The impact of calcium phosphate on FITC-BSA loading of sonochemically prepared PLGA nanoparticles for inner ear drug delivery elucidated by two different fluorimetric quantification methods

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ARTICLE INFO

Keywords: Intratympanic Protein loading capacity Nanoparticles Calcium phosphate Quenching FITC-BSA

ABSTRACT

Although therapeutically active proteins are highly efficacious, their content in protective nanoparticles is often too low to elicit adequate plasma levels. A strategy to increase protein loading is the in-situ generation of calcium phosphate as a protein adsorbent. To verify this approach, a highly sensitive and reliable fluorimetric method for quantification of incorporated fluorescein-labelled bovine serum albumin (FITC-BSA) as a model protein drug was developed. Dequenching the fluorescein label by pronase E, which digests the protein backbone, and dissolving the nanoparticle matrix in acetonitrile enabled FITC-BSA quantification in the nanogram per milliliter range. This test was confirmed by a second assay involving alkaline hydrolysis of FITC-BSA and the matrix. Nanoparticles prepared with calcium phosphate contained 40 µg FITC-BSA/mg and nanoparticles without calcium phosphate only 15 µg FITC-BSA/mg, representing a 2.7-fold increase in model protein loading. In this work the nanoparticle preparation procedure was optimized in terms of size for administration in the inner ear, but the range of applications is not limited.

1. Introduction

As reflected by 26 % of the approvals by the FDA in 2020 [2] and a growing global market share [3], proteins proved to be valuable drugs with low toxicity [4], but at the same time show high susceptibility to inactivation associated with short plasma half-life and poor bioavailability [5]. To face these challenges, protein drugs are protected and stabilized by encapsulation into smallest scaled drug delivery systems. It is widely accepted that such nanoparticles can overcome absorptive barriers and release their protein cargo at the intended site of action in the body [6].

Especially in case of inner ear drug delivery, most recently nanocarriers have gained increasing attention [7–10]. Restricted accessibility within the petrous bone together with the blood-perilymph barrier [11] make systemic drug delivery to the inner ear particularly challenging and emphasizes the potential benefits of locally applied drug delivery systems for the inner ear. In the case of intratympanic administration, the drug delivery system is placed in the middle ear, preferably into the round window niche, offering a volume of only a few microliters. Thereby escaping the rapid drainage through the Eustachian tube into the nasopharynx, the nanocarrier still has to surmount the round window membrane to finally distribute and release the proteinaceous drug within the perilymph fluid of the cochlea. According to the current, still limited physiological knowledge, there are a few issues to be considered for successful drug delivery: The hydrodynamic diameter of the nanocarrier should be smaller than 200 nm and the polydispersity index (PDI) lower than 0.2 [12–14].

For the preparation of nanocarriers, poly (D,L-lactic-co-glycolic acid) (PDG,)...
(PLGA), a biodegradable and biocompatible FDA-approved polyester, is most frequently used. However, “a high loading efficacy of active protein is very difficult to achieve for PLGA-nanoparticles” according to Mäder, Lehner, Liebau and Plontke [7], leading to different approaches to increase the content of hydrophilic proteins. Based on the work by Tang et al. [15] for increasing the DNA encapsulation efficiency in nanoparticles, Dördemann et al. [1] deployed calcium phosphate to leverage its protein adsorbing ability. The underlying mechanisms have been thoroughly investigated [16,17]: Alkaline proteins are retained by a cation exchange reaction at the negatively charged phosphate-site of calcium phosphate, whereas acidic proteins such as bovine serum albumin (BSA) strongly interact with the positively charged calcium-site, forming metal coordination complexes at acidic and neutral pH [18]. In addition to its protein adsorbing properties, calcium phosphate is also biocompatible [19,20] and thus an ideal auxiliary agent to entrap a hydrophilic protein within a hydrophobic matrix.

In this work, nanoparticles are prepared by employing an ultrasound-supported W/O/W double emulsion solvent evaporation technique. The underlying mechanism of ultrasound contribution can be described – in simple terms – as the generation and collapse of gas cavities [21] due to acoustic cavitation [22] that result in high localized shearing forces associated with an increase in temperature [23]. One of the benefits of an ultrasound-based particle preparation protocol is the generation of particles with both a desired average particle size and a narrow size distribution [24], two very important parameters for targeted drug delivery. The preparation of the final W/O/W double emulsion includes a three-step ultrasound-driven emulsification protocol, that starts by preparation of two separate W/O emulsions. Each contains either the calcium- or the phosphate component of the to-be-precipitated calcium phosphate and FITC-BSA in the water (W) phase, that is sonically dispersed in the outer, continuous oil (O) phase, containing the polymer PLGA in ethyl acetate. Subsequently, both W/O emulsions are combined and subjected to a second sonication step, facilitating the ultrasound-supported in-situ precipitation of calcium phosphate and the formation of calcium phosphate/FITC-BSA complexes. Physical shearing and acoustic cavitation further disrupt the disperse aqueous phase to smallest droplets within the continuous ethyl acetate/PLGA phase, that will finally form the nanoparticle matrix. The generation of these W/O emulsions is most probably supported and stabilized by the presence of FITC-BSA, which acts as a surfactant. During the third and final sonication step, the W/O emulsion is dispersed in a threefold larger continuous aqueous phase containing the non-ionic surfactant poloxamer 188. This facilitates decreasing the solubility of the hydrophobic polymer PLGA due to a far larger outer continuous water-phase, and it is stabilized by the presence of poloxamer 188, supporting the emulsification process. As a result, PLGA starts to solidify, simultaneously entrapping the highly dispersed nanodroplets containing calcium phosphate/FITC-BSA complexes within the nanoparticle PLGA matrix.

To verify the protein-loading effect of calcium phosphate, the fluorescein-5-isothiocyanate isomer-I-labelled bovine serum albumin (FITC-BSA) served as model protein. Fluorescein is a widespread fluorophore in research that can enable visualization of the labelled protein’s distribution in drug delivery systems, cells, and tissues as well as its quantification [25–27]. Most frequently, FITC-BSA encapsulation efficiency is estimated by UV/Vis spectroscopy either directly or as part of protein quantification assays [28–31]. For a sensitive detection, however, in this work a fluorimetric quantification approach was followed instead. Unfortunately, Wischke and Borchert [32] concluded after investigating the fluorescent properties of FITC-BSA encapsulated into PLGA microparticles that “the quantification of protein by fluorescence spectroscopy failed”. The problem addressed is fluorescence quenching, a multi-faceted phenomenon occurring due to interaction of spatially close fluorophores one to another [33], with certain amino acids [34–36] and/or nucleic acids [37], leading to a loss of fluorescence quantum yield. A strategy to solve this problem is to release each fluorophore by degradation of possibly interacting structures and to harvest the full quantum yield.

This work is aimed to optimize the ultrasound-supported preparation protocol and classification of FITC-BSA-loaded PLGA-nanospheres for intratympanic administration but at the same time to increase the model protein loading by presence of calcium phosphate. Additionally, with a reliable quantification assay being a prerequisite to enable systematic optimization of the ultrasound-based preparation protocol, two reliable assay designs are presented to confirm the beneficial effect of calcium phosphate on protein entrapment, finally yielding nanoparticles appropriate for intratympanic administration in animals and a protein content suitable for therapy and imaging.

2. Materials and methods

2.1. Materials

Resomer® RG 503H (poly (D,L-lactic-co-glycolic acid; PLGA), with uncapped carboxylic end groups and a 50:50 lactide : glycolide ratio was purchased from Evonik Nutrition & Care GmbH (Essen, Germany). Fluorescein-5-isothiocyanate isomer-I-labelled bovine serum albumin (FITC-BSA; F/P ratio = 7–12), poloxamer 188, sodium phosphate dibasic dihydrate and sodium dodecyl sulfate (SDF) were obtained from Sigma-Aldrich Corporation (St. Louis, MO, USA). Calcium chloride dihydrate as well as pronase E (Pro E; from Streptomyces griseus, 4,000,000 PU/g) were purchased from Merck KGaA (Darmstadt, Germany). Ethyl acetate and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were obtained from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). All other chemicals were of analytical grade and used as supplied.

2.2. Methods

2.2.1. Preparation of FITC-BSA-loaded PLGA nanoparticles

2.2.1.1. Synthesis of calcium phosphate-FITC-BSA-PLGA nanoparticles.

The method applied for the preparation of calcium phosphate-FITC-BSA-PLGA nanoparticles (CP-FITC-BSA-PLGA) was a double emulsion solvent evaporation technique with basic modifications to a protocol according to Dördemann et al. [1]. In the first step, two FITC-BSA solutions were prepared by dissolving 8.0 mg of FITC-BSA in either 1600 µl of 10 mM Na₂HPO₄ × 2 H₂O/distilled water (solution I) or 1600 µl of 16 mM CaCl₂ × 2 H₂O/distilled water (solution II). Solution I and II were mixed separately with 4800 µl of 4 % (w/v) PLGA/ethyl acetate, followed by ultrasoundication on ice for 20 s using a Sonopuls HD 2070 instrument with sonotrode MS 73 (40 % amplitude, pulse cont.; BANDELIN electronic GmbH & Co. KG, Berlin, Germany) forming the W/O emulsions I and II. In the next step, W/O emulsions I and II were combined by ultrasoundication for 2 min and added immediately to 38.4 ml of 10 % (w/v) poloxamer 188/distilled water, followed by another sonication step for 2 min yielding the W/O/W emulsion. The W/O/W emulsion was dispersed in 100 ml of 0.1 % (w/v) poloxamer 188/distilled water and stirred overnight to allow evaporation of the organic solvent and hardening of the nanoparticles.

Calcium phosphate-free FITC-BSA-PLGA nanoparticles (FITC-BSA-PLGA) were prepared as described above but omitting the addition of Na₂HPO₄ × 2 H₂O and CaCl₂ × 2 H₂O.

2.2.1.2. Purification and stabilization of nanoparticles.

Subsequently, the nanoparticle batches were sorted according to a centrifugation/filtration protocol that was optimized to obtain a nanoparticle fraction with < 150 nm in size. After the first centrifugation step at 10,490 × g for 30 min and 4 °C (Sorvall™ LYNX 6000 centrifuge equipped with a Fiberlite™ F21-8 × 50y fixed-angled rotor; Thermo Fisher Scientific Inc., Waltham, MA, USA) the supernatants were collected and subjected to a
second centrifugation step (30 min, 32,322 × g, 4 °C). Subsequently, the purification process was completed by a third step at 40,632 × g for 30 min and 4 °C. The pellets obtained after the second and the second the centrifugation step were redispersed in 0.1 % (w/v) poloxamer 188 and the nanoparticle suspension was filtered through a glass fiber membrane syringe filter (1.0 μm pore size; MilliporeSigma, Billerica, MA, USA).

For nanoparticle characterization, aliquots of the suspensions were frozen at −80 °C and lyophilized for at least 24 h at 0.37 mbar and −10 °C using an Alpha 1–4 LD plus instrument (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany).

2.2.2. Nanoparticle characterization

The nanoparticle yield was calculated according to the following equation:

\[
yield \% = \frac{m_p(\text{nanoparticles})}{m_m(\text{PLGA}) + m_i(\text{FITC-BSA}) + m_i(\text{calcium}) + m_i(\text{phosphate})} \times 100
\]

with the mass (m) of materials initially added (m₀) and NP mass after purification (mₚ). The loading capacity (LC) and encapsulation efficiency (EE) were evaluated according to:

\[
LC \% = \frac{m_i(\text{FITC-BSA})}{m_p(\text{nanoparticles})} \times 100 \tag{2}
\]

and

\[
EE \% = \frac{LC}{LC_{\text{max}}} \times 100 \tag{3}
\]

respectively. The maximum loading capacity (LCmax), describing the theoretical maximum protein loading of NP, was calculated as follows:

\[
LC_{\text{max}} \% = \frac{m_i(\text{FITC} – \text{BSA})}{m_i(\text{matrix}) + m_i(\text{FITC-BSA})} \times 100 \tag{4}
\]

The hydrodynamic diameter (size) and particle size distribution (polydispersity index, PDI) were analyzed via dynamic light scattering and the zeta potential was calculated according to the Smoluchowski approximation. Measurements were performed with a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) using distilled water containing 10% (w/v) poloxamer 188 (particle size) or 0.05% (w/v) poloxamer 188 (zeta potential) as a medium.

2.2.3. Scanning electron Microscopy (SEM)

Fifty microliters of an aqueous nanoparticle suspension were distributed dropwise on a polycarbonate membrane (0.1 µm pore size; Isopore™, Merck Millipore Ltd, Cork, IRL). After drying under vacuo, samples were sputter-coated with gold for subsequent examination in a FlexSEM 1000 scanning electron microscope (Hitachi High-Technologies Corporation, Tokyo, Japan) at 20 kV.

2.2.4. Quantification of incorporated FITC-BSA

The protein content of the nanoparticles was assessed by quantification of the fluorescein labels after degrading the protein backbone by two basically different methods.

2.2.4.1. Enzymatic digestion with pronase E (pronase E/acetoniitrile method). Considering enzyme characteristics such as optimum pH for substrate turnover, sensitivity against organic solvents and time dependent activity, the assay was done as follows: Either a mixture of 100 µl aqueous nanoparticle suspension and 400 µl acetoniitrile (AcN) or a solution of 1.0 mg nanoparticles lyophilized in 500 µl acetoniitrile were incubated with 1500 µl 0.1 % (w/v) pronase E solution in 0.15 M HEPES/NaOH buffer pH 7.4 at 37 °C for 24 h. After appropriate dilution with HEPES, the fluorescence intensity was determined at 488/522 nm (exc/em; gain 81) using an Infinite M200 PRO instrument (Tecan Group Ltd., Männedorf, Switzerland). The blank was prepared as described above but without addition of nanoparticles.

The protein content was calculated from a calibration curve prepared by digestion of 40 µg FITC-BSA as above but with a 20 – 400-fold dilution of the samples with HEPES prior to read out. All samples were analyzed at least in triplicate.

2.2.4.2. Unfolding and chemical hydrolysis with SDS/NaOH (SDS/NaOH method). Contrary to the mild environment of enzymatic proteolysis, a second technique with harsh conditions was applied. For determination of entrapped FITC-BSA, 1.0 mg of lyophilized FITC-BSA nanoparticles was dispersed in 2000 μl of 5% (w/v) SDS in 0.1 M NaOH. After incubation for 24 h at 37 °C the samples were analyzed fluormetrically at 485/525 nm (exc/em; gain 80) in an Infinite M200 PRO instrument microplate reader.

The calibration curve was established by hydrolysis of a solution containing 500 µg/ml FITC-BSA as described above. Prior to fluorescence reading the samples were diluted to contain 0.05 – 4.0 µg FITC-BSA/ml.

2.2.5. Statistical analysis

Data are presented as mean ± SD of four different batches. Error bars represent SD. Data analysis was performed using GraphPad Prism version 9.0.1 software (GraphPad Software, San Diego, CA, USA). Nanoparticle characteristics (i.e. NP yield, z-average size, polydispersity index and zeta potential; n = 4) were compared between groups (CP-FITC-BSA-NP vs. FITC-BSA-NP) employing an unpaired, two-tailed t-test. Results of FITC-BSA quantification (FITC-BSA per mg NP, loading capacity and encapsulation efficiency; n = 4) were statistically evaluated between CP-FITC-BSA-NP and FITC-BSA-NP using a Welch t-test. Results of FITC-BSA quantification were compared between pronase E/ acetoniitrile and NaOH/SDS method (n = 4) using an unpaired t-test. All statistical tests were two-tailed and results were considered statistically significant if p ≤ 0.05.

Table 1

| Surfactant type | Organic solvent | Ratio water/organic solvent | Ratio [W/O]/W → [W/O/W] | Centrifugation settings | Determination of FITC-BSA content of NP/µl supernatants | Redispersion of pellets/medium | Determination of FITC-BSA | Current work |
|-----------------|-----------------|-----------------------------|-------------------------|-------------------------|------------------------------------------------------|-----------------------------|--------------------------|-------------|
| Hepes/NaOH 1:3 | Poloxamer 188 1% | 1:3                         | 1:3                     | 1.3, 14,800 rpm/30 min | Free FITC-BSA/UV/Vi (460 nm) analysis of supernatants | Ultrapurified water         | Bound FITC-BSA            | 10 mM       |
| Acetic acid 1:3 | Poloxamer 188 10% | 1:3                         | 1:3                     | 1.3, 14,800 rpm/30 min | Free FITC-BSA/UV/Vi (460 nm) analysis of supernatants | Ultrapurified water         | Bound FITC-BSA            | 100 %      |
| Acetic acid 1:3 | Polyvinyl alcohol | 1:3                         | 1:3                     | 1.3, 14,800 rpm/30 min | Free FITC-BSA/UV/Vi (460 nm) analysis of supernatants | Ultrapurified water         | Bound FITC-BSA            | 100 %      |
| Acetic acid 1:3 | Acetic acid 1%   | 1:3                         | 1:3                     | 1.3, 14,800 rpm/30 min | Free FITC-BSA/UV/Vi (460 nm) analysis of supernatants | Ultrapurified water         | Bound FITC-BSA            | 100 %      |

Table 1 Comparison of parameters for nanoparticle preparation and purification

Used in the publication by Dordelmans et al. [1] and in this work. AcN actonitrile; F-BSA fluorescein-5-isothiocyanate isomer-l-labelled bovine serum albumin; PLGA poly (D,L-lactic-co-glycolic acid); SDS sodium dodecyl sulfate.
3. Results and discussion

3.1. Optimization of the nanoparticle preparation protocol

Basically, the nanoparticles were prepared by an ultrasound-mediated double emulsion technique roughly based on the protocol of Dördelmann et al. [1], which in turn follows the method published by Tang et al. [15]. As experiments done by Feczko et al. [38] revealed an improved encapsulation efficiency of bovine serum albumin (BSA) when the PLGA concentration was 2.5–4.0 % and the BSA/PLGA ratio was strongly increased, the PLGA-concentration was set at 4 % and the BSA/PLGA ratio was increased threefold to 1:24 (Table 1).

Another issue towards optimization was decreasing the concentration of CaCl$_2$ from 1.25 M (originally based on the publication by Tang et al. [15]) to 16 mM, leading to a reduction of the molar calcium/phosphate ratio from 125 to 1.6. On the one hand, this way an unnecessary calcium exposure of cochlear structures is avoided, on the other hand the ratio of 125 is by far out of range for formation of calcium orthophosphates [39]. A calcium/phosphate ratio of 1.6 and a neutral to slightly acidic pH during precipitation from an aqueous solution would suggest formation of octacalcium phosphate or calcium deficient hydroxyapatite [39]. However, it is most unlikely that hydroxyapatite is formed under these prevailing experimental conditions since hydroxyapatite is formed at a calcium/phosphate ratio of 1.67 and is only stable within a pH range of 9.5–12 [40].

Ongoing from preliminary experiments, ethyl acetate instead of toxicological harmful dichloromethane and poloxamer 188 instead of polyvinyl alcohol yielded nanoparticles that best met our requirements. The volume ratio of 1:3 between inner aqueous and outer organic phase of the emulsion remained unchanged.

3.2. Nanoparticle characteristics

Narrowing the size range of the nanoparticles by the centrifugation protocol presented reduced the yield of both calcium phosphate-containing and calcium phosphate-free FITC-BSA-nanoparticles; FITC-BSA-NP calcium phosphate-containing FITC-BSA-nanoparticles; CP-FITC-BSA-NP calcium phosphate-free FITC-BSA-nanoparticles. **p ≤ 0.01.

![Fig. 1. Size and polydispersity index of nanoparticles after purification. Bars represent hydrodynamic diameter (left y-axis, nm), triangles show PDI values (right y-axis). CP-FITC-BSA-NP calcium phosphate-containing FITC-BSA-nanoparticles; FITC-BSA-NP calcium phosphate-free FITC-BSA-nanoparticles. **p ≤ 0.01.](image-url)

![Fig. 2. Overview SEM images of nanoparticles. (A) calcium phosphate-containing FITC-BSA-nanoparticles (B) calcium phosphate-free FITC-BSA-nanoparticles. Magnification 5,000×; scale bar 10.0 µm.](image-url)
was observed that 300 nm and 150 nm particles migrated faster into the cochlea than 80 nm particles within the first 30 min. However, 24 h after application, no size-dependent differences could be detected [41]. Interestingly, experiments on the translocation of 160 nm PLGA NP after intratympanic injection performed by Zhang, Xu, Cao, Xie, Wen and Chen [14] revealed that intact nanoparticles reached the perilymph within 30 min. They suggested that the transport of nanoparticles across the round window membrane is regulated by micropinocytosis and by caveolae-mediated endocytosis. Thus, the classified nanoparticles are promising tools for pharmacokinetic studies. SEM images confirm the presence of a homogenous particle population with spherical nanoparticles and nanoparticle diameters within the order of magnitude of size measurements by Dynamic Light Scattering (Fig. 2).

In contrast to the diameter, the polydispersity of the particle preparations was independent from the type of particles (Fig. 1). At first sight, the purification of the particles shifted the preparations from a narrow, nearly monodisperse size range to a moderate polydisperse one as referred to by Nobbmann [42]. However, this effect is only due to the smaller average particle size as statistical evaluation revealed no difference in PDI between particles of the same group (CP-FITC-BSA-NP or FITC-BSA-NP) before and after purification. As confirmed by animal experiments, particles with these size characteristics crossed the round window membrane [13,14].

The zeta potential of CP-FITC-BSA-NP was −30.47 ± 4.20 mV and insignificantly lower than that of FITC-BSA-NP amounting to −37.44 ± 4.47 mV (Table 2). As recently reported, positive surface charges of nanocarriers seem to result in a more pronounced accumulation in the inner ear compared to neutral and negatively charged ones [8]. Nevertheless, in a study by Youm, Musazzi, Gratton, Murowchick and Youan [43] negatively charged intratympanically applied nanoparticles of comparable size and surface charge (153.4 ± 8.7 nm; −22.1 ± 1.1 mV) migrated across the round window membrane followed by distribution in the basal and middle turns, even reaching lower parts of the third cochlear turn.

3.3. Assays for FITC-BSA quantification in nanoparticles

To verify that calcium phosphate can increase the protein content of the nanoparticles, FITC-BSA was used as a model protein which allows indirect quantification of the protein drug content. The excellent fluorescence quantum yield, good water solubility, matching the spectral line of an argon-ion laser for excitation as well as simple derivatization chemistry makes fluorescein a highly preferred label. However, pH-sensitivity of fluorescence quantum yield, a moderate rate of photo-bleaching and especially a high tendency towards quenching upon conjugation to biopolymers raises difficulties in practice. Quenching can occur due to either a short distance mechanism (photoinduced electron transfer) below 1 nm or due to a long-distance mechanism (Förster resonance energy transfer) in the range of 1–10 nm [44–46]. Thus, the degree of substitution plays a pivotal role, e.g. FITC-BSA with a fluorescein-to-protein ratio of up to 3 is not susceptible to distance quenching, but steeply increases beyond especially in commercially available FITC-BSA containing 7–12 fluorescein moieties per protein molecule [33]. Furthermore, cluster formation of fluorophores can be observed, which also contributes to self-quenching as a special form of concentration quenching [47]. Apart from homo-Förster resonance energy transfer between two fluorescein molecules or self-quenching, interactions between the label and other molecules such as the amino acids methionine, histidine, tryptophan, and tyrosine can occur and are not predictable [34].

The basic concept for dequenching is to spatially separate the fluorescein molecules by degradation of the polymeric backbone to avoid interactions between the fluorophores as well as fluorophores and other acceptor molecules. The polymeric molecules can be degraded either enzymatically or chemically to facilitate reliable and reproducible quantification of multi-labelled FITC-BSA by fluorescence spectroscopy.
the same as those obtained in the absence of acetonitrile although there was a slight delay within the first two hours in the presence of acetonitrile (Fig. A.1). Obviously, the low amount of acetonitrile was high enough to dissolve the matrix and concurrently low enough to prevent denaturation of the enzyme. On the other hand, the high water content would increase the conformational mobility of the protein, potentially favoring the loss of enzyme activity. [50].

As indicated by the calibration curves, the organic solvent negligibly influenced the performance of the assay since the limit of detection and limit of quantification were 18.54 ng/ml and 56.48 ng/ml in absence of acetonitrile as compared to 18.33 ng/ml and 55.54 ng/ml in presence of acetonitrile. Both calibration curves had identical coefficients of determination ($R^2 = 0.9999$).

3.3.2. SDS/NaOH-method

At first sight, 0.1 M NaOH seems to be the ideal for dequenching as it degrades the polyester PLGA by alkaline hydrolysis as well as the protein backbone of FITC-BSA. Preliminary assays, however, revealed non-reproducible dequenching most probably due to incomplete denaturation despite a pH of 13. In accordance with the literature, treatment with 5 % aqueous SDS alone also did not fully unfold the protein’s structure [51] and therefore did not enable reliable quantification of FITC-BSA.

Treatment of the particles with 0.1 M NaOH/5 % SDS is a method known for protein quantification, however, by using colorimetric assays such as the Bradford test, and not for sensitive fluorimetric assessment [52-54]. The anionic surfactant SDS unfolds the globular structure of BSA by electrostatic repulsion and hydrophobic interactions [51]. Similarly, the alkali sets the pH value at 13 and causes negative charge of amino acids that repel each other and unfold the secondary and tertiary structure of BSA [55,56].

All in all, the harsh conditions resulted in a sensitive method with a limit of detection of 14.54 ng/ml and a limit of quantification of 44.06 ng/ml, being about 4 ng/ml and 10 ng/ml lower than those of the pronase E/acetonitrile method, respectively. The coefficient of determination was 1.000.

3.4. FITC-BSA content of the nanoparticles

Despite minor differences in limit of quantification and limit of detection, both methods can be seen as equally suitable concepts for fluorimetric quantification of multi-FITC-labelled BSA in nano- and microparticles. The FITC-BSA content of PLGA nanoparticles was about 15 µg/mg, whereas calcium phosphate increased the protein content 2.7-fold to about 40 µg FITC-BSA/mg nanoparticles (Table 3, Fig. 3).

The loading capacity specifies the proportion of incorporated FITC-BSA in relation to the total nanoparticle mass. In the absence of calcium phosphate, the loading capacity of FITC-BSA nanoparticles was only 1.52 ± 0.08 % representing 37.9 % of the amount of FITC-BSA incorporated into calcium phosphate nanoparticles. In the latter type of nanoparticles 95 % of the theoretical maximum loading capacity was reached as opposed to 35 % in absence of calcium phosphate. Calculation of the encapsulation efficiency allows the assessment of a nanoparticle preparation protocol from an economic point of view, as it puts in context the maximum amount of active pharmaceutical ingredient that can be incorporated into the nanoparticle and the quantity that actually got entrapped. In presence of calcium phosphate, the encapsulation efficiency was about 94 % in contrast to 35 % in absence of calcium phosphate. Thus, highly expensive active pharmaceutical ingredients from the biotech pipeline might be fully incorporated into nanoparticles with the aid of calcium phosphate. Dördelmann et al. [1] achieved an encapsulation efficiency of 78 % in the presence of calcium phosphate, suggesting that our alterations to the protocol further increased it by 16 %. As shown in preliminary experiments, both FITC-BSA and the PLGA-NP (regardless of the presence of calcium phosphate) exhibit a negative zeta potential during preparation (data not shown), which represents the surface charge in an aqueous suspension. These results suggest that the adsorption of FITC-BSA onto the NP surface is counteracted by electrostatic repulsion, indicating that the extent of protein adsorption to the nanoparticle surface is negligible. Therefore, the increase in encapsulation efficiency can only be attributed to the protein adsorbing characteristics of calcium phosphate [16].

4. Conclusions

Against expectations, a sensitive and reliable fluorimetric quantification of entrapped multi-labelled FITC-BSA in PLGA nanoparticles is possible. The concept of enzymatic dequenching by pronase E/acetonitrile can easily be adapted to other FITC-labelled proteins and for application in microparticles. To the best of our knowledge, the use of pronase E as part of a quantification method for multi-FITC-labelled BSA incorporated into PLGA nanoparticles has not been published yet. Additionally, the amount of entrapped protein is determined directly and not indirectly by quantification of the non-entrapped protein, which is highly susceptible to errors.

This assay and an additional one confirmed undoubtedly that the in situ generated calcium phosphate during ultrasound-mediated nanoparticle formation indeed acts as an adsorber for a protein imitating a therapeutic one. The 2.7-fold higher protein content is impressive but at the same time means that the nanoparticle is composed of 40 µg protein

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Table 3
Results of protein quantification using two different fluorimetric quantification methods (pronase E/acetonitrile and SDS/NaOH). AcN acetonitrile; CP-FITC-BSA-NP calcium phosphate-containing FITC-BSA-nanoparticles; EE encapsulation efficiency; FITC-BSA-NP calcium phosphate-free FITC-BSA-nanoparticles; LC loading capacity; Pro E pronase E; SDS sodium dodecyl sulfate. * p ≤ 0.01; *** p ≤ 0.001.

|                | quantification method | µg FITC-BSA/mg NP | LC [%]     | EE [%]     | p value |
|----------------|-----------------------|-------------------|------------|------------|---------|
|                 | CP-FITC-BSA-NP         |                   |            |            |         |
| Pro E/AcN      | 40.05 ± 5.47          | 4.01 ± 0.55       | 94.16 ± 12.86 | **       |
|                 | (p=0.0024)            |                   |            |            |         |
| SDS/NaOH       | 40.70 ± 4.24          | 4.07 ± 0.42       | 96.71 ± 10.08 | ***      |
|                 | (p=0.0010)            |                   |            |            |         |
|                 | FITC-BSA-NP           |                   |            |            |         |
| Pro E/AcN      | 15.21 ± 0.84          | 1.52±0.08         | 35.52 ± 1.97 |         |
| SDS/NaOH       | 15.43 ± 0.75          | 1.54 ± 0.08       | 36.02 ± 1.76 |         |


drug and 960 µg matrix material. On the one hand, this amount is by far too low to reach a therapeutic level in case of conventional drugs. On the other hand, this ratio might be promising for future drug delivery especially in view of highly potent drugs from the biotech pipeline. Additionally, the optimization of the ultrasound-supported preparation protocol resulted in nanoparticles particularly suitable for local inner ear administration via intratympanic injection.

CRediT authorship contribution statement

Julia Clara Gausterer: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing – original draft Visualization. Clara Schüßler: Methodology, Validation, Investigation, Data Curation, Writing – original draft, Visualization. Franz Gabor: Conceptualization, Methodology, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A: Effect of incubation time and acetonitrile addition on FITC-BSA degradation and pronase E activity

100 µl of FITC-BSA solution (500 µg/ml FITC-BSA in 150 mM HEPES/NaOH buffer pH 7.4) were added to 400 µl of acetonitrile and mixed with 1500 µl of 0.1 % (w/v) pronase E/150 mM HEPES/NaOH buffer pH 7.4 followed by subsequent incubation at 37 °C. For the negative control, acetonitrile was replaced with 400 µl of 150 mM HEPES/NaOH buffer pH 7.4. Samples were taken every hour and fluorimetrically analyzed at 488/522 nm (exc/em; gain 81) until maximum relative fluorescence intensity values were reached. To ensure that its absolute maximum had been measured, additional samples (after 24 h and 1 week of incubation) were analyzed. Although the specimens were incubated with pronase E in this experimental setting for at least 24 h, evaluation of the time-fluorescence intensity curve suggested that an incubation time of at least 4 h was required for complete enzymatic degradation of FITC-BSA, which is indicated by fluorescence intensity values increasing to a maximum plateau. The results showed an initial 1-2 h lasting reduction in proteolytic activity recognizable by a less steep increase of relative fluorescence intensity values in the presence of acetonitrile which is undone after 3-4 h of incubation time. This led to the conclusion that acetonitrile addition had no effect on the overall proteolytic activity of pronase E and the final degradation grade of FITC-BSA, indicating that the effect of acetonitrile is negligible in this experimental setting (Fig. A1).

Appendix B: Effect of the pH value on relative fluorescence intensity of FITC-BSA

To assess the pH dependency of the fluorescence intensity of FITC-BSA, 1.0 mg of FITC-BSA was proteolyzed by addition of 2000 µl 0.1 % (w/v) pronase E/150 mM HEPES/NaOH buffer pH 7.4 and incubated for at least 24 h at 37 °C. Afterwards, the FITC-BSA solution was split into 14 aliquots and each aliquot was pH-adjusted to a different value within the range of 1 to 14 (dilution factor 1:100). Immediately after that, fluorescence spectroscopy was carried out at 488/522 nm (exc/em; gain 81). Based on these findings, all fluorimetric measurements linked to the pronase E method were performed at a pH value of 7.4, therefore not bearing the risk of interfering with the pH optimum of pronase E (Fig. B1).
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