Optimisation of one-step desolvation and scale-up of gelatine nanoparticle production

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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 here focusing 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. 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.

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 (Devereux, 2012). Evidence also indicates that there is an increasing number of domestic animals, which are afflicted with allergic pulmonary disorders. For example, recurrent airway obstruction (RAO) is currently the most common airway disease in horses (Pirie, 2014). RAO shares many similarities with human asthma and is described as a genetically predisposed allergic immune response to inhaled environmental allergens (Kirschvink and Reinhold, 2008). The allergic response leads to the development of major clinical signs, such as bronchoconstriction, mucus hypersecretion and inflammation of the lower airways (Léguillette, 2003; Pirie, 2014).

Conventional therapies include corticosteroids or ß2-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-ODN) have been identified to redirect the immune response from the pro-allergic Th2 pathway to the pro-inflammatory Th1 via the toll-like receptor 9 (TLR9) stimulation (Krieg, 2006). 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 (Foged et al., 2005; Hanagata, 2012). Several nanoparticles have provided promising results as carrier systems for CpG-ODNs, such as mesoporous silica nanoparticles (MSN) (Zhu et al., 2011), protamine nanoparticles (proticles) (Pali-Schöll et al., 2013) or gelatine nanoparticles (Klier et al., 2011, 2012, 2015a, 2015b). MSN could successfully prevent CpG-ODNs from degradation and enhance cellular uptake (Zhu et al., 2011). However, in vitro studies using MSN revealed complications, such as formation of reactive oxygen species or inhibition of cellular respiration (Vivero-Escoto et al., 2010). Proticles loaded with CpG-ODNs showed the ability to prevent an allergen-induced Th2 immune response in mice (Pali-Schöll et al., 2013). Nevertheless, protamine may induce severe side effects such as histamine release or anaphylactic reactions (Park, 2004).

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 functionalised 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 cationized 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 (Fuchs et al., 2012; Klier et al., 2012, 2015a, 2015b). 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 (Klier et al., 2015b). However, the co-application of allergens did not further increase the efficacy of this treatment (Klier et al., 2015a).

Besides RAO in horses, CpG-GNPs also showed the first positive results in the treatment of allergy-derived canine atopic dermatitis (Praelaud et al., 2013; Wagner et al., 2016). 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 GNP s is two-step desolvation (Coester et al., 2000). 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 (Marty et al., 1978; Coester et al., 2000). 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 (Klier et al., 2012, 2015b). 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 (Zwiorek, 2006; Zillies, 2007; Ahlers et al., 2007), which allowed to neglect the first irreproducible desolvation step. A previous study by Ofokansi and co-workers (Ofokansi et al., 2010) 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 (Zwiorek, 2006). A higher gelatine concentration promotes higher inter-molecular interactions and co-aggregation of gelatine during desolvation. As a result larger nanoparticles are formed (Zwiorek, 2006). 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 (Azarmi et al., 2006; Zwiorek, 2006). 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 (Azarmi et al., 2006). Azarmi et al. (2006) could show that GNPs prepared with ethanol showed particles which were 100–150 nm larger in size than GNPs prepared with acetone.

Two-step desolvation has become the standard preparation process for gelatine nanoparticles, but a reliable scale-up method has not yet been established (Fuchs, 2010). 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 (Wacker et al., 2011). 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.

Micro- and nanoparticles are commonly prepared through glutaraldehyde cross-linking of gelatine (Young et al., 2005; Elzoghby, 2013). 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 (Kari, 1993; Ballantyne and Jordan, 2001). 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 (Won and Kim, 2008), transglutaminase (Fuchs et al., 2010) or glyceraldehyde (Zhao et al., 2012) 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 manufacturing process in order to improve reproducibility, as well as the rate of yield. A screening of factors such as gelatine type, concentration and pH value was performed on small scales. To evaluate the effect of alternative cross-linking agents, we studied incubation time, cross-linking degree and colloidal stability.

Materials and methods

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). Glutaraldehyde (25% solution), glyceraldehyde, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (2-Aminoethyl)trimethylammonium chloride hydrochloride (Cholamine) and 2,4,6-Trinitrobenzenesulfonic acid (TNBS) 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.

Preparation of gelatine nanoparticles

Optimisation of gelatine nanoparticle preparation

Gelatine nanoparticles were prepared either by two-step desolvation (Coester et al., 2000) or one-step desolvation (Ahlers et al., 2007), 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
Table 1. Concentrations and pH conditions of alternative cross-linking agents.

| Gelatine  | Cross-linking agent | pH value | Concentration (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                 |

*Referred to volume of gelatine solution.

stirred overnight and purified by two-fold centrifugation (20 000 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.

Characterisation of gelatine nanoparticles

Cationisation of GNPs was performed according to the standard protocol (Zwiorek et al., 2005). In brief, GNP dispersion was diluted with HPW (1–2 mg/mL) and the pH was adjusted 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 (16 000 g for 15 min; Sigma Laborzentrifugen, Osterode, Germany).

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.

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 1). After incubation, GNPs were purified by two-fold centrifugation and dispersed 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.

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.

Characterisation of gelatine bulk material by asymmetric flow field-flow fractionation (AF4)

Characterisation of gelatine bulk material was performed by asymmetric field-flow 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, CA) 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 Na2HPO4 + 2H2O) pH 6.0 was chosen as running buffer. According to Schultes et al. (2009) 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 min.

Characterisation of gelatine nanoparticles

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.

Particle concentration

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

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% NaHCO3 (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 h 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 spectrophotometer, Agilent Technologies, Santa Clara, CA). 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:

$$ CL(\%) = \left(1 - \frac{A(\text{sample}) - A(\text{blank})}{A(\text{control}) - A(\text{blank})}\right) \times 100\% \quad (1) $$

Scanning electron microscopy (SEM)

Gelatine nanoparticles were freeze dried according to the protocol of Zillies et al. (2008) 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, OR) at 2.0 kV and a working distance of 4.0–4.2 mm.

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).
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 1).

Preparation of gelatine nanoparticles by one-step desolvation

Effect of gelatine concentration and pH value

Type A 300 bloom As reported in a previous study (Zwiorek, 2006), 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 2(a). The particles obtained had diameters between 150 and 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.

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 (Babel et al., 2000) 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 2(a)). 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.

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 S1). This includes particle sizes between 150 and 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 1. Schematic representation of the optimised gelatine nanoparticle preparation process and scale-up.

Figure 2. Particle characteristics of GNPs prepared by one-step desolvation. (A) Effect of concentrations on size and yield for gelatine type A 300 bloom (particle size: ●, relative particle yield: △) and type B 300 bloom (particle size: ○, relative particle yield: △) compared to two-step desolvation (particle size: - - - -, relative particle yield: - - - - ). (B) Particle size (●) and relative particle yield (△) of GNPs prepared at different pH values by one-step desolvation from gelatine type B 300 bloom compared to two-step desolvation (particle size: - - - -, relative particle yield: - - - - - ). Data is presented as mean ± SD (n = 3).
The evaluation of different pH values during desolvation showed smaller particle sizes as well as decreasing particle yields with increasing pH (Figure 2(b)). 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.

**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 3). 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.

**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 maximise 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 4(a)). 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.

![Figure 3](image3.png)

**Figure 3.** 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).

![Figure 4](image4.png)

**Figure 4.** (A) Particle yields of GNPs prepared by one-step desolvation from gelatine type A 300 bloom. Comparison of standard batch size and purification by centrifugation, scaled batch size and purification by centrifugation, standard batch size and purification by ultrafiltration, and scaled batch size and purification by ultrafiltration. Data are presented as mean ± SD (n = 3). **p < .001. (B) Preparation of GNPs in large scale using glyceraldehyde. Comparison of particle size (●) and particle yield (▲) 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).
To increase the particle yield further and also to lower the particle loss during purification by centrifugation, ultrafiltration was performed. The purification of GNPs 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 4(a), p < .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.

**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 5). 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 cationization, 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 cationization. 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 cationization process generated a permanent positive surface charge on GNPs from gelatine type B, which is comparable to the zeta potential of cationized GNPs from gelatine type A.

**Evaluation of alternative cross-linking agents**

**Glyceraldehyde**

Glyceraldehyde is commonly used to increase the mechanical strength of the sclera via cross-linking collagen (Wollensak and Spoerl, 2004). 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 2 summarises the parameters that were examined for the preparation of stable nanoparticles, as well as the resulting particle characteristics. An extended cross-linking time of 65 h was necessary to stabilise the nanoparticles from type A 300 bloom, compared to 15 h 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 2). Stable and monodisperse GNPs in a particle size range of 200–250 nm with high cross-linking degree and particle yield were prepared.

In scale-up experiments it could be shown that glyceraldehyde is suitable for large scale production of GNPs (Figure 4(b)). Using a five-fold amount of gelatine for the production of 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).

**Genipin**

In addition to glyceraldehyde, the naturally occurring cross-linking agent genipin was evaluated for its suitability to stabilise GNPs (Table 2). 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 h 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 and 370 nm. In comparison to glutaraldehyde (ca. 85%) or glyceraldehyde (ca. 75%), these particles showed a decrease in the degree of cross-linking, 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.

**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 6). 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 (Boottz et al., 2004).

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 20 mg particle yield with the standard two-step desolvation to a maximum output of 2.6 g GNPs with one-step desolvation. Furthermore, two alternative cross-linking agents were evaluated to substitute the critical substance glutaraldehyde.

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) (Ahlers et al., 2007). 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. (2010), 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. (Zwiorek, 2006), 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 and 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 \( p < .001 \) higher when compared to two-step desolvation. This is most likely due to the subjectivity of the

Figure 6. 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.
first of the 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%) (Ahlers et al., 2007). However, the mean MW of this gelatine, as measured by Schultes et al. (Schultes et al., 2009), 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 (Digenis et al., 1994). 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 desolation step. Based on their findings, they defined a mean molecular weight of ~400–500 kDa as the threshold for the one-step desolvation (Schultes et al., 2009), 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. (Ahlers et al., 2007), 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 cationization process. The cationisation reagents react with free carboxyl groups, free amine groups as well as glutaraldehyde residues (Zwiorek, 2006). 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 cationization and for electrostatic loading of CpG-ODNs onto their surface (loading efficiency >95%, Figure S1).

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. (Wacker et al., 2011) showed that a stirring bar and a small paddle stirrer (21 × 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 × 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 (Zwiorek et al., 2008). Here, we demonstrated an efficient way to apply stirred ultrafiltration cells, which are commonly used for protein concentration and purification (Stradner et al., 2004). 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.

Evaluation of alternative cross-linking agents

Glutaraldehyde is well known as cross-linking agent for proteinous nanoparticles, but presents safety issues for the patient and during manufacture (Kari, 1993). 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 (Fuchs et al., 2010), genipin (Won and Kim, 2008) and glyceraldehyde (Zhao et al., 2012), 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 h (Fuchs et al., 2010). However, high costs of the recombinant enzyme and reports indicating potential immunogenicity of transglutaminase residuals due to incomplete removal limit its applications (Schloegl et al., 2012). Moreover, previous studies showed successful cross-linking of nanoparticles from recombinant human gelatine with genipin (Won and Kim, 2008). Stable GNPs with a uniform size distribution and particle sizes between 200 and 300 nm were obtained after a cross-linking time of 72 h. 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 (Sung et al., 2001). 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 188 (Zhao et al., 2012). 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 keto-enol tautomerism of glyceraldehyde resulting in a di-aldehyde, which is similar to the more reactive glutaraldehyde (Gerrard et al., 2002). 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 (Figure S1). 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.

**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.

**Disclosure statement**

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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