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Poly(malic acid) bearing Doxorubicin and N-Acetyl Galactosamine as a site-specific prodrugs for targeting HepatoCellular Carcinoma

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Poly(malic acid) bearing Doxorubicin and N-Acetyl Galactosamine as a site-specific prodrug for targeting HepatoCellular Carcinoma

Abstract: In the past, several systems of drug delivery carriers have been designed with a high capacity to target specific cells and/or tissues and a reduced non-specific toxicity. In this context, we synthesized and characterized novel poly(malic acid) derivatives bearing Doxorubicin (Dox), Poly(ethylene glycol) (PEG) and/or N-Acetyl Galactosamine (NAcGal) for drug delivery. These poly(malic acid) derivatives were obtained by chemical modification of the carboxylic acid lateral groups of poly(malic acid) (PMLA). The resulting nanoplateforms were evaluated for their *in vitro* cytotoxicity using the human HepaRG hepatoma cell line. Results reveal that the PMLA nanoplateform modified with PEG and Dox has an IC_{50} of 936 nM corresponding to a Dox concentration of 47 nM, while the grafting of NAcGal onto the nanoplateform reduced the IC_{50} to 527 nM corresponding to a Dox concentration of 26 nM. The presence of the targeting moiety, NAcGal, thus improves the cellular toxicity of the Dox.

Keywords: Poly(malic acid) prodrug, Doxorubicin, N-Acetyl Galactosamine, hepatocellular carcinoma.

Introduction

Hepatocellular carcinoma (HCC) is one of the most lethal cancers widely prevalent in the world with only limited treatment options that depend on the stage of the disease, namely very early/early, intermediate or advanced/late stage [1,2]. The available therapies range from surgical resection, liver transplantation to chemotherapy with only limited success [1,3]. Lin et al. have summarized the available treatments of HCC in a recent review and report that, despite the existence of several curative treatments for very early/early stage, only transarterial therapy or chemotherapy (mainly based on Sorafenib) can be proposed to patients with intermediate or advanced/late stage of HCC with medium to short survival time [1]. Therefore, there is an urgent need for new treatments better suited not only to the patient but also to the stage of the HCC, especially for intermediate and advanced/late stages. The aims of designing new therapeutic forms for HCC treatments are to ameliorate the efficiency of the anticancer drug by increasing its accumulation at the cancer site in the liver and decreasing its uncontrolled biodistribution as well as the drug efflux by membrane channels while reducing its systemic toxicity and improving the quality of the patient's life [3]. These goals, however, represent major challenges.

To answer these challenges, several drug delivery systems (DDS) have been developed in the past and some of these have been approved by the Food and Drug Administration (FDA) for clinical uses or are under clinical trials [4].

Among the several DDS developed, polymer-drug conjugates might be considered as promising nanovectors. They are based on the model proposed by Ringsdorf in 1975 which consists of a polymeric backbone bearing a solubilizer (mainly PEG), a drug (linked through a degradable bond), a targeting molecule and/or a fluorescence probe allowing to follow the biodistribution of the prodrug [5]. Tumor cell targeting is usually achieved by both passive targeting, through the Enhanced Permeability Retention (EPR) effect [6,7] and active

targeting, using the ligand/receptor interaction [4]. Several targeting agents (TA) have been described for their capacity to target human hepatic cancer cells and to promote cellular uptake of the carriers thus improving the drug efficiency [8-12]. Among the HCC specific cell membrane receptors, the Asialoglycoprotein (ASGP) receptor is largely present on the hepatic cell surface and more specifically in human HCC [3]. Moreover, ASGP receptor is known to be capable of specifically recognizing and binding to galactose and galactosamine residues [3,13]. For example, the NAcGal has been shown to bind the ASGP receptor expressed on the surface of human hepatic cancer cells and trigger efficient cellular uptake via the receptor-mediated endocytosis [14]. Therefore, NAcGal can be used as a targeting moiety in the design of site-specific drug carriers for HCC treatments.

Besides, a DDS needs to be protected against non-specific recognition by the Reticuloendothelial System (RES) or macrophage system in order to increase its circulation time in the body. A hydrophilic polymer, PEG, approved by the FDA in Humans has been widely used for this purpose [15-17]. The stealth property, conferred to a DDS thanks to the presence of PEG, depends on both the molar mass and the density of PEG [15-17]. In view of the published results, a PEG having an amine end group (for the chemical coupling to the polymer backbone) and a molar mass of 5,056 g/mol may be selected for designing a long term circulating DDS.

Various biocompatible and/or (bio)degradable polymers have been evaluated as potential drug carriers [18,19]. Among them, polyesters represent an attractive family for biomedical applications as a result of their good biocompatibility and (bio)degradability. In this context, PMLA attracts our attention because, in addition of being (bio)degradable, biocompatible, non-toxic and non-immunogenic, it also has carboxylic acid lateral functional groups, which can be used to chemically bind various molecules of interest such as drugs, targeting moieties, etc. PMLA can be obtained either from the slime mold (*Physarum polycephalum*) [20] or by

the deprotection of the benzyl lateral groups of the poly(benzyl malate) prepared by anionic ring opening polymerization of the corresponding monomer, the benzyl malolactonate (MLABe) [21]. The chemical synthesis of PMLA is of interest because a large family of polymers can be obtained by varying the nature of the lateral groups, the molar masses, and the nature of the polymers (homopolymers, random or block copolymers) [22].

In the present study, we have chemically modified a PMLA obtained by chemical synthesis in order to introduce several molecules of interest: (i) Doxorubicin, an anti-cancer drug, (ii) NAcGal, a targeting moiety for liver cancer cells and (iii) PEG, a polymer allowing to confer stealth properties to the DDS. The synthesized polymer-drug nanoconjugates were well characterized and evaluated *in vitro* on a hepatoma cell line, the HepaRG. The cytotoxicity of the Dox linked to the PMLA was thus evaluated and related to the presence or absence of the NAcGal targeting moiety.

Experimental part

Materials

The racemic benzyl malolactonate (MLABe) was synthesized from DL-aspartic acid according to the previously reported method [21]. In our method, we used dichloromethane instead of benzene.

All the chemicals were used as received. Anhydrous THF was obtained from Aldrich. Anhydrous ethanol was prepared just before use by distillation over sodium under N₂ atmosphere. Nuclear magnetic resonance spectra (¹H NMR) were recorded on a Bruker ARX400 instrument (¹H at 400 MHz). Data are reported as follows: chemical shift (multiplicity, number of hydrogen). The chemical shifts are reported as parts per million (ppm) referenced to the appropriate residual solvent peak. Abbreviations are as follows: s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublet), m (multiplet).

Weight average molar masses (\overline{Mw}) and molar mass distribution ($\text{Đ} = \overline{Mw}/\overline{Mn}$) of PMLABe were determined by size exclusion chromatography (SEC) in THF at 40°C (flow rate=1.0mL/min) on a GPC2502 Viscotek apparatus equipped with a refractive index detector, a LT5000L mixed medium org 300*7.8mm gel column, a TGuard Org Guard Col 10*4.6 mm and a GPC/SEC OmniSEC software. The polymer samples were dissolved in THF at a concentration of 2 mg/mL. All elution curves were calibrated with poly(styrene) standards.

\overline{Mw} and $\text{Đ} = \overline{Mw}/\overline{Mn}$ of the PMLA were determined by size exclusion chromatography (SEC) in a 0.15 M phosphate buffer solution of pH7.4, 1 M NaCl at 35°C (flow rate=0.5mL/min) on a GPC/SEC Malvern apparatus equipped with a refractive index detector (VE3580) and a LALS-RALS 270 Dual Detector, a A4000-300*8 mm gel column, a 50*6 mm guard column and a GPC/SEC OmniSEC software. The polymer samples were dissolved in 0.15 M phosphate buffer solution pH 7.4, 1 M NaCl at a concentration of 2 mg/mL. All elution curves were calibrated with Pullulan standards.

The size (average diameter obtained by the CONTIN method), polydispersity and zeta potential, (the measured electrophoretic mobility (μ) was converted to zeta potential (ζ) using the Smoluchowski approximation) of the formulations were measured by Dynamic Light Scattering using a Delsa™ Nano Beckman Coulter apparatus at 25°C.

Fourier Transform Infra-Red (FTIR) spectra were recorded using a Fourier Nicolet 250 apparatus at 25°C.

UV spectra were recorded using a microplate reader (BioTek) at 485 nm for quantification of Dox in the nanoconjugates and on a Secoman apparatus at 515 nm for MTT assays.

For the UV characterization of the nanoconjugates modified with Dox, we first realized a calibration curve using Dox dissolved into PBS at pH7 with concentrations ranging from

0.0625 mg/mL to 0.00098 mg/mL (Figure S1). The equation giving the concentration of Dox (mg/mL) as a function of the absorbance (Abs) at 485 nm is the following:

$$[Dox] = 0.0833 * Abs - 0.0045$$

with $R^2 = 0.9998$ (Figure S1).

The HepaRG hepatoma cells [23-25] were cultured as previously described in William's E medium (Lonza) supplemented with 2 mM of glutamine (Gibco), 5 mg/L of insulin (Sigma), 10^{-5} M hydrocortisone hemisuccinate and 10% of fetal calf serum (Lonza).

Synthesis of poly(benzyl malate), PMLABe:

Poly(benzyl malate) was synthesized as described previously [21] starting from 3 g (1.46×10^{-2} mol) of racemic MLABe in presence of benzoate tetrabutylammonium (5.02×10^{-5} mol) as initiator in bulk at 37°C. The polymerization was followed by FTIR and stopped when the band, characteristic of MLABe, at $1,850 \text{ cm}^{-1}$ had totally disappeared from the spectrum. The resulting PMLABe was solubilized in a minimum volume of acetone and precipitated using a large excess of ethanol. The precipitate was recovered and dried under vacuum leading to 2.53 g (yield= 84%) of PMLABe which was then analyzed by ^1H NMR (structure) and SEC in THF (molar masses and molar mass distribution).

^1H NMR (CD_3COCD_3 , δ ppm, 400MHz): 2.84-3.10 (m, 2nH, CHCH_2CO_2), 5.09-5.19 (m, 2nH, $\text{CO}_2\text{CH}_2\text{C}_6\text{H}_5$), 5.47-5.63 (m, 1nH, CHCH_2CO_2), 7.25-7.46 (m, 5nH, $\text{CO}_2\text{CH}_2\text{C}_6\text{H}_5$).

SEC (THF, polystyrene standards, 1 mL/min, 40°C): $\overline{M}_w = 5,540 \text{ g/mol}$, $\overline{D} = 1.63$

Synthesis of PMLA:

The PMLA was obtained by catalytic hydrogenolysis of the synthesized PMLABe. PMLABe (2.53 g) was solubilized in 10 mL of acetone and 20 wt% of palladium on activated charcoal (Pd/C, 0.51 g) were added to the solution. The round bottomed flask containing the PMLABe and the Pd/C was placed under hydrogen atmosphere. The mixture was vigorously stirred for 12 h under hydrogen atmosphere. The solution was then filtered over celite and the acetone

was evaporated under vacuum. The obtained crude PMLA was solubilized in water, poured into a dialysis bag (Molecular weight cut off, MCWO, of 3,500 g/mol) and dialyzed against water overnight. The solution contained into the dialysis bag was lyophilized leading to a white powder of PMLA (0.99 g, yield= 78%). It was characterized by ^1H NMR and SEC in 0.15M phosphate buffer solution (PBS) pH7.4 containing 1M of NaCl.

^1H NMR (CD_3COCD_3 , δ ppm, 400MHz): 2.86-3.12 (m, 2nH, CHCH_2CO_2), 5.44-5.58(m, 1nH, CHCH_2CO_2), 6.78-7.85(m, 1nH, CO_2H).

SEC (0.15 M PBS pH7.4, 1 M of NaCl, Pullulan standards): $\overline{M}_w = 7,910$ g/mol, $\text{Đ} = 1.2$.
Degree of polymerization= $7,910/116 = 68$.

Activation of PMLA with N-hydroxysuccinimide (NHS):

PMLA (0.84 g, 7.24×10^{-3} mol, $M_{\text{repeat unit}} = 116$ g/mol) was dissolved in 12 mL of anhydrous acetone under N_2 atmosphere and the flask was placed in an ice bath. In parallel, 2 eq. (1.45×10^{-2} mol) of N-hydroxysuccinimide (NHS) were dissolved in 5 mL of anhydrous dimethyl formamide (DMF) and transferred into the PMLA solution under N_2 atmosphere. Then, 2 eq. (1.45×10^{-2} mol) of dicyclohexylcarbodiimide (DCC) were dissolved into 5 mL of anhydrous DMF and transferred under N_2 atmosphere into the PMLA/NHS solution maintained in the ice bath. The mixture was stirred at room temperature (RT) for 4 h. The dicyclohexyl urea (DCU) formed was eliminated by filtration and the acetone was evaporated under vacuum. The activated polymer, PMLA-NHS, was precipitated using a large excess of ether. The precipitate was recovered, dried under vacuum and solubilized in acetone. This solution was poured into a dialysis bag (MWCO=3,500 g/mol) and was dialysis against acetone overnight. The solution contained into the dialysis bag was evaporated under vacuum leading to 0.54 g of PMLA-NHS (yield= 64%). The purified polymer was characterized by ^1H NMR.

^1H NMR (CD_3COCD_3 , δ ppm, 400MHz): 2.67 (s, 4H, NHS, 55%), 2.86-3.12 (m, 2nH, CHCH_2CO_2), 5.45-5.58 (m, 1nH, CHCH_2CO_2).

Grafting PEG onto activated PMLA-NHS:

The activated polymer (0.3 g, $1.43 \cdot 10^{-3}$ mol, $M_{\text{repeat unit}} = 213$ g/mol) was dissolved in 4mL of anhydrous DMF under N_2 atmosphere. α -methoxy, ω -amino PEG ($\text{PEG}_{5056}\text{-NH}_2$; $\overline{M}_w = 5,056$ g/mol; number of repeating unit: 113; 5 mol%; $7.04 \cdot 10^{-5}$ mol) and trimethylamine (TEA, 5 mol%, $7.04 \cdot 10^{-5}$ mol), dissolved in 2 mL of anhydrous DMF, were transferred to the PMLA-NHS solution under N_2 atmosphere. The mixture was stirred under N_2 atmosphere for 1 h. The solution was then separated into three batches of 2 mL each, containing 0.1 g of PMLA-NHS- PEG_{5056} . Two batches were used for further chemical modifications as described in the following paragraphs.

One batch [containing 0.1 g ($4.69 \cdot 10^{-4}$ mol) of PMLA-NHS + 5mol% of PEG_{5056} (0.119 g)] was stirred overnight at room temperature. Then 5 mL of sodium buffer solution pH5.5 were added to the PMLA-NHS- PEG_{5056} to transform the residual NHS groups into carboxylic acid functions. This solution was stirred at room temperature for 1 h and then centrifuged at 1,500 g for 10 min. The supernatant was lyophilized and the resulting polymer was dialyzed against water ($\text{MWCO} = 3,500$ g/mol) for 12 h. The solution contained into the dialysis bag was then lyophilized leading to 0.113 g of PMLA- PEG_{5056} (yield= 52 %). This polymer obtained was characterized by ^1H NMR.

^1H NMR (CD_3COCD_3 + 2 drops of D_2O , δ ppm, 400MHz): 2.86-3.12 (m, 2nH, CHCH_2CO_2), 3.60 (s, $4 \cdot 113\text{H}$, PEG_{5056} , 5 mol%), 5.45-5.58 (m, 1nH, CHCH_2CO_2).

Molar mass of PMLA- $\text{PEG}_{5056} = 23,053$ g/mol [Molar mass= $68 \cdot 0.95 \cdot 116 + 68 \cdot 0.05 \cdot (115 + 5,056)$].

Grafting NAcGal onto PMLA-NHS-PEG₅₀₅₆

Five mol% of NAcGal (M= 221.21 g/mol, 2.35×10^{-5} mol) dissolved in 1 mL of anhydrous DMF, was added to one batch of PMLA-NHS (0.1 g, 4.69×10^{-4} mol) + 5 mol% of PEG₅₀₅₆. The solution was stirred overnight at room temperature. Then, 5mL of sodium buffer solution of pH5.5 were added to the PMLA-NHS-PEG₅₀₅₆-NAcGal to transform the NHS groups into carboxylic acid functions. This solution was stirred at room temperature for 1 h and centrifuged at 1,500 g for 10 min. The supernatant was lyophilized and the resulting polymer was dialyzed against water (MWCO= 3,500 g/mol) for 12 h. The solution contained into the dialysis bag was then lyophilized leading to 0.129 g of PMLA-PEG₅₀₅₆-NAcGal (yield= 58 %). The polymer obtained was characterized by ¹H NMR.

¹H NMR (CD₃COCD₃ + 2 drops of D₂O, δ ppm, 400MHz): 2.86-3.12 (m, 2nH, CHCH₂CO₂), 3.60 (s, 4*113H, PEG₅₀₅₆, 5 mol%), 5.45-5.58 (m, 1nH, CHCH₂CO₂).

Molar mass of PMLA-PEG₅₀₅₆-NAcGal= 23,714 g/mol [Molar mass= $68 \times 0.90 \times 116 + 68 \times 0.05 \times (115 + 5,056) + 68 \times 0.05 \times (115 + 221.21)$].

Grafting Dox onto PMLA-NHS-PEG₅₀₅₆

Five mol% of Dox (M= 579.98 g/mol, 2.35×10^{-5} mol), dissolved into 2mL of anhydrous DMF containing 3 μL of trimethylamine (NEt₃), were added to one batch of PMLA-NHS (0.1 g, 4.69×10^{-4} mol) + 5 mol% of PEG₅₀₅₆. The solution was stirred for 1 h at room temperature under N₂ atmosphere in the dark. This solution was then separated into two batches of 1 mL, each containing 0.115 g of PMLA-NHS-PEG₅₀₅₆-Dox. One batch was used to link the NAcGal as described in the following paragraph.

The other batch was stirred overnight at room temperature in the dark. Then, 2.5 mL of sodium buffer solution pH5.5 were added to the PMLA-NHS-PEG₅₀₅₆-Dox to transform the NHS groups in carboxylic acid functions. This solution was stirred at room temperature for 1 h in the dark and centrifuged at 1,500 g for 10 min. The supernatant was lyophilized and the

resulting polymer was dialyzed against water (MWCO= 3,500 g/mol) for 12 h. The solution contained into the dialysis bag was then lyophilized leading to 0.102 g of PMLA-PEG₅₀₅₆-Dox (yield= 87 %). This polymer obtained was characterized by ¹H NMR and UV at 485 nm (quantification of Dox).

¹H NMR (CD₃COCD₃ + 2 drops of D₂O, δ ppm, 400MHz): 2.86-3.12 (m, 2nH, CHCH₂CO₂), 3.60 (s, 4*113H, PEG₅₀₅₆, 5 mol%), 5.45-5.58 (m, 1nH, CHCH₂CO₂).

UV at 485 nm: Abs= 0.180, [Dox]= 0.0136mg/mL, Dox: 2.34*10⁻⁵ mol (5mol%).

Molar mass of PMLA-PEG₅₀₅₆-Dox= 24,790 g/mol [Molar mass= 68*0.90*116 + 68*0.05*(115+5,056) + 68*0.05*(115+579.98)].

Grafting NAcGal onto PMLA-NHS-PEG₅₀₅₆:

Five mol% of NAcGal (M= 221.21 g/mol, 2.35*10⁻⁵ mol) dissolved in 1 mL of anhydrous DMF were added to one batch of PMLA-NHS (0.1 g, 4.69*10⁻⁴ mol) + 5 mol% of PEG₅₀₅₆ + 5 mol% of Dox. The solution was stirred overnight at room temperature in the dark. Then, 2.5 mL of sodium buffer solution of pH5.5 were added to the PMLA-NHS-PEG₅₀₅₆-Dox-NAcGal to transform the NHS groups into carboxylic acid functions. This solution was stirred at room temperature for 1 h in the dark and centrifuged at 1,500 g for 10 min. The supernatant was lyophilized and the resulting polymer was dialyzed against water (MWCO= 3,500 g/mol) for 12 h. The solution contained into the dialysis bag was then lyophilized leading to 95 mg of PMLA-PEG₅₀₅₆-Dox-NAcGal (yield= 81 %). The polymer obtained was characterized by ¹H NMR and UV at 485 nm (quantification of Dox).

¹H NMR (CD₃COCD₃ + D₂O, δ ppm, 400MHz): 2.86-3.12 (m, 2nH, CHCH₂CO₂), 3.60 (s, 4*113H, PEG₅₀₅₆, 5 mol%), 5.45-5.58 (m, 1nH, CHCH₂CO₂).

UV at 485 nm: Abs= 0.179, [Dox]= 0.0135mg/mL, Dox: 2.33*10⁻⁵ mol (5mol%).

Molar mass of PMLA-PEG₅₀₅₆-Dox-NAcGal= 25,103 g/mol [Molar mass= 68*0.85*116 + 68*0.05*(115+5,056) + 68*0.05*(115+579.98) + 68*0.05*(115+221.21)].

Preparation of the nanoconjugate solutions:

The nanoconjugates obtained were directly dissolved into a phosphate buffer solution (pH 7.5) at a concentration of 1 to 2 mg/mL. The resulting clear solutions were filtered through a 0.2 μm pore membrane and analyzed using a DelsaNano apparatus to determine their average diameter (\bar{D}), size dispersity (PDI) and zeta potential (ζ). Results obtained are shown in Table 2 in Results & Discussion section.

The stability of the nanoconjugates was followed by DLS measurements after 24h, 48h and 72h of storage at 4°C. The results obtained are shown in Table 3 in Results & Discussion section.

In vitro cytotoxic studies:

The prepared nanoconjugates were evaluated for their *in vitro* cytotoxicity on HepaRG hepatoma cells using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. During sub-culturing, 2×10^6 cells were seeded in a 75 cm^2 flask. The medium was renewed every 48 h. The sub-culturing was realized by trypsinization every 2 weeks in order to maintain the progenitor phenotype. For an optimal differentiation, the cells were maintained at confluence after the 2 weeks and the medium was supplemented with 2% of dimethylsulfoxide (DMSO) [25]. For the cytotoxicity assays, 96 well culture plates were seeded 10^5 cells per well. The nanoconjugate preparations were then added to the wells at concentrations ranging from 2 μM to 11 nM as shown in Table 1. The initial solutions of nanoconjugates at a concentration of 2 mg/mL were prepared by diluting the nanoconjugate with PBS.

Table 1

The cells were incubated for 24, 48 and 72h. After incubation, 100 μL of MTT solution were added to each well. The culture plate was incubated for 2 h. The medium was then removed by vacuum and a few μL of PBS were added to each well. This PBS was then removed by

vacuum and 100 μ L of DMSO were added to each well to solubilize the formazane crystals. The absorbance of each well was read by UV at 515 nm.

Results and Discussion

Synthesis and characterization of PMLA derivatives

As shown in Scheme 1, the PMLABe was synthesized by anionic ring opening polymerization of MLABe which was obtained in four steps starting from aspartic acid, as described previously [21].

Scheme 1

The molar mass of PMLABe ($\overline{M}_w = 5,540$ g/mol) measured by SEC in THF was lower than the theoretical value because the analytical technique involves a calibration curve (to link the retention time or volume to the molar mass) based on poly(styrene) standards, that have a chemical structure different from PMLA. Moreover, the solvent (THF) used to realize this SEC analysis is not the best solvent for PMLABe. On the other hand, it was not possible to determine the molar mass using the ^1H NMR spectrum of the PMLABe because the end chains, a benzoate group at one end and a carboxylic acid at the other hand, are not visible on the spectrum. Consequently, we are only able to conclude that the dispersity of the synthesized PMLABe ($\mathcal{D} = 1.63$) is in good agreement with the data given in the literature for this kind of polymerization [21].

The catalytic hydrogenolysis of benzyl lateral groups of the synthesized PMLABe gives access to the expected PMLA without any degradation of the ester groups of the polymer backbone, as shown by the ^1H NMR spectrum of the obtained PMLA (Figure 1).

Figure 1

Indeed, such catalytic deprotection of lateral benzyl ester groups is known to be specific and does not affect the ester groups in the main chain [26]. The number of units of the PMLA is,

therefore, the same as the ones of the PMLABe and no degradation of the polymer occurs under such conditions. The molar mass of PMLA was measured by SEC in a 0.15 M phosphate buffer solution (PBS) at pH7.4 in presence of 1 M NaCl with Pullulan as standards (Figure 2).

Figure 2

As shown by Figure 2, only one peak was observed in the chromatogram at a molar mass of 7,950 g/mol and a quite narrow dispersity. The number of malic acid units was calculated from the molar mass (7,950 g/mol) and the molar mass of the repeating unit (116 g/mol) as follow: $n = 7,950/116 = 68$.

Starting with this PMLA, we have used the lateral carboxylic acid functions to introduce the molecules of interest we have selected for this study, namely the PEG₅₀₅₆ to confer stealth properties to the nanoconjugate [15-17], Dox, an anticancer drug, as a drug model [27] and the NAcGal as the targeting agent for cancer cells [3,13,14] (Scheme 2).

Scheme 2

The carboxylic acid lateral functions of the PMLA were first activated by NHS through the reaction between the carboxylic acids and NHS in the presence of DCC [28]. After purification, the PMLA-NHS was analyzed by ¹H NMR. The NMR spectrum showed that the chemical modification is successful (Figure 3). However, only 55% of the lateral carboxylic acid functions are activated under NHS form. Such a result is not surprising because the chemical modification of lateral groups of polymers is hardly quantitative [29].

Figure 3

However, even if only 55% of the carboxylic acid functions are activated, it is enough to continue the modification of the PMLA because we have chosen to introduce only 5 mol% of each selected molecules. This amount was selected based on the results obtained by Ljubimova et al. with natural PMLA [28+ references cited within]. These authors have

highlighted that the modification of the natural PMLA with 5 mol% of each selected molecule (Dox, Temozoline Hydrazide, anti-sense oligonucleotides, PEG, Alexa Fluor 680, etc.) leads to the expected results, both *in vitro* and *in vivo*. These nanoconjugates, called Polycephin®, were designed to treat brain and/or breast cancers [30,31]. As shown in Scheme 1, we introduced first a telechelic PEG with a molar mass of 5,056 g/mol with an amine group at one end and a methoxy group at the other end. Starting from this PMLA-NHS-PEG₅₀₅₆ polymer, we introduced 5 mol% of NAcGal and/or 5 mol% of Dox (Scheme 2), to the remaining activated carboxylic acid functions in order to obtain, four PMLA derivatives, namely PMLA-PEG₅₀₅₆, PMLA-PEG₅₀₅₆-NAcGal, PMLA-PEG₅₀₅₆-Dox and PMLA-PEG₅₀₅₆-Dox-NAcGal from the same PMLA batch. Each time, the remaining NHS groups were eliminated under mild conditions by the addition of sodium buffer solution at pH5.5. Such mild conditions avoid the possible degradation of the ester groups of the PMLA main chain [28]. The synthesized macromolecular pro-drugs were characterized by ¹H NMR. The ¹H NMR spectrum of the PMLA-PEG₅₀₅₆ (Figure 4) shows the peak characteristic of the two methylene (CH₂) protons at 3.6 ppm, and only the peak characteristic of the methine (CH) protons of the PMLA backbone between 5.45-5.58 ppm, the peak characteristic of the methylene protons of the PMLA backbone being hidden by the peak of water.

Figure 4

Nevertheless, we were able to calculate the percent of PEG linked to the PMLA backbone from the relative integration of the PMLA's methine protons and the PEG's methylene protons as follow:

$$\%PEG = \left(\frac{[\text{Integration of CH}_2 \text{ of PEG}]/4}{[\text{Integration of CH of PMLA}]} \right) * 100$$

We found that 5 mol% of PEG were effectively linked to the PMLA backbone, as expected. Unfortunately, the ¹H NMR spectra of the three other nanoconjugates do not allowed us to determine the percent of Dox and/or NAcGal linked to the PMLA backbone because the

peaks characteristics of these molecules were hidden either by the peaks of the solvent used for the NMR ($\text{CD}_3\text{CODC}_3 + 2$ drops of D_2O) or by the ones of the PEG. We realized the proton NMR of our nanoconjugates in other deuterated solvents, D_2O and DMSO-d_6 , but the resulting spectra showed only the characteristic peak of the methylene protons of the PEG as a result of the specific conformation adopted by the nanoconjugates in these solvents. Indeed, water and DMSO promote the aggregation of the nanoconjugates and only the more hydrophilic parts of the nanoconjugates are visible. However, because the binding of the PEG chains to the PMLA backbone was shown to be as expected, we can reasonably assume that the binding of Dox and/or NAcGal, which are small molecules, is also successful. Consequently, in view of the purification steps done on our nanoconjugates (dialysis allowing to eliminate all the unbounded small molecules) and results obtained for PMLA modification with PEG, we considered that 5 mol% of Dox and/or NAcGal were effectively linked to the PMLA backbone. To confirm that 5 mol% of Dox were effectively linked to the PMLA backbone, we analyzed the nanoconjugates containing Dox by UV at 485 nm.

Table 2

Indeed, such analysis allows to link the absorbance of the solution containing Dox to its concentration using a calibration curve (Figure S1). As shown by the results gathered in Table 2, the amount contained in the PMLA-PEG₅₀₅₆-Dox and PMLA-PEG₅₀₅₆-Dox-NAcGal nanoconjugates were $2.34 \cdot 10^{-5}$ mol and $2.33 \cdot 10^{-5}$ mol, respectively, corresponding to around 5 mol% of Dox in each nanoconjugate.

Nevertheless, we are still working on the characterization of the PMLA-PEG₅₀₅₆-NAcGal, PMLA-PEG₅₀₅₆-Dox and PMLA-PEG₅₀₅₆-Dox-NAcGal nanoconjugates (structure, percent of molecules linked to the PMLA backbone). Therefore, we are currently looking for a solvent to realize the proton NMR in which all the parts of the nanoconjugates can be visible and thus analyzed. Moreover, we are also investigating the possibilities to use MALDI and ESI to

characterize our nanoconjugates with a major difficulty coming from the presence of the carboxylic acid lateral function which can prevent the correct ionization of the nanoconjugates together with the multiple ways for the nanoconjugates to be degraded under the conditions used for MALDI and ESI measurements. Finally, HPLC is an analytical method which we also intend to use to determine the amounts of Dox and NAcGal present in our compounds. The results obtained thanks to these three analytical methods will have to be compared in order to confirm: *i*). that the Dox and/or NAcGal were effectively linked to the PMLA backbone and *ii*). the amount of Dox and/or NAcGal present in our nanoconjugates.

Preparation of the nanoconjugates

The four prodrugs synthesized were then solubilized in a phosphate buffer solution at pH7.5 containing 0.15N NaCl at a concentration of 1 to 2 mg/mL. The resulting clear solutions were analyzed by dynamic light scattering (DLS) to obtain the average diameter and dispersity of the nanoconjugates, in addition to zeta potential measurements to obtain the charge on the nanoconjugates. As shown in Table 3, we were able to obtain aggregates from all the macromolecular prodrugs with apparent hydrodynamic diameter ranging from 70 to 140 nm.

Table 3

The dispersity of the nanoconjugates obtained, however, are quite high, ranging from 0.34 to 0.71, denoting that they form aggregates rather than well-defined nano-objects. Such large dispersity values are in good agreement with the nature of the polymers used in this study. Indeed, the synthesized macromolecular prodrugs are made up of macromolecular chains with different side groups with various hydrophilicity/hydrophobicity properties and distributed randomly throughout the chain. Such structure can only lead to the formation of loose aggregates in aqueous media, unlike amphiphilic block copolymers known to self-aggregate in aqueous media leading to the formation of nanoparticles [32].

The behavior of the nanoconjugates was obtained under storage at 4°C in PBS pH7.5, 0.15 N NaCl by measuring the average diameter and dispersity by DLS. Results obtained (Table 4) reveal a significant increase in the average diameter for two nanoconjugates, namely the PMLA-PEG₅₀₅₆ and the PMLA-PEG₅₀₅₆-NACGal, from 70/80 nm to 180/290 nm after 72 h of incubation at 4°C together with a stability of the dispersity at around 0.60. Such observation might be due to the formation of very loose aggregates that undergo severe changes upon incubation such as the fusion of aggregates. Further studies are, however, required using other techniques such as the Asymmetrical Field-Flow Fractionation (AFFF), which allows not only to characterize nanoparticles [33] but also the behavior of macromolecules in solution [34]. For the nanoconjugate PMLA-PEG₅₀₅₆-Dox, a significant decrease in the average diameter, from 140 to 60 nm, was first observed after 48 h of incubation at 4°C together with an increase in the dispersity (from 0.34 to 0.67), probably showing the formation of nanoparticles-like structures as a result of the presence of hydrophobic Dox molecules.

Table 4

After 72 h of incubation at 4°C, however, the average diameter reached the initial value, showing possible degradation of the aggregates as a result of too weak hydrophobic interactions within the nanoconjugate in comparison to that observed for nanoparticles. In this case also, the AFFF techniques might bring more information on the behavior of this nanoconjugate in aqueous solutions. Finally, the nanoconjugate PMLA-PEG₅₀₅₆-Dox-NACGal seems to be quite stable in aqueous media over 72 h of incubation at 4°C, probably as a result of the presence of both the Dox and NACGal increasing the forces driving the formation of aggregates.

We are currently studying the Dox release from the PMLA-PEG₅₀₅₆-Dox and PMLA-PEG₅₀₅₆-Dox-NACGal nanoconjugates under different conditions of pH and temperature.

In vitro cytotoxicity assays

As the nanoconjugates were synthesized to be used as site-specific drug carriers to treat hepatocellular carcinoma (HCC), we first evaluated their *in vitro* cytotoxicity on the human HepaRG hepatoma cell line [23-25]. The nanoconjugates were incubated at concentrations ranging from 7 nM to 1.52 μ M with HepaRG cells and the cell viability was measured using the MMT assay after 1, 3 and 7 days of incubation (Figure 5 & Figure 6). The red line, plotted on each graph, is the half maximal inhibitory concentration (IC_{50}), a concentration at which 50% of the cells are dead, and allow to determine the cytotoxicity of the material studied. No cytotoxicity effect was observed for the PMLA-PEG₅₀₅₆-NACGal (Figure 5 B), at the different time points of incubation even at a very high concentration as the cell viability stayed above 80%. We can conclude, therefore, that this compound does not have an acute toxicity on the HepaRG cell line.

Figure 5

The nanoconjugate, PMLA-PEG₅₀₅₆ does not show *in vitro* cytotoxicity up to a concentration of 1.2 μ M (Figure 5 A). Surprisingly, the cell viability decreases to about 80% for a concentration of 1.52 μ M after 1 and 3 days of incubation and about 40% for a concentration of 1.52 μ M after 7 days of incubation. An IC_{50} of 1.25 μ M was obtained for the PMLA-PEG₅₀₅₆ nanoconjugate after 7 days of incubation. Since the PMLA is biocompatible even at high concentrations [35], this cytotoxicity might come from the presence of PEG chains. Indeed, in a recent study on the comparison of the *in vitro* cytotoxicity and hemocompatibility of PEG and poly(2-ethyl-2-oxazoline), Fischer et al. have reported that PEGs with molar masses ranging from 400 to 1,000 g/mol show an effect on cell viability after 12 and 24 h of incubation. This effect was shown to be depending on PEG concentration and the molar mass [23]. It is, therefore, not surprising that we also observe a cytotoxicity effect of the PMLA-PEG₅₀₅₆ nanoconjugate at a higher concentration and longer incubation time.

The PMLA-PEG₅₀₅₆-Dox nanoconjugate showed a cell viability decreasing in a dose dependent manner (Figure 6 A) after 7 days of incubation, while the cell viability stays stable regardless of the concentration after 1 or 3 days of incubation. The IC₅₀ for this PMLA-PEG₅₀₅₆-Dox nanoconjugate is 936 nM corresponding to a concentration in Dox of 47 nM.

Figure 6

Interestingly, the nanoconjugate of PMLA-PEG₅₀₅₆-Dox-NAcGal induces a significant decrease in the cell viability after 3 and 7 days of incubation in a concentration dependent manner (Figure 6 B). The cell viability decreases to around 60% after 3 days of incubation for a concentration of 1.43 μ M. After 7 days of incubation, an IC₅₀ of 527 nM was obtained for the nanoconjugate, PMLA-PEG₅₀₅₆-Dox-NAcGal corresponding to a concentration of 26 nM in Dox.

The IC₅₀ obtained for free Dox is around 94 nM after 3 days of incubation and below 20 nM after 7 days of incubation while almost no cytotoxic effects were observed after 1 day of incubation for the free Dox with HepaRG cells (data not shown).

The results reveal that, the presence of NAcGal on the nanoconjugate, PMLA-PEG₅₀₅₆-Dox-NAcGal improves the effect of Dox since the IC₅₀ of this nanoconjugate (IC₅₀= 26 nM) is lower than the one of the corresponding nanoconjugate without the NAcGal moieties (IC₅₀= 47 nM). NAcGal groups seem therefore to improve the cellular uptake of the nanoconjugates thus leading to a more efficient of cytotoxicity this Dox conjugate. Nevertheless, such conclusion has to be proven by further experiments demonstrating the mechanism of the nanoconjugate uptake by HepaRG cells and whether the NAcGal really plays a role in the nanoconjugate internalization.

Conclusion

As hypothesized, the present work clearly demonstrates that PMLA is a suitable carrier for targeting anticancer drugs to cancer cells. Moreover, the synthesized PMLA-PEG₅₀₅₆-Dox-NAcGal pro-drug nanoconjugate has a higher cellular toxicity when compared to the PMLA-PEG₅₀₅₆-Dox nanoconjugate without the targeting moiety. The targeting moiety NAcGal may enhance the cellular uptake of the nanoconjugate PMLA-PEG₅₀₅₆-Dox-NAcGal, thus improving the cytotoxic effect of the bound Dox *in vitro* against the HepaRG hepatoma cell line. Further works, using confocal fluorescence microscopy and flow cytometry, are presently in progress to prove the target specificity and the cellular uptake of the PMLA based nanoconjugates substituted by the NAcGal targeting molecule.

Acknowledgement

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Figures

Figure 1. ^1H NMR spectra (400 MHz) of PMLA in CD_3COCD_3 .

Figure 2. ^1H NMR spectra (400 MHz) of PMLA-NHS in CD_3COCD_3 .

Figure 3. ^1H NMR spectra (400 MHz) of PMLA-PEG₅₀₅₆ in CD_3COCD_3 + 2 drops of D_2O .

Figure 4. SEC chromatogram of PMLA into 0.15 M phosphate buffer solution, pH7.4 + 1 M NaCl, Pullulan standards.

Figure 5. *In vitro* cell viability measured by the MMT assay after incubation with HepaRG cells of PMLA-PEG₅₀₅₆ (A) and PMLA-PEG₅₀₅₆-NACGal (B).

Figure 6. *In vitro* cell viability measured by the MMT assay after incubation with HepaRG cells of PMLA-PEG₅₀₅₆-Dox (A) and PMLA-PEG₅₀₅₆-Dox-NACGal (B).

Tables

Table 1. Concentration of the nanoconjugates used for the cytotoxicity assays.

Table 2. Results of UV analysis (485 nm) of the nanoconjugates containing Dox.

Table 3. Characteristics of the prepared nanoconjugates in PBS pH7.5, 0.15 N NaCl measured by DLS.

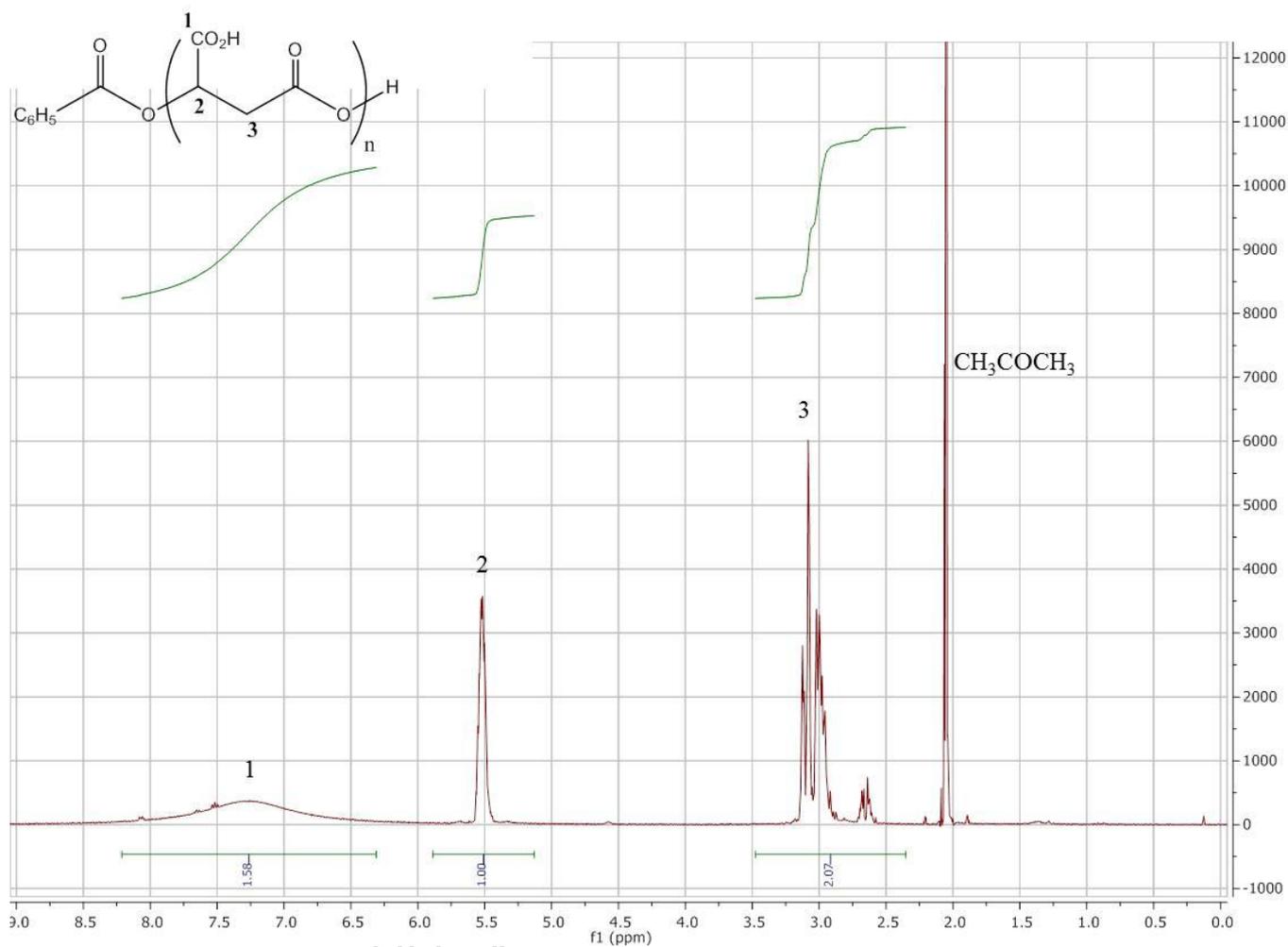
Table 4. Evolution of the average diameter and dispersity of the nanoconjugates upon incubation in PBS pH7.5, 0.15 N NaCl at 4°C followed by DLS.

Schemes

Scheme 1. Synthetic route to PMLA.

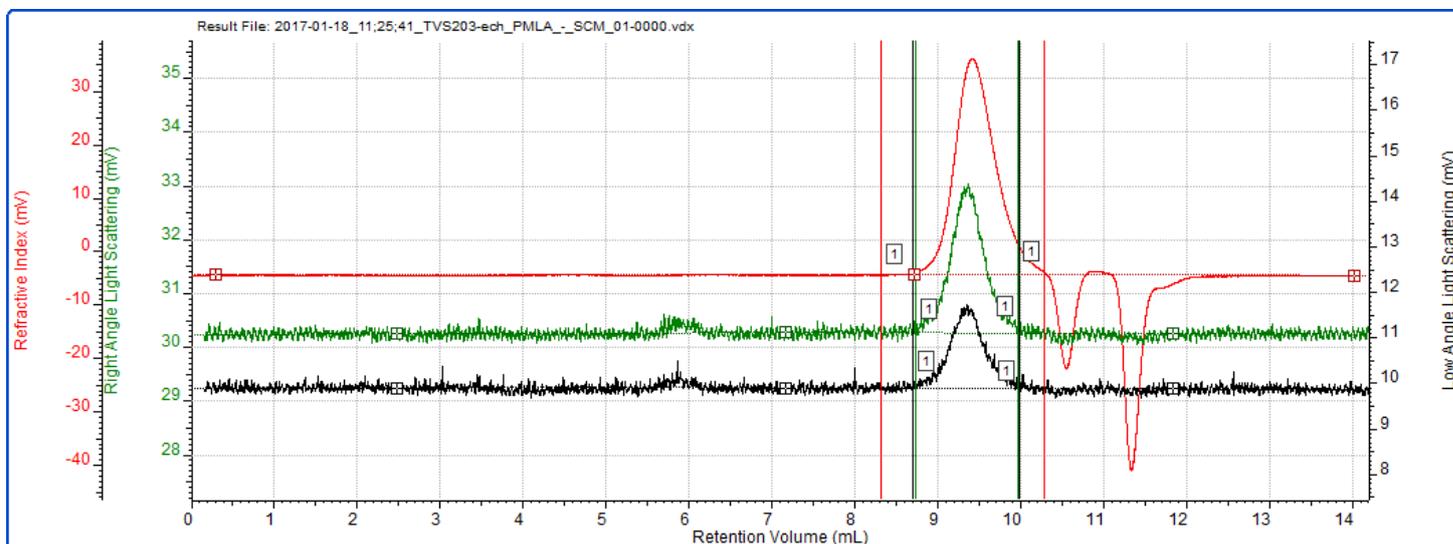
Scheme 2. Synthetic route to PMLA-based nanoconjugates.

Figure 1. ^1H NMR spectra (400 MHz) of PMLA in CD_3COCD_3 .



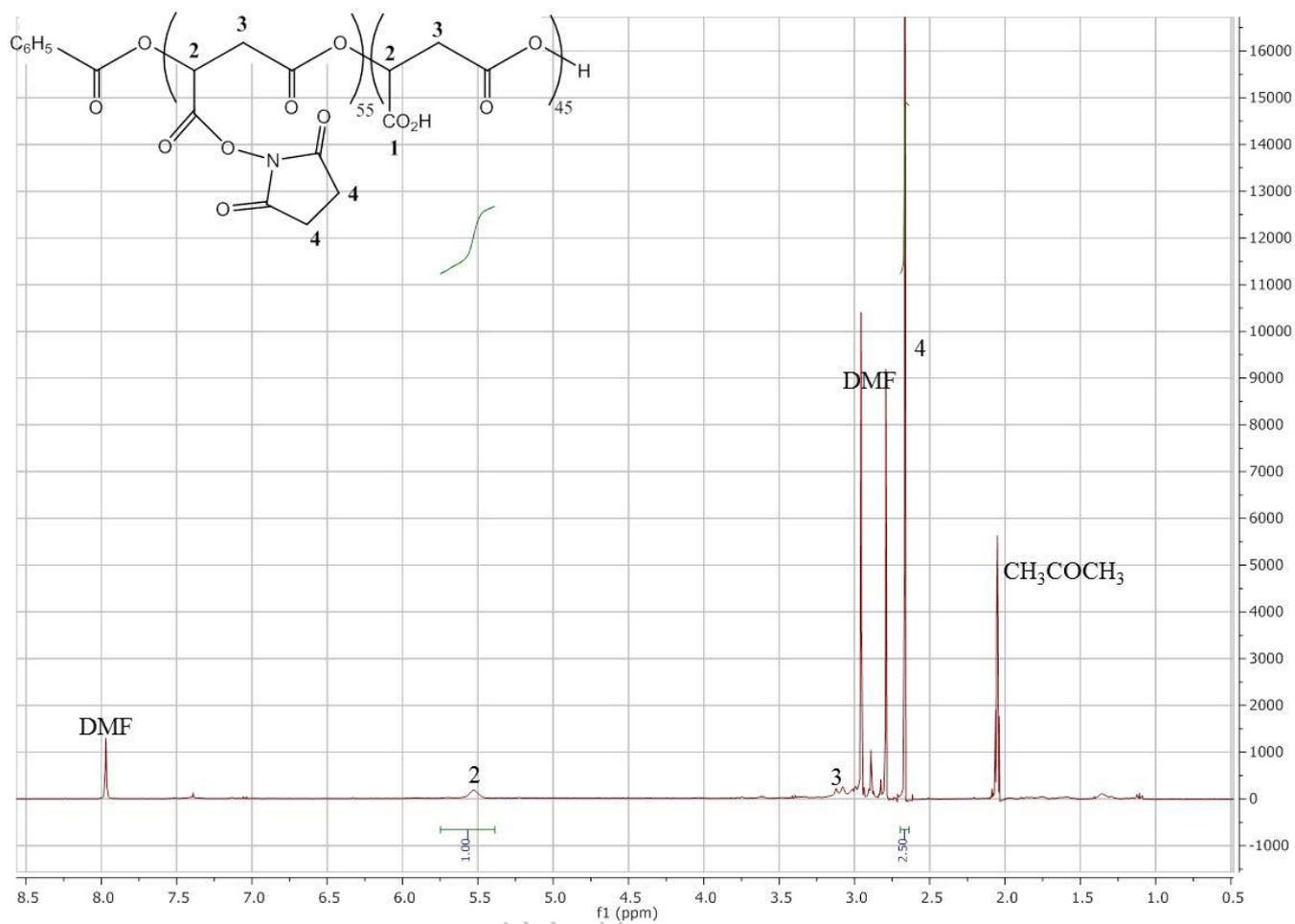
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Figure 2. SEC chromatogram of PMLA into 0.15 M phosphate buffer solution, pH7.4 + 1 M NaCl, Pullulan standards.



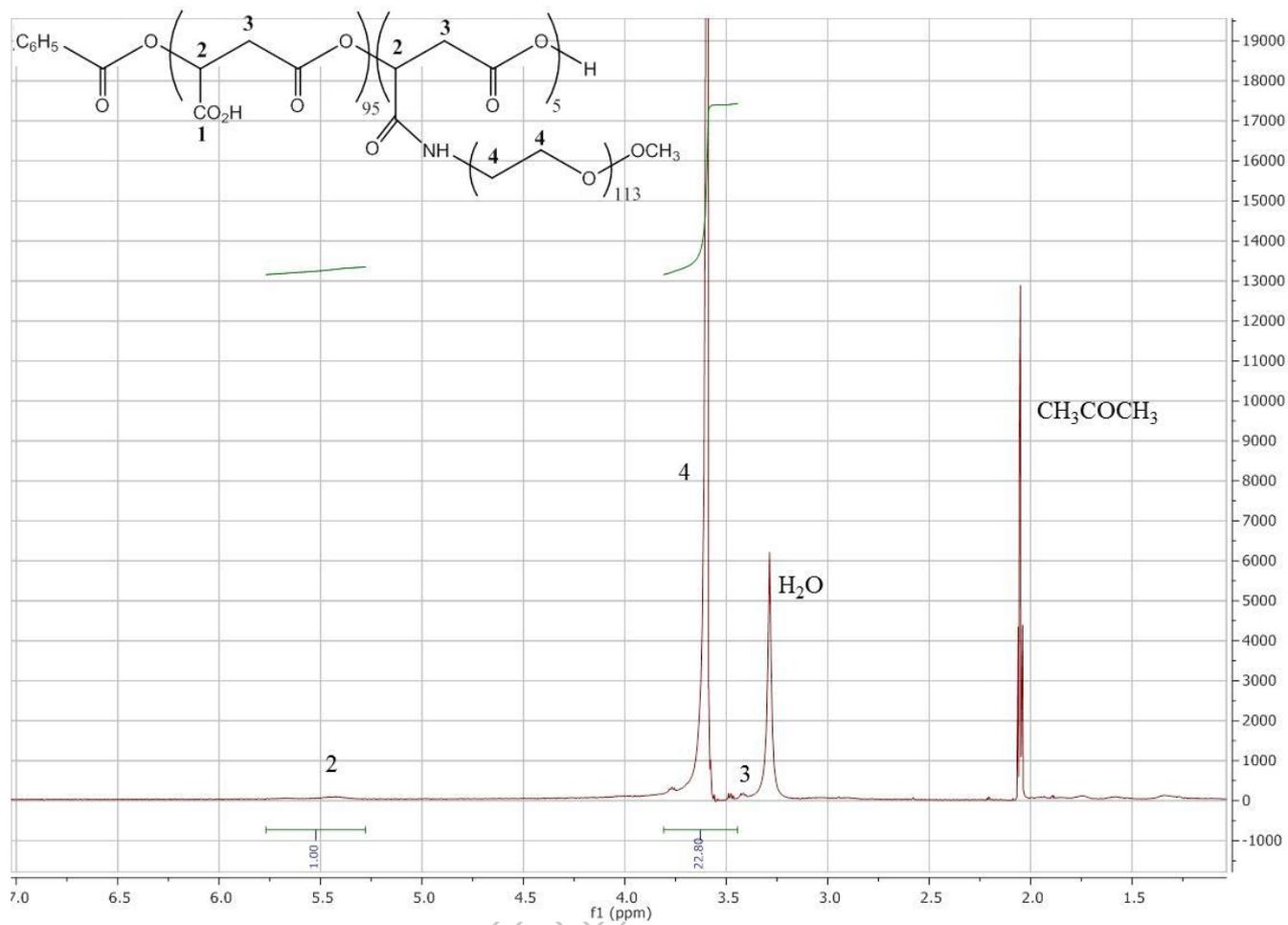
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Figure 3. ^1H NMR spectra (400 MHz) of PMLA-NHS in CD_3COCD_3 .



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Figure 4. ^1H NMR spectra (400 MHz) of PMLA-PEG in $\text{CD}_3\text{COCD}_3 + 2$ drops of D_2O .



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Figure 5. *In vitro* cell viability measured by the MMT assay after incubation of the nanoconjugates with HepaRG cells.

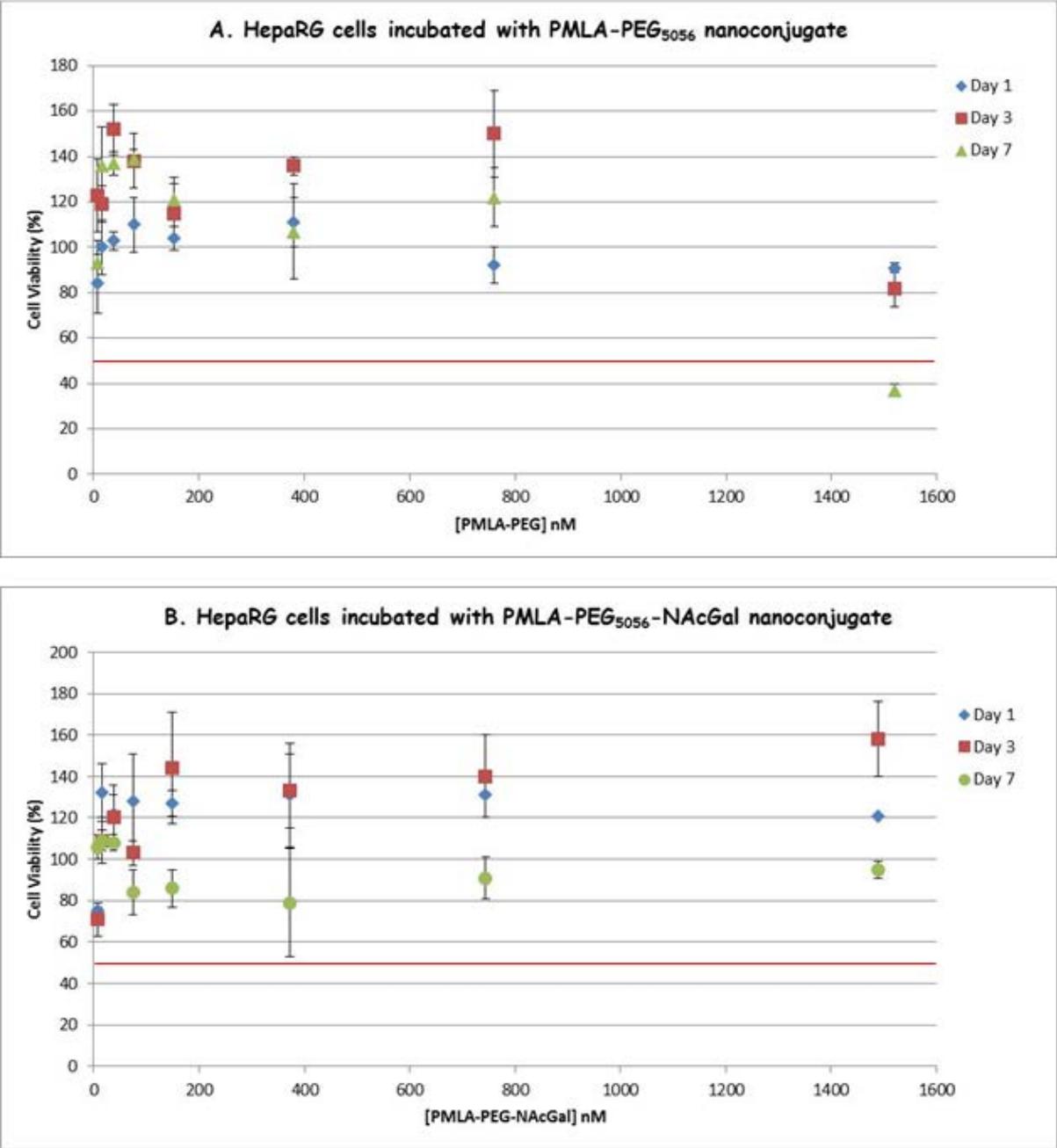


Figure 6. *In vitro* cell viability measured by the MMT assay after incubation of the nanoconjugates with HepaRG cells.

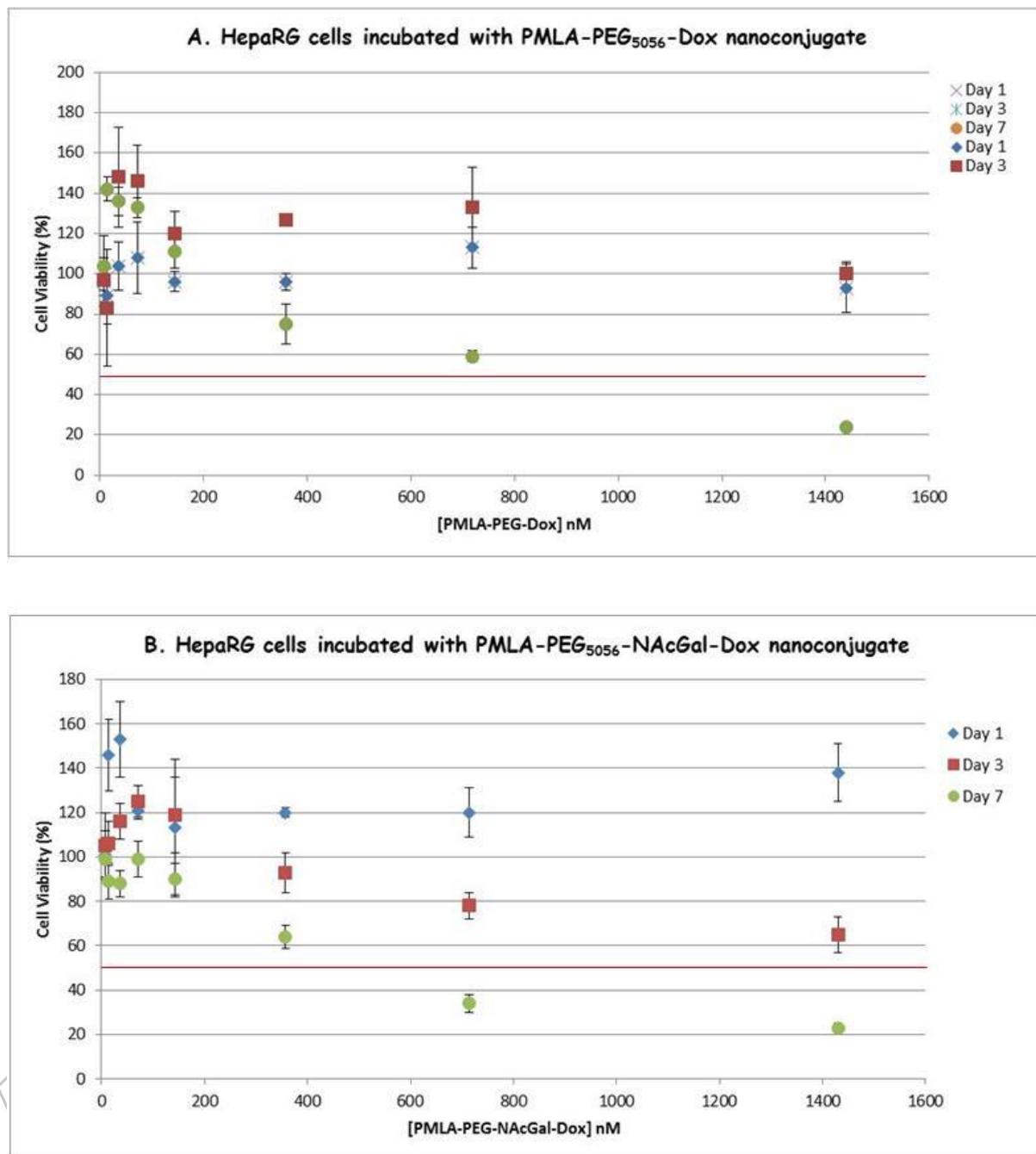


Table 1. Concentration of the nanoconjugates used for the cytotoxicity assays.

PMLA-PEG ₅₀₅₆	1.52 μ M	760 nM	380 nM	152 nM	76 nM	38 nM	15 nM	8 nM
PMLA-PEG ₅₀₅₆ -NACGal	1.49 μ M	744 μ M	372 nM	149 nM	74 nM	37 nM	15 nM	7 nM
PMLA-PEG ₅₀₅₆ -Dox	1.44 μ M	719 nM	359 nM	144 nM	72 nM	36 nM	14 nM	7 nM
PMLA-PEG ₅₀₅₆ -Dox-NACGal	1.43 μ M	714 nM	357 nM	143 nM	71 nM	36 nM	14 nM	7 nM

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Table 2. Results of UV analysis (485 nm) of the nanoconjugates containing Dox.

	Abs at 485 nm	[Dox] Mg/mL	Number of mol	mol% of Dox in the nanoconjugate
PMLA-PEG ₅₀₅₆ -Dox	0.180	0.0136	2.34×10^{-5}	5
PMLA-PEG ₅₀₅₆ -Dox-NAcGal	0.170	0.0135	2.33×10^{-5}	5

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Table 3. Characteristics of the prepared nanoconjugates in PBS pH7.5, 0.15 N NaCl measured by DLS.

Nanoconjugates	\bar{D} (nm)	PDI	ζ (mV)
PMLA-PEG ₅₀₅₆	80	0.61	+ 11
PMLA-PEG ₅₀₅₆ -NAcGal	70	0.71	- 46
PMLA-PEG ₅₀₅₆ -Dox	140	0.34	+ 11
PMLA-PEG ₅₀₅₆ -Dox-NAcGal	120	0.34	+ 5

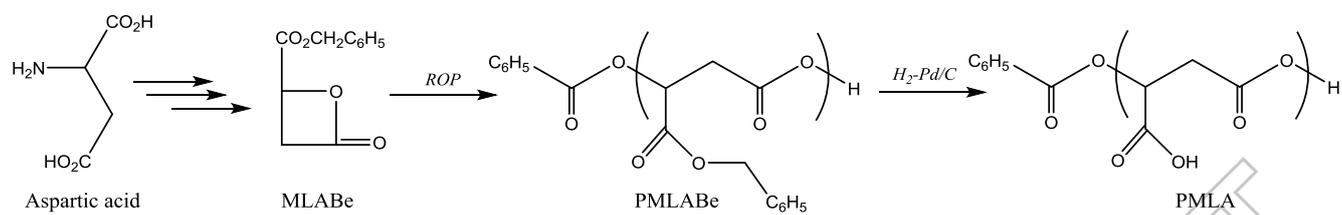
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Table 4. Evolution of the average diameter and dispersity of the nanoconjugates upon incubation in PBS pH7.5, 0.15 N NaCl at 4°C followed by DLS.

Nanoconjugates	T0		24 hours		48 hours		72 hours	
	\bar{D} (nm)	PDI						
PMLA-PEG ₅₀₅₆	80	0.61	90	0.60	150	0.60	180	0.54
PMLA-PEG ₅₀₅₆ -NACGal	70	0.71	110	0.50	130	0.60	290	0.33
PMLA-PEG ₅₀₅₆ -Dox	140	0.34	90	0.40	60	0.67	130	0.41
PMLA-PEG ₅₀₅₆ -Dox-NACGal	120	0.34	110	0.40	130	0.75	150	0.36

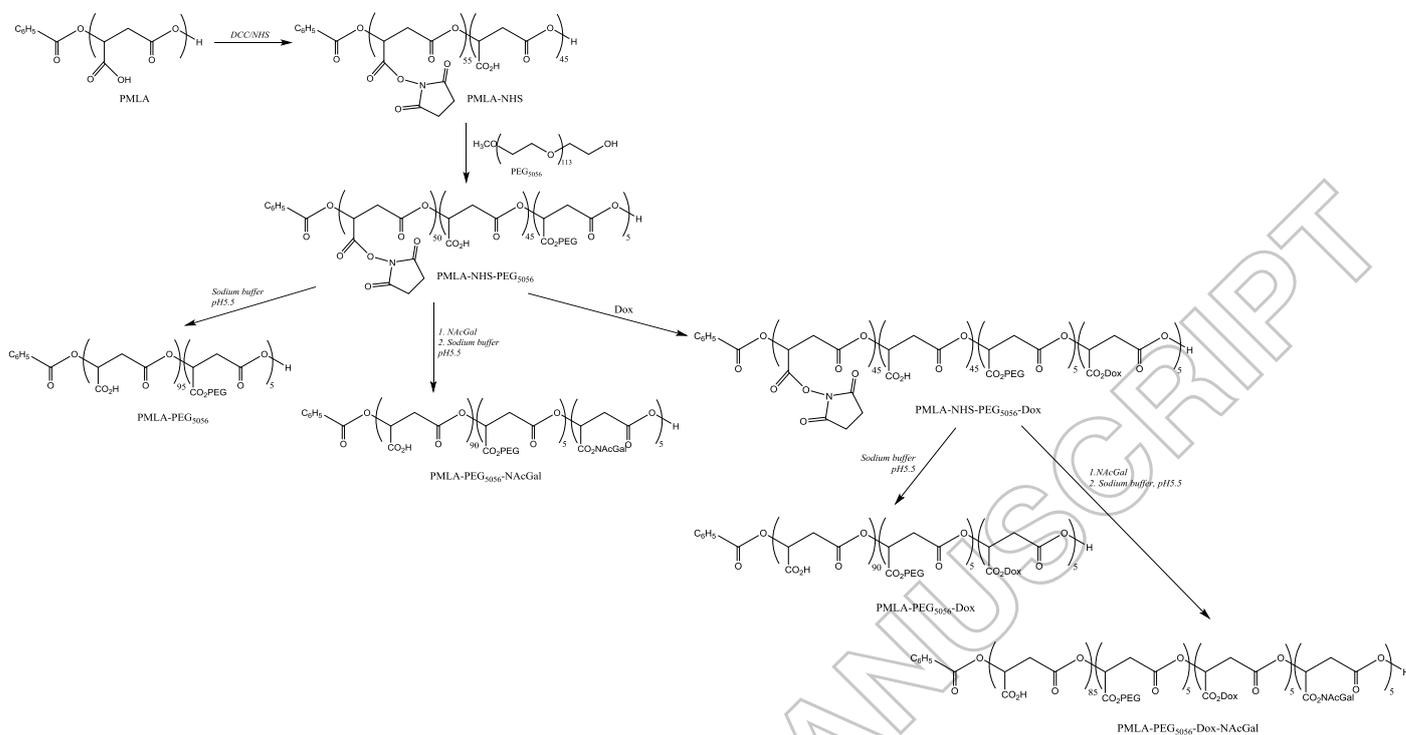
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Scheme 1. Synthetic route to PMLA.



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Scheme 2. Synthetic route to PMLA-based nanoconjugates.



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Supplementary Materials

Poly(malic acid) bearing Doxorubicin and N-Acetyl Galactosamine as a site-specific prodrugs for HepatoCellular Carcinoma

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Figure S1. Calibration curve of Dox solubilized in PBS pH7 giving the Dox concentration as a function of absorbance of the Dox solution at 485 nm.

