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Electrospun fiber mats containing shikonin and derivatives with potential biomedical applications

Konstantinos N. Kontogiannopoulos[a], Andreana N. Assimopoulou[a], Ioannis Tsivintzelis[b], Costas Panayiotou[b], Vassilios P. Papageorgiou[a,*]

[a] Organic Chemistry Laboratory, Chemical Engineering Department, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece
[b] Physical Chemistry Laboratory, Chemical Engineering Department, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece

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ABSTRACT

Alkannin, shikonin (A/S) and their derivatives are naturally occurring hydroxynaphthoquinones with a well-established spectrum of wound healing, antimicrobial, anti-inflammatory, antioxidant and anti-tumor activity. Clinical studies over the years revealed that A/S derivatives-based wound healing preparations (such as HELIXDERM®) are among a very small group of therapeutics that modulate both the inflammatory and proliferative phases of wound healing and present significant tissue regenerative activity. The purpose of the present work was to combine the biological properties of A/S and the advantages of electrospun meshes to prepare a potent topical/transdermal biomaterial for A/S. Four biocompatible polymers (cellulose acetate, poly(L-lactide), poly(lactide-*co*-glycolide) LA/GA:50/50 and 75/25) were used for the first time, to produce electrospun fiber mats containing either shikonin or A/S mixture in various amounts. Both drugs were effectively loaded into the above biomaterials. The incorporation of drugs did not considerably affect fibers morphology and their mean diameter size varied from 315 to 670 nm. High drug entrapment efficiencies (ranged from 74% to 95%) and appropriate release profiles were achieved, that render these fibers as potential A/S topical/transdermal wound healing dressings. Given the multifunctional activity of the natural products alkannins and shikonins, their consideration as bioactive constituents for tissue engineering scaffolds seems a promising strategy for repairing and regenerating tissues and mainly skin.

1. Introduction

Polymeric drug delivery systems are able to improve therapeutic efficacy, reduce toxicity, and enhance compliance of the patients by delivering drugs at a controlled rate over a period of time to the site of action (Kenawy et al., 2002; Langer, 1998). Over the past few years, there has been increasing interest in polymer ultrafine micro/nanofibers for biomedical applications that are produced by several fabrication techniques, such as electrospinning, phase separation, template synthesis and self-assembly (Puppi et al., 2010). These techniques result in reproducible scaffolds for the regeneration of specific tissues (Lanza et al., 2007).

Electrospinning has gained widespread interest for applications in tissue engineering and drug delivery systems (DDSs) the last years, after the first report in 2002 (Kenawy et al., 2002). Reported electrospun DDSs include transdermal, oral sustained, targeted, implantable ones, DDSs for tissue engineering and trans-membrane DDSs (Bidone et al., 2009; Cui et al., 2010; Huang et al., 2003; Puppi et al., 2010; Sill and von Recum, 2008; Suwantong et al., 2008; Xu et al., 2008; Yu et al., 2009a). Electrospinning technique possesses great advantages, such as simplicity of use, adaptability and potential scale up, versatility in spinning a wide variety of polymeric fibers and the ability to fabricate fibers with diameters on the nanometer size scale (Sill and von Recum, 2008). These e-spun fibers have shown severe outstanding properties, such as high surface area, high length/diameter ratio, flexible surface functionality, tunable surface morphologies and superior mechanical performance, which could enhance cell attachment, drug loading, mass transfer properties and are suitable for replicating the physical structure of the extracellular matrix of biological tissues (wound dressing materials) (Cui et al., 2010; Puppi et al., 2010). Localized drug therapy of skin and wounds, using appropriate electrospun fibers as delivery vehicles and tissue engineering scaffolds, significantly reduce the systemic absorption of the drugs and also provide

Abbreviations: DDSs, drug delivery systems; CA, cellulose acetate; PLLA, poly(L-lactide) acid; PLGA, poly(lactic-*co*-glycolic acid); A, alkannin; S, shikonin; A/S mixture, the mixture of A/S pigments isolated from *Alkanna tinctoria* roots; A/S, alkannins and shikonins; SLS, sodium lauryl sulfate; SEM, scanning electron microscopy; DMF, dimethylformide.

* Corresponding author. Tel.: +30 2310 996242; fax: +30 2310 996252.
E-mail address: vaspap@eng.auth.gr (V.P. Papageorgiou).

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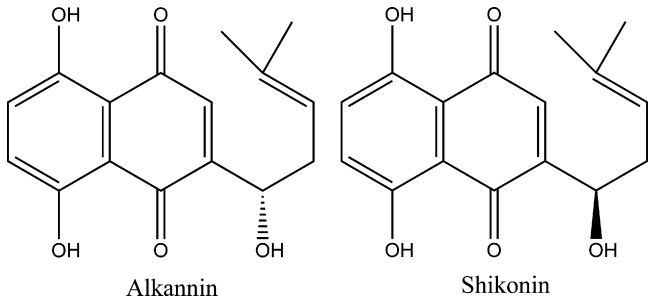

Fig. 1. The chiral pair alkannin and shikonin that possess major biological activity.

localized therapeutic effect at lower drug concentration (Cui et al., 2010).

Cellulose-based materials, such as cellulose acetate (CA), are widely used in the biopharmaceutical processing industry (Zhang et al., 2008). E-spun CA fiber mats have been developed as carriers for topical/transdermal delivery of drugs (Suwantong et al., 2007, 2008; Taepaiboon et al., 2007; Tungprapa et al., 2007). Specifically, e-spun CA fiber mats were prepared as carriers for topical/transdermal delivery of four different non-steroidal anti-inflammatory drugs (Tungprapa et al., 2007), curcumin (Suwantong et al., 2007), asiaticoside and *Centella asiatica* L. extract (Suwantong et al., 2008).

Poly(lactic acid) (PLA), poly(glycolic acid) (PGA) and poly(lactic-co-glycolic acid) (PLGA) are typical polymers utilized in various biomedical applications and tissue engineering scaffolds, due to their biodegradability and biocompatibility. They have also been used as the base materials for implant devices, such as suture fibers and scaffolds for tissue engineering (Langer and Vacanti, 1993; Zong et al., 2002). Recently, the electrospinning of these polymers has attracted a great deal of attention due to their potential applications in drug delivery, surgical implantation, tissue engineering and prevention of post-operative induced adhesion (Bini et al., 2004; Luu et al., 2003; Pan et al., 2008; Puppi et al., 2010; Zong et al., 2005).

Alkannin and shikonin (A/S; Fig. 1) are optical antipodes of plant origin. They are found in the outer surface of the roots of at least 150 species that belong to the genera *Alkanna*, *Lithospermum*, *Echium*, *Onosma*, and *Anchusa* of the Boraginaceae family (Papageorgiou et al., 1999, 2006).

Biological investigations over the last 30 years have shown that alkannin, shikonin and their derivatives possess strong wound healing, antimicrobial, anti-inflammatory, tissue regenerative, antioxidant and antitumor properties (Papageorgiou, 1980; Papageorgiou et al., 1999, 2006, 2008). These were originally introduced as wound healing agents by Prof. Papageorgiou and a wound healing pharmaceutical ointment is already commercially available under the trademark HELIXDERM®, and the medical devices HELIXFILM, HELIX GEL and HELIX SPRAY (wound healing collagen film, gel and spray respectively) are under development. Clinical studies over the years revealed that A/S based wound healing preparations are among a very small group of therapeutics that modulate both the inflammatory and proliferative phases of wound healing (Fig. 2), while delivering an antimicrobial and analgesic effect in a broad range of ulcers (i.e. indolent and chronic ulcers, leprotic ulcers, burns and anal fissures) (Papageorgiou et al., 2008). Therefore, the administration of A/S by means of polymeric drug delivery systems represents a good approach to achieve a therapeutic effect and/or promote tissue regeneration. A recent study (Han et al., 2009) with shikonin-loaded PCL/PTMC fiber mats showed that medicated fibers retained shikonin biological functionality (possessed equal antioxidant and antibacterial activity) and thus could be used as promising materials for wound healing and treating surfaces that contain pathogenic microorganisms.

The aim of the present study was to prepare and characterize several electrospun sub-micron sized fibers as carriers for topical/transdermal delivery of either shikonin or a mixture of A/S pigments isolated from *Alkanna tinctoria* roots, as tissue engineering biomaterials and drug delivery systems. The mixture of A/S pigments is equally active with shikonin, regarding the wound healing and anti-microbial activity (Papageorgiou et al., 1999, 2006, 2008) and can be isolated after a few steps procedure more easily and at low cost. Therefore, the entrapment of A/S mixture in medicated fibers could result in lower cost for the final biomaterial. Cellulose acetate, PLLA and PLGA (50:50 and 75:25) were used to fabricate A/S-loaded electrospun sub-micron sized fibers for the first time. Scanning electron microscopy (SEM) and differential scanning calorimetry (DSC) were applied to investigate the structure and morphology of the e-spun fibers. Additionally, fibers were characterized in terms of their total drug content and drug release kinetics.

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. With the present paper we contribute to the field of tissue and skin engineering and the prepared biomaterials seem promising strategies for repairing and regenerating different tissues and mainly skin.

2. Materials and methods

2.1. Materials

Shikonin was purchased from Ichimaru Pharcos Co., Ltd. (Tokyo, Japan). The mixture of pigments (A/S mixture) was isolated from *A. tinctoria* roots [kindly donated by the pharmaceutical Company PNG Gerolymatos, Greece], according to the procedure proposed by Prof. Papageorgiou (Assimopoulou et al., 2009). Cellulose acetate (Mn = 30,000, 39.8 wt.% acetyl content), PLLA (inherent viscosity 2 dL/g, Mn = 99,000), PLGA (50:50 lactide/glycolide,

Table 1
Composition of fiber mats prepared.

Sample	Polymer type	Polymer solution (% w/v)	Drug (wt.% polymer)	Sample	Polymer type	Polymer solution (% w/v)	Drug (wt.% polymer)
1	CA	20.0%	1.0%	10 neat	PLLA	5.0%	–
1 neat	CA	20.0%	–	12	PLLA	5.0%	5.0%
2	CA	20.0%	2.0%	14	PLLA	5.0%	10.0%
3	CA	20.0%	3.0%	11	PLGA 50:50	17.5%	3.0%
4	CA	20.0%	4.0%	11 neat	PLGA 50:50	17.5%	–
5	CA	20.0%	5.0%	13	PLGA 50:50	17.5%	5.0%
6	CA	20.0%	10.0%	15	PLGA 50:50	17.5%	10.0%
7	CA	20.0%	3.0%	20	PLGA 75:25	17.5%	3.0%
8	CA	20.0%	5.0%	20 neat	PLGA 75:25	17.5%	–
9	CA	20.0%	10.0%	21	PLGA 75:25	17.5%	5.0%
10	PLLA	5.0%	3.0%	22	PLGA 75:25	17.5%	10.0%

Samples 1–6 contain shikonin and samples 7–22 contain A/S mixture. CA samples were diluted in acetone:DMF 2:1 and all other samples in $CHCl_3$:DMF 3:1.

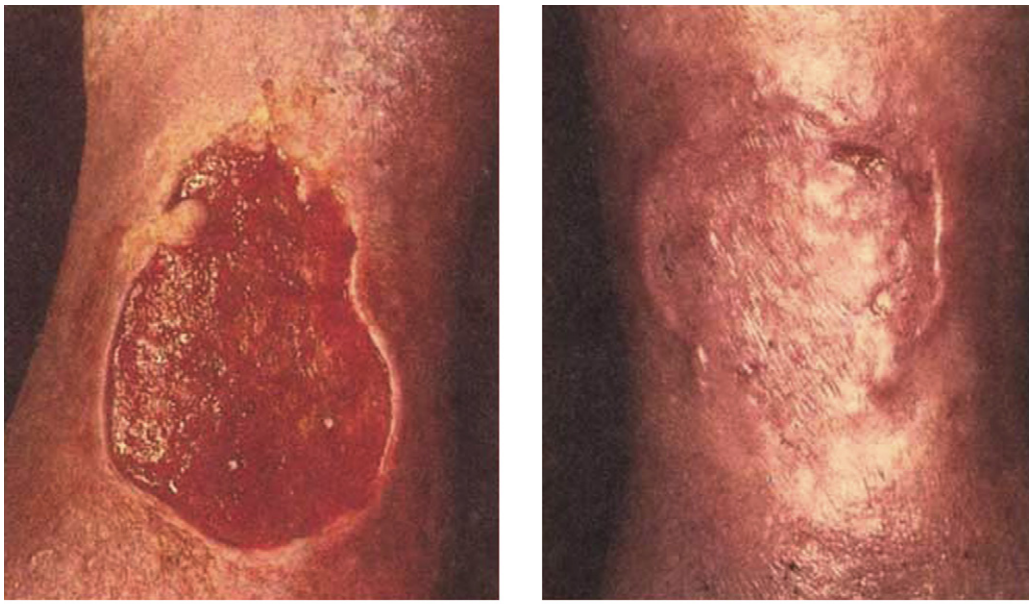

Fig. 2. An indolent ulcer (a) before and (b) after 6 weeks treatment with HELIXDERM® (Papageorgiou et al., 2008).

mol wt. 40,000–75,000), PLGA (75:25 lactide/glycolide, mol wt. 66,000–107,000) and sodium lauryl sulfate (SLS) were purchased from Sigma–Aldrich (St. Louis, USA). Acetone, dimethylformide (DMF) and chloroform were of analytical grade and were purchased from Merck (Darmstadt, Germany).

2.2. Preparation of polymer solutions

2.2.1. Cellulose acetate

All CA solutions were prepared at a fixed concentration of 20.0% w/v cellulose acetate in 2:1 v/v acetone/dimethylformide (DMF). Drug was incorporated into the above polymer solution at several concentrations (0%, 1%, 2%, 3%, 4%, 5%, 10% wt.% based on the weight of the CA).

2.2.2. PLLA

All PLLA solutions were prepared at a fixed concentration of 5.0% w/v PLLA in 3:1 v/v chloroform/DMF. Drug was incorporated into the above polymer solution at several concentrations (0%, 3%, 5% and 10% wt.% based on the weight of PLLA).

2.2.3. PLGA

All PLGA solutions were prepared at a fixed concentration of 17.5% w/v PLGA in 3:1 v/v chloroform/DMF. Drug was incorporated into the above polymer solution at several concentrations (0%, 3%, 5% and 10% wt.% based on the weight of PLGA).

2.3. Electrospinning experiments

Two groups of experiments were designed. The first group (samples 1–6; Table 1) concerned the incorporation of shikonin into CA e-spun fibers and the second one (samples 7–22; Table 1) the incorporation of A/S mixture into e-spun fibers prepared with different polymers (CA, PLLA, PLGA 50:50 and PLGA 75:25).

Prior to electrospinning, the solutions prepared as in Section 2.2, were stirred for 6 h to obtain a homogenous solution. The electrospinning setup used for the mats' manufacturing is a homemade system shown in Fig. 3. The apparatus consists of a syringe pump (HARVARD APPARATUS, model 2274) for the injection of the polymer solution, an aluminum-covered rotating drum as a grounded substrate for the collection of the fibers, and a high voltage power supply (Spellman High Voltage DC SUPPLY, model RHR30P30). All experiments were performed using a glass syringe of 10 mL internal volume and 1 mm (18G) needle diameter. The flow rate of the polymer solution was controlled by a syringe pump and set to 0.76 mL/h. The needle was connected to a high-voltage supply, and the ground electrode was connected on the conductive surface of the aluminum plate. The distance between the needle and the collector's surface was 5 cm and the applied voltage was different for each polymer (16.5 kV for CA, 12.5 kV for PLLA and 10 kV for PLGA), after preliminary tests performed. The temperature of the experiments was around 25 °C, and ambient relative humidity was recorded for each experiment. After preparation, all the electrospun samples were vacuum-dried (320 Pa, Shanghai Laboratory Instrument Work Co. Ltd., Shanghai, China) for 24 h (at 80 °C for CA fibers and at 40 °C for PLLA and PLGA fibers) in order to remove the residual organic solvent and moisture (Tsimpliaraki et al., 2009).

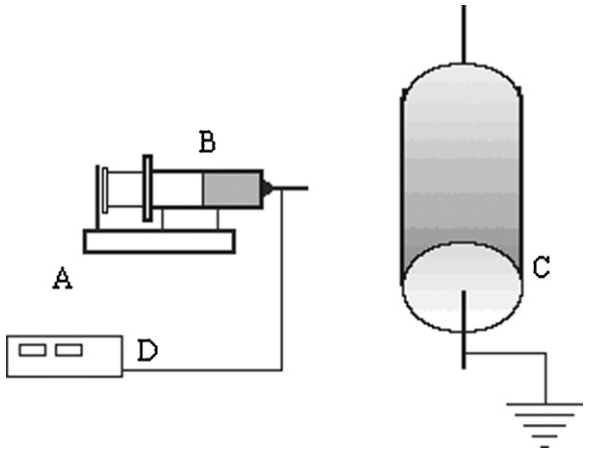

Fig. 3. Schematic diagram of the electrospinning setup: A, syringe pump; B, syringe containing polymer solution; C, rotating drum; D, high voltage supply.

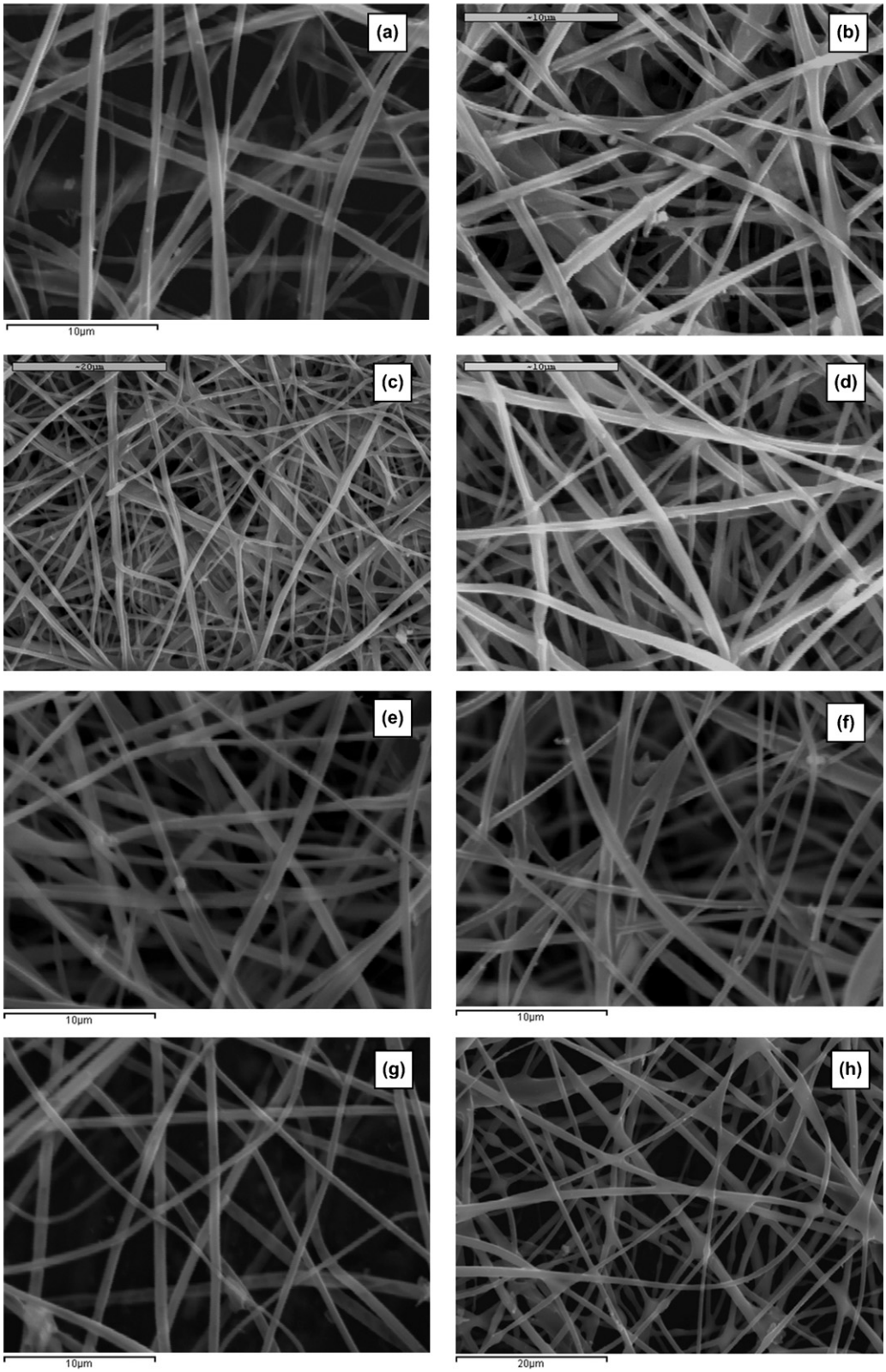

Fig. 4. Selected scanning electron micrographs of the shikonin-loaded and A/S mixture-loaded electrospun CA fibers: (a) sample 1 neat, (b) sample 1 (shikonin 1%), (c) sample 2 (shikonin 2%), (d) sample 3 (shikonin 3%), (e) sample 6 (shikonin 10%), (f) sample 7 (A/S mixture 3%), (g) sample 8 (A/S mixture 5%) and (h) sample 9 (A/S mixture 10%).

2.4. Characterization of the e-spun fibers

2.4.1. Morphological characterization

Samples of the fibrous structures were examined by scanning electron microscopy (SEM) (JEOL, mod. JSM-840A). All surfaces were coated with graphite to avoid charging under the electron beam. The fiber size distributions were obtained by image analysis with the use of appropriate software (ImageJ software, National Institutes of Health, U.S.A.). Two samples were examined under the microscope from each experimental run, and at least two rep-

Table 2
Physicochemical characteristics of the prepared fiber mats.

Sample	Mean diameter size (μm)	Total drug content (%)	Total drug release (72 h) (%)	Drug release at 60 min (%)	$t_{50\%}$ (h)	k ($s^{-0.5}$)	R^2
1 neat	0.382 ± 0.14	–	–	–	–	–	–
1	0.500 ± 0.18	81.77 ± 1.05	63.02 ± 1.79	18.94 ± 4.09	7.1	0.0029	0.978
2	0.499 ± 0.23	81.66 ± 1.72	67.04 ± 1.14	26.16 ± 4.80	6.3	0.0041	0.962
3	0.508 ± 0.21	84.37 ± 1.17	72.27 ± 1.78	32.69 ± 2.04	2.3	0.0055	0.983
4	0.523 ± 0.22	90.49 ± 1.83	79.98 ± 1.12	40.13 ± 1.52	1.5	0.0064	0.993
5	0.565 ± 0.22	91.53 ± 2.39	88.11 ± 1.57	47.11 ± 4.76	1.3	0.0074	0.985
6	0.595 ± 0.22	94.61 ± 3.77	93.30 ± 2.27	51.72 ± 1.77	0.9	0.0092	0.955
7	0.390 ± 0.12	73.86 ± 4.65	74.62 ± 1.71	59.17 ± 4.48	1.6	0.0074	0.970
8	0.440 ± 0.14	77.90 ± 2.55	87.86 ± 2.00	63.80 ± 4.76	0.9	0.0090	0.975
9	0.532 ± 0.21	83.74 ± 2.26	94.27 ± 3.43	66.01 ± 4.60	0.6	0.0105	0.970
10 neat	0.542 ± 0.16	–	–	–	–	–	–
10	0.650 ± 0.22	87.47 ± 2.65	65.59 ± 4.75	43.62 ± 4.47	6.9	0.0047	0.967
11 neat	0.303 ± 0.08	–	–	–	–	–	–
11	0.315 ± 0.08	80.36 ± 1.93	80.67 ± 2.56	42.89 ± 4.94	3.9	0.0059	0.996
12	0.649 ± 0.18	91.38 ± 1.51	78.08 ± 3.96	51.25 ± 4.69	1.8	0.0066	0.996
13	0.335 ± 0.09	83.37 ± 4.04	87.58 ± 2.48	46.41 ± 1.19	2.2	0.0073	0.970
14	0.671 ± 0.22	94.85 ± 3.60	87.72 ± 1.82	58.40 ± 4.61	0.9	0.0083	0.946
15	0.370 ± 0.07	87.20 ± 1.58	93.74 ± 1.95	55.28 ± 4.16	0.8	0.0090	0.969
20 neat	0.379 ± 0.09	–	–	–	–	–	–
20	0.382 ± 0.09	83.49 ± 1.66	80.01 ± 1.31	37.45 ± 4.17	5.5	0.0052	0.986
21	0.494 ± 0.12	86.59 ± 3.98	88.65 ± 1.69	49.05 ± 4.58	2.4	0.0081	0.932
22	0.558 ± 0.13	91.58 ± 1.23	95.19 ± 1.58	61.11 ± 1.01	0.7	0.0095	0.962

Data are shown as mean ± SD of $n = 3$ independent experiments. $t_{50\%}$ represents the required time to release the 50% of the drug. The last two columns represent the analyses of the release kinetics of both shikonin and A/S mixture from the prepared drug-loaded electrospun fiber mats based on the Fickian diffusion type of release mechanism (k is the release rate parameter).

resentative areas from each sample were chosen to determine the average sizes of the fibers. Three magnifications were recorded for each area (1000×, 2000× and 4000×). In all cases, the average value of the distribution is reported, while the error bars refer to the standard deviation.

2.4.2. Total drug content

The "total drug content" in the fibers (represents the drug incorporated both inside the fibers and on their surface compared to the initially used drug), was quantified by UV/vis spectrophotometry (UV–1900 spectrophotometer, Hitachi, Tokyo, Japan) at the characteristic wavelength of each drug (514 nm for shikonin and 516 nm for the A/S mixture). A pre-weighed amount of each fiber (3–5 mg) was diluted in 5 mL acetone (under vortexing) in order to destroy the fiber structure, releasing the drug into the organic phase. Absorbance of the organic phase was afterwards measured. Drug concentration was determined using the following calibration curves:

For shikonin:

$$\text{drug concentration (mg/mL)} = 0.03947 \cdot \text{absorbance} - 0.00036; \quad (R^2 = 0.99997) \tag{1}$$

For A/S mixture:

$$\text{drug concentration (mg/mL)} = 0.04742 \cdot \text{absorbance} - 0.00040; \quad (R^2 = 0.99989) \tag{2}$$

The total drug content (entrapment efficiency) was calculated using the following equation:

$$\text{total drug content}\,(\%) = \left(\frac{F_i}{F_t}\right) \times 100 \tag{3}$$

where F_i is the amount of drug incorporated into fibers and F_t is the initially added amount of drug.

Moreover, in order to determine the uniformity of the drug content throughout each fiber, the total drug content from random parts of the polymeric construct, was calculated as described above. Three replicate meshes were analyzed each time.

2.4.3. In vitro drug release studies

The release profile of each drug from the e-spun fibers was studied in buffer solution with pH 5.5 + 1% SLS at 37 °C. Each drug-loaded fiber sample (25–30 mg) was incubated at 37 °C in 30 mL of the aforementioned release medium under magnetic stirring. Aliquots of samples (3 mL) were taken from the release medium at specific time intervals and that volume was replaced with fresh one. The amount of drug released at various times, up to 72 h, was determined using UV–vis spectrophotometry at 518 nm for shikonin and 520 nm for the A/S mixture, with the aid of the following calibration curves of each drug in the same release medium:

For shikonin:

$$\text{drug concentration (mg/mL)} = 0.06358 \cdot \text{absorbance} - 0.00009; \quad (R^2 = 0.99939) \tag{4}$$

For A/S mixture:

$$\text{drug concentration (mg/mL)} = 0.08989 \cdot \text{absorbance} - 0.00046; \quad (R^2 = 0.99988) \tag{5}$$

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

$$\%\ \text{cumulative drug released}_t = \left(\frac{\text{drug released}_t}{\text{total entrapped drug}}\right) \times 100 \tag{6}$$

2.4.4. Thermal analysis

Calorimetric measurements were carried out with a Shimadzu differential scanning calorimeter (DSC-50, Shimadzu, Japan). DSC measurements were performed using a heating rate of 10 °C/min under a constant nitrogen flow of 20 cm^3/min. The sample weight was kept at low levels (<5 mg) in order to minimize any possible thermal lag during the scans.

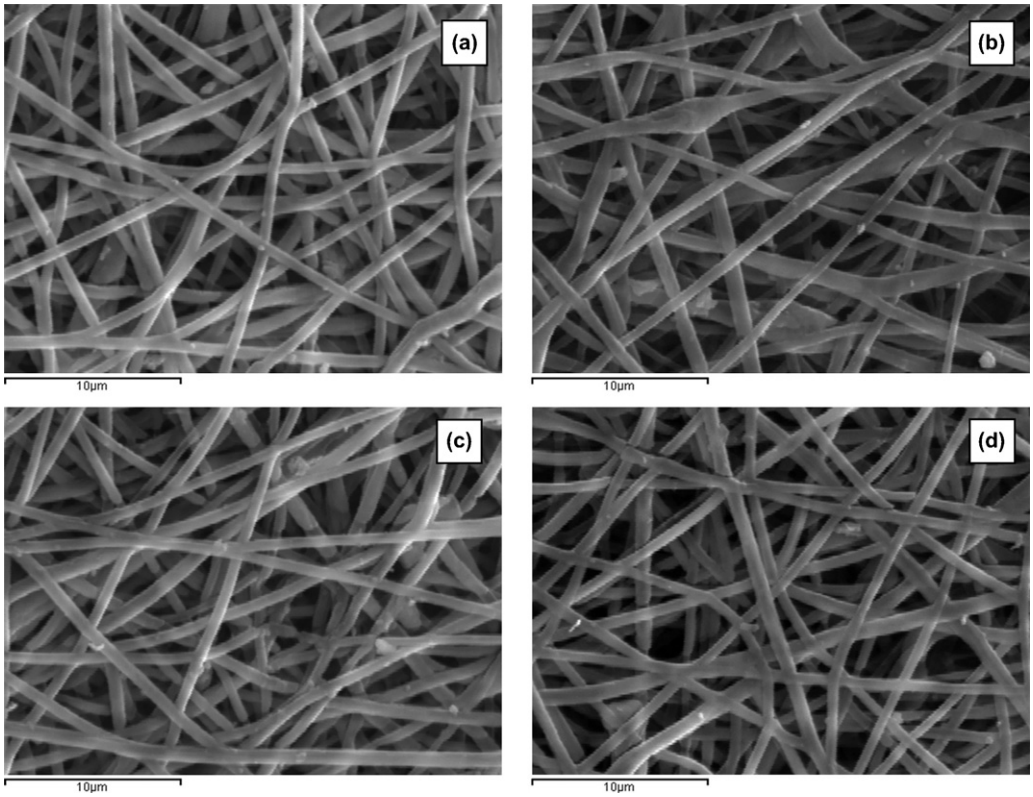

Fig. 5. Selected scanning electron micrographs of the A/S mixture-loaded electrospun PLLA fibers: (a) sample 10 neat, (b) sample 10 (A/S mixture 3%), (c) sample 12 (A/S mixture 5%) and (d) sample 14 (A/S mixture 10%).

2.5. 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

Shikonin is a well-known wound healing agent and the mixture of A/S pigments isolated from *A. tinctoria* roots are the active ingredients of a strong wound healing pharmaceutical ointment, commercially available under the trademark HELIXDERM®, as well as of wound healing medical devices. The incorporation of shikonin and A/S mixture into electrospun polymeric fibers promotes the combination of the biological properties of the loaded agent and the structural advantages of ultrafine fibers. In order to prepare a potent topical/transdermal drug delivery system for shikonin and A/S derivatives, which could be used as a wound healing dressing, several drug-loaded electrospun sub-micron sized fibers were prepared using four different polymers.

3.1. Preparation of electrospun fiber mats

Two groups of experiments were designed for the fabrication of fiber mats. In the first group (samples 1–6; Table 1) shikonin was incorporated into CA e-spun fibers and in the second one (samples 7–22; Table 1) A/S mixture was entrapped into several e-spun fibers prepared with different polymers (CA, PLLA, PLGA 50:50 and PLGA 75:25). In all cases, sub-micron sized fibers impregnated with A/S were produced and their characterization was performed by SEM, DSC, release and efficiency studies. The colour of the produced fiber mats varied from light pink to dark red.

3.2. Morphological characterization of e-spun fibers

3.2.1. Shikonin-loaded fibers

Both neat and drug-loaded e-spun fibers were evaluated for their morphology. Selected SEM images of the e-spun CA fibers are shown in Fig. 4((b)–(e)). Cross-sectionally round fibers were obtained in all cases. Minor shikonin aggregates were observed on the surface of these fibers, implying that a small portion of shikonin was not perfectly incorporated within the fibers, but left upon their surface. The average diameters of the neat CA fibers were 0.382 ± 0.14 μm, while those of the shikonin-loaded CA fibers ranged between 0.5 and 0.595 μm (Table 2). The presence of shikonin caused a small increase in the average fiber diameter, but it did not affect their morphology, since their surface was also smooth as previously described (Taepaiboon et al., 2007; Tungprapa et al., 2007). Additionally, increasing the drug loading did not cause a significant effect on the mean fiber size, although it is a common effect of the drug loading, as reported elsewhere (Deitzel et al., 2001; Puppi et al., 2010; Takano-Ohmuro et al., 2008).

3.2.2. A/S mixture-loaded fibers

After the successful incorporation of shikonin into CA e-spun fibers (for total drug content and release profile see Sections 3.3 and 3.4 respectively), we further examined the possibility of incorporating a mixture of A/S pigments isolated from *A. tinctoria* roots. This mixture is well known for its wound healing properties and is much cheaper and easier to isolate and purify than pure shikonin. As a result, the final product will have a much lower production cost. Both neat and drug-loaded e-spun fibers were evaluated for

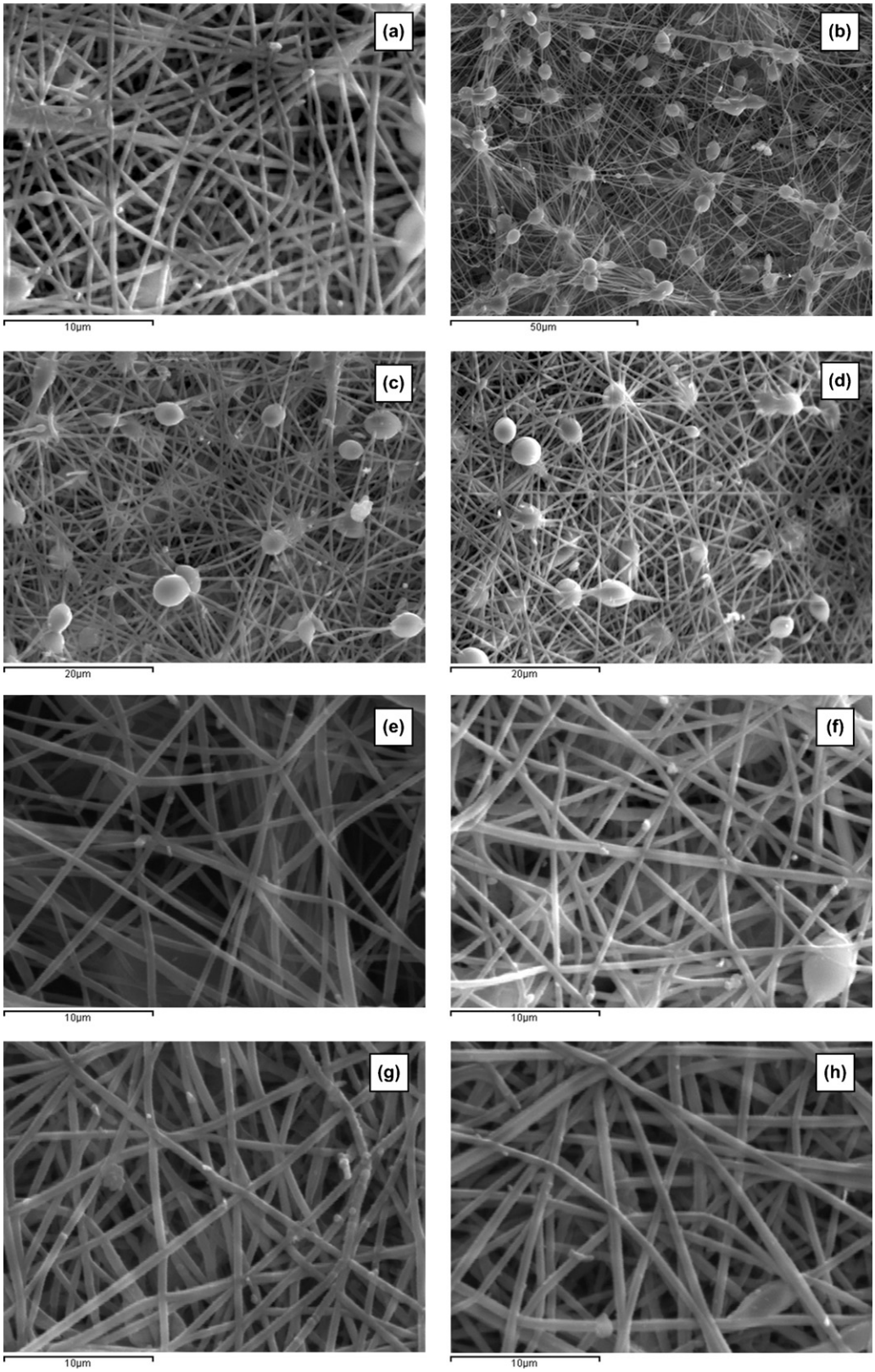

Fig. 6. Selected scanning electron micrographs of the A/S mixture-loaded electrospun PLGA fibers [(a)–(d) PLGA 50:50 and (e)–(h) PLGA 75:25]: (a) sample 11 neat, (b) sample 11 (A/S mixture 3%), (c) sample 13 (A/S mixture 5%), (d) sample 15 (A/S mixture 10%), (e) sample 20 neat, (f) sample 20 (A/S mixture 3%), (g) sample 21 (A/S mixture 5%) and (h) sample 22 (A/S mixture 10%).

their morphology. Selected SEM images of the A/S mixture-loaded e-spun CA fibers are shown in Fig. 4((f)–(h)).

From SEM images (Fig. 4(f)–(h)) it is indicated that the incorporation of A/S mixture, instead of shikonin, did not affect the morphology of the CA fibers. The average diameter size ranged between 0.390 and 0.532 μm and as shown in Table 2, A/S mixture-loaded fibers were slightly smaller than the respective ones with shikonin. This could be attributed to the lower surface tension and/or higher viscosity of the solution caused by the incorporation of A/S mixture instead of shikonin (Fong et al., 1999; Puppi et al., 2010). Concerning the influence of drug loading upon the fiber size, results were similar to those of shikonin-loaded CA fibers, showing no statistically significant effect.

After the successful fabrication of shikonin and A/S mixture-loaded CA e-spun fibers, the incorporation of A/S mixture was examined into three widely used biocompatible polymers (PLLA and PLGA in two different blends) that have already been utilized for drug delivery applications (Lee et al., 2010; Li et al., 2006; Puppi et al., 2010; Suwantong et al., 2008; Xu et al., 2009). PLLA loaded with A/S mixture was fabricated into smooth fibers, more rounded than CA ones and with no beads, as shown by SEM analysis (Fig. 5). Concerning their size, PLLA fibers showed the larger average diameter among all examined polymers (0.650–0.671 μm; Table 2).

The use of PLGA 50:50 for the fabrication of e-spun fibers (samples 11, 13 and 15; Table 1) did not result to fine, cross-sectionally round fibers similar to CA and PLLA fibers, but characterized by beaded fiber morphology (Fig. 6(a)–(d)). Their average diameter sizes varied from 0.315 μm to 0.370 μm (Table 2) with rounded and spindle-like beads formed between fibers (Fig. 6). This beaded fiber morphology could be attributed to high surface tension of the solution used for electrospinning (Huang et al., 2003). When PLGA 75:25 was used to produce A/S mixture-loaded e-spun fibers (samples 20–22; Table 1), it also resulted into beaded fiber morphology, but with significantly fewer number of beads (Fig. 6(e)–(h)). The average size of the fibers ranged between 0.382 μm and 0.558 μm.

Concerning the influence of drug loading upon the size of PLLA, PLGA 50:50 and PLGA 75:25 fiber mats, statistical analysis showed that increasing the drug concentration did not significantly increased diameter mean size (Table 2).

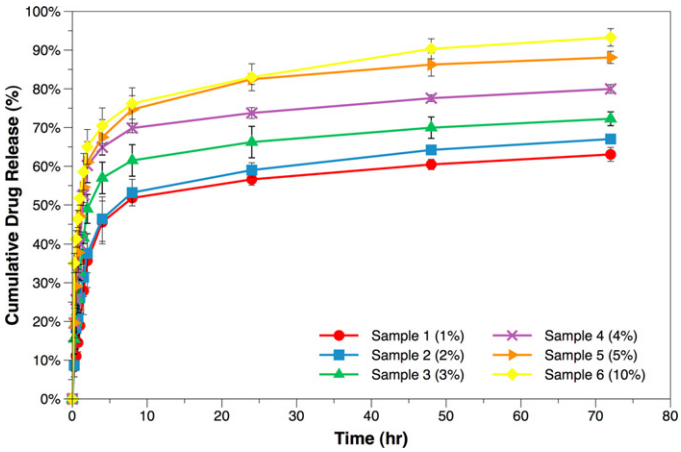

Fig. 7. Shikonin release profile from CA fibers.

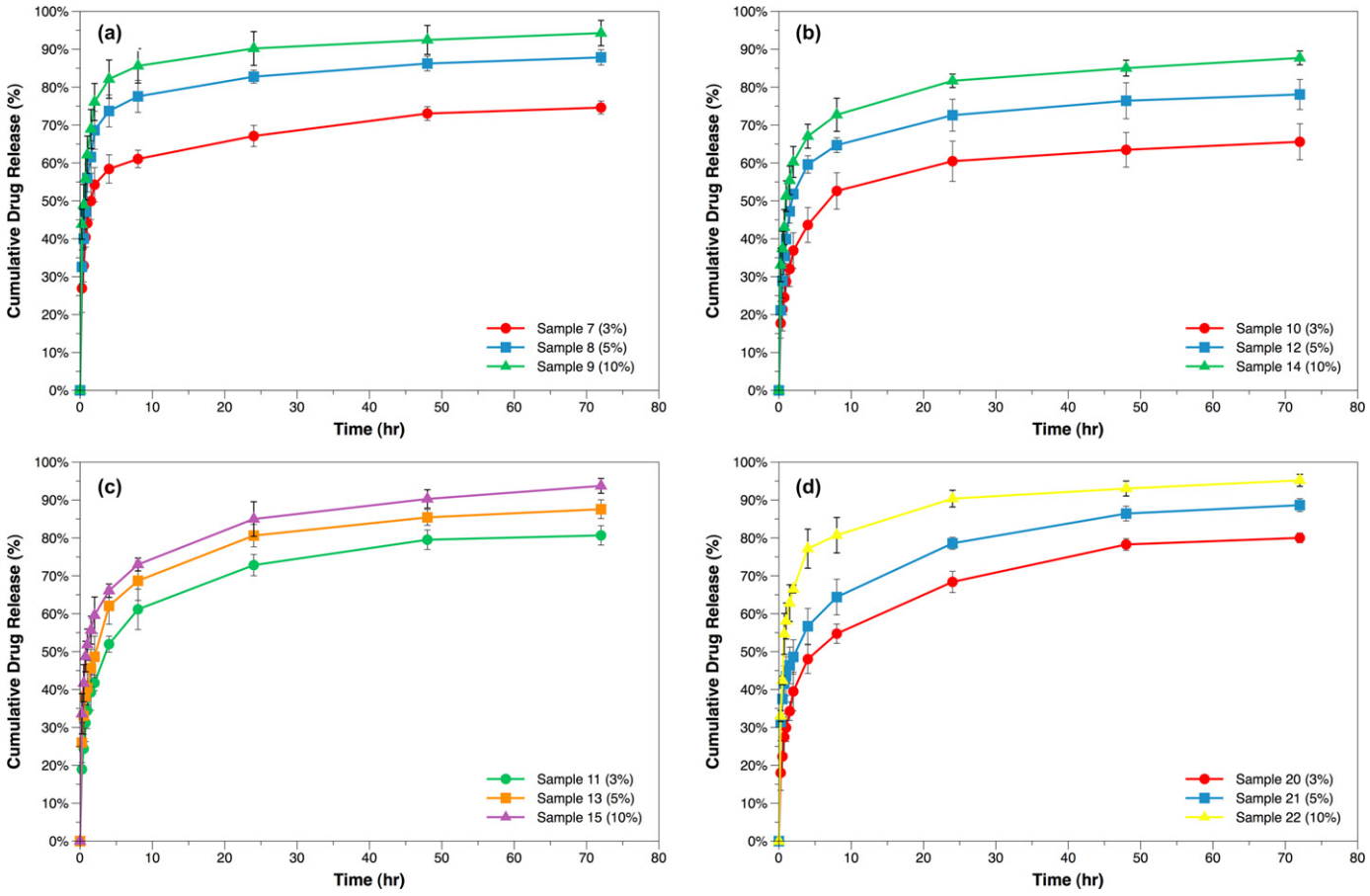

Fig. 8. A/S mixture release profile from the electrospun fibers (CA: samples 7–9; PLLA: samples 10, 12, 14; PLGA 50:50: samples 11, 13, 15 and PLGA 75:25: samples 20–22).

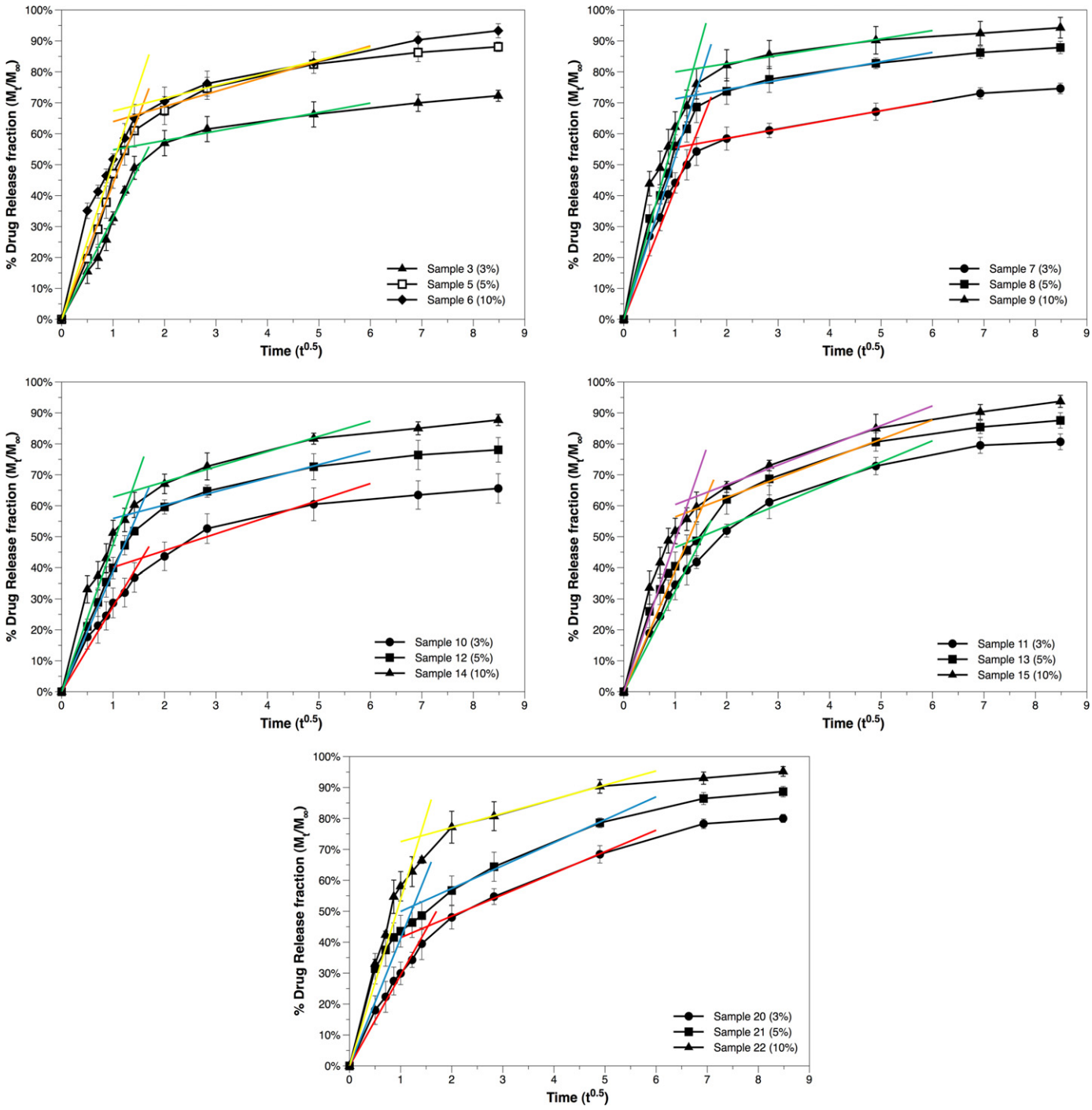

Fig. 9. Drug release curves of samples, re-plotted versus square root of time for kinetic studies.

3.3. Total drug content

The "total drug content" of each of the resulted fiber mats was additionally estimated. As depicted in Table 2, high drug-loading efficiencies were successfully achieved for both shikonin and A/S mixture of pigments. As reported elsewhere (Zeng et al., 2005) where a lipophilic drug (paclitaxel) was highly soluble in the polymer solution (PLLA/chloroform/acetone), when the solution jet was rapidly elongated and the solvent evaporated quickly, phase-separation (between the drug and the polymer) was difficult to take place and the drug tended to remain inside the fiber where sufficient solvent was left. Thus, when the fiber became dry, the drug was encapsulated inside. Similarly in our case, where shikonin and A/S mixture appeared to have good compatibility with the matrix/solvent system, due to their lipophilicity and high solubility in all solvents used for the experiments, it could be assumed that fibers are impregnated with most of the initially applied drug.

3.3.1. Shikonin-loaded fiber mats

Shikonin-loaded CA fiber mats showed quite high drug entrapment efficiency values (between 81.66 and 94.61%). Comparing the total drug content for samples 1–6 (Table 2), it can be concluded that increasing the initial shikonin loading used for the preparation of the electrospun solution, increased the final amount of drug incorporated into the e-spun fibers. Table 2 reveals that this increase is more pronounced at low drug concentrations (1–4%),

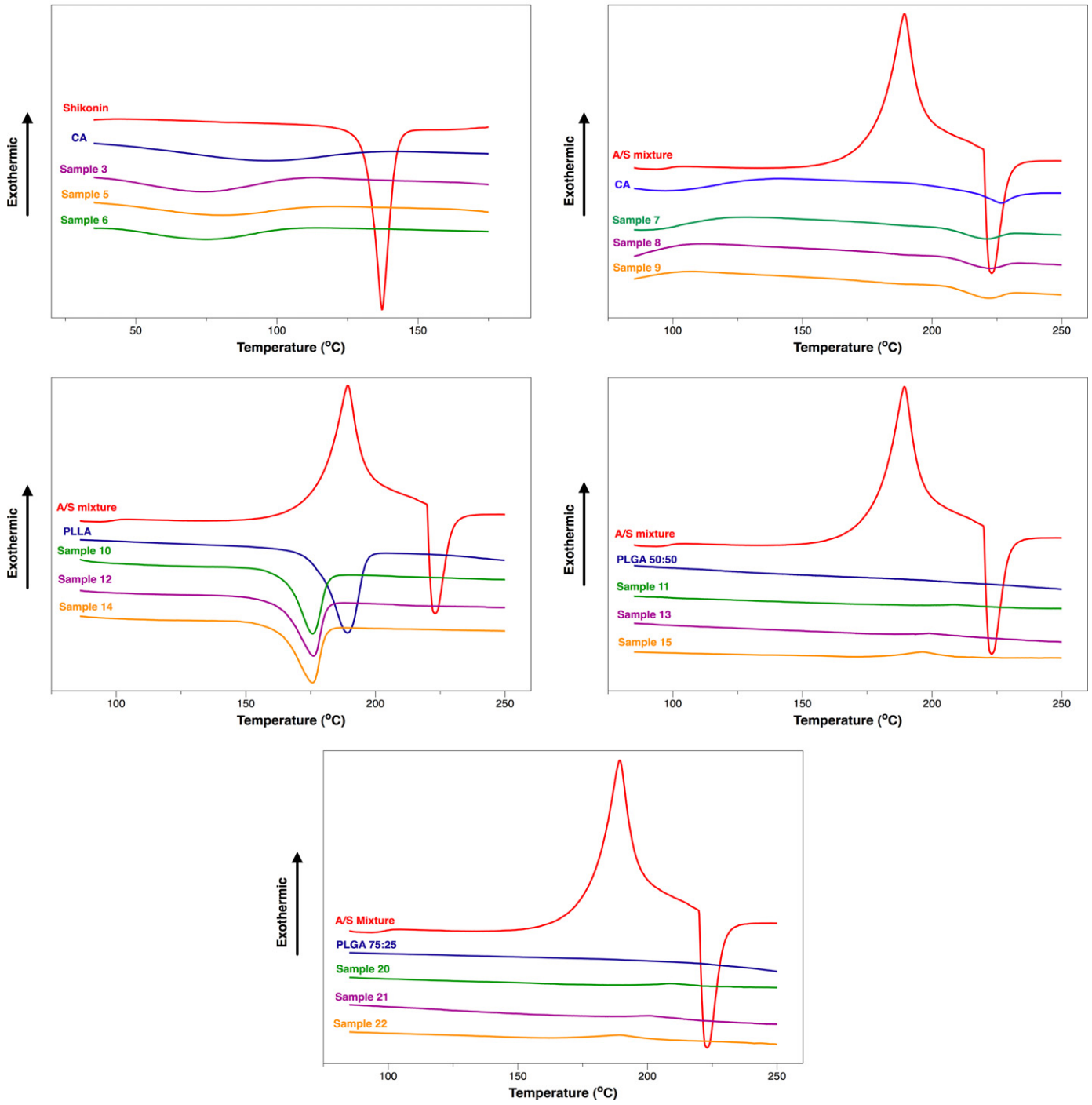

Fig. 10. DSC thermographs of e-spun fibers.

above which there is a less intense increase rate. For that reason, only selected drug concentrations (3%, 5% and 10%) were used in the following experiments for the incorporation of A/S mixture into e-spun fibers.

3.3.2. *A/S mixture-loaded fibers*

After the successful incorporation of shikonin into CA fibers and the determination of their total drug content, A/S mixture-loaded CA e-spun fibers were prepared. The mixture of pigments isolated from *A. tinctoria* roots has well established strong wound healing properties and comprise the active ingredient of an already approved pharmaceutical formulation under the trademark HELIXDERM®. A/S mixture can be isolated after a few steps procedure easily and at low cost. Therefore, further experiments focused on the fabrication of A/S mixture into electrospun fibers using different polymers, such as CA, PLLA, PLGA 50:50 and PLGA 75:25. Their total drug content values ranged from 73.86% to 94.85% (Table 2). The entrapment efficiency values of the A/S mixture of pigments into CA were slightly lower than the corresponding ones of shikonin-loaded CA fibers.

As shown in Table 2, the quantity of A/S mixture incorporated into the PLLA fibers depends in some way on the initial drug load-

ing and significant differentiation was observed between samples with 3% and 10% drug loading. Same results arise for the total drug content for PLGA 50:50 and PLGA 75:25 fibers.

Summarizing, between the four polymers studied for the fabrication of sub-micron sized fibers incorporating A/S mixture (specifically CA, PLLA, PLGA 50:50 and PLGA 75:25), using the electospinning technique, the polymer with the highest total drug entrapment was PLLA, followed by PLGA blends 75:25 and afterwards 50:50, while cellulose acetate showed the lower total drug content among all the polymers tested.

It should be mentioned that inclusion of both shikonin and A/S mixture in several polymers resulted in uniform fiber structures, displaying no significant differences in entrapment efficiencies among different regions along side their constructs.

3.4. In vitro drug release

In order to investigate the drug release characteristics from the drug-loaded e-spun mats, experiments were carried out using a pH 5.5 buffer solution with 1% SLS as the release medium, at a controlled temperature of 37 °C. Since shikonin and A/S mixture are hydrophobic substances with scarce aqueous solubility, the release investigation was carried out under sink conditions, keeping drug concentration below its solubility limit. The accumulative amount of shikonin released from the drug-loaded CA fibers is depicted in Fig. 7.

The amount of drug finally released varied from sample to sample (Table 2) and in Fig. 7 it is indicated that the percentage of shikonin released depends on the total drug incorporated into the fibers. Specifically, the higher the fiber content in shikonin, the greater the percentage of drug released divided by the totally entrapped drug, during the dissolution experiment. The sample with the lowest drug content (sample 1, 1 wt.% polymer) released only 62.5% of its total drug within 72 h, whereas the sample with the highest shikonin content (sample 6, 10 wt.% polymer) released 92.7% of its entrapped drug.

Shikonin released from the medicated electrospun fibers showed a burst release during the first 2 h, releasing a considerable amount of drug during the first 60 min of the experiment (Table 2). Afterwards, a gradual increase in the cumulative release followed, over the next hours, reaching a plateau at 48 h. This burst release could be attributed to the presence of shikonin on or near the surface of the fibers. This portion of shikonin could be easily diffused into the release medium and lead to burst effect. Similar release profiles for shikonin were reported from PCL/PTMC fiber mats (Han et al., 2009).

Similar conclusions could be drawn from Fig. 8 and Table 2 concerning the release characteristics of A/S mixture from the medicated CA e-spun fibers. Samples with higher drug concentrations released greater amounts of A/S mixture, divided by the totally entrapped drug, during the dissolution experiments. Fibers with the lower amount of drug (sample 7, 3 wt.% polymer) released only 74.6% of its total entrapped A/S mixture in 72 h, while sample 9 (10 wt.% polymer) released 94.3%. Likewise shikonin-loaded fibers, A/S mixture-fibers showed a burst release during the first 2 h, followed by a gradual increase in the cumulative release until reaching a plateau at 48 h, similar to previously reported fiber meshes loaded with several drugs (Bidone et al., 2009; Puppi et al., 2010; Suwantong et al., 2007; Taepaiboon et al., 2006).

Fig. 8 also reveals that PLLA and PLGA e-spun fibers presented a similar release profile with the corresponding A/S mixture-loaded CA fibers. Concerning the PLLA fibers, sample 14 (10 wt.% polymer) released the greater amount of drug (87.7%) during the 72 h, while 58.4% was released during the first 60 min. Compared with sample 14, sample 10 (3 wt.% polymer) released 20% less drug (65.6%) during the dissolution experiment. Concerning the total amount of drug released from the fibers, PLGA samples with both blend ratios and afterwards CA released the greater amounts of A/S mixture among all the polymers tested, whereas PLLA ones released the least amount of drug and CA ones presented the fastest release rate. This fact could be attributed to the crystallinity of the samples (PLLA is semicrystalline, while PLGA 50:50 is completely amorphous), as well as to the slow degradation profile of PLLA, which can be increased by the incorporation of structurally similar but faster degrading glycolide components in the polymer chains through copolymerization (random or block) (Kim et al., 2003; Park and Jonnalagadda, 2006). Therefore, PLGA 50:50 and PLGA 75:25 possess faster degradation profiles and thus release greater amounts of drug compared to PLLA samples.

As shown in Table 2, CA fibers released on average 85.6% of their total entrapped A/S mixture in 72 h, PLLA fibers released 77.1%, PLGA 50:50 fibers 87.3% and finally PLGA 75:25, released the greater percentage, 87.9%. Additionally, Table 2 reveals that CA fibers released on average 63.0% of their total entrapped drug in the first 60 min, PLLA 51.1%, PLGA 50:50 48.2% and PLGA 75:25 ones 49.2%. Similar profile arise from the time required for each sample to release 50% of its entrapped drug (Table 2). Therefore, it could be concluded that while PLGA 75:25 fibers are those releasing the greatest amount of drug, CA are showing the fastest release rate, with significant difference among all others.

3.4.1. Release kinetics of drugs from drug-loaded e-spun mats

Drug release from polymer devices occurs by drug diffusion through the polymer, by matrix erosion or by a combination of both mechanisms and is affected by various factors such as drug physicochemical properties, polymer composition, M_w and crystallinity, matrix–drug interaction and morphology of the carrier (e.g. porosity). The mechanism governing drug release kinetics from biodegradable matrices is complex due to alterations in polymer phase properties during degradation, leading to changes in drug diffusivity and permeability with time (Puppi et al., 2010).

The release kinetics of drugs from a carrier is often characterized using an equation of the following form (Ritger and Peppas, 1987; Taepaiboon et al., 2006):

$$\frac{M_t}{M_\infty} = kt^n, \quad \text{for} \quad \frac{M_t}{M_\infty} < 0.6 \tag{7}$$

where M_t is the accumulative amount of drug released at an arbitrary time t, M_∞ is the accumulation amount of drugs released at an infinite time (or equivalently the total amount of drug entrapped), n is an exponent characterizing the mechanism with which the release kinetics can be described and k is the release rate of the drugs that incorporates physical characteristics of the matrix/drug system.

For $n = 0.5$, the release mechanism can be described as Fickian diffusion (Taepaiboon et al., 2006; Verreck et al., 2003). To further assess the mechanism of drug release, the released fraction of drugs (M_t/M_∞) was plotted as a function of $t^{0.5}$ (Fig. 9). On the basis of a theoretical analysis (Higuchi, 1963; Verreck et al., 2003; Xu et al., 2008), when a drug release is controlled by a diffusion mechanism, this plot should be a linear line. As shown in Fig. 9, each of the drug release profiles consisted of two sequential stages. Approximately, linear relationships ($R^2 > 0.950$) between M_t/M_∞ and $t^{0.5}$ were obtained for both stages.

The slope of the linear lines for the first stage was steeper than that of the second one. This is probably due to drug molecules absorbed or loosely bind on or near the surface of the polymer fibers, which would diffuse out quickly at initial release times. The slower release rate for the second phase may be attributed to the drug being entrapped into the inner core of the fiber matrix, which would definitely need a longer distance to diffuse through and therefore take longer time to be released. Furthermore, due to the

decline of the diffusion driving force induced by the reduction of the drug molecules inside the inner space of the medicated fibers, the release rate gradually decreased until reaching a plateau.

It should be noted that the release rate of both shikonin and A/S mixture increased with the increasing drug content in the fibers. Similar results concerning the release characteristics of shikonin from PCL/PTMC e-spun fibers have been reported (Han et al., 2009).

3.5. Thermal analysis

Thermal analysis of shikonin, mixture of A/S pigments, each polymer and the respective sub-micron sized fibers impregnated with the above drugs, was additionally performed for the characterization of the produced fiber mats. The DSC curve of shikonin exhibited a single endothermic response corresponding to the melting point of shikonin at 146 °C (Fig. 10). A/S mixture's DSC curve exhibited an exothermic and an endothermic response at 190 °C and 225 °C respectively. DSC thermograms of shikonin-loaded CA fibers did not show any melting peak of the drug, but a broad endotherm ranging from 60 °C to 90 °C (Fig. 10). This indicated that shikonin was no longer present as a crystalline material but has been converted into an amorphous state (Yu et al., 2009b) and was uniformly dispersed in the CA matrix (Zhu et al., 2005). PLLA indicated an endothermic peak at 190 °C corresponding to its melting point. A/S mixture-loaded PLLA fibers exhibited a decrease in PLLA melting point temperature. PLGA fibers exhibited a tiny exothermic peak at approximately 200 °C (temperature varied with drug concentration). This tiny peak could be attributed to A/S mixture, but the absence of the drug's main peaks indicated that the drug was present in amorphous state and had been dispersed in the polymer matrix.

4. Conclusions

To meet the diverse needs for tissue and organ reconstruction and replacement, tissue engineering strategies attempt to provide viable options. Scaffolding is essential in this endeavor to act as a three-dimensional template for tissue ingrowth by mimicking the extracellular matrix for cell adhesion and proliferation. The inclusion of bioactive molecules in scaffolds for cell adhesion, cell signalling and drug or gene delivery is a major consideration in their design. Local drug delivery to promote cell migration, proliferation and differentiation is an enormous tool to improve the required time and quality of tissue regeneration. This is a really important contribution, especially when the incorporated bioactive constituents have well approved regenerative and wound healing activities, such as alkannins and shikonins.

In the present study, several polymeric electrospun fiber meshes with controlled A/S release features were prepared and characterized for potential applications in tissue engineering, as drug delivery systems and wound healing dressings. The entrapment of A/S and their derivatives into electrospun fibers take advantage of the biological properties of A/S and the structural characteristics of sub-micron sized fibers for tissue regeneration applications. Electrospinning was used in combination with biocompatible and biodegradable polymers (CA, PLLA, PLGA 50:50 and PLGA 75:25) in order to fabricate e-spun fiber mats containing either shikonin or a mixture of A/S pigments isolated from *A. tinctoria* roots. All electrospun scaffolds exhibited sub-micron sized fiber diameters, ranging from 315 to 670 nm, depending on the polymer type and the drug type and concentration used. With both shikonin and A/S mixture as bioactive constituents, uniform fibrous structures were formed with all polymers, and there were no significant differences in entrapment efficiencies among different regions along side their constructs. Fiber DSC thermographs indicated that the drug was no longer present as a crystalline material, but had been dispersed in the polymer matrix and converted into an amorphous state in each polymer used. High total drug contents were achieved for all types of polymers used. Increasing the initial drug concentration used for the preparation of the electrospun solution increased the final amount of drug incorporated into the e-spun fibers, while fiber diameters were not significantly affected by the initial drug content. The *in vitro* drug release studies demonstrated an initial rapid release of the drug followed by a slower second stage until reaching a plateau after 48 h. This is probably due to drug molecules absorbed or loosely bound on or near the surface of the polymer fibers. Furthermore, the release rate of both shikonin and A/S mixture increased with the increasing drug content in the fibers and could be tailored by the LA/GA blend ratio.

The prepared A/S-loaded e-spun sub-micron sized fiber mats appear advantageous compared to conventional wound healing devices and preparations and could be exploited to increase the therapeutic index of the incorporated drugs. Given the potential of the multifunctional natural products alkannin and shikonin, their consideration as bioactive constituents for tissue engineering scaffolds seems appealing.

The present study may serve as the basis for a further systematic investigation and a deeper understanding of the behavior, the polymer–drug interactions and the biological activities of the A/S-loaded biomaterials, which can be the subject of our future studies.

Conflict of interest

The authors have no conflict of interest to declare.

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