

# Antimicrobial wound dressing film utilizing cellulose nanocrystal as drug delivery system for curcumin

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Received: 16 August 2017 / Accepted: 7 November 2017 / Published online: 10 November 2017  
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**Abstract** Diabetic patients with foot ulcer showed 150-fold increased risk of amputation, which is primarily caused by microbial infection. Silver ions are commonly incorporated into wound dressing to enhance the antimicrobial property. However, concerns have been expressed about the development of bacterial resistance to heavy metals. In this study, we evaluate the *in vitro* and *in vivo* efficacy of cellulose nanocrystal film to be used as antimicrobial drug delivery system in a diabetic wound dressing. Cellulose nanocrystals were successfully isolated from medical grade cotton fibers. We observe needle-like cellulose nanocrystals with an average length of 159 nm under transmission electron microscope. The developed film with curcumin shows a uniform yellow color, with a thickness of 0.4 mm. The film obtained is

soft and flexible, based on the mechanical characterization study of the film. For the curcumin release test, the release reaches plateau condition at 36 h with a total release of 98.9% from the cellulose nanocrystal film. No burst release effect was detected during the test period. The film exhibited significant inhibitory activity on 3 Gram positive bacteria, 2 Gram negative bacteria and 1 yeast. On Hohenstein challenge test, all test microorganisms showed significant growth reduction, with the treatment of curcumin loaded film. 5 of 6 test microorganisms showed 99% of growth reduction relative to growth control. We also notice that the antimicrobial activity of the film sustained even after 15 washes. In the *in vivo* study using diabetic rat models, a significant reduction of wound size was observed from Day 7 with the topical application of curcumin loaded film. At the end of the study, the lesion was covered by epithelial tissue and the hair started to grow from the skin. A bacterial growth reduction of 99.99% was observed from the skin sample excised from the animal models. The histological examination of skin sample also showed that curcumin loaded film significantly improved the regeneration of hair follicles and sebaceous glands of the skin. Our results indicate that the curcumin load cellulose nanocrystal films can be used for diabetic wound healing applications.

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**Keywords** Cellulose nanocrystal · Curcumin ·  
Antimicrobial activity · Diabetic wound ulcer

## Introduction

Diabetes mellitus is defined as a metabolic disease characterized by hyperglycemia resulting from defect in insulin action or insulin secretion (Barbagallo and Dominguez 2007). Fernandes et al. (2016) stated that in 2010, the world diabetic prevalence was 6.4%, which affecting 285 million adults. The figure is predicted to rise to 7.7% in 2030 with a total of 439 million patients worldwide. Foot ulcer is very common among diabetic patients. Complications that caused by these ulcers include necrotizing fasciitis, soft tissue gangrene, septic arthritis, and osteomyelitis (Bader 2008). Diabetic patients with foot ulcer showed 150-fold increased risk of amputation, which is primarily caused by poor wound management (Pednekar et al. 2015). The high microbial load at the surface of the wound wrappings is the main cause of the delayed healing time. Thus, preventing the infection is very crucial to improve diabetic ulcer healing process. One of the effective way to prevent microbial growth on wound is topical application of antimicrobial substance-loaded film as wound dressing (Bowler et al. 2001).

Nanocellulose is a nano-scaled biopolymer which can be derived from plants or bacteria. Nanocellulose shows good biocompatibility, biodegradability and low toxicity compared to traditional materials (Maneerung et al. 2008; Lin and Dufresne 2014). Nanocellulose is an excellent material for wound dressing due to its good compaction property and excellent ability to absorb exudates during the dressing process (Moritz et al. 2014). Besides, it promotes wound recovery by accelerating the contraction via accumulation of external matrix. Nanocellulose has been applied in clinical practice for dressing of deep wound, burn wound and diabetic ulcer (Lin and Dufresne 2014).

Several antimicrobial compounds were incorporated into wound dressing material to prevent wound infection. Silver ions are commonly incorporated into cotton material to enhance the antimicrobial property of the wound dressing. However, concerns have been expressed about the development of bacterial resistance to heavy metals (Biter et al. 2014). Besides chronic ingestion or inhalation of heavy metal preparations can lead to deposition of heavy metal particles in the skin, eyes, kidneys and livers (Joaquim et al. 2015). Thus, an alternative is necessary to replace

silver as antimicrobial dressing. In this study, by using curcumin as model drug, we evaluate the in vitro and in vivo efficacy of cellulose nanocrystal film to be used as antimicrobial drug delivery system in a diabetic wound. Besides, we also use polyvinyl alcohol (PVA) as a polymeric binder for the film formation. To date, no study was conducted to assess the antimicrobial efficacy of curcumin loaded cellulose nanocrystal film, especially by using diabetic rat models.

Curcumin (PubChem CID 969516) is the principal yellow orange compound isolated from *Curcuma longa* (Turmeric). The compound consists two methoxylated phenols connected by two  $\alpha$ ,  $\beta$  unsaturated carbonyl groups that exist as a stable enol form (Anand et al. 2007). It is a lipophilic phytopolyphenol that is insoluble in water but stable for topical application. Curcumin exhibits a spectrum of pharmacological activities, particularly on its good inhibitory effect on several metabolic enzymes. To date, it has not been shown to cause any toxicity effect. We hypothesize that the cellulose nanocrystal film is an excellent wound dressing material as it allows controlled release of the curcumin particle from the film, which contribute to the long lasting antimicrobial effect of the film.

## Experimental

### Isolation of cellulose nanocrystal

The pulping process was conducted by soaking 5 g of medical grade cotton fiber (Premiera, Malaysia) in 1 M sodium hydroxide solution at a ratio of 1:20 (w/v) at 160 °C for 2 h. The pulp was washed thoroughly with distilled water to remove excessive sodium hydroxide. Then, the cotton fibers were filtered with Whatman No 1 filter paper and dried at 50 °C until a constant weight was obtained. To remove lignin from the cotton fiber, the fibers were acidified with 30% sulfuric acid (1:20, w/v) for 6 h with continuous agitation. Refrigerated water (4 °C) was then added to stop the reaction. The cotton fibers were then sonicated in a water bath (4 °C) for 20 min at sonication power 1000 W in an ultrasonic generator. After that, the mixture was centrifuged at 3500 g for 15 min at room temperature. The supernatant was decanted to remove the excess water. The pellet was collected as nanocellulose fibers.

### Microscopic analysis

The images of the nanocellulose suspension were checked optically using transmission electron microscopy (TEM; Philips, Model CM12, Eindhoven, Netherlands), operating at 120 kV. To prepare samples for TEM observation, a clean dropper was used to transfer a droplet of sample on a carbon-coated copper grid. The sample was allowed to air-dry about 3 min at room temperature. Then, a small drop of uranyl acetate stain is applied to the grid. After 1 min, the excess stain is removed and the grid is examined with TEM.

### Development of cellulose nanocrystal film with curcumin

5 g of nanocellulose fibers was first suspended in 100 mL of 5% polyvinyl alcohol (PVA) solution. Then, 50 µg of curcumin (Sigma, CAS number 458-37-3) was added into the mixture. The mixture was then homogenized at 8000 g for 10 min. The mixture was finally cast on a circular container with diameter of 20 mm and left to dry at room temperature.

### Mechanical characterization of cellulose nanocrystal film with curcumin

The tensile strength, Elastic modulus and Elongation at break of the film was measured using Universal Testing Machine (Llyod LR30K Plus). The tests were conducted according to standard test method of tensile properties analyses for thin plastic sheeting (ASTM D882-00). The films were kept at 25 °C and 55% relative humidity for conditioning purposes. The strips have an initial grip separation of 100 mm and tested at crosshead speed of 12.5 mm/min.

### Curcumin release test

The assay was performed according to Shaikh et al. (2009). Samples of cellulose nanocrystal films were inserted into the conical flasks containing artificial sweat solution (Sodium chloride 5 g/L, Urea 1 g/L, Lactic acid 1 g/L; adjusted to pH 5.5 with 1 M sodium hydroxide) at a ratio of 1:100 (w/v). All the samples were placed at 37 °C, on an orbital shaker at 80 rpm. At the predetermined time point (0, 2, 4, 6, 8, 10, 12, 18, 24, 36, 48 h), a flask was withdrawn and the

absorbance was taken at 460 nm with spectrophotometer (Shidmazu). The curcumin released from the film was determined based on a standard curve developed using curcumin standard (Sigma). The experiments were done in triplicate. A control was included with curcumin loaded PVA film, without addition of cellulose nanocrystal.

### Test microorganisms

The test bacteria used in this study include 4 Gram positive bacteria [*Bacillus cereus*, *Bacillus coagulans*, *Streptococcus* sp. and Methicilin-resistant *Staphylococcus aureus* (MRSA)], 4 Gram negative bacteria [*Escherichia coli*, *Proteus mirabilis*, *Yersinia* sp. and *Pseudomonas aeruginosa*] and 2 yeasts [*Candida albicans* and *Candida utilis*]. All the test microorganisms were isolated from diabetic patient in Hospital Seberang Jaya, Penang, with a cohort of chronic wounds. The test microorganisms were sub-cultured on nutrient agar prior to use for every 2 weeks in order to maintain its viability. The microbial inoculums were prepared as per protocols described by Yenn et al. (2014).

### Agar diffusion test

The test was performed by streaking 0.1 ml of the inoculums to the surface of Mueller–Hinton agar (Merck) using cotton swab (Yenn et al. 2014). The cellulose nanocrystal film with curcumin, excised to a square disc (20 mm) was then placed on the agar medium. Cellulose nanocrystal film, without addition of curcumin was served as negative control. The experiment was done in triplicate in separate occasions. All plates were incubated at 37 °C for 24 h. After the incubation period, the diameters of inhibition zone surrounding discs were measured.

### Hohenstein challenge test (AATCC-100)

A total of 6 bacteria were selected for this test based on their susceptibility to the film on agar diffusion test. The antimicrobial effectiveness of the curcumin loaded cellulose nanocrystal film was assessed according to the standard test method by American Association of Textile Chemists and Colourists (AATCC) standard (Vaideki et al. 2008). Firstly, 100 µl of bacterial inoculum was inoculated to 25 ml of sterile

nutrient broth. Then, 0.1 g of the film was added into the culture. All the flasks were incubated at 37 °C, with a rotational speed of 120 rpm for 24 h. After the incubation period, viable cell count was performed by inoculating the diluted sample on nutrient agar (Merck). The antimicrobial efficiency of the sample in term of percentage of growth was determined relative to negative control (cellulose nanocrystal film without loading with curcumin).

#### Wash durability test (AATCC-147)

For the wash durability test, the films were dipped into the 1% detergent solution (Breeze, Malaysia) for 5 and 15 washes, respectively. The films were finally dipped into distilled water to remove any remaining detergent. The antibacterial efficacy of the wash film was tested according to protocol mentioned in section “[Hohenstein challenge test](#)”.

#### In vivo diabetic wound healing study

The in vivo study was approved by Animal Ethics Committee, Universiti Sains Malaysia. Efforts were made to minimize the suffering and numbers of rats used. A total of 9 male Sprague Dawleys rats were tested. The rats were bred and supplied by Animal Research and Service Centre, Universiti Sains Malaysia. At the beginning of the study, the rats were 12 weeks old and their body weights were ranged from 220 to 250 g. They were kept in a 12 h light–dark cycle and were cared for in accordance with the regulations for the protection of laboratory animals. The animal cages were sanitized by 70% ethanol daily and they were allowed ad libitum access to fresh water. Streptozotocin (Abcam) was injected intraperitoneally at a dose of 50 mg/Kg of body weight per day, for 5 consecutive days, to induce diabetes in rats. The glucose level of the blood sample withdrawn from tail vein was measured with glucometer (Accu Chek). The rats with a blood glucose level higher than 350 mg/dL were selected for the experiments.

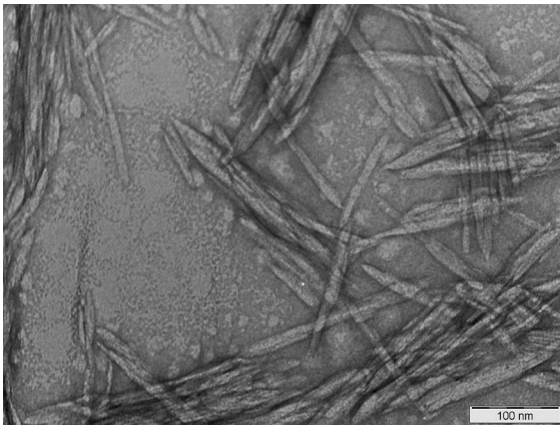
Four weeks after streptozotocin injection (Day 1), rats were anesthetized with ketamine (Abcam). Using a 6 mm punch biopsy instrument, a full thickness skin wound was created on the dorsal thorax of the animal. Rats were administrated with ketoprofen (Abcam) (4 mg/kg) subcutaneously immediately after the surgery. The treatment was started on Day 4, which

is 48 h after the establishment of the infection. Rats were divided into 3 groups, consisting of 3 animals each. The first group receive no treatment, the second group was treated with cellulose nanocrystal film (placebo control) and the third group was treated with curcumin-loaded cellulose nanocrystal film (test group). The treatments were done by dermal application with a film with size of 9 cm<sup>2</sup>. The change of wound dressing was done on Day 7 and 10. Diameter of the wound was measured using a digital caniper on Day 1, 4, 7, 10. At the end of the treatment (Day 12), animals were euthanized with carbon dioxide and the skin sample was excised at the infection site.

To estimate the viable bacterial cells in the skin sample, 1 g of the skin sample excised from the infection site was dissected and transferred into micro-centrifuge tube with 1 mL of sterile physiological saline. The samples were then centrifuged at 1500 g for 10 min. The suspension was serially diluted and aliquots from each of the diluents were plated on Mueller–Hinton agar and incubated at 37 °C for 24 h. The colony forming units of each the sample was calculated. Hematoxylin and eosin staining was performed and the slides were then observed under light microscope.

## Results and discussion

The hydrolysis of cellulosic fibers from medical grade cotton with sulfuric acid yields cellulose nanoparticles. Sulfuric acid hydrolysis cleaves the amorphous region of the cellulosic fibers longitudinally, which causing a reduction of the fibers to a size of nanometers (Moritz et al. 2014). The hydrolysis with sulfuric acid causes the disintegration of cellulose fibers with high shearing and impact forces. In this study, ultrasonic was utilized after the acid hydrolysis to promote the efficiency of the hydrolysis by preventing the aggregation of nanocellulose. The application of ultrasonic treatment after hydrolysis for nanocellulose formation was previously reported by Tang et al. (2014). The acid hydrolysis and sonication processes yield an aqueous colloid suspension of nanocellulose. Figure 1 shows the TEM micrograph of the nanocellulose suspension. The measurements of individual nanocellulose shows a dimension of  $159 \pm 31$  nm in length and 2 nm in width. We observe needle-like cellulose nanocrystals,



**Fig. 1** The TEM micrograph of the nanocellulose sample. Each rod appears as a rigid nanocellulosic crystal

where each rod appears as a rigid nanocellulosic crystal, with no apparent defect.

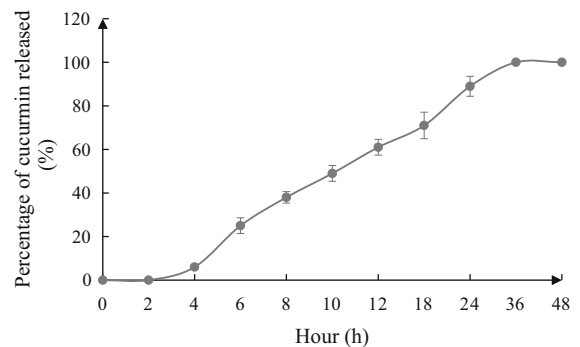
The visual observation of the micrograph shows significant morphological similarities with cellulose nanocrystal reported by Neto et al. (2013). Cellulose nanocrystals are hydrophilic crystalline-biopolymer with high surface area, renewability and nanoscale dimensions (Lu and Hsieh 2010). Cellulose nanocrystal are commonly incorporated with wide range of polymeric matrix as a nanocomposite material, and currently used as tablet binder in pharmaceutical industry.

The developed film shows a uniform yellow color, with a thickness of 0.4 mm. PVA was utilized as polymer matrix to improve the mechanical properties of the films, even at a low filler loading. Besides, the polymeric matrix also affects the drug release kinetic from the film. PVA is selected due to its good water solubility, film-forming ability and biodegradability (Pal et al. 2007). PVA has been used as polymeric matrix for nanocellulose film by Lee et al. (2009). Tensile testing is crucial to provide an indication of the strength and elasticity of the film developed. Based on our observation of the force required to break the film, a tensile strength of  $17.13 \pm 1.8$  MPa was obtained. The film obtained is soft and flexible. However, the film has low ductility, as the percentage elongation at break was only  $1.2 \pm 0.04\%$ . The property can be improved by addition of glycerol to the mixture (25). The film suitable for wound dressings should be strong but flexible and elastic. It is noteworthy that a modulus of elasticity of  $883 \pm 140$  MPa was observed. Elastic

modulus can be used to measure the stiffness and rigidity of the film (Lee et al. 2009). The elasticity of the developed film is important to hold the wound dressing in place for a period of time, in order to provide a therapeutic effect to a diabetic wound.

The release of curcumin from the cellulose nanocrystal film was evaluated at skin temperature for a duration of 48 h. The model used is an established method to investigate the drug release kinetic for an active wound dressing. No burst release effect was detected during the test period (Fig. 2). The curcumin release reaches plateau conditions at 36 h with a total release of  $98.9 \pm 1.4\%$  from the cellulose nanocrystal film. The developed film provides a constant drug release in a wound, without a rapid increase of drug concentration. It provides a therapeutically threshold amount by constantly release the drug to maintain the drug level in the wound. The condition favors the application as wound dressing. The control film, without cellulose nanocrystal showed instant burst release effect in artificial sweat solution. The cellulose nanocrystals in the film serve as a diffusion barrier, which retard the burst release effect of curcumin from the film (Lu and Hsieh 2010). The control release property is crucial to ensure long term antimicrobial effect of the film.

The curcumin loaded film showed broad spectrum antimicrobial activity. The film exhibited significant inhibitory activity on 3 Gram positive bacteria, 2 Gram negative bacteria and 1 yeast (Table 1). A negative control was included by using the cellulose nanocrystal film without curcumin finishing. The results showed that the control films did not exhibit any inhibitory effect on all test microorganisms. The antimicrobial activity of the developed film was



**Fig. 2** The release of the curcumin from cellulose nanocrystal film

**Table 1** The antimicrobial activity of the developed films on agar diffusion test

Test bacteria	Diameter of inhibition zone (mm)	
	Curcumin loaded film	Negative control
Gram positive bacteria		
MRSA	42.0 ± 2.7	–
<i>Streptococcus</i> sp.	49.0 ± 9.5	–
<i>B. cereus</i>	–	–
<i>B. coagulans</i>	67.0 ± 4.4	–
Gram negative bacteria		
<i>E. coli</i>	53.7 ± 3.5	–
<i>P. mirabilis</i>	62.3 ± 2.5	–
<i>Yersinia</i> sp.	–	–
<i>P. aeruginosa</i>	–	–
Yeasts		
<i>C. albicans</i>	25.7 ± 1.2	–
<i>C. utilis</i>	–	–

The curcumin-load film exhibited broad spectrum antibacterial activity

indicated by the clear zone surrounding the films. The largest size of inhibition zone was observed on *B. coagulans*, which commonly infects surgery wounds (Tehrani et al. 2016). Besides, it is noteworthy that the film showed significant antimicrobial properties against 1 yeast (*C. utilis*). In agreement with Soheil et al. (2014), the study showed that curcumin is a high potential photosensitizer compound for fungicidal photodynamic therapy especially against *Candida* species.

On Hohenstein challenge test, all test microorganisms showed significant growth reduction, with the treatment of curcumin loaded film (Table 2). 5 of 6 test microorganisms showed 99% of growth reduction relative to control. The results obtained are in agreement with previous studies. Prasad et al. (2014) stated that curcumin showed significant antimicrobial activity against Gram positive bacteria (MRSA) and Gram negative bacteria (*E. coli* and *P. aeruginosa*). Due to the control release property of the film, no regrowth of microorganisms was observed throughout the test period. We also notice that the antimicrobial activity of the film sustained even after 15 washes, on all test microorganisms. The results reveal a strong binding of curcumin to the cellulose nanocrystals, which withstand the effect of washing with detergent. Besides,

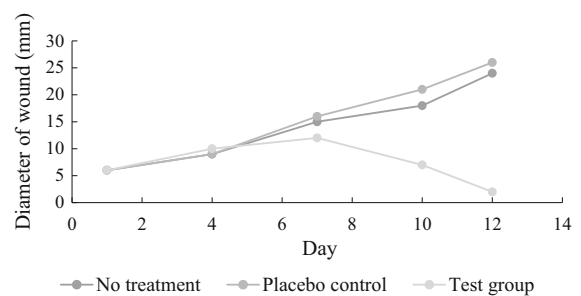
**Table 2** All test microorganisms showed significant growth reduction with the treatment of curcumin loaded film on Hohenstein challenge test

Test bacteria	Percentage reduction (%)		
	0 wash	5 washes	15 washes
MRSA	99	99	99
<i>B. coagulans</i>	99	99	99
<i>Streptococcus</i> sp.	88.4	86.5	85.3
<i>E. coli</i>	99	99	99
<i>P. mirabilis</i>	99	99	99
<i>C. albicans</i>	99	99	99

The antimicrobial activity sustained even after 15 washes

the excellent binding mechanism from PVA also increased the laundering durability.

In the in vivo study using diabetic rat models, no animal death was observed for all test groups during the experimental period. On the second day after the wound creation, the wound area appeared as a red and swollen bump. The formation of yellowish colored pus was observed. The rats received no treatment and placebo control showed no significant difference in the wound size throughout the experimental period (Fig. 3). On Day 12, the pus formation was still observed on the rats receiving placebo control, and the symptom of inflammation was obvious on the infection site. The application of cellulose nanocrystal film, without the curcumin, did not improve the healing of wound in diabetic rat models. However, with the topical application of curcumin loaded film, a significant reduction of wound size was observed from Day 7. At the end of the study, the lesion was covered by epithelial tissue and the hair started to grow from the

**Fig. 3** The diameter of wound recorded for the diabetic rat models for a duration of 12 days. The rats treated with curcumin loaded film showed a significant reduction in wound size

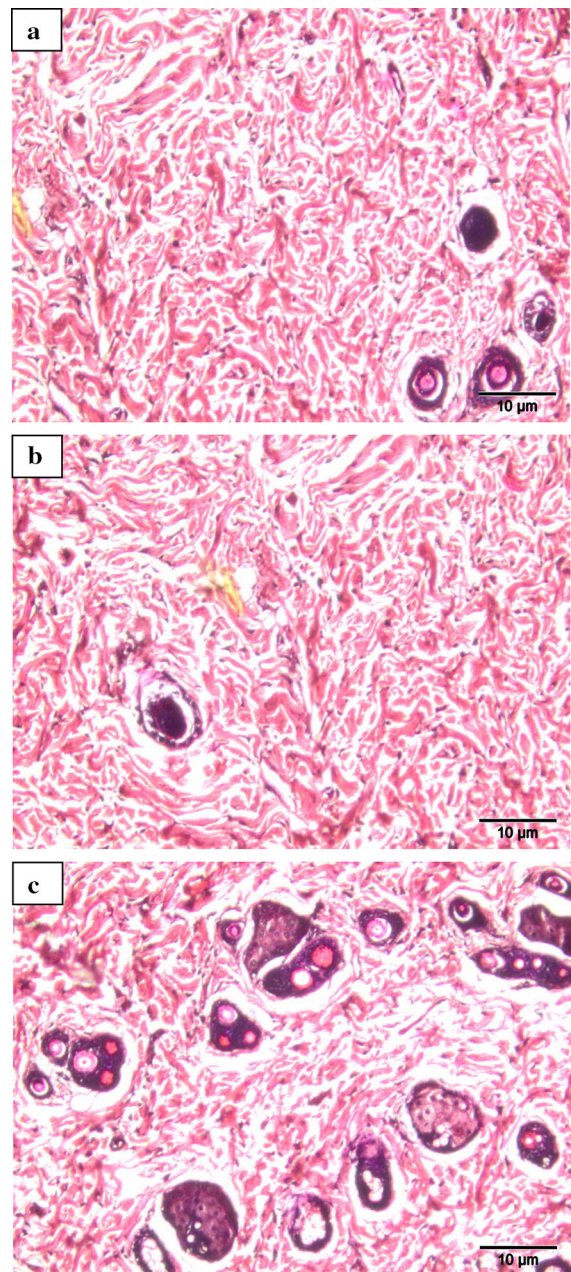
skin. We observed a faster wound closure of punch wound compared to controls.

The delayed wound healing in diabetic models is generally caused by peripheral neuropathy, altered hemoglobin rheology and the glycation of the cell membrane components (Kunkemoeller and Kyriakides 2017). The use of topical antimicrobial agent is necessary to prevent wound sepsis and infection. Curcumin loaded cellulose nanocrystal film significantly improve wound healing of diabetic rat models, with full thickness skin wound. Curcumin improves the recovery of the wound by increasing the level of transforming growth factor  $\beta$ 1, which is a protein involved in cell growth and proliferation (Shaikh et al. 2009). Besides, the compound also protects the skin cells from oxidative damages.

The viable cell count was conducted to enumerate the bacterial cells present in 1 g of skin sample excised from the infection site (Table 3). The topical application of the curcumin loaded film significantly reduced the bacterial count in the rat models ( $p < 0.05$ ). A bacterial growth reduction of 99.99% was observed. Bacterial colonization of wound slows the healing, hence the recovery of wound can be accelerated by reducing the bacterial load of the wound. The histological analysis of the non-treated and placebo control group (Fig. 4) showed the structure of damaged skin, with no presence of hair follicle and sebaceous glands. The findings were in agreement with Nichols and Florman (2001) where the hair follicle and sebaceous gland of the skin tissue were ruptured after infected with bacteria. Besides, the non-treated and placebo control groups showed significantly higher numbers of inflammatory cells, compared to the test group. The histological studies of the skin for test group (with curcumin loaded film) showed widespread of hair follicles and sebaceous glands in the dermis layer of the skin. We also observed well developed dermis layer under the light microscope. The results showed

**Table 3** The bacterial load of the skin sample excised from the diabetic animal models

Group	Bacterial load (CFU/g of tissue)
No treatment	$7.23 \times 10^7$
Placebo control	$6.48 \times 10^7$
Test group (Curcumin loaded film)	$1.24 \times 10^2$



**Fig. 4** The histological examination of the skin sample excised from diabetic rats received **a** no treatment **b** Placebo control **c** Test group with curcumin loaded film

that curcumin loaded film significantly improved the regeneration of hair follicles and sebaceous glands of the skin, by inhibiting the growth of bacteria. The effective healing was also proved by the presence of matured fibrous tissues. This clearly indicates the

curcumin load cellulose nanocrystal films can be used as diabetic wound healing applications.

**Acknowledgments** The authors are thankful to Universiti Kuala Lumpur. The study is funded by Fundamental Research Grant Scheme (FRGS/1/2017/STG05/UNIKL/02/5), Ministry of Higher Education, Malaysia.

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