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Synthesis and characterization of functionalized poly(γ-benzyl-L-glutamate) derivates and corresponding nanoparticles preparation and characterization

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ABSTRACT

For being fully efficient a targeted delivery system should associate simultaneously multiple functionalities. In this context, the association of several polymeric materials to form composite multifunctional particles can be foreseen. The present work describes the synthesis of different derivates of poly(γ-benzyl-L-glutamate) and their use for the preparation of nanoparticles exhibiting different properties, including surface hydrophilization by PEG, fluorescence imaging by FITC and target recognition through easy attachment of desired ligands by using the avidin–biotin interaction, after the nanoparticles preparation. Four PBLG derivates were successfully obtained by ring-opening polymerization (ROP) of NCA, using various initiators corresponding to the molecules to be introduced into the copolymers. Further, nanoparticles smaller than 100 nm could be prepared using a nanoprecipitation technique and the presence of the active moieties introduced within the particles as well as their functionality has been checked. Very interestingly, it has been shown that biotin molecules could be efficiently introduced at the surface of the nanoparticles, which (for 75% of the theoretical amount) could be engaged in a complexation with avidin. It is suggested that this strategy offers the possibility to easily decorate these nanoparticles with various recognition ligands for specific targeting applications by using the well known biotin–avidin sandwich technique.

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1. Introduction

To overcome the multiple physical–chemical and biochemical barriers which are raised between the site of administration of a drug and its therapeutic target, a common strategy consists in associating the molecule to a targeted delivery system that will be able to mask unfavorable properties of the drug in the body and help to target the desirable organ. Obviously, for being efficient, targeted delivery systems need to combine simultaneously various mechanical and biological functionalities and among others durability, compatibility with blood and/or tissues, efficient targeting of specific organs into the body, capacity to control the drug release at/or close to the site of action. Nanoparticles made of hydrophobic or amphiphilic polymers (Janes et al., 2001; Labarre et al., 2005) present a real potential in these applications and are currently widely investigated in targeted delivery.

A possible strategy for conferring simultaneously the desired properties to the nanoparticles can consist in self-assemblying on demand different amphiphilic copolymers each one bearing a specific functionality, depending on the application. It requires to develop a family of polymers or copolymers constituted by a single backbone and bearing at one of the chain extremity adapted chemical groups (Martínez Barbosa et al., 2007). For being able to self-associate easily, these different polymers should present an invariant moiety in their structure, while another part of the chain bears the functional moiety. Polypeptides are particularly interesting for creating this invariant block in the copolymers because of their capacity to form secondary structures (Block, 1983). Indeed, compared to amorphous copolymers such as aliphatic polyesters which form coil structures, polypeptides can adopt ordered conformations such as α-helices or β-strands, which may be useful to direct the formation of nanoscale structures, while functional ligands can be presented by more flexible chains. Further, polypeptides are biocompatible (Jagur-Grodzinski, 1999).

Among many constraints, these polymers have to be able to form efficiently and easily nanoparticles, which necessitates the use of sufficiently water-insoluble copolymers (Bilati et al., 2005;

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Martínez Barbosa et al., 2007). Poly(γ-benzyl-L-glutamate) has been selected in this purpose to constitute the hydrophobic block of the copolymers because of its ability to form easily rods stabilized by regular intramolecular hydrogen bonding (Carraher and Seymour, 2003; Sperling, 2006). Because of their hydrophobicity, such copolymers can be easily nanoprecipitated by the solvent displacement method (Martínez Barbosa et al., 2007), which presents numerous advantages as it is a straightforward technique, rapid and easy to perform.

The polypeptides can be synthesized by various routes, including solid-phase synthesis, solution-phase coupling and by ring-opening polymerization (ROP) of N-carboxy-α-amino acid anhydrides (NCAs) also referred Leuchs's anhydrides (Odian, 2004). This latter approach has the greatest potential for synthesizing high molecular weight polypeptides with good yields and with no detectable racemization at the chiral centers (Deming, 1997). Initiators of NCA ring-opening polymerization (ROP) include a broad range of nucleophiles and bases, such as primary amines, tertiary amines, and alkoxide and hydroxide ions (Deming, 1997, 2000; Odian, 2004). Depending on the nucleophilicity/basicity ratio of the initiator, the polymerization of NCAs is thought to proceed along two main pathways, the amine mechanism (or protic mechanism) and the activated-monomer mechanism. For copolymers synthesis, the primary amine mechanism is desired because the initiator is linked to the growing chain, which is not the case in the activated-monomer mechanism, where the initiator is not linked to the growing chain and which leads to the formation of unwanted homopolymers. Noteworthy, the polymerization rate in the primary amine mechanism is directly dependent on the concentrations of monomer and amine (Deming, 1997, 2000, 2006).

The present work describes the synthesis of different copolymers of PBLG using different amino initiators to allow the preparation of biocompatible nanoparticles possessing different specific functionalities, including lowered non-specific recognition properties, fluorescent markage for tracking the vector system *in vivo* and the possibility of attaching easily various ligands for target recognition by using the avidin–biotin non-covalent interaction. For that purpose, PBLG derivates were synthesized by ring-opening polymerization (ROP) of γ-benzyl-L-glutamate N-carboxyanhydride using selected primary amine terminated initiators. The resulting polymers were analyzed by FT-IR spectroscopy, viscosimetry, ^{1}H NMR, and SEC. Finally, because these polymers have been conceived for different drug targeting applications, their individual capacity to form nanoparticles has been evaluated and the functionality of the molecules of interest within the nanoparticles has been checked.

2. Materials and methods

2.1. Reagents

N,N-dimethylformamide (DMF, Acros, 99%) and benzylamine (Bz, Janssen Chimica) were purified following standard procedures as described elsewhere in the literature (Perrin et al., 1980). Briefly, just before utilization, they were distilled (taking the middle fraction onto Linde type molecular sieve 4A) under reduced pressure over $MgSO_4$ and NaOH, respectively, and stored under argon atmosphere. γ-Benzyl-L-glutamate N-carboxyanhydride (H-Glu(OBzl)-NCA or NCA, ISOCHEM-SNPE) was used as received. α-Methoxy-ω-amino poly(ethylene glycol) (MeO-PEG-NH_2) and α-biotin-ω-amino poly(ethylene glycol) (biotin-PEG-NH_2) with PEG-MW = 5000 Da from Irish Biotech GmbH, and fluorescein isothiocyanate isomer I (FITC) from Sigma–Aldrich were dried separately under vacuum over KOH at 38 °C for several hours. 4′-Hydroxyazobenzene-2-carboxylic acid (HABA, Pierce – part of Thermo Fisher Scientific) and avidin (Sigma–Aldrich) were used as received.

Water was purified by reverse osmosis (Milli-Q, Millipore). Diethyl ether, methanol and other solvents were analytical grade and all other chemicals were commercially available reagent grade.

2.2. Preparation of initiator solutions

All initiator solutions were prepared under argon atmosphere and used immediately.

Benzylamine (Bz) solution: A 0.1 mol L^{-1} solution was prepared by diluting freshly distilled benzylamine in freshly distilled DMF.

MeO-PEG-NH_2 solution: Dried MeO-PEG-NH_2 was dissolved, at 30 °C, into freshly distilled DMF to prepare a 0.025 mol L^{-1} solution.

Biotin-PEG-NH_2 solution: A 0.020 mol L^{-1} solution was prepared, at 30 °C, by dissolving biotin-PEG-NH_2 in freshly distilled DMF.

Fluorescein isothiocyanate (FITC) solution: A 0.03 mol L^{-1} solution was prepared by dissolving FITC in freshly distilled DMF.

2.3. Synthesis and purification of the PBLG derivates

Four PBLG derivates with an molecular weight near of 50,000 g mol^{-1}: PBLG-Bz, PBLG-PEG, PBLG-PEG-Bt and Bz-PBLG-FITC, were obtained by ring-opening polymerization (ROP) of H-Glu(OBzl)-NCA in DMF initiated by Bz, MeO-PEG-NH_2, biotin-PEG-NH_2 or initiated by Bz and terminated by addition of FITC, respectively, using a lightly modified method described elsewhere (Fontaine et al., 2001; Martínez Barbosa et al., 2007). Briefly, *N* millimoles of NCA were weighed under argon atmosphere in a degassed three necks roundbottomed flask equipped with thermometer, mechanical stirring, refrigerant with a silica gel guard and a bubble detector. NCA was dissolved in DMF at room temperature under mechanical stirring and argon flux (volume was adjusted to obtain a 1 mol L^{-1} solution of NCA taking into account the volume of initiator solution needed to obtain the final initiator concentration). After about 15 min, the argon flux was stopped, the solution was heated at 30 °C, and the absence of NCA auto-polymerization was checked after some minutes by visual inspection looking for the lack of CO_2 production and by analyzing the reaction medium by Fourier transform infrared spectroscopy (FT-IR). Then, the initiator solution was added and immediately a gaseous CO_2 emission was observed. The reaction mixture was stirred at 30 °C and sometimes bubbled with argon until the characteristic NCA bands disappeared from FT-IR spectrum (which lasted from 72 to 144 h, depending on the reaction). The mixture was precipitated in an excess of cold diethyl ether to give a white solid. The precipitate was filtrated and washed five times with diethyl ether and dried under vacuum at 35 °C for at least 12 h. A second dissolution (but in THF), precipitation, purification and drying procedure were performed for all polymers. In addition, the precipitates of PBLG-PEG and PBLG-PEG-Bt were purified also by washing three times with methanol. For the Bz-PBLG-FITC polymers, close to the end of the reaction, a solution containing three times more moles of FITC than initiator (Bz) was added and after 24 h the precipitation step, described before, was made.

2.4. Characterization of PBLG derivates

2.4.1. Fourier transform infrared spectroscopy (FT-IR)

A Fourier Transform Perkin-Elmer 1750 spectrometer was used for collecting FT-IR spectra to monitor NCA auto-polymerization and to follow the completion of the reactions. The structures of polymer powders were also confirmed by FT-IR measurements at room temperature.

2.4.2. Viscosity measurements

Specific viscosity was determined in DMF at 25 °C using an automatic Ubbelohde viscometer (Schott-Geräite GmbH, Germany), capillary tube no. I, 0.63 mm of internal diameter, for which the flow time of pure solvent was 88 s. Solutions of different concentrations were prepared by dilution of a stock solution and filtrated through a Whatman Anatop 25 0.2 μm filter. The specific viscosity, ηsp, was given by the ratio of the difference between flow time of solution and flow time of solvent to the flow time of solvent, $(t - t_0)/t_0$ (number of repeats: $n = 10$). Reduced specific viscosity, ηsp/C, was plotted against concentration (in g/mL) and the intrinsic viscosity was determined by extrapolation to zero concentration.

2.4.3. ^{1}H NMR

^{1}H NMR spectra of the copolymers (PBLG-PEG, PBLG-PEG-Bt) were measured in solvent deuterated chloroform ($CDCl_3$) to estimate the copolymers composition and the molecular weight of PBLG blocks using a Bruker Advance 400 apparatus, operating at 400 MHz. For molecular weight estimation, integration values of ^{1}H NMR (δ, $CDCl_3$): 7.26 (br, s, 5H, Ph–) or 5.04 (br, s, 2H, $-CH_2-$benzyl) for PBLG segment, and 3.65 (br, s, 4H, $-OCH_2-CH_2O-$) for PEG segment were used.

2.4.4. Size Exclusion Chromatography (SEC)

SEC was performed at room temperature with a system equipped with a Spectra SYSTEM AS1000 auto sampler, a guard column (Polymer Laboratories, PL gel 5 μm guard, 50 mm × 7.5 mm) followed by 2 columns (Polymer Laboratories, 2 PL gel 5 μm MIXED-D columns, 2 mm × 300 mm × 7.5 mm), a Spectra SYSTEM RI-150 detector. THF was used as eluant at a flow rate of 1 mL min^{-1} at 35 °C. Polystyrenes (580–483 × 10^3 g mol^{-1}) were used as standards.

2.5. Nanoparticles preparation and characterization

Nanoparticles from PBLG-Bz, PBLG-PEG, PBLG-PEG-Bt and Bz-PBLG-FITC were prepared using a modified nanoprecipitation method described elsewhere (Thioune et al., 1997; Alvarez-Román et al., 2001; Martínez Barbosa et al., 2007). Briefly, 15 mg of polymer were dissolved in 5 mL of THF at 30 °C during 18 h, without stirring. This solution was added by dripping to 10 mL of Milli-Q water under magnetic stirring. The mixture was left under magnetic stirring for 15 min and then transferred in a Teflon® coated recipient. The solvent was evaporated, under a light air flux. In order to eliminate residual solvent, nanoparticles were washed with 5 mL of Milli-Q water and evaporation was carried out to lead to 10 mL of nanoparticles suspension.

2.5.1. Dynamic laser light scattering (DLS) measurements

Nanoparticles mean diameter and zeta (ζ) potential ($n = 8$) was determined, after suitable dilution of bulk suspensions of nanoparticles in Milli-Q water or 1 mM NaCl, using DLS (Zetasizer 4, Malvern Instrument).

2.5.2. Transmission electron microscopy (TEM)

Nanoparticles were observed by TEM in a Philips EM 208 transmission electron microscope in order to examine their morphology and to confirm their size. 3 μL of a nanoparticles dilution was placed on a formvarcarbon film previously coated on a nickel grid (400 mesh). After 5 min of deposition at room temperature, a phosphotungstic acid solution (1%) was added and staining was allowed for 30 s, and finally the non-adherent nanoparticles were eliminated. TEM bright field imaging was performed under a 60 kV accelerating voltage.

2.5.3. Fourier transform infrared spectroscopy (FT-IR)

Fresh and 20-day-old nanoparticle suspensions (kept in ambient temperature) were lyophilized and their FT-IR spectra were recorded in a Perkin Elmer FT-IR Spectrometer Spectrum 2000.

2.5.4. Fluorescence measurements

Fluorescence spectra were recorded using a LS 50 B Perkin Elmer Luminescence Spectrometer in the right-angle geometry (90° collecting optics). A proper dilution of Bz-PBLG-FITC nanoparticle suspensions was used for determining the maximum excitation and emission wavelengths. Also, fluorescence was compared between fresh and 135- or 195-day-old nanoparticle suspensions stored at 4 °C in the dark.

2.5.5. UV–vis measurements

Biotin availability and specificity in the nanoparticles surface was colorimetrically detected at 500 nm using the HABA/avidin method as described by Pierce (Pierce, part of Thermo Fisher Scientific) in a Shimadzu UV-2101PC UV-VIS Scanning Spectrophotometer. The HABA dye (4′-hydroxyazobenzene-2-carboxylic acid) binds specifically to avidin to produce a yellow-orange colored complex which absorbs at 500 nm. Free biotin will displace the HABA dye and cause the absorbance to decrease.

3. Results and discussion

3.1. Synthesis and characterization of PBLG derivates

Polymeric nanoparticles can be functionalized in various manners. Functionalization may be a critical step and generally, the functionalization of the building polymers previously to the preparation of the particles may be highly preferable to a functionalization of the preformed particles, mostly because chemical steps necessitate the use of chemical reagents and request numerous washings, which are all likely to alter encapsulated drugs. Thus, the present study has focused on the preparation of a set of copolymers derived of poly(γ-benzyl-L-glutamate) and bearing different desirable functionalities, in order to be able to adapt the structure of the nanoparticles to the active drug and the requirements in term of drug delivery.

A traditional, easy to conduct and metal free synthesis method has been chosen (Deming, 2000) to give the desired structures and also because of its simplicity, the ease of purification of the polymers, etc. In order to be able to prepare block copolypeptides, polymerization solely through the amine mechanism is desired, but these traditional ROP reactions have some limitations because may exist polymerization conditions where chain transfer and chain termination reactions are present, which could results in a broad distribution of polymer chain lengths (Deming, 1997). Although other more controlled methods of polymerization exist, these are complex routes and/or use metals (as Ni for example) as catalysts that could not be completely removed (Deming and Curtin, 2000; Vayaboury et al., 2004; Steig et al., 2005), which may impair their use in many applications needing biocompatibility (Aliferis et al., 2004; Knoop et al., 2008).

The major point is that the chosen method requires special care in purifying solvent and chemicals used, for preserving adequate reaction conditions, because it has been probed that for ROP of NCA by the amine mechanism, the purity of the reacting mixture is a necessary and a sufficient prerequisite for effective control of NCA polymerization (Aliferis et al., 2004).

In the present work, several batches of PBLG-Bz, PBLG-PEG, and PBLG-PEG-Bt, were prepared by ring-opening polymerization (ROP) of H-Glu(OBzl)-NCA (NCA) in DMF using Bz, MeO-PEG-NH_2 or biotin-PEG-NH_2 as initiators, respectively (Fig. 1). In addition, it has been taken advantage of the fact that it is well known that

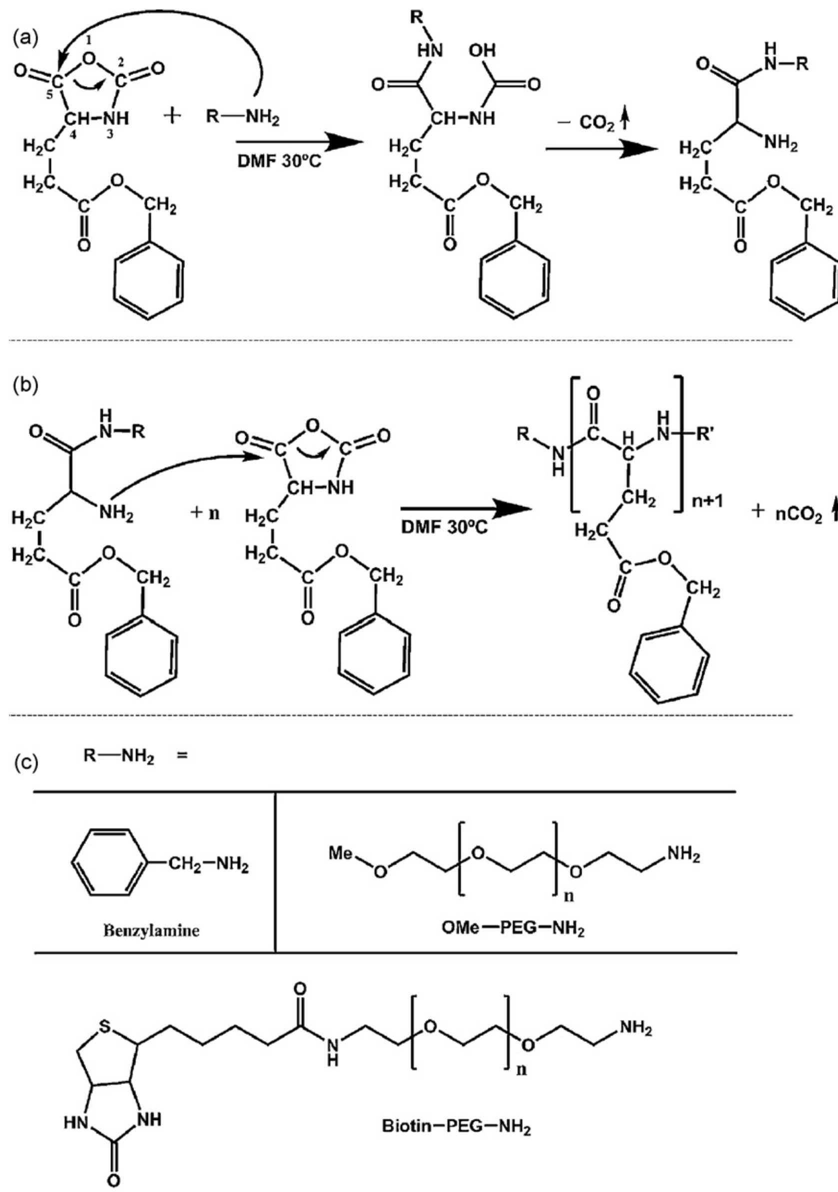

Fig. 1. Ring-opening polymerization (ROP) of γ-benzyl-L-glutamate N-carboxyanhydride (NCA) by the "normal-amine mechanism". (a) The primary amines, strong nucleophiles, attack the 5-CO of the NCA monomer; the resulting carbamic acid releases CO_2 from the 1-O and 2-CO to give the free amino group and (b) which continues the polymerization in the absence of active impurities. (c) Initiators used for the polymerization reactions to obtain PBLG-Bz, PBLG-PEG, and PBLG-PEG-Bt (R′ = H).

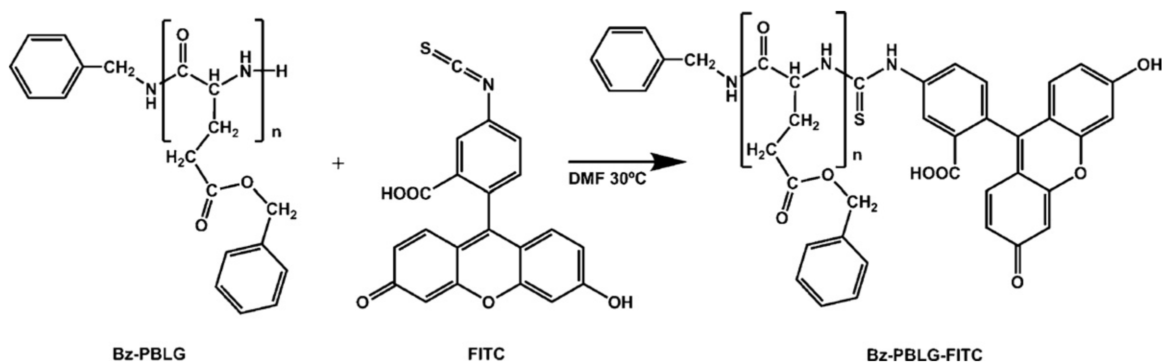

Fig. 2. Poly γ-benzyl-L-glutamate, Bz-PBLG (polymerized by ROP of their NCA by the "normal-amine mechanism" initiated by benzylamine), and further fluorescein isothiocyanate, FITC, coupling at the terminal free amino group for obtaining Bz-PBLG-FITC.

in NCA polymerizations, electrophiles such as isocyanates act as chain-terminating agents because they react with the propagating amine chain-ends (Deming, 2006). Two batches of Bz-PBLG-FITC were also synthesized by ROP of NCA initiated by Bz and terminated by the addition of FITC (Fig. 2). This strategy takes advantage of the fact that the ROP of a NCA generates a terminal amino group at the end of the PBLG polypeptide chain which possesses a rigid α-helical conformation in DMF as a solvent. Due to this conformation, this N-terminal group is exposed by the extremity of the polypeptide chain (Kim et al., 2006b) and has been used for further coupling of FITC.

Molecular weights of polymers were controlled by adjusting the concentration ratio of NCA to initiator. It was necessary to adjust the initial concentration of each initiator in DMF because of differences in solubilities. Polymerizations were followed by FT-IR spectroscopy. The disappearance of absorption bands at ~1850 cm^{-1} and ~1775 cm^{-1} corresponding to the $C^5{=}O$ and $C^2{=}O$ of the anhydride, respectively, and ~920 cm^{-1} corresponding to C–O–C of the anhydride (Pretsch et al., 1989), was considered as the end of the polymerization reaction. Moreover, before the addition of initiator, no auto-polymerization of the different NCA solutions was observed. DMF was used because it is a polar solvent in which PBLG adopts an α-helix conformation but without aggregation (Balik and Hopfinger, 1978), which permits the growing of the chain in development in an organized manner.

3.1.1. Copolymers characterization

The position of amide absorption bands in the FT-IR spectra may be used to characterize the conformation of a polypeptide. For a polypeptide in a α-helix conformation, the amide I band and the amide II band are located near to 1656 cm^{-1} and 1548 cm^{-1}, respectively (Miyazawa and Blout, 1961; Fontaine et al., 2001; Higashi et al., 2005; Martínez Barbosa et al., 2007), while for a polypeptide in a β-sheet conformation, the amide I band and the amide II band are located near to 1630 cm^{-1} and 1536 cm^{-1}, respectively (Fontaine et al., 2001; Martínez Barbosa et al., 2007). Moreover, an absorption band near to 1260 cm^{-1} corresponding to the amide III band may be observed in the FT-IR spectrum of a polypeptide in a α-helix conformation (Martínez Barbosa et al., 2007). In the present case, continuous monitoring of the polymerization reaction showed in all FT-IR spectra for all the polymers, a peak at 1544 cm^{-1} and another at 1260 cm^{-1} which could be assigned to amide II and amide III bands, both characteristics of an α-helix conformation of the polypeptide (Fig. 3), and which were already noticeable in the time course of the polymerization reactions. It confirmed that DMF is an helicogenic solvent for PBLG (Balik and Hopfinger, 1978), which may drive the growing of the chain in development in an organized manner.

After purification and washing steps, the PBLG derivates were analyzed by FT-IR spectroscopy, SEC, ^{1}H NMR spectroscopy, and viscosity measurements. FT-IR spectra (spectra not presented) showed for all the polymers characteristic peaks that could be assigned to amide I, II and III bands, all of them being characteristic of the α-helix conformation.

Attempts were made to determine the molecular weight of the copolymers by SEC in THF but these determinations were not reliable because PBLG aggregated in this solvent (Balik and Hopfinger, 1978; Block, 1983; Kros et al., 2005), and thus gave rise to a broad peak. In SEC the aggregates had a smaller elution volume, which indicated an apparently higher molecular weight (Kros et al., 2005) presumably due to an increase in the hydrodynamic volume owing to rigid helical conformation of the PBLG block (Kim et al., 2005, 2006a,b). The rigid nature of the copolymers prevented calibration with standards such as polystyrene (Kros et al., 2005). Consequently, the PBLG derivates molecular weights were evaluated by ^{1}H NMR or by viscosimetry.

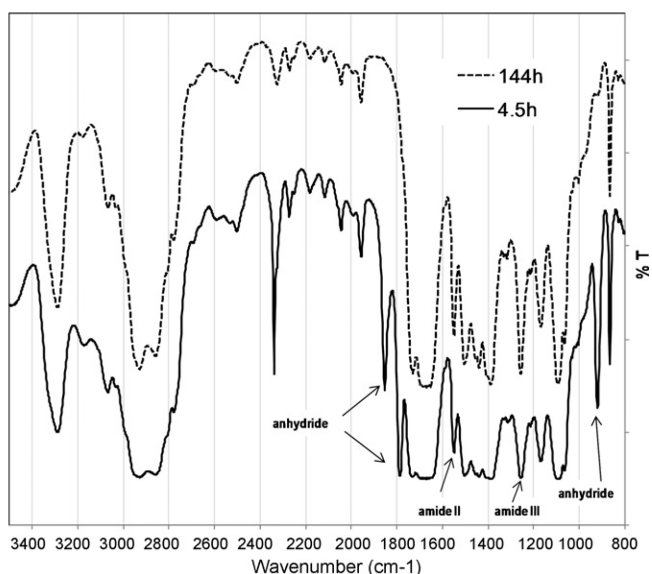

Fig. 3. Typical FT-IR spectra for the PBLG-PEG polymers recorded at 4.5 and 144 h after the beginning of the reaction of polymerization. Similar spectra were observed for other polymers (data not shown).

For PBLG-PEG and PBLG-PEG-Bt copolymers, the degree of polymerization (DP_n) and subsequently the average molecular weights (MW) of the PBLG block were determined by ^{1}H NMR, analyzing the peak intensities of the protons of the phenyl or benzyl groups of the PBLG chain, and the ethylene protons ($O–CH_2–CH_2–O$) of the PEG segment (Park et al., 2005) (Fig. 4).

For PBLG-Bz and Bz-PBLG-FITC polymers, the average molecular weight has been determined by viscosimetry using the Mark–Houwink equation. Intrinsic viscosity [η], was measured in DMF at 25 °C using an Ubbelohde viscosimeter. Molecular weights were determined using the Mark–Houwink equation in DMF at 25 °C (Temyanko et al., 2001):

$$[\eta] = 1.58 \times 10^{-5} \times MW^{1.35}$$

Results are gathered in Table 1 and show that measured molecular weights, either by ^{1}H NMR or by viscosimetry, were in good agreement with theoretical molecular weights.

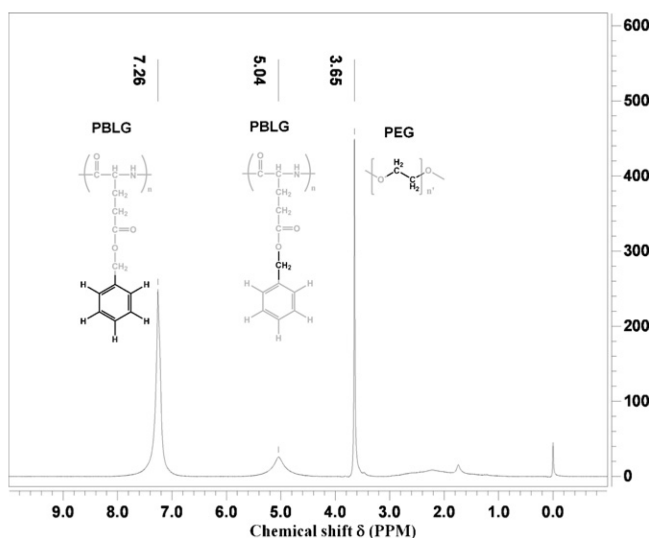

Fig. 4. Typical ^{1}H NMR spectra at 400 MHz (in $CDCl_3$) for the PBLG-PEG-Bt polymers. Very similar spectra were obtained for the other synthesized polymers (data not shown).

Table 1
Characteristics of polymer batches prepared by ring-opening polymerization of γ-benzyl-L-glutamate N-carboxyanhydride in DMF at 30 °C.

Polymer	$Mn_{(t)}$	$DP_{n(t)}$	Reaction time (days)	Yield (%)	$MW_{(exp)}$ (g mol^{-1})	Mass obtained (g)
PBLG-Bz	51,700	236	4	83	45,700[a]	0.70
PBLG-Bz	51,900	237	3	81	43,900[a]	2.63
PBLG-Bz	51,700	236	6	87	45,500[a]	2.93
PBLG-PEG	60,900	278	6	72	49,100[b]	1.17
PBLG-PEG	60,100	275	6	85	50,800[b]	2.77
PBLG-PEG	60,000	274	3	86	51,800[b]	4.85
PBLG-PEG	60,000	274	5	87	52,500[b]	5.35
PBLG-PEG-Bt	60,900	278	6	83	49,700[b]	0.98
PBLG-PEG-Bt	60,000	274	6	83	47,600[b]	3.95
Bz-PBLG-FITC	50,300	230	1	72	40,000[a]	0.69
Bz-PBLG-FITC	60,000	274	2	75	43,300[a]	2.63

(t), theoretical; (exp), experimental.
[a] Molecular weight determined by viscosity measurements in DMF at 25 °C using an automatic Ubbelohde viscometer (n = 10).
[b] Molecular weight (only for the PBLG segment) determined by integration of the ^1H NMR spectrum measured in $CDCl_3$ at 400 MHz.

Batches could be prepared which were characterized by reproducible molecular weights, close to the theoretically expected values. It could be concluded that the synthesis process was controlled and reproducible and can be usefully employed for the synthesis of functional polypeptides.

3.2. Preparation and characterization of nanoparticles

Before investigating the possibility to associate simultaneously two or more PBLG copolymers to form multifunctional particles, it was necessary to check individually the possibility of forming nanoparticles with the prepared copolymers. The different PBLG derivates were nanoprecipitated and nanoparticles were successfully prepared from PBLG-Bz, PBLG-PEG, PBLG-PEG-Bt and Bz-PBLG-FITC.

It was found that nanoparticles from the different polymers, PBLG-Bz, PBLG-PEG, PBLG-PEG-Bt and Bz-PBLG-FITC, could be easily and reproducibly obtained without the addition of stabilizing agents such as surfactants. In addition, it was found that the morphology, size and zeta potential of nanoparticles, were influenced by the type of copolymer. Whatever the polymer used, the nanoparticles were under 100 nm in hydrodynamic diameter and characterized by a narrow distribution (Table 2). Comparable sizes were obtained from TEM microphotographs. However, their morphology depended on the copolymer. As shown by TEM microphotographs (Fig. 5), nanoparticles containing PEG (PBLG-PEG or PBLG-PEG-Bt) exhibited a spherical form but on the contrary, nanoparticles made from PBLG-Bz, or the fluorescent Bz-PBLG-FITC derivate had an ellipsoidal morphology (Fig. 5a).

Whatever the polymer, the nanoparticles were characterized by a negative zeta potential, which intensity depended on the nature of the initiating moiety employed for the PBLG copolymer synthesis (Table 2). PEG-containing nanoparticles showed a substantial decrease of the ζ potential, probably due to the presence of a neutral PEG corona at the surface of the particles. Indeed, it is expected that due to the amphiphilic nature of the copolymers, the water-soluble PEG chains (MW = 5000 Da) would orientate at the surface of the particles, while the water-insoluble and more hydrophobic PBLG blocks (MW in the range of 50,000 Da), would be arranged to form the core of the nanoparticles.

Table 2
Morphological characteristics of nanoparticles prepared by nanoprecipitation.

Polymer	Mean hydrodynamic diameter (nm)[a]	Mean size from TEM (nm)[b]	ζ potential (mV)[c]
PBLG-Bz	67 ± 8	67 ± 13	−22.4 ± 3.2
PBLG-PEG	50 ± 7	37 ± 7	−16.7 ± 1.9
PBLG-PEG-Bt	59 ± 3	43 ± 7	−15.3 ± 1.5
Bz-PBLG-FITC	79 ± 6	67 ± 9	−29.2 ± 3.5

[a] Measured using dynamic laser light scattering, n = 3.
[b] Determined from TEM measurement as the mean between the maximal length and the maximal width of each nanoparticle, n = 100.
[c] Measured using a Zetasizer 4, Malvern Instrument, n = 10.

The exact organization of the polypeptidic chains in the core of the nanoparticles has not been elucidated yet. As was shown here and also earlier by Martínez Barbosa (Martínez Barbosa et al., 2007) it is known from FT-IR spectra of copolymers directly obtained after synthesis, that the PBLG blocks form α-helix structures, which are quite rigid. The comparison of FT-IR spectra of both fresh and 20-day-old lyophilized nanoparticles from each of the four different PBLG derivates shown also peaks characteristics for a polypeptide forming an α-helix conformation, suggesting that PBLG existed in nanoparticles in the form of quite rigid polymer chains, leading to stable and organized structures (spectra not presented). It is in agreement with previous works which reported that PBLG polypeptides having molecular weights higher than about 28,000 (degree of polymerization (D.P.) ~ 128) exist in the α-form in solid films and in many solvents. However, this degree of polymerization may not be the minimum D.P. where the α-form exists (Blout and Asadourian, 1956). Further, Papadopoulos concluded that PBLG chains with degrees of polymerization higher than 18, only form α-helix structures (Papadopoulos et al., 2004).

One of the aims of this work was to check the possibility of preparing a set of functional PBLG derivates bearing different moieties of interest at the end of the chains. For targeting applications, these specific building-block are intended to be used on demand for preparing tailored multifunctional nanoparticles, exhibiting adjusted properties for site specific drug delivery applications.

In many applications, whatever the route of administration, the nanoparticles will be placed in contact with very complex biological fluids and it is nowadays well known that in these situations, initial surfaces are promptly (within minutes) covered by molecules such as proteins, which adsorb at the particle surface, making these particles to be easily recognized by immune cells, phagocytosed and therefore diverted from their route to the target. For example, when delivered by the parenteral IV route, many drug delivery applications request the carrier system once in the body to be able to persist in the blood circulation for a sufficient period of time, without being uptaken by the reticuloendothelial system (RES). Although this phenomenon can be considerably reduced for small enough particles (usually under 100 nm), uptake (responsible of the RES response) occurs generally very rapidly with unmodified nanoparticles because adsorption of opsonins on the surface of this kind of particles is very fast (Nakada et al.,

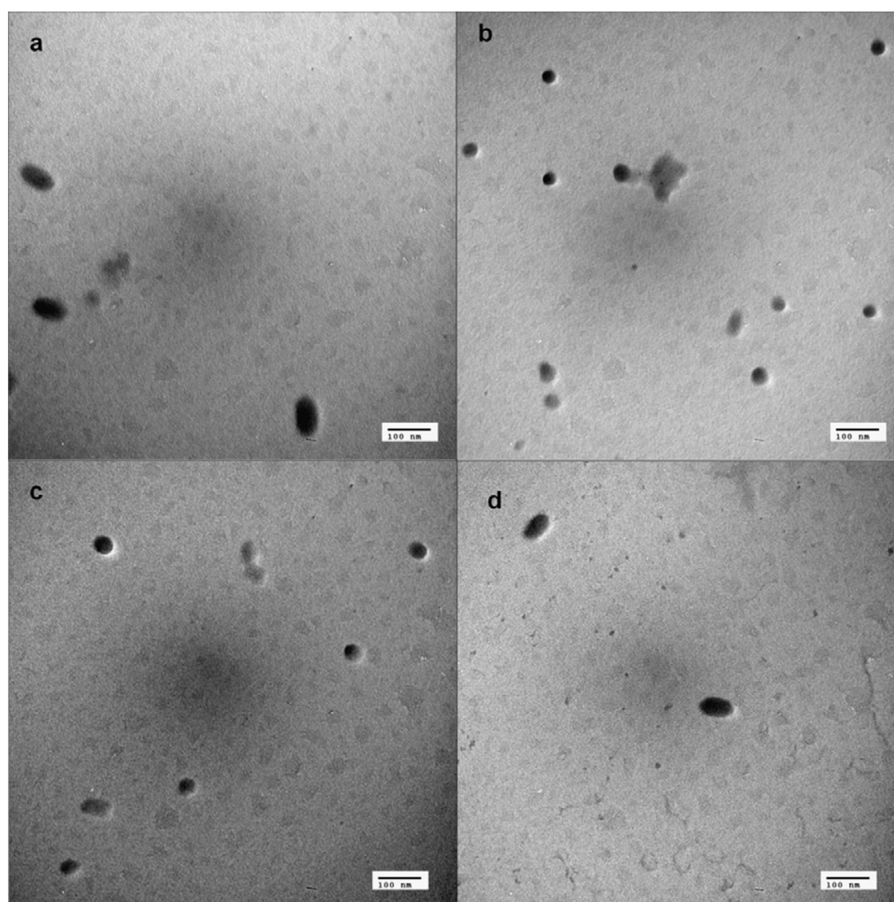

Fig. 5. TEM photographs of nanoparticles obtained from the following derivates. (a) PBLG-Bz, (b) PBLG-PEG, (c) PBLG-PEG-Bt and (d) Bz-PBLG-FITC.

1998). Such phenomenon can be dramatically retarded by grafting hydrophilic poly(ethylene glycol) on particles surface (Leroux et al., 1995; Hrkach et al., 1997). For this purpose, methoxy poly(ethylene glycol) amine (MeO-PEG-NH_2), MW = 5000 Da has been selected as initiator of polymerization because this chain length seems optimal for conferring stealth properties to particles (Marjan and Allen, 1996; Gref et al., 2000; Avgoustakis et al., 2002). As discussed before, the introduction of a PEG block conferred an amphiphilic nature to the PBLG-PEG copolymers. When forming nanoparticles, the hydrophilic PEG blocks were certainly exposed at the surface of the particles, resulting in the decrease of the zeta potential when compared to pure PBLG nanoparticles. Further, previous experiments conducted on similar particles demonstrated their capacity to reduce complement protein activation (Martinez-Barbosa et al., 2009).

Looking for "easily" tracking the carrier system in future applications, Bz-PBLG-FITC polymer was conceived to prepare fluorescent nanoparticles for further visualization of the distribution of nanoparticles *in vivo* or *in vitro* (for example by confocal laser scanning microscopy) (Alvarez-Román et al., 2004). Nanoparticle suspensions in water were made from each one of the two batches of Bz-PBLG-FITC synthesized polymers and analyzed by fluorescence spectroscopy. It has been found that the maximum excitation and emission values were close to 486 nm and 515 nm, respectively (Fig. 6). These values are in agreement with experimental values reported by Sigma–Aldrich for free FITC (λ_{ex} 492 nm; λ_{em} 518 nm) (Green, 1990).

Free FITC water solutions are not very stable. To determine if a water suspension of fluorescent nanoparticles had a stable fluorescence, fluorescences of fresh, 135- and 200-day-old nanoparticles (stored a 4 °C in the dark) were compared and it was observed that there were not significant differences in their fluorescence intensities. Even, the 135-day-old formulation showed a slightly higher fluorescence than the fresh preparation. These results suggest that Bz-PBLG-FITC nanoparticle suspensions in water were very stable, making this polymer suitable for being included in little amount in multifunctional nanoparticles of interest for visualization and tracking purposes.

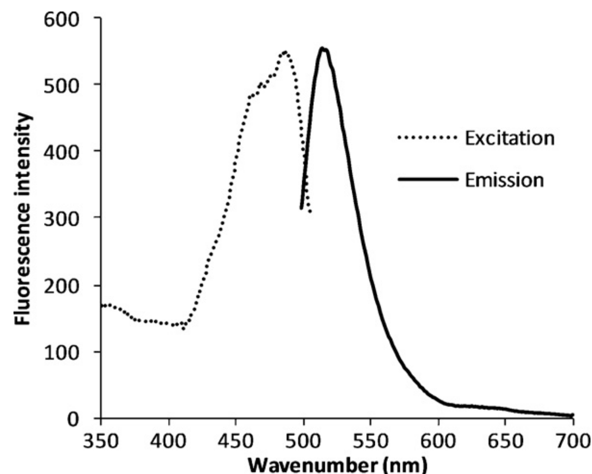

Fig. 6. Fluorescence excitation and emission spectra of Bz-PBLG-FITC nanoparticles in water suspension.

Because nanoparticles in drug delivery applications are expected to modify favorably the distribution of the drug within the body, there exists a considerable interest to be able to decorate the surface of the particles with ligands intended to recognize specifically a target localized in the ideal site for drug delivery (e.g. an organ, a specific cells, a specific sub-cellular compartment). This strategy, known as active targeting, necessitates the grafting and the presentation of the ligand at the surface of the particles in sufficient amount and in an active configuration, to make the ligand able to recognize efficiently the target receptor. This is not an easy task and therefore, various strategies have been imagined to do it properly. In the present study, it has been imagined to use the biotin–avidin system (Diamandis and Christopoulos, 1991) for achieving easily the presence of a ligand on nanoparticles surfaces easily after their preparation.

Avidin (p*I* of 10.5, ~67 kDa, 5.6 nm × 5 nm × 4 nm (Vermette et al., 2003)) is a basic, highly stable, homo-tetrameric glycoprotein, isolated from bird egg-white, binding up to four molecules of vitamin H, D-biotin, by a non-covalent interaction with extremely high affinity ($K_d \sim 10^{-15}$ M). This interaction is extremely tight, being three to six orders of magnitude higher than that of typical antigen:antibody complexes (Rosano et al., 1999). This complexation property represents the basis for the exploitation of avidin (and similar derivates such as streptavidin) as a molecular tool in many biotechnological applications (Morpurgo et al., 2004). The high affinity of avidin for biotin ensures that, once formed, the complex will not be disturbed by changes in pH, the presence of chaotropes, or manipulations such as multiple washings when the complex is immobilized (Diamandis and Christopoulos, 1991). Therefore, by introducing biotin moieties at nanoparticle surface by the mean of a PBLG-PEG-biotin polymer, it can be expected that it will be possible to attach firmly avidin to the surface of these particles. Further, because avidin is a tetrameric protein, unoccupied sites are likely to remain available after immobilization at the surface, which in turn can accommodate another biotin moiety linked to a ligand of interest. Indeed, this situation is favored because in the tetrameric form of avidin, two biotin-binding sites are located toward a side while the other two sites are on the opposite side (Qin et al., 1995). This strategy is illustrated in Fig. 7, explaining how the "sandwich" structure would lead finally to the firm association of a desired biotinylated ligand at the surface of the nanoparticles. The major advantage of this strategy for decorating the particle surface would be that the introduction of the ligand of interest at the surface of the particles would not require any further chemical step once the nanoparticles have been prepared and loaded with drugs. Moreover, biotinylation of numerous biological ligands has been applied to many molecules so far, and which are commercially available, thus forming a wide library of available ligands of interest in many targeting applications.

For preparing such constructions, a PBLG-PEG-Bt copolymer has been synthesized. A PEG chain was used not only because of its hydrophilicity and "stealth" properties but also to act as a spacer. Indeed, the active binding site for biotin within each avidin subunit is located in a deep pocket, at the center of a β-barrel and as the biotin-binding site is buried deep in the protein core, a spacer may be used for improving the complex formation (Qin et al., 1995; Haugland and You, 2008). In addition, as it has been suggested that PBLG in α-helix conformation was quite rigid, it became really necessary to use a "flexible" spacer between PBLG and biotin.

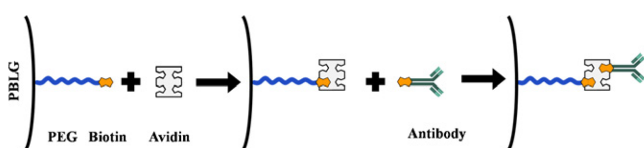

Fig. 7. Schematic representation of the strategy for decorating the surface of a PEG-Bt containing nanoparticle by constructing an avidin–biotin-ligand sandwich. An antibody as been used as an example of a biotinylated ligand of interest (components are not scaled).

Biotin availability and its ability to bind avidin on the nanoparticles surface elaborated from both of the PBLG-PEG-Bt synthesized polymers, has been colorimetrically detected using a competition between HABA and biotin. In this technique, the HABA dye binds to the biotin-binding sites in avidin. It has a broad absorption peak at 500 nm in the complex state and only a very low absorbance in the free state. In presence of HABA–avidin complexes, added biotin (free or covalently bound to a protein of interest), will quantitatively displace the bound HABA because of its very higher affinity for avidin and a decrease in absorbance at 500 nm will result, which can be used to determine the amount of added biotin. In the present case, it was found that approximately 76% and 72% of biotin in nanoparticles prepared from two PBLG-PEG-Bt batches (corresponding to the PBLG segment of 49,700 g/mol, or 47,600 g/mol, respectively) were available and accessible at the surface of the particles for the avidin protein in solution. As a control, biotin was not detected in nanoparticles made only from PBLG-PEG but without biotin, suggesting that the HABA/avidin technique is specific and that PEG did not interfere in the colorimetrical detection of biotin. This result, suggests that the synthesis of the biotinylated copolymer has been quite efficient. Indeed, it should be reminded that it has been supposed for PBLG-PEG-Bt synthesis calculations that synthesis followed only an "amine mechanism" and that there were little if no formation of PBLG homopolymers as a byproduct of the reaction. Further, the rigid structure of the hydrophobic PBLG block combined to the hydrophilicity and flexibility of PEG-Bt chains certainly favored the location of biotin in the surface of nanoparticles. These biotin moieties were probably sufficiently exposed to avidin in solution and because of the length (MW = 5000 g/mol) and flexibility of the spacer arm, PEG-biotin could be accessible to avidin as suggested by the experimental results. Finally, the fact that about only 25% of biotin was not engaged in a complexation with avidin could also be simply related to sterical limitations at the surface of the particles. Avidin is a large protein (MW ~ 67 kDa, in the tetrameric form) and sterical limitations may occur, making possible that some available biotin moieties located on the nanoparticle surface could not physically be reached by avidin molecules.

4. Conclusions

Several batches of PBLG-Bz, PBLG-PEG, PBLG-PEG-Bt and Bz-PBLG-FITC, biocompatible derivates of PBLG were successfully synthesized using an easy, controlled and reproducible ring-opening polymerization method. This synthesis strategy offered a convenient means to introduce functional moieties at the end of PBLG blocks, which molecular weight could be easily adjusted, and which can be probably quite easily extended to other molecules of interest.

Because of the water-insolubility of the PBLG block, these polymers could be used to prepare by an easy to conduct modified nanoprecipitation technique, different types of small (lower than 100 nm) and stable nanoparticles with multiple and specific functionalities, in order to solve specific problems in different drug targeting applications. Interestingly, biotin can be very efficiently presented at the surface of the nanoparticles and it is suggested that the biotin–avidin complexation reaction will offer a convenient mean for introducing targeting ligands in a controlled manner at the surface of poly(γ-benzyl-glutamate) nanoparticles.

Finally, because of the constant characteristics of the PBLG block for each derivates, it is expected that these polymers will be able

to easily self-associate in various proportions and form on demand multifunctional particles.

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