Pharm Res (2014) 31:2593–2604
DOI 10.1007/s11095-014-1355-x

RESEARCH PAPER

Nanoparticles Based on a Hydrophilic Polyester with a Sheddable PEG Coating for Protein Delivery

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Received: 15 January 2014 / Accepted: 27 February 2014 / Published online: 14 March 2014

ABSTRACT

Purpose To investigate the effect of polyethylene glycol (PEG) in nanoparticles based on blends of hydroxylated aliphatic polyester, poly(D,L-lactic-co-glycolic-co-hydroxymethyl glycolic acid) (PLGHMGA) and PEG-PLGHMGA block copolymers on their degradation and release behavior.

Methods Protein-loaded nanoparticles were prepared with blends of varying ratios of PEG-PLGHMGA (molecular weight of PEG 2,000 and 5,000 Da) and PLGHMGA, by a double emulsion method with or without using poly(vinyl alcohol) (PVA) as surfactant. Bovine serum albumin and lysozyme were used as model proteins.

Results PEGylated particles prepared without PVA had a zeta potential ranging from ~ −3 to ~−35 mV and size ranging from ~200 to ~600 nm that were significantly dependent on the content and type of PEG-block copolymer. The encapsulation efficiency of the two proteins however was very low (<30%) and the particles rapidly released their content in a few days. In contrast, all formulations prepared with PVA showed almost similar particle properties (size: ~250 nm, zeta potential: ~− 1 mV), while loading efficiency for both model proteins was rather high (80–90%). Unexpectedly, independent of the type of formulation, the nanoparticles had nearly the same release and degradation characteristics. NMR analysis showed almost a complete removal of PEG in 5 days which explains these marginal differences.

Conclusions Protein release and particle degradation are not substantially influenced by the content of PEG, likely because of the fast shedding of the PEG blocks. These PEG shedding particles are interesting system for intracellular delivery of drugs.

Electronic supplementary material The online version of this article (doi:10.1007/s11095-014-1355-x) contains supplementary material, which is available to authorized users.

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KEY WORDS biodegradable · nanoparticles · polyethylene glycol (PEG) · protein release · shedding

INTRODUCTION

Biodegradable nanoparticles based on aliphatic polyesters are presently under investigation as injectable colloidal systems for the targeted (intracellular) delivery of classical drugs as well as biotherapeutics (1–3). It is well known that to improve the biodistribution of nanoparticles, *e.g.* tumor accumulation, their surface properties should be modified to give them a stealth character. One of the most commonly used strategies to increase the circulation half-life of i.v. injected nanoparticles is to cover the surface with a hydrophilic, flexible and non-ionic polymer, such as poly(ethylene glycol) (PEG) (4–6). The PEG coating, however, may obstruct the entry of nanoparticles into the target cells (7). But even when the nanoparticles are endocytosed, the PEG layer may adversely affect endosomal escape (8). The therapeutic efficacy of sterically stabilized nanoparticles can be enhanced by means of PEG shedding after arrival of the nanoparticles at the target site. Different approaches have been used for the design of PEG sheddable nanoparticles such as shedding by degradation of the coating material itself (9); shedding by cleavage of a chemical bond between the stabilizing polymer and its anchor (7) and shedding by spontaneous extraction of stealth polymer from the nanoparticles (10). The shedding kinetics should however be optimized: when the stealth coating is shed too fast, the circulation kinetics will be negatively affected whereas a too slow shedding will hamper cellular uptake.

In addition to providing a protective hydrophilic layer, some other characteristics of the nanoparticles such as particle surface charge and geometry, drug loading and release

behavior of encapsulated compounds are affected by PEG (11–13). It should be mentioned that PEG is not only present at the particle surface but might also be present in the bulk of the particles. The hydrophilic nature of PEG results in a greater water absorbing capacity of the matrix, thereby increasing the hydrolysis kinetics of the polymers and thus degradation rate of the particles (14–16). The release of encapsulated biomacromolecules such as proteins or peptides from PLGA micro and nanoparticles is essentially governed by matrix degradation/erosion (17–20). It has been also reported that polymer–protein interactions and the nature of such interactions (ionic, hydrophobic/hydrophilic) are also determinative factors for both loading and release of the therapeutic agent (21–23). We have recently reported on protein loaded 10–15 μm microparticles and nanoparticles of 400–600 nm in diameter, based on an aliphatic polyester with pendant hydroxyl groups (poly(D,L-lactic-co-glycolic-co-hydroxymethyl glycolic acid), PLGHMGA), that were prepared using an emulsion solvent evaporation technique with PVA as a surfactant. It was demonstrated that release patterns of bovine serum albumin as a model protein and octreotide as model peptide was modulated by varying the density of pendant hydroxyl groups (24–27). However, for i.v. administration and intracellular delivery of therapeutic proteins, stealth particles with smaller size (<400 nm) are preferred (28).

Therefore, in the present study we explored the preparation of PEGylated nanoparticles, based on the same polyester, and studied the effect of PEG content and molecular weight on particle properties (size, charge), particle degradation and release behavior. Nanoparticles were prepared from blends of PLGHMGA with two different PEG-PLGHMGA block copolymers varying in molecular weight of the PEG block, by a double emulsion solvent evaporation method. The effect of blend composition, *i.e.* the amount and type of PEG-PLGHMGA block copolymer, on nanoparticles properties, degradation and protein release was investigated. BSA and lysozyme, proteins with different molecular weights and isoelectric points, were chosen as model proteins.

MATERIALS AND METHODS

Materials

O-Benzyl-L-serine was purchased from Senn Chemicals AG (Switzerland). Bovine serum albumin (BSA), poly(vinyl alcohol) (PVA; MW 30,000–70,000; 88% hydrolyzed), tin(II) 2-ethylhexanoate (SnOct2) and poly(ethylene glycol) monomethyl ether (MePEG) with molecular weights of 5,000 and 2,000 were obtained from Sigma Chemical Company (USA). Hen egg-white lysozyme was purchased from Fluka (Belgium). D,L-lactide was obtained from Purac, The Netherlands. *N*,*N*-dimethylformamide (DMF), peptide grade dichloromethane (DCM), methanol, ethyl acetate, chloroform and tetrahydrofuran (THF) were purchased from Biosolve (The Netherlands). Benzyl alcohol, disodium hydrogen phosphate (Na_2HPO_4) and sodium dihydrogen phosphate (NaH_2PO_4) were obtained from Merck (Germany). Toluene from Acros (Belgium) was stored over 3 Å molecular sieves. *N*,*N*-Dimethylaminopyridine (DMAP) and sodium azide (NaN_3, 99%) were obtained from Fluka (The Netherlands). BCA reagent was from Interchim, USA. Pd/C (Palladium, 10 wt.% on activated carbon, Degussa type E101 NE/W) was purchased from Aldrich (The Netherlands). Unless otherwise stated, all chemicals were used as received.

Synthesis of PEG-PLGHMGA and PLGHMGA Copolymers

Random copolymers of benzyl protected hydroxymethyl glycolide (BMG) and D,L-lactide were synthesized by ring opening polymerization in the melt using benzyl alcohol (BnOH) as initiator and stannous octoate as catalyst, respectively, essentially as described previously (29). Briefly, a mixture of D,L-lactide (1.5 g) and BMG (1.4 g) was loaded into a Schlenk tube followed by the addition of stannous octoate in dry toluene (11 mg, 32 μl from a 338 mg/ml stock solution in toluene) and 6 mg BnOH (M/I ratio of 300/1). After removal of toluene by applying vacuum, the Schlenk tube was closed and subsequently transferred into an oil bath of 130°C. The melt polymerization proceeded overnight and after cooling to room temperature, the crude product was dissolved in chloroform, precipitated into an excess of methanol and vacuum dried overnight. The protecting benzyl groups were removed in a hydrogenation reaction using Pd/C as catalyst (29). PEG-PLGHMGA block copolymers were synthesized with the same method as PLGHMGA using $MePEG_{2000}$ and $MePEG_{5000}$ as initiator. The amounts of MePEG used in polymerization were 109 mg for $MePEG_{2000}$ and 283 mg for $MePEG_{5000}$ (corresponding with M/I ratio of 300/1). The synthesized polymers are denoted as PLGHMGA for the copolymers of D,L-lactic acid, hydroxymethyl glycolic acid (HMG) and glycolic acid and PEG-PLGHMGA for the block copolymers of MePEG and PLGHMGA.

Polymer Characterization

^{1}H NMR analysis of the polymers dissolved in DMSO was performed using a Gemini-300 MHz spectrometer at 298 K. The composition of the PEG-PLGHMGA copolymers as well as their molecular weight and PEG content were determined by ^{1}H NMR, using the following equations:

The molar % of composing units (lactic acid (%L), glycolic acid (%G) and hydroxymethyl glycolic acid (%HMG)) was determined according to the following formulas:

$$I_{HMG} = [(I_{3.8})/2 + (I_{4.2-4.5})/3]$$
$$I_G = (I_{4.7-5.0})/2$$
$$I_L = [I_{5.2-5.4} - (I_{3.8})/2]$$
$$\%\,L = I_L/(I_{HMG} + I_G + I_L) \times 100$$
$$\%\,G = I_G/(I_{HMG} + I_G + I_L) \times 100$$
$$\%HMG = I_{HMG}/(I_{HMG} + I_G + I_L) \times 100$$

where I_{HMG}, I_G and I_L are the peak integrals per one proton of each monomer unit, and I_{number} are the integrals obtained from the NMR spectra at the indicated peak shifts (ppm).

Molecular weight of the diblock copolymers:

$$(I_{HMG}/I_{PEG} \times MW\,HMG\,unit) + (I_L/I_{PEG} \times MW\,L\,unit) + (I_G/I_{PEG} \times MW\,G\,unit) + PEG\,molecular\,weight\,(2000\,or\,5000),$$

where I_{PEG} is the peak integrals per one proton of PEG, %PEG is PEG molecular weight/calculated diblock molecular weight × 100%

The relative molecular weights and molecular weight distributions of the obtained polymers were determined using GPC (Waters Alliance system), with a Waters 2695 separating module and a Waters 2414 refractive index detector. Two PL-gel 5 μm Mixed-D columns fitted with a guard column (Polymer Labs, MW range 0.2–400 kDa) were used and calibration was done using polystyrene standards with narrow molecular weight distributions. THF was used as the mobile phase and the elution rate was 1 ml/min. The thermal properties of the different polymers were measured using differential scanning calorimetry (TA instrument, Q2000). Approximately 5 mg polymer sample was loaded into an aluminum pan, and after heating from room temperature to 120°C, with a heating rate of 10°C/min, the sample was cooled down to −50°C. Thereafter, the sample was heated to 120°C with temperature modulation at ±1°C and a ramping rate of 2°C/min. The second cycle was used to determine the glass transition temperature (T_g) of the synthesized polymers.

Nanoparticle Preparation

Nanoparticles were prepared by a double emulsion solvent evaporation technique (30). Briefly, a solution of protein (BSA or lysozyme) in reverse osmosis water (300 μl, 50 mg/ml) was emulsified in 3 ml dichloromethane (DCM) containing PEG-PLGHMGA or different blends of PEG-PLGHMGA and PLGHMGA (total polymer concentration was 5% w/v) in an ice-bath using an ultrasonic homogenizer (LABSONIC P, B.Braun Biotech) for 1 min at 40% amplitude. The w/o emulsion thus formed was then emulsified into an external aqueous phase (30 ml) with or without surfactant [poly(vinyl alcohol) (5% w/v) in NaCl (0.9% w/v), filtered through 0.2 μm Millipore filter], in an ice-bath using the same ultrasonic homogenizer for 2 min at 60% amplitude to form a water-in-oil-in-water (w/o/w) emulsion. Next, DCM was evaporated at room temperature under reduced pressure for 1 h. The obtained nanoparticles were collected by ultracentrifugation (20,000×*g* for 20 min, J-26XP, Beckman Colter, Avanti ®) and washed twice with 45 ml of 0.9% NaCl in water. Finally, the particles were suspended in a certain volume of sodium phosphate buffer (NaCl 6 mM, Na_2HPO_4 99 mM, NaH_2PO_4 49 mM, NaN_3 4 mM, pH 7.4) to obtain a dispersion of 4–6 mg particles for release and degradation studies. Empty (placebo) nanoparticles were prepared in the same way, but using water without protein as the internal aqueous phase.

Characterization of the Nanoparticles

Nanoparticles were suspended in distilled deionized water and their average size and size distribution were measured using Dynamic Light Scattering (DLS; Zetasizer 4000, Malvern Instruments, Malvern, UK) at 25°C at an angle of 90° (Z-average). The zeta-potential of the nanoparticles, suspended in 10 mM sodium phosphate buffer (NaCl 0.4 mM, Na_2HPO_4 6.6 mM, NaH_2PO4 3 mM, pH: 7.4), was determined by laser Doppler electrophoresis using a Zetasizer Nano-Z (Malvern Instruments Ltd.). The morphology of the nanoparticles was studied by Transmission Electron Microscopy (TEM, Tecnai 10, Philips, 100 kV). The samples for TEM visualization were prepared according to the following procedure: 25 μl of nanoparticle suspension was pipetted onto parafilm, and a formvar/carbon-coated copper grid was placed on top of the sample droplet for 2 min to adsorb particles on the grid. Excess liquid was removed by filter paper. Subsequently, the grid was negatively stained by placing them on top of a 20 μl droplet of 2% uranyl acetate in demineralized water on parafilm for 2 min. Excess liquid was removed by filter paper and the grid was dried for 5 min at room temperature before the measurement.

In Vitro Release of Proteins

The obtained particles (described in "Nanoparticle Preparation" section) were suspended in sodium phosphate buffer and samples of 1 ml of the homogeneous particle suspension were aliquoted into 1.5 ml eppendorf tubes. Two aliquots were taken and particles were washed twice with reverse osmosis water (centrifuged for 20 min at 20,000×*g*; Hermle Z233MK-2 centrifuge) and the obtained pellets were resuspended in deionized water, aliquoted and freeze dried at −50°C and at 0.5 mbar in a Chris Alpha 1–2 freeze-drier (Osterode am Harz,

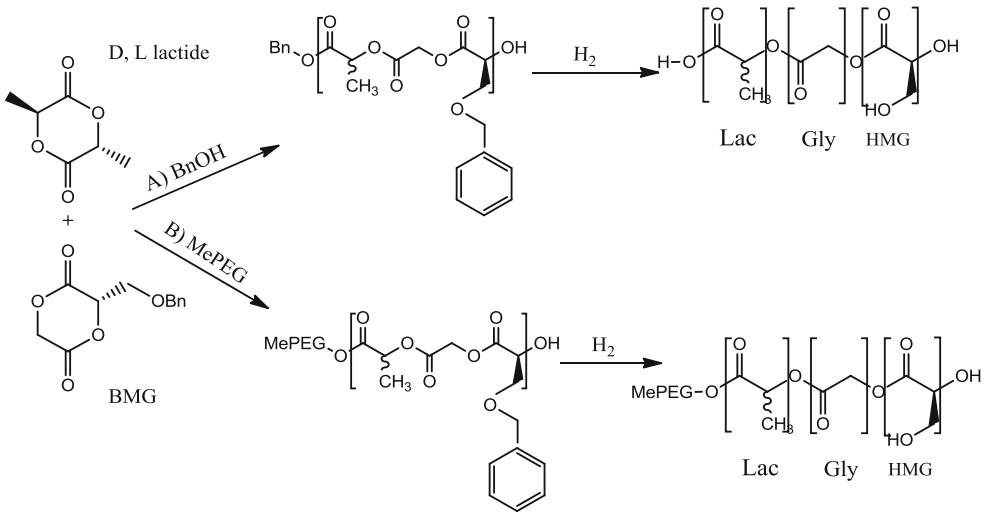

Fig. 1 Synthesis of hydrophilic aliphatic polyesters with pendant hydroxyl groups based on lactic acid, glycolic acid and hydroxymethylglycolic acid: poly(lactic-co-glycolic-co-hydroxymethyl glycolic acid, PLGHMGA). (**a**) PLGHMGA copolymer using Bn-OH as initiator (**b**) PLGHMGA blockcopolymers with PEG using MePEG-OH as macroinitiator.

Germany) for 12 h. Freeze-dried particles were used to determine the protein loading efficiency and particle concentration of the dispersion. The other aliquots were incubated at 37°C under mild agitation. At different time points, one tube was taken and the particles were centrifuged at 20,000×*g* for 20 min and the pH of supernatants was determined. The amount of protein in the supernatant was measured by UPLC (Acquity UPLC®) equipped with a BEH C18 1.7 µm column, using a linear elution gradient starting at 100% solvent A (95% H2O, 5% ACN and 0.1% Trifluoroacetic acid (TFA)) to 60% solvent A and 40% solvent B (100% ACN and 0.1% Trifluoroacetic acid (TFA)) over 6 min, followed by re-equilibration to 100% solvent A in 4 min. The flow rate was 0.25 ml/min, and typically 7.5 µl of sample was injected. Detection was performed by measuring the UV absorbance at 280 nm. Protein standard solutions (10–300 µg/ml) were used for calibration.

Protein Loading Efficiency and Loading %

Protein loading of the nanoparticles was determined by a BCA protein assay, essentially as described by Hongkee *et al.* (31). Briefly, about 10 mg of freeze-dried nanoparticles was dissolved in 1 ml DMSO. Next, 5 ml of a 0.05 M NaOH solution containing 0.5% (w/v) SDS (sodium dodecyl sulfate) was added. After an overnight incubation at 37°C a clear solution was obtained, which was analyzed for protein content. Protein loading efficiency (LE) is defined as the amount of protein entrapped divided by the nominal protein × 100%. The protein loading % (L%) is calculated as the encapsulated amount of protein divided by the dry weight of the loaded particles × 100%.

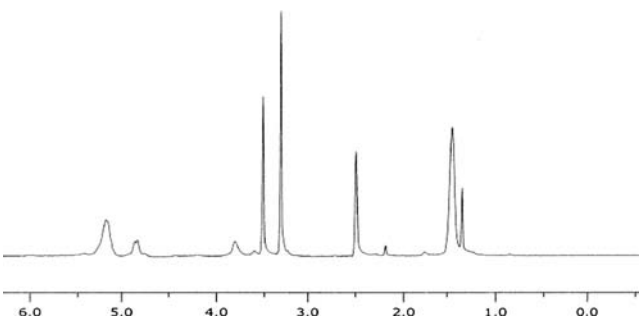

Fig. 2 ^{1}H NMR spectrum of PEG_{5000}-PLGHMGA in deuterated DMSO. δ= 1.3–1.5 (m, 3H, $-CH_3$), 2.5 (s, CH_3, DMSO), 3.3 (s, H_2O), 3.5 (O-$\underline{CH_2}$-$\underline{CH_2}$ of MePEG), 3.7–3.9 (m, 2H, CH-$\underline{CH_2}$-OH), 4.7–5.0 (m, 2H, O-$\underline{CH_2}$-C(O)O), 5.2–5.4 (m, 2H, 1H ($-\underline{CH}$-$\underline{CH_3}$ of lactic acid) plus 1H ($\underline{CH}$-$\underline{CH_2}$-OH of HMG)).

In Vitro Degradation of BSA-Loaded Nanoparticles

Samples of 1 ml of BSA-loaded particles suspension in sodium phosphate buffer at concentration of 4–6 mg/ml ("Nanoparticle Preparation" section) were transferred into eppendorf tubes. The samples were incubated at 37°C while gently shaken. At different time points, one tube was taken and the particles were collected after centrifugation at 20,000 ×*g* for 20 min and washed twice with reverse osmosis water. After freeze-drying, the remaining weight of the samples was measured, and NMR and GPC (see "Polymer Characterization" section) were used to analyze the remaining insoluble residues.

RESULTS AND DISCUSSION

Synthesis and Characterization of the Synthesized Polymers

Random copolymers of benzyl protected hydroxymethyl glycolide (BMG) and D,L-lactide, using BnOH or MePEG (molecular weight of 2,000 or 5,000 Da) as initiators and stannous octoate as catalyst, were synthesized by ring opening polymerization in the melt at 130°C (Fig. 1). After removal of

Table I Characteristics of the Polymers Used in this Study

Polymer	Composition x:y[a]		Molecular weight (Kg/mol)				PEG	Measured
	Feed ratio	Polymer ratio (NMR)	GPC		NMR	Theoretical	W%	T_g (°C)
			M_w	M_n				
$MePEG_{2000}$-PLGBMGA	35:65	38:62	30	19	38	56	5.2	ND
$MePEG_{2000}$-PLGHMGA		31:69	26	16	42	45	4.7	47
$MePEG_{5000}$-PLGBMGA		31:69	23	15	44.5	59	11	ND
$MePEG_{5000}$-PLGHMGA		37:63	20	13	54.4	48	9	42
PLGBMGA		36:64	57	27	–	54	–	ND
PLGHMGA		36:64	44	24	–	43	–	58

ND not determined

[a] x:y denotes the molar ratio of BMG/D,L-lactide or HMG/D,L-lactide

the protective benzyl groups by hydrogenation, the polymers (MePEG-PLGHMGA and PLGHMGA) were obtained in high yields (>70%). The copolymer compositions as well as number average molecular weights for the diblock copolymers were determined by ^{1}H NMR. Figure 2 shows a representative NMR spectrum of one of the synthesized PEG-PLGHMGA block copolymers which demonstrates complete removal of the protecting benzyl groups, in line with previous experiences (29). The characteristics of the synthesized copolymers are given in Table I which demonstrates that the copolymer compositions are close to the monomer feed ratios. Further, the number average molecular weights of the diblock copolymers based on NMR are in good agreement with the theoretical molecular weights.

DSC analysis showed that the synthesized polymers were fully amorphous (Supplementary Fig. 1). PLGHMGA had a T_g at 58°C, and PEG_{5000}-PLGHMGA and PEG_{2000}-PLGHMGA diblock copolymers showed T_g at 42°C and 47°C, respectively (see Table I). Like the diblock copolymers, also the blends of PEG-PLGHMGA and PLGHMGA revealed only one T_g between that of the two components. In polymer blends, with increasing PEG-PLGHMGA weight fraction from 10 to 100%, a decrease of T_g from 58 to 47°C was observed for PEG_{2000}-PLGHMGA blends and from 57 to 42°C for PEG_{5000}-PLGHMGA blends. The observed single

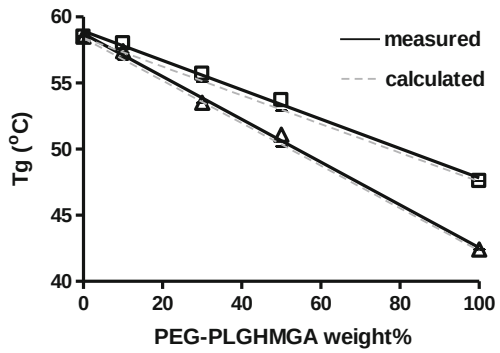

Fig. 3 Measured (DSC) and calculated T_g (Fox equation) of nanoparticles made of Δ) blends of PEG_{5000}-PLGHMGA and □) blends of PEG_{2000}-PLGHMGA.

T_g's both for the diblock copolymers and polymer blends demonstrates miscibility of the PEG and PLGHMGA blocks in the solid state. This is supported by calculating the theoretical T_g for polymer blends using the Fox equation: $1/T_g = W_{PLGHMGA}/T_{g\ PLGHMGA} + W_{diblock}/T_{g\ diblock}$ (31). As illustrated in Fig. 3, the observed linear decrease in experimental T_g values are indeed in good agreement with the values calculated by the Fox equation, demonstrating indeed full miscibility of PLGHMGA and PEG-PLGHMGA (32). Based on DSC data it was previously shown that PEG is also (partially) miscible with structurally related polymers like PLA or PLGA (29).

Preparation and Characterization of Placebo and Protein-Loaded Nanoparticles Without PVA

Nanoparticles with and without protein loading and based on different blends of PEG-PLGHMGA and PLGHMGA were prepared by a double emulsion solvent evaporation method (see Table II). PEG-PLGHMGA is an amphiphilic polymer with surface-active properties. Therefore, nanoparticles were prepared without using a surfactant (PVA) in the external water phase. It was confirmed by NMR that the diblock copolymer was quantitatively incorporated in the particles. Particles prepared without PEG-PLGHMGA diblock copolymers and with low content of PEG_{2000}-PLGHMGA (*i.e.* 10%) showed aggregation, whereas small particles were obtained for all formulations containing PEG_{5000} diblock copolymers. This implies higher shielding of the nanoparticles with PEG 5000 than with PEG 2000, suggesting that even at the lowest contents of diblock copolymer (10%) and thus very low PEG_{5000}-PLGHMGA contents (*i.e.* 0.9%) a PEG corona most likely covers the surface, rendering sterically stabilized particles in aqueous medium. Figure 4 shows that with increasing PEG-PLGHMGA content the particle size as well as polydispersity of particles decreased. This effect is more pronounced for particles prepared using PEG_{2000}-PLGHMGA blends (size decrease from 636 to 213 nm) than for the blends that contained PEG_{5000}-PLGHMGA, which showed a decrease in

Table II Characteristics of Protein Loaded Nanoparticles Formulated with PVA ($n = 2$)

Protein	PEG	PEG-PLGHMGA w%	Hydrodynamic diameter	PDI	L% (BCA)	LE%
BSA	2000	10	289 ± 7	0.10 ± 0.05	7.9 ± 0.0	79 ± 0
		30	263 ± 16	0.08 ± 0.03	7.0 ± 0.1	70 ± 1
		100	247 ± 1	0.08 ± 0.01	7.3 ± 0.0	73 ± 0
	5000	10	246 ± 5	0.06 ± 0.03	8.0 ± 0.4	79 ± 5
		30	238 ± 5	0.07 ± 0.06	7.9 ± 0.1	79 ± 2
Lysozyme	2000	10	268 ± 5	0.05 ± 0.01	10.0 ± 1.0	95 ± 5
		30	262 ± 2	0.06 ± 0.02	9.0 ± 1.0	92 ± 8

particle size from 393 to 249 nm. This decrease in size can be explained as follows: the size of polymeric particles is dependent on the size of the droplets formed during the emulsification process. PEG-PLGHMGA, due to its amphiphilicity and surface active properties, likely locates at the interface of the emulsified droplets and water. As a result, the interfacial tension between the two immiscible phases decreases with increasing PEG content of the formulation resulting in smaller particles. Zeta-potential values of placebo nanoparticles (Fig. 5) demonstrated a significant effect of PEG. As depicted in Fig. 5, the nanoparticles prepared using blends of PEG-PLGHMGA and PLGHMGA showed a decrease in zeta potential with increasing PEG content demonstrating shielding of the surface charge and points to, as expected, localization of PEG chains on the surface of the particles. This figure also shows that at similar block copolymer contents, nanoparticles containing PEG_{5000}-PLGHMGA showed significantly lower zeta potentials than particles containing PEG_{2000}-PLGHMGA (*i.e.* −3 *versus* −24 mV at 30% PEG-PLGHMGA content). In line with expectation, the surface charge masking effect of PEG is dependent on the thickness of the PEG corona, which increases with molecular weight (4). The zeta potential of PLGHMGA nanoparticles before centrifugation was highly negative (−76 mV) which is in agreement with previous findings for PLGA particles (33). The negative zeta-potential of PLGHMGA nanoparticles is due to the charged carboxylic end-groups of the polymer (see Fig. 1a) at pH 7. However, despite their high negative surface charge, PLGHMGA nanoparticles could not be redispersed after centrifugation. Colloids can be stabilized by either electrostatic repulsion or steric stabilization (34). Since stable dispersions were only observed for PEGylated and not for bare PLGHMGA particles, it can be assumed that steric stabilization by PEG is the main contributing factor for the stability of the particle dispersions.

For most of the blend particles prepared in this study, TEM analysis revealed non-porous, spherical particles with a smooth surface (see Fig. 6a for a typical example). Interestingly, a mixture of worm shape and spherical particles were found for the formulation based on only PEG_{5000}-PLGHMGA, that contained the highest amount of PEG of all formulations studied here (*i.e.* 9 w% of PEG; see Fig. 6b). The other particles that all had lower PEG content, including PEG_{2000}-PLGHMGA based particles with 4.7 w% of PEG (see Fig. 6c), gave spherical particles. It has been reported that amphiphilic diblock copolymers self-assemble in dilute aqueous solution into three basic morphologies: spherical micelles, worm-like micelles, and vesicles. The assembly of amphiphilic diblock copolymers into these differently shaped nanostructures depends on the weight fraction of the hydrophilic block (13,35)

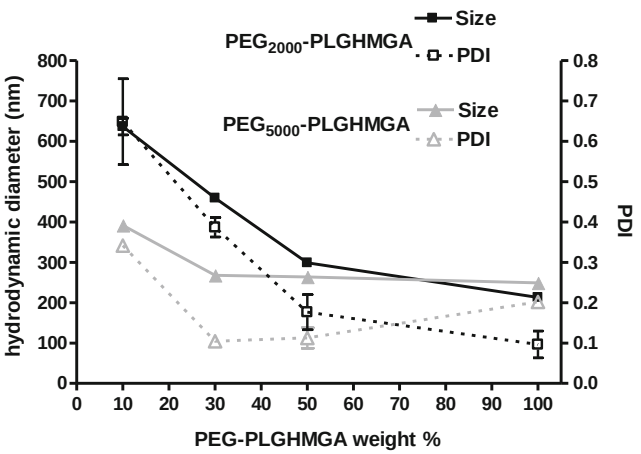

Fig. 4 Size and poly dispersity index of placebo nanoparticles based on blends of PEG-PLGHMGA formulated without PVA ($n = 3$).

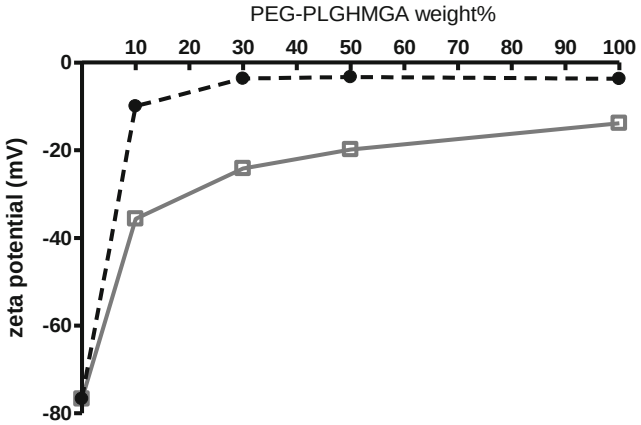

Fig. 5 Zeta-potential of placebo nanoparticles formulated without PVA as a function of the PEG-PLGHMGA weight%. □: PEG_{2000}-PLGHMGA, ●: PEG_{5000}-PLGHMGA.

as well as on the applied processing route (*e.g.*, solvent exchange, film rehydration, pH switch, *etc.*) (36). For PEG-PLA based particles it was previously observed that with a weight fraction of the PEG block less than ~50%, the hydrophilic corona imparts such curvature to the copolymer assembly that worm-like micelles are the predominant morphology (37). Probably, when the PEG content becomes too low (*i.e.* <5%, as for most of our formulations), PEG will not be able to control the particle morphology, and regular round-shaped droplets will be formed during the emulsification process. A detailed influence of block length ratio's on the particle morphology would need further investigation which is however beyond the scope of the present paper.

For protein encapsulation (both lysozyme and BSA) it was observed that the formulations containing 50 and 100% PEG-PLGHMGA, showed no protein incorporation. This can be due to high hydrophilicity of the formulation resulting in high water penetration during particle preparation and thus migration of protein to external water phase. Therefore, in the further experiments lower contents of diblock polymer (*i.e.* 10 and 30%) were used. However, the particles that were prepared using formulations containing 10 and 30% of PEG-PLGHMGA, still showed low encapsulation efficiency (10–30%) and showed complete release of the loaded protein in 1 day (Supplementary Fig. 2).

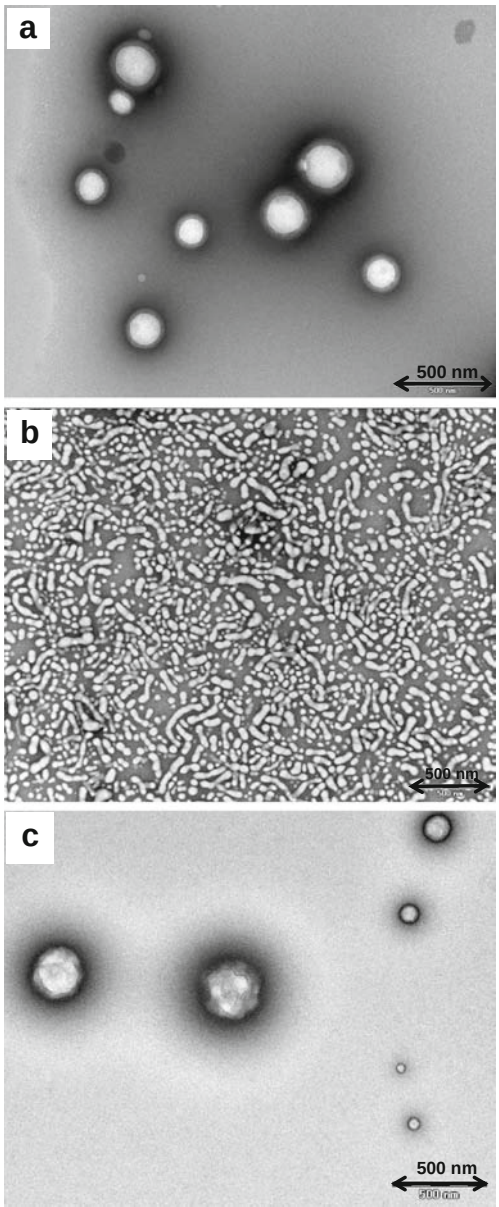

Fig. 6 TEM pictures of particles prepared from (**a**) blend of 30% PEG_{5000}-PLGHMGA and PLGHMGA (**b**) 100% PEG_{5000}-PLGHMGA (**c**) 100% PEG_{2000}-PLGHMGA.

Preparation and Characterization of Protein-Loaded Nanoparticles with PVA

In order to improve protein incorporation and release duration, nanoparticles were prepared using PVA (5% w/v) in the external water phase. PVA (the most common surfactant used in emulsion solvent evaporation method for the preparation of PLGA nano and microparticles) (30,38) and other surfactants such as polysorbates have been used to stabilize PEG-PLGA nanoparticles (3,39). It has been shown that an increasing PVA concentration (and thus increasing viscosity of the external water phase) used for the preparation of BSA loaded PLGA NPs, resulted in a higher resistance for entrapped proteins to diffuse from the internal to the external water phase and therefore yielded particles with a higher protein loading. Probably more important, the presence of PVA at the interface of the organic and the aqueous phase acts as barrier for protein diffusion not only during particle formation but also during release from the solidified nanoparticles (40,41). The size of the particles prepared in the presence of PVA was

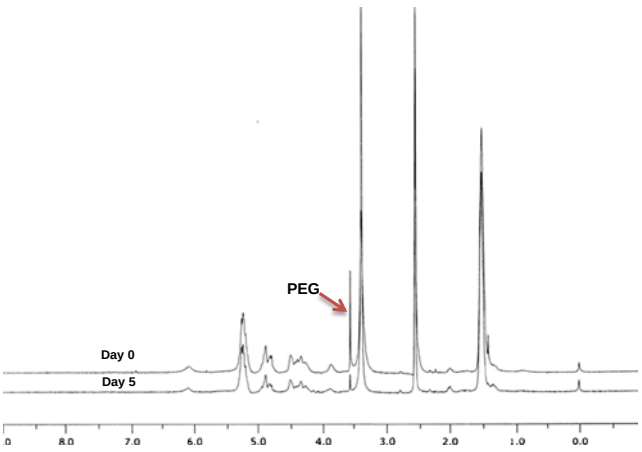

Fig. 7 ^{1}H NMR spectra of nanoparticles made of a blend of PEG_{5000}-PLGHMGA (30%) and PLGHMGA (70%) at day 0 and after 5 days incubation at 37°C and pH 7.4. The nanoparticle samples were dissolved in deuterated DMSO.

Table III Change in Copolymer Composition of Different BSA Loaded Nanoparticles During Incubation in Sodium Phosphate Buffer at 37°C

Formulation	Day	Copolymer composition (%)		
		HMG	Glycolic acid	Lactic acid
PEG_{5000}-PLGHMGA: 30 w%	0	17	17	66
	5	15	15	70
	10	13	13	74
	16	11	11	78
	21	10	11	79
	31	11	9	80
PEG_{2000}-PLGHMGA: 30 w%	0	17	17	66
	5	13	13	74
	18	11	11	78
	23	13	10	77
PEG_{5000}-PLGHMGA: 10 w%	0	17	17	66
	10	12	12	76

around 240–280 nm and PDI was <0.1, which were not profoundly influenced by the PEG content (Table II). These results demonstrate that smaller sized particles with lower polydispersity were formed in the presence of PVA as compared to particles prepared without this surfactant. The zeta-potential of placebo and protein-loaded nanoparticles was around −1 mV for all formulations demonstrating excellent shielding of the surface charge by combination of PEG and PVA. This table also shows that BSA was efficiently encapsulated in the nanoparticles (~80%) and an even higher loading efficiency of 90–95% was obtained for lysozyme. It has been reported that protein–polymer ionic interactions contribute to the extent of protein incorporation in polymer matrices (42). The relatively high molar ratio of carboxylic end groups to lysozyme (*e.g.* mol COOH/mol lysozyme >5 in the formulation containing 30% PEG_{2000}-PLGHMGA) therefore likely explains the higher encapsulation efficiency of lysozyme.

To further study the colloidal stability, placebo nanoparticles containing 30% PEG_{5000}-PLGHMGA formulated with and without PVA were dispersed in buffer of pH 7.4, incubated at 37°C and particle size was measured at different time points. All formulations showed a gradual increase of both particle size and PDI indicating particle aggregation; however, this was more severely observed for formulations without PVA (Supplementary Fig. 3). Besides, a decrease in zeta potential from −1 to −3 mV and from −3 to −40 mV at day 6 was observed for nanoparticles prepared with and without PVA, respectively, implying removal of the particle surface coating and exposure of carboxylic end groups. The low negative zeta potential of the nanoparticles suggests that the stability of these particles is likely due to their surface coverage by either PVA or combination of PEG and PVA

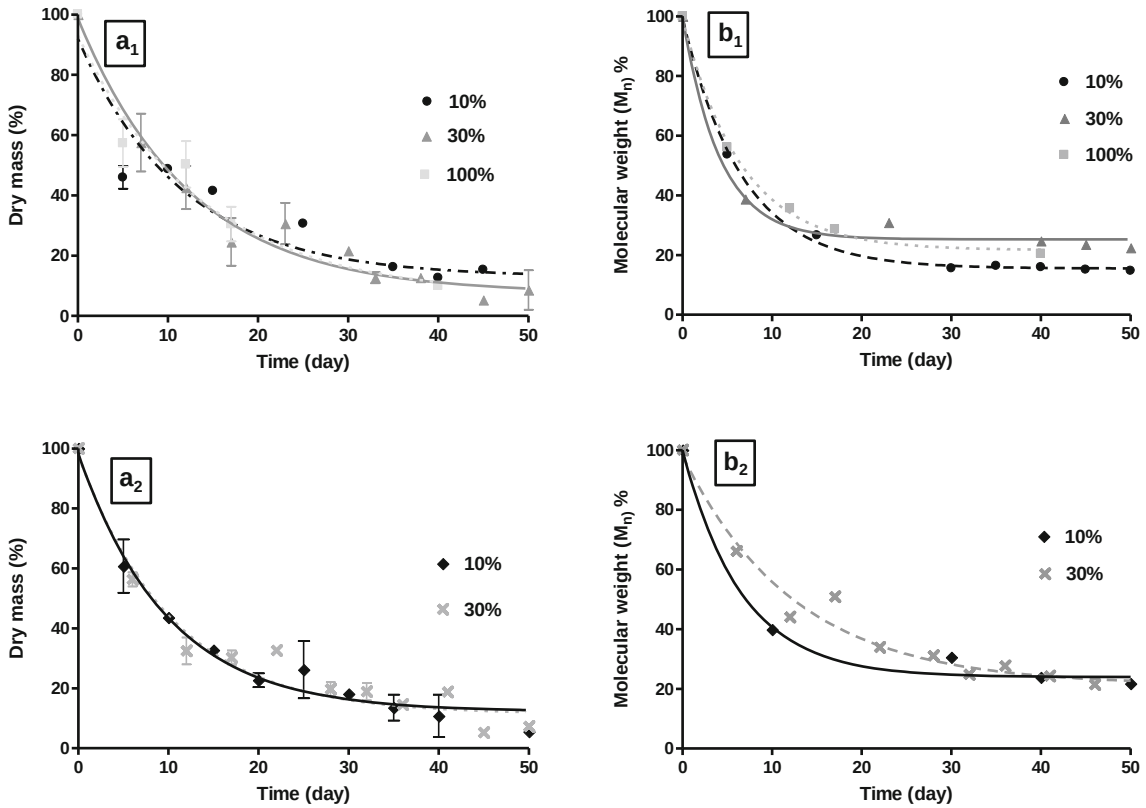

Fig. 8 ($a_{1,2}$) Relative dry mass ($n = 2$); ($b_{1,2}$) number average molecular weight (M_n) % of BSA-loaded nanoparticles based on different blends: ●, ▲, ■: PEG_{2000}-PLGHMGA and ♦, ×: PEG_{5000}-PLGHMGA.

providing steric stabilization. It has previously been shown that even after several washing steps PVA remains associated with PLGA particles (30,41,43–45). However, during incubation PVA is slowly removed from the particle surface (46). ^{1}H NMR spectra of placebo and protein loaded particles with and without PVA (Fig. 7) demonstrated a significant decrease of the PEG content at day 5. Altogether, these results indicate that the observed particle aggregation is likely caused by removal of the PEG corona as well as PVA. The removal of PEG points to a preferential cleavage of the ester bond connecting PEG to the PLGHMGA block which is in agreement with previous observation on particles based on PLGA-PEG blockcopolymers (15,16). A possible explanation is that the ester bonds that connect PEG and PLGHMGA are mainly located at the surface of the nanoparticles and consequently are more accessible for surrounding water molecules resulting in (fast) hydrolysis (44).

In Vitro Degradation of the Nanoparticles Prepared with PVA

^{1}H NMR analysis of the degrading samples showed a shift of the proton peaks attributed to the HMG units at 3.8 ppm (CH_2) and 5.2 ppm (CH, coinciding with CH of lactide) in the original structure, to 4.2–4.5 ppm. This was previously explained by the occurrence of an intramolecular transesterification reaction (25). Moreover, in line with our previous findings, a gradual decrease in both HMG and glycolic content in the copolymer was observed (Table III). As pointed out in our previous paper (25) the HMG ester bonds in PLGHMGA are more susceptible to hydrolysis which subsequently results in a relatively fast release of HMG units from the degrading polymer matrices. As also indicated in the previous section, NMR analysis also revealed a substantial decrease in PEG content (*e.g.* ~10 mol% of its initial content remained at day 5).

It is generally accepted that steric stabilization by PEG is not required for all steps in the drug targeting process. A PEG coating favors circulating kinetics but also hampers (target) cell/nanoparticle interactions and can therefore be an obstacle for internalization of the drug loaded nanoparticles (47). In general, loss of the PEG coating after arrival of drug-loaded nanoparticles at their target site is desirable, allowing enhanced target cell binding and internalization. This is particularly of interest for drugs that do not pass cellular membranes passively (7). PEG-PLGA and PEG-PLA nanoparticles have also been shown to shed PEG during incubation, which however, occurs in a couple of weeks (15,16) which is not desirable for efficient delivery. The fact that our particles loose PEG at a much faster rate is a clear advantage for the design of delivery systems with prolonged circulation time and yet a sufficient interaction with target cells. The explanation for relatively faster shedding of PEG-PLGHMGA systems as compared to related ones (*e.g.* PEG-PLGA and PEG-PLA systems) can be due to greater hydrophilicity of the PLGHMGA matrix causing faster hydrolysis of the PEG-PLGHMGA ester bonds both at the surface and within the matrix. The BSA-loaded nanoparticles demonstrated a continuous weight loss accompanied by continuous decrease in number average molecular weight in time (Fig. 8a, b). Nanoparticles were fully degraded in

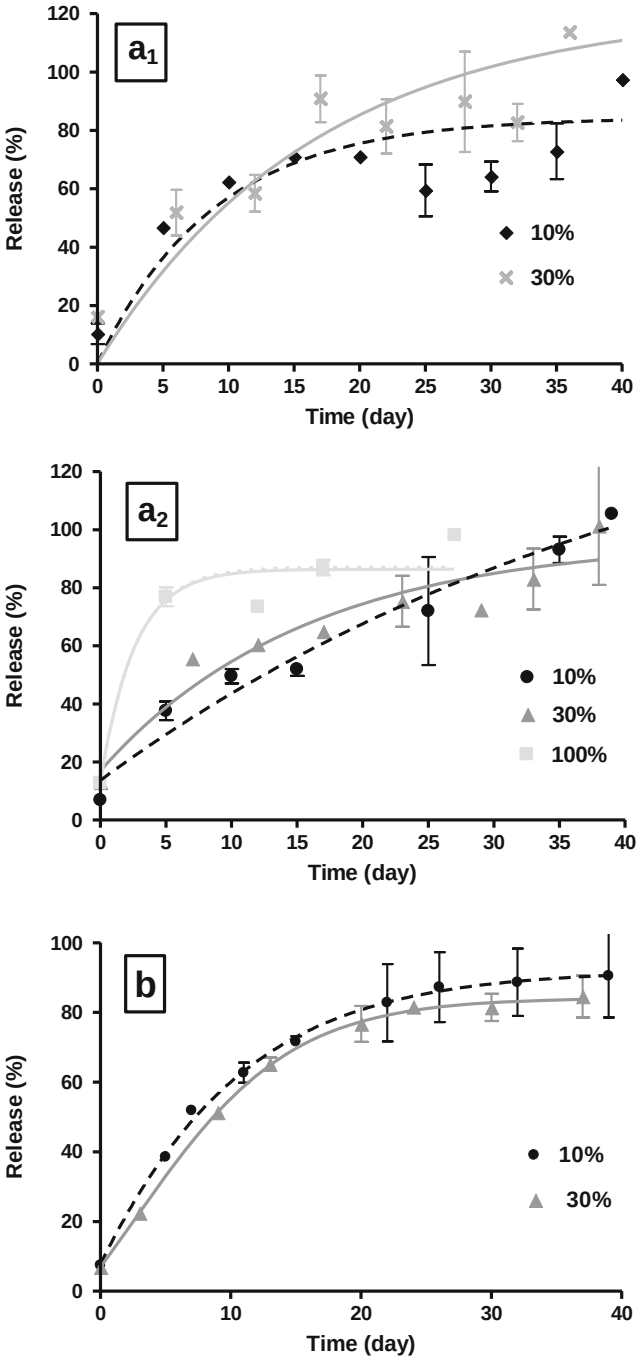

Fig. 9 ($a_{1,2}$) BSA and (b) lysozyme release from nanoparticles based on different blends ($n = 2$). ●, ▲, ■: PEG_{2000}-PLGHMGA and ♦, ×: PEG_{5000}-PLGHMGA. The percentage of the released protein is relative to the amount of protein encapsulated in the nanoparticles.

60 days and, in contrast to expectations, there were no significant differences between the degradation rates of different formulations. Nanoparticle degradation is initiated by water-uptake followed by hydrolysis of the ester bonds of the PLGHMGA block. So, it can be expected that more hydrophilic matrices (thus matrices with more PEG) initially swells to a greater extent than matrices of lower PEG content (48). However, with the loss of PEG, which occurs in the early stage of degradation for all nanoparticle formulations, degradation of the matrix is dominated by hydrolysis of PLGHMGA resulting in degradation patterns that are hardly affected by the initial PEG content of the particles.

In-Vitro Protein Release from Nanoparticles Prepared with PVA

The release profiles of BSA and lysozyme from different formulations are presented in Fig. 9a and b. For the different BSA-loaded particles based on blends of 10% and 30% of PEG-PLGHMGA, the release patterns consisted of an initial burst of the encapsulated protein (6–12%) followed by a continuous release reaching completion in 40 days. Nanoparticles of 100% PEG_{2000}-PLGHMGA, however, showed faster release and around 80% of BSA was released in 5 days. In an ongoing and previous studies, using the same method for particle preparation, we have demonstrated, using advanced spectroscopic techniques, that the structural integrity of the released proteins was preserved (25,49,50). Since proteins do not dissolve in hydrophobic polymeric matrices and have a low (or absent) mobility in such matrices, their release from PLGA based systems and matrices of related polymers is mainly governed by diffusion through water-filled pores that are initially present or formed during degradation (51,52). Since the degradation rate of 100% PEG_{2000}-PLGHMGA nanoparticles was not significantly different from particles prepared from the blends, their fast protein release in early stage can be explained as follows. For the particles based on 100% PEG_{2000}-PLGHMGA, a considerable amount of PEG is present in the bulk, because of the good miscibility of PEG and PLGHMGA blocks (DSC analysis, Fig. 3). This consequently results in strong hydration of the matrix allowing relatively fast diffusion and thus release of the protein, as also observed for the release of BSA from PEGylated PLGA particles (14).

Lysozyme-loaded nanoparticles showed a low burst of approximately 5% and over the next 35 days the protein was completely released. In line with the BSA release data, no significant difference in lysozyme release patterns for the different formulations was observed. Besides polymer degradation, protein–matrix interactions are also reported as an important controlling factor for protein release (22,53). Taking into account the different physicochemical characteristics of the two proteins in this study (BSA and lysozyme), different release patterns could be also expected. However, our results demonstrate nearly similar release rates for both proteins meaning that the release is mainly governed by particles' degradation and other factors have only a minor effect.

CONCLUSIONS

Nanoparticles based on blends of PEG-PLGHMGA and PLGHMGA were successfully prepared without using PVA as surfactant. Differences in particle size, morphology and surface charge were observed by changing the PEG-PLGHMGA content. High protein loading and sustained release was however only achieved when PVA was used in the external water phase. The different nanoparticles, independent of their composition, showed almost a similar protein release and degradation behavior demonstrating that the controlling factors of the degradation and protein release characteristics of the nanoparticles were determined by the relatively hydrophobic polyester core (PLGHMGA), whereas the initial PEG content had no major effect. The observed shedding of the PEG coating of the nanoparticles in around 5 days is attractive for the design of polymeric particulate nanocarriers. Such systems will likely remain sufficiently long in the circulation to accumulate in *e.g.* tumors at good levels. The PEG coating cleavage on the other hand is fast enough to allow cellular binding and internalization.

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