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Galactosylated polymeric carriers for liver targeting of sorafenib

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ABSTRACT

In this paper, we describe the preparation of liver-targeted polymeric micelles potentially able to carry sorafenib to hepatocytes for treatment of hepatocarcinoma (HCC), exploiting the presence of carbohydrate receptors, ASGPR. These micelles were prepared starting from a galactosylated polylactide-polyaminoacid conjugate. This latter was obtained by chemical reaction of α,β-poly(*N*-2-hydroxyethyl) (2-aminoethylcarbamate)-D,L-aspartamide (PHEA-EDA) with polylactic acid (PLA), and subsequent reaction with lactose, leading to PHEA-EDA-PLA-GAL copolymer. Liver-targeted sorafenib-loaded micelles were obtained in aqueous media at low PHEA-EDA-PLA-GAL copolymer concentration value with nanometer size and slightly positive zeta potential. Biodistribution studies on mice demonstrated, after oral administration of sorafenib loaded PHEA-EDA-PLA-GAL micelles, the preferential sorafenib accumulation into the liver. This finding raises hope in terms of future drug delivery strategy of sorafenib-loaded micelles targeted to the liver for the HCC treatment.

1. Introduction

Liver diseases are the major causes of disability and mortality worldwide (Li et al., 2010). In the absence of a satisfactory curative option, the pursuit of alternative pharmacological interventions, therefore, remains extremely pressing. Many potent drugs are often not effective enough in vivo or exhibit adverse effects. Although they accumulate rapidly in the liver, they do not accumulate in the proper intrahepatic cell-type; thus drug targeting to the liver may represent a new promising strategy (Poelska et al., 2012). For several diseases, hepatocytes represent the most relevant target cell, playing a crucial role in viral hepatitis, steatohepatitis, some genetic diseases and several other metabolic disorders. However, the major issue is not drug uptake by hepatocytes; but other competing processes such as the first pass effect or drug uptake by other cell-types may occur. Many efforts have been dedicated to the targeted delivery of drugs and genes in particular to hepatocytes by the development of galactosylated polymeric or lipid carriers in order to enhance the therapeutic effects (in case of hepatocellular carcinoma, viral hepatitis or gene-based therapies) or to reduce side-effects of drugs (in case of antiviral or anticancer drugs) (Poelska et al., 2012). Galactosylation is a speculated strategy in this field because targeting via galactosylated carriers exploit highly specific interactions of galactose ligands with endogenous lectin receptors such as asialoglycoprotein receptor (ASGPR), which is specifically and abundantly present on hepatocytes (Jain et al., 2012; Rensen et al., 2001; Wu et al., 2004). This receptor has been used to deliver to hepatocytes all kinds of therapeutic compounds ranging from therapeutic proteins, antiviral agents (Di Stefano et al., 1997) to anticancer drugs (Di Stefano et al., 2006; Fiume et al., 2005). Active targeting by coupling galactose (GAL) residues or lactose moieties to proteins and polymers or by their introduction on the colloidal surface of nanoparticles or polymeric micelles, allow enhancing the uptake of drug-loaded systems into hepatocytes with high degree of selectivity (Fiume and Di Stefano, 2010; Li et al., 2010; Poelstra et al., 2012; Suo et al., 2010; Suriano et al., 2010; Wu et al., 2010; Yang et al., 2011; Zheng et al., 2011). In well-differentiated forms of hepatocellular carcinoma (HCC), hepatocytes over-express on the surface the ASGP-R (Hyodo et al., 1993) and many drug delivery systems have been already developed to deliver drugs to this receptor using lactosaminated or galactosaminated substituted drug carriers,

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i.e. based on synthetic polymers (Julyan et al., 1999) or on modified albumins (Fiume and Di Stefano, 2010).

Sorafenib (Nexavar®) is the first drug currently approved by the US Food and Drug Administration (FDA) for the first-line treatment of unresectable HCC (Kane et al., 2009; Keating and Santoro, 2009; Llovet et al., 2008; URL: http://www.cancer.gov/cancertopics/druginfo/fda-sorafenib-tosylate). It is the first systemic therapy to prolong median survival and the time progression by nearly 3 months in HCC patients, representing the new reference standard for systemic treatment in HCC patients. The recommended dosage is 400 mg twice daily, but dose reduction or temporary interruption of sorafenib therapy may be needed to manage adverse events, such as dermatological hand-foot skin reactions and diarrhoea, that may significantly affect patients quality of life (Chaparro et al., 2008; Keating and Santoro, 2009; Llovet et al., 2008).

In this paper, we described the construction of polymeric micelles bearing GAL moieties on the surface. Moreover, their capability to carry efficiently Sorafenib to the liver is demonstrated by in vivo experiments. Starting polymer was the α,β-poly(*N*-2-hydroxyethyl)-D,L-aspartamide (PHEA) (Craparo et al., 2010; Giammona et al., 1987) to which a proper amount of ethylenediamine (EDA) chains was linked to obtain PHEA-EDA copolymer (Licciardi et al., 2006) in order to increase reactivity towards further successive functionalization. Poly-lactic acid (PLA) molecules were subsequently grafted in order to obtain an amphiphilic copolymer (PHEA-EDA-PLA) (Craparo et al., 2008) able to give self-assembling polymeric micelles. This derivative subsequently reacted with lactose, to obtain the PHEA-EDA-PLA-GAL copolymer. Polymeric micelles were successfully obtained in aqueous media starting from PHEA-EDA-PLA-GAL copolymer. Physical–chemical and biological studies were performed on polymeric micelles obtained from this copolymer in order to demonstrate their colloidal size, biocompatibility, and their capability to act as controlled and liver-targeted drug delivery systems.

2. Materials and methods

2.1. Chemicals

SEC poly(ethylene oxide) standards, anhydrous *N*,*N*-dimethylformamide (DMF_a), anhydrous dimethylsulfoxide ($DMSO_a$), methanol, DMSO-d_6 (isotopic purity 99.9%), *N*-hydroxysuccinimide (NHS), diethylamine (DEA), acetone, diethyl ether, sodium cyanoborohydride, α-lactose, anthrone, were purchased from Sigma–Aldrich (Italy) and were used as received. Sorafenib was purchased at L.C. Laboratories (USA); Nexavar was a gift of a HCC patient. RESOMER R 202 (D,L-Polylactic acid, PLA 8000 Da) from Bidachem-Boehringher Ingelheim (Italy) was also used. SpectraPor dialysis tubing was purchased from Spectrum Laboratories, Inc. (Italy).

Weight-average molecular weight ($\overline{M}_w$) of each copolymer was determined by size exclusion chromatography (SEC) analysis. SEC system (Waters, Mildford, MA) was equipped with a pump system, two Phenogel columns from Phenomenex (5 μm particle size, 103 Å and 104 Å of pores size), and a 410 differential refractometer (DRI) as concentration detector. Analyses was performed with tris buffer solution 0.1 M at pH 8 as eluent at 37 °C with a flux of 0.8 mL/min and poly(ethylene oxide) standards (range 145–1.5 kDa) to obtain the calibration curve.

The ^{1}H NMR spectra were recorded by using a Bruker Avance II 300 spectrometer operating at 300 MHz. Centrifugation was performed using a Centra MP4R IEC centrifuge.

α,β-poly(*N*-2-hydroxyethyl)-D,L-aspartamide (PHEA) was prepared via polysuccinimide (PSI) by polycondensation of D,L-aspartic acid in the presence of H_3PO_4 at 180 °C, followed by reaction with ethanolamine in DMF solution, and purified according to a procedure elsewhere reported (Giammona et al., 1987). Spectroscopic data were in agreement with the attributed structure. ^{1}H NMR (300 MHz, D_2O, 25 °C, TMS): δ = 2.78 (m, 2H, —$CHCH_2CONH$—), δ = 3.32 (m, 2H, —$NHCH_2CH_2OH$), δ = 3.63 (m, 2 H, —$NHCH_2CH_2OH$), δ = 4.68 (m, 1H, —$NHCH(CO)CH_2$—. Weight average molecular weight of PHEA was 40.3 kDa (M_w/M_n = 1.58), determined by SEC analysis.

The synthesis of α,β-poly(*N*-2-hydroxyethyl)(2-aminoethyl-carbamate)-D,L-aspartamide (PHEA-EDA) copolymer was performed according to the procedure previously published and spectroscopic data were in agreement with the attributed structure (Craparo et al., 2013a; Licciardi et al., 2006).

^{1}H NMR (300 MHz, D_2O, 25 °C, TMS): δ = 2.60 (m, 2H, —$CHCH_2CONH$—), δ = 2.84 (m, 2H, —$NHCH_2CH_2NH_2$), δ = 3.16 (m, 2H, —$NHCH_2CH_2OH$), δ = 3.27 (m, 2H, —$NHCH_2CH_2NH_2$), δ = 3.47 (m, 2H, —$NHCH_2CH_2OH$), δ = 3.93 (m, 2H, —$NHCH_2CH_2O(CO)NHCH_2CH_2NH_2$), δ = 4.52 (m, 1H, —$NHCH(CO)CH_2$—). The derivatization degree in EDA (DD_{EDA}), calculated according to the method reported, was 30.0 ± 0.5 mol%. $\overline{M}_w$ of PHEA-EDA was 23.3 kDa ($\overline{M}_w/\overline{M}_n = 1.66$).

The derivatization of PHEA-EDA with D,L-polylactic acid (PLA) to obtain the copolymer PHEA-EDA-PLA copolymer was performed according to a procedure previously published and spectroscopic data were in agreement with the attributed structure (Craparo et al., 2008). The derivatization degree in PLA (DD_{PLA}) was calculated according to the method reported, resulted to be 0.73 ± 0.12 mol%. $\overline{M}_w$ of PHEA-EDA-PLA, determined by SEC analysis, was found to be 19.3 kDa ($\overline{M}_w/\overline{M}_n = 1.60$).

2.2. PHEA-EDA-PLA-GAL synthesis

Galactosylated PHEA-EDA-PLA copolymer (PHEA-EDA-PLA-GAL) was synthesized by a reductive amination of lactose with primary amine functions of PHEA-EDA-PLA in the presence of sodium cyanoborohydride, according to the procedure previously published and spectroscopic data were in agreement with the attributed structure (Craparo et al., 2013a).

^{1}H NMR (300 MHz, D_2O, 25 °C, TMS): δ = 0.79 and δ = 1.24 (2d, $3H_{PLA}$, —$OCOCH(CH_3)O$—), δ = 2.75 (m, $2H_{PHEA}$, —$CHCH_2CONH$—), δ = 2.99 (m, $2H_{EDA}$, —$NHCH_2CH_2NH_2$), δ = 3.28 (m, $2H_{PHEA}$, —$NHCH_2CH_2OH$), δ = 3.40 (m, $2H_{EDA}$, —$NHCH_2CH_2NH_2$), δ = 3.58 (m, $2H_{PHEA}$, —$NHCH_2CH_2OH$), δ = 3.62–3.77 (m, 12 H_{sugar}, —CH_2OH and —CH(OH)— sugar protons), δ = 4.06 (m, $2H_{PHEA}$, —$NHCH_2CH_2O(CO)NHCH_2CH_2NH_2$), δ = 4.41 (s, 1 H_{GAL}, anomeric proton), δ = 4.64 (m, $1H_{PHEA}$, —$NHCH(CO)CH_2$—).

The $\overline{M}_w$ was found to be 24.3 kDa ($\overline{M}_w/\overline{M}_n = 1.66$).

The amount of GAL grafted onto PHEA-EDA-PLA-GAL copolymer measured by using anthrone-sulphuric acid colorimetric method was 0.86 wt% (corresponding to 1.22 mol%) (Craparo et al., 2013a; Song et al., 2009).

The critical aggregation concentration (CAC) of PHEA-EDA-PLA-GAL copolymer, determined by collecting steady-state fluorescence spectra of pyrene probe in the presence of increasing concentrations of that copolymer, resulted to be 4.1×10^{-7} M (Craparo et al., 2013a).

2.3. Preparation of sorafenib-loaded PHEA-EDA-PLA-GAL and PHEA-EDA-PLA micelles

To obtain sorafenib-loaded micelles, the evaporation method was chosen according to the published procedure (Craparo et al., 2009; Craparo et al., 2011; Ngawhirunpat et al., 2009). In particular, these micelles were prepared by closely mixing an appropriate amount of PHEA-EDA-PLA-GAL or PHEA-EDA-PLA and sorafenib to obtain a final copolymers/drug weight ratio equal to 10:1. Then, aliquots of ethanol (total volume = 1.5 mL) were added and, after evaporation of the solvent at room temperature under a gentle

stream of nitrogen, aliquots of water were added until reaching 25 mL of volume. The obtained dispersions were submitted to ultrasounds for 10 min, centrifuged at 4000 rpm, at 25 °C for 10 min, filtered and lyophilised.

2.4. Drug loading determination

The amount of sorafenib blended into PHEA-EDA-PLA-GAL and PHEA-EDA-PLA micelles (drug loading, DL%), was determined by the HPLC method. The column was a Gemini C18 (μBondpack, 5 μm, 250 × 46 mm i.d., obtained from Waters); the mobile phase was a mixture of methanol:K_2HPO_4 (0.1 M, pH 8.7) 90:10 v/v with a flow rate of 0.7 mL/min, the column temperature was 25 °C, and the detection wavelength was 264 nm. 40 μL sample was injected into the column. The obtained peak area (t_r = 5.68 min) corresponding to sorafenib amount blended into PHEA-EDA-PLA-GAL or PHEA-EDA-PLA micelles was compared with a calibration curve obtained by plotting areas versus standard solution concentrations of sorafenib in methanol in the range of 40–0.1 μg/mL ($y = 4E+08x$, $R^2 = 0.996$). Results were expressed as the weight percent ratio between the loaded sorafenib and the dried system (micelles + sorafenib).

2.5. Dimensional analysis

The mean diameter and width distribution of each sample were determined by Photon Correlation Spectroscopy (PCS) by using a Zetasizer Nano ZS (Malvern Instrument, Malvern, UK) that utilizes the Non-Invasive Back-Scattering (NIBS) technique. The measurements were carried out at a fixed angle of 173° and at 25 °C on each dispersion obtained by using NaCl 0.9 wt% and phosphate buffered saline (PBS) aqueous solutions at pH 7.4 as suspending media filtered on nylon 5 μm.

Each dispersion was kept in a cuvette and analyzed in triplicate. The deconvolution of the measured correlation curve to an intensity size distribution was accomplished by using a non-negative least squares algorithm.

2.6. Zeta potential measurements

The zeta potential values were measured using principles of laser doppler velocitometry and phase analysis light scattering (M3-PALS technique) by using Zetasizer Nano ZS Malvern Instrument. Samples were dispersed in filtered NaCl 0.9 wt% and PBS at pH 7.4 aqueous solutions, filtered on nylon 5 μm and analysed in triplicate.

2.7. Transmission electron microscopy (TEM) analysis

TEM micrographs were acquired by using a JEM-2100 (JEOL, Japan) electron microscope, operating at a 200 kV accelerating voltage. A few tens of a milligram of the freeze-dried samples were dispersed in 2 mL of bi-distilled water and a small drop of the dispersion was deposited on a 300 mesh carbon-coated copper grid, which was introduced into the TEM analysis chamber after complete solvent evaporation.

2.8. Drug release studies

The stability of PHEA-EDA-PLA and PHEA-EDA-PLA-GAL micelles was studied at pH 1.6, 6.5 and 7.4 in sink conditions by evaluating the release of sorafenib by a dialysis method.

To evaluate gastric stability, a fluid simulating conditions in the stomach in the fasted state (FaSSGF) was used by preparing a solution of NaCl (30 mM), pepsin (0.1 mg/ml), sodium taurocholate (80 μM) and lecithin (20 μM), at pH 1.6 by addition of HCl (Vertzoni et al., 2005).

To evaluate intestinal stability, a fluid simulating the proximal small intestine conditions in fasted state (FaSSIF) was used by preparing a solution of monobasic potassium phosphate (3.9 g/l), KCl (7.7 g/l), sodium taurocholate (3 mM) and lecithin (0.75 mM), at pH 6.5 by addition of NaOH (Galia et al., 1998).

To evaluate plasma and interstitial stability, phosphate buffered saline (PBS, NaCl, Na_2HPO_4, KH_2PO_4) at pH 7.4 containing 50% fetal bovine serum (FBS) was used (Zhang et al., 2011).

Each sample (total drug amount = 0.48 mg) was dispersed in aliquots of the proper medium (2 mL), placed in a dialysis bag (MWCO 12–14 kDa) immersed in 48 mL of the same medium, and then incubated in a thermostatic shaker (100 rpm, 37 °C). At scheduled time-points, aliquots of the receiver medium (0.5 mL) was taken and replaced by fresh medium (0.5 mL). The sorafenib amounts in each sample, after adding methanol (1 mL) to precipitate proteins and extract the drug, were determined by the HPLC method mentioned above (to calculate the cumulative Sorafenib release percentages).

A control experiment to determine the release behavior of the free drug was also performed: an appropriate amount of sorafenib was dispersed in the proper medium, in order to have a sorafenib final concentration equal to which of micelles, put into a dialysis tube (MWCO 12–14 kDa) and immersed into the proper medium. The amount of sorafenib was detected as reported above.

2.9. Haemolytic test

Human erythrocytes isolated from fresh citrated-treated blood were collected by centrifugation at 2200 rpm for 10 min at 4 °C. The pellet was washed four times with PBS at pH 7.4 by centrifugation and suspended in the same buffer. Afterwards, it was diluted in PBS at pH 7.4 to a final concentration of 4% erythrocytes. This stock dispersion was always freshly prepared and used within 24 h after preparation. Sorafenib, empty or sorafenib-loaded PHEA-EDA-PLA and PHEA-EDA-PLA-GAL micelle dispersions (with a drug concentration ranging between 0.1–0.5 mg/ml) were added to the erythrocyte suspension and incubated for 2 h at 37 °C under constant shaking. After centrifugation, the release of haemoglobin was determined by photometric analysis of the supernatant at 540 nm. Complete haemolysis was achieved by using a 1 wt% aqueous solution of Triton X-100 (100% control value). Each experiment was performed in triplicate and repeated twice. The erythrocyte lysis percentage was calculated according to the following formula:

$$\%\ \text{lysis} = \frac{(A_{\text{sample}} - A_{\text{blank}})}{(A_{100\%\ \text{lysis}} - A_{\text{blank}})} = \times 100$$

where A_{sample} is the absorbance value of the haemoglobin released from erythrocytes treated with sorafenib, empty or sorafenib-loaded micelle dispersion; $A_{\text{blank}}$is the absorbance value of the haemoglobin released from erythrocytes treated with PBS buffer, and $A_{100\%\ \text{lysis}}$ is the absorbance value of the haemoglobin released from erythrocytes treated with 1% Triton X-100 solution.

2.10. Animals

Experiments were performed using adult female mice (C57BL/6SnJ) weighting 20–25 g obtained from Harlan Laboratories (San Pietro al Natisone, Udine, Italy). Animals were kept under environmentally controlled condition, ambient temperature 24 °C, humidity 40% and 12 h light/dark cycle with food and water ad libitum. Procedures involving animals and their care were conducted in conformity with institutional guidelines which are in compliance with national (D.L. n. 116, G.U., suppl. 40, 18 Feb. 1992) and international laws and policies (NIH Guide for the Care and Use

of Laboratory Animals, NIH Publication no. 80–23, 1985; EEC Council Directive 86/609, OJ L 358,1,1 December 12, 1987; Giles, 1987).

2.11. Experimental protocol

Mice received by gavage a single oral dose of 5.7 mg/kg of drug (as sorafenib tosylate, or sorafenib-loaded micelles), after dispersion in isotonic normal saline solution. The dose selected was an approximate equivalent conversion from the recommended daily dose for adults with refractory cancers. Control mice received an equal volume of isotonic normal saline solution.

Animals were then sacrificed at different time-points (1, 2, 4 h) following drug administration. Blood was taken by cardiac puncture, and the following organs were collected: lungs, spleen, liver and kidneys. Specimens were then weighted and stored at −80 °C until drug analyses were performed.

To extract and quantify the amount of drug, each organ or blood sample was mixed with Tris buffer (2 mL, 1 M, pH 8) in a 15 ml glass tube, and homogenized using a Ultraturrax for 15 min (not for blood). Then, methanol (1 mL) was added to precipitate proteins. Samples were extracted three time with diethyl ether (2 mL), followed by centrifugation at 4000 rpm (5 min). After every solvent addition, the centrifuge tubes were shaken for 20 min at room temperature, and centrifuged for 5 min at 4000 rpm. The organic layers were transferred into a glass tube and evaporated to dryness at 35 °C under a gentle stream of nitrogen. The dried residue was reconstituted in methanol (0.6 mL) and a 40 μL volume was injected into the HPLC system in conditions described above.

The extraction efficiency in each organ or blood was previously determined by spiking known amounts of sorafenib in blood or homogenate organs obtained from un-administered animals and by analysing each mixture as described above.

2.12. Statistical analysis

The statistical analysis of the samples was performed by using a Student's *t*-test. A p-value < 0.05 was considered to be indicative of statistical significance while a p-value < 0.01 was considered as highly significant. All data were reported as mean ± SD, unless otherwise stated.

3. Results and discussion

α,β-poly(*N*-2-hydroxyethyl)(2-aminoethylcarbamate)-D,L-aspartamide (PHEA-EDA) copolymer is an appropriate starting macromolecule to design novel amphiphilic copolymers for biomedical application (Craparo et al., 2008; Craparo et al., 2013a; Licciardi et al., 2006).

In order to develop new colloidal liver-targeted polymeric carriers for sorafenib, a PHEA-EDA amphiphilic derivative able to self-assemble in aqueous media was prepared (Craparo et al., 2013a). In particular, poly-lactic acid (PLA) and lactose were chemically grafted onto the amine groups of PHEA-EDA to obtain the PHEA-EDA-PLA-GAL graft copolymer. In this way, the grafting of each PLA residue permits to introduce a hydrophobic biodegradable tail that contributes to obtain stable micelles. The subsequent grafting of GAL moieties onto the PHEA-EDA-PLA backbone permits to obtain a sugar-targeted polymeric conjugate for a specific therapy of liver diseases, due to the presence of carbohydrate receptors in the liver, i.e., the asialoglycoprotein receptors (ASGPR) in hepatocytes (Craparo et al., 2013b; Fiume and Di Stefano, 2010; Li et al., 2010; Medina et al., 2011).

3.1. Synthesis and characterization of PHEA-EDA-PLA-GAL graft copolymer

The derivatization reaction of PHEA-EDA with PLA was carried out in organic solvent using the NHS derivative of PLA (PLA-NHS), in the presence of diethylamine (DEA), as described previously (Craparo et al., 2008; Craparo et al., 2013a). The derivatization degree in PLA residues (DD_{PLA}) for PHEA-EDA-PLA graft copolymer was expressed as mean value of eight determinations and resulted to be 0.73 ± 0.12 mol, indicating that about one PLA tail is inserted on the PHEA-EDA backbone every one hundred repeating units. The weight-average molecular weight ($\overline{M}_w$) and the polydispersity index ($\overline{M}_w/\overline{M}_n$) values of PHEA-EDA-PLA graft copolymer, obtained in aqueous environment by SEC analysis, were found to be equal to 19.3 kDa and 1.60, respectively.

In order to develop a hepatocyte-targeted carrier, GAL residues were introduced in the PHEA-EDA-PLA side chains by leaving to react it with lactose moieties (Craparo et al., 2013a).

This derivatization reaction was carried out by a single synthetic step in organic solvent involving the reductive amination of lactose with PHEA-EDA-PLA primary amine functions, in the presence of sodium cyanoborohydride for 4 h at 40 °C (Craparo et al., 2013a). The chemical structure of PHEA-EDA-PLA-GAL graft copolymer is depicted in Fig. 1.

The amount of GAL grafted onto PHEA-EDA-PLA-GAL copolymer measured by using anthrone–sulphuric acid colorimetric method was 0.86 wt% (corresponding to 1.22 mol%) (Craparo et al., 2013a; Song et al., 2009). The ($\overline{M}_w$) and the ($\overline{M}_w/\overline{M}_n$) values of PHEA-EDA-PLA-GAL graft copolymer, obtained in aqueous environment, were found to be equal to 24.3 kDa and 1.66, respectively.

The critical aggregation concentration (CAC) of PHEA-EDA-PLA-GAL copolymer, determined by collecting steady-state fluorescence spectra of pyrene probe in the presence of increasing concentrations of that copolymer resulted to be 4.1×10^{-7} M, while PHEA-EDA-PLA copolymer showed a CAC equal to 2.6×10^{-6} M in the same experimental conditions (Craparo et al., 2013a).

3.2. Preparation and characterization of empty and sorafenib-loaded micelles

To obtain colloidal nanostructures in aqueous media starting from obtained PHEA-EDA-PLA-GAL copolymer, the evaporation method was chosen according with the published procedure (Craparo et al., 2009; Craparo et al., 2011; Craparo et al., 2013a; Ngawhirunpat et al., 2009).

PHEA-EDA-PLA-based micelles were prepared following the same procedure in order to obtain nontargeted systems to use for comparison with targeted ones in further experiments. Briefly, this technique involved the simple dispersing of each dry copolymer (in the presence of the drug, to obtain drug-loaded systems) by adding aliquots of ethanol, then its evaporation at room temperature under a gentle stream of nitrogen to form a copolymer/prodrug film. This latter was then dispersed in bi-distilled water and the amount of drug exceeded the solubilisation capacity of micelles was removed by centrifugation and filtration. Obtained aqueous dispersions were lyophilised to recover drug-loaded micelles.

The drug loading (DL%), determined by HPLC and expressed as the weight percent ratio between the loaded sorafenib and the dried system, resulted to be 3.0 and 3.6 wt%, respectively, for PHEA-EDA-PLA-GAL or PHEA-EDA-PLA micelles.

Empty and sorafenib-loaded PHEA-EDA-PLA-GAL or PHEA-EDA-PLA micelles, after re-dispersion in a proper medium, were characterised in terms of mean size and width of distribution values by using photon correlation spectroscopy (PCS). Analytical data are reported in Table 1.

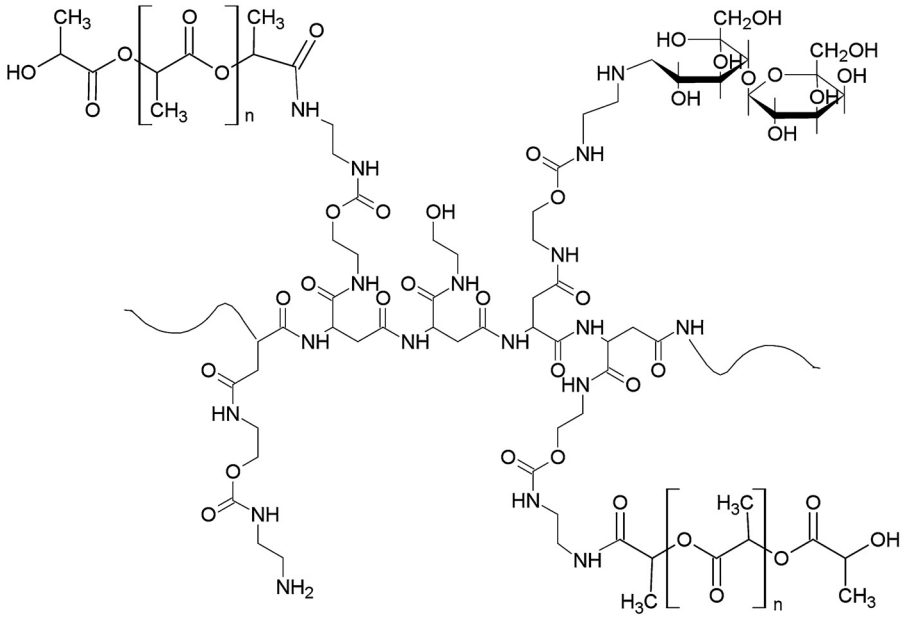

Fig. 1. Chemical structure of PHEA-EDA-PLA-GAL graft copolymer ($n = 111$).

Table 1
Mean size, width of distribution and ζ potential values in PBS and NaCl 0.9 wt% of empty and sorafenib-loaded PHEA-EDA-PLA and PHEA-EDA-PLA-GAL micelles.

Sample	Dispersing medium	Mean size (nm)(±S.D.)	ζ potential (mV)(±S.D.)
PHEA-EDA-PLA micelles	PBS	21.6 ± 3.2	−2.2 ± 3.5
	NaCl 0.9%	29.5 ± 7.2	−1.2 ± 2.8
PHEA-EDA-PLA-GAL micelles	PBS	21.5 ± 6.8	−1.7 ± 3.0
	NaCl 0.9%	28.2 ± 7.9	−3.9 ± 1.9
Sorafenib-loaded PHEA-EDA-PLA micelles	PBS	120.2 ± 76.8	+5.1 ± 2.7
	NaCl 0.9%	147.4 ± 40.8	+3.3 ± 2.6
Sorafenib-loaded PHEA-EDA-PLA-GAL micelles	PBS	125.7 ± 53.7	+4.3 ± 3.2
	NaCl 0.9%	101.8 ± 64.3	+1.9 ± 2.1

Either empty PHEA-EDA-PLA or PHEA-EDA-PLA-GAL nanostructures showed a size distribution with an average diameter of about 30 nm in NaCl 0.9%, with non-significant differences in diameters when these were measured in PBS aqueous solution.

When sorafenib was entrapped into PHEA-EDA-PLA and PHEA-EDA-PLA-GAL micelles (during the micelle formation), the mean size of both nanostructures was strongly affected. In particular, drug-loaded PHEA-EDA-PLA nanostructures showed a size distribution with an average diameter of 147.41 nm in NaCl 0.9%, while drug-loaded PHEA-EDA-PLA-GAL nanostructures showed an average diameter of 101.82 nm in the same medium. Therefore, the drug entrapment increased the mean size of either PHEA-EDA-PLA or PHEA-EDA-PLA-GAL micelles presumably for a size increase of the hydrophobic core due to the drug presence. Moreover, also for drug-loaded systems, in PBS aqueous solution, mean size values showed small differences compared to those obtained in NaCl 0.9%.

The ζ potential values of these structures, also reported in Table 1, were slightly negative for empty PHEA-EDA-PLA and PHEA-EDA-PLA-GAL systems and changed to slightly positive for drug-loaded systems in either NaCl 0.9% or in PBS aqueous solution.

In order to confirm the nanometric size and to investigate the morphology of empty or sorafenib-loaded PHEA-EDA-PLA-GAL micelles, TEM was used and representative TEM images of PHEA-EDA-PLA-GAL micelles, empty and loaded with sorafenib have been reported in Fig. 2.

These images were consistent with findings obtained from dimensional analysis, greater drug-loaded mean size than that of empty micelles, and also revealed a spherical shape of investigated samples.

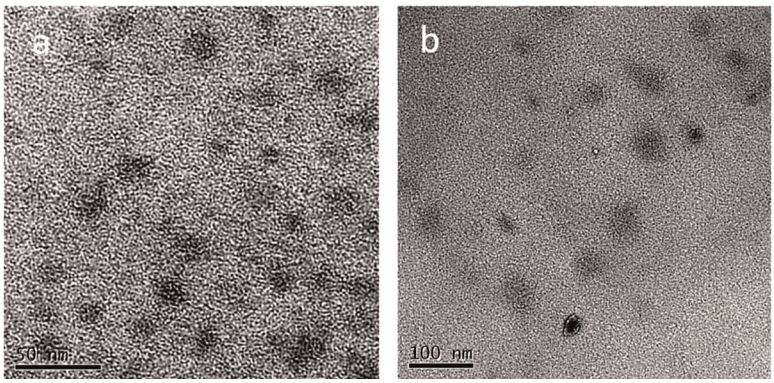

Fig. 2. Representative TEM images of PHEA-EDA-PLA-GAL micelles, empty (a, 80,000×) and loaded with sorafenib (b, 30,000×). The bars represent: 50 nm in image (a) and 100 nm in image (b).

3.3. Drug release studies

The micelle stability after administration, before they reach the target site to release the entrapped drug, is a need to obtain optimization of bioavailability and reduction of side effects.

In order to evaluate the potential stability of obtained micelles, a drug release study was carried out in physiological – mimicking fluids in sink conditions to assess their capability to retain the encapsulated drug under sink conditions and/or to release it slowly in physiological media. In particular, these studies were carried out in fasted state simulated gastric fluid (FaSSGF) at pH 1.6 for 4 h to mimic gastric conditions, in fasted state simulated intestinal fluid (FaSSIF) at pH 6.5 for 24 h to mimic intestinal conditions, and in phosphate buffer solution (PBS) at pH 7.4/foetal calf serum (FCS) (50:50) for 24 h to mimic extracellular and plasmatic conditions (Francis et al., 2005; Roger et al., 2010; Zhang et al., 2011).

The micelle stability was deduced by evaluating the amount of released drug from micelles at prefixed time intervals across a dialysis tube. Moreover, the sorafenib diffusion profile alone was investigated in each medium in order to determine the diffusion rate of the free drug across the dialysis membrane. The amount of released sorafenib was expressed as percentage ratio between the weight of released drug at the prefixed time and the total amount of sorafenib loaded into micelles.

Fig. 3a and b show the drug dissolution and release profiles of sorafenib from PHEA-EDA-PLA and PHEA-EDA-PLA-GAL micelles in FaSSGF, and in FaSSIF and PBS, respectively.

As shown in Fig. 3a, after 4 h incubation in FaSSGF, the cumulative release of sorafenib was about 4% from both PHEA-EDA-PLA and PHEA-EDA-PLA-GAL micelles. As show in Fig. 3b, after 24 h incubation, the cumulative release of sorafenib was about 6.6% and 4.2% in FaSSIF, and 12.9% and 11.6% in PBS from PHEA-EDA-PLA and PHEA-EDA-PLA-GAL micelles, respectively.

These results reveal that micelles were adequately stable in all the investigated media at all the incubation period. Moreover, the burst release was not observed from both micelles in all incubation conditions, very remarkable observation since fast release of drug may result in fast elimination of drug in vivo, leading to a lower antitumor efficacy.

3.4. In vitro biological characterization

Hemocompatibility is an essential requirement for a successful use of PHEA-EDA-PLA-GAL micelles as carriers for targeted sorafenib delivery into hepatic cells, not only after intravenous administration but also after oral administration, being the bloodstream the main path for these systems to reach and target the liver after gastrointestinal absorption. Therefore, the potential sorafenib-containing micelle interaction with erythrocyte membranes and their hemolytic effects were investigated by hemolysis experiments, incubating under proper conditions empty or sorafenib-loaded PHEA-EDA-PLA-GAL or PHEA-EDA-PLA micelles, together with erythrocytes and quantifying the haemoglobin release.

Erythrocytes were treated with 1 wt% Triton X-100 and PBS at pH 7.4 in order to obtain the values corresponding to 100% and 0% of lysis, respectively. Erythrocytes were incubated with aqueous dispersions of empty or sorafenib-loaded nanoparticles (at micelle concentrations of 1.6, 3.3 and 6.6 mg/ml) for 1 h at 37 °C. Under these conditions, empty or prodrug-loaded micelles showed no significant haemolytic effects, indicating no detectable interaction with red blood cell membranes. In fact, the percentage of haemolysis at the highest copolymer concentration was always less than 6.0%. In particular, haemolysis effect at the highest copolymer concentration resulted to be equal to 2.6 and 5.9% for empty and drug-loaded PHEA-EDA-PLA micelles, respectively; to 1.3 and 3.4% for empty and drug-loaded PHEA-EDA-PLA-GAL micelles, respectively. Moreover, no erythrocyte aggregation, after incubation with empty or prodrug-loaded micelles, was detected by microscopic observations (data not shown).

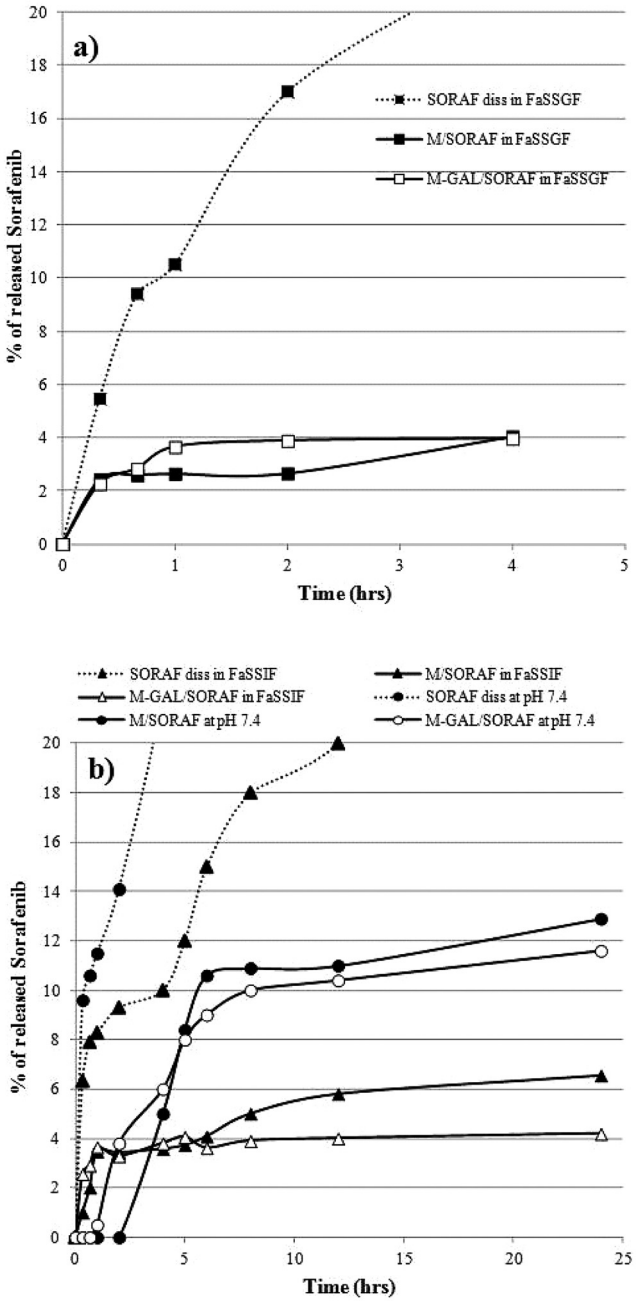

Fig. 3. Sorafenib diffusion profile across the dialysis membrane and release profiles of sorafenib from PHEA-EDA-PLA and PHEA-EDA-PLA-GAL micelles in: (a) FaSSGF (pH 1.6) for 4 h; (b) FaSSIF (pH 6.5) and in PBS (pH 7.4)/FCS mixture for 24 h. Data represent mean ± S.D., ($n = 3$).

These results demonstrate that micelles do not cause lysis of red blood cell membranes under chosen experimental conditions. The absence of cellular toxicity of empty micelles was already demonstrated (Craparo et al., 2013a).

3.5. In vivo experiments

To evaluate whether the entrapment of sorafenib into PHEA-EDA-PLA-GAL micelles could increase the drug bioavailability and

targeting to the liver, in vivo biodistribution studies were carried out. Oral administration route was chosen to carry out these experiments, since it is the best route in terms of patient compliance and currently sorafenib is administer in patients as Nexavar tablet.

In the field of oral drug delivery, drug encapsulation into polymeric carriers, such as polymeric micelles, could reduce several drawbacks usually associated with oral administration, which dramatically decrease the bioavailability of the drug. In particular, encapsulation could protect sensitive drugs from degradation in stomach and gut lumen, enhance absorption in the intestine by increasing water solubility, overcome drug resistance mechanisms (MDR) by altering the absorption pathway from transcellular to paracellular or transcytosis routes (Gaucher et al., 2010; Roger et al., 2010). Moreover, the capability of some polymers to increase the drug adsorption through the gastro-intestinal mucosa either by

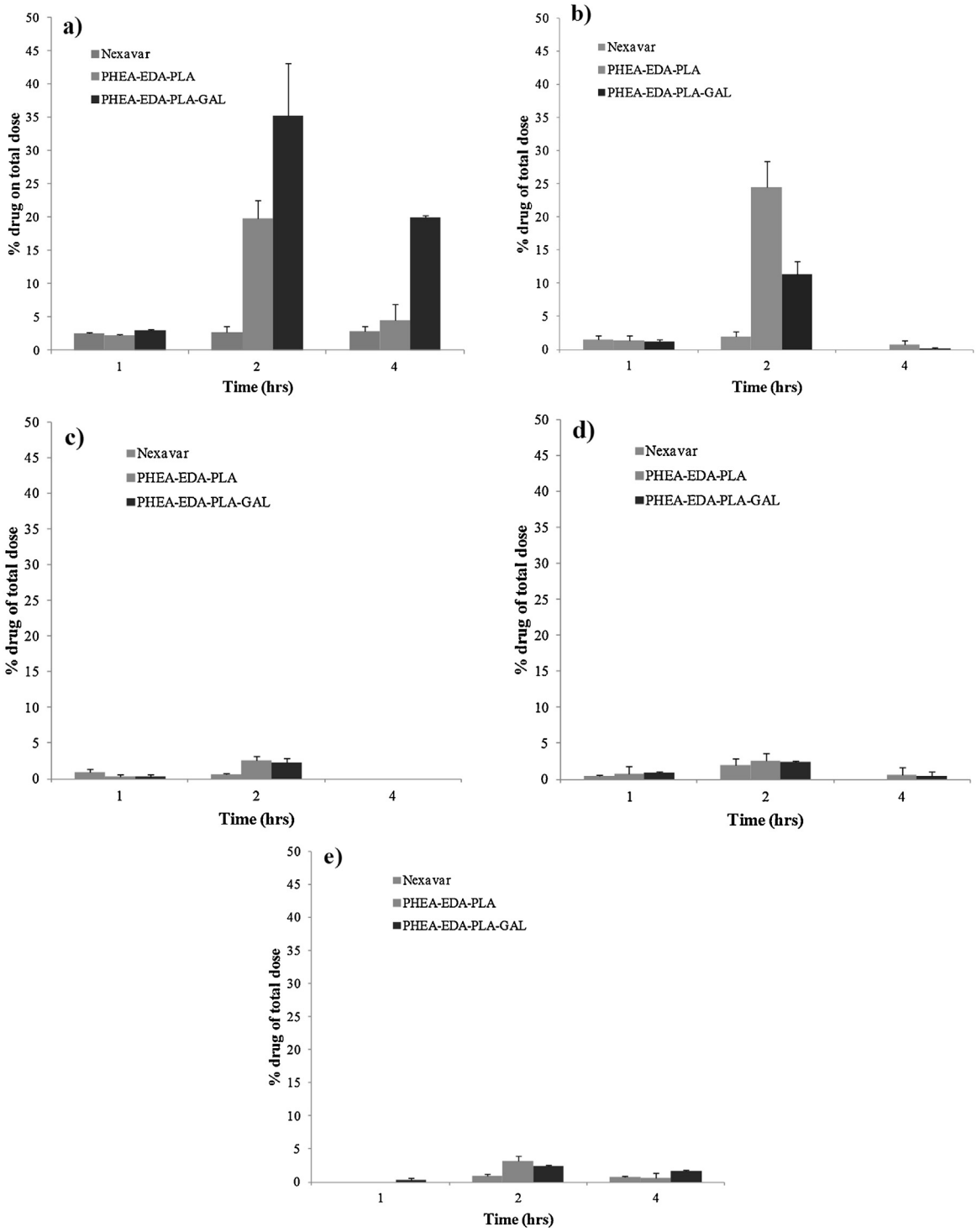

Fig. 4. % of sorafenib amount on total administered dose 1, 2 and 4 h post oral administration to adult female mice. ■, Nexavar; ■, sorafenib-loaded PHEA-EDA-PLA; and ■, sorafenib-loaded PHEA-EDA-PLA-GAL micelles, in (a) liver, (b) kidneys, (c), spleen, (d) lung and (e) blood, ($n = 2$ animals/time point).

increase the membrane permeability to the drug and/or to the carrier has been also reported (Benny et al., 2008; Gaucher et al., 2010; Mathot et al., 2007).

In our experiments, three formulations were used for the treatment: the powdered commercial tablet ("Nexavar", containing sorafenib tosylate) and sorafenib entrapped into both PHEA-EDA-PLA-GAL and PHEA-EDA-PLA micelles. These formulations were orally administered by gastric gavage, after dispersion in the vehicle (isotonic saline solution) at the single dose of 5.7 mg kg^{-1} of the body weight.

After 1, 2 and 4 h post administration, the concentration of sorafenib in liver, spleen, lung, kidney and blood was determined by HPLC analysis, as described in the experimental section. Data (expressed as drug weight percentage on the total administered dose) are summarized in Fig. 4a–e, respectively for liver, kidneys, spleen, lung and blood, as a function of time.

In the liver, 1 h post administration (Fig. 4a), the recovered drug amounts were very low and no significant differences were found depending on the administered sample. Indeed, 2 h post administration, the sorafenib concentration in the liver, when it was administered into targeted drug-loaded micelles, was significantly higher than when administered into non-targeted drug-loaded micelles ($p < 0.05$), or as free drug (sorafenib tosylate) ($p < 0.02$). In particular, when the sorafenib-loaded PHEA-EDA-PLA-GAL and PHEA-EDA-PLA micelles were administered, the amounts of drug found into the liver were equal respectively to 35 and 19.8 wt% on total administered dose, while when sorafenib was administered as Nexavar, the amount of drug found into the liver was equal to 2.7 wt% on total administered dose.

4 h post administration, the amounts of sorafenib in the liver were significantly decreased compared to those found after 2 h administration; but in the case of the targeted sample (drug-loaded galactosylated micelles), the amount was significantly higher than those found when Nexavar or drug-loaded non galactosylated micelles were administered ($p < 0.02$). In particular, when the sorafenib-loaded PHEA-EDA-PLA-GAL micelles were administered, the amount of drug found into the liver was equal to 19.9 wt% on total administered dose, while when sorafenib was administered as Nexavar or loaded into PHEA-EDA-PLA micelles the amounts of drug found into the liver were equal to 2.8 and 4.5% p/p on total administered dose, respectively. This result means that administered sorafenib entrapped into targeted micelles reached the target organ, the liver, to a much higher extent than the drug administered as free or loaded into non-targeted micelles, and it accumulated in the same organ.

As reported in Fig. 4b an accumulation into kidneys after 2 h post administration was appreciated when the administered drug is loaded into micelles compared to Nexavar. However, in the case of galactosylated micelles the drug percentage is halved compared to non-galactosylated micelles. Moreover the disappearance of the drug in kidneys 4 h post administration is in agreement with the hypothesis that the drug, as administered as micelles, is following the normal elimination pattern by kidney.

In Fig. 4b–e, the drug amounts found into kidneys, spleen, lung and blood are also reported as function of administration time. Although some significant differences in recovered drug amounts were almost evidenced in these organs, the significantly increased liver accumulation obtained with targeted micelles could allow to a reduction of administered dose.

From these graphics, the total amount of recovered drug 1, 2 and 4 h post administration in investigated organs and blood for each sample was calculated, and it resulted to be respectively 5.5, 53.6 and 22.2 wt% on total administered dose for galactosylated micelles, 4.7, 52.6 and 6.4 wt% for non-galactosylated micelles and 5.5, 8.2 and 3.5 wt% for Nexavar. This fact could be explained considering both the increased effect on bioavailability due to entrapment into polymeric colloidal carriers and also their potential absorption towards the gastro-intestinal mucosa (Benny et al., 2008; Gaucher et al., 2010; Mathot et al., 2007; Roger et al., 2010).

Therefore, all together these results seem to demonstrate that the use of galactosylated PHEA-EDA-PLA-GAL micelles increased the total absorbed drug and the liver targeting of sorafenib, and that this could be due to the contributor of the ASGR to internalization.

4. Conclusions

In this paper, we describe the synthesis and the characterization of a novel galactosylated graft amphiphilic copolymer, PHEA-EDA-PLA-GAL, able to self-assemble and form hepatocytes-targeted micelles carrying sorafenib. Empty and drug-loaded micelles were obtained and characterised in terms of zeta potential, that results positive, and colloidal mean size, showing values in the order of few ten nanometres. These sizes were confirmed by TEM analysis that showed also spherical shape of investigated samples. The DL% of prodrug loaded-galactosylated micelles resulted to be 3.0 wt%.

Preliminary in vitro studies demonstrated that these carriers have no haemolytic activity.

Biodistribution studies demonstrated that the amount of sorafenib entrapped into the galactosylated systems reaching the liver was significantly greater than both free sorafenib and sorafenib-loaded into non-targeted micelles, and it is more evident at longer times. Therefore, the presence of GAL on PHEA-EDA-PLA-GAL micelles confers them the capability to give an enhanced accumulation of sorafenib into the liver compared with that obtained by non-galactosylated systems, demonstrating the potential contribution of ASGPR to the internalization process.

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References

Benny, O., Fainaru, O., Adini, A., Cassiola, F., Bazinet, L., Adini, I., Pravda, E., Nahmias, Y., Koirala, S., Corfas, G., D'Amato, R.J., Folkman, J., 2008. An orally delivered small-molecule formulation with antiangiogenic and anticancer activity. Nature Biotechnology 26, 799–807.

Chaparro, M., Gonzàlez Moreno, L., Trapero-Marugàn, M., Medina, J., Moreno-Otero, R., 2008. Review article: pharmacological therapy for hepatocellular carcinoma with sorafenib and other oral agents. Alimentary Pharmacology and Therapeutics 28, 1269–1277.

Craparo, E.F., Ognibene, M.C., Casaletto, M.P., Pitarresi, G., Teresi, G., Giammona, G., 2008. Biocompatible polymeric micelles with polysorbate 80 for use in brain targeting. Nanotechnology 19, 485603.

Craparo, E.F., Cavallaro, G., Ognibene, M.C., Teresi, G., Bondì, M.L., Giammona, G., 2009. Amphiphilic poly(hydroxyethylaspartamide) derivative-based micelles as drug delivery systems for ferulic acid. Journal of Drug Targeting 17, 78–88.

Craparo, E.F., Teresi, G., Ognibene, M.C., Casaletto, M.P., Bondì, M.L., Cavallaro, G., 2010. Nanoparticles based on novel amphiphilic polyaspartamide copolymers. Journal of Nanoparticle Research 12, 2629–2644.

Craparo, E.F., Teresi, G., Bondì, M.L., Licciardi, M., Cavallaro, G., 2011. Phospholipid-polyaspartamide micelles for pulmonary delivery of corticosteroids. International Journal of Pharmaceutics 406, 135–144.

Craparo, E.F., Triolo, D., Pitarresi, G., Giammona, G., Cavallaro, G., 2013a. Galactosylated micelles for a ribavirin prodrug targeting to hepatocytes. Biomacromolecules 14, 1838–1849.

Craparo, E.F., Teresi, G., Licciardi, M., Bondì, M.L., Cavallaro, G., 2013b. Novel composed galactosylated nanodevices containing a ribavirin prodrug as hepatic cell-targeted carriers for HCV treatment. Journal of Biomedical Nanotechnology 9, 1107–1122.

Di Stefano, G., Colonna, F.P., Bongini, A., Busi, C., Mattioli, A., Fiume, L., 1997. Ribavirin conjugated with lactosaminated poly-L-lysine. Selective delivery to the liver and increased antiviral activity in mice with viral hepatitis. Biochemical Pharmacology 54, 357–363.

Di Stefano, G., Fiume, L., Baglioni, M., Bolondi, L., Busi, C., Chieco, P., Kratz, F., Manaresi, F., Pariali, M., 2006. A conjugate of doxorubicin with lactosaminated albumin enhances the drug concentrations in all the forms of rat hepatocellular carcinomas independently of their differentiation grade. Liver International 26, 726–733.

EEC Council Directive 86/609, OJ L 358,1,1 December 12, 1987.

Fiume, L., Bolondi, L., Busi, C., Chieco, P., Kratz, F., Lanza, M., Mattioli, A., Di Stefano, G., 2005. Doxorubicin coupled to lactosaminated albumin inhibits the growth of hepatocellular carcinomas induced in rats by diethylnitrosamine. Journal of Hepatology 43, 645–652.

Fiume, L., Di Stefano, G., 2010. Lactosaminated human albumin, a hepatotropic carrier of drugs. European Journal of Pharmaceutical Sciences 40, 253–262.

Francis, M.F., Cristea, M., Yang, Y., Winnik, F.M., 2005. Engineering polysaccharide-based polymeric micelles to enhance permeability of cyclosporin A across caco-2 cells. Pharmaceutical Research 22, 209–219.

Galia, E., Nicolaides, E., Horter, D., Lobenberg, R., Reppas, C., Dressman, J.B., 1998. Evaluation of various dissolution media for predicting in vivo performance of class I and II drugs. Pharmaceutical Research 15, 698–705.

Gaucher, G., Satturwar, P., Jones, M.-C., Furtos, A., Leroux, J.-C., 2010. Polymeric micelles for oral drug delivery. European Journal of Pharmaceutics and Biopharmaceutics 76, 147–158.

Giammona, G., Carlisi, B., Palazzo, S., 1987. Reaction of α,β-poly(*N*-2-hydroxyethyl)-D,L-aspartamide with derivatives of carboxylic acids. Journal of Polymer Science Part A: Polymer Chemistry 25, 2813–2818.

Giles, A.R., 1987. Guidelines for the use of animals in biomedical research. Thrombosis and Haemostasis 58, 1078–1084.

Hyodo, I., Mizuno, M., Yamada, G., Tsuji, T., 1993. Distribution of asialoglycoprotein receptor in human hepatocellular carcinoma. Liver 13, 80–85.

Jain, K., Kesharwani, P., Gupta, U., Jain, N.K., 2012. A review of glycosylated carriers for drug delivery. Biomaterials 33, 4166–4186.

Julyan, P.J., Seymour, L.W., Ferry, D.R., Daryani, S., Boivin, C.M., Doran, J., David, M., Anderson, D., Christodoulou, C., Young, A.M., Hesslewood, S., Kerr, D.J., 1999. Preliminary clinical study of the distribution of HPMA copolymers bearing doxorubicin and galactosamine. Journal of Controlled Release 57, 281–290.

Kane, R.C., Farrell, A.T., Madabushi, R., Booth, B., Chattopadhyay, S., Sridhara, R., Justice, R., Pazdur, R., 2009. Sorafenib for the treatment of unresectable hepatocellular carcinoma. The Oncologist 14, 95–100.

Keating, G.M., Santoro, A., 2009. Sorafenib – a review of its use in advanced hepatocellular carcinoma. Drugs 69, 223–240.

Li, L., Wang, H., Ong, Z.Y., Xu, K., Ee, P.L.R., Zheng, S., Hedrick, J.L., Yang, Y.Y., 2010. Polymer- and lipid-based nanoparticle therapeutics for the treatment of liver diseases. Nano Today 5, 296–312.

Licciardi, M., Campisi, M., Cavallaro, G., Cervello, M., Azzolina, A., Giammona, G., 2006. Synthesis and characterization of polyaminoacidic polycations for gene delivery. Biomaterials 27, 2066–2075.

Llovet, J.M., Ricci, S., Mazzaferro, V., Hilgard, P., Gane, E., Blanc, J.F., de Oliveira, A.C., Santoro, A., Raoul, J.L., Forner, A., Schwartz, M., Porta, C., Zeuzem, S., Bolondi, L., Greten, T.F., Galle, P.R., Seitz, J.F., Borbath, I., Häussinger, D., Giannaris, T., Shan, M., Moscovici, M., Voliotis, D., Bruix, J., 2008. SHARP investigators study group: sorafenib in advanced hepatocellular carcinoma. New England Journal of Medicine 359, 378–390.

Mathot, F., des Rieux, A., Ariën, A., Schneider, Y.-J., Brewster, M., Préat, V., 2007. Transport mechanisms of mmePEG750P(CL-co-TMC) polymeric micelles across the intestinal barrier. Journal of Controlled Release 124, 134–143.

Medina, S.H., Tekumalla, V., Chevliakov, M.V., Shewach, D.S., Ensminger, W.D., El-Sayed, M.E.H., 2011. *N*-acetylgalactosamine-functionalized dendrimers as hepatic cancer cell-targeted carriers. Biomaterials 32, 4118–4129.

Ngawhirunpat, T., Wonglertnirant, N., Opanasopit, P., Ruktanonchai, U., Yoksan, R., Wasanasuk, K., Chirachanchai, S., 2009. Incorporation methods for cholic acid chitosan-g-mPEG self-assembly micellar system containing camptothecin. Colloids and Surfaces B: Biointerfaces 74, 253–259.

NIH Guide for the Care and Use of Laboratory Animals, NIH Publication no. 80-23 (1985).

Poelstra, K., Prakash, J., Beljaars, L., 2012. Drug targeting to the diseased liver. Journal of Controlled Release 161, 188–197.

Rensen, P.C.N., Sliedregt, L.A.J.M., Ferns, M., Kieviet, E., van Rossenberg, S.M.W., van Leeuwen, S.H., van Berkel, T.J.C., Biessen, E.A.L., 2001. Determination of the upper size limit for uptake and processing of ligands by the asialoglycoprotein receptor on hepatocytes in vitro and in vivo. Journal of Biological Chemistry 276, 37577–37584.

Roger, E., Lagarce, F., Garcion, E., Benoit, J.P., 2010. Biopharmaceutical parameters to consider in order to alter the fate of nanocarriers after oral delivery. Nanomedicine 5, 287–306.

Song, B., Zhang, W., Peng, R., Huang, J., Nie, T., Li, Y., Jiang, Q., Gao, R., 2009. Synthesis and cell activity of novel galactosylated chitosan as a gene carrier. Colloids and Surfaces B: Biointerfaces 70, 181–186.

Suo, A., Qian, J., Yao, Y., Zhang, W., 2010. Galactosylated poly(ethylene glycol)-b-poly (L-lactide-co-β-malic acid) block copolymer micelles for targeted drug delivery: preparation and in vitro characterization. International Journal of Nanomedicine 5, 1029–1038.

Suriano, F., Pratt, R., Tan, J.P.K., Wiradharma, N., Nelson, A., Yang, Y.-Y., Dubois, P., Hedrick, J.L., 2010. Synthesis of a family of amphiphilic glycopolymers via controlled ring-opening polymerization of functionalized cyclic carbonates and their application in drug delivery. Biomaterials 31, 2637–2645.

US Food and Drug Administration. FDA approval for sorafenib tosylate. 2010-10-01. Available from: URL: http://www.cancer.gov/cancertopics/druginfo/fda-sorafenib-tosylate.

Vertzoni, M., Dressman, J., Butler, J., Hempenstall, J., Reppas, C., 2005. Simulation of fasting gastric conditions and its importance for the in vivo dissolution of lipophilic compounds. European Journal of Pharmaceutics and Biopharmaceutics 60, 413–417.

Wu, D.-Q., Li, Z.-Y., Li, C., Fan, J.-J., Lu, B., Chang, C., Cheng, S.-X., Zhang, X.-Z., Zhuo, R.-X., 2010. Porphyrin and galactosyl conjugated micelles for targeting photodynamic therapy. Pharmaceutical Research 27, 187–199.

Wu, Y.-T., Lin, K.-G., Huang, C.-M., Chang, C.-H., Sun, Y.-L., Fan, K.-H., Hsu, W.-C., Wang, H.-E., Lin, S.-B., 2004. A new *N*-acetylgalactosamine containing peptide as a targeting vehicle for mammalian hepatocytes via asialoglycoprotein receptor endocytosis. Current Drug Delivery 1, 119–127.

Yang, R., Meng, F., Ma, S., Huang, F., Liu, H., Zhong, Z., 2011. Galactose-decorated cross-linked biodegradable poly(ethylene glycol)-b-poly(ε-caprolactone) block copolymer micelles for enhanced hepatoma-targeting delivery of paclitaxel. Biomacromolecules 12, 3047–3055.

Zhang, J.-Y., He, B., Qu, W., Cui, Z., Wang, Y.-B., Zhang, H., Wang, J.-C., Zhang, Q., 2011. Preparation of the albumin nanoparticle system loaded with both paclitaxel and sorafenib and its evaluation in vitro and in vivo. Journal of Microencapsulation 28, 528–536.

Zheng, H., Zhang, X., Xiong, F., Zhu, Z., Lu, B., Yin, Y., Xu, P., Du, Y., 2011. Preparation, characterization, and tissue distribution in mice of lactosaminated carboxymethyl chitosan nanoparticles. Carbohydrate Polymers 83, 1139–1145.