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Chimeric advanced drug delivery nano systems (chi-aDDnSs) for shikonin combining dendritic and liposomal technology

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ABSTRACT

The interest of drug delivery has focused on the creation of new formulations with improved properties, taking much attention to the drug release from the carrier. Liposomes have already been commercialized, while dendrimers and hyperbranched polymers are emerging as potentially ideal drug delivery vehicles. Chimeric advanced drug delivery nano systems (chi-aDDnSs) are mixed nanosystems combining different biomaterials that can offer advantages as drug carriers. Alkannin and shikonin (A/S) are naturally occurring hydroxynaphthoquinones with a well-established spectrum of wound healing, antimicrobial, anti-inflammatory, antioxidant and recently established antitumor activity. In this work three generations of hyperbranched aliphatic polyesters were used for the first time to form complexes with shikonin, as well as liposomal chi-aDDnSs. Characterization of the shikonin-loaded chi-aDDnSs was performed by measuring their particle size distribution, ζ-potential, drug encapsulation efficiency and the *in vitro* release profile. The analysis revealed sufficient drug encapsulation and appropriately featured release profiles. Chi-aDDnSs were also examined for their physical stability at 4 °C. The results are considered promising and could be used as a road map for designing *in vivo* experiments.

1. Introduction

In the last 20 years, there have been numerous efforts on the development of a specific drug delivery system which can maintain continuous drug levels in a desired range, while reducing any side effects by improving the tissue or organ selectivity (Kolhe et al., 2003; Langer, 1998). Nanotechnology emerges as the multidisciplinary scientific field that will deeply change the philosophy and the modalities by which drugs are administered to patients. The new drug carriers in nano-scale offer the possibility of increasing the therapeutic index (TI) of the known or new drug molecules by increasing their effectiveness, diminishing their toxicity against physiological tissues and achieving controlled therapeutic levels of the drug for prolonged time (Alexis et al., 2008).

Dendrimers and hyperbranched polymers (Hp) represent a novel class of multifunctional macromolecules derived from a branches-upon-branches structural motif (Tomalia et al., 1990). As vehicles for drug delivery, they can be used either for encapsulation of bioactive compounds or for drug attachment at the periphery, offering various potential advantages such as increased water solubility, prolongation of drug circulation time, protection of a drug from its surroundings, increase in drug stability (and possibly effectiveness) and the ability to target diseased tissue (Esfand and Tomalia, 2001; Gillies and Frechet, 2005). This solubility enhancement is promoted either via covalent attachment of the active compounds onto the surface functional groups or through their encapsulation inside the inner cavities sustained by the combined action of steric hindrance and specific interactions (D'Emanuele et al., 2007). Dendrimers have been used to form complexes with anticancer drug resulting in conjugates that exhibited higher accumulation in solid tumors and lower toxicity compared to the free drug (Astruc et al., 2010; Boas and Heegaard, 2004; Svenson and Tomalia, 2005).

In this context, a promising idea is the replacement of dendrimers with Hp, which represent highly branched, polydisperse macromolecules with a treelike topology and a large number of functional groups (Kolhe et al., 2003; Suttiruengwong et al., 2006; Voit, 2000). The tedious and complex multistep synthesis

Abbreviations: Hp, hyperbranched polymers; Chi-aDDnSs, chimeric advanced drug delivery nano systems; Alkannin, A; Shikonin, S; MCRnSs, modulatory controlled release nano systems; EPC, egg phosphatidylcholine; PBS, phosphate buffer saline pH 7.4; SLS, sodium lauryl sulfate; DMSO, dimethyl sulfoxide; PDI, polydispersity index.

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of dendrimers results in expensive products with limited use in large-scale industrial applications. In contrast, randomly branched hyperbranched polymers with similar properties can be easily synthesized via one-step reaction and thus represent economically promising products for both small and large-scale industrial applications. During the last years, a wide variety of applications which originally seemed conceivable only for dendrimers, were investigated for hyperbranched polymers, such as in the field of life sciences and more specifically for the development of new drug delivery systems (Suttiruengwong et al., 2006). Among these polymeric materials, the commercially available hyperbranched aliphatic polyesters bearing the commercial name Boltorn® are considered promising candidates for the development of novel drug delivery systems as demonstrated in recent experiments (Klee et al., 2001). Given the potential of these multifunctional polymers to act as solubility enhancement agents, their consideration as carriers for poorly water soluble but therapeutically active pharmaceutical compounds seems appealing. Gao and coworkers suggested a water-soluble hyperbranched polyester as a novel polymer for drug delivery (Gao et al., 2003), while the Kolhe group has studied the ability of hyperbranched polyols to form carrier systems encapsulating the drug ibuprofen (Kolhe et al., 2003).

Liposomes are spherical vesicles of phospholipid bilayers with an entrapped aqueous phase and due to their biphasic character can act as carriers for both lipophilic and hydrophilic drugs. They have been proved to ameliorate the pharmacokinetic and pharmacological profile of many drugs giving way to the appearance of several liposomal formulations in the market, some concerning anticancer drugs (Abraham et al., 2005; Schiffelers and Storm, 2008; Woodle, 1995).

The combination of dendrimers and liposomes in liposomal *locked-in* dendrimers (LLDs) and the study of their component interactions has been reported for the first time in 2001 (Purohit et al., 2001). Such nanocarriers have been classified as chimeric advanced drug delivery nano systems (chi-aDDnSs) due to the combination of two different nanobiomaterials and two independent technologies for producing an overall system with unique properties (Demetzos, 2010a,b), which could have advantages over conventional nanocarriers. The attention of the scientific community to chi-aDDnSs is caused by two significant advantages over conventional nanocarriers and especially liposomes: (a) the increase of the drug load uptaken by the system and (b) the modification of the release profile of the drug from the chi-aDDnS compared to that of the conventional liposomal formulation, leading to a higher therapeutic index (Gardikis et al., 2010a; Khopade et al., 2002; Tekade et al., 2009). In this context, chi-aDDnSs have been classified as modulatory controlled release nano systems (MCRnSs) (Demetzos, 2010a,b).

Alkannin and shikonin (A/S; Fig. 1) are chiral-pair of naturally occurring isohexenylnaphthazarins. They are found in the external layer of the roots of at least a 150 species that belong mainly to the genera *Alkanna*, *Lithospermum*, *Echium*, *Onosma* and *Anchusa* of the Boraginaceae family (Papageorgiou et al., 1999, 2006).

Alkannin, shikonin and their derivatives were originally introduced and established as wound healing agents by Prof. Papageorgiou. A wound healing pharmaceutical ointment is already commercially available under the trademark HELIXDERM® and the medical devices HELIX*FILM*, HELIX*GEL* and HELIX*SPRAY* (wound healing collagen film, gel and spray respectively) are under development. Further biological investigations over the last 35 years have shown that A/S are potent pharmaceutical substances with a well-established wide spectrum of antimicrobial, anti-inflammatory and antioxidant activity (Papageorgiou, 1980; Papageorgiou et al., 1999, 2006, 2008). Extensive scientific research has been conducted the last years on cancer chemotherapy, focusing on A/S effectiveness on several tumors and mechanism(s) of antitumor action (Chen et al., 2002; Komi et al., 2009; Lee et al., 2008; Papageorgiou et al., 1999, 2006; Yang et al., 2009; Yao and Zhou, 2010; Zeng et al., 2009).

The scarce aqueous solubility of A/S (0.00002 M) (He, 2009) is a barrier for their oral and internal administration, since they cannot be easily dissolved and further absorbed from the receptor. A/S are also oxidized (Cheng et al., 1995), polymerized (Assimopoulou and Papageorgiou, 2004c,d; Papageorgiou et al., 2002) and internally metabolized (Meselhy et al., 1994a,b). Regarding the toxicity of the active compounds, alkannin was found to bear a LD_{50} of 3 g/kg in mice and less than 1 g/kg in rats, when administered orally in a feeding study (Majlathova, 1971). Shikonin, on the other hand, was found to be rather more toxic to mice by intraperitoneal administration, with a LD_{50} of 20 ± 5 mg/kg (Hayashi, 1977). In addition, during *in vivo* testing (mice, intraperitoneal administration), shikonin showed toxicity at dosages higher than 15 mg/kg/day (Sarcoma-180) and at 10×5 mg/kg/day (L-1210) (Sankawa et al., 1977). Both the solubility and instability matters could be overcome by delivering A/S through a drug delivery nano-system, such as Hp–drug complexes or chi-aDDnSs formulations, which could furthermore enhance their antitumor activity through toxicity decrease and targeted delivery.

In this work, new formulations of shikonin were prepared in order to combine the advantages of the applied drug carriers with the biological properties of shikonin and mainly its antitumor one. Shikonin was incorporated into hyperbranched polymers (Boltorn®, PFH-16-OH, PFH-32-OH, PFH-64-OH) forming complexes (samples 1–12; Table 1) and furthermore some of these complexes were *locked-in* liposomes consisting of EPC, forming chi-aDDnSs (LipoA-32-OH, LipoA-64-OH, LipoB-32-OH and LipoB-64-OH; Table 2), in order to enhance selectivity to cancer cells, reduce any side effects and protect shikonin from internal biotransformations. This is the first time that hyperbranched polymers are used to form drug delivery system for shikonin, either as complexes or as mixed liposomal systems (aDDnSs) and are characterized in terms of their physicochemical behaviour and drug encapsulation. Stability for both shikonin–Hp complexes and aDDnSs was studied, while the *in vitro* release rate of the aDDnSs was evaluated as well. A theoretical approach on the complexation of shikonin with the same hyperbranched polymers has been published by our group, studying in detail the mechanisms involved in the non-covalent association of shikonin with the Hp, in ethanol solutions (Tanis et al., 2011).

This research is a continuation study of the authors on exploiting the biological properties of A/S and other naphthoquinones through the preparation of DDSs, such as microcapsules (Assimopoulou et al., 2003; Assimopoulou and Papageorgiou, 2004b), cyclodextrins (Assimopoulou and Papageorgiou, 2004a), liposomes (Kontogiannopoulos et al., 2011a) and electrospun fiber mats (Kontogiannopoulos et al., 2011b).

2. Materials and methods

2.1. Materials

Shikonin was used after purification from a commercial sample (Ikeda Corporation, *Tokyo, Japan*), by silica gel column chromatography (gradient mixtures of *n*-hexane:dichloromethane:chloroform) followed by recrystallization (*n*-hexane), according to the procedure proposed by Prof. Papageorgiou (Assimopoulou et al., 2008) (purity obtained 100%).

Egg phosphatidylcholine (EPC), phosphate buffer saline pH 7.4 (PBS), sodium lauryl sulfate (SLS), Sephadex G75 were purchased from Sigma–Aldrich (*St. Louis, USA*). The hyperbranched polymers Boltorn® (PFH-16-OH, PFH-32-OH, PFH-64-OH) with

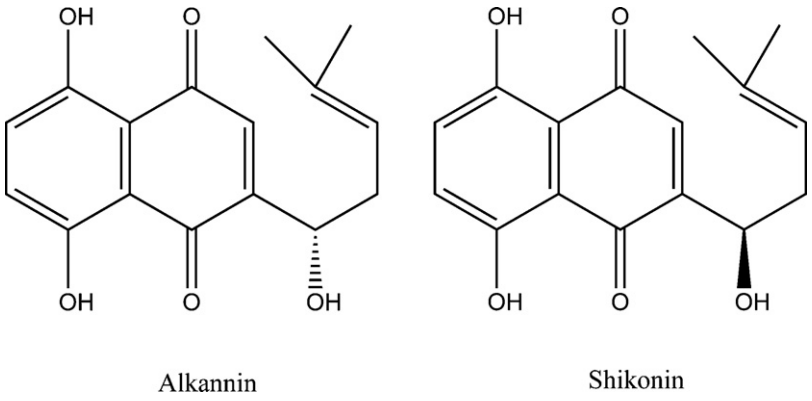

Fig. 1. The chiral pair alkannin and shikonin that possesses major biological activity.

Table 1
Results of the entrapment efficiency of shikonin into Hp complexes.

Sample no.	Hyperbranched polymer type	Structure maker solvent	Shikonin/Hp molar ratio	Drug entrapment efficiency (%)
1	PFH-16-OH	Ethanol	2:1	0.62 ± 0.17
2	PFH-32-OH	Ethanol	2:1	1.28 ± 0.10
3	PFH-64-OH	Ethanol	2:1	2.63 ± 0.23
4	PFH-16-OH	Methanol	2:1	1.07 ± 0.15
5	PFH-32-OH	Methanol	2:1	2.36 ± 0.27
6	PFH-64-OH	Methanol	2:1	3.79 ± 0.27
7	PFH-16-OH	Ethanol	0.2:1	4.27 ± 0.51
8	PFH-32-OH	Ethanol	0.2:1	5.32 ± 0.59
9	PFH-64-OH	Ethanol	0.2:1	6.44 ± 0.93
10	PFH-16-OH	Methanol	0.2:1	3.97 ± 0.68
11	PFH-32-OH	Methanol	0.2:1	7.10 ± 0.69
12	PFH-64-OH	Methanol	0.2:1	8.59 ± 0.56

Data are shown as mean ± SD of $n = 3$ independent experiments.

average molecular weights (M_w) of 2100, 3500, and 5100 g/mol, respectively were purchased from Perstorp (*Sweden*). All organic solvents were of analytical grade and were purchased from Sigma–Aldrich (*St. Louis, USA*). Water used in all experiments was of HPLC grade.

2.2. Preparation of shikonin–Hp complexes

Three different generations of hyperbranched polymers (PFH-16-OH, PFH-32-OH and PFH-64-OH; Fig. 2) were used and various process parameters were tested in order to optimize the polymer/drug complexes' preparation technique and to achieve sufficient encapsulation efficiency.

2.2.1. Incorporation technique

Two different organic solvents (ethanol and methanol) were used for the complexation process (will be referred as *structure-maker solvent*) for the incorporation of shikonin in Hp, in order to examine the effect of the solvent in the entrapment efficiency of the drug. An organic solution of shikonin was mixed with a Hp solution (varying generations) in the same organic solvent (2:1 and 0.2:1 molar ratio of shikonin:Hp) and the solutions were stirred for 22 h. The organic solvent was evaporated to dryness at 35 °C in vacuum, using rotary evaporator (*Rotavapor R-114, Buchi, Switzerland*) and left overnight under vacuum. The dry residue was dissolved in PBS (final Hp concentration 10 mg/mL) and left 24 h for hydration at 37 °C. Non-entrapped shikonin was removed through centrifugation (4000 rpm for 15 min, using a *SORVAL T-880* centrifuge with fixed angle rotor) and the amount of shikonin incorporated into Hp complexes was calculated as described in Section 2.2.2.

2.2.2. Determination of entrapment efficiency

The percentage of shikonin incorporated into complexes with hyperbranched polymers was estimated by UV–vis spectrophotometry (*UV-1700, UV-Visible spectrophotometer, Shimadzu, Tokyo, Japan*) at the characteristic wavelength of shikonin (516 nm). 0.3 mL of each complex solution in PBS were suspended in 2.7 mL of organic solvent to destroy the complex structure, releasing the drug into the organic phase. Absorbance of the organic phase was measured. Shikonin content was determined using a calibration curve.

The entrapment efficiency was calculated using the following equation:

$$\text{Entrapment efficiency } (\%) = \frac{F_i}{F_t} \times 100 \quad (1)$$

where F_i is the amount of shikonin incorporated into shikonin–Hp complexes and F_t is the initially added amount of shikonin.

Table 2
Physicochemical characteristics of prepared aDDnSs with shikonin.

Sample no.	Mean particle size (nm)	PDI	ζ-potential (mV)	Encapsulation efficiency (%)
LipoA-32-OH	170.3 ± 29.0	0.426 ± 0.03	−0.75 ± 3.32	5.41 ± 0.70
LipoA-64-OH	169.4 ± 23.7	0.411 ± 0.06	−1.00 ± 3.11	12.96 ± 0.60
LipoB-32-OH	177.9 ± 21.5	0.462 ± 0.07	0.50 ± 2.69	45.61 ± 3.03
LipoB-64-OH	185.0 ± 10.9	0.482 ± 0.02	1.97 ± 0.75	47.60 ± 1.12

The results are the mean of three independent experiments ± S.D.

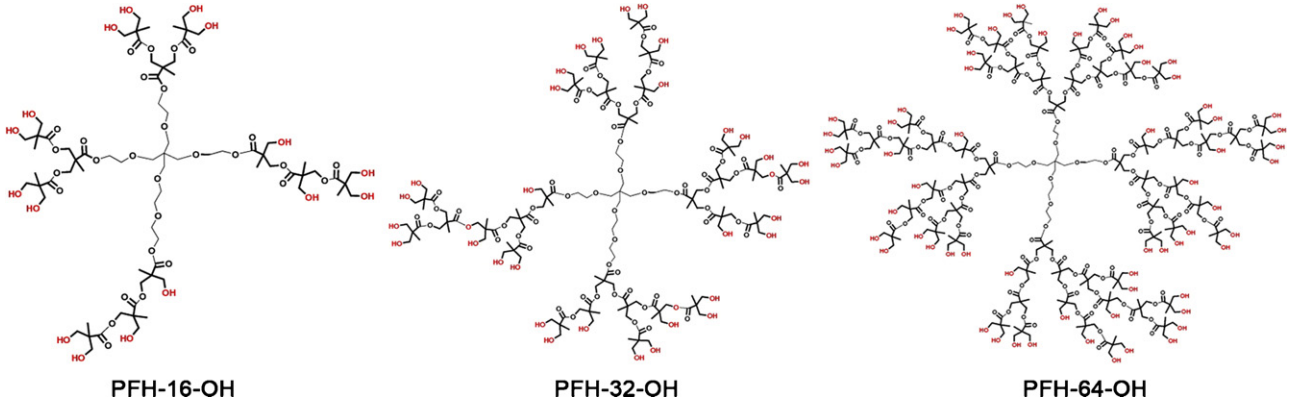

Fig. 2. Structures of PFH-16-OH, PFH-32-OH and PFH-64-OH hyperbranched polymers.

Various organic solvents were tested for the determination of the entrapment efficiency, in order to destroy the complex structure and release the drug into the organic phase (ethanol, DMSO:H_2O 1:1.8 (v/v) and methanol).

2.2.3. Drug leakage

Shikonin–Hp complexes were evaluated for drug leakage at 4 °C for 48 h. Aliquots of samples (1 mL) were taken at specific time intervals and centrifuged at 4000 rpm for 15 min; under these conditions the complexes remained suspended and the released shikonin precipitated. The supernatant was analyzed for shikonin concentration in order to determine the leakage rate as described in Section 2.2.2.

2.3. Shikonin-loaded chi-aDDnSs preparation

Two different procedures were tested for the incorporation of shikonin–Hp complexes into EPC liposomes, in order to produce chi-aDDnSs. In both cases liposomes were formed using the thin film hydration method (Bangham et al., 1965). In the first method an aqueous solution of shikonin–Hp complexes (0.2:1 molar ratio of shikonin:Hp) was prepared as described in 2.2.1. A lipid film was prepared by dissolving EPC (0.195 mmol of total lipids) in chloroform followed by evaporation of the solvent in a rotary evaporator (*Rotavapor R-114, Buchi, Switzerland*). The film was left under vacuum overnight and then hydrated with the aqueous solution of shikonin–Hp complexes prepared as above (final lipid concentration 30 mg/mL), under stirring for 1 h at 45 °C in order to prepare multilamellar vesicles (MLVs). These liposomal formulations will be referred to as LipoA.

In the second method, a specific volume of shikonin solution in methanol was mixed with an Hp solution in methanol (0.2:1 molar ratio of shikonin:Hp) and the solution was stirred for 22 h. Afterwards, a chloroform solution of EPC (0.195 mmol of lipid) was added, stirred for 1 h and the solvent was slowly evaporated in a rotary evaporator to form the lipid film. The lipid film was dried under vacuum overnight and multilamellar vesicles (MLVs) were prepared by hydrating the lipid film with PBS (final lipid concentration 30 mg/mL) and stirring for 1 h at 45 °C. These formulations will be referred later as LipoB.

In both methods, small unilamellar vesicles (SUVs) were prepared from the resultant liposomal suspension (MLVs), after sonication for two 5 min periods interrupted by a 5 min resting period, using a probe sonicator (amplitude 0.7; pulser 50%; *UP 200S, dr. Hielsher GmbH, Berlin, Germany*). The resultant vesicles were allowed for 30 min to anneal any structural defects. Non-encapsulated shikonin was removed by passing the liposomal suspensions through a Sephadex G75 column that was swollen with water overnight

2.3.1. Particle size measurement and ζ-potential

Size and ζ-potential of liposomes are crucial parameters that indicate their physical stability. The hydrodynamic diameter of all liposomal formulations was measured by light scattering. 50 μL of each liposomal formulation was 60-fold diluted in PBS (pH 7.4) immediately after preparation and *z*-average mean and ζ-potential were measured. Measurements were made at 25 °C and at a 90° angle in a photon correlation spectrometer (Zetasizer 3000HS, Malvern Instruments, *Malvern, UK*) and analyzed by the CONTIN method (MALVERN software).

2.3.2. Determination of entrapment efficiency

To remove the non-encapsulated shikonin, liposomal suspensions were passed through a Sephadex G75 column prior to the determination of the entrapment efficiency as described in Section 2.2.2. Shikonin concentration was measured by UV–vis spectrometer at λ_{max} = 516 nm after the addition of methanol to the liposomal suspension, with the aid of the following shikonin calibration curve in methanol:

$$\text{Drug concentration (mg/mL)} = 0.03825 \times \text{absorbance} + 0.00002; \quad (R^2 = 0.99992) \qquad (2)$$

2.3.3. In vitro drug release

The release profile of shikonin from aDDnSs was studied in (PBS + 1% SLS) at 37 °C. Liposomal formulations (1 mL of each sample containing 0.024 nmol shikonin for LipoA-32-OH, 0.022 nmol for sample LipoA-64-OH, 0.25 nmol for LipoB-32-OH and 0.129 nmol for sample LipoB-64-OH) were placed in dialysis sacks (molecular weight cut off 13,000; *Sigma–Aldrich*). Dialysis sacks were inserted in 20 mL (PBS + 1% SLS) in shaking water bath (*Selecta*) set at 37 °C. Aliquots of samples (3 mL) were taken from the external solution at specific time intervals and that volume was replaced with fresh release medium in order to maintain sink conditions. The amount of shikonin released at various times, up to 72 h, was determined using UV–vis spectrometer at λ_{max} = 518 nm with the aid of the following calibration curve of shikonin in the release medium:

$$\text{Drug concentration (mg/mL)} = 0.04779 \times \text{absorbance} - 0.00001; \quad (R^2 = 0.99998) \qquad (3)$$

The cumulative percentage of drug release was calculated and plotted versus time using the equation:

$$\%\ \text{Cumulative drug released}_t = \frac{\text{Drug released}_t}{\text{Total entrapped shikonin}} \times 100 \quad (4)$$

2.3.4. Stability studies

aDDnSs incorporating shikonin–Hp complexes were tested for their stability by means of drug leakage, mean particle size, polydispersity index (PDI), and ζ-potential. Specifically, immediately after preparation liposomes formulations were placed in glass vials and stored at 4 °C for five days. Aliquots of samples were taken at specific time intervals and mean particle size, ζ-potential and entrapment efficiency were measured as described earlier.

2.4. Statistical analysis

Results are shown as mean value ± standard deviation (S.D.) of three independent experiments. Statistical analysis was performed using Student's *t*-test and multiple comparisons were done using one-way ANOVA. *P* values <0.05 were considered statistically significant. All statistical analyses were performed using "SPSS 14.0".

3. Results and discussion

Dox has been incorporated into a liposomal aDDnSs with PAPAM dendrimer (Papagiannaros et al., 2005), as well as with a synthesized poly-ester-ether PG1 dendrimer (Gardikis et al., 2010a). In the present study three generations of hyperbranched polymers are used for the first time, as drug delivery system for shikonin, either as complexes or as mixed liposomal systems (aDDnSs).

3.1. Shikonin–Hp complexes

Shikonin is a potent anticancer drug with an extensive scientific research focusing the last years on its effectiveness on several tumors (Chen et al., 2002; Papageorgiou et al., 1999, 2006; Zeng et al., 2009). In order to achieve a sufficient controlled release system of shikonin, several shikonin–Hp complexes were prepared and consecutively these complexes were attached to lipidic bilayers of liposomes composed of EPC.

In the present study, the formation of shikonin–Hp complexes has been successfully achieved, for the first time, using three generations of hyperbranched polymers (i.e. PFH-16-OH, PFH-32-OH and PFH-64-OH), various organic solvents (ethanol and methanol) and varying shikonin:Hp molar ratios for all three generations (i.e. 2:1 and 0.2:1). The ability of hyperbranched polymers to encapsulate various amounts of drug was studied. The complexation of shikonin into Hp was successfully carried out, exhibiting acceptable entrapment efficiency values (similar drug-loaded Hp-complexes exhibited entrapment efficiency values between 4% and 20% (Suttiruengwong et al., 2006)), but the amount of shikonin incorporated into Hp complexes varied from one Hp generation to another. Table 1 reveals that PFH-64-OH (samples 3, 6, 9 and 12) results, in all cases, to higher entrapment efficiencies compared to the other two lower generation hyperbranched polymers.

3.1.1. Determination of entrapment efficiency

For the proper determination of the entrapment efficiency, it is crucial to use the appropriate organic solvent in order to destroy the complex structure and release the drug into the organic phase.

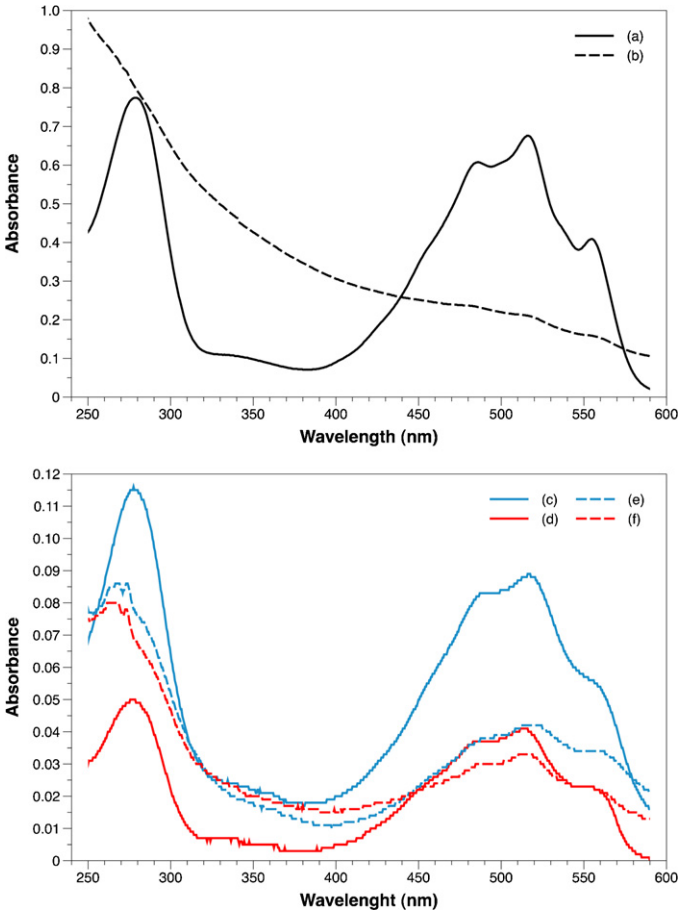

Fig. 3. UV–vis spectra of (a) purified shikonin in ethanol, (b) shikonin–Hp complexes in ethanol (c) purified shikonin in DMSO:H_2O, (d) purified shikonin in methanol, (e) shikonin–Hp complexes in DMSO:H_2O and (f) shikonin–Hp complexes in methanol.

Drug must be highly soluble into the organic solvent and the solvent must destroy complexes immediately and effectively. Three different solvents were tested for the entrapment efficiency determination of the complexes: ethanol, DMSO:H_2O 1:1.8 (v/v) and methanol. Shikonin is highly soluble in all of them. Dissolving the same sample to each of the three solvents and calculating the amount of the encapsulated drug, as described in Section 2.2.2, resulted in different values of entrapment efficiency. Although ethanol is widely used for the determination of entrapment efficiency, when it comes to shikonin–Hp complexes, it proved to be inefficient. Fig. 3 shows the spectroscopic characterization of pure shikonin and a shikonin–Hp sample after they have been dissolved in ethanol. The two spectra are distinctly different revealing that ethanol did not act towards destroying the complexes and as a result no shikonin was dissolved in the organic phase.

On the contrary, the other two organic solvents tested showed satisfactory results. Fig. 3 also reveals that shikonin–Hp complexes when diluted in both methanol and DMSO:H_2O 1:1.8 (v/v) exhibited a similar spectrum with pure shikonin dissolved in the same organic solvent.

In order to examine which of the two solvents (methanol and DMSO:H_2O) is more appropriate to be utilized for the estimation of entrapment efficiency of the samples, both solvents were tested. The results of entrapment efficiency values for methanol are presented in Table 1 while those for DMSO:H_2O were 0.24 ± 0.04 for sample 1, 0.78 ± 0.07 for sample 2 and 2.18 ± 0.09 for sample 3. Therefore, in all cases methanol rendered higher entrapment efficiency values. This might be due to the lower ability of DMSO:H_2O

to destroy the shikonin–Hp complexes. As a result, methanol appears as the most proper solvent for the determination of the entrapment efficiency and therefore was used in the following experiments for the determination of entrapment efficiency.

3.1.2. Effect of the Hp generation and the organic solvent used as the structure-maker on the complexation process

The organic solvent used as the structure-maker for the complexes, is a crucial parameter for the successive incorporation of the drug into a hyperbranched polymer. Both polymer and drug must be soluble in the organic solvent, while it should possess –OH groups to act as a structure-maker and enhance complexation. The work presented here is the first attempt to incorporate shikonin in some new commercial hyperbranched polymers. In order to examine the effect of the solvent in the entrapment efficiency of the drug, two different solvents (ethanol and methanol) were tested during the complexation process. Table 1 reveals that methanol offers a significant advantage when used as the structure-maker solvent enhancing the complexation of shikonin into Hp resulting in higher entrapment efficiency values.

Previous studies showed that the drugs which do not interact with the end groups can be encapsulated in the interior of the polymer and the extent of encapsulation can be increased by increasing the generation number, or by attaching long chain molecules to the end groups of the hyperbranched polymer (Kojima et al., 2000; Kolhe et al., 2003). Similarly, in our case, the increase of the generation results in increased entrapment efficiency. Since PFH-64-OH molecule is bigger in size compared to PFH-32-OH and PFH-16-OH, some shikonin molecules could have been encapsulated in the core of the hyperbranched polymer in addition to the complexation with the surface end groups. This encapsulation could be a result of geometric constriction combined with weak interactions between the polymer core and the drug, as pointed out previously (Kojima et al., 2000; Tanis and Karatasos, 2009a; Tanis et al., 2011). Furthermore, as demonstrated by recent molecular dynamic simulations (Tanis and Karatasos, 2009b; Tanis et al., 2011) different generations of the hyperbranched polyesters, exhibit markedly different intramolecular hydrogen bonding patterns, which might affect (e.g. act antagonistically) the polymer/drug association. In fact, the low-generation hyperbranched polymers appear to form a stronger intramolecular hydrogen bonding network, which results to more "closed" conformations that might inhibit the drug/Hp association.

Regardless the organic solvent used for the determination of the entrapment efficiency, the higher generation polymer (PFH-64-OH) in any case resulted in complexes with the highest entrapment efficiency of the drug, as indicated in Table 1. This observation can be understood in the context described in the recently published simulational work (Tanis et al., 2011). PFH-16-OH showed the smallest complexation ability among the three generations of PFH hyperbranched polymers and therefore it was not further considered for the preparation of aDDnSs.

Table 1 also reveals the correlation between the entrapment efficiency and the shikonin–Hp molar ratio used. Regardless the generation of the polymer and the organic solvent used, the use of 0.2:1 shikonin:Hp molar ratio resulted, in all cases, into higher entrapment efficiency values.

3.1.3. Drug leakage

Drug leakage is a crucial characteristic for the evaluation of a drug delivery system. Shikonin–Hp complexes were tested for their drug leakage over a 48 h period at 4 °C. Most of the drug leakage occurred at the first 8 h of incubation followed by minor drug precipitation. PFH-64-OH complexes retained 86.8% of their initial drug content at the first 8 h of the testing period (Fig. 4). PFH-32-OH complexes were less stable and retained only 55.5% of their initial drug content. Drug leakage in plasma and buffers is a known drawback of drug–dendrimers complexes that has been previously reported (Astruc et al., 2010). The average amount of shikonin leaked after one day of incubation was 17.9% for PFH-64-OH (sample 12) and 50% for PFH-32-OH (sample 11) complexes, while after two days increased to 23.5% and 51.3%, respectively (Fig. 4).

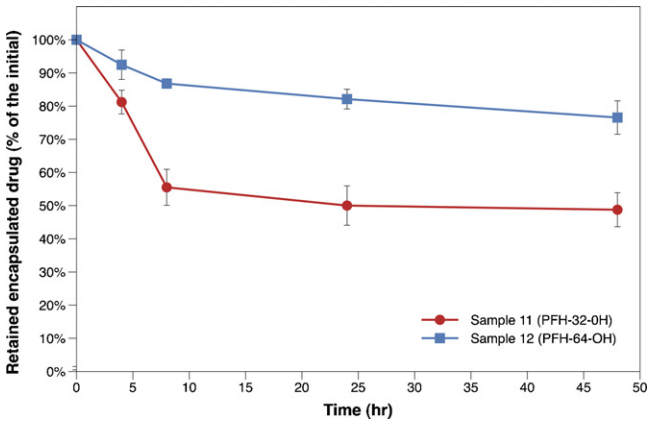

Fig. 4. Drug retention in shikonin–Hp complexes (samples 11 and 12) in storage conditions at 4 °C (each value represents the mean ± S.D. of $n = 3$ independent experiments).

3.2. Shikonin-loaded chi-aDDnSs

To the best of our knowledge, this is the first time that hyperbranched polymers are used to form drug delivery system for shikonin, either as complexes or as mixed liposomal systems (aDDnSs). In a recent study, the same hyperbranched polyesters were combined with liposomes to form a drug-free aDDnSS and the polymer–lipid interactions along with their dependence on the polyesters pseudogeneration number and the liposomal lipid composition were examined (Mourelatou et al., 2011). In the present study, PFH-32-OH and PFH-64-OH shikonin complexes were used for the development of new aDDnSs, at 0.2:1 shikonin:Hp molar ratio, since this molar ratio resulted in higher drug entrapment efficiency (Section 3.1.2). Shikonin–Hp complexes were successfully incorporated into liposomes composed of EPC using two different techniques. The percentile entrapment efficiency of shikonin into aDDnSs was 5.41–12.96% for the first technique and 45.61–47.60% for the second one (Table 2). Evidently, the second preparation technique was more effective concerning the entrapment of shikonin regardless the generation of the hyperbranched polymer used. This could be attributed to the inherent advantage of the second technique, which enables the incorporation of those shikonin molecules that were not complexed to the hyperbranched polymer, into the lipid bilayer of liposomes. On the contrary, in the first technique the non-complexed shikonin was removed through centrifugation prior to the incorporation of the shikonin–Hp complexes into liposomes.

3.2.1. Mean particle size and ζ-potential

Size measurements of aDDnSs indicated an average diameter of 169.9 nm for LipoA liposomes and 181.4 nm for LipoB formulations. This slight variation might be ascribed to the higher amount of drug being encapsulated in LipoB. Since shikonin is hydrophobic, it is incorporated into the lipid bilayer of the liposomes, increasing thus the average size of the liposome formulations. Additionally, the size of these aDDnSs is similar to that of conventional shikonin-loaded liposomes prepared recently (Kontogiannopoulos et al., 2011a). The mean particle size, PDI index and ζ-potential of all liposome formulations are indicated in Table 2.

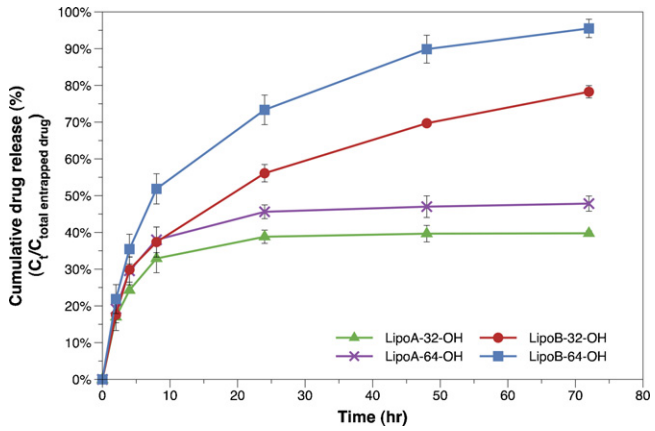

Fig. 5. Cumulative drug release from chi-aDDnSs (each value represents the mean ± S.D. of $n = 3$ independent experiments).

3.2.2. *In vitro drug release*

The *in vitro* release of the drug from the prepared aDDnSs is quite slow for the formulations prepared with the first technique (39.76% at 72 h for LipoA-32-OH and 47.84% within 72 h for LipoA-64-OH) (Fig. 5). On the other hand, liposomes prepared with the second technique exhibited a faster release rate. The formulation prepared with PFH-64-OH (LipoB-64-OH) released 95.51% of the entrapped drug at 72 h and LipoB-32-OH released 78.27% of the encapsulated drug at 72 h (Fig. 5). The average amount of shikonin released after one day of incubation was 56.10% for LipoB-32-OH, 73.35% for LipoB-64-OH, 38.82% for LipoA-32-OH and 45.61% for LipoA-64-OH (Fig. 5). Due to their preparation procedure, LipoB formulations incorporate both shikonin–Hp complexes and free shikonin as previously stated. As shown in Fig. 5, LipoA formulations almost complete their release procedure at the first 24 h. On the other hand, LipoB formulations continue to release drug up to 72 h. This observation could serve as a control parameter for the preparation of liposomal formulations with the desired release profile, modulating the release rate of the drug, improving its therapeutic index and finally decreasing any unwanted side effects.

In this context the combination of hyperbranched polymers with liposomes for the development of a novel nanocarrier appears very promising, mostly due to the fact that the hyperbranched polymer acts as a modulator for the release rate of the encapsulated drug. Though the exact mechanism of release modulation is still under investigation, there are several studies that shed light on the physicochemical interactions between the components of such nanosystems comprised by different nanobiomaterials (Gardikis et al., 2010a,b,c; Klajnert and Epand, 2005).

3.2.3. *Stability studies*

The stability of aDDnSs was studied for a period of five days. The liposomal suspensions were kept under dark at 4 °C. No sedimentation was observed, while their average size did not increased significantly. As shown in Fig. 6, LipoB-32-OH reached a size of 184.3 nm (3.6% increase) after five days, LipoB-64-OH a size of 194.2 (4.97% increase), LipoA-32-OH 176.4 nm (3.55% increase) and LipoA-64-OH 175.6 nm (3.66% increase). Liposomes incorporating shikonin–Hp complexes found to be physically stable unlike similar liposomal formulations incorporating doxorubicin-PAMAM complexes where the average size of liposomes increased up to 500 nm (from 115 nm) within three days (Papagiannaros et al., 2005).

Similar to shikonin–Hp complexes, the prepared chi-aDDnSs were tested for their drug leakage over a five days period at 4 °C while they were kept under dark. Both LipoA and LipoB formulations were found quite stable for the first three days of the experiment (Fig. 6). After day 3, a major drug leakage occurred for

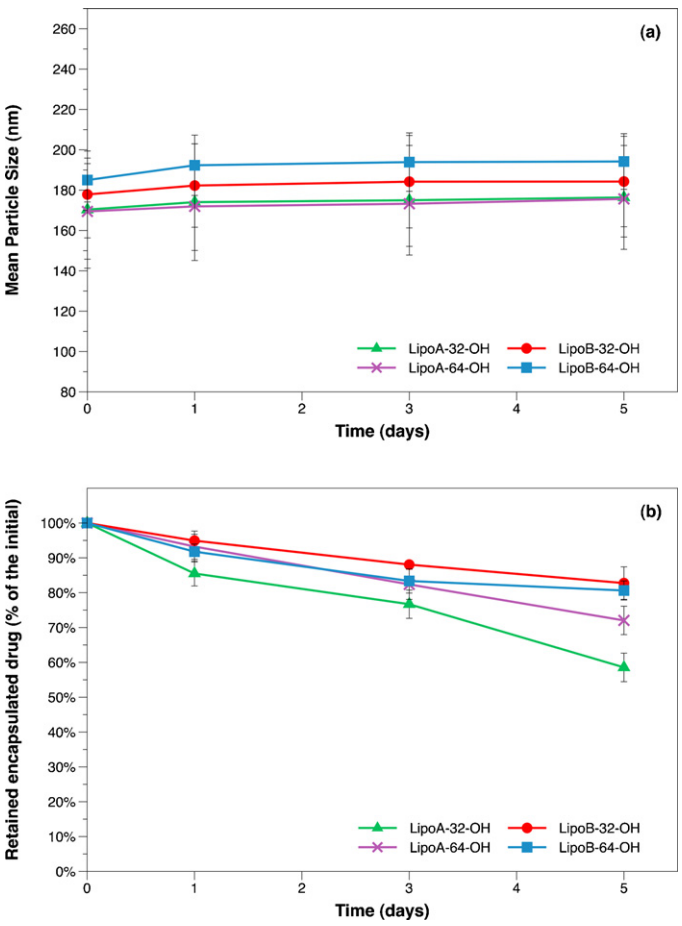

Fig. 6. Stability study of shikonin-loaded chi-aDDnSs. (a) mean particle size and (b) shikonin retention over time (each value represents the mean ± S.D. of $n = 3$ independent experiments).

LipoA-32-OH leaving the liposomal formulation with only 58.56% of the initial encapsulated drug at the end of day 5. LipoB liposomes proved to be more stable retaining 82.75% (LipoB-32-OH) and 80.63% (LipoB-64-OH) of their initial drug content. LipoA-64-OH was more stable than LipoA-32-OH retaining 72.04% of its initial drug content. The average amount of shikonin leaked after three days of incubation was 11.94% for LipoB-32-OH, 16.63% for LipoB-64-OH, 23.30% for LipoA-32-OH and 17.62% for LipoA-64-OH (Fig. 6).

4. Conclusions

Dendrimers and hyperbranched polymers are emerging as potentially ideal drug delivery vehicles providing a remarkable versatility in the chemical modification of their active groups combined with multifunctionality. In the present study we report for the first time an attempt to incorporate shikonin into hyperbranched polymer (PFH-16-OH, PFH-32-OH and PFH-64-OH) complexes, and to characterize these systems for their capacity to be utilized as new drug delivery agents. Shikonin–Hp complexes exhibited acceptable entrapment efficiencies (similar drug-loaded Hp-complexes exhibited entrapment efficiency values between 4% and 20%). PFH-64-OH showed the higher complexation ability among the three generations of PFH hyperbranched polyesters and methanol proved to be more effective as the structure-maker solvent compared to ethanol. Acceptable physical stability was observed for all shikonin–Hp complexes, regarding their known drug leakage instability in buffers (Astruc et al., 2010), which was

observed for the first 8 h of incubation, followed by minor drug precipitation.

The combination of liposomes and dendrimers in a single drug carrier is gaining attention in the pharmaceutical nanotechnology field of research. Barriers like fast or unsustained drug release and low drug encapsulation seem to be able to be overcome by using the concept of chi-aDDnSs. Careful tailoring of the physicochemical properties of the carrier components may lead to products with higher bioavailability, effectiveness and fewer side effects. Specifically chimeric systems of dendrimers and liposomes have been reported but it is the first time that hyperbranched polymers were combined with liposomes forming chi-aDDnSs. These chi-aDDnSs could be considered as an efficient carrier with a great potential for carrying a high drug load, modifying the drug release rate from the system and thus increasing the therapeutic index of the carried bioactive substance.

To the best of our knowledge shikonin has never been incorporated into chi-aDDnSs. Four liposomal formulations (incorporating two shikonin–Hp complexes) were prepared. These aDDnSs, exhibited such physicochemical characteristics and release properties that could be further used as a road map for designing *in vivo* experiments. The stability of the liposomal formulations at 4 °C was quite satisfactory considering the fact that they were in hydrated state. Their overall stability could be further improved with the use of lyophilisation as reported elsewhere (Gardikis et al., 2010a,b,c). The two different techniques applied for the preparation of the liposomal formulations resulted in significantly different entrapment efficiency values of the drug. In the first method the non-complexed shikonin was removed through centrifugation prior to the incorporation into liposomes. In the second technique, shikonin was initially complexed to hyperbranched polymers and the non-complexed shikonin was left in the mixture in order to be incorporated into the liposomal lipid bilayers increasing in that way the overall entrapment efficiency.

The final chi-aDDnSs formulations appear advantageous compared to commonly used delivery systems and could be further exploited to increase the therapeutic index of the incorporated drug. However, physicochemical interactions of such complicated systems should be further considered in order to rationally design stable, safe and efficient drug formulations.

Conflict of interest

The authors have no conflict of interest to declare.

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