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Release of anti-restenosis drugs from poly(ethylene oxide)-poly(DL-lactic-co-glycolic acid) nanoparticles

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Abstract

Dexamethasone- or rapamycin-loaded nanoparticles based on poly(ethylene oxide) and poly(DL-lactic-co-glycolic acid) block copolymers (PEO-PLGA) were prepared without additional stabilizer using the salting-out method. A fast release of drug in PBS (pH 7.4) at 37 °C resulting in 100% release within 5 h was observed for both drugs. The rate of drug release was substantially reduced by treating the particles with gelatin or albumin after drug loading, resulting in a linear drug release in time. It was shown that the rate of drug release is related to the amount of protein associated with the nanoparticles. After gelatin treatment of drug-loaded nanoparticles, sustained release of dexamethasone for 17 days and of rapamycin for 50 days could be achieved.
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1. Introduction

After percutaneous transluminal coronary angioplasty (PTCA), restenosis of 30 to 50% of the treated coronary arteries occurs [1]. Attempts have been made to decrease the incidence of restenosis. A successful approach was the introduction of a stent, which led to a decrease of 10–15% in the incidence of restenosis [2–5]. Nevertheless, stented small arteries tend to reocclude more easily than stented large arteries and conditions where excessive smooth muscle cell response occurs (e.g., in diabetics) lead to relatively high in-stent restenosis [3]. Due to the decrease in the incidence of restenosis, stents are nowadays also used in more complex lesions, resulting in an overall in-stent restenosis of 10 to 50% of the stented lesions [6]. The use of polymer-coated, drug-eluting stents reduced the incidence of (in-stent) restenosis. Besides as a drug depot, the polymer coating can be used to regulate the drug release rate [7]. It has been shown that the use of rapamycin-eluting stents, stents coated with a drug containing non-erodable polymer layer, which released the drug for more than 28 days, leads to complete inhibition of restenosis [7,8]. This

is due to the inhibition of vascular smooth muscle cell proliferation [9]. Also after 18 months, no delayed restenosis was observed [10]. Although drug-eluting stents inhibit restenosis completely, they are not biodegradable and are thus permanently present, which could lead to long-term adverse tissue reactions. Biodegradable drug-loaded nanoparticles may be a good alternative for, or complementary to, the use of drug-eluting stents. It has been shown, that drug-loaded nanoparticles can be locally delivered to the site of the atherosclerotic lesion [11]. Furthermore, in an in vitro model using a microporous balloon catheter and testing particles of different sizes (120–230–1000 nm), it was shown that the size of the nanoparticles mainly determines the particle localization in the arterial wall [12]. Particles of 120 nm in size were present in all layers of the arterial wall, whereas particles of 230 and 1000 nm were mainly introduced to the intima of the arterial wall. So by using nanoparticles of different sizes, drug delivery to specific layers in the arterial wall can be achieved simultaneously.

Besides rapamycin, dexamethasone has also been applied to reduce the incidence of restenosis [13]. Both drugs are well soluble in various organic solvents [14,15], but differ in terms of molecular weight and hydrophobicity. Rapamycin has a higher molecular weight (914.2 g/mol) than dexamethasone (392.5 g/mol) and a

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lower water solubility (2.6 μg/ml for rapamycin [16] compared to 100 μg/ml for dexamethasone [14]). This difference may influence drug loading and drug release characteristics.

This study is an initial evaluation of the suitability of PEO-PLGA nanoparticles as carriers for anti-restenosis drugs, and describes the preparation of these particles loaded with dexamethasone or rapamycin and the release of these active agents in PBS (pH 7.4) at 37 °C. Since in an in vivo application of these nanoparticles, protein interaction will occur and may affect drug release, the effect of a protein incubation step using to model proteins (albumin and gelatin) on the in vitro drug release was also studied.

2. Materials and methods

2.1. Materials

DL-Lactide and glycolide were purchased from Purac Biochem B.V. (Gorinchem, The Netherlands). Stannous octoate, gelatin B (bovine skin, 75 bloom, approximate $\bar{M}_w = 22 \times 10^3$ g/mol), bovine serum albumin (BSA) (approximate $\bar{M}_w = 66 \times 10^3$ g/mol; minimum 98% pure), dexamethasone and rapamycin were purchased from Sigma (St. Louis, USA) and used as received. Monomethoxy poly(ethylene glycol) (MPEG) ($\bar{M}_n = 3.0 \times 10^3$ g/mol) was obtained from Shearwater Polymers (Huntsville, USA) and sodium dodecyl sulfate (SDS) and deuterated dimethylsulfoxide were purchased from Aldrich (Milwaukee, USA). Phosphate-buffered saline (PBS; pH 7.4) was purchased from NPBI (Emmer Compascuum, The Netherlands). All solvents used were of analytical grade (Biosolve, Valkenswaard, The Netherlands). All other reagents were obtained from Merck (Darmstadt, Germany).

Poly(ethylene oxide)-poly(DL-lactic-co-glycolic acid) block copolymer (PEO-PLGA) (molar ratio of lactyl:glycolyl = 52:48; $\bar{M}_n$, PEO-block = 3.0×10^3 g/mol; $\bar{M}_n$, PLGA-block = 8.2×10^3 g/mol and polydispersity index = 1.24) was synthesized by ring-opening polymerization of DL-lactide and glycolide using MPEG as initiator and stannous octoate as a catalyst at 130 °C for 24 h as described previously [17].

2.2. Nanoparticle preparation

Nanoparticles were prepared using the salting-out method [17] in which acetone was chosen as the water-miscible organic solvent, because of its pharmaceutical acceptance with regard to toxicity [18]. Typically, an acetone solution (3.5 g) containing 3 wt.% PEO-PLGA and various amounts (0–1.2 wt.%) of drug was emulsified under mechanical stirring (20,500 rpm; 40 s; T25 Ultraturrax equipped with an S25 dispersing tool, Ika-Labortechnik, Staufen, Germany) in an aqueous phase (8.75 g) containing 60 wt.% MgCl2·6H2O as the salting-out agent (in a glass beaker; 3.5 cm diameter; 6.6 cm height). After the fast addition (5 s) of pure water (7.5 g) under mechanical stirring (20,500 rpm) causing acetone to diffuse into the water phase, nanoparticles were formed and stirring was continued (20,500 rpm; 20 s). The nanoparticles were purified by rinsing with water. First, the nanoparticles were separated by ultracentrifugation (65,000×g for 30 min; Centrikon T-2180, Kontron Instruments, Watford, UK) and the supernatant was

removed. The nanoparticles were redispersed in water, centrifuged and the supernatant was removed. This procedure was repeated three times.

All nanoparticle preparations were performed in duplo, unless stated otherwise.

2.3. Nanoparticle treatment with protein

After the first purification step by ultracentrifugation (see nanoparticle preparation), the nanoparticles were redispersed in 5 ml of a protein solution (0.02, 0.1 or 0.5 mg/ml) in millipore water (MilliQ, Molsheim, France) for 1 h and centrifuged (65,000×g for 30 min). After removal of the supernatant, the protein-treated particles were rinsed with water twice by redispersion in millipore water, centrifugation and subsequent removal of the supernatant. The proteins used were gelatin and BSA.

2.4. Particle size analysis

The nanoparticle size was determined by dynamic light scattering (DLS) (Zetasizer 4000, Malvern Instruments Ltd., Malvern, UK) at 25 °C at an angle of 90°, taking the average of three measurements. The particle dispersion was diluted with water to such degree that the desired number of counts was obtained. The desired number of counts is the number of counts that is high enough to get the highest possible signal to noise ratio, yet small enough to prevent multiple scattering to occur.

First, the polydispersity index (P.I.) is determined by the cumulants method. The P.I. is a dimensionless number indicating the width of the size distribution, and has a value between zero and one, being zero for monodisperse particles. If the P.I. is small enough (<0.08), the particle size can be determined by the cumulants method and the size distribution obtained is based on a log normal distribution characterized by a mean and width. For polydispersity indices higher than 0.08, the CONTIN-method is used to determine the particle size. The CONTIN-method, developed by Provencher et al. [19], describes bimodal and smooth distributions without the need for information such as an initial estimate for the particle size.

To examine the size and morphology of the nanoparticles in the dry state, samples were analyzed by scanning electron microscopy (SEM) using a LEO 1500 (LEO Electron Microscopy Ltd., Cambridge, UK). Silicon substrates (∅ 15 mm) were cleaned ultrasonically, successively in isopropanol (10 min, two times), in methanol (10 min, two times) and in acetone (10 min, two times). The nanoparticle samples were prepared by dropping an aqueous particle dispersion on a freshly cleaned silicon substrate and drying for 2 h at ambient temperature. SEM analysis was performed at 1 kV at magnifications ranging from 2000× to 15,000×. The particle size in the dry state was determined by averaging the size of 35 particles.

2.5. Determination of protein content of the nanoparticles

The surface of protein-treated and untreated drug-loaded particles was analyzed by X-ray photoelectron spectroscopy (XPS). Nanoparticle samples were prepared on silicon substrates

as described above for SEM analysis. Spectra of the nanoparticle samples were obtained using a Quantum 2000 Scanning ESCA Microprobe (Physical Electronics, Eden Prairie, MN, USA) using monochromatized Al K-α (25 W) X-rays and an electron take off angle of 45°. The X-ray spot size was 100 μm. A single survey spectrum (0–1100 eV) was recorded on each sample on three different spots using a pass energy of 187.85 eV and an acquisition time of 5 min. Charge neutralization was performed using a 1 eV electron source and a 5 eV ion source. The measured peak areas were converted into atomic percentages by using sensitivity factors known from literature [20].

The nitrogen content of lyophilized, protein-treated and untreated dexamethasone-loaded nanoparticles, gelatin and albumin was determined by elemental analysis using an EA 1108 (Fisons Instruments, Interscience B.V., Breda, The Netherlands). From the percentage of nitrogen in the nanoparticle samples and in the gelatin and albumin samples, the amount of gelatin and albumin associated with the nanoparticles was calculated. The analysis of all samples was performed in duplo.

2.6. Determination of the rapamycin content of nanoparticles

The rapamycin content of the nanoparticles was analyzed by high-performance liquid chromatography (HPLC). Lyophilized nanoparticles were dissolved in acetonitrile (0.75 mg/ml) and 20 μl of this solution was injected (Injector 20 μl loop Valco) on a Polaris C18-A column (150×4.6 mm; 5 m; Ansys Technologies, Torrance, USA). Acetonitrile/water (80/20 v%) was used as an eluent at a flow rate of 2 ml/min (Varian HPLC pump 2510). A Varian variable λ detector 2550 was used to detect rapamycin at 278 nm. The amount of rapamycin in the sample was calculated using a calibration curve of rapamycin in acetonitrile at various concentrations.

2.7. Determination of the dexamethasone content of nanoparticles

The dexamethasone content of the nanoparticles was determined by proton nuclear magnetic resonance (^{1}H-NMR). A known amount of drug-loaded nanoparticles (approximately 5 mg) was dissolved in 1 ml of deuterated DMSO. Spectra were obtained using a Varian Inova (Varian, Palo Alto, USA) operating at 300 MHz. The dexamethasone content of the nanoparticles was calculated from the integral of a dexamethasone peak (d, 2H, $\delta = 7.2$ ppm) and the integral of a glycolyl peak (m, 2H, $\delta = 4.6–4.9$ ppm), using the integrals of the peaks of dexamethasone and of the glycolyl units of polymer solutions in deuterated DMSO containing known amounts of dexamethasone and polymer.

2.8. Drug release study

Dexamethasone release from the nanoparticles was studied by dispersing nanoparticles in PBS containing 0.02% (w/v) of sodium azide (NaN₃) at 37 °C. For rapamycin-loaded nanoparticles, PBS containing 0.02% (w/v) of NaN₃ and 1 mM SDS was used as the release medium. SDS was used to increase the solubility of rapamycin in PBS to levels well detectable by HPLC. Drug-loaded nanoparticles were redispersed in 2 ml of release medium at a

known concentration (approximately 10 mg/ml) and transferred to a dialysis tube (1 cm width, 20 cm length; Spectra/Por® 6 Membrane; MWCO: 25,000; Medicell International Ltd., London, UK). One end of the dialysis tube was tied up. After transfer of the nanoparticle dispersion, the other end of the dialysis tube was clamped. The tube was incubated in Erlenmeyer flasks in 500 ml or 67 ml release medium at 37 °C for dexamethasone- and rapamycin-loaded particles, respectively. These volumes were chosen to ensure that the maximum concentration of the drug in the release medium would always be less than 10% of the maximum solubility, i.e., sink conditions [21]. The Erlenmeyer flasks were continuously shaken. At different time points, 1.5 ml of the eluate was removed for analysis and replaced by fresh release medium.

2.9. Drug concentration in release medium

The concentration of drug in the release medium was determined by HPLC. In the case of dexamethasone, 20 μl of eluate was injected (Injector 20 μl loop Valco) on an RP-18e column (100×4.6 mm; 5 μm; Merck, Darmstadt, Germany). Water/tetrahydrofuran/acetonitrile (80/12/8 v%) was used as an eluent at a flow rate of 2 ml/min (Varian HPLC pump 2510). Dexamethasone was detected at 240 nm using a Varian variable λ detector 2550. In the case of rapamycin, 20 μl of eluate was injected on a Polaris C18-A column (150×4.6 mm; 5 μm; Ansys Technologies, Torrance, USA). Acetonitrile/water (80/20 v%) was used as an eluent at a flow rate of 2 ml/min. Rapamycin was detected at 278 nm. The drug concentration in the release medium was calculated using a calibration curve of the drug in the corresponding release medium at various concentrations.

2.10. In vitro degradation of dexamethasone-loaded nanoparticles

The in vitro degradation of dexamethasone-loaded PEO-PLGA nanoparticles was studied by dispersing nanoparticles in PBS containing 0.02% (w/v) NaN₃. The nanoparticle dispersions in closed ultracentrifugation tubes were kept at 37 °C. At different time points (0–24 days), the particle size was determined and subsequently the nanoparticles were separated from the medium by ultracentrifugation ($65,000 \times g$ for 40 min). The sediment was lyophilized and analyzed with respect to the molecular weight of the polymer. The molecular weight was determined by gel permeation chromatography (GPC) using chloroform at 25 °C and a flow rate of 1.5 ml/min. The GPC setup consisted of a Waters Model 510 pump, a HP Ti-Series 1050 autosampler, a Waters Model 410 Differential Refractometer, and a Viscotek H502 Viscometer Detector with HR0.5, HR2 and HR4 Waters Ultra-Styragel columns (Waters, Milford, USA) placed in series. Polystyrene standards with narrow molecular weight distributions (PSS, Mainz, Germany) were used for calibration.

3. Results and discussion

3.1. Particle analysis

Dexamethasone- and rapamycin-loaded PEO-PLGA nanoparticles were prepared and some of these nanoparticle formulations

Table 1
The drug content, swelling and polydispersity index (P.I.) of untreated and protein-treated PEO-PLGA nanoparticles in water and the size of protein-treated and untreated PEOPLGA nanoparticles in the wet and in the dry state

Nanoparticle type[a]	Drug during NP preparation (wt.%)[b]	Drug loading (wt.%)[c]	Size (nm)[d]	P.I. (−)[d]	Size (nm)[e]	Swelling (%)[f]
NP-0	0	0	230±10	0.16±0.05	190±70	77±28
NP-dex-0	17	2±0	210±2	0.33±0.04	nd	na
NP-dex-0	29	29±2	197±3	0.31±0.08	170±50	56±16
NP-dex-gel-0.5	29	29±2	193±3	0.45±0.06	nd	na
NP-dex-gel-14	29	29±2	194±3	0.42±0.05	nd	na
NP-dex-alb-0.5	29	29±2	199±5	0.44±0.03	nd	na
NP-rap-0	0.3	0.1±0	163±6	0.49±0.04	nd	na
NP-rap-0	1.0	0.4±0	192±3	0.46±0.03	nd	na
NP-rap-gel-0.5	1.0	0.4±0	193±3	0.52±0.05	nd	na
NP-rap-gel-3	1.0	0.4±0	192±5	0.38±0.02	nd	na

[a] Nanoparticles (NP) loaded with dexamethasone (dex) or rapamycin (rap); the numbers denote the weight percentage of gelatin (gel) or albumin (alb) relative to the polymer weight in the particle preparation procedure. The data of NP-dex are of two nanoparticle preparations, the data of NP-rap are of one nanoparticle preparation.
[b] The wt.% of drug relative to the total drug and polymer weight during particle preparation.
[c] The wt.% of drug in the nanoparticle preparation after purification as determined by HPLC.
[d] Nanoparticle size in the wet state as determined by DLS; the standard deviations denote the variation in size within three size measurements.
[e] Nanoparticle size in the dry state as determined by SEM by averaging the diameter of 35 particles of a representative part of the sample; nd=not determined.
[f] Calculated by dividing the hydrodynamic volume (from DLS) by the volume in dry state (from SEM); na=not applicable.

were treated with an aqueous protein solution. The drug concentration of the organic phase during particle preparation was varied and its influence on drug loading and particle size was determined. The drug content and the particle characteristics in the wet state of untreated and protein-treated nanoparticles and the particle characteristics in the dry state of untreated nanoparticles are shown in Table 1.

The dexamethasone content of the nanoparticles strongly depends on the relative amount of dexamethasone being present during particle preparation. For a low amount of dexamethasone, the drug content of the nanoparticles is rather low, which is in agreement with the results of Hickey et al. [22] who prepared dexamethasone loaded nanoparticles of a blend of PLGA and PEO (9:1), by an oil-in-water emulsification–evaporation method. They determined a dexamethasone content of 3 wt.% using 16 wt. % of drug relative to the weight of drug and polymer during particle preparation. The relatively high solubility of dexamethasone in water (100 µg/ml [14]) causes dexamethasone to diffuse into the aqueous phase during particle formation and could explain the low content of dexamethasone for low amounts of drug.

For nanoparticle preparations in which 29 wt.% of dexamethasone was used, the dexamethasone content was 29 wt.%. To make sure that this high dexamethasone content was not due to the formation of dexamethasone particles, the nanoparticle preparation procedure was performed in the absence of PEO-PLGA polymer. In this experiment, no particles were obtained. The high efficiency of drug incorporation might be explained by crystallization of dexamethasone in the particles. In lidocaine-loaded poly(lactic acid) nanoparticles, lidocaine crystals were only observed at high lidocaine contents (approximately 30 wt.%) and not at low contents (approximately 10 wt.%) [23]. A possible method to study the state of dexamethasone in the nanoparticle could be differential scanning calorimetry (DSC). However, because the decomposition temperature of PEO-PLGA is lower than the melting temperature of dexamethasone, DSC analysis of the PEO-PLGA nanoparticles was not possible. An alternative technique to study the state of dexamethasone in the nanoparti-

cles, which is not explored thus far, could be X-ray diffraction analysis.

For both weight fractions drug/(drug and polymer) that were applied during nanoparticle preparation, the rapamycin content of the nanoparticle is approximately 40% of this weight fraction (Table 1). For a relatively low amount of drug during particle preparation, the rapamycin content is relatively high compared to dexamethasone, which might be due to the low solubility of rapamycin in water (2.6 µg/ml [16]) and in the mixture of acetone and aqueous salt solution.

The hydrodynamic diameter of drug-loaded nanoparticles is slightly smaller than that of unloaded nanoparticles (Table 1). A possible reason for this might be that the hydrophobic drug decreases the interfacial tension between the organic and aqueous phase, which results in an increase of the area to volume ratio and thus in smaller particles.

Both unloaded and loaded PEO-PLGA nanoparticles appear to have a spherical shape in the dry state as was determined by SEM (Fig. 1).
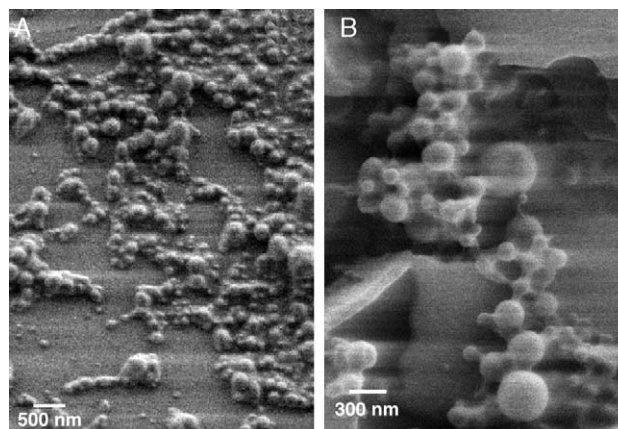

Fig. 1. Scanning electron microscopy image of (A) unloaded and (B) dexamethasone-loaded PEO-PLGA nanoparticles on a silicon substrate.

3.2. Drug release from nanoparticles

For the drug release from degrading PLGA or PEO-PLGA particles, a triphasic profile has been described in literature [24–26]. The first phase is a burst effect, caused by the release of drug that is adsorbed onto the outer particle surface. The second phase is characterized by a relatively slow release due to diffusion of drug out of the matrix. The third phase is a phase of increased drug release, caused by (extensive) polymer degradation, resulting in an increased permeability of the drug in the polymer matrix.

The release of dexamethasone and rapamycin from untreated and protein-treated PEO-PLGA nanoparticles in PBS is depicted in Fig. 2A and B, respectively. A rapid drug release was observed for untreated drug-loaded nanoparticles, being complete within 5 h (second data point, Fig. 2). The rapid release of all drug could indicate that all drug is present at the surface. To verify this, the surface of drug-loaded nanoparticles was analyzed by XPS. As the ratio of carbon and oxygen atoms (C/O-ratio) of dexamethasone (4.40) and of rapamycin (3.92) is much higher than of PEO-PLGA (1.50) or unloaded PEO-PLGA nanoparticles (1.50), the C/O-ratio can be used to determine whether all drug is present at the surface. Assuming that all dexamethasone is present at the surface of dexamethasone-loaded nanoparticles (29 wt.%; 197 nm (Table 1)) and the density of dexamethasone and the copolymer are the same, the thickness of the dexamethasone layer would be approximately 11 nm, which equals the depth of analysis. The C/O-ratio of nanoparticles loaded with 29 wt.% of dexamethasone was determined to be 1.57±0.06. Although some reorganization due to the drying process might occur, it is highly unlikely that all dexamethasone is present at the surface. In the case of rapamycin-loaded particles (0.4 wt.%; 192 nm (Table 1)) the thickness of the rapamycin layer would be approximately 0.1 nm, which is much lower than the depth of analysis. Since the C/O-ratio of nanoparticles loaded with 0.4 wt.% of rapamycin was determined to be 1.57±0.08 it cannot be concluded whether rapamycin was preferentially present at the surface.

The rapid drug release is in accordance with the rapid release of savoxepine and estradiole from poly(DL-lactic acid) (PDLLA) nanoparticles prepared by the salting-out method [27,28] and the rapid release of propranolol hydrochloride and lidocaine from PEO-PLLA microparticles that were prepared by an emulsification−evaporation method [29]. The rapid release was explained by the presence of pores in the nanoparticles [28,29]. The presence of pores increases the total surface area that is available for diffusion of drug out of the matrix, resulting in a relatively rapid drug release. Similarly, the rapid release of drug in this study could indicate that the PEO-PLGA particles contain pores. The presence of pores and the high water uptake of PEO-PLGA nanoparticles (Table 1) [30] could explain the high permeability of the drug in the polymer matrix [31]. During nanoparticle preparation, liquid−liquid demixing resulting in a polymer poor and a polymer rich phase may occur, which will lead to pore formation. In principle, two kinds of particles can be formed, namely particles with a phase-separated PEO and PLGA polymer phase or with a mixed polymer phase. Whether phase separation occurs cannot be concluded from the data presented in this study. A reason that no pores were observed by SEM analysis might be that these have collapsed during drying of the particles.

The time to release all drug was extended by redispersion of drug-loaded nanoparticles in an aqueous gelatin or albumin solution (Fig. 2). First, a small burst effect was observed, possibly resulting from desorption of drug from the nanoparticle surface. This was followed by a linear release of dexamethasone over a period of 8 (for 0.5 wt.% protein) to 17 (for 14 wt.% protein) days and of rapamycin over a period of 25 (for 14 wt.% gelatin) to 50 (for 3 wt.% gelatin) days. No difference in the release profile or release time was observed between gelatin- and albumin-treated dexamethasone-loaded particles (for 0.5 wt.% protein).

The in vitro degradation study of dexamethasone-loaded PEO-PLGA nanoparticles showed that during the first 3 weeks of drug release the particle size and PEO-PLGA molecular weight as a function of drug release time were similar to the particle size and PEO-PLGA molecular weight of unloaded PEO-PLGA nanoparticles [17]. This means that the $\bar{M}_n$ of PEO-PLGA decreases during the first 2 weeks, is stable during the following few weeks and decreases again to reach a value of 2×10^3 g/mol after 8 weeks. The initial decrease of $\bar{M}_n$ was due to the preferential cleavage of the ester linkage between PLGA and PEO. The particles retained their size in the first 2 weeks but then (partially) aggregated as a result of the release of PEO.
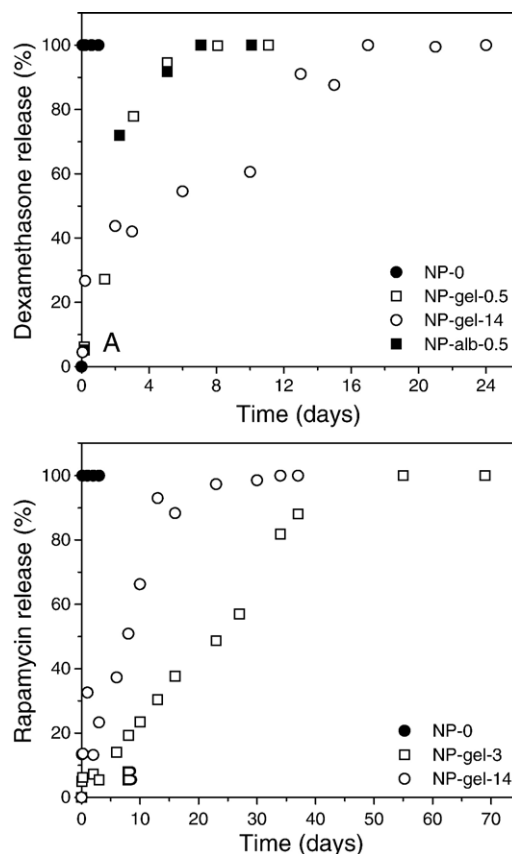

Fig. 2. Drug release in PBS (pH 7.4) at 37 °C from PEO-PLGA nanoparticles loaded with (A) 29 wt.% dexamethasone and (B) 1 wt.% of rapamycin as a function of time and amount of protein (gelatin (gel) or albumin (alb)). The numbers in the nanoparticle code represent the wt.% of protein relative to the initial polymer weight (n=1).

Table 2
The protein content (wt.%) of dexamethasone-loaded (29 wt.%) (dex) and unloaded PEO-PLGA nanoparticles after treatment with an aqueous solution of gelatin (gel) or albumin (alb)

Nanoparticle formulation[a]	Protein content of particles (wt.%)
NP-dex-0	<0.07[b]
NP-dex-alb-0.5	0.76±0.35
NP-dex-gel-0.5	0.63±0.19
NP-dex-gel-14	2.46±0.69
NP-0	<0.07[b]
NP-alb-0.5	<0.07[b]
NP-gel-0.5	<0.07[b]
NP-gel-14	1.70±0.44

[a] The numbers denote the amount of protein relative to the initial polymer weight.
[b] Below detection limit (=0.01% N, which corresponds to 0.07 wt.% protein).

Classical equations describing diffusion of drug out of a spherical matrix, such as the Baker-Lonsdale equation [32,33] cannot be applied to describe drug release from these particles for several reasons. First of all, mass loss occurs due to the preferential cleavage of the ester linkage between PLGA and PEO [17]. This results in an increase of hydrophobicity of the polymer matrix in time. As the particle size remains constant in time, this means that the porosity increased in time, resulting in an increased diffusion of drug out of the matrix. Secondly, the molecular weight of the block copolymer decreases in time, which leads to an increase in the diffusion coefficient of the drug [34]. Thirdly, the particle size distribution plays a role in the drug release. Smaller particles release drug at a higher rate than larger particles [27,31,35], probably caused by the higher surface to volume ratio.

Several effects of the protein treatment on the drug release characteristics might play a role. Protein can be adsorbed onto the surface, thereby forming a coating that decreases diffusion of the drug out of the polymer matrix. The protein can also be incorporated in the nanoparticles and can interact with drug and/or polymer, decrease the degree of swelling or reduce the porosity, which all result in a lower diffusion coefficient of the drug.

Since the amount of protein that is associated with the nanoparticle is very small and the hydrodynamic diameter of protein-treated and untreated drug-loaded particles is equal (Table 1), the effect of the protein treatment on drug release is not caused by a difference in swelling of the particles.

The amount of protein associated with the nanoparticle was calculated by determining the nitrogen content using elemental analysis. The results are shown in Table 2. It can be seen that the higher the protein concentration during treatment of the nanoparticles, the more protein is associated with the unloaded and dexamethasone-loaded nanoparticles. No protein was present in the unloaded nanoparticles after treatment with an aqueous solution containing 0.5 wt.% of protein. If it is assumed that all protein is present at the outer particle surface, the surface concentration of protein is 0.09 µg/cm^2 at the most, which is less than the surface concentration of a monolayer of albumin [36].

The protein content of the dexamethasone-loaded particles was higher than that of the unloaded particles that were treated with the same amount of protein, irrespective of the amount of protein in the aqueous protein solution with which the particles

were treated. This indicates that dexamethasone influenced the uptake of protein in the nanoparticles. The protein might coat or bind to the dexamethasone present in the nanoparticles or a combination of both. The effect of the protein incorporated in the nanoparticles on the release of drug from the nanoparticles will probably depend on the state of the drug in the nanoparticles. If dexamethasone is homogeneously dispersed in the nanoparticle, the protein is likely to interact with dexamethasone, either through hydrophobic interaction or through hydrogen bonding [29]. In the case that dexamethasone is present in the nanoparticle as dexamethasone crystals, the protein may also be present as a coating on the dexamethasone crystals. This probably reduces the dissolution rate of the crystals, leading to a lower drug release rate. The more protein is associated with the nanoparticles, the higher is the probability that the protein coats the dexamethasone or interacts with dexamethasone and the slower is the dexamethasone release, as seen in Fig. 2A. However, it has to be noted that the amount of protein present in the nanoparticles in relation to the amount of drug present is rather small.

Therefore, a more likely explanation for the effect of protein treatment on the drug release is that the protein molecules penetrate and/or block the pores of the particles, thereby decreasing diffusion of drug through the pores, as also was suggested by Huang et al. [29]. Due to the presence of protein, the viscosity of the aqueous phase in the pores will increase resulting in a decrease of diffusion of the drug through the pores [21]. Consequently, the drug release rate is decreased. At this point, it cannot be excluded that protein aggregation and the rinsing procedure using ultracentrifugation play a role in the decreased drug release.

For rapamycin, the same trend is observed for the effect of protein treatment on drug release. However, the total drug release time of particles treated with an aqueous gelatin solution containing low amounts of gelatin was longer than that of particles treated with an aqueous gelatin solution containing higher amounts of gelatin. As the amount of gelatin in rapamycin-loaded nanoparticles is not known, it is difficult to give an explanation for this observation. Besides, the presence of SDS in the release medium might play a role in the release of rapamycin as it can complex with the protein or desorb protein.

The degree of interaction between the protein and the drug is dependent on drug characteristics, such as hydrophobicity, molecular weight and ability to form hydrogen bonds. In this respect, the interaction between rapamycin and protein is expected to be stronger than between dexamethasone and protein and the diffusion of rapamycin through the pores is expected to be slower than of dexamethasone. This explains the longer drug release times of protein-treated rapamycin-loaded particles compared to dexamethasone-loaded particles.

In this study, a sustained rapamycin release from biodegradable PEO-PLGA nanoparticles containing 0.4 wt.% of rapamycin for 50 days was observed. This means that the release time is potentially long enough to inhibit smooth muscle cell proliferation and thus restenosis. From a comparison with rapamycin-eluting stents [7,8] it can be concluded that the rapamycin content probably has to be increased to be efficient in this respect. This might be achieved by using relatively high rapamycin amounts during nanoparticle preparation.

4. Conclusions

Dexamethasone- and rapamycin-loaded PEO-PLGA nanoparticles were prepared without stabilizer using the salting-out method. High dexamethasone loadings (29 wt.%) were obtained by using 29 wt.% of dexamethasone during nanoparticle preparation. The rapamycin content of the PEO-PLGA nanoparticles (0.1–0.4 wt.%) was 40% of the amount of rapamycin during nanoparticle preparation, irrespective of the absolute amount of rapamycin. The release of dexamethasone and rapamycin from the nanoparticles dispersed in PBS at 37 °C reached 100% within 5 h. This rapid drug release was largely reduced by redispersion of the particles in an aqueous gelatin or albumin solution. This approach resulted in a linear dexamethasone release for 17 days and in a linear rapamycin release for 50 days.

It is concluded that biodegradable PEO-PLGA nanoparticles, prepared without additional stabilizer, have the potential to be used for the intravascular delivery of anti-restenosis drugs.

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