Phomopsidione loaded chitosan polyethylene glycol (PEG) nanocomposite dressing for pressure ulcers

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Abstract

Pressure ulcers are commonly associated with microbial infections on the wounds which need an effective wound dressing. However, the silver dressings have shown promising result but they have toxicity and argyria. Hence, this study aimed to develop and characterize chitosan-polyethylene glycol (PEG) nanocomposite hydrogel loaded with phomopsidione as an antimicrobial dressing. The hydrogel being synthesized was analyzed with transmission and scanning electron microscopes. Drug release and mechanical properties were studied having confirmed the functional groups with Fourier transform infrared (FTIR) spectroscopy. Finally, antimicrobial activities were evaluated against the clinical wound pathogens. The developed hydrogel was soft, flexible and elastic, having nanospheres of chitosan-PEG but no sign of aggregation under the electron microscopes. Releasing of phomopsidione from the nanocomposite hydrogel was slow and gradual following the first order of kinetic. On average, 34 µg/mL phomopsidione released per hour and 67.9% active ingredients delivered into the surrounding medium over the study period. Although, the bioactivity activity of the hydrogel was narrow-spectrum, it showed significant results against all Gram-negative bacteria and Candida utilis with 99.99% reduction of microbial growth. The findings reveal that the phomopsidione loaded hydrogel has a great promise to act as an antimicrobial dressing for chronic wounds.

1 INTRODUCTION

National Pressure Ulcer Advisory Panel (NPUAP) defined pressure ulcers as locally damaged soft tissue or skin over a bony part or due to a device (Unver et al., 2017). In the United States, pressure ulcers have affected about 2.5 million patients annually and the treatments are estimated to cost between 9 and 11.5 billion dollars. Due to a direct result of pressure ulcers, about 60,000 patients died annually (Zapirain et al., 2017). Pressure ulcers are often associated with disabilities where 70% of the time occurring in people above 70 years old. Prolonged periods of recovery are due to chronic deep wounds suffered by several elderly patients. The mortality rate of patients who suffered from serious infective complications such as bacteremia was reported to be greater than 50%. Even though preventable, pressure ulcer is a common find in bed-ridden, elderly patients who are immobilized by their acute illness. The percentage is foretold to be more than 11% of the population by 2020 (Khor et al., 2014). As the skin damages, it loses its functions such as preventing water vapor loss, physical protection and thermal regulators. In this situation, wound infection is better prevented by declining bacterial entry into the open wound rather.

The microorganisms recurrently found in colonized pressure ulcers are*Staphylococcus aureus* and Gram-negative bacilli such as Enterobacteriaceae, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* . The

patients possessing colonized ulcers with developing infections are often seriously ill (Braga et al., 2013). Consequently, this leads to prolonged hospital stays and the need to use invasive medical devices such as central vascular catheter, tracheal tube and urinary catheter.To tackle the issue, a range of wound dressing products were created in order to return the functions to damaged skin (Vowden & Vowden, 2017). Unfortunately, these wound dressings come with their fair share of drawbacks. Silver wound dressings have been reported to cause argyria from extensive use of silver-containing wound dressing materials and medications (Berger et al., 2013). In addition to anti-inflammatory effects, nanosilver has been shown to control collagen deposition leading to a proper alignment of collagen fibrils that accelerates wound healing and have shown their efficacy in bypassing drug-resistance mechanisms (Song et al., 2015).

In wound healing, the wound dressing acts as a protection to the wound while the dermal and epidermal tissues heal. Thus, natural polymers such as polysaccharides; chitosan, alginates, heparin, and cellulose are widely selected in wound treatments and management because they are biologically compatible, biodegradable and similar to macromolecules that are familiar to the human body (Capanema et al., 2017). Phomopsidione ($C_7H_{10}O_4$) is a novel ketone derivative isolated from *Diaporthe flaxinii*ED2. In this study, we designed a chitosan-polyethylene glycol (PEG) nanocomposite wound dressing hydrogel using phomopsidione as antimicrobial finishing. The mechanical and physical properties of the developed hydrogel were characterized and *in vitro* antimicrobial efficiency of the hydrogel was evaluated on the clinical wound microorganisms.

2 MATERIALS AND METHODS

2.1 Test Compound

The test compound phomopsidione was generously donated by Professor Darah Ibrahim, Universiti sains Malaysia. The compound was first discovered by her research team (Tong et al., 2017).

2.2 Synthesis of Chitosan-PEG Nanocomposite

The chitosan-PEG nanocomposite was synthesized according to Liu and Kim (Liu & Kim, 2012). Firstly, aqueous medium molecular weight chitosan (Bio Basic) of 0.4% (w/v) was prepared in 2% (v/v) acetic acid solution. Next, 0.1 g of PEG (Merck) and 0.1 g of phomopsidione were added into the chitosan solution to obtain a Chitosan/PEG/Phomopsidione (C/PEG/Ph) solution. Then, 3 mL of 0.4% genipin (Sigma) cross-linker solution was added into the C/PEG/Ph solution. The final solution was then homogenized for 30 min at room temperature using a homogenizer (Heidolph Silent Crusher M) to allow proper mixing and bridging of the polymeric components, then cast onto sterile Petri dishes. The hydrogels were dried at room temperature for 48 hours. Lastly, the resulting nanocomposite hydrogels were then stored at room temperature for further experiments. Besides, a standard GC/PEG nanocomposite hydrogel was also prepared as the negative control.

2.3 Morphological Study

The morphology of the nanocomposite hydrogel with phomopsidione was observed under the scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The surface morphology of the nanocomposite hydrogel was observed under SEM (Jeol 6010Plus). For TEM observation, the sample was stained using uranyl acetate and placed on a copper grid. The sample was then examined using TEM (Phillips CM12), operating at 120 kV.

2.4 Fourier Transform Infrared (FTIR) Spectroscopy

The characteristic functional groups of the nanocomposite hydrogels were studied by FTIR. The FTIR spectra of chitosan, PEG, nanocomposite hydrogel with phomopsidione and negative control nanocomposite hydrogel were studied. The analysis was carried out on an FTIR spectrometer (Thermo Scientific Nicolet IS10, USA). The spectra were obtained at room temperature over the range of 4000 cm^{-1} to 500 cm^{-1} wave number range.

2.5 Drug Release Study

Four swatches of hydrogel (20mm × 20mm) were added into 50 ml artificial sweat solution with pH5.5. The solution was prepared by dissolving 1g lactic acid (Bendosen), 1g urea (Biofroxx) and 5g sodium chloride (Mallinckrodt) in 1L of distilled water. Then, 50 µL of the sample was withdrawn from each flask at 0, 2, 4, 6, 8, 12, 24, 48 and 72 hours. The sample was filtered using a 0.22 µm syringe filter. Then, the amount of phomopsidione released was analyzed using the High-Performance Liquid Chromatogram (Sahadan et al., 2019). The mobile phase used was chloroform/methanol at a ratio of 3:7 (v/v) with an HPLC (Perkin Elmer Series 200) coupled with Breeze software. The HPLC was set up with a wavelength of 245 mm, column (C_{18}) particle size 5 µm, length 120 mm, and injection volume of 20 µL. The amount of phomopsidione was determined based on the calibration curve (0 to 1000 µg/ml phomopsidione).

2.6 Study of Mechanical Properties

The tensile strength, elongation at break and Young's Modulus of elasticity were measured using a universal testing machine (INSTRON 3366). The nanocomposite hydrogels were cut to a size of 100 mm × 20 mm length by width. The extension rate speed was set at 10 mm/min. The tensile strength, elongation and Young's modulus of elasticity were obtained from the load extension curves (Liu & Kim, 2012). Two types of nanocomposite hydrogels were used; negative control and nanocomposite hydrogel with phomopsidione. Any defect shown or unintentional breakage of the nanocomposite hydrogel was removed and the results from the three trials were taken to average. A t-test was conducted to evaluate the statistical significance of the average values between the two test samples.

2.7 Swelling Study

The standard test method for water absorption (ASTM D570-98) was tested through immersion of the samples in a container filled with water at room temperature (Liu & Kim, 2012). The hydrogels were excised into strips measuring 76.2 mm long and 25.4 mm wide with a thickness of 0.02 mm and were conditioned for 24 hr. Repeated immersion test was done which started with 24 hours of immersion in phosphate-buffered saline, pH7 (Sigma). At the end of the immersion period, the samples were removed from the water one by one and gently wiped with Whatman No 1 filter paper to remove the surface water. They were immediately weighed to the nearest 0.001 g. The swelling ratio was calculated using the equation below.

$$\%\ Swelling\ ratio = \frac{\text{Weight of sample in swollen state } \times \text{Weight of sample in dry state}}{\text{Weight of sample in dry state}} \times 100\%$$

2.8 Test Microorganisms

The antimicrobial activity of the nanocomposite hydrogel was tested against 8 test microorganisms. They were previously identified as Methicillin-resistant *Staphylococcus aureus* (MRSA),*Bacillus cereus* , *Escherichia coli* , *Acinetobacter baumannii* , *Pseudomonas aeruginosa* , *Proteus mirabilis* ,*Candida albicans* , and *Candida utilis* . The test microorganisms were previously isolated from patients with chronic wounds at Hospital Seberang Jaya, Pulau Pinang, Malaysia. The test microorganisms were sub-cultured on nutrient agar for every 2 weeks in order to maintain its viability. To prepare the microbial inoculums, a loopful of microbial colonies using a wire loop into 5 ml of sterile distilled water. Then, the inoculum was mixed vigorously. The turbidity of the suspensions was adjusted match to 0.5 McFarland standard. The final inoculum size of bacteria was 1×10^8 cfu/mL and 1×10^6 cfu/mL for yeast.

2.9 Parallel Streak Test

The assay was performed following American Association of Textile Chemists and Colorists (AATCC TM147) standard (Rejendra et al., 2011). Nutrient agar (Merck) was prepared in 90 mm Petri dishes for bio-assay. Parallel lines were drawn at the back of the petri dish with measurements of 6.5 cm in length with 1 cm space between each line. Before proceeding with the test, both the negative control and nanocomposite hydrogel with phomopsidione were excised to square hydrogels of 20 mm × 20 mm and sterilized under UV light for 30 min. Using sterile cotton swabs, the microbial inoculum was streaked on the nutrient agar (Merck) based on the parallel lines drawn. Next, the square nanocomposite hydrogels were placed symmetrically on

the parallel lines, then gently pressed to allow proper contact between the hydrogel and the inoculated agar medium. The experiment was done in triplicate in separate occasions. All plates were incubated at 37@C for 24 hours. After the incubation period, the diameters of inhibition zone surrounding the hydrogel were observed and measured.

3.0 Hohenstein Challenge Test (AATCC TM100)

In vitro antimicrobial activities against test microorganisms were conducted for both the negative control and nanocomposite hydrogels with phomopsidione (Rejendra et al., 2011). At the start, one square hydrogel measuring 20 mm by 20 mm was added into each flask containing nutrient broth (Merck). In the meantime, the flasks were inoculated with 100 µL of test microorganism where it was allowed to grow aerobically in the incubator shaker at 120 RPM, 37@C for 24 hours. Then, serial dilution was done before spreading the solution on nutrient agar plates. The dilution factor of 10^{-2} was used for the flasks containing nanocomposite hydrogels with phomopsidione and the dilution factor of 10^{-7} was used for flasks containing negative control. The diluted bacterial solution was then distributed evenly on the nutrient agar (Merck) plates using an L-shaped spreader. Each test microorganism was cultured for 24 hours and counted (Liu & Kim, 2012). The Colony Forming Unit (CFU)/mL and percentage bacterial reduction for each sample was calculated using the following formulae.

$$\%\ Bacterial\ Reduction = \frac{\text{CFU before treatment } \times \text{CFU after treatment}}{\text{CFU before treatment}} \times 100\%$$

4 RESULTS AND DISCUSSION

Recently, research attention has drawn towards polymer nanocomposite due to its various applications and capabilities like biocompatibility, degradability, and non-toxicity (Ma et al., 2017). Nanocomposites are defined typically as novel materials where at least one of the constituents is in between the number range of 1 to less than 100 nm. Due to the nanocomposites' small size effect, the physical and chemical properties are better than the traditional composites. With the advancement of nanoscience and nanotechnology, remarkable innovations towards nanocomposites have been accomplished (Huang & Cheng, 2017). Nanocomposites are also known as one of the most promising materials in the 21st century for its rapid development and is vastly utilized in many disciplines because of its unique polymer attributes such as lightweight and helps ease production along with processing (Ma et al., 2017).

In this study, PEG-chitosan nanocomposite was selected as a wound dressing and drug delivery medium. The developed hydrogel showed a uniform light blue color. The hydrogel was transparent. PEG-chitosan nanocomposites are widely recognized as biodegradable polymers with outstanding safety and biocompatibility. They are used in association with other polymers to produce controlled-release drugs. The second prolific polysaccharide in nature is chitosan, the cationic (1-4)-2-amino-2-deoxy-β-D-glucan. Chitosan holds great significance due to its biodegradability, non-toxicity, and biocompatibility (Gilani et al., 2018). Surface coating by means of biodegradable and biocompatible polymers with low toxicity like chitosan and PEG were applied to curb the phagocytic reactions and improve the lifespan of the nanoparticles. Intriguingly, chemical alteration of chitosan and PEG does not only enhance the biocompatibility of chitosan, but also decreases the adsorption of circulating plasma proteins upon the material surface (Rabou & Ahmed, 2017).

Microscopic examination is important to observe the surface morphology of the developed hydrogels. Figure 1 represents the SEM micrographs of the nanocomposite hydrogel with phomopsidione. The hydrogel showed a smooth and uniform surface. Phomopsidione was successfully embedded in the smooth-textured surface of the hydrogel. Figure 2 depicts the TEM micrographs of the hydrogel produced. On the micrograph, nanospheres of various sizes were observed, with no sign of aggregation. Nanospheres ranging from 16.9 ± 3.8 nm represented chitosan whereas nanospheres ranging from 4.8 ± 1.6 nm represented PEG, based on the comparison with the previous studies (Liu & Kim, 2012). The mixing of chitosan and PEG was homogenous. All nanospheres observed were less than 100 nm in diameter. Supporting these results, Huang and Cheng reported that nanocomposites are component ranges from 1 to less than 100 nm (Huang & Cheng, 2017).

The FTIR spectra of chitosan, PEG, hydrogel with phomopsidione and negative control hydrogel were studied (Figure 3). The spectrum of chitosan showed characteristic absorptions at 3283 (overlap of O-H and N-H stretching), 1646 (C-N stretching) and 1558 (N-H bending) cm^{-1} (Manuela et al., 2016). For PEG, absorption bands at 1359 and 2879 cm^{-1} were due to the bending and stretching vibrations of C-H (Shameli et al., 2012). In the spectrum of negative control, the molecular interactions between the chitosan and genipin-crosslinked PEG caused the shifting in the absorption bands. The characteristic absorptions of chitosan were shifted to 1636 and 1556 cm^{-1} while the absorption at 3283 cm^{-1} became stronger at 3278 cm^{-1}.With the presence of phomopsidione in the nanocomposite, the shift in the peaks towards lower frequency at 1600 and 1554 cm^{-1}compared to the negative control. The results suggest the successful binding of phomopsidioneto the nanocomposite hydrogel.

This drug release study is important to determine the drug release behavior of phomopsidione form the hydrogel, by quantifying the phomopsidione released with HPLC. The test medium was artificial sweat solution prepared at pH 5.5 as to mimic the condition of the skin (Manuela et al., 2016). Figure 4 shows the pattern of phomopsidione released from the hydrogel for a period of 72 hours. Overall, no burst release was observed throughout the whole experimental period. The release of phomopsidione was slow and gradual, with an average amount of 34 µg/mL phomopsidione released per hour. The release followed the first order of kinetic, where 67.9 ± 6.4% of phomopsidione was released. This is due to excellent drug carrier properties from the two polymers, chitosan, and PEG. Nano-sized chitosan showed great promise in drug delivery as they possess high surface to volume ratio and porosity which enhances its drug loading capacity and deliver applications. Moreover, Parveen and Sahoo mentioned that surface coating by polymers such as chitosan and PEG were used to enhance the lifespan of the nanoparticles (Parveen & Sahoo, 2011). Chitosan accepts protons at low pH upon adding cationic polymers and becomes positively charged (Gupta, Vermani, & Garg, 2002; Saikia, Gogoi, & Maji, 2015). This type of polymer swells at low pH mediums. Since an acidic (pH 5.5) medium was used in this experiment, it caused swelling of the polymeric matrix which in turn released the loaded drug into it. Genipin was added in the nanocomposite as a crosslinker between phomopsidione and polymers by forming physical bonding such as van der Waal forces, hydrogen bonding and ionic interaction (Manickam, Sreedharan, & Elumalai, 2014). It plays a significant role in controlling the drug release from the hydrogel. The drug release behavior study showed that genipin is an excellent crosslinker for phomopsidione.

The mechanical properties of the hydrogels were determined to evaluate their suitability as a wound dressing material for chronic wounds. The average tensile strength for hydrogel with phomopsidione was 15.46 MPa which showed an increment of 29.62% in average tensile strength as compared to that of the negative control (Table 1). In addition, the results showed a 5.41% increment in elongation for the hydrogel with phomopsidione. Lastly, Young's modulus for hydrogel with phomopsidione was 1152.69 MPa which showed an increment of 13.76% from the negative control. The developed nanocomposite hydrogel was soft, flexible and elastic, which is ideal as a wound dressing material (Tong et al., 2017). The elasticity of the nanocomposite hydrogel is crucial to hold the wound dressing in place for a long period of time, in order to provide an excellent therapeutic effect for the wound recovery (Ali & Ahmed, 2018). The loading of phomopsidione into the hydrogel did not cause a significant change in the elongation at break and Young's modulus of the hydrogel (p [?]0.05). However, the tensile strength of the hydrogel was significantly improved with the addition of phomopsidione (p [?]0.05). The improved tensile strength of the nanocomposite hydrogel means that it is able to endure greater force before it breaks. Pressure ulcers are constantly exposed to pressure or external forces hence a wound dressing with better tensile strength is favorable.

By comparing to Liu and Kim, the Chitosan-PEG blend hydrogels were found to be more flexible and possess better mechanical properties, as compared to pure chitosan films (Liu & Kim, 2012). A study by Suyatma*et al* . reported that a pure chitosan film exhibited a tensile strength of 82.4 MPa with 5.2% elongation at break whereas the study by Liu and Kim showed that the Chitosan-PEG exhibited a tensile strength of 85.5 MPa with elongation at break of 25.5%, which justified enhanced properties upon mixing (Suyatma et al., 2004; Liu & Kim, 2012). This is because when the two polymers are blended together, the chitosan helped in improving mechanical properties while PEG improves its flexibility (Huang & Cheng, 2017). In comparison to other blends, Zivanovic, Davidson & Kit reported that chitosan-polyethylene oxide blend of

the same mixing ratio exhibited a mean tensile strength of 55.4 MPa with an elongation at break of 10.8% (Zivanovic, Davidson & Kit, 2007). Ultimately, the addition of phomopsidione has shown improvement in the nanocomposite hydrogels' mechanical properties. Therefore, the nanocomposite hydrogels, comprised of chitosan and PEG blends, have shown great potential to serve as antimicrobial wound dressings. The mechanical properties of the nanocomposite hydrogels can be further improved. Hydrogel layering can help improve the overall mechanical properties. Also, manipulating the thickness of the nanocomposite hydrogels can ultimately find the optimum mixing ratio giving the best mechanical properties (Zivanovic, Davidson & Kit, 2007).

Various natural polymers were utilized as wound dressing material. However, the application of chronic wounds is limited due to poor swelling activity. Overall, the maximal swelling ratio of the nanocomposite was high, which make it an ideal wound dressing material. The high swelling ratio of the hydrogel allows the absorption of a large amount of exudates in chronic wounds (Qin, 2008). The high swelling ratio is comparable to carboxymethyl cellulose hydrogel (Chang et al., 2010). The excellent swelling ratios are contributed by many hydrophilic groups in the hydrogel, particularly the ionic bond between water molecule and the amino groups of chitosan (Kiuchi, Kai, & Inoue, 2007). The difference in swelling ratios for nanocomposite hydrogel with phomopsidione and negative control were not statistically significant (p [?]0.05). The addition of phomopsidione into the hydrogel did not affect its swelling ratio.

The antimicrobial study was performed to evaluate the antimicrobial efficiency of the developed hydrogel on wound pathogens. The study was done through the parallel streak method with 20 mm by 20 mm hydrogels. The inhibitory activity on all test microorganisms is shown in Table 2**.** The hydrogel with phomopsidione showed significant antimicrobial activity against all Gram-negative bacteria and one yeast (*C. utilis*). The antimicrobial activity of the hydrogel was a narrow spectrum. The largest inhibition zone observed was against*P. aeruginosa* with an average inhibition zone diameter of 41.2 mm. *P. aeruginosa* is most frequently isolated from chronic wounds treated with antibiotic therapy (Serra et al. 2015). Due to its high virulence, the bacterium often prolongs the hospitalization of patients with the chronic wounds. Negative control hydrogels were also tested on and were done as comparative studies. It was observed that the hydrogels without phomopsidione showed no antimicrobial effects. This has proven that the synthesized hydrogels were able to release efficiently onto the agar medium as well as to suppress any microbial growth.

Besides, opposing with the previous reports by Fayaz *et al* . and Bhawana *et al* . the hydrogel with phomopsidione exhibited inhibitory activity only on Gram-negative bacteria (Fayaz et al., 2010; Bhawana et al., 2011). However, the inhibitory activity of phomopsidione on Gram-negative bacteria was previously reported earlier (Sahadan et al., 2019). Phomosidione did not target the bacterial cell wall. Figure 5 depicts the antimicrobial effect of the nanocomposite hydrogels against *C. utilis* . Similarly, the presence of clear zones from phomopsidione against Candida *species* also reported (Tong et al., 2017).

The quantitative evaluation of the antimicrobial efficiency was performed with the Hohenstein challenge test. Four test microorganisms showed 99.9% of bacterial reduction, relative to the negative control. The results were tabulated in Table 3. Based on the results, the hydrogel with phomopsidione has proven to exhibit a significant reduction in the growth of Gram-negative bacteria and yeast. The outcome of this study is in agreement with Tong *et al* . who reported phomopsidione's biocidal effect towards yeast, though a similar effect was observed form Gram-negative bacteria (Tong et al., 2017). To support the claim, the hydroxyl groups which are present in a bioactive compound served to reduce free radicals from the microorganisms by donating their hydrogen atoms and this inhibits microbial growth (Feng & Liu, 2009). Since phomopsidione contains hydroxyl groups, free radicals from the microorganisms were able to be scavenged resulting in the inhibition of microorganisms. Mirroring the results of parallel streak test, the highest percentage of inhibition was observed on *P. aeruginosa* . The results showed the potential use of this hydrogel in the treatment of Gram-negative infection on chronic wounds.

5 CONCLUSIONS

In conclusion, the chitosan-PEG nanocomposite hydrogels loaded with phomopsidione were successfully syn-

thesized and characterized. The characterization results have revealed that the nanocomposite hydrogels have great promise to act as antimicrobial dressings for chronic wounds. The testing of the nanocomposite hydrogel should be further extended to*in vivo* study to evaluate the efficacy with the animal models simulating the chronic pressure ulcers.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest.

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