

One-step preparation of polyelectrolyte-coated PLGA microparticles and their functionalization with model ligands

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Abstract

This work aimed at the development of a novel surfactant-free, one-step process for the concomitant formation of poly(lactide-*co*-glycolide) (PLGA) microparticles (MP) and surface coating with the polyelectrolyte chitosan, which is suitable for subsequent covalent conjugation of bioactive ligands. The technology is based on solvent extraction from an O/W-dispersion using a static micromixer. Surface coating occurred through interaction of the negatively charged, nascent PLGA MP with the polycationic chitosan, which was dissolved in the aqueous extraction fluid. Particles of 1–10 μm in diameter were produced with excellent reproducibility. The chitosan-coated PLGA MP were spherical and showed a smooth surface without pores, as demonstrated by scanning electron microscopy (SEM). The chitosan coatings were characterized by zeta potential measurements and X-ray photoelectron spectroscopy (XPS). The functional amino groups of chitosan were used to conjugate two model ligands to the coating, i.e. fluorescamine and NHS-PEG-biotin. The presence of the conjugated ligands was revealed by confocal laser scanning microscopy (CLSM) and fluorescence activated cell sorting (FACS). Evidence for biotinylation was demonstrated through binding of fluorescently labelled streptavidin. The developed platform technology is straightforward and flexible. Future studies will focus on the design of microparticulate carriers with bioactive surfaces, e.g. as antigen delivery systems.

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1. Introduction

Biodegradable microparticles made of poly(lactide-*co*-glycolide) (PLGA) are well-established delivery systems for therapeutics, and have high potential for peptide, protein and nucleic acid vaccines [1–3]. Thanks to its established safety record, PLGA continues to be prevalently used in the field, although the stability of entrapped protein remains a major issue. Nevertheless, one of the typical deficiencies of aliphatic polyesters such as PLGA is their lack of suitable functional groups for efficient and stable deposition or conjugation of bioactive agents under mild conditions. Surfaces coated with functional polymers or bearing bioactive ligands represent an increasingly desirable feature for drug and antigen delivery through microparticulate or nanoparticulate systems besides controlled release and protection of bioactive agents from premature clearance and degradation.

Various approaches have been proposed to functionalize the surface of biodegradable microparticles (MP) and nanoparticles (NP). The negatively charged surface of PLGA MP has been functionalized by the electrostatic binding of cationic surfactants such as cetyltrimethylammonium bromide (CTAB) [4]. An alternative to cationic surfactants is the electrostatic coating with polycationic polymers such as chitosan, poly(lysine) or poly(ethyleneimine) (PEI) [5–7]. Typically, the establishment of cationic NP or MP surfaces may be performed by incubation of previously prepared particles in an aqueous solution of the respective cationic agent. Direct coating of nascent particles in the course of a typical solvent evaporation/extraction process is achieved by addition of cationic surfactants or polymers to the aqueous extraction phase [8,9]. A third approach to obtain PLGA particles with cationic surface charge is the co-encapsulation of a cationic lipid or a polyelectrolyte through spray drying [10].

The purpose of PLGA NP or MP bearing a cationic surface charge may be, e.g. (i) to provide a positively charged binding site for the adsorption, condensation and stabilization of

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nucleic acid biopharmaceuticals, such as plasmid DNA, mRNA, siRNA or antisense oligonucleotides, through electrostatic interaction [11,12], or (ii) to render the particles muco-adhesive, e.g. in view of nasal delivery [13].

An alternative approach to obtain NP or MP with functional surfaces encompasses the direct use of a polyelectrolyte such as chitosan as matrix material. Chitosan is highly bioadhesive and has been reported to enhance the permeability of the nasal mucosa [14–16]. Thus, besides offering a substrate for the adsorptive deposition or conjugation of bioactive ligands, chitosan NP or MP feature intrinsic bioadhesive and permeation enhancing properties, which make them particularly suitable for e.g., nasal administration. A drawback of pure chitosan particles versus chitosan coated PLGA particles is the need to cross-link chitosan to exclude premature dissolution upon application. Further, chitosan forms a hydrogel in aqueous environments and, therefore, cannot sustain the release of bioactive materials over prolonged periods of time [17]. Finally, chitosan, as well as other polycationic materials, may cause safety concerns when intended for parenteral administration. Thus, the use of polycationic materials for forming or coating micro- or nanoparticles will eventually require careful toxicity studies.

An interesting concept to functionalize the surface of negatively charged PLGA MP was recently proposed by Faraasen et al. [18]. The PLGA MP were coated with positively charged poly(ethylene oxide)-grafted poly(L-lysine) (PLL-g-PEG) constructs via electrostatic interaction. This endeavour aimed at ligand-specific phagocytosis using the integrin ligand, RGD, which was conjugated to the distal end of the PEG chains to form PLL-g-PEG-RGD. Coatings with PLL-g-PEG drastically inhibited unspecific phagocytosis by antigen presenting cells, whereas PLL-g-PEG-RGD coatings enhanced phagocytosis, probably by ligand specific interactions [18]. The immunological benefit of such systems for the delivery of vaccines awaits demonstration.

The objective of the present study was the development of a novel surfactant-free, one-step process for the concomitant formation of PLGA MP and their surface coating with chitosan, applying a previously reported micromixer-based solvent extraction protocol [19]. We also demonstrated the feasibility to functionalize such chitosan coated MP by covalent conjugation of two model ligands, fluorescamine and NHS-PEG-biotin, under mild conditions. The quality of the chitosan coatings was analyzed by zeta potential measurements, scanning electron and confocal laser scanning microscopy, as well as X-ray photoelectron spectroscopy. The model ligands were successfully conjugated to the PLGA MP.

2. Materials and methods

2.1. Materials

End-group uncapped 35 kDa PLGA50:50 (Resomer[®] RG503H) was purchased from Boehringer-Ingelheim (Ingelheim, Germany), and three chitosan types of different viscosity grade (Seacure[®] CL110, CL210 and CL310) were kindly offered by Pronova Biopolymer (Drammen, Norway). The

apparent dynamic viscosity values of the three chitosan types CL110, CL210 and CL310 were 6, 86 and 296 mPa·s, respectively, as given by the manufacturer and measured by Brookfield LVT viscometer at 25°C and 30 rpm (1%, w/w, chitosan in 1% acetic acid, 2 h after dissolution; Brookfield Engineering Laboratories, Inc., Middleboro, MA, USA). *N*-hydroxysuccinimidyl-poly(ethylene glycol)-biotin (NHS-PEG-biotin, 3.4 kDa) was from Nektar Therapeutics (Huntsville, USA), and the fluorescent streptavidin conjugate Streptavidin Oregon Green[®] 514 was from Molecular Probes (Leiden, The Netherlands). Fluorescamine was from Sigma-Aldrich (Steinheim, Germany). Synthesis grade dichloromethane (DCM) was from EGT Chemie (Tägerig, Switzerland), poly(vinylalcohol) (PVA, Mowiol[®] 4-88) was a gift from Hoechst (Frankfurt/M., Germany), and bovine serum albumin (BSA, fraction V) was from Fluka (Buchs, Switzerland). Phosphate buffered saline (10 mM, PBS) of pH 7.4 was from Sigma-Aldrich (Steinheim, Germany).

2.2. Preparation of microparticles (MP)

PLGA was dissolved at 5% (w/w) in DCM (O-phase), and chitosan at various concentrations in 0.1 N HCl (extraction phase), with 0.5% (w/w) chitosan being used for standard preparations. For some preparations, pure water was used as extraction phase. MP were prepared by microextrusion-based solvent extraction using a static micromixer as previously described [19]. The aqueous extraction phase (water or chitosan solution) and the PLGA solution were individually fed into the two inlets of the micromixer. For standard preparations, Resomer[®] RG503H and Seacure[®] CL110 were used; the flow rates were 0.5 ml/min for the organic phase and 14.0 ml/min for the aqueous extraction phase. Passage through the micromixer produced a dispersion of organic phase droplets in the extraction phase with concomitant extraction of the organic solvent and solidification of the nascent PLGA particles. The PLGA MP were collected in purified water or 0.5% (w/w) aqueous solution of PVA (collection fluid). Typically, 500 ml collection fluid were used for batch sizes of 150–250 mg PLGA. Under gentle stirring, the MP slurry was placed under laminar airflow for 30 min for solvent removal and solidification of the MP. The particle suspension was filtered over a 0.2- μ m mixed cellulose ester membrane (Schleicher and Schuell, Dassel, Germany) and the particles were dried at 20 mbar and room temperature for 24 h.

For microencapsulation of BSA, an aqueous 5% (w/w) solution of BSA in 10 mM PBS of pH 7.4 was dispersed and homogenized by ultrasonication in the organic phase at a phase ratio (aqueous/organic) of 1:20 (w/w) to form a primary emulsion for subsequent passage through the micromixer. Further processing was as described above for unloaded MP.

2.3. Particle size distribution

Particle size distributions were determined by laser light scattering (Mastersizer X, Malvern, Worcestershire, UK,

equipped with a 100-mm lens) using a Fraunhofer diffraction model. The particles were analysed prior to their final drying. All size distributions are presented in the volume-weighted mode and characterized by their 10%, 50%, and 90% undersize diameters, i.e. $d(v, 0.1)$, $d(v, 0.5)$ and $d(v, 0.9)$. The 50% undersize diameter is referred to as the mean diameter, and $d(v, 0.1)$ and $d(v, 0.9)$ are used to characterize the span of the distribution.

2.4. Scanning electron microscopy (SEM)

Samples of dried MP were placed on a double-sided adhesive carbon sticker (Provac AG, Balzers, Liechtenstein) and coated with 10 nm of gold/palladium. The micrographs were taken on a Hitachi S-4100 (Hitachi Scientific, Düsseldorf, Germany) field emission scanning electron microscope.

2.5. Zeta potential

The surface charge of the MP was determined by zeta potential measurement (Malvern Zetasizer 3000 HSA, Malvern, UK). Approx. 1 mg of MP was suspended either in 10 ml of 1 mM KCl or 10 mM PBS buffer (pH of 7.4; 10 mM phosphates; 138 mM NaCl; 2.7 mM KCl). Mean values were calculated on the basis of five measurements per sample.

2.6. Conjugation of model ligands

Fluorescamine and NHS-PEG-biotin were used as model ligands for covalent conjugation to PLGA MP coated with Seacure® CL210. Uncoated PLGA MP served as negative control. For conjugation of fluorescamine, 1 mg MP was incubated under light protection and vigorous shaking at 37 °C for 1 h in a solution consisting of 900 µl borate buffer of pH 9 and 100 µl of 1.107 nM fluorescamine in acetone. After incubation, the MP were centrifuged at 10,600×g; the supernatant was discarded and the MP were washed thrice with 10 mM PBS of pH 7.4.

Persistence of the chitosan coatings was demonstrated by performing the conjugation with fluorescamine after extended incubation times of the particles in PBS (6, 24, 48 h) prior to conjugation.

For biotinylation with NHS-PEG-biotin, 1 mg MP was inoculated in a 1 µM NHS-PEG-biotin in 10 mM PBS of pH 7.4 at 37 °C under vigorous shaking for 1 h. Then, the MP were centrifuged at 10,600×g for 5 min, the supernatant was discarded, and the MP were washed thrice with 10 mM PBS of pH 7.4. The MP were then incubated in 1 ml of 0.166 µM Streptavidin Oregon Green® 514 at room temperature for 30 min and subsequently washed twice with 10 mM PBS of pH 7.4.

2.7. FACS analysis of MP

Fluorescent MP were analysed by FACS analysis (FacScan, Becton Dickinson, Franklin Lakes, NJ) using a total number of approx. 2×10^4 MP per sample. The median fluorescence

intensity of labelled chitosan coated MP was compared to uncoated PLGA MP subjected to the same fluorescence labelling procedure. Chitosan coated MP that were not subjected to fluorescence labelling served as second control.

2.8. Confocal laser scanning microscopy (CLSM)

Fluorescent MP were suspended in PBS at 0.1 mg/ml and diluted, if necessary. Preparations were examined using a Zeiss CLSM 410 inverted microscope with a 20×1.4 NA plan apochromatic lens (lasers: He–Ne 543 nm, Ar 488/514 nm). Optical sections were taken at increments of 0.8 µm, and 3-D multichannel processing was performed using the IMARIS software (Bitplane AG, Zurich, Switzerland) on a Silicon Graphics workstation.

2.9. X-ray photoelectron spectroscopy (XPS)

XPS analyses were performed using a PHI 5700 spectrophotometer equipped with a concentric hemispherical analyzer (CHA) (Physical Electronics, Eden Prairie, MN). Spectra were acquired at a base pressure of 10^{-9} mbar using a non-monochromatic Al K α source operating at 350 W and positioned 10 mm away from the sample. MP samples were pressed on an indium foil. The instrument was run in the minimum-area mode using an aperture of 0.4 mm diameter. The CHA was used in the fixed analyzer transmission mode. Pass energies used for survey scans and detailed scans were 187 and 23 eV, respectively. Data were analyzed using the program CASA XPS. The signals were fitted using Gaussian-Lorentzian functions and least squares fit routines following Shirley iterative background subtraction. Sensitivity factors were calculated using published ionization cross sections [20] corrected for the energy dependency of the transmission function of the instrument and the attenuation length dependency with kinetic energy. Charging of the samples was compensated by calibration of the spectra to the aliphatic hydrocarbon peak at 285 eV.

3. Results

3.1. Particle size and morphology of PLGA MP

With each of the chitosan types (Seacure CL 110, CL 210, CL 310) we produced three batches of BSA-free, chitosan-coated PLGA MP. Three uncoated MP batches were also produced, with all other parameters kept constant. MP size distributions were highly reproducible within these formulations (Fig. 1A and B). Mean diameters of chitosan coated PLGA MP made with the lowest viscosity grade chitosan (CL110) were only marginally larger than those of uncoated PLGA MP (Fig. 1A,B; Table 1). In contrast, the use of higher viscosity chitosan types (CL210 and CL310) led to significantly increased mean diameters of the resulting MP (Fig. 2; Table 1). Moreover, the particle-size distributions broadened significantly. Further, microparticle size also appeared to increase slightly upon BSA encapsulation, although this effect

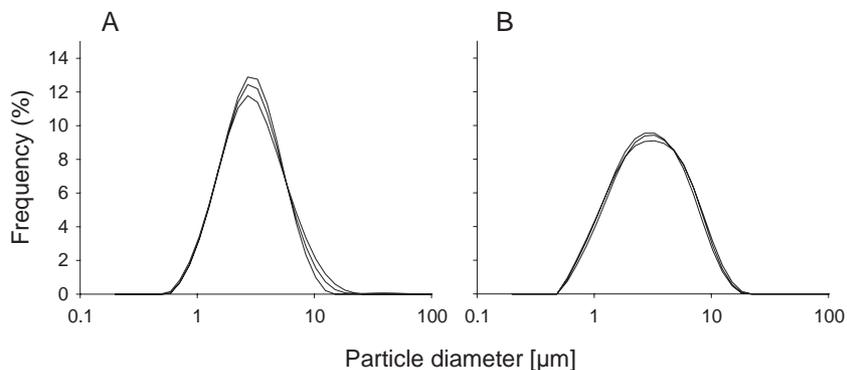


Fig. 1. Reproducibility of MP size distributions (volume weighted) between three identically prepared batches; (A) uncoated PLGA MP, (B) chitosan-coated (CL110) PLGA MP. All preparations were collected in purified water. Preparation conditions were standard, as described under Materials and methods.

became more pronounced only with the highest viscosity grade chitosan coating (Table 1).

Chitosan coated (CL 110) and uncoated PLGA MP (each collected in either purified water or 0.5% PVA) were examined for their surface morphology. Scanning electron micrographs revealed smooth surfaces without pores (Fig. 3) for all batches irrespective of the composition of the aqueous extraction phase (water or 0.5% chitosan) and collection fluid (water or 0.5% PVA).

3.2. Zeta potential of the microparticles

As zeta potential measurements depend on a variety of parameters, we first examined if the presence of uncoated or chitosan-coated PLGA MP in the measuring media (1 mM KCl and 10 mM PBS) exerted a pH-effect. The pH was measured before particle dispersion and 1 h after particle incubation. In the unbuffered 1 mM KCl (pH=7.66), the dispersion of uncoated and coated PLGA MP raised the pH slightly to 8.17 ± 0.06 and 7.94 ± 0.06 , respectively. In the pH 7.40 PBS, the pH was maintained within the range of 7.40 and 7.44 after particle incubation. Thus, the minor effects of the particles on pH of the dispersion media can be neglected.

The zeta potential of the MP differed significantly between uncoated PLGA MP and chitosan-coated PLGA MP (Table 1). Chitosan coating shifted the strongly negative zeta potential of pure PLGA MP to positive values, irrespective of the chitosan type used. Encapsulation of BSA generally attenuated the zeta potential. For instance, encapsulation of

BSA shifted the zeta potential of uncoated PLGA MP from -78.3 ± 2.62 mV to -39.1 ± 6.31 mV, whereas that of chitosan-coated MP dropped from 25.4 ± 2.72 mV to 11.8 ± 2.82 mV, and from 29.4 ± 2.87 mV to 9.9 ± 9.51 mV for chitosan type CL 110 and CL 210, respectively (Table 1). Particles coated with chitosan CL 310 showed only a slight increase in zeta potential upon encapsulation of BSA (Table 1). BSA encapsulation also affected the mean diameter of the MP, which increased only slightly for the uncoated PLGA MP and chitosan CL 110- and CL 210-coated MP, but substantially for the chitosan CL 310-coated particles (Table 1).

Moreover, the zeta potential of the PLGA MP was affected by the concentration of chitosan (CL 110) in the extraction phase (Fig. 4). Increasing chitosan concentrations shifted the zeta potential from highly negative to slightly positive values. A plateau of maximal surface charge was reached at a concentration of 0.5% chitosan (w/w) in the extraction phase.

Finally, the zeta potential was affected by the composition of the collection fluid. With a 0.5% (w/w) PVA solution, a commonly used extraction and collection phase in PLGA MP production, the zeta potential values of chitosan-coated particles were close to zero and significantly lower than those of the chitosan-coated PLGA MP collected in purified water (Fig. 5), indicating interference of adsorption between chitosan and PVA.

A crucial quality aspect of MP coating is the persistence of the chitosan coating upon particle incubation. Here, we assessed this feature by monitoring the zeta potential of chitosan-coated MP upon incubation in 1 mM KCl, the

Table 1
Effect of chitosan coating and of BSA-encapsulation [5%, relative to PLGA mass] on the ζ -potential and volume-weighted mean diameter of PLGA MP

Chitosan coating	Unloaded PLGA MP		BSA-loaded PLGA MP	
	ζ -potential [mV]	Mean diameter [μ m]	ζ -potential [mV]	Mean diameter [μ m]
None	-78.3 ± 2.62	2.55 ± 0.10	-39.1 ± 6.31	3.92 ± 0.13
CL110	25.4 ± 2.72	3.10 ± 0.09	11.8 ± 2.86	4.60 ± 0.20
CL210	29.4 ± 2.87	5.55 ± 0.13	9.9 ± 9.51	7.05 ± 0.34
CL310	14.6 ± 8.66	11.50 ± 0.32	17.5 ± 4.17	21.88 ± 1.59

The PLGA MP were coated with chitosan types of different viscosity (CL110, CL2210 and CL310). All other conditions were standard. Particles were collected in purified water. The zeta potentials was measured 1 mM KCl. Indicated standard deviations are for variability ($n=3$).

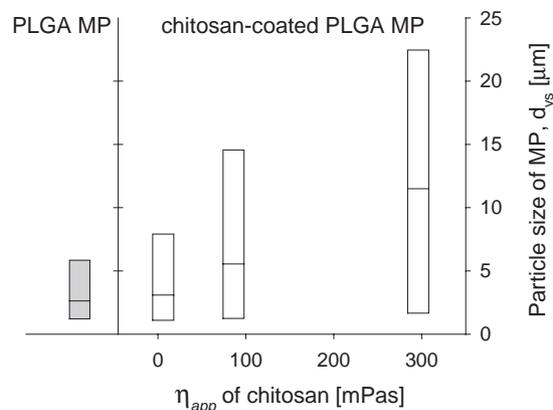


Fig. 2. Influence of the chitosan viscosity grade (given as apparent viscosity, η_{app}) on chitosan-coated PLGA MP size distributions. The span of the distributions is characterized by the 10 and 90% undersize diameters $d(v, 0.1)$ and $d(v, 0.9)$, respectively; the 50% undersize diameter is referred to as the mean diameter ($n=3$). The three viscosity grade chitosan types were Seacure CL110, CL210 and CL310, respectively (see under Materials and methods). For reference, size distribution data of uncoated PLGA MP are also given (grey bar).

medium used for zeta potential measurement, or in 10 mM PBS of pH 7.4 (Fig. 6), for a period of 7 days. Upon incubation in the KCl solution, the zeta potential of uncoated and chitosan-coated PLGA MP gradually shifted from -78 to -37 mV and from $+25$ to -10 mV, respectively. In PBS, however, the recorded zeta potentials were more moderate from the beginning of incubation, i.e. -33 mV and -6 mV for uncoated and chitosan-coated PLGA MP, respectively, on day 0 of incubation. These values changed only slightly over seven days. The differences in the particles' zeta potential values observed between 1 mM KCl and 10 mM PBS dispersion and incubation media must be ascribed to the differences in ionic strength, which is well known to affect zeta potential values.

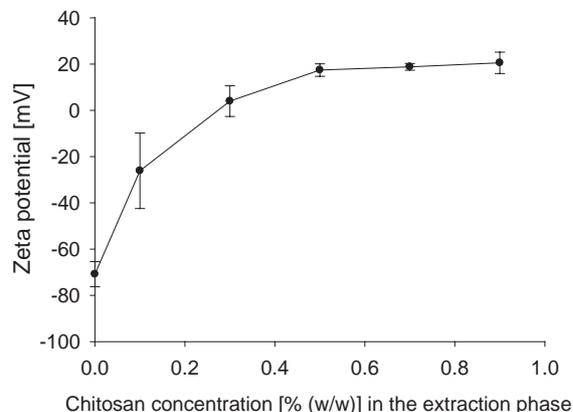


Fig. 4. Effect of chitosan (Seacure® CL110) concentration in the extraction phase on the zeta potential (measured in 1 mM KCl) of PLGA MP prepared under standard conditions. Error bars stand for batch-to-batch variability ($n=3$).

3.3. Conjugation of model ligands to chitosan-coated PLGA MP

Free fluorescamine is non-fluorescent but becomes fluorescent upon conjugation to primary amino groups. FACS analysis of chitosan-coated PLGA MP after conjugation with fluorescamine demonstrated a large increase in median fluorescence intensity as compared to uncoated PLGA MP (Fig. 7A). To demonstrate the persistence of the chitosan coatings on MP as a platform for covalent conjugation, fluorescamine coupling was also performed after extended incubation times of 6 up to 48 h of the particles in PBS prior to conjugation. Even after MP incubation in PBS over 48 h, median fluorescence intensity upon fluorescamine conjugation was substantially elevated as compared to control (Fig. 7B). To exclude chitosan-related false-positive effects, chitosan-coated PLGA MP without fluorescamine conjugation were also analysed by FACS. The resulting fluorescence intensity was

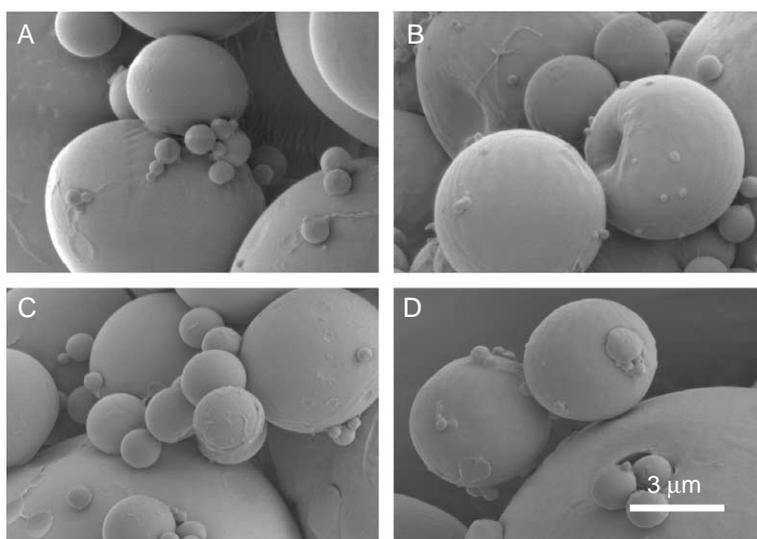


Fig. 3. SEM micrographs of unloaded PLGA MP (A, B) and of chitosan-coated PLGA MP (C, D). The MP were prepared using as extraction phase either purified water (A, B) or 0.5% chitosan solution (C, D), and as collection fluid either purified water (A, C) or 0.5% PVA (B, D). No difference in surface morphology could be observed between the four preparations.

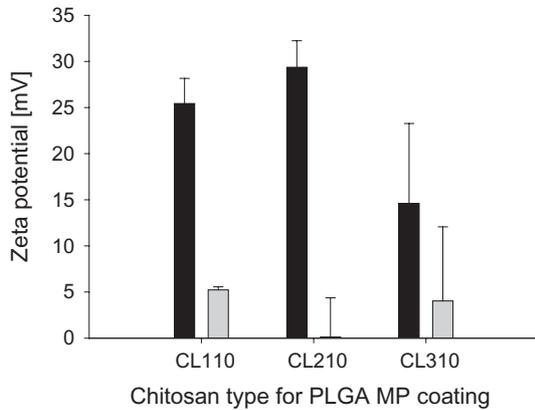


Fig. 5. Effect of the collection fluid on the zeta potential of chitosan-coated PLGA MP (measured in 1 mM KCl). Black and grey bars indicate the zeta potential of MP collected in purified water or 0.5% PVA solution, respectively. Error bars are for batch-to-batch variability ($n=3$).

as low as that measured with uncoated PLGA MP treated with fluorescamine.

Conjugation of NHS-PEG-biotin to the chitosan coatings on PLGA MP also proved to be successful, as the coupled biotin remained accessible for subsequent binding with Streptavidin Oregon Green® 514. In the FACS analysis, the median fluorescence intensity of chitosan-coated MP after such treatment was approx. five times higher than that of uncoated PLGA MP (Fig. 7A). Analysis by CLSM revealed a strongly fluorescent corona on chitosan-coated PLGA MP after biotinylation and subsequent treatment with streptavidin Oregon Green® 514 (Fig. 8A). Again, to exclude false-positive results due to adsorption of reagents, the experiment was also performed with uncoated PLGA MP (Fig. 8B), and with chitosan-coated PLGA MP exposed to either NHS-PEG-biotin alone (Fig. 8C) or streptavidin Oregon Green® 514 alone (Fig. 8D). None of the controls showed indications for the presence of ligand on the MP.

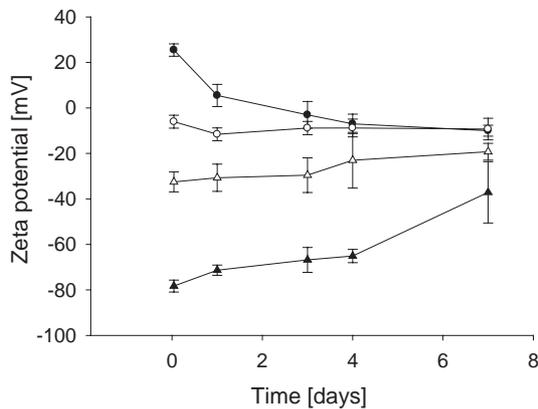


Fig. 6. Change of the zeta potential of uncoated PLGA MP (triangles) and chitosan-coated PLGA MP (circles) during incubation in 10 mM PBS of pH 7.4 (empty symbols) or in 1 mM KCl (filled symbols). Error bars are for batch-to-batch variability ($n=3$). Before incubation, the zeta potential values were -78 mV for uncoated PLGA MP and $+25$ mV for chitosan-coated particles in 1 mM KCl; in 10 mM PBS the values were -33 mV and -6 mV, respectively.

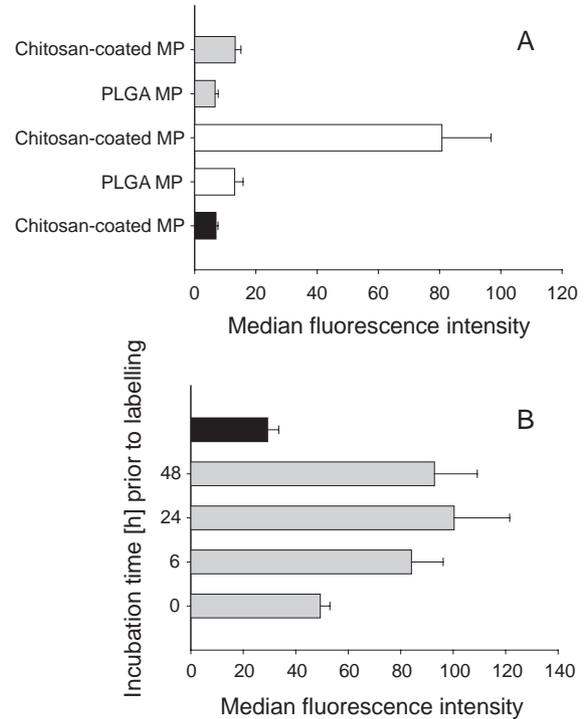


Fig. 7. Median fluorescence intensity, as measured by FACS, of PLGA MP treated with fluorescamine (grey columns), or with NHS-PEG-biotin followed by fluorescent streptavidin (empty columns), or left untreated (black columns). Panel (A): The chitosan-coated PLGA MP exhibited significantly higher labelling with both fluorescamine and NHS-PEG-biotin/fluorescent streptavidin than uncoated PLGA MP. Panel (B): Median fluorescence intensity after conjugation of chitosan coated MP with fluorescamine after extended incubation times of the particles in PBS (6, 24, 48 h) prior to the labelling. Even after 48 h, fluorescence intensity of chitosan-coated PLGA MP was significantly higher than that of untreated control. Differences in median fluorescence intensity between (A) and (B) upon fluorescamine labelling are due to different instrument settings.

3.4. Surface chemistry of chitosan-coated and uncoated PLGA MP

For chitosan-coated PLGA MP, photoelectrons from carbon C1s, oxygen O1s and nitrogen N1s were detected by means of XPS (Table 2). The survey scan gave elemental contents of 38.2% C1s, 61.4% O1s and 0.4% N1s. The N1s signal was quite low and close to the limit of detection, whereas the N1s signal of a chitosan control film was prominent. When collecting chitosan-coated PLGA MP in a solution of 0.5% (w/w) PVA instead of purified water, the nitrogen peak was undetectable, and the elemental content was 42.4% C1s and 57.6% O1s. Control particles prepared from pure PLGA (Resomer® 503H) showed elemental contents of 37.0% C1s and 63.0% O1s when the particles were collected in purified water and of 43.5% C1s and 56.5% O1s when using PVA solution as collection fluid. The theoretical elemental content of PLGA 50:50 is 55.6% C1s and 44.4% O1s and that of the used chitosan (degree of deacetylation: 83%) 55.1% C1s, 36.2% O1s and 8.69% N1s. Measured values for the chitosan CL210 type were 61.4% C1s, 32.9% O1s and 5.7% N1s. When considering the C1s envelope contents, the use of PVA as collection fluid increased the aliphatic C1s signal in the C1s

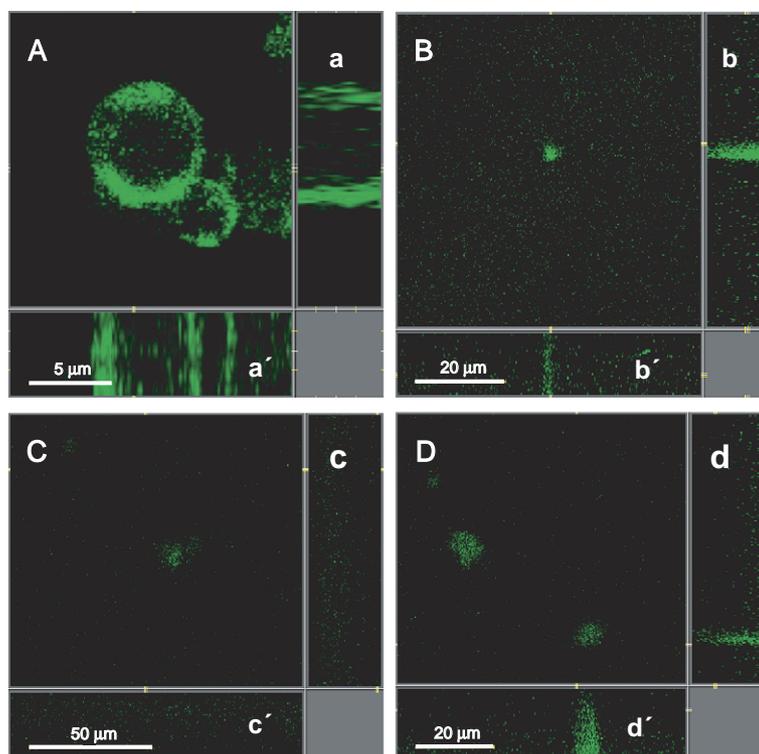


Fig. 8. Confocal laser scanning micrographs of chitosan-coated PLGA MP (A, C, D) or uncoated PLGA MP (B) after subsequent treatment with NHS-PEG-biotin and streptavidin Oregon Green[®] 514 (A, B), or after treatment with NHS-PEG-biotin alone (C) or streptavidin Oregon Green[®] 514 alone (D). a, b, c and d show yz sections, a', b', c' and d' show xz sections.

envelope content for both formulations (pure PLGA MP and chitosan-coated MP), i.e. from 32% to 50% for uncoated PLGA and from 33% to 44% for chitosan-coated PLGA MP (Table 2). For both formulations, the C1s CON, COO envelope content decreased when the particles were collected in PVA solution rather than purified water. After coupling of NHS-PEG-biotin to the chitosan-coated particles, only slight changes in elemental and C1s envelope contents were detectable. The element S remained undetectable.

4. Discussion

The use of biodegradable microparticles has traditionally and primarily been motivated by the sustained release properties for periods of weeks to months and by the potential of protecting drugs from (enzymatic) degradation. More recently, composite core-shell type microparticles have been proposed to add further functionalities, e.g., by creating a surface charge or a protein-resistant corona [21–24]. Particles

Table 2
XPS analysis of PLGA MP and chitosan-coated PLGA MP after collection in water or in 0.5% PVA solution

Theoretical values	Elemental content (%)				
	C	O	N	S	
PLGA 50:50	55.6	44.4			
Chitosan CL210	55.1	36.2	8.7		
PVA	66.7	33.3			
NHS-PEG-Biotin	40.5	19.8	1.1		0.3

Formulation/collection medium	Elemental content (%)				C1s envelope content (%)		
	C	O	N	S	Aliphatic	CO	CON, COO
PLGA MP/water	37.0	63.0			32	33	35
PLGA MP/0.5% PVA	43.5	56.5			50	30	19
Chit-coated PLGA MP; water	38.2	61.4	0.4		33	34	33
Chit-coated PLGA MP; 0.5% PVA	42.4	57.6			44	31	25
PLGA MP/water; +NHS-PEG-biotin coupling	37.2	62.8			31	33	36
Chit-coated PLGA MP/water; +NHS-PEG-biotin coupling	37.2	62.1	0.7		30	36	34
Chitosan film	61.4	32.9	5.7		30	55	15

Coupling of NHS-PEG-biotin was done only with particles collected in water. Pure PLGA MP and a chitosan film served as controls.

coated with polycationic compounds have been conceived basically to achieve muco-adhesiveness for nasal [25] or other routes of administration [26], or bind DNA [12]. Particles with functional surfaces can be produced either directly from single polymers carrying reactive groups, e.g. poly(anhydrides) [27], or from blends of polymers with and without reactive groups, e.g., poly(ethyleneimine) and PLGA [28], or from adsorptive coating of particles with polymers carrying reactive groups [5].

In this proof-of-concept study, we developed core-shell type microparticles of a PLGA core for controlled drug release and a chitosan coating exposing free amino groups for the conjugation of bioreactive entities. The generation of microparticles by micromixer technology [19] proceeded concomitantly to their coating with chitosan, which was dissolved in the solvent extraction phase. This process produced particles with mean diameters between 1 and 10 μm (Figs. 1–3), which is, for example, a useful size range for phagocytic uptake by dendritic cells and macrophages. The chitosan coating was evidenced by zeta potential and XPS measurements as well as by the feasibility of conjugating fluorescent ligands that required the presence of amine groups (Figs. 4–8). The chitosan coating was well anchored in the PLGA core and persisted for several days upon incubation of MP in 10 mM PBS or 1 mM KCl (Fig. 6). Thus, the prepared chitosan-coated PLGA MP may offer opportunities for future covalent conjugation of biologically active ligands.

The here developed micromixer-based solvent extraction process enabled us to prepare chitosan-coated PLGA microparticles in a single step. This was feasible, because the micromixer-based process did not require the presence of a surfactant in the extraction phase, which is necessary in beaker/stirrer-based processes to stabilize the PLGA droplets and which is commonly PVA [29]. Thus, the non-surfactant chitosan could be added directly into the solvent extraction phase, so that direct adsorption and anchoring on the PLGA core was achieved, a process that was hindered in the presence of PVA. So far, only few authors have described a surfactant-free solvent extraction process for microparticle production [30,31]. Although the hydroxyl groups of surface-bound PVA or the uncapped end-groups of PLGA have been used as reactive sites for coupling of lectins [32], the availability of primary amino groups on PLGA MP offers a wider spectrum of conjugation reactions with relatively safe reagents and under mild conditions.

The PLGA particle size increased substantially with the viscosity grade of chitosan used for coating (Fig. 2) and generally only slightly when BSA was microencapsulated. The effect of chitosan on particle size must be attributed to the increased viscosity of the chitosan phase. The increased viscosity has probably lowered the shear force between chitosan solution and PLGA solution, which is responsible for PLGA microdroplet formation in the micromixer. Alternatively, we cannot exclude entirely that the thickness of the adsorbed chitosan layer also contributed to the MP size, although neither electron microscopic observations nor zeta potential measurements (Table 1) provided any evidence for this latter hypothesis. The generally slightly increased size of

BSA-loaded PLGA MP can be ascribed to the additional volume the aqueous BSA-solution displaces inside the PLGA microdroplets upon formation. It remains, however, unclear why BSA entrapment increased the PLGA MP size more substantially when the high viscosity grade chitosan was used.

The zeta potential values of chitosan-coated PLGA MP did not appear to vary significantly with the chitosan type, although the absolute value for the unloaded PLGA MP coated with the high viscosity grade chitosan (CL310) was inconsistently low. BSA loading, however, decreased the positive surface charge of the PLGA MP, except for the particles coated with the high viscosity grade chitosan, for which we cannot provide an explanation. Thus, BSA appeared to attenuate the surface charge of the microparticles, most likely because some BSA was also present in the surface layer, as a common result of encapsulation. At pH of approx. 7 (1 mM KCl for zeta potential measurement), BSA ($\text{pI}=4.7$) was negatively charged and counterbalanced some of the surface charge imposed by chitosan.

Extent and persistence of the chitosan coatings on the PLGA MP was only indirectly assessed, as we faced technical hurdles to quantify reliably the amount of adsorbed polyelectrolyte. Nonetheless, the nicely parabolic saturation curve of the particles' zeta potential suggests that the surface coverage of the negatively charged PLGA MP is chitosan concentration-dependent (Fig. 4). Evidently, this concentration dependency relates only to the measured surface charge, but does not provide information on the amount of adsorbed chitosan or the extent of surface coverage. Means to analyze quantitatively the amount of adsorbed chitosan have to be developed in future studies. The persistence of the adsorbed chitosan coat was assessed by zeta potential measurements of incubated particles [13,33] (Fig. 6). For control purposes, zeta potential of uncoated PLGA MP was also monitored over time. In 1 mM KCl, the absolute values of zeta potential decreased over 7 days of incubation for both the uncoated (from -78 to -37 mV) and coated (from $+25$ to -10 mV) particles. For the uncoated PLGA MP, the drop of zeta potential might be ascribed to minor particle aggregation and some release of acidic moieties that re-adsorb on the particle surface and neutralize gradually the surface charge. Nonetheless, we ascertained that the possibly released small amounts of lactic and glycolic acid did not affect the pH of the incubation medium, so that an undesired pH-effect on the zeta potential measurement can be excluded. For the coated PLGA MP, we interpret the drop of zeta potential as limited desorption of chitosan combined with re-adsorption of released acidic moieties from the slowly degrading polymer. Importantly, even after 7 days incubation, the chitosan coated particles maintained a zeta potential that was significantly less negative than that of the uncoated particles, suggesting persistence of chitosan on the particles' surface. In 10 mM PBS, similar trends in zeta potential development were observed, although the zeta potential values were generally closer to zero as a result of the relatively important ionic strength of this buffer. The persistence of chitosan, i.e., the availability and reactivity of primary amino groups, on the particles' surface was also confirmed by the

feasibility of fluorescamine-labeling after exposure for up to 48 h of chitosan-coated particles in PBS buffer (Fig. 7B). Incidentally, the higher fluorescence intensity measured with particles pre-incubated in PBS for 6, 12 and 48 h prior to fluorescamine-labeling, as compared to those labeled without pre-incubation, might be ascribed to increased hydration and mobility of the chitosan chains; this very likely facilitated the accessibility of primary amino groups for the coupling reagent.

As neither zeta potential measurements nor covalent labeling can prove directly the presence or extent of surface coating, we also used XPS for characterizing the particles' surface chemistry. Scholes et al. used XPS to detect PVA and poloxamer on biodegradable PLGA nanospheres [29]. In their study, the XPS spectra revealed not only the elemental composition of the surface coating, but also that of the PLGA substrate. Their findings are in agreement with our own observations: XPS spectra were dominated by elemental signals from PLGA, while the chitosan nitrogen was detected only at low intensity (Table 2). The reasons for this observation might lie in either incomplete coverage of the MP-surface or the relatively important penetration depth (50–100 Å) of XPS signals [34]. Therefore, alternative methods with a lower penetration depth like time-of-flight secondary ion mass spectrometry (TOF-SIMS) might provide more information about the actual surface composition of MP coatings.

Future work will have to address the quality of peptide or protein encapsulation in terms of stability of entrapped material, encapsulation efficiency, and in vitro release kinetics. The first two issues may be particularly critical, because the solvent extraction phase containing chitosan has low pH, which may be detrimental for the protein in the inner water phase, and because the encapsulated material may be partially eluted during the subsequent bio-conjugation step. Thus, attempts should be made to bioconjugate ligands directly in the collection medium of the microparticles rather than drying and re-exposing them to another aqueous reaction medium.

Finally, we expect this modular technology to be suitable for manufacturing biodegradable PLGA microparticles with different types of functional surfaces. Besides chitosan, other cationic or amphoteric synthetic or biopolymers may be of interest as coating materials. In preliminary experiments, we coated particles with poly(ethyleneimine), poly(L-lysine) and protamine sulfate and tested the subsequent coupling of a model ligand (S. Fischer, unpublished data). Diverse biological functionality can be achieved by bio-conjugating ligands or targeting agents such as peptidomimetic agonists, adhesion peptides, carbohydrate clusters, receptors or antibody fragments. Conjugation of a medium-chain PEG corona, with or without terminal ligands, to avoid rapid clearance of the microparticles by the mononuclear-phagocytic system, may add further interesting features to these particles. Many groups have reported on covalent surface modifications of nano- and microparticles [32,35]. One purpose was to enhance the intestinal transport of incorporated drugs through lectin-mediated bioadhesion upon oral administration. In the field of antigen delivery systems, the motivation for creating

bioactive surfaces lies in the targeting of DC and concomitant camouflage to macrophages, or in the exposure of danger signals to induce specific activation and maturation patterns, e.g., through specific interaction with selected surface receptors [36]. Nevertheless, it remains to be shown whether MP bearing bio-active ligands are superior to unmodified or polymer coated MP that contain encapsulated or adsorbed immunostimulatory molecules. Diwan et al. have recently shown that the simple incorporation of CpG oligodeoxynucleotides into biodegradable PLGA nanoparticles induced high T cell response in vitro [37]. Thus, the actual benefit of MP with bioactive surfaces awaits demonstration in vitro and in vivo.

5. Conclusions

We propose a one-step process for concomitant PLGA MP formation and surface coating with the cationic polyelectrolyte chitosan, which bears primary amino groups. The underlying technology to prepare MP was solvent extraction from an O/W-dispersion using a static multilamination-based micromixer. The produced microparticles were in the size range of 1–10 µm. The surface coating of the nascent MP occurred through electrostatic interaction between the negatively charged PLGA MP surface and the positively charged chitosan, which was dissolved in the aqueous extraction fluid. The chitosan coatings resisted elution by aqueous buffer for several days. Our approach enabled a surfactant-free production of PLGA MP. We further demonstrated the successful conjugation of two model ligands, fluorescamine and NHS-PEG-biotin to the primary amino groups of the chitosan coating under mild conditions. Upon conjugation, both ligands remained functional. As a platform technology with flexible options regarding the choice of core and coating materials, surface coated MP have attractive potential towards the design of, e.g., microparticulate antigen delivery systems.

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