Synthesis of Pegylated Immunonanoparticles

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Purpose. This work describes the synthesis of pegylated immunonanoparticles by conjugation of an anti-transferrin receptor monoclonal antibody (MAb) to maleimide-grafted pegylated nanoparticles prepared from poly(lactic acid) (PLA) and a bi-functional polyethyleneglycol (PEG).

Methods. Maleimide-PEG_{3500}-PLA_{40000} and methoxyPEG_{2600}-PLA_{40000} copolymers were synthesized by ring opening polymerization of L-lactide using stannous octoate as catalyst. Pegylated nanoparticles were prepared from these copolymers by a multiple emulsion/solvent evaporation method and thiolated OX26 MAb was conjugated through the maleimide function located at the distal end of the PEG spacer. The pegylated immunonanoparticles were characterized by quasi-elastic light scattering, gel permeation chromatography, turbidimetry assays, and transmission electron microscopy.

Results. NMR spectroscopy confirmed the synthesis of both copolymers and the preservation of the maleimide function. The pegylated immunonanoparticles had an average diameter of 121 ± 5 nm and appeared spherical by transmission electron microscopy. The number of OX26 MAb molecules conjugated per individual pegylated nanoparticle was 67 ± 4. The MAb conjugated to the surface of the pegylated immunonanoparticle was visualized directly by electron microscopy using a conjugate of 10 nm gold and an anti-mouse immunoglobulin secondary antibody.

Conclusion. Pegylated immunonanoparticles can be synthesized with bifunctional PEG derivatives that bridge the nanoparticle and the targeting MAb. This novel formulation may enable the targeted delivery of small molecules, protein drugs, and gene medicines.

KEY WORDS: polyethylene glycol; maleimide; immunonanoparticle; monoclonal antibody; brain drug targeting.

INTRODUCTION

Targeted drug delivery using nanocontainers, such as liposomes or nanoparticles, requires that the nanocontainer be delivered to the target organ in parallel with restricted uptake by nontargeted tissues. Liposomes or nanoparticles are rapidly removed from the bloodstream by cells lining the reticulo-endothelial system (RES), and this greatly reduces the area under the plasma concentration curve (AUC) and pharmacological effect. The rapid RES uptake can be reduced, and the plasma AUC increased, by conjugating to the surface of the liposome several thousand polymeric strands, such as polyethyleneglycol (PEG) (1). Pegylated liposomes are not specifically targeted to tissues, but this can be accomplished by tethering to the tips of 1% to 2% of the PEG strands a targeting ligand, such as a receptor-specific monoclonal antibody. The pegylated immunoliposome (PIL) can be delivered across microvascular barriers by receptor-mediated transcytosis (2).

Nanoparticles have certain advantages relative to liposomes since nanoparticles can be freeze-dried for long term storage. Nanoparticles are prepared from polymeric precursors such as poly(lactic acid) (PLA) homopolymer or poly(lactic-co-glycolic acid) (PLGA) heteropolymer. PLA and PLGA are biocompatible and biodegradable polyesters (3). Similar to liposomes, nanoparticles are rapidly removed from the blood by the RES, and this uptake is inhibited by conjugation of PEG to the nanoparticle surface (4–6). Pegylated nanoparticles (PN) are formed by a hydrophobic PLA core that is surrounded by a hydrophilic PEG "corona" layer (5). These can be freeze-dried (4) and loaded with small molecules (7), proteins (8), or plasmid DNA (9). Nanoparticles made of methoxyPEG_{2000}-PLA_{30000} with respective molecular weights of 2000 and 30,000 Daltons for the methoxyPEG and PLA moieties were shown to have an acceptable safety profile in rats (10). Pegylated PLA nanoparticles have a prolonged plasma half-life ($t_{1/2}$ = 6 h) in comparison with PLA nanoparticles coated with albumin or with poloxamer F68 ($t_{1/2}$ = 2–3 min) (6).

Pegylated nanoparticles, like pegylated liposomes, are not specifically targeted to tissues *in vivo*, as this requires the attachment of a targeting ligand to the tip of the PEG strands (2). Targeting ligands, such as endogenous peptides or peptidomimetic MAbs, can be conjugated to the PEG strands of preformed PNs, if a fraction of the PEG strands contains a terminal reactive group. Yasugi *et al.* (11) synthesized sugar-terminated PEG-PLA block copolymers, using a multi-step method that was not adapted to the conjugation of protein ligands, such as antibodies. Most targeting ligands are peptide or protein macromolecules, and mild reaction conditions are necessary to preserve their biologic and receptor binding activity. Pegylated immunoliposomes (PIL) are prepared by conjugating thiolated antibodies to pegylated liposomes, wherein a small fraction of the PEG lipid is comprised of a bifunctional PEG, that contains a lipid polymer such as distearoylphosphatidylethanolamine (DSPE) at one terminus and a reactive maleimide moiety at the other terminus (2). The maleimide function reacts with thiolated proteins in conditions that maintain their biologic and binding properties. The present work describes the synthesis of pegylated immuno-nanoparticles (PIN). The PIN synthesis was enabled by the initial synthesis of a novel copolymer comprised of maleimide-PEG_{3500}-PLA_{40000}, which was produced from a bifunctional PEG_{3500} containing a maleimide moiety at one terminus and a free hydroxyl group at the other terminus (Fig. 1A). The maleimide-PEG_{3500}-PLA_{40000} copolymer was blended with methoxyPEG_{2600}-PLA_{40000} to prepare pegylated nanoparticles, which were then conjugated to a thiolated targeting MAb to obtain the final PIN product (Fig. 1B). The targeting MAb used in these studies is the murine OX26 MAb to the rat transferrin receptor. This MAb undergoes receptor-mediated transcytosis across the brain microvascular barrier, which forms the blood-brain barrier (BBB) *in vivo*,

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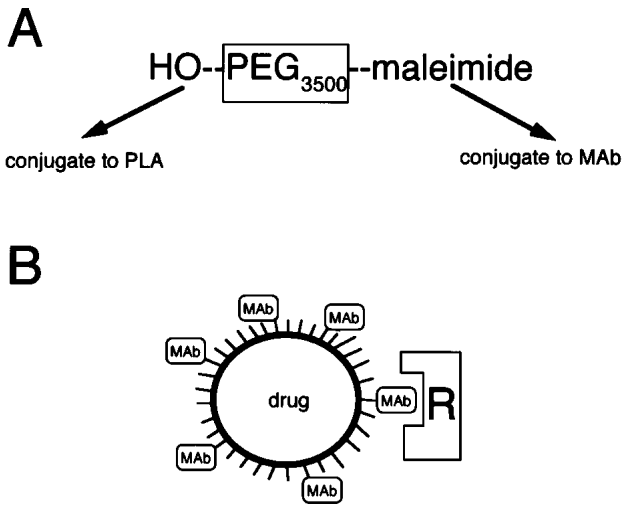

Fig. 1. (A): Schematic diagram of bi-functional PEG_{3500} containing a free hydroxyl group at one terminus, for conjugation to the PLA, and a maleimide moiety at the other terminus, for conjugation to a thiolated targeting ligand. (B): Diagram of a pegylated immunonanoparticle showing drug encapsulated within an 100–150 nm nanoparticle containing a hydrophilic corona of several thousand strands of PEG. About 1–2% of the PEG strands are conjugated with a receptor (R)-specific targeting monoclonal antibody (MAb).

via the endogenous BBB transferrin transport system. Therefore, PINs prepared with anti-transferrin receptor (TfR) MAbs could be used for targeted brain drug delivery. The present studies describe the synthesis of the PIN and use electron microscopy to visualize the targeting MAb conjugated to the surface of the pegylated nanoparticle.

MATERIALS AND METHODS

Materials

Ethyl acetate (99.5% pure) and stannous octoate (95% pure) were obtained from Sigma Chemical Co.(St.Louis, MO, USA). Toluene (99.8% pure), dichloromethane (>99.9% pure), ethyl ether (98% pure), calcium hydride (95% pure), L-lactide (98% pure) were purchased from Aldrich (Milwaukee, WI, USA). Methoxypolyethyleneglycol, Mn = 2600 Daltons ($MethoxyPEG_{2600}$) was obtained from Shearwater Polymers, Inc (Huntsville, AL, USA). Hydroxy-polyethyleneglycol–maleimide (Fig. 1A), Mn = 3501 ($Maleimide\text{-}PEG_{3500}$) was custom-synthesized by Shearwater Polymers, Inc. The number average molecular weights (Mn) are per the manufacturer's specifications. Anti-mouse immunoglobulin (IgG) gold (10nm) conjugate (1.4×10^{13} particles/ml) was obtained from Sigma. Ethyl acetate and toluene were dried and distilled under argon over calcium hydride. L-lactide was dried under vacuum (180 mm Hg) at 70°C for 30 min, recrystallized from dry ethyl acetate, and then dried at 70°C under an argon flow. Stannous octoate was distilled under vacuum, the fraction distilling at 160°C and 30 mm Hg corresponding to the purified compound as assessed by ^{1}H-NMR. $MethoxyPEG_{2600}$ was dried under vacuum and under magnetic stirring at 70°C for 30 min. $Maleimide\text{-}PEG_{3500}$ was dried under vacuum at room temperature for 30 min.

Copolymer Synthesis and Characterization

The $methoxypolyethyleneglycol_{2600}$-$poly(lactic\ acid)_{40000}$, designated $methoxyPEG_{2600}$-PLA_{40000} copolymer, and the $maleimide\text{-}polyethyleneglycol_{3500}$-$poly(lactic\ acid)_{40000}$, designated $maleimide\text{-}PEG_{3500}$-$PLA_{40000}$, were synthesized by ring opening polymerization in dry toluene under moisture-free high-purity argon atmosphere, according to a method adapted from Bazile *et al.* (12). For the $methoxyPEG_{2600}$-PLA_{40000} synthesis, 3.75 g recrystallized L-lactide was combined with 208 mg $methoxyPEG_{2600}$ and 20 mg stannous octoate, as solutions in dry toluene (respective concentrations: 62.5 and 20 mg/ml). For the $maleimide\text{-}PEG_{3500}$-$PLA_{40000}$ synthesis, 3.75 g purified L-lactide was combined with 294 mg $maleimide\text{-}PEG_{3500}$ and 20 mg stannous octoate, as solutions in dry toluene (respective concentrations: 150 and 20 mg/ml). In both cases, the final volume of dry toluene was 10 ml. The polymerization was then carried out under magnetic stirring and under moisture-free high-purity argon atmosphere at 110°C for 2 h. At the end of the polymerization step, the copolymers were purified and recovered as follows. To the reaction medium, slightly cooled dichloromethane (10 ml) was added. The copolymers were then precipitated by addition of 30 ml ethyl ether and recovered on a Buchner funnel with a fritted disk. They were re-dissolved in 20 ml dichloromethane, precipitated by 20 ml ethyl ether, and recovered on the same filter. They were then dried at 70°C under an argon flow for 30 min, and under vacuum at 70°C for 1h. The two copolymers were analyzed by ^{1}H and ^{13}C NMR spectroscopy at 300.0 K using a Brucker Advance 500 spectrometer operating at 500 MHz and with deuterochloroform as the solvent. Chemical shifts in ppm (δ) were determined using the chloroform signals at 7.26 ppm (^{1}H) or at 77.00 ppm (^{13}C) as references. The integrals of the peaks corresponding to the PLA methylene protons (δ 5.1 ppm) and the PEG methylene protons (δ 3.6 ppm) were used to determine the weight ratio of PLA to PEG and to calculate the average number molecular weight (Mn) of the PLA moiety (13). The integral of the peak corresponding to the maleimide protons (δ 6.7 ppm) was used to check for the preservation of the maleimide function in the synthesized $maleimide\text{-}PEG_{3500}$-$PLA_{40000}$.

Pegylated Nanoparticle Preparation

Nanoparticles were made of a blend of $methoxyPEG_{2600}$-PLA_{40000} and $maleimide\text{-}PEG_{3500}$-$PLA_{40000}$. They were prepared using the emulsion/solvent evaporation technique (14). Fifty micro liters of water (aqueous inner phase) was emulsified by sonication (30 s) on ice using a probe sonicator (Branson sonifier cell disruptor) at the maximum power in 1ml of a solution of $methoxyPEG_{2600}$-PLA_{40000} (24.2mg/ml) and $maleimide\text{-}PEG_{3500}$-$PLA_{40000}$ (0.8 mg/ml) in dichloromethane (intermediate organic phase). This primary emulsion was then emulsified by sonication (30 s) on ice in 2 ml of a 1% sodium cholate aqueous solution (aqueous outer phase). The w/o/w emulsion obtained was diluted into 38 ml of a 0.5% sodium cholate aqueous solution under rapid magnetic stirring. After 1 min, dichloromethane was evaporated at low pressure and at 40°C using a Büchi R-3000 rotary evaporator (rotation speed set at 90% maximum speed). Nanoparticles were then centrifuged at 45000 *g* (using a J2–21

Beckman Centrifuge equipped with a JA–20 rotor) and, after discarding the supernatant, they were resuspended in 0.5 ml of a 0.01M HEPES buffer pH 7 containing 0.15 M NaCl and 0.1 mM EDTA. The mean (number based) diameter of the nanoparticles was determined by quasi-elastic light scattering (QELS) using a Microtrac Ultra Particle Analyzer (Leeds-Northrup, St. Petersburg, FL, USA). Nanoparticles were also observed by transmission electron microscopy. Nanoparticle concentration was determined by turbidimetry.

Pegylated Immunonanoparticle Preparation

The anti-rat transferrin receptor OX26 MAb was harvested from serum-free OX26 hybridoma-conditioned media as described and purified by protein G Sepharose affinity chromatography (15). OX26 MAb was radiolabelled with N-succinimidyl[2,3-^{3}H]propionate as described previously (16). The [^{3}H]-OX26 MAb had a specific activity of 0.12 mCi/mg and a TCA precipitability of 95%. OX26 MAb (1 mg, 6.65 nmol) supplemented with [^{3}H]-OX26 MAb (1 μCi) was thiolated by reacting for 60 min with a 40:1 molar excess of 2-iminothiolane (Traut's reagent) in 0.15 M sodium borate buffer, pH = 8 supplemented with 0.1 mM EDTA, as described previously (2). The buffer was exchanged with 0.01 M HEPES pH = 7 containing 0.15 M NaCl and 0.1 mM EDTA using a Centricon YM-30 concentrator tube (Amicon) and thiolated OX26 MAb was concentrated to a volume of 50 μl. Thiolated OX26 MAb was then mixed with nanoparticles at a thiolated OX26 MAb:maleimide molar ratio of 1:3. The volume of the mixture was 1 ml and the conjugation of the MAb to the pegylated nanoparticle was performed overnight on a rotating plate set at a low speed. The reaction mix was then applied to a 1.5 × 20cm Sepharose CL-4B column and was eluted with 0.05 M HEPES buffer pH = 7 (2). To determine the OX26 MAb concentration in each collected fraction, aliquots (100 μl) of the column eluate were analyzed by scintillation counting using a Tricarb 2100TR Liquid Scintillation Analyzer (Packard Instrument Co., Downers Grove, IL, USA). The PIN fractions were milky and were visually identified and collected, and the nanoparticle concentration was determined by turbidimetry. The average number of OX26 MAb molecules conjugated per nanoparticle was calculated by dividing the number of OX26 MAbs in each fraction by the calculated average number (n) of nanoparticles using the following equation:

$$n = 6m/(\Pi \times D^3 \times \rho)$$

where m is the nanoparticle weight, D the number based mean nanoparticle diameter determined by QELS, ρ the nanoparticle weight per volume unit (density), estimated to be 1.1 g/cm^3.

Electron Microscopy of Gold-Labeled Pegylated Immunonanoparticles

PIN-conjugated OX26 MAb was examined using a conjugate of 10 nm gold and an anti-mouse secondary antibody (Sigma). The PIN (10–30 μg) was incubated with undiluted IgG gold conjugate (5–15 × 10^{11} gold particles) for 1 h in 0.018 M Tris buffered saline, pH = 8, with 0.9% bovine serum albumin, and 17% glycerol in a total volume of 58 μL, and then examined directly with electron microscopy.

Morphological evaluation was also performed for the pegylated nanoparticles without gold staining. Transmission electron microscopy was performed using a Jeol JEM-100CX II electron microscope at 80 kV. The nanoparticles were deposited on a 200 mesh formvar-coated copper grid followed by negative staining with 1%–2% (w/v) phosphotungstic acid solution, pH = 7. Negatives, taken at a 14,000 and 29,000 magnification, were scanned and enlarged in Adobe Photoshop 5.5 on a G4 Power Macintosh.

Dry Weight Content and Turbidimetry Measurements

The weight percent yield of the nanoparticle preparation method was calculated from the dry-weight content of the suspensions after nanoparticle washing with water. Three nanoparticle batches were prepared as described, except that after preparation nanoparticles were resuspended with 1 ml water. Half was used as standard nanoparticle suspension for calibrating the turbidimetry method, the other half was used

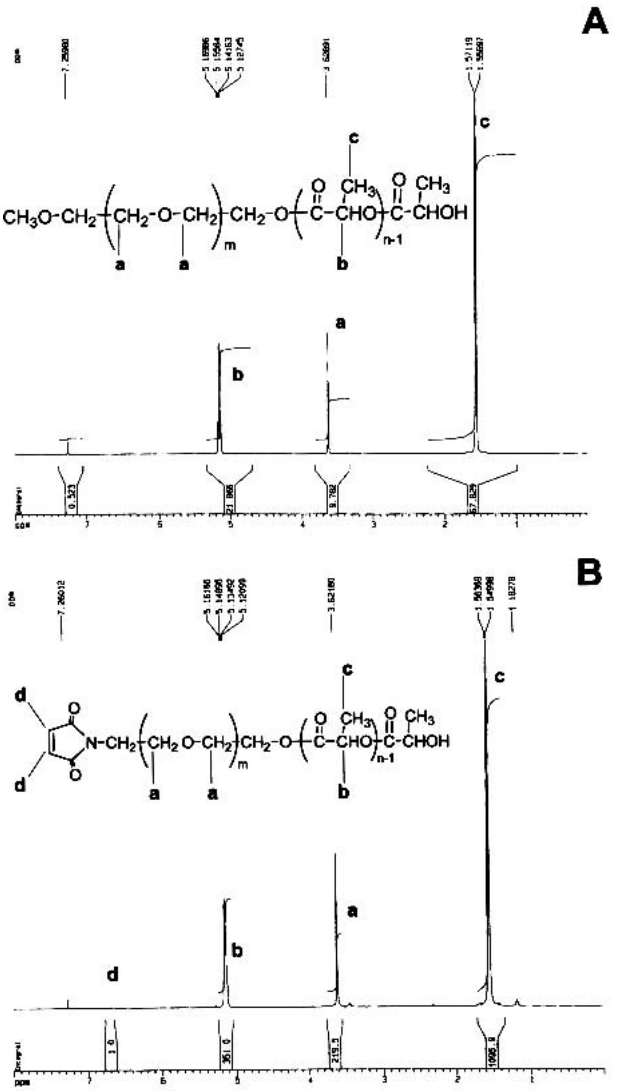

Fig. 2. (A) Formula and ^{1}H NMR spectrum of methoxyPEG$_{2600}$-PLA$_{40000}$ copolymers in deuterochloroform at 300.0K. (B) Formula and ^{1}H NMR spectrum of maleimide-PEG$_{3500}$-PLA$_{40000}$ block copolymers in deuterochloroform at 300.0K.

Table I. ^{1}H and ^{13}C NMR Chemical Shifts of Protons and Carbons of the Two Copolymers Synthesized

Groups	Peak assignments on Fig. 2A and 2B (^{1}H NMR spectra)	Chemical shifts δ (ppm)			
		MethoxyPEG-PLA		Maleimide-PEG-PLA	
		^{1}H NMR	^{13}C NMR	^{1}H NMR	^{13}C NMR
CH_3[a]	c	1.56 (doublet)	16.58	1.56 (doublet)	16.56
CH[a]	b	5.15 (quartet)	68.95	5.14 (quartet)	68.93
C = O[a]			169.54		169.51
CH_2[b]	a	3.63 (singlet)	70.51	3.62 (singlet)	70.49
CH of maleimide	d	none	none	6.69 (singlet)	too low signal

Note. Signals from the methoxy end groups of the methoxyPEG-PLA copolymer, from the α-methylene groups of PLA-connecting ethylene oxide units, from the hydroxylated lactyl end units or from the carbons involved in the maleimide end groups of the maleimide-PEG-PLA copolymer were not detected by the method.

[a] From lactyl main units of PLA.

[b] From ethylene oxide units of PEG.

for dry-weight content determination. The nanoparticles were diluted with 50-ml water, centrifuged at 45000 *g* and at 4°C for 45 min and, after discarding the supernatant, was re-dispersed in 250-μl water. Nanoparticles were then dried at 80°C for 16 h in a pre-weighed glass tube. The dry-weight was then measured and used to calculate the percent yield and the nanoparticle concentration of the standards.

Nanoparticle concentration was determined by turbidimetry measurements at 400 nm. The method was calibrated using the nanoparticle preparations the dry-weight contents of which were determined. The turbidity versus concentration response was linear over a concentration ranging from 0.05 to 1.00 mg/ml dry-weight content (r = 0.9998, slope = 0.9192, intercept = 0.0014).

RESULTS

The synthesized methoxyPEG$_{2600}$-PLA$_{40000}$ and maleimide-PEG$_{3500}$-PLA$_{40000}$ copolymers had typical ^{1}H (Fig. 2A and 2B) and ^{13}C NMR (not shown) spectra and chemical shifts (Table I) were in agreement with previously published data on PEG-PLA copolymers (17) and with NMR reference data available on maleimide functions (18,19). The number average molecular weights (Mn_{PLA}) of methoxyPEG$_{2600}$-PLA$_{40000}$ and of maleimide-PEG$_{3500}$-PLA$_{40000}$ as determined by ^{1}H NMR analysis are given in Table II. Determined Mn_{PLA} for both copolymers were around 80% of values expected from the feed. For maleimide-PEG$_{3500}$-PLA$_{40000}$ the ratio of the peak area for maleimide-PEG$_{3500}$ methylene protons versus the peak area for maleimide protons was 220, which was close to the ratio of 189 determined for the maleimide-PEG$_{3500}$ used for the synthesis. This indicated that the maleimide function was mostly preserved in the final product.

The pegylated nanoparticles were generally spherical and of regular size when examined by TEM (Fig. 3). Most pegylated nanoparticles were around 100 nm or below in diameter. A few were 200 to 300 nm in diameter. By QELS, the nanoparticle size distribution was monodisperse. The number-based nanoparticle diameter determined by QELS was 121 ± 5 nm (n = 3) with a standard deviation (describing the width of the nanoparticle size distribution) of 34 ± 3 nm (n = 3). The percent yield based on the dry weight content (17.8 ± 0.8 mg/ml) determined by turbidimetry was calculated to be 63.7 ± 4.9% (n = 3).

Figure 4 is an example of elution profile of the PINs obtained by Sepharose CL-4B gel filtration chromatography. Due to their sizes, the PINs eluted in fractions corresponding to the void volume of the column (from 8 to 11 ml), whereas the unconjugated OX26 MAb eluted in later fractions (from 17 to 32 ml). The average number of OX26 MAbs conjugated per nanoparticle of 121 nm in diameter was calculated for each fraction (Table III), which gives a mean of 67 ± 4 OX26 MAb per nanoparticle. Figure 5 is a transmission electron micrograph where the OX26 MAb conjugated to the pegylated nanoparticle was visualized by binding to a conjugate of 10 nm gold and an anti-mouse IgG. The PIN is surrounded by a corona of gold particles owing to the presence of the OX26 MAb conjugated to the surface of the pegylated nanoparticles.

DISCUSSION

Pegylated immunonanoparticles were prepared from PLA and are designed to permit specific drug targeting to organs *in vivo* based on the receptor specificity of the targeting MAb. The PIN was prepared with two copolymers: methoxyPEG-PLA, as the major component, and maleimide-PEG-PLA, as a novel functionalized copolymer to which can be conjugated any thiolated targeting ligand (Figs. 1 and 2). The molecular weight (2600) of the methoxyPEG moiety was selected to form a PEG "corona" around the PLA nanoparticle core that inhibits the nanoparticle uptake by the RES, with an expected *in vivo* blood half-life of several hours (6). A higher molecular weight was chosen for the PEG spacer

Table II. Number Average Molecular Weight Data for the Copolymers

Copolymers	Copolymer weight ratio (Mn_{PEG}:Mn_{PLA})[a]	
	Theoretical (feed)	Determined by ^{1}H NMR
MethoxyPEG-PLA	2600:46900	2600:37900
Maleimide-PEG-PLA	3501:45700	3501:36900

a Mn_{PEG} of methoxyPEG and of Maleimide-PEG are provider's data, Mn_{PLA} were determined by ^{1}H NMR.

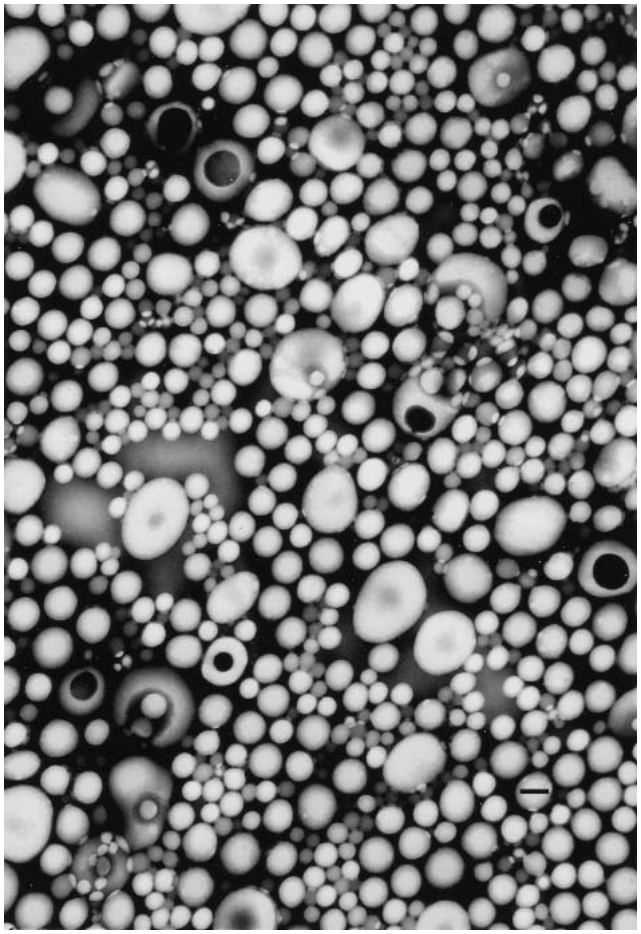

Fig. 3. Transmission electron micrograph of pegylated nanoparticles negatively stained with phosphotungstic acid solution. Magnification bar in lower right hand corner is 120 nm.

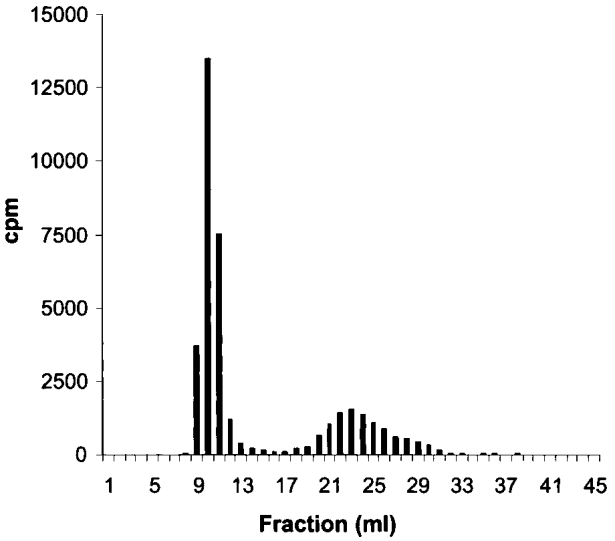

Fig. 4. Elution profile of [^{3}H]-OX26 MAb -conjugated pegylated immunonanoparticles obtained by Sepharose CL-4B gel filtration chromatography. Fractions 8 to 11 correspond to [^{3}H]-OX26 MAb-conjugated PINs and fractions 17 to 32 to unconjugated [^{3}H]-OX26 MAb.

(3500) of the maleimide-PEG-PLA so that the maleimide function would protrude from the corona to be available for conjugation. Copolymers were synthesized by ring-opening polymerization of L-lactide, using stannous octoate as a catalyst. The polymerization initiators were methoxyPEG$_{2600}$ or maleimide-PEG$_{3500}$ to synthesize methoxyPEG$_{2600}$-PLA and maleimide-PEG$_{3500}$-PLA, respectively. Performed in an organic solvent (toluene or xylene), this method allows control of the length of the PLA chain and a low polydispersity (20). The NMR spectra confirmed the synthesis of both copolymers and permitted the calculation of the number average molecular weight (Mn) of the PLA moieties (Fig. 2A and 2B, and Table I). The maleimide protons are observed at δ 6.69 with a surface area ratio vs. the PEG methylene proton (δ3.62) close to the ratio calculated for the maleimide-PEG$_{3500}$. This result shows that the maleimide function was not altered during the synthesis. Molecular weights of the PLA moieties calculated from the ^{1}H-NMR spectra for both copolymers were almost equal and were 80% of the values expected from the L-lactide feed (Table II).

Nanoparticles were prepared with methoxyPEG$_{2600}$-PLA$_{40000}$ and maleimide-PEG$_{3500}$-PLA$_{40000}$ by the multiple emulsion/solvent evaporation method (14). The inner aqueous phase can dissolve hydrophilic drugs to be entrapped within the nanoparticle core, including small molecules, proteins, and nucleic acids (7–9,14). Sodium cholate was chosen as surfactant as previous work showed that cholate did not interfere with blood coagulation factors (21) and caused a reduction in both the hydrodynamic diameter and polydispersity index (5). Due to the hydrophilicity of PEG and to the hydrophobicity of PLA, resulting in a phase-separation of the two blocks in water, PEG moieties orient themselves toward the aqueous phase forming a "corona" layer around the PLA nanoparticle core (7,12). Some PEG chains may have an orientation toward the inner phase of the nanoparticles, when the multiple emulsion is prepared, and may be trapped within the inner aqueous phase during the hardening of the polymeric PLA core (14). The maleimide-PEG$_{3500}$-PLA$_{40000}$ was blended with methoxyPEG$_{2600}$-PLA$_{40000}$, according to a methoxyPEG:maleimide-PEG ratio of 1:30. This ratio was based on methods used for preparing pegylated immunoliposomes (2,22). Stable nanoparticles with satisfactory yields and concentrations were obtained (Results). Pegylated nanoparticles had a diameter of 121 ± 5 nm and appeared spherical by TEM (Fig. 3).

The procedure for antibody conjugation to the pegylated nanoparticle was derived from the method used to prepare pegylated immunoliposomes (2). The OX26 MAb, directed against the rat transferrin receptor, was chosen for the conjugation because prior work with PILs showed this MAb allowed for targeted drug delivery to transferrin receptor-rich organs such as brain of either small molecules (2,22) or non-viral plasmid DNA (23). The OX26 MAb was thiolated with 2-iminothiolane (2). A ratio of MAb:2-iminothiolane of 1:40 (mol/mol) was shown to provide the thiolation of an average of one primary amine per MAb (2). The thiolation of the OX26 MAb does not interfere with its target recognition (24). The OX26 MAb was conjugated to pegylated nanoparticles by formation of a thioether bond between the thiol groups of the MAb and the maleimide moiety at the distal end of maleimide-PEG$_{3500}$-PLA$_{40000}$. A reproducible coupling was achieved using a OX26 MAb:maleimide ratio of 1:3 (Results).

Table III. Concentrations in Immunonanoparticles and in OX26 MAb, and the Calculated Average Number of OX26 MAb per an Average Nanoparticle of 121nm

Experiment number	Fraction number[a]	Nanoparticle amount (mg)[b]	Amount of OX26 MAb (nmol)[c]	Average number of OX26 MAb per nanoparticle
1	9	2.8	0.31	65
	10	10.1	1.14	68
	11	5.3	0.64	71
2	9	1.9	0.18	60
	10	8.8	0.95	65
	11	3.4	0.36	64
3	8	0.5	0.06	76
	9	7.4	0.83	67
	10	5.4	0.60	67
	11	0.7	0.07	65

Note. The values were determined in the fractions containing immunonanoparticles and obtained by a Sepharose CL-4B gel filtration chromatography.
[a] 1 ml per fraction.
[b] Determined by turbidimetry.
[c] Determined by radioactivity measurements.

Surface-linked OX26 MAb was detected by electron microscopy using a conjugate of anti IgG secondary antibody and 10 nm gold (Fig. 5). The number of OX26 MAbs conjugated per nanoparticle of 121 nm in diameter was calculated to be 67 ± 4 (Table III). This number should be sufficient for brain targeting, since comparable degrees of MAb conjugation to pegylated liposomes with the OX26 MAb allows for effective drug or gene targeting to the brain (2,23).

In conclusion, a novel maleimide-PEG-PLA copolymer was synthesized from maleimide-PEG and lactide dimers by ring opening polymerization, without altering the maleimide function. This copolymer was blended with methoxyPEG-PLA to prepare functionalized pegylated PLA nanoparticles. A thiolated monoclonal antibody directed against the rat transferrin receptor was conjugated to these nanoparticles to obtain PIN. Such structures may prove useful in future applications of targeted drug delivery to organs *in vivo* of a variety of pharmaceuticals including small molecules, proteins, and gene medicines.

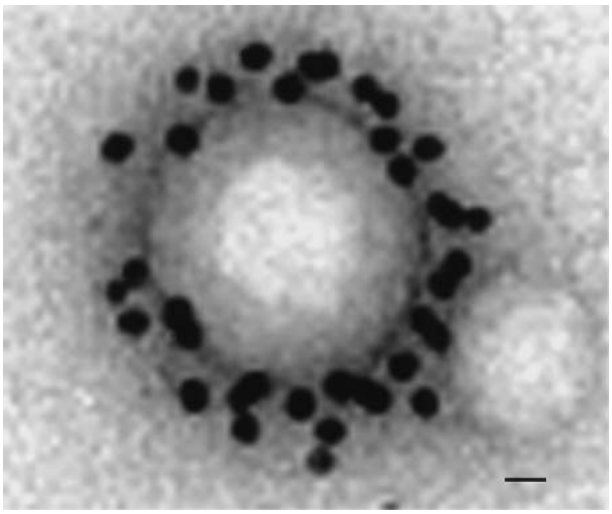

Fig. 5. Transmission electron micrograph of pegylated immunonanoparticles negatively stained with phosphotungstic acid solution. The OX26 antibodies conjugated to the pegylated nanoparticles are revealed by binding with a conjugate of 10 nm gold and an anti-mouse IgG secondary antibody. The magnification bar is 15 nm.

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