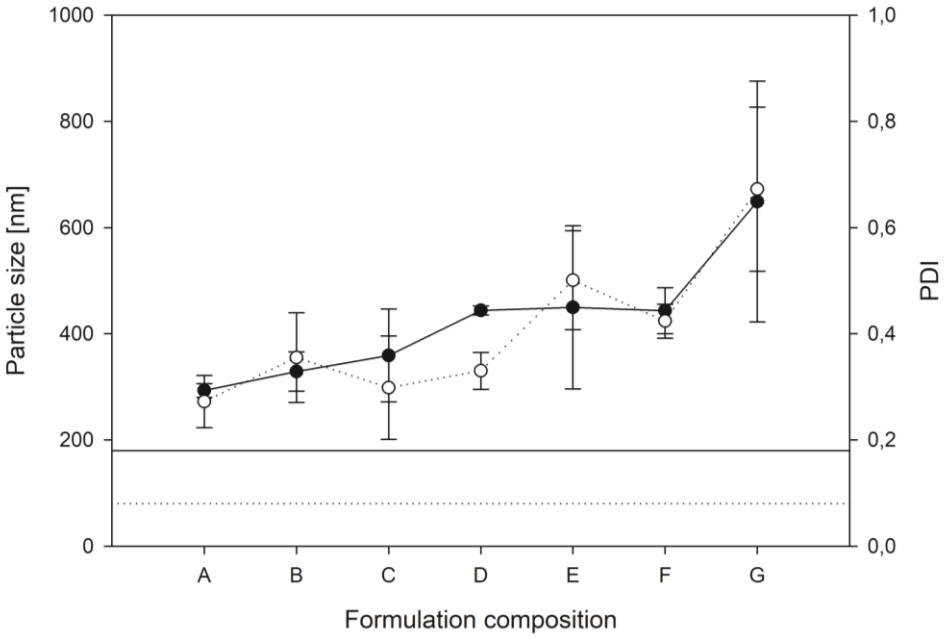

Figure II-9 Particle characteristics of GNPs prepared by nanoprecipitation (particle size: black dots, PDI value: white dots, compared to standard two-step desolvation (particle size: solid line, PDI value: dotted line) A: Gelatine A 175, 25 mg/ml, 7% stabiliser; B: Gelatine A 175, 25 mg/ml, 10% stabiliser; C: Gelatine A 100, 25 mg/ml, 7% stabiliser; D: Gelatine A 100, 25 mg/ml, 10% stabiliser; E: Gelatine B 75, 25 mg/ml, 7% stabiliser; F: Gelatine B 75, 25 mg/ml, 10% stabiliser, G: Gelatine B 75, 20 mg/ml, 10% stabiliser. Data is presented as mean ± SD (n=3).

3.5 EVALUATION OF ALTERNATIVE CROSS-LINKING AGENTS

3.5.1 GLYCERALDEHYDE

Glyceraldehyde is commonly used to increase the mechanical strength of the sclera via cross-linking collagen [44]. Here we applied glyceraldehyde for cross-linking of gelatine nanoparticles in order to substitute the commonly used glutaraldehyde. Glyceraldehyde was evaluated for its ability to cross-link GNPs made from either type A 300 bloom or type B 300 bloom. Various conditions, such as concentration of cross-linking agent and incubation time, were screened. Table II-3 Results of cross-linking (CL) GNPs from different types of gelatine (type A 300 bloom and type B 300 bloom) with glyceraldehyde and genipin (n=3) summarises the parameters that were examined for the preparation of stable particles, as well as the resulting particle characteristics. An extended cross-linking time of 65 hours was necessary to stabilise the nanoparticles from type A 300 bloom, compared to 15 hours required for glutaraldehyde. Additionally, particle sizes and PDI values strongly increased. Only a glyceraldehyde concentration of 16 mg/mL gave GNPs with acceptable characteristics; however, this forfeited the particle yield.

In comparison to the standard reagent glutaraldehyde, similar particle characteristics were achieved when GNPs made from type B 300 bloom were cross-linked with glyceraldehyde (Table II-3). Stable and monodisperse GNPs in a particle size range of 200 – 250 nm with high cross-linking degree and particle yield were prepared.

Table II-3 Results of cross-linking (CL) GNPs from different types of gelatine (type A 300 bloom and type B 300 bloom) with glyceraldehyde and genipin (n=3).

Gelatin Type	CL agent	CL agent [mg/mL]	Incuba-tion [h]	Particle size [nm]	PDI	CL degree [%]	Yield [%]	Colloidal stability
A300	Glutaraldehyde	1.75	15	≈ 200	< 0.2	≈ 85	≈ 10	yes
B300	Glutaraldehyde	1.75	15	150 - 200	< 0.15	≈ 85	≈ 15	yes
A300	Glyceraldehyde	16	65	300 - 350	< 0.2	≈ 40	≈ 2.5	yes
B300	Glyceraldehyde	20	19	200 - 250	< 0.1	≈ 75	≈ 20	Yes
A300	Genipin	10 - 30	24 - 48	gelation	---	---	---	---
B300	Genipin	20	19	280 - 370	< 0.2	≈ 40	≈ 15	no

In scale-up experiments, it could be shown that glyceraldehyde is suitable for large scale production of GNPs (Figure II-10). Using a five-fold amount of gelatine to produce particles combined with ultrafiltration gave similar particle sizes and PDI values to the standard procedure (200-250 nm, PDI < 0.15). A considerable increase in particle yield was obtained (2517 mg ± 411.8 mg vs. 112 mg ± 30 mg).

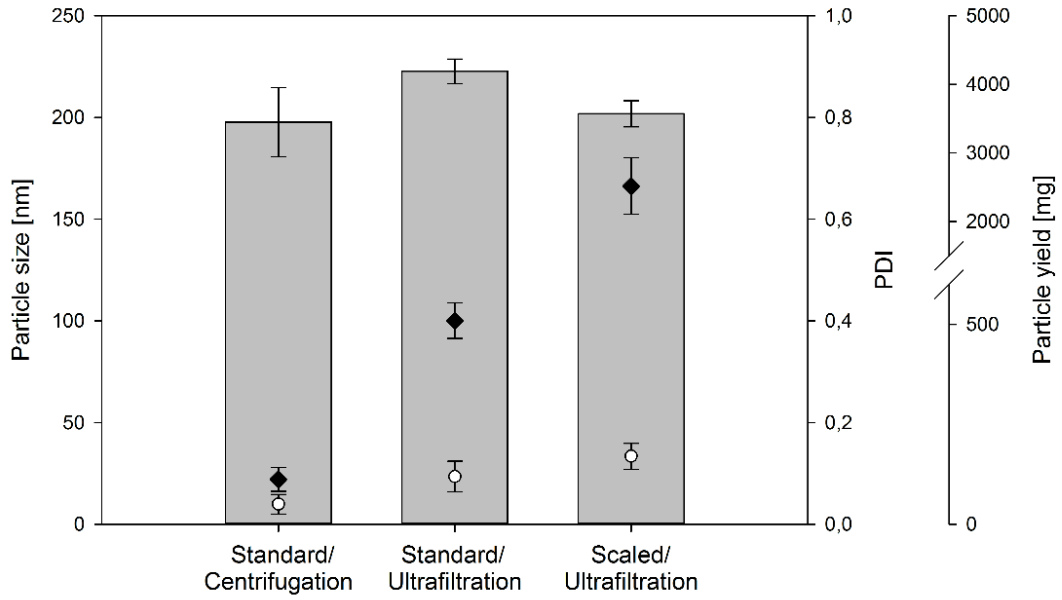

Figure II-10 Preparation of GNPs in large scale using glyceraldehyde. Comparison of particle size (bars), PDI value (white dots) and particle yield (black dots) of a standard batch purified by centrifugation or ultrafiltration, and a scaled batch size purified by ultrafiltration. GNPs were prepared using gelatine type B 300 bloom. Data is presented as mean ± SD (n=3).

3.5.2 GENIPIN

In addition to glyceraldehyde, the naturally occurring cross-linking agent genipin was evaluated for its suitability to stabilise GNPs (Table II-3). In case of gelatine type A, no stable GNPs were obtained with the various parameters studied. Incubation of GNPs with genipin over a maximum of 48 hours led to gel formation. On the other hand, genipin enabled the preparation of monodisperse GNPs based on type B in a particle size range between 280 – 370 nm. In comparison to glutaraldehyde (ca. 85%) or glyceraldehyde (ca. 75%), these particles showed a decrease in the degree of cross-linking (ca. 40%), resulting in reduced colloidal stability. Further increase of the genipin concentration or the incubation time led to gel formation. Consequently, scale-up experiments with GNPs cross-linked by genipin were not performed.

3.6 EVALUATION OF DIFFERENT TYPES OF GNPS BY SEM

To visualise the different types of GNPs and analyse their morphology SEM was performed. In the micrographs, all GNPs appeared to be smooth particles with a spherical shape (Figure II-11). With respect to the size, the particle diameters obtained with SEM differed by approximately 100 nm from the sizes recorded with DLS. This was expected as the freeze-drying process caused a modest shrinking of the particles. Furthermore, in contrast to SEM, which determines the particle diameter in a dry state, DLS measures the hydrodynamic radius of a nanoparticle [45].

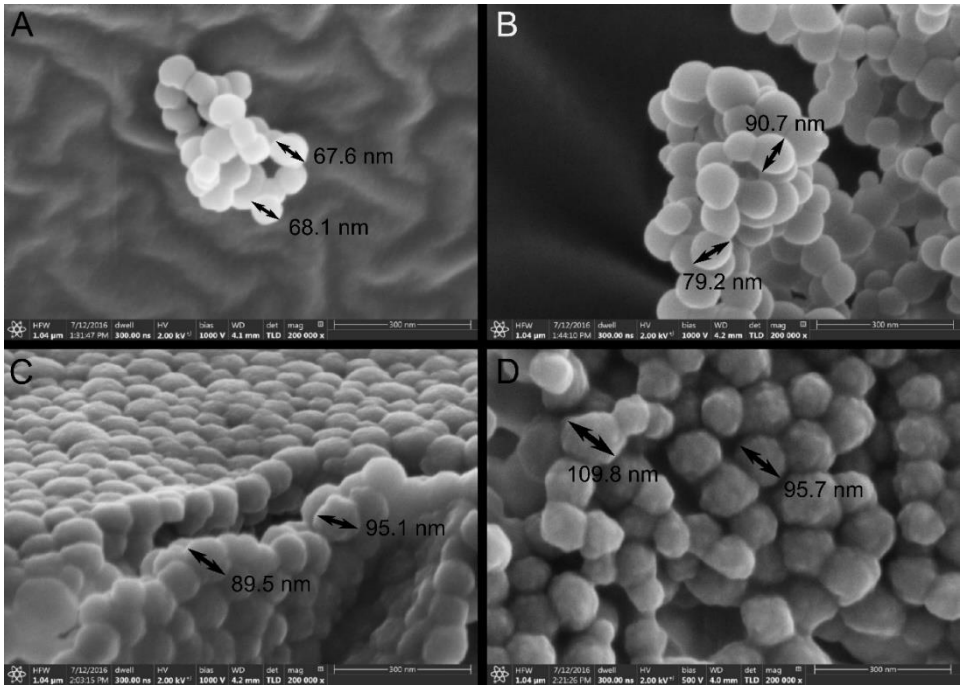

Figure II-11 SEM images of GNPs prepared by (A) two-step desolvation using gelatine type A175, (B) one-step desolvation using gelatine type A300, (C) one-step desolvation using gelatine type B300. These formulations were stabilised with glutaraldehyde. An image of GNPs prepared by (D) one-step desolvation using gelatine type B300, in which the particles were stabilised with glyceraldehyde, was added for comparison.

4 DISCUSSION

The purpose of this study was to improve the commonly used two-step desolvation for GNP preparation and to develop a straightforward and reproducible protocol. This, we hoped would allow us to provide a toolbox to establish large-scale processes. By eliminating the first unreliable desolvation step, as well as introducing new process parameters and purification techniques, we were able to scale the procedure from 15-20 mg particle yield with the standard two-step desolvation to a maximum output of 2.6 g GNPs with one-step desolvation. Moreover, further approaches were investigated for their potential to scale common two-step desolvation. This included an enlarged contact area between gelatine and acetone as well as nanoprecipitation. Furthermore, two alternative cross-linking agents were evaluated to substitute the critical substance glutaraldehyde.

4.1 PREPARATION OF GELATINE NANOPARTICLES BY ONE-STEP DESOLVATION

In the interest of circumventing the irreproducible first desolvation step, a one-step desolvation method has previously been developed, which uses a customised gelatine type A (VP413-2, reduced LMW fraction) [24]. As this gelatine is not regularly available, there was a need to establish a one-step desolvation process with a standard gelatine. Significant contributions towards achieving this were made by Ofokansi et al. [25], who successfully prepared GNPs from gelatine type B 225 bloom applying ethanol as the desolvation agent. However, this method was accompanied by several incubation steps and a strong effect of pH on particle sizes. Despite those efforts, none of the methods has been proven to be feasible. Towards this aim, we were able to successfully establish a robust and straightforward one-step desolvation method with two commercially available gelatine types (type A and B 300 bloom).

To identify optimal conditions, GNP preparations were performed with different initial gelatine concentrations. Interestingly, with increasing gelatine concentrations, particle sizes of GNPs also increased. This effect has previously been

shown by Zwiorek et al. [22], where a higher amount of the gelatine sediment resulted in larger nanoparticles during a two-step desolvation. This may be caused by a denser packing of gelatine molecules during desolvation, which promotes inter-molecular interactions and co-aggregation of gelatine, resulting in larger particle sizes. However, in our study, all nanoparticles made from both gelatine types showed diameters between 143.4–281.7 nm and were therefore acceptable for our purposes. The similar sizes and shapes of GNPs prepared by one-step or two-step desolvation were additionally verified by SEM.

Furthermore, particle yields obtained from one-step desolvation were significantly ($p < 0.001$) higher when compared to two-step desolvation. This is most likely due to the subjectivity of the first of two desolvation steps, in, which the amount of the HMW fraction (sediment) is determined visually and the supernatant discarded manually. This led to an uncontrolled loss of starting material and extensive between- and within-person variations. By circumventing this step, the entire particle preparation can be conducted in a more controlled and reproducible manner. A further increase in yield was achieved with gelatine type B. The initial pH value of 6 of this solution was found to be optimal for particle preparation and thus pH adaption was not required.

With respect to the optimal pH during particle production, in a solution of type B the pH value can be much closer to its IEP compared to type A. Thus, the lower overall net charge of the gelatine molecules led to decreased repulsion forces and stronger inter-molecular interaction resulting in larger particles with a higher yield. Nevertheless, the lower net charge is strong enough to prevent aggregation. This hypothesis is supported by the observation of lower particle yields when pH values were increased or decreased for gelatine type B and gelatine type A, respectively.

Due to the highest particle output with the required parameters and morphology, an initial gelatine solution of 3.0% (w/v) was chosen to be optimal for one-step desolvation with both gelatine qualities.

The analysis of the fractionation experiment provided insight into the molecular weight distribution of several gelatine samples and may help to understand, which

properties are required for successful particle formation. Gelatine type A 175 is a mixture of HMW and LMW fractions, whereby the relatively high content of the latter led to the formation of large particles with a broad size distribution, making it unsuitable for one-step desolvation. On the other hand, the customised gelatine (VP413-2) with a mean MW of 700 kDa has previously been shown to produce particles due to its low LMW fraction (< 20%) [24]. However, the mean MW of this gelatine, as measured by Schultes et al. [41], was lower than the mean MW determined in our study. This higher mean MW may be explained by self-cross-linking during storage of VP413-2, a phenomenon known from gelatine capsules [46]. Furthermore, Schultes et al. showed a mean MW of the sediment that was by one order of magnitude higher than in our measurements. This confirmed the issue of batch-to-batch variability of the first desolvation step. Based on their findings, they defined a mean molecular weight of ~400 – 500 kDa as the threshold for the one-step desolvation [41], which is in the range of the mean MW of gelatine type A and B 300 bloom. In conclusion, the HMW fraction included in an overall MW of 400 – 500 kDa is sufficient to prepare stable GNPs, whereas the LMW fraction is low enough to not affect GNP preparation and colloidal stability.

Consistent with the results of Ahlers et al. [24], the one-step desolvation with type A 300 bloom was successfully performed over the complete pH range used in two-step desolvation (pH 2.5 – 3.0). On the other hand, type B 300 bloom had an optimal pH value of 6.0. Although, GNPs from gelatine type B show an overall negative surface charge, we were able to permanently cationise the particles via the standard cationisation process. The cationisation reagents react with free carboxyl groups, free amine groups as well as glutaraldehyde residues [22]. Zeta potential values measured for gelatine type B were comparable to those of type A, indicating that the free functional groups on the surface of GNPs from gelatine type B are similar to those from type A. GNPs from either gelatine type A or gelatine type B are suitable for cationisation and for electrostatic loading of CpG ODNs onto their surface (loading efficiency > 95%).

4.2 Scale-Up of GNP Preparation and Ultrafiltration

Here, we demonstrated that the large-scale production of GNPs by one-step desolvation can be achieved via an increase in stirring intensity to ensure homogenous distribution of the gelatine molecules during desolvation. In a similar fashion, Wacker et al. [28] showed that a stirring bar and a small paddle stirrer (21 x 16 mm) are inappropriate for the preparation of HSA particles due to ineffective homogenisation of large volumes of albumin solutions and greater variability. By contrast, the usage of a larger paddle stirrer (30 x 25 mm) ensured homogeneous protein distribution and allowed scale-up in a reproducible manner.
Furthermore, by employing ultrafiltration to remove acetone and unreacted glutaraldehyde, the high particle loss and the low product outcome seen with centrifugation and redispersion could be overcome [47]. Here, we demonstrated an efficient way to apply stirred ultrafiltration cells, which are commonly used for protein concentration and purification [48]. Through the combination of a pressure-driven membrane process and gentle stirring, the proportion of particle loss was decreased remarkably and, as a result, the yield improved by 60-70%. This study reports, for the first time, the possibility for a large-scale production of GNPs in gram ranges by linking a maximised one-step desolvation process with ultrafiltration.

4.3 Alternative Approaches to Increase Particle Yield in Two-Step Desolvation

4.3.1 Increasing The Contact Area Between Gelatine and Acetone

Besides the simplification of GNP preparation, it was also followed the approach to optimise the standard two-step desolvation to enlarge GNP yield. It was stated that an increasing contact area between gelatine solution and desolvation agent could result in a higher particle amount [29]. Based on the assumption that GNPs are only formed at the liquid-liquid interface due to interfacial turbulences, when acetone gets in contact with the gelatine molecules [30], acetone should be added to the gelatine solution in a more distributed way. By spreading the acetone over a larger area, more gelatine molecules should be desolvated, resulting in an increasing

number of particles. An initial approach in this direction was the separation of the acetone addition tubes during the second desolvation step. As this did not improve in particle yield, the acetone addition area was further increased by six tubes evenly distributed above the gelatine solution. However, no increase in GNP amount could be observed, but a trend to larger and more inhomogeneous GNPs. This may be explained by the fact that a larger amount of gelatine gets in contact with a reduced amount of acetone compared to the standard method. This results in a slowed down desolvation process and an apparently higher gelatine density. Consequently, inter-molecular interactions are enhanced and larger and more polydisperse particles can be formed, but overall yield does not increase [22]. This could be probably circumvented by an accelerated pump rate of acetone. However, this approach was not further pursued.

Instead, a dual syringe pump system was tested, which is an established method for the preparation of spider silk particles [40]. This technique allows a maximization of contact area between protein solution and desolvation agent, as well as a more controllable pump rate and contact time compared to a peristaltic pump. These features enabled the preparation of GNPs in a more reproducible size and extended particle yield. However, PDI values were still elevated compared to standard procedure. This may be due to higher shearing forces in the T-shaped mixing element leading to more irregularities. Further optimisation could solve this issue, but this technique has not been further pursued due to limited filling volume of the syringes of the used system. By using a tailored system, this method could be applicable for continuous manufacturing of GNPs combined with one-step desolvation.

4.3.2 NANOPRECIPITATION

Another concept to facilitate GNP preparation is nanoprecipitation. According to Khan and Schneider nanoprecipitation is rapid, easy and straightforward [31]. In this technique an aqueous gelatine solution is added dropwise to a desolvating agent that contains a stabiliser. Consequently, nanoparticles are formed and stabilised.

The main postulated advantage of this preparation method is that only one step is necessary to form stable and uniform GNPs. Furthermore, in contrast to desolvation, no adaption of the pH value below the isoelectric point is required.

In the study performed by Khan and Schneider [31] GNPs with a particle size of 200 - 300 nm and unimodal size distribution (PDI < 0.15) were prepared via nanoprecipitation. These results could not be confirmed in our study. Particle formation via nanoprecipitation was principally possible, however particle characteristics were not comparable to GNPs prepared by two-step or one-step desolvation. GNPs showed considerably larger particle sizes and appreciably higher PDI values. The trend to higher particle sizes was already observed by Khan and Schneider and explained by the different principles of GNP formation [49]. Furthermore, utilising a stabiliser ensures the arrangement of a stable emulsion droplet and consequently attachment of the stabilising agent to the GNP surface [50]. Due to this shell of molecules, particle sizes may be larger and less uniform compared to plain GNPs prepared by desolvation. This statement is confirmed by the fact that in a direct comparison, GNPs prepared with 10% stabiliser were larger and more polydisperse than those with 7%. However, this is in contrast to the findings by Khan and Schneider where 10% stabiliser resulted in smaller particles [31].

Another explanation for these larger and polydisperse GNPs could be the heterogeneity of the used gelatine types. Nanoprecipitation is performed with gelatine qualities with a low bloom number and consequently a higher LMW fraction. From two-step desolvation, it is known that monodisperse GNPs can only be formed from the HMW fraction of gelatine. The LMW fraction would disturb this process [20, 21]. This may also have an impact on GNP formation by nanoprecipitation. The presumption can be strengthened by the observation that increasing bloom numbers, meaning increasing HMW fractions, resulted in more adequate GNPs. However, this would also contrast with the assertion of Khan and Schneider. They developed the nanoprecipitation method for GNP preparation as a

straightforward one-step preparation option. Further experiments should be performed to clarify these issues and distinct findings.
Even though nanoprecipitation resulted in high particle yields, this method was not further pursued due to the worse particle characteristics. However, by putting some effort into optimisation (e.g. test of gelatine with 300 bloom), this procedure could be an alternative for GNP preparation by desolvation.

4.4 Evaluation of Alternative Cross-Linking Agents

Glutaraldehyde is well known as cross-linking agent for proteineous nanoparticles, but presents safety issues for the patient and during manufacture [34]. Due to its consumption during manufacturing, and adequate purification of the GNPs, no adverse effects have been reported. Nevertheless, there is a need to find an alternative cross-linking agent. So far, several groups have studied alternative cross-linking agents for GNPs such as transglutaminase [37], genipin [36] and glyceraldehyde [38], but no alternatives have been found that are sufficiently effective under the tested conditions.
For instance cross-linking with transglutaminase gave monomodal GNPs with a particle size of 150 – 200 nm after an incubation of 48 hours [37]. However, high costs of the recombinant enzyme and reports indicating potential immunogenicity of transglutaminase residuals due to incomplete removal limit its applications [51]. Moreover, previous studies showed successful cross-linking of nanoparticles from recombinant human gelatine with genipin [36]. Stable GNPs with a uniform size distribution and particle sizes between 200 and 300 nm were obtained after a cross-linking time of 72 hours. In our study, these results could not be reproduced with porcine gelatine type A 300, which showed gel-like structures and no particle formation. The problem here lies in the low pH necessary for desolvation: The amine groups of gelatine are protonated at pH 2.5-3 and are therefore not available for the cross-linking reaction. The pH conditions required for gelatine type B, are optimal for the genipin reaction resulting in monodisperse GNPs. However, the reduced cross-linking degree in comparison to glutaraldehyde (ca. 40% vs. ca. 85%) led to

instability of the nanoparticles. This could be explained by the complex reaction between genipin and a protein and of several ring-opening steps that must take place [52]. Longer cross-linking times and higher genipin concentrations had no positive effect on stability, but induced gelation. Consequently, this study indicated that genipin is not suitable in large scale GNP production.

Recent studies with a focus on cross-linking GNPs with glyceraldehyde showed that the preparation of stable GNPs was successful only in the presence of a high content of Poloxamer 407 [38]. In this study, we were able to demonstrate that glyceraldehyde is suitable for GNP cross-linking without the addition of a stabiliser. Due to different pH conditions during desolvation and, therefore the number of free amines present, gelatine type A and type B required different cross-linking durations. Glyceraldehyde seems to be more reactive compared to genipin. This may be explained by the possible water elimination and following keto-enol tautomerism of glyceraldehyde resulting in reactive malondialdehyde [53]. Nevertheless, only gelatine type B gave GNPs that met the required characteristics due to more optimal reaction conditions for glyceraldehyde. In addition, glyceraldehyde is also a suitable cross-linking agent in large scale productions of GNPs. Although the cross-linking degree of type B particles was lower than for GNPs cross-linked with glutaraldehyde (ca. 75% vs. ca. 85%), the particles showed adequate colloidal stability over 35 days. Furthermore, the particle morphology of GNPs cross-linked by glyceraldehyde appeared to be less smooth compared to the GNPs cross-linked by glutaraldehyde, which could also be a consequence of the lower cross-linking degree.

5 CONCLUSION

The research presented successfully shows for the first time that GNP preparation by one-step desolvation is scalable and that the cross-linking agent glutaraldehyde can be substituted without significant effects on physicochemical characteristics of the nanoparticles. Providing large amounts of GNPs in a reproducible quality is the first step to become a standard drug delivery system in the treatment of RAO in horses and potentially in the treatment of various diseases in humans.

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CHAPTER III

PROGRESS IN FORMULATION DEVELOPMENT OF FREEZE-DRIED OLIGODEOXYNUCLEOTIDE LOADED GELATINE NANOPARTICLES

Parts of the following chapter are intended to be published in *European Journal of Pharmaceutics and Biopharmaceutics*:

Katharina J. Geh, Madlen Hubert, Gerhard Winter. Progress in formulation development and sterilisation of freeze-dried oligodeoxynucleotide-loaded gelatine nanoparticles. *Submitted to European Journal of Pharmaceutics and Biopharmaceutics.*

ABSTRACT

Oligodeoxynucleotide (ODN)-loaded gelatine nanoparticles (GNPs) have proven their outstanding potential in the treatment of allergic diseases, such as equine asthma and canine atopic dermatitis, which are appropriate models for the corresponding human diseases. To encourage the development of a marketable product, long term stability need to be ensured. In this work, freeze-drying options to stabilise these nanoparticles were advanced. Firstly, matrix-assisted laser desorption/ionisation mass spectrometry time-of-flight (MALDI-TOF) was implemented as versatile tool to assess ODN stability. Then, long term storage stability of lyophilised ODN-loaded GNPs formulated in sucrose or trehalose could be shown. Controlled nucleation was introduced in order to optimise lyophilisation processes. Freeze thaw experiments confirmed stability of ODN-loaded GNPs following controlled nucleation. In comparison to standard freeze-drying process, shortening of the freeze-drying process was achieved, but no further advantages were observed. Particle sizes, PDI values, ODN stability, residual moisture and glass transition temperature were maintained upon storage. Excipient portfolio was enlarged by novel amino acid containing formulations for lyophilisates. Histidine emerged as appropriate excipient in stabilising lyophilised ODN-loaded GNPs, whereas addition of arginine and glycine revealed to be inadequate at accelerated conditions.

KEYWORDS

Gelatine nanoparticles, oligodeoxynucleotides, lyophilisation, freeze-drying, controlled nucleation, amino acid, MALDI-TOF

1 INTRODUCTION

Gelatine nanoparticles (GNPs) provide an exceptional potential for the application as drug delivery systems. In contrast to other polymers, gelatine is an excellent starting material for nanoparticles due to its biodegradability, biocompatibility and physiological tolerance [1]. Additionally, it is known for its long and successful history of clinical use for medical applications [2]. Moreover, gelatine offers unfunctionalised amino groups for electrostatic attachment of different kinds of active substances, such as cytosine phosphate guanosine oligodeoxynucleotides (CpG ODNs). By induction of intracellular TLR 9, ODNs are able to rebalance the disrupted homeostasis between Th1 and Th2 immune response in order to combat the principle of allergic diseases [3]. GNPs proved their ability to protect ODNs from degradation and implant them into cells [4-6]. Recent *in vivo* studies showed the exceptional potential of GNPs as drug delivery system in the treatment of equine recurrent airway obstruction (RAO), an allergic airway disruption similar to human asthma [7-10]. In several equine studies an improvement of clinical parameters such as bronchoconstriction, mucus hypersecretion and inflammation of the lower airways was described by inhalative application of ODN-loaded GNPs [7-10]. ODN-loaded GNPs have also proven to be superior compared to free ODNs or placebo. Additionally, in contrast to conventional treatment, this therapy led to a sustained effect and causal treatment of the disease [8, 10].
Besides these findings, further *in vitro* and *in vivo* studies indicated that ODN-loaded GNPs are also active in the treatment of canine atopic dermatitis (CAD), an allergy driven skin disorder in dogs comparable to human atopic dermatitis [11, 12]. In a preliminary canine *in vivo* study, the subcutaneous application of ODN-loaded GNPs showed significant improvement of clinical parameters, such as pruritus or skin lessons. These results were confirmed by a decreased serum concentration of pro-allergic IL-4 [12].

Due to the outstanding success and effectiveness in several kinds of applications of ODN-loaded GNPs, there was a strong need to prepare GNPs in large quantities for

further clinical studies and future commercialisation. A big step in this direction was to achieve a more efficient particle production process. This could already be realised by our group by the optimisation of GNP production and subsequent scale-up of the process [13]. A commercially available gelatine type was found, which enabled the conversion and simplification of GNP preparation from a critical two-step process into an easy and straightforward one-step desolvation process. This allowed a more reproducible and homogeneous production process and enabled scaling up. Large particle quantities up to a 130-fold increase of the initial particle gain using the previous method were accomplished. Furthermore, no limitations for a further increase in batch size could be observed. This is an important fact for future industrial large-scale productions of GNPs.

Besides scalability, a requirement for the wide use of GNPs in different clinical applications and the possibility to enter the market is to provide a storage stable product. However, in liquid formulations ODN-loaded GNPs show high tendency to aggregate. This leads to a maximal verified storage stability of 48 to 72 hours depending on ODN sequence [12, 14]. A first step into stabilisation has already been done by Zillies et al. They could show the possibility to freeze-dry NF-κB decoy ODN-loaded GNPs without any loss in physicochemical quality or biological function [15]. In the present work, we could confirm these findings regarding the stability of ODN-loaded GNPs. Furthermore, we established an additional analytical method for ODN integrity evaluation, matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF). Moreover, storage stability could be extended to six months.

Standard lyophilisation is a versatile procedure to stabilise sensitive drug formulations such as proteins or as described here ODN-loaded GNPs. However, it is also related to some drawbacks, such as high time- and energy-consumption. Therefore, lot of research is carried out to shorten lyophilisation processes. Promising approaches include controlled nucleation [16], aggressive freeze-drying

[17] or collapse freeze drying [18, 19]. Among the mentioned techniques, controlled nucleation is the most advanced and most investigated approach.

Conventional shelf-ramped freezing induces a stochastic ice nucleation in the formulation, leading to different ice crystal growing and consequently high vial-to-vial variability within one batch. This causes unpredictable sublimation rates and great quality differences within the final product [20]. Furthermore, many small ice crystals are formed that slow down drying due to high specific surface area, small pore size and high dry layer resistance. Via a controlled nucleation, simultaneous freezing of all vials can be achieved resulting in uniform and large ice crystals in all vials. Consequently, specific surface area and dry layer resistance are reduced. This leads to a shortened drying time. Besides economic factors, controlled nucleation is also beneficial for an increased batch homogeneity [20, 21]. Different methods to achieve a controlled ice nucleation, their effects on product quality and stability are intensively studied in the field of therapeutic proteins [16, 21, 22]. It is known from protein research that methods controlling ice nucleation can be beneficial in stabilising the protein due to reduced ice-water interfaces and thus lower protein damage [20, 23]. Still, there is only few research on nanoparticles regarding controlled nucleation in freeze-drying. The work of Kasper et al. revealed that in principle controlled nucleation in lyophilisation of nanoparticulate polyplexes is possible without the loss of particle quality. However, DNA concentration is crucial, and high additive concentrations are necessary to stabilise them [24]. Interestingly, initial freeze-thaw experiments showed that ODN-loaded GNPs are not sensitive to the aforementioned factors.

This work investigates for the first time the applicability of controlled nucleation in lyophilisation of gelatine nanoparticles in order to accelerate the freeze-drying procedure without adverse influences on product quality or stability.

Additionally, we could expand the portfolio of possible formulations from conventional lyophilisation excipients, such as sugars, to amino acid formulations. Amino acids were investigated due to their beneficial features in stabilising proteins

during lyophilisation and irradiation-based sterilisation [25-27]. Histidine showed a clear benefit in stabilisation of ODN-loaded GNPs. Arginine and glycine also stabilised particles by preserving sizes and PDI values. However, ODN integrity was affected at accelerated storage temperature.

Summarising, this work opens the way for this innovative medicinal approach towards marketable products in human and veterinary application.

2 Material and Methods

2.1 Materials

Gelatine type A 300 bloom was obtained from Gelita AG (Eberbach, Germany). Acetone was supplied by Fisher Chemicals (Loughborough, UK). Glutaraldehyde (25% solution), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, (2-Aminoethyl) trimethylammonium chloride hydrochloride, L-arginine base, L-histidine base and L-glycine base were acquired from Sigma (Taufkirchen, Germany). D-(+)-Sucrose and D-(+)-Trehalose dihydrate were purchased from VWR International (Leuven, Belgium). Highly purified water (HPW), which was produced by a PURELAB Plus device (conductivity < 0.055 µS/cm, Elga Labwater, Celle, Germany), was used in all experiments. Oligodeoxynucleotides (ODNs) were synthesized by biomers.net GmbH (Ulm, Germany).

2.2 Preparation of Cationised Gelatine Nanoparticles

GNPs were prepared according to one-step desolvation [13]. In brief, after an amount of 750 mg gelatine type A (300 bloom) was dissolved in HPW (3.0% w/v) under constant stirring at 50°C, the pH was adjusted to a value between 2.5 and 3.0. In order to initiate desolvation and nanoparticle formation, acetone was added dropwise. A volume of 175 µL glutaraldehyde solution was added to cross-link GNPs. The dispersion was stirred overnight and purified via three-fold ultrafiltration using a solvent resistant stirred cell and an ultrafiltration disc of regenerated cellulose and a molecular weight cut-off of 100'000 kDa (Millipore S.A.S., Molsheim, France).

Cationisation of GNPs was performed according to the standard protocol [28] with some modifications. Briefly, GNP dispersion was diluted with HPW (~ 10 mg/mL) and pH was adjusted (4.5 - 5.0). Following, 1000 mg of each 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and (2-Aminoethyl) trimethylammonium chloride hydrochloride (Cholamine) were added. After

incubation for 30 min, the reaction mixture was purified by two-fold centrifugation (16000*g for 15 min; Sigma Laborzentrifugen, Osterode, Germany). Finally, nanoparticle concentration was determined gravimetrically using a UMX2 ultra-microbalance (Mettler Toledo, Greifensee, Switzerland).

2.3 Oligodeoxynucleotide Loading of Gelatine Nanoparticles

ODN loading was performed in solutions of different kind of excipients to prepare formulations for lyophilisation. GNP dispersion was diluted to a final concentration of 1.5 mg/mL and incubated with 5% (w/w) ODNs for 60 min at 21°C and under continuous shaking at 350 rpm (Thermomixer Comfort, Eppendorf AG, Hamburg, Germany). The excipient-to-ODN ratios for sugar containing samples were 100:1, 500:1 and 1333:1. The latter represents an isotonic formulation. The ratio is used to identify the samples, e.g. “S500” is a formulation consisting of a sucrose-to-ODN of 500:1.

For the stability study, amino acids (L-arginine, L-histidine and L-glycine) were used in excipient-to-ODN ratio of 333:1 (one amino acid, representing 2.5% [w/v]), 667:1 (two amino acids, equally mixed) and 1000:1 (three amino acids, equally mixed). Sugar amino acid combinations were based on a sugar-to-ODN ratio of 500:1, amino acids were added at a ratio of 333:1.

2.4 Freeze-Drying of ODN-Loaded GNPs and Storage Conditions For Stability Study

Freeze-drying was performed using a volume of 500 µL in 2R glass vials according to the following freeze-drying cycle (Figure III-1) adapted from Zillies et al. [15] using an EPSILON 2-6D pilot scale freeze dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterrode, Germany) and type T thermocouples (Newport Electronics, Deckenpfronn, Germany). Upon finishing the cycle, the product chamber was vented with nitrogen and samples were stoppered at a chamber pressure of 800 mbar. The sealed vials were stored at 2-8°C and 20-25°C

for six months, as well as at 40°C for four weeks. Analytics was performed directly after freeze-drying and after one, three and six months of storage.

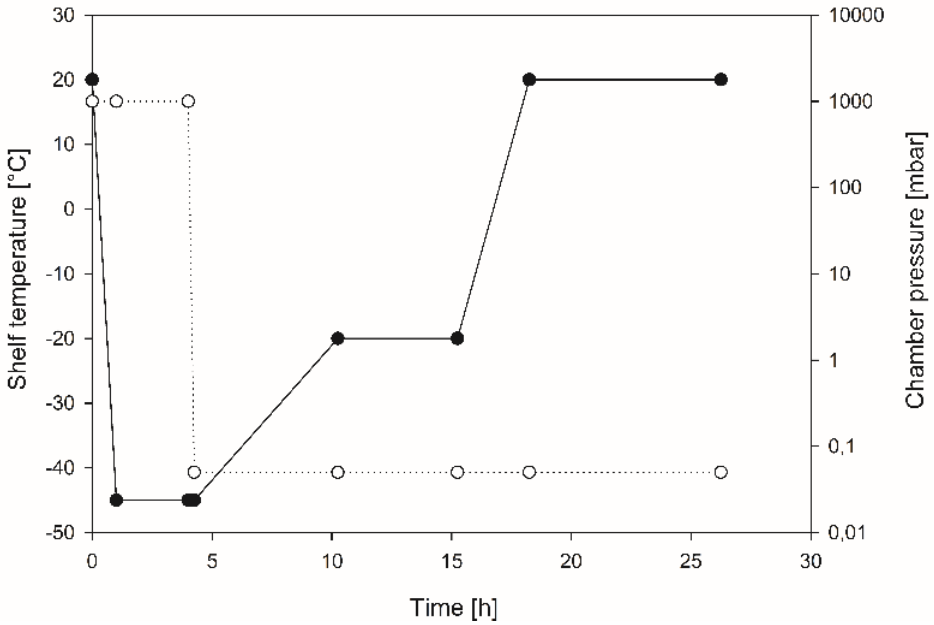

Figure III-1 Freeze-drying cycle with shelf temperature (black dots) and chamber pressure (white dots) for lyophilisation of ODN-loaded GNPs for storage stability.

2.5 Freeze Thaw Experiments using Different Freezing Protocols

Freeze thaw experiments were performed in an EPSILON 2-6D pilot scale freeze dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterrode, Germany) equipped with thermocouples (Newport Electronics, Deckenpfronn, Germany) for temperature monitoring. The following formulations were investigated in triplicates: S100, S500, S1333, T100, T500, T1333 and the corresponding sugar free formulation (HPW). A volume of 500 µL of each formulation was filled into 2R vials. Vials were positioned in the middle of the shelf and surrounded by two rows of vials filled with 5% sucrose solution. All samples were equilibrated at 20°C for 15 minutes prior to freezing until a shelf temperature of -45°C. In all freeze thaw experiments, samples were kept frozen for 10 hours and subsequently thawed by a ramp of 2.5°C/min until 20°C, followed by a hold step at 20°C for 60 minutes.

2.5.1 Conventional Shelf Ramped Freezing

Conventional shelf ramped freezing was conducted with a shelf ramp rate of -1°C/min or -1.5°C/min until -45°C.

2.5.2 Controlled Nucleation

Controlled nucleation was performed as described by Geidobler et al. [29]. Shelves were cooled at a ramp rate of -1°C/min until -4°C and samples were equilibrated at that temperature. As soon as all sample reached the equilibrium temperature, the product chamber was depressurized to a vacuum of 3.69 mbar. In order to initiate controlled ice nucleation, the vacuum was immediately released by opening the drain valves and ice fog was brought from the condenser into the product chamber. After ice nucleation, shelf temperature was either kept at -4°C for 15 minutes and then cooled to -45°C at-1°C/min (w/ hold time) or directly cooled to -45°C at -1°C/min (w/o hold time).

2.6 Freeze-Drying Using Controlled Nucleation

Controlled ice nucleation was performed as previously described by our group [29]. The applied process is displayed in Figure III-2. The shelves of the freeze-dryer (EPSILON 2-6D, Martin Christ Gefriertrocknungsanlagen GmbH, Osterrode, Germany) were cooled until a constant product temperature of -4°C was reached. The freeze-dryer was then depressurized to a vacuum of 3.69 mbar and immediately brought to atmospheric pressure by opening the drain valves. This induced repressurization via the cold condenser and ice fog was released into the product chamber initiating ice nucleation. Complete solidification was achieved by ramping down to −45°C with a ramp of 1°C/min followed by three hours primary drying at -20°C and a chamber pressure of 0.05 mbar. Secondary drying was performed for eight hours at 20°C. The product temperatures were monitored via type T thermocouples (Newport Electronics, Deckenpfronn, Germany). Stoppering and sealing was operated as mentioned above. Storage was executed at 2-8°C and 20-

25°C for three months, as well as four weeks at 40°C. Analytics were carried out directly after freeze-drying, as well as after one month and three months of storage.

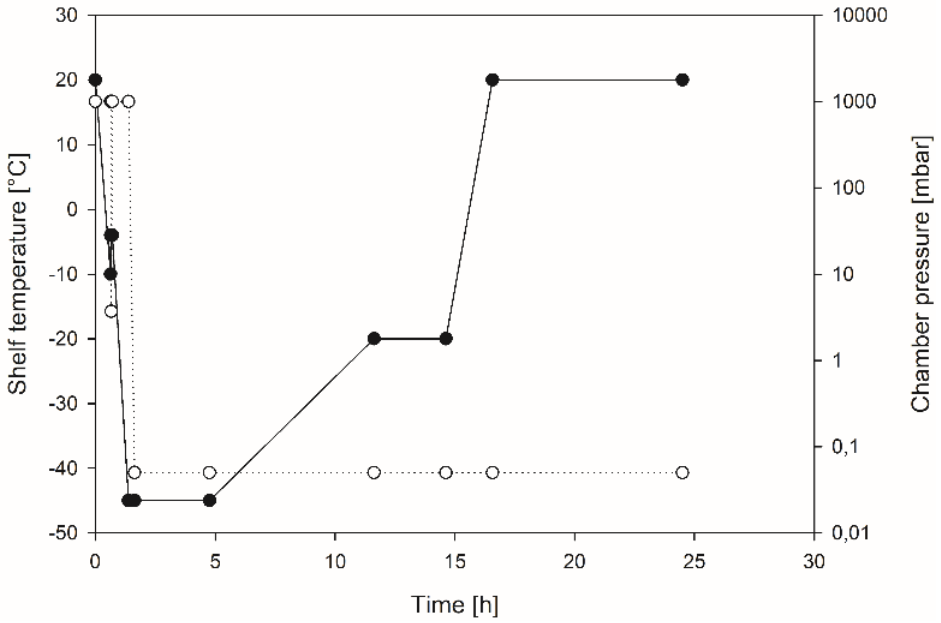

Figure III-2 Freeze-drying cycle using controlled nucleation with shelf temperature (black dots) and chamber pressure (white dots) for lyophilisation of ODN-loaded GNPs for storage stability.

2.7 Freeze-Drying of Amino Acid Formulations

Amino acid containing formulations were lyophilised using a more cautious freeze-drying protocol (Figure III-3) due to very low glass transition temperatures of the maximally freeze concentrated solution (Tg' values, down to -53°C). After freezing the samples at -60°C for three hours, the chamber pressure was reduced to 0.009 mbar. Primary drying was carried out at -40°C for 52 hours, followed by a pressure increase to 0.03 mbar and a two-step temperature ramp of 0.1°C/min to 0°C and then 0.33°C/min to 20°C. Secondary drying was performed at 20°C for ten hours. Stoppering, sealing, storage and analytics were conducted analogous to the aforementioned processes.

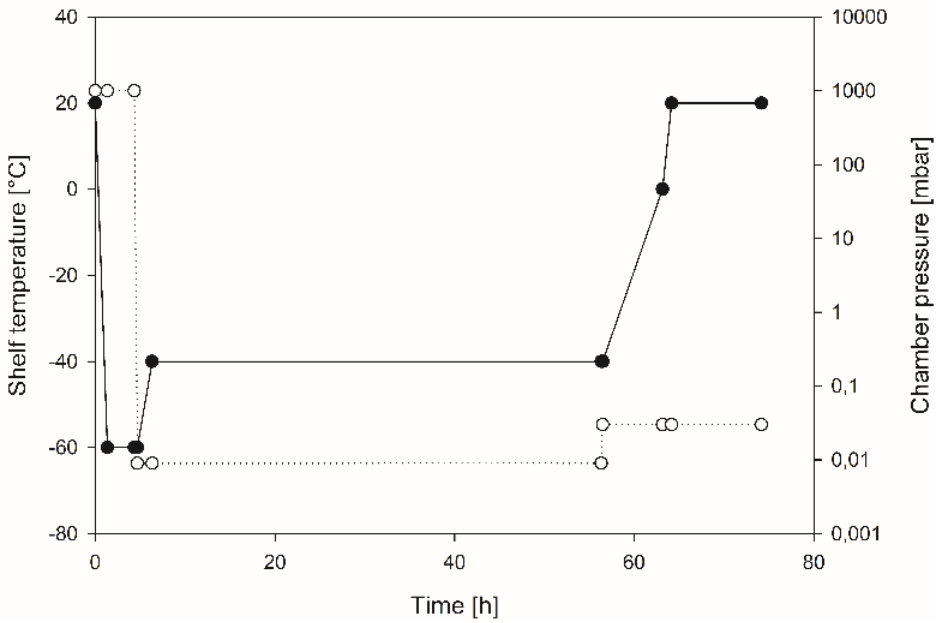

Figure III-3 Freeze-drying with shelf temperature (black dots) and chamber pressure (white dots) for lyophilisation of amino acid containing ODN-loaded GNPs for storage stability.

2.8 GELATINE NANOPARTICLE CHARACTERISATION

Particle size and polydispersity index (PDI) were determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). Zeta potentials were evaluated by electrophoretic light scattering (ELS) in a sodium chloride solution of 10 mM (Zetasizer Nano ZS, Malvern Instruments, Worcestershire, UK).

2.9 DETERMINATION OF LOADING EFFICIENCY

Loading efficiency was indirectly determined by centrifugation and following measuring of UV absorbance at 260 nm (Agilent 8453 UV-visible spectrophotometer, Agilent Technologies, Santa Clara, California, USA) in the supernatant of the ODN-loaded GNPs, a GNP control and an ODN-control [12]. Loading efficiency was calculated using the following equation:

$$\text{Loading efficiency } [\%] = 1 - \left(\frac{\text{A (ODN-loaded GNP)} - \text{A (GNP control)}}{\text{A (ODN control)}}\right) x\ 100\ \%.$$

2.10 Matrix-Assisted Laser Desorption/Ionisation Time-of-Flight Mass Spectrometry (MALDI TOF)

Prior to measurement, the samples were desalted on a 0.025 µm VSWP membrane (Merck Millipore, Darmstadt, Germany) and co-crystallised in a 3-hydroxypicolinic acid matrix (HPA). Matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectra were recorded on a Autoflex II (Bruker Daltonics, Germany) and a AnchorChip®-Target (Bruker MTP var/384) in negative mode.

2.11 Karl-Fischer Titration

Coloumetric Karl-Fischer titration using Aqua 40.00 titrator with a headspace module (Analytik Jena AG, Halle, Germany) was used to determine residual moisture. The lyophilised ODN-loaded GNPs were heated to 100°C. The evaporated water was transferred into the titration solution and the water content was determined.

2.12 Dynamic Scanning Calorimetry (DSC)

Glass transition temperatures (Tg) and glass transition temperatures of the maximally freeze-concentrated solution (Tg') were determined using a Mettler DSC 821e (Mettler Toledo, Columbus, OH, USA). An amount of 1-15 mg lyophilisate was weighed into aluminium crucibles. To ascertain Tg values, samples were analysed at a heating and cooling rate of 10 K/min from 0 to 150 °C in a first and from -10 to 150°C in a second cycle against an empty crucible as reference. For measuring of Tg' values samples were heated from -60°C to 20°C. Tg and Tg' values were evaluated from heating scans.

2.13 BIOACTIVITY ASSAY IN EQUINE BRONCHOALVEOLAR (BAL) CELLS

This part of the study was performed in close collaboration with the veterinarian Dr. med. vet. John Klier. Equine BAL cells were harvested by Dr. Klier, following experiments were conducted either together or by me, depending on the individual case.

Bronchoalveolar lavage fluids (50 mL sterile, warm, isotonic NaCl solution per 100 kg bodyweight) were taken from two healthy horses and two horses affected from RAO. Fluids were immediately centrifuged to collect BAL cells (10 min at 1200**g*; Sigma Laborzentrifugen, Osterode, Germany). An amount of 2 x 10^5 cells in RPMI medium (Biochrom AG, Berlin, Germany; 10% FCS, 67.8 mg/mL penicillin, 113 mg/mL streptomycin) was seeded per well in 96 well plates and incubated in triplicates with the different formulations for 24 h at 37°C and 5% (v/v) CO_2 atmosphere. Investigated formulations were lyophilised S100, S500, S1333, T100, T500 and T1333 after six months storage at 2-8°C or 20-25°C, as well as corresponding freshly prepared formulations and corresponding ODN-free placebos. Reference samples contained freshly prepared ODN-loaded GNPs in water (standard formulation used in previous *in vitro* and *in vivo* studies) as well as corresponding placebo.

Following incubation, well plates were centrifuged (10 min at 1000**g*; Sigma Laborzentrifugen, Osterode, Germany) and supernatants were collected in order to analyse cytokine concentrations. Supernatants were either directly investigated or stored at -80°C until measurements.

The cytokine determination was conducted using equine ELISAs (R&D Systems, Minneapolis, USA) and included evaluation of IFN-γ, IL-4 and IL-10. ELISAs were performed according to the manufacturer's instructions. Cell viability was tested using alamarBlue™ Cell Viability Reagent (Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to the manufacturer's protocol.

3 Results and Discussion

3.1 Conventional Lyophilisation

Excipient selection was based on common knowledge and the findings of Zillies et al. [15]. Sugars and sugar alcohols, such as sucrose, trehalose or mannitol are widely used lyo- and cryoprotectants or bulking agents for freeze-dried biomaterials [30]. Sucrose is known for its ability to stabilise biopharmaceuticals, such as proteins [31]. Trehalose is stated to be even superior due to a higher glass transition temperature [32]. However, trehalose is high-priced compared to sucrose. This may be an issue in large scale production, especially when it comes to veterinary use. Mannitol is particularly known for its properties as bulking agent. Due to its crystallisation during freeze-drying, it is not suitable to be used as lyo- or cryoprotectant for proteins. Therefore, it is often mixed with sucrose to combine the features of good stabilisation and appropriate cake performance [33].
Zillies et al. could show equivalent stabilising properties for freeze-dried placebo GNPs of all their chosen excipients. ODN-loaded GNPs were sufficiently preserved by sucrose and trehalose, whereas mannitol and a mannitol-sucrose mixture were not beneficial [15]. Furthermore, they evaluated a minimal ODN-to-sugar ratio of 1:100 as necessary to stabilise ODN-loaded GNPs. Hence, we decided to choose sucrose and trehalose at ODN-to-sugar ratios 1:100, 1:500 and 1:1333 (isotonic) for our experiments.

3.1.1 Particle Sizes and PDI Values

DLS measurements of the rehydrated formulations revealed particle sizes of around 200 nm and monodisperse PDI values of around 0.2 (Figure III-4). No change in particle sizes or PDI values was observed over six months storage at 2-8°C or 20-25°C. Used sugar types and sugar concentrations were shown to be equivalent. Furthermore, storage at accelerated conditions (40°C, four weeks) had no impact on

particle characteristics (Figure III-4 C and F). This indicates GNPs as a very stable drug delivery system and prolongs already stated storage stability [15].
However, it can be noted that PDI values may depend on used oligodeoxynucleotide batch as the utilisation of different batches led to varying PDI values already before freeze-drying (same ODN batch for 2-8°C and 20-25°C, different batch for 40°C).

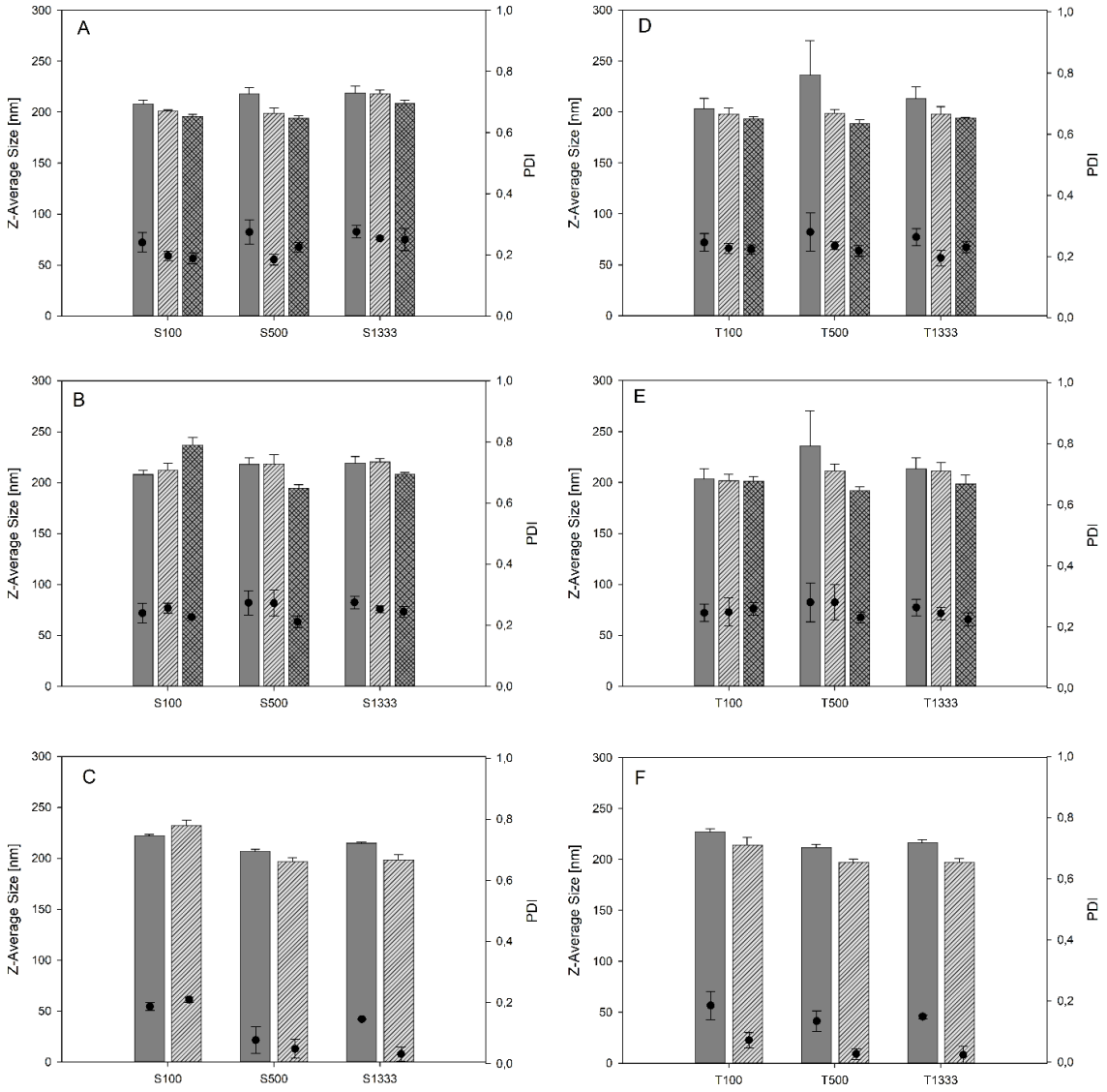

Figure III-4 Particle sizes (bars) and PDI values (dots) of freeze-dried ODN-loaded GNPs directly after lyophilisation (dark grey), after four weeks of storage (light grey striped) and six months of storage (dark grey chequered). Sucrose formulations at A: 2-8°C, B: 20-25°C and C: 40°C. Trehalose formulations at D: 2-8°C, E: 20-25°C and F:40°C. Results are represented as mean + or ± SD (n=3).

Interestingly, after freeze-drying and reconstitution, particle sizes were smaller than before. Similar results has already been observed for lyophilised GNPs [15] and PCL (Poly(epsilon-caprolactone)) nanocapsules [34]. This has been explained by a shrinking of the particles during freeze-drying followed by an incomplete rehydration [15].
Furthermore, most samples showed a tendency to smaller sizes during storage. This is only partly comparable to literature. For ODN-loaded GNPs in sucrose, a reduction of particles sizes was ascertained after storage of four weeks, whereas preservation or even a slight increase in particle sizes is reported for ODN-loaded GNPs in trehalose and other types of nanoparticles [35-38].

Moreover, particle sizes and PDI values after reconstitution of all lyophilised GNPs were stable for 48 hours similar to freshly prepared formulations. This was found for samples directly after freeze-drying as well as after six months of storage at 2-8°C or 20-25°C.

3.1.2 Loading Efficiencies

It is important to point out that the electrostatic interaction between oligodeoxynucleotides and GNPs results a sensitive system of loaded NPs. Therefore, it is crucial to evaluate changes in loading efficiency after any kind of processing.
Loading efficiencies remained stable after freeze-drying in all formulations and all storage conditions (see annex). This stability of high loading efficiency was persistent for 48 h after reconstitution. Consequently, it can be assumed that lyophilisation has no impact on the positive charge of GNPs and the electrostatic interactions of GNPs and oligodeoxynucleotides are not disturbed by lyophilisation.

3.1.3 Oligodeoxynucleotide Integrity

It should be recognized that the integrity of the oligodeoxynucleotides is one of the most important points regarding bioactivity of freeze-dried ODN-loaded GNPs. To

evaluate ODN stability after freeze-drying and storage, we used matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF).

MALDI-TOF is a common method to asses oligodeoxynucleotide identity and quality in synthesis, which offers rapid and direct analysis of oligodeoxynucleotides [39]. Furthermore, MALDI-TOF can be used to analyse degradation of ODNs [40, 41]. Intact ODNs can be identified by a sharp peak at the m/z value of the oligodeoxynucleotide's molecular weight. Following degradation the peak of the intact ODN would disappear, whereas peaks of degradation products would appear [41].

We could show that MALDI-TOF is a suitable method to asses ODN integrity even if the ODNs are loaded onto GNPs. GNPs are not ionized by the laser and do not desorb from the matrix, whereas laser power is high enough to release electrostatically bound ODNs from GNPs. However, high laser power may induce depurination of the oligodeoxynucleotide, meaning cleavage of the last base, which can be seen by a second small peak on the left side of the main peak [39]. This typical phenomenon should not be misinterpreted as a degradation product. A representative spectrum is displayed in Figure III-5.

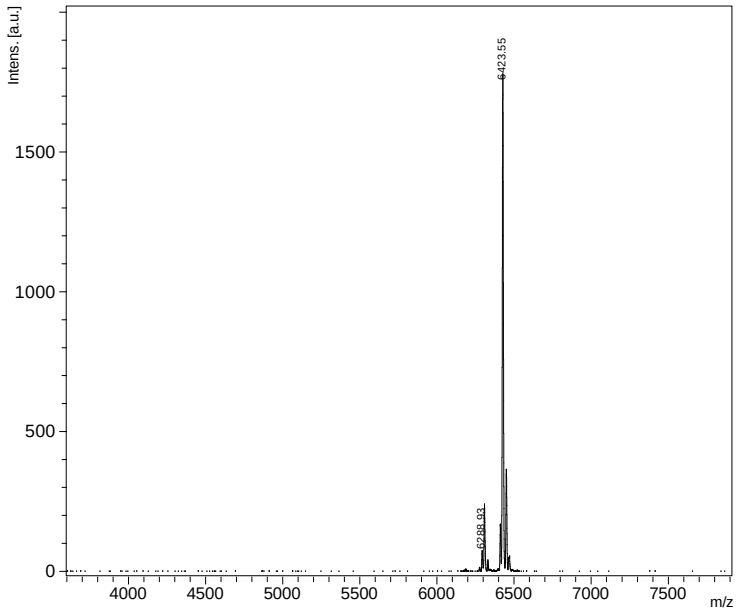

Figure III-5 Representative MALDI-TOF spectrum of investigated oligodeoxynucleotide.

Results demonstrate that ODNs are stable during lyophilisation, more precisely stress during freezing and drying does not degrade ODNs (Table III-1). Furthermore, ODNs were stable for six months during storage at 2-8°C and 20-25°C. In higher sugar concentrations (S500, S1333, T500, T1333) they were even resistant for four weeks at accelerated conditions (40°C). However, at low sugar concentrations (S100 and T100) first signs of a starting degradation of ODNs, such as an increase of a small additional peak or low signal intensity, were observed (see annex). Hence, for long time storage of ODN-loaded GNPs formulations with higher sugar contents are recommended.

Table III-1 Oligodeoxynucleotide integrity after conventional lyophilisation and subsequent storage. ODN integrity is represented with symbols: ✓ stable ODN, × degraded ODN, ± indications for starting degradation.

Formulation	Conventional lyophilisation		
	2-8°C 6 months	20-25°C 6 months	40°C 4 weeks
S100	✓	✓	±
S500	✓	✓	✓
S1333	✓	✓	✓
T100	✓	✓	±
T500	✓	✓	✓
T1333	✓	✓	✓

3.1.4 Residual Moisture and Glass Transition Temperature

In addition to particle and ODN characteristics, residual moisture as well as glass transition temperature of the lyophilised samples are important parameters to asses storage stability. High residual moisture values are expected to negatively influence nanoparticle stability in freeze-dried samples due to higher mobility resulting in particle aggregation [42]. Furthermore, ODN degradation may be more likely with increasing water content [42]. The relation between water content and

glass transition temperature (Tg) of solids in their amorphous states is well known [43]. More water absorption leads to lower Tg values. Evaluation of Tg values is crucial for stability predictions, as storage far below the Tg is important to preserve the glassy state of the lyophilisates and to reduce molecular mobility [44]. Consequently, low residual moisture is essential to ensure Tg values above the storage temperature [30].

Evaluation of residual moisture contents in our lyophilisates revealed low starting values (< 1%) that increased upon storage depending on excipient content and storage temperature. The higher the sugar content, the less pronounced the relative water uptake was, whereas the higher the storage temperature was, the stronger the increase of residual moisture. However, values did not reach a critical treshold inducing particle aggregation. On the other hand, the increased water content may be related to slight ODN degradation starting in S100 and T100 after storage at 40°C. Tg values agreed with values from literature and remained stable in the higher sugar concentrations (S500, S1333, T500 and T1333). The formulations with the lowest amount of sugar (S100 and T100) showed a drop in Tg according to their increasing water absorption (for further details see annex).

Taken together, residual moisture values and glass transition temperatures developed as expected [15]. Regarding stability, no critical values were exceeded.

3.1.5 Bioactivity Assay of Lyophilised ODN-Loaded GNPs

Although immunological processes of recurrent airway obstruction (RAO) are still controversially discussed, a Th2 derived pathogenesis is mainly presumed as its origin [45]. By stimulation of pro-inflammatory Th1 pathways and consequent suppression of Th2 activation, CpG ODNs are able to redirect the balance between Th1 and Th2 derived immune processes [3]. Furthermore, regulatory T-cells (Treg) producing IL-10, which acts as beneficial modulator in allergic diseases, are assumed to be activated [3]. These effects have been demonstrated in multiple

equine *in vitro* and *in vivo* studies addressing RAO therapy by the application of ODN-loaded GNPs [7-10, 14]. Thus, IL-4 is used as main marker for Th2 dependent processes, whereas IFN-γ reflects Th1 derived immune mechanisms. Lastly, IL-10 is considered as indicator for Treg activation.

Formulations were assessed for the bioactivity in equine bronchoalveolar (BAL) cells to give further information regarding stability. This particular cell type was chosen as the main usage of these formulations would be the inhalative treatment of RAO in horses. Based on previous studies, the ability of formulations to stimulate the release of key cytokines IFN-γ and IL-10 as well to inhibit the production of IL-4 were evaluated in BAL cells harvested from healthy and RAO-affected horses [14].

Results of IFN-γ and IL-10 expression are demonstrated in Figure III-6. Lyophilised and subsequently stored ODN-loaded GNPs are able to induce INF-γ release in healthy (Figure III-6 A) and RAO-affected horses (Figure III-6 B) similar to freshly prepared formulations and the standard formulation in highly purified water (HPW). This indicates Th1 stimulation by all formulations. Interestingly, in healthy horses freeze-dried formulations stored for six months at 20-25°C even induced highest levels of IFN-γ. However, this could be a bias caused by the low number of available horses as cell donors and overall high variations in the results of ELISA measurements. Nonetheless, the ascertained IFN-γ concentrations are in the same range as reported in a previous study [14].

Additionally, IL-10 concentrations induced by lyophilised or freshly prepared ODN-loaded GNPs were comparable (Figure III-6 C and D). This reinforces that lyophilisation and subsequent storage does not affect bioactivity of the formulations. Nevertheless, one should admit that IL-10 levels were 10-fold lower than in the previously performed study by Klier et al. [14].

Values for IL-4 were below the detection limit of the ELISA, whereas values for placebo formulations were in a quantifiable range. Hence, one can say that freshly

prepared as well as lyophilised formulations decreased IL-4 levels indicating a Th2 suppression.

Cell viability after incubation with freshly prepared or lyophilised and stored formulations did hardly vary (81.8% ± 9.3% vs. 85.2% ± 9.2%).

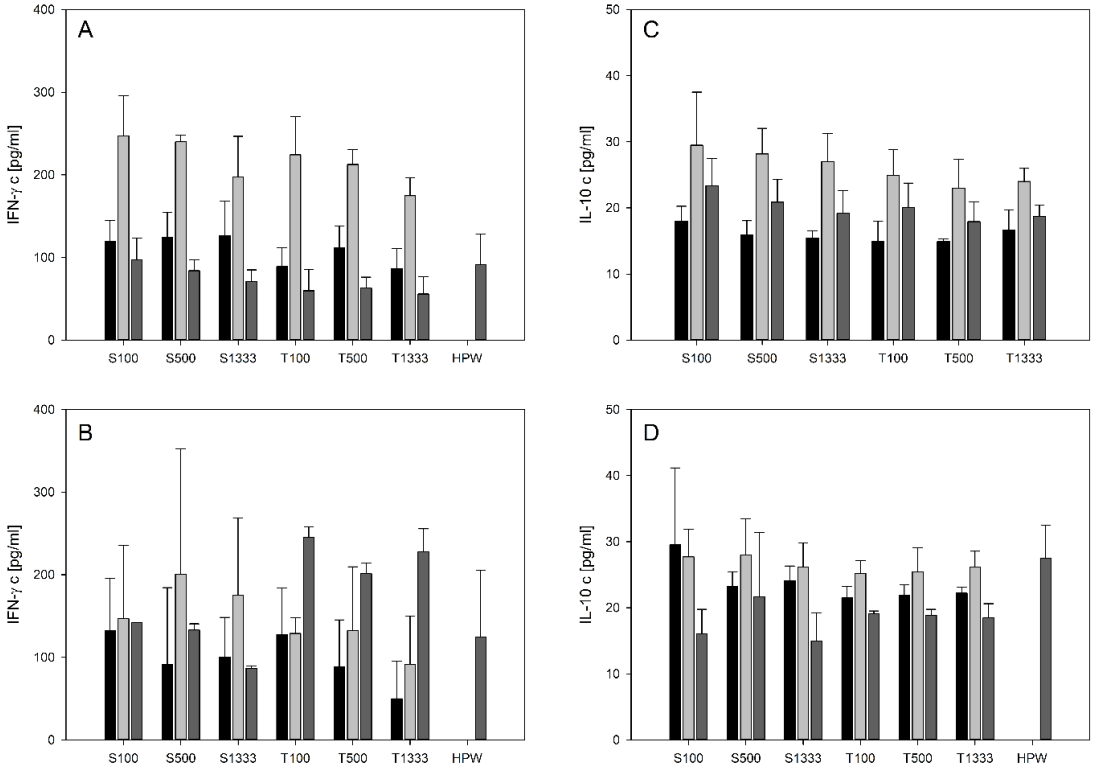

Figure III-6 IFN-γ expression in equine BAL cells from healthy (A) and RAO-affected horses (B) or IL-10 expression in equine BAL cells from healthy (C) and RAO-affected horses (D) after incubation with lyophilised ODN-loaded GNPs stored for six months at 2-8°C (black), 20-25°C (light grey) and freshly prepared formulations (dark grey). Data is represented as mean + SD, n=3 evaluated in two cell cultures each.

The main assertion of this experiment is that lyophilised and stored ODN-loaded GNPs are still able to induce their immunomodulatory effects comparable to freshly prepared formulations. This finding is independent of the used excipient, their concentration or storage condition. However, due to the very limited number of cell donors, measured values should be rated as hints and not as absolute values. Nonetheless, the findings of intact ODNs after lyophilisation and storage using MALDI-TOF confirm bioactivity of the processed formulations.

3.2 CONTROLLED NUCLEATION

Controlled nucleation was investigated as a possibility to shorten lyophilisation process time of ODN-loaded GNPs. Furthermore, in a stability study, controlled nucleation was evaluated as a potential tool to increase ODN stability compared to conventional lyophilisation. For a direct comparison, same formulation compositions were chosen.

3.2.1 FREEZE THAW EXPERIMENTS

As already mentioned, controlled nucleation has been reported to show positive effects on stability of proteins compared to standard ramp freezing [46, 47]. However, Kasper et al. found different results for polyplex formulations [24]. To stabilise these PEI/plasmid formulations during controlled nucleation, higher cryoprotectant concentrations were necessary compared to standard shelf-ramp freezing. In order to investigate the effect of controlled nucleation on ODN-loaded GNPs, samples with increasing sucrose or trehalose/ODN ratios were frozen via controlled nucleation or via conventional shelf-ramp freezing (-1°C/min or -1.5°C/min).

The most divergent product temperature profiles and nucleation temperatures are displayed in Figure III-7 and Figure III-8. Using conventional freezing, ice nucleation occurred stochastically at random product temperatures (-8.1°C ± 2.0°C or -8.7°C ± 3.0°C) as well as at random time points (standard deviation nucleation times: ± 4.2 min or ± 5.1 min). In contrast, when the controlled nucleation method was applied, ice nucleation occurred simultaneously at a temperature of -2.7°C ± 0.2°C and a distinct time point (standard deviation nucleation time ± 0 min). This confirms that controlled ice nucleation occurred reliably.

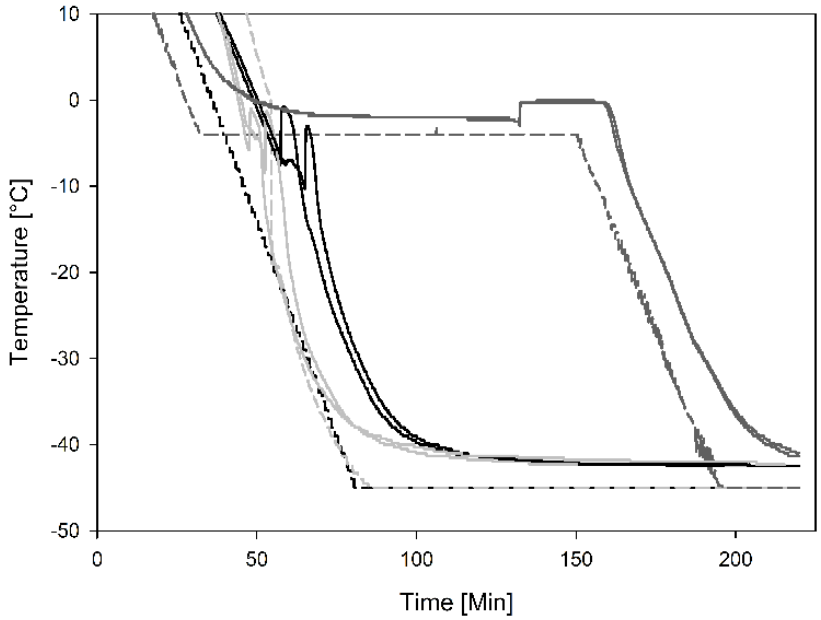

Figure III-7 Temperature profiles of shelves (dashed) and formulations (solid) during conventional freezing at -1°C/min (dark grey) or at -1.5°C/min (light grey) and during controlled nucleation (middle grey).

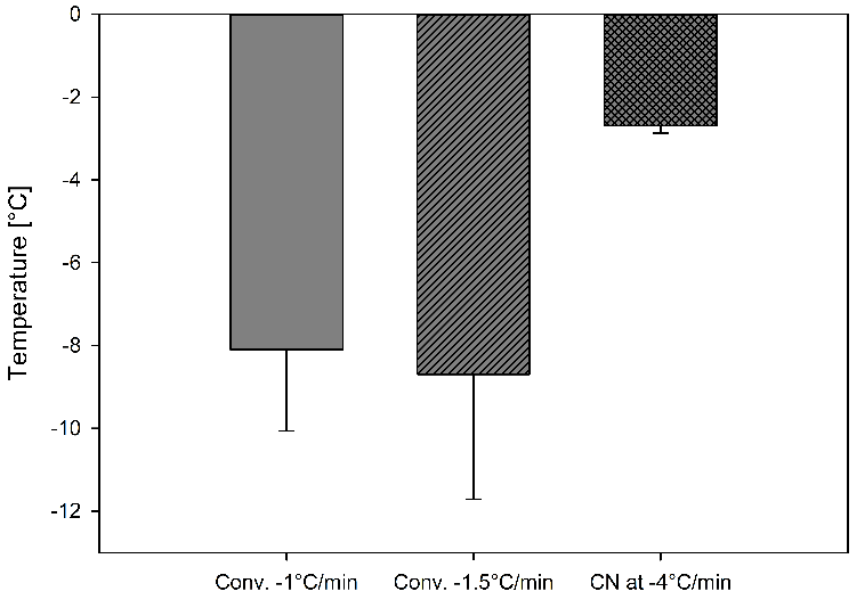

Figure III-8 Nucleation temperatures of samples frozen by conventional freezing at -1°C/min (left bar) or -1.5°C/min (middle bar) and controlled nucleation at a shelf temperature of -4°C (right bar). Data is represented as mean - SD (n=3).

Particle size and PDI values of ODN-loaded GNPs before and after freeze-thawing using different freezing methods is displayed in Figure III-9A. In general, particle sizes and PDI values were preserved by the addition of sugars as cryoprotectant, whereas an excipient free formulation led to aggregation of the ODN-loaded GNPs. This reinforces that the vitrification and particle isolation hypothesis as explanation for stabilisation of non-viral gene vectors by cryoprotectants can also be applied to ODN-loaded GNPs. Briefly, the vitrification hypothesis states that non-viral gene vectors, such as lipoplexes or polyplexes, are stabilised by sugars due to their immobilisation in the glassy state. This is combined with the isolation of the particles in the freeze concentrated solution by the sugar molecules and consequent reduced aggregation [48, 49]. Hence, a certain sugar concentration is necessary to achieve sufficient stabilisation.

Hardly any difference between sucrose and trehalose formulations could be observed. A higher cryoprotectant concentration had slightly beneficial effect on particle sizes after freeze-thawing. A tendency to narrower particle size distributions could be observed with increasing sugar concentrations indicating better stabilisation, which further supports the particle isolation hypothesis [48, 49].

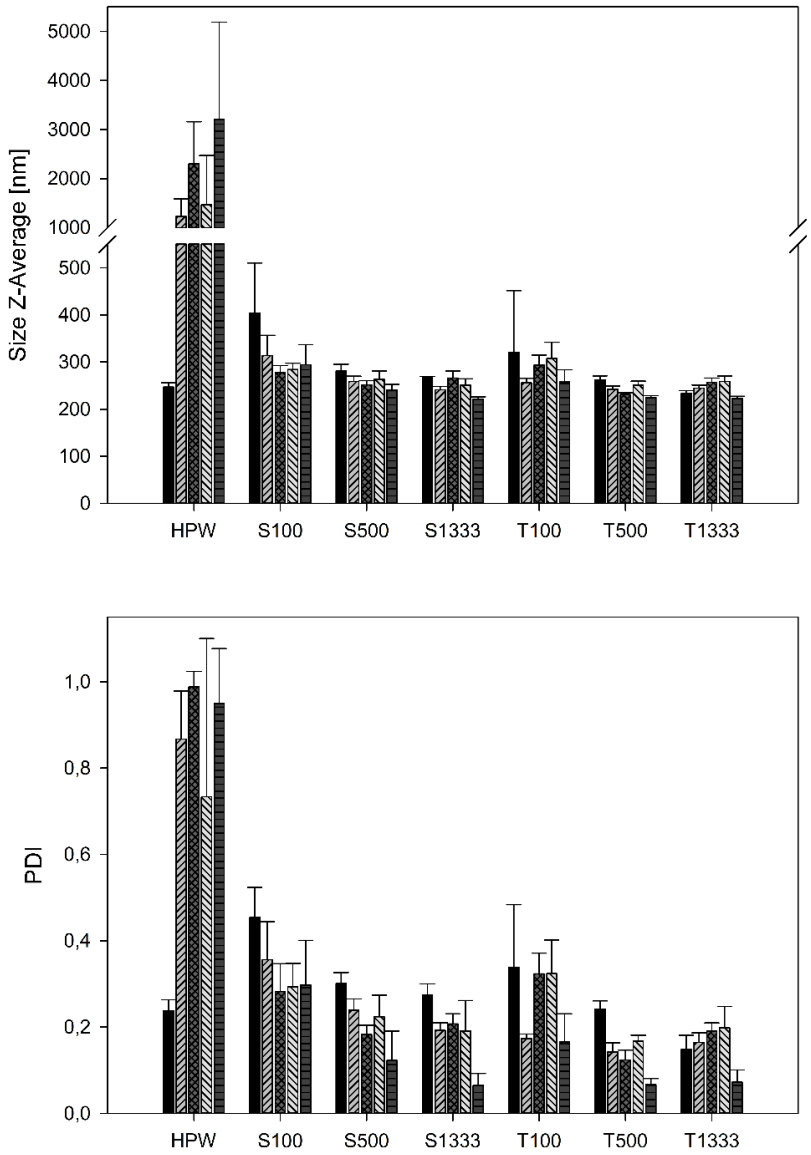

Figure III-9 Particle sizes (top) and PDI values (bottom) of ODN-loaded GNPs in different formulations before freezing (black bar), frozen by conventional freezing at -1°C/min (light grey bar upwards striped) or -1.5°C/min (dark grey bar chequered) and frozen by controlled nucleation with hold time (light grey bar downwards striped) or w/o hold time (dark grey bar stiped across). Data is represented as mean + SD (n=3).

In contrast to polyplexes, different freezing methods have no impact on particle sizes and PDI values of ODN-loaded GNPs. During ice formation, strong electric fields are generated due to favoured inclusion of one ionic species into the ice [50, 51].

Among others, this phenomenon is dependent on freezing rate, type of ions as well as concentrations [50, 51]. For polyplexes, it was stated that destabilisation during freezing is related to this charged ice formation and the fact that polyplexes are formed due to electrostatic interactions [24]. Thus, their integrity can be disturbed leading to aggregation. This is supported by the observation of lipoplexes being more stable during freezing due to hydrophobic interactions in addition to electrostatic interactions [24, 49]. Upon these nanoparticle types GNPs are most stable, because they are covalently cross-linked. Hence, their stability may be less disrupted by electric fields and aggregation is less likely. Furthermore, the used GNP formulations did not compose any buffer salts. Accordingly, the formation of electric fields at the ice interface is less pronounced. In summary, this enables that even low sugar/ODN rations are sufficient to prevent ODN-loaded GNPs from aggregation during freezing.

On the basis of the observations during freeze thawing studies no isothermal equilibration step (hold time) after controlled nucleation was applied for the following lyophilisation experiments.

3.2.2 Lyophilisation Using Controlled Nucleation

By applying controlled nucleation, the drying time could be shortened by only 7%. This could save some time, energy and costs. However, it is questionable if these fractional savings are worth implementing a more complex freezing method.

Regarding particle sizes, PDI values, loading efficiencies, residual moisture values, and glass transition temperatures no relevant differences of storage time compared to a conventional freeze-drying cycle could be found. Only ODN stability (Table A 3, annex) seemed to be slightly enhanced in low concentration sugar formulations at elevated temperatures (for further details see annex). In contrast to reports in literature, controlled nucleation neither has a positive effect as assumed from protein research [23], nor has negative impact on the product as reported for polyplexes [24].

3.3 AMINO ACIDS

The intention of this part of the work was to identify excipients for lyophilisation of ODN-loaded GNPs apart from classical sugars. Furthermore, keeping a later sterilisation via gamma irradiation in mind, the portfolio of potential stabilising excipients should be enlarged. It is known form literature that amino acids can have stabilising effects on protein formulations and lyophilisates [25, 26, 52, 53], as wells as protective properties to shield against sterilising irradiation [27]. The aim of the study was to evaluate if this can be transferred to lyophilisation of ODN-loaded GNPs.

Excipient choice was based either on charge of the amino acid and/or known ability to stabilise biomolecules. Positively charged amino acids were chosen in order to preserve high loading efficiencies. We know from previous experiments, that ODN-loaded GNPs are very sensitive to the addition of negatively charged molecules leading to tremendous loss in loading efficiency. This is based on interaction of the negatively charged molecule and the positively charged GNPs and resulting competition of ODNs and excipient. Positively charged excipients do not have this strong impact on loading efficiency. From the group of basic amino acids, arginine and histidine were selected, as they are known to prevent proteins from aggregation and form amorphous cakes after freeze-drying [26, 53, 54].

Furthermore, a neutral amino acid was chosen. Neutral amino acids result in a crystalline state after freeze-drying [26]. Therefore, they are not suitable to stabilise biomolecules such as proteins, but can be used as bulking agents [33]. For this study, we selected glycine.

Additionally, we evaluated mixtures of these amino acids, as well as combinations of a sugar and an amino acid in order to enhance their potential stabilising effect.

Pre-experiments revealed the following formulations as promising regarding particle sizes and PDI values of ODN-loaded GNPs in the liquid state: His, His + Arg, His + Gly, Arg + Gly, Arg + His + Gly, Suc + Gly, Tre + Gly.

3.3.1 DRYING TIME

In order to prevent collapse of the formulations due to very low glass transition temperatures of the maximally freeze concentrated solution (Tg' values as low as -53°C), process changes in the lyophilisation cycle were necessary. However, this led to a strongly prolonged drying time. Further process development is recommended to optimise lyophilisation cycles for the amino acids.

3.3.2 PARTICLE SIZES AND PDI

In contrast to sugar formulations, no change in GNP particle sizes before and after freeze-drying in pure amino acid formulations was observed. Sugar amino acid (glycine) combinations showed smaller particle sizes after lyophilisation and rehydration similar to plain sugar formulations. This leads to the suggestion that either GNPs formulated in amino acids do not shrink to the same extent as in sugar formulations, or sugars are involved in incomplete swelling after rehydration. Furthermore, sugar glycine combinations showed clearly higher particle sizes (~300 nm before and 280 nm after freeze-drying) than amino acid or sugar formulations (~200 nm). PDI values of all formulations were acceptable directly after freeze-drying, the chosen amino acids had no impact on particle size distribution (Figure III-10). During storage at 2-8°C and 20-25°C particle sizes and PDI values remained unchanged for three months in all formulations. At accelerated conditions, the particle characteristics of the formulations do neither change, except for Arg + Gly. This formulation shows a clear particle aggregation after four weeks storage at 40°C.

Regarding particle characteristics, it can be concluded that amino acids are equivalent to sugars in stabilising freeze-dried ODN-loaded GNPs, except for the combination of arginine and glycine. The aggregation could be caused by the crystallisation of glycine after lyophilisation and subsequent destabilisation of the nanoparticles. Amorphous arginine was not able to compensate the negative effects of crystalline glycine.

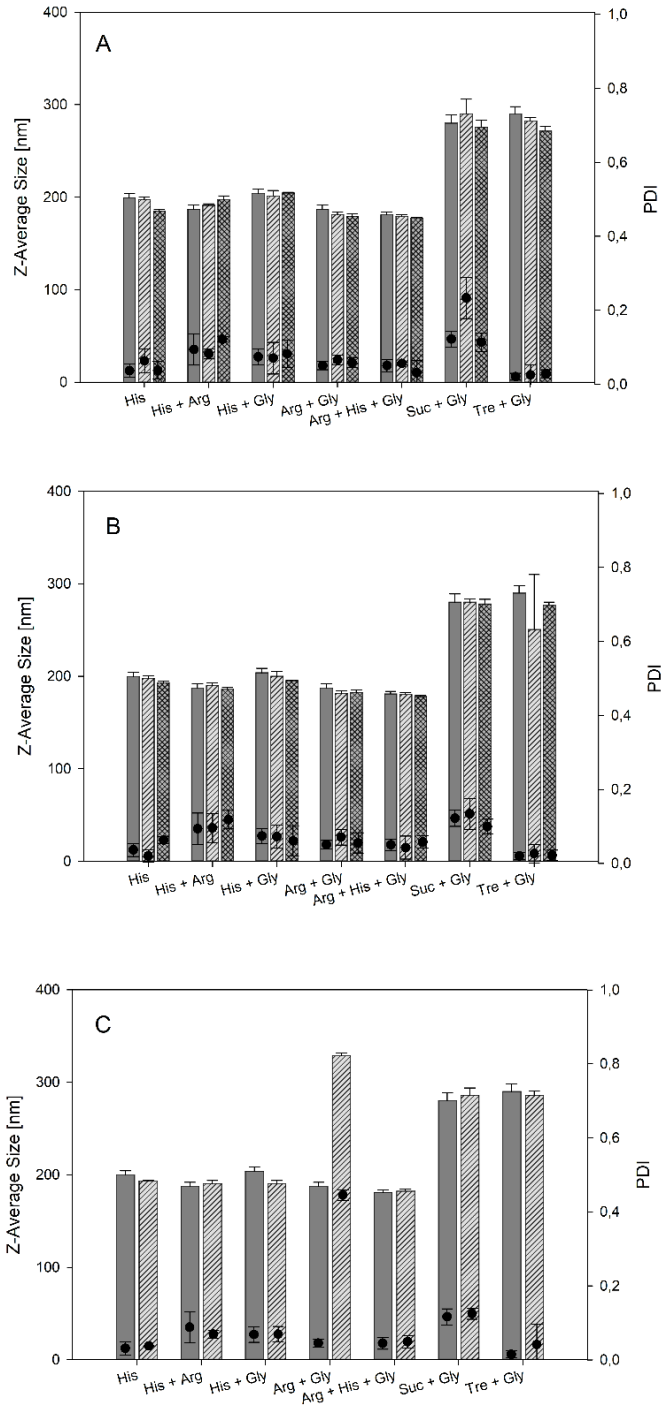

Figure III-10 Particle sizes (bars) and PDI values (dots) of freeze-dried ODN-loaded GNPs in amino acid containing formulations directly after lyophilisation (dark grey), after four weeks of storage (light grey striped) and three months of storage (dark grey chequered). Stored at A: 2-8°C, B: 20-25°C and C: 40°C. Results are represented as mean + or ± SD (n=3).

3.3.3 Loading Efficiencies

Investigation of the loading efficiencies is important and interesting, as charged amino acids may have a stronger impact than uncharged sugars. Compared to sugar formulations and sugar containing amino acid formulations, pure amino acid formulations showed slightly reduced loading efficiencies (annex). This may be because of an interaction between positively charged amino acids and negatively charged ODNs. Nonetheless, loading efficiencies persisted above 80% and remained stable during storage at all conditions. However, His as well as Arg + Gly showed a trend to stronger loss in loading efficiency with increasing storage temperature.
Summarising, amino acids seem to interact with the charged surface of GNPs leading to a competitive reduction in ODNs loading efficiency.

3.3.4 ODN Integrity

The resistance of ODNs loaded onto GNPs has already been shown for sugar formulations in previous sections. The focus of this part was to study if the stability is transferrable to amino acid formulations. Results are listed in Table III-2.
ODN integrity was not affected in amino acid formulation after storage for three months at 2-8°C or 20-25°C. However, the MALDI-TOF signal in the Tre + Gly combination was low, which may indicate starting ODN degradation even if no additional peak was detected.
At accelerated temperature, only His was adequate to stabilise ODNs. In the other formulations, ODNs showed degradation by reduced signal intensity or complete degradation by a not detectable signal.

Table III-2 Oligodeoxynucleotide integrity after lyophilisation of amino acid containing formulations. ODN integrity is represented with symbols: ✓ stable ODN, × degraded ODN, ± indications for starting degradation.

Formulation	**2-8°C** 3 months	**20-25°C** 3 months	**40°C** 4 weeks
His	✓	✓	✓
His + Arg	✓	✓	×
His + Gly	✓	✓	±
Arg + Gly	✓	✓	×
Arg + His + Gly	✓	✓	±
Suc + Gly	✓	✓	±
Tre + Gly	±	±	×

Several reasons are conceivable for ODN degradation in these formulations and shall be discussed: I.) Lower ODN protection because of reduced loading efficiency, II.) ODN degradation induced by particle aggregation, III.) pH dependent ODN degradation during holding time between rehydration and MALDI-TOF measurement and IV.) ODN degradation at elevated temperature by increasing residual moisture content.

I.) No correlation between ODN degradation and loading efficiency was found. The only formulation that protected ODNs at 40°C showed the lowest loading efficiency, whereas no intact ODN was detectable in the formulation with the highest loading efficiency.

II.) The formulation Arg + Gly showed strong particle aggregation after storage at 40°C and was not suitable to stabilise ODNs. However, other formulations with degraded ODNs did not tend to aggregate. This implies that particle aggregation may be involved in ODN degradation, but not the only reason for it.

III.) ODN stability tests at different pH values between 4.5 and 10.5 revealed that ODN degradation after 24 hours was only detectable at pH 10.5, whereas the highest pH of the examined formulations was 9.09 (annex). Furthermore, there is no correlation between pH and ODN degradation.

Regarding pH value, ODN degradation seems to be randomly distributed. This leads to the conclusion that a pH driven reaction is not the reason for ODN degradation in these formulations.

IV.) A correlation between ODN degradation at accelerated temperature and the residual moisture content was already discussed in the sections dealing with sugar formulations. Amino acid formulations indicated lower hygroscopicity compared to sugar formulations and more stable Tg values (for more information see annex). However, no relation between the increase in residual moisture and the extend of ODN degradation was observed for the amino acid formulations. This leads to the conclusion that in contrast to sugar formulations, residual moisture content is not the driving factor of ODN degradation after freeze-drying in the amino acid containing formulations.

In summary, it can be stated that amino acids can generally be used as excipients for lyophilisation of ODN-loaded GNPs. However, due to low Tg' values, process time, costs and energy consumption are affected. Additionally, except for histidine, the investigated amino acids seem to be inferior for long term stability compared to standard sugars, such as sucrose and trehalose.

For glycine, this can be related to its crystalline state after lyophilisation [26]. It is well known that crystallising excipients are not able to protect proteins during lyophilisation [30]. However, in the field of nanoparticles there is disagreement in literature about the effects of crystalline agents, such as glycine or mannitol. On the one hand, the particle isolation hypothesis conveys that a spatially separation of the particles is sufficient to prevent them from aggregation [49]. This can also be achieved by crystalline excipients. On the other hand, e.g. a study on albumin nanoparticles showed a reduced stabilisation capacity of mannitol compared to amorphous sugars that was attributed to crystallisation [36]. For GNPs, Zillies et al. demonstrated that mannitol is sufficient to stabilise unloaded nanoparticles [15]. However, for oligonucleotide-loaded GNPs mannitol failed to prevent

aggregation [15]. Our study confirms for amino acids that crystallisation of the excipients is disadvantageous, at least for ODN-loaded GNPs.
Negative effects of arginine are further discussed in Chapter IV.

4 CONCLUSION

Stability of lyophilised ODN-loaded GNPs was proved for at least six months at 2-8° and 20-25°C. MALDI-TOF was found to be a versatile tool to investigate ODN integrity.
Freeze thaw studies using conventional shelf ramped freezing versus controlled nucleation showed overall stability of ODN-loaded GNPs to stresses induced during freezing.
Controlled ice nucleation leads to slightly reduced drying time in lyophilisation of ODN-loaded GNPs. Nevertheless, no further clear advantages compared to standard lyophilisation were noticed.
Amino acids can be used as excipients in freeze-drying of ODN-loaded GNPs. However, ODNs stability during storage is reduced at accelerated temperatures compared to sugar based formulations. Amongst the investigated formulations, only pure histidine is adequate to completely stabilise ODN-loaded GNPs upon storage.

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6 ANNEX

6.1 CONVENTIONAL LYOPHILISATION

6.1.1 LOADING EFFICIENCIES

Loading efficiencies related to section 3.1.2 of the main text are listed in Table A 1

Table A 1 Loading efficiencies of ODN-loaded GNPs before freeze-drying, directly after freeze-drying, after six months storage at 2-8°C, after six months storage at 20-25°C and after four weeks storage at 40°C. Results are represented as mean ± SD (n=3).

Formulation	Loading efficiency [%]				
	Before lyophilisation	After lyophilisation	6 months 2-8°C	6 months 20-25°C	4weeks 40°C
S100	98.4 ± 0.8	99.4 ± 0.4	100.1 ± 0.1	101.1 ± 0.2	99.8 ± 0.6
S500	97.9 ± 0.3	99.8 ± 0.3	100.5 ± 0.1	100.8 ± 0.3	101.7 ± 0.4
S1333	95.1 ± 0.7	94.7± 0.3	97.6 ± 0.3	99.4 ± 0.2	101.8 ± 0.8
T100	95.6 ± 3.7	97.6 ± 0.8	100.2 ± 0.2	100.9 ± 0.4	101.8 ± 0.6
T500	96.7 ± 2.2	97.3 ± 0.2	100.6 ± 0.5	100.5 ± 0.5	93.5 ± 0.7
T1333	92.0 ± 2.6	91.5 ± 3.6	97.5 ± 0.3	92.1 ± 4.9	98.6 ± 0.4

6.1.2 ODN INTEGRITY

A representative MALDI-TOF spectrum of ODNs that show indications for starting degradation is displayed in Figure A 1. Related discussion can be found in section 3.1.3 of the main text.

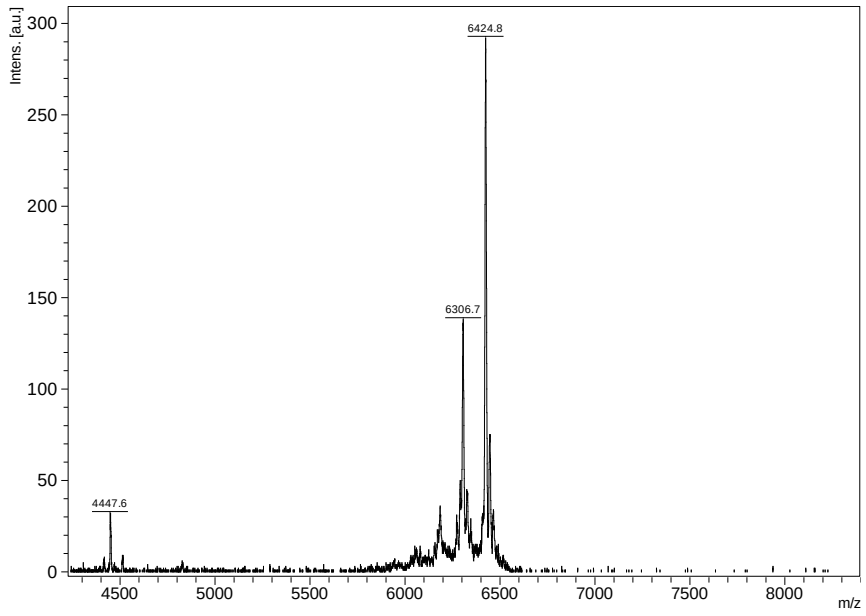

Figure A 1 MALDI-TOF spectrum of ODNs in formulation S100 after storage of four weeks at 40°C. Additional peak represents starting degradation of ODNs.

6.1.3 RESIDUAL MOISTURE AND GLASS TRANSITION

The following section is a detailed description of the results discussed in section 3.1.4 of the main text.

Initially measured water contents of all lyophilised ODN-loaded GNPs were below or close to 1% (Figure A 2). However, upon storage, an increase in residual moisture content of the sucrose containing formulations could be observed. This was most pronounced in the low concentration samples and hardly detectable in the highest sugar concentration. This finding is in accordance with observations of Zillies et al. [1]. They also noticed increasing residual moisture contents upon storage of ten weeks. However, they saw no further increase after four weeks. An equilibrium moisture content was reached after four weeks of storage. For the S100 formulation we saw a similar steady state residual moisture after four weeks of storage. Furthermore, a correlation between storage temperature and moisture increase is observed. The lower the storage temperature, the less distinct is the increase of residual moisture content.

The same trend can be seen in the trehalose formulations (Figure A 2), whereas water sorption was not that marked, especially the samples stored at 2-8°C showed hardly increase in residual moisture. This is in contrast to previous results that demonstrated slightly higher hygroscopicity of trehalose containing freeze-dried GNPs [1]. However, our results are in accordance with other research that stated trehalose to be less hygroscopic compared to other sugars [2].

It is known from the work of Zillies et al. that aggregation of lyophilised GNPs is initiated at residual moisture values of 5% [1]. Below this threshold, no particle aggregation was observed. These findings are confirmed by our study, as we did not detect increasing PDI values even in samples with high residual moisture of around 3%. However, the starting ODN degradation noticed in the lowest concentration sugar formulations at 40°C storage may be related to the increasing water content and elevated temperature [3].

Measured initial Tg values (Figure A 2) of all sucrose (~ 60°C) and trehalose (~ 100°C) formulations agree with values from literature [4].

Sucrose formulations S500 and S1333 showed stable Tg values upon six months storage independent of storage conditions. Only the lowest sucrose concentration demonstrated a decrease of Tg, which is stronger the more pronounced is the increase in residual moisture and the higher is the storage temperature. The Tg values of samples stored at 2-8°C stayed above 40°C during the complete storage duration, whereas Tg values of samples stored at room temperature dropped just below 40°C. Measured Tg values of S100 samples at 40°C were even below 35°C after four weeks. As this value is below the storage temperature, particle aggregation and ODN degradation are more likely. This assumption is supported by the observation of starting ODNs depletion in MALDI-TOF spectra. However, particle aggregation is not yet induced.

Similar trends can be noticed for the trehalose formulations (Figure A 2). The higher concentrated formulations (T500 and T1333) showed hardly changes in Tg values in all storage conditions, whereas T100 samples behaved comparable to S100 samples. Admittedly, Tg remained overall at higher values than the sucrose samples and did not fall below storage temperatures. However, the formulation with the highest residual moisture and the lowest Tg (T100) also showed first evidence of ODN degradation expressed in a very low MALDI-TOF signal. Equally to S100, no GNP aggregation was observed.

These findings regarding residual moistures and Tg values are in accordance with results reported by Zillies et al. [1, 5].

Taking residual moistures and Tg values into account, it can be stated that for long term stability trehalose is superior to sucrose and storage at 2-8°C is recommended for lyophilised ODN-loaded GNPs.

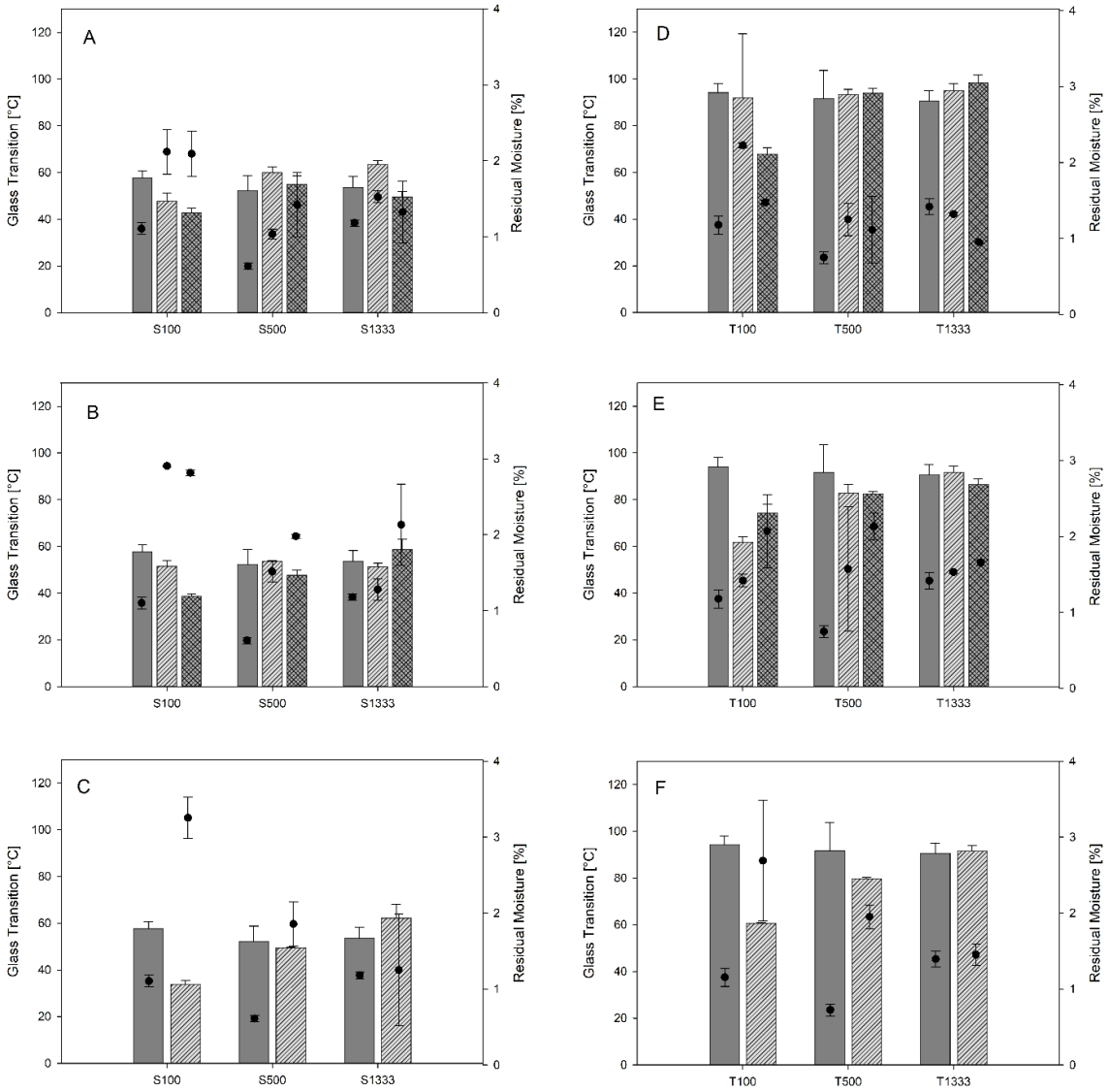

Figure A 2 Glass transition temperatures (bars) and residual moisture contents (dots) of freeze-dried ODN-loaded GNPs directly after lyophilisation, after four weeks of storage and six months of storage. Sucrose formulations at A: 2-8°C, B: 20-25°C and C: 40°C. Trehalose formulations at D: 2-8°C, E: 20-25°C and F: 40°C. Results are represented as mean + or ± SD (n=2).

6.2 CONTROLLED NUCLEATION

The following sections represent a detailed report about the results regarding controlled nucleation given in section3.2.2 of the main text.

6.2.1 PARTICLE SIZES AND PDI VALUES

Particle sizes of ODN-loaded GNPs freeze-dried after controlled nucleation (Figure A 3) were comparable to conventional lyophilisation. This supports our initial results of GNPs being more stable during controlled nucleation than polyplexes. Similar to conventionally freeze-dried ODN-loaded GNPs, particle sizes were smaller after lyophilisation than before. The hypothesis of no full rehydration of GNPs after freeze-drying [5] is again confirmed.

Furthermore, particle sizes remained stable upon storage. This again indicates no damage of the nanoparticles during controlled ice nucleation, which would lead to aggregation during storage. Beyond this, PDI values did not change during storage, which also indicates that no aggregation occurs (Figure A 3).

However, formulation S100 showed increasing particle size and PDI value at accelerated temperature indicating starting aggregation. Consequently, similar to conventional lyophilisation, it can be stated that trehalose is superior in stabilising ODN-loaded GNPs compared to sucrose. Besides this, results of different sugar types and storage conditions were equivalent.
Hence, the main finding of that part of the study is that controlled nucleation allows only minor process shortage and has no negative impact on particle sizes and monodispersity of ODN-loaded GNPs.

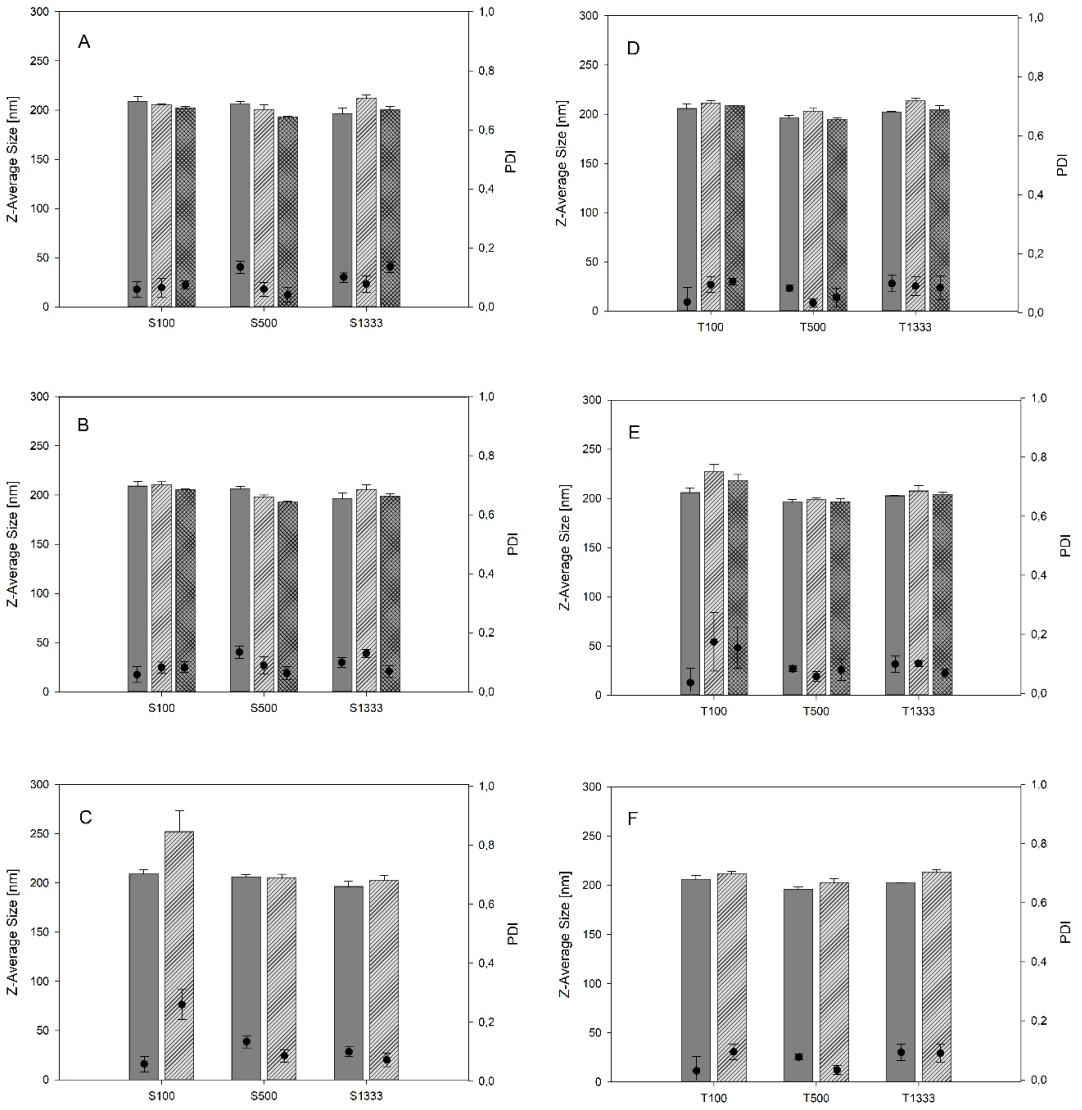

Figure A 3 Particle sizes (bars) and PDI values (dots) of ODN-loaded GNPs freeze-dried via controlled nucleation directly after lyophilisation (left bar), after four weeks of storage (middle bar) and three months of storage (right bar). Sucrose formulations at A: 2-8°C, B: 20-25°C and C: 40°C. Trehalose formulations at D: 2-8°C, E: 20-25°C and F: 40°C. Results are represented as mean + or ± SD (n=3).

6.2.2 LOADING EFFICIENCIES

As a further parameter for stability, loading efficiencies were evaluated after freeze-drying and storage. As one can see from Table A 2, loading efficiencies remained stable during freeze-drying with controlled nucleation and following storage at different conditions. Loading efficiencies behaved comparable to those of the conventionally lyophilised samples. The positive net charge of GNPs and

their interaction with ODNs was not affected by controlled nucleation, which is independent of sugar type or storage temperature. This further shows the superior stability of ODN-loaded GNPs compared to ODN-loaded polyplexes [6].

Table A 2 Loading efficiencies of ODN-loaded GNPs before freeze-drying, directly after freeze-drying using controlled nucleation, after three months storage at 2-8°C, after three months storage at 20-25°C and after four weeks storage at 40°C. Results are represented as mean + SD (n=3).

Formulation	Loading efficiency [%]				
	Before lyophilisation	After lyophilisation	3 months 2-8°C	3 months 20-25°C	4 weeks 40°C
S100	98.8 ± 1.9	95.4 ± 0.6	93.0 ± 0.6	92.9 ± 0.5	96.5 ± 6.0
S500	98.9 ± 3.5	96.5 ± 1.6	93.7 ± 0.6	97.6 ± 0.5	96.2 ± 0.2
S1333	102.1 ± 2.2	95.5± 0.6	97.1 ± 0.2	98.5 ± 0.6	89.0 ± 0.3
T100	96.9 ± 3.4	95.5 ± 0.6	92.0 ± 1.2	92.5 ± 0.6	94.8 ± 0.6
T500	100.7 ± 0.4	97.0 ± 1.0	94.5 ± 0.6	97.9 ± 0.3	93.2 ± 0.6
T1333	100.3 ± 1.5	101.9 ± 0.7	96.6 ± 0.3	97.0 ± 0.3	98.9 ± 0.3

6.2.3 ODN INTEGRITY

The important factor regarding activity of ODN-loaded GNPs is the integrity of the oligodeoxynucleotides. Table A 3 depicts that the ODNs were not affected by controlled nucleation followed by lyophilisation in all formulations and were stable upon storage at all conditions. In contrast to conventional lyophilisation, no ODN degradation in the low excipient containing formulations S100 and T100 was apparent at 40°C. Hence, at least for low sugar content formulations, freezing via controlled nucleation prior to lyophilisation seems to be advantageous regarding ODN integrity.

Table A 3 Oligodeoxynucleotide integrity after lyophilisation combined with controlled nucleation. ODN integrity is represented with symbols: ✓ stable ODN, × degraded ODN, ± indications for starting degradation.

Formulation	Controlled nucleation		
	2-8°C 3 months	20-25°C 3 months	40°C 4 weeks
S100	✓	✓	✓
S500	✓	✓	✓
S1333	✓	✓	✓
T100	✓	✓	✓
T500	✓	✓	✓
T1333	✓	✓	✓

6.2.4 Residual Moisture and Glass Transition

In the conventional lyophilisation experiment, ODN degradation was associated with a higher residual moisture. To assess this correlation, residual moisture contents of the controlled nucleated samples were analysed and discussed in this section.

Residual moisture values of controlled nucleated (Figure A 4) and conventional frozen samples (Figure A 2) hardly differed directly after freeze-drying and at the end of the storage period. Intriguingly, residual moistures of the lowest sugar concentrations reached their maximum value after three months, whereas in the conventional samples the maximum was already attained after four weeks. This suggests a slower water uptake rate of the controlled nucleated samples, which may be explained by a lower specific surface area. However, final residual moistures after three months were even higher than of the conventionally freeze-dried products after six months. Nonetheless, increased water absorption seems not to be critical regarding three months stability at 2-8°C and 20-25°C, as it did not lead to particle aggregation or ODN degradation. However, for an industrial production and long-term storage, methods to control residual moisture contents are highly recommended.

It is important to be careful when comparing ODN stability in S100 and T100 formulations at accelerated conditions. In the controlled nucleated samples ODNs is stable for four weeks, but residual moisture is obviously lower than in the conventional samples. This supports the presumption that ODN degradation at elevated temperature is directly related to the water content of the lyophilisates.

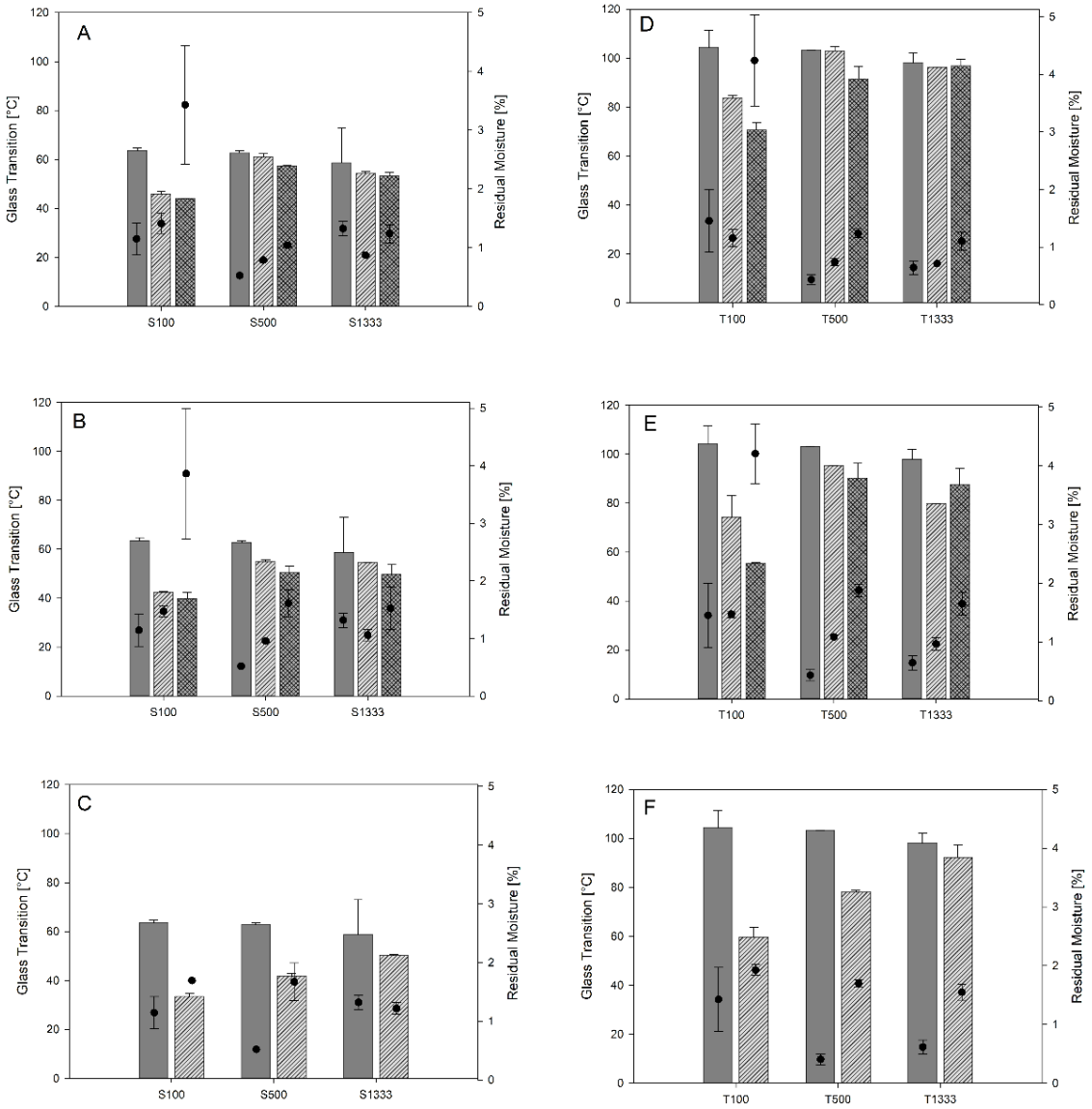

Figure A 4 Glass transition temperatures (bars) and residual moisture contents (dots) of ODN-loaded GNPs freeze-dried via controlled nucleation directly after lyophilisation, after four weeks of storage and three months of storage. Sucrose formulations at A: 2-8°C, B: 20-25°C and C: 40°C. Trehalose formulations at D: 2-8°C, E: 20-25°C and F: 40°C. Results are represented as mean + or ± SD (n=2).

Evaluated Tg values (Figure A 4) are in accordance to literature [4] and comparable to Tg values of conventionally lyophilised samples (Figure A 2), whereas there can be seen a trend to slightly higher initial values.

Apart from that, Tg values of S500, S1333, T500 and T1333 were stable over the whole storage period at storage temperatures of 2-8°C and 20-25°C. Results were comparable to conventional freeze-drying. Interestingly, at 40°C S500 and T500 showed stronger Tg decrease than the corresponding

conventional samples. Tg values of the lowest sugar formulations behaved also comparable to conventionally dried samples resulting in a strong decrease associated with a strong increase in residual moisture.

6.3 AMINO ACIDS

6.3.1 LOADING EFFICIENCIES AND PH VALUES

Loading efficiencies related to section 3.3.3 of the main text are displayed in Table A 4.
In section 3.3.4 of the main text pH values of the formulations are discussed. These values are listed in Table A 5

Table A 4 Loading efficiencies of ODN-loaded GNPs in amino acid containing formulations before freeze-drying, directly after freeze-drying using controlled nucleation, after three months storage at 2-8°C, after three months storage at 20-25°C and after four weeks storage at 40°C. Results are represented as mean ± SD (n=3).

Formulation	Loading efficiency [%]				
	Before lyophilisation	After lyophilisation	3 months 2-8°C	3 months 20-25°C	4 weeks 40°C
His	94.6 ± 0.7	81.0 ± 1.0	92.5 ± 0.6	89.7 ± 0.5	83.6 ± 0.6
His + Arg	94.3 ± 0.6	84.8 ± 0.6	96.1 ± 0.6	90.0 ± 0.6	95.1 ± 0.3
His + Gly	96.0 ± 1.7	93.9 ± 0.6	94.9 ± 0.7	90.1 ± 0.7	92.9 ± 0.7
Arg + Gly	93.0 ± 0.5	96.3 ± 0.6	92.1 ± 0.7	87.8 ± 0.3	85.4 ± 0.7
Arg + His + Gly	93.8 ± 0.6	87.7 ± 0.6	92.3 ± 0.5	88.8 ± 0.9	90.0 ± 0.7
Suc + Gly	97.2 ± 0.8	99.6 ± 0.6	100.4 ± 0.5	100.7 ± 0.5	97.8 ± 0.7
Tre+Gly	98.2 ± 0.3	100.2 ± 0.6	101.4 ± 1.6	101.2 ± 1.1	99.1 ± 0.4

Table A 5 pH values of all investigated formulations. Results are represented as mean ± SD (n=3).

Formulation	pH value	Formulation	pH value	Formulation	pH value
S100	4.33 ± 0.04	Suc + Gly	5.96 ± 0.09	Gly 2.5	6.00 ± 0.04
S500	4.56 ± 0.08	Suc + His	7.82 ± 0.06	Gly 5.0	5.74 ± 0.05
S1333	4.51 ± 0.07	Tre + Arg	10.60 ± 0.06	His	7.59 ± 0.04
T100	4.46 ± 0.11	Tre + Gly	5.98 ± 0.05	Arg + His	9.09 ± 0.03
T500	4.69 ± 0.17	Tre + His	7.57 ± 0.07	Arg + Gly	9.08 ± 0.06
T1333	4.52 ± 0.11	Arg 2.5	10.59 ± 0.07	His + Gly	7.38 ± 0.05
Suc + Arg	10.53 ± 0.06	Arg 5.0	10.77 ± 0.06	Arg + His + Gly	9.02 ± 0.07

6.3.2 Residual Moisture and Glass Transition

The following section gives further information on residual moisture contents and glass transition values mentioned in section 3.3.4 of the main text.

Freeze-drying of amino acid containing formulation led to residual moisture contents below 1%, except for formulation Arg + Gly with a higher residual moisture content of ~1.4% (Figure A 5). This high initial water content may have triggered particle aggregation in this formulation during storage at 40°C due to higher product mobility.

Upon storage none of the formulations, independent of storage temperature, exceeds a residual moisture content above 2% (Figure A 5). This indicates lower hygroscopicity or a slowed down water sorption rate compared to sugar formulations.

A stable residual moisture content is normally related to a higher product stability. There is evidence to suggest that this pertains for the sugar formulations of ODN-loaded GNPs at higher temperature. However, it could not be confirmed by our study regarding the amino acids. A starting ODN degradation in the amino acid formulations at 40°C was detected, whereas ODN were stable in more hygroscopic sugar formulations. Furthermore, there was no relation between the increase in residual moisture and the extend of ODN degradation observed. The formulations with the highest water content after 4 weeks at 40°C were Arg + His, His + Gly, Arg + His + Gly, but in only one of them no ODN signal could be detected. On the other hand, Tre + Gly was overall one of the driest formulations, but ODN degradation started already at 2-8°C.

So far, there is little information on glass transition temperatures of sugar free lyophilised amino acids available. For lyophilised arginine a Tg of 42°C was reported by Mattern et al., whereas a Tg of 37°C was declared for histidine [7]. This is not consistent with our findings. The measured Tg of the ODN-loaded GNP formulation with pure histidine was found to be much higher,

at 105°C (Figure A 5). However, Mattern et al. did not comment if they used histidine base or a salt. This would be important information, as the counterion may have an influence on glass transition temperatures [8, 9].

Initial Tg values of Suc + Gly were lower than for pure sucrose. This could be expected due to the Tg lowering effects of glycine [10]. The same phenomenon was observed for the Tre + Gly and His + Gly formulations. A Tg lowering effect can also be supposed for arginine, as the Arg + His formulation revealed a clearly lower Tg of 67°C compared to pure histidine. However, the combination of all three amino acids did not further lower the Tg value. Nonetheless, the combination of Arg + Gly strongly increased the standard deviation (Figure A 5).

During storage at 2-8°C and 20-25°C, no decrease in Tg could be detected in the amino acid containing formulations, except for pure histidine. This is in correlation with the low increase in residual water content. Only at accelerated conditions a reduction in Tg was annotated in most of the formulations (Figure A 5).

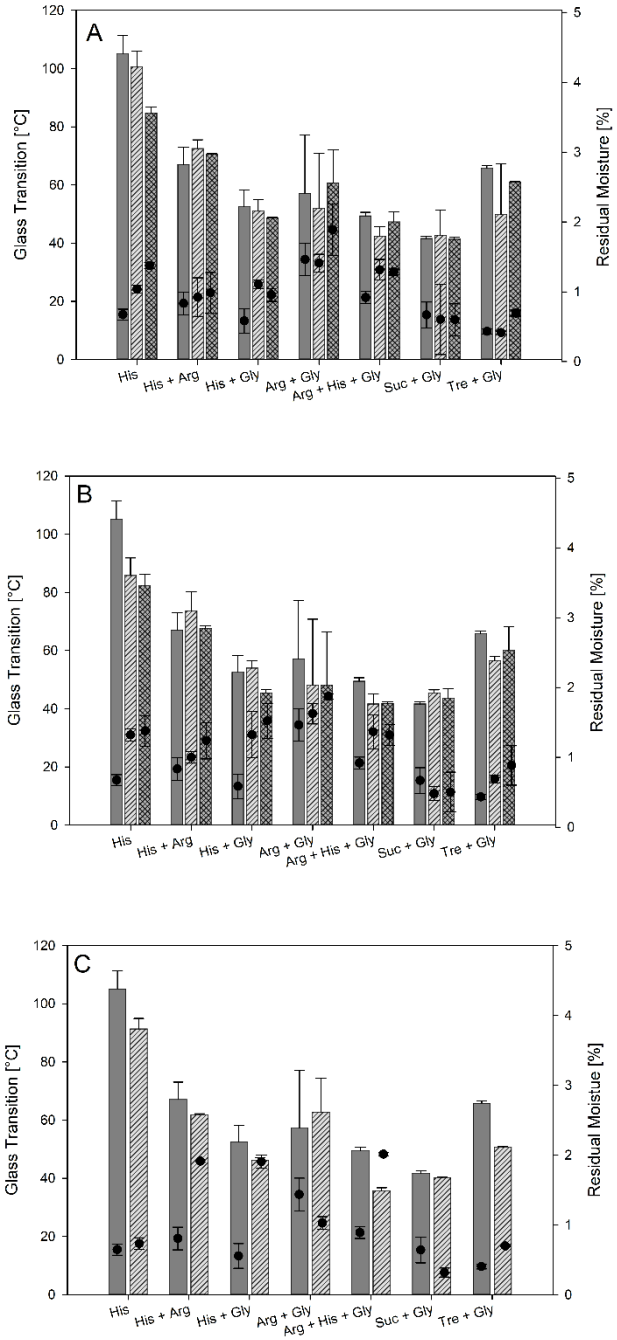

Figure A 5 Glass transition temperatures (bars) and residual moisture contents (dots) of freeze-dried ODN-loaded GNPs in amino acid containing formulations directly after lyophilisation (left bar), after four weeks of storage (middle bar) and three months of storage (right bar). Storage at A: 2-8°C, B: 20-25°C and C: 40°C. Results are represented as mean + or ± SD (n=2).

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CHAPTER IV

STERILISATION OF GELATINE NANOPARTICLES

Parts of the following chapter are intended to be published in *European Journal of Pharmaceutics and Biopharmaceutics*:

Katharina J. Geh, Madlen Hubert, Gerhard Winter. Progress in formulation development and sterilisation of freeze-dried oligodeoxynucleotide-loaded gelatine nanoparticles. *Submitted to European Journal of Pharmaceutics and Biopharmaceutics.*

ABSTRACT

Sterilisation is an important prerequisite for drug products applied via the parenteral route. Steam sterilisation is the most common method and recommended by pharmaceutical authorities for aqueous formulations. This work investigated steam sterilisation for its applicability to sterilise gelatine nanoparticles (GNPs). GNP dispersions were subjected to different autoclave treatments and subsequently analysed for particle sizes, size distributions, particle concentrations, cross-linking degrees and protein secondary structures. GNPs mostly remained stable during standard steam sterilisation conditions (121°C,15 min), whereas harsher conditions led at least partly to degradation. The second part of the study included the investigation of gamma irradiation for sterilisation of lyophilised ODN-loaded GNPs. Different excipients, such as sugars and amino acids, were analysed for their suitability to stabilise GNPs and ODNs during irradiation. Analytics included particle characteristics, size distributions, loading efficiencies, and ODN integrity. Gamma irradiation has proven to be a versatile sterilisation method for ODN-loaded GNPs. Additionally, sugars have shown be superior in stabilising and protecting during gamma irradiation compared to amino acids.

KEYWORDS

Steam sterilisation, sterilisation, gamma irradiation, MALDI-TOF, gelatine nanoparticles

1 INTRODUCTION

Nanoparticles are widely researched as drug delivery systems for different kinds of drugs. Amongst a variety of starting materials, such as poly (lactic-co-glycolic) acid (PLGA), albumin, chitosan or lipids, gelatine has proven to be very promising. Gelatine nanoparticles (GNPs) have been successfully used as carriers for immunomodulatory oligodeoxynucleotides (ODNs) in several veterinary clinical studies treating allergic diseases, such as equine recurrent airway obstruction or canine atopic dermatitis [1-5].

An important critical quality attribute and prerequisite for medicinal application via the inhalative and parenteral route is sterility. So far, an aseptic particle production and loading process is necessary to ensure appropriate product quality. However, working aseptically is always critical due to a lot of potential contamination risks caused by inadequate handling, which cannot completely eliminated by validation or monitoring [6, 7]. Consequently, aseptic preparation should be circumvented if possible in order to ensure reliable patient safety [7]. The most common and safest way to achieve a sterile product is steam sterilisation, preferably performed in the final product container. The reason for using this approach is a controllable, validatable and calculatable sterility assurance level [6]. However, steam sterilisation of nanoparticles is challenging due to different stability issues. For instance, it is known from literature that the harsh conditions during autoclaving induce degradation and hydrolysis of PLGA [8]. This results in the loss of structural integrity of the particles. Furthermore, an acidic microclimate emerges due to the immediate release of lactic and glycolic acid, which may cause degradation of the loaded drug [9]. Information on effects of steam sterilisation on lipidic composites, such as solid lipid nanoparticles (SLNs) or liposomes is contradictory. Depending on the lipid composition or buffer system, steam sterilisation may be applicable to these systems or cause particle aggregation and lipid degradation [10-12]. On the other hand, nano- and microparticles prepared from recombinant spider silk protein showed excellent resistance during steam sterilisation [13]. Even extended

sterilisation conditions, such as prolonged or repeated autoclaving did not negatively impact on the spider silk particles.
First aim of this study was to evaluate the effects of steam sterilisation on gelatine nanoparticles. We could show that standard autoclaving conditions (121°C, 15 min) can be applied to sterilise GNPs without negative impact on particle properties.

However, due to thermal sensitivity of oligodeoxynucleotides, steam sterilisation after particle production and ODN loading is not advisable [14]. Aside from this, particle sizes of GNPs do not allow sterile filtration of the final formulation. Hence, even if pre-sterilised GNPs can be provided, an aseptic loading process would be still essential to guarantee adequate product quality for parenteral application. Accordingly, there is a need to establish a possibility of sterilisation of ODN-loaded GNPs. An alternative procedure to sterilise pharmaceutical products accepted by the European Pharmacopeia and United States Pharmacopeia is gamma irradiation [15]. Gamma irradiation is an advantageous method due to high penetration depth, low temperature rise and no accumulation of toxic residues [15]. It has been reported to be a versatile method to sterilise polymeric nano- and microparticles. Several studies using synthetic (e.g. PLGA) or natural occurring polymers (e.g. casein or chitosan) showed the appropriate use of gamma rays without impact on these drug delivery systems. However, most of these particles were loaded with small molecules instead of biopharmaceuticals. It is known that gamma irradiation can cause degradation and functional loss of biomolecules, such as proteins [16, 17]. Nonetheless, the addition of protection agents, such as amino acids [18, 19] or antioxidants [17], allows successful sterilisation of proteineous products. Furthermore, sterilisation of the protein in a dry state, such as lyophilised or spray dried, is beneficial to maintain its function [17, 19].
In the present work, we could show that lyophilised ODN-loaded GNPs can be sterilised in their final container by gamma irradiation without any adverse effects on particle characteristics or ODN integrity.
Summarising, this work provides different approaches to sterilise unloaded and ODN-loaded GNPs, which is an important step into commercialisation of GNPs.

2 MATERIALS AND METHODS

2.1 MATERIALS

Gelatine type A 300 bloom was provided from Gelita AG (Eberbach, Germany). Acetone was obtained from Fisher Chemicals (Loughborough, UK). Glutaraldehyde (25% solution), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, (2-Aminoethyl) trimethylammonium chloride hydrochloride, L-arginine base, L-histidine base, L-glycine base, TNBS (2,4,6-trinitrobenzene sulfonic acid) and sodium bicarbonate were supplied by Sigma (Taufkirchen, Germany). D-(+)-Sucrose and D-(+)-Trehalose dihydrate were acquired from VWR International (Leuven, Belgium). In all experiments highly purified water (HPW) was used, which was produced by a PURELAB Plus device (conductivity < 0.055 µS/cm, Elga Labwater, Celle, Germany). Oligodeoxynucleotides (ODNs) were synthesized by biomers.net GmbH (Ulm, Germany).

2.2 PREPARATION OF CATIONISED GELATINE NANOPARTICLES

GNPs were prepared according to one-step desolvation [20]. Briefly, 750 mg gelatine type A (300 bloom) was dissolved in 25 mL HPW (3.0% w/v) under constant stirring at 50°C. Afterwards, pH was adjusted to a value between 2.5 and 3.0. Following, acetone was added dropwise to initiate GNP formation. A volume of 175 µL glutaraldehyde solution was added to cross-link GNPs. After overnight stirring, the dispersion was purified via three-fold ultrafiltration using a solvent resistant stirred cell and an ultrafiltration disc of regenerated cellulose and a molecular weight cut-off of 100'000 kDa (Millipore S.A.S., Molsheim, France).
For cationisation, GNP dispersion was diluted with HPW (~ 10 mg/mL) and pH was adjusted (4.5 - 5.0). Subsequently, EDC and Cholamine were added. After incubation for 30 min, the reaction mixture was purified by two-fold centrifugation (15000*g for 60 min; Sigma Laborzentrifugen, Osterode, Germany). Finally, nanoparticle

concentration was determined gravimetrically using a UMX2 ultra-microbalance (Mettler Toledo, Greifensee, Switzerland).

2.3 Oligodeoxynucleotide Loading of Gelatine Nanoparticles

For ODN loading, GNP dispersion was diluted with the respective excipient stock solution to a final concentration of 1.5 mg/mL and incubated with 5% (w/w) ODNs for 60 min at 21°C and under continuous shaking at 350 rpm (Thermomixer Comfort, Eppendorf AG, Hamburg, Germany). Sugar containing samples were prepared in excipient-to-ODN ratios of 100:1, 500:1 and 1333:1. This ratio is used to identify the samples in the following text. Amino acid formulations (L-arginine, L-histidine and L-glycine) were prepared at an excipient-to-ODN ratio of 333:1 (one amino acid, representing 2.5% [w/v]), 667:1 (two amino acids, equally mixed) and 1000:1 (three amino acids, equally mixed). Sugar amino acid combinations were based on a sugar-to-ODN ratio of 500:1, amino acids were added in the ratio 333:1.

2.4 Lyophilisation of ODN-Loaded Gelatine Nanoparticles

Freeze-drying of ODN-loaded GNPs was performed with a volume of 500 µL in 2R glass vials according to the conventional freeze-drying cycle described in chapter III, section 2.4 adapted from Zillies et al. [21]. An EPSILON 2-6D pilot scale freeze dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterrode, Germany) equipped with type T thermocouples (Newport Electronics, Deckenpfronn, Germany) for temperature monitoring was used. Upon finishing the cycle, the product chamber was vented with nitrogen and samples were stoppered at a chamber pressure of 800 mbar.

2.5 Steam Sterilisation

GNP dispersions (5 mg/mL) were steam sterilised in glass vials (DIN 2R, closed with stoppers and crimped with aluminium caps) using a GTA 50 autoclave (Fritz

Gössner, Hamburg, Germany). Samples were autoclaved for 15, 30 and 45 min at 121°C or 3 min at 134°C. Repeated sterilisation cycles were performed for 2 x 15 min and 3 x 15 at 121°C. Control samples were stored at 2-8°C.

2.6 Sterilisation by Gamma Irradiation

Lyophilised ODN-loaded GNP formulations were sterilised via gamma irradiation by a cobalt-60 source and an absorbed dose of 25 kGy (STERIS AST Allershausen GmbH, Germany).

2.7 Gelatine Nanoparticle Characterisation

Particle yield was determined gravimetrically using a UMX2 ultra-microbalance (Mettler Toledo, Greifensee, Switzerland). Particle size and polydispersity index (PDI) were ascertained by DLS using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). Derived countrates were calculated from DLS measurements using DTS Nano software (Malvern Instruments, Worcestershire, UK).

2.8 Determination of Loading Efficiency

Loading efficiency was indirectly determined by measuring UV absorbance at 260 nm (Agilent 8453 UV-visible spectrophotometer, Agilent Technologies, Santa Clara, California, USA) in the supernatant of centrifuged ODN-loaded GNPs, a GNP control and an ODN control [5]. Loading efficiency was calculated using the following equation:

$$\text{Loading efficiency } [\%] = 1 - \left(\frac{\text{A (ODN}-\text{loaded GNP)}-\text{A (GNP control)}}{\text{A (ODN control)}}\right) x\ 100\ \%.$$

2.9 Determination of Cross-Linking Degree

TNBS assay was performed to determine cross-linking degrees of GNPs before and after steam sterilisation procedures. In brief, the GNP dispersion was diluted with water to a concentration of 4 mg/mL. A volume of 0.25 mL GNP dispersion was mixed with a volume of 0.25 mL 0.05% TNBS (v/v) and 0.25 mL of 4% $NaHCO_3$ (w/v) (pH 8.5). Subsequently, the samples were incubated at 40°C in a Thermomixer (Eppendorf, Hamburg, Germany) for 2 hours under constant shaking (500 rpm). Afterwards, a volume of 750 µL of HCl 6M was added to each sample. Further incubation for 90 min at 60°C under constant shaking at 500 rpm followed. After that, samples were diluted with water for photometric determination of the reaction product at 349 nm (Agilent 8453 UV-visible sprectrophotometer, Agilent Technologies, Santa Clara, CA, USA). Blank samples of gelatine (≙ 0% cross-linking) and control samples of gelatine (≙ 100% cross-linking) were prepared. Control sample preparation included HCl addition prior to TNBS solution to avoid the reaction between TNBS and free amino groups of gelatine. Cross-linking degree was calculated by the following equation:

$$CL\ [\%] = \left(1 - \frac{A(sample) - A(blank)}{A(control) - A(blank)}\right) * 100\%$$

2.10 Scanning Electron Microscopy (SEM)

Gelatine nanoparticles were lyophilised according to the protocol described in chapter III, section 2.4 and immobilised on an aluminium sample grid. Following carbon sputtering, samples were analysed by a Helios NanoLab G3 UC scanning electron microscope (FEI, Hillsboro, Oregon, USA) at 3.0 kV and a working distance of 3.2 – 4.2 mm.

2.11 Dynamic Scanning Calorimetry (DSC)

Glass transition temperatures (Tg) were analysed using a Mettler DSC 821e (Mettler Toledo, Columbus, OH, USA). GNP dispersions were dried overnight in a vacuum drying cabinet at 10 mbar and 25°C and weighed into aluminium crucibles. To determine Tg values, samples were measured at a heating and cooling rate of 10 K/min from 0 to 150°C against an empty crucible as reference.

2.12 Fourier Transform Infrared Spectroscopy (FT-IR)

Fourier transform infrared spectroscopy (FT-IR) using the Bruker Tensor 27 FT-IR spectrometer (Billerica, USA) was performed to analyse protein secondary structure. Particle dispersions were examined by adding 20 µl into a BioATRCell II (Harrick Scientific, Pleasantville, USA) at a temperature of 25°C. Each spectrum comprises the average of 120 scans at a resolution of 4 cm^{-1} and was performed in triplicate. Data was analysed with the Bruker OPUS software (version 6.5).

2.13 Matrix-Assisted Laser Desorption/Ionisation Time-of-Flight Mass Spectrometry (MALDI TOF)

After desalting on a 0.025 µm VSWP membrane (Merck Millipore, Darmstadt, Germany), samples were co-crystallised in a 3-hydroxypicolinic acid matrix (HPA). Matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry was performed in negative mode using a Autoflex II (Bruker Daltonics, Germany) and a AnchorChip®-Target (Bruker MTP var/384).

3 Results and Discussion

3.1 Steam Sterilisation

Providing a sterile product is essential for the inhalative or parenteral application of a drug product. According to the European Medicines Agency, steam sterilisation is the method of choice for aqueous formulations [6]. Based on these authority requirements, the aim of this study was to evaluate the effects of steam sterilisation on gelatine nanoparticles.

3.1.1 Visual Inspection

Visual inspection of the autoclaved samples revealed no change in colour or turbidity of GNPs treated at 121°C, but a tremendous change in optical appearance of GNPs autoclaved at 134°C (Figure IV-1). These samples became almost clear after autoclave treatment. Consequently, it can be assumed that particle integrity was damaged during steam sterilisation and GNPs subsequently dissolved.

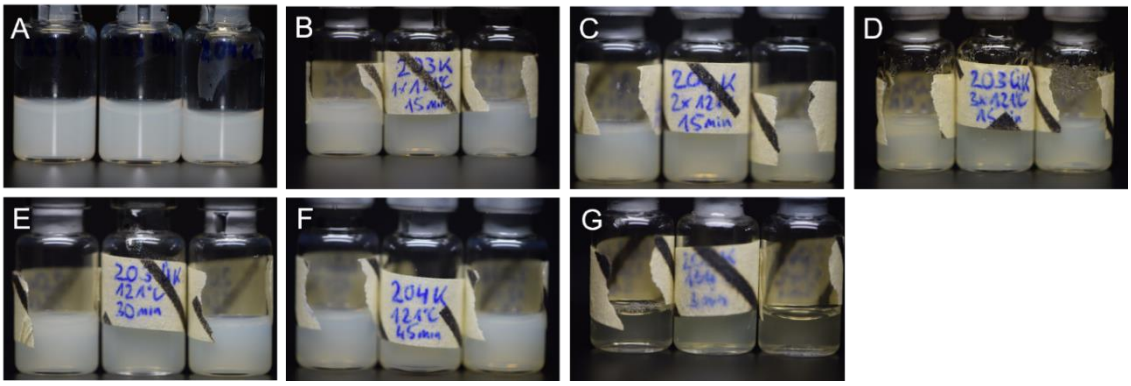

Figure IV-1 Optical appearance of GNPs after different steam sterilisation procedures. A: Not autoclaved reference sample, B: 15 min at 121°C, C: 2x 15 min at 121°C, D: 3x 15 min at 121°C, E: 30 min at 121°C, F: 45 min at 121°C, G: 3 min at 134°C.

3.1.2 Particle Sizes and PDI Values

Particle sizes and PDI values of GNPs after different procedures of steam sterilisation were analysed (Figure IV-2). A slight increase in particle sizes was

noticed after the standard autoclaving process for 15 minutes at 121°C, whereas monomodal size distribution remained stable (PDI < 0.1). This indicates a certain swelling of the particles caused by moist heat. Repeated (2-fold or 3-fold) or prolonged (30 min or 45 min) sterilisation cycles at 121°C did not further influence particle characteristics. However, autoclaving at 134°C and consequent elevated pressure of 3 bar resulted in an escalation in particle sizes and PDI values. Nonetheless, PDI values were still around 0.2. Based on these results, particle aggregation is not likely, but a stronger and more irregular swelling of the GNPs compared to standard autoclave treatment.

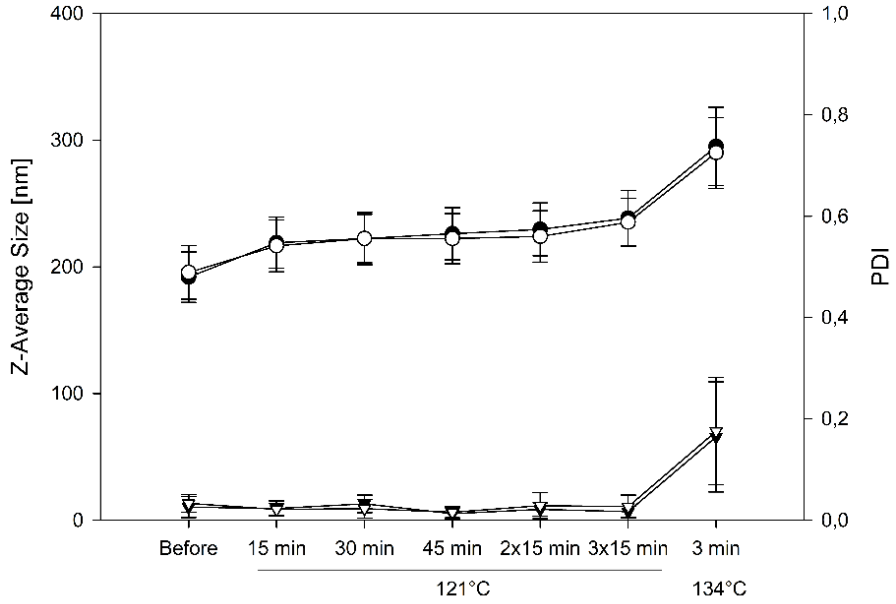

Figure IV-2 Particle sizes (dots) and PDI values (triangles) of plain GNPs after different steam sterilisation procedures (black) and subsequent two weeks storage at 2-8°C (white). Data is represented as mean ± SD (n=3).

All particle sizes and PDI values of GNPs after autoclaving remained stable during subsequent storage at 2-8°C for two weeks (Figure IV-2). Furthermore, optical appearance did not further change. This implies that no damage occurred, which would impact storage stability of steam sterilised GNPs.

Findings from DLS measurements can be supported by SEM micrographs (Figure IV-3). All particles retained their round shape and smooth surface after autoclave treatment. However, steam sterilisation at 134°C caused intense swelling of the GNPs.

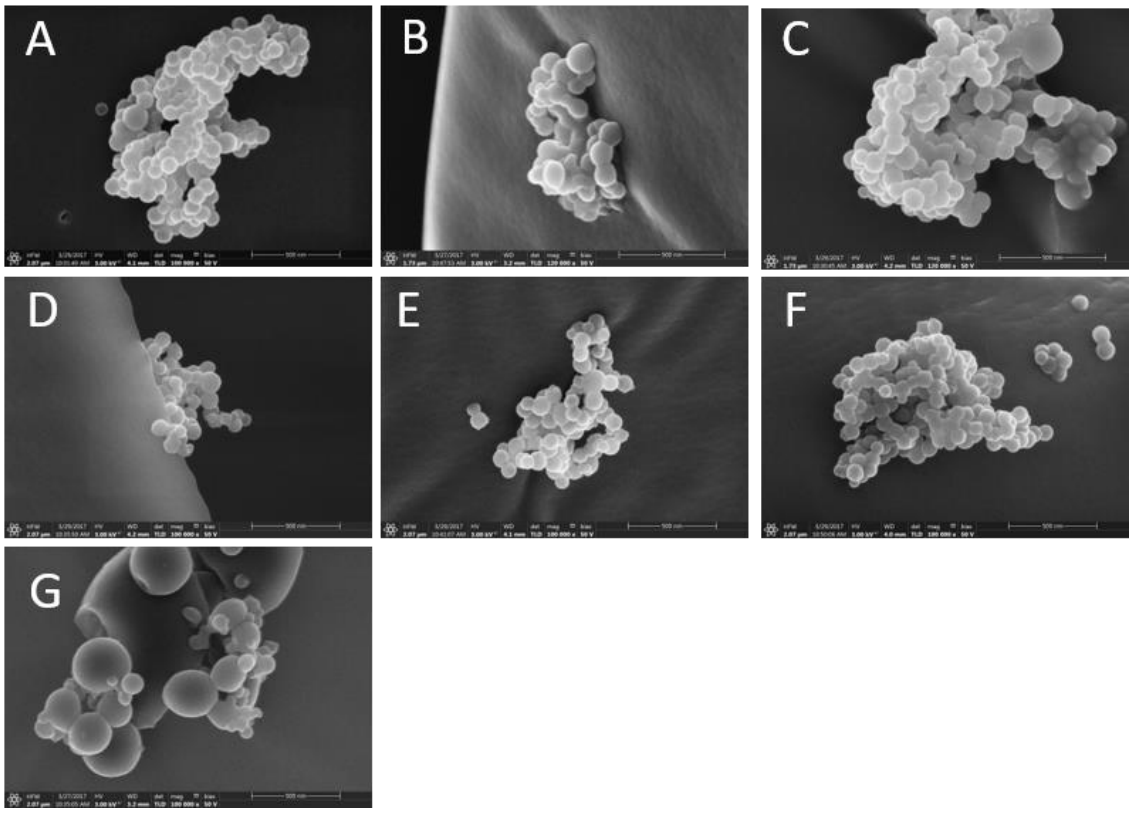

Figure IV-3 SEM micrographs of GNPs after different procedures of steam sterilisation. A: Not autoclaved reference sample, B: 15 min at 121°C, C: 2x 15 min at 121°C, D: 3x 15 min at 121°C, E: 30 min at 121°C, F: 45 min at 121°C, G: 3 min at 134°C. Size bar represents 500 nm in each individual image.

3.1.3 Derived Count Rates and Cross-Linking Degrees

These results demonstrate that steam sterilisation at 134°C had a negative impact on GNPs compared to steam sterilisation at 121°C. However, so far, one cannot explain the discolouration of the samples. To clarify this phenomenon, the derived countrates of the particular DLS measurements were analysed. The derived countrate is a theoretic value that describes the respective light scattering without

laser attenuation calculated from values obtained by attenuated laser intensity [22]. This allows to directly compare the countrates of different samples and consequently infer on particle concentrations. Figure IV-4 displays the derived countrates of GNPs treated by steam sterilisation. From these data it can be concluded that autoclave treatment causes particle damage paired with particle loss and particle dissolution. This effect is more pronounced the stronger is the stress during steam sterilisation. Interestingly, a repeated autoclaving procedure, including multiple heating and cooling steps, is more harmful to GNPs than the corresponding prolonged process (e.g. 2x15 min vs. 30 min). Furthermore, steam sterilisation at 134°C results in a tremendous loss in derived countrate of ~ 95%. Consequently, one can expect an immense decrease in particle concentration, which provoked that the particle dispersions became almost clear.

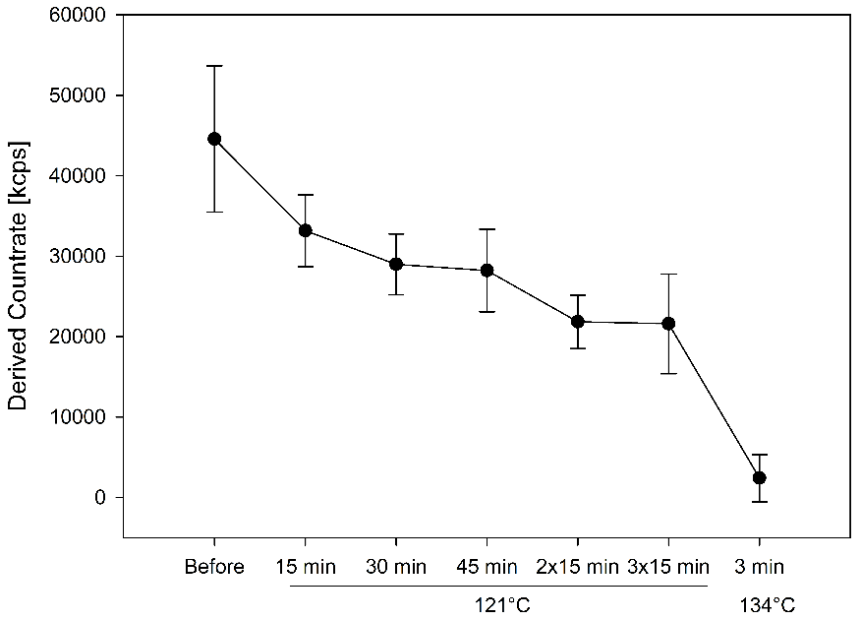

Figure IV-4 Derived countrates in DLS measurements of GNPs before and after different steam sterilisation procedures. Data is represented as mean ± SD (n=3).

The described particle dissolution induced by steam sterilisation was further confirmed by evaluation of cross-linking degrees (Figure IV-5). One can deduce a reduction in cross-linking degree, which was more pronounced the harsher the

sterilisation conditions were. A reduced cross-linking degree signifies an increased number of free amino groups in the sample. Consequently, this implies degradation of GNPs. Considering these data, it can be presumed that steam sterilisation at 134°C led to an almost complete disintegration of GNPs, whereas a large population of GNPs withstood autoclave treatment at 121°C.

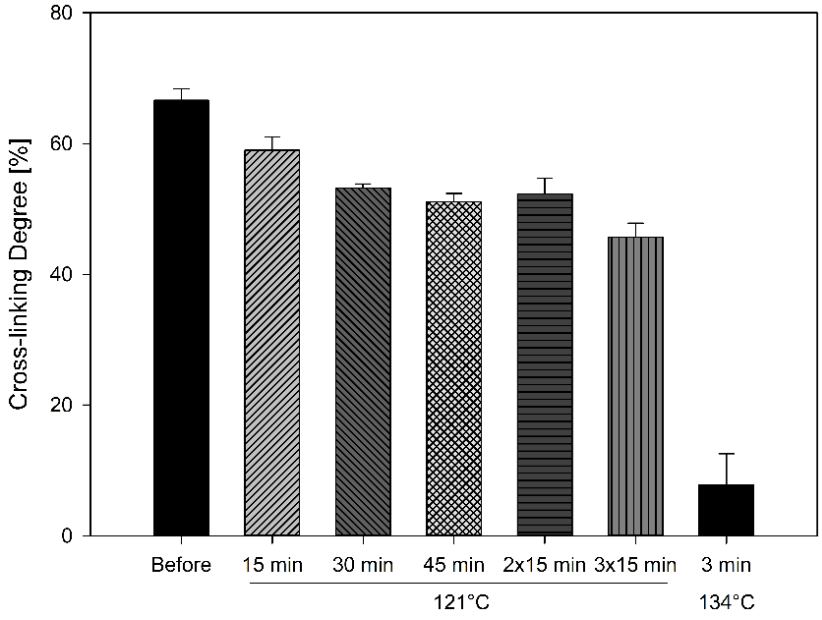

Figure IV-5 Cross-linking degree of GNPs before and after different steam sterilisation procedures. Data is represented as mean + SD (n=3).

3.1.4 Secondary Structure of Gelatine Nanoparticles

FT-IR measurements of the samples were performed to analyse effects of steam sterilisation on secondary structure of GNPs. Figure IV-6 shows the spectra of the amide I band between 1600 cm^{-1} and 1700^{-1} and the amide II band between 1510 cm^{-1} and 1580 cm^{-1} in the second derivative. The large number of minima in the region of amide I indicate a combination of different structures in GNPs. This includes β-sheet (band at 1695 cm^{-1}, two bands between 1620 and 1630 cm^{-1}), random coil (band at 1646 cm^{-1}), β-turn (band at 1680 cm^{-1}) and 3^{10}-helix [23]. On the other hand, amide II region illustrates mainly peaks

demonstrating a β-sheet structure (bands at 1515 cm^{-1}, 1534 cm^{-1}, 1552 cm^{-1}, 1569 cm^{-1}) [24]. After autoclave treatment, intensity changes of the respective bands were noticed. However, no shift of the local minima was observed. To conclude, although GNPs at least partially degraded during steam sterilisation, no drastic changes in secondary structure were detectable by FT-IR.

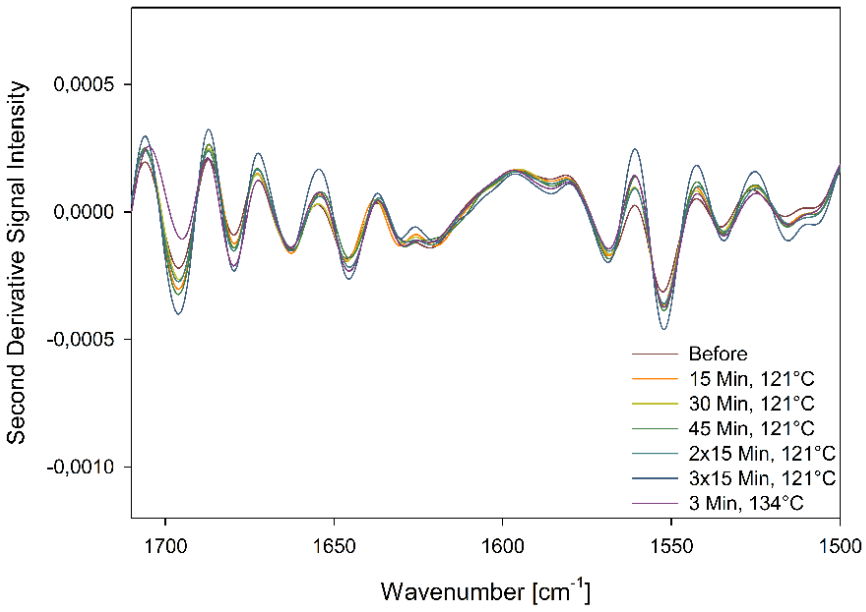

Figure IV-6 Second derivative of the averaged FT-IR spectra in the amide I and amide II region of GNPs after different sterilisation conditions (n=3).

GNPs are subjected to higher stress during autoclaving at 134°C and a pressure of 3 bar than at 121°C and 2 bar, even though the exposure time is shorter. Furthermore, data from dynamic scanning calorimetry show a glass transition of GNPs at 134.57°C ± 0.96°C. This may also contribute to the degradation of GNPs during the hasher steam sterilisation process.

On the other hand, literature showed that steam sterilisation is generally not applicable for collagen and collagen derived proteins, such as gelatine, due to degradation [25-27]. Gelatine microparticles did not withstand steam sterilisation and led to degradation of the particles [26]. However, these microcarriers seemed

to have strongly reduced initial cross-linking degrees compared the gelatine nanoparticles used in our study. No absolute values were stated, but a ten-fold lower amount of glutaraldehyde was used to cross-link the gelatine microparticles compared to GNPs. Consequently, besides the applied temperatures, one can assume that a high degree of cross-linking is an important prerequisite to enable resistance of GNPs against stress during steam sterilisation.

Summarising, steam sterilisation of GNPs under standard conditions of 15 minutes at 121°C can be performed. However, one has to keep in mind that this treatment already causes slight particle degradation. Consequently, alternative concentration determination methods need to be evaluated for steam sterilised GNPs, as common gravimetric analysis would lead to false high results due to residual gelatine fragments. This could include concentration determination using absorption of UV light, turbidity or derived countrates from DLS measurements. However, all these methods require calibration curves. On the other hand, a purification method to remove gelatine residues from autoclaved GNP dispersions should be developed. Furthermore, steam sterilisation can only be applied to plain GNPs or GNPs loaded with heat resistant drugs, whereas alterations of drug release characteristic might be considered and evaluated.

3.2 GAMMA IRRADIATION

From the aforementioned section, it can be ascertained that steam sterilisation of GNPs is in principle possible. However, ODN loading would still be necessary to be performed under aseptic conditions, due to the heat sensitivity of ODNs [14]. Moreover, sterile filtration is not applicable due to particle sizes larger than the pore size of a 0.2 µm sterile filter. Therefore, the aim of this part of the study was to establish a method to sterilise ODN-loaded GNPs without impact on particle attributes and ODN integrity.

In this section, gamma irradiation is evaluated to be a suitable sterilisation method for lyophilised ODN-loaded GNPs. Four different formulation principles were

investigated: sugar-based and amino acid-based formulations, as well as combinations of a sugar and an amino acid or combinations of different amino acids.

3.2.1 Visual Inspection

The energy introduced into the samples by gamma rays did not change the optical appearance of the lyophilisates and did not induce collapse of the cakes. However, a colouration of the glass vials was observed. This is a common phenomenon, as glass is sensitive to radiation induced coloration due to its amorphous structure [28, 29]. This is a reversible metastable change in the glass and has no impact on its physical properties or the product [29].

3.2.2 Particle Sizes and PDI Values

Data of DLS measurements reveal that gamma irradiation had hardly any impact on particle sizes and PDI values in most of the formulations (Figure IV-7). All sucrose concentrations (S100, S500, S1333), the higher trehalose concentrations (T500, T1333), pure histidine, most sugar amino acid combinations (Suc + Gly or His, Tre + Arg or Gly or His) and the amino acid mixtures (Arg + His, Arg + Gly, His +Gly, Arg + His + Gly) stabilised ODN-loaded GNPs.

On the other hand, pure glycine at low concentration (2.5%) was not adequate to prevent ODN-loaded GNPs from aggregation indicated by a strongly increasing particle size and PDI value. Furthermore, highly concentrated glycine (5.0%), both arginine concentrations (2.5% and 5.0%) as well as the combination of sucrose and arginine (Suc + Arg) and low concentrated trehalose (T100) showed higher PDI values after sterilisation suggesting aggregation. This leads to the conclusion, that glycine and arginine and low amounts of trehalose are not suitable to retain particle stability during gamma irradiation.

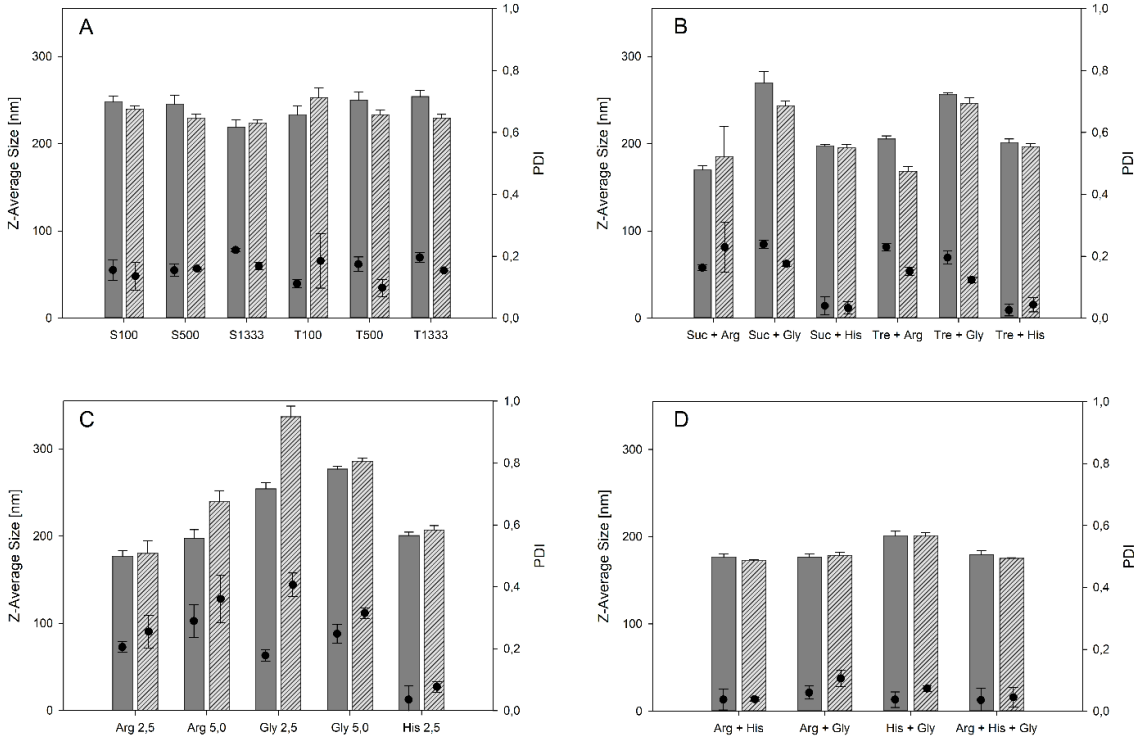

Figure IV-7 Particle sizes (bars) and PDI values (dots) of ODN-loaded GNPs in different lyophilised formulations before and after gamma irradiation. A: Pure sugar formulations, B: Sugar amino acid combinations, C: pure amino acid formulations, D: amino acid combinations. Data is represented as mean + or ± SD (n=3).

3.2.3 Loading Efficiencies and ODN Integrity

Loading efficiencies remained stable in most formulations indicating no breakage of the permanently positive charged side chains of GNPs by radiation (Table IV-1). However, a tremendous drop in loading efficiency was recognized in formulation Gly 2.5% (from 96.6% to 68.5%). This loss in loading efficiency may be related to the strong particle aggregation.

Investigation of ODN integrity revealed that ODNs endured gamma irradiation in all sugar formulations (Table IV-1). Additionally, ODNs are stable in all amino acid containing formulations free from arginine. Of all the formulations containing arginine only high arginine (5.0%) and its combination with trehalose stabilised the ODNs.

Table IV-1 ODN integrity after gamma sterilisation and loading efficiencies before and after gamma irradiation. ODN integrity is represented with symbols: ✓ stable ODN, × degraded ODN. Loading efficiency is represented as mean ± SD (n=3).

Formulation	ODN integrity after γ-irradiation	Loading efficiency [%]	
		Before γ-irradiation	After γ-irradiation
S100	✓	98.2 ± 0.8	99.8 0.2
S500	✓	98.6 ± 0.9	102.2 2.2
S1333	✓	97.4 ± 0.5	93.6 ± 0.1
T100	✓	98.4 ± 0.6	100.7 ± 0.3
T500	✓	98.9 ± 0.2	96.6 ± 0.6
T1333	✓	96.3 ± 0.9	95.2 ± 0.6
Suc + Arg	×	100.3 ± 0.4	97.4 ± 2.9
Suc + Gly	✓	98.1 ± 0.5	90.1 ± 1.0
Suc + His	✓	96.3 ± 0.4	96.8 ± 0.6
Tre + Arg	✓	95.4 ± 0.4	92.5 ± 0.4
Tre + Gly	✓	98.5 ± 0.9	96.0 ± 0.6
Tre + His	✓	94.5 ± 0.6	89.3 ± 0.6
Arg 2.5	×	99.1 ± 0.7	97.9 ± 0.3
Arg 5.0	✓	91.9 ± 0.9	98.6 ± 0.2
Gly 2.5	✓	96.6 ± 0.4	68.5 ± 1.2
Gly 5.0	✓	96.5 ± 0.6	96.2 ± 0.6
His	✓	95.6 ± 0.9	97.4 ± 0.6
Arg + His	×	95.1 ± 0.5	91.3 ± 0.3
Arg + Gly	×	93.3 ± 0.8	87.5 ± 6.1
His + Gly	✓	98.3 ± 0.6	97.3 ± 0.5
Arg + His + Gly	×	94.8 ± 0.4	102.0 ± 0.5

This leads to the conclusion that arginine is disadvantageous as excipient for ODN-loaded GNPs during gamma irradiation. This was not expected as arginine is well known to stabilise proteins [30]. On the other hand, destabilising effects of arginine have already been noticed in the lyophilisation and storage stability study (see Chapter III). We hypothesize that this negative impact of arginine is related to its

guanidinium group. This group shows a high affinity to the negatively charged backbone of nucleic acids and highest binding capacity to DNA motifs consisting of guanine rich residues, which are represented in our ODN [31, 32]. Consequently, the arginine binding induces conformational changes in the secondary structure of the DNA sequences [31]. Lastly, the change in conformation makes the ODNs more susceptible for degradation.

In summary, we could show that gamma irradiation is a suitable method to sterilise GNPs. Previously, gamma irradiation has shown to induce disintegration of non-crosslinked gelatine nanoparticles (~ 300 nm) and subsequent reformation of smaller ones (~ 10 nm) in aqueous formulations [33]. In our study, covalent cross-linking and sterilisation in solid state prevented degeneration of GNPs into smaller particles.

However, gamma irradiation aims to eliminate microorganisms by damaging their DNA. Therefore, evaluation of ODN integrity was a critical part of this study. Interestingly, we could show for the first time that rather simple lyophilised formulations were adequate to stabilise ODNs loaded onto GNPs during gamma irradiation. A mixture of two amino acids was sufficient for stabilisation, whereas arginine had a negative impact on the stability of ODN-loaded GNPs. On the other hand, if histidine was used, one amino acid was sufficient to protect ODNs from degradation. This beneficial effect of histidine was already noticed during the storage stability study of lyophilised ODN-loaded GNPs (see chapter III, section 3.3). Furthermore, the addition of a sugar to a pure amino acid formulation was advantageous. Similar observations have already been reported for a spray dried influenza vaccine, where the addition of trehalose to an amino acid composition was found to be favourable [19]. Surprisingly, pure sugar formulations were also appropriate and even superior to amino acids for stabilisation of ODN-loaded GNPs during gamma irradiation. This was not expected as it was published that complex formulation compositions of five to eight excipients, mostly based on amino acids, are necessary provide irradiation stability of a dry biomolecular product [18, 19]. In

another study, RNA oligonucleotides encapsulated into spray dried albumin nanoparticles were found to be stable upon radiation without any excipients [34]. Here the RNA was really entrapped inside the particle matrix, and therefore albumin may have acted as protecting agent. Based on this, it can be hypothesised that GNPs in general may also have protective features, but in our case ODNs are attached to the GNP surface and therefore additional excipients are be necessary to stabilise the oligodeoxynucleotides.

Summarising, it can be assumed that sugars are at least equivalent in protecting ODN-loaded nanoparticles from gamma rays compared to amino acids. However, long term stability of gamma irradiated ODN-loaded GNPs should be studied to provide a final recommendation on excipients.

4 CONCLUSION

Steam sterilisation is an acceptable method to sterilise plain GNPs. However, due to thermal stress a certain particle degradation was even be detected under standard conditions.

Gamma irradiation is a suitable method to sterilise lyophilised ODN-loaded GNPs, whereas sugar formulations were superior to amino acid mixtures and arginine was even detrimental in terms of ODN stability.

Amongst the two investigated sterilisation approaches, gamma irradiation of lyophilised GNPs is preferable.

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CHAPTER V

PRELIMINARY EVALUATION OF CPG OLIGODEOXYNUCLEOTIDES BOUND TO GELATINE NANOPARTICLES AS IMMUNOTHERAPY FOR CANINE ATOPIC DERMATITIS

The following chapter has been published in *Veterinary Record*:

Wagner, I., Geh, K.J., Hubert, M., Winter, G., Weber, K., Classen, J., Klinger, C., Mueller, RS. (2017) Preliminary evaluation of cytosine-phosphate-guanine oligodeoxynucleotides bound to gelatine nanoparticles as immunotherapy for canine atopic dermatitis. *Veterinary Record* 181 (5), 118.

This work was conducted in close cooperation with the Clinic for Small Animal Medicine, Centre for Clinical Veterinary Medicine, LMU Munich. The personal contribution covers GNP preparation, loading and characterisation including written parts of these experiments. Treatment of the dogs, diagnosis of clinical symptoms and cytokine quantification was conducted by the veterinarian Dr. med. vet. Iris Wagner-Storz.

ABSTRACT

Cytosine-phosphate-guanine oligodeoxynucleotides (CpG ODNs) are a promising new immunotherapeutic treatment option for canine atopic dermatitis (AD). The aim of this uncontrolled pilot study was to evaluate clinical and immunological effects of gelatine nanoparticle (GNP)-bound CpG ODNs (CpG-GNP) on atopic dogs. Eighteen dogs with AD were treated for eight (group 1, n=8) or 18 weeks (group 2, n=10). Before inclusion and after two, four, six (group 1+2), eight, 12 and 16 weeks (group 2) 75 µg CpG ODNs/dog (bound to 1.5 mg GNP) were injected subcutaneously. Pruritus was evaluated daily by the owner. Lesions were evaluated and serum concentrations and mRNA expressions of interferon-γ, tumour necrosis factor-α, transforming growth factor-β, interleukin-10 and interleukin-4 (only mRNA expression) were determined at inclusion and after eight (group 1+2) and 18 weeks (group 2).

Lesions and pruritus improved significantly from baseline to week eight. Mean improvements from baseline to week 18 were 23% and 44% for lesions and pruritus respectively, an improvement of ≥50% was seen in 6/9 and 3/6 dogs, respectively. Interleukin-4 mRNA expression decreased significantly. The results of this study show a clinical improvement of canine AD with CpG GNP comparable to allergen immunotherapy. Controlled studies are needed to confirm these findings.

KEYWORDS*:*

Allergy, atopy, dogs, immunomodulation, TLR9

1 INTRODUCTION

Canine atopic dermatitis (AD) is an inflammatory allergic skin disease in genetically predisposed dogs associated with distinctive clinical signs [1]. The allergy is mostly directed against environmental allergens though food allergens might contribute to the disease [2, 3]. In most, but not all dogs, IgE antibodies against those allergens can be found [4].

The pathogenesis of AD is complex and not fully understood [5]. Besides skin barrier impairments, alterations of the immune system seem to play a central role in the development of the disease [6]. Atopic dogs as well as humans show a tendency to T helper type 2 (Th2)-polarized immune reactions [7-10]. However, although a Th2 phenotype predominates in early stages of inflammation, chronic lesions show a more mixed pattern of lymphocytes and cytokines with a slight trend towards Th1-polarization [8, 9, 11]. Regulatory T cells (Tregs) and the regulatory cytokines transforming growth factor (TGF)-β and interleukin (IL)-10 can modulate the immune response to allergens by directly and indirectly suppressing T cells. Although still not fully understood, there might be a Treg cell deficiency or an impairment of Treg function in AD [4, 12, 13].

To date, allergen immunotherapy (AIT) is the only causative therapy [14]. However, there are certain disadvantages of AIT. For each dog allergens contributing to the disease must be identified, and then an individual allergen extract has to be formulated. Allergen testing and extracts are costly [15]. Furthermore, there is a subset of dogs not showing positive test reactions excluding them from this treatment option [4]. In addition, it may take several months before clinical improvement is seen and up to half of the patients may fail to respond to AIT [16, 17]. Thus, an efficacious immunomodulation of AD that does not require allergen identification would be desirable.

Cytosine phosphate guanine oligodeoxynucleotides (CpG ODNs) offer such a new immunotherapeutic approach. CpG ODNs are synthetic DNA oligodeoxynucleotides containing at least one unmethylated cytosine guanine (CG) dinucleotide with certain flanking bases. Unmethylated CG dinucleotides are relatively common in

microbial DNA and represent a pathogen-associated molecular pattern (PAMP), which is bound by Toll-like receptor (TLR) 9. They initiate various immune responses [18-20].

In humans, stimulation of TLR 9 by CpG ODNs leads to a polarization of the immune response to a Th1 phenotype, which suppresses Th2 responses, increases the secretion of regulatory cytokines such as IL-10 and suppresses IgE antibody production. Furthermore, differentiation of B-cells to plasma cells and isotype switching to IgG is promoted [20-22]. In atopic dogs, CpG ODNs also induce a Th1-biased immune response and increase the expression of IL-10 mRNA *in vitro* [23-25]. These effects resemble those observed in the course of AIT [26-28].

Adsorption of CpG ODNs onto cationised gelatine nanoparticles (GNPs) protects the CpG ODNs from early enzymatic degradation and enhances uptake into target cells, thereby increasing and prolonging the immunostimulatory effects of the CpG ODNs [23, 29, 30]. Gelatine as a carrier matrix is biocompatible, biodegradable and safe [31]. Unloaded GNPs do not show immunostimulatory activity [30]. Repeated inhalation of an aerosol formulation of GNP-bound CpG ODNs (CpG-GNPs) increased IL-10 and IFN-γ expression, but also reduced clinical parameters of allergic inflammation in horses with recurrent airway obstruction [32, 33]. The CpG-GNP used in this study increased secretion of IL-10 *in vitro* in peripheral blood mononuclear cells (PBMCs) obtained from atopic dogs [23].

The aims of this study were (1) to evaluate the effects of CpG-GNPs on the clinical lesions and pruritus of dogs with nonseasonal atopic dermatitis and (2) to examine the influence of the treatment on gene expression and serum concentrations of selected Th1, Th2 and regulatory cytokines in these dogs.

2 MATERIALS AND METHODS

2.1 STUDY DESIGN

The study was conducted as an uncontrolled, prospective pilot study in the setting of the Clinic of Small Animal Medicine, LMU, Munich, Germany.

2.2 STUDY DRUG PREPARATION

ODNs with the sequence 5'-GGTGCATCGATGCAGGGGGG-3' were provided with a full phosphorothioate backbone (Biomers.net, Ulm, Germany).

The GNPs were prepared using a two-step desolvation method as previously described [34]. Nanoparticles were stabilised by cross-linking with glutaraldehyde. Cationisation was performed by attaching permanent positive charges from quaternary amines to the surface of GNP according to an established protocol [30]. Aseptically prepared GNP were then incubated with CpG ODNs in isotonic sorbitol solution to a final concentration of 5 mg/mL GNP and 0.25 mg/mL CpG ODNs for 1 h at 22°C under gentle shaking using a thermomixer (Eppendorf, Hamburg, Germany). The loading efficiency was calculated by photometric determination at a wavelength of 260 nm (Agilent 8453 UV-visible spectrophotometer, Agilent Technologies, Santa Clara, California, USA) as follows. Samples (CpG-GNPs) and controls of plain GNPs and pure CpG ODNs were centrifuged at 14000*g for 30 min (Sigma 4K15 centrifuge, Osterode, Germany). The absorbance (A) of the supernatants was then measured and the loading efficiency was calculated using the following equation:

$$\text{Loading efficiency } [\%] = 1 - \left(\frac{\text{A (CpG GNP)} - \text{A (GNP control)}}{\text{A (CpG} - \text{ODNs control)}}\right) x\ 100\ \%.$$

A loading efficiency of at least 95% (w/w) was acceptable. Particle size, polydispersity index (PDI) and zeta potential were analysed using a Zetasizer ZS Nano (Malvern Instruments, Malvern, UK) before and after loading with CpG ODNs. The study drug was then stored at 3 to 7 °C for not longer than 72 hours until

injection to ensure that particle size, PDI, zeta potential and loading efficiency meet the required quality criteria.

2.3 PATIENT SELECTION

Eighteen client-owned dogs with non-seasonal AD were included. Atopic dermatitis was diagnosed by history, clinical examination and ruling out differential diagnoses with appropriate tests and treatments. Within the last month prior to and during the study every dog was treated with appropriate flea control products and its diet was unchanged for at least two months prior to inclusion and during the trial. Injectable depot formulation of glucocorticoids had to be discontinued at least six weeks prior to participation, any oral glucocorticoid or other immunosuppressive agent four weeks prior. Patients receiving other medication, such as antihistamines or shampoo therapy continued this medication unchanged in the last two weeks prior to and during the trial.

Dogs were withdrawn from the study when requiring any form of additional anti-inflammatory therapy or when showing unacceptable discomfort or adverse effects of immunotherapy. Poor owner compliance also led to exclusion.

2.4 TREATMENT PROTOCOL

The procedure was approved by the local animal welfare authorities (District Government of Upper Bavaria, Field of interest 54, 80534 Munich, Germany; reference number 55.2-1-54-2532-122-13, date of approval: 9/18/2013). Prior to treatment of each dog, written informed consent was obtained from the owner.

Recruitment and treatment of patients started in 04/2014 and ended (for reasons of time) in 09/2015. The first group of eight dogs (group 1) was treated over a period of eight weeks, whereas the following ten dogs (group 2) received immunotherapy over a prolonged period of 18 weeks. The study drug (300 µL of the final formulation, which corresponds to 75 µg CpG ODNs bound to 1.5 mg GNPs per dog) was injected subcutaneously in the area of the popliteal lymph nodes at

inclusion and after two, four and six weeks. Patients of group 2 received additional injections after eight, 12 and 16 weeks.

2.5 Clinical Evaluation

At inclusion and after eight (group 1+2) as well as 18 weeks (group 2) skin lesions were evaluated using the validated Canine Atopic Dermatitis Extent and Severity Index (CADESI)-03 which evaluates 62 body sites for erythema, lichenification, excoriation and alopecia [35].

Pruritus was assessed by the dog´s owner on a daily basis using a validated visual analogue scale of zero (no pruritus) to ten (extremely severe and continuous pruritus) [36, 37]. For each dog individual weekly values of pruritus were calculated by forming the arithmetic mean of the scores of week one, eight and 18 (if available), respectively.

Any given medication or treatment as well as any adverse effects had to be recorded by the owner.

2.6 Quantification of Serum Cytokine Concentrations

Blood samples were collected at the beginning of the study and after eight (group 1+2) and 18 weeks (group 2). The latter blood samplings were both performed two weeks after the last CpG-GNP injection. Serum was obtained by centrifuging the clotted blood samples for 5 minutes at 2540*g (centrifuge Universal 320R, Andreas Hettich, Tuttlingen, Germany) and stored at -80 °C until further usage.

Serum concentrations of IFN-γ, TNF-α, TGF-β and IL-10 were measured using Milliplex MAP Canine Cytokine Magnetic Bead Kits (Merck Millipore, Darmstadt, Germany) according to manufacturer's instructions. Quantification was performed by the Bio-Plex 200 Reader, Results were calculated using the Bio-Plex Manager Software 4.1 (both BioRad, Hercules, California, USA). The sensitivity limits of the

assays were 18 ng/L for IFN-γ, 6.1 ng/L for TNF-α, 8.5 ng/L for IL-10 and 11.4 ng/L for TGF-β.

2.7 Relative Quantification of Cytokine mRNA

Blood samples of group 2 were collected in RNAprotect Animal Blood Tubes (500 µL) (Qiagen, Hilden, Germany) at the beginning of the study, after eight and 18 weeks (two weeks after the last CpG-GNP injection, each) and stored at -20°C until further usage.

Quantitative RT-PCR was performed to assess mRNA expression of IFN- γ, TNF-α, IL-4, TGF-β and IL-10. Genes for ribosomal protein L13a (RPL13A) and succinate dehydrogenase complex subunit A, flavoprotein (SDHA) were used as reference genes [38, 39]. Briefly, mRNA was extracted from the samples using the RNeasy Protect Animal Blood Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Samples were evaluated for quality and quantity of mRNA via NanoDrop 1000 Spectrophotometer (PEQLAB Biotechnology, Erlangen, Germany). Using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany), mRNA was transcribed to cDNA. Real-time PCR was performed on an Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific, Waltham, Massachusetts, USA) using the QuantiTect SYBR Green PCR Kit and QuantiTect Primer Assays for all target and reference genes (both Qiagen, Hilden, Germany). The PCR conditions were 95 °C for 15 min and 40 cycles of 94 °C for 15 s, 55 °C for 30 s and 72 °C for 35 s. Data were analysed via Applied Biosystems DataAssist Software v3.01 (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Results were normalized using RPL13A and SDHA as references [40].

2.8 Statistical Analysis

Data for pruritus, CADESI, serum cytokine concentrations and mRNA expressions were tested for normality using the D'Agostino-Pearson omnibus normality test.

Individual data pairs of CADESI, pruritus and serum cytokine concentrations at the beginning and end of the study were compared by Wilcoxon matched-pairs signed-rank tests. Results of gene expression analysis (2-(ΔCT) values) were compared by Friedman-tests followed by Dunn's multiple comparisons tests. Statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software, San Diego, California, USA). For all comparisons, a P-value < 0.05 was considered significant.

3 RESULTS

3.1 STUDY POPULATION

Of the 18 dogs, a total of 15 dogs completed the study (group 1: n=6, group 2: n=9). One dog (group 1) was excluded from the study because of severe vomitus after the second appointment. The two other dogs (one of group 1 and 2 each) were excluded, because clinical signs of AD were severe enough to warrant immunosuppressive drugs. The mean age of the patients was 4.7 years (median: 4.8 years). Body weight ranged from 8 kg to 49 kg. Ten participating dogs were male (eight of them neutered) and eight female (seven of them spayed). Ten breeds were represented in this study (two boxers, and one of the following breeds each: Beagle, Doberman Pinscher, German Shepherd, French Bulldog, Irish Setter, Jack Russel Terrier, Rhodesian Ridgeback, Golden and Labrador Retriever). Seven mixed-breed dogs also participated.

3.2 CONCURRENT MEDICATIONS

All concurrent medications and treatments were performed according to study guidelines, i.e. dosage and frequency of administration remained unchanged. They mostly consisted of shampoo therapy, local disinfection and topical and oral supplementation of polyunsaturated fatty acids. Only one dog in group 1 received an antihistamine (cetirizine) during and before study.

3.3 CLINICAL PARAMETERS

Lesions showed a statistically significant improvement in week 8 (P = 0.037). Mean CADESI decreased from 21.93 in week 0 to 14.00 in week 8. Although CADESI showed further improvement until week 18 (mean CADESI: 11.11), this decrease failed to reach statistical significance (Figure V-1 A).

Mean pruritus in the first study week was 5.58. Pruritus was significantly reduced to 4.46 in week 8 (P = 0.008) and 3.19 in week 18 (P = 0.031) (Figure V-1 B). Mean

values, standard deviations, confidence intervals and medians of CADESI and pruritus at the different time points of the treatment can be found in Table V-1. Mean percentage reductions of lesions and pruritus as well as the proportion of dogs showing at least 25% and 50% improvement in week 8 and 18 are summarised in Table V-2.

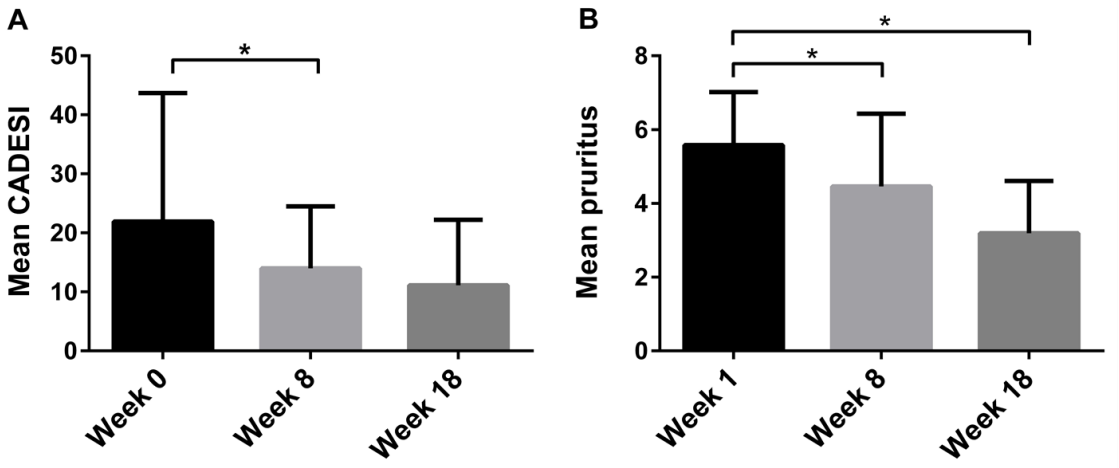

Figure V-1 Development of mean CADESI (Canine Atopic Dermatitis Extent and Severity Index) (A) and pruritus (B) in dogs with atopic dermatitis treated with gelatine nanoparticle-bound CpG oligodeoxynucleotides. *P < 0.05.

Table V-1 Means, standard deviations (SD), confidence intervals (CI) and medians of CADESI (Canine Atopic Dermatitis Extent and Severity Index) and pruritus at different time points of the immunotherapy of dogs with atopic dermatitis with gelatine nanoparticle-bound CpG oligodeoxynucleotides.

	Week	Mean Value ± SD	95% CI	Median
CADESI	0	21.93 ± 21.78	9.87 – 33.99	13
	8	14.00 ± 10.49	8.19 – 19.81	11
	18	11.11 ± 11.12	2.57 – 19.66	4
Pruritus	1	5.58 ± 1.43	4.79 – 6.38	5.79
	8	4.46 ± 1.97	3.37 – 5.55	4.43
	18	3.19 ± 1.42	1.7 – 4.68	3

Table V-2 Improvement of clinical signs in comparison to baseline values in dogs with atopic dermatitis at different time points of immunotherapy with gelatine nanoparticle-bound CpG oligodeoxynucleotides.

	Clinical parameter	Mean	≥ 25%	≥ 50%
Improvement in week 8	CADESI [a]	23.07%	8/15 dogs	5/15 dogs
	Pruritus	24.11%	7/15 dogs	3/15 dogs
Improvement in week 18	CADESI [a]	22.98%	6/9 dogs	6/9 dogs
	Pruritus	44.01%	4/6 dogs	3/6 dogs

[a] CADESI, Canine Atopic Dermatitis Extent and Severity Index

3.4 SERUM CYTOKINE CONCENTRATIONS

Concentrations of IFN-γ, TNF-α and IL-10 in most of the blood samples were under the detection limits of the assay. TGF-β did not significantly change during the study.

3.5 EXPRESSION OF CYTOKINE MRNA

There were no significant changes in mRNA expression of IFN-γ, TNF-α, TGF-β and IL-10. In contrast, mRNA expression of IL-4 was decreased 3.08-fold in week 18 compared to baseline ($P = 0.048$). The observed changes in expression of all measured cytokines are displayed in Figure V-2.

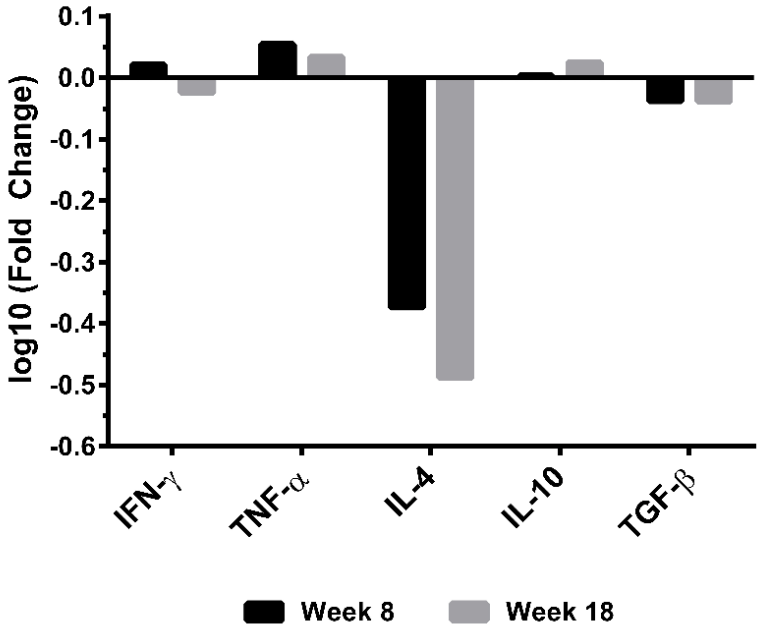

Figure V-2 mRNA expression of interferon (IFN)-γ, tumour necrosis factor (TNF)-α, interleukin (IL)-4, transforming growth factor (TGF)-β and IL-10 in dogs suffering from atopic dermatitis during immunotherapy with gelatine nanoparticle-bound CpG oligodeoxynucleotides. Depicted are the changes in expression in samples of week 8 and 18 in comparison to the expression in samples collected at the beginning of the study (fold changes).

3.6 ADVERSE EFFECTS

Nine of 18 dogs experienced at least one adverse event. The observed reactions included vomitus (6/18 dogs), diarrhoea (4/18 dogs), swelling of the popliteal lymph node (1/18 dogs), and swelling at the injection site (3/18). In two cases vomitus was heavy, frequent and associated with diarrhoea. One of those two dogs was excluded from the study. The other dog initially showed only mild gastrointestinal adverse effects and completed the study, but deteriorated further after the end of the study. Both dogs recovered completely after symptomatic treatment. In the other affected dogs, gastrointestinal symptoms were mild and occurred only occasionally and independent of the injections. The swellings of the popliteal lymph nodes and at the injection sites were mild, painless and subsided within a few days in all cases.

4 DISCUSSION

In this pilot study, immunotherapy with CpG-GNPs reduced pruritus and lesions of canine AD. Studies evaluating the efficiency of AIT found that 52 to 65% of the treated dogs show a clinical improvement of at least 50% [16, 17, 41]. In a prospective blinded study evaluating allergen immunotherapy, improvement of CADESI scores and pruritus of at least 50% was reported in 7/11 dogs (64%) and in 5/11 dogs (45%), respectively [42]. In our study, immunotherapy with CpG-GNPs, performed for 18 weeks, led to a clinical improvement of at least 50% in a similar proportion of patients (Table V-2).

In this study, immunotherapy with CpG-GNPs was only conducted for four months while cited studies evaluated the efficacy of AIT after at least 12 months of treatment. It may take quite long for clinical signs to improve with AIT [14, 16]. In a retrospective study about AIT, 21% of the dogs showed first signs of clinical improvement in the first two months of AIT, 45% in the period between two and five months and 17% of patients later than five months [41]. Unfortunately, only an overall assessment of the owner was given and pruritus and CADESI scores were not obtained in that study. If prolonged application of CpG-GNPs results in further improvement of symptoms, has to be evaluated in further studies, but the fast clinical improvement is rather encouraging.

The adverse effects observed in the course of the study can be divided into gastrointestinal symptoms and local reactions, both of which are reported in humans treated with CpG ODNs [43-45]. In humans, systemic adverse events such as gastrointestinal reactions generally occurred 12 to 24 hours after application of CpG ODNs and subsided after a few days [43, 45]. In the two dogs in our study suffering from severe and frequent vomitus and diarrhoea, these signs persisted for as late as two weeks after the last injection. It is unclear if they were associated with the study medication or not. Occasional vomiting or diarrhoea is extremely common in otherwise healthy dogs and often spontaneously resolving [46]. In some human patients allergic to red meat, hypersensitivity reactions to small amounts of intravenously administered gelatine were observed, even when the regularly

consumed red meat only caused overt reactions occasionally [47]. It seems possible, that gastrointestinal adverse effects could occur in response to porcine gelatine in dogs as well. One of the two dogs expressed similar gastrointestinal reactions after administration of Fenistil dragées (Novartis, Basel, Switzerland), which also contain gelatine. In all other cases, vomitus and diarrhoea were mild and occurred in time intervals, which the owners considered 'normal' for their dogs. The painless and temporary local reactions observed in the course of the study are compatible with the mechanism of action of CpG ODNs and can be considered as mild adverse effects [43].

Overall, during the immunotherapy with CpG-GNPs adverse reactions were observed in 50% of the patients. In AIT, the incidence of adverse events is reported to range between 5% and 50% [16, 48, 49]. Increased pruritus after injection of the immunotherapy is the most commonly observed adverse effect [14, 48]. Systemic reactions have been reported in approximately 1% of the treated dogs [49]. They include not only gastrointestinal symptoms, but also weakness, anxiety, urticaria/angioedema and severe reactions such as collapse and anaphylaxis [16, 48, 50]. None of the latter ones could be observed in our study. It is assumed that by omitting the allergens in immunotherapy of AD, the risk of potentially life threatening anaphylactic reactions can be reduced or even eliminated [51]. However, it remains to be seen if the adverse effects seen here (exclusive local swellings and gastrointestinal signs) using CpG-GNPs will be confirmed in larger placebo-controlled studies.

The mRNA expression of IL-4 significantly decreased in the course of the study. IL-4 is known as a key cytokine in allergic inflammation, increasing the differentiation of naïve T-cells to Th2-cells, inducing antibody class switching to IgE and stimulating the activation of mast cells [52]. Hence a reduction in IL-4 mRNA expression, as observed in this study, can be regarded as beneficial in the treatment of atopic dogs. This observation is in accordance with a study using liposome-DNA complexes as an adjuvant in AIT [53]. In another study, conventional AIT augmented the Th1 to Th2 cytokine ratio, although by an increase in IFN-γ [28].

In contrast to *in vitro* studies evaluating the effects of CpG ODNs on the PBMCs of atopic dogs [23, 24], neither an increase in Th1 nor in regulatory cytokine serum concentration and mRNA expression could be detected. However, apart from TGF-β, cytokine concentrations in the serum samples were below the detection threshold, thus a thorough evaluation of the immunological effects of CpG-GNP immunotherapy on serum cytokines was not possible. As blood samples were obtained two weeks after the last injection of CpG-GNPs, effects of CpG ODNs on cytokine serum concentrations and mRNA expression may already have diminished due to this period of time.

Limitations of this study include the absence of a control group, the small number of treated dogs and the short duration of treatment. As to the authors' knowledge this was the first time CpG-GNPs were administered exclusively to atopic dogs, the optimal dosage was unknown. In human medicine, safety of CpG ODNs application was assessed over a dose range from 0.0025 mg/kg to 0.81 mg/kg [43]. Since little empirical data is available for dogs [54-56], the dosage was chosen at the low end of the doses assessed in humans. Immunotherapy with CpG-GNPs administered at a higher dosage may have resulted in more pronounced clinical improvement but also has the risk of more frequent and severe adverse effects.

In AIT the same dose of allergen extract is typically used for each dog regardless of body weight [14]. The same concept was applied in this study. It cannot be ruled out that administration of individually adapted doses may have yielded greater clinical improvement as well, although the results of our study do not suggest any correlation between body weight and grade of improvement in this limited number of dogs.

The injection site (near the popliteal lymph nodes) was selected to deposit the CpG-GNPs in close proximity to their target, i.e. immune cells. Injection directly into the lymph nodes may also have enhanced clinical improvement. However, at this point (long term) safety of intralymphatic CpG-GNP administration in dogs is unknown.

Performance of intradermal testing or allergen-specific serum IgE testing was not mandatory for study participation. However, 5/6 dogs in group 1 and 6/9 dogs in

group 2 did receive either one independently of the study. All dogs tested showed positive reactions to house dust mite (Dermatophagoides farinae), most of them also to other environmental allergens. Since, by definition, IgE antibodies directed against environmental allergens must be documented to classify the disease as canine atopic dermatitis [1], it cannot be excluded that one or more of the remaining five dogs were suffering from atopic-like dermatitis rather than from atopic dermatitis. This must be considered another limitation of the study.

A major downside of the immunotherapy of atopic dogs with CpG-GNPs is that gelatine nanoparticle-bound CpG ODNs are not commercially available yet. In addition, at the time of the execution of the study, the CpG-GNPs were only stable for 72 hours. Recent studies indicate, however, that the stability can be extended to six months by lyophilisation, enabling upscaling of the process and therefore better availability.

5 CONCLUSIONS

Results of the present study suggest that immunotherapy with CpG-GNPs can lead to significant clinical improvement of canine atopic dermatitis. Administration over a period of 18 weeks reached an efficacy similar to that reported for allergen immunotherapy. Additionally, treatment with CpG-GNPs reduced expression of the Th2-cytokine IL-4 in atopic dogs. However, these results need to be confirmed in controlled, randomised, double-blinded studies.

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CHAPTER VI

SUMMARY OF THE THESIS

1 SUMMARY OF THE THESIS

Nanoparticles are intensively researched as drug delivery systems since the 1970s. Amongst a variety of starting materials for nanoparticles, gelatine has proven to be versatile due to its biodegradability, biocompatibility and low immunogenicity. Furthermore, gelatine provides several functional groups, which allow cross-linking and surface modifications of gelatine nanoparticles (GNPs) [1].

Besides different small molecules, GNPs were sucessfully investigated for their potential as drug delivery system for macromolecules, such as therapeutic proteins or nucleic acids [2, 3]. Several studies showed the effective treatment of allergic diseases, such as equine recurrent airway obstruction, by cytosine phosphote guanosine oligodeoxynucleotides (CpG ODNs) bound to gelatine nanoparticles [4-7]. Following recognition of the innate immune system via toll-like receptor 9 (TLR9), CpG ODNs are able to restore the disrupted balance between Th1 and Th2 immune response in allergy driven diseases [8]. Furthermore, regulatory T cells (Treg), which control T helper cell reactions in general, can be activated [8]. GNPs are able to protect these sensitive oligodeoxynucleotides from degradation and enhance their cellular uptake by antigen presenting cells due to their particle sizes similar to microorganisms [9, 10].

The work presented in this thesis focused on the optimisation of the preparation process of gelatine nanoparticles, their stabilisation and sterilisation. Moreover, a preliminary clinical evaluation of CpG ODN-loaded GNPs in canine atopic dermatitis is described.

Chapter I contains the general introduction of the thesis. Different starting materials for nanoparticles including gelatine are discussed. GNPs are presented as promising drug delivery system for CpG ODNs. Furthermore, the mechanism of action of CpG ODNs and their potential as immunomodulatory therapeutic option in the allergic diseases are described. Lastly, the aims of the thesis are stated.

Chapter II focuses on the optimisation of the GNP preparation process and its scale up. The establishment of a more straightforward one-step desolvation process compared to the common delicate two-step desolvation process is demonstrated. A commercially available high molecular weight gelatine for one-step desolvation was found that omitted the need of customised gelatine qualities. Beyond that, the scale up of this improved preparation method is shown. Using the improved one-step desolvation process, a 130-fold increase of particle gain was available. This opens the possibility for further industrial large-scale production of GNPs.

Moreover, alternative methods to scale GNP production are discussed in this chapter. This includes enlarging the contact area between gelatine solution and desolvation agent during the desolvation process, the use of a dual-syringe pump system or the alternative preparation method nanoprecipitation. However, no satisfying results could be obtained using these alternative approaches.

Besides the optimisation and scale up of GNP preparation, this chapter also describes the investigation of alternative non-toxic cross-linking agents to common glutaraldehyde. This includes the sugar derivative glyceraldehyde as well as the naturally occurring genipin. Glyceraldehyde could successfully be used as alternative to cross-link GNPs, whereas genipin did not result in high cross-linking degrees, which would be able to stabilise GNPs.

Matrix-assisted laser desorption/ionisation mass spectrometry (MALDI MS) was successfully established as analytical tool to evaluate the integrity of ODNs loaded onto GNPs as described in **Chapter III.** Furthermore, this chapter deals with the stabilisation of ODN-loaded GNPs via lyophilisation, which is an important topic due to the limited stability of 2-3 days in the liquid state. Long-term stability of lyophilised ODN-loaded GNPs for six months at 2-8°C and 20-25°C in sugar-based formulations is shown. Particle characteristics, such as particle sizes and PDI values,

remained stable upon storage and ODN integrity is not affected. Additionally, stability at accelerated storage conditions was shown.

Moreover, controlled nucleation was investigated as potential freezing method prior to lyophilisation in order to shorten lyophilisation process and increase batch homogeneity. ODN-loaded GNPs resisted the stress induced by freezing via controlled nucleation equally to standard ramp freezing, which was shown in freeze-thaw studies. However, using controlled nucleation prior to freeze-drying has hardly benefits on the drying time and the stability of the product. Contrary to expectations from literature, controlled nucleation has neither negative impact on ODN-loaded GNPs as reported from polyplexes, nor beneficial effects as known from proteins [11, 12].

Additionally, amino acids are discussed as alternative excipients in lyophilisation of ODN-loaded GNPs. Histidine offers excellent potential in stabilising ODN-loaded GNPs, whereas crystallisation of glycine is unfavourable and initiates particle aggregation. Furthermore, in glycine formulations starting ODN degradation was detected at accelerated storage temperature. Besides, arginine is even detrimental and favours ODN degradation during storage. This may be due to the strong binding affinity of its guanidinium group to the negatively charged backbone of the ODNs and consequent disruption of the secondary structure of the nucleic acid. This change in secondary structure makes the ODNs more vulnerable to degradation.

Sterility is a main prerequisite of parenterally applied drug products. So far, GNP preparation and ODN loading were performed under aseptic conditions to avoid microbial contamination. However, aseptic working is prone to failure and difficult to validate. Therefore, **Chapter IV** approaches the sterilisation of GNPs. Firstly, steam sterilisation is shown to be possible for unloaded GNPs under standard conditions (121°C for 15 minutes). However, due to high stresses induced by temperature and pressure, a certain degradation of GNPs was noticed indicated by

loss in derived countrate during dynamic light scattering (DLS) measurements and reduced cross-linking degrees. This was more pronounced when repeated sterilisation cycles at 121°C (2fold or 3fold) or extended sterilisation periods (30 and 45 minutes) were applied. Steam sterilisation for three minutes at 134°C caused even almost complete particle dissolution.

Due to heat sensitivity of oligonucleotides, autoclaving of ODN-loaded GNPs is not applicable. This still entails an aseptic loading process of GNPs. Consequently, gamma irradiation is represented as option to sterilise lyophilised ODN-loaded GNPs. A variety of excipients was tested for protecting ODN-loaded GNPs during gamma irradiation. Interestingly, simple sugar formulations were most appropriate. Particle characteristics and ODN integrity could completely be preserved. Amongst the investigated amino acids histidine was comparable to sugars, whereas glycine and arginine based formulations did not or less protect ODNs from degradation. These observations are in common with our findings from lyophilisation studies.

Canine atopic dermatitis (CAD) is a genetically predisposed allergic skin disease, mostly directed against environmental allergens. The immunological process is still not fully understood, but early stage Th2 activation followed by a chronic Th1 mediated immune reaction with Treg dysfunction are discussed [13]. Consequently, CpG ODNs are stated to be a promising therapy approach. **Chapter V** describes the successful preliminary clinical evaluation of ODN-loaded GNPs in the treatment of canine atopic dermatitis (CAD). After 18 weeks of subcutaneous application of ODN-loaded GNPs a clinical improvement of pruritus and Canine Atopic Dermatitis Extent and Severity Index (CADESI) of up to ≥ 50% were noticed. Furthermore, a significant reduction in allergy mediated IL-4 mRNA expression was observed. This study opens the way for further promising placebo controlled clinical trials using ODN-loaded GNPs to cure canine atopic dermatitis.

Taking together the conclusions of all chapters, GNP production process was successfully optimised and scaled. Different lyophilisation options were evaluated to find optimal process conditions and formulation excipients for long term stability of ODN-loaded GNPs. MALDI MS was evaluated as a versatile analytical approach to study integrity of ODNs loaded onto GNPs. Steam sterilisation and gamma irradiation were auspiciously investigated to sterilise unloaded and loaded GNPs. A preliminary clinical evaluation proved ODN-loaded GNPs to be a promising treatment in canine atopic dermatitis.

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CHAPTER VII

APPENDIX

1 LIST OF ABBREVIATIONS

AD ..... *Atopic dermatitis*
AF4 ..... *Asymmetric field flow-field fractionation*
APCs ..... *Antigen presenting cells*
Arg ..... *L-Arginine*
ASIT ..... *Allergen-specific immunotherapy*
BAL ..... *Bronchoalveolar lavage*
CAD ..... *Canine atopic dermatitis*
CADESI ..... *Canine Atopic Dermatitis Extent and Severity Index*
CG ..... *Cytosine guanine*
Cholamine ..... (2-Aminoethyl) trimethylammonium chloride hydrochloride
CL ..... *Cross-linking*, *Cross-linking*
CpG ODNs ..... *Cytosine phosphate guanine oligonucleotides*
CpG-GNPs *Cytosine phosphate guanine oligodeoxynucleotide loaded gelatine nanoparticles*
DLS ..... *Dynamic light scattering*
DNA ..... *Desoxyribonucleic acid*
EDC ..... 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide
ELS ..... *Electrophoretic light scattering*
FDA ..... *United States Food and Drug Administration*
FT-IR ..... *Fourier transform infrared spectroscopy*
Gly ..... *L-Glycine*
GNPs ..... *Gelatine nanoparticles*
GRAS ..... *Generally Recognised as Safe*
His ..... *L-Histidine*
HMW ..... *High molecular weight*
HPW ..... *Highly purified water*
HSA ..... *Human serum albumin*
IAD ..... *Inflammatory airway disease*
IEP ..... *Isoelectric point*
IFN-γ ..... *Interferon γ*
Ig ..... *Immunoglobulin*
IL ..... *Interleukine*
LMW ..... *Low molecular weight*
MALDI MS ..... *Matrix-assisted laser desorption/ionisation mass spectrometry*
MALS ..... *Multi-angle light scattering*
MSN ..... *Mesoporous silica nanoparticles*
MW ..... *Molecular weight*
NF-κB ..... *Nuclear factor κB*
PAMP ..... *Pathogen-associated molecular pattern*
PBMCs ..... *Peripheral blood mononuclear cells*
PCL ..... *Poly(epsilon-caprolactone)*
PDI ..... *Polydispersity index*
PEG ..... *Polyethylene glycol*
PEI ..... *Polyethylenimine*

PLGA ..... *Poly(lactic-co-glycolic) acid*
PTO ..... *Phosphorothioate*
RAO ..... *Recurrent airway obstruction*
rHG ..... *recombinant human gelatine*
RNA ..... *Ribonucleic acid*
RPL13A ..... *Ribosomal protein L13a*
RT-PCR ..... *Real time polymerase chain reaction*
SDHA ..... *Succinate dehydrogenase complex subunit A*
SEM ..... *Scanning Electron Microscopy*
SLNs ..... *Solid lipid nanoparticles*
Tg ..... *Glass transition temperature*
Tg' ..... *Glass transition temperature of the maximally freeze-concentrated solution*
TGF ..... *Transforming growth factor*
Th1 ..... *T helper cell type 1*
Th2 ..... *T helper cell type 2*
TLR ..... *Toll-like receptor*
TNBS ..... *2,4,6-Trinitrobenzenesulfonic acid*
TNF- α ..... *Tumour necrosis factor α*
TOF ..... *Time of flight*
Treg ..... *Regulatory T cells*

2 PUBLICATIONS

PUBLICATIONS ASSOCIATED WITH THIS THESIS:

J. Klier, S. Geis, J. Steuer, K. Geh, S. Reese, S. Fuchs, R.S. Mueller, G. Winter, H. Gehlen
A Comparison of Nanoparticulate CpG Immunotherapy With and Without Allergens in Spontaneously Equine Asthma-Affected Horses, an Animal Model.
Immunity, Inflammation and Disease (2018), 6: 81–96.

I. Wagner, K.J. Geh, M. Hubert, G. Winter, K. Weber, J. Classen, C. Klinger, R.S. Mueller
Preliminary Evaluation of CpG Oligodeoxynucleotides Bound to Gelatine Nanoparticles as Immunotherapy for Canine Atopic Dermatitis.
Veterinary Record (2017) 181: 118.

Geh K.J., Hubert M., Winter G.
Optimisation of One-Step Desolvation and Scale-Up of Gelatine Nanoparticle Production.
Journal of Microencapsulation (2016), 33: 595-604

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Progress in Formulation Development and Sterilisation of Freeze-Dried Oligodeoxynucleotide-Loaded Gelatine Nanoparticles.
Manuscript submitted to European Journal of Pharmaceutics and Biopharmaceutics.

J. Klier, C. Zimmermann, S. Geuder, K. Geh, S. Reese, L.S. Goehring, G. Winter, H. Gehlen
Immunomodulatory Inhalation Therapy of Equine Asthma-Affected Horses: A Dose-Response Study and Comparative Study of Inhalative Beclometasone Therapy.
Manuscript in preparation.

ADDITIONAL PUBLICATIONS WRITTEN DURING THE RESEARCH PERIOD (NOT DIRECTLY ASSOCIATED WITH THIS THESIS):

Geh K.J., Stelzl A., Godl K., Engelke L., Förster B., Winter G.
Development of a Sprayable Hydrogel Formulation as Drug Carrier for the Skin Application of Therapeutic Antibodies.
Manuscript in preparation.

PROCEEDINGS AND ABSTRACTS CONTRIBUTED TO INTERNATIONAL CONFERENCES

Geh K.J., Hubert M., Winter G.
Stabilisation of Oligonucleotide-loaded Gelatin Nanoparticles by Lyophilization
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4-7 October 2016

Geh K.J., Hubert M., Winter G.
Stabilisation of Oligonucleotide-loaded Gelatin Nanoparticles by Lyophilization
43rd Annual Meeting & Exposition of the Controlled Release Society, Seattle, Washington, USA, 17-20 July 2016

Geh K.J., Hubert M., Winter G.
Progress in Scale Up of Gelatin Nanoparticle Production
10th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology, Glasgow, Scotland, UK, 4-7 April 2016

Geh K.J., Hubert M., Winter G.
Novel Approaches for Gelatin Nanoparticle Preparation
1st European Conference on Pharmaceutics: Drug Delivery, Reims, France,
13-14 April 2015

Wagner I., Geh K., Winter G., Weber K., Mueller R.S.

Immunotherapy of Canine Atopic Dermatitis with CpG Oligodeoxynucleotides Bound to Gelatine Nanoparticles

European Academy of Allergy and Clinical Immunology Congress, Vienna, Austria, 11-15 June 2016