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Note

Cell interaction studies of PLA-MePEG nanoparticles☆

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Abstract

Poly(D,L-lactic acid)-methoxypoly(ethylene glycol) (PLA-MePEG) copolymers were synthesized by ring-opening polymerization of D,L-lactide in the presence of MePEG of different molecular weights and stannous octoate as the catalyst. The chemical composition of the diblock-copolymer PLA-MePEG was confirmed by ^{1}H-NMR and the molecular weight and distribution were assessed by gel permeation chromatography. Nanoparticles containing Nile red as a fluorescent dye were prepared using poly(D,L-lactic acid) (PLA), blends of PLA and PLA-MePEG or PLA-MePEG alone. Incubation of nanoparticles with human blood monocytes was performed in serum or in PBS and the cell-associated fluorescence was analyzed by flow cytometry. In serum, a protective effect was obtained and the interaction of particles with mononuclear leukocytes decreased to 40%.

Keywords: Nanoparticles; Surface modification; PLA-MePEG; Flow cytometry; Cell interaction; MPS

Nanoparticles (NP) having methoxypoly(ethylene glycol) (MePEG) at their surface have been shown to undergo reduced interaction with cells of the mononuclear phagocytic system (MPS) and to remain in the systemic circulation for a prolonged period (Gref et al., 1994; Basile et al., 1995). Biodegradable AB diblock copolymers such as poly(lactate-*b*-ethylene oxide) can be used to prepare particles in which the MePEG chains are covalently bound to the core of the NP (De Jaeghere et al., 1999).

The aims of this study were to synthesize PLA-MePEG copolymers of different composition to produce NP with these polymers and to evaluate their interactions with mononuclear leukocytes.

Poly(D,L-lactic acid) (PLA) R202H with an average molecular weight of 16,000 Da was a gift from Boehringer Ingelheim (Germany) and was used as received. D,L-Lactide (Purac Biochem B.V., The Netherlands) was recrystallized twice in toluene and dried under vacuum overnight. Methoxypoly(ethylene glycol) with M_w of 550, 2000 and 5000 Da were a gift from Union Carbide (Danbury, CT, USA) and were washed twice with an excess of dioxane and dried overnight in the melt (75 °C, 0.05 mbar). Poly(vinyl alcohol) (PVAL) with a M_w of 26,000 Da (Mowiol® 4–88, Hoechst, Germany) and magnesium chloride hexahydrate (Fluka, Switzerland) were used as the emulsifier and salting-out agent, respectively. Stannous octoate was purchased from Sigma (St. Louis, MO, USA), trehalose and all other chemicals were of analytical grade and used as received.

The PLA-MePEG diblock copolymers were synthesized by ring-opening polymerization of D,L-lactide

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in the presence of MePEG (M_w of 550, 2000 or 5000 Da). Stannous octoate was used as a catalyst as described previously (Tobío et al., 1998). Typically, polymerization was conducted in a glass flask, where 10.0 g of the monomer were mixed with 1.6 g of MePEG 2000 and stannous octoate (0.1%). The feed was degassed through vacuum/argon cycles applied to the molten mixture at 135 °C. The flask was then sealed under vacuum (4×10^{-2} mbar). After 12 h of stirring at 145 °C, the flask was opened and the resulting polymer was dissolved in 20 ml of chloroform. It was then precipitated in a 10-fold excess of ethanol and dried under vacuum (20 mbar) during 2 days at 40 °C. ^{1}H-NMR was performed with a 500 MHz Bruker spectrometer in $CDCl_3$. Molecular weights (M_w, M_n and polydispersity) were determined by size exclusion chromatography (SEC) using a Waters liquid chromatography system equipped with Styragel HR columns (MA, USA). Data was expressed relative to a calibration curve generated from polystyrene standards. The mobile phase was chloroform (1 ml/min).

Nanoparticles were produced by the salting-out process, as described elsewhere (Allémann et al., 1993). Briefly, an aqueous phase (10 g) containing 60% (w/w) of magnesium chloride hexahydrate and 10% (w/w) of PVAL was added under mechanical stirring (2000 rpm) to an acetone solution (5 ml) containing 0.2 g of polymer, namely blends of PLA and PLA-MePEG of various ratios. During emulsification, the miscibility of both phases was avoided by the high concentration of salt in the aqueous phase. Subsequently, 40 g of pure water was added to induce the diffusion of acetone into the aqueous phase and the formation of the nanoparticles. Fluorescent nanoparticles were also produced by dissolving Nile red (0.02%, w/w) in the organic phase. Centrifugation was used to remove the organic solvent, free PVAL and electrolytes from the raw nanoparticle suspensions. Purification of NP was performed five times at 13,500 × g for 15 min (Beckman, CA, USA). NP were freeze-dried with trehalose as lyoprotective agent using a ratio (NP:trehalose, 1:4, w/w).

The mean particle size, and polydispersity index were determined by photon correlation spectroscopy (PCS) using a Zetasizer® 5000 (Malvern, Worcestershire, UK).

Mononuclear leukocytes (mixture of lymphocytes and monocytes) were isolated from fresh human blood using the density gradient separation procedure Accuspin™ System-Histopaque®-1077 (Sigma) (De Jaeghere et al., 2000). The cells were washed by centrifugation in phosphate buffer saline (PBS) and redispersed in human serum (10×10^6 cells/ml). Aliquots of 100 μl were withdrawn and added to 50 μl of a 0.01% (w/w) suspension of Nile red-loaded nanoparticles in PBS (100 NP/cell). Incubation was performed for 30 min at 37 °C under gentle stirring. Cells were washed twice in PBS and analyzed using a FACScan® flow cytometer (Becton Dickinson, USA) (De Jaeghere et al., 2000; Leroux et al., 1994). The excitation of Nile red-loaded NP was performed at 488 nm and only the fluorescence associated with the monocytes was analyzed because these cells act as efficient phagocytes (Leroux et al., 1994). Each determination was performed in triplicate and normalized as a percentage of maximal fluorescence emission recorded during the experiment.

Results and discussion

The composition and the molecular weight of PLA-MePEG were characterized by size exclusion chromatography (SEC) and by ^{1}H-NMR spectroscopy. The measured molecular weight and the molecular weight distribution of PLA and PLA-MePEG are summarized in Table 1. After precipitation of the synthesized PLA-MePEG using a 10-fold excess of ethanol, the peak of D,L-lactide at 5.43 ppm could no longer be detected, indicating that the synthesized copolymer contained almost no residual monomer (results not shown).

Results from SEC measurements exhibited relatively similar values to the calculated molecular weights of PLA-MePEG. This demonstrated that PLA-MePEG of varying molar composition could be synthesized in the bulk state. SEC chromatograms exhibited a monomodal distribution of molecular weight (results not shown).

The mean particle size and polydispersity index of NP were determined by photon correlation spectroscopy using a Zetasizer®. From the PCS measurements, the mean particle sizes of different batches of NP made of PLA alone or blends of PLA and PLA-MePEG after resuspension in PBS ranged from 294 to 343 nm. As shown in Table 2, full redispersion of nanoparticles in PBS could be achieved after

Table 1
Composition and molecular weight distribution of PLA and PLA-MePEG

Polymer	Polymer composition (kDa)[a]		Molecular weight, M_w ($\times 10^{-3}$)[b]	Polydispersity (M_w/M_n)
	PLA	MePEG		
PLA	–	–	14.8	1.34
PLA-MePEG 550	10.5	0.6	11.0	1.27
PLA-MePEG 2000	9.5	1.9	11.5	1.35
PLA-MePEG 5000	11.0	5.8	16.6	1.22

[a] According to ^{1}H-NMR results.
[b] Determined by SEC.

rehydration, but freeze-dried NP of blends of PLA and PLA-MePEG or PLA-MePEG showed a slightly increased size (up to 25 nm) compared to control batches (pure PLA nanoparticles). These results confirm that MePEG-containing NP must be freeze-dried in the presence of a lyoprotective agent such as trehalose to allow their redispersion (De Jaeghere et al., 1999).

In serum, the protective effect of MePEG was dependent on its content in the nanoparticles. The cell-associated fluorescence decreased linearly, to 40% of the normalized value, with increasing levels of MePEG (Fig. 1). The ability to reduce cellular interactions was even more pronounced, when the longer chain length MePEG (5000 Da) was incorporated into the NP. This study has demonstrated that the protective efficiency of MePEG is directly related to both the MePEG chain length (i.e. molecular weight) and chain density at the nanoparticle surface. The interaction of NP with mononuclear leukocytes was only 20% in PBS compared to control batches of PLA nanoparticles incubated in serum. This effect was independent of the polymer composition employed (see Fig. 2). The results obtained suggest that the MePEG layer acts by interfering with the opsonization process (Leroux et al., 1995) (see Fig. 2).

In this study, PLA-MePEG copolymers having a PLA moiety of approximately 10 kDa but exhibiting different MePEG chain lengths were synthesized. Using these copolymers or blends of these copolymers with PLA, it was possible to produce surface-modified nanoparticles. For long term storage, freeze-drying was investigated, but this process had a negative effect on particle redispersion, leading to massive aggregation. Particle aggregation was successfully prevented by the addition of trehalose as a lyoprotective agent. By increasing the amount of PLA-MePEG copolymer in the blend, it was possible to create a stronger steric barrier, leading to decreased capture by mononuclear leukocytes. The ability to reduce interaction in serum was directly dependent on the MePEG chain length

Table 2
Nanoparticle formulation

Batch no.	Composition	PLA % (w/w)	PLA-MePEG % (w/w)	Particle size ± S.D.[a] (nm)	
				Before freeze-drying	After freeze-drying[b]
1	PLA	100	–	292 ± 6	294 ± 7
2	MePEG 550	–	100	281 ± 5	289 ± 7
3	PLA-MePEG 2000	–	100	307 ± 9	319 ± 11
4	PLA + PLA-MePEG 2000	25	75	302 ± 6	313 ± 8
5		50	50	323 ± 8	334 ± 9
6		75	25	340 ± 8	343 ± 8
7	PLA-MePEG 5000	–	100	306 ± 5	331 ± 11
8	PLA + PLA-MePEG 5000	25	75	328 ± 6	341 ± 8
9		50	50	305 ± 7	316 ± 9
10		75	25	311 ± 6	328 ± 8

[a] Standard deviation, $n = 3$.
[b] NP freeze-dried with trehalose (1/4), after resuspension in PBS.

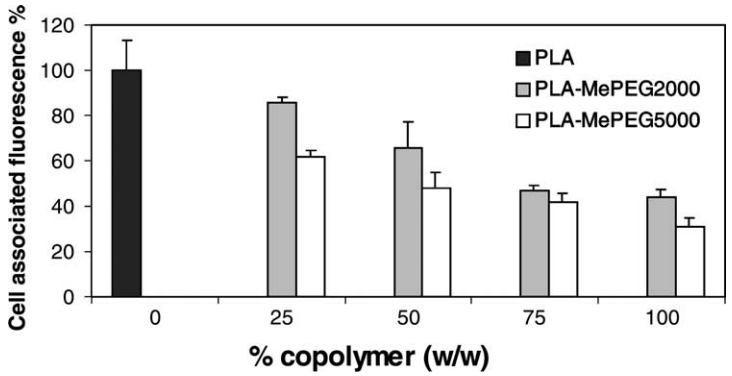

Fig. 1. Type and content of MePEG in NP: interaction with human monocytes in serum (mean ± S.D., $n = 3$).

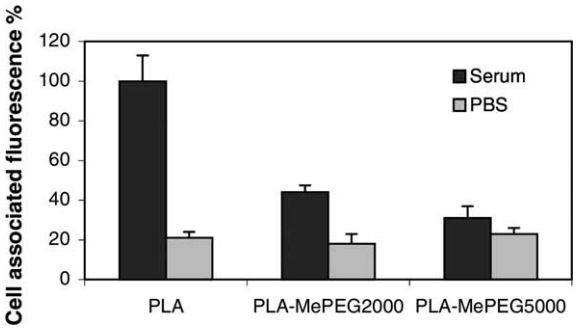

Fig. 2. Type of PLA-MePEG in NP and interaction with human monocytes in serum and in PBS (mean ± S.D., $n = 3$).

(i.e. molecular weight) and chain density at the surface of nanoparticles. In PBS, no reduction was observed, suggesting that the MePEG layer interferes with the opsonization process. Further investigations are in progress in order to evaluate the importance of MePEG molecular weight and surface density with respect to cellular uptake in vitro through more detailed surface characterization of these particles.

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