Research paper

Zinc–alginate microparticles for controlled pulmonary delivery of proteins prepared by spray-drying

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ABSTRACT

The aim of this study was to prepare novel Zn^{2+}-cross-linked alginate microparticles for controlled pulmonary delivery of protein drugs via a simple one-step spray-drying process and to physicochemically characterize these systems. Microparticles were prepared by spray-drying aqueous alginate solutions, containing the model protein BSA, $Zn(NH_3)_4SO_4$, and optionally additional excipients. Upon ammonia evaporation, the alginate was cross-linked by Zn^{2+}-ions. The microparticles were characterized by SEM, laser and X-ray diffraction, gel electrophoresis, aerodynamic particle size, and drug release measurements. Particles in a size range suitable for deep lung administration were obtained. Pure alginate microparticles were spherical in shape, whereas the addition of zinc led to a more collapsed geometry. Protein release depended on the (i) alginate:$ZnSO_4$ ratio (minimum release rate at 2:1); (ii) BSA content (decreasing release rate and extent with decreasing BSA content); (iii) type of release medium (increasing release rate with increasing phosphate concentration). The emitted microparticle dose was high for all formulations (~90%). Fine particle fractions (FPF, depositing in the deep lung) up to 40% could be achieved. The FPF was affected by the BSA content, alginate:$ZnSO_4$ ratio and presence/absence of poloxamer. Thus, novel Zn^{2+}-cross-linked alginate microparticles were prepared via a simple one-step process, providing an interesting potential for controlled pulmonary delivery of proteins.

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1. Introduction

Pulmonary delivery offers an interesting, non-invasive option for the systemic therapy with high molecular weight drugs like peptides and proteins (e.g., insulin, growth hormones, and calcitonin) [1,2]. The large surface area available for absorption, very thin and highly permeable epithelia, relatively low metabolic activity as well as the avoidance of the hepatic first-pass effect make the lung more efficient in systemic absorption than other non-injection-based application sites [3,4].

However, a major challenge for many inhalable drugs is the relatively short duration of the clinical effects due to the rapid drug elimination from the systemic circulation. Different approaches have been proposed to overcome this restriction aiming to reduce the dosing frequency and to improve patient compliance [5]. One attempt is to use dry powder aerosols consisting of microparticles, in which the drug is incorporated within biocompatible matrix materials controlling the release [6,7]. These microparticles must not rapidly be cleared from the lung in order to be able to deliver the

drug over prolonged periods of time. This is challenging, because the human lung provides very efficient clearance mechanisms for foreign particles. If the particles have an aerodynamic diameter >5 μm, they are mainly deposited in the upper airways via impaction. The "mucociliary escalator" (ciliated epithelia) then sweeps them quickly toward the mouth [8]. On the other hand, too small particles are exhaled upon pulmonary administration. The optimal aerodynamic particle size allowing for deposition within the alveolar region of the lung (site of absorption) is 1–5 μm. Unfortunately, particles of this dimension are rapidly eliminated by alveolar macrophages [9]. Importantly, the phagocytotic activity decreases with increasing foreign particle size [10].

In 1997, Edwards et al. [11,12] proposed a very interesting concept, using large, porous poly(lactic acid)(PLA)/poly(lactic-co-glycolic acid)(PLGA)-based particles (diameter >5 μm, density <0.4 g/cm³) for the pulmonary deliver of insulin over extended periods of times (resulting in elevated systemic drug levels for up to 96 h). This concept is based on the observation that the deposition of particles in the lung depends on the *aerodynamic* diameter, whereas the macrophage uptake is a function of the *geometric* particle diameter. The proposed highly porous particles are physically sufficiently large to avoid the engulfment by macrophages, but aerodynamically they behave like smaller particles (1–3 μm) with a density of 1 g/ml and can reach the deep lung. However,

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several drawbacks are related to the use of PLA/PLGA as matrix materials. The slow breakdown and clearance of the biodegradable polymers ($t_{1/2}$ is generally equal to several weeks) can lead to an accumulation in the lung upon multiple dosing. Furthermore, the use of organic solvents during drug encapsulation (e.g., w/o/w emulsion solvent evaporation methods), polymer hydrophobicity, and potential formation of acidic microclimates upon polymer degradation can affect peptide/protein stability, resulting in a loss of the biological activity [13–15]. A promising alternative to overcome these restrictions is the use of hydrogel-forming materials [16] as carriers in low density microparticles. Due to their hydrophilic nature, they can offer a preferable aqueous environment for peptide and protein drugs [17–19]. Furthermore, certain hydrophilic polymers are able to open tight junctions between epithelial cells [20]. This might enhance absorption, but potentially also cause toxic effects.

The approach chosen in this study is to rapidly dry swollen hydrogel particles in order to obtain highly porous microparticles with a relatively large geometric, but small aerodynamic diameter. This type of system also offers the advantage of having hydrophilic surfaces, being less prone to opsonization and phagocytosis than PLA/PLGA surfaces [10,21]. In addition, due to in situ swelling within the human lung, the microparticle size further increases and, thus, reduces the engulfment by macrophages. A major challenge with this type of peptide/protein delivery systems is the appropriate control of drug release, because swollen polymer networks are generally freely permeable for most drugs.

Alginates are natural, linear, unbranched polysaccharides containing 1,4′-linked beta-D-mannuronic and alpha-L-guluronic acid residues. They are able to form water-insoluble gels upon cross-linking with divalent cations (e.g., Ca^{2+}, Zn^{2+}) [22,23]. Due to this mild gelation process, the relatively inert aqueous environment within the matrix, and its high biocompatibility, alginate has been widely used as matrix material for the encapsulation of bioactive peptides/proteins and even living cells [24,25]. Common preparation methods include (water-in-oil) emulsification and atomization techniques. Generally, emulsion methods are rather complex, involving several steps and the use of organic solvents [26,27]. Furthermore, it is difficult to produce microparticles in a size range that is suitable for pulmonary administration with these techniques without sonication (which is likely to destroy native protein structures) [28]. Preliminary studies showed that atomization of alginate–drug solutions into solutions of divalent cations results in very low encapsulation efficiencies of the model protein BSA and low yields of spherical particles (data not shown). Alternatively, alginate–drug solutions can be spray-dried and the obtained microparticles subsequently cross-linked in salt-solutions [29]. However, this involves several production steps and can lead to significant drug loss and particle aggregation [30].

To overcome these problems, the following approach was chosen in this study: Aqueous solutions of alginate, $Zn(NH_3)_4SO_4$, and the model protein bovine serum albumin (BSA) were spray-dried, eliminating water and NH_3. Thus, Zn^{2+} ions are freed and cross-link the negatively charged alginate chains. In addition, Zn^{2+} ions form insoluble complexes with many peptides and proteins (e.g., insulin, BSA, human growth hormone). This can be beneficial to sustain drug release. Furthermore, zinc complexation has been shown to stabilize proteins during drying, for instance BSA [31] and recombinant human growth hormone [32] (however, note that high Zn concentrations might also be toxic). The major objectives of this study were to prepare such Zn^{2+} cross-linked alginate microparticles in a size range suitable for pulmonary application using a simple one-step process and to characterize these systems in vitro.

2. Materials and methods

2.1. Materials

Bovine serum albumin (BSA, Mw 69 kDa; Carl Roth GmbH + Co. KG, Karlsruhe, Germany), terbutaline sulfate (Welding GmbH, Hamburg, Germany), diprophylline (Knoll AG, Ludwigshafen, Germany), sodium alginate (low viscosity; Sigma–Aldrich Chemie GmbH, Steinheim, Germany), zinc sulfate heptahydrate ($ZnSO_4$; Caesar & Loretz, Hilden GmbH, Germany), ammonia (ammonia solution, 25% NH_3; Riedel-de Haën Laborchemikalien + Co. KG, Seelze, Germany), poloxamer 407 (polyoxypropylene–polyoxyethylene block copolymer, Lutrol® F127; BASF AG, Ludwigshafen, Germany), sodium hyaluronate (sodium hyaluronate pharma grade 80; NovaMatrix/FMC BioPolymer, FMC Corp., Oslo, Norway), chondroitine sulfate (chondroitine sulfate A + C, oral), glucosamine sulfate (D-glucosamine sulfate potassium salt; Kraeber & Co GmbH, Ellerbek, Germany), carrageenan (iota-carrageenan, Genuvisco carrageenan type TPH-1; Copenhagen Pectin A/S, Lille Skensved, Denmark), sodium citrate (tri-sodium citrate dehydrate; Merck KGaA, Darmstadt, Germany), and Coomassie assay (Coomassie Plus Protein Assay Kit; Pierce Biotechnology Inc., Rockford, IL, USA).

2.2. Microparticle preparation

Alginate and optionally other hydrogel formers (poloxamer 407, sodium hyaluronate, chondroitine sulfate, carrageenan, glucosamine sulfate) and drug (BSA, terbutaline sulfate or diprophylline) were dissolved in deionized water and mixed with an aqueous $Zn(NH_3)_4SO_4$ solution. These solutions contained 1% (w/w) alginate and optionally 1% (w/w) of a second hydrogel former (except for sodium hyaluronate: 0.5%, because of the high viscosity), 2.5% (1.25%) ammonia and 0.5% (0.25%) $ZnSO_4$, corresponding to the following weight ratios: alginate:$ZnSO_4$ = 2:1, alginate:second hydrogel former:$ZnSO_4$ = 2:2:1, and NH_3:$ZnSO_4$ = 5:1. The theoretical drug loading was 5%, 10%, 20%, or 30% (w/w), based on the total solids content. The aqueous solutions were spray-dried (Buechi 190 mini spray-dryer, BUECHI Labortechnik AG, Switzerland) using the following conditions: inlet temperature = 140–145C; pump flow = 5–7 g/min; spray flow = 600–650 nl/h, aspirator pressure = 50 mbar; outlet temperature = 70–80 °C. The particles were sieved (100 µm sieve) and stored at room temperature in a desiccator until further use. For reasons of comparison, particles consisting only of $ZnSO_4$ and BSA were prepared by spray-drying aqueous solutions of 1%(w/w) $ZnSO_4$, 2.5%(w/w) ammonia, and BSA (theoretical loading = 10% or 25%w/w, based on the total solids content).

2.3. Powder X-ray diffraction

Powder X-ray diffraction was used to study the crystallinity of the raw materials and the microparticles. Diffraction patterns were measured using a Philips X-ray generator PW 1830 equipped with a copper anode (λ = 1.5418 Å, 40 kV, 20 mA) coupled to a computer-interfaced Philips PW 1710 diffractometer control unit. The scattered radiation was measured with a vertical goniometer (Philips PW 1820; Royal Philips Electronics N.V., Philips Industrial & Electro-Acoustic Systems Division, Almelo, The Netherlands).

2.4. Scanning electron microscopy (SEM)

SEM was used to characterize the external morphology of the microparticles (S-4000; Hitachi High-Technologies Europe GmbH, Krefeld, Germany). Microparticles were fixed on a sample holder

with double-sided tape and coated under argon atmosphere with a fine gold layer (10 nm, SCD 040, Bal-tec GmbH, Witten, Germany).

2.5. Particle size analysis

The volume-based mean diameter and the size distribution of the microparticles were measured by laser diffraction (LD, Coulter LS 230; Beckmann Coulter GmbH, Krefeld, Germany). The particles were suspended in ethanol (96% v/v) to avoid swelling effects in aqueous media.

2.6. Drug release studies

In vitro drug release from the microparticles was studied using a previously described, self-made modified Franz diffusion cell adapted from Bertram and Bodmeier [33] in order to simulate the release conditions on mucosal surfaces [27]. Briefly, at the lower end of a polypropylene tube (inner diameter = 2.7 cm), a regenerated cellulose filter membrane [pore size = 0.45 µm, d = 47 mm; RC-fleece supported (Sartorius AG, Göttingen, Germany)] and a thin cloth (Flinka Allzwecktuch; Kornbusch & Starting GmbH + Co. KG, Borken, Germany) were tightly attached. This tube was vertically placed into a plastic vessel filled with 10 ml water, phosphate buffer pH 7.4 (USP 34), or modified simulated lung fluid (composition see Table 1) [34]. All media were preserved with 0.05% (w/w) sodium azide. The tube position was adjusted so that the filter was wetted, but not submersed by release medium. Microparticles (∼20 mg, accurately weighed) were manually sprinkled on the filter membrane, and the whole system was sealed with Parafilm® "M" (American National Can Company, Chicago, IL, USA) to avoid evaporation of release medium during the experiment and to guarantee a constant relative humidity the microparticles were exposed to (85–95% rh). The diffusion cells were placed in a horizontal shaker (GFL 3033; Gesellschaft für Labortechnik GmbH + Co. KG, Burgwedel, Germany, 75 rpm, 37 °C). At predetermined time points, samples of 2 ml were withdrawn and replaced with fresh medium. The BSA content of the samples was determined spectrophotometrically using a Coomassie assay (λ = 595 nm, UV-2101 PC; Shimadzu Deutschland GmbH, Duisburg, Germany). Drug-free microparticles were used as a reference and allowed to account for polymer absorption. The 100% release values were experimentally verified by adding 10 ml of a 6% (w/w) sodium citrate solution into the cells at the end of each experiment in order to completely dissolve the alginate. All measurements were taken in triplicate (mean ± SD).

Table 1
Composition of physiological lung fluid, simulated lung fluid (SLF), modified SLF, and the applied SLF (meq./l) [adapted from Davies and Feddah [34]].

Ions	Physiological	Simulated lung fluid	Modified SLF, with 0.02% DPPC	Applied SLF
Calcium, Ca^{2+}	5.0	5.0	5.0	–
Magnesium, Mg^{2+}	2.0	2.0	2.0	2.0
Potassium, K^+	4.0	4.0	4.0	4.0
Sodium, Na^+	145.0	145.0	145.0	150.0
Total cations	156.0	156.0	156.0	156.0
Bicarbonate, $HCO3$	31.0	31.0	31.0	31.0
Chloride, Cl^-	114.0	114.0	114.0	115.0
Citrate, $H_5C_6O_7^{3-}$	–	1.0	1.0	–
Acetate, $H_3C_2O_2$	7.0	7.0	7.0	7.0
Phosphate, HPO_4^{2-}	2.0	2.0	2.0	2.0
Sulfate, SO_4^{2-}	1.0	1.0	1.0	1.0
Protein	1.0	–	–	–
DPPC	–	–	200 mg	–
Total anions	156.0	156.0	156.0	156.0
pH	7.3–7.4	7.3–7.4	7.3–7.4	7.3–(8.7[a])

DPPC: dipalmitoylphosphatidylcholine.
[a] After 48 h, due to CO_2 loss.

2.7. LDS–polyacrylamide gel electrophoresis

The molecular weight integrity of encapsulated BSA was determined by lithium dodecyl sulfate polyacrylamide gel electrophoresis (LDS–PAGE) (Invitrogen Corp., Carlsbad, USA). Samples were dissolved in 10 ml 3% (w/w) aqueous sodium citrate solution and deep frozen to allow for storage until measurement. For reasons of comparison, solutions of spray-dried BSA (frozen/thawed) and BSA as received (frozen/thawed or freshly prepared) were also analyzed. The electrophoresis procedure was conducted (under non-reducing or reducing conditions) according to the instructions and protocols of the manufacturer. The samples and a molecular weight reference marker (10–250 kDa, Precision Plus Protein™ Standard; Bio-Rad Laboratories Inc., Hercules, CA, USA) were loaded onto a pre-cast acrylamide/bis-acrylamide gel (NuPAGE® 10% Bis-Tris gel). The gels were run in NuPAGE® MES running buffer (2-morpholinoethanesulfonic acid) for 45 min in an XCell SureLock™ Mini-Cell using a programmable power supply (PowerEase™ 500, pre-programmed method for NuPAGE® gels).

2.8. Determination of drug loading

Microparticles (∼10 mg, accurately weighed) were dissolved in 10 ml sodium citrate solution (3% w/w). The BSA content was determined via a Coomassie assay as described in Section 2.6.

2.9. In vitro aerosol deposition

The pulmonary deposition of the dry powders was investigated in vitro using a Multi (5)-stage Liquid Impinger (MSLI; Erweka International AG, Battwil, Switzerland, USP 34, Apparatus 4). Twenty milliliters of an aqueous sodium citrate solution (3% w/w) was dispensed into each of the four upper stages of the apparatus. A glass fiber filter (type A/E, d = 76 mm; Pall Corp., Ann Arbor, Michigan, USA) was placed in the filter stage (stage 5). Hydroxypropyl methylcellulose capsules (HPMC capsules size 4; Hovione FarmaCiencia SA, Loures, Portugal) were filled to approximately halfway to two-thirds of their volume (∼20–25 mg) with microparticles (previously stored in a desiccator) and placed in a Flow-Caps® dry powder inhaler (Hovione). The capsules were pierced at both ends by inhaler actuation. The mouthpiece was connected via a silicone rubber adaptor to the induction port of the impinger. The powder was aerosolized and drawn through the impinger with a flow rate of 30 l/min (=airflow stated by Hovione, Ref. FlowCaps® Technical Pack, that creates the pressure drop of 4 kPa required by the pharmacopeias) for 8 s, which allowed the aspiration of 4 l of air as recommended by pharmacopeias. The fraction of powder emitted from the capsule ("Emitted Dose" = ED) was determined gravimetrically as the percent of total particle mass leaving the capsule.

$$ED = \frac{\text{Weight of filled inhaler before−after aerosolization}}{\text{Weight of filled inhaler before aerosolization}} \times 100\%$$

After discharging 4–5 capsules into the apparatus, the mouthpiece adaptor/induction port unit, filter, and inlet jet tube to stage 1 were washed thoroughly with an appropriate volume of sodium citrate solution. When the microparticles were completely dissolved, samples were drawn from each stage for analysis. Their BSA content was determined spectrophotometrically using a Coomassie assay as described in Section 2.6. The fine particle fraction (FPF, "respirable fraction") of the aerosolized powder was calculated by dividing the BSA amount recovered from the stage 4 and the filter stage (≤cut-off aerodynamic diameter 4.4 µm) by the total BSA amount recovered in the impinger.

3. Results and discussion

A dry powder aerosol formulation of Zn^{2+} cross-linked, alginate-based microparticles could be a promising controlled delivery system for the systemic administration of peptide and protein drugs via the pulmonary route. Such microparticles were prepared in a one-step procedure by spray-drying aqueous solutions of alginate, $Zn(NH_3)_4SO_4$, drug, and if indicated other additives. During spray-drying, the ammonia evaporated, thereby setting Zn^{2+} ions free (from the zinc-tetra-ammonium complexes) to cross-link the negatively charged alginate chains (Fig. 1). In addition, the Zn^{2+} ions form poorly water-soluble complexes with proteins such as BSA.

3.1. Morphology, size, and physical state of the microparticles

Non-cross-linked alginate microparticles containing 10% BSA were homogeneous and spherical in shape (Fig. 2A) with a volume mean geometric diameter of 2.8 (±2.3) μm (Table 2). The addition of $ZnSO_4$ led to a more crumpled, raisin-like shape (Fig. 2B) and increased size (Table 2). Particles consisting of only $ZnSO_4$ and BSA were even more collapsed (Fig. 2C) with a size of 3.6 (±1.9) μm. This is in good agreement with the structure of spray-dried zinc–BSA microparticles reported by Prinn et al. [35]. The impact of formulation and processing parameters of spray-dried microparticles on the latter's shape was recently reviewed by Vehring [36]. A decrease in the alginate:$ZnSO_4$ ratio led to an increase in particle size from 2.9 (±2.1) μm (10:1 ratio) to 5.0 (±2.2) μm (1:1 ratio) (Table 2). Increasing the BSA content from 1% to 30% also resulted in larger particles [3.4 (±2.2) vs. 4.4 (±2.2) μm]. Both effects might be explained by the increased solid content of the respective spray-drying solutions. All mean particle sizes were between 2 and 5 μm (geometric diameter) and, thus, fulfilled the size prerequisite for deep lung deposition (at appropriate porosities). The same was true for most of the other investigated hydrophilic carriers and their blends with alginate (Table 3). Only microparticles containing poloxamer 407 were exceptionally large [12.9 (±2.6)/6.5 (±2.4) μm in the absence/presence of $ZnSO_4$]. This can probably be attributed to particle aggregation/fusion (Fig. 2D) during spray-drying caused by molten poloxamer 407 (Tm = 56 °C). The presence of $ZnSO_4$ reduced this effect. Microparticles containing either Na-hyaluronate or poloxamer 407 in addition to alginate were less crumpled than microparticles containing only alginate (Fig. 2), probably due to their *relatively* lower $ZnSO_4$ content.

Powder X-ray diffraction revealed that the raw materials $ZnSO_4$, poloxamer 407, and glucosamine sulfate showed crystalline structures (Fig. 3), whereas alginate-$ZnSO_4$-containing, spray-dried microparticles did not. Thus, the formation of the respective crystals is efficiently suppressed (probably due to rapid solvent evaporation
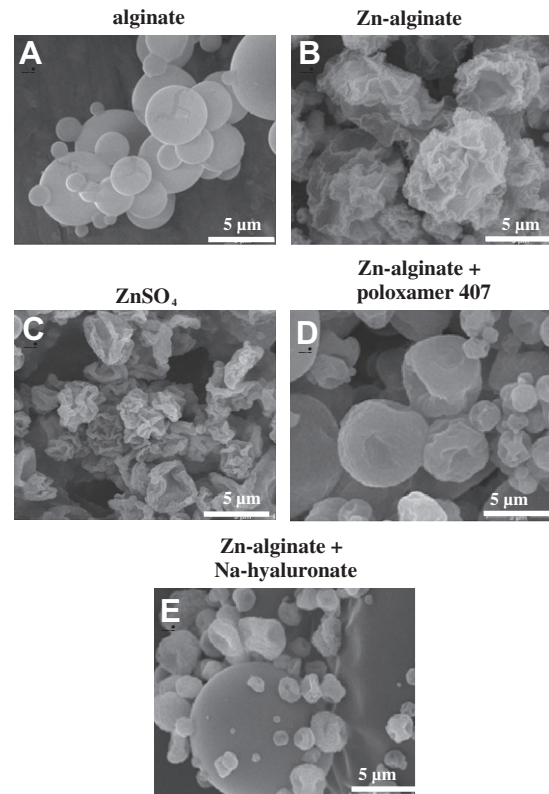

Fig. 2. Morphology (SEM pictures) of microparticles prepared by spray-drying aqueous solutions of BSA, NH$_3$, and (A) alginate; (B) alginate:$ZnSO_4$ (2:1); (C) $ZnSO_4$; (D) alginate:poloxamer 407:$ZnSO_4$ (2:2:1); and (E) alginate:Na-hyaluronate:$ZnSO_4$ (2:2:1).

Table 2
Effects of the alginate:$ZnSO_4$ ratio and BSA content on the size, Emitted Dose (ED), and fine particle fraction (FPF) of the microparticles (n.d. = not determined).

Alginate: $ZnSO_4$ ratio	BSA content (%)	Size, (μm) (±s)	ED (%) (±s)	FPF (%) (±s)
1:0	10	2.8 (2.3)	93.8 (1.3)	23.3 (3.7)
10:1	10	2.9 (2.1)	91.1 (n.d)	32.8 (n.d.)
2:1	1	3.4 (2.2)	87.3 (n.d.)	20.8 (n.d.)
2:1	5	4.2 (2.3)	96.5(1.7)	34.1 (0.2)
2:1	10	3.9 (2.3)	96.6 (0.6)	40.4 (1.0)
2:1	20	4.3 (2.3)	95.5 (0.8)	33.4 (2.1)
2:1	30	4.4 (2.2)	95.5(1.2)	38.9 (3.4)
1:1	10	5.0 (2.2)	94.7(0.1)	35.1 (2.4)
0:1	10	3.6(1.9)	90.1 (1.3)	17.6 (0.9)
0:1	25	4.0 (2.0)	88.2(5.5)	22.4 (4.4)

and/or the presence of the alginate). Microparticles consisting of $ZnSO_4$ and BSA only showed some small crystalline peaks. The addition of poloxamer 407 to alginate-$ZnSO_4$ microparticles also resulted in two small crystalline peaks, indicating the presence of poloxamer 407 crystals.

3.2. In vitro drug release

The erosion of Zn^{2+} cross-linked alginate networks is induced by the removal of Zn^{2+} ions either due to the exchange with other cations (e.g., Na^+ ions) or by zinc binding agents like phosphate ions (formation of a water-insoluble precipitate, the fate of which should be addressed if also formed in vivo). The latter process is likely to be the faster one. For this reason, the influence of the type of release medium on the resulting BSA release patterns was investigated. The protein release rate increased with increasing
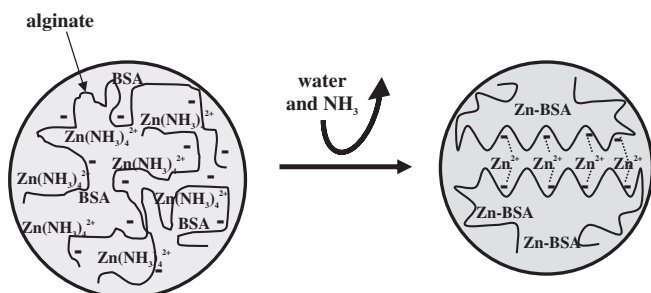

Fig. 1. Schematic presentation of the cross-linking process within aqueous droplets containing dissolved alginate, $Zn(NH_3)_4SO_4$, and BSA during spray-drying.

Table 3
Effects of the type of matrix-former and absence/presence of ZnSO₄ on the size, Emitted Dose (ED), and fine particle fraction (FPF) of spray-dried, BSA-loaded microparticles.

Type of matrix-former	ZnSO$_4$	Size (µm) (±s)	ED (%)	FPF (%) (±s)
Alginate	–	2.8 (2.3)	93.8 (1.3)	23.3 (3.7)
Alginate	+	3.9 (2.3)	96.6 (0.6)	36.2 (0.2)
Alginate + poloxamer	–	12.9 (2.6)	95.7 (0.1)	1.9 (0.4)
Alginate + poloxamer	+	6.5 (2.4)	94.0 (6.7)	4.2 (0.7)
Alginate + HPMC E5	+	4.4 (2.3)	92.2 (0.5)	21.4 (1.4)
Alginate + HMPC E50	+	4.2 (2.2)	86.2 (3.2)	18.9 (3.3)
Alginate + glucosamine-SO$_4$	–	3.5 (2.6)	96.5 (1.5)	13.3 (0.9)
Alginate + glucosamine-SO$_4$	+	4.2 (2.4)	96.3 (0.1)	19.2 (1.4)
Na-hyaluronate	–	4.2 (3.3)	89.5 (8.6)	19.9 (1.0)
Na-hyaluronate	+	4.7 (2.8)	94.6 (0.5)	28.5 (6.1)
Alginate + Na-hyaluronate	–	3.5 (2.8)	94.8 (2.5)	26.9 (4.8)
Alginate + Na-hyaluronate	+	4.3 (2.8)	95.4 (1.7)	29.4 (1.3)
Chondroitine-SO$_4$	–	2.7 (1.9)	89.9 (1.7)	32.0 (2.6)
Chondroitine-SO$_4$	+	4.8 (2.2)	97.4 (1.6)	33.0 (0.9)
Alginate + chondroitine-SO$_4$	–	2.8 (2.3)	94.4 (0.3)	27.6 (1.3)
Alginate + chondroitine-SO$_4$	+	4.8 (2.5)	97.2 (0.6)	31.1 (0.2)

$Zn_3(PO_4)_2$ crystals was observed on top of the filter membrane (optical microscopy, data not shown). BSA release in 5-fold-diluted USP 34 phosphate buffer pH 7.4 was consistently slower (~35% protein release after 8 h, Fig. 4). Interestingly, the release rate in modified simulated lung fluid (Table 1, modified SLF) was similar to that in the 5-fold-diluted phosphate buffer, despite the 10-fold lower phosphate ion concentration in the modified SLF. This can probably be attributed to the much higher concentration of other cations in the modified SLF (e.g., sodium and potassium ions), which compete with zinc for alginate binding sites. Simulated lung fluid, which much better represents the in vivo conditions at the absorption site in the lung than the other release media, was used for all further in vitro release studies.

Decreasing the alginate:ZnSO₄ ratio from 10:1 to 2:1 led to decreasing BSA release (Fig. 5). This can be attributed to denser gel structures (containing more cross-links), slower gel erosion, and more pronounced BSA complexation. However, when further decreasing the alginate:ZnSO₄ ratio to 1:1, the resulting BSA release rate increased. This is likely due to the lower *relative* alginate content, leading to a less dense polymer networks. At elevated zinc contents, BSA release was incomplete (Fig. 5), indicating that a certain portion of the protein remained complexed and did not dissolve under the investigated conditions. This was confirmed by visual observation (residues on the filter membrane). This explanation is consistent with the fact that the rate and extent of BSA release significantly increased with increasing protein content (Fig. 6): less Zn^{2+} ions are available per protein molecule.

Protein release was measured from microparticles consisting of (i) pure BSA; (ii) BSA and alginate; (iii) BSA and ZnSO₄; and (iv) BSA, alginate and ZnSO₄ (Fig. 7) to better understand the underlying
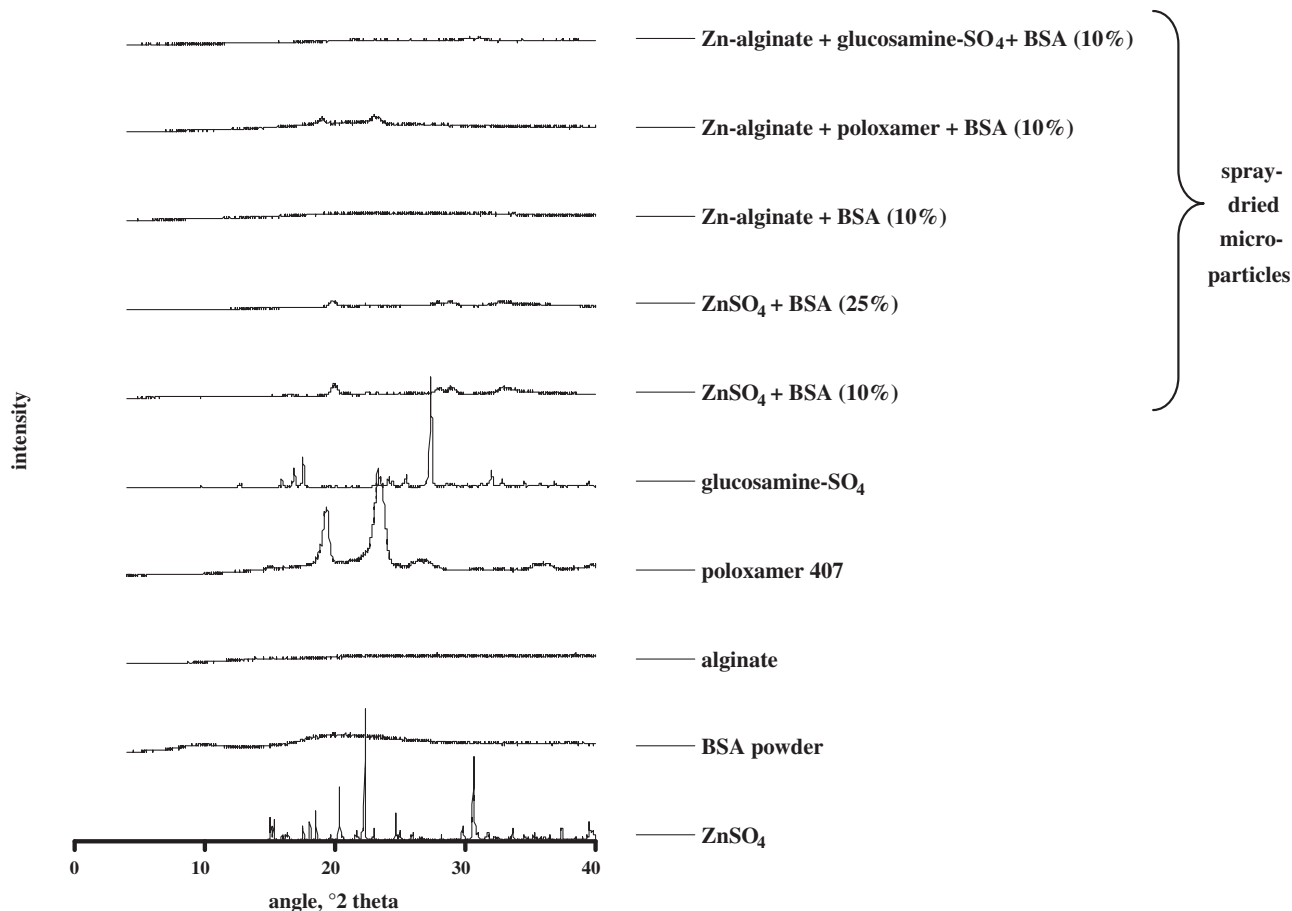
phosphate ion content (Fig. 4). In water, where no gel degradation was possible, almost no BSA was released during 48 h, indicating that diffusion through the gel network did not occur. Dissolved BSA is able to diffuse through aqueous alginate gels [37,38]. However, in the present case, zinc–BSA complexes are formed, which are too poorly soluble/permeable to allow a faster release. In fact, about 60% protein was released after 8 h in USP 34 phosphate buffer pH 7.4 containing 0.05 mol/l phosphate and 0.04 mol/l sodium ions. Formation of a white precipitate consisting of needle-shaped

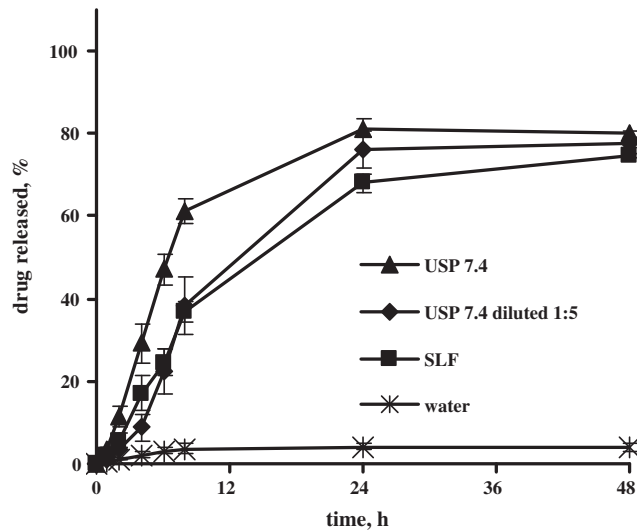**Fig. 3.** X-ray diffraction patterns of the raw materials and spray-dried microparticles.

Fig. 4. Effect of the type of release medium (indicated in the figure) on protein release from microparticles prepared by spray-drying aqueous solutions of alginate, $Zn(NH_3)_4SO_4$, and BSA (n = 3, mean ± s).
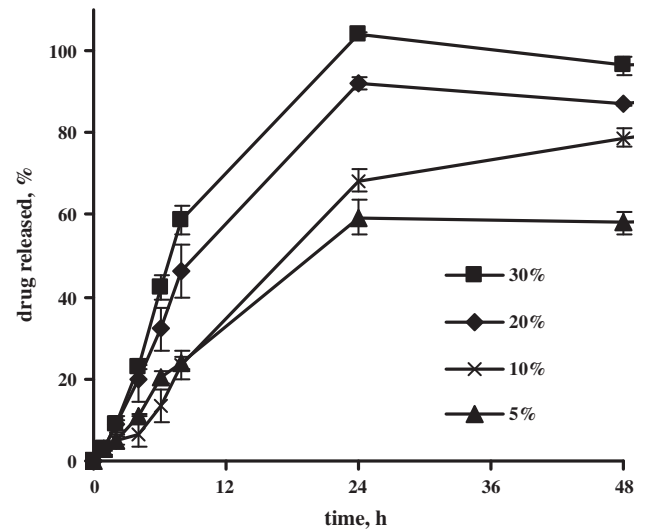

Fig. 6. Effect of the initial protein loading (indicated in the figure) on BSA release from microparticles prepared by spray-drying an aqueous solution of alginate, $Zn(NH_3)_4SO_4$, and BSA (alginate:$ZnSO_4$ ratio = 2:1) (n = 3, mean ± s).
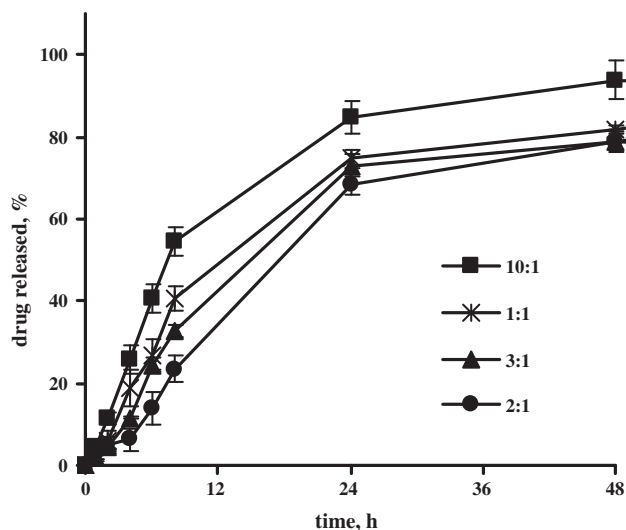

Fig. 5. Effect of the alginate:$ZnSO_4$ ratio (indicated in the figure) on protein release from microparticles prepared by spray-drying aqueous solutions of alginate, $Zn(NH_3)_4SO_4$, and BSA (n = 3, mean ± s).
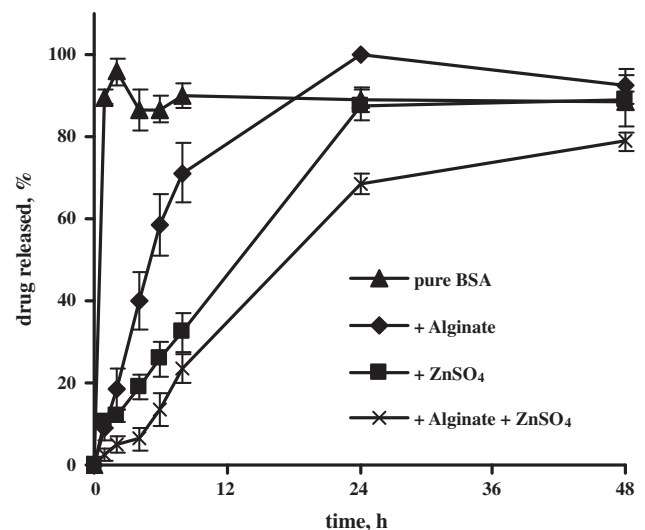

Fig. 7. Protein release from microparticles consisting of (i) pure BSA; (ii) BSA and alginate; (iii) BSA and $ZnSO_4$; and (iv) BSA and Zn-alginate, prepared by spray-drying (n = 3, mean ± s).

protein release mechanisms, in particular the relative importance of protein diffusion through the alginate gel, Zn^{2+} cross-linking and zinc–BSA complexation. Pure protein particles rapidly dissolved, and drug release was fast with the applied diffusion cell. The presence of both the alginate network and the $ZnSO_4$ significantly slowed down protein release. The fact that BSA release from microparticles prepared from alginate and BSA and $ZnSO_4$ showed the slowest release rate of all formulations indicates that both phenomena, namely protein diffusion through the hydrogel network as well as protein complexation with zinc are of importance in these novel drug delivery systems.

Zinc–alginate microparticles containing either terbutaline sulfate (interaction with alginate) or diprophylline (neither interaction with alginate nor with zinc) were compared with BSA-containing particles (interaction with zinc) to further clarify the role of zinc–drug, zinc–alginate, and drug–alginate interactions with regard to drug release. In the absence of $ZnSO_4$, the release

followed the following order: diprophylline > terbutaline sulfate ≫ BSA (Fig. 8). The lower release rate of terbutaline sulfate compared to diprophylline was probably due to electrostatic interactions between the positively charged amino group of the terbutaline and the negatively charged carboxylic groups of the alginate. The markedly slower release of the BSA could be explained by its higher molecular weight (69.000 Da vs. 323.4 Da for terbutaline sulfate or 254.3 Da for diprophylline), resulting in a lower diffusion coefficient. In the presence of $ZnSO_4$, the order of release rates was slightly changed: terbutaline > diprophylline ≫ BSA (Fig. 8). The cross-linking of alginate by Zn^{2+} ions slowed down the dissolution of alginate and generated a stronger diffusion barrier, resulting in a decreased release rate for diprophylline and BSA. Thereby, diprophylline, which did not interact with zinc or alginate, was supposedly released mainly by diffusion before the zinc–alginate gel started to erode following Zn^{2+} ion removal. In contrast to diprophylline, there was assumingly almost no diffusional release
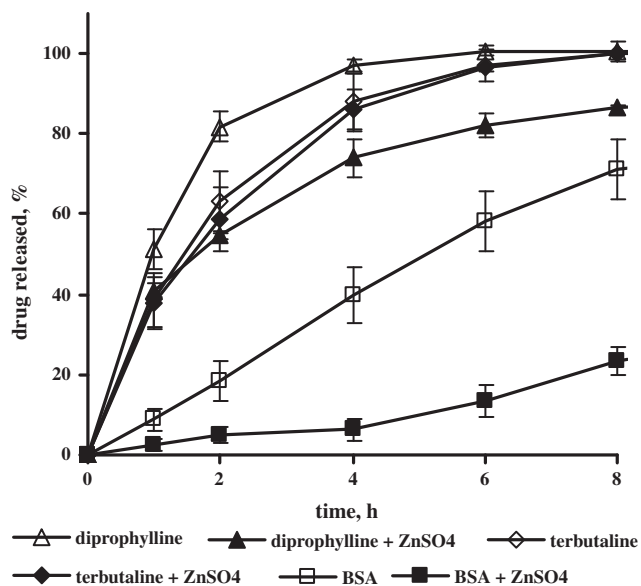
Fig. 8. Effect of ZnSO$_4$ and drug on release from alginate microparticles prepared by spray-drying an aqueous solution of alginate + drug (bovine serum albumin – BSA, terbutaline sulfate – TS) + NH3 ± ZnSO$_4$ (10% drug w/w, diffusion cell, SLF, 37 °C) (n = 3, mean ± s).

of BSA, which was trapped as an insoluble zinc complex inside the alginate gel until the Zn^{2+} ion removal by phosphate and cations led to gel degradation and complex dissolution. Surprisingly, the presence of ZnSO$_4$ had no influence on the terbutaline release rate.

The following materials were studied as alternative matrix-formers for the protein-loaded microparticles: (i) chondroitine sulfate, (ii) sodium hyaluronate, (iii) carrageenan, and (iv) polox-amer 407 as hydrogel-forming polymers and glucosamine sulfate as a low molecular weight compound (for reasons of comparison). Chondroitine sulfate, sodium hyaluronate, and glucosamine sulfate are endogenous to the human body and, thus, highly biocompatible. Poloxamer 407 is a synthetic, non-toxic polymer forming thermore-versibly gels at body temperature. Similar to alginate, carrageenan can form cross-links with Zn^{2+} ions. The respective microparticles were prepared by spray-drying aqueous solutions of the matrix-for-mer, BSA and optionally Zn(NH$_3$)$_4$SO$_4$. Under the selected spray-drying conditions, it was not possible to obtain discrete microparticles based on poloxamer 407 and glucosamine sulfate (probably due to the low melting point of poloxamer 407 and high water binding capacity/hygroscopicity of glucosamine sulfate). Protein release was fastest from chondroitine sulfate-based micro-particles, followed by alginate-, sodium hyaluronate- and carra geenan-based systems (Fig. 9A). The observed ranking corresponds to the dissolution rate and viscosity of the polymers in water for chondroitine sulfate, alginate, and sodium hyaluronate. The fast dissolving chondroitine sulfate did not even sustain the release compared to pure BSA (Fig. 7). The relatively slow release from carrageenan-based microparticles can be explained by the fact that this sulfated polysaccharide forms water-insoluble gels with potassium ions (double-helix formation), which are present in the release medium. The addition of Zn(NH$_3$)$_4$SO$_4$ to the spray-drying solutions led to a significant decrease in the release rate, except for carrageenan-based microparticles (the release remained unal-tered) (Fig. 9B). In the latter case, the removal of Zn^{2+} ions did not lead to gel erosion. Instead, a more stable potassium-carrageenan gel was formed controlling the diffusional release of BSA. Sodium hyaluronate and chondroitine sulfate do not form complexes with zinc or potassium ions. Hence, the decrease in release rate upon Zn(NH$_3$)$_4$SO$_4$ addition can be attributed to the formation of zinc–BSA complexes.
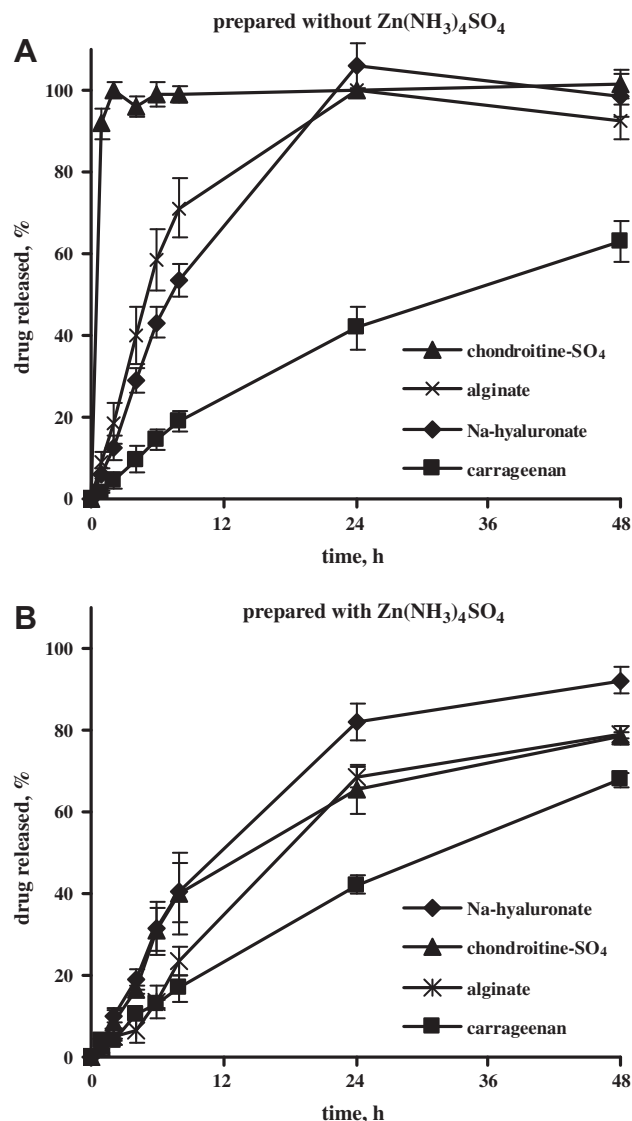

Fig. 9. Effects of the type of matrix-former (indicated in the figures) on protein release from BSA-loaded microparticles prepared by spray-drying aqueous solu-tions of (A) matrix-former and BSA only; and (B) matrix-former, BSA and Zn(NH$_3$)$_4$SO$_4$ (n = 3, mean ± s).

Furthermore, microparticles based on alginate:glucosamine sulfate, alginate:poloxamer 407, alginate:chondroitine sulfate, algi-nate:sodium hyaluronate, and alginate:carrageenan 1:1 blends were prepared with or without Zn(NH$_3$)$_4$SO$_4$. The protein release rate from zinc-free systems decreased as follows: glucosamine sulfate ≈ poloxamer 407 > chondroitine sulfate > sodium hyaluro-nate > carrageenan (Fig. 10A). Again, this correlated well with the observed dissolution rates in water/viscosities of aqueous solutions. As in the case of the pure matrix-formers (Fig. 8), the addi-tion of Zn(NH$_3$)$_4$SO$_4$ resulted in decreasing release rates (Fig. 10B) except for carrageenan (for the same reasons as discussed above).

3.3. BSA stability

A critical point when developing protein carrier systems is the preservation of the structural integrity of the drugs. For most proteins, keeping the native state is an essential prerequisite for full biological activity. During microparticle preparation, BSA was ex-posed to potentially harsh conditions, such as a basic pH due to
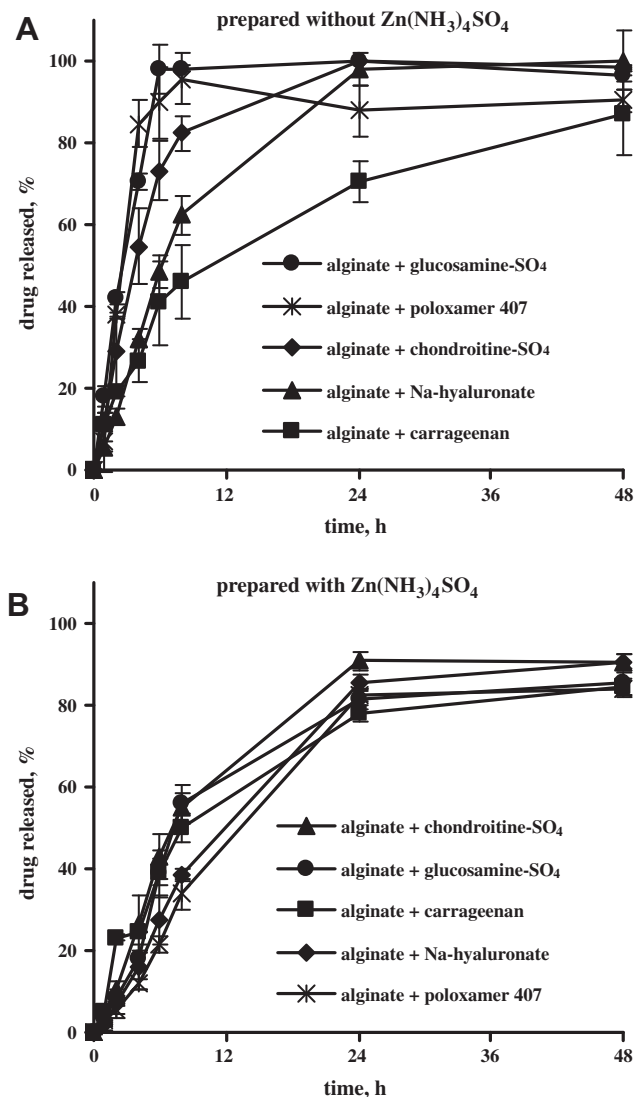
Fig. 10. Effects of the type of matrix-former blend (indicated in the figures) on protein release from BSA-loaded microparticles prepared by spray-drying aqueous solutions of: (A) matrix-former blend and BSA; and (B) matrix-former blend, BSA, and $Zn(NH_3)_4SO_4$ ($n = 3$, mean ± s).

ammonia addition (around pH 11), shear forces, heat, and contact with air–water interfaces during spray-drying. This could result in protein hydrolysis or aggregation, both leading to a change in the molecular weight. Non-reducing polyacrylamide gel electrophoresis showed intact BSA in all the investigated microparticle formulations after dissolution of the particles in 3% (w/w) sodium citrate solution, when compared to freshly dissolved BSA (data not shown) (however, alterations like deamidation and oxidation are not assessed with this method). Low molecular weight hydrolysis products were not visible. Direct spray-drying of BSA from an aqueous ammonia solution and freezing and thawing of the samples also appeared to have no deleterious effect. Small traces of higher molecular weight aggregates (dimers and trimers) were also visible in the gel. However, since those traces were found in all samples, including freshly prepared BSA solution, they were probably present already in the commercially obtained BSA powder or formed immediately after dissolution. These aggregates were therefore not caused by the microparticle preparation procedure. Samples of BSA released after 24 h from the different formulations showed comparable gel electrophoresis patterns (data not shown).

3.4. Aerosolization and aerodynamic flow behavior

From an economic perspective, an efficient delivery of the formulation from the inhaler device during inhalation (=minimal drug loss) is highly desirable. The fraction of particles emitted from the capsule in the Flowhaler® was high (∼87–97%) for all formulations (Table 2). The fine particle fraction (FPF) of BSA-loaded (10% w/w) alginate microparticles prepared without $Zn(NH_3)_4SO_4$ was low (only 23.3%, Table 2), although their geometric diameter was 2.8 μm. This indicated that agglomerates could not sufficiently be dispersed in the air stream during aerosolization. The addition of $Zn(NH_3)_4SO_4$ to the spray-drying solution during microparticle preparation resulted in a remarkable increase in the FPF (to 32.8% at an alginate:ZnSO$_4$ ratio of 10:1, and to 40.4% at an alginate:ZnSO$_4$ ratio of 2:1). This can be explained by the effect of $Zn(NH_3)_4SO_4$ addition on the particle morphology as discussed above (Fig. 2). Due to the increasing surface roughness, the true area of contact between particles is reduced and, thus, cohesion is lowered. Furthermore, the reduced inter-particulate point-to-point contact reduces the influence of van der Waals forces, which play a major role in particle adhesion and cohesion [39]. This observation is in good agreement with the results reported by Chew and Chan [40], who found 10–25% higher FPFs for corrugated compared to smooth BSA microparticles. Recently, the idea to improve particle dispersion and flow by minimizing the contact area was also applied to poly(lactic-co-glycolic acid) (PLGA)-based microparticles with dimpled surfaces. They were produced by an w/o/w-double emulsion–solvent evaporation technique using Pluronic® copolymers, showed promising aerosol properties and were suggested for the pulmonary delivery of DNA [41]. A further increase in the ZnSO$_4$ content (alginate:ZnSO$_4$ ratio 1:1) did not improve the FPF anymore. Chew et al. [42] reported similar results for corrugated BSA microparticles: A slight increase in surface roughness enhanced the FPF significantly from 27% to 41%, but a further roughness increase led to a plateau. In contrast, the use of only ZnSO$_4$ as carrier material (resulting in the most crumpled particle surface, Fig. 2) led to a significant decrease in the FPF (to 17.6%). The microparticles' surface became very rough and uneven, resulting in physical entanglement between the particles. An increase in the BSA content from 1% to 5% (alginate:ZnSO$_4$ ratio 2:1) resulted in a pronounced increase in the FPF from 20.8% to 34.1% (Table 2). Similar observations were described by French et al. [39], who found that mannitol powders containing recombinant human granulocyte-colony stimulating factor exhibited improved performance (dispersion, deaggregation, and deposition in the model lung) compared to pure mannitol powders. Bosquillon and coworkers [43] reported that the incorporation of albumin in sugar-dipalmitoylphosphatidylcholine (DPPC)-based formulations improved the aerosolization properties. Both groups attributed this phenomenon to a reduced bulk density leading to smaller aerodynamic diameters ($d_{aer} = d_{geom} \times \sqrt{\rho}$, ρ = particle density) and to limited point-to-point contacts due to a highly indented surface geometry. However, Bosquillon et al. suggested also that the known reduction in the Hamaker constant (a material constant representing the strength of the van der Waals attraction) by proteins and their surfactant properties likely contribute to a reduced inter-particulate cohesion. This is in good agreement with our finding that a further increase in the BSA content (up to 30%) did not lead to a further increase in the FPF. Probably the particle surface was already saturated with protein at lower loadings.

Formulations containing alternative hydrophilic carriers or blends of alginate with those carriers generally possessed high Emitted Doses (90–97%, Table 3). As for alginate-based microparticles, the addition of $Zn(NH_3)_4SO_4$ to the spray-drying solutions increased the FPF, despite an increase in particle size (except for poloxamer 407-based formulations). Again, this might be explained by
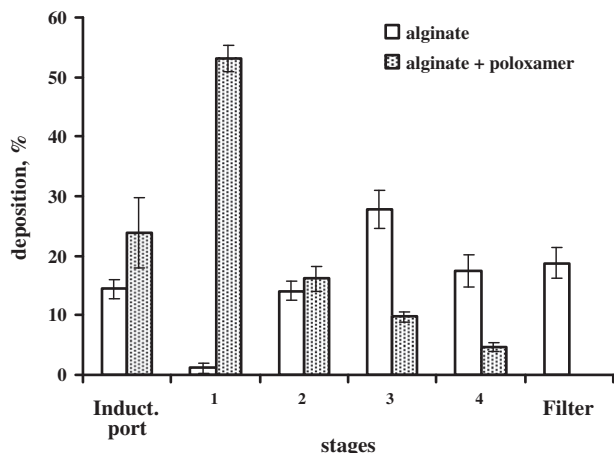
Fig. 11. Influence of poloxamer 407 on multistage liquid impinger deposition characteristics of alginate microparticles produced by spray-drying aqueous solutions of alginate, BSA, NH_3, and if indicated poloxamer 407 and $Zn(NH_3)_4SO_4$ (flow rate $\approx$ 30 l/min) ($n = 3$, mean $\pm$ s).

the reduced inter-particulate contact area of either larger or more surface crumpled particles. The $Zn(NH_3)_4SO_4$ effect was least pronounced for chondroitine sulfate-based microparticles, having a comparatively high FPF already without $ZnSO_4$ (32.0%). In the absence of $Zn(NH_3)_4SO_4$, blending of alginate with sodium hyaluronate (FPF 27.4%) or chondroitine sulfate (FPF 27.6%) slightly enhanced the FPF compared to pure alginate microparticles (FPF 23.3%). On the other hand, blending with poloxamer 407 (FPF 1.9%) or glucosamine sulfate (FPF 13.3%) impaired the fraction of particles potentially capable of reaching the deep lung. The FPF of microparticles based on alginate:second carrier material blends prepared with $Zn(NH_3)_4SO_4$ increased in the following ranking order: poloxamer 407 (4.2%) $\ll$ glucosamine sulfate (19.2%) $\approx$ HPMC E50 (20.5%) $\approx$ HPMC E5 (23.4%) < chondroitine sulfate (31.1%) $\approx$ sodium hyaluronate (31.7%) (Table 3). Obviously, the presence of poloxamer 407 had a detrimental effect on the respirable particle fraction. This is at least partially due to the larger particle diameter (6.5 μm) resulting from fusion/aggregation of the particles during spray-drying as discussed above.

A comparison of the deposition patterns of zinc–alginate-based microparticles with and without poloxamer 407 measured with the multistage liquid impinger showed major differences
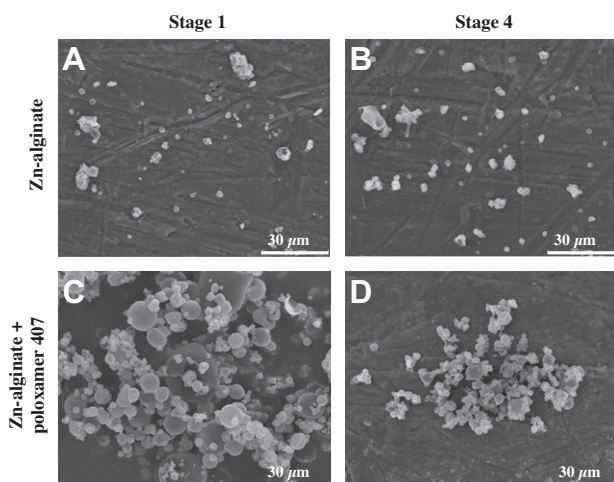
especially on stage 1 and the filter stage (Fig. 11). Without poloxamer 407, only 1.2% of the particles emitted from the inhaler deposited on stage 1, but 18.8% on the filter stage. When poloxamer 407 was added, 53.0% deposited on stage 1, whereas no detectable particles reached the filter stage (cut-off diameter 2.4 μm). Scanning electron microscopy of the respective powders deposited on these stages showed big particle lumps consisting of larger and smaller particles sticking together on stage 1 when poloxamer 407 was present (Fig. 12). This might be attributable to particle fusion during spray-drying and/or the presence of particle agglomerates that cannot be dispersed in the air stream.

4. Conclusion

Zinc–alginate microparticles in a size range suitable for pulmonary administration were successfully prepared using a simple one-step spray-drying process. The microparticles provide an interesting potential for the controlled pulmonary delivery of peptide and protein drugs.

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Fig. 12. Morphology (SEM pictures) of alginate- and alginate-poloxamer 407-based microparticles prepared by spray-drying aqueous solutions of alginate, BSA, NH_3, and if indicated poloxamer 407 and $Zn(NH_3)_4SO_4$ and deposited on multistage liquid impinger stages 1 and 4 (flow rate $\approx$ 30 l/min).

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