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Research paper

Alginate–poloxamer microparticles for controlled drug delivery to mucosal tissue

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ABSTRACT

Purpose: The aim of this study was to prepare and characterize novel hydrogel-based delivery systems allowing for the controlled release of drugs to mucosal surfaces. *Methods:* Terbutaline sulfate and bovine serum albumin (BSA)-loaded alginate–poloxamer microparticles were prepared by a w/o-emulsion- and external gelation method. The microparticles were characterized by optical and scanning electron microscopy, laser light diffraction, atomic absorption spectroscopy, energy-dispersive X-ray analysis, via complexation with 1,9-dimethyl methylene blue and using dialysis bags as well as modified Franz diffusion cells for in vitro drug-release measurements. *Results:* Using heptane as organic phase, homogeneous and almost spherical microparticles were obtained with a high-loading efficiency (>90%). The resulting drug-release patterns could effectively be adjusted by varying the "alginate:poloxamer" blend ratio. In addition, the particle size, morphology, calcium and chloride content as well as alginate-release rates could be altered. Erosion was the predominant release mechanism for BSA. Special attention needs to be paid to the microparticle recovery procedure, which can significantly affect key properties such as the resulting drug-release patterns. *Conclusions:* The novel hydrogel-based microparticles offering mild conditions for incorporated drugs (e.g., proteins) provide an interesting potential as controlled delivery systems for mucosal surfaces.

1. Introduction

Most peptide and protein drugs have to be administered by injection, since the preferred oral application is often not feasible due to degradation, low permeation and significant first pass metabolism. The increasing number of available recombinant peptide and protein therapeutics [1,2] has enhanced the search for alternative administration routes to parenteral applications (e.g., rectal, buccal, nasal, pulmonary [3–5]). Peptides and proteins are generally rapidly eliminated from the systemic circulation. To reduce the application frequency, microparticulate carrier systems providing sustained delivery have been proposed [6,7]. For example, polylactides [poly(lactic acid), poly(glycolic acid), and poly(lactic-co-glycolic acid)] (PLGA) have been studied extensively as carrier materials for depot formulations [8,9]. However, the PLGA degradation times (several weeks to months) are not suitable for all administration routes, e.g., via mucosal surfaces. Furthermore, the use of organic solvents during drug encapsulation (e.g., in w/o/w emulsion solvent extraction and evaporation methods), the polymer hydrophobicity, and the potential formation of acidic microclimates upon polymer degradation often results in a loss of the biological activity of the protein [6,10,11].

The use of hydrogel-forming polymers (e.g., alginates and poloxamers) as encapsulation materials presents a promising alternative to overcome these restrictions. These polymers swell and gel in water, and retain a significant fraction of water in their structure without dissolving [12,13]. Often, hydrogels are highly biocompatible. They usually possess a shorter duration of release and degradation compared to, e.g., PLGA-based formulations. Due to their aqueous, hydrophilic nature and mostly mild preparation procedures, they offer a preferable environment for peptide and protein drugs and have been shown to stabilize the complex structure of protein drugs [13–16]. However, the appropriate control of drug release from these highly swollen networks is challenging.

Poloxamers/Pluronics are a series of synthetic block copolymers of poly(ethylene oxide-b-propylene oxide-b-ethylene oxide) (PEO–PPO–PEO) with varying molecular weights and block ratios. They are non-ionic surfactants possessing excellent wetting, antifoaming and solubilizing properties. Poloxamer 407 (Pluronic F127) is an ABA-type triblock copolymer consisting of poly(oxyethylene) units ($A = 70\%$) and poly(oxypropylene) units ($B = 30\%$), which is transformed from a low-viscosity solution to a semisolid gel upon heating from 4 °C to room or body temperature in aqueous solutions at concentrations of $\geqslant 20\%$ [17].

This (reverse) thermal gelation and the low toxicity are properties that have made poloxamer 407 an attractive matrix-former in in situ gel-forming, controlled drug-delivery systems [18–20]. Such injectable depot formulations have enhanced the stability and sus-

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tained the delivery of biologically active peptide/protein drugs, e.g., interleukin-2 [21], urease [22], deslorelin and GnRh [23], insulin [24], human growth hormone [25] and the MSH-analog melanotan-I [26]. Furthermore, the suspension of protein precipitates in a poloxamer 407 gel matrix was reported to result in an enhanced protein stability [27]. Poloxamer 407 has also been investigated as release-sustaining additive in buccal, nasal, ophthalmic and rectal delivery systems [18,19,28,29]. However, due to the dissociation of packed poloxamer 407 micelles in an excess of water, the gel integrity does not persist during prolonged periods of time. Most formulations show sustained release kinetics only during several hours (e.g., 8 h for IL-2 [21], and 4 h for melanotan-I [26]). And most of the described systems are liquids (injectable at low temperature). However, with respect to long-term stability, dry powder systems would be preferable. Also, evidence for the safety of poloxamer for delivery to mice lungs was provided [30].

Alginates are naturally occurring, linear unbranched polysaccharides which contain various amounts of 1,4-linked β-D-mannuronic and α-L-guluronic acid units arranged as blocks along the chain, where homopolymeric regions are interdispersed with regions of alternating structure. They are able to form water-insoluble gels by cross-linking with divalent cations (e.g., Ca^{2+}). Due to this mild gelation process, a relatively inert aqueous environment within the matrix and the high biocompatibility of alginate, this polymer has been widely used as a matrix-former for the microencapsulation of bioactive peptides and proteins as well as living cells [13,15,31,32]. However, alginate gels degrade and precipitate in 0.1 M phosphate buffer (as the calcium ions are removed and a calcium phosphate precipitate is formed), leading to rapid drug release. Furthermore, alginate gels show a high porosity resulting in high-diffusion rates. The formation of stable complexes of alginate with polycations such as poly(L-lysine), chitosan or polyethyleneimine has therefore been proposed. The calcium alginate gels are, thus, protected against Ca^{2+} chelators. The porosity is reduced, resulting in a more sustained drug delivery [33–35]. However, an additional coating step with the polycations is required.

The combination of poloxamer 407 and calcium alginate gels in a dry powder microparticulate system could be a promising strategy to overcome the restrictions of the individual polymers. The idea is to reinforce the poloxamer 407 gel via a calcium cross-linked alginate network. Hence, the rapid dissociation of poloxamer 407 micelles should be hindered. In addition, the poloxamer 407 (upon gelation at body temperature) could fill the pores of the alginate gel and act as diffusion barrier for entrapped drug as well as for the dissolution medium, thereby slowing down the exchange of calcium ions, and consequently the calcium alginate gel degradation.

The objective of this study was to prepare drug-loaded calcium alginate–poloxamer 407 microparticles by a w/o-emulsion method, and to investigate the influence of formulation and processing parameters on the encapsulation efficiency, particle size, morphology and in vitro drug-release kinetics of the microparticles. A self-made diffusion cell was used to better simulate the release conditions on mucosal surfaces, where only a small amount of release medium is available.

2. Materials and methods

2.1. Materials

Terbutaline sulfate (Welding, Hamburg, Germany), bovine serum albumin (BSA, M_w 69 kDa; Carl Roth, Karlsruhe, Germany), sodium alginate (low-viscosity grade; Sigma–Aldrich, Steinheim, Germany), poloxamer 407 (polyoxypropylene–polyoxyethylene block copolymer; Lutrol F127, BASF, Ludwigshafen, Germany), sodium hyaluronate (sodium hyaluronate pharma grade 80; NovaMatrix/FCM BioPolymer, Oslo, Norway), hydroxypropyl methylcellulose (HPMC E50; Methocel E50; Colorcon, Dartford, UK), calcium chloride, heptane and peanut oil (Carl Roth), sorbitan trioleate (Span® 85; Merck-Schuchardt, Hohenbrunn, Germany), polyoxyethylene sorbitan trioleate (Tween® 85; ICI Surfactants, Everberg, Belgium), sodium citrate (tri-sodium citrate dehydrate; Merck, Darmstadt, Germany), Coomassie assay (Coomassie Plus Protein Assay Kit; Pierce Biotechnology, Rockford, IL), 1,9-dimethyl methylene blue (DMMB; Sigma–Aldrich).

2.2. Microparticle preparation

Microparticles were prepared by a w/o-emulsion external cross-linking procedure (Fig. 1) adapted from Wan et al. [36], and Chan and Heng [37]. Heptane or peanut oil was used as organic phase:

(1) *Heptane:* Twenty gram aqueous solution of 3% (w/w) alginate, 3% poloxamer 407 and (if indicated) the drug (terbutaline sulfate or BSA, 10% w/w based on total solids) were dispersed in 30 g heptane (w/o volume ratio ~1:2) containing 3.3% w/w Span 85 (1 g) using a magnetic stirrer (1000 rpm) for 10 min. Two gram aqueous solution of 25% Tween 85 (0.5 g, resulting in a ratio of Span 85: Tween 85 = 2:1) was added, and the emulsion was stirred for another 5 min. The system was heated to 40–45 °C under continuous stirring on a magnetic heating plate. Then, 8 g of an aqueous $CaCl_2$ solution (25% w/w) was added dropwise (during ~4 min) using a syringe with needle (Sterican® Gr.1 0.90 × 40 mm; Braun, Melsungen, Germany). Stirring was continued for another 15 min. The system was then allowed to cool down for 10 min (under stirring at 200 rpm). Subsequently, the microparticles were recovered by two different procedures: (i) by vacuum filtration without further washing, or (ii) by centrifugation in 15 mL screw-capped tubes (5 min/1500 rpm, Ecco-Praxa-2, Theodor Karow, Berlin, Germany) and washing of the settled microparticles by redispersion in water followed by another centrifugation step. The obtained microparticles were dispersed in 2 mL water, immediately stored in a −70 °C freezer for at least 1 h and freeze dried for 48 h (Alpha I-5, Martin Christ Gefriertrocknungsanlagen, Osterode am Harz, Germany). Parameters such as the drug loading (BSA: 5, 10 and 20% w/w) and the alginate:poloxamer 407 ratio in the aqueous phase (1:5, 3:3, 5:1, 5:0) were varied. Additionally, an alternative method was used to load terbutaline sulfate more efficiently into the microparticles. Blank microparticles were prepared by the same procedure (without drug in the aqueous phase). Five-hundred milligram blank microparticles were dispersed in 2.5 g prewarmed (40–45 °C) terbutaline sulfate solution (0.5, 2.8 or 6.25% w/w, leading to theoretical drug loadings of 2.5, 12.2 and 23.8%) under vortex stirring (1 min, 2500 rpm). Microparticles were recovered by

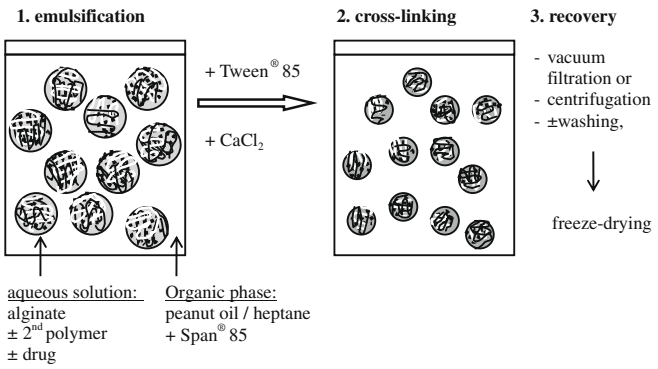

Fig. 1. Schematic presentation of the microparticle preparation techniques.

centrifugation (5 min, 3000 rpm), and again redispersed in 5 mL warm (40 °C) water, frozen and freeze dried as described above.

(2) *Peanut oil:* In principal, the above-described preparation procedure for heptane was followed. However, the entire process was conducted at room temperature under propeller stirring at 1000 rpm (EUROSTAR power control-visc; IKA). Ten gram aqueous polymer solution and 20 g peanut oil were used (w/o volume ratio 1:2). The peanut oil contained 3.3% w/w Span 85 (~0.67 g), and 1.3 g of 25% Tween 85 solution was added (Span 85:Tween 85 ratio 2:1). The microparticles were recovered by vacuum filtration. The adhering peanut oil was removed by extensive washing with isopropanol (3 × 30 mL). If indicated, certain processing parameters like the type of stirrer (magnetic or propeller) and washing liquid (ethanol or isopropanol), stirring speed, emulsifier concentration and the way of $CaCl_2$ addition [4 g of 25% aqueous or 8 g of 12.5% isopropanolic solution or emulsion of an aqueous solution in peanut oil using a homogenizer/Ultra-Turrax® (T 25; IKA, at 10,000 rpm for 2 min)] as well as formulation parameters, e.g., theoretical BSA loading, polymer concentration in the aqueous phase and type of polymer blend, were varied.

2.3. Determination of drug, calcium and alginate contents

Microparticles (~10 mg, accurately weighed) were dissolved under horizontal shaking (130 rpm, HS 501 digital; IKA) for 24 h at room temperature in 10 mL sodium citrate solution (3% w/w). The terbutaline sulfate content was determined by UV spectroscopy (UV 2101 PC; Shimadzu, Duisburg, Germany, λ = 276 nm), the BSA content via a Coomassie assay according to the instructions of the manufacturer (test tube procedure, λ = 595 nm). The Ca^{2+} ion content was measured by flame atomic absorption spectroscopy in an acetylene/air flame (λ = 422.7 nm, bandpass: 0.5 nm, PU9100X; Philips Industrial & Electro-Acoustic Systems Division, Amlelo, The Netherlands). The amount of alginate was determined using a method based on cationic dye complexation with 1,9-dimethyl methylene blue (DMMB) described by Richardson et. al. [38]. Briefly, 1 mL sample solution was mixed with 1 mL of 0.8 M sodium hydroxide, and neutralized after 5 min with 120 μL of 2.25 M citric acid. Thereafter, 40 μL of 1 mM DMMB was added, the sample was vortexed and incubated at room temperature for 60 min. The absorbance was measured UV-spectrophotometrically at 520 and 650 nm (= absorbance maxima of bound and unbound DMMB), and the 520:650 nm absorbance ratio was calculated.

2.4. Optical microscopy

Microparticles were dispersed in ethanol or water and observed with an optical light microscope (Axiotrop; Carl Zeiss Jena, Jena, Germany) equipped with an image analysis system (InteQ Informationstechnik, Berlin, Germany) consisting of a digital camera (type MC1) and the EasyMeasure software (version 1.0.15).

2.5. Scanning electron microscopy (SEM) and energy-dispersive X-ray measurement (EDX)

Scanning electron microscopy (SEM; S-4000 and S-2700; Hitachi High-Technologies Europe, Krefeld, Germany) was used to characterize the external and internal morphology of the microparticles. For surface imaging, the microparticles were fixed on a sample holder with double-sided tape. To investigate the inner structure, the particles were dispersed in non-aqueous glue (UHU® extra Alleskleber; UHU, Baden, Germany) and after drying in a desiccator, the hardened matrix was cut with a razor blade. All samples were coated under an argon atmosphere with a fine gold layer (8 nm, SCD 040, Baltec, Witten, Germany). Furthermore, energy-dispersive X-ray mapping (EDX; DFix; SAMx, Levens, France) was conducted on the cross-section samples after carbon coating, using an accelerating voltage of 20 keV to analyse the calcium and chloride distribution. Additionally, EDX spectra were measured at the edge and in the centre of the particles. No absolute values for the calcium and chloride content were determined, but the electron counts per second detected for different formulations were compared.

2.6. 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, Krefeld, Germany). The particles were suspended in ethanol (96% v/v) to avoid swelling effects in aqueous media.

2.7. Drug-release studies

In vitro drug release from the microparticles was studied using two experimental setups, based on (a) a dialysis bag; or (b) a diffusion cell (Fig. 2). The two methods are briefly described as follows:

(a) Accurately weighed amounts (~50 mg) of terbutaline sulfate-loaded microparticles were filled into dialysis bags (6 cm long, 2.2 cm wide, molecular weight cut-off 12–14 kDa; Medicell International, London, UK). The dialysis bags were placed into glass test tubes containing 10 mL phosphate buffer of pH 7.4 (USP XXX) preserved with 0.05% sodium azide at 37 °C in a horizontal shaker (GFL 3033; Gesellschaft für Labortechnik, Burgwedel, Germany) (75 rpm, n = 3). At predetermined points, 2 mL samples were withdrawn and replaced with fresh medium. The terbutaline sulfate content was determined by UV spectroscopy at 276 nm.

(b) A self-made, modified Franz diffusion cell was used to simulate the release conditions on mucosal surfaces. The experimental setup was adapted from Bertram and Bodmeier [39]. Briefly, a regenerated cellulose filter membrane [pore size = 0.45 μm, d = 47 mm; RC – fleece supported (Sartorius, Goettingen, Germany)] and a thin cloth (Flinka Allzwecktuch; Kornbusch & Starting, Borken, Germany) were tightly attached at the lower end of a polypropylene tube (inner diameter = 2.7 cm). This tube was vertically placed in a plastic vessel filled with 10 mL phosphate buffer of pH 7.4 + 0.05% sodium azide. The tube position was adjusted so that the filter was wetted, but not submersed by the release medium. Microparticles (~20 mg, accurately weighed) were sprinkled on the filter membrane and the whole system was sealed with a Parafilm "M" (American National Can Company, Chicago, IL) to avoid evaporation of water during the experiment and to guarantee a constant relative humidity to which the microparticles were exposed to (85–95% RH). The diffusion cells were placed in a hor-

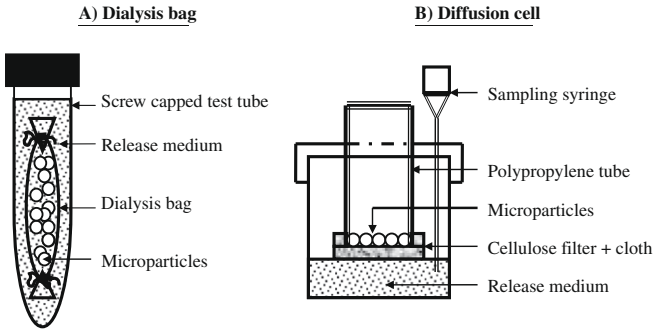

Fig. 2. Schematic presentation of the two experimental setups used for the in vitro drug-release measurements: (A) dialysis bags, (B) diffusion cells.

izontal shaker (GFL 3033; 75 rpm, 37 °C). At predetermined time points, 2 mL samples were withdrawn and replaced with fresh medium. The drug content was determined as described in Section (a). The BSA content was measured spectrophotometrically using a Coomassie assay (λ = 595 nm). Drug-free microparticles were used as reference to correct for polymer absorption. The 100% release values were experimentally verified by adding 10 mL of a 6% (w/w) sodium citrate solution in the release cells at the end of the experiments in order to completely dissolve the calcium alginate. All measurements were performed in triplicate (mean values ± SD shown in figures).

3. Results and discussion

The combination of poloxamer 407 and calcium alginate within one drug carrier system could be a tool to control the release kinetics of hydrophilic drugs, such as peptides and proteins. Therefore, alginate–poloxamer 407-based microparticles were prepared by a w/o-emulsion method. Lemoine et al. [40] also used an emulsification technique to encapsulate BSA in alginate microparticles and reported that it did not affect the molecular weight or the antigenicity of the protein. Aqueous emulsion droplets, containing both the polymers and the dissolved drug, transformed into solid microparticles upon addition of $CaCl_2$. The divalent calcium ions are bound in a highly cooperative manner to the guluronic acids units of the alginate (cross-linking), leading to the formation of water-insoluble gel particles. Since alternatives to the common parenteral routes are desirable, these microparticles are intended for the application of peptide and protein drugs on mucosal surfaces (e.g., buccal, nasal, and pulmonal).

3.1. Model drug terbutaline sulfate and release setup

Terbutaline sulfate was used as a low-molecular weight model drug to study the effect of the type of release setup. A high drug loss occurred during microparticle preparation (encapsulation efficiency = 25%). This can probably be attributed to a significant loss of the highly water-soluble drug (250 mg/mL) upon expulsion of the water during cross-linking inducing microparticle shrinking and to drug loss during the washing step. Low encapsulation efficiencies were also reported for pure alginate microparticles, e.g., in the case of theophylline [36] and indomethacin [41]. In order to avoid such drug loss, blank calcium alginate–poloxamer 407 microparticles were produced using the emulsion technique (Fig. 1), and subsequently soaked with equal amounts of aqueous drug solutions of varying concentration. As observed by optical microscopy, spherical and fairly homogeneous particles were obtained (data not shown), but also a few agglomerates were visible. They were likely due to the fusion of particles during soaking and led to a quite broad (volume based) size distribution (Table 1), especially for particles with a low drug loading (2.5%). Interestingly, the loading efficiency increased with increasing drug concentration of the soaking solution (Table 1). A possible reason for this phenomenon is the electrostatic interaction of the positively charged terbutaline (amino group) and the negatively charged alginate (carboxylic group). An increasing drug concentration can be expected to lead to an increasing replacement of Ca^{2+} ions during drug loading resulting in less dense polymer networks, able to take up more aqueous medium. This is in good agreement with the observation that the amount of supernatant after soaking decreased with increasing drug concentration of the soaking solution. A similar observation was reported by Segi et al. [42] for propranolol soaked into alginate beads. The drug loading increased in a sigmoidal manner with increasing bulk drug concentration when loaded at a pH >2.5 accompanied by an abrupt contraction of the beads.

Drug release from the microparticles was much faster when using the dialysis bag setup compared to the diffusion cell (Fig. 3). This can be explained by the higher volume of release medium the microparticles are exposed to with the dialysis bag setup. The diffusion cell setup is more realistic than the dialysis bag method because of better simulating the quantities of water on mucosal surfaces in vivo. Also, the diffusion cell setup is more discriminative than the dialysis bag method: Varying the initial drug loading, the resulting drug-release kinetics were very similar when dialysis bags were used, whereas differences were observed when diffusion cells were applied. Therefore, the diffusion cell release setup was used for all further studies (more realistic simulation of the in vivo conditions and higher discriminative power).

Table 1
Theoretical and actual terbutaline sulfate contents, encapsulation efficiencies and mean particle size of microparticles prepared by soaking drug-free particles (produced by an emulsion/external gelation technique, 3% alginate + 3% poloxamer 407 in aqueous phase) with aqueous drug solution of different concentrations.

Drug content (%) theoretical	Drug content (%) actual	Encapsulation efficiency (%)	Size (μm) (±s) median
2.5	2.1	**82.8**	52.6 (116.0) 15.0
12.2	10.5	**86.4**	23.8 (37.9) 11.8
23.8	21.8	**91.6**	24.0 (26.1) 17.3

3.2. Alginate:poloxamer 407 blend ratio

The microparticles are intended to control the release of peptide and protein drugs on mucosal surfaces. Therefore, BSA was used as model protein for all further studies. The polymer blend ratio was investigated as a potentially important parameter controlling the microparticle properties. The alginate:poloxamer 407 ratio significantly affected the particle shape and the morphology (Figs. 4 and 5). Particles with high poloxamer 407 contents were rather fragile, transparent and distorted in shape (Figs. 4 and 5A), exhibiting an uneven (but not porous) surface (Fig. 5D). With increasing alginate content, microparticles became more spherical, opaque and smaller in size (Figs. 4 and 5). Very broad particle size distributions were measured for all formulations. One main peak was accompanied by one or two smaller peaks at larger sizes likely indicating agglomerates. Since a few agglomerates strongly affect the mean volume-based particle size, the *median* was chosen instead to compare different particle batches.

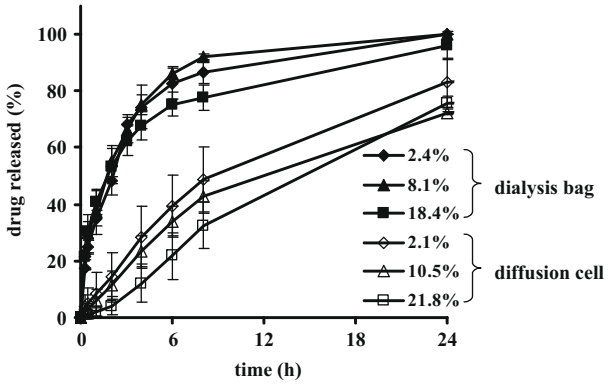

Fig. 3. Effect of the initial drug loadings (indicated in the diagram) and type of release setup on the release of terbutaline sulfate from calcium alginate–poloxamer 407 microparticles prepared by an emulsion/external gelation procedure, and subsequent soaking of aqueous drug solutions (closed symbols dialysis bag, open symbols diffusion cell, USP buffer, pH 7.4, 37 °C).

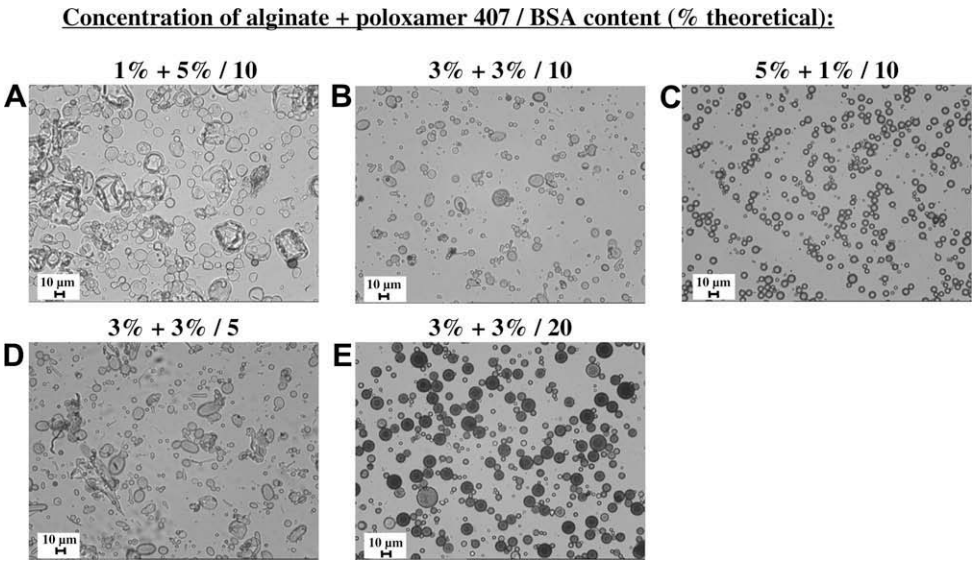

Fig. 4. Optical microscopy pictures (in ethanol) of alginate:poloxamer 407 microparticles prepared using a w/o-emulsion/external gelation method with varying concentrations/ratios of alginate and poloxamer 407 in the aqueous phase and varying BSA loadings as indicated.

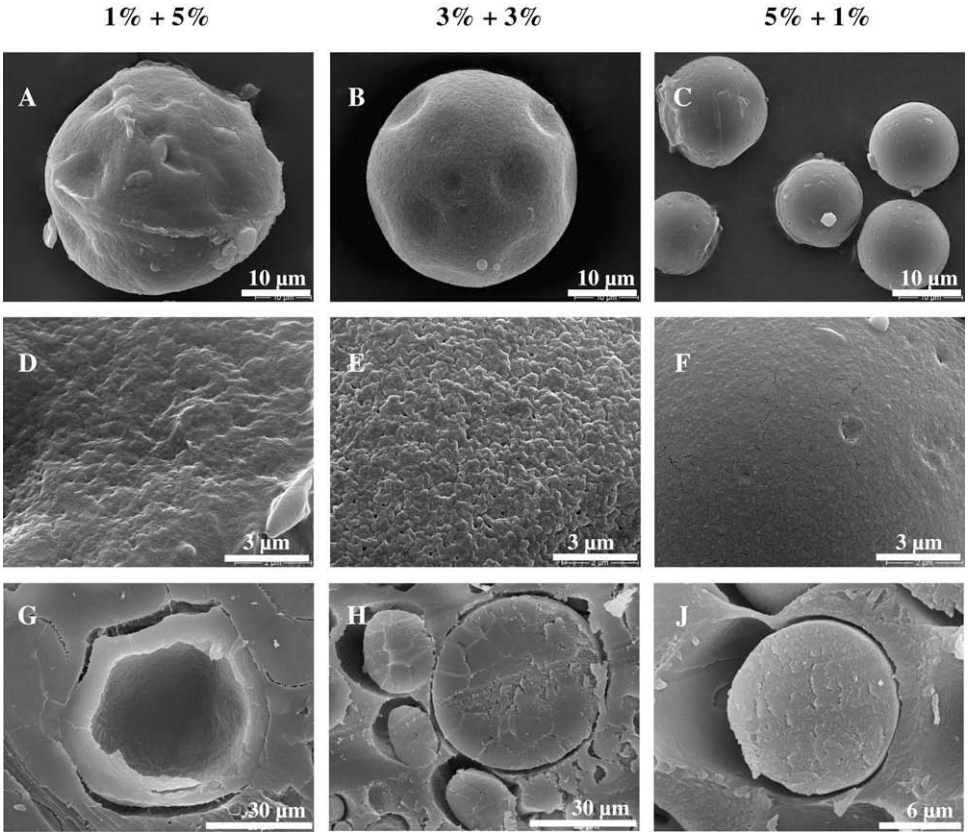

Fig. 5. SEM pictures of alginate:poloxamer 407 microparticles with varying concentrations/ratios of alginate and poloxamer 407 (left column: 1 + 5%, middle column: 3 + 3%, right column: 5 + 1%) in the aqueous phase during microparticle preparation (theoretical BSA content 10% w/w) (top row: surface/lower magnification, middle row: surface/higher magnification, bottom row: cross-section).

Two different methods were used to recover the microparticles after cross-linking with calcium: (i) repeated centrifugation and washing with water and (ii) vacuum filtration without washing. Particles recovered by centrifugation and washing showed no clear relationship between the polymer blend ratio and the particle size (Table 2). In contrast to terbutaline sulfate, almost no BSA was lost during microparticle preparation (with only one exception at high poloxamer content). This can be explained by the higher molecular weight of BSA, resulting in more hindered diffusion through the polymeric network. The low BSA encapsulation efficiency of microparticles containing high poloxamer 407 amounts can be attributed to the low alginate content of the systems, leading to more permeable polymeric networks. Interestingly, the actual alginate content of poloxamer 407-containing particles that were centri-

Table 2
Effects of the alginate and poloxamer 407 concentrations in the aqueous phase during microparticle preparation (with an emulsion/external gelation procedure) and centrifugation/washing on the size, actual BSA, calcium, alginate and poloxamer 407 contents (theoretical BSA loading: 10% w/w).

Conc. (%) alginate + poloxamer	Centrifugation + washing	Size-median (μm)	BSA content (%) (±s)	Calcium content (%) (±s)	Alginate content (%)		Poloxamer content (%)	
					Theoretical	Actual (±s)	Theoretical	Actual* (max.)
1 + 5	Yes	76.1	5.2 (0.1)	5.4 (0.1)	14.9	42.6 (7.0)	74.5	46.8
3 + 3	Yes	31.1	10.5 (0.5)	7.8 (0.2)	40.9	76.4 (1.1)	40.9	5.3
5 + 1	Yes	43.1	10.6 (1.0)	5.5 (0.4)	69.9	77.2 (9.3)	14.0	6.7
5 + 0	Yes	41.5	10.5 (1.0)	6.1 (0.7)	83.5	91.2 (12.9)	–	–
1 + 5	No	37.1	9.1 (0.3)	6.0 (0.1)	14.2	27.2 (3.0)	70.8	57.7
2 + 4	No	32.3	9.8 (0.3)	6.7 (0.3)	27.8	34.1 (3.5)	55.7	49.4
3 + 3	No	28.8	9.3 (0.2)	9.1 (0.4)	40.8	37.5 (4.0)	40.8	44.1
4 + 2	No	14.8	10.5 (0.7)	6.4 (0.2)	55.4	50.5 (3.8)	27.7	32.6
5 + 1	No	12.0	10.4 (0.4)	6.5 (0.4)	69.3	64.4 (1.8)	13.9	18.7

* Indirectly determined via the measurement of the alginate contents as described in Section 2 – Determination of drug, calcium and alginate contents.

fuged and washed was higher than the theoretically calculated one. This is due to a significant poloxamer 407 loss during microparticle preparation (Table 2). Vacuum filtration without washing was studied as an alternative recovery technique in order to avoid poloxamer 407 loss. These microparticles were generally smaller than centrifuged and washed ones. Their size decreased with increasing alginate:poloxamer 407 ratio, indicating a more pronounced particle shrinkage upon cross-linking due to the higher density of the alginate network (Table 2). As expected, washed microparticles contained less calcium than unwashed ones. With both recovery techniques microparticles prepared using 3% alginate and 3% poloxamer 407 in the aqueous phase exhibited the maximum calcium content (7.8% and 9.1% in washed and unwashed microparticles, respectively). Calcium within the particles can either be bound to alginate or exist as $CaCl_2$. It was expected that the $CaCl_2$ content increases with decreasing alginate content. This could be confirmed by EDX analysis (Fig. 6A): The chloride counts increased with an increasing poloxamer 407 content, whereas the calcium counts were similar for all polymer blend ratios. Furthermore, EDX analysis showed a homogeneous distribution of calcium and chloride throughout the particles (Fig. 6B shows 5 + 1% microparticles as an example, being representative for all other investigated formulations). A significant poloxamer 407 loss could be avoided when the cross-linked particles were vacuum filtered and not washed (Table 2). Due to the low viscosity of heptane, the organic phase was almost completely removed by vacuum filtration. Remaining heptane evaporated during subsequent drying (volatile solvent).

Drug diffusion through the polymer network plays a major role in the control of drug release from the microparticles. The mobility of the low-molecular weight drug terbutaline sulfate is likely to be much less restricted than that of BSA. In the present case, the removal of Ca^{2+} ions (either due to an exchange against other cations, e.g., sodium ions, or via a precipitation of poorly soluble calcium salts, e.g., calcium phosphate) can be expected to strongly affect the resulting polymer, and thus drug mobility upon exposure to the release medium.

The polymer blend ratio and microparticle recovery method strongly affected the BSA and alginate-release kinetics (Fig. 7). The relative release rate decreased with increasing alginate content, irrespective of the type of recovery method. This can be explained by the increasing density of the polymeric networks. The differences in drug release were more pronounced in the case of microparticles recovered by vacuum filtration without further washing (unwashed). This might be attributable to the higher calcium content and/or to the higher poloxamer content (Table 2), rendering the system less permeable for the drug if the poloxamer polymer gels and is entrapped within the particles. The relatively fast release from the particles with high poloxamer contents is likely to be caused by a quite loose, rapidly eroding calcium alginate gel structure. A more and more efficient diffusion barrier is formed, and gel dissolution prolonged with increasing alginate content. All formulations showed a significant increase in the drug-release rate after a certain time period, which increased with increasing alginate content of the microparticles (Fig. 7 and Table 2). The sudden increase in the release rate might be explained by abrupt loosening of the polymer network upon calcium removal (due to ion exchange and/or calcium phosphate precipitation). The alginate-release rate also decreased with increasing alginate content (Fig. 7C and D). This can again

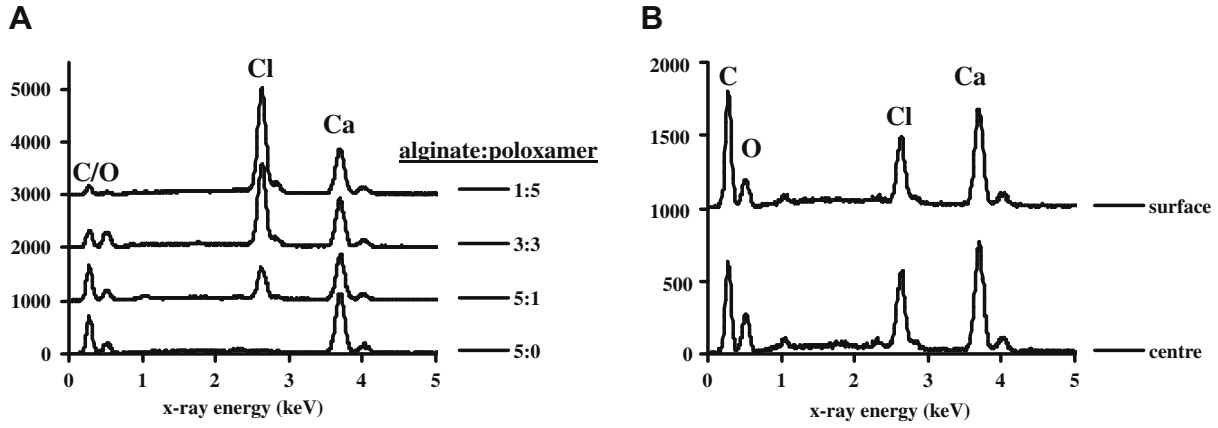

Fig. 6. Calcium and chloride counts (EDX analysis) in cross-sections of alginate–poloxamer 407 microparticles. Effects of: (A) polymer blend ratio (surface counts), and (B) localization within the particle (5:1 alginate:poloxamer).

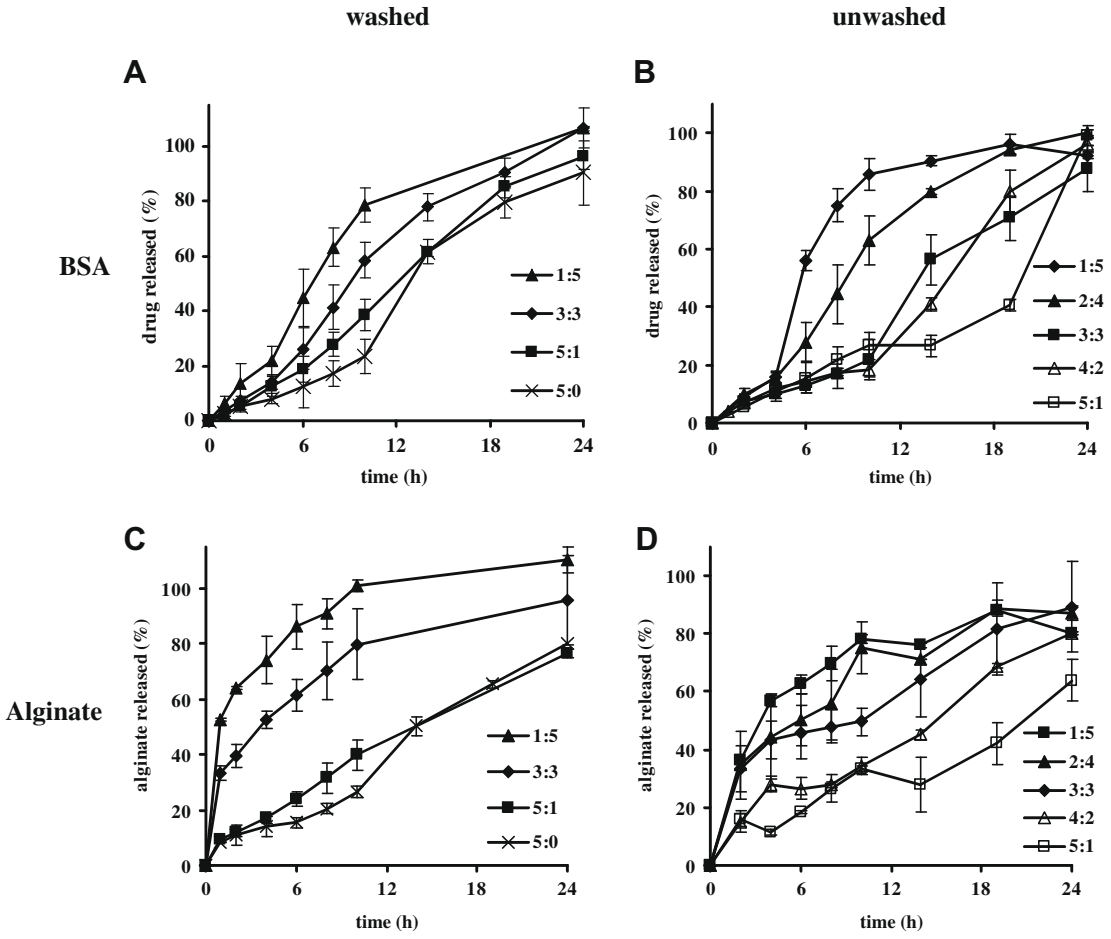

Fig. 7. Effects of the polymer blend ratio on BSA (A and B) and alginate (B and C) release from washed and unwashed alginate:poloxamer microparticles (diffusion cell, USP buffer, pH 7.4, 37 °C). The alginate:poloxamer blend ratio is indicated in the diagrams.

be attributed to the decreasing density of the polymeric networks.

3.3. Drug loading

The drug loading is another important parameter that might influence the microparticle characteristics. Often, it is desired that the properties of microparticles are independent of the drug loading in order to have a flexible, broad formulation range. Microparticles that are recovered by centrifugation and washing showed no clear correlation between the BSA content and the particle size (Table 3). In contrast, the size decreased with increasing BSA loading in the case of unwashed microparticles. This was confirmed by optical microscopy (Fig. 4D and E), which also revealed pronounced differences in particle morphology when varying the drug loading. From very irregularly shaped, rather translucent 5% BSA containing particles (Fig. 4D), the appearance changed to perfectly spherical and very dense, opaque 20% BSA containing microparticles (Fig. 4E). This phenomenon might be attributable to the surface activity of the protein, leading to a decreased emulsion droplet size, and/or to BSA-alginate interactions [43]. Apparently only little or no BSA was lost during microparticle preparation (Table 3), except for the 20% BSA-loaded, washed microparticles. In the latter case, the alginate content is likely to be too low to prevent significant drug loss during washing. Again, the washing step led to lower calcium and higher alginate contents (Table 3), the latter indicating a pronounced poloxamer 407 loss.

The relative protein-release rates of washed formulations were not significantly affected by the initial BSA content (Fig. 8A), irrespective of the type of recovery method. Also the alginate-release

Table 3
Effects of BSA content and type of recovery method on the size, actual BSA, calcium, alginate and poloxamer 407 contents of the microparticles (theoretical BSA loading: 10% w/w).

BSA content (%) theoretical	Centrifugation + washing	Size-median (μm)	BSA content (%) (±s)	Calcium content (%) (±s)	Alginate content (%)		Poloxamer content (%)	
					Theoretical	Actual (±s)	Theoretical	Actual* (max.)
5	Yes	19.9	5.6 (0.1)	8.0 (0.3)	43.2	64.1 (3.3)	43.2	22.3
10	Yes	37.1	10.5 (0.5)	7.8 (0.2)	40.9	76.4 (1.1)	40.9	5.3
20	Yes	23.6	15.3 (1.1)	7.7 (1.1)	38.5	76.4 (1.0)	38.5	0.6
5	No	36.1	5.4 (0.3)	9.1 (0.1)	42.8	38.0 (3.5)	42.8	47.5
10	No	28.8	9.3 (0.4)	9.1 (0.4)	40.8	37.5 (4.0)	40.8	44.1
20	No	21.4	19.9 (0.6)	4.7 (0.1)	37.7	45.1 (0.9)	37.7	26.4

* Please refer to Table 2.

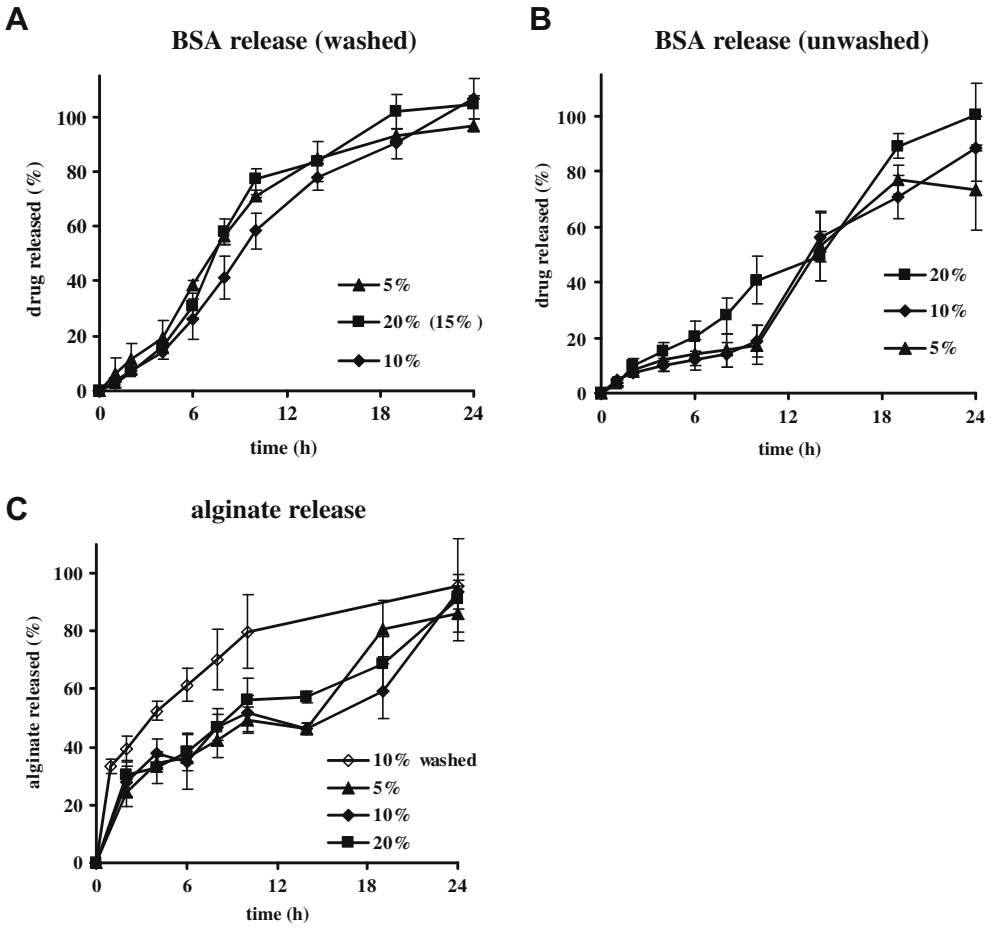

Fig. 8. Influence of the initial drug loading and microparticle recovery procedure on BSA (A and B) and alginate (C) release from alginate–poloxamer 407 microparticles (diffusion cell, USP buffer, pH 7.4, 37 °C).

rates were higher for washed than for unwashed formulations (Fig. 8D). Unwashed microparticles showed longer lag times prior to gel erosion, which might be attributable to the higher poloxamer 407 content, the latter polymer filling the network pores, and thus slowing down the diffusion of water, BSA and alginate.

3.4. Organic phase

According to the ICH guideline Q3C and the USP monograph (USP XXX, section <467>) concerning residual solvents' content in drug products, heptane belongs to the class III solvents, which should be limited by GMP or other quality-based requirements (<50 mg/day or 5000 ppm = 0.5%). Therefore, peanut oil was investigated as an alternative organic phase in the following studies. The influence of several process and formulation parameters on the particle composition and especially on the particle size was investigated. A mean geometric diameter of less than 5 μm is necessary for a potential pulmonary application of these microparticles as dry powder aerosol. Preliminary trials (data not shown) indicated that the particle size did not depend on the temperature of the emulsion system during microparticle preparation (in contrast to heptane). Consequently, all particles in the following studies were prepared at room temperature. The use of a propeller instead of a magnetic stirrer led to a significant decrease in particle size (from 34.4 to 12.7 μm, Table 4), probably due to a more effective dispersion of the aqueous phase within the organic one. Furthermore, the use of the propeller led to an increased calcium content of the microparticles. This might be attributable to the increased surface area of the smaller emulsion droplets that are accessible for the calcium ions. However, the peanut oil needed to be removed completely to obtain a free-flowing (non-sticking) powder. The use of ethanol as washing agent led to a significant loss of poloxamer 407 as indicated by the high alginate content of the microparticles (~70%; Table 4). In order to reduce this poloxamer loss, ethanol was substituted by isopropanol as washing liquid (poloxamer is less soluble in isopropanol). The resulting poloxamer loss could, thus, significantly be reduced. An increase in the propeller speed (from 1000 to 2000 rpm) led to smaller particles (14.6 vs. 11.7 μm). Increasing emulsifier concentrations led to decreasing microparticle sizes, which can be attributed to the reduced surface energy of the system, permitting smaller emulsion droplets (Table 4). Increasing the BSA loading resulted in significantly increasing calcium contents (Table 4), which might be explained by the high water binding ability of this protein, keeping higher quantities of the aqueous phase (containing $CaCl_2$) inside the swollen particles. The mean particle size increased when decreasing the polymer:polymer concentrations from 3% to 1% (Table 4). The 1% polymer particles showed a more flake-like and fairly aggregated morphology (Fig. 9A), whereas particles produced using a 2% or 3% polymer solution appeared less distorted and agglomerated (Fig. 9B and C). Probably, concentration of 1% alginate in the aqueous phase was too low to form a stable gel network, indicated also by lower calcium and BSA contents.

So far, the cross-linking of the alginate in the aqueous emulsion droplets had been induced by the dropwise addition of an aqueous $CaCl_2$ solution. This procedure potentially destabilizes the emul-

Table 4
Effects of different processing and formulation parameters on the size, actual BSA, calcium, alginate contents of microparticles produced by an emulsion/external gelation procedure using peanut oil as organic phase (unless otherwise stated).

Parameter	Value	Size-median, (μm)	BSA content (%) (±s)	Calcium content (%) (±s)	Content (%)		
					Each polymer theoretical	Alginate actual (±s)	Poloxamer actual[3]
Stirrer type (1000 rpm) and wash medium	Magnetic/ethanol	34.4	7.0(1.2)	4.8(0.1)	44.1	69.9 (4.7)	18.3
	Propeller/ethanol	12.7	5.9(0.1)	7.8 (0.6)	43.2	69.3 (2.5)	17.0
	Propeller/isoprop.	14.6	6.3 (0.2)	7.7 (0.3)	43.0	53.2(2.1)	32.8
Stirring speed (propeller)	1000	14.6	6.3 (0.2)	7.7 (0.3)	43.0	53.2 (4.7)	32.8
	2000	11.7	8.7 (0.3)	8.3(0.1)	41.5	48.5 (4.7)	34.5
Span® 85, % (w/w in peanut oil)	0	18.9	5.6 (0.7)	8.9 (0.2)	42.8	48.7 (12.2)	36.8
	3	14.6	6.3 (0.2)	7.7 (0.3)	43.0	53.2 (4.7)	32.8
	5	14.4	5.2(0.1)	8.5 (0.2)	43.2	48.6 (2.8)	37.7
BSA content, % theoretical	3.8	14.2	2.2(0.1)	6.7 (0.2)	45.6	58.6(1.6)	32.5
	10	14.6	6.3 (0.2)	7.7 (0.3)	43.0	53.2 (4.7)	32.8
	20	15.1	12.4(0.3)	9.6 (0.3)	39.0	46.4(2.1)	31.6
Polymer conc.,% alginate + poloxamer	1 + 1	24.3	3.0(0.1)	5.2 (0.2)	45.9	48.2 (5.5)	43.6
	2 + 2	21.4	3.5(0.1)	5.2 (0.2)	45.7	49.9 (6.5)	41.4
	3 + 3	14.6	6.3 (0.2)	7.7 (0.3)	43.0	53.2 (4.7)	32.8
$CaCl_2$ addition	Aqueous sol.	14.6	6.3 (0.2)	7.7 (0.3)	43.0	53.2 (4.7)	32.8
	w/o emulsion	14.6	5.9(0.1)	7.5 (0.4)	43.3	50.3 (2.4)	36.3
	Isoprop. sol.	20.5	11.5(0.1)	7.5 (0.3)	40.5	49.7(1.4)	31.3
[1]Alginate + ?	Poloxamer 407	19.1	5.0 (0.3)	9.3(0.1)	42.9	45.4 (2.6)	40.3
	HPMCE50	14.2	6.5 (0.3)	9.3(0.1)	41.9	48.1 (5.8)	36.1
	Na hyaluronate	85.6	6.9 (0.2)	7.5(0.1)	42.8	42.5 (0.7)	43.1
[2]Organic phase	Peanut oil	19.1	5.0 (0.3)	9.3(0.1)	42.9	45.4 (2.6)	40.3
	Heptane	60.3	9.6 (0.9)	4.1 (0.4)	43.2	41.8 (2.4)	44.5

Standard procedure: external phase 20 g peanut oil + 3% Span® 85, internal phase 10 g alginate 3% + poloxamer 407 3%, + 1.3 g 27% Tween® 85, w:o ratio 1:2 v/v, Span® 85:Tween® 85 2:1 w/w, propeller stirring 1000 rpm, 4 g aqueous $CaCl_2$ solution 25% w/w dropwise, vacuum filtration, washing with isopropanol, freeze drying.
[1] 2% Polymers in aqueous phase, emulsion of aq. $CaCl_2$ solution in peanut oil.
[2] 2% Alginate/poloxamer in aqueous phase, emulsion of aq. $CaCl_2$ solution in organic phase.
[3] Please refer to Table 2.

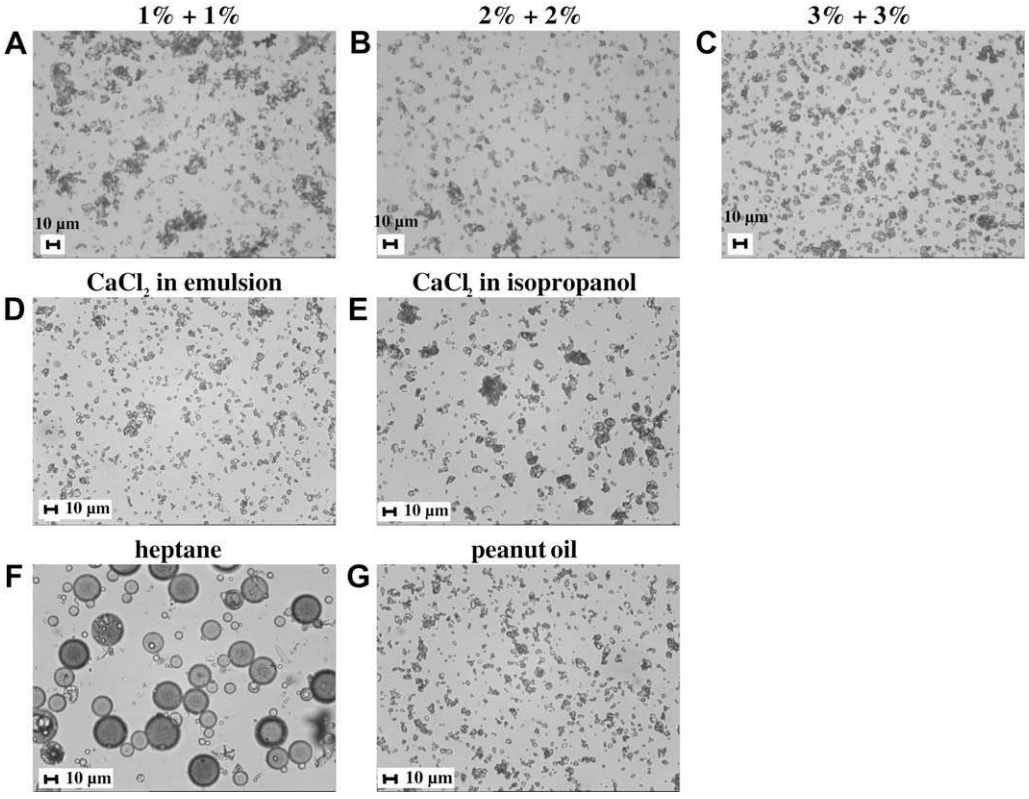

Fig. 9. Optical microscopy pictures (in ethanol) of alginate:poloxamer 407 microparticles prepared using a w/o-emulsion/external gelation method. Influence of the polymer concentration in the aqueous phase (A–C), method of $CaCl_2$ addition (D and E) and type of organic phase (F and G) (theoretical BSA content 10% w/w).

sion, and might lead to larger particle sizes. In addition, the drug and poloxamer 407 can partially get lost into this aqueous phase. Therefore, alternative methods for $CaCl_2$ addition were investigated. In a first attempt, the aqueous $CaCl_2$ solution was emulsified into peanut oil using the same phase ratio and emulsifier amounts as for the emulsification of the polymer–drug solution in peanut oil. An Ultra-Turrax was used for this purpose in order to provide very small $CaCl_2$-containing droplets that can subsequently fuse with the polymer/drug-containing droplets upon combination of both emulsions. Light microscopy revealed that the microparticles were smaller and less agglomerated than particles prepared by the direct dropping method (Fig. 9D vs. C). However, this was not reflected in the particle size measurements using laser diffraction (Table 4). The overall microparticle composition was not significantly affected. Alternatively, a solution of $CaCl_2$ in isopropyl alcohol was added dropwise. The idea was to decrease the solubility of BSA in this phase, and thus to limit drug loss during cross-linking. The resulting microparticles were more agglomerated than particles cross-linked using an aqueous $CaCl_2$ solution (Fig. 9E vs. C). This was confirmed by laser diffraction size measurements (Table 4). As intended, a loss of BSA could be avoided (11.5% actual BSA loading). However, although the microparticles' calcium content did not significantly differ, BSA release was faster when isopropanol was used as solvent for $CaCl_2$ (Fig. 10A). This might be ex-

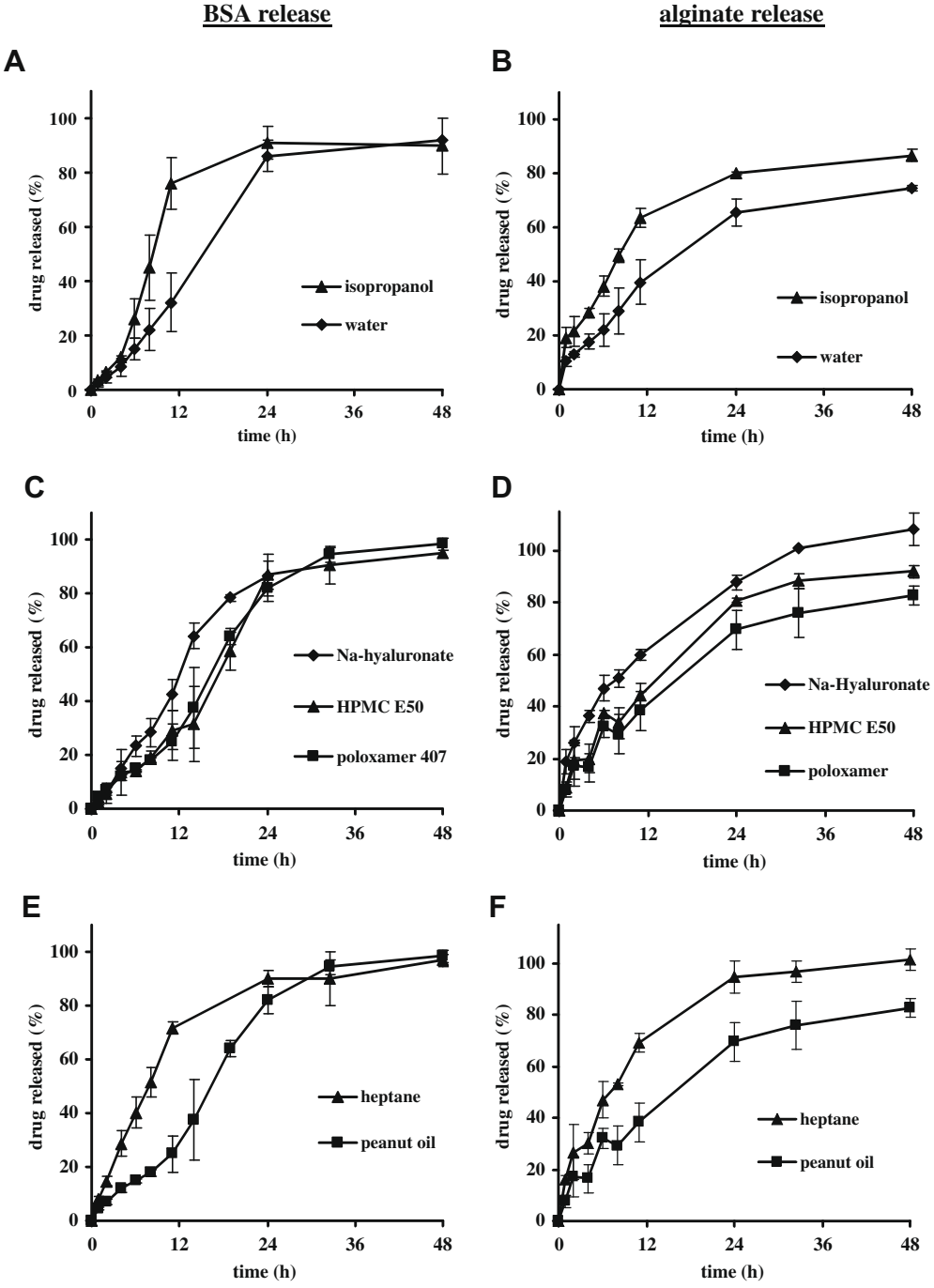

Fig. 10. Effects of the type of solvent used for $CaCl_2$ (A and B), type of blend polymer (C and D) and type of organic phase (E and F) on BSA (left column) and alginate (right column) release from alginate microparticles produced using an emulsion/external gelation method (diffusion cell, USP buffer, pH 7.4, 37 °C).

plained by a weaker calcium–alginate network, resulting from the fact that alginate is less soluble in isopropanol–water mixtures and that isopropanol is a weaker Broenstedt base than water, leading to a decreased $-COO^-$ concentration, and thus formation of Ca^{2+} cross links. This hypothesis is in good agreement with the experimentally observed faster alginate release from these systems (Fig. 10B). As a further tool to modify BSA release and to get deeper insight into the role of poloxamer 407, HPMC E50 and sodium hyaluronate were used as alternative polymer additives. Since these two polymers form highly viscous solutions already at low concentrations, aqueous solutions of 2% of these polymers and 2% alginate were used. The replacement of use of poloxamer 407 by HPMC E50 led to a slight decrease in the mean particle size (Table 4). However, when sodium hyaluronate was used, very large (85.6 μm) and flake-like particles were formed (data not shown), probably as a result of the extremely high viscosity of the inner aqueous phase. The higher molecular weight of HPMC E50 (M_W = 50,000) and sodium hyaluronate (0.62–1.15 MDa) (responsible for the high viscosity) led to the formation of a more effective diffusion barrier, decreasing the BSA loss during microparticle preparation [BSA content ~7%, compared to poloxamer 407 (M_W = 12,500, BSA content ~5%)]. The very large sodium hyaluronate chains probably even hindered the inward diffusion of calcium ions, as indicated by the significantly lower calcium content (Table 4). Consequently, the alginate was cross-linked only to a low extent. This in turn can explain the above-mentioned irregular shape of the hyaluronate-containing particles as well as the higher release rates of BSA and alginate (Fig. 10C and D). In contrast, similar (and lower) release rates were observed for HPMC E50 and poloxamer 407 (Fig. 10C and D).

Formulation and process conditions that were found to be optimal to produce very small particles with peanut oil were finally used to prepare microparticles using heptane as external phase in order to directly evaluate the influence of the organic phase type. The use of heptane resulted in large (60.5 μm) but very spherical microparticles (Fig. 9F), whereas peanut oil led to significantly smaller particles (19.1), which were more irregular in shape (Fig. 9G). A possible reason could be the higher viscosity of peanut oil. Furthermore, particles produced with heptane as organic phase showed a higher BSA (9.6% compared to 5.0% with peanut oil) and a lower calcium content (4.1% compared to 9.3%). Both the lower BSA loss and calcium uptake might be due to the lower surface area. Probably, the lower content of calcium also caused the faster alginate and consequently BSA release from particles prepared using heptane (Fig. 10E and F).

4. Conclusions

The novel hydrogel-based microparticles containing thermogelling poloxamer and cross-linked alginate offer an interesting potential as controlled protein delivery systems. The mild microenvironment within the systems is combined with the possibility to provide a wide range of drug-release profiles. Microparticles that are appropriate for mucosal administration can be prepared in suitable size ranges by this method.

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