Polymer Chemistry

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ARTICLE

Triglycerol-based hyperbranched polyesters with an amphiphilic branched shell as novel biodegradable drug delivery systems.

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The synthesis of biodegradable triglycerol-based hyperbranched polyesters (HBPEs), characterized by different hydrophobicity, has been optimized and described. A new amphiphilic branched shell (ABS) was developed; PEGylated amphiphilic chains were attached to the external corona of the HBPEs, with the aim of enhancing the encapsulation efficiency of hydrophobic drugs while additionally encouraging the solubilization of the HBPEs in aqueous media. Pyrene was tested as a template to evaluate potential drug transport capacity and to obtain information about the microenvironment and binding sites of the drug carriers. Experimental tests have displayed the excellent capabilities of the aforementioned systems as drug delivery systems (DDS); it was possible to load up to 4.1 wt-% of pyrene, evenly released from the system, within 9 days in the presence of *Candida Antarctica* lipase B (CALB). Subsequently the anticancer drug Doxorubicin and the anti-inflammatory steroidal drug Dexamethasone were efficiently encapsulated in the ABS-nanocarriers.

Introduction

Due to the poor solubility and short lifetime of most clinically used drugs, new drug delivery concepts that rely on the use of polymeric drug delivery systems have been developed. These are of crucial importance for maximizing bioavailability and for targeting the active molecules at the desired location in the body, whilst reducing side effects.[1-3] The most prominent examples for this type of nanocarrier systems are polymeric micelles and liposomes commonly formed after linear amphiphilic polymers aggregate. However, a drawback of such systems is the low stability under harsh conditions *i.e.* dilution, temperature *etc.*, that might lead to a rapid disassembly of the structure, accompanied by an instantaneous release of the encapsulated drug.[4] Dendrimers and hyperbranched polymers can overcome this problem. The high intrinsic stability and solubility, low viscosity, and their high number of functional groups - allowing for various modifications - make these systems interesting candidates for drug delivery applications.[5-6] One of the most promising classes of compounds are based on hyperbranched or dendritic polyglycerol (dPG) that can be synthesized by a simple ring-opening multi-branching (ROMB) polymerization of glycidol.[7-9] The hydrophobicity and/or hydrophilicity of dPGs can be tailored by the post-synthetic chemical modification of external hydroxyl groups. For years dPGs has been successfully exploited as an efficient nanocarrier platform for drug/dye conjugation and encapsulation.[10-12]

A series of nanocarriers based on dPG or dPEI,[13] characterized by a core-multishell architecture inspired by the polarity gradient of liposomes, ranging from a polar interior with a hydrophobic lipid inner-shell to a polar exterior, had previously been described. By attaching α–ω octadecane dicarboxylic acid comprising of water-soluble and biocompatible mPEG on one end to the polymeric core, highly versatile core-multishell systems (CMS) able to efficiently encapsulate polar and unpolar dyes and drugs were able to be developed. By employing these systems it was possible to significantly increase the solubilization of hydrophobic molecules such as doxorubicin (DOX), dexamethasone (DXM), pyrene and Nile red.[14]

Hyperbranched polymers functionalized with an amphiphilic branched shell have been previously described and employed as DDSs. Chen *et. al.* reported the synthesis of amphiphilic biodegradable hyperbranched copolymers, using PEI as backbone, functionalized with a cationic (PEG - poly-γ-benzyl L-glutamate)[15]and a non-cationic (PEG – poly-ε-benzyloxycarbonyl-L-lysine)[16] branched shell, and studied their behavior in solution and their potential medical application in

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drug and gene delivery. More recently Liu *et. al*., reported the synthesis of pH-responsive core-branched shell nanocarriers employing PEI as core backbone to were it was attached a poly(L-glutamic)[17] acid or poly(L-lysine)[18] inner shell and a PEG outer shell. These novel core-shell systems were used to efficiently transport and release drugs as doxorubicin and protein as insulin. Polymeric carrier systems that are not biodegradable may accumulate in different organs causing long-term toxic effects.[19] Biodegradability thus became a crucial parameter for polymeric as well as unimolecular micelles used in drug delivery applications.

Despite the high biocompatibility displayed by dPGs on in vitro and in vivo tests,[20] the polymeric backbone is unfortunately not biodegradable and due to this fact its usage is restricted to certain (lesser) molecular weights.

With the aim of obtaining hyperbranched polymers having similar characteristics to dPG but following the biodegradation criteria, and in order to tailor the polarity of the polymers, three novel hyperbranched triglycerol-based polyesters, characterized by different hydrophobicity, have been developed and described in this paper as such (Scheme 1). Using alkaline-catalysis esterification, the hydroxyl groups of the triglycerol moieties reacted with a α–ω dicarboxylic acid such as succinic acid (SA), adipic acid (AA) or dodecane dicarboxylic acid (DDCA). It was possible to obtain a hydrophilic, a partially hydrophilic and a lipophilic hyperbranched polyester respectively. The high number of free hydroxyl groups in the polymeric backbone is a key aspect of further functionalization.

Seen the improvement in loading capacity brought by the presence of the multishell attached to a polymeric core, in this paper, a novel amphiphilic branched shell is described and linked to the functionalized surfaces of the biodegradable HBPEs. By reacting a succinic anhydride bearing a long alkenyl chain with a water-soluble mPEG chain it was possible to obtain an amphiphilic molecule characterized by a central free carboxyl group; this can be used as anchor for the attachment of the amphiphile to the functionalized surfaces of the HBPEs. The further attachment of those molecules via ester bond to the hyperbranched triglycerol-based polyester cores, enabled an innovative set of nanocarriers (Figure 3). These new macromolecules are characterized by different hydrophobicity of the inner core and an amphiphilic shell to promote the encapsulation of hydrophobic molecules while encouraging the solubilization of the HBPEs in aqueous media. Hydrophobic guest molecule such as pyrene and the drugs DOX and DXM were successfully encapsulated in the novel core-ABS nanocarrier. Furthermore, the kinetic of the enzymatic release of the encapsulated dye was also carried out.

Experimental section

Materials:

All reagents, which are commercially available from Sigma-Aldrich, Acros and Alfa Aesar, were used without further purification. Polyglycerol-3® (triglycerol) was kindly donated by Solvay and used for the polymerization step without any purification. The reactions were carried out under dry argon atmospheres using standard Schlenk-line technique.

Characterization:

Dynamic Light Scattering (DLS): the size of HBPE-PEG nanoparticles in aqueous solution was obtained using a Zetasizer Nano ZS analyzer with integrated 4 mW He-Ne laser at wavelength 633 nm with backscattering detector angle 173° (Malvern Instruments Ltd, UK) at 25 °C. To measure the size, an aqueous solution of polymer with different concentrations was prepared in Milli-Q water and vigorously stirred for 18 hours at room temperature (25 °C). Solutions were filtered via 0.45 μm polytetrafluoroethylene (PTFE) filters and used for dynamic light scattering measurements. Disposable UV-transparent cuvettes (Sarstedt AG & Co, Germany) were used for all the experiments.

Cryogenic - Transmission Electron Microscopy (cryo-TEM): the samples for cryo-TEM were prepared at room temperature by placing droplets (≈5 μL) of the solution on hydrophilized perforated carbon-filmed grids (60s Plasma treatment at 8 W using a BALTEC MED 020 device). The excess fluid was blotted off to create an ultra-thin layer (typical thickness of 100-200 nm) of the solution spanning the holes of the carbon film. The grids were immediately vitrified in liquid ethane at its freezing point (-184 °C) using a standard plunging device. Ultra-fast cooling is necessary for an artifact-free thermal fixation (vitrification) of the aqueous solution avoiding crystallization of the solvent or rearrangement of the assemblies. The vitrified samples were transferred under liquid nitrogen into a Tecnai F20 transmission electron microscope (FEI company, Oregon, USA) using the Gatan cryo holder and stage (Model 626). Microscopy was carried out at -175 °C sample temperature using the microscope's low dose protocol at a primary magnification of 50k ×. The defocus was chosen in all cases to be 5 μm.

UV-VIS and Fluorescence Measurements: absorption spectra were recorded using a Scinco S-3150 UV/VIS spectrophotometer. All measurements were carried out in Milli-Q water in a thermostated UV-cell (1 cm). Fluorescence emission spectra were taken with a Jasco FP-6500 spectrofluorimeter equipped with a thermostated cell holder at room temperature (25 °C). For pyrene, emission spectra were recorded from 350 to 600 nm after excitation at 317 nm. Both excitation and emission slits were set at 1 nm. For the release study, the intensity of the emission spectrum of pyrene was monitored as a function of time at physiological pH (7.4) by maintaining the temperature at 37.4 ± 0.1 °C. Data were fitted to the equation $It = I_0 \cdot \exp(-k_{obs} \cdot t)$ with It as the intensity measured at time t and I0 as the initial fluorescence intensity. The half-life time is given by the simple equation $t_{1/2} = \ln(2)/k_{obs}$.

Gel Permeation Chromatography (GPC): when water or THF were used as eluent, weight average molecular weight (M_w) and number average molecular weight (M_n) of the polymers were determined using a GPC equipped with an Agilent 1260 organic pump, refractive index detector, and Mixed-C gel (THF) and Suprema (H_2O) columns with a flow rate of 1.0 mL/min. Measurements in water were carried out using aqueous

solution of $NaNO_3$ (0.1 M) as mobile phase. The molecular weights were calibrated with pullulan (water) and polystyrene (THF) standards. Measurements performed using DMF as eluent, were carried out using a GPC equipped with a Nexera XR LC20 AD XR pump, refractive index (RID-10A) detector and 3x PPS Polarsil column (particle size: 5μm) with a flow rate of 1.0 mL/min. The eluent used was DMF with 3 g/L LiBr and 6 g/L Acetic acid. The molecular weights were calibrated with polystyrene standards.

Nuclear Magnetic Resonance (NMR): NMR spectra were recorded on a Jeol ECX 400 or a Jeol Eclipse 700 MHz spectrometer. Proton and carbon NMR were recorded in ppm and were referenced to the indicated solvents. NMR data were reported as follows: chemical shift, multiplicity (s=singlet, d=doublet, t=triplet, m=multiplet) and integration. Multiplets (m) were reported over the range (ppm) at which they appear at the indicated field strength.

High Pressure Liquid Chromatography (HPLC): HPLC measurements for the detection of Dexamethasone were carried out using a Knauer Smartline-HPLC system with an internal UV absorption detector (λ = 254 nm) and Chromgate software. A Gemini RP C18 column (Phenomenix, 250 mm × 4.6 mm, particle Size: 5 μm). Acetonitrile–water (40 : 60) was used as the mobile phase at a flow rate of 1.0 mL min^{-1} under isocratic regime.

Synthesis:

General procedure for the synthesis of HBPEs: The three hyperbranched polyesters characterized by different polarity were synthesized by charging the α−ω dicarboxylic acid and triglycerol in a 1 : 1 molar ratio in a three-necked flask equipped with mechanical stirrer and vacuum outlet. The mixture was stirred at 200 rpm at 160 °C for 1 hour in an open flask and 1 hour at 10 mmHg in order to extract the water formed during the polyesterification. A catalytic amount of $Ti(OBu)_4$ (0.18 mol-%) was than added and the mixture was further stirred at 160 °C under argon flow and at atmospheric pressure for 1 hour and then at 10 mmHg until the desired polymer molecular weight was obtained. The polymers were purified twice via precipitation to eliminate smaller oligomers and then characterized via ^{1}H-NMR, ^{13}C-NMR and GPC (ESI).

Synthesis of the amphiphilic branched shell (ABS): Poyethylene glycol mono-methyl ether (mPEG-500 Da) was dissolved in acetonitrile followed by the addition of (2-dodecen-1-yl) succinic anhydride (1.05 equiv.) and catalytic amount of DMAP (0.1 equiv.).[21] The reaction mixture was stirred for 24 h at room temperature. The solvent was evaporated and the crude product re-dissolved in chloroform and washed (2x) with brine solution. The organic phase was dried over magnesium sulfate. The drying agent was removed by filtration, and the solvent was evaporated to yield a yellowish oil in 84% yield, characterized by ^{1}H-NMR and ^{13}C-NMR (ESI).

General procedure for the synthesis of the core-ABS HBPEs: in a round bottom flask equipped with magnetic stirrer, the HBPE was dissolved in dry DMF. At 0 °C the amphiphilic shell (500 wt-%), dicyclohexylcarbodiimide (DCC) (1.1 equiv.) and catalytic amount of 4-dimethylaminopyridine (DMAP) (0.1 equiv.) were subsequently added.[22] The mixture was stirred for 3 hours at 0 °C and 72 hours at room temperature. The insoluble dicyclohexylurea (DCU) formed during the reaction was filtered off and the solvent evaporated under reduced pressure to afford a cloudy oil. The crude was purified via precipitation in diethyl ether – hexane mixture (2 : 1) and ultrafiltration in methanol (MWCO = 3000 Da) affording the desired core-shell HBPE that was characterized by ^{1}H-NMR, ^{13}C-NMR and GPC (ESI).

Enzymatic Degradation of the HBPE; several samples were prepared following a standard procedure; 2 mL of polymer C6-HBPE solutions (5 mg/mL) in 10 mM phosphate buffer (pH 7.4) were prepared. Novozym-435 (200 wt-% of wt of polymer) and a few drops of *n*-butanol were added and the samples were incubated at 37°C using a BioShake XP® (Biometra) and shaken at 1000 rpm for 9 days. Every so often, a sample was taken, filtered and evaporated to dryness. The solid residue was dissolved in DMSO-d_6 and filtered through a syringe filter (CA, 0.45 μm pore size) to remove insoluble materials and analyzed by ^{1}H-NMR to monitor the degradation process.[23]

Solubilization of Guest Molecules: for the encapsulation experiments of the guest molecules (*e.g.* pyrene), a 20 mM pyrene solution was freshly prepared by dissolving an appropriate amount of the dye in dry THF. Aliquots (20 μL) were taken in vials and the organic solvent was removed. 1.5 mL of the aqueous polymer stock solutions with various concentrations (0,5 – 0,0125 mg/mL) in Milli-Q water was added and the resulting mixture was stirred at 500 rpm for 18 hours at room temperature. The mixture was filtered through a syringe filter (PTFE, 0.45 μm) to remove the insoluble excess of pyrene. The aqueous solutions of the polymers were then analyzed by UV-Vis and fluorescence spectroscopy. Guest loading capacity was calculated based on the following expression:

$$Loading\ capacity\ (LC) = \frac{\text{mass of guest encapsulated}}{\text{mass of host}}$$

Release of pyrene under enzymatic conditions; Pyrene was encapsulated into 0.5 mg/mL nanocarrier solutions, using a 10 mM phosphate buffer solution (pH 7.4) instead of milliQ water. After stirring at 500 rpm for 18 hours, the samples were filtrated to eliminate any insoluble pyrene, and the enzyme *Candida antartica* Lipase B (200 wt% of wt of polymer) and a few drops of *n*-butanol were added. The samples were incubated at 37°C using a BioShake XP® (Biometra) and shaken at 1000 rpm for 9 days. To evaluate the stability of the nanocarriers under physiological pH, samples were prepared and incubated without the addition of the enzyme. All experiments were carried out in duplicate. The kinetics of the experiments were followed every day by fluorescence spectroscopy monitoring the intensity decrement of the pyrene signal.[23]

Results and discussion

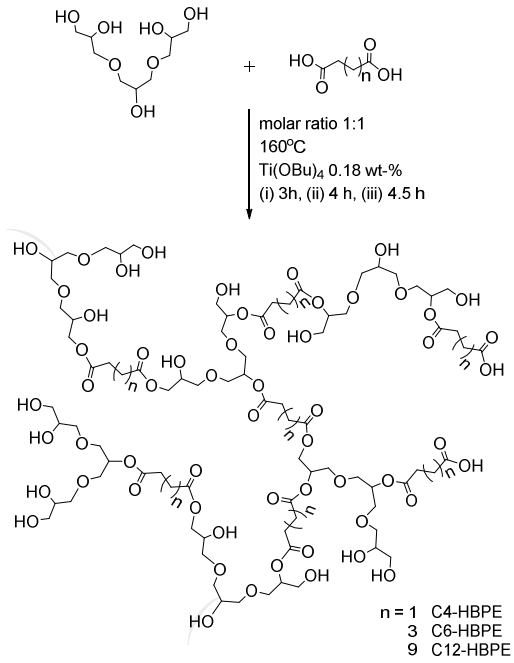
Synthesis of the Hyperbranched Polyesters.

The synthesis of the hyperbranched triglycerol-based polyesters, characterized by different polarity, that were used as core molecules of the novel nanocarriers was optimized adopting different catalysts and polymerization parameters (e.g. temperature, pressure, reaction time) in order to obtain the suitable conditions (ESI). The hyperbranched polyesters were synthetized by reacting a mixture of glycerol-oligomers based on triglycerol, (polyglycerol-3®) with different α–ω dicarboxylic acids (SA, AA and DDCA) in a 1 : 1 mole ratio at 160 °C in the presence of catalyst $Ti(OBu)_4$ (Scheme 1).

In order to obtain comparable characteristics in the HBPEs, the polymerization's conversions were evaluated via NMR spectroscopy by monitoring the ratio between the signals of the protons in alpha position to the unreacted and reacted carbonyl groups. The polymerizations were stopped, by cooling the reactions at room temperature, when evenly 90% of the free carboxylic acids were reacted (ESI). The average molecular weights of the HBPEs were evaluated via GPC analysis. Employing these reaction conditions it was possible to obtain three hyperbranched polyesters having similar molecular weight (evenly 30 KDa) but characterized by different polarity (Table 1).

After the A5-B2 polyesterification using a 1-1 monomer molar ratio, a large excess of free hydroxyl groups is still available for further functionalization.

The triglycerol moiety is composed of three glycerol molecules condensated and in its structure it is possible to distinguish different structures; terminal units (T) and linear units (L) (Figure 1).



Scheme 1. Synthesis of the HBPEs and schematic representation of the HBPEs. (i) C4-HBPE, (ii) C6-HBPE, (iii) C12-HBPE.

When the hydroxyl groups of these units are reacted with the dicarboxylic acid, the T units forms three possible conformation denominated as L1,2 , L 1,3 and D1,2, and the L unit forms only one possible conformation denominated as D2.

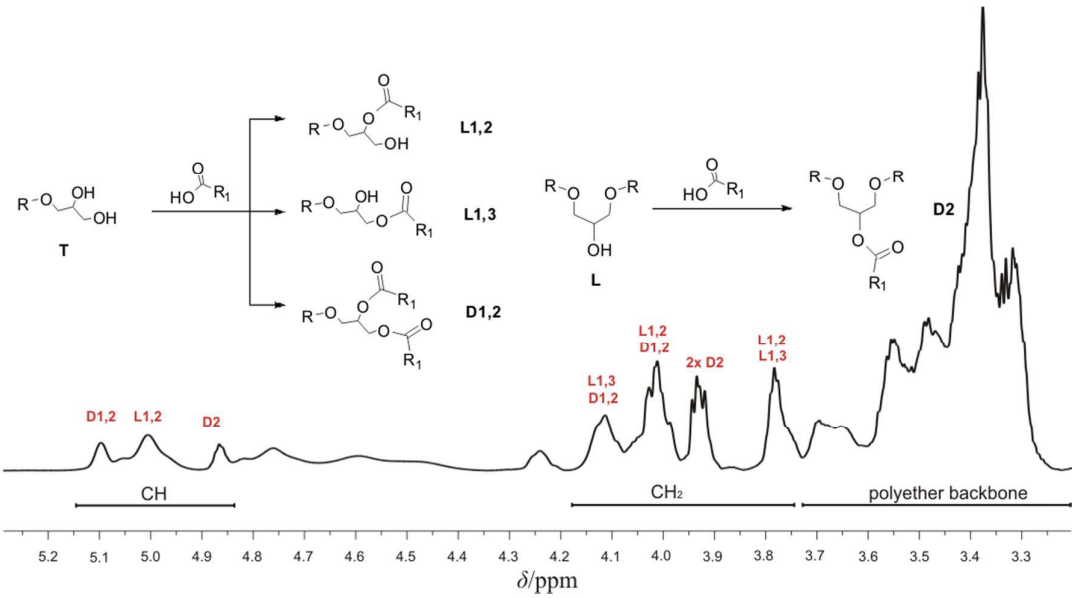


Figure 1: ^{1}H-NMR magnification of C6-HBPE with the different conformation units.

Table 1. Characterization of the HBPEs

Polymer	Solubility (H_2O)	M_w (g/mol)[a]	M_n (g/mol)[a]	D[b]	Conversion (%)[c]
C4-HBPE	soluble	27758	12456	2.2	90
C6-HBPE	slightly soluble	34761	17671	1.9	89
C12-HBPE	insoluble	28633	12935	2.2	90

[a] M_w and M_n from GPC analysis. [b] Dispersity (M_w/M_n). [c] Calculated via ^{1}H NMR.

In figure 1 is displayed the ^{1}H-NMR of C6-HBPE with the relative peaks assignment where it is possible to define three main parts. Between 3.25 ppm and 3.70 ppm are expressed the classical peaks of the triglycerol moiety derived from unreacted linear and terminal units. When the triglycerol reacts with the carboxylic acid, the CH and CH_2 signals of the T and L1,3 units, shift at lower field; the proton of the tertiary carbon shifted between 5.10 and 4.18 ppm and the protons of the secondary carbons shifted between 4.30 and 3.72 ppm.

Additional information about the conformation of the hyperbranched polyesters was obtained by comparing the IG ^{13}C-NMR of the triglycerol and the final polymer (C6-HBPE) (ESI). The integration of the terminal (T, 63.16 ppm) and the linear units (L, 61.00 ppm) of the triglycerol decreased respectively of 44 and 50 % after polymerization (Table 1 ESI). This fact confirmed the imperfect structure of the final hyperbranched polymer in which over 50 % of both linear and terminal units did not react during the polymerization.

Enzymatic Degradation of the Hyperbranched Polyester.

Enzyme-mediated degradation of the hyperbranched polyester backbone was performed with a de-esterification method using *Candida Antarctica* Lipase B that had been immobilized on acrylic resin, commercially known as Novozym-435.[23]

Samples were prepared following a general procedure reported above and analyzed every so often by ^{1}H-NMR to monitor the degradation process.

To evaluate the degree of degradation, the ratio between the signal of the protons in alpha position to the carbonyl groups was monitored (DMSO-d_6, 2.32 ppm related to the ester and 2.20 ppm related to the carboxylic acid) and displayed in figure 2. Over time, a decrease of the peak at 2.32 ppm with the corresponding increase of the peak at 2.20 ppm becomes visible. The ester linkages are hydrolyzed with the formation of the corresponding carboxylic acid.

After only 12 hours the enzymatic polymer breakdown allowed over 12 % of the ester bonds present in the polymeric structure to hydrolyze, increasing to 30 % after 24 hours. The experiment was carried out for 9 days, after which the enzyme had hydrolyzed 55% of the polymeric structure's ester bonds (Table 2).

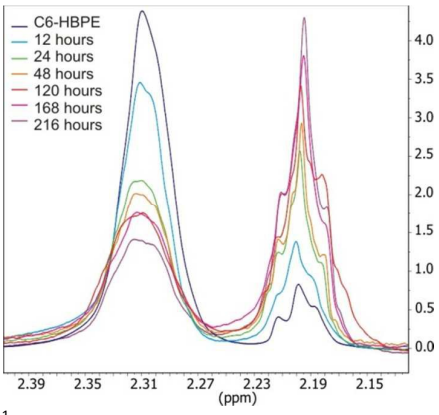


Figure 2. ^{1}H-NMR magnification of the protons in alpha position to the carbonyl groups of C6-HBPE during the degradation process in presence of CALB at 37°C. Signal at 2.32 ppm is related to the ester and 2.20 ppm is related to the carboxylic acid.

Table 2. Degradation profile of C6-HBPE.

Time (days)	Carboxylic acid (%)	Degree of ester degradation (%)
0	11	0
0.5	21	12
1	34	33
2	40	37
5	52	46
7	55	49
9	60	55

Synthesis of the Amphiphilic Branched Shell

The amphiphilic branched shell (ABS) was synthesized and furthermore attached to the accessible free hydroxyl groups of the HBPEs in order to obtain a strategic gradient of polarity in the nanocarriers, useful for increasing the encapsulation of hydrophobic drug molecules, whilst guaranteeing water-solubility at the same time.

Amphiphilic molecules derived from alkenyl succinic anhydrides have already been described in prior art literature. Compounds which are derived from alkenyl succinic

anhydrides (ASA) having C6-C50 carbon atoms in the substituent and a nonionic water-soluble compound, preferably mPEG chain, have been used for decades as surfactants, emulsifiers, paper sizing agents and as dispersing agents for dyes or pigments.[25]

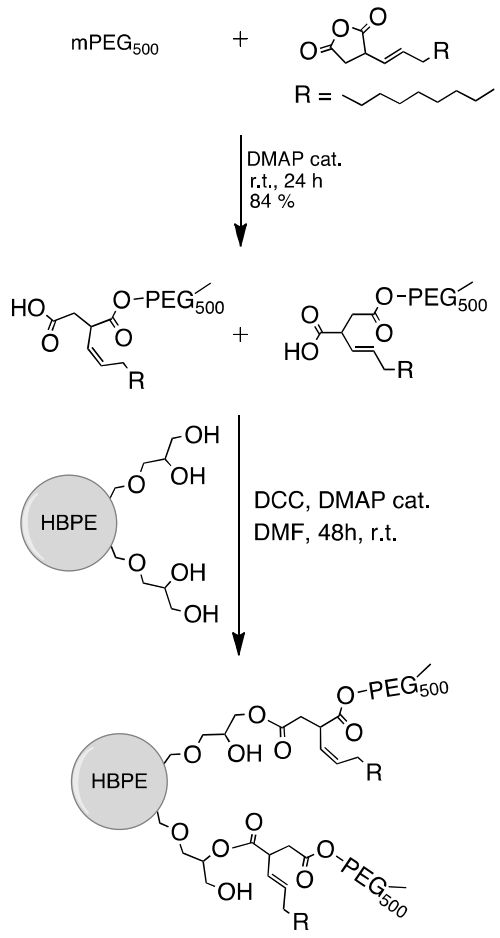
These amphiphilic molecules are characterized by a central free carboxyl group that can be strategically used as an anchor for its chemical attachment to the functionalized surfaces of the HBPEs.

With this aim a methoxy polyethylene glycol (mPEG 500 Da) chain was made to react with an alkenyl succinic anhydride (ASA) (dodecen-1-yl succinic anhydride) in the presence of catalytic amount of DMAP [21] to obtain a mixture of the two isomers of the desired amphiphilic molecule (Scheme 2 upper) that were not isolated but used directly for further functionalization.

Synthesis of the Core-ABS nanocarriers

The amphiphilic branched shell was covalently bonded to the functionalized surfaces of the biodegradable HBPEs. The unreacted accessible hydroxyl groups of the triglycerol moieties preferentially located in the peripheries of the globular hyperbranched polymers, were reacted, using different methodologies, with the free carboxylic acid of the ABS to form enzyme label ester bonds.

Using Steglich esterification conditions, it was possible to obtain core-ABS nanocarriers with a similar degree of functionalization. The coupling agent dicyclohexylcarbodiimide (DCC) and catalytic amount of 4-dimethylaminopyridine (DMAP) were used to enable ester formation.[22] After 72 hours of reaction at room temperature, the insoluble side product dicyclohexylurea (DCU) that had formed during the reaction was filtered off and the solvent evaporated under reduced pressure to afford the crude mixtures that were purified via



Scheme 2. Synthesis of the core-ABS nanocarrier: Schematic synthesis of the amphiphilic shell and further reaction with the hyperbranched polyesters.

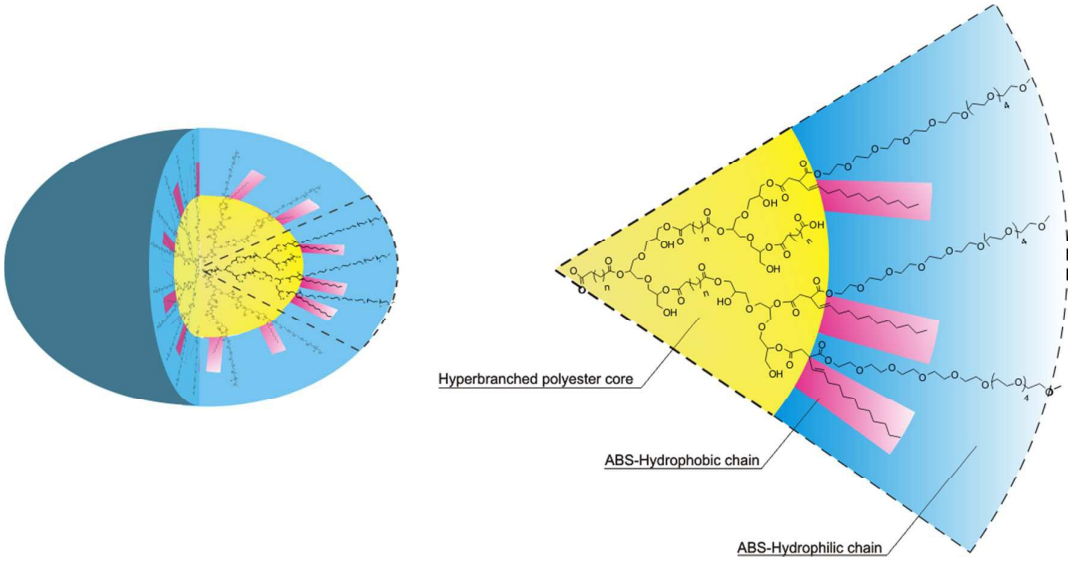


Figure 3: Schematic representation of the Core-ABS nanocarrier

Table 3. Characterization of core-AB-shell HBPEs

Polymer	Solubility in H_2O (mg/mL)[b]	Tot. OH Funct. (%)	N° ABS per core	Shell (wt-%)[a]	NMR-M_w (g/mol)[a]	GPC-M_w (g/mol)
C4-HBPE-ABS	> 20	14	36	102	48000	27700
C6-HBPE-ABS	10	11	32	71	56000	29400
C12-HBPE-ABS	2	14	28	77	49000	26700

[a] Shell wt-% and approximate molecular weights were calculated via ^{1}H NMR spectroscopy. [b] Measured at 25°C.

precipitation in diethyl ether / hexane mixture and ultrafiltration in methanol (MWCO = 3000 Da), affording the desired core-ABS HBPEs C4-HBPE- ABS, C6-HBPE- ABS and C12-HBPE-ABS (Scheme 2, Figure 3) that were analyzed via ^{1}H-NMR, ^{13}C-NMR and GPC (ESI).

Figure 4 shows the comparison between the ^{1}H-NMR spectra of the hyperbranched polyester core (C6-HBPE) and the spectra after reaction with the AB-shell (C6-HBPE-ABS) were it is possible to detect the characteristic peaks of the amphiphilic molecules bounded to the polymeric structure.

By integrating the signal related to the CH_3 of the alkyl chain at 0.85 ppm with signal related to the CH_2 in alpha position to the ester´s carbonyl group of the polymeric structure at 2.31 ppm it was possible to evaluate the percentage of hydroxyl groups that effectively reacts with the AB-shell (Table 3) (ESI).

Further information about the conformation of the core-shell nanocarriers was obtained comparing the IG ^{13}C-NMR of the polymeric core and the core-shell molecule (ESI). The integration of the terminal (T, 63.16 ppm) and the linear units (L, 61.00 ppm) of the HBPE decreased respectively by 6.5 and 6.7% after reaction (Table 2 ESI), confirming that only a minor percentage of the hydroxyl groups of the HBPEs effectively reacted with the AB-shell.

Determining the molecular mass of the nanocarriers was carried out via ^{1}H-NMR and by GPC using DMF as eluent with reference to conventional polystyrene calibration standards of low polydispersity. Between the two methodologies however we observed a great discrepancy. Its known that GPC analysis effects separation according to molecular volume rather than molecular mass; the presence of the amphiphilic shell is most likely making the macromolecules shrink when dissolved in water, thus leading to an underestimated result from the GPC analysis as a direct consequence. The GPC measurements were, however, useful to ensure the absence, after purification, of any non-bonded ABS molecules.

Dynamic Light Scattering (DLS) and Cryo-TEM

To achieve a better understanding of the conformation of the nanocarriers when dissolved in aqueous media, DLS and Cryo-

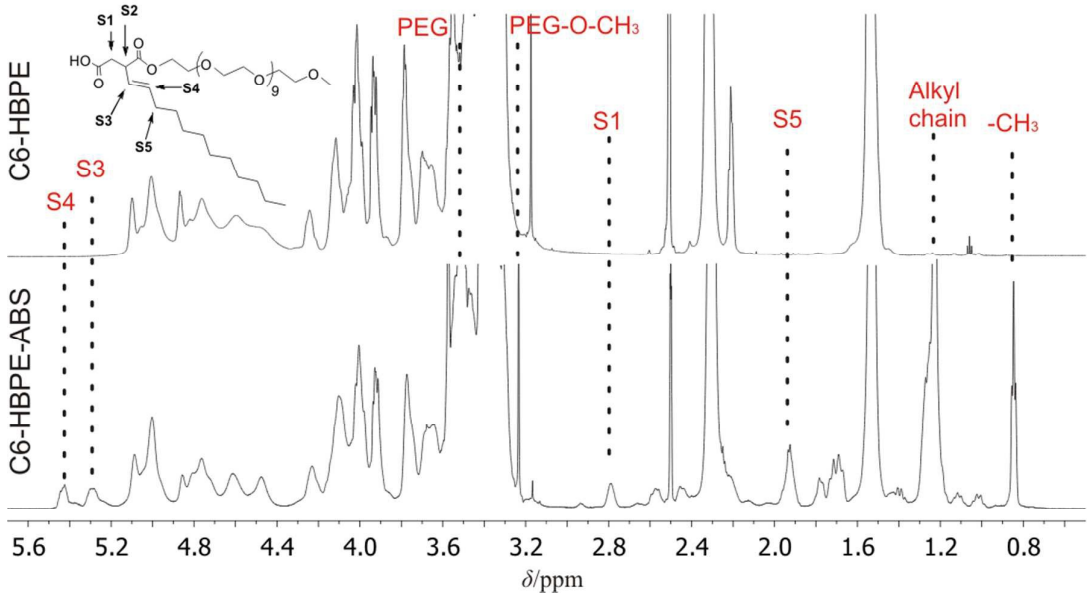


Figure 4: ^{1}H-NMR spectra comparison between C6-HBPE ans C6-HBPE-ABS.

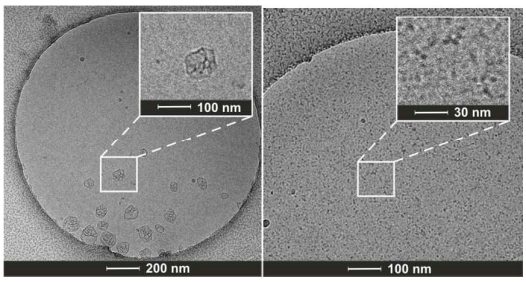


Figure 5. Cryo-TEM microscopic images of C12-HBPE-ABS in water (left) and C6-HBPE-ABS in water (right).

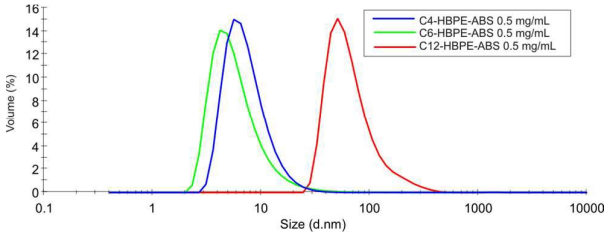


Figure 6. DLS diagram of 0.5 mg/mL solutions of the nanocarriers C4-HBPE-ABS, C6-HBPE-ABS and C12-HBPE-ABS in water.

TEM measurements were carried out. The summary of DLS measurements can be seen in Table 4.

The polarity of the hyperbranched core displayed its fundamental importance in the conformation adopted by the nanocarrier in solution. In figure 5 we present the cryo-TEM microscopic images of the aqueous solutions of the nanocarrier C12-HBPE-ABS and C6-HBPE-ABS at a concentration above CMC (0.5 mg/mL).

According to DLS measurements, when dissolved in water, the nanocarrier characterized by a highly hydrophobic core (C12-HBPE-ABS) aggregates, forming considerable micellar assembly with an average diameter of 80 nm. The hydrophobic interaction between the polymeric cores, derived from the presence of long aliphatic alkyl linkers in the structure, is proposed to be the driving force that induces the nanocarrier to form bigger macromolecular aggregates (Figure 5 left).

It is interesting to notice that the cryo-TEM image of the nanocarrier characterized by a more hydrophilic polymeric core (C6-HBPE-ABS) (Figure 5 right) shows the formation of smaller aggregates consisting of a few molecules, with an average diameter of 7 nm on the surface. These values fit well with the DLS results (Figure 6).

Host-Guest Interaction of core-AB-shell HBPEs

To understand the carrier properties of the novel nanocarriers, the hydrophobic guest molecule pyrene was used as a model drug to obtain information about the microenvironment and binding sites of the drug carrier. Furthermore, the anti-inflammatory drug Dexamethasone (DXM) and the antitumor drug Doxorubicin (DOX) were encapsulated and the drugs loading capacities (LC) were determined by HPLC and UV-vis analysis respectively.

Solubilization of Pyrene

Pyrene was applied as a model drug for delivery investigation. Tests to evaluate the LC (loading capacity), CMC (critical micelle concentration) and the guest release profile were successfully carried out via fluorescence and UV-Vis spectroscopy (Table 4). A low concentration of pyrene was used for the encapsulation study to avoid the formation of excimer complexes. The absorbance and fluorescence spectra of pyrene in different polymer concentration solutions (C12-HBPE-ABS) are shown in figure 7 a-b. Using the Lambda maximum at 338 nm the concentration of solubilized pyrene was calculated. LCs of 0.8, 1.3 and 4.2 wt-% of pyrene were determined for polymer C4-HBPE-ABS, C6-HBPE-ABS and C12-HBPE-ABS respectively (ESI).Pyrene is a common hydrophobic dye that can be used to calculate the CMC of surfactants.[26] Using the intensity ratio of pyrene (I_1/I_3 ratio with I_1 = 374 nm and I_3 = 385 nm), the CMC of a surfactant can be extrapolated. By using this strategy, it was possible to confirm that our nanocarrier systems have different intrinsic polarity; in fact, the polarity index of pyrene (I_1/I_3) can be used as an indicator of hydrophobicity of the environment in which the pyrene is located.[27] The fluorescence spectroscopy studies indicated that the CMC of the pyrene loaded nanocarriers were 2.92 x 10^{-3}, 8.93 x 10^{-4} and 4.08 x 10^{-4} mM respectively for the polymers C4-HBPE-ABS, C6-HBPE-ABS and C12-HBPE-ABS (Figure 8) (ESI). The nanocarrier with a highly hydrophobic core (C12-HBPE-

Table 4. Solubilization data of pyrene in the core-AB-shell HBPEs

Polymer	DLS		Pyrene loading			
	Unloaded[a] (nm)	Pyrene[a] (nm)	LC[b] mg_{guest}/g_{host}	LC[b] wt-%	CMC[c] mg/mL	CMC[c] mM
C4-HBPE-ABS	6.9	7.7	7.9	0.8	0.215	2.92 x 10^{-3}
C6-HBPE-ABS	6.2	6.7	13.2	1.3	0.099	8.93 x 10^{-4}
C12-HBPE-ABS	72.9	75.1	41.7	4.2	0.021	4.08 x 10^{-4}

[a] in a 0.5 mg/mL nanocarrier solution. [b] Loading capacity. [c] Critical micelle concentration

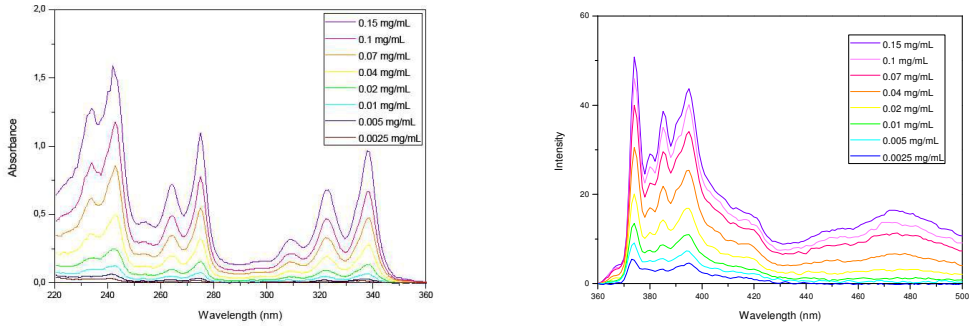


Figure 7. (a) UV-Vis spectra of pyrene in C12-HBPE-ABS (left) and **(b)** Fluorescence spectra of pyrene in C12-HBPE-ABS (right).

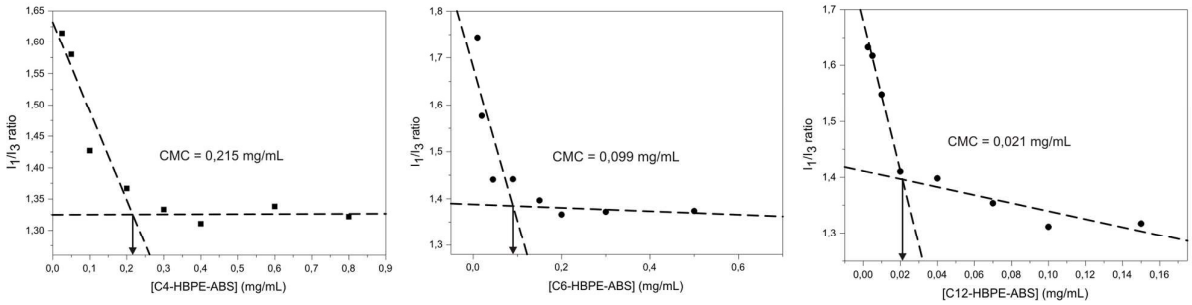


Figure 8. Calculation of the CMC of the nanocarriers using I1/I3 diagrams.

ABS) displays an intrinsic CMC averagely 6 times smaller than the nanocarrier with a hydrophilic core (C4-HBPE-ABS). It is of interest to notice the relationship between the CMC of a nanotransporter with its pyrene loading capacity; in fact, C12-HBPE-ABS allows to encapsulate over 5 times more pyrene than the nanocarrier C4-HBPE-ABS. It is possible to confirm the importance of the amphiphilic branched shell to encapsulate hydrophobic molecules, especially for the nanocarrier characterized by a polar polymeric core (C4-HBPE-ABS).

Nevertheless, from the data collected, the lipophilicity of the polymeric core displayed its fundamental importance, that allow the formation of big aggregates (C12-HBPE-ABS), to enable high drug loading capacity. The size of the aggregates formed by the nanocarriers when dissolved in aqueous media, displayed previously in Figure 5 and confirmed by DLS measurements (Figure 6), is certainly a key factor to explain the higher loading capacity of the nanocarrier C12-HBPE-ABS. The aggregates formed by this carrier, in average 73 nm, allow loading between 3 to 5 times more pyrene than the nanocarriers C6-HBPE-ABS and C4-HBPE-ABS, which forms smaller aggregates of 7 nm when dissolved in water. Surprisingly, no significant changes in size were recorded after encapsulation of the guest molecules. DLS measurements showed that when pyrene was loaded the average diameters of the aggregates formed by the nanocarrier C12-HPBE-ABS increased by 2.2 nm (Table 4). The aggregates formed by the nanocarriers characterized by a more hydrophilic core C4-HBPE-ABS and C6-HBPE-ABS did not substantially change their size after the encapsulation of the hydrophobic dye pyrene.

In a 1 mg/mL solution of C12-HBPE-ABS it was possible to solubilize 42 mg/L of pyrene; this value is 311 times higher than the solubility of pyrene in water reported in literature of 0.135 mg/L. at 25°C.[28]

Enzymatic induced release of pyrene under physiological conditions.

The biodegradability of the hyperbranched polyester core under enzymatic conditions has been successfully displayed above. Using the same methodology, a general de-esterification method using *Candida antarctica* Lipase B,[23] we investigated the time-dependent release of solubilized pyrene from the core-shell nanocarriers, in the presence of an enzyme by means of fluorescence spectroscopy.

The samples for the experiments were prepared following the general procedure. Pyrene was encapsulated into 0.5 mg/mL nanocarrier solutions of C4-HBPE-ABS, C6-HBPE-ABS and C12-HBPE-ABS in a 10 mM phosphate buffer solution (pH 7.4). After encapsulation, enzyme *Candida antartica* Lipase B (200 wt% of wt of polymer) and a few drops of *n*-butanol were added. The samples were incubated at 37°C using a BioShake® for 9 days and the kinetics of the experiments were monitored by fluorescence spectroscopy. To evaluate the stability of the loaded nanocarriers under physiological conditions, samples were prepared and incubated without the addition of the enzyme.

In figure 9 we display the cumulative release profile of pyrene encapsulated in C4-HBPE-ABS during 9 days of the experiment

under physiological conditions in the presence and absence of the enzyme. It is interesting to notice the stability of the loaded nanocarrier during the experiment under physiological conditions in absence of the enzyme; in fact non-relevant amounts of pyrene were released from the systems adopting these conditions. In contrary, the nanocarrier, placed in an environment similar to the human body, in the presence of the enzyme, shows an exponential release profile, characterized by a fast initial release that decreases with the time of the experiment. After 48 hours, 50 % of the pyrene encapsulated was released from the nanocarrier, and only after 6 days it was possible to obtain a release of 85%. The hyperbranched polyester core degraded into lower molecular weight polymer fragments due to the hydrolysis process of ester linkages, allowing the release of the hydrophobic dye.

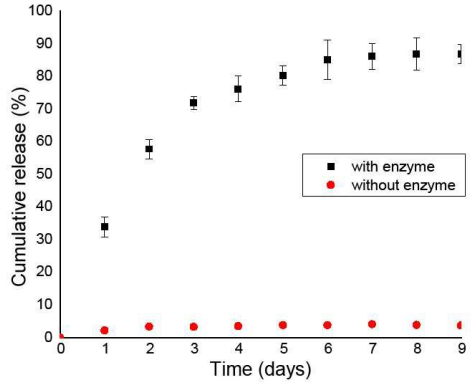


Figure 9. Cumulative release profile from C4-HBPE-ABS of pyrene under physiological conditions, in the presence and absence of enzyme.

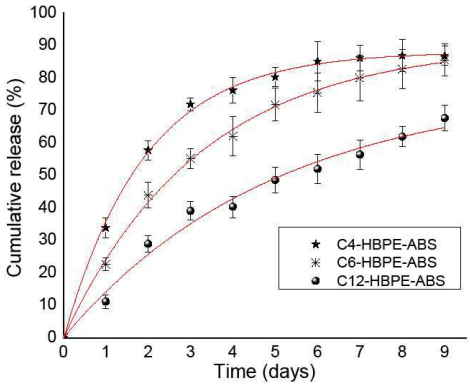


Figure 10. Cumulative release profile of pyrene under physiological conditions in the presence of enzyme from the nanocarriers C4-HBPE-ABS, C6-HBPE-ABS and C12-HBPE-ABS.

Figure 10 shows the different cumulative release profile of the nanocarriers C4-HBPE-ABS, C6-HBPE-ABS and C12-HBPE-ABS. A relevant comparison of the pyrene release profile obtained from the three different nanocarriers can allow for a better understanding of the mechanisms involved during the guest release. The fastest release was obtained when pyrene was encapsulated into C4-HBPE-ABS, characterized by a hydrophilic polymeric core. This can be a direct cause of the highest activity of the enzyme in a polar environment.

Similar behavior is displayed by the nanocarrier C6-HBPE-ABS characterized by a partially hydrophilic polymeric core. A lower initial decay, together with a half-life of 60 hours, enabled the release of over 85% of the pyrene encapsulated in 9 days.

An ideal and almost linear pyrene release was achieved by employing the nanocarrier C12-HBPE-ABS. 50% of the pyrene encapsulated was released after 5 days, and 70% after 9 days using the nanocarrier characterized by a highly hydrophobic polymeric core. The difficulty for the enzyme to reach the unpolar environment can be an explanation for the slow release obtained. The possible formation of amphiphilic molecules after degradation of the polymeric core, that can aggregate to form micellar structures able to encapsulate guest molecules, can be a second valid explanation for the interesting pseudo-linear release profile.

The non perfect dendritic architecture of the polymeric core and the low degree shell functionalization has brought to obtain highly flexible structures that may facilitate the enzymatic degradation process. More compact and inflexible polymeric structures certainly can inhibit the enzymatic activity due to the difficulties for the enzyme to reach the hindered polymeric core.

In order to obtain reproducible experiments, it was decided to use a fixed nanocarrier concentration of 0.5 mg/mL. As above-mentioned, the pyrene loading capacity of the nanocarriers is not constant and thus the amount of pyrene solubilized in the samples is different; this value range from 0.004 mg/mL in C4-HBPE-ABS to 0.021 mg/mL in C12-HBPE-ABS. The hydrophobicity of the pyrene molecules encapsulated into the nanocarriers, certainly increase the lipophilicity of the systems and can lead to a decreased activity of the water-soluble enzyme in degrading the polymeric structure of the nanocarrier.

Solubilization of Doxorubicin and Dexamethasone

To investigate the suitability of the novel biodegradable nanocarriers as possible drug delivery systems, it is of importance to exploit them using non water-soluble drug molecules in order to understand the transport capacity of the different systems. Two widely used and poorly water-soluble drugs, the anticancer Doxorubicin and the anti-inflammatory steroid drug Dexamethasone, were selected for this purpose.

Both drugs are partially soluble in water and for this reason particular attention was paid in order to evaluate the amount of drug effectively encapsulated into the polymer from the free drug.

Commercially available Doxorubicin hydrochloride (DOX·HCl) was transformed into the free base doxorubicin (DOX) similar

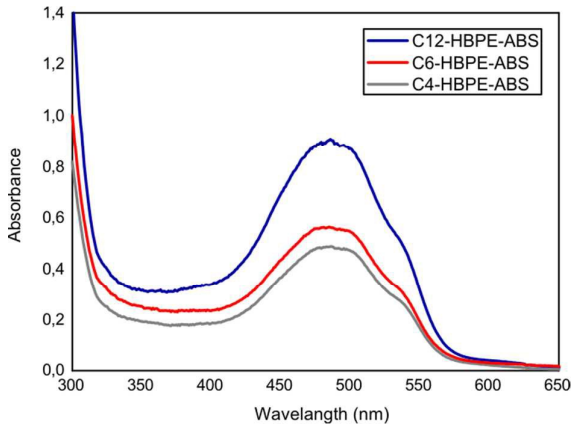


Figure 11. UV-Vis spectra of DOX loaded in 1 mg/mL solution in PBS of C4-HBPE-ABS, C6-HBPE-ABS and C12-HBPE-ABS.

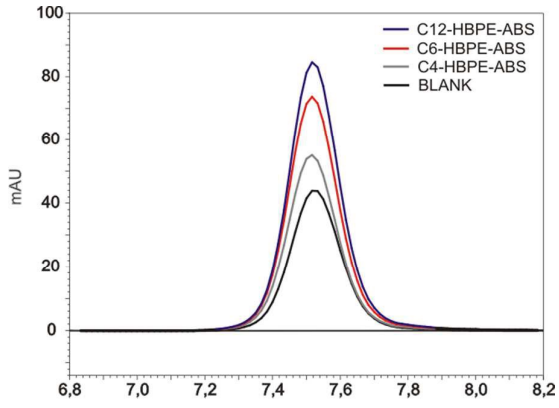


Figure 12. HPLC chromatogram of DXM loaded in 1 mg/mL solution of C4-HBPE-ABS, C6-HBPE-ABS and C12-HBPE-ABS, using a water/acetonitrile mixture (60/40) as an eluent.

to the published method.[26,29] DOX·HCl was dissolved in methanol and stirred with 2 eq. of triethylamine for two hours. Both DOX and DXM were encapsulated into the nanocarriers adopting a general film method.

An excess of drug (50 wt-% -wt of polymer) was dissolved in acetone (DXM) or dimethyl formamide (DOX) and subsequently dried under vacuum in order to obtain a uniform layer (film) of drug in the flask. Aqueous nanocarrier solutions (1 mg/mL) were added and the samples were stirred for 18 hours at 500 rpm. An excess of drug (50 wt-% -wt of polymer) was dissolved in acetone (DXM) or dimethyl formamide (DOX) and subsequently dried under vacuum in order to obtain a uniform layer (film) of drug in the flask. Aqueous nanocarrier solutions (1 mg/mL) were added and the samples were stirred for 18 hours at 500 rpm.

Due to its partial solubility in water, the non-loaded DOX was removed using Sephadex® G-20 columns.[30] The loading capacities of the carriers C4-HBPE-ABS, C6-HBPE-ABS and C12-HBPE-ABS were determined by UV-Vis spectrometry; using the Lambda maximum at 495 nm and an extinction coefficient (ε) of 1,0645 M^{-1} cm^{-1} in PBS, the concentration of solubilized DOX was able to be calculated. The absorbance spectra of DOX in the different carriers are shown in Figure 11. A loading capacity of 3.7, 4.3 and 6.8 wt-% of DOX was encapsulated respectively into a 1 mg/mL polymer solution of C4-HBPE-ABS, C6-HBPE-ABS and C12-HBPE-ABS (Table 5).

Due to difficulties in separating the non-loaded DXM from the loaded drug, a blank sample was prepared to evaluate the amount of dexamethasone soluble in water, by which dexamethasone was stirred in water without the nanocarrier.

After encapsulation, the samples and the blank were filtered, diluted with acetonitrile (40%) and directly analyzed via HPLC using a water/acetonitrile mixture (60/40) as an eluent. A calibration curve was prepared (ESI) in order to evaluate the solubility of DXM in water and the loading capacity of the nanocarriers.

Similar to a literature reported value, we determined a solubility of Dexamethasone in water of 0,08 mg/mL.[31] In figure 12 the HPLC chromatograms are shown, representing the peaks of the DXM loaded in C4-HBPE-ABS, C6-HBPE-ABS and C12-HBPE-ABS and the blank sample.

The best loading performance was once again displayed by the nanocarrier characterized by a highly hydrophobic core. In a 1 mg/mL aqueous solution of C12-HBPE-ABS, it was possible to solubilize 0,15 mg/mL of DXM. As shown in Table 5, we were able to double the solubility of dexamethasone in water.

Conclusions

A set of novel biodegradable triglycerol-based hyperbranched polyesters characterized by different hydrophobicity has been

Table 5. Solubilization data of DXM and DOX in the core-AB-shell HBPEs.

Polymer	Dexamethasone			Doxorubicin	
	DXM solubilized (mg/mL)	LC_{DXM} (wt-%)	LC_{DXM} (mg_{DEX}/g_{host})	LC_{DOX} (wt-%)	LC_{DOX} (mg_{DEX}/g_{host})
C4-HBPE-ABS	0,10	2,2	22	3,7	37
C6-HBPE-ABS	0,14	5,5	55	4,3	43
C12-HBPE-ABS	0,15	7,4	74	6,8	68

optimized and described. Biodegradation tests in presence of the enzyme CALB showed that it was possible to hydrolyze over 50% of the ester linkages of C6-HBPE in 9 days. A novel amphiphilic branched shell was developed and attached, via ester bond, to the accessible hydroxyl groups of the HBPEs in order to obtain a polarity gradient in the nanocarriers.

The core-AB-shell nanocarriers characterized by different polymeric cores, forms aggregates of different sizes when dissolved in aqueous media. The hydrophobicity of the polymeric core is certainly the driving force for the formation of these architectures and proved its fundamental importance in order to significantly increase the loading capacity of hydrophobic guest molecules. The amphiphilic branched shell displayed a double important role on the development of the novel nanocarriers. It is increasing the loading capacity of the nanocarriers characterized by a polar polymeric core as well as helping to solubilize in aqueous media the nanocarrier having a hydrophobic polymeric core.

The nanocarrier C12-HBPE-ABS, characterized by a highly hydrophobic core consisting of long aliphatic alkyl linkers, when dissolved in water is forming big aggregates of an average diameter of 73 nm. This proved to be the best candidate for drug delivery applications; it was possible to load up to 5 times more pyrene than the nanocarriers that forms smaller aggregates C4-HBPE-ABS, C6-HBPE-ABS.

In a 1 mg/mL C12-HBPE-ABS solution, it was possible to increases the solubility of pyrene in water by a factor of over 300. Furthermore, a pseudo-linear delivery profile in the presence of enzyme (CALB) allowed a release of over 60% of the encapsulated pyrene in 9 days.

Finally, the anticancer drug Doxorubicin and the anti-inflammatory steroidal drug Dexamethasone were efficiently encapsulated in the nanocarriers.

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Table of contents

A set of biodegradable nanocarriers characterized by a hyperbranched polyester core and an amphiphilic branched shell was developed and employed to efficiently solubilize hydrophobic drugs in aqueous media.