Cellulose acetate/poly(vinyl alcohol) hybrid fibrous mat containing tetracycline hydrochloride and phenytoin sodium: Morphology, drug release, antibacterial, and cell culture studies

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Abstract
The objective of this study was to introduce an electrospun hybrid fibrous mat (a dual-fiber drug delivery system) based on cellulose acetate and poly(vinyl alcohol) containing tetracycline hydrochloride and phenytoin sodium, respectively. Characterization of samples was carried by morphology, drug release, cell cytotoxicity, adhesion, antibacterial property, and wettability investigations. The results showed a uniform shape and a narrow diameter distribution of fibers (between 160 ± 20 nm) for fabricated cellulose acetate/poly(vinyl alcohol) hybrid fibrous mat. The tetracycline hydrochloride release from cellulose acetate significantly decreased due to gel formation of poly(vinyl alcohol) in aqueous media. The best fit for drug release kinetic of hybrid sample was Higuchi model. Sample with tetracycline hydrochloride and phenytoin sodium drugs showed improved cell growth, viability, and antibacterial activity against Escherichia coli (≈89%) and Staphylococcus aureus (≈98%) in comparison with sample without drugs. The hydrophilic property

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of cellulose acetate/poly(vinyl alcohol) fibrous sample containing the drugs was also remarkable (~45°). To consider the obtained results, the presented hybrid fibrous mat shows a high potent for biomedical applications.

Keywords
Electrospinning, hybrid fibrous mat, drug delivery system, cellulose acetate, poly(vinyl alcohol)

Introduction

Over the past two decades, electrospinning of natural and synthetic polymers has received a great deal of attention. It has been used for fabrication of fibers ranging from nanometers to micrometers for industrial applications such as textiles,1–5 heterostructured materials,6–8 electromagnetic interference (EMI) shielding,9–13 sensing,14–19 membrane and filters,20–27 absorbents,28–30 smart and responsive materials,31–35 and biomedical.36–39

In recent years, many developments related to electrospun fibers have been made in terms of drug delivery,6,40–42 scaffolds,43–46 self-healing materials, and wearable sensors.15,18 Schkarpetkin et al.47 investigated the drug release behavior of different fiber systems based on poly(1-lactide-co-d/1-lactide). They compared single-fiber drug delivery system with metronidazole and ampicillin combined in a fiber with a dual-fiber drug delivery system, in which two single fibers, each with one antibiotic, were spun simultaneously into one fiber mat. They concluded that the dual-fiber drug delivery system was a potential drug delivery system for use in periodontal and endodontic infections. Similar results have also confirmed the unique characteristics of these drug delivery systems for sustained control release of multidrug48,49 because of their high specific surface area, controlling hydrophilic/hydrophobic properties, and the ease of production using electrospinning technique.

Xue et al.50 studied the drug-loaded homogeneous electrospun poly(ε-caprolactone) (PCL)/gelatin hybrid fibrous structures for anti-infective tissue regeneration. Their results exhibited that the samples containing metronidazole have good mechanical properties and an appropriate biodegradation rate, and that the controlled release of the drug significantly prevented from the colonization of anaerobic bacteria. In another study, Shi et al.51 investigated the pH- and electro-responsive characteristics of bacterial cellulose fibers/sodium alginate hybrid hydrogels for ibuprofen-controlled drug delivery. They showed that the release of drug could be controlled by deprotonating or protonating sodium alginate in hydrogels under different pH conditions in which the trend was faster in alkaline conditions and slower in acidic conditions.

Up to now, many biopolymer-based electrospun fibrous mats have been introduced for drug delivery systems, and they have been loaded with a variety of herbal and synthetic drugs. Furthermore, they have also been used as a substrate for the growth and proliferation of peripheral nerves, mesenchymal, and fibroblast cells, signifying their use in wound dressings and nerve conduits.52–57 As the most abundant renewable carbon sources on the earth and their intrinsic properties, cellulose and lignin (including their derivatives) have been used in this field extensively, for example, in an innovated application for absorption of lead from blood.58 Cellulose acetate (“CA”) is one the most well-known derivate of cellulose obtained by esterification of cellulose. It is insoluble in water and it has been used as a film in photography, coating and as a synthetic fiber for cigarette filters. Recently, it has been emphasized as a good candidate for developing electrospun fibrous scaffolds owing to its ability to enhance the cellular interaction between the scaffolds and fibroblasts.59
In addition to the above-mentioned natural polymers, poly(vinyl alcohol) (“PVA”), as a water-soluble, non-toxic, biodegradable, biocompatible, and cost-effective synthetic polymer, has been used in electrospinning process abundantly. Although this polymer has different advantages, it has been found with low mechanical properties which can be negligible in biomedical applications. PVA is well-suited as a topical carrier for many drugs. Opanasopit et al. fabricated capsicum extract-loaded PVA and CA fibrous patches to investigate their properties for use as a transdermal drug delivery system. The researchers showed that the release rate of capsicum from PVA part was faster than that of CA part.

Despite presenting a variety of drug delivery systems through electrospinning (blend and/or core-sheath two polymeric systems), rare reports have been found to consider hybrid systems for studying their characteristics in in vitro and in vivo conditions. Besides, there is no evidence of research in the literature about the electrospinning of CA and PVA either as combination or as hybrid system in the presence of drugs especially used for a multidrug delivery system and cell cultivation application.

In this study, a new dual-fiber drug delivery system is presented. Two single fibers simultaneously, each with one drug, were electrospun and fabricated into one hybrid fibrous mat. Fibers were based on PVA and CA as a water-soluble polymer and a water-insoluble polymer, respectively. PVA (as a water-soluble polymer) and CA (as a water-insoluble polymer) fiber polymers were selected for fabrication of each of fibers individually. It was supposed that PVA in aqueous media such as wound exudate would be a fast disintegrated matrix showing a burst release manner, while CA would be a more stable matrix and it was showing a gradual drug release behavior. Tetracycline hydrochloride (TC) as a well-known antibiotic and phenytoin sodium (PHT-Na) as an accelerating drug for cell growth were loaded into CA and PVA solutions, respectively. Each of polymer solutions (with or without drug) was injected via one syringe placed in opposite directions in an electrospinning apparatus and fibers were collected on an aluminum target. The hybrid fibrous mat characterization was carried out by observing the morphology and evaluating the drugs release, antibacterial assay, and cell culture studies. Finally, the results of fabricated hybrid fibrous mat with or without drugs were compared and discussed.

**Experiment**

**Materials**

CA (acetylation 39.8%, molecular weight (Mw) ~30 kDa) was purchased from Sigma-Aldrich Co. (Zwijndrecht, Netherlands). PVA (Gohsenol™ GH-17, Mw ~98 kDa, DH ~88 mol%) was obtained from Nippon Gohsei Co. (Osaka, Japan). TC antibiotic and PHT-Na with 99.99% purity were supplied from Merck (Darmstadt, Germany) and Katwijk Chemie (Katwijk aan Zee, Netherlands), respectively. All the other chemicals were of analytical grade and were used without further purification.

**Fabrication of CA/PVA hybrid fibrous mat with or without TC and PHT-Na**

**PVA spinning dope.** PVA powder (8% (w/v)) was dissolved in distilled water at 80°C for 2 h. To load the drug, after cooling the solution down to room temperature, PHT-Na powder (1% (w/v)) was added to the PVA solution and stirred for 1 h.

**CA spinning dope.** CA powder (17% (w/v)) was added to a mixed solvent of acetic acid:distilled water (8:2 (v/v)) gradually and stirred until a uniform solution obtained. To prepare CA-loaded
solution with drug, TC powder (500 μg mL⁻¹) was dissolved in the mixed solvent (by vigorously stirring) first. Then, CA powder (17% (w/v)) was added to the drug solution gradually and stirred gently to obtain a uniform mixture.

**Electrospinning.** Prepared spinning dopes (PVA and CA solutions) with or without drugs were inserted to two syringes placed in opposite directions facing a collector. Electrospinning parameters were adjusted briefly as follows: Flow rate was 0.8 mL h⁻¹ for PVA and it was 1 mL h⁻¹ for CA, the distance between syringe needle tip and the collector was 10 cm, and the voltage was set at 19.5 kV for both polymers. Electrospinning was carried out for 4 h and the electrospun CA/PVA (50/50) layer or the hybrid fibrous mat was removed from the aluminum collector. The processing parameters of electrospinning were the same for with or without drugs spinning dopes.

**Cross-linking PVA electrospun fibers.** A glutaraldehyde solution (25% (v/v)) at room temperature for 12 h was used for cross-linking PVA electrospun fibers with and without drugs. Samples were impregnated with the solution and were heat treated in an oven at 40°C overnight to accomplish the cross-linking reaction.

**Morphological study**

The morphology of electrospun hybrid fibrous mats was observed using a field-emission scanning electron microscopy (FE-SEM, SU 8040; Hitachi, Tokyo, Japan) at 10 kX magnification. Samples were gold-sputtered using a Bio-Rad E5200 auto sputter coater (Bio-Rad, Herts, England). The mean values of the fibers diameters were measured using ImageJ software for at least 10 different sections, and the mean results were reported.

**Water contact angle measurements**

To investigate the hydrophilicity of mat samples, water contact angles of samples were determined by direct measurement of the tangent angles at the three-phase contact point on a sessile drop. A contact goniometer (model OCA 15 Plus; DataPhysics Co., Filderstadt, Germany) fitted with a CCD camera with high frame-rate photographic capability was used. Water droplets (distilled water and was 1 μL in volume) onto the surfaces of the samples were photographed after 3 s.

**In vitro drug release study of the mat samples along with their release kinetic models**

The concentrations of released TC and PHT-Na in phosphate-buffered saline (PBS, pH 7.2) were determined using ultraviolet (UV)-vis spectrophotometry (UNICAM, series 8700 model; Philips Co., Amsterdam, Netherlands) and high-performance liquid chromatography (HPLC), respectively. HPLC was equipped with a UV detector (λ= 254 nm, model 486; Waters Associates, Milford, MA, USA). The detector was coupled with a C₁₈ analytical column (Bond clone 30 cm × 3.9 mm i.d., 5 μm; Phenomenex, Macclesfield, Cheshire, UK) for separation.

First, solutions of known concentrations for both drugs were prepared and their absorbance values versus concentrations were plotted and their straight line equation obtained (equations (1) and (2)). Where A is the absorbance and C is the corresponding concentrations

\[ A_{TC} = 0.03 \times C_{TC} \]  

(1)
A_{\text{PHT-Na}} = 3.6 \times C_{\text{PHT-Na}} \quad (2)

Then, to determine the releasing profile for each drug, sample was immersed into PBS. An amount of 3 mL of samplings were taken at pre-determined time intervals up to 48 h. After each sampling, the same volume of PBS was added to the release medium (PBS) to keep the volume of solution constant. The concentration or the release amount of the drugs in each 3 mL of sampling was calculated according to obtained equations (1) and (2). Eventually, the cumulative drug release was reported by equation (3):

\[
\text{Cumulative drug release (\%)} = \sum_{t=0}^{\infty} \frac{M_t}{M_0} \times 100
\]

where “M_t” and “M_0” are the cumulative amounts of TC and PHT-Na released at each sampling point and the initial weights of the drugs loaded into the samples, respectively. The release kinetic of the drugs from the electrospun samples can be explained by Korsmeyer–Peppas equation as follows (equation (4))

\[
\frac{M_t}{M_\infty} = k t^n, \quad \frac{M_t}{M_\infty} \leq 0.6
\]

where “M_t” is the cumulative amount of the drug released at time “t”, “M_\infty” is the initial drug loading, “k” is the constant characteristic of the polymer–drug system, and “n” is the diffusion exponent suggesting the nature of release mechanism. In addition, four other models were used to further analyze the profile of drug release such as zero-order, first-order, Higuchi, and Hixon–Crowell, which are listed below (equations (5) to (8))

\[
\text{Zero-order : } Q_t = Q_0 + K_0 t
\]

\[
\text{First-order : } \ln Q_t = \ln Q_0 + K_1 t
\]

\[
\text{Higuchi : } Q_t = K_H \sqrt{t}
\]

\[
\text{Hixon–Crowell : } Q_0^{\frac{1}{3}} - Q_t^{\frac{1}{3}} = K_{HC} t
\]

Cell cytotoxicity and adhesion onto the surfaces of the mat samples

The cell cytotoxicity of electrospun CA/PVA hybrid fibrous mats with and without the drugs was investigated. In brief, after being sterilized with gamma irradiation using Cobalt-60 with dosage of 25 kGy, the mat samples were immersed into the Roswell Park Memorial Institute (RPMI) medium for 72 h (1 mL of medium per 6 cm² in area of the sample). Finally, the supernatant was removed using a syringe and passed through a 0.2-μm microporous filter. The extract of electrospun CA/PVA hybrid fibrous mat with and without the drugs and RPMI medium as control was inserted to a three-well plate containing fibroblast cells L929 with 5000 cells mL⁻¹ and were kept in an incubator at 37°C for 14 days. After this time period, the wells including the neat and drug-loaded samples as well as control (the cells’ environment RPMI) were fixed with glutaraldehyde
solution (4% (v/v)) for 30 min. The solution in the wells was then replaced with a crystal violet solution for 45 min and stained cell colonies were observed under an inverted biological microscope. Then, 1 mL of methanol was added to each well until crystal violet was dissolved, and the optical density of solution was detected using an enzyme-linked immunosorbent assay (ELISA) reader at 570 nm.

Non-toxicity of samples was also investigated through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In this test, the reduction level of the yellow-colored MTT to purple formazan dye depends on the alive cells. As the samples were in the 96-well plate for cell culture, 100 μL of MTT solution was added to each well. After 24 h at 37°C, 200 μL of dimethyl sulfoxide (DMSO) solution was added to the well until formazan crystals were dissolved, after which the solution was shaken for 10 min. The optical density of formazan solution was detected at 570 nm.

Regarding the qualitative observation of cell adhesion onto the surfaces of the samples, the morphology of fibroblast L929 cells was studied using SEM (AIS 2100; Seron Technology, Gyeonggi-do, Korea) at different magnifications. In short, after fixing the cells with glutaraldehyde solution (5% (v/v)), the samples were dehydrated gradually in an ethanol–distilled water mixture from 50% to 100% in steps of 10% at 10-min intervals. After that, the SEM micrograph images were taken and reported.

**Antibacterial evaluation of the samples**

Two different bacteria, namely, *Escherichia coli* (gram-negative, ATCC 25922) and *Staphylococcus aureus* (gram-positive, ATCC 25023) were used to investigate the antibacterial properties of electrospun CA/PVA hybrid fibrous mat in the presence and absence of TC and PHT-Na. Accordingly, bacterial suspensions with a concentration of 1.5 × 10⁸ CFU mL⁻¹ were prepared and added directly to the samples with a dimension of 2.5 × 2.5 cm² for 1 h. They were then incubated at 37°C for 72 h, and the viable bacteria were counted based on equation (9):

$$\text{Bacteria reduction} (\%) = \frac{B-A}{B} \times 100$$

where “A” and “B” denote the amounts of alive bacteria in the Petri dishes containing the samples and without them (control), respectively.

**Statistical analysis**

All data were expressed as mean ± SD (standard deviation) during statistical comparisons performed using SPSS software, and the differences were considered significant when p value was less than 0.05.

**Results and discussion**

**The effect of polymer solution concentrations on the morphology of mat samples**

The morphology for different electrospun samples is depicted in Figure 1(a) to (e).

The lower concentration of PVA (6% (w/v)) led to the formation of non-uniform fibers with a few beads along it (Figure 1(a)). When the PVA concentration was increased from 6% to 8% (w/v), no more beads were seen in the morphology (Figure 1(b)). This was due to the stability of the polymer solution jet during the electrospinning process between the syringe needle tip and the collector.
Similar results could be observed for the CA mat samples with higher sensitivity by altering the polymer solution concentration (Figure 1(c) and (d)). The SEM micrograph of fabricated CA/PVA fibrous hybrid mat is been depicted in Figure 1(e). It shows the morphology with proper uniformity and smoothness. In this respect, the average fiber diameter of the hybrid sample was measured about 160 ± 20 nm.

Water contact angle measurements of the samples

The hydrophilicity of fibers is one of key roles that make them suitable for cell growth and proliferation. Therefore, the water contact angle of CA/PVA hybrid mat sample containing TC and PHT-Na was determined. The photograph of the water droplet when contacting with the surface of the sample after 3 s is shown in Figure 2. The observed contact angle was about 45°, which implied a proper hydrophilic property of the sample. It is worth noting that the CA/PVA hybrid mat with no drugs showed a superior hydrophilic property, which meant that the water droplet immediately absorbed and disappeared upon coming into contact with the surface. It was concluded that the all fabricated samples in the presence and absence of the drugs have a great hydrophilic property. It is an acceptable value for a drug delivery system in similar works. For example, Zhang et al.72
investigated the water contact angle of poly(ε-lactic acid) (PLLA)/zein fibers and reported the starting contact angle about 130° decreasing with contact time. In another report, the water contact angles of neat chitosan (CS) and its blends with hyaluronic acid (HA) were measured and the maximum and minimum amounts were about 88° and 73°, respectively. It seemed that the higher hydrophilic property of the sample, herein, was due to using PVA.

In vitro drug release along with kinetic model study for the mat samples

In Figure 3, the TC and PHT-Na release profiles from TC-loaded CA, TC-loaded CA/PVA, TC-loaded CA/cross-linked PVA, PHT-Na–loaded CA/cross-linked PVA, and PHT-Na–loaded cross-linked PVA mat samples are shown over 48 h in PBS at pH 7.2. The difference between trends was due to the cumulative release of each drug from the different samples. The PHT-Na release from the cross-linked PVA mat sample occurred over 10 h with an initial burst release, whereupon a plateau level was observed. In contrast, the release trend of this drug from the electrospun CA/PVA hybrid mat sample was slower and gradual while reaching the ultimate release amount of 73%. The presence of CA fibers besides PVA fibers alone (physical barrier effect) could postpone the rapid release of PHT-Na compared to the drug release from cross-linked PVA mat sample. Similarly, the release of TC could be also controlled in the CA/PVA hybrid mat sample in which the TC release percentages declined about 16% after 48 h while cross-linked PVA was added. Furthermore, the bimodal behavior of TC release was seen from the CA/PVA mat sample before and after 10 h. It can be concluded that the drug release from the samples was governed by a diffusion mechanism that resulted in that a sustained control release of the drugs during the release time.

The chemical structures of TC and PHT-Na are shown in Figure 4(a) and (b). First, the presence of many hydroxyl groups on TC molecule might cause more interactions with the matrix than the PHT-Na, as the polymer matrices, either PVA or CA, have many hydroxyl groups too. Then, TC molecular size was greater than PHT-Na, hence justifying the higher released amount of PHT-Na in PBS.
By applying the drug release data in the Korsmeyer–Peppas equation, the constant exponent “n” was calculated with the value of 0.5, which concluded that the predominant release mechanism was Fickian diffusion, particularly for the electrospun CA/PVA hybrid mat sample (Table 1).

Furthermore, the release data correspondence with the four kinetic models with the reported regression coefficients is listed in Table 2, whereby the observation followed good fitting to Higuchi model to confirm the dominant mechanism of the drugs release was diffusion, as well.
Cytotoxicity, MTT results, and the cell adhesion study of the samples

The results of cytotoxicity analysis of electrospun CA/PVA hybrid fibrous mat with and without the drugs and RPMI medium as control are depicted in Figure 5(a). The microscopic pictures of these three plates assayed by crystal violet staining are depicted in Figure 5(a). It showed that the level of cell colonies’ formation in the well containing the drugs was higher than the well without the drugs. It was concluded that the presence of drugs not only reduced the cell colony formation ability of fabricated sample but also increased it due to the presence of cell growing drug or PHT-Na. The MTT assay results or the number of living cells present in the supernatants extracted from CA/PVA hybrid mat samples with and without the drugs, as well as the control environment (the cells’ feed, RPMI), are demonstrated in Figure 5(b). The number of living cells in the neat CA/PVA fibers extract was acceptable after three days; however, this value was greater for the sample containing the drugs and the control sample as well. Therefore, the electrospun CA/PVA hybrid mat sample containing TC and PHT-Na drugs has enough capability of suitable environment for the cells viability without surface cytotoxicity. In other words, the presence of drugs in the supernatant of the mat sample could help to keep the viability of the cells in which their viability has increased to 13% with respect to the neat CA/PVA mat sample.

The adhesion of the fibroblast L929 cells onto the surfaces of CA/PVA hybrid fibrous mat samples and its comparison with the control sample can be observed in Figure 6(a) and (c). The growth of cells on the surface of fibrous hybrid mat with drugs was remarkably higher than that of sample without drugs after 24 h. This could be attributed to the presence of drugs especially PHT-Na resulting in rapid cell growth and adhesion. Moreover, the accumulation of the cells was greater for the sample containing the drugs, specifically PHT-Na. On the other hand, the amount of cell adhesion onto the surface of neat CA/PVA hybrid mat sample was at the lowest level due to the absence of drugs in the formulation. Nevertheless, this sample also could help the adhesion of cells.
Antibacterial evaluation of the samples

The antibacterial activity results of the fibrous CA/PVA hybrid mat with or without drugs are reported in Table 3. No significant bacterial growth inhibition was observed for samples without the drugs, at three different time intervals of 10 min, 30 min, and 1 h. In contrast, its values were outstanding for the sample containing the drugs at all three different time intervals.

The percentage of bacterial growth reduction for sample with drugs increased from 75.68% to 88.73% and from 84.85% to 97.63% for *E. coli* and *S. aureus*, respectively, from 10 min to 1 h time periods.

Conclusion

In this study, a new dual-fiber drug delivery system has been presented and characterized. It was based on a fibrous CA/PVA hybrid mat containing TC and PHT-Na drugs, respectively. PVA as a fast soluble matrix (in aqueous media such as wound exudate) provides a burst release ability for its drug content. On the other hand, CA dissolves gradually and it provides a gradual
release ability for its content drug. Hence, one of the drugs releases quickly and the other drug releases during time, gradually. Furthermore, CA would be surrounded physically by dissolved PVA and its drug releasing would be even more delayed.

The drug release of presented fibrous CA/PVA hybrid mat was best fitted with Higuchi mathematical model. Its water contact angle values showed a high hydrophilic property and its surface exhibited non-cytotoxicity regarding the fibroblast L929 cells, which results in a suitable environment for cell growth and adhesion. In addition, dual-drug-loaded CA/PVA hybrid fibrous mat could appropriately inhibit the bacterial growth. Finally, the presented CA/PVA hybrid fibrous mat can be considered as an advanced wound dressing with controlled delivery system.

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