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ORIGINAL CONTRIBUTION

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Preparation, characterization and application of pyrene-loaded methoxy poly(ethylene glycol)–poly(lactic acid) copolymer nanoparticles

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Abstract Pyrene-loaded biodegradable polymer nanoparticles were prepared by incorporating pyrene into the polymer nanoparticles formulated from amphiphilic diblock copolymer, methoxy poly(ethylene glycol)–poly(lactic acid) (MePEG–PLA). Their morphological structure and physical properties were characterized by nuclear magnetic resonance (NMR), dynamic light scattering, fluorescence spectroscopy, transmission electronic microscopy and zeta potential measurements. Further, MePEG–PLA nanoparticles containing pyrene as fluorescent marker were administered intranasally to rats, and the distribution of nanoparticles in the nasal mucosa and the olfactory bulb were visualized by fluorescence microscopy. NMR results confirmed that MePEG–PLA copolymer can form nanoparticles in water, and hydrophilic PEG chains were located on the surface of the nanoparticles. The particle size, zeta potential and pyrene loading efficiency of MePEG–PLA nanoparticles were dependent on the PLA block content in the copolymer. Following nasal administration, the absorption of nanoparticles across the epithelium was rapid, with fluorescence observed in the olfactory bulb at 5 min, and a higher level of fluorescence persisted in the olfactory mucosa than that in the respiratory mucosa. These results show that pyrene could serve as a useful fluorescence probe for incorporation into polymer nanoparticles to study tissue distribution and MePEG–PLA nanoparticles might have a great potential as carriers of hydrophobic drugs.

Keywords Biodegradable polymer · Pyrene-loaded nanoparticle · Methoxy poly(ethylene glycol)–poly(lactic acid) · Fluorescent marker · Tissue distribution

Introduction

In recent years biodegradable polymer nanoparticles have attracted much attention for their potential in biomedical applications, such as drug and vaccine delivery [1, 2], gene technique [3], etc. For a lot of these applications, it is necessary to study the intracellular distribution as well as the tissue uptake of the nanoparticles to optimize the efficacy of the encapsulated agent [4]. Understanding of the intracellular and tissue distribution of the nanoparticles is also useful to elucidate the mechanism of enhanced therapeutic efficacy of the nanoparticle-encapsulated therapeutic agents [5, 6].

Towards these objectives, the fluorescence probe technique is widely used. However, conventional fluorescent labels are restricted in some biological fields

because of some limitations, e.g. the detection sensitivity is not so high because only a few fluorophores can be coupled to one biomolecule etc. Recently, some polymer nanoparticles as fluorescent markers have attracted great interest. For example, fluorescent polystyrene nanoparticles are used as a model for PLGA/PLA nanoparticles so that nanoparticle uptake and distribution can be visualized by either confocal or fluorescence microscopy or quantified by analyzing the extracted fluorescent dye [7]. However, the physical properties of these nanoparticles including their hydrophobicity, surface charge and protein adsorption could be different from that of PLGA/PLA nanoparticles. Hence, the results obtained with polystyrene nanoparticles might not truly represent PLGA/PLA nanoparticles. Panyam et al. [8]. have characterized 6-coumarin, incorporated in poly(D,L-lactide-co-glycolide) (PLGA) nanoparticles as a marker to study nanoparticle uptake in cells. The results indicate that 6-coumarin could serve as a useful fluorescence probe for studying nanoparticle uptake, retention and distribution in vivo and in vitro.

Recently, a progressive interest has arisen for applying amphiphilic block copolymers to formulating nanoparticles. Amphiphilic block copolymers are classified into several types by sequential arrangement of component segment, such as AB-type diblock copolymer, ABA-type triblock copolymer, $(AB)_n$ type multiblock copolymer and star block copolymer. Compared with other type of amphiphilic block copolymers, AB-type amphiphilic diblock copolymers are the most appropriate candidates for forming polymer nanoparticles in size design, aggregation number, and nanoparticle stability due to the simple architecture of their molecules [9]. Among the amphiphilic diblock copolymers, biodegradable copolymers, such as methoxy poly(ethylene glycol)–poly(lactic acid) (MePEG–PLA) and methoxy poly(ethylene glycol)–poly(ϵ-caprolactone) [10, 11], are very attractive for drug delivery applications. The nontoxic and non-immunogenic nature of PEG provides a great advantage in utilizing it as a component of PLA materials for medical purposes, and its strong hydration power contributes to regulation of the hydrophilicity of the materials [12, 13]. MePEG–PLA can self-assemble into polymeric nanoparticles in aqueous systems with a diameter ranging from 10 to 100 nm depending on the length of blocks. The copolymer nanoparticles are characterized by a core–shell architecture, in which a segregated core of associated hydrophobic segments (PLA) is surrounded by a hydrophilic and sterically stabilized shell (PEG), and the shell causes the nanoparticles to have a long half-life in the blood due to the reduced interaction with biological components [14].

In our previous study [15], nimodipine, a hydrophobic drug was incorporated into MePEG–PLA nanoparticles and administered intranasally to rats. A brain distribution study confirmed that nimodipine could be transported directly to the brain (data not shown), which indicated that MePEG–PLA nanoparticles might be a promising delivery system to enhance brain uptake of drugs after nasal administration. In order to directly visualize the transport pathway of drug-loaded MePEG–PLA nanoparticles from nasal cavity to brain, in this paper, pyrene was selected as a model drug and the fluorophore to incorporate into the nanoparticles, formed from diblock copolymer MePEG–PLA by physical entrapment. Pyrene is a hydrophobic fluorescent dye which is frequently applied in fluorescence studies of labeled polymers [16, 17, 18, 19] and also frequently used as a model for hydrophobic compounds [20]. Different methods such as dynamic light scattering (DLS), transmission electron microscopy (TEM) and ^{1}H-nuclear magnetic resonance (NMR) were employed to study the formation of biodegradable fluorescent nanoparticles, and the localization of pyrene-loaded nanoparticles in the nasal cavity was also investigated to identify its suitability as a fluorescent marker for studying tissue distribution.

Experimental

Materials

Methoxy poly(ethylene glycol) (MePEG, Mn = 5,000) was supplied by Aldrich and dried under vacuum in a desiccator with P_2O_5 overnight before use. D,L-Lactide (purity:99.5%) was purchased from PURAC and purified by recrystallization twice from dried ethyl acetate. Stannous octoate (stannous content: 26.5–27.5%) was supplied by Shanghai Chemical Reagent Company. Pyrene was purchased from Fluka. Hemapun 948 A, B (glycol methacrylate, GMA) was supplied by Shanghai Sixth Hospital. Propidium iodide was kindly gifted by Department of Molecular Virology of Fudan University (1 mg/ml in water, Sigma) and diluted to 5 μg/ml in phosphate buffered saline (PBS) prior to use. Bouin's fluid (saturated picric acid, 40% formaldehyde and concentrated acetic acid; 15:5:1) was used for perfusion and immersion fixation. All other chemicals used were reagent grade and used without further purification. A cellulose dialysis bag (molecular weight cut off: 14,000) was supplied by Luniao Technology Co..

Synthesis and composition of MePEG–PLA diblock copolymer

MePEG–PLA block copolymers were synthesized as described elsewhere [15]. In brief, the block copolymer was obtained by the polymerization of D,L-lactide initiated by MePEG using stannous octoate as catalyst. A predetermined amount of MePEG and D,L-lactide (the ratio of MePEG to D,L-lactide was varied from 1:1 to 1:6) were placed in a dried round-bottomed bottle connected with a vacuum joint, and the appropriate amount of stannous octoate was added as a solution in dried toluene. The reactants were dried under reduced pressure at 70 °C for 1 h, and then the reaction was allowed to proceed under vacuum at 160 °C for 2 h. The cooled product was dissolved in dichloromethane, recovered by precipitation into excess mixed solvent of ethyl ether and petroleum ether, and then the precipitant was redissolved in acetone and precipitated into excess water. The purified copolymers were dried in a vacuum oven at 40 °C for 24 h and then stored in a desiccator under vacuum.

Preparation of pyrene-loaded nanoparticles

MePEG–PLA copolymeric nanoparticles were prepared by the phase-separation/dialysis method. 200 mg MePEG–poly (D,L-lactide) diblock copolymers were dissolved in 6 ml DMF, then 14 ml water was added to induce micellization under agitation. The aqueous solution was placed in a dialysis bag and dialyzed against doubly distilled water for 2 days to remove DMF. After dialysis, the nanoparticle dispersion was diluted to 0.67 wt%.

For the preparation of a solution of pyrene-loaded nanoparticles, the pre-dialyzed nanoparticle dispersion was put into the dialysis bag and equilibrated with 500 ml saturated pyrene solution for 4 days under shielded light. The solution was replaced at times 2, 6, 10, 16, 24, 32, 40, 48, 72 and 96 h during the experiment with fresh saturated medium. The resulted nanoparticles containing pyrene were lyophilized for 24 h (Advantage freeze dryer, Virtis, USA) by using 1% sucrose as cryoprotectant so that the nanoparticles can redisperse, and then the dry powder was stored in a desiccator at 4 °C.

Characterization of the pyrene-loaded polymer nanoparticles

Various techniques were employed to determine the formation, morphology, dispersibility and surface properties of the nanoparticles.

The structure of polymer nanoparticle was analyzed by ^{1}HNMR. The ^{1}HNMR spectra of MePEG–PLA nanoparticles dispersed in $CDCl_3$ and D_2O was obtained using a 500-MHz NMR spectrometer (DMX-500, Bruker).

Fluorescence measurements were carried out on a fluorometer (RF-540, Shimadzu). The measured polymer dispersion was diluted and the content of solids was $\sim 10^{-2}$ wt%. The excitation wavelength was 339 nm, and the excitation and emission slits were both 5 nm wide.

The hydrodynamic radii of nanoparticles were determined by DLS measurement at 25 °C using a light scattering spectrophotometer (Autosizer 4700, Malvern) with a vertically polarized incident beam at 532 nm supplied by an argon ion laser. A scattering angle of 90°was used in this study. Before measurement, all samples were filtered through a 0.45-μm filter (Millipore).

The morphological examination of nanoparticles was performed using a transmission electron microscope (Hitachi H-600) following negative staining with phosphotungstic acid.

Zeta potential measurements of the nanoparticles were performed employing a Nicom Zeta potential apparatus. (Nicomp 380ZLS, USA), all the samples were analyzed at the concentration of 0.23 wt%.

The pyrene loading efficiency was determined using a UV spectrophotometer (Lambda35, PE). An aliquot of the pyrene-loaded nanoparticle dispersion was disrupted by the addition of THF, and the UV absorbance intensity at 335 nm was used for quantitative analysis. It was verified that the trace amount of water did not affect this calibration [21].

Pyrene-loaded content was calculated according to the following equation:

$$\text{pyrene loading content (mg/g)} = \frac{\text{weight of pyrene in nanoparticles (mg)}}{\text{weight of pyrene - loaded nanoparticles (g)}} \quad (1)$$

Their application as a fluorescent marker

Male Sprague–Dawley rats (252 g ± 35 g, Experimental Animal Center, Fudan University) were anesthetized with an intraperitoneal injection of urethane (1.2 g/kg) and kept on a heating pad to maintain the body temperature. For intranasal administration, the lyophilized powder of pyrene-loaded nanoparticles (MLP16) was dispersed in a certain volume of PBS (pH 7.4), then 50 μl (6% w/v) PBS solution was dosed via a PE 10 tube attached to a microliter syringe, which was inserted approximately 1 cm into the right nostril of a rat. At 5 min, 15 min, 1 h and 4 h after administration, the rats were fixed by perfusion of saline (0–4 °C) containing 10 IU/ml heparin by the left ventricle for 5 min, and then cooled using Bouin's fluid via the heart. The right nasal cavity, including the olfactory bulb, was isolated and immersed in Bouin's fluid for 48 h. The experiment was repeated for 2–3 times at every time point.

After decalcification of the tissue specimen in 4-M formic acid for 2 days, the nasal cavity was cut into slices approximately 2–3 mm thick according to a schedule reported by Jansson et al. [22]. The slices were washed with 0.2-M cold sodium cacodylate buffer solution for 20 min and dehydrated twice sequentially in a graded series of ethanol solutions (60, 70, 80, 95 and 100%); they were then soaked overnight in Hemapun 948 A solution, and then 2–3 drops Hemapun 948 B solution was added for polymerization of the resin according to the manufacture's instructions.

The polymerized blocks were sectioned (5 μm) using a Leica microtome (Leica 5a, Germany). The sections were floated out with distilled water, transferred to microscope slides and dried at room temperature overnight, then stained with propidium iodide for 10 min, and finally washed with PBS twice prior to visualization under a fluorescence microscope (Nikon DIAPHOT, Japan).

Results and discussion

The composition and molecular weight of MePEG–PLA block copolymers

A series of amphiphilic diblock copolymers, MePEG–PLA, were synthesized by a ring opening polymerization of D,L-lactide initiated with the hydroxyl group of methoxy poly(ethylene glycol) using stannous octoate as catalyst. Their chemical structure was identified by FTIR, NMR and GPC, as described in our previous study [15]. Their number average molecular weights measured by NMR are listed in Table 1, and the result of NMR approximated to the theoretical value calculated from the feed recipe in the synthesis of MePEG–PLA copolymers. As the amount of D,L-lactide in the feed recipe increased, the molecular weight of MePEG–PLA copolymers increased.

The structure of MePEG–PLA nanoparticles

NMR can be employed to study the structure of polymer nanoparticles due to the fact that the nanoparticle system is in the same conformational state whether the PLA-PEG copolymeric nanoparticles are dispersed in water or in D_2O. ^{1}HNMR spectra of polymeric nanoparticles dissolved in $CDCl_3$ (a) and dispersed in D_2O (b) are shown in Fig. 1. Figure 1a shows that in $CDCl_3$, a nonselective solvent for the MePEG block and PLA block, peaks at 5.2, 1.7 and 3.7 ppm [23] are observed, which are assigned to methane protons and methyl protons of PLA component and methylene protons of MePEG component

Table 1 Molecular weight and composition of MePEG-PLA diblock copolymers

Sample	Feed weight ratio Lactide/MePEG	Mn($\times 10^{-3}$)		$\bar{M}_{n(PLA)}/\bar{M}_{n(PEG)}$ in the copolymer
		Calc[a]	NMR[b]	
ML11	1/1	10	9.15	4,150/5,000
ML12	1/2	15	14.9	9,900/5,000
ML14	1/4	25	26.3	21,300/5,000
ML16	1/6	35	35.4	30,400/5,000

[a]Number average molecular weight calculated from feed recipe
[b]Number average molecular weight calculated by NMR

respectively. However, as shown in Fig. 1b, only the signal of the PEG component is seen in the spectra of polymeric nanoparticles, which indicates that the PEG segment is in an extended solvated state to form the hydrophilic outer shell of the nanoparticles and stabilize the colloidal nanoparticles. The protons in PLA component have disappeared from the spectra, implying that PLA segments are entrapped in the central solid-like hydrophobic core to minimize their interaction with water due to their hydrophobic character.

Characterization of pyrene-loaded polymer nanoparticles

Using MePEG–PLA copolymer with various molecular weights, a series of pyrene-loaded nanoparticles were prepared. As shown in Table 2, the mean diameter of the nanoparticles was around 100 nm and increased with the content of PLA component in the copolymer. In addition, it could be seen that the particle size of the nanoparticles increased with the increasing polymer concentration. This result is in accordance with the report on preparing biodegradable nanoparticles by a precipitation method [10].

Figure 2 is a TEM image of the nanoparticle solution of MLP12, in which the fluorescent nanoparticles can be seen with a spherical shape and they have a narrow size distribution on the nano scale.

Zeta potential is a concept used to describe the electrokinetic property of a colloid under the influence of an applied electric field. It was observed that all the nanoparticles present a negative zeta potential (Table 1), which may be attributed to the presence of ionized carboxyl groups of PLA segments on the surface [24]. When the weight percentage of PLA component decreases (from MLP16 to MLP11), a marked decrease in the surface charge for MePEG–PLA nanoparticles is observed (from −14.98 mV to −4.42 mV). This result is therefore, an indication of the presence of the MePEG at the surface of the nanoparticles. When the weight percentage of the PLA in MePEG–PLA copolymer decreases, the core becomes smaller, and the percentage

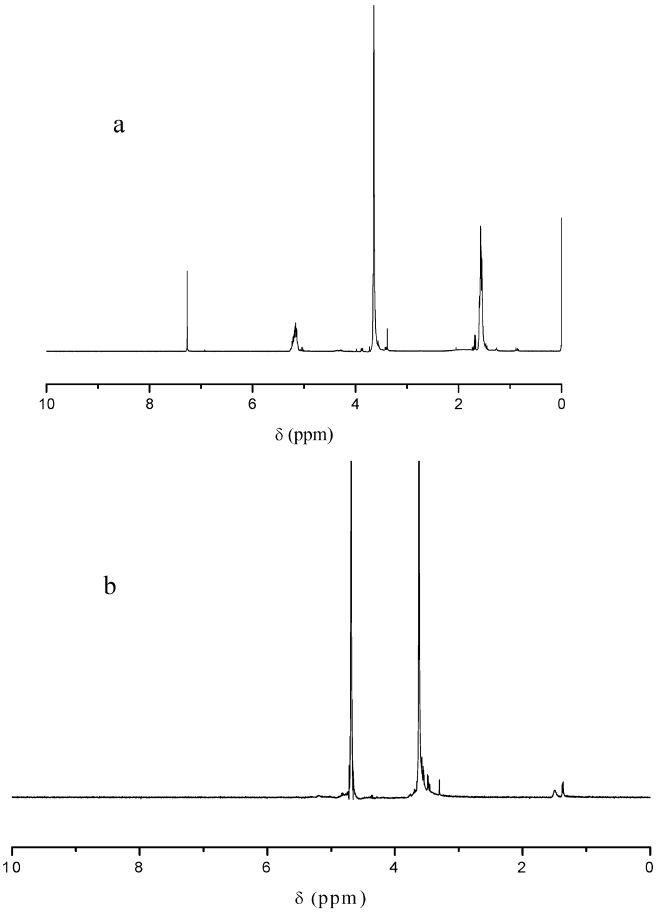

Fig. 1 **a** ^{1}HNMR of MePEG–poly(D,L-lactide) in $CDCl_3$. **b** ^{1}HNMR of MePEG–poly(D,L-lactide) in D_2O; sample was MLP11

of PEG on the surface increases. The increase of the thickness of the MePEG barrier reduces the ionization degree of carboxyl groups, resulting in a lower zeta potential.

In order to study the capacity of the nanoparticles of MePEG–PLA for loading pyrene, we dialyzed the nanoparticle dispersion against saturated pyrene solution for 4 days, and during this process the pyrene solution was changed for ten times in order to keep the solution saturated. As shown in Table 2, the pyrene loading content depends mainly on the copolymer composition ratio of MePEG to PLA, which is due to the fact that the longer the PLA block is in the amphiphilic block copolymers, the stronger is the hydrophobicity of the inner core of the formed nanoparticles, causing the enhancement of compatibility between hydrophobic pyrene and the nanoparticles. However, with the increase of the polymer concentration, the loading capacity of per unit polymer decreases (Table 2). Perhaps, the core of the nanoparticles becomes more compact when the polymer concentration increases, and it is difficult for pyrene to be incorporated into nanoparticles [25].

Table 2 Hydrodynamic diameter, loading efficiency of pyrene-loaded MePEG-PLA nanoparticles

sample	Feed weight ratio Lactide/MePEG	Polymer concentration (%)	Zeta potential (mV)[a]	Particle size (nm)	Loading efficiency (mg/g)
MLP11	1/1	0.67	−4.42	53	3.7
MLP12	1/2	0.67	−6.94	75	4.0
MLP14	1/4	0.67	−10.45	88	4.2
MLP16	1/6	0.67	−14.98	107	4.4
MLP161	1/6	0.33	n.m[b]	78	8.0
MLP162	1/6	1.00	n.m	112	3.8

[a]The zeta potential of the blank nanoparticles
[b]Not measured

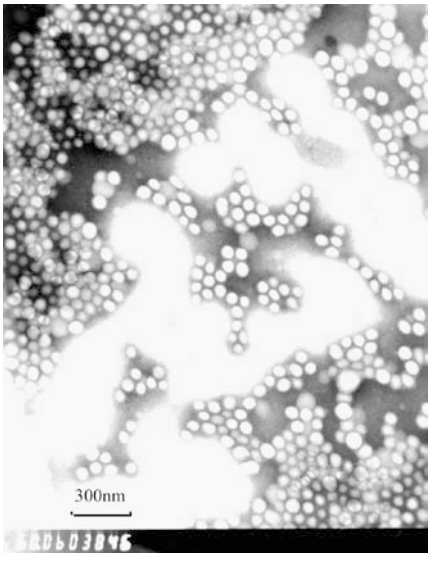

Fig. 2 TEM image of pyrene-loaded nanoparticles; sample was MLP12

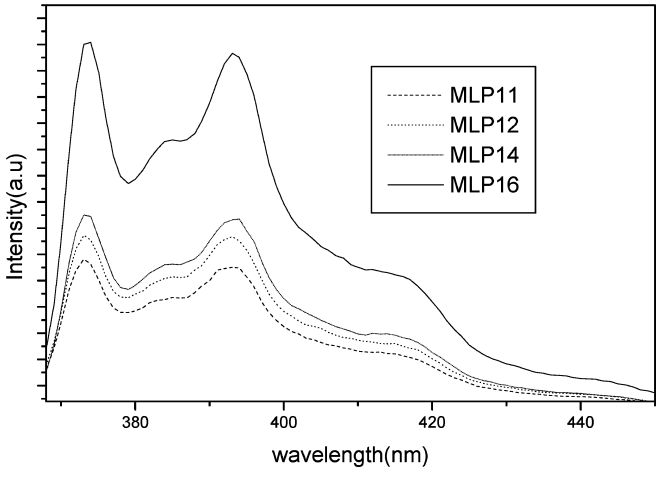

Fig. 3 The emission spectra of various nanoparticles (samples were MLP11–MLP16)

Steady-state fluorescence measurements

Pyrene is sensitive to the environmental polarity, which is widely used in fluorescence marker studies of nanoparticles. Figure 3 shows the fluorescence emission spectra of various diluted nanoparticles dispersion. With increasing PLA component in the copolymer, the intensity of the peak I_1 increases. Since the nanoparticle solutions have the same content of solids, this indicates that the longer the PLA block is in the amphiphilic block copolymers, causing the enhancement of compatibility between hydrophobic pyrene and the nanoparticles, the more pyrene was incorporated into the hydrophobic core of the nanoparticles; the enrichment of more pyrene molecules enhances the emission intensity.

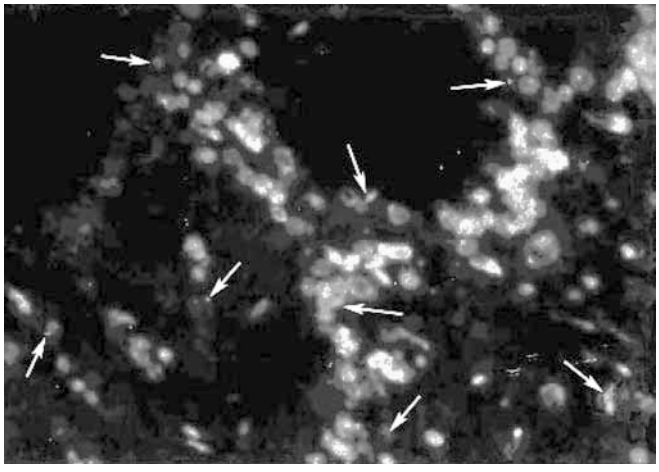

Fig. 4 The olfactory bulb region in level V of a rat euthanized 5 min after administration of pyrene-loaded nanoparticles (→ represents pyrene-loaded nanoparticles)

Application of pyrene-loaded polymer nanoparticles as a fluorescent marker

In order to clearly present the transport pathway of MePEG–PLA nanoparticles from nasal cavity to brain after nasal administration, pyrene-loaded nanoparticles were administered intranasally to rats. The microscopic examination of the sections of nasal cavity showed that the fluorescent nanoparticles were distributed extensively. The nanoparticles can be found in olfactory mucosa, respiratory mucosa, fatty cells, muscles cells, substitution bone cells, the olfactory bulb, and so on (Fig. 4). Absorption across the epithelium was rapid and

nanoparticles were observed in the olfactory bulb at the first time point (the 5th minute). In addition, a higher level of pyrene fluorescence was exhibited in the olfactory mucosa than in the respiratory mucosa, which strongly support the hypothesis that olfactory mucosa could be more permeable for nanoparticles. These results led us to deduce that the nanoparticles came across the mucosa epithelium mainly via the endocytosis, and the olfactory epithelium pathway was used to transfer nanoparticles from the nasal cavity to the olfactory bulb. Thus, it is obvious that pyrene-loaded polymer nanoparticles as a fluorescent marker can present direct evidence to prove the transport pathway of MePEG–PLA nanoparticles after nasal administration, and these nanoparticles could serve as useful probes for studying the uptake, retention and distribution of nanoparticles in the nasal cavity.

Conclusion

Biodegradable fluorescent nanoparticles were obtained by incorporating highly hydrophobic pyrene into MePEG–PLA nanoparticles using a physical entrapment method. A series of techniques, including ^{1}H NMR, fluorescence spectroscopy, TEM and zeta potential were used to characterize the fluorescent nanoparticles. The results show that they are spherical in shape with a narrow size distribution. When the length of the hydrophilic PEG block was identical, the particles size and zeta potential were dependent on the PLA segment length. Microscopic examination of the nasal tissue presents direct evidence to prove the transport pathway of MePEG–PLA nanoparticles after nasal administration. These nanoparticles could serve as useful probes for studying the uptake, retention and distribution of nanoparticles in the nasal cavity, and MePEG–PLA nanoparticles may have potential for intranasal drug delivery.

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