Synthesis And Characterization of Thermo-Responsive Particles of Poly(Hydroxybutirate-Co-Hydroxyvalerate)-B-Poly(N-Isopropylacrylamide)

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A new kind of thermo-responsive particles were prepared by the self-assembly technique, comprising poly(hydroxyvalerate-co-hydroxybutirate)-b-poly(N-isopropylacrylamide)/ (PHBHV-b-PNIPAAm) block copolymers. The hydrophilic part PNIPAAm was synthesized by Reversible Addition- Fragmentation chain Transfer (RAFT) polymerization. Particles with core-shell morphology were obtained with hydrophobic outer shells and hydrophilic inner cores. Dexametasone acetate (DexAc) was used as a model drug with an encapsulation efficiency of 77%. The release of DexAc in aqueous solution was strongly dependent on temperature, suggesting that PHBHV-b-PNIPAAm particles can be used as a thermo-responsive carrier material with external control in a drug release system.

Keywords: thermo-responsive particles, dexametasone acetate, drug release, block copolymer, RAFT

1. INTRODUCTION

Stimuli-responsive polymers are defined as polymers that undergo relatively large and abrupt physical or chemical changes in response to small external changes in the environmental conditions. There are many different stimuli to modulate the response of polymer systems. These stimuli can be classified as either physico-chemical or biological [1]. Ionic strength, pH and chemical agents can be considered as chemical stimuli, whereas temperature, electric or magnetic fields and mechanical forces are pointed out as physical stimuli [2]. Temperature and pH are the most widely used stimuli in environmental responsive polymer systems. The change of temperature is not only relatively easy to control, but also easily applicable for in vitro and in vivo drug delivery experiments [3].

Thermo-sensitive polymers, such as PNIPAAm and their copolymers, have been well-exploited in drug delivery systems [4]. PNIPAAm is a water-soluble polymer exhibiting an extended chain conformation below the Lower Critical Solution Temperature (LCST) of 32 °C, and coalescing above this temperature forming hydrophobic and insoluble aggregates [5, 6].

Polyhydroxybuturate (PHB), a biodegradable polymer, and its copolymers can be used as raw materials for many applications due to their physical-chemical and biocompatible characteristics [7].

Previous works have shown the utilization of PHB or its copolymers in drug delivery systems [7, 8].

Recently, self-assembly technique have provided an efficient and rapid pathway for the synthesis of objects in the nanometers to micrometers range, which are difficult or even impossible, to be obtained by conventional chemical reactions. A recent approach looks into the possibility of reducing tedious and time consuming synthetic steps by engineering oligomers or polymers of relatively small size that can self-organize by only physical forces, simulating the folding of peptides segments in proteins [9, 10]. Among the various aggregation processes, the most extensively studied concerns the self-assembly of block or graft copolymers. Amphiphilic block copolymers have been used extensively due to the properties of the self-assembling micelles formed in the presence of a suitable solvent. In an aqueous environment, the hydrophobic parts of the block copolymers form the core of the micelle, while the hydrophilic ones form the corona or outer shell. The hydrophobic micelle core acts as a microenvironment for the incorporation of lipophilic drugs, while the corona shell works as a stabilizing interface between the hydrophobic core and the external medium [11-14].

Block copolymers consisting of a PNIPAAm segment and a hydrophobic segment can form core-shell micelle structure below the LCST. This polymeric micelle structure comprises a hydrophilic outer shell of hydrated PNIPAAm segments and a hydrophobic inner core. The inner core can be loaded with hydrophobic drugs, while PNIPAAm outer shell plays the role of aqueous solubilization and temperature responsiveness. The hydrophilic outer shell, which prevents the inner core from interacting with biocomponents and other micelles, can suddenly become hydrophobic in specific conditions depending on the temperature [13,15-20].

In this work a block copolymer was synthesized by coupling reaction between PHBHV and PNIPAAm using N,N'-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) as coupling agents. The block copolymer was characterized by 1H NMR and Gel Permeation Chromatography (GPC). The thermo-responsive nanoparticles were prepared by dialysis method and analyzed by light scattering (LS) and transmission electron microscopy (TEM). The thermo-responsive behavior of nanoparticles was studied by turbidimetry and LS. Additionally, nanoparticles
were loaded with Dexametason acetate (DexAc) and the drug release behavior was determined *in vitro* in order to evaluate its application in drug delivery systems.

2. EXPERIMENTAL

2.1. Materials

N-isopropylacrilamide (NIPAAm, 97% from Aldrich) was used after purification through recrystallization in a mixture of 60:40 toluene/hexane. 2,2’-Azobisisobutyronitrile (AIBN) was purified by recrystallization in absolute ethanol. The RAFT agent (2-((2-Phenyl-1-thioxoethyl)thio)propanoic acid (PTET PA) was synthesized according to the methodology described in the literature [21]. N,N-dicyclohexyl carbodi-imide – DCC (Aldrich, 99%) and 4-dimethyl aminopyridine - DMAP (Aldrich, 99%) were used as received. The solvents chloroform (Anidrol, 99%), tetrahydrofuran (THF) (Vetec, 99%), diethyl ether (Anidrol, 99%), methanol (Anidrol, 99%), Dimethylformamide (DFM) (Vetec, 99%) and 1,4-dioxane (Anidrol, 99%) were used as received. Dexametason acetate (DexAc), was kindly furnished by Henrifarm. and utilized without further purification. The PHBHV used in this work was supplied by PHB Industrial S.A. After the reduction of the molecular weight by means of a methodology described in the literature [22], the resulted PHBHV was characterized by 

2.2. Synthesis and characterization of PNIPAAm

The NIPAAm polymerization was performed in a glass reactor capped with a septum. The reaction vessel was loaded with 1,4-dioxane, NIPAAm (2 M), the RAFT agent (2-((2-Phenyl-1-thioxoethyl)thio)propanoic acid (PTETPA) (5.0 mmol), and AIBN in a 4:1 [RAFT]/[AIBN] molar ratio. The mixture was purged with nitrogen for 20 min at 4°C. The temperature was then raised to 70°C using a thermostated water bath. Polymerization was carried out under a nitrogen atmosphere for 5 hours. PNIPAAm was precipitated out with diethyl ether and recovered by filtration. PNIPAAm was characterized by 1H NMR (Varian, 300MHz) using chloroform as solvent. For this polymer the GPC analysis was performed using a 0.25% (w/v) solution of tetrabutylammonium bromide in tetrahydrofuran (HPLC grade) as eluent, at the same concentration of flow rate and temperature, as was used in the analysis of PHBHV. The results for PNIPAAm were: $M_n = 35282$ g/mol $MWD = 1.36$. 

2.3. Synthesis and characterization of PHBHV-b-PNIPAAm

The block copolymer PHBHV-b-PNIPAAm, was obtained reacting hydroxyl–caped PHBHV with carboxyl-caped PNI-PAAm (Scheme 1), as follows: PHBHV (2.3 $\times 10^{-4}$ mol) and DMAP (1 $\times 10^{-3}$ mol) were dissolved in 50 mL of chloroform under nitrogen and agitation, at room temperature. After 4 hours, PHBHV (6.0 $\times 10^{-6}$ mol) and DCC (9.2 $\times 10^{-4}$ mol) were dissolved in 50 mL of chloroform and added to the reaction medium. This reaction was carried out during 7 days at room temperature. After this time the block copolymer was precipitated in an excess of cold methanol. The block copolymer was characterized by 1H NMR and by GPC using a Viscotek, model 302 Triple Detector. A 0.02 M LiBr solution in N,N’-Dimethylformamide (DMF) was used as eluent. The analysis was performed with a flow rate of 1 mL/min, at 60°C.

2.4. Preparation and characterization of nanoparticles

PHBHV-b-PNIPAAm block copolymer was dissolved in DMF and dialyzed in water at room temperature using dialysis bag with a cut-off of 6,000-8,000 Da, for 24 hours.

2.4.1. Particles size

The particles size and particles size distribution were determined by 1000, in aqueous medium.

2.4.2. Particles morphology

A drop of the particle suspension was placed on a copper grid and dried before analysis by TEM, in a Philips CM120 transmission electron microscope, at an acceleration voltage of 80 kV (Centre Technologique des Microstructures (CT), Claude Bernard University, Lyon, France).
2.4.3. Optical characterization of particles aqueous suspension

Optical characterization of PHBHV-b-PNIPAAm particles in aqueous suspension was carried out using a Turbiscan Lab (Formulation, France) equipment. The particle suspensions were transferred to a glass cylindrical cell and analyzed by a light beam, emitted in near infrared light source (880 nm), which scanned the sample cell from bottom to top. Two synchronous optical sensors, transmission (T) and backscattering (BS), received the light that goes across the sample and that scattered backward, respectively, by the sample. The samples in the cell were scanned at two different conditions, heating from 25 to 60°C and cooling from 60 to 25°C, for 24 hours. The change in transmittance as a function of time was taken as a measure in order to evaluate the thermo-responsive properties of nanoparticles.

2.5. Drug loading

PHBHV-b-PNIPAAm block copolymer (11.8 mg) and DexAc (10.3 mg) were dissolved in 1 mL of DMF and added, drop by drop, in 40mL of water and dialyzed against water at room temperature. After dialysis, the DexAc-loaded particles were separated and frozen by a freeze dryer system (Liobras, L101). The encapsulation efficiency was calculated using the following equation:

$$\% E = \frac{A_{CN}}{A_{CI}} \times 100$$

Where $A_{CN}$ is the mass of drug in nanoparticles after dialysis and $A_{CI}$ is the initial mass of drug.

2.6. Drug delivery assay

The release experiments of DexAc loaded particles were conducted in a buffer solution (pH 7.4) at temperatures of 4 and 40°C. PHBHV-b-PNIPAAm particles loaded with DexAc (1 mg) were placed in a dialysis bag (cut-off 6,000-8,000 Da) and immersed in 200 mL of the buffer solution. Samples were withdrawn for analyses of DexAc in the external buffer solution using a functionalized chain transfer agent (PTETPA). This reaction was successfully conducted due to the use of DCC and DMAP as coupling agents. Figure 1 shows the 1H NMR spectra of PHBHV-b-PNIPAAm. The analysis of this spectrum allows identifying all signals of both segments, PHBHV and PNIPAAm, confirming the formation of the block copolymer.

These images were used to qualitatively verify the presence of particles of different sizes. This qualitative analysis corroborates the results obtained by LS, where an average particle diameter of 511 nm and a high polydispersity index (PI = 0.3) were observed. Additional and very important information from the TEM analyses is the particles morphology. An accurate analysis of Figure 3 shows the presence of particles with a core-shell morphology, more precisely, a PNIPAAm low contrast (green in the picture) outer shell and a dark inner core constituted of PHBHV segments. This finding suggests that PNIPAAm has stabilized the nanoparticles in the aqueous medium and turned these particles thermo-responsive.

To evaluate the instability of the dispersions, we used the transmission profiles produced by Turbiscan Lab equipment. The profiles are related to particle migration (creaming and sedimentation) and to the increase in particle size (coalescence and flocculation). Figure 4 shows the variation in the transmittance (AT) of an aqueous particle dispersion, as a function of a heating process (from 25°C to 60°C) for 1 day, whereas Figure 5 shows the cooling process of the same sample (from 60°C to 25°C).

Figure 4 shows that the transmittance decreases by 36% with the increase of the temperature. When heating the suspension, the PNIPAAm segments precipitated onto the par-
In contrast, when the sample was cooled from 60°C to 25°C (Figure 5), the transmittance increased around 36%. It has occurred due to the increase of solubility of PNIPAAm segments with the decrease of temperature. Important information is that the particle size ($D_p$) remains unchanged with the heating/cooling cycle, indicating that the nanoparticles coalescence is reversible. By heating the particles suspension, the solubility of PNIPAAm segments in water decreases, allowing the agglomeration of nanoparticles and, when the suspension is cooled again, the hydrophilic segment returns to the original conformation (extended chains). This is a very specific property of nanoparticles obtained by means of self-assembly of amphiphilic block copolymers. This mechanism of agglomeration/deagglomeration allows a very subtle and sophisticated control of the mechanism of drug delivery from nanoparticles. This phenomenon has been usually characterized by means of fluorescence technique [13, 17]. At the best of our knowledge, the utilization of turbidimetry for such purpose has never been reported before in the literature.

A model drug (dexametazone acetate) was encapsulated by self-assembly technique to study the thermo-responsive property of nanoparticles in drug delivery system. This encapsulation was realized by diffusion of solvent (dialysis) [18]. In this case, the drug was mainly located in the core of nanoparticles due to the physical and chemical affinity between the drug (hydrophobic) and the also hydrophobic PHBHV segments of the block copolymers. The efficiency of the encapsulation was of 77%. The assays of in-vitro drug delivery were realized in phosphate buffer ($pH = 7.4$) at two different temperatures, 4°C and 40°C, respectively, below and above the LCST of the PNIPAAm (32°C). For comparison, a drug delivery assay was realized at 40°C with a sample of free DexAc, i.e., with no encapsulation of the drug by the block copolymer. Figure 6 shows the release kinetics of DexAc from the nanoparticles as a function of the temperature. The DexAc concentration released was determined as previously described in 2.6, using UV/vis spectrometry. As it can be seen, the drug release profile shows drastic changes with temperature alterations, below and above the LCST. In fact, the PNIPAAm segments are located on the shell of the PHBHV core of the nanoparticles and the thermal sensitivity of the PHBHV-PNIPAAm nanoparticles can control the reversible structural changes of the nanoparticles by temperature cycles through the LCST of PNIPAAm. At 4°C, the highly hydrated PNIPAAm shell stabilizes the drug loaded in the nanoparticle core, allowing a slower diffusion rate. At 40°C, the PNIPAAm segments collapsed onto the particles surface, facilitating the DexAc diffusion through the outer shell as illustrated in Figure 7.

The oscillating drug release that can be obtained from the PHBHV-PNIPAAm nanoparticles developed in this work is shown in Figure 8, where a fast/slow thermo-responsive switching is controlled by the temperature cycles through the LCST.

4. CONCLUSION

Core-shell nanoparticles have been prepared using a biodegradable block of PHBHV (hydrophobic segment) and PNIPAAm, a thermo-responsive polymer (hydrophilic segment), that was synthesized by RAFT polymerization. Self-assembly of PHBHV-b-PNIPAAm in water enabled to encapsulate DexAc, a hydrophobic drug, in order to protect...
FIG. 6: Drug release from thermo-responsive PHBHV-b-PNIPAAm nanoparticles containing dexametazone acetate. Effect of the temperature above and below the LCST.

FIG. 7: Schematic illustration of the release mechanism of DexAc from thermo-responsive PHBHV-PNIPAAm nanoparticles. When the temperature is above the LCST of the PNIPAAm, the expanded form of the PNIPAAm in the shell part is changed into the compact one and the drug is ejected.

FIG. 8: DexAc released from thermo-responsive and partially biodegradable nanoparticles in response to temperature switching between 4 and 40°C.

system for site-specific drug release induced by local temperature changes.

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