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Research paper

Preparation of preformed porous PLGA microparticles and antisense oligonucleotides loading

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ABSTRACT

The objective of this study was to load preformed highly porous microparticles with drug. The microparticles were prepared by a modified multiple emulsion (w/o/w) solvent evaporation method with the addition of pore formers (NaCl into the internal aqueous phase or of glycerol monooleate to the poly(lactide-co-glycolide) (PLGA) polymer phase). The drug-free solidified microparticles were then washed with either water (for NaCl) or hexane (for glycerol monooleate) to extract the pore formers. The drug was then loaded into the preformed porous microparticles by incubation in aqueous drug solutions followed by air- or freeze-drying. The drug was strongly bound to the polymeric surface with air-dried microparticles. A biphasic drug release with an initial rapid release phase (burst effect) was followed by a slower release up to several weeks. The initial burst was dependent on the drug loading and could be significantly reduced by wet (non-aqueous) temperature curing.

1. Introduction

The most widely used methods for the microencapsulation of macromolecules are w/o/w solvent extraction/evaporation, coacervation and spray drying techniques [1–3]. These drugs require prolonged treatment and due to their short half-life and poor systemic and oral bioavailability are interesting candidates for microencapsulation into PLGA microparticles. However, the macromolecular drug can degrade during the preparation of the microparticles, during storage and also during in vitro or in vivo release. For example, the drug may be exposed to heat, shear forces, pH-extremes, organic solvents, interfaces, freezing and drying during preparation of the microparticles.

One strategy to overcome these processing problems is to load the drug into preformed porous microparticles. The drug can be loaded by a simple and mild procedure, i.e. the preformed porous microparticles incubated in an aqueous drug solution followed by drying. The drug may remain in the solid state within the porous microparticles until release in vivo [4–10]. Further, it could be possible to prepare sterile products without terminal sterilization by loading a sterile-filtered drug solution into pre-sterilized porous microparticles.

Several techniques have been investigated in recent years to form highly porous biodegradable systems (microparticles and scaffolds) for use in tissue engineering [11–15], solvent casting/particulate leaching [16–18], gas-foaming [19] and phase separation [20–22].

The interaction of recombinant human bone morphogenic protein-2 (rhBMP-2) with poly(D,L-lactide-co-glycolide) porous microparticles was investigated [5]. The loading equilibrium was attained within 8 h. Increasing the concentration of the rhBMP-2 solution increased both the amount of rhBMP-2 adsorbed and free rhBMP-2 (physically entrapped). A higher ionic strength and buffer concentration decreased the rhBMP-2 adsorption. Unbound (free) rhBMP-2 was released during an initial period of 72–96 h. This phase was followed by a phase with no significant release of rhBMP-2 up to 7 days. The bound protein was released slowly upon erosion of the polymer. A biphasic release of BSA from preformed porous poly(L-lactic acid) microparticles was reported [8]. The initial burst was inversely proportional to the BSA loading and correlated well with the water penetration. The sustained release phase was independent of the water penetration kinetics. The BSA-polymer interaction was a major contributing factor to the overall release kinetics. Recently, recombinant human growth hormone was loaded into porous microparticles, and an extended release profile was obtained by closing pores using ethanol vapour in a fluidized bed reactor [10]. The release of dextran and mannitol (loading level between 0.5% to 2.5%) from porous L-PLA microparticles was sustained for an extended period of time (160 days) [4].

The objective of this study was to prepare highly porous microparticles by a w/o/w multiple emulsion solvent evaporation method followed by leaching of pore formers. The microparticles were then loaded with oligonucleotides and investigated with respect to drug uptake and release.

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2. Materials and methods

2.1. Materials

The following materials were used as received and were at least of reagent grade: phosphorothioate oligodeoxynucleotide (ISIS Pharmaceuticals Inc., Carlsbad, CA, USA), poly(D,L-lactide) (PLA, Resomer® R 206, Mw 1,25,000, inherent viscosity 1.0; poly (D,L-lactide-co-glycolide) (PLGA, Resomer® RG 756, Mw 89,000, inherent viscosity 0.8; Resomer® RG 503, Mw 42,800, inherent viscosity 0.4; Boehringer Ingelheim KG, Ingelheim Germany), polyvinyl alcohol (PVA, Mowiol® 40–88, Clariant GmbH, Frankfurt, Germany), glycerol monooleate (GMOrphic® 80; Eastman Chemical Company, Kingsport, TN USA), ethanol, *n*-hexane, methylene chloride, potassium dihydrogen phosphate, sodium hydroxide, sodium chloride, sodium azide (Merck KGaA, Darmstadt, Germany).

2.2. Preparation of porous microparticles

Porous microparticles were prepared by the w/o/w multiple emulsion methods with NaCl or glycerol monooleate as pore formers. (1) NaCl: An aqueous solution of NaCl (0, 25, 50, 75 and 100 mg in 1 ml water) was emulsified into a solution of polymer (0.30 g) in methylene chloride (4.0 ml with R 206 and RG 756 and 2.0 ml with RG 503) by probe sonication (Sonoplus® HD 250, Bandelin Electronic, Berlin, Germany) for 30 s under ice cooling in order to obtain the primary w/o emulsion. (2) Glycerol monooleate: 0.5 ml water was emulsified into a solution of polymer (0.30 g) in methylene chloride (4.0 ml) containing glycerol monooleate (100 mg) by probe sonication (Sonoplus® HD 250, Bandelin Electronic, Berlin, Germany) for 30 s. The primary w/o emulsion was subsequently dispersed into 800 ml external aqueous phase (0.25% w/v PVA) by a propeller stirrer (Heidolph Elektro, Kehlheim, Germany) and stirred for 3 h. The solidified microparticles were separated from the external aqueous phase by wet-sieving (stainless steel test sieves; 50, 100 and 150 μm). The porous microparticles prepared with NaCl as pore former were washed with 200 ml water and were then re-dispersed in 1000 ml water and stirred with a magnetic stirrer (Variomag-Electronicrührer Multipoint HP 6, H + P Labortechnik GmbH, Oberschleißheim, Germany) for 24 h to remove NaCl. The resulting porous microparticles were separated from water again by wet-sieving, followed by desiccator-drying for 48 h. The porous microparticles prepared with glycerol monooleate as pore former were washed three times with *n*-hexane (100 ml) for 1 h to extract glycerol monooleate, followed by desiccator-drying for 48 h.

2.3. Drug loading of porous microparticles

Porous microparticles (200 mg) were pre-wetted with 5 ml of 20–50% (v/v) ethanol in a glass vial prior to drug loading. The wetted microparticles were subsequently rinsed three times with 5 ml water to remove the ethanol. The pre-wetted and washed microparticles were then loaded with drug by incubating the microparticles in 4 ml of an aqueous oligonucleotide solution at room temperature under agitation in a horizontal shaker (IKA HS 501 digital horizontal Shaker, Janke & Kunkel & Co. IKA Labortechnik, Staufen, Germany) for different periods of time (0.5, 1, 2, 4 and 6 h). Five different concentrations of oligonucleotide solutions were used: 0.1, 0.5, 1.0, 1.5 and 2.0% w/v. The microparticles were removed by filtration and dried by freeze- or air-drying.

2.4. Washing of the drug-loaded porous microparticles

Drug-loaded microparticles were rinsed with water to remove loosely bound drug. The microparticles (200 mg) were placed in a 4 ml glass vial and 2 ml distilled water was added. The vial was shaken for 30 s followed by removal of water with a micropipette. The washed microparticles were dried in a desiccator for 48 h.

2.5. Curing of porous microparticles

100 mg drug-loaded microparticles were dispersed in 25 ml of liquid paraffin oil (+2% w/v Tween 80) with a propeller stirrer (Heidolph Elektro, Kehlheim, Germany). The resulting microparticle suspension was heated and stirred in a water bath at 60 or 80° C for different periods of time (1, 5 and 15 min). After cooling to room temperature, the cured microparticles were collected by filtration and washed with 100 ml *n*-hexane three times to remove excess oil, and stored in desiccator.

2.6. Scanning electron microscopy (SEM)

The external and internal morphology of the porous microparticles was studied by scanning electron microscopy (Philips SEM 515, type PW 6703, Philips Optical Electronics, Eindhoven, Netherlands). The microparticles were dispersed in a solvent-free glue (UHU GmbH, Bühl, Germany) followed by cutting the dried matrix with a razor blade in order to observe the internal structure. The microparticles were coated for 230 s with gold–palladium under an argon atmosphere using a gold sputter module in a high-vacuum evaporator (Sputter coater device 040, Blazers Union, Liechtenstein). The coated microparticles were then observed with a scanning electron microscope.

2.7. Particle size measurement

The size distribution of the microparticles was examined by laser light scattering including the Polarization Intensity Differential Scattering (PIDS) with a Coulter® LS 230 (Coulter Electronics, Krefeld, Germany) in deionized water. The volume distribution of the particle size was calculated based on relative frequency of the particles ($n = 3$).

2.8. Drug content

Microparticles (40–50 mg, accurately weighed) were dissolved in 8 ml of 0.5 M NaOH solution under agitation in a horizontal shaker (IKA HS 501 digital horizontal Shaker, Janke & Kunkel & Co, IKA Labortechnik, Staufen, Germany) for 12 h. The drug concentration in the aqueous phase was determined by UV-spectrophotometry at $\lambda = 260$ nm (Shimadzu UV 2101 PC UV–vis scanning spectrophotometer, Kyoto, Japan) [23]. The oligonucleotide actual loading was calculated as

$$\text{Actual drug loading}(\%) = \text{mass of extracted drug/mass of microparticles} \times 100\%$$

2.9. In vitro drug release

Microparticles (40–50 mg, accurately weighed) were placed into a 10 ml glass vial followed by the addition of 8 ml prewarmed release medium (0.1 M phosphate buffer, pH 7.4, with 0.1% sodium azide as preservative; 37° C), followed by horizontal shaking in a temperature-controlled shaker (37 °C, 75 rpm, $n = 3$; GFL 3033, Gesellschaft für Labortechnik mbH, Burgwedel, Germany). At predetermined time intervals, 2 ml samples were withdrawn and replaced with fresh medium. The drug concentration was measured UV-spectrophotometrically at $\lambda = 260$ nm (Shimadzu UV 2101 PC UV-Vis scanning spectrophotometer, Kyoto, Japan) [23].

3. Results and discussion

3.1. Preparation of porous microparticles

Highly porous microparticles were prepared by a modified multiple emulsion (w/o/w) solvent evaporation technique. Two alternative procedures to incorporate a pore former were used: (1) addition of NaCl to the internal water phase or (2) addition of glycerol monooleate to the PLGA phase. Microparticles were obtained after emulsification of the primary w/o emulsion into the external phase. The solidified microparticles were washed for 24 h with deionized water in the case of NaCl, and with hexane in the case of glycerol monooleate to extract the pore formers.

3.2. Morphology and particle size of the microparticles

The addition of NaCl to the inner water phase led to a porous, sponge-like structure (Fig. 1). The porosity appeared to increase (by visual observation) with increasing molecular weight and lactide content of the PLGA polymer (Resomer R 206 > RG 756 > RG 503). This is probably because of the faster polymer precipitation of the higher molecular weight and lactide-containing polymers. The pres-

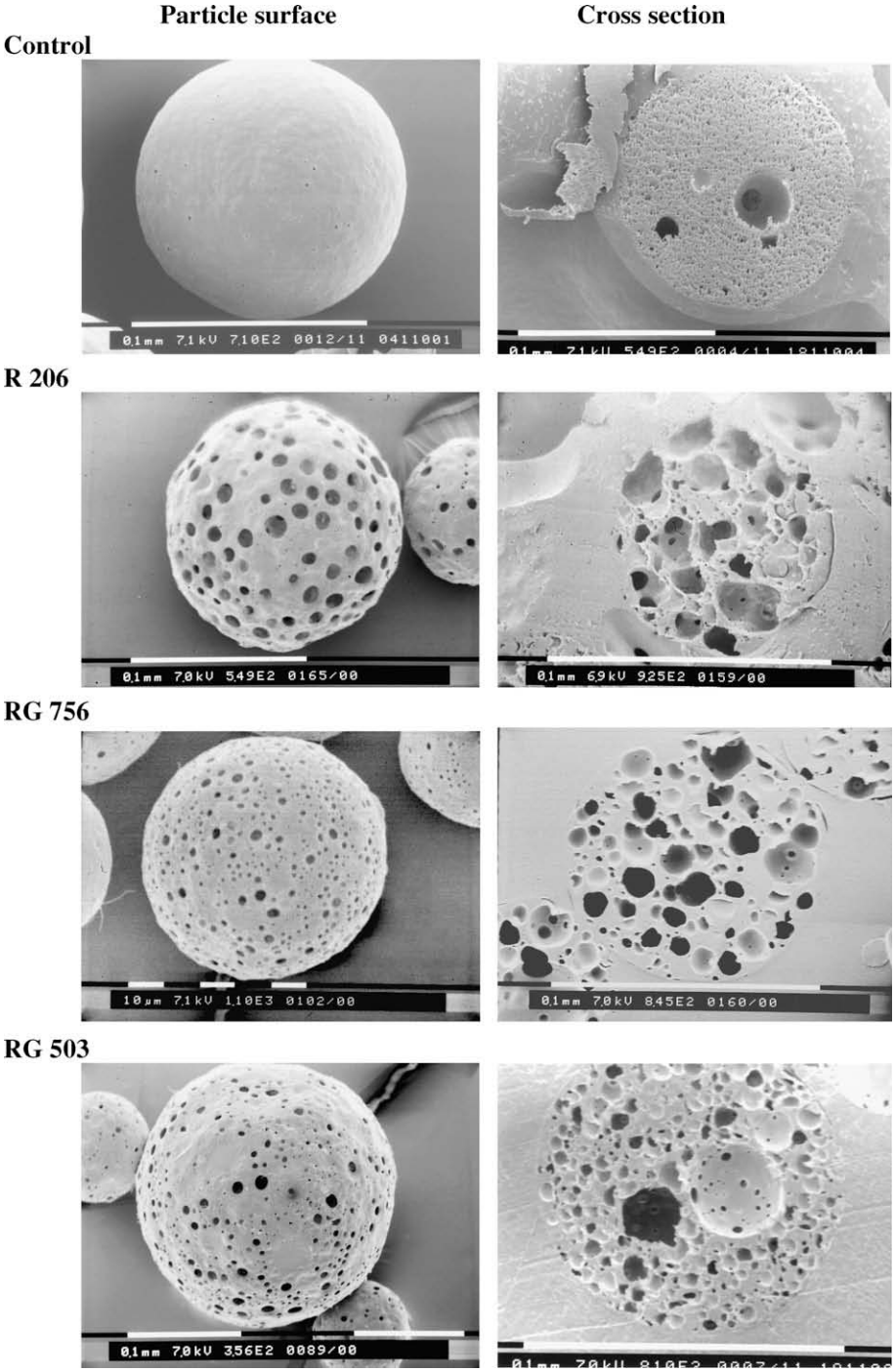

Fig. 1. Scanning electron micrographs of porous microparticles: Control (no NaCl, RG 756), R 206, RG 756 and RG 503 (internal water phase contains NaCl 100 mg/ml).

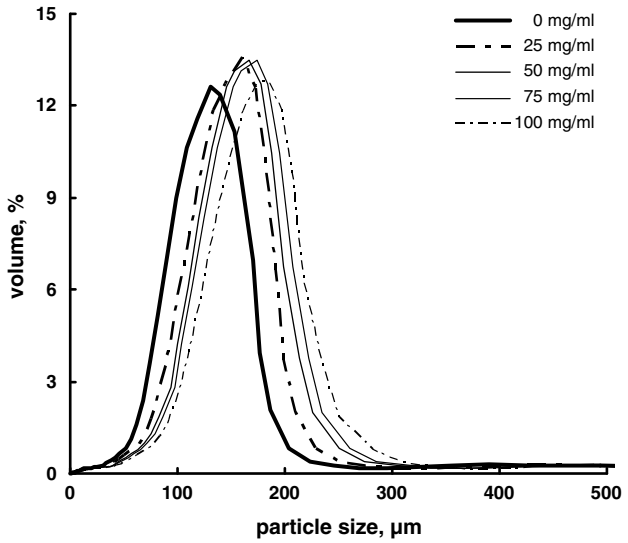

Fig. 2. Effect of the NaCl concentration in the internal water phase on the particle size distribution of porous microparticles (polymer: PLA R 206, w/o 1:4, $n = 3$).

ence of salt in the inner water phase attracts water from the external phase to penetrate along the osmotic pressure gradient, resulting in swelling of the internal droplets. The solvent extraction to the external phase caused the precipitation of the polymer thereby immobilizing and encapsulating the inner aqueous phase within the polymeric matrix. The entrapped aqueous droplets evaporated during the drying process and left pores behind. The mean particle size increased with increasing NaCl concentration (Fig. 2).

Highly porous microparticles were also obtained with glycerol monooleate as pore-former (Fig. 3). Microparticles were irregular and less spherical in shape may be due to rapid solvent movement. Pores formation can be explained by the fact that the solvent diffused out and the non-solvent (water) diffused into the embryonic microparticle droplets upon emulsification of the primary emulsion with the external aqueous phase. A portion of monoglyceride also diffused out with the solvent. The monoglyceride entrapped inside the embryonic microparticle droplets absorbed additional water from the external aqueous phase and swelled into an isotropic cubic phase within the polymeric network [24]. Consequently phase separation between the swollen cubic phase and the polymer occurred. Washing of solidified microparticles with hexane dissolved glycerol monooleate, thus resulting in pore formation.

3.3. Drug loading

Porous microparticles were loaded with oligonucleotide by incubation in an aqueous drug solution (Fig. 4). The drug loading increased with increasing incubation time. An equilibrium of approximately 3–4% was reached after 4 h incubation with all

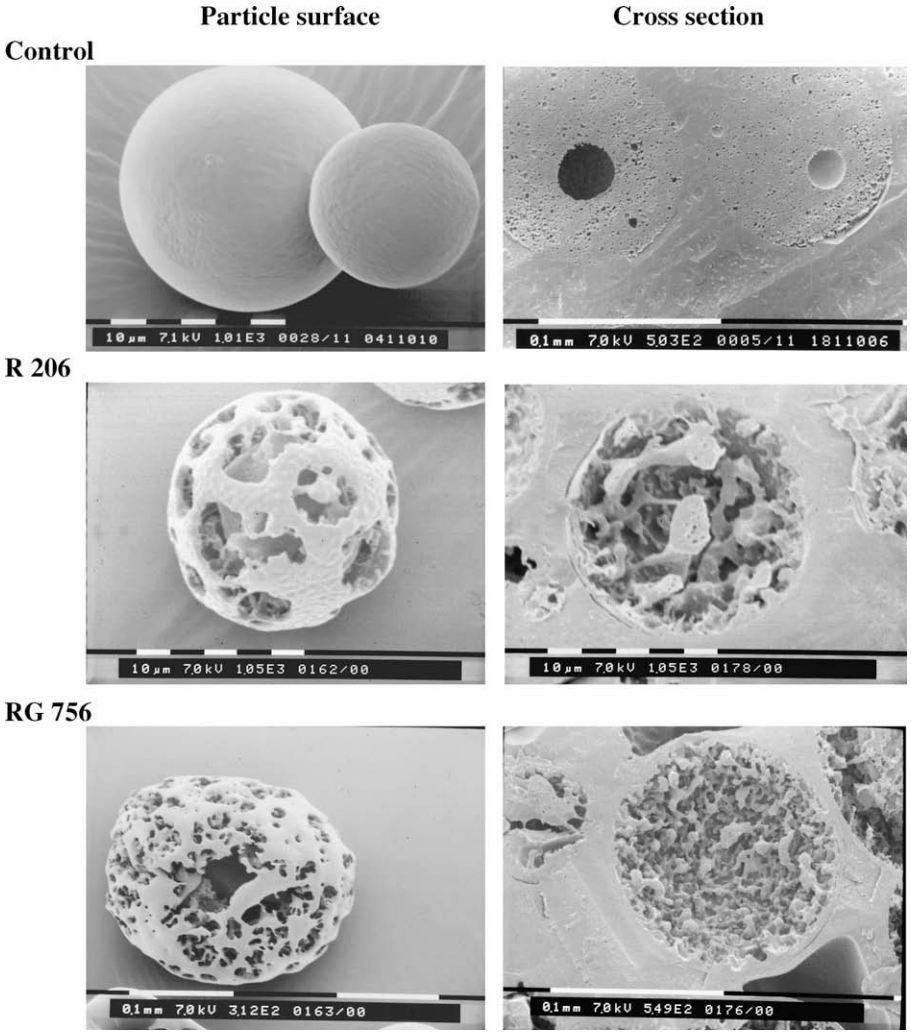

Fig. 3. Scanning electron micrographs of porous microparticles: Control (no glycerol monooleate, RG 756), R 206 and RG 756 (oil phase contains glycerol monooleate 25 mg/ml).

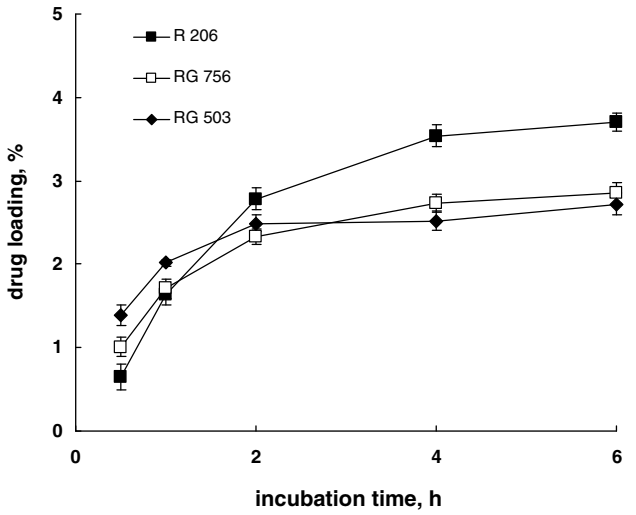

Fig. 4. Effect of the incubation time and polymer type on drug loading onto preformed porous microparticles (drug solution: 1% w/v, $n = 3$).

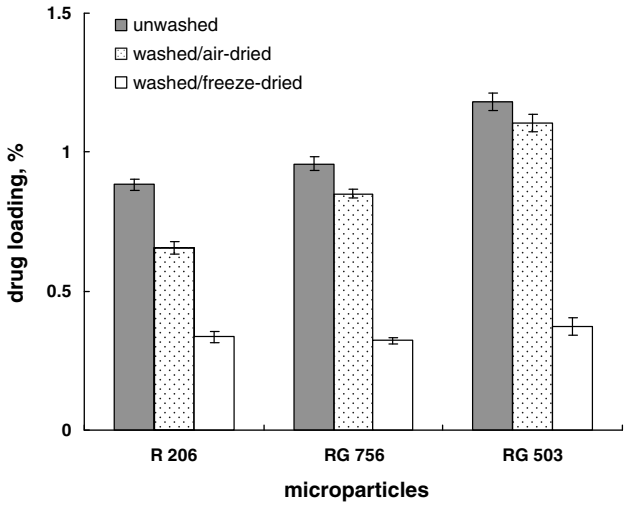

Fig. 5. Effect of drying process on the drug loading to the porous microparticles ($n = 3$).

polymers. The drug loading increased with increasing drug concentration in the incubation medium with all polymers (R 206, RG 756 and RG 503) (Table 1).

3.4. Effect of drying process

The drug-loaded microparticles were either freeze- or air-dried. The microparticles were then washed with deionized water to determine/remove the fraction of loosely bound drug. A significant drug loss was observed after washing the freeze dried microparticles independent of the type of polymer (Fig. 5). In contrast, a comparatively low drug loss was observed after washing the air-dried microparticles. A portion of oligonucleotide was adsorbed to the surface of the porous microparticles during the incubation period in the adsorption medium (aqueous drug solution). The microparticles were separated from the adsorption medium by filtration. Microparticles to be freeze dried were frozen at −70° C prior to the freeze-drying process. During the freezing period, non-adsorbed drug from the aqueous drug solution was entrapped inside the ice. After sublimation of the water, the drug was physically entrapped inside the pores of microparticles without entrapment in the polymer. The non-entrapped drug quickly dissolved in the washing medium (water). Consequently, a significant amount of drug was lost during this washing process. In contrast, the drug was entrapped in the porous microparticles during the air drying. The polymer swelled and softened due to the presence of aqueous medium [25] and shrunk again upon air drying, thus entrapping the drug within the polymer matrix. As a result, a lower amount of the drug was lost during washing.

Table 1
Effect of oligonucleotide concentration on drug loading into preformed porous microparticles prepared by using pore-former NaCl or glycerol monooleate (incubation time 60 min, $n = 3$)

Drug concentration, % w/v	Actual drug loading, %			
	NaCl			Glycerol monooleate
	R 206	RG 756	RG 503	R 206
0.1	0.14 ± 0.02	0.22 ± 0.01	0.27 ± 0.03	0.27 ± 0.01
0.5	0.52 ± 0.02	0.42 ± 0.01	0.44 ± 0.01	0.58 ± 0.03
1.0	1.63 ± 0.01	1.71 ± 0.03	2.02 ± 0.01	1.75 ± 0.01
1.5	2.35 ± 0.03	2.41 ± 0.03	2.34 ± 0.06	2.56 ± 0.07
2.0	2.63 ± 0.02	2.64 ± 0.06	2.49 ± 0.07	2.94 ± 0.02

3.5. In vitro drug release

The drug release was biphasic with an initial rapid release (burst effect) followed by a slower second phase of release (Fig. 6). The initial burst increased with increasing drug loading. During the dissolution test, the release medium penetrated into the porous microparticles and dissolved unbound drug. At higher drug loadings, a higher amount of unbound drug was present. The tightly entrapped oligonucleotide was then released over a period of several weeks after the initial burst.

After the initial burst, the oligonucleotide release was not rate-limited by the water uptake, which only controls the initial release phase [8]. Once the porous matrix is saturated with the release medium, the slow oligonucleotide release over several weeks could be due to several factors: (1) interactions between oligonucleotide and microparticle surface. The release of oligonucleotide at loading levels of 0.5 and 1% (w/w) could be dramatically increased by the addition of 0.1% Tween 20 (w/v) to the release medium (Fig. 7). The microparticles were rapidly wetted thus resulting in an increased release. (2) The drug, which was entrapped inside the polymer matrix during the drying process, was released slowly by diffusion through the polymer prior to erosion of the polymer.

The effect of the polymer type on the drug release was investigated by preparing porous microparticles of different polymers R 206, RG 756 and RG 503 (Fig. 8). The drug release was biphasic with R 206 and RG 756. In contrast, microparticles prepared from RG 503 (low molecular weight, higher glycolide containing polymer) showed a triphasic drug release, an initial burst, followed by a very slow release phase of almost no release and a third phase with extended release. R 206 (PLGA 100/0), RG 756 (PLGA 75/25) and RG 503 (PLGA 50/50) have half-lifes of 12, 6 and 1 month, respectively [26]. The third release phase with the RG 503 microparticles could thus be explained with the drug being released through erosion of the polymer.

3.6. Reduced initial burst release

Porous microparticles showed a high initial burst release (approximately 90%) after 24 h. The microparticles were temperature cured in order to close the pores after the drug loading process. A non-aqueous curing technique was applied because of the high water solubility of the drug. The drug-loaded porous microparticles were subjected to an oil at a temperature near or above the glass transition temperature of the polymer for several minutes

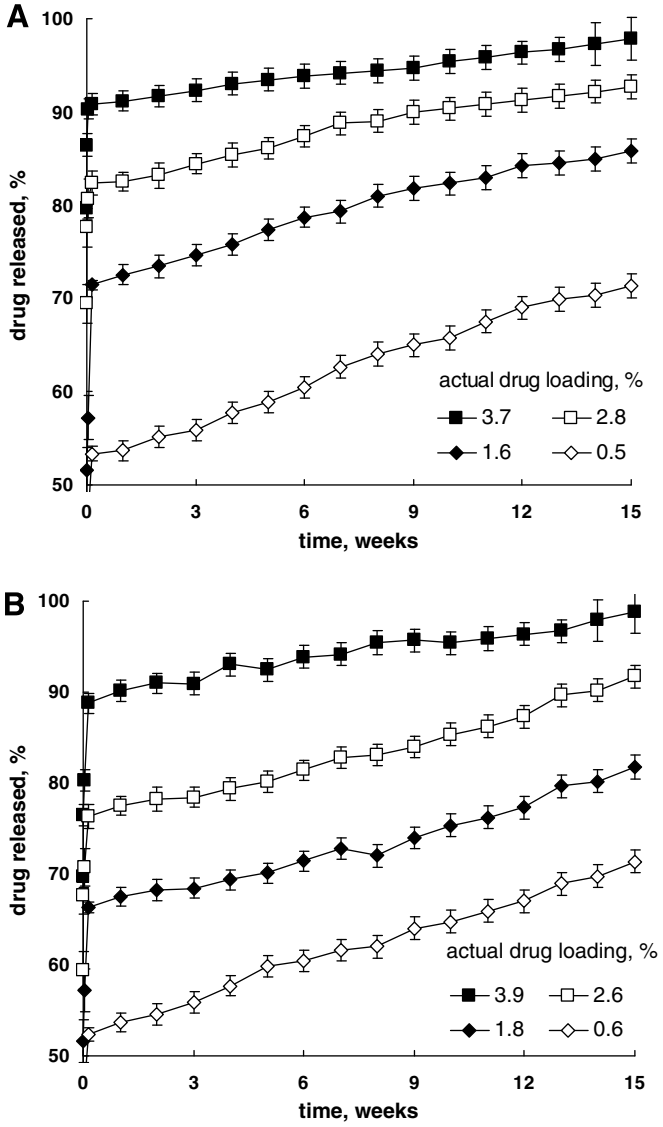

Fig. 6. Effect of drug loading on drug release from the preformed porous microparticles prepared with pore formers [A] NaCl and [B] glycerol monooleate (PLA R 206, unwashed porous microparticles, $n = 3$).

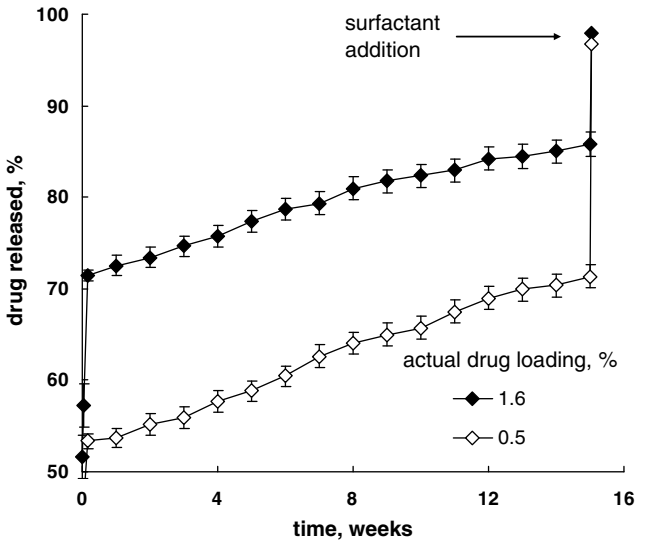

Fig. 7. Effect of Tween 20 on the release of oligonucleotide from porous microparticles with different drug loadings (surfactant was added after 15 weeks, $n = 3$).

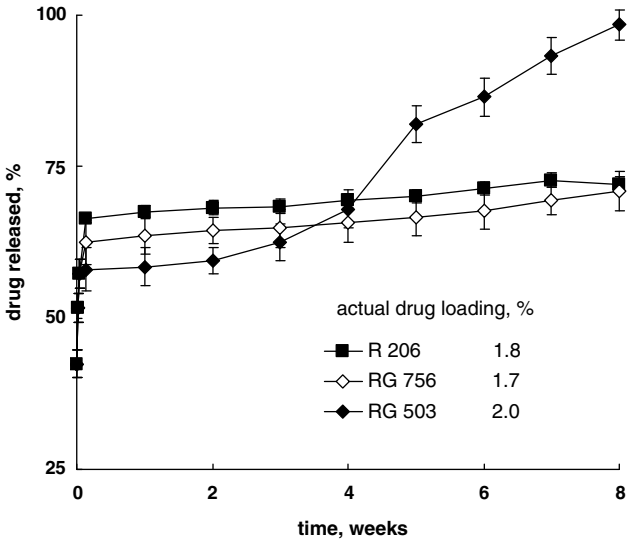

Fig. 8. Drug release from the preformed porous microparticles prepared from different polymers ($n = 3$).

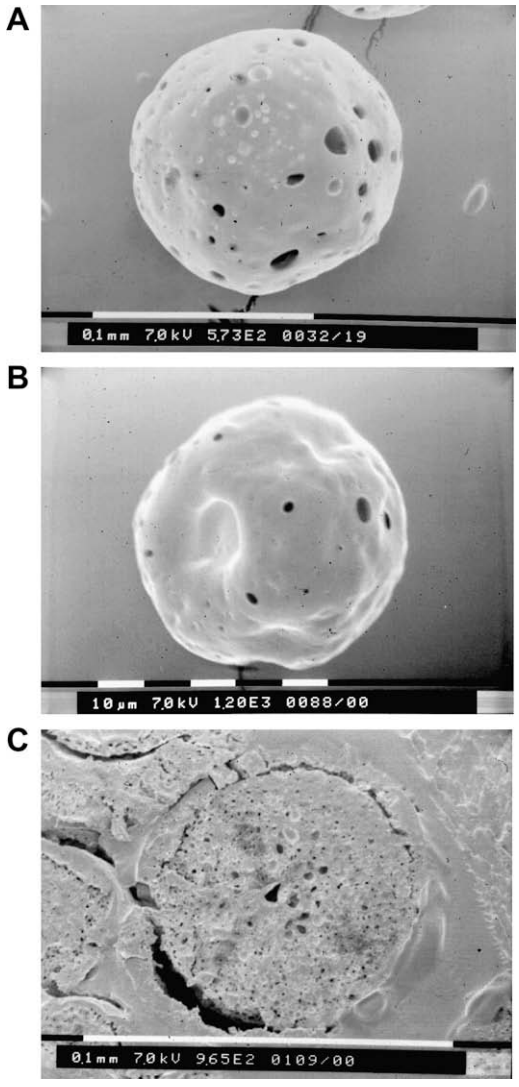

Fig. 9. Scanning electron micrographs of porous microparticles cured for 5 min by non-aqueous method at [A] 60° C, [B] 80° C and [C] 80° C (cross-section) (PLA R 206).

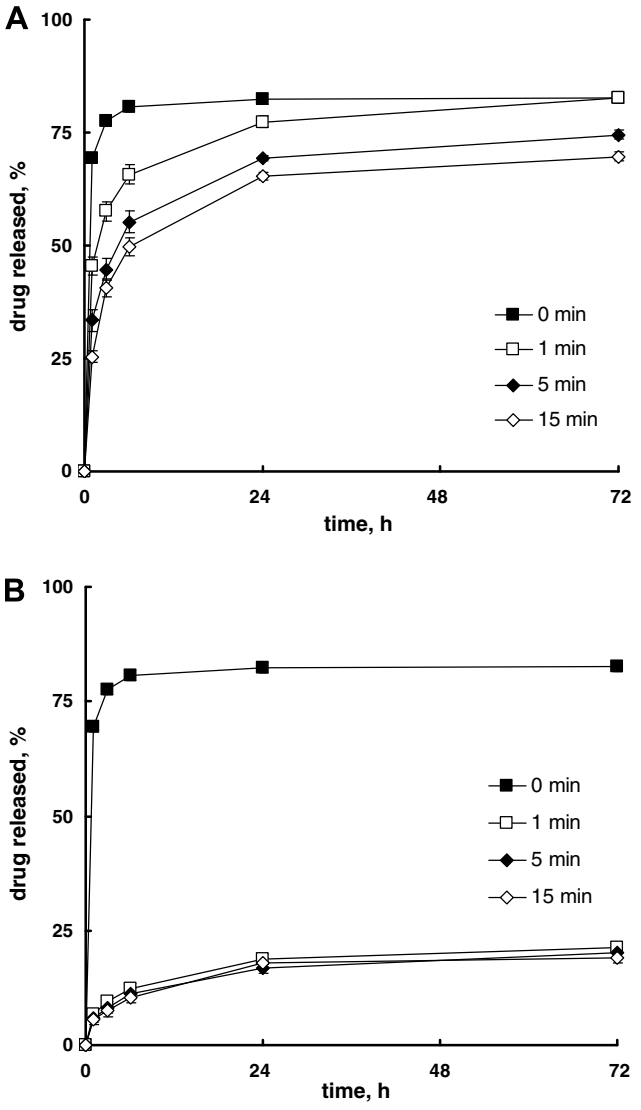

Fig. 10. Drug release from porous microparticles cured by non-aqueous method at different temperatures: [A] 60° C and [B] 80° C (PLA R 206, $n = 3$).

followed by washing with hexane. The external and internal pores were almost completely closed by curing at 80° C compared to curing at 60° C (Fig. 9A–C).

The initial burst decreased with increasing curing time at 60° C, indicating a continued densification of the polymer matrix (Fig. 10A). However, overall, the release was still fairly rapid after curing at 60° C, which is slightly above the T_g of the polymer. The initial burst could be significantly reduced to <20% with curing at 80° C (Fig. 10B).

4. Conclusions

Porous microparticles can easily be prepared by multiple emulsion (w/o/w) solvent extraction/evaporation method with NaCl or glycerol monooleate as pore formers. The drug loading of preformed porous microparticles is interesting for drugs, which are highly sensitive to the encapsulation conditions of the classical encapsulation methods. By non-aqueous temperature curing the higher initial burst can be controlled.

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