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Characterisation and stability studies of a hydrophilic decapeptide in different adjuvant drug delivery systems: A comparative study of PLGA nanoparticles versus chitosan-dextran sulphate microparticles versus DOTAP-liposomes

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ABSTRACT

Poly[lactic-co-glycolide] (PLGA) nanoparticles, chitosan-dextran sulphate microparticles, and DOTAP-liposomes were prepared as vaccine adjuvants and drug carriers for a small hydrophilic model peptide, and their different physico-chemical properties (size, PDI, zeta-potential, pH-value and peptide loading) were investigated. The model peptide's encapsulation efficiency (EE) in PLGA particles amounted to 15%, for DOTAP-liposomes to 20% and for chitosan particles up to 90%. The structural appearance of the particles was visualized by SEM and TEM. The stability of the aqueous formulations and the corresponding lyophilisates was monitored for 12 weeks (stored at $T = 2–8\,^{\circ}C$). The freeze-drying process and the addition of an appropriate cryoprotective agent (sucrose) proved to be essential for all carrier systems. As a result of this study, three different peptide-loaded drug delivery systems with different properties were successfully manufactured and showed sufficient product stability of their freeze-dried formulations over 12 weeks of storage.

1. Introduction

Vaccines besides antigens, isotonic and stabilizing compounds may also consist of adjuvants. These adjuvants are used to enhance the body's immune response to an infection or to a foreign body without initiating a specific immune response against themselves. Vaccines without adjuvant are very often not sufficiently immunogenic and unable to induce a significant antibody level. The most potent and oldest known adjuvant, which even today is referred to as a "gold standard" in terms of high adjuvant activity, was developed by Freund in 1937. This emulsion of water and mineral oil containing inactivated mycobacteria is called Freund's complete adjuvant (FCA) and is not applicable for human use due to potent local dermal reactions. Hence, a great demand in vaccine research areas for potent and well-tolerated adjuvant systems exists, and, accordingly, many different adjuvant systems are evaluated. At present aluminium-based systems are the most widely used adjuvants (Gupta, 1998) and are licensed in Europe and USA for human use. Next to these two types of adjuvants polymeric particles, liposomes and other emulsion based systems (Montanide™ or MF59®) raised an increasing interest in vaccine research (Schijns, 2006).

Along these lines, the polymers PLGA, chitosan and DOTAP lipid were selected in the present study as adjuvant carrier systems for a hydrophilic model peptide. These materials were chosen because of their variety in physico-chemical nature: PLGA [poly(D,L-lactide-co-glycolide)] represents one of the most frequently studied polymers with adjuvant activity. It is biodegradable and biocompatible, and therefore it is approved for human use in sutures or implants for sustained drug delivery (Frazza and Schmitt, 1971). The antigen, which is incorporated or adsorbed onto the PLGA's surface, can gradually be released from the matrix as a function of the used polymer's degradation rate. Hence, PLGA formulations act like depot reservoirs with concurrent protection of the encapsulated antigen (Peek et al., 2008). The potent immunological adjuvant effect of PLGA was successfully demonstrated by several scientists using a number of model antigens (O'Hagan et al., 1998) such as diphtheria (Singh et al., 1991) or tetanus toxoid, and an immunological effect comparable to aluminium hydroxide was demonstrated (Men et al., 1995; Singh et al., 1991). Moreover, a growing interest in chitosan-based particulate drug delivery systems and as an adjuvant matrix arose over the past decade (Agnihotri et al., 2004; Arca et al., 2009; Fini and Orienti, 2003). Chitosan is a natural, biocompatible, nontoxic polysaccharide which is obtained by deacetylation of chitin, a product of marine crustacean shells. The structure of chitosan is similar to cellulose, and the presence of additional primary amine groups yield hydrophilic properties which are beneficial for pharmaceutical applications. For example,

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a charged antigen could easily interact with the polycationic chitosan molecule and consequently can be incorporated in the matrix or absorbed on its surface. Because of these structural benefits and its availability as low-cost resource, chitosan represents an alternative to the synthetic PLGA polymer, and an immunological effect of tetanus toxoid loaded chitosan microspheres comparable to PLGA microspheres was shown by Jaganathan et al. (2005). Additionally Gordon et al. (2008) supported chitosan's potent immunological effects by showing similar amounts of generated T-cells and antibody titres of a chitosan hydrogel formulation compared to the common aluminium based antigen system.

In addition to particulate adjuvant systems, especially liposomes have many advantages concerning their applications as vaccine adjuvants because of their structural character. The positively charged DOTAP lipid in addition to cholesterol was chosen for a liposomal adjuvant formulation in the present study. The positively charged ammonium group in the head region of the lipid enables an easy penetration through negative charged cell membranes, and therefore the drug could be transferred easily and more selectively into targeted cells, e.g. dendritic or antigen presenting cells (Nakanishi et al., 1997). Its potent immunological adjuvant effect was demonstrated by several scientists (Cui et al., 2004; Fujimura et al., 2006; Vangasseri et al., 2006). Moreover, the DOTAP lipid may feature cell-mediated immune response and anti-tumor activity (Chen et al., 2008).

Next to its immunity enhancing effect, the manufacturability and stability of the formulation represents a main challenge for the development of a suitable and potent adjuvant. The objective of this study was the development of three different adjuvant systems for a hydrophilic model peptide and its physico-chemical evaluation over an observation time of 12 weeks (stored at $T = 2–8\,^{\circ}C$). Aqueous dispersions as well as lyophilised powders of these adjuvants were investigated. The model peptide used was a linear and hydrophilic decapeptide, which could be successfully incorporated in all three carrier systems and its entrapped amount between the three systems was compared. As indicators of sufficient system stability, the morphology, size, PDI, zeta-potential, pH, and the determination of the incorporated peptide amount were selected. Sucrose was chosen as the cryoprotective agent for all lyophilised formulations and added in appropriate amounts to stabilize the freeze-dried products.

2. Materials and methods

2.1. Materials

Poly(D,L-lactic-co-glycolic acid) 50/50 (PLGA), inherent viscosity 0.16–0.24 dl/g, was purchased from Boehringer Ingelheim (Ingelheim, Germany). Polyvinylalcohol (PVA) (average mol wt 30,000–70,000) and dextran sulphate (sodium salt, from *Leuconostoc* spp., average mol wt 9000–20,000) was obtained from Sigma–Aldrich (Steinheim, Germany). Chitosan (Chitosan 90/10, Chitoscience, 13 mPa s) was produced by Heppe Medical Chitosan GmbH (Halle, Germany). For all liposomal experiments the lipid 1,2-dioleoyloxy-3-trimethylammoniumpropane (DOTAP) chloride (MW: 698.54 g/mol) was used and obtained from Merck & Cie (Schaffhausen, Switzerland). Cholesterol (MW: 386.67 g/mol) was purchased from Avanti Polar Lipids (Alabaster, USA).

Methanol, acetonitrile (both LiChrosolv®, gradient grade), chloroform (SeccoSolv®, max 0.003% H_2O), methylene chloride (pro analysi), ammonia acetate (pro analysi), ammonia solution 25% (EMSURE®), acetic acid (glacial, pro analysi), and sucrose (biochemistry purpose) were kindly donated by Merck KGaA (Darmstadt, Germany). Purified water of MilliQ quality (MILLIPORE® Q-POD™) was used for all experiments. The peptide (IATFKNWPFL-OH, MW: 1236.5 g/mol, purity determined by HPLC 99.2%) was purchased from Peptisyntha, Inc. (Torrance CA, USA). For purifying the drug delivery systems by dialysis Spectra/Por® dialysis membrane (MWCO 6-8000, Spectrum Laboratories Inc., USA) was used.

2.2. Methods

2.2.1. Preparation of peptide-loaded PLGA nanoparticles

The hydrophilic decapeptide was encapsulated in PLGA nanoparticles using the double water/oil/water solvent evaporation technique at room temperature (Coombes et al., 1998; Feczkó et al., 2008). Briefly, the hydrophilic peptide (2 mg/ml) was dissolved in 1% (w/v) PVA solution to form the inner aqueous phase. The organic phase was obtained by dissolving 5% (w/v) PLGA in methylene chloride. To form the w/o emulsion the two phases were combined and sonicated for 60 s (amplitude 42%) by a SONOPLUS HD 2070 (Bandelin). This w/o emulsion thereafter was poured into the external aqueous phase containing 1% (w/v) PVA solved in deionised water and also was sonicated for 30 s (amplitude 42%) to obtain the final w/o/w emulsion. The three phases were used in a ratio of 16/4/1 for outer aqueous/organic/inner aqueous phase. Finally, the organic solvent was evaporated 3.5 h under stirring at room temperature, and nanoparticles were formed due to polymer precipitation. To restore the initial concentrations, the amount of evaporated organic solvent was replaced by 1% (w/v) PVA solution. Afterwards, the nanoparticle suspension was dialyzed 24 h with 5 l of milli-Q water to wash out unbound peptide.

2.2.2. Preparation of peptide-loaded chitosan-dextran sulphate microparticles

Chitosan microparticles were prepared in very mild conditions based on the polyelectrolyte complexation technique at room temperature by modifying the method of Chen and Calvo (Calvo et al., 1997; Chen et al., 2003, 2007). Various preliminary experiments were performed (data not shown) in order to determine optimal concentration conditions for all solutions.

Firstly, a 0.175% (w/v) acetic acid solution was prepared as already used by Chen et al. (2003). 0.1% (w/v) chitosan was dissolved in the acetic acid solution, and 0.72 mg/ml peptide was added. Due to the pH 3.2 of the acetic acid solution, the chitosan molecule became positively charged and soluble. Separately, 0.1% (w/v) dextran sulphate also was dissolved in a defined volume of the 0.175% (w/v) acetic acid solution to form the oppositely charged polymer solution. Next, the two phases were combined drop wise by means of an Eppendorf pipette under magnetic stirring and room temperature the 1.4 fold volume of acid dextran sulphate solution slowly into the acid chitosan-peptide solution. The dispersion turned turbid and particles were formed spontaneously. The final peptide concentration in the acidic chitosan-dextran sulphate solution thus was 0.3 mg/ml.

After 30 min of stirring, the particle suspension was sonicated for 60 s (amplitude 42%) with a SONOPLUS HD 2070 (Bandelin) to homogenize the particles. To remove unentrapped peptide, the particles were centrifuged (14,000 rpm, 15 min, 21 °C, Eppendorf 5804R), the supernatant was collected for peptide entrapment determination, and the obtained sediment afterwards was suspended in the same volume of milli-Q water. This purification step was repeated three times (14,000 rpm, 8 min, 21 °C, Eppendorf 5804R).

2.2.3. Preparation of peptide-loaded DOTAP-liposomes

To embed the hydrophilic decapeptide in DOTAP-liposomes, the lipid film hydration method (Bangham et al., 1965) with minor modifications was used. On the basis of previous results, the presence of cholesterol to stabilize the liposome membrane was beneficial and therefore it was employed for liposome preparation.

Briefly, the total lipid amount of 28.3 μmol/ml DOTAP and cholesterol 1:1 (μmol/μmol) was dissolved in a defined volume of chloroform and filled in a 100 ml round-bottom flask. A thin film was formed on the inner surface of the glass vessel by removing the organic solvent under reduced pressure (1–10 mbar) on a rotary evaporator (Rotavapor R-134, Büchi, Essen, Germany) at 40 °C (water bath) for 1 h. The film then was stored at least for 12 h at 2–8 °C. The next day, the film was hydrated in a defined volume (twice the volume for chloroform) of an aqueous peptide solution (0.25 mg/ml, set to pH 9 with ammonia solution 25%) containing 7% (w/v) sucrose as cryoprotector. The mixture was shaken by hand until all lipid film was dissolved. The dispersion turned turbid and liposomes were formed. The liposome suspension was homogenized with an Ultra-turrax (IKA®T10 basic Ultra-turrax®) for 10 min (grade 6, motor speed about 30,000/min) to obtain a homogenous dispersion.

After preparation, all drug delivery system formulations were analyzed and immediately stored or freeze-dried and stored at $T = 2–8$ °C.

2.2.4. Physico-chemical characterisation of the drug delivery systems

The size, polydispersity index (PDI), and zeta-potential of the particle systems before and after freeze-drying were determined by photon correlation spectroscopy (PCS) using a Malvern Zetasizer Nanoseries-ZS (Malvern Instruments, Worcestershire, UK). Briefly, a defined volume of the particle dispersions (20 μl of the PLGA nanoparticles and 2 μl of the DOTAP-liposome suspension) were diluted in 980 or 998 μl, respectively deionised water (filtered before usage trough a 0.22 μm polystyrene filter) and filled in a 1.5 ml semi-micro disposable cuvette. For the zeta-potential measurements folded capillary cells were used. The measurements were performed at $T = 25$ °C with a detection angle of 173°. Data analysis was accomplished by the Malvern DTS software and reported as the *z*-average, the mean hydrodynamic diameter of the particles (average of two measurements, each involving 10 runs).

The micro-flow-imaging (MFI) technique with a DPA 4100 flow microscope (BrightWell Technologies Inc., Ottawa, Canada) was used for the size characterisation of the chitosan particle formulations. The technique is based on digital microscopy. Particles in colloidal formulations pass trough a flow-cell while images were captured by a digital camera. In each image every individual particle was analyzed in size as well as morphology and the mean size average and the size distribution was determined with the system software (MVSS). The instrument is configured for particle size range of 0.75–300 μm. 25 μl of the chitosan-dextran sulphate particle solution was diluted in 600 μl of milli-Q water. 350 μl of the diluted sample was analyzed with a flow rate of 0.1 ml/min. The size of a particle was measured as the equivalent circular diameter (ECD) in microns.

The PLGA nanoparticle and chitosan-dextran sulphate microparticle morphology was characterised by a scanning electron microscope (SEM Supra 35, Carl Zeiss NTS GmbH, Germany), whereas for DOTAP-liposome morphology analysis a transmission electron microscope (TEM, CM20, limitation 2.3 A, FEI®, Netherlands) and SEM (for the lyophilised end product) were used. For the SEM analysis of the PLGA nanoparticles and the lyophilised DOTAP-liposomes, the freeze-dried cakes were placed on a conductive sample appliance made of aluminium and sputtered with platinum. The chitosan microparticles were transferred in liquid form to the sample holder. In order to obtain a very thin suspension for TEM imaging, the liposome dispersion was diluted 1:5 (v/v) with milliQ-water and contrasted 1:1 (v/v) with 4% of a phosphotungstic acid (pH = 7.2). This dispersion was transferred to a copper grid and images were taken after drying.

A 780 pH meter with a micro glass electrode (pH 1–11, Metrohm AG; Zofingen, Switzerland) was used to determine potentiometrically the pH value of the different delivery systems before and after freeze drying after calibration with known standards.

The residual moisture content in the freeze-dried drug delivery systems was measured by indirect coulometric Karl–Fischer-titration using a 756 KF coulometer with a 774 oven sample processor (Metrohm AG, Switzerland). 70 mg of the lyophilised samples were determined in duplicate at different temperatures (150 °C for PLGA nanoparticles; 121 °C for chitosan micropartciles; 100 °C for freeze-dried liposomes) with a gas stream (flow rate 69 ml/min) of dry 0.3 nm nitrogen/air. The average values reported are percentage of residual water content in the samples after freeze-drying.

If not otherwise stated, all test results were run in triplicate and reported as mean ± S.D.

2.2.5. Quantification of the chitosan-dextran sulphate microparticle amount by gravimetry

To quantify the microparticle amount of the chitosan-dextran sulphate formulation, 50 μl of the purified particle dispersion was weighed into an aluminium pan and stored for 2 h at $T = 80$ °C in a compartment dryer (Trockenschrank Serie ED 53, Firma Binder, Germany). For cooling down, the pan was placed in a desiccator for 30 min. After drying and cooling, the pan was weighed again, and the particle amount in the dispersion was calculated by the difference between the weights. In order to determine the amount of microparticles in the formulation, the particle dispersion was filtered through a polypropylen filter with a pore size of 1.2 μm to separate the nanoparticle from the microparticle fraction. The nanoparticle fraction was then determined gravimetrically with a volume of 50 μl as described above and the microparticle fraction was calculated by the following equation afterwards:

$$\text{Microparticle fraction [\%]} = \left[1 - \frac{\text{nanoparticle amount [mg]}}{\text{amount of all particle [mg]}}\right] \times 100 \quad (1)$$

2.2.6. Encapsulation efficiency and drug load of the hydrophilic decapeptide into the drug delivery systems

To determine the encapsulation efficiency (EE) and drug load (DL) after lyophilisation of the drug delivery systems, different strategies were used in accordance with the different properties of the three systems. In case of the freeze-dried formulations the lyophilised cakes were reconstituted in 1 ml of milliQ-water prior to analysis.

To quantify the peptide amount in PLGA nanoparticles, 800 μl of the particle suspension was centrifuged (14,000 rpm, 15 min, Eppendorf 5804R) and 700 μl supernatant was removed. 350 μl acetonitrile was added to the nanoparticle sediment to dissolve the PLGA polymer and after that the same amount of water to dissolve the hydrophilic peptide and to precipitate the polymer. The suspension was vortexed, centrifuged again, and 700 μl of the supernatant containing redundant peptide was analyzed by HPLC (LaChrome Elite, Merck Hitachi High-Technologies Corporation, Japan).

In the case of chitosan-dextran sulphate particles, it was not possible to dissolve the particles. Therefore, the peptide was quantified indirectly by HPLC determination of the non-entrapped amount in the collected supernatants during particle purification.

For the determination of entrapped peptide in DOTAP-liposomes, the liposome dispersion was dialyzed for 24 h with 5 l of a 7% (w/v) sucrose solution. This dialysis under constant osmotic conditions maintained the stability of the liposomes. Subsequently, 500 μl acetonitrile was added to 250 μl purified liposome

Table 1
HPLC parameters for peptide quantification.

Parameters	Drug delivery system		
	PLGA nanoparticles	Chitosan-dextran sulphate microparticles	DOTAP-liposomes
Column	ZIC-HILIC (250 mm × 4.6 mm, 5 μm, 200 A)	Phenomenex (250 mm × 2.0 mm, Jupiter 4 μm Proteo 90 A)	ZIC-HILIC (250 mm × 4.6 mm, 5 μm, 200 A)
Column oven temperature [°C]	30	50	20
Flow rate [ml/min]	1	0.4	1
Detection wave length [nm]	205	210	205
Sample injection volume [μl]	20	20	20

dispersion to dissolve the DOTAP lipid and precipitate the cholesterol. The samples were vortexed and placed for 10 s in an ultrasonic bath (Ultrasonic cleaner USC2100TH, VWR International GmbH, Germany). To separate the released peptide from the destroyed liposomes, the samples were centrifuged (14,000 rpm, 15 min, $T = 21\,^{\circ}C$, Eppendorf 5804R, VWR International GmbH, Germany) and the peptide was determined in the supernatant by HPLC.

In Table 1 the parameters for HPLC analysis of the peptide content in the different drug delivery systems are summarised.

Results were calculated from linear regression of the peptide concentration range of 5 μg/ml to 0.5 mg/ml in milliQ-water. All peptide quantification tests were run in triplicate and the mean values are reported. The encapsulation efficiency (EE) and drug load (DL) of the peptide in the delivery systems are calculated according to Eqs. (2)–(4).

Encapsulation efficiency for PLGA nanoparticles and DOTAP-liposomes.

$$EE\,[\%] = \frac{\text{effective peptide concentration [mg/ml] in drug delivery system}}{\text{inital peptide concentration [mg/ml] in drug delivery system}} \times 100 \quad (2)$$

Encapsulation efficiency for chitosan-dextran sulphate microparticles.

$$EE\,[\%] = \left[1 - \frac{\text{effective peptide concentration [mg/ml] in supernatant}}{\text{inital peptide concentration [mg/ml] in drug delivery system}}\right] \times 100 \quad (3)$$

Drug load calculation for all three particle systems.

$$DL\,[\%] = \frac{\text{entrapped peptide amount [mg]}}{\text{total polymer or lipid amount [mg]}} \times 100 \quad (4)$$

2.2.7. Freeze-drying of peptide-loaded drug delivery systems

For the PLGA and chitosan particles the cryoprotector sucrose (3% (w/v) for PLGA, respectively 5% (w/v) for chitosan) was weighed into 2 ml glass vials (Fiolax, HGB 1/ISO 719, MGlas AG, Münnerstadt, Germany) and 1 ml of the particle dispersion was added. Thus, the sucrose was not incorporated in the PLGA and chitosan particles in contrast to the DOTAP-liposomes. For the DOTAP-liposomes it was essential to integrate the cryoprotector sucrose (7% (w/v)) during liposome preparation in order to ensure the stability of the liposomes during lyophilisation by presence of the sucrose inside and outside of the liposomes.

All vials were then closed with a lyophilisation stopper, shortly vortexed, and placed in the freeze-drier afterwards (Com 6011; Hof Sonderanlagenbau GmbH, Lohra, Germany). The freeze-drying protocols are given in Table 2.

In process I an annealing step was additionally involved, in that the temperature was increased up to −15 °C for 2 h during freezing, followed by cooling down to its originally adjusted temperature. This step was included to allow the ice crystals to grow in order to achieve more porous and extra dry cakes. For the liposome lyophilisation (process II) this procedure was not carried out because of unfavourable stress conditions for the liposomes discovered in previous experiments.

The primary drying temperature was −38 °C for all lyophilisation processes in accordance with the collapse temperature (T_C) of the sucrose which is around −31 °C varying with the chosen indications for onset of collapse (Cook, 2009; Duddu and Dal Monte, 1997; Meister and Gieseler, 2009; Tang and Pikal, 2004). The T_C is defined as the maximum allowable product temperature during primary drying where an amorphous product in the frozen state can maintain its structure. In order to ensure that the samples maintained the temperature for complete solidification during primary drying, a safety factor was added in the freeze-drying experiments.

After lyophilisation all samples were stored at $T = 2$–$8\,^{\circ}C$. Samples were reconstituted with 1 ml of milliQ-water for stability determination.

2.2.8. Stability studies of peptide-loaded drug delivery systems

The different preparations were stored at 2–8 °C for a period of 12 weeks. Samples were taken after 0, 1, 2, 4, 6, 8, 12 weeks and the following physico-chemical properties have been examined: (1) particle/liposome size and size distribution (=PDI), (2) zeta-potential, (3) pH value and (4) incorporated peptide.

After consideration and description of the respective drug delivery system itself, a comparison to the other drug delivery systems were performed.

All determinations were run in triplicate and reported as mean ± S.D.

Table 2
Freeze-drying cycles of the different drug delivery systems.

Process step	Process no.	
	(I) PLGA nanoparticles/ chitosan-dextran sulphate microparticles	(II) DOTAP-liposomes
Freezing		
Plate temperature [°C]	−50	−50
Rate [°C/min]	3.7	0.5
Duration [h]	3	2
	Annealing step involved	
Primary drying		
Plate temperature [°C]	−38	−38
Chamber pressure [mbar]	0.04	0.08
Duration [h]	48	48
Secondary drying		
Plate temperature [°C]	25	35
Chamber pressure [mbar]	0.04	0.08
Duration [h]	12	6

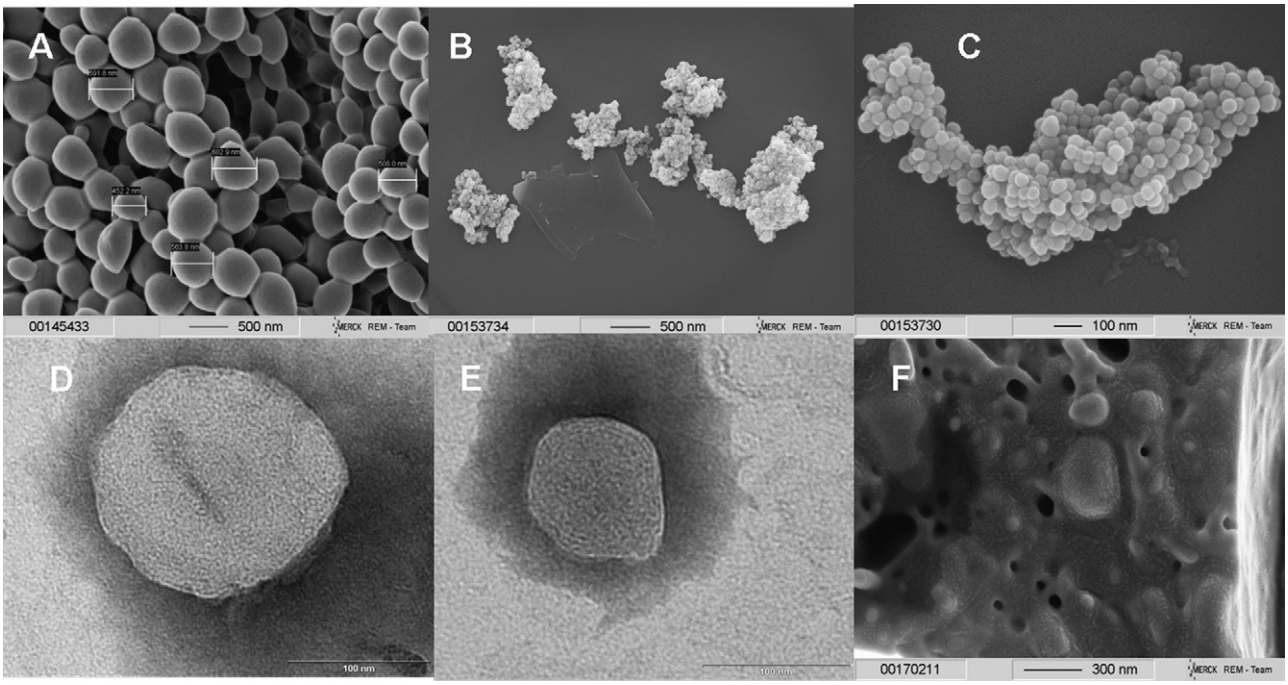

Fig. 1. Morphology of the different peptide-loaded adjuvant drug delivery systems captured with SEM (A, B, C and F) and TEM (D and E). (A) shows freeze-dried PLGA nanoparticles; (B) and (C) chitosan-dextran sulphate microparticle agglomerates; (D) and (E) single DOTAP-liposomes (aqueous formulation); (F) lyophilised DOTAP-liposomes in the presence of 7% sucrose (w/v).

3. Results and discussion

3.1. Morphology of adjuvant drug delivery systems

For the characterisation of the morphologies of the three peptide-loaded drug delivery systems (poly[lactic-co-glycolide] (PLGA) nanoparticles, chitosan-dextran sulphate microparticles, and DOTAP-liposomes), different microscopic techniques for vesicle demonstration were used. The images are given in Fig. 1.

Freeze-dried PLGA nanoparticles (in presence of 3% (w/v) sucrose) were analyzed by scanning electron microscope (SEM, Fig. 1A). Small spherical vehicles in the nanometer size range (400–600 nm) with a smooth surface and without any irregularities were observed. All particles formed individual units and no particle agglomeration appeared to have occurred.

The SEM pictures of the chitosan-dextran sulphate particles (aqueous formulation) are shown in Fig. 1B and C. The chitosan particles formed particle agglomerates with different sizes ranging from 500 nm up to microns (Fig. 1B). These microparticle agglomerates consisted of small individual and spherical nanoparticles, each with smooth surfaces (Fig. 1C). After lyophilsation the chitosan particles had a similar agglomerated appearance, with microparticles scattered all over the cyroprotector matrix (sucrose or trehalose, respectively).

Firstly, the liposomes were analyzed by transmission microscope (TEM, Fig. 1D and E) to demonstrate the membrane structure of the hollow vehicles. For this purpose, single DOTAP-liposomes (placebo) were selected (Fig. 1D and E). These mapped liposomes were mostly spherical with sizes around 100 nm. Furthermore, to demonstrate the liposome's morphology after lyophilisation SEM analysis was investigated additionally and Fig. 1F confirmed the existence of intact liposomes after freeze-drying in the presence of 7% (w/v) sucrose. Individual spherical elevations and holes could be seen in the picture (Fig. 1F) scattered over the sucrose matrix. These spherical elevations showed intact liposome vehicles with diversity in size (50–300 nm). The holes next to the liposomes were artefacts caused by sample preparation.

3.2. Determination of the residual moisture content after freeze-drying

The residual moisture content after freeze-drying determined for all formulations are shown in Table 3. For all systems the water content was < 1%. Therefore, freeze-drying processes I and II led to suitable dried products with a reasonable stability and long shelf life (see below).

3.3. Stability studies of freeze-dried peptide-loaded drug delivery systems

3.3.1. Size and size distribution (PDI) during 12 weeks of storage (2–8 °C)

PLGA nanoparticles without sucrose were manufactured with a size of 430 ± 30 nm and a PDI of 0.30 ± 0.04 before freeze-drying. The aqueous suspension of the PLGA-particles was stable for only a few days (at T = 2–8 °C), and already after one week the polymer precipitated resulting in increasing size and PDI followed by the dispersion's collapse. Therefore, for the PLGA drug delivery system the lyophilisation of the particle dispersion was necessary. The presence of a sugar-based cryoprotector with an amount of at least 2% (w/v) was essential to manufacture a stable PLGA product because in the absence of a cryoprotector during freeze-drying the resulting product cake showed insufficient stability (collapse, Fig. 2A) followed by a decrease in particle homogeneity after freeze-drying ($PDI_{without\ cryoprotector}$ 0.46 ± 0.09). In contrast, in the presence of 3% (w/v) sucrose (lyophilised with process I) the nanoparticle

Table 3
Residual moisture contents after freeze-drying of the three colloidal drug delivery systems (mean ± S.D.; n = 2).

Drug delivery system	Residual moister content [%]
PLGA particles	0.19 ± 0.01
Chitosan-dextran sulphate particles	0.53 ± 0.02
DOTAP-liposomes	0.76 ± 0.2

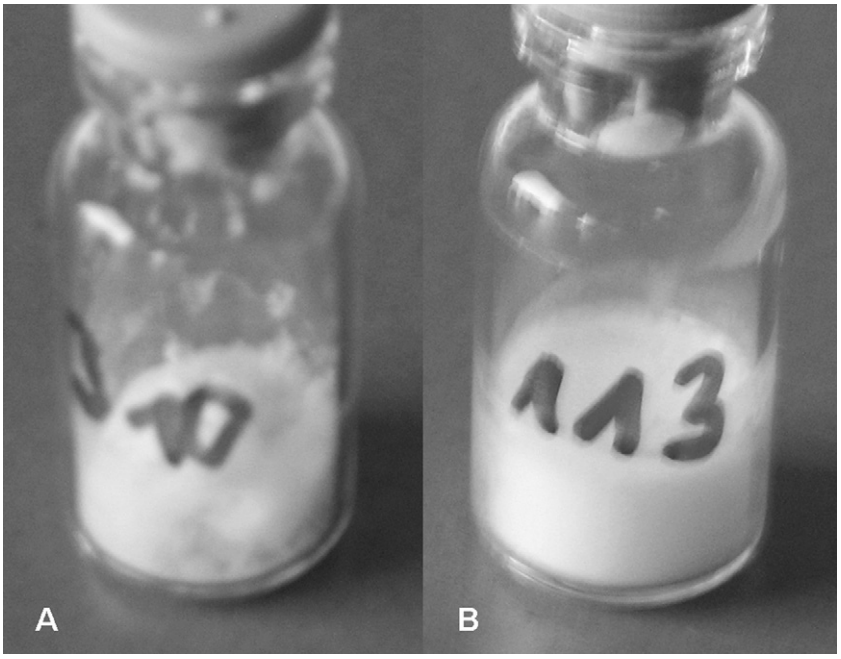

Fig. 2. Lyophilised PLGA cakes freeze-dried by process I. (A) shows a collapsed cake structure obtained if sucrose is absent during freeze-drying, whereas (B) represents a homogeneous PLGA cake, which was lyophilised in presence of 3% sucrose (w/v).

size and PDI after freeze-drying (Fig. 3, Day 0, size = 410 ± 23 nm; PDI = 0.23 ± 0.01) and a compact and homogenous cake structure (Fig. 2B) were maintained. Furthermore, these data disclosed constancy over the 12 weeks period (Fig. 3).

SEM analysis (Fig. 1B) indicated that the chitosan-dextran sulphate particles formed nanoparticle agglomerates in the micron size range. For this reason, the respective percentages of the nanoparticle content relative to the microparticle amount were quantified gravimetrically after separation by filtration with a 1.2 μm filter. About 80% of all peptide-loaded chitosan-dextran sulphate particles were larger than 1.2 μm. Therefore, the complete microparticle fraction without prior filtration was analyzed for stability testing. This formulation exhibited microparticles with an average size of 1.9 ± 0.1 μm before freeze-drying. Fig. 3 shows the microparticle size distribution over the time period of 12 weeks. On Day 0, the preparation was composed of 81% of particles with sizes in the range between 1 and 2 μm, 15% with sizes between 2 and 5 μm and 3.4% between 5 and 10 μm. The average size of the chitosan formulation after freeze-drying with process I was 1.7 ± 0.07 μm. The addition of 5% sucrose (w/v) as cryoprotective agent after particle preparation was required for stabilisation but had no influence on the particle sizes as with PLGA. In presence of 5% (w/v) sucrose the peptide-loaded chitosan-dextran sulphate particle formulation showed a great cake homogeneity, and during 12 weeks the particle size also stayed constant (Fig. 3). The corresponding aqueous formulation (without sucrose) equally stayed stable in particle size during 12 weeks (~2.15 μm).

The DOTAP-liposomes prepared in the presence of 7% (w/v) sucrose yielded sizes of 460 ± 40 nm and a PDI of 0.32 ± 0.09 before freeze-drying. In contrast to the solid particle systems, the sugar based cryoprotective agent sucrose had to be already present during liposome preparation, because as previously observed it was essential to maintain constant conditions inside and outside of the liposome core. If the sugar was not incorporated into the liposome interior, freeze-drying resulted in complete peptide leakage due to insufficient membrane stability. After lyophilisation (process II) the DOTAP-liposome size decreased around 100 nm (Fig. 3, Day 0; 360 ± 4 nm) accompanied by a decrease in homogeneity (PDI 0.43 ± 0.04). These two parameters remained constant over the 12 weeks (Fig. 3). The corresponding aqueous liposomal formulation in the presence of 7% (w/v) sucrose showed similar constant sizes (~425 nm) and PDI (~0.29) during storage (T = 2–8 °C) over this time period.

3.3.2. Zeta-potential during 12 weeks of storage (2–8 °C)

Table 4 shows the zeta-potential of all three formulations before freeze-drying. In all cases the zeta-potential was high (irrespective of the positive or negative charge) thus leading to a high stability against coalescence. PLGA and chitosan-dextran sulphate formed negatively charged particle systems whereas DOTAP-liposomes were positively charged. The high negative charge of the chitosan-dextran sulphate particles was due to an excess of free (SO_4^-)-groups. This excess of (SO_4^-)-groups was also necessary to entrap the hydrophilic peptide into these particles via electrostatic bounds. Higher chitosan concentrations compared to the amount of dextran sulphate resulted in positively charged particles (chitosan/dextran sulphate 0.15/0.1% (w/v): 36 ± 0.3 mV; chitosan/dextran sulphate 0.2/0.1% (w/v): 47 ± 1 mV) as also observed by the group of Sarmento et al. (2006) which in our case after a short time led to a collapse of the dispersion and in the precipitation of the polymer. In contrast, the DOTAP-liposomes were positively charged because of the positively charged quaternary ammonium group in the head region of the DOTAP lipid. At a pH of 9 the hydrophilic peptide was negatively charged due to its isoelectric point of pKI = 7, and hence it could easily interact with the

Table 4
Zeta-potential and pH values before freeze-drying of the colloidal drug delivery systems (mean ± S.D.; n = 3).

Drug delivery system	Zeta-potential [mV] before freeze-drying	pH before freeze-drying
PLGA particles	−30 ± 0.6	4.8 ± 0.2
Chitosan-dextran sulphate particles	−50 ± 1	4.8 ± 0.04
DOTAP-liposomes	+55 ± 0.6	7.9 ± 0.3

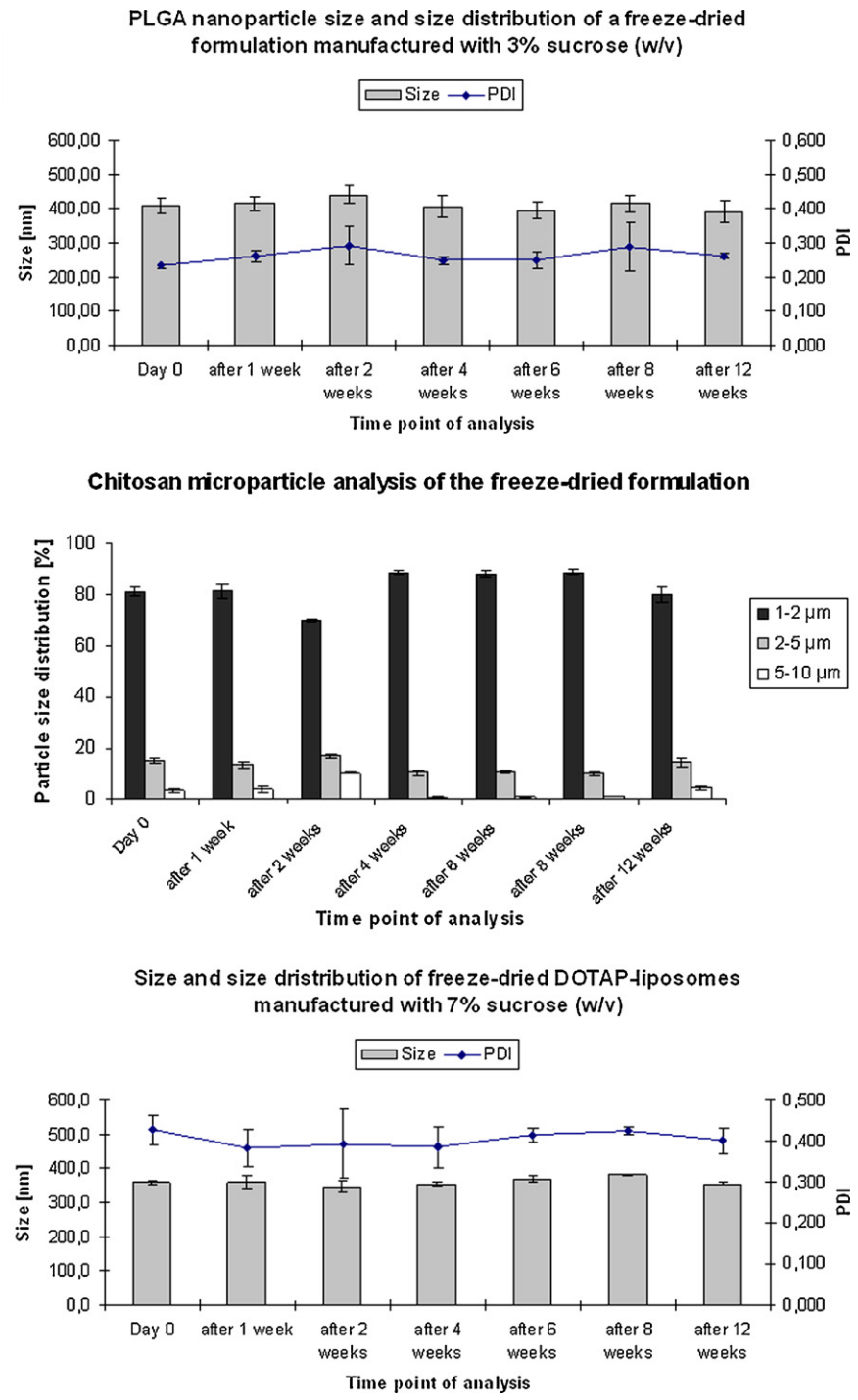

Fig. 3. Particle size and size distribution of different colloidal freeze-dried drug delivery system formulations during 12 weeks of storage (T = 2–8 °C, mean ± S.D.; n = 3). PLGA and DOTAP size were analyzed using a Malvern Zetasizer Nanoseries-ZS, the chitosan microparticles by micro-flow-imaging (MFI).

DOTAP lipid resulting in its encapsulation into the hollow aqueous liposome core.

All drug delivery systems maintained their original surface charges after freeze-drying (Fig. 4, Day 0). Consequently the addition of sucrose did not influence the initial surface charge of the drug delivery systems.

During 12 weeks of storage (T = 2–8 °C) all freeze-dried drug delivery systems exhibited constant zeta-potential results. Aqueous preparations of chitosan and DOTAP formulations also showed no significant differences in zeta-potential behaviour compared to their respective freeze-dried formulations during 12 weeks of storage.

3.3.3. *pH value observation during 12 weeks of storage (2–8 °C)*

In Table 4 the initial pH values of all three drug delivery systems after preparation and before freeze-drying are presented. PLGA and chitosan peptide-loaded particle based systems showed similar acidic pH values before freeze-drying. The pH was adjusted to 9 with 25% ammonia solution to ensure beneficial alkaline conditions for peptide incorporation into the liposomal DOTAP formulation.

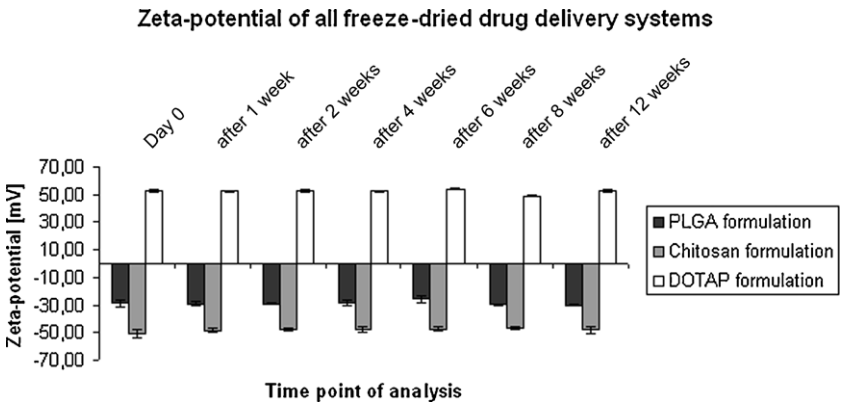

Fig. 4. Zeta-potential of different colloidal freeze-dried drug delivery system formulations during 12 weeks of storage (T = 2–8 °C, mean ± S.D.; n = 3).

After peptide addition and liposome preparation the pH dropped to 7.9 (Table 4).

The resuspended freeze-dried PLGA and chitosan-dextran sulphate formulations maintained their pH after lyophilisation and during storage (Fig. 5). Consequently the cryoprotector sucrose did not influence the pH of the freeze-dried formulation (Table 4 versus Fig. 5, Day 0). In contrast to the sizes and zeta-potential the pH dropped in aqueous chitosan particle formulation during storage. After 8 weeks of storage a small pH decrease was observable, and already after 12 weeks the pH dropped to 4.4 ± 0.04.

The pH of the reconstituted DOTAP-liposome formulation decreased to a pH of 6.6 ± 0.07 (Day 0, Fig. 5) after freeze-drying. The addition of a buffer system in combination with the hydrophilic peptide was not possible because flocculation accompanied by a rupture of the liposomes occurred. Nevertheless the pH of the freeze-dried liposomal DOTAP formulation remained constant at ~6.6 for 12 weeks of storage (Fig. 5). In contrast, the aqueous liposomal formulation showed a decrease from pH 6.6 to 5.7 ± 0.04 after 4 weeks and to 5.2 ± 0.04 after 12 weeks.

3.3.4. *Determination of incorporated peptide during 12 weeks of storage (2–8 °C)*

The three drug delivery systems showed substantial differences in peptide loading respectively the encapsulation efficiency (EE) due to their different physico-chemical properties. The EE into PLGA particles was the lowest compared to all three carrier systems with a value of about 15% and a drug load (DL) of 0.15% before freeze-drying due to the required high polymer amount (initial peptide/polymer ratio 1/100 (w/w)). The positively charged DOTAP-liposomes facilitated the entrapment of the peptide. The adjustment of the pH to 9 resulted in an entrapment of the peptide with an EE of about 20% before freeze-drying. The corresponding DL was 0.33 ± 0.03%. The highest entrapment of the peptide was achieved with the chitosan-dextran sulphate particles. Up to 90% (EE) of the initial peptide amount was incorporated into the chitosan particles with a DL of 27 ± 0.6%.

The encapsulation efficiencies after freeze-drying of all drug delivery systems are given in Fig. 6 on Day 0. After lyophilisation the entrapment of the hydrophilic peptide decreased slightly around 15–17% for both, PLGA and chitosan-dextran sulphate particles. This can be explained by the centrifugation for peptide entrapment analysis (explained in Section 2.2 above), whereby peptide could have been squeezed out due to the high centrifugation forces. Regarding the encapsulation of the hydrophilic peptide into DOTAP-liposomes after freeze-drying, the entrapment was actually slightly improved with the support of peptide from the outer media.

The encapsulation efficiencies of the peptide in all drug delivery systems remained constant for 12 weeks of storage (T = 2–8 °C). In contrast, the corresponding aqueous formulations showed no adequate peptide encapsulation stability except for the DOTAP-liposomes. The liquid PLGA particle dispersion was not stable for 2 weeks. After 8 weeks of storage (T = 2–8 °C) the aqueous chitosan-dextran sulphate dispersion released about 30% of the peptide. Only the aqueous DOTAP-liposome formulation showed no significant difference compared to the freeze-dried formulation (EE ~20%) when peptide also was present in the outer media. The peptide content remained constant without structure degradation in aqueous media at a pH of 5 or 7 during 12 weeks (T = 2–8 °C).

Interestingly, if the lyophilisation process was performed at a primary drying temperature above the critical temperature of the products ($T_{C\ sucrose}$ = −31 °C), the peptide binding was much less stable resulting in leakage after 8 weeks similar to the corresponding aqueous formulations, although the optical appearance of the

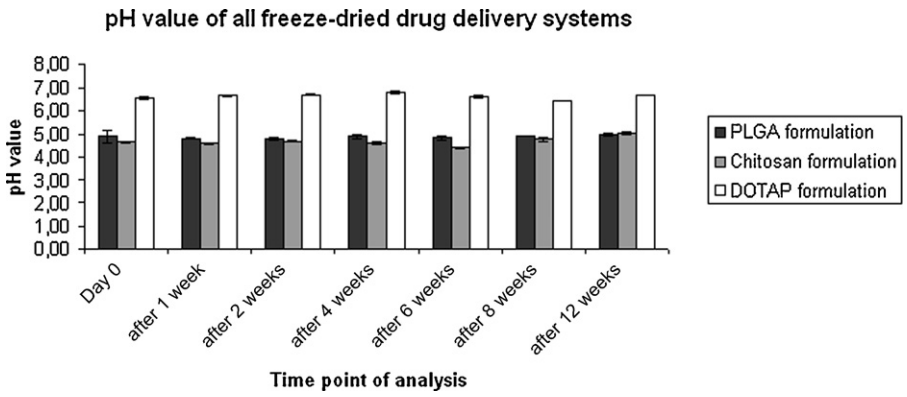

Fig. 5. pH values of different colloidal freeze-dried drug delivery system formulations during 12 weeks of storage (T = 2–8 °C, mean ± S.D.; n = 3).

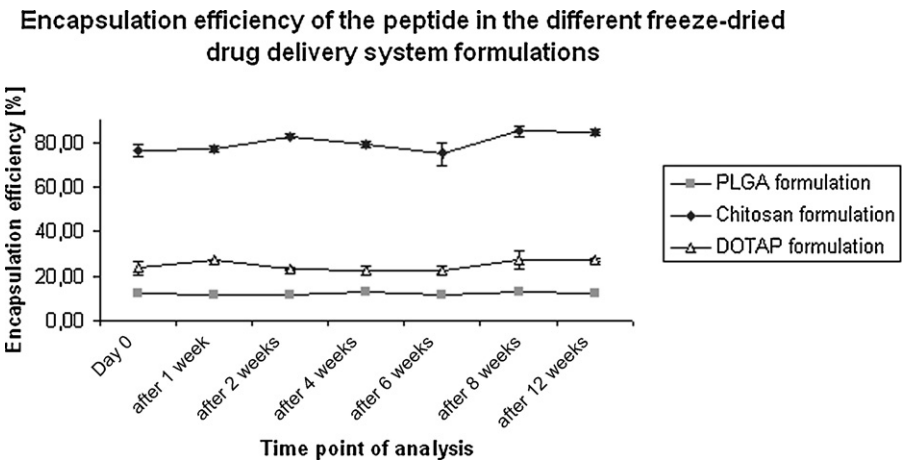

Fig. 6. Encapsulation efficiencies of different colloidal freeze-dried drug delivery system formulations during 12 weeks of storage (T = 2–8 °C, mean ± S.D.; n = 3).

lyophilised cake was homogenous and no structural deficits were observable. This was seen in PLGA and chitosan exploratory formulation experiments when freeze-drying was performed at a primary drying temperature of −23 °C. Consequently, also the lyophilisation process by itself has an influence on the long term stability of a lyophilised product, and, hence, adequate conditions of the lyophilisation parameters have to be developed and ensured for successful product stability.

4. Conclusions

This study represents a comparison of the successful encapsulation of a hydrophilic model peptide within three different drug delivery systems, to be used as immunity enhancing adjuvant. PLGA nanoparticles, chitosan-dextran sulphate microparticles, and DOTAP-liposomes were effectively loaded with the hydrophilic model peptide. All three systems were stable after lyophilisation at a primary freezing temperature below −38 °C and addition of sucrose as a cryoprotector. The respective high negative and positive charges also ensured a good colloidal stability. Concerning entrapment efficacy the optimal system appeared to be the chitosan particles. Only in the case of the DOTAP-liposomes it was possible to also formulate aqueous preparations that showed sufficient stability concerning peptide encapsulation for at least 12 weeks. The chitosan-dextran sulphate system as well as the DOTAP-liposomes may be preferred over the PLGA systems from a pharmaceutical perspective for the encapsulation of the hydrophilic model peptide due to a more efficient preparation procedure. In summary, this study has shown a successful and stable peptide loading of different delivery systems which may be considered for vaccination.

References

Agnihotri, S.A., Mallikarjuna, N.N., Aminabhavi, T.M., 2004. Recent advances on chitosan-based micro- and nanoparticles in drug delivery. J. Control. Release 100, 5–28.
Arca, H.Ã.I, Günbeyaz, M., Åženel, S., 2009. Chitosan-based systems for the delivery of vaccine antigens. Expert Rev. Vaccines 8, 937–953.
Bangham, A.D., Standish, M.M., Watkins, J.C., 1965. Diffusion of univalent ions across the lamellae of swollen phospholipids. J. Mol. Biol. 13, 238–252, IN226–IN227.
Calvo, P., Remuñán-López, C., Vila-Jato, J.L., Alonso, M.J., 1997. Novel hydrophilic chitosan-polyethylene oxide nanoparticles as protein carriers. J. Appl. Polym. Sci. 63, 125–132.
Chen, W., Yan, W., Huang, L., 2008. A simple but effective cancer vaccine consisting of an antigen and a cationic lipid. Cancer Immunol. Immunother. 57, 517–530.
Chen, Y., Mohanraj, V., Parkin, J., 2003. Chitosan-dextran sulfate nanoparticles for delivery of an anti-angiogenesis peptide. Int. J. Peptide Res. Ther. 10, 621–629.
Chen, Y., Mohanraj, V., Wang, F., Benson, H., 2007. Designing chitosan-dextran sulfate nanoparticles using charge ratios. AAPS PharmSciTech 8, 131–139.
Cook, I., 2009. Why, What and How? Understanding the Freeze Drying Process. http://www.biopharma.co.uk/wp-content/uploads/2010/08/why_what_how.pdf.
Coombes, A.G.A., Yeh, M.-K., Lavelle, E.C., Davis, S.S., 1998. The control of protein release from poly(-lactide co-glycolide) microparticles by variation of the external aqueous phase surfactant in the water-in oil-in water method. J. Control. Release 52, 311–320.
Cui, Z., Han, S.-J., Vangasseri, D.P., Huang, L., 2004. Immunostimulation mechanism of LPD nanoparticle as a vaccine carrier. Mol. Pharm. 2, 22–28.
Duddu, S.P., Dal Monte, P.R., 1997. Effect of glass transition temperature on the stability of lyophilized formulations containing a chimeric therapeutic monoclonal antibody. Pharm. Res. 14, 591–595.
Feczkó, T., Tóth, J., Gyenis, J., 2008. Comparison of the preparation of PLGA-BSA nano- and microparticles by PVA, poloxamer and PVP. Colloids Surf. A: Physicochem. Eng. Aspects 319, 188–195.
Fini, A., Orienti, I., 2003. The role of chitosan in drug delivery: current and potential applications. Am. J. Drug Deliv. 1, 43–59.
Frazza, E.J., Schmitt, E.E., 1971. A new absorbable suture. J. Biomed. Mater. Res. 5, 43–58.
Fujimura, T., Nakagawa, S., Ohtani, T., Ito, Y., Aiba, S., 2006. Inhibitory effect of the polyinosinic-polycytidylic acid/cationic liposome on the progression of murine B16F10 melanoma. Eur. J. Immunol. 36, 3371–3380.
Gordon, S., Saupe, A., McBurney, W., Rades, T., Hook, S., 2008. Comparison of chitosan nanoparticles and chitosan hydrogels for vaccine delivery. J. Pharm. Pharmacol. 60, 1591–1600.
Gupta, R.K., 1998. Aluminum compounds as vaccine adjuvants. Adv. Drug Deliv. Rev. 32, 155–172.
Jaganathan, K.S., Rao, Y.U.B., Singh, P., Prabakaran, D., Gupta, S., Jain, A., Vyas, S.P., 2005. Development of a single dose tetanus toxoid formulation based on polymeric microspheres: a comparative study of poly(D,L-lactic-co-glycolic acid) versus chitosan microspheres. Int. J. Pharm. 294, 23–32.
Meister, E., Gieseler, H., 2009. Freeze-dry microscopy of protein/sugar mixtures: drying behavior, interpretation of collapse temperatures and a comparison to corresponding glass transition data. J. Pharm. Sci. 98, 3072–3087.
Men, Y., Thomasin, C., Merkle, H.P., Gander, B., Corradin, G., 1995. A single administration of tetanus toxoid in biodegradable microspheres elicits T cell and antibody responses similar or superior to those obtained with aluminum hydroxide. Vaccine 13, 683–689.
Nakanishi, T., Kunisawa, J., Hayashi, A., Tsutsumi, Y., Kubo, K., Nakagawa, S., Fujiwara, H., Hamaoka, T., Mayumi, T., 1997. Positively charged liposome functions as an efficient immunoadjuvant in inducing immune responses to soluble proteins. Biochem. Biophys. Res. Commun. 240, 793–797.
O'Hagan, D.T., Singh, M., Gupta, R.K., 1998. Poly(lactide-co-glycolide) microparticles for the development of single-dose controlled-release vaccines. Adv. Drug Deliv. Rev. 32, 225–246.
Peek, L.J., Middaugh, C.R., Berkland, C., 2008. Nanotechnology in vaccine delivery. Adv. Drug Deliv. Rev. 60, 915–928.
Sarmento, B., Ribeiro, A., Veiga, F., Ferreira, D., 2006. Development and characterization of new insulin containing polysaccharide nanoparticles. Colloids Surf. B: Biointerfaces 53, 193–202.
Schijns, V.E.J.C., 2006. Immunopotentiators in Modern Vaccines. Elsevier Academic Press.
Singh, M., Singh, A., Talwar, G.P., 1991. Controlled delivery of diphtheria toxoid using biodegradable poly(D,L-lactide) microcapsules. Pharm. Res. 8, 958–961.
Tang, X., Pikal, M., 2004. Design of freeze-drying processes for pharmaceuticals: practical advice. Pharm. Res. 21, 191–200.
Vangasseri, D.P., Cui, Z., Chen, W., Hokey, D.A., Falo, L.D., Huang, L., 2006. Immunostimulation of dendritic cells by cationic liposomes. Mol. Membr. Biol. 23, 385–395.