Available online at www.sciencedirect.com
ScienceDirect
European Journal of Pharmaceutics and Biopharmaceutics 69 (2008) 476–485
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

Preparation and evaluation of microparticles from thiolated polymers via air jet milling

Herbert Hoyer, Wolfgang Schlocker, Kafedjiiski Krum, Andreas Bernkop-Schnürch *

Leopold-Franzens-University Innsbruck, Innsbruck, Austria

Received 3 April 2007; accepted in revised form 14 January 2008
Available online 19 January 2008

Abstract

Microparticles were formulated by incorporation of the model protein horseradish peroxidase in (thiolated) chitosan and (thiolated) poly(acrylic acid) via co-precipitation. Dried protein/polymer complexes were ground with an air jet mill and resulting particles were evaluated regarding size distribution, shape, zeta potential, drug load, protein activity, release pattern, swelling behaviour and cytotoxicity. The mean particle size distribution was 0.5–12 μm. Non-porous microparticles with a smooth surface were prepared. Microparticles from (thiolated) chitosan had a positive charge whereas microparticles from (thiolated) poly(acrylic acid) were negatively charged. The maximum protein load for microparticles based on chitosan, chitosan–glutathione (Ch–GSH), poly(acrylic acid) (PAA) and for poly(-acrylic acid)–glutathione (PAA–GSH) was 7 ± 1%, 11 ± 2%, 4 ± 0.2% and 7 ± 2%, respectively. The release profile of all microparticles followed a first order release kinetic. Chitosan (0.5 mg), Ch–GSH, PAA and PAA–GSH particles showed a 31.4-, 13.8-, 54.2- and a 42.2-fold increase in weight, respectively. No significant cytotoxicity could be found. Thiolated microparticles prepared by jet milling technique were shown to be stable and to have controlled drug release characteristics. After further optimizations the preparation method described here might be a useful tool for the production of protein loaded drug delivery systems.

Keywords: Air jet mill; Co-precipitation; Microparticles; Chitosan; Poly(acrylic acid)

1. Introduction

Carrier technology offers an intelligent approach for oral drug delivery by associating the drug to a carrier particle such as microspheres, nanospheres or liposomes, which modulates the release and absorption characteristics of the drug [1]. On the one hand different oral administration experiments in animals revealed that size and morphology of particles carrying the drug can largely influence the absorption of drugs after oral administration [2]. On the other hand Longer et al. showed that a delayed gastrointestinal transit induced by mucoadhesive polymers could also lead to an increased oral bioavailability of a drug [3]. Hence there will be several perceived advantages to combine both strategies and to develop a mucoadhesive drug delivery system: by using specific mucoadhesive molecules, it will be possible to target a particular site or tissue in the gastrointestinal tract and increased residence time in combination with controlled release of a drug may lead to lower administration frequency [4]. A promising strategy to improve mucoadhesion is the use of thiolated polymers which are divided in two different groups: cationic and anionic thiolated polymers which are mainly based on chitosan and poly(acrylic acid), respectively. Their improved mucoadhesive features are reasoned by covalent attachment of their thiol bearing side chains with cysteine-rich subdomains of glycoproteins in the mucus [5]. As this adhesion process goes hand in hand with an interpenetration process of the mucus and the adhesive polymer, not only thiol groups on the surface but also inside the particles are responsible for improved mucoadhesion. Another

* Corresponding author. Institute of Pharmacy, Leopold-Franzens-University Innsbruck, Innrain 52, Josef Möller Haus, A-6020 Innsbruck, Austria. Tel.: +43 512 507 5371; fax: +43 512 507 2933.
E-mail address: andreas.bernkop@uibk.ac.at (A. Bernkop-Schnürch).

0939-6411/$ - see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejpb.2008.01.009

likely mechanism being responsible for the improved mucoadhesive properties is based on their in situ cross-linking properties. During and after the interpenetration process, which could be verified for mucoadhesive polymers, such as poly(acrylic acid) recently [6], disulfide bonds are formed within the thiolated polymer itself leading to additional anchors via chaining up with the mucus gel layer. In addition disulfide bonds are also formed within the polymer itself leading to cohesive properties [7]. Altogether these properties result in a prolonged residence time at the site of application.

Accessorily it has already been demonstrated that thiolated polymers exhibit improved controlled release, permeation enhancing [8], protease as well as efflux pump inhibitory properties [9,10].

Within this study microparticles were prepared by a grinding technique which consists of three consecutive steps of precipitation, pregrinding and jet milling. Jet milling offers various advantages such as the achievement of micron-sized particles with narrow size distributions, the avoidance of contamination, the ability of grinding heat-sensitive materials and the scalability from small batches to large volume commercial production. The production of micro- and nanoparticles is on the one hand in most cases extensive in the realisation, material- and cost-intense and on the other hand problems often occur at the scale up process for the production of industrial quantities. It was therefore the objective of this study to develop a production technique for the preparation of a microparticulate drug delivery system being based on thiolated polymers in commercial quantities without loosing activity of the incorporated therapeutic agent during the manufacturing process.

Peroxidase from horseradish was used as model peptide. Drug-loaded solid particles were generated by co-precipitation of the model peptide with chitosan, Ch–GSH, PAA and PAA–GSH, representing the carrier matrix and grinding via an air jet mill. Different polymers of cationic chitosan and anionic poly(acrylic acid) were utilized in order to evaluate the differences in the characteristics of the resulting particles. Both polymers are usable for thiomer preparation and can be used as carrier matrix for particle preparation. Resulting microspheres were analysed regarding particle size, shape, stability, zeta potential, protein activity, drug load, drug release, swelling behaviour and cytotoxicity. Control microparticles of unmodified PAA and chitosan were prepared under the same conditions.

2. Materials and methods

2.1. Materials

Chitosan (medium molecular mass: 400 kDa; degree of deacetylation: 83–85%) was obtained from Fluka Chemie (Buchs, Switzerland). Poly(acrylic acid) (exclusively linear, MM: 450 kDa), L-glutathione reduced form (GSH), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), 5,5-dithiobis(2-nitrobenzoic acid), poly (acrylic acid) (medium molecular mass: 450 kDa); peroxidase from horseradish (55.000 U/g), o-phenylendiamine dihydrochloride and minimum essential medium (MEM) were all purchased from Sigma (St. Louis, MO, USA). *N*-hydroxysuccinimide (NHS) was obtained from Acros (Geel, Belgium). All chemicals were of analytical grade.

2.2. Synthesis of chitosan–glutathione

The covalent attachment of GSH to chitosan was achieved via the formation of amide bonds between carboxylic acid moieties of GSH and amine groups of chitosan.

First, 1 g of chitosan was hydrated in 8 ml of 1 M HCl and then dissolved by the addition of demineralized water in order to obtain a 1% (w/v) polymer solution. The pH was adjusted to 6.0 by the addition of 5 M NaOH. Afterwards, 5 g of reduced glutathione in 10 ml demineralized water was added under stirring. Then, EDAC dissolved in 5 ml demineralized water was added in a final concentration of 200 mM. Thereafter, 200 mM NHS dissolved in 5 ml demineralized water was added into the reaction mixture under vigorous stirring. The pH was readjusted to 6.0 with 5 M NaOH. The reaction mixture was incubated for 7 h at room temperature under permanent stirring. The resulting polymer conjugate was dialysed in tubings (molecular weight cut-off 12 kDa) first against 5 mM HCl, twice against 5 mM HCl containing 1% NaCl and finally two times against 1 mM HCl. Finally, the frozen aqueous polymer solutions (samples, controls and complex) were lyophilized at −50 °C and 0.01 mbar (Lyolab B; Inula, Austria) and stored at 4 °C until further use. The synthesis was accomplished as described by Kafedjiiski et al. [11].

2.3. Synthesis of poly(acrylic acid)–glutathione

Reduced glutathione was attached covalently to poly (acrylic acid) via the formation of amide bonds between the amino group of glutathione and a carboxylic acid group of the polymer. First, 1 g of PAA was hydrated in 80 mL demineralized water and the pH value of the obtained polymer solution was adjusted to 5.5 by the addition of 5 M NaOH. EDAC dissolved in 5 ml demineralized water was added in a final concentration of 50 mM, in order to activate the carboxylic acid moieties of poly(acrylic acid). After 15 min incubation time under stirring at room temperature, 2 g of reduced glutathione in 10 ml demineralized water was added and the pH was readjusted to 5.5. The reaction mixture was then allowed to proceed for 3 h at room temperature under stirring. The resulting PAA–GSH conjugate was isolated by dialysing first against 1 mM HCl, two times against 1 mM HCl but additionally containing 1% NaCl and then two times against 1 mM HCl. Controls were prepared and isolated in the same way as the polymer conjugate but omitting carbodiimide (EDAC) during the coupling reaction. After dialysis, the pH value of the polymers and

corresponding controls was readjusted to 4 and frozen aqueous polymer solutions were lyophilized at −50 °C and 0.01 mbar (Lyolab B; Inula, Austria). Samples were stored at 4 °C until further use [12].

2.4. Microparticle preparation

First, 2.00 g of each polymer was hydrated in 50 ml of demineralised water until a homogeneous gel was formed. Peroxidase (20 mg) was dissolved in 2 ml of demineralised water and was added to this gel. Afterwards 1 ml of 5% (v/v) H_2O_2 solution was added dropwise in order to initiate a cross-linking process via the formation of disulfide bonds. Finally the gel was precipitated by the addition of 250 ml of isopropanol, which was chosen because of its non-solvent character for the polymers and the protein, low toxicity and cost effectiveness. First, 250 ml of non-solvent was added under stirring. The solution was incubated for 14 h at 4 °C in order to achieve a quantitative co-precipitation. The liquid phase was substituted three-times by 250 ml of non-solvent followed by the proceeding as described above. The precipitate was dried in a desiccator under vacuumization at 4 °C with Silica blue gel up to the constant weight. The dried model protein/polymer complex was ground carefully in a mortar to a size of approximately 1.5 mm (the highest particle size for the jet mill) and stored at −18 °C until further use.

2.5. Preparation of microparticles via air jet milling

The experiments were accomplished on a Hosokawa Alpine Aeroplex spiral jet mill 50 AS (diameter and height of grinding chamber 50 and 4.5 mm; standard blowing out nozzle; number of nozzles 4; nozzle diameter 0.8 mm; nozzle pitch 50°; solid feed rate 0.1–1 kg/h) equipped with a temperature sensor (Lutron, DH-802C, Ming Chuan, Taiwan). The injector air pressure was 7.5 bar and the grinding air pressure (GAP) was 2.5 bar, respectively. Dependent on the injector pressure, each 1 g of the protein/polymer complex was ground for 10 min. The mill material was kept at −18 °C until further use.

2.6. Determination of the thiol/disulfide content

The amount of thiol groups immobilized on Ch–GSH and PAA–GSH microparticles before and after the addition of H_2O_2 solution and after jet milling was determined photometrically via Ellman's reagent (5,5′-dithio-bis(nitrobenzoic acid)) as described previously [8]. Disulfide content was measured after reduction with $NaBH_4$ and addition of 5,5′-dithio-bis(nitrobenzoic acid) as described by Habeeb [13].

2.7. Particle size distribution

The particle size distribution was determined using a laser diffraction particles size analyzer (''analysette 22'' compact version, Fritsch GmbH, Idar Oberstein). Low viscous silicone oil WACKER AK 10 (viscosity 10 mPas ± 10% (25 °C), Wacker/Hüls, Nünchritz, Germany), was used as dispersing vehicle. The particle suspensions were prepared with an ultrasonic stick (Dr. Hielscher GmbH, ultrasonic processor UP200H) prior to analysis for 1 min. In the measurement cell, the use of a propeller mixer (dispersion equipment, Fritsch GmbH, Idar Oberstein) facilitated the continuous flux of particles. For calculations of particle size distribution the Fraunhofer model was used.

2.8. Determination of particle morphology

The morphology of the samples was visualized with a scanning electron microscope (JSM 5310LV, Jeol, Japan) operating at 25 keV.

2.9. Electrokinetic potential

The zeta potential of particles was measured in double-distilled water. The zeta potential was analysed by measuring the electrophoretic mobility using a PSS NICOMPTM 380 DLS/ZLS. All measurements were carried out at room temperature.

2.10. Disintegration studies

The stability of microparticles was analysed visually in 10 ml of 0.2 mM PBS with a pH of 6.7 at 37 °C over 24 h.

2.11. Determination of model protein load

The amount of peroxidase incorporated in the dried protein/polymer complex was determined via TNBS (2,4,6-trinitrobenzenesulfonic acid) test.

After washing the particles three times with distilled water 5 mg of each protein/polymer complex were hydrated in 500 μl of 0.5% (m/v) sodium chloride solution. Thereafter, 100 μl of each sample were transferred in the first well of a microtitration plate (Greiner 96, Greiner Bio-one, Vienna) and diluted in 1:2 steps with 0.5% (m/v) sodium chloride in the following five wells. Then, 100 μl of TNBS solution (200 μl 5% TNBS (m/v) and 9.8 ml 8% (m/v) $NaHCO_3$ solution) were added to each well. The reaction was allowed to proceed in the dark at 37 °C for 90 min. Afterwards the absorption was measured at 450 nm with a microtitration plate reader (Fluostar/Polarstar Galaxy, BMG Labtechnologies, Vienna). Increasing amounts of peroxidase dissolved in 0.5% sodium chloride solution (m/v) served as standard curve.

2.12. Measurement of peroxidase activity

Peroxidase activity in microparticles was determined in the same way as described for the determination of model protein load. Merely instead of the TNBS solution 150 μl of a peroxidase substrate solution (36 mg of o-phenylendi-

amine dihydrochloride dissolved in 18 ml of 0.1 M phosphate buffers saline (PBS), pH 6.7 and 36 μl 30% hydrogen peroxide) was added. After 5 min incubation at room temperature the enzymatic reaction was stopped by the addition of 50 μl of 2 M HCl. The absorption was measured at 492 nm with the microtitration plate reader. Increasing amounts of peroxidase dissolved in 0.1 M PBS, pH 6.7, and analysed as described above served as standard curve.

2.13. In vitro release studies of test compound

Microparticles (5 mg) were placed in a beaker containing 10 ml of 0.2 mM PBS with a pH of 6.7 at 37 °C. Beakers were closed up and continuously shaken on an oscillating water bath. Every hour aliquots of 0.2 ml were withdrawn and replaced with an equal volume of phosphate buffer solution, pH 6.8, equilibrated at 37 °C. Sink conditions were maintained throughout the study. The amount of released peroxidase was analysed as described above and was calculated from an according standard curve obtained by solutions with increasing concentrations of peroxidase.

2.14. Swelling behaviour

The water-absorbing capacity was determined by a gravimetric method. First 0.5 mg of microparticles was incubated in 200 μl of 100 mM PBS, pH 6.0, preequilibrated to 37 °C. After 1, 2 and 3 h of incubation under continuous shaking on an oscillating water bath (GFL 1092; 100 rpm) at 37 °C the samples were centrifuged for 5 min at 24,000*g* and the supernatant was removed. The weight of remaining swollen microparticles was determined and the water uptake was calculated.

2.15. Cytotoxicity assay

Caco-2 cells were subcultured on 12-well plates at a density of 1×10^4 cells/ml in MEM medium for 24 h. Thereafter, 1 ml fresh medium along with 260 μl of particle suspension (25 μg/ml) was added to the cell culture. Untreated cells were used as negative control and cells cultured in 2% Triton X-100 used as positive control. After 1, 2, 3 and 4 h 100 μl medium from each well was collected (ca. 1×10^4 cells/ml). Damage to plasma membrane by microparticles was studied by measuring the release of lactic acid dehydrogenase (LDH) from injured cells. LDH leakage into medium and total LDH activity (LDH leakage to medium plus LDH in remaining cells) were measured with an in vitro cytotoxicity assay kit and corrected by the activity already present in the medium of untreated cells. The assay is based on the reduction of NAD to NADH by LDH. NADH is utilized to convert a tetrazolium dye in the assay kit to a coloured compound with an absorption maximum at 490 nm. The intensity of the colour is indicative for LDH activity in the assay medium, and the LDH activity was measured photometrically (UV-1202, Shimadzu, Japan) at 490 nm. LDH activity in the supernatant was determined as a percentage of the total LDH activity.

2.16. Statistical data analysis

Statistical data analysis was performed using the Student *t*-test or ANOVA with $p < 0.05$ as the minimal level of significance.

3. Results and discussion

3.1. Preparation of microparticles via air jet milling

Due to the fact that synthetic particles of appropriate size and surface characteristic can pass intact through intestinal membranes, such absorbable particles loaded with drug, could be used as carriers of labile drug molecules, such as proteins or other poorly absorbed materials [14]. The most popular techniques are the double emulsion technique methods, solvent evaporation or the organic phase separation [15]. Microparticulate drug delivery systems based on thiomers were prepared by the emulsification/solvent evaporation method and displayed reproducible and promising results [16,17]. Besides the time consuming preparation step up to two days, the oxidation of thiol groups of the polymer into disulfide bonds during the microparticle preparation synthesis imply critical disadvantages due to the fact that thiomers are dependent on free thiol groups being immobilized on the polymers. Therefore jet milling was chosen and results obtained within this study could be directly compared to microparticles synthesized by emulsification/solvent evaporation technique. Both methods lead to stable particles with a smooth and non-porous surface; however, particles from jet milling are considerably smaller and display a more narrow size distribution. Mean size for particles prepared by emulsification/solvent evaporation technique was ~15 μm with a broad size distribution [16,17] in contrast to particles prepared by jet milling with a mean size of ~5 μm with a narrow size distribution. This is the more important as it is well known that particles smaller than 10 μm are taken up by the M-cells and transported into the Peyer's patches. Larger particles than 5 μm stay in the Peyer's patches while smaller particles are transported through the efferent lymphatics [18]. In addition particles prepared by emulsification/solvent evaporation technique display an amount of thiol groups in the range of 70 μmol/g polymer [16,17] or lower in contrast to particles prepared by jet milling displaying 100 μmol/g polymer. One reason for the smaller amount of thiol groups on particles prepared by emulsification/solvent evaporation technique might be the oxidation of thiol groups into disulfide bonds which takes place to some extent during the microparticles preparation. It was demonstrated that free thiol moieties of the microparticles were inversely proportional to the pH value of the aqueous phase [19]. The decrease of thiol content with increase of the pH of the aqueous phase is attributed, on the one hand,

to the formation of the interpolymer disulfide bonds which lead to decreased flexibility of the polymeric chains and, on the other hand, to the reduced possibility of disulfide bridging of thiol groups of the polymer with the thiol groups of the cysteine-rich subdomains of mucus glycoproteins [16]. In contrast, results within this study evidence that free thiol groups on microparticles will not be oxidized during the milling process. The drug encapsulation yield of microspheres synthesized by emulsification/solvent evaporation technique was determined to be almost 100% whereas nearly 100% were liberated during an in vitro dissolution test. However, due to the evaluation method of the drug load, it was not clear if the model compound was incorporated in the particles or just adsorbed on the surface [16,17]. In contrast particles prepared by jet milling displayed a drug load of 11%, whereas 70% were liberated. Due to the washing process before the evaluation of the drug load it could be assumed that the model compound was really incorporated in particles and not just adsorbed on the surface. However, an improvement of the drug load should be envisaged.

The main features affecting the grinding ratio in jet mills can be classified into two types. On the one hand the geometrical parameters which concern the mill design such as diameter of the grinding chamber, shape, number and angle of grinding nozzles and on the other hand the operational conditions e.g., solid feed rate, grinding pressure, injector pressure and, of course, material to grind [20]. Therefore a preparation method for protein-loaded microparticles was used which has been evaluated in a preliminary study by Schlocker et al. [21].

Orientating studies showed that thiomers were suitable for the co-precipitation process and results of this study demonstrated that the used polymer has a great impact on the resulting drug load. Unfortunately microparticles produced in preliminary studies (data not shown) were not stable in buffer solutions and disintegrated within a couple of minutes. Through the addition of H_2O_2 to the peroxidase containing gel prior to the precipitation step microparticles significantly improved stability. Results from preliminary studies showed that the more H_2O_2 was added, the more disulfide bonds were formed within the particles [10]. On the one hand disulfide bonds are responsible for the stability of microparticles and on the other hand free thiol groups are essential for improved mucoadhesive and permeation enhancing properties [19,22]. Thiolated particles should therefore exhibit both disulfide bonds and free thiol groups. After the precipitation step the dried protein/polymer complexes were ground with an air jet mill.

3.2. Determination of the thiol/disulfide content

Thiol groups can be oxidized to disulfides by either intra- or intermolecular reaction. Thiol groups being located closely to each other can form disulfide bonds more rapidly than remaining isolated thiol groups. The total content of thiol groups is necessary because the formation of intra- and intermolecular disulfide bonds is strongly dependent on the amount of free thiol groups available. Results from prior studies confirmed the theory of intra-molecular disulfide bond formation, as the increase of viscosity correlated with the amount of sulfhydryl moieties on the polymer. The increase in viscosity is dependent on the amount of covalently attached ligands to the polymer [23]. During the preparation process thiol groups were already oxidized forming inter- and intrachain disulfide bond by oxidation due to the addition of H_2O_2. The amount of free thiol groups for Ch–GSH and PAA–GSH before the oxidation with H_2O_2 was 178 ± 22 μmol/g and 125 ± 18 μmol/g and the amount of disulfide bonds was 85 ± 21 μmol/g and 93 ± 18 μmol/g. After oxidation the amount of free thiol groups for Ch–GSH decreased to 107 ± 12 for Ch–GSH and 78 ± 14 μmol/g for PAA–GSH and the amount of disulfide bonds increased up to 85 ± 21 μmol/g for Ch–GSH and 93 ± 18 μmol/g for PAA–GSH. In contrast results showed that no significant decrease in the thiol group and disulfide content could be observed after jet milling. The amount of free thiol groups for Ch–GSH was 104 ± 16 for Ch–GSH and 71 ± 18 μmol/g for PAA–GSH and the amount of disulfide bonds was 92 ± 21 μmol/g for Ch–GSH and 88 ± 18 μmol/g for PAA–GSH.

3.3. Particle size distribution

Small particle sizes with a narrow particle size distribution can be achieved by the use of air jet mills [24]. The grinding air pressure (GAP) is thereby the dominating parameter for the resulting particle size [25]. Results obtained within this study are in good accordance with this theory. Analyses of microparticles revealed that a higher GAP leads to a more pronounced fineness and a narrower particle size distribution. Even the products ground with a GAP of 2.5 bar led to particles of a mean size <10 μm as listed in Table 1. Differences between mean size distribution of thiomers and unmodified polymers can be observed. Thiomer particles display a broader distribution and a higher mean size than particles consisting of unmodified polymers. Due to the formation of disulfide bonds within thiomers higher cohesiveness and stability in comparison to the corresponding unmodified polymers is provided in the milling process. In Figs. 1 and 2 the size distribution of different microparticles obtained via jet milling is shown.

Table 1
Influence of material on particle size distribution of microparticles

Microparticle	Mean particle size $D_{50} \pm$ SD [μm], $n = 3$	$\bar{s}_r$
Chitosan	5.2 ± 0.2	2.3
Ch–GSH	4.6 ± 0.4	2.0
PAA	5.5 ± 0.9	2.3
PAA–GSH	4.5 ± 0.2	2.1

$\bar{s}_r$, mean standard deviation of the particle size distributions.

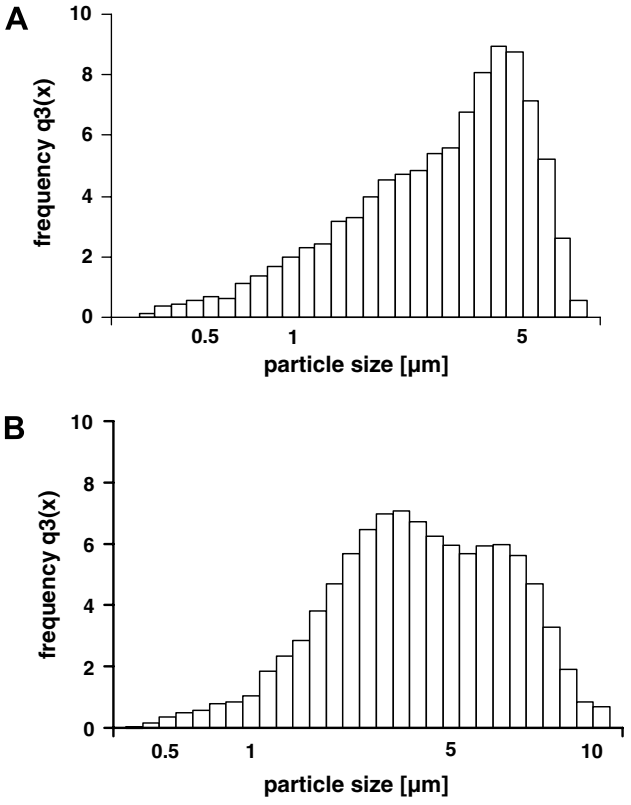

Fig. 1. Size distribution of chitosan (A) and Ch–GSH microparticles (B).

No significant differences were obtained between different batches. The sizes listed in Table 1 are also confirmed by scanning electron microscopy. As shown in Fig. 3 microparticles display a non-porous and smooth surface.

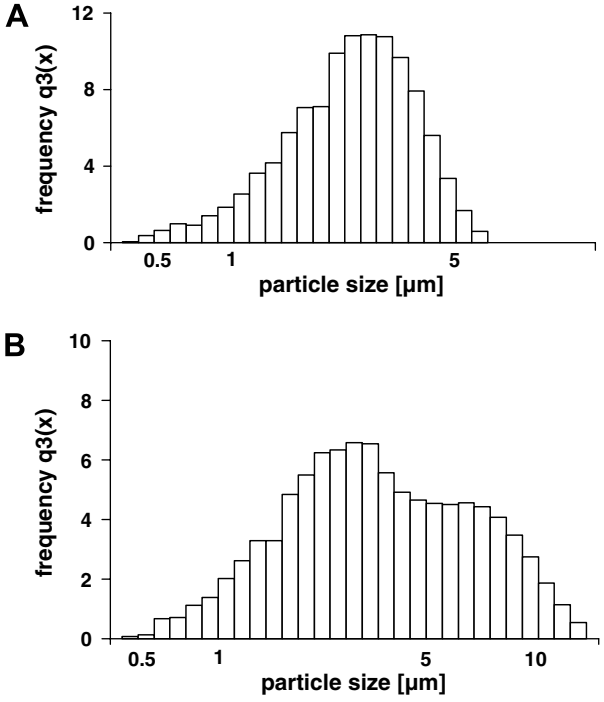

Fig. 2. Size distribution of carbopol (A) and PAA–GSH microparticles (B).

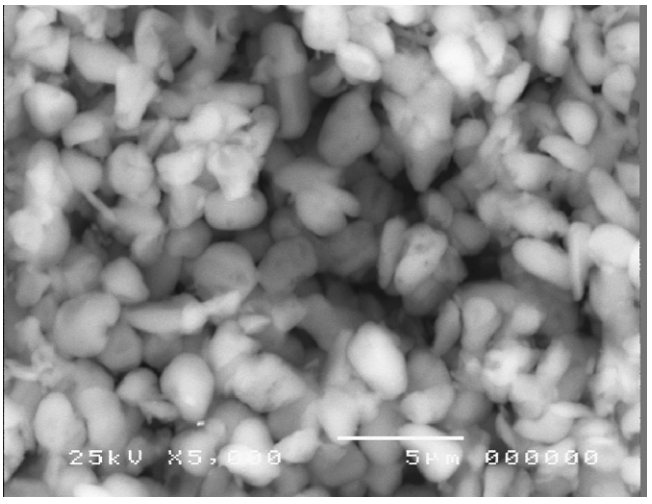

Fig. 3. SEM of an average protein/polymer complex ground in a jet mill at GAP 2.5 bar.

3.4. Electrokinetic potential

It was demonstrated that positive charge on surface of chitosan could give rise to a strong electrostatic interaction with mucus or with a negatively charged mucosal surface. In addition the uptake of particles may be initiated by electrostatic interactions between the particle and the membrane [26]. More precisely particles with a higher zeta potential would exhibit a stronger affinity for the negatively charged cell membrane, accounting for its higher cellular uptake. Therefore by measuring the zeta potential of microparticles, an insight into behaviour of the particle can be obtained. The surface charge of the microparticles was determined in double distilled water. Chitosan possess a primary amino and two free hydroxyl groups for each C_6 building unit. Due to the easy availability of free amino groups in chitosan, chitosan and Ch–GSH microparticles display a positive charge [27]. The zeta potential of the chitosan and Ch–GSH microparticles was +8.54 (mean ± SD; $n = 3$) and +18.91 mV (mean ± SD; $n = 3$), respectively. Due to carboxylic groups PAA and PAA–GSH microparticles were negatively charged and amount −28.84 (mean ± SD; $n = 3$) and −31.90 mV (mean ± SD; $n = 3$), respectively. The cross-linking process, changes in the morphology of the surface and the higher hydrophilicity have an influence on the zeta potential and may explain the different results of thiolated polymer particles in comparison to unmodified polymer particles. It was found that cross-linking with GSH resulted in an increase in the zeta potential of the chitosan microspheres from +8.54 to nearly +18.91 mV. In contrast, the zeta potential of the PAA and PAA–GSH microspheres seemed to show only a small difference. Taking into consideration that particles with a zeta potential of ±20 mV are more stable than particles with a zeta potential above or below, results obtained within this study are in good accordance

with this theory because chitosan particles are less stable than the PAA and the thiolated particles.

3.5. Disintegration studies

The disintegration studies on chitosan, Ch–GSH, PAA and PAA–GSH microparticles displayed significant differences in terms of stability. A more pronounced displacement of chitosan microparticles in comparison to Ch–GSH, PAA and PAA–GSH microparticles could be observed. Thiolated microparticles and PAA did not disintegrate in physiological buffer within a time period of 24 h, whereas unmodified chitosan particles disintegrated within 90 min. By reason that thiolated matrices formed inter- and intramolecular disulfide bonds during the microparticle preparation process an increased stability of the resulting microparticles was obtained. The stability of PAA microparticles might be based on the ionic interactions between the cationic peroxidase (IEP > 7) and the anionic PAA matrix.

3.6. Determination of model protein load

As the drug load is an important parameter for microparticle preparation providing valuable information about the efficacy of the preparation method used, it was determined within this study. Schlocker et al. demonstrated that the polymer used as well as the type of added non-solvent has a great impact on the resulting drug load [21]. Apart from the influence of the polymer also the way of co-precipitation had a significant influence on protein load. Results within this study confirmed this theory by reason that microparticles comprising thiolated polymers showed a higher drug load and activity than control microparticles. For the determination of drug load a subsequent washing step is required to remove peroxidase which is not incorporated into microparticles. Results obtained before the washing step display a theoretical drug load of nearly 100% for all microparticles (data not shown). After the washing step results showed that by using chitosan a maximum protein load of $7 \pm 1\%$ (mean $\pm$ SD; $n = 3$) could be achieved, whereas in case of Ch–GSH a maximum protein load of $11 \pm 2\%$ (mean $\pm$ SD; $n = 3$) was feasible. Microparticles comprising PAA had a maximum protein load of $4 \pm 0.2\%$ (mean $\pm$ SD; $n = 3$) and microparticles based on PAA–GSH had a maximum protein load of $7 \pm 2\%$ (mean $\pm$ SD; $n = 3$). Results indicate that most of peroxidase was adsorbed to the surface instead of incorporated into particles. However, due to the washing step effective drug load without adsorbed particles could be determined. Results are shown in Fig. 4a.

3.7. Measurement of peroxidase activity

Generally peroxidase is a relatively unstable protein and its stability is dependent on temperature [28], pH, grinding air pressure and non-solvent [8]. Utilizing isopropanol for co-precipitation and a GAP of 2.5 bar the remaining activity of the determined incorporated amounts of peroxidase was determined to be $30.0 \pm 9.2\%$ (mean $\pm$ SD; $n = 3$) for chitosan, $52.8 \pm 3.4\%$ (mean $\pm$ SD; $n = 3$) for Ch–GSH, $52.1 \pm 4.8\%$ (mean $\pm$ SD; $n = 3$) for PAA and $60.0 \pm 2.3\%$ (mean $\pm$ SD; $n = 3$) for PAA–GSH. At higher GAP the measured loss in activity was even higher [29]. Results are shown in Fig. 4b. In particular at a low grinding air pressure of 2.5 bar no activity at all was lost during the milling process, when peroxidase was incorporated in polymer via co-precipitation.

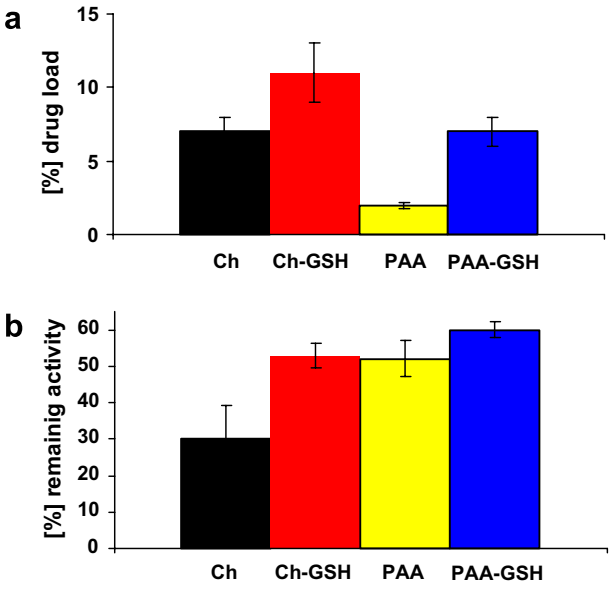

Fig. 4. Comparison of drug load (a) and peroxidase activity (b) in peroxidase/polymer complex after grinding via air jet mill at a GAP of 2.5 bar. Indicated values are means $\pm$ SD of at least three experiments.

Generally, peroxidase activity decreased during the grinding process due to its relatively low stability. Schlocker et al. could show that peroxidase degrades in solution over time. So the non-solvent which was used had a great impact on the protein activity. Isopropanol as best precipitant leads to a remaining activity of approximately 90% [20]. Apart from solvent-dependent instability, peroxidase is also sensitive to higher temperatures and pH. Lige et al. showed that Peroxidase lost about 30% of its activity at 55 °C and even the glycosylation level of a protein can have an effect on its stability [30]. So the summation of natural degradation and the influence of pH value, temperature and milling conditions lead to a higher loss of activity during the production of the microparticles.

3.9. In vitro release studies of test compound

Previous studies demonstrated that a controlled drug release out of microparticles having been produced in different ways can be easily achieved by making use of ionic inter-

actions between the polymeric excipient and the embedded protein drug. Leitner et al., for instance, showed a controlled release of human growth hormone out of poly(acrylate) microparticles within 6 h [31]. Because all tested particles were hydrated and swelled, the incorporated model compound was released rapidly from the delivery system. Diffusion of the model compound occurs gradually slower after an initial burst release by virtue of the forming swelling gel barrier. The release profile of the highly drug-loaded thiolated microparticles was similar to that of the non-thiolated microparticles showing a lower drug load. However, the total amount of released peroxidase was found to be higher from thiolated microparticles in comparison to non-thiolated microparticles. The release profile indicates a fast release of peroxidase within 1 h following by a controlled release over the remaining time period. Due to this controlled and sustained release profile, objectionable interactions between the thiolated polymer matrix and peroxidase can be excluded. 98.86 ± 2.49% (mean ± SD; $n = 3$) of peroxidase was released from microparticles consisting of chitosan. Contrary, drug release from microparticles consisting of Ch–GSH was 95.18 ± 6.92% (mean ± SD; $n = 3$). 99.35 ± 11.72% (mean ± SD; $n = 3$) of peroxidase was released from microparticles consisting of PAA and 93.32 ± 4.18% (mean ± SD; $n = 3$) from microparticles consisting of PAA–GSH (mean ± SD; $n = 3$) within 8 h (Fig. 5).

3.10. Swelling behaviour

Impetus to the swelling is most of all the tendency to compensate the density of charge between gel and solvent phase. In this case the surface of the gel phase works as semipermeable membrane. Water and ions get through it into the gel phase, but the charged cross-linking chains cannot diffuse to the outside because of the crosslinking [32]. Good absorbers are polymer chains which are slightly crosslinked and which have a high number of charged groups which can be dissociated and are firmly fixed on the polymer. Consequently they can absorb the multiple of their own volume of liquid and can fix it firmly to them. The degree of swelling depends on the number of fixed charges on the polymer, the density of crosslinking and the salt concentration in the solvent. It increases with the number of charges on the polymer and decreases if the density of crosslinking increases [32]. Results within this study could confirm this theory due to the fact that in general non-thiolated microparticles swelled more rapidly than thiolated ones. It was shown within the current study that non-thiolated microparticles are more erodible and more swellable as the swelling microparticles is strongly dependent on the cross-linking ratio with the heavier cross-linked particles exhibiting a significantly slower swelling ratio. The swelling ratio of thiomers was dependent on the pH of the surrounding medium. Whereas at pH 4 thiol groups of thiomers remained stable towards oxidation, a significant decrease in the thiol group content could be observed at pH 5 and 6.5. This observation can be explained by the decreasing $H^{\pm}$ concentration on raising pH-values which leads in turn to a higher amount of negative thiolated anions, S^{-}, representing the active form for oxidation which lead to crosslinking [33]. The weight of microparticles with chitosan increased 31.4-fold (mean ± SD; $n = 3$), respectively, a 12.4 mg uptake of water within 120 min and of microparticles with Ch–GSH 13.8-fold (mean ± SD; $n = 3$), respectively, 6.9 mg. The weight of microparticles with PAA increased 54.2-fold (mean ± SD; $n = 3$), respectively, 27.1 mg and of microparticles with PAA–GSH 42.2-fold (mean ± SD; $n = 3$), respectively, 21.1 mg uptake of water. At the end of the experiment, no erosion or dissolution of the microparticles consisting of thiomers and PAA was observed. Contrary, microparticles based on unmodified chitosan eroded (Fig. 6).

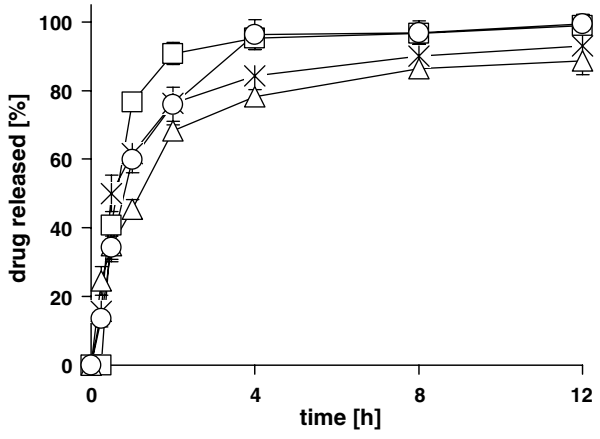

Fig. 5. Release profile of peroxidase from microparticles comprising PAA (○) in comparison to microparticles comprising PAA–GSH (Δ) and release profile of peroxidase from microparticles comprising chitosan (□) in comparison with microparticles made out of Ch–GSH (×). Studies were performed with phosphate buffer solution with a pH of 6.8. Indicated values are means (±SD) of at least three experiments.

3.11. Cytotoxicity assay

Chitosan and poly(acrylic acid) are often used as pharmaceutical excipients because of their very safe toxicity

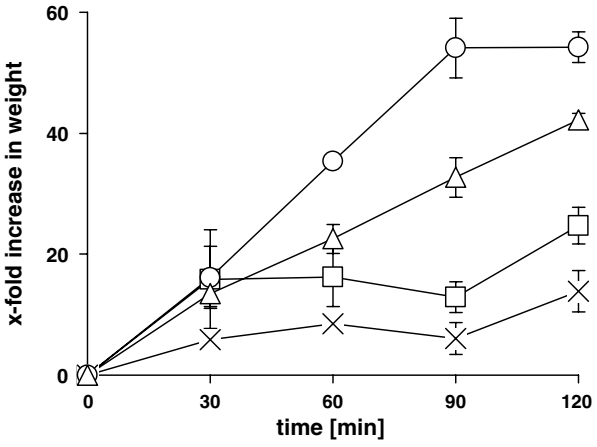

Fig. 6. Swelling behaviour of Ch–GSH (×), PAA–GSH (Δ), chitosan (□) and PAA (○) in 0.1 M phosphate buffer solution pH 6.8 at 37 °C; indicated values are means (±SD) of at least three experiments.

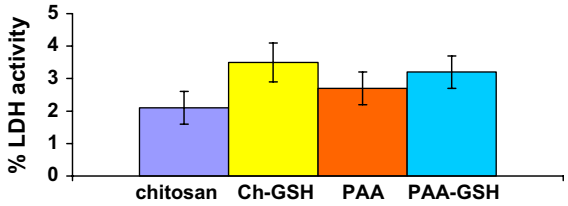

Fig. 7. Lactic acid dehydrogenase activity in the assay medium as a percentage of total cell LDH activity. Caco-2 cell cultures were incubated with different microparticles preparation chitosan, Ch–GSH, PAA and PAA–GSH for 4 h. Each point represents the mean ± SD of three determinations.

profiles [34,35]. Regarding thiomer safety first orientating studies demonstrated a clear tendency that their toxic profile does not change significantly in comparison to the unmodified polymers [36]. IC_{50} values could be evaluated to be 1.2 mg/ml for particles consisting of chitosan, 1.1 mg/ml for particles consisting of Ch–GSH, 1.1 mg/ml for particles consisting of PAA and 0.9 mg/ml for particles consisting of PAA–GSH particles. However, the greater surface area per mass compared with larger-size particles of the same renders micro-, and submicron particles more active biologically [37]. This activity includes a potential for inflammatory and pro-oxidant, but also antioxidant, activity in the case of micro-, and submicron particles. Although further toxicological studies of these formulations would be of great value for this branch of research. The Caco-2 cell line, which exhibits a well differentiated brush-border on the apical surface and tight junctions, and expresses typical small-intestinal microvillus hydrolases and nutrient transporters, has proven to be the most popular in vitro model to assess toxicological effects of drug or formulation component on this biological barrier [38]. Nevertheless additional tests with other cell lines are needed as Caco-2 cells are well known for their robustness. LDH tests with Caco-2 cells revealed that the extent of LDH release from the cells is less than 4%. The cells remained intact and only minimal LDH release was observed after incubation with microparticles for 8 h. Particles comprising chitosan, Ch–GSH, PAA and PAA–GSH were shown to be to some extent harmless for the cells. The viability of the cells was about 90–100% in comparison to control. Untreated cells were used as control. Results are shown in Fig. 7. Within this study no significant cell toxicity could be observed.

4. Conclusion

Within this study, the potential of jet milling for large scale protein-loaded microparticle production has been evaluated. Thiolated microparticles prepared by jet milling technique were shown to be stable and to have controlled drug release characteristics following a first order release kinetic. The loss of remaining activity of peroxidase was caused by accumulation through several preparation steps and the instability of peroxidase in principle. So further work needs to be carried out for the optimization of this preparation technique. Nevertheless these studies suggest that the microparticle preparation method described here might be a useful tool for the production of protein loaded particulate drug delivery systems.

Acknowledgements

The Austrian Nano-Initiative co-financed this work as part of the Nano-Health project (No. 0200), the sub-project NANO-N-0204 being financed by the Austrian FWF (Fonds zur Förderung der Wissenschaftlichen Forschung) (Project No. N-0204-NAN).

References

[1] J.-K. Vasir, K. Tambwekar, G. Garg, Bioadhesive microspheres as a controlled drug delivery system, Int. J. Pharm. 255 (2003) 13–32.
[2] G. Ponchel, J.-M. Irache, Specific and non-specific bioadhesive particulate systems for oral delivery to the gastrointestinal tract, Adv. Drug Del. Rev. 34 (1998) 191–219.
[3] M.-A. Longer, H.-S. Ch'ng, J.-R. Robinson, Bioadhesive polymers as platforms for oral controlled drug delivery III: Oral delivery of chlorothiazide using a bioadhesive polymer, J. Pharm. Sci. 74 (1985) 406–411.
[4] J. Woodley, Bioadhesion: new Possibilities for drug administration? Clin. Pharmacokin. 40 (2) (2001) 77–84.
[5] A. Bernkop-Schnürch, Thiomers: a new generation of mucoadhesive polymers, Adv. Drug Deliv. Rev. 57 (2005) 1569–1582.
[6] M.-E. Imam, M. Hornof, C. Valenta, A. Bernkop-Schnürch, Evidence for the interpenetration of mucoadhesive polymers into the mucus gel layer, STP Pharma 13 (2003) 171–176.
[7] A. Bernkop-Schnürch, A. Weithaler, K. Albrecht, A. Greimel, Thiomers: Preparation and in vitro evaluation of a mucoadhesive nanoparticulate drug delivery system, Int. J. Pharm. 317 (2006) 76–81.
[8] A. Bernkop-Schnürch, V. Schwarz, S. Steininger, Polymers with thiol groups: a new generation of mucoadhesive polymers? Pharm. Res. 16 (1999) 876–881.
[9] M. Werle, M. Hoffer, Glutathione and thiolated chitosan inhibit multidrug resistance P-glycoprotein activity in excised small intestine, J. Control. Release 111 (1-2) (2006) 41–46.
[10] F. Föger, H. Hoyer, K. Kafedjiiski, M. Thaurer, A. Bernkop-Schnürch, In vivo comparison of various polymeric and low molecular mass inhibitors of intestinal P-glycoprotein, Biomaterials 27 (34) (2006) 5855–5860.
[11] K. Kafedjiiski, F. Föger, M. Werle, A. Bernkop-Schnürch, Synthesis and in vitro evaluation of a novel chitosan–glutathione conjugate, Pharm. Res. 22 (2005) 1480–1488.
[12] K. Kafedjiiski, M. Werle, F. Föger, A. Bernkop-Schnürch, Synthesis and in vitro characterization of a novel poly(acrylic acid)–glutathione conjugate, J. Drug Del. Sci. Technol. 15 (6) (2005) 411–417.
[13] A.-F. Habeeb, A sensitive method for localization of disulfide containing peptides in column effluents, Anal. Biochem. 56 (1973) 60–65.
[14] G. Volkheimer, Hematogenous dissemination of ingested polyvinyl chloride particles, Ann. N. Y. Acad. Sci. 246 (1975) 164–171.
[15] D.M. Brown, Drug Delivery Systems in Cancer Therapy, Humana Press Inc., Totowa, NJ, 2003, pp. 117–136.
[16] M.E. Imam, A. Bernkop-Schnürch, Controlled drug delivery systems based on thiolated chitosan microspheres, Drug Dev. Ind. Pharm. 31 (2005) 557–565.
[17] K. Maculotti, I. Genta, P. Perugini, M. Imam, A. Bernkop-Schnürch, F. Pavanetto, Preparation and in vitro evaluation of thiolated chitosan microparticles, J. Microencapsul. 22 (5) (2005) 459–470.

[18] I.M. van der Lubben, J.C. Verhoef, A.C. van Aelst, G. Borchard, H.E. Junginger, Chitosan microparticles for oral vaccination: preparation, characterization and preliminary in vivo uptake studies in murine Peyer's patches, Biomaterials 22 (2001) 687–694.
[19] M. Roldo, P. Hornof, P. Caliceti, A. Bernkop-Schnürch, Mucoadhesive thiolated chitosans as platforms for oral controlled drug delivery: synthesis and in vitro evaluation, Eur. J. Pharm. Biopharm. 57 (2004) 115–121.
[20] N. Midoux, P. Hosek, L. Pailleres, J.-R. Authelin, Micronization of pharmaceutical substances in a spiral jet mill, Powder Technol. 104 (1999) 113–120.
[21] W. Schlocker, S. Gschließer, A. Bernkop-Schnürch, Evaluation of the potential of air jet milling of solid protein-poly(acrylate) complexes for microparticle preparation, Eur. J. Pharm. Biopharm. 62 (3) (2006) 260–266.
[22] A. Bernkop-Schnürch, D. Guggi, Y. Pinter, Thiolated chitosans: development and in vivo evaluation of a mucoadhesive permeation enhancing oral drug delivery system, J. Control. Release 94 (2004) 177–186.
[23] G. Nykamp, U. Carstensen, B.-W. Müller, Jet milling – a new technique for microparticle preparation, Int. J. Pharm. 242 (2002) 79–86.
[24] Hosokawa Alpine AG & Co OHG, D-Augsburg, Technische Daten und Versuchsdaten für Spiralstrahlmühle 50AS (2000).
[25] T.F. Palmberger, K. Albrecht, B. Loretz, Bernkop-Schnürch, Thiolated polymers: Evaluation of the influence of the amount of covalently attached L-cysteine to poly(acrylic acid), Eur. J. Pharm. Biopharm. 66 (2007) 405–412.
[26] M. Huang, K. Khor, L.-Y. Lim, Uptake and cytotoxicity of chitosan molecules and nanoparticles: effects of molecular weight and degree of deacetylation, Pharm. Res. 21 (2) (2004) 344–353.
[27] P. He, S.S. David, L. Illum, Chitosan microspheres prepared by spray drying, Int. J. Pharm. 187 (1999) 53–65.
[28] M.-F. Machado, J.-M. Saraiva, Thermal stability and activity regain of horseradish peroxidase in aqueous mixtures of imidazolium-based ionic liquids, Biotechnol. Lett. 27 (2005) 1233–1239.
[29] M. Thanou, J.-C. Verhoef, H.-E. Junginger, Oral drug absorption enhancement by chitosan and its derivatives, Adv. Drug Deliv. Rev. 52 (2) (2001) 117–126.
[30] B. Lige, S. Ma, R.-B. van Huystee, The effects of the site-directed removal of *N*-glycosylation from cationic peanut peroxidase on its function, Arch. Biochem. Biophys. 386 (1) (2001) 17–24.
[31] V.-M. Leitner, G. Guggi, A. Bernkop-Schnürch, Thiomers in non-invasive peptide delivery: in vitro and in vivo characterisation of a polycarbophil-cysteine/glutathione gel formulation for hGH, J. Pharm. Sci. 93 (2004) 1682–1691.
[32] J. Stachetzki, Untersuchungen zu mechanischen Eigenschaften und Quellung von mit Schwefelverbindungen vernetzten Stärkemaleaten. PhD thesis. University of Stuttgart, 2003.
[33] CE. Kast, A. Bernkop-Schnürch, Thiolated polymers-thiomers: development and in vitro evaluation of chitosan–thioglycolic acid conjugates, Biomaterials 22 (2001) 2345–2352.
[34] L. Illum, Chitosan and its use as a pharmaceutical excipient, Pharm. Res. 15 (1998) 1326–1331.
[35] V. Dodane, V.D. Vilivalam, Pharmaceutical applications of chitosan, Pharm. Sci. Technol. Today 1 (1998) 246–253.
[36] D. Guggi, N. Langoth, A. Bernkop-Schnürch, A comparative evaluation of cytotoxicity of a glucosamine-TBA conjugate and a chitosan–TBA conjugate, Int. J. Pharm. 278 (2) (2004) 353–360.
[37] G. Oberdörster, E. Oberdörster, J. Oberdörster, Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles, Environ. Health Perspect. 113 (7) (2005) 823–840.
[38] R.B. Shah, A. Palamakula, M.A. Khan, Cytotoxicity Evaluation of Enzyme Inhibitors and Absorption Enhancers in Caco-2 Cells for Oral Delivery of Salmon Calcitonin, J. Pharm. Sci. 93 (4) (2004) 1070–1082.