Tunable Degradation Behavior of PEGylated Polyester-Based Nanoparticles Obtained Through Emulsion Free Radical Polymerization.

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INTRODUCTION

The use of nanotechnology has enabled new and safer administration routes for pharmaceuticals through the use of carriers which can selectively deliver drugs only to specific organs, enhancing their efficacy and reducing the side effects.[1,2] Toward this goal numerous types of carriers have been developed, such as liposomes,[3] polymersomes,[4] micelles,[5] and polymer nanoparticles (NPs).[6] Due to their well-investigated biocompatibility and biodegradability, which arise from the simple clearance of the polymer matrix by the metabolic pathways, the most commonly used materials for polymer-based carriers are poly(lactic acid) (PLA), poly(lactic-co-glycolic acid) (PLGA), and poly(caprolactone) (PCL).[6−8] Polymer NPs are widely studied in the literature as drug carriers due to their high drug loading efficiency and the possibility to have both triggered and controlled release; also possessing a longer shelf life than other carriers.[9] NPs must satisfy various requirements in order to successfully fulfill their role of drug delivery vehicles; for instance, it has been shown that parameters such as size or surface charge greatly influence the behavior of the system in terms of cellular uptake and drug release.[10] Another key characteristic of a successful drug carrier is its stability, both in terms of residence time in the bloodstream and in a chemical and colloidal sense and, since a possible cause of NPs failure is the aggregation in in vivo condition, the colloidal stability must be assured at least in vitro conditions.[11] A further challenge that NPs must overcome once injected intravenously is their well-documented removal by the mononuclear phagocytic system from the bloodstream. This clearance occurs through the opsonization process which involves blood proteins binding on the surface of the NPs, making them detectable by phagocyte cells. A correlation between surface charge and binding of blood proteins has been assessed, and it was found out that noncharged NPs have a much lower opsonization rate than charged ones.[12,13] Therefore a common way to mitigate opsonization is the use of surface-functionalized NPs with moieties that can reduce hydrophobic interactions and shield the surface charge. Among these materials the most used is poly(ethylene glycol) (PEG) which is hydrophilic and neutrally charged and thus allows a longer circulation time for the NPs, reducing the macrophage uptake.[14]

The great majority of polyester-based NPs are produced using bulk polymers of medium or high molecular weight through processes such as nanoprecipitation, solvent evaporation, and salting out.[6,15,16] In these methods, the polymer is dissolved in a suitable organic solvent together with the drug that must be encapsulated; NPs are formed by mixing this organic phase with an aqueous phase which contains a stabilizing agent (for example a surfactant); a common drawback of these techniques lies in the fact that the removal of the organic solvent after NPs formation can be difficult and incomplete.[17] The degradation process of NPs produced from bulk polymers has been assessed to be function of the type of material used as well as its molecular weight and can be as long as several months.[18,19] Therefore it would be appealing to

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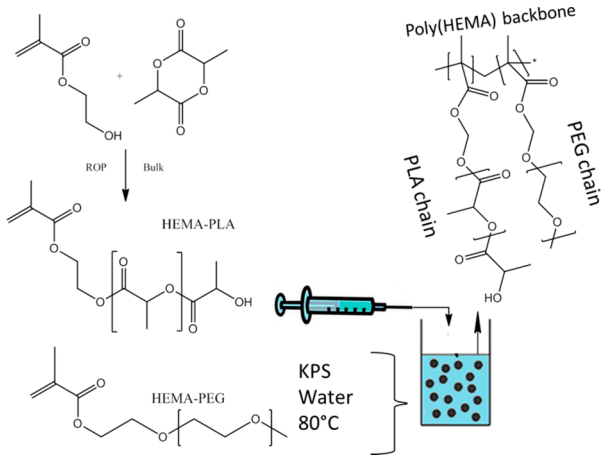
reduce and tune the degradation time of the NPs to have an additional parameter to control the drug release.[20]

Recently a new kind of NPs based on biodegradable polyesters, such as PCL,[21] PLA,[22] and PLGA[23] has been developed. The process used to synthesize the NPs consists in two steps; first a ring-opening polymerization (ROP) of lactide (LA), caprolactone (CL), or glycolide (GA) using tin octoate ($Sn(Oct_2)$) as a catalyst and 2-hydroxyethyl methacrylate (HEMA) as a cocatalyst is performed. The products of this step are functionalized macromonomers composed of a polyester chain of controlled length and composition functionalized with a vinyl bond coming from HEMA molecule; these macromonomers are then used in an emulsion free radical polymerization. The overall procedure is schematized in Scheme 1 for the case of PLA-based NPs.

Scheme 1. Different Steps Used to Obtain PLA-Based NPs[a]



[a]The final polymer is composed of a poly(HEMA) backbone with PLA side chains of different length as well as PEG chains.

As shown in Scheme 1, no solvent is required in any part of the synthesis; this process also has other advantages. For example it was possible to add a fluorescent monomer based on Rhodamine B to the free radical polymerization step to covalently attach a dye to the NPs themselves in order to make them detectable for biological applications.[24] Furthermore, through the use of commercially available materials consisting in a PEG chain functionalized with a terminal HEMA molecule (HEMA-PEG) a strategy has been adopted to obtain PEGylated NPs with PEG chains covalently bonded to the NPs themselves.[25] In this case PEG chains act as a surfactant and NPs have been therefore produced in a surfactant-free monomer starved semibatch emulsion polymerization (MSSEP) where the hydrophilic HEMA-PEG is loaded in the reactor and the hydrophobic macromonomer is slowly added during the reaction. The structure of these NPs consists in a poly(HEMA) backbone grafted with polyester chains of different lengths attached to it, as well as PEG chains. PEGylated NPs have been proved to be noncytotoxic even at high concentration and the presence of the dye has allowed the study of their internalization by tumor cells in in vitro conditions.[26] In vivo experiments have been performed to study the pharmacokinetic and biodistribution in murine tumor model; showing a time-stable NPs concentration in the tumor and only a transient accumulation in the liver.[27] Moreover, due to their ability to convey hydrophobic drugs these NPs have been used in a combined hydrogel-NPs treatment for spinal cord injury.[28]

In this work the degradation behavior of the PEGylated NPs is studied in different environments. PEGylated PLA and PCL-based NPs with different average chain length have been synthesized and fully characterized. Then, the stability and degradation of these NPs have been studied in media such as isotonic phosphate buffer solution (PBS), cell medium, gastric and intestinal solution through dynamic light scattering (DLS) measurements. The NPs long-term colloidal stability in isotonic environment, which is strictly required for biomedical application for the storage of the NPs themselves, has been assessed in PBS for all the synthesized materials. Measurements performed in cell medium confirmed that, by changing the type of polyester and the chain length, the degradation time can be tuned from over one month down to about two days. This allows the NPs to reduce their degradation time, thus minimizing the risk of unwanted long-term accumulation in organs such as liver, and also ensuring the complete release of the drug after a selected time. These materials also show good stability in gastric and intestinal solution, therefore making possible candidates for oral delivery.

EXPERIMENTAL SECTION

Materials. ε-Caprolactone (CL, 99%), (3*S*)-cis-3,6-dimethyl-1,4-dioxane-2,5-dione (lactide, LA, 98%), 2-hydroxyethyl methacrylate (HEMA, ≥ 99%), 2-ethylhexanoic acid tin(II) salt ($Sn(Oct)_2$,~ 95%), potassium persulfate (KPS; ≥ 99%), $CDCl_3$ (used for ^1H NMR analysis, 99.6% atom D), and poly(ethylene glycol) methyl ether methacrylate (HEMA-PEG_{19}, molecular weight: ca. 950 Da) were purchased from Sigma Aldrich and used without further treatment. The cell medium is composed by high glucose DMEM/F12 (Biowest, Nuaillé, France) supplemented with 10% fetal bovine serum (Lonza, Basel, Switzerland). Phosphate buffered saline solution (PBS, Biowest), hydrochloric acid (37%), sodium chloride, and sodium hydrogen carbonate were bought from Sigma Aldrich and used to produce the gastric and intestinal solution without further treatment.

Production and Characterization of Macromonomers and NPs. Macromonomers were synthetized through a previously described ring-opening polymerization process.[21,23] Briefly, CL or LA was heated to 130 °C in a stirred flask to which a mixture of $Sn(Oct)_2$ and HEMA (molar ratio 1:200) was added in a specified molar ratio in order to obtain the desired chain length: 2, 3, and 5 for PCL-based materials and 4, 6, and 8 for PLA-based ones. Each macromonomer was characterized by ^1H NMR analysis in $CDCl_3$ performed on a 500 MHz apparatus (Bruker, Switzerland). Macromonomers were also analyzed by using a maXis ESI-Q-TOF (Bruker, Switzerland) mass spectrometer equipped with an automatic syringe pump for sample injection (KD Scientific, US). The ESI-Q-TOF mass spectrometer was operated at 4500 V with a desolvation temperature of 200 °C, while positive ion mode was implemented for the mass spectrometer with nitrogen as a nebulizer and drying gas. The standard electrospray ion (ESI) source was used to generate ions and chloroform (CH_3Cl) was used as a solvent. Sixty shots from each spot were averaged to obtain one mass spectrum. The ESI-Q-TOF MS instrument was calibrated in the m/z range 50−1300 with the use of an external calibration standard (Tunemix solution), supplied by Agilent.

NPs were synthetized using a monomer starved semibatch emulsion polymerization (MSSEP) of the produced macromonomers. In this process the hydrophilic monomer (HEMA-PEG_{19}) is loaded in the reactor together with 45 mL of deionized water, the solution is the heated up to 80 °C and purged using nitrogen/vacuum cycles. The hydrophobic monomer (HEMA-PCL_n or HEMA-PLA_m), produced via ROP in the previous step, was then injected in the reactor with a syringe pump (Model NE-300, New Era Pump System, US) at a feeding rate of 2 mL/h; KPS (0.02 g, dissolved in 2.5 mL of deionized water) was used as a water-soluble initiator and added with syringe at the beginning of the lipophilic monomer injection. For all the reaction, 2.1 g of the proper macromonomers were used together with 0.4 g of HEMA-PEG_{19}; the final solid content for the produced latexes is 5%. It is important to note that among of all the produced macromonomers only HEMA-PCL_5 and HEMA-PLA_8 are solid at room temperature and therefore were dissolved in 1 mL of EtOH before injection; in this case the feeding rate has been changed accordingly to ensure a constant monomer feeding time;[26] for all the other materials this step was unnecessary due to the fact that they are liquid at room temperature and therefore could be injected without any solvent. In all the cases, the reaction was carried out for 3 h.

After the synthesis, NPs are characterized in terms of average diameter and particle size distribution by dynamic light scattering (Malvern, Zetanano ZS) using the cumulant method as defined by ISO (standard document 13321:1996E); all the reported data are an average between two measures of the same sample.

Degradation Studies. The produced NPs have been employed in further studies concerning degradation in the following buffers: cell medium, gastric, intestinal solution, and PBS (10 mM, pH 7.4). A 1 mL portion of diluted NP suspension (with a polymer/water ratio of 0.1% w/w) was dissolved in 1 mL of the selected medium and maintained in an heating block at 37.0 ± 0.1 °C. The evolution of size particle and particle size distribution of the samples was studied through dynamic light scattering (DLS) measurements. Simulated gastrointestinal fluids were prepared according to U.S. Pharmacopeia XXIV, 2006 (gastric juice is composed of 300 mM of sodium chloride and 840 mM of hydrochloric acid in water for a final pH value of 1.2 while intestinal solution is 800 mM of sodium hydrogen carbonate with a pH of 8.1);[29] cell medium and PBS were used as received as already specified. No aggregates were detected during the degradation both visually and by DLS measurements.

The morphology study of the degrading NPs in cell medium were carried out by transmission electron microscopy (TEM) using a Zeiss EFTEM Leo 912AB transmission electron microscope working at 80 kV. Samples were prepared placing a 5 μL drop of NPs colloidal suspension on a Formvar/carbon-coated copper grid and dried overnight. Digital images were acquired by a charge coupled device (CCD) Esi Vision Proscan camera.

RESULTS AND DISCUSSION

Macromonomer Characterization. Macromonomers were characterized by ^{1}H NMR analysis in order to evaluate the average chain length by following an already established procedure.[21,22] As shown in Table 1, the chain lengths of the produced macromonomers are in good agreement with their corresponding target values. Detailed ^{1}H NMR and ESI spectra are provided in the Supporting Information section.

Table 1. ^{1}H-NMR Analysis of the Produced Macromonomers

	target		^{1}H NMR	
macromonomer	n	M_n [Da]	n	M_n [Da]
HEMA-PCL_2	2	358	2.4	404
HEMA-PCL_3	3	473	3.3	507
HEMA-PCL_5	5	701	4.9	689
HEMA-PLA_4	4	418	3.8	404
HEMA-PLA_6	6	562	5.6	533
HEMA-PLA_8	8	706	8.3	727

Table 2. Size and PDI of the Produced NPs

material	particle size [nm]	PDI [−]
Poly(HEMA-g-PCL_2-g-PEG_{19})	135	0.101
Poly(HEMA-g-PCL_3-g-PEG_{19})	124	0.098
Poly(HEMA-g-PCL_5-g-PEG_{19})	112	0.085
Poly(HEMA-g-PLA_4-g-PEG_{19})	158	0.184
Poly(HEMA-g-PLA_6-g-PEG_{19})	110	0.108
Poly(HEMA-g-PLA_8-g-PEG_{19})	98	0.128

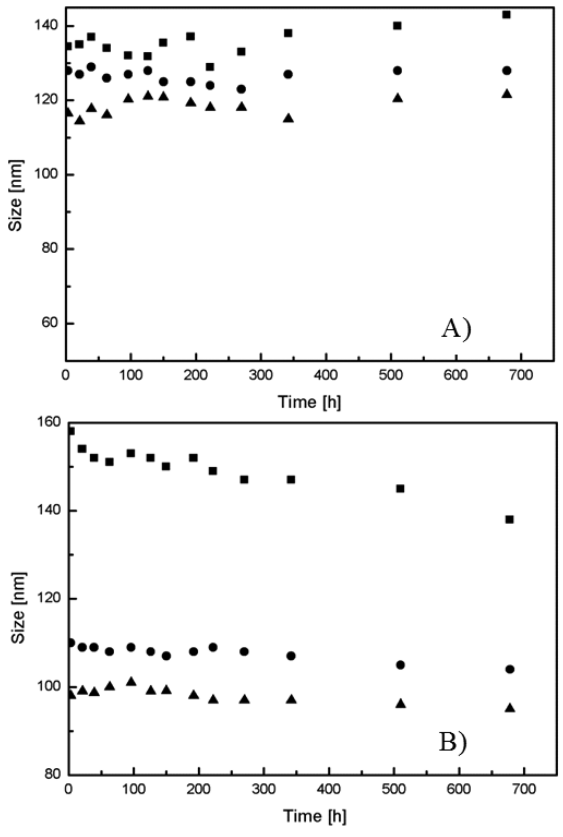


Figure 1. Evolution of NPs diameter as a function of time in time in PBS: For PCL-based materials (panel A): (■) poly(HEMA-g-PCL_2-g-PEG_{19}), (●) poly(HEMA-g-PCL_3-g-PEG_{19}), and (▲) poly(HEMA-g-PCL_5-g-PEG_{19}). For PLA-based materials (panel B): (■) poly-(HEMA-g-PLA_4-g-PEG_{19}), (●) poly(HEMA-g-PLA_6-g-PEG_{19}), and (▲) poly(HEMA-g-PLA_8-g-PEG_{19}).

NP Characterization. The products of the ROP processes consist of polyester chains functionalized with vinyl end group preserved from the HEMA cocatalyst, thus making the macromonomers suitable to free radical polymerization. NPs

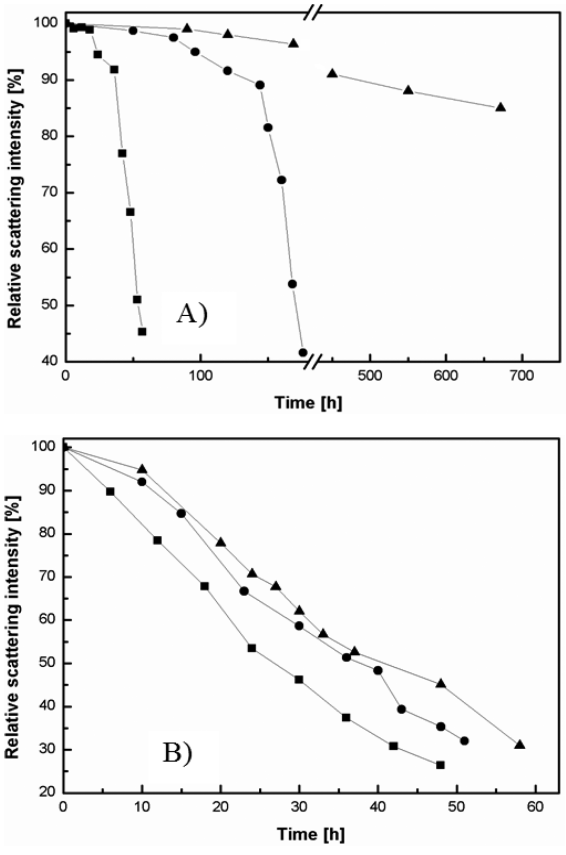


Figure 2. Evolution of the scattering intensity (expressed as a percentage of the initial value) in cell medium. For PCL-based materials (panel A): (■) poly(HEMA-g-PCL_2-g-PEG_{19}), (●) poly(HEMA-g-PCL_3-g-PEG_{19}), and (▲) poly(HEMA-g-PCL_5-g-PEG_{19}). For PLA-based materials (panel B): (■) poly(HEMA-g-PLA_4-g-PEG_{19}), (●) poly(HEMA-g-PLA_6-g-PEG_{19}), and (▲) poly(HEMA-g-PLA_8-g-PEG_{19}).

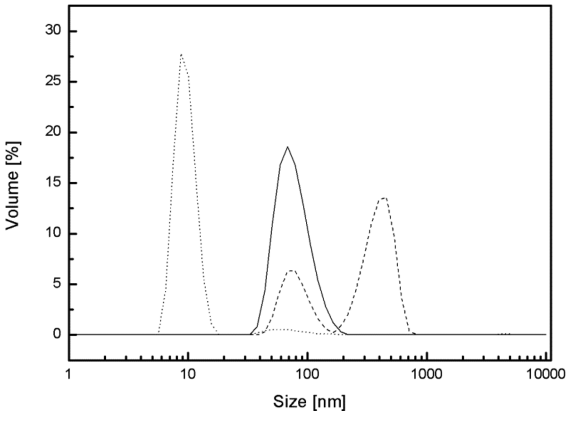


Figure 3. Evolution of PSD for poly(HEMA-g-PLA_6-g-PEG_{19}) during degradation in cell medium at 37 °C. Solid line represents the beginning of the test (0 h), dashed line is after 36 h, and dotted line after 53 h.

were synthetized as described in the Experimental Section, and their size and polydispersity index (PDI) were determined by light scattering measurements and are reported in Table 2.

It is possible to observe that the adopted synthetic route allows to obtain monodispersed latexes. The NP diameter is related to the molecular weight of the hydrophobic macro-

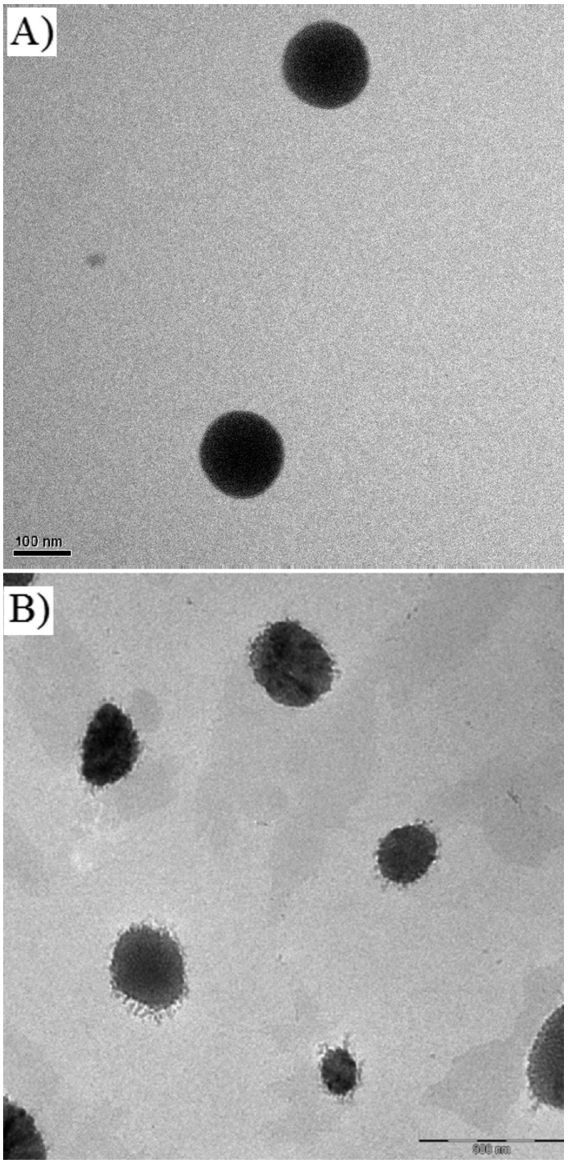


Figure 4. TEM picture of the degrading poly(HEMA-g-PLA_6-g-PEG_{19}): (panel A) 0 and (panel B) 36 h of degradation.

monomer where for both LA- and CL-based NPs, an increase in polyester chain length results in a decrease in NPs size, in agreement with previously reported results.[26]

NP Stability and Degradation. Degradation studies in different fluids were carried out at 37 °C to evaluate the colloidal and chemical stability of the NPs in biological media of interest and to make preliminary predictions about their in vivo behavior. The study of NP behavior in isotonic PBS is relevant since it is the medium in which they are stocked, and it is also used for their intravenous injection. The particle size evolution of different NP formulations in PBS is presented in Figure 1.

Upon inspection of Figure 1 it is clear that after one month there is no significant change in NP size for both PCL and PLA-based NPs confirming the effectiveness of the attached PEG chains to both sterically stabilize the NPs in isotonic environment and to prevent their aggregation.

Cell medium is used to grow cells which are then employed for in vitro experiments such as for example the determination of the cytotoxicity and cellular uptake of the produced NPs;

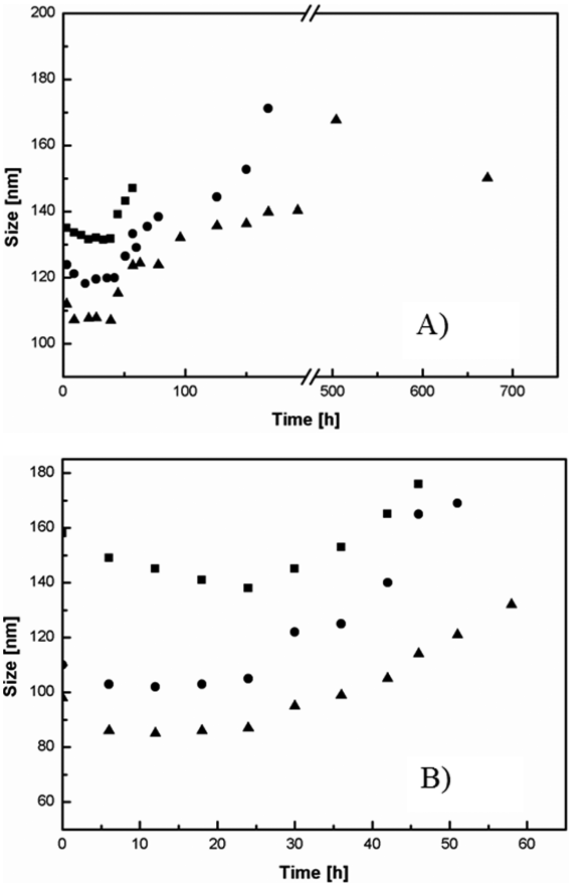


Figure 5. Evolution of the diameter for PCL-based NPs and PLA-based NPs in cell medium. For PCL-based materials (panel A): (■) poly(HEMA-g-PCL_2-g-PEG_{19}), (●) poly(HEMA-g-PCL_3-g-PEG_{19}), and (▲) poly(HEMA-g-PCL_5-g-PEG_{19}). For PLA-based materials (panel B): (■) poly(HEMA-g-PLA_4-g-PEG_{19}), (●) poly(HEMA-g-PLA_6-g-PEG_{19}), and (▲) poly(HEMA-g-PLA_8-g-PEG_{19}).

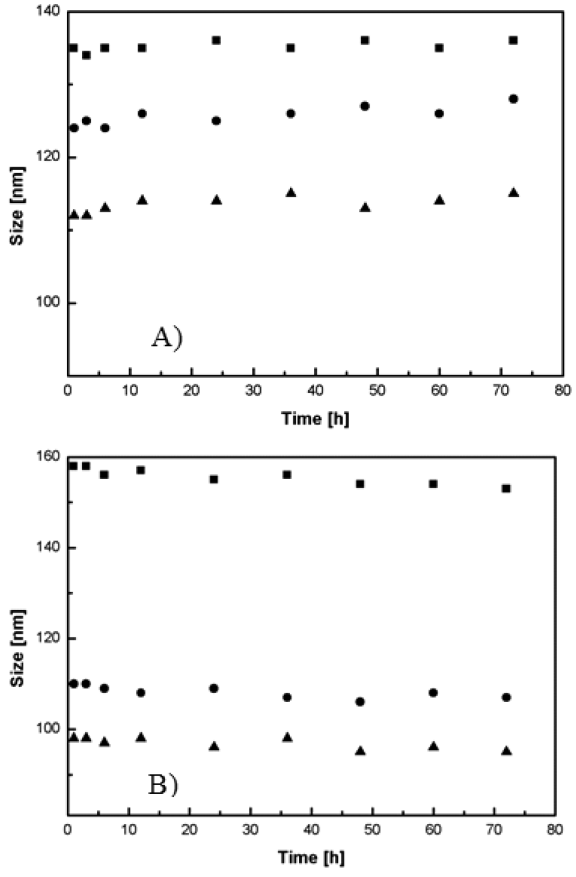


Figure 6. Evolution of NP diameter in gastric solution: (panel A) for PCL-based NPs; (panel B) for PLA-based ones. For PCL based materials: (■) poly(HEMA-g-PCL_2-g-PEG_{19}), (●) poly(HEMA-g-PCL_3-g-PEG_{19}), and (▲) poly(HEMA-g-PCL_5-g-PEG_{19}). For PLA based materials: (■) poly(HEMA-g-PLA_4-g-PEG_{19}), (●) poly-(HEMA-g-PLA_6-g-PEG_{19}), and (▲) poly(HEMA-g-PLA_8-g-PEG_{19}).

therefore is a relevant medium in which the behavior of the NPs has to be studied.[26] It also contains proteins such as albumin, which is also present in the blood, and it is therefore a good choice to test how the NPs would behave in a protein rich environment, i.e. after intravenous injection. NPs have been primary monitored by studying the evolution of the relative scattering light intensity, a technique used in protein containing media, which is related to the NPs concentration.[30,31] The results of this analysis are reported in Figure 2.

From Figure 2, a remarkable lowering of the scattering intensity is observed for the different NPs, revealing their degradation in cell medium. This process occurs through the hydrolysis of ester bonds of the PLA or PCL chains and subsequent release of acidic species, leaving the water-soluble poly(HEMA-co-HEMA-g-PEG_{19}) as a secondary product. Also, the proteins in the medium act as amphiphilic molecules increasing the solubility of the oligomers released from the degrading NPs in the water phase. In this way the mass transfer of oligomeric species from the NPs to the bulk phase is increased, thus increasing the overall degradation rate. The absence of free PEG chains or ethylene glycol in the media has been proved,[25] confirming the absence of hydrolysis in the poly(HEMA) backbone.

By looking at panel A of Figure 2, where the results for PCL-based NPs are reported, it is immediately possible to see how the degradation time of these NPs is a function of the polyester chain length since it greatly increases by increasing the average chain length of the side chains. For poly(HEMA-g-PCL_2-g-PEG_{19}) NPs, a significant decrease in the scattering light intensity is reached after about 50 h. By increasing the macromonomer chain length by one unit, the resulting NPs, poly(HEMA-g-PCL_3-g-PEG_{19}), achieve the same result after one week. Poly(HEMA-g-PCL_5-g-PEG_{19}) NPs do not show significant decrease in the scattering light intensity up to one month. Therefore it is possible to conclude that for PCL-based NPs the macromonomer chain length is the main parameter that directly influences the degradation of the NPs and a wide range of degradation rates can be obtained for these NPs. The reliability of the data obtained in this experiments have been confirmed also in in vitro condition, where poly(HEMA-g-PCL_3-g-PEG_{19}) NPs internalized in cells were no longer detected after 6 days.[28] For PLA-based materials (panel B of Figure 2), it is immediately possible to see that the characteristic time for the degradation is shorter than that of PCL-based materials; also the influence of the macromonomer chain length is less pronounced since the range of degradation rate is narrower. Nevertheless chain length is still the parameter that govern the behavior since poly(HEMA-g-PLA_4-g-PEG_{19}) NPs are the fastest degrading material among the selected ones.

NPs synthesized using these novel macromonomers show a peculiar degradation behavior since the process is controlled by

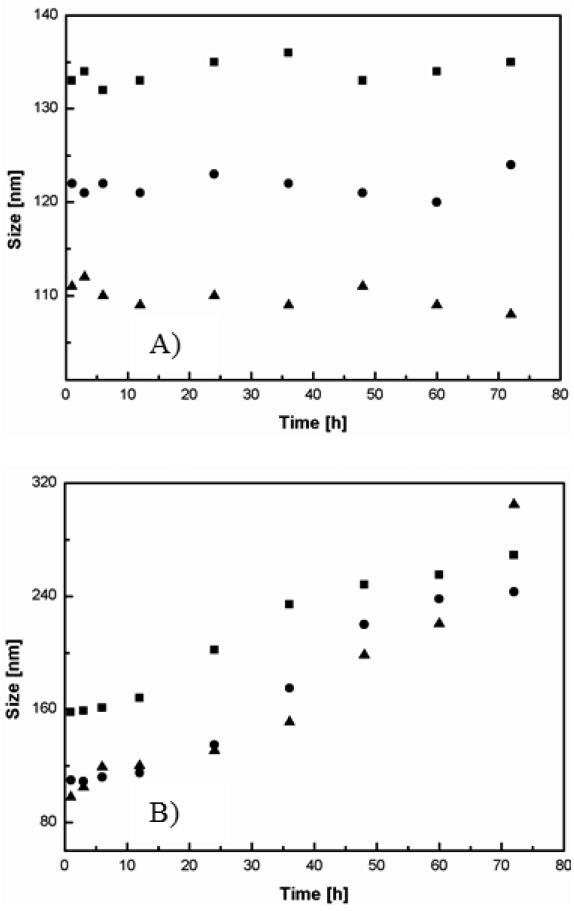


Figure 7. Evolution of NPs diameter in intestinal solution: panel A for PCL-based NPs while panel B for PLA-based ones: (panel A) (■) poly(HEMA-g-PCL_2-g-PEG_{19}), (●) poly(HEMA-g-PCL_3-g-PEG_{19}), and (▲) poly(HEMA-g-PCL_5-g-PEG_{19}); (panel B) (■) poly-(HEMA-g-PLA_4-g-PEG_{19}), (●) poly(HEMA-g-PLA_6-g-PEG_{19}), and (▲) poly(HEMA-g-PLA_8-g-PEG_{19}).

the composition and length of the side chains and not by the molecular weight of the whole polymer. In fact, since the degradation rate is strictly dependent upon water concentration in the particle, the NPs lipophilicity plays a relevant role in the overall process. The more hydrophilic the NP (i.e., PLA-based with shorter average chain length), the faster the degradation rate. As a result these NPs show a short degradation time compared to polyester-based NPs obtained via physical methods such as nanoprecipitation. It is relevant to point out that the duration of the process is similar to that of polymer micelles and comparable with the residence time of these NPs in the body.[31,32] This feature allows the mediation of the release of drug loaded into NPs with their degradation.

Through DLS it is also possible to study the evolution of the particle size distribution (PSD) in time as well as their average diameter. In Figure 3 are reported the PSDs for poly(HEMA-g-PLA_6-g-PEG_{19}) NPs.

At the beginning of the experiment only a single peak is observed (straight line in Figure 3), which reflects the monodispersity of the NPs. As the degradation progresses, the polyester chains are hydrolyzed and therefore the NPs become more and more hydrophilic and are swollen by water as this process continues. This is reflected by the bimodality of the particle size distribution represented by the dashed lined after 36 h. The presence of the bimodality is due to the fact the process is not homogeneous, as highlighted also by TEM picture of the degrading NPs, reported in Figure 4.

In Figure 4, it is possible to observe that due to the hydrolysis of the polyester side chains NPs spherical shape is lost. After 53 h of degradation, the majority of the PSD is related to the peak centered at 11 nm (dot line of Figure 3) which can be ascribed to the hydrophilic poly(HEMA-co-HEMA-g-PEG_{19}), organized into micelles. The degradation behavior is reflected in the evolution of the average diameter of the degrading NPs reported in Figure 5.

Figure 5 shows the evolution of the average diameter of the NPs in cell medium during degradation. A common trait for all the produced materials is a slight shrinking during the first hours, which is due to the change of the environment in which the NPs are dispersed and the subsequent spatial rearrangement of the solvated PEG chains. As the degradation occurs, NP behavior is the same as the one observed in Figure 3. In particular the NPs start swelling, due to the loss of CL and LA oligomers, leading to an increase in NP hydrophilicity and prompts further NPs swelling by water. This process continues until NP disappearance leaving poly(HEMA-co-HEMA-g-PEG_{19}) chains as the final water-soluble product of the process.

Gastric and intestinal solution were used to evaluate the stability of the particle concerning their possible use as oral delivery carriers and to study their behavior in different pH values, the evolution of the NPs characteristics was followed closely for 72 h in order to mimic the maximum persistence in the gastrointestinal tract after oral administration.[29] To create the different chemical environments of the gastrointestinal tract (i.e., stomach and intestine) fluids usually employed to evaluate the stability of drug formulations were prepared as described in the Experimental Section. The results obtained through DLS analysis for the NP behavior in gastric and intestinal solutions are reported in Figures 6 and 7, respectively.

Concerning gastric environment, it can be seen in Figure 6 that both PLA and PCL-based materials exhibit no significant changes in the diameter for the duration of the measurements; therefore showing good chemical stability in acidic conditions. A different behavior is detectable in intestinal solution (Figure 7), where it can be seen that PCL-based NPs remain stable, like in the gastric solution, while the more hydrophilic PLA-based NPs swell consistently during time due to the basic environment, in agreement with literature results showing faster degradation at higher pH values;[33] however the monodispersity of these samples ensures the absence of any aggregation process. The gastric resistance and swelling behavior in intestinal environment could make these materials a good candidate for gastro-resistant oral delivery.

■ CONCLUSION

In this work ring-opening polymerization was employed to produce functionalized polyester macromonomers suitable for further polymerization in a MSSEP process to obtain PEGylated NPs with tunable degradation time. The final NPs have a comblike structure composed by a poly(HEMA) backbone grafted with chains of either PCL or PLA of different length and PEG. The stability of the NPs in isotonic condition have been followed in PBS for a month and no particle degradation nor aggregation were detected, thus confirming their long-term stability in isotonic environment. The degradation behavior of the NPs was followed in cell medium, gastric, and intestinal solution in order to obtain data in media of biological interest. Degradation occurs in cell medium and

the main parameter that govern the process, other than the type of polyester, is the chain length of the starting macromonomer for both PLA-based and PCL-based materials. Therefore a wide range of degradation times can be obtained, which could lead to the possibility of controlling the drug release with degradation itself. All the produced NPs show a good stability in gastric solution at low pH while in intestinal solution the basic pH induces a swelling for the more hydrophilic PLA-based materials.

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Notes

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