Drug release, cell adhesion and wound healing evaluations of electrospun carboxymethyl chitosan/polyethylene oxide nanofibres containing phenytoin sodium and vitamin C

Mohammad Amin Zarandi1, Payam Zahedi1✉, Iraj Rezaeian1, Alireza Salehpour1, Meihdi Gholami2, Behrooz Motealleh1

1School of Chemical Engineering, College of Engineering, University of Tehran, P. O. Box: 11155-4692, Tehran, Iran
2Faculty of Pharmacy, and Pharmaceutical Sciences Research Center and Endocrinology and Metabolism Research Center, Tehran University of Medical Sciences (TUMS), Tehran, Iran
✉ E-mail: phdzahedi@ut.ac.ir

Abstract: In this work, N,O-carboxymethyl chitosan (CMCS) samples from virgin chitosan (CS) were synthesised and CMCS/polyethylene oxide (PEO) (50/50) blend nanofibrous samples were successfully electrospun from their aqueous solution. The electrospinning conditions to achieve smooth and fine diameter nanofibrous mats were optimised via D-optimal design approach. Afterwards, vitamin C and phenytoin sodium (PHT-Na) were added to these samples for producing wound dressing materials. H-nuclear magnetic resonance, scanning electron microscopy and Fourier transform infrared tests for the evaluation of functionalised CS, morphology and biodegradability studies of CMCS/PEO blend nanofibrous samples were applied. The kinetic and drug release mechanism for vitamin C and PHT-Na drug-loaded electrospun samples were also investigated by UV-vis spectrophotometer and high performance liquid chromatography, respectively. The results showed an approximately similar drug release rate of the two drugs and followed Higuchi’s kinetic model. The stem cells viability and their adhesion on the surface of the samples containing PHT-Na and vitamin C were carried out using MTT assay and the best cells’ biocompatibility was obtained using both drugs into the CMCS/PEO nanofibrous samples. Moreover, the in vivo animal wound model results revealed that the electrospun samples containing vitamin C and PHT-Na (1%) had a remarkable efficiency in the wounds’ closure and their healing process compared with vitamin C/PHT-Na (50/50) ointment. Finally, the histology observations showed that the wound treated with optimised electrospun samples containing two drugs enabled regeneration of epidermis layers due to collagen fibres accumulation followed by granulating tissues formation without necrosis.

1 Introduction

Skin is relatively soft, the greatest organ in the integumentary system of the body and normally covers the interior parts and protects them against the outside environment. It accounts for 8% of the total weight of the human body. Every year, thousands of people need to repair and/or graft their skin because of some injuries due to burns or cutaneous wounds, heat, electricity and chemicals [1].

Vitamin C (ascorbic acid) has a key role as a part of enzymes that synthesise collagen and cartilage. Vitamin C is a dominant water-soluble antioxidant within the body and can control cholesterol and reduce blood pressure. This vitamin is extensively used for prevention of colds and bacterial and viral illnesses. Due to collagen synthesis stimulation by using vitamin C, it can have a vital role in the wound healing and its reparation [2–4]. Collagen is an extracellular protein in the human body and is produced by fibroblast cells. This protein consists of three main amino acids including hydroxylysine, hydroxyproline and hydroxylysine, formed by a vitamin C-dependent process that entails the enzymatic transfer of hydroxyl groups to selected proline and lysine residues in the nascent procollagen chains. The fundamental reaction for collagen synthesis is the hydroxylation of proline to convert 4-hydroxyproline and this reaction can be stabilised by using hydroxylase enzymes in role of a catalyst. During this reaction, vitamin C is capable of promoting the collagen growth as a co-catalyst. In addition to this main role for vitamin C, it can also stimulate the collagen fibres’ growth in another way that returns to activate the lipid and aldehyde groups’ peroxidation and subsequently induces the mRNA surface of collagen. Gene expression of collagen is under effect of the lipid peroxidation and/or acetaldehyde formation that can be increased via gene expression of human collagen’s fibroblast cultivation [5].

A wound healing process is a complex procedure containing cells and biochemical phenomena in terms of inflammation, immigration and proliferation of the epithelial growth cells, formation of extracellular matrices (ECMs) of proteins, new and fresh vascular cells production etc. The wound repair is carried out by a series of different phases and they might have an overlap to each other at the same time. Promoting these phases can be controlled via growth factors, cytokines and some hormones [6, 7].

One of the appropriate techniques to improve and repair severely injured tissues is the development of some engineered tissues that can be substituted instead of the dead skin cells. In recent years, electrospun nanofibres have shown a great promise for developing tissue engineered scaffolds. These nanofibrous scaffolds have some unique properties such as high porosity, oxygen transmission capability, different pore size distribution and a high surface to volume ratio. Besides these interesting properties, a significant characteristic of the electrospun nanofibrous scaffolds is their morphological similarity with the biological tissue of the body that leads them to have good compatibility with ECM cells. These special properties of biopolymer nanofibres cause them to have a great deal of mesenchymal stem cells proliferation, adhesion and viability [8–10].

Some techniques, including phase separation, self-assembly and electrospinning, have been developed to produce nanofibres with their exclusive properties. Among these methods, the electrospinning technique as a common and cost-effective method
is extensively focused for preparing the electrospun nanofibrous mats from a polymer solution or melt (Fig. 1) [11, 12].

A broad range of biopolymers such as chitosan (CS) [13, 14], gelatin [15], poly (lactide-co-glicolide) [16], dextran [17], collagen [14] and some synthetic polymers containing polyethylene oxide (PEO) [13], poly (ε-caprolactone) (PCL) [1] and polyurethane [18] have been electrospun. CMCS has been highly attractive for tissue engineering applications. Their measured cellular activity results on the samples suggested that the used acetic acid as a solvent did not cause cytotoxicity and blend; in particular 50% PCL and 75% PCL were superior to individual polymers. Finally, 50/50 CS/PCL blend mats showed improved mechanical properties as well as cellular support. These are interesting properties for CS, but the solution of this polymer in acetic acid or formic acid is not suitable for electrospinning process. So far, limited research works for preparing electrospun CS derivatives nanofibres has been carried out. Zhou et al. [29] studied electrospun water-soluble CMCS/poly (vinyl alcohol) (PVA) nanofibrous mats as potential wound dressings for skin regeneration. They showed that the CMCS/PVA nanofibres were successfully prepared by electrospinning of aqueous CMCS/PVA in different weight ratios. Their results also revealed electrospun CMCS/PVA nanofibrous samples with mouse fibroblasts (L929) have a good capability through in vitro experiments. In spite of their in vitro studies, they did not investigate in vivo animal model and drug release characteristics for these samples as a wound dressing material.

The aim of this work was to synthesise and characterise water-soluble N, O-CMCS and then optimise the electrospun CMCS/PVA nanofibrous samples via D-optimal design method. In the next stage, these optimised samples containing vitamin C and PHT-Na were investigated through a series of in vitro and in vivo experiments in terms of the drug release, cell adhesion and animal wound model along with histology observation. Some useful information and interesting results with their reasons were critically discussed.

2 Materials and methods

2.1 Materials

CS medium molecular weight (degree of deacetylation 75–85%, viscosity 200–800 cP for the solution concentration 1 (wt. %) in acetic acid (1%) at 25°C, Brookfield) and PEO (average viscosity molecular weight 10^5 kDa, viscosity 400–800 cP for the solution concentration 2 (wt. %) in H2O at 25°C, Brookfield) were purchased from Aldrich Chemical Co., United States. PHT-Na (white, fine powder, particle size max 180 μm, purity 99.99%) was obtained from Katwick Chemie (The Netherlands). Ascobic acid (powder, white colour, pH 2.3 for 5% solution in water) was provided by DSM Nutritional Products Ltd., UK. Water and methanol (high performance liquid chromatography [HPLC] grade) were purchased from Dae-Jung Chemicals & Materials Co. Ltd., South Korea. Stem cells were dedicated from the Department of Pharmacy, Tehran University of Medical Sciences, Iran. All the other chemicals were analytical reagent grade and were used without further purification.
Water-soluble N, O-CMCS samples were synthesised according to Hayes’s method [30]. Briefly, 10 g of CS was added to 100 ml of isopropyl alcohol and then the mixture was stirred for about 20 min at 25°C. Then, 50 ml of NaOH solution 40 (％w/v) was added to the mixture and was stirred again for about 2 h until the alkylation reaction was completed. While stirring the mixture at 60°C, 20 g of monochloroacetic acid was gradually added to the solution and the finalised mixture was stirred for 3 h to complete the carboxymethylation reaction (Fig. 2b). This mixture was then dispersed in water and centrifuged. Subsequently, the polymer precipitate was washed by using ethanol 70% and drip-dried using Whatman filter paper. In the next step, the samples were washed again for two times by the use of anhydrous ethanol and were dried within a vacuum oven at 60°C overnight. The reaction efficiency for the above procedure to synthesise CMCS was about 34.8% and degree of substitution for carboxyl group was about 82% [31].

3 Experimental

3.1 Electrospinning of CMCS/PEO containing vitamin C and PHT-Na

To provide CMCS (0.5–1 (％w/v)) and PEO (0.5–1.5 (％w/v)) solutions, weighed polymer powders were dissolved in distilled water and stirred separately at 25°C for 3 h. Then, a mixture of 50/50 from each polymer solution was prepared and stirred for 1 h. Owing to provide the samples with vitamin C (1%) and PHT-Na (1%), first, 50 mg of PHT-Na powder was added to 5 ml of 50/50 CMCS/PEO solution and the mixture was stirred at 25°C for 1 h. Similarly, 50 mg of vitamin C powder was added to another 5 ml of CMCS/PEO (50/50) solution and the mixture was vigorously stirred at 25°C for 30 min. When a uniform solution of both systems was obtained, a syringe containing 50/50 CMCS/PEO/PHT-Na and another syringe containing 50/50 CMCS/PEO/vitamin C were prepared for the fabrication of a hybrid nanofibrous sample. Since two separate systems containing the drugs were used for the electrospinning process, there was no interaction between the chemical structures of the drugs after evaporating the solvents and collecting the nanofibres as the shape of a solid on the collector.

The operation conditions of the electrospinning device (eSpinner NF-CO ENII, Asian Nanostructures Technology Co., Tehran, Iran) for the preparation of the samples were as follows: PEO concentration 1.5 (％w/v), CMCS concentration 0.5(％w/v), solution flow rate 0.5 ml/h, applied voltage 20 kV and the distance between the syringe needle tip and collector 18 cm. To obtain fully dried electrospun nanofibrous samples, they were taken from the aluminium foil and dried at room temperature for 24 h.

The electrospun CMCS/PEO (50/50) with and without the drugs were then cross-linked by using 20 ml of saturated vapour of glutaraldehyde solution (25％w/v) at ambient temperature for 1 h. To ensure the evaporation of the residual glutaraldehyde from the spun fibres, the samples were dried in a vacuum chamber at 40°C for 12 h.

3.2 D-optimal design

Optimisation of electrospinning conditions for the preparation of CMCS/PEO to achieve the morphology with smooth and ultrafine fibres was carried out by D-optimal design methodology which is one of the beneficial tool boxes in Design Expert V.6 software (Stat-Easy Inc., Minneapolis, MN). According to Table 1, six independent different parameters (x1 to x6) and y for the nanofibrous average diameter as an answer were considered. The morphology analysis of the nanofibrous samples and measurement of their average diameter were studied by using scanning electron microscopy (SEM) and ImageJ software, respectively. Finally, a generalised polynomial model was fitted on the obtained experimental results and a unique equation regarding the relation between the independent parameters and nanofibrous average diameter of CMCS/PEO was attained.

3.3 (H-nuclear magnetic resonance) H-NMR studies

To characterise carboxymethyl groups provided by the use of Hayes’s method and their substitution instead of hydrogen elements in –OH and –NH2 on the surface of CS chemical structure, H-NMR spectroscopy (Ultra Shield 500 MHz, Bruker Co., United States) was applied. An aqueous solution of CMCS with a concentration of 2 M was prepared by using deuterium oxide (Aldrich Chemical Co., Milwaukee, WI, US) and injected into the device chamber.

3.4 FE-SEM studies

The morphology and fibre diameters of the electrospun samples with and without the drugs were carried out by using field-emission SEM (FE-SEM) (SU8040, Hitachi, Japan) and ImageJ software, respectively. The mean values of the nanofibres’ diameters from 100 different sections were measured and then the nanofibre’s diameters distribution was separately calculated and plotted. The magnification of the FE-SEM micrographs samples was 5000 × with scale bars of 1 μm.

3.5 Samples’ biodegradability

The biodegradability of electrospun CMCS/PEO nanofibrous samples by immersing them in phosphate buffered solution (PBS, pH 7.2) at 37°C during an overnight was investigated. For analysing the samples before and after the immersion in PBS, Fourier transform infrared (FTIR) spectroscopy (model EQUINOX 55, Bruker Co., United States), with an attenuated total reflection (ATR) technique at room temperature was utilised. The wave number range used in FTIR spectroscopy studies was 500–4000 cm⁻¹.

3.6 HPLC and UV spectroscopy

PHT-Na release rate from the electrospun samples to PBS environment was analysed by an HPLC system containing a UV detector (λ = 254 nm) (SCL, 10 AVP, Shimadzu, Japan). The detector was coupled with a C18 analytical column. The mobile phase consisted of MeOH/H2O/acetonitrile/triethylamine (1%)/CH3COOH (270:500:230:5:1) and was sonicated for about 15 min. The mobile phase was delivered at a flow rate of 1 ml/min and the eluent was evaluated at a wavelength of 254 nm.

For detecting the amount of released vitamin C, a UV-vis spectrophotometer (model mini-1240, Shimadzu, Japan) was used. According to the literature [2], released vitamin C solution needs to be derivatised until is detected by UV. Briefly, the following steps were carried out on the released vitamin C to PBS environment: (i) 10 ml of sulfuric acid 9 N, 0.04 g of thiourea, 0.005 g of CuSO4.5H2O and 0.3 g of 2,4-dinitrophenyl hydrazide were mixed with each other (as an indicator), (ii) a 5/1 ratio of

| Table 1 Range of independent variables for experimental design |
|------------------|------------------|------------------|
| Factor | Name | Low actual | High actual |
| x1 | PEO concentration, ％w/v | 0.5 | 1.5 |
| x2 | CMCS concentration, ％w/v | 0.5 | 1 |
| x3 | applied voltage, kV | 12 | 20 |
| x4 | distance between the syringe needle and collector, cm | 12 | 18 |
| x5 | flow rate, mL/h | 0.3 | 0.7 |
| x6 | crosslinking time, h | 1 | 11 |
released vitamin C/indicator was prepared and was then put into a water bath at 37°C for about 3 h. (iii) 1.5 times of the above prepared solution, H2SO4 (65%) was added to the solution obtained from step (ii) and was then vigorously stirred and, (iv) the finalised mixture was kept at ambient temperature for 30 min and its UV absorbance at 498 nm was recorded.

The release kinetics of PHT-Na and vitamin C from all the samples can be described by using Korsmeyer–Peppas, (1)

\[
\frac{M_t}{M_\infty} = K t^n
\]  

where \(M_t\) is the accumulative amount of drug released at time \(t\), \(M_\infty\) is the initial drug loading, \(K\) is a constant characteristic of the drug-polymer system and \(n\) is the diffusion exponent suggesting the nature of release mechanism. In addition to Fickian theory, three more models were used to further analyse the profile of drug release including zero order, first order and Higuchi models (Table 2) [32].

<table>
<thead>
<tr>
<th>Model name</th>
<th>Mathematical equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>zero order</td>
<td>(M_t = K t)</td>
</tr>
<tr>
<td>first order</td>
<td>(\ln M_t = K t)</td>
</tr>
<tr>
<td>Higuchi</td>
<td>(M_t = K t / t)</td>
</tr>
</tbody>
</table>

### 3.7 In vitro cell culture and adhesion

For the investigation of mesenchymal stem cells’ morphology and their adhesion, the electrospun CMCS/PEO nanofibres were cut into a disc of 1.5 cm in diameter and sterilised under UV light for 2–3 h. The growth cells in DMEM medium were then cultivated. After fixing, the samples were dehydrated ethanol/distilled water mixtures from 50 to 100% in steps of 10% at 10 min intervals. The resulting samples were then observed by using FE-SEM (model SU8040, Hitachi, Japan) at 1000 × magnification.

Cytotoxicity of the electrospun nanofibres according to ISO 10993-5 and the viability of cultured cells on the nanofibres were characterised after 6 days using 3-[4(dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT). The rate of reduction in the level of MTT to formazan depends on the cell metabolism. As can be seen from Table 2, a generalised polynomial equation was calculated using an ELISA reader (Dana, 3200, Iran) at 570 nm.

### 3.8 In vivo wound healing

Male wistar rats (250 ± 25 g) were used in this study (\(n = 6\)). After anaesthetisation by ketamine and xylazine, the hairs on their back were removed using depilatory cream. A full-thickness square wound (2 × 2 cm) was cut from the back of each rat. The depth of the wound was the same for all the rats; moreover, the tissues were removed from the back surface to reach the muscle tissue. The wounds were covered with a neat CMCS/PEO nanofibrous mat, the nanofibrous sample containing PHT-Na and vitamin C (50/50). It was noted that the concentrations of the drugs loaded in the nanofibres and ointment samples were the same and their average diameters (\(y\)) was 1%. The area of the wounds was measured every day up to 14 days. The percentage of wound healing is defined by (2)

\[
\text{Wound area} (\%) = \frac{A_i}{A_j} \times 100
\]

where \(A_i\) is the initial wound area and \(A\) is the wound area after a fixed time interval.

After day 7 and day 14, the rats were euthanised and their wound tissues were dissected fixed with 10% formalin and stained with hematoxylin and eosin (H&E) reagents for the histologic observations of epithelialisation and collagen fibres accumulation [33].

### 4 Results

#### 4.1 H-NMR studies

H-NMR spectroscopy is an efficient technique for characterising a synthetic polymer. In this work, Fig. 3 shows the H-NMR spectrum of prepared N, O-CMCS in D2O. As can be seen from Fig. 3, the main chemical shifts for proton (H) were considered as follows: signals at 1.97 and 2.46 ppm referred to the presence of CH2COOR) and O-substitution (−O−CH2COOR) at C2 and C6, respectively, in both CS and CMCS at C2 (carbon number 2), multiple signals between 3.3 to 3.8 were related to (−CH2−CH) at C3 to C6 of the acetil group (−COCH3) and hydrocarbon (−CH), respectively.

#### 4.2 Optimisation of CMCS/PEO nanofibrous mats

After optimising the six independent parameters which were mentioned in Table 1, a generalised polynomial equation was calculated by D-optimal design method. (3) shows the relationship in respect to PEO concentration (\(x_1\)), CMCS concentration (\(x_2\)), applied voltage (\(x_3\)), distance between needle tip and collector (\(x_4\)), flow rate (\(x_5\)) and crosslinking time (\(x_6\)) and the nanofibres' average diameters (\(y\)) as the answer

\[
y = 1542.5 + 78.6x_1 + 314.1x_2 - 10.2x_3 - 119.4x_4 \\
- 1730x_5 - 28.2x_6 - 185.1x_1x_2 - 18.5x_1x_4 \\
+ 484.1x_1x_5 + 338x_2x_5 - 40.7x_2x_6 + 2x_3x_6 \\
- 128x_5x_6 + 6.2x_1^2 + 3178.6x_2^2 + 2.2x_6^2
\]  

Fig. 3  H-NMR spectrum of prepared CMCS

| Table 2 Mathematical models used to describe drug dissolution curves |
|----------------|--------------------------|
| Model name    | Mathematical equation    |
| zero order    | \(M_t = K t\)            |
| first order   | \(\ln M_t = K t\)        |
| Higuchi       | \(M_t = K t / t\)        |
The regression coefficient ($r^2$) of (3) was estimated to be 0.91. Generally every parameter eliminated in this equation was due to their slight effects on the answer, with a $P$-value $> 0.05$.

4.3 Morphology studies of the optimised electrospun CMCS/PEO (50/50) blend nanofibrous mats

Morphology of two polymers' electrospun nanofibres and a variation of their diameters depend on the type of the polymers solution combination. Fig. 4 shows FE-SEM micrographs of neat optimised electrospun CMCS/PEO (50/50) nanofibrous sample, the sample containing PHT-Na, the sample containing vitamin C and the one containing both PHT-Na and vitamin C. All the samples' morphologies except those including vitamin C have uniform and smooth nanofibres. The resulting information from Fig. 5 shows that maximum peaks of diameter curves distribution for the optimised CMCS/PEO (50/50) composite nanofibres with and without PHT-Na are about 125 and 165 nm, respectively.

4.4 Biodegradability of the optimised electrospun samples

Hydrolytic degradation occurs when water molecules penetrate into electrospun CMCS/PEO nanofibrous sample and react with the hydroxyl group (–OH) of the hydrophilic polymer chains. Fig. 6 shows the FTIR spectrum of the samples before and after immersing in PBS environment at 37°C for 24 h. A broad peak was observed between 3100–3500 cm$^{-1}$ which corresponded to stretching vibration of –OH bond, extension vibration of –N–H bond and the intermolecular H-bonds of the polysaccharide moieties [34]. During hydrolysis, a hydrogen bonding was formed between the water molecules and –OH and –NH groups of the nanofibrous samples. Therefore, the intensity of the FTIR peak at wave numbers between 3100 and 3500 cm$^{-1}$ was increased with increasing the immersing time of the nanofibre sample in PBS environment (Fig. 6b).

4.5 Drugs release rate and kinetic investigation

Figs. 7a–d show chromatogram peaks of PHT-Na at 0.5, 6, 24 and 48 h for electrospun CMCS/PEO (50/50) blend nanofibrous samples. As can be observed from these figures, the retention time of PHT-Na is about 9.1 min. Figs. 8a and b shows the calibration curves of vitamin C and PHT-Na obtained by UV-vis spectrometer and HPLC, respectively. The accumulative release rate profiles of PHT-Na and vitamin C from the optimised electrospun samples to PBS environment were plotted in Fig. 8c. The release rates for these samples containing 1% PHT-Na and 1% vitamin C after 48 h were 73.25 ± 4.5% and 68.63 ± 3.8%, respectively.

For further studies on PHT-Na and vitamin C release rate mechanisms, the release data were analysed by Korsmeyer–Peppas (11). Based on this equation, the regression coefficients were calculated and the results are represented in Table 3. Moreover, Table 4 shows the results of regression coefficients for different drug release rates from different kinetic models. This table shows that in CMCS/PEO nanofibres, the levels of PHT-Na and vitamin...
C release rate during the released time are probably related to the diffusion of the drugs located near the nanofibres surfaces.

4.6 In vitro cell culture, adhesion and proliferation on the electrospun samples

The morphology of seeded mesenchymal stem cells after 6 days' incubation time was observed by FE-SEM and the results are shown in Figs. 9a–c. In Fig. 9a, the cells are seeded on PHT-Na loaded CMCS/PEO nanofibres. Fig. 9b shows the stem cells seeded on CMCS/PEO nanofibres containing vitamin C and also Fig. 9c exhibits the cells seeded on the electrospun nanofibrous samples containing 1% PHT-Na and vitamin C.

Fig. 10 shows the UV absorbance at 570 nm, illustrating the viability of stem cells. After 3 and 6 days, cell seeding on electrospun CMCS/PEO (50/50) nanofibres samples with and

---

**Fig. 5** Normalised distribution curves of electrospun CMCS/PEO nanofibres diameters

a) Neat samples

b) Samples containing PHT-Na (1%)

---

**Fig. 6** FTIR spectrum of CMCS/PEO nanofibrous samples without drug

a) Before immersing PBS

b) After immersing PBS

---

*IET Nanobiotechnol.*, 2015, Vol. 9, Iss. 4, pp. 191–200

© The Institution of Engineering and Technology 2015
without the drugs can be seen. As a result, after 3 days, the level of cell viability on the samples’ surface, containing vitamin C was higher compared with the other samples. This trend was changed after 6 days as the samples containing both PHT-Na and vitamin C show remarkable cell growth and their adhesion on the surfaces of the samples containing PHT-Na and vitamin C compared with the samples containing PHT-Na and also those samples containing vitamin C.

4.7 In vivo wound healing and histology

Figs. 11a and b show the photographic pictures from the wound surfaces made on the back of the rats under investigation and the wounds’ area closure after 1, 6, 10 and 14 days post-treatment time. As can be seen from Fig. 11a, the rate of wound closure for day 6 in the wounds treated with CMCS/PEO nanofibres containing both PHT-Na and vitamin C, neat CMCS/PEO nanofibres and the combined drugs ointment changed from 100% on the first day to about 60, 65 and 70%, respectively. This trend of behaviour when examined on days 10 and 14 showed satisfactory results in Fig. 11b. On day 14, the rates of wound closure and healing for the above samples were reduced to 3.8, 8.5 and 12.5%, respectively.

**Table 3** Regression coefficient of released PHT-Na and vitamin C from 50/50 CMCS/PEO nanofibres calculated by Korsmeyer–Peppas equation (\(M_t/M_\infty = Kt^n\)) (\(n = 0.5\))

<table>
<thead>
<tr>
<th>Sample</th>
<th>(R^2 \pm \text{SD})</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMCS/PEO nanofibrous samples + PHT-Na (1%)</td>
<td>0.9 ± 0.04</td>
</tr>
<tr>
<td>CMCS/PEO nanofibrous samples + vitamin C (1%)</td>
<td>0.88 ± 0.04</td>
</tr>
</tbody>
</table>

SD = Standard deviation
Table 4  Regression coefficient of different mathematical models fitted to the release of PHT-Na and vitamin C (1%) from electrospun 50/50 CMCS/PEO blend nanofibrous samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Zero order</th>
<th>First order</th>
<th>Higuchi</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMCS/PEO nanofibrous samples + PHT-Na</td>
<td>0.47 ± 0.02</td>
<td>0.33 ± 0.01</td>
<td>0.7 ± 0.03</td>
</tr>
<tr>
<td>CMCS/PEO nanofibrous samples + vitamin C</td>
<td>0.45 ± 0.02</td>
<td>0.35 ± 0.01</td>
<td>0.7 ± 0.03</td>
</tr>
</tbody>
</table>

Fig. 9  FE-SEM micrographs of mesenchymal stem cells after 6 days seeded on

a CMCS/PEO nanofibres containing 1% PHT-Na,
b CMCS/PEO nanofibres containing 1% vitamin C,
c CMCS/PEO nanofibres containing vitamin C and PHT-Na (the scale bars are 20 μm)

Fig. 10  Effect of PHT-Na and vitamin C (1%) on electrospun CMCS/PEO nanofibres viability after 3 and 6 days

Fig. 11  Wound surfaces made on the back of the rats under investigation and the wounds area closure

a Photographic images from skin wounds treated with electrospun CMCS/PEO containing PHT-Na and vitamin C, neat electrospun CMCS/PEO nanofibres and the drugs ointment at day 1, day 6, day 10 and day 14 post-treatment periods
b The closure percentage of the wounds treated with electrospun CMCS/PEO containing PHT-Na and vitamin C, neat electrospun CMCS/PEO nanofibres and the drugs ointment during 14 days post-treatment time
PHT-Na and vitamin C reduces the average diameters of the drugs were about 125 and 165 nm, respectively. Similar to many, the drug penetration is the determining factor in its release. On the other hand, the drug release from a polymer matrix can be from drug release out of polymer matrix or/and drug release because of the degradation of polymer matrix [36]. From release data of CMCS/PEO nanoﬁbrinous samples, when in vivo, the Korsmeyer–Peppas equation, the main penetration mechanism is of Fickian type which is applicable for the nanoﬁbrinous samples in this work.

An advanced and ideal wound dressing must not only help wound healing, it should also not produce toxic materials. In comparison with the accumulation of initial growth stem cells about of $1 \times 10^6$ cells/mL for the neat electrospun CMCS/PEO nanofibres samples, the samples containing vitamin C, the samples containing PHT-Na and those containing both vitamin C and PHT-Na have the following results after 6 days, respectively: $1.8 \times 10^5$, $4.1 \times 10^5$, $2.8 \times 10^5$ and $8.3 \times 10^5$.

The in vitro animal wound model results showed that the optimised electrospun CMCS/PEO nanoﬁbrinous samples containing PHT-Na and vitamin C had a remarkable efﬁciency for healing of the wounds. The histology results after 14 days post treatment period showed that when the wound treated with the ointment had a few fresh granulation tissues, this treatment was unable to cover throughout the wound surface (Fig. 12b). On the other hand, for the wound covered by the neat 50/50 CMCS/PEO nanoﬁbrons, an epithelial regeneration was observed that can ﬁll a little upon the granulation tissue, but the two edges of the wound were left open. Moreover, some inﬂammatory cells existed at the top and right-hand side of the picture (Fig. 12d). According to Fig. 12c, by using PHT-Na and vitamin C loaded electrospun CMCS/PEO nanoﬁbrons a complete epithelium layer followed by mitosis in the cells of basal layer are formed and resulted in two edges of the wound connected to each other. The thickness of the epithelium layer seemed to be appropriate and led to decreased granulation tissues. Consequently, there were no inﬂammatory cells and the wound healing process seemed to be approximately accomplished. The reason for high effectual CMCS/PEO nanoﬁbrinous samples containing both the drugs can be because of the following reasons: PHT-Na is an effective drug because it has especial properties, that is, as growth promoter for collagen ﬁbrils and the formation

For histology observations, Figs. 12a–f show the trends of the wound treated with PHT-Na/vitamin C ointment, the wound covered by neat 50/50 CMCS/PEO nanoﬁbrous samples and those treated with electrospun CMCS/PEO (50/50) nanoﬁbrinous containing PHT-Na and vitamin C.

5 Discussion

H-NMR spectrum studies revealed that the N and O– substitutions by the carboxymethylation reaction had a signiﬁcant efﬁciency according to Hayes’s method. The reason for this claim can be related to the peaks area for N and O– substitutions, which were calculated to be about 3.26 and 3.85, respectively, compared with the –COCH3 (acytel group) for primary CS before modiﬁcation. Therefore, it can be concluded that the prepared CMCS from CS was successfully carried out.

According to (3), the optimisation of electrospinning conditions for CMCS/PEO (50/50) blend nanoﬁbrous samples via D-optimal design method was calculated as follows: $x_1 = 1.5$ (w/v), $x_2 = 1$ (w/v), $x_3 = 20$ kV, $x_4 = 18$ cm, $x_5 = 0.5$ mL/h and $x_6 = 1$ h. When these optimised conditions were applied, the mean average diameters of CMCS/PEO nanoﬁbrons samples with and without the drugs were about 125 and 165 nm, respectively. Similar to many other drugs, the morphology studies reveal that the addition of PHT-Na and vitamin C reduces the average diameters of the nanoﬁbrons [35]. On the other hand, some beads appeared in the morphology of the samples when vitamin C was added to the CMCS/PEO solution. This was due to the low pH value of vitamin C, about 2.3, which resulted in a burst reduction in the polymer viscosity solution. The resulting average diameter of CMCS/PEO nanoﬁbrons containing vitamin C was about 95 nm, whereas this result for the electrospun samples containing PHT-Na was about 165 nm.

Generally, the drug release rate from a polymer matrix is explained by the Fickian diffusion mechanism. In this mechanism the drug penetration is the determining factor in its release. On the
of granulation tissues as well as wound healing accelerator [37]. Moreover, in the presence of PHT-Na, myofibroblast and fibroblast proliferation, the production of the ECM and its protein and the activity of growth factors and their mediators may be increased within the wound. Overall, PHT-Na appears to reduce collagenase activity, edema, wound exudates and bacterial load [27]. On the other hand, ascorbic acid plays a critical role in wound repair and the healing/regeneration process as it stimulates collagen synthesis. It has been suggested that there will be rapid utilisation of ascorbic acid for the synthesis of collagen at the site of wound/burns during the post-operative period [38].

6 References
