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European Journal of Pharmaceutics and Biopharmaceutics 65 (2007) 175–187
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

Development of a spray congealing process for the preparation of insulin-loaded lipid microparticles and characterization thereof

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Received 22 December 2005; accepted in revised form 8 August 2006
Available online 23 August 2006

Abstract

A spray congealing process for the preparation of protein-loaded microparticles was developed. The influence of the process parameters atomization pressure and spraying temperature on particle size and process yield was investigated by experimental design. The employed spray congealing technique enabled the production of microparticles with yields ranging from 79% to 95% and median particle sizes ($d(0.5)$) from 182.2 to 315 μm. Insulin lipid microparticles could be prepared without any loss of insulin during the preparation process and the protein stability was not affected by the spray congealing process as investigated by HPLC-MS analysis. The stability of insulin encapsulated in lipid microparticles under release conditions over 28 days was assessed by investigating the residual insulin content. Starting after 3 days of release, a continuous increase of desamidoinsulin in the remaining particles of up to 7.5% after 28 days was observed. An additional degradation product was detected by HPLC and HPLC-MS analysis and identified as a covalent insulin dimer by MALDI-ToF. The microparticles did not show a burst release and testing the insulin lipid microparticles in a fibrin gel chondrocyte culture revealed that the released insulin was bioactive and had a significant effect on chondrocyte extracellular matrix production.

Keywords: Spray congealing; Lipid microparticles; Controlled release; Protein; Insulin; Experimental design

1. Introduction

The development of delivery systems for peptides and proteins is a challenging task as it is necessary to maintain protein integrity and bioactivity during the encapsulation and the intended release time [1]. For the parenteral release of peptides and proteins, controlled release systems based on biodegradable polymers have been developed. Although this concept works very well for various proteins, it might not be suited for all. Therefore, there is an increasing interest in alternative matrix materials for controlled release purposes that are able to enhance protein stability during manufacture and release. Previous studies with lipid microparticles and lipid matrices confirmed that triglycerides – highly biocompatible materials [2] – represent a suitable alternative matrix material for controlled release applications. Different proteins and peptides, like insulin [3], somatostatin [4], thymocartin [5], BSA [6] and lysozyme, have been incorporated in compressed lipid cylinders and/or microparticles and displayed long-term release profiles over days to months. Insulin-loaded lipid microparticles prepared by melt dispersion, for example, released 20–50% of the protein within 14 days [3].

Lipid microparticles can be prepared using solvent evaporation and melt dispersion techniques by incorporating a protein solution in the molten/dissolved lipid and

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doi:10.1016/j.ejpb.2006.08.008

dispersing the preemulsion in an aqueous phase [7]. The microparticles are formed by solidification due to congealing of the molten lipid droplets or evaporation of the organic solvent from the lipid phase. Another approach to prepare lipid microparticles is the spray drying of an O/W emulsion or an organic lipid solution [8,9].

Although all these preparation processes have been successfully used for the production of microparticles, they have disadvantages regarding protein stability and encapsulation efficiency, resulting from the large interfaces generated during the preparation process. Often stabilizers have to be implemented in the manufacturing process to ensure high encapsulation efficiencies and increase protein stability. Although incorporating the proteins in the formulation process in solid form proved to increase protein stability in the aforementioned preparation methods by reducing liquid interfaces [10], a technique eliminating interfaces from the production of lipid microparticles altogether will further enhance protein stability. Therefore, spray congealing, also termed spray chilling, represents a very attractive method for the preparation of lipid microparticles as the microparticles are formed by atomizing the molten lipid into droplets, which subsequently solidify upon cooling. The microparticles can be prepared without the need of an aqueous phase or organic solvents and droplet size can be controlled by the atomizing conditions without additional stabilizers. Spray congealing was successfully employed for preparing lipid microparticles loaded with drugs such as clarithomycin [11], theophyllin [12], diclofenac [13], verapamil [14] and indomethacin [15], but the preparation of protein lipid microparticles by spray congealing has been rarely reported.

So far little work has been done on the development of a spray congealing process suitable for the production of lipid microparticles on the lab-scale with high yields [16]. Therefore, the aim of this study was to develop and evaluate a new spray congealing process by modifying a spray-dryer for the production of protein lipid microparticles. The performance of the apparatus was investigated by a central composite face centered design (CCF) to evaluate the effect of atomization pressure and temperature on particle size and production yield thus enabling to choose the appropriate preparation conditions for the production of protein-loaded lipid microparticles. As the particle size of microparticles intended for parenteral application by injection should be smaller than 150 mm to avoid blockage of the injection needle [3], the optimal process parameter combinations can be selected from the experimental design to maximize the yield of such particles. In the second part of our investigations, insulin served as a model protein for incorporation into microparticles for determining the suitability of the preparation process for protein loaded lipid microparticles. The microparticles were characterized regarding encapsulation efficiency, particle size, morphology and protein stability. Additionally, the stability of the encapsulated insulin under release conditions was monitored over 28 days by analyzing the residual insulin content of the microparticles. To determine the influence of the spray congealing method on the bioactivity of insulin and to test the ability of insulin lipid microparticles to achieve a long-term release of bioactive insulin in a biological system, the effect of insulin lipid microparticles on cartilage formation was tested in an insulin-sensitive fibrin gel chondrocyte culture system over 5 weeks.

2. Materials and methods

2.1. Materials

Crystalline human insulin was a gift from Sanofi-Aventis (Frankfurt, Germany) and glycerol tripalmitate (Dynasan116®) was provided by Sasol AG (Witten, Germany). Chloroform p. a. grade was purchased from Merck (Darmstadt, Germany) and acetonitrile in HPLC-grade from Baker (Deventer, The Netherlands), trifluoroacetic acid was from Riedel-De-Haen (Sigma–Aldrich, Seelze, Germany). Double-distilled water was filtered through a cellulose nitrate filter (pore size 0.2 μm, from Sartorius, Göttingen, Germany) prior to use. Fibrinogen was purchased from Sigma (Sigma, Hannover, Germany) and aprotinin from Bayer (Trasylol®, Bayer, Leverkusen, Germany). The fetal bovine serum (FBS) was from Gibco (Gibco/Invitrogen, Karlsruhe, Germany) and cell culture medium was prepared and used in a composition as described in [17].

2.2. Description of the spray congealing apparatus

The microparticles were prepared by spraying the molten lipid with a temperature-controlled pressure nozzle with a swirl chamber (nozzle orifice 300 μm) (Fig. 1) into a customized spray congealing apparatus. The spray congealing apparatus was composed of the nozzle in the heating jacket and the conventional glassware of a spray-dryer (Mini-Spray Dryer; B290, Büchi Labortechnik, Flawil, Switzerland) consisting of a spray cylinder (15 cm diameter and 60 cm length), a high performance cyclone and an aspirator. To ensure solidification of the particles upon their passage through the spray cylinder, the spraying distance of the cylinder was increased to 150 cm by placing a tube on top of the glassware. In addition, a continuous flow

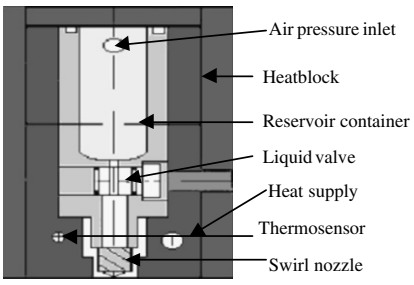

Fig. 1. Schematic drawing of the temperature controlled single substance nozzle constructed for small batches of 4 g lipid (scale 5.4 cm × 6.4 cm).

of cooled air (approximately −40 °C) was installed on the top of the apparatus.

2.3. Preparation of lipid microparticles by spray congealing

Four grams of the lipid was molten at 70 °C. Subsequently, the spray congealing apparatus was cooled by applying a flow of cooled air for approximately 10 min until the temperature at the connection of the spraying tower with the cyclone had reached at least −20 °C. The molten lipid was filled into the vessel of the nozzle (heated to 70 °C), the nozzle placed on top of the apparatus and the lipid was atomized within the desired temperature range controlled by the nozzle temperature (70–80 °C), applying an atomization pressure of 5–6 bar. The microparticles were collected in the production vessels, frozen in liquid nitrogen and were subsequently freeze-dried for 23.5 h at 6 °C and a pressure of 0.120 mbar, followed by a second drying step under minimal pressure (30 min) to remove adherent condensed water (Christ-Beta 2-16; Martin Christ Gefriertrocknungsanlagen, Osterode am Harz, Germany). Insulin loaded microparticles were prepared by dispersing weighed amounts of insulin in the molten lipid (total batch size 4 g) with an ultraturrax (TP 18/10 equipped with a S 25 N 10 G dispersing tool; IKA Laboratory Technology, Staufen, Germany) for 2 min at 10,000 rpm in the reservoir container of the nozzle. Subsequently, the insulin lipid suspensions were atomized at 70 °C with 6 bar spraying pressure. The resulting particles were freeze-dried as described above. Finally, the resulting particles were sieved through a sieve with a mesh size of 560 μm to separate other loose aggregates.

2.3.1. Spray congealing-process yield

The product yield was determined by dividing the quantity of microparticles obtained in the production vessels by the amount of lipid or insulin and lipid loaded into the spraying nozzle. The result is the unit percent by weight (% w/w).

2.4. Characterization of excipients and microparticles

2.4.1. Viscosity of glycerol tripalmitate at different temperatures

The kinematic viscosity of glycerol tripalmitate was determined in a temperature range of 70–90 °C with an Ubbelohde capillary (capillary no. I, capillary constant 0.009726; Schott-Geräte GmbH, Mainz, Germany). Each experiment was performed 5 times.

2.4.2. Particle size distribution and morphology

The particle size distributions were determined by laser diffraction (Mastersizer 2000 Hydro2000; Herrenberg, Germany). Two-hundred milligrams of microparticles was dispersed in 2 ml of an ethanol–water mixture (64% V/V, density (20 °C) 0.90 g/cm^3) on a vortex mixer (Vortex-Genie®; Scientific Industries, New York, USA) for 10 s. The sample was added to the dispersion unit, filled with the ethanol–water -mixture, under constant stirring at 1850 rpm. The particle sizes were calculated with the Fraunhofer approximation and are given as d (0.1), d (0.5) and d (0.9), representing the percentage of particles smaller than the indicated value.

The particle morphology was investigated by scanning electron microscopy (SEM). The particles were mounted on aluminum stubs using conductive carbon tape and coated with a layer of 1.4 nm gold-palladium (Polaron SC515; Fisons surface systems, Grinstead, UK). The micrographs were obtained at 4.0 kv on a DSM-950 (Zeiss; Oberkochen, Germany).

2.4.3. Thermal behavior/physical state of lipid microparticles

To determine the crystal modification of the lipid matrix, samples of 3.5 mg ± 0.05 mg of the bulk lipid or microparticles were sealed into AutoDSC aluminum sample pans (TA Instruments, Alzenau, Germany) and DSC thermograms were acquired on a 2920 differential scanning calorimeter (TA Instruments, Alzenau, Germany) with an empty pan as a reference. Scans were recorded between −20 °C and 90 °C with a rate of 5 K/min after equilibration at −20 °C for 5 min. The obtained data were evaluated with the DSC-software Universal Analysis for Windows 95/98/NT, version 2.5 H (TA instruments, Alzenau, Germany). The influence of release conditions on thermal behavior and morphology of protein lipid microparticles was investigated with insulin-lipid microparticles. The particles were incubated in 0.1 M phosphate buffer, pH 7.4, in a shaking water bath at 37 °C for 1 month. At various time points samples were drawn, the microparticles were washed with water as described in Section 2.4.5, subsequently freeze-dried and investigated by SEM and DSC.

2.4.4. Encapsulation efficiency of insulin-loaded lipid microparticles

Weighed amounts of microparticles (9.7 mg ± 0.3 mg, $n = 3$) were dissolved in 600 μl chloroform vortexed for 10 s; the samples were allowed to further dissolve for 10 min and vortexed for more 10 s. After 10 min the incorporated insulin was then extracted with 600 μl of 0.01 M HCl. After 10 s of mixing on a vortex mixer, phase separation was achieved by letting the mixture settle for 10 min. This procedure was repeated twice followed by centrifugation at 15,974g. Three-hundred microliters of the aqueous phase was diluted with 700 μl water and this sample was finally analyzed for insulin using a previously described HPLC gradient method applying an injection volume of 50 μl [18]. Briefly the sample was analyzed with a C_{18}-reversed phase precolumn (LC318, 4.6 mm × 20 mm; Supelco, Bellefonte, USA) combined with an analytical C_{18}-reversed phase column (Supelcosil, LC318, 4.6 mm × 250 mm) at 37 °C applying a linear gradient (mobile phase A: 90% H_2O, 10% acetonitrile, 0.1% TFA; mobile phase B: 90% acetonitrile, 10% H_2O and 0.1% TFA; flow rate of 1 ml/min) by changing the mobile phase

B from 20% to 36% over 24 min and a total run time of 34 min.

The HPLC-System consisted of a degasser (Knauer, Berlin, Germany) LC-10AT pump, FCV-10ATvp gradient mixer, SIL-10Advp autosampler, CTO-6a oven, SPD-10AV UV-Detector, RF-551 fluorescence detector and SCL-10Avp controller (all from Shimadzu, Duisburg, Germany). Insulin was detected by UV–vis analysis at 210 and 274 nm as well as fluorescence detection (274 nm excitation and 308 nm emission). HPLC data acquisition and analysis were performed with the Class VP 5.0.1 software (Shimadzu).

The concentration of total insulin (insulin and desamidoinsulin) was calculated for calibration curves of solutions of known insulin concentrations (8.8–110 μg/ml). The calculation was performed by analyzing the area under the curve (AUC) of the desamidoinsulin and insulin peaks for the calibration samples. The insulin content was determined by calculating the AUC of the insulin peaks and determining the percentage in relation to the total insulin concentration [19]. From these experiments, the influence of the preparation conditions on the desamidoinsulin content was determined.

2.4.5. In vitro stability during release and release behavior of insulin lipid microparticles

Ten micrograms of lipid microparticles ($n = 3$ for each time point) was incubated in 1 ml of release medium (0.1 M phosphate buffer, pH 7.4, containing 0.02% sodium azide) and kept in a shaking water bath at 37 °C for up to 28 days. At predetermined time points, microparticles were withdrawn, frozen in liquid nitrogen and kept at −80 °C until further analysis. For HPLC-analysis the samples were thawed and the particles were separated from the release medium by a filtration step using a 0.4 μm membrane filter unit (Isopore™; Millipore GmbH, Schwalbach, Germany). Subsequently, the particles were washed 3 times with 1.0 ml water to remove adsorbed protein and buffer salts. Following freeze-drying of the microparticles for 24 h at 6 °C (as described in Section 2.3), the residual insulin was extracted and the insulin content was analyzed as described in Section 2.4.4.

For HPLC-MS analysis the analytical method was carried out in positive ion mode using a Hewlett Packard HPLC system with Series 1100 degasser, binary pump, autosampler, column oven and diode array detector (all from Hewlett Packard, Waldbronn, Germany), coupled with a TSQ7000 electrospray-mass spectrometer (ThermoQuest, San José, CA, USA) with API2-source (capillary temperature: 350 °C, spray voltage: 4.0 kV). The Xcalibur software package (ThermoQuest, San José, CA, USA) was used for data acquisition and analysis. The sample was analyzed with a RP_{18}-column (Jupiter, 5 μm, 300 Å, 2 mm × 250 mm; Phenomenex, Torrance, USA) and a flow rate of 0.3 ml/min. Insulin and desamidoinsulin were detected in the total ion chromatogram of the mass spectrometer as triple-charged ions.

After 28 days of release, insulin extracted from 2% insulin microparticles was further analyzed by MALDI-ToF (Applied Biosystems 4700 Proteomics Analyzer; Foster City, CA 94404, USA). For the analysis, the sample was fractionated by HPLC, lyophilized and reconstituted in 50 μl aqua bidest. 1 μl of the sample was mixed with 50 μl of 33 mM solution of α-cyano-4-hydroxycinnamic acid in 50% ACN/H_2O (0.1% TFA). The MALDI-spectra were acquired in the linear mode.

2.4.6. Bioactivity assessment of insulin lipid microparticles

The ability of lipid microparticles to release bioactive insulin over a long time period was investigated in a recently developed insulin-sensitive fibrin gel chondrocyte culture [17]. 1.5×10^6 freshly isolated bovine femoral chondrocytes were suspended in 0.25 ml fibrinogen solution (100 mg/ml fibrinogen dissolved in 10,000 KIE/ml aprotinin solution); subsequently, gel discs were prepared by adding 0.25 ml of thrombin dissolved in 40 mM $CaCl_2$ buffer in a stabilizing glass ring. After 1 h of gelation at 37 °C, the glass ring was removed, the gels were covered with 4 ml culture medium and cultured for 5 weeks ($n = 3$ for each group, 12-well plates; incubator at 37 °C, 5% CO_2 and 95% humidity). Medium was exchanged 3 times per week. The negative control group was cultured with medium containing 5% FBS, the positive control with medium containing 5% FBS and 2.5 μg/ml insulin. In the third group, 0.375 mg (per gel) of lipid microparticles loaded with insulin (2% w/w) was suspended in thrombin solution before mixing with fibrinogen cell suspension and was cultured with medium containing 5% FBS. After 5 weeks of culture, collagen content of the constructs was determined spectrophotometrically at 550 nm with *p*-dimethylaminobenzaldehyde (Merck, Darmstadt, Germany) after hydrolysis (6 M HCl at 110 °C for 18 h) and reaction with chloramin-T (Merck, Darmstadt, Germany).

2.5. Experimental design and statistical analysis

A randomized central composite face-centered design (CCF) with 11 experiments [20,21] was performed to investigate the effect of the spraying temperature and atomization pressure on the particle size distribution, the product yield and to evaluate the percentage of particles smaller than 150 μm. The experimental matrix with the chosen values for pressure and temperature is given in Table 1. The applied nozzle temperature ranged from 70 to 80 °C and the atomization pressure was varied from 5 to 6 bar. For generating the experimental design as well as analysis thereof, the PC software MODDE 7.0 (Umetrics AB, Umeå, Sweden) was applied. For the evaluation of the experimental design, the model was fitted with multiple linear regression and the regression coefficients were calculated based on the full model with all effects. The validity and significance of the model and the regression coefficients was determined by ANOVA.

Table 1
Experimental design matrix and results

Factors		Responses (y_i)				
Pressure (bar)	Temperature (°C)	Yield (%)	d (0.1) (μm)	d (0.5) (μm)	d (0.9) [μm]	Particles <150 μm %
5	70	78.70	179.77	315.00	507.43	5.29
5	75	87.56	136.96	253.38	440.30	13.62
5	80	93.96	118.17	229.54	406.50	20.08
5.5	70	90.09	160.65	304.65	535.64	7.87
5.5	75	93.61	110.19	225.02	415.08	22.78
5.5	80	91.18	96.28	209.23	390.96	27.81
5.5	75	94.31	106.51	215.14	388.97	24.58
5.5	75	95.10	104.86	217.05	398.98	24.81
6	70	91.91	152.20	283.57	505.69	9.47
6	75	94.08	112.67	228.57	409.97	21.29
6	80	89.31	93.28	182.19	324.48	34.77
	Range	78.7	93.28	182.19	324.48	5.29
		95.10	179.77	315	535.64	34.77

3. Results and discussion

3.1. Investigation of the spray congealing process by experimental design

The resulting particle size of a liquid atomized by a pressure nozzle is mainly influenced by the nozzle orifice, the applied pressure, surface tension, density and viscosity of the liquid [22]. Therefore, a face-centered central composite design with 11 runs (see Table 1) was used to investigate the influence of the spraying temperature (as a method for controlling the viscosity of the liquid) and atomization pressure on the particle size distributions, the percentage of particles smaller 150 μm and the yield. The atomization pressure was varied from 5 to 6 bar and the effect of temperature was investigated from 70 to 80 °C. In this temperature range, the kinematic viscosity of glycerol tripalmitate decreased from 17.58 mm^2/s at 70 °C to 13.74 mm^2/s at 80 °C and allows thus for the investigation of the impact of viscosity on the resulting particle size.

By statistical evaluation of the obtained experimental data (Table 1), the significance and validity of the model was investigated by analysis of variance (ANOVA) (data not shown). The model was valid for all investigated responses and the data fitted the model well validated by the large regression coefficients (R2, variation explained by the model). The predictive power Q2 (predicted variation) of the model was good for all responses, thus showing no lack of fit on a significance level of 95%, meaning that the model error is of same magnitude as the pure error. Therefore, the fitted polynomial equations for the responses were generated by calculating the scaled and centered coefficients (Table 2) enabling to evaluate the contribution of the coefficients to the response and to visualize the influence of the investigated factors in the corresponding response surface and contour plots.

Increasing temperature and atomization pressure resulted in smaller particle sizes. Changing the applied pressure from 5 to 6 bar and the temperature from 70 to 80 °C reduced the median particle size d (0.5) from 315 to 182 μm (Fig. 2A). Investigation of the coefficients of the fitted polynomial equation (Table 2) revealed that both factors significantly affected the median particle size d (0.5). For the particle size distribution parameters d (0.1) (Fig. 2B) and d (0.9) (Fig. 2C), the same tendencies were detected in the surface and the corresponding contour

Table 2
Scaled and centered coefficients of the investigated responses (y_i) d (0.1), d (0.5), d (0.9), fraction of particles <150 μm and yield (y) for the polynomial equation (1)

Polynomial equation for the chosen model
$y_i = const + b_1 \times p + b_2 \times t + b_3 \times p^2 + b_4 \times t^2 + b_5 \times p \times t$ (1) p, pressure; t, temperature

Coefficient		Responses				
		d (0.1)	d (0.5)	d (0.9)	<150 μm	Yield
	Constant	109.34	224.60	411.83	22.76	94.05
b_1	pre[a]	**−12.79**	**−17.27**	−19.02	**4.42**	**2.52**
b_2	temp[b]	**−30.82**	**−47.04**	**−71.14**	**10.00**	**2.29**
b_3	pre * pre	**12.24**	8.08	−2.93	−3.36	**−2.80**
b_4	temp * temp	**15.89**	**24.04**	**35.24**	−2.97	**−2.99**
b_5	pre * temp	0.67	−3.98	−20.07	2.63	**−4.47**

Significant contribution of the coefficients to the response are printed in bold.
[a] Atomization pressure.
[b] Atomization temperature.

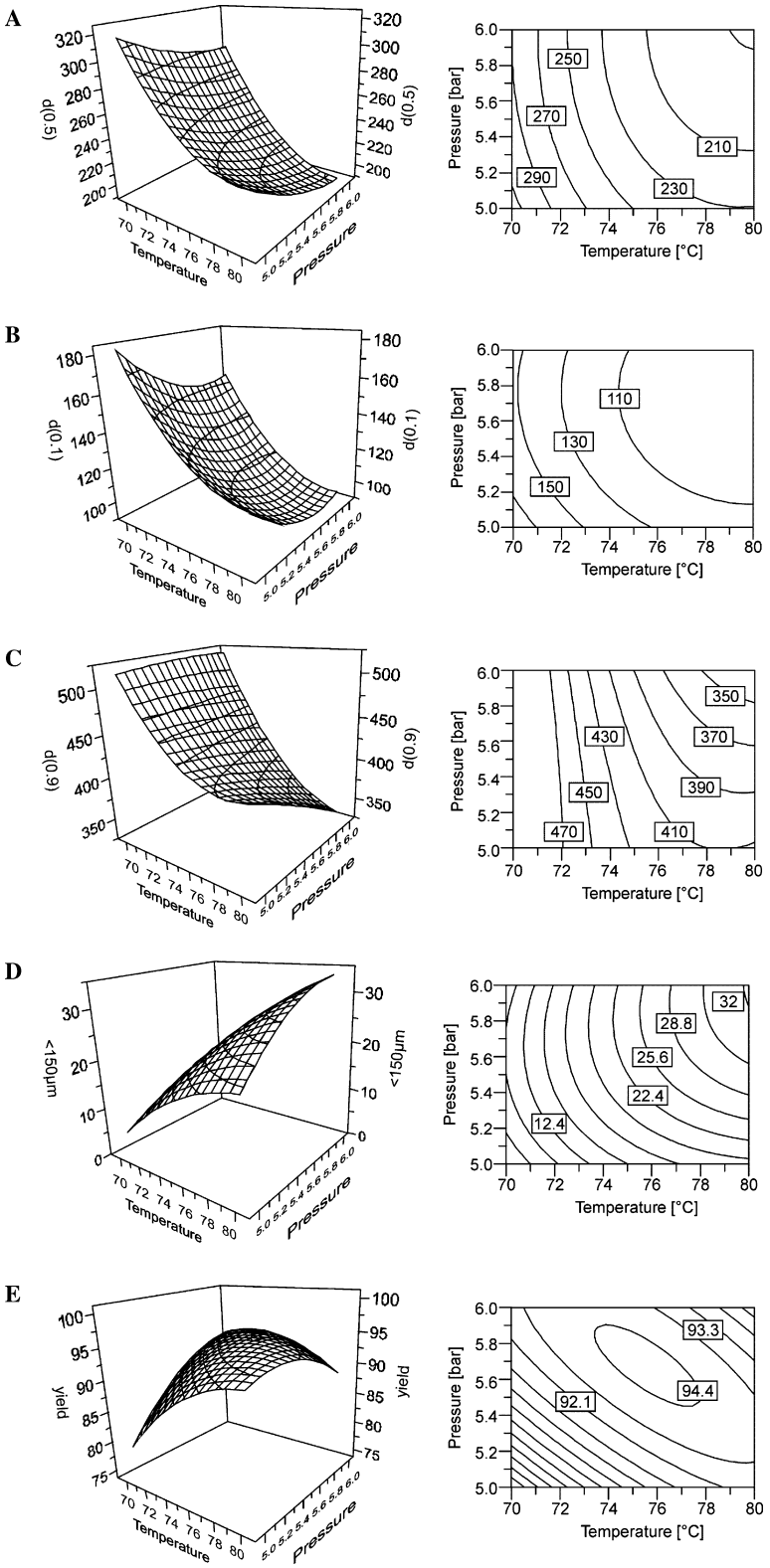

Fig. 2. Response surface plots (left) and the corresponding contour plots (right) for (A) d (0.5); (B) d (0.1); (C) d (0.9) in [μm], (D) for the resulting percentage of particles smaller 150 μm in [%] and (E) for the process yield [%].

plots, although the reduction of d (0.9) was significantly affected by the applied temperature only. The influence of higher temperature on the particle size reduction was larger than the effect of raising the atomization pressure. Therefore, the percentage of particles smaller than 150 μm increased from 5.3 to 35% by setting the levels of the inves-

tigated factors to their upper limit (Fig. 2D). No local minimum for the particle size or maximum for the percentage of particles <150 μm was detected within the investigated area. All of the lipid microparticle batches prepared for the investigation exhibited an even particle size distribution with a small amount of particles below 50 μm.

Another aspect of the experimental design was to investigate the influence of atomization pressure and temperature on the product yield. For all experiments, a high yield of particles between 78.7% and 95.10% was achieved. Increasing the temperature as well as the atomization pressure led to a significant yield increase. Evaluation of the resulting response surface diagram and contour plot revealed that within the investigated area a local optimum of the yield of particles was found close to the center point, displayed as an elliptic area in the contour plot and a peak in the resulting surface diagram (Fig. 2E). Therefore, highest yields can be obtained by choosing an atomization pressure between 5.5 and 5.9 bar in combination with a spraying temperature of 73–78 °C. Examination of the scaled and centered coefficients for the polynomial equation showed that the effect of temperature and pressure are of same magnitude (Table 2). Additionally, interaction of pressure and temperature could be observed, so that a synergistic effect of the factors led to an increase in yield within the experimental matrix. The resulting local optimum can be explained by a decreasing spraying angle as the viscosity of the liquid decreases, so that fewer droplets collided with the walls of the spraying tower and were lost due to solidification. Furthermore, spraying the lipid at increased pressure and with reduced viscosity led to the formation of smaller droplets (see Table 1) and their faster solidification minimized loss of lipid droplets on the walls of the spraying tower. However, the yield dropped at the high levels of the factors due to the greater adhesion tendency of small particles on the walls of the spraying tower and the smaller particles were not blown of the walls during particle collection.

Although the smallest particles were obtained at higher temperatures, for the preparation of protein loaded particles a temperature of 70 °C was chosen in order to minimize the impact of elevated temperatures on protein stability. The highest possible atomization pressure was used to achieve a high yield of particles with diameters smaller than 150 μm.

3.2. Characterization of blank and insulin lipid microparticles

3.2.1. Particle morphology and size distribution

The morphology of unloaded and insulin-loaded lipid microparticles was examined by SEM (Fig. 3). On the left side of Fig. 3, representative particles were scanned at a magnification of 500; on the right a close look at individual particles is shown. All particles were round shaped. The surface of pure lipid microparticles exhibited a rough structure (Fig. 3A). Incorporation of insulin in the lipid microparticles led to a smoother surface enhanced by increasing insulin content. No insulin crystals were observed in any of the particle batches, indicating that insulin had been efficiently entrapped in the microparticles.

Smaller particles loosely adhering to larger ones were also detected in the SEM micrographs (Fig. 3). These particles were found to represent a small fraction of particles below 30 μm as determined by laser light scattering. The particle size distributions of insulin-loaded microparticles revealed that higher insulin loading increased the particle size (d (0.5)) from 227 μm of 0.5% insulin lipid microparticles to 236 μm for 2% insulin lipid microparticles, respectively (data not shown).

3.2.2. Crystallinity of spray congealed microparticles

Glycerol tripalmitate is known to exhibit polymorphic behavior and may crystallize in three different forms: the unstable α-modification with a loosely packed hexagonal structure and a melting point of 44.7 °C, the metastable β′-modification with orthorhombic structure and a melting point of 56.6 °C and the stable β-modification with a triclinic subunit structure melting at 66.4 °C [23–25]. Upon fast cooling of the molten lipid, a large fraction of the glyceryl tripalmitate crystallizes in the unstable α-modification, as has been reported for lipid microparticles before [26]. In the DSC-thermograms of lipid microparticles prepared by the presented spray congealing method, the endothermic melting peak of the α-modification and β-modification were observed for all formulations including insulin-loaded lipid microparticles, whereas the melting peak of the metastable β′-modification at 52 °C was only visible in the thermograms of insulin-loaded microparticles (Fig. 4).

3.2.3. Encapsulation efficiency and insulin stability

No loss of insulin resulting from the manufacturing was detected regardless of the level of drug loading in the lipid microparticles (Table 3). Insulin was completely encapsulated with an efficiency of 107.7% ± 3.36%, 106.7% ± 1.32% and 103.5% ± 3.75% for 0.5%, 1% and 2% insulin lipid microparticles, respectively. An encapsulation efficiency greater than 100% resulted from loss of lipid within the nozzle. The impact of the chosen process conditions on insulin stability was investigated by HPLC-MS analysis (data not shown). In the total ion chromatogram of the samples, two peaks were observed and the triple charged ions of the related substances were used for calculating the mass of the corresponding uncharged ion. The substance eluting at a retention time of 19.30 min with a mass of 5808.6 Da was identical to insulin. The substance eluting at a retention time of 20.19 min matched the mass of desamidoinsulin with 5809.5 Da. No additional degradation products were detected, indicating that structural stability of insulin was maintained during the preparation process. However, the desamidoinsulin content slightly increased from 1.1% to at least 1.3%, most likely as a result of the high temperature applied in the spraying process.

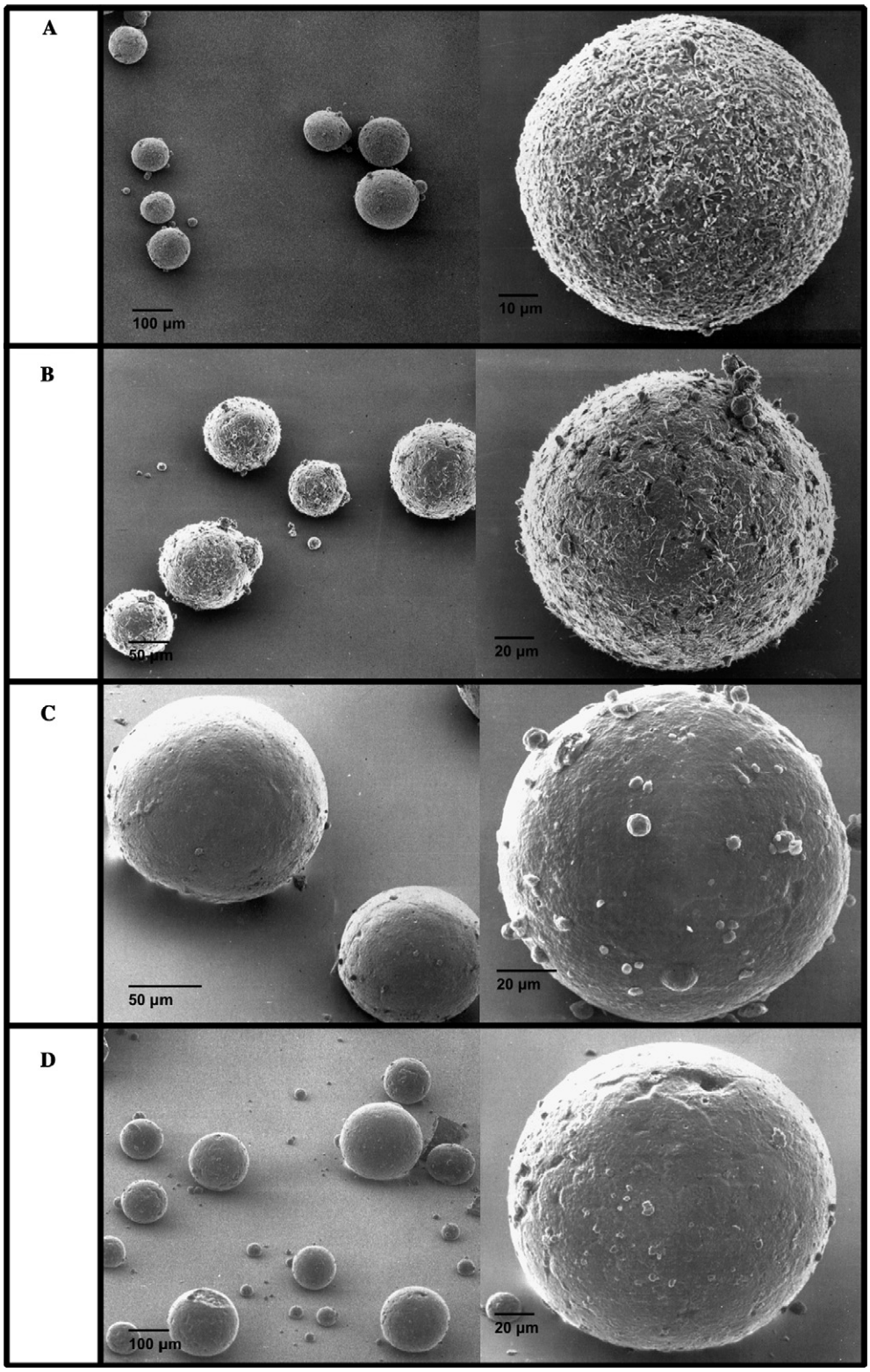

Fig. 3. Scanning electron microscopy pictures of (A) pure lipid microparticles and microparticles loaded with (B) 0.5%, (C) 1% (D) and 2% insulin.

3.3. Investigation of insulin lipid microparticles during release

3.3.1. Morphology and thermal behavior

The presence of the unstable α-modification in lipid microparticles prepared by spray congealing can have an impact on the release behavior as recrystallization in the stable β-modification occurs upon storage and application as a long-term delivery system at 37 °C [27,28]. The rearrangement of the lipids from the loosely packed α-modification to a triclinic substructure of the β-modification may not only lead to expulsion of the encapsulated drug from the matrix

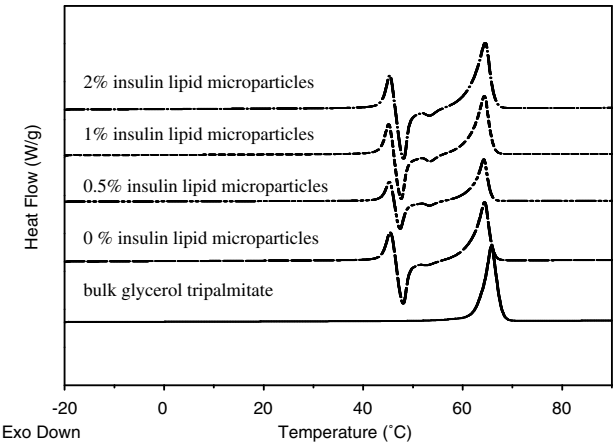

Fig. 4. DSC thermograms of glycerol tripalmitate as bulk and lipid microparticles recorded at a heating rate of 5 K/min.

Table 3
Encapsulation efficiency and desamidoinsulin content

Theoretical drug loading (%)	Experimental drug loading	Encapsulation efficiency	Desamidoinsulin content (bulk insulin 1.09%)
0.49	0.51% ± 0.02%	107.66% ± 3.36%	1.47%
0.93	0.98% ± 0.01%	106.76% ± 1.32%	1.59%
1.93	2.07% ± 0.07%	103.53% ± 3.75%	1.32%

[29], but release characteristics may change as the diffusion of the incorporated drug can be affected by alteration of the lipid matrix density [27,29]. Therefore, the alterations in morphology and crystallinity during the release of insulin from lipid microparticles were assessed by SEM and DSC. In the SEM-pictures of insulin-loaded lipid microparticles incubated for 3, 14 and 28 days, changes in the surface structure were detectable (Fig. 5). After 3 days of incubation, the previously snug surface of insulin microparticles (Fig. 3D) displayed a rougher structure with some areas still showing a sheet-like smooth area. During 28 days of release, the surface had completely changed to a more crystalline appearance. A similar trend was recently reported for lipid microparticles with gelucire as matrix material and found to be related to the degree of crystallinity [27]. Therefore, DSC-thermograms of lipid microparticles after different times of incubation were analyzed. The endothermic peak of the α-modification at 45 °C was barely detectable after 3 days, while the thermal melting enthalpy attributed to the stable β-modification increased over the investigated incubation time to a value close to that of the bulk lipid (Fig. 6). These results indicated that for the most part the lipid recrystallized within 3 days, and that the recrystallization will affect the release behavior only in the first release period. To avoid the influence of the lipid recrystallization on the release profile, lipid microparticles can be subjected to a thermal treatment slightly above the melting temperature of the α-modification (45 °C) after the production, if the protein stability is not affected by the chosen temperature.

3.3.2. Stability of insulin under release conditions

The stability of insulin in microparticles under release conditions was investigated over 28 days by determining

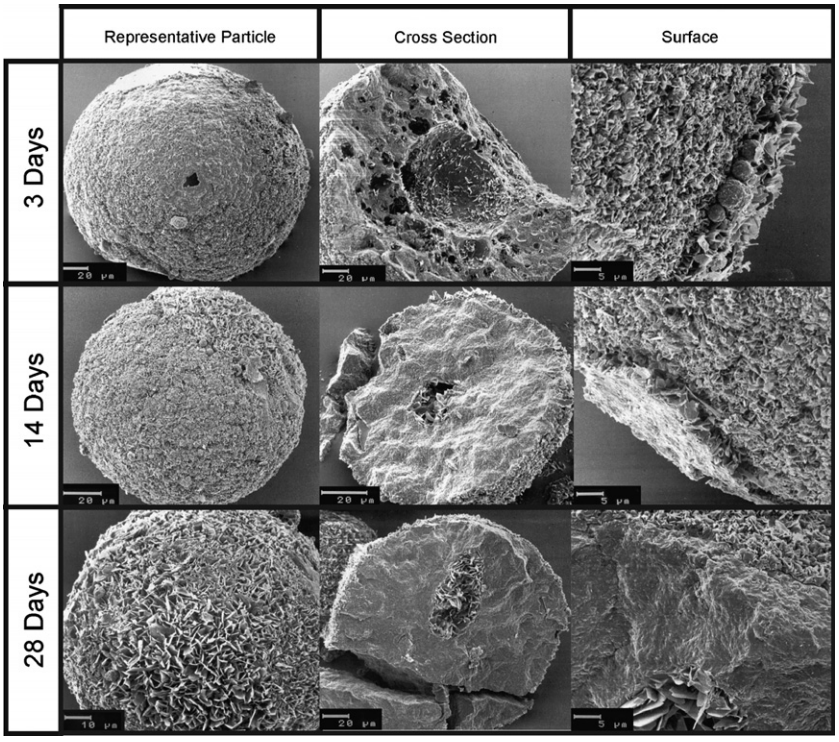

Fig. 5. Scanning electron microscopy pictures of insulin lipid microparticles incubated for 3, 14 and 28 days. In the left column representative particles are shown, in the middle cross-sections and on the right detailed surface structure of the particles.

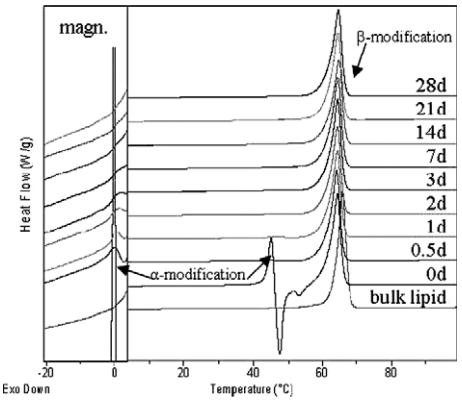

Fig. 6. Thermograms (recorded at a heating rate of 5 K/min) of insulin lipid microparticles incubated in release medium for 28 days. For better visualization a magnification of the melting peak related to the α-modification of the lipid is displayed on the left-hand side.

the residual content of insulin and its degradation products. When samples were analyzed by HPLC, analysis of the HPLC chromatograms showed that besides desamidoinsulin, additional degradation products formed. For their identification, insulin was extracted from microparticles (initial drug loading of 2%) after 28 days of release and was subjected to HPLC-MS analysis (Fig. 7). In the UV-chromatogram the degradation products were eluted at retention times (RT) of 22.6, 24 and 25.4 min (Fig. 7A). The substances eluting at 19.35 and 20.19 min could be ionized and were detected in the total ion chromatogram (Fig. 7B). Calculation of the triple charged ions of those peaks confirmed that they corresponded to insulin with a mass of 5808.6 Da (RT 19.35 min, Fig. 7C) and to desamidoinsulin with a mass of 5809.5 Da (RT 20.19 min, Fig. 7D). As the additional peaks could not be identified by HPLC-MS, the corresponding fractions were isolated by HPLC and analyzed via MALDI-Tof. The substances eluting at retention 22.6 and 24 min had masses of 5810.7–5811.03 Da, as detected for native desamidoinsulin in Maldi-Tof spectra, thus indicating mono-, di- and trideamidated isoforms of insulin, that had been described to elute after the A21-desamidoinsulin in RP-HPLC [30]. The peak at 25.4 min was identified as a covalent dimer with a mass of 11607.7 Da (data not shown). The mass difference of 15.6 Da to the mass of two insulin molecules results most likely from a new peptide bond formation via a cyclic intermediate, a precursor for deamidation as well as new peptide bond formation. This mechanism was found to occur in acidic medium leading to dimer formation with the linkage of insulin molecules at A21-B1, identified by HPLC-MS after enzymatic digestion of the dimer with endoproteinase Glu-c [31,32]. After the major degradation products were identified to be desamidoinsulin and the covalent dimer, their formation in lipid microparticles and the residual content of insulin were monitored over 28 days under release conditions. Within 28 days, the residual insulin content continuously dropped to 79.8% for 1% and 2% loaded insulin lipid microparticles

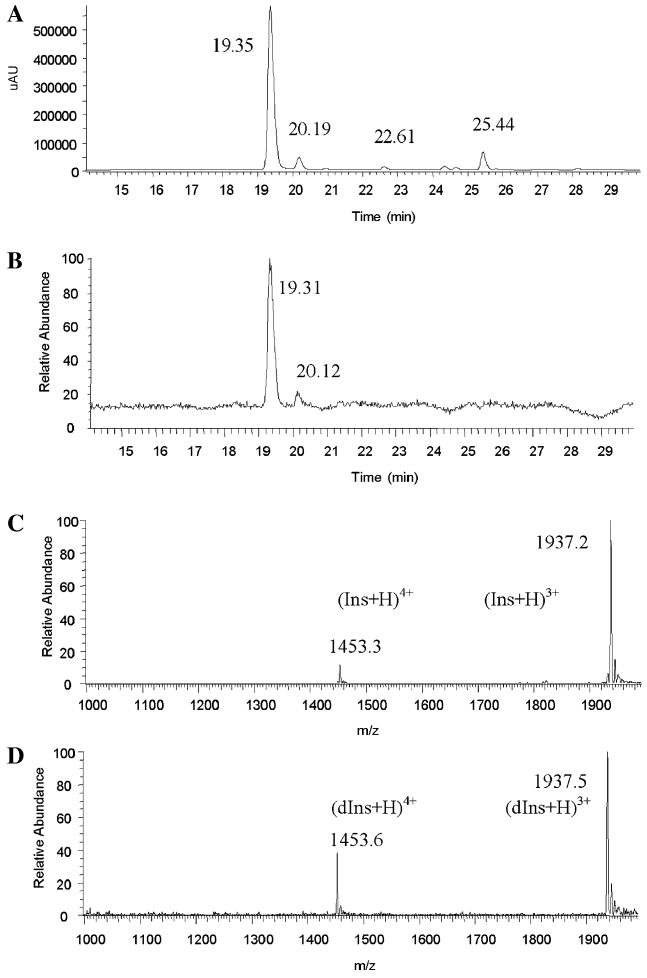

Fig. 7. Identification of the degradation products by HPLC-MS of insulin inside lipid microparticles after 28 days of release (A) UV210 nm HPLC chromatogram (B) total ion chromatogram (C) mass spectrum of the peak at a retention time of 19.31 (D) mass spectrum of the peak at a retention time of 20.19 min.

and to 74.5% for 0.5% microparticles (Fig. 8). Concomitantly, the desamidoinsulin content increased slowly to 7.49%, 5.5% and 5.4% in 0.5%, 1% and 2% insulin lipid microparticles, respectively. In all formulations a similar linear mechanism of desamidoinsulin formation was observed, with the absolute amount dependent on the initial insulin content as the slope of the regression lines had identical values (0.0015). The residual insulin content data demonstrate that insulin lipid microparticles exhibited a small burst release, followed by a long-term release of insulin. During 28 days of release, the dimer levels continuously increased in all formulations (Fig. 9).

Comparing the results with the stability of insulin in microparticulate controlled release systems based on biodegradable polymers such as PLGA and PLA under release conditions, a similar degradation profile with desamidoinsulin and dimer as the major degradation products can be observed as well [30,33]. Although the biological potency of bovine desamidoinsulin is still 85% and is 92% for porcine desamidoinsulin [34], the covalent insulin dimers

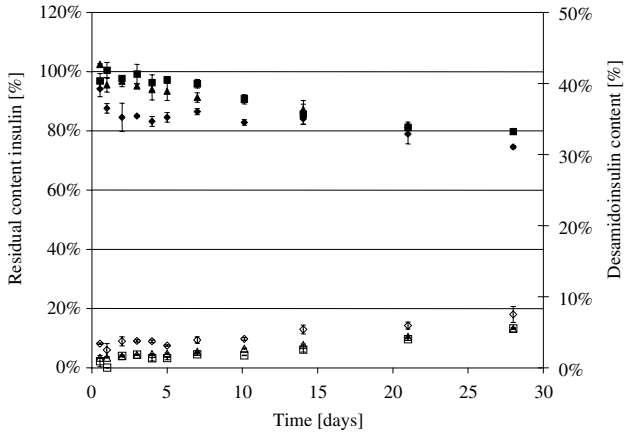

Fig. 8. Stability of insulin in lipid microparticles under release condition investigated over 28 days. Insulin content of 2% insulin lipid microparticles (▲) 1% (■) and 0.5% (◆). Residual content of desamidoinsulin is shown with the same but open symbols.

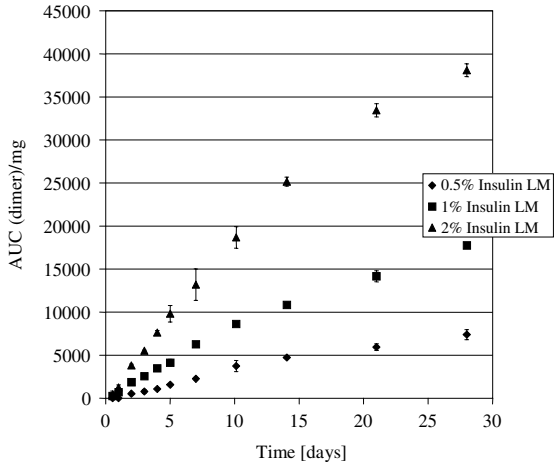

Fig. 9. Increase of dimer content within insulin lipid microparticles (Insulin LM) incubated in release medium over 28 days as determined by plotting the area under the curve in correlation to the absolute amount of microparticles.

have a reduced biological activity of approximately 15% [34] and their formation has to be limited to avoid an antigenic response [35,36]. Due to the long-term release profile achieved by the lipid matrix material and the enhanced degradation rate of crystalline insulin at higher humidity [32], the increase of degradation products is most probably related to dissolved protein not yet released from microparticles.

To fully profit from the potential of lipid microparticles to release proteins over a long period, additional formulation strategies should be considered to reduce protein degradation within microspheres, focussing on the accelerated diffusion of the dissolved protein. Reduced accumulation of degradation products in microparticles can be achieved by using lyophilized proteins, higher drug loading or the addition of polyols/proteins as porogens for creation of a continuous porous structure through the matrix material. Furthermore, the incorporation of hydrophilic additives, such as sugars and polyols, can prevent protein aggregation caused by high local protein concentration through preferential exclusion and reduce interaction of the dissolving protein with the hydrophobic matrix that may lead to unfolding of the protein [37].

3.3.3. Bioactivity of insulin released from lipid microparticles

A three-dimensional fibrin gel chondrocyte culture was used to investigate the influence of the preparation process on the bioactivity of insulin encapsulated in lipid microparticles and to determine the ability of insulin lipid microparticles to release bioactive insulin over an extended time period.

To this end, insulin lipid microparticles were suspended with chondrocytes in a fibrin gel and cultivated over 5 weeks; as control groups chondrocytes were cultivated in a fibrin gel without and with the supplementation of exogenous insulin. The effect of insulin on cartilage extracellular matrix formation such as collagen and glycosaminoglycan (GAG) was determined. The absolute collagen fraction in constructs cultivated with insulin or insulin lipid microparticles was significantly increased as well as the collagen content per cell compared to the control group cultivated without insulin ($p < 0.01$; Fig. 10). The same tendency could be observed for the glycosaminoglycan formation within the constructs (data not shown). These results demonstrate that the bioactivity of insulin was maintained dur-

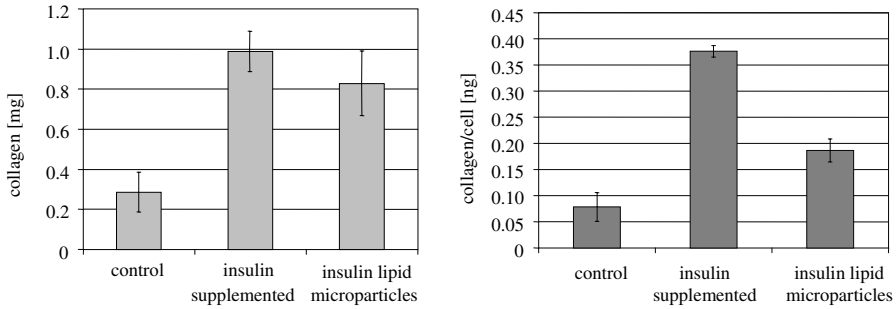

Fig. 10. Increase of collagen production in a three-dimensional fibrin gel chondrocyte culture after 5 weeks of cultivation without insulin, with insulin supplementation each 3 days and cultivation in the presence of insulin lipid microparticles. Insulin released from microparticles significantly increased the amount of collagen per cell ($p < 0.01$).

ing manufacturing and that bioactive insulin was released increasing the quality of the cartilaginous fibrin gel constructs.

4. Summary and conclusions

A spray congealing apparatus was developed that enables the production of lipid microparticles in a lab-scale batch size of 4 g with product yields up to 95%.

The influence of the processing parameters atomization pressure and spraying temperature on the particles size distribution, percentage of particles smaller 150 μm and product yield were investigated with the central composite design (CCF) and both factors significantly affected the responses. Increasing spraying temperature and pressure reduced the particle size, with temperature being the most efficient factor. The smallest particles (d (0.5) = 182.2 μm; 35% < 150 μm) were prepared by applying a spraying temperature of 80 °C and atomization pressure of 6 bar. As the particle size of microparticles intended for parenteral application by injection should be smaller than 150 μm to avoid blockage of injection needle [3], smaller particles could be obtained by increasing the spraying temperature, choosing a lipid with a lower melting point or a twin-fluid nozzle for atomizing the lipid [38].

The suitability of the spray congealing method for the production of protein lipid microparticles was investigated with insulin as a model substance. Insulin lipid microparticles of different drug loading (0.5, 1% and 2%) with high yields of 77–84%, encapsulation efficiency and a good control over particle size were prepared and the stability of insulin was not affected during the preparation process. The spray congealing method therefore represents a most promising approach for the formulation of protein-loaded microparticles, especially for proteins sensible to degradation caused by interfaces. The fact that no organic solvents are used within the preparation process will facilitate the acceptance of the formulation by regulation authorities.

The potential of lipid microparticles for long-term release of protein was investigated with insulin as a model substance. Insulin was released over a period of at least 28 days, indicating that a release period of more than 6 months is possible. Application of insulin lipid microparticles in a fibrin gel cell culture showed that bioactive insulin was released from lipid microparticles and improved cartilage formation after the investigated period of 5 weeks.

The presented spray congealing technique, therefore, provides an excellent method to produce protein-loaded lipid microparticles and to take full advantage of lipids as an alternative material for the controlled release of proteins.

Acknowledgements

This work was sponsored by the 'German Ministry of Economics and Labour (BMWA)' through the 'Arbeitsgemeinschaft industrieller Forschungsvereinigungen Otto von Guericke' e.V.: (AIF, Grant No. 12711) and the 'Forschungsvereinung der Arzneimittelhersteller' (FAH). Thanks are due to Sanofi-Aventis for providing the insulin.

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