Electrospun matrices for localised controlled drug delivery: release of tetracycline hydrochloride from layers of polycaprolactone and poly(ethylene-co-vinyl acetate)

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Abstract We report the controlled release of tetracycline (Tet) HCl from a three layered electrospun matrix for the first time. Five formulations of electrospun poly-ε-caprolactone (PCL) and poly(ethylene-co-vinyl acetate (PEVA) have been designed, prepared as micro/nanofibre layers, and assayed for the controlled release of the clinically useful antibiotic Tet HCl with potential applications in wound healing and especially in complicated skin and skin-structure infections. Tet HCl was also chosen as a model drug possessing a good UV chromophore and capable of fluorescence together with limited stability. Tet HCl was successfully incorporated (essentially quantitatively at 3% w/w) and provided controlled release from multi-layered electrospun matrices. The Tet HCl release test was carried out by a total immersion method on 2×2 cm square electrospun fibrous mats in Tris or PBS heated to 37 ºC. The formulation PCL/PEVA/PCL with Tet HCl in each layer gave a large initial (burst) release followed by a sustained release. Adding a third layer to the two layered formulations led to release being sustained from 6 days to more than 15 days. There was no detectable loss of Tet chemical stability (as shown by UV and NMR) or bioactivity (as shown by a modified Kirby-Bauer disc assay). Using Tet HCl-sensitive bacteria, *Staphylococcus aureus* (ATCC 25,923), the Tet HCl loaded three layer matrix formulations were still showing significantly higher antibacterial effects on days 4 and 5 than commercially available Antimicrobial Susceptibility Test Discs of Tet HCl. Electrospinning provides good encapsulation efficiency of Tet HCl within PCL/PEVA/PCL polymers in micro/nanofibre layers which display sustained antibiotic release.

Keywords Drug delivery, Electrospinning, Multilayer, Nanofibre, Polycaprolactone, Poly(ethylene-co-vinyl acetate), Tetracycline, Wound dressing

Introduction

In recent years, micro/nanofibrous matrices have gained widespread attention for their diverse potential in many biomedical applications, e.g. wound dressing, tissue engineering, enzyme immobilization, and drug (including gene) delivery [1-3]. Such matrices are polymeric, therefore potentially chemically diverse, potentially biodegradable, and we have selected to work with two biocompatible and FDA approved polymers, poly-ε-caprolactone (PCL) and poly(ethylene-co-vinyl acetate) (PEVA). The interesting and distinct pharmaceutical properties of these matrices follow from their being on the micro-nano scale so they mimic the extracellular matrix (ECM) and therefore they also have the potential of enhancing cell attachment as their high surface-to-volume ratio can be subject to surface modification by the addition of amine or carboxylic acid functional groups. These nanofibres [4] also have tuneable porosity, malleability (to form fibres, threads, sheets, and tubes), and their more recent possible biomedical applications include use in the construction of effective drug delivery systems [3]. The topographical similarity of these micro-nano scale electrospun matrices with the ECM [3] is particularly interesting and it bodes well for their potential use as medical implants and in tissue engineering applications [5-7].

As potential local controlled drug delivery systems, such electrospun fibres demonstrate good mechanical properties compared to hydrogels. They can also be used as carriers for both hydrophobic and hydrophilic drugs [7]. Among the methods of preparing nanofibres e.g. drawing, template synthesis, phase separation, and self-assembly, electrospinning has emerged as a technology which is adaptable, reproducible, might be scalable, and should allow for fine control of the fibre dimensions [8]. These properties render the combination of an electrospun nanofibrous matrix and therapeutic agents beneficial for local delivery along with wound healing, tissue regeneration, or other biomedical applications [9-11].

Wound healing and ulcer treatments require antibiotic therapy, intensive medical care, frequent hospitalization and even surgery. In 2004, the direct cost of wound care in the USA exceeded $15 bn [12]. Wounds are either acute or chronic. Acute wounds happen because of burns, physical or chemical injuries, while chronic wounds, which tend to heal slowly, develop with nutritional deficiencies, diabetes, persistent infections or some genetic disorders [13, 14]. Traditional wound therapy depends largely on the wound-dressing materials and the application of aseptic reagents. However, there is an increasing demand for biomaterials that

not only provide physical and structural support, but also are bioactive for wound healing [15]. Micro/nanofibrous matrices appear to be promising for wound dressings. As thin films, they combine light weight with good mechanical properties and a large surface area that prevents the compression of the injured tissue. Practically they can cover large areas like burns [15]. As they are structurally ECM-like, they can promote adhesion, proliferation, and differentiation of cells at the wound site [16]. In addition, the incorporation of an antibacterial agent stops or prevents bacterial infection that significantly compromises the healing process. It can also overcome several problems associated with the systemic administration of antibacterial agents e.g. liver and renal toxicity, drug resistant bacteria, and low levels of the antibiotic in the granulating tissue [17]. The release of an antibiotic drug has been studied from nanofibrous matrices made from different polymers such as PEVA [18], poly-L-lactic acid (PLLA) [18] and blends of these two polymers [18], polyvinyl alcohol (PVA) [19] and polylactide-co-glycolide (PLGA) [20]. The main challenge is that nanofibrous matrices possess a high surface area-to-volume ratio which leads to a large initial burst-release of the drug. Several attempts have been made to reduce this initial burst and elicit more control over a subsequent sustained release using formulations such as emulsions [21], core-shell systems [22], and nanotubes [23]. However, these formulations render the nanofibre preparation more complicated and they require control over a wide set of parameters.

These current studies are designed to demonstrate the incorporation and controlled release of an antibiotic drug, tetracycline hydrochloride (Tet HCl), from multilayered electrospun fibrous matrices for wound dressings. We have varied the polymer, the number of layers, and the drug location, resulting in different release profiles that may find uses in a variety of applications. Tet HCl was chosen as a clinically relevant antibiotic and also as a model drug possessing a good UV chromophore and capable of fluorescence together with limited stability. One facet of a multilayered fibrous system is the combination of different polymers with different properties and different release profiles. This could pave the way to engineering complex tissues with different structures e.g. for tissue engineering [24, 25]. The layered matrices were made from two polymers, PCL and PEVA [18]. The use of PCL nanofibrous scaffolds as a dermal substitute has been reported and showed enhancement of cell proliferation [26-28]. PEVA (40 wt % vinyl acetate) is a rubbery, biocompatible, and substantially amorphous copolymer. At 40 wt % vinyl acetate, the percentage polyethylene crystallinity is no more than about 5% (see: http://www.vitaldose.com/blog/ crystallinity 07.30.12, accessed 09.11.12). PEVA is commonly used for rate-controlling membranes in

drug delivery devices [29]. Five formulations have been designed and prepared, two formulations of one layered matrices, one formulation of a two layered matrix, and two formulations of three layered matrices. This is the first time Tet HCl or indeed any small molecule drug has been reported in controlled release studies from an electrospun three layered matrix.

Materials and Methods

Materials

Poly-ε-caprolactone (PCL) (Mn 70 000-90 000), poly(ethylene-co-vinyl acetate) (PEVA) (40 wt % vinyl acetate), tetracycline HCl (Tet HCl) and other chemicals and solvents were purchased from Sigma Aldrich, UK. *Staphylococcus aureus* (ATCC 25,923) and Antimicrobial Susceptibility Test Discs were from Oxoid, UK.

Polymer solutions

PCL solution was dissolved to a fixed concentration of 12% (w/v) in a 9:1 (v/v) mixture of chloroform ($CHCl_3$):methanol (MeOH). Tet HCl was dissolved in the MeOH at 3% of the weight of the polymer. PEVA solution was prepared similarly at 12% (w/v) in $CHCl_3$:MeOH (9:1 v/v) and Tet HCl (3% of the weight of PEVA) was likewise incorporated in the MeOH.

Micro/nanofibre fabrication via electrospinning

Each polymer solution was loaded into a syringe and electrospun at 16.5 kV and a flow rate of 1.5 mL/h, with a distance between the tip of the needle and the collector of 13 cm. The flow rate was controlled by a syringe infusion pump (Cole Parmer). The electrospun micro/nanofibrous matrices were collected on two parallel metal electrodes covered with aluminium foil. To fabricate layered mats, each polymer solution was electrospun for a fixed deposition time (40 min) in a layer-by-layer manner. After 20 min, the rectangular collector was rotated through 180^o for a second collection of 20 min. Five formulations were designed: one layer PCL matrix with 3% Tet HCl, one layer PEVA matrix with 3% Tet HCl, 2 layered PCL/PEVA matrix with 3% Tet HCl in each layer, 3 layered PCL/PEVA/PCL

matrix with 3% Tet HCl in each layer, and 3 layered PCL/PEVA/PCL with 3% Tet HCl only in the PEVA layer.

Morphology

The surface morphology and the cross-sections of the electrospun layered matrices were observed by scanning electron microscopy (SEM; JEOL JSM-6480LV). The matrices were cut into small cm^2 sized pieces, and cross-sections of the layers obtained were freeze-fractured in liquid nitrogen. The samples were sputter-coated with gold (Edwards Sputter Coater 5150B) and then analyzed by SEM with an accelerating voltage from 15 kV. The average fibre diameter was determined in electrospun layered matrices containing (3%) Tet HCl and in a PCL layer electrospun without the antibiotic. Some fibre diameter measurements were also taken after drug release for comparison. The average fibre diameter was determined by randomly selecting 20 fibres and measuring their diameters using Image J software (NIH, Bethesda, MD; http://rsb.info.nih.gov/ij/ accessed 09.11.12.). The average thickness of each layer was determined by measuring the thickness of the cross-sections from seven different points of two SEM images using the same software. The homogeneity of the deposition of each layer on the collector was observed by adding a dye (Rhodamine B) to the PEVA solution (which will become the middle layer) and then electrospinning the layers. After spinning each layer, the collector was imaged with a digital camera. Autofluorescence of the electrospun PCL fibres (without Tet HCl) and fluorescence of Tet HCl (3% in PCL) were determined on a Leica DMI4000B inverted fluorescent microscope.

Encapsulation efficiency

To determine the encapsulation efficiency of the nanofibres, the electrospun mats were cut into small pieces (~ 1 cm^2), weighed and then dissolved in $CHCl_3$:CH_3OH (9:1 v/v, 10 mL). The UV absorbance was measured at λ = 360 nm and the amount of Tet HCl in the fibres was then calculated using a Tet HCl calibration curve and compared to the theoretical value (3%) (n = 8).

Chemical integrity of Tet HCl in the matrices and following release

The chemical integrity of Tet HCl in the PCL and PEVA electrospun nanofibres was investigated by ^{1}H NMR spectroscopy (Bruker Avance spectrometer operating at 500 MHz for ^{1}H, 5 mm probe). The matrices were prepared with 10% Tet HCl rather than 3%. The matrices were cut into small pieces (5 mg) and then dissolved in deuteriated chloroform $CDCl_3$ (n = 2). A similar PCL (one layer matrix) was prepared and the Tet HCl was released over 48 h, shaking at 37 ^{o}C and 200 rotations per min (rpm) into D_2O (1 mL).

In vitro drug release studies

The micro/nanofibrous matrices were cut into 4 cm^2 squares. Each sample was placed in Tris buffer (10 mL; pH = 7.4) [18] or in PBS (10 mL; pH = 7.4) [22] in a glass vial. The vials were immersed in a water bath in a thermostatic shaking incubator at 37 ^{o}C and 200 rpm. At specific time intervals (typically 24 h), each matrix sample was introduced into a new glass vial with buffer (10 mL). The samples were assayed by UV spectroscopy at λ = 360 nm and the amount of Tet HCl released at each specific time was then calculated using a Tet HCl calibration curve and subsequently compared to the theoretical value (3%) (n = 3). In initial studies we established the consistency of results by cutting 3 similar sized squares from within a single mat (n = 3).

Antibacterial efficacy of Tet HCl-loaded micro/nanofibrous layered matrices

Commercially available Tet HCl-sensitive bacteria, *S. aureus* (ATCC 25,923), were used to investigate the efficacy of Tet HCl-loaded in the designed formulations. For this a modified Kirby-Bauer test was used [30]. The *S. aureus* suspension was incubated for 16 h and diluted with sterile Mueller-Hinton broth to obtain an absorbance value between 0.1 and 0.2 at 625 nm (equivalent to 0.5 McFarland standard). Then, 200 μL of the diluted bacteria solution was streaked onto the surface of the Mueller-Hinton agar plate (prepared previously according to the manufacturer's instructions) followed by placing the micro/nanofibrous matrices on the agar plates. Five groups of samples were tested: PCL containing no Tet HCl, PCL containing 3% Tet HCl, PCL/PEVA containing 3% Tet HCl in each layer, PCL/PEVA/PCL containing 3% Tet HCl in each layer, and PCL/PEVA/PCL containing 3% Tet HCl in the PEVA layer only. The matrices were cut (punched) into circular discs containing 30 μg Tet HCl which is equivalent to the amount in each commercially available

Antimicrobial Susceptibility Test Disc containing Tet HCl. 3 replicates of one formulation and one commercially Tet HCl disc as a positive control were placed on each agar plate. After incubation at 37 ^{o}C for 24 h, the inhibition zones were measured. The discs were then introduced to new Mueller-Hinton agar plates pre-streaked with fresh bacteria and incubated for another 24 h and the new inhibition zones were measured. This test took place for 5 successive days. The inhibition zone sizes (n = 3) were averaged and expressed as the mean ± standard deviation. Student's t-test (Microsoft Excel) was used to determine any statistically significant differences between the formulations and the commercially available discs. $P < 0.01$ is considered to be significant.

Results and Discussion

Morphology of electrospun micro/nanofibrous matrices

Optical images

The homogeneity of the deposition of the upper layer over the previous layer was investigated by adding Rhodamine B to the PEVA second layer and observing the coloured coverage. The optical images of the matrices (Fig. 1) clearly show the first PCL layer (Fig. 1a) is white, while the second PEVA layer (Fig. 1b) is pink due to Rhodamine B and it shows full coverage over the first PCL layer. Finally, the third PCL layer completely hides the pink layer (Fig. 1c).

(a) (b) (c)

Fig. 1. Optical images (top views of the 9 cm long mats) showing the steps in electrospinning layered mats: (a) PCL first layer; (b) PEVA second layer spun over the first

layer with Rhodamine B added to monitor the deposition over the first layer; (c) PCL third layer spun over the PEVA (Rhodamine B) layer.

Microscopy

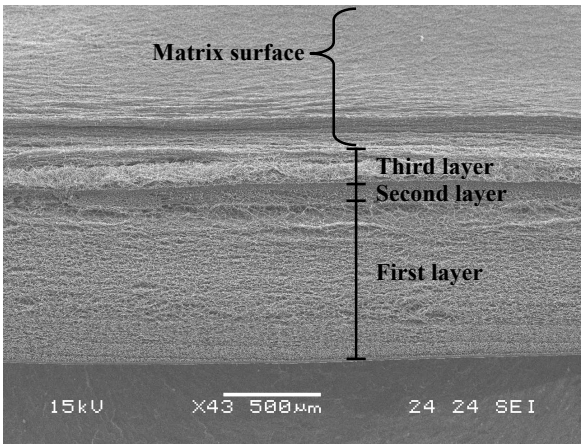
Fig. 2 shows the SEM morphologies of the electrospun triple-layered PCL-PEVA-PCL micro/nanofibre matrix. The total thickness of the matrix is 1050 ± 50 μm (Fig. 2a), and the thickness of each layer differs from one to another. The first electrospun layer was PCL with a thickness of 767 ± 17 μm (Fig. 2b). This decreases sharply to 77 ± 14 μm for the middle PEVA layer (Fig. 2c) and 121 ± 18 μm for the PCL upper electrospun layer (Fig. 2d). The diameter of the fibres also differs. The diameter of the PEVA fibres is 1246 ± 221 nm and the fibres fuse together at several points (Fig. 2e). This might be explained by the solvent not evaporating completely upon the stretching of the PEVA fibres during the electrospinning process as any residual solvent may allow the fibres to fuse together, although (by NMR spectroscopy) there is only a small amount of residual $CHCl_3$ solvent. Comparing PEVA layers, there was no apparent difference between the fibres and their morphology in the second layer (Fig. 2f) and in an electrospun single layer of PEVA (Fig. 2e). The diameter of the PCL fibres in the first layer is 702 ± 120 nm with a smooth shape (Fig. 2g). The diameter of the PCL fibres in the third layer is 1916 ± 532 nm. Comparing the PCL layers, the thickness of the third layer is much less than the first PCL layer, however, the diameter of the fibres in the third layer is larger than the first PCL layer. The accumulated charges of the electrospun fibres interrupt the build-up of the later deposited fibres which renders them less stretched, thus of larger diameter and with reduced layer thickness [31, 32]. With regard to before and after Tet HCl release from a three layered matrix, the diameter of the PCL fibres in the first layer was slightly reduced on average from 702 ± 120 nm (Fig. 2g) to 666 ± 125 nm (Fig. 2h). The electrospun PCL layer containing no drug was of thicker (double) fibre diameter 1578 ± 257 nm (Fig. 2i). Whilst a similar shape is maintained, the morphology of the fibres is changed due to the absence of the ionic (charged) Tet HCl species during the electrospinning which increases the conductivity and thus decreases the diameter. Fluorescence microscopy demonstrates the fluorescence of Tet HCl in PCL nanofibres (Fig. 2j).

Drug loading determination

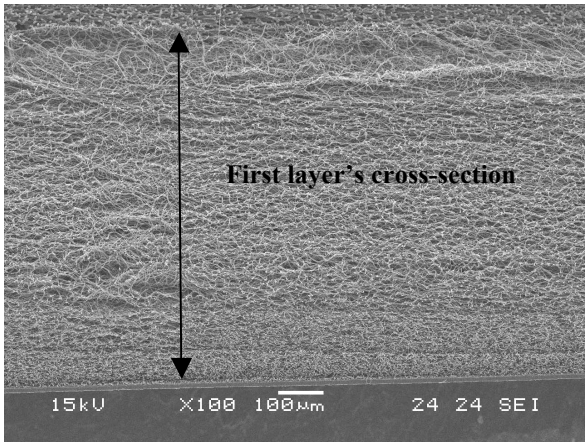
Tet HCl loading in PCL electrospun fibres and PEVA electrospun fibres was 3.1 ± 0.1% and 2.95 ± 0.1% respectively compared to 3% w/w initial strength. Essentially quantitative drug encapsulation efficiencies were achieved, 104 ± 5% and 98 ± 5%, respectively.

Chemical stability of Tet HCl

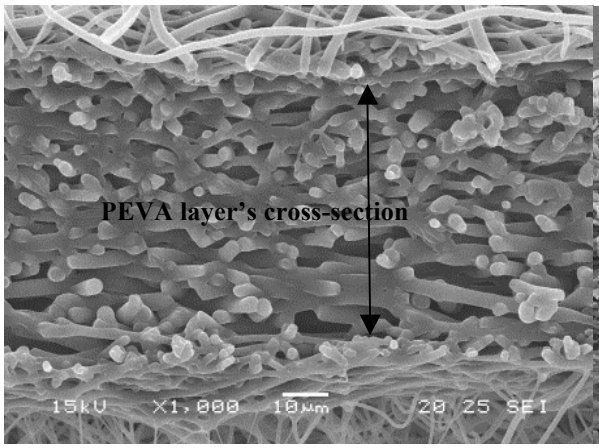
It is well known that light, temperature, moisture, and duration of storage influence the stability of Tet leading to a decrease in its microbiological activity and an increase in its toxicity [33, 34]. Changes in shape or hydrolysis of the acid or alkali labile functional groups which decorate the periphery of the Tet molecule may result in changes in the ^{1}H NMR [35] and UV spectroscopic data. The chemical integrity of Tet HCl after being spun under high electrical potential (16.5 kV) was therefore analysed. Tet HCl loaded PCL electrospun nanofibres and Tet HCl loaded PEVA electrospun nanofibres were dissolved in $CDCl_3$ and characterized by ^{1}H NMR spectroscopy. Solutions in $CDCl_3$ of drug-free electrospun PCL, PEVA, and Tet HCl, were used as reference samples. Based on comparisons of the obtained spectra (Figs. 3 and 4), the chemical integrity of loaded Tet HCl was maintained after the electrospinning process as all the signals of Tet HCl and the polymers could be observed, comparable with the literature data [34]. Such a spectroscopic measure of chemical stability after electrospinning has been reported in the analysis of nanofibres containing the antibiotic cefoxitin sodium (Mefoxin) [36], a range of NSAIDs [37], and even the protein bovine serum albumin (BSA) [38].


Matrix surface
Third layer
Second layer
First layer
15kV X43 500µm 24 24 SEI

(a)


First layer's cross-section
15kV X100 100µm 24 24 SEI

(b)


PEVA layer's cross-section
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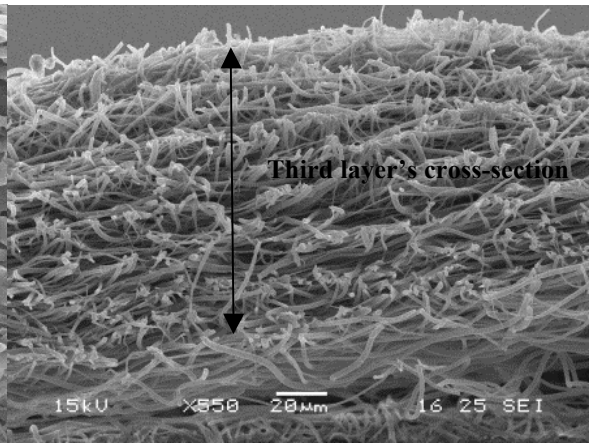
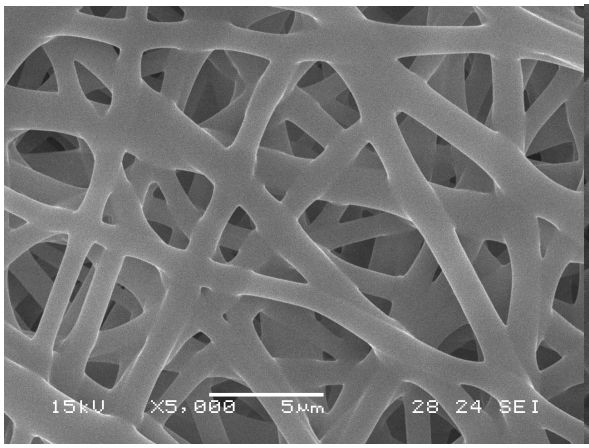
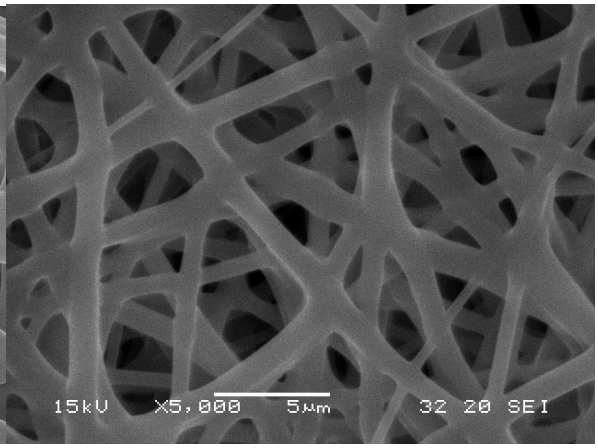
(c)


Third layer's cross-section
15kV X550 20µm 16 25 SEI

(d)


15kV X5,000 5µm 28 24 SEI

(e)


15kV X5,000 5µm 32 20 SEI

(f)

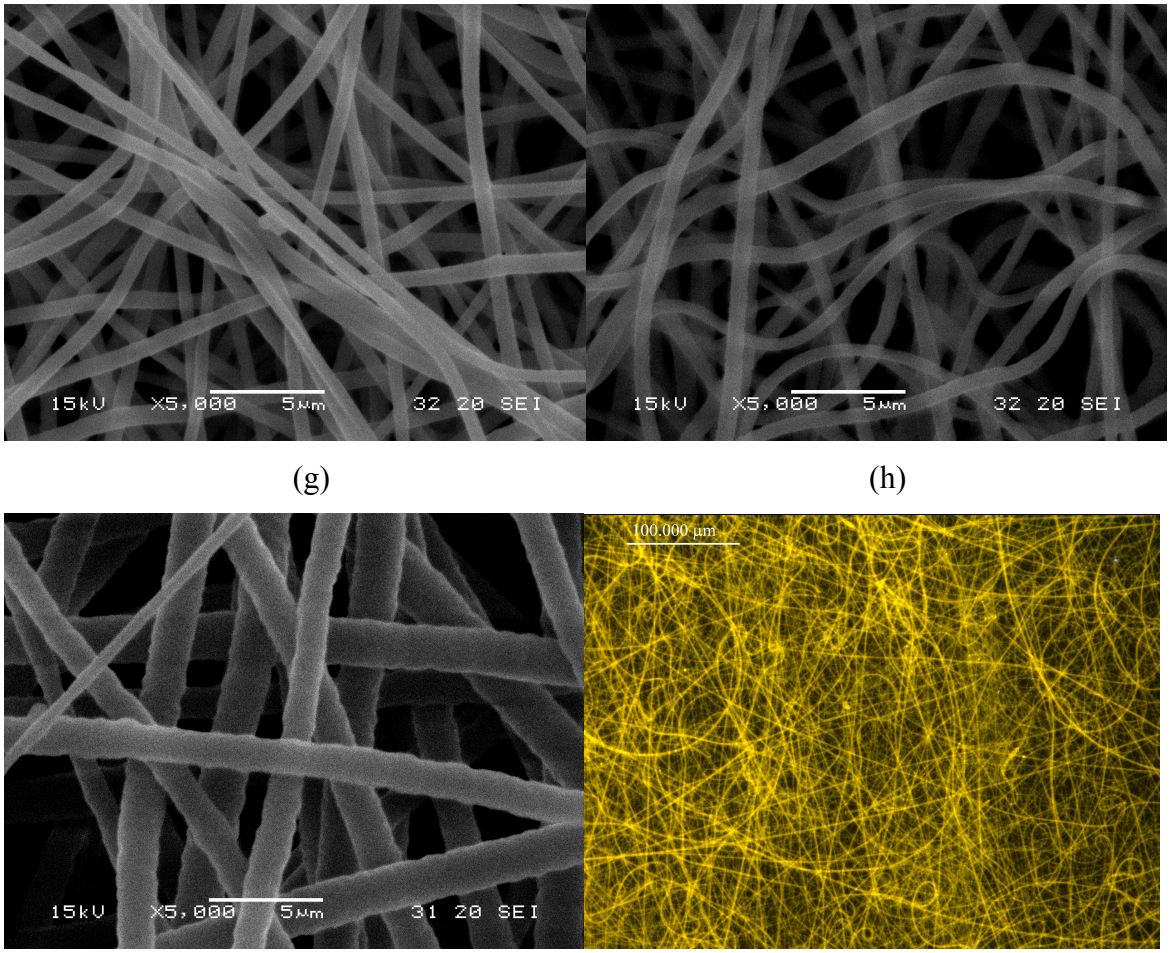
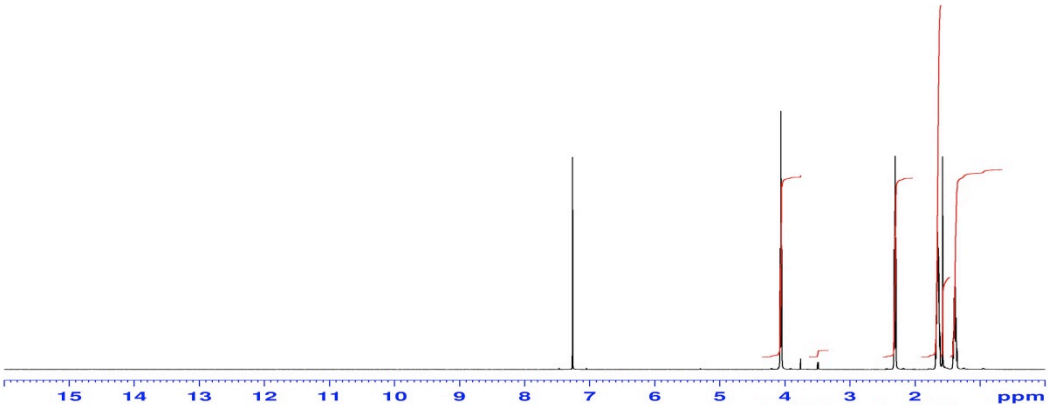
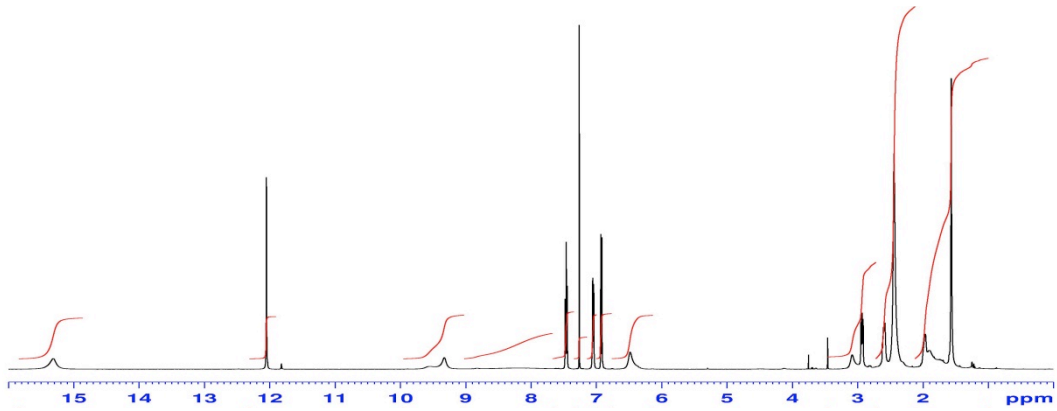


Fig. 2. SEM images of: (a) cross-section of electrospun fibrous mat (3% Tet HCl) consisting of three layers with a total thickness of 1050 ± 50 µm; (b) the first layer (PCL) with a thickness of 767 ± 17 µm; (c) the second layer (PEVA) with a thickness of 77 ± 14 µm; (d) the third layer (PCL) with a thickness of 121 ± 18 µm; (e) PEVA fibres (single layer) electrospun with Tet HCl with diameter of 1246 ± 221 nm; (f) PEVA fibres (second layer) electrospun with Tet HCl; (g) PCL fibres (first layer) electrospun with Tet HCl with diameter of 702 ± 120 nm; (h) PCL fibres as in (g) but after the release of Tet HCl with diameter of 666 ± 125 nm; (i) PCL fibres electrospun without Tet HCl with diameter of 1578 ± 257 nm. (j) Fluorescence microscopy of Tet HCl in PCL nanofibres.



(a)



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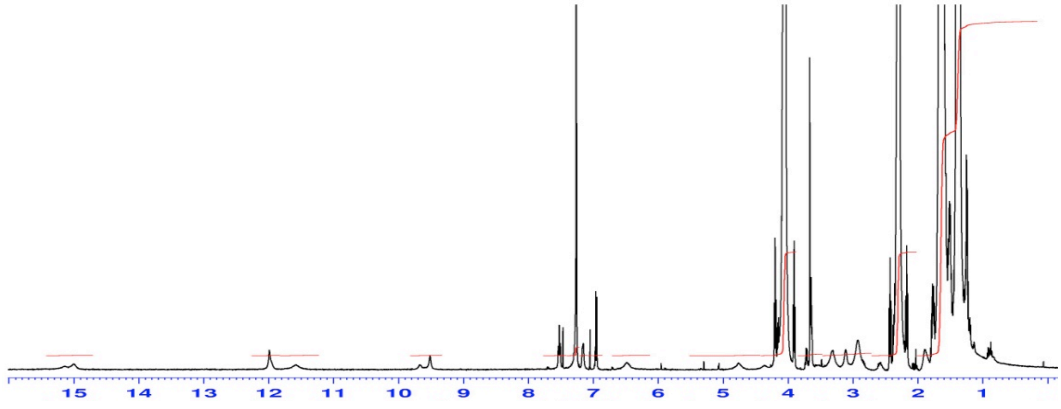
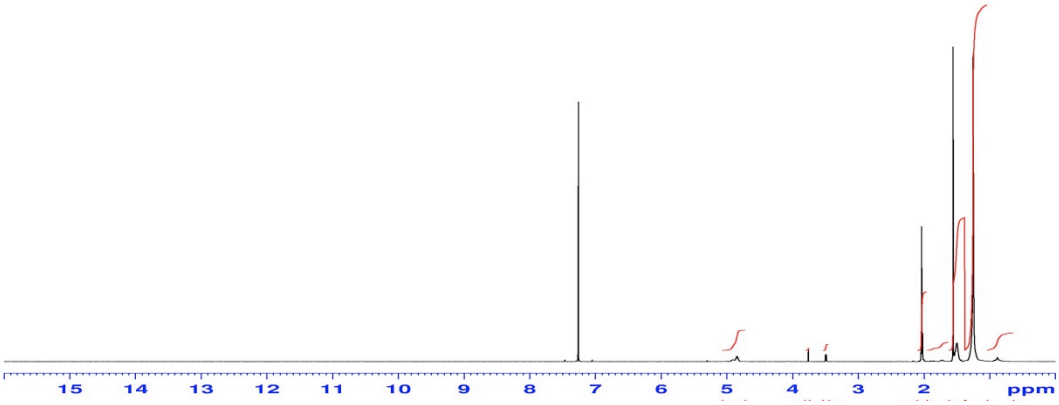
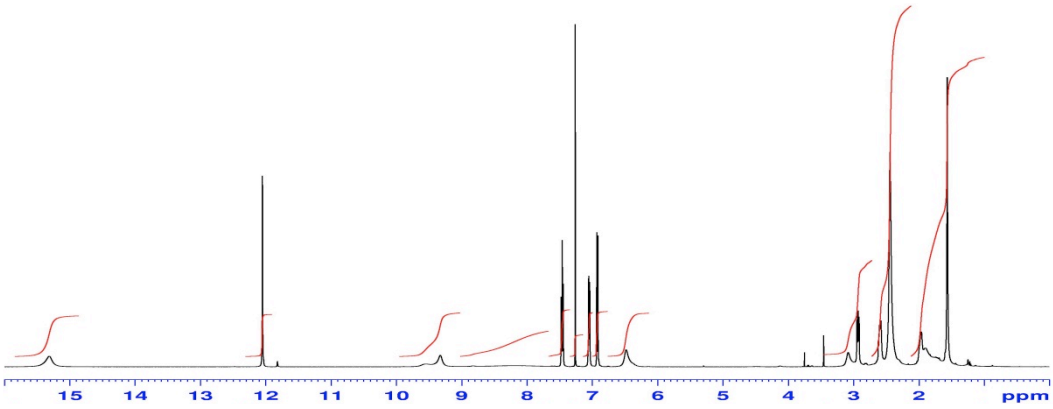


(c)

Fig. 3. ^{1}H NMR spectra, obtained in $CDCl_3$, of (a) drug-free electrospun PCL; (b) tetracycline HCl; (c) electrospun PCL mat containing Tet HCl (10%).



(a)



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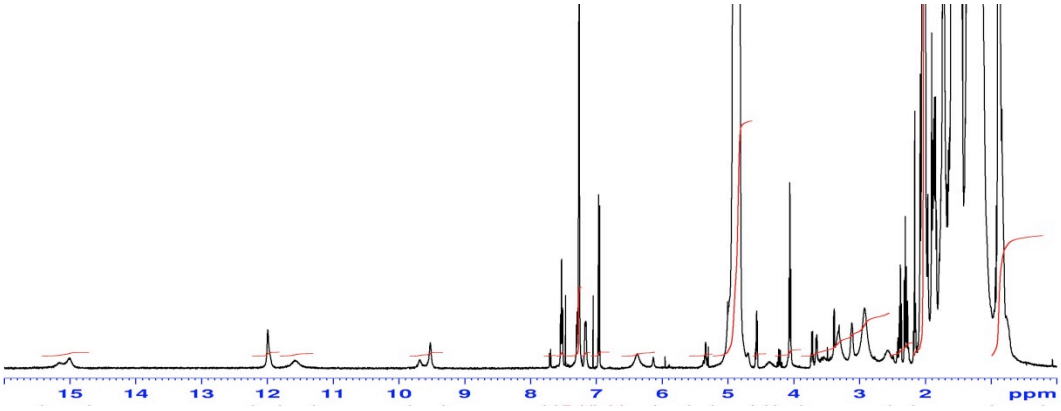
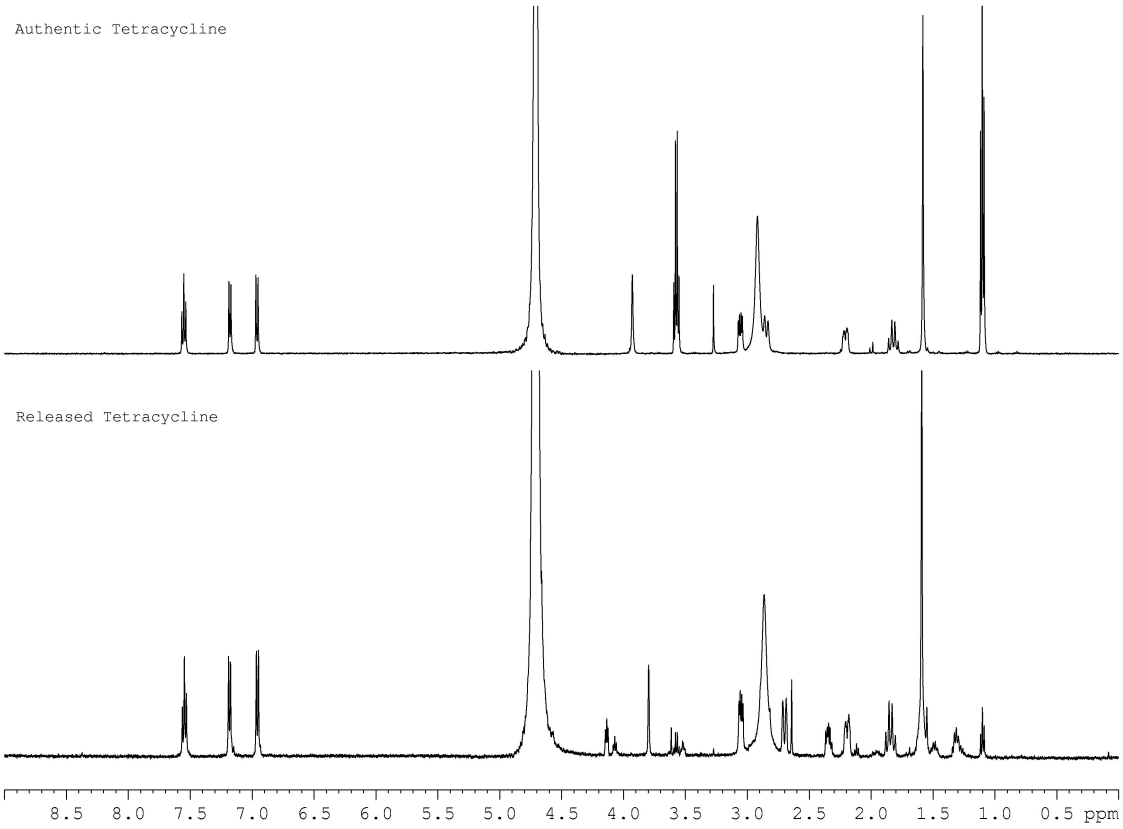
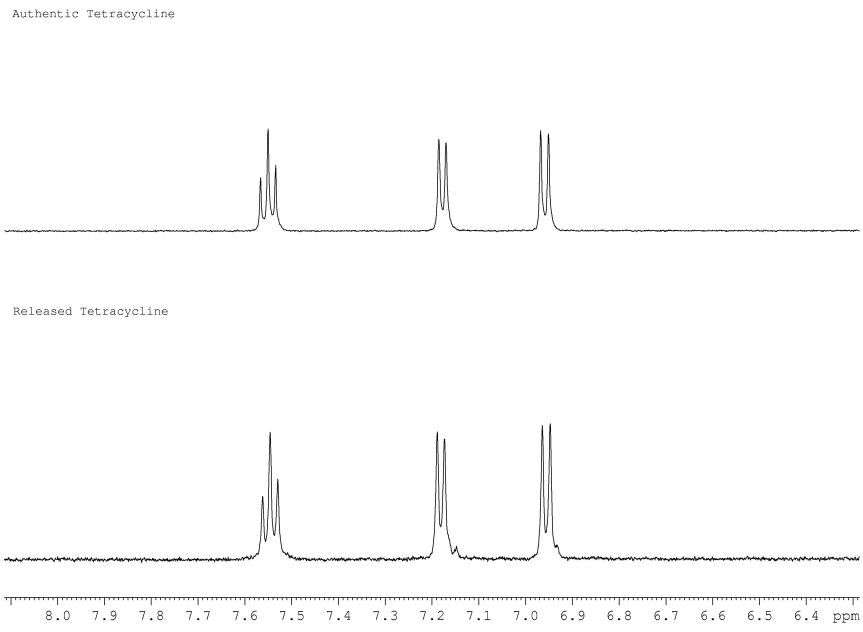


(c)

Fig. 4. ^{1}H NMR spectra, obtained in $CDCl_3$, of (a) drug-free electrospun PEVA; (b) tetracycline HCl; (c) electrospun PEVA mat containing Tet HCl (10%).


Authentic Tetracycline
Released Tetracycline
8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 ppm

(a)


Authentic Tetracycline
Released Tetracycline
8.0 7.9 7.8 7.7 7.6 7.5 7.4 7.3 7.2 7.1 7.0 6.9 6.8 6.7 6.6 6.5 6.4 ppm

(b)

Fig. 5. ^{1}H NMR spectra of (a) authentic tetracycline HCl stacked above tetracycline HCl released from PCL into D_2O; (b) detail in the stack of the aromatic regions obtained in D_2O (authentic shown above released).

In vitro drug release studies

Release profiles of one layer matrices

The Tet HCl release test was carried out by a total immersion method, in Tris at 37 ºC and 200 rpm, on electrospun fibrous mats cut from 2×2 cm squares of aluminium foil. PEVA monolayers were difficult to handle if not intractable. The absorbance of Tet HCl at λ = 360 nm was monitored as a function of time. The ^{1}H NMR spectra of authentic tetracycline HCl and tetracycline HCl released from PCL into D_2O are essentially equivalent (Fig. 5a); the detail in the stack of the aromatic regions (Fig. 5b) is superimposable showing signals at δ 6.95 (d, J = 8 Hz), 7.18 (d, J = 8 Hz) and 7.54 (t, J = 8 Hz) ppm for H-9, H-7, and H-8 respectively, entirely consistent with both an authentic sample and the literature data [34].

Fig. 6 shows the cumulative Tet HCl release profile from electrospun fibrous PCL compared to electrospun fibrous PEVA, both one layer matrices. As shown in Fig. 6, PCL released approximately 92% of Tet HCl within the first 3 h, whereas, PEVA released only 19% within the same time. Following a small burst release, PEVA sustained the release of the Tet HCl over 8 days compared to PCL where the drug was liberated completely in the first day. It is likely that the difference in the release profile is due to the difference in the chemical and physical properties of the two polymers.

PCL is a hydrophobic and semi-crystalline polymer [39]. During the electrospinning process, the solvent evaporates as the polymer travels from the needle tip to the collector. In the case of the PCL, it appears that Tet HCl, being hydrophilic in nature in comparison to PCL, exhibits a greater affinity for the solvent than for the polymer and migrates with the solvent to the surface of the polymer during the solvent evaporation process. This results in the drug being mainly distributed on the surface and, consequently, a burst release. This phenomenon has also been observed in Tet HCl release from nanofibres of poly(lactic acid), a polymer with similar properties to PCL [18]. Similar behaviours have been proposed in order to speculate upon drug release profiles from PCL microparticles prepared by the solvent evaporation method [40, 41].

PEVA is a rubbery polymer [42] with a polar group of vinyl acetate, conferring a greater degree of hydrophilicity in comparison to PCL. Indeed, the air-water contact angle of PEVA membranes has been reported to be 56.1º [43] in comparison 81.2º reported for PCL [44], indicating PEVA's more hydrophilic nature. When electrospinning 12% PEVA from a 9:1 chloroform/methanol mixture, the solvents did not evaporate completely (or evaporated slowly) from the polymer, causing the deposited fibres to fuse (Fig. 2e and 2f). However, based on the release profile and physicochemical properties, it appears that hydrophilic Tet HCl was distributed more evenly through the more hydrophilic PEVA rather than partitioning into the solvent. This drug distribution resulted in a more sustained release. To further understand how PEVA sustains drug release, Peppas and co-workers observed a substantial pore collapse and proposed that the main mechanism of release from PEVA matrix systems is solute dissolution and diffusion through the generated water-filled pore structure [45, 46].

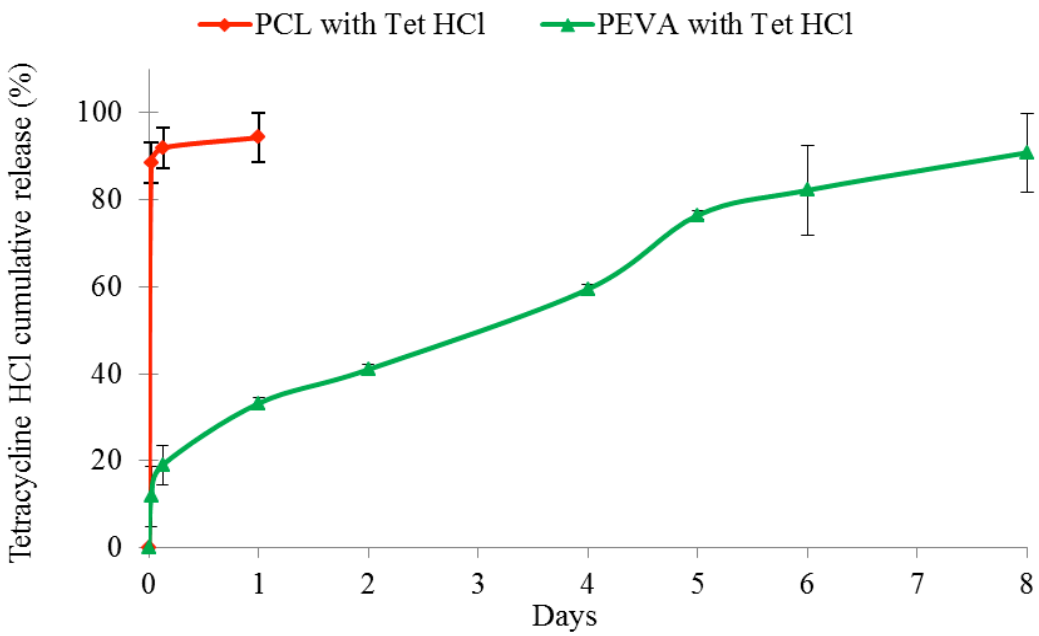


Fig. 6. The Tet HCl release profile from the electrospun one-layered fibrous mat.

Release profiles of layered matrices

In Fig. 7, the electrospun mats peeled from the aluminium foil show that the three layered matrix, PCL/PEVA/PCL, retains its shape better than the ruffled two layered matrix with one face composed of PEVA.

Fig. 7. Electrospun mats peeled from the aluminium foil. The three layered matrix, PCL/PEVA/PCL (left), the two layered matrix with PCL face up (right lower) and PEVA face up (right upper).

Fig. 8 displays the release profiles of layered matrices using our total immersion method on 2×2 cm square electrospun fibrous mats in Tris buffer and heated to 37 ºC. A two layered matrix released 85% of its content within the first 3 h and the rest a sustained release over the next 5 days. Upon the introduction of the third layer, the burst release was reduced to ~55% within the first 3 h and the release of the remaining drug was sustained to reach ~80% at day 15. The three layered matrix PCL/PEVA/PCL with Tet HCl in the PEVA layer only, however, produced no initial burst, resulting in prolonged release reaching 50% at day 15. Taking into the account the single layer PEVA matrix release profile, 20% release of its Tet HCl contents in a burst, the two layered matrix was expected initially to show a burst release of the loaded drug with ~50% from release of almost all the drug in the PCL layer and then the remaining drug coming from the PEVA layer. The differences between the release from a PEVA one layered matrix (of an intractable rubbery polymer) and the case of a two layered matrix are due to the sticky properties of the PEVA which caused the one layer PEVA sample to roll-up on handling and stick to itself in an irregular manner, whereas the two layered matrix, with the PEVA layer supported by the PCL layer, essentially maintained its shape when cut and then peeled from the aluminium foil (Fig. 7), making the PEVA surface of the two layers more available to interact with the buffer.

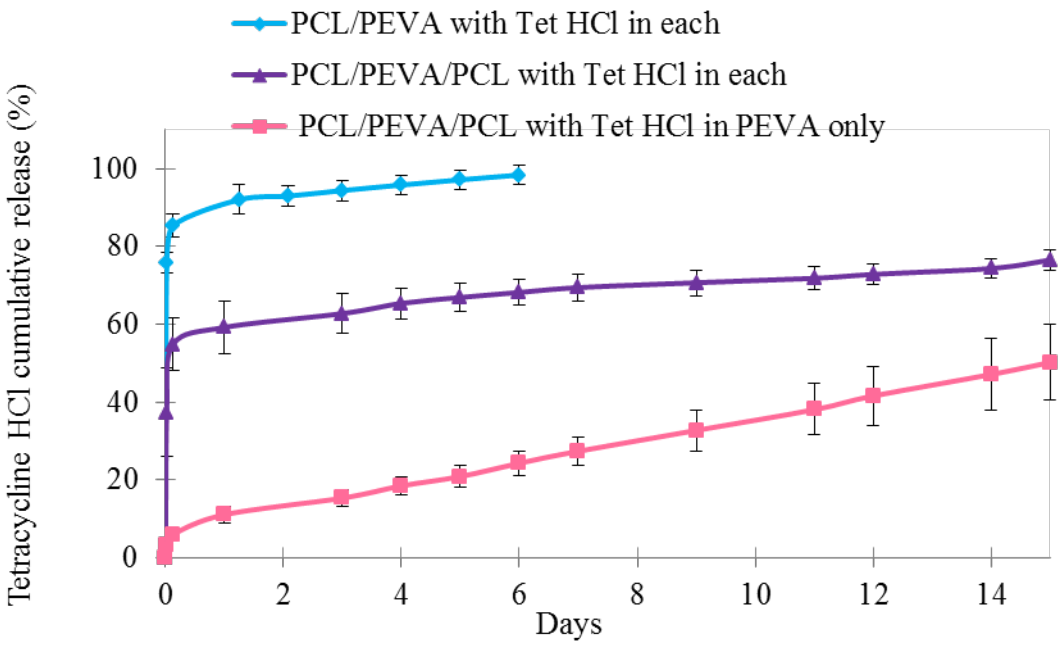


Fig. 8. The Tet HCl release profile from the electrospun multi-layered fibrous mat.

From the three layered matrix, PCL/PEVA/PCL with Tet HCl in each layer, the initial burst was close to our prediction (about 60%, 30% coming from each PCL layer). However, there was then a sustained release for more than 8 days to reach about 80% of the total loading released by day 15. We conclude that the PCL layer plays the role of a physical barrier which delays the diffusion of the buffer into the PEVA layer. PCL is a hydrophobic polymer and PEVA is a known rate-controlling drug-release polymer [39, 42]. The water molecules have to diffuse through the hydrophobic polymer to reach the PEVA, then to diffuse through the PEVA and dissolve the Tet HCl incorporated in the PEVA, and diffuse out again. To further investigate this concept, we studied the release from PCL/PEVA/PCL three layered matrix where the Tet HCl is only in the PEVA layer. We found that sandwiching the PEVA between two layers of PCL clearly sustained the release from about 3 days (where a one layered matrix of PEVA continued releasing 50%, green line in Fig. 8) to 15 days (where a three layered matrix with Tet only in the PEVA layer also continued releasing 50%, pink line in Fig. 8). For a successful antibacterial wound dressing, an initial burst release would help to eliminate any invading bacteria, whereas, a sustained release antibiotic will secure the prevention of any relapse [36]. Achieving a delivery system capable of a burst release

followed by a sustained release can be usefully incorporated in orthopaedic-related devices, potentially enhancing the wound healing processes, and further other applications may be in organ transplantation, periodontal devices, intravascular devices and vascular grafts [46, 47]. Of our obtained release profiles, the formulation PCL/PEVA/PCL with Tet HCl in each layer is most promising. However, each of the release profiles might be useful for a specific application, for example, the three layered matrix with Tet HCl in the PEVA layer only, gave a release profile that could be favourable where no burst release, but rather a slow sustained release is required. Moreover, the layered design of the electrospun fibrous matrices is a platform for engineering tissues with several layers e.g. blood vessels and skin. Employing this design to control drug release will enable an easy combination between a layered engineered tissue and the controlled release of a bioactive which are necessary requirements in support of organ regeneration. PCL and PEVA are FDA approved biocompatible polymers. The layered matrices neither swell nor shrink on contact with and during immersion in heated Tris or PBS buffer; there was no obvious erosion. The mats are not visibly frayed after 15 days under such conditions, with daily manipulation and constant heating and shaking. Whereas many papers on electrospinning nanofibres from a blend of a polymer and a drug end with a UV or related spectrophotometric release assay, and our UV, fluorescence, and NMR data are all entirely consistent with unchanged Tet HCl being released, the acid test of Tet HCl surviving electrospinning and then being biologically available by exiting the matrix must be antibacterial efficacy.

Antibacterial efficacy of Tet HCl release from the fibrous layered matrices

The biological activity of the released Tet HCl was investigated using *S. aureus* bacteria inhibition experiments. Formulations of PCL/PEVA with Tet HCl in both layers, PCL/PEVA/PCL with Tet HCl in all layers, PCL/PEVA/PCL with Tet HCl only in the PEVA layer, and one layer of PCL with Tet HCl, were chosen. However, one layered PEVA with Tet HCl has poor mechanical properties, in that it is such a sticky material, so it was omitted from the antibacterial assay as it was not practical to punch the PEVA circular discs which often tore on handling; robustness was achieved with a 2 layer formulation i.e. adding a PCL layer. Two controls were used, commercially available Antimicrobial Susceptibility Test Tet HCl-loaded discs (positive control) and electrospun nanofibrous PCL without any antibiotic (negative control). The samples were punched into circles containing ~30 μg Tet HCl,

chosen as this is the dose in the positive control discs. The test was conducted for 5 days to show any sustained release of the drug and its antibacterial activity. The results of the microorganism susceptibility tests are shown in Fig. 9. On day 1, all the samples exhibited inhibition zones comparable to the positive control apart from the negative control, which unsurprisingly showed no inhibition zone and the formulation PCL/PEVA/PCL with Tet HCl in the PEVA only, which indicated a smaller inhibition zone demonstrating that no burst release was taking place. On day 2, the size of the inhibition zones decreased, however all our samples were comparable to the positive control. On day 3, the inhibition zones of the positive control and of the one layer electrospun PCL containing Tet HCl decreased sharply, whereas the multilayered formulations were still showing significantly higher antibacterial effects ($P < 0.01$). This shows that the inhibition zones were due to the sustained release of Tet HCl. On days 4 and 5, the multi-layered formulations were still showing significantly higher antibacterial effects than the commercially available discs ($P < 0.01$) (Fig. 9). In addition to the above UV and NMR data, these biological activity results confirm that Tet HCl loaded into the micro/nanofibrous matrices maintains its biological function, unaffected by the 16.5 kV electrospinning process showing antimicrobial activity sustained over 5 days.

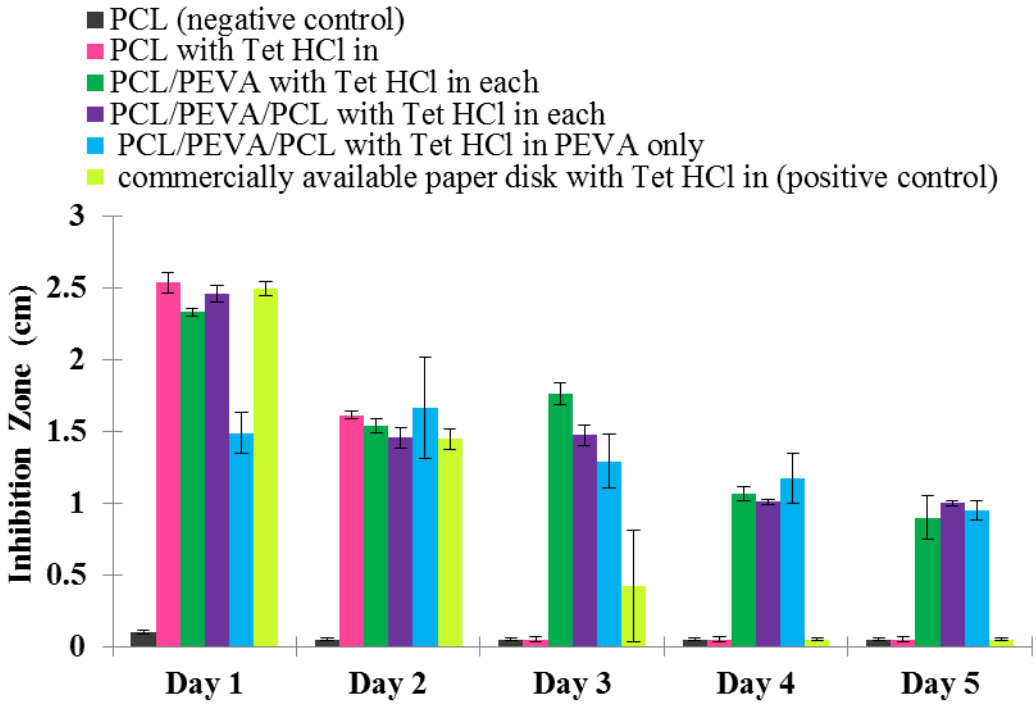


Fig. 9. Diameter of inhibition zone in the *S. aureus* susceptibility test of electrospun nanofibres. The errors bars represent the standard deviation of the mean (n = 3).

Earlier this year, the potential for electrospun matrices in localized drug delivery and related biomedical applications was highlighted [3]. We are only aware of one other research group that has published on a triple layered mat. Yoon, Kim, and co-workers have reported the release of Rhodamine B as a model drug from various thicknesses of layered mats consisting of electrospun PCL and polyethylene oxide (PEO) micro/nanofibres [48]. They reported the release of a cationic peptide, a synthetic 15-mer (FKRLKKLFKKIWNWK, ~2.06 kDa) called HPA3NT3 which carries 7 positive charges at physiological pH and is a pore-forming and membrane disrupting antibiotic, acting in a similar manner to melittin [49]. The 15-mer peptide was encapsulated in the PEO layer with variation in the thickness of the PCL layers [48]. They continue their work on PCL and PEO monitoring the release of Rhodamine B [50], earlier this year reporting ways to overcome edge-effects in layer-by-layer electrospun micro/nanofibrous mats of PCL and PEO with the ultimate goals of soft and hard tissue regeneration [51].

In our studies, the first small molecule drug, Tet HCl has been successfully incorporated in and its release controlled from multi-layered electrospun matrices without any loss in its chemical stability or bioactivity. Electrospinning provides good encapsulation efficiency of Tet HCl within the polymers. The introduction of the third layer to the two layered formulation led to an increase in sustained release from 6 days to more than 15 days. The formulation PCL/PEVA/PCL with Tet HCl in each layer could potentially be a successful wound dressing system as it gives a large initial (burst) release which is important to deal with the immediately invading bacteria, followed by a sustained release which will help to prevent any relapse. These biocompatible triple-layered electrospun matrices are also of interest as they can be further developed and studied for multi-layered bioartificial replacement tissues e.g. blood vessels and skin, where bioactive molecules can be incorporated and their release controlled.

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