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Mechanical and Antibacterial Activities Study of Gellan Gum/Virgin Coconut Oil Film Embedded Norfloxacin

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Abstract. Biofilm based on gellan gum, was prepared by evaporative casting technique, and its properties were evaluated. The tensile strength (TS) and elongation-at-break (EAB) of the GGVCO-NOR films were found to be 9 MPa and 7.3%, respectively. The swelling properties and water vapor transmission rates of the prepared films were found to be 278% and 212 g m-2 d-1, respectively, after 24 hours. Surface morphology of the film revealed the presence of the pores. From the anti-bacterial study, it was found that the inhibition zone of GG-NOR and GG-VCONOR film were 5.3±0.06 mm and 5.7±0.06 mm against gram-positive (Staphylococcus aureus) and 5.0±0.01 mm and 6.3±0.06 mm againts gram-negative bacteria (Escherichia coli).

1. Introduction

Mechanical and antibacterial properties were considered as an important in the development of qualified wound dressing materials. The mechanical properties were essential to define the strength of the material while the antibacterial properties will prevent the wound from the bacteria infection. Fluoroquinolones group such as norfloxacin is among an effective antibacterial agent against gram-positive and gramnegative bacteria. Norfloxacin (1-ethyl-6-fluoro-1, 4-dihydro-4-oxo-7-(piperazin-1-yl) quinolone-3 carboxilic acid) is a broad spectrum antimicrobial [1] analog of nalidixic acid [2, 3]. Norfloxacin is derived from fluoroquinolones and it is among the rapid growing class of an antimicrobial agent. Norfloxacin has been reported to be more active than other fluoroquinolones against Gram-positive (*i.e.*,

Staphylococcus epidermidis, and *Staphylococcus aureus*) and Gram negative bacteria (*i.e.*, *Pseudomonas aeruginosa* and *Escherichia coli*) [4]. At the same time, norfloxacin had appeared to be the least cytotoxic compared to other fluoroquinolones such as levofloxacin and ofloxacin [5, 6]. Previous study has reported that norfloxacin has potent activity against gram-positive and gramnegative bacteria but limited against anaerobes [2]. Norfloxacin is also used for common and complicated urinary tract infections treatment including cystitis and prostitis [7, 8]. The norfloxacin has been applied in therapy treatment for gonorrhoea infections and was became the first choice drug to treat diseases caused by *Campylobacter, E-coli, Salmonella, Shigella,* and *V. colera* [9]. In the past few years, norfloxacin has become a choice in the drug determination for biological samples and medicine [10-13]. Besides that, norfloxacin is effective in the treatment of gonococcal urethritis and infectious diarrhea [14].

Gellan gum (GG) received great attention, particularly in the field of biomedicine, due to their biocompatibility and biodegradability properties. Gellan gum is produced by *Pseudomonas elodea* and

consists of a repeating unit of tetrasaccharide: 1,3-linked -D-glucose, 1,4-linked - D-glucuronic acid, 1,4-linked -D-glucose, and 1,4-linked - L-rhamnose. Gellan gum has been explored as stomach-specific delivery [15] and it is also a candidate material for tissue engineering application [16-19]. In addition, gellan gum also has been applied in cosmetics, lotions, creams, make-ups, face masks, hair care products, and various toothpastes. Besides that, previous studies have been reported that gellan gum can be used in the bioremediation of contaminated soils and aquifers [20, 21].

On the other hand, virgin coconut oil (VCO) is one of the recent promising candidates in promoting antibacterial activities. St-Onge and Jones (2002) and Assunção *et al.* (2009) have been reported that the virgin coconut oil has potential to be used as anti-obesity treatment due to its medium chain fatty acid component of lauric acid [22, 23]. The lauric acid will increases the energy expenditure, then directly absorbed and burnt as energy in the liver, resulted in early satiety and lastly leading to weight loss [24]. The lauric acid also responsible to destroyed all the microorganisms, thus promotes collagen production on the skin and directly speeds the healing process to reduce scarring [25]. The virgin coconut oil also reported to have an anti-viral activity due to its medium chain fatty acid was converted to the monolaurin by the body [26].

2. Experiments

2.1. Materials

Low-acyl gellan gum (Gelzan™ CM, Mw ≈ 2-3 x 10^5 Da, product number-G1910, lot number SLBB0374V), and Triton™ X-102 (product number-1001318460, lot number MKBDD4707V) were obtained from Sigma-Aldrich, Malaysia. The virgin coconut oil (VCO) (product number-617488-D) was obtained from BioNeutraceutical (M) Sdn. Bhd., and the commercial antibiotic, Norfloxacin (product number-N9890) was from Fluka, USA. All materials were used as initially received.

2.2. Film Formation

A stock solution of gellan gum (GG) was made by dissolving 1% (w/v) of GG in 100 mL deionized water (18.2 MΩ) with continuous stirring for 2 h at 70 °C. GG-NOR solutions were prepared by adding 0.01% (w/w) norfloxacin (NOR) into the stock solution of GG. Meanwhile, the GG-VCO solutions were prepared by adding 0.3% (w/w) of VCO into the stock solution of GG, followed by 1.0% (w/v) of nonionic surfactant, i.e. Triton X-102. The GG-VCO solutions were then stirred at 500 rpm at 70 °C for 2 h to obtain a stable emulsion. For GG-VCO-NOR solutions, 0.01% (w/w) of norfloxacin were added into GG-VCO solution and stirred for 2 h at 70 °C. The GG, GG-NOR, GG-VCO and GG-VCO-NOR solutions were next deposited onto petri dishes (90 mm x 15 mm) and placed inside Venticell oven at 30 °C for at least 24 h. All films were pre-conditioned in a desiccator (24 °C, 50 % relative humidity (RH)) for at least 2 days prior to testing.

2.3. Characterization of Film

ATR-FTIR spectra were collected using a Perkin Elmer Spectrum 100 FT-IR spectrophotometer with PIKE Miracle ATR accessory (single-bounce beam path, 45 º incident angle, 16 scans, 4 cm^{-1} resolution) and all spectra were corrected by the Perkin Elmer spectrum 100 software. Stress-strain measurements were obtained using an Instron Universal Testing machine (model 3366) with ± 10 kN grips and the cross-speed set at 20 mm/min. Film thickness (2.0 cm x 6.0 cm) was measured by a hand-held micrometer (Mitutoyo). Young's modulus (E), tensile strength (TS), and toughness (T) were calculated from the slope of the linear part of the stress-strain curve, maximum stress, and through integration of the area under the curve, respectively. Elongation-at-break (EAB) was also recorded. Scanning electron microscopy (SEM) images were obtained by using a JOEL JSM 6360 LA electron microscope. SEM images of cross-sections were obtained by freeze-drying the samples in liquid nitrogen (-160 °C). Then, the samples were fractured at -150 °C. Later coated with Auto Fine Coats (JFC-1600), and imaged by SEM.

2.4. Bacterial study

Gram-positive (*Staphylococcus aureus - S. aureus*) and gram-negative (*Escherichia coli – E. coli*) bacteria were used for the antibacterial assay. The standard growth medium (Muller-Hinton, MH, Difco™) agar was prepared by sterilized with an autoclave (15 min, 120 °C). *S. aureus* and *E. coli* were grown in MH agar and incubated aerobically (37 °C, 24 h). The suspensions were measured by using spectrophotometer (Biomerieux Densicheck Plus, USA) at 600 nm with optical density at 0.5. Inoculants of both *S. aureus* and *E. coli* were spread on sterile petri plates contained the MH agar. Using sterile cotton swab, both bacteria were swabbed over the surface of the agar plates. The film samples and control was gently pressed on the agar. Prior inserting the samples discs with diameter 6 mm, the plates containing bacterial were let to dry. The plates contained film samples and agar with bacteria were incubated at 37 °C for 24 h in triplicates. The plates were examined for the presence of zones of growth inhibition after 24 h of incubation at 37 °C.

3. Results and Discussion

The ATR spectra of GG-VCO-NOR spectrum in Figure 1 shows an absorption band at 3377 cm^{-1} which is refer to –N-H stretching. This absorption band is absent in virgin coconut oil (VCO) and appeared in gellan gum (GG), norfloxacin (NOR), and GG-VCO-NOR spectra. A strong absorption band of alkane groups also have been observed at 2855 and 2924 cm^{-1} in GG-VCO-NOR film due to the interaction of hydrogen bonding formation between the blends [27] as proposed in Figure 2. Besides that, the peak at 1745 cm^{-1} indicated the formation of esterification and conjugation of carbonyl ester group [28] due to the interaction occurred and the particular peak almost absent in pure norfloxacin. Further, the appearance peak at 1615 cm^{-1} in GG-VCO-NOR films proved that ~N-H bending vibration of quinolones group successfully interact in the blends in which the group is absent in pure gellan gum, virgin coconut oil and also in GG-VCO films.

The chemical interaction of ester group stretching vibration at 1246 cm^{-1} also strongly supported the interaction between the blends in GG-VCO-NOR film due to this peak absent in pure norfloxacin and GG-NOR but appeared in GG-VCO-NOR films. Last but not least, the GG-VCO-NOR interaction was supported by the appearance of fluoroquinolones group in the blends at 1111 cm^{-1}. However, this peak was shifted to the left compared to pure norfloxacin films. This might be due to the formation of βketoesters [29]. This is expected due to the chemically interaction between the carboxylic acid group of norfloxacin and the polymer (GG) also might be with the virgin coconut oil. The nitrogen atoms of quinolone groups at ortho position are less of electron rich due to the deficient of flouroquinolone ring. So, it is are not likely involved in the reaction and sterically hinder the reaction also due to the methoxy and piperazinyl groups [28].

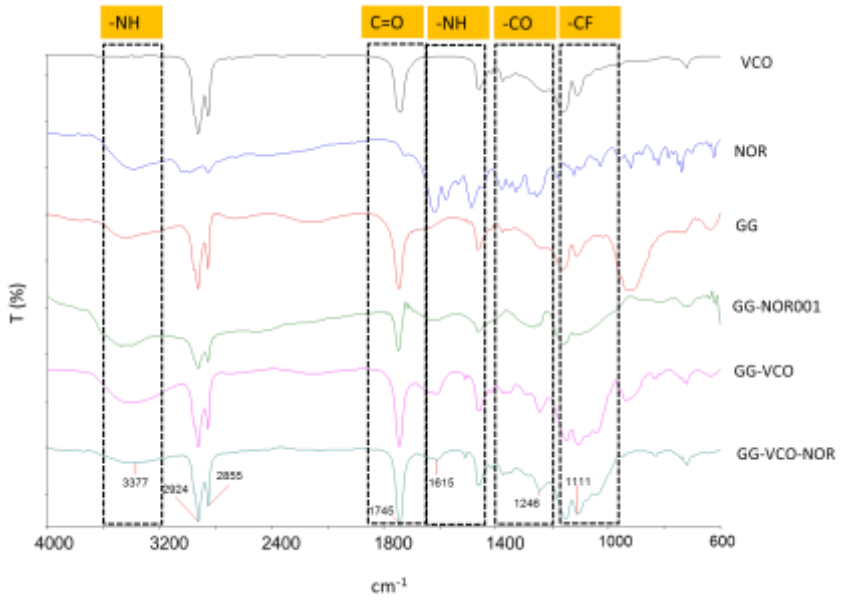

Figure 1. ATR spectra of pure virgin coconut oil (VCO), pure norfloxacin (NOR), gellan gum (GG) film, gellan gum film containing norfloxacin (GG-NOR), gellan gum film with virgin coconut oil (GGVCO) and gellan gum film containing virgin coconut oil and norfloxacin (GG-VCO-NOR).

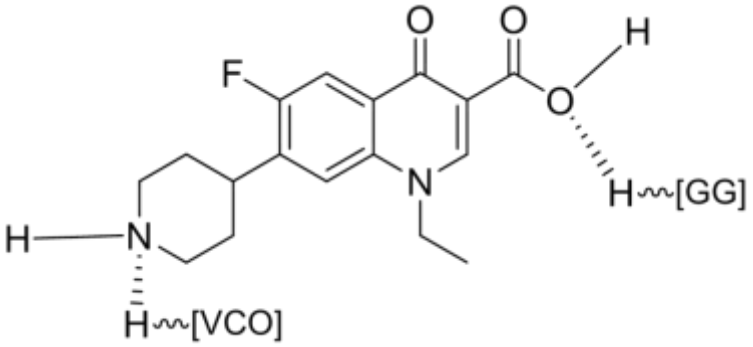

Figure 2. Proposed norfloxacin structured for hydrogen bonding formation between GG and VCO.

The stress-strain diagram of GG, GG-VCO, GG-NOR and GG-VCO-NOR films are shown in Figure 3. The addition of virgin coconut oil (VCO) improved the flexibility or elongation-at-break and inclusion of norfloxacin (NOR) increased the toughness values. The addition of VCO into GG film improved the T and EAB value of composite film to 0.67 J g^{-1} and 13.5 %. Our result show that the reason as to the increase of T and EAB of GG-VCO film was due to the formation of hydrogen bonds by reaction of carbonyl group of virgin coconut oil with gellan gum as proved by ATR spectroscopy. Meanwhile, the addition of norfloxacin in gellan gum films increased the toughness (T) values, however decreased the tensile strength (TS) and Young's modulus (E) of films as summarized in Table 1. The inclusion of norfloxacin in gellan gum films improved the toughness to 0.28 J g^{-1} compared to gellan gum film at 0.26 J g^{-1} which equivalent to 1-fold increase. However, the TS and E value of the GG-NOR films were decreased to 59±11 MPa and 3671±639 MPa; respectively, compared to gellan gum film. This is due to the modification imposed on the composite films lead to weakening the TS of the

composite films. For GG-VCO-NOR films, the strain-at-break improved to 7.3 % compared to GG-NOR at 2.1 %, but lowered than GG-VCO at 13.5 % as shown in Figure 3 and summarized in Table 1.

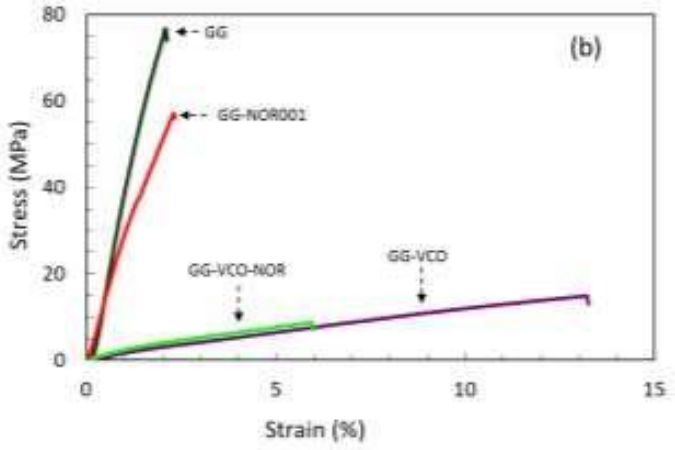

Figure 3. Stress-strain curves of comparison between gellan gum (GG) film, gellan gum film containing norfloxacin (GG-NOR), gellan gum film with virgin coconut oil (GG-VCO) and gellan gum film containing virgin coconut oil and norfloxacin (GG-VCO-NOR).

Table 1. Summary of the tensile strength (TS), Young's modulus (E), toughness (T), and elongation-atbreak (EAB) of gellan gum containing of norfloxacin (GG-NOR) films, gellan gum with virgin coconut oil (GG-VCO) and gellan gum containing virgin coconut oil and norfloxacin (GG-VCO-NOR) film (mean±SD) (n=3)

Sample	Thick (µm)	TS (MPa)	E (MPa)	T (J g^{-1})	EAB (%)
GG	30±0.01	80±12	4600±330	0.26±0.09	2.1±0.2
GG-VCO	80±0.06	14±2	130±19	0.67±0.33	13.5±2
GG-NOR	27±0.01	59±11	3671±639	0.28±0.06	2.1±0.5
GG-VCO-NOR	87±0.01	9±1	195±61	0.25±0.19	7.3±3

In contrast, incorporated both of VCO and NOR decreased the TS and YM values to 9 MPa and 195 MPa; respectively. The addition of virgin coconut oil and norfloxacin into the blends promote the changes of chemical interaction due to the hydrogen bonding formation, thus influenced the mechanical properties. The micelles formation appeared as in the GG-VCO film, then exhibit the flexibility to the materials film. Gellan gum, like many anionic polysaccaharide forms a physical gel by undergoing a random coil-helix to double-helix transition upon cooling [30]. During this transition, the emulsion does not have enough time to settle off, thus micelles formation was produced as shown in Figure 4 (c) and (d) compare to smooth cross section appearance of GG and GG-NOR film (Figure 4 (a) and (b)). The formation of micelles interfered the chemical structure thus enhanced the toughness of the GG-VCONOR films.

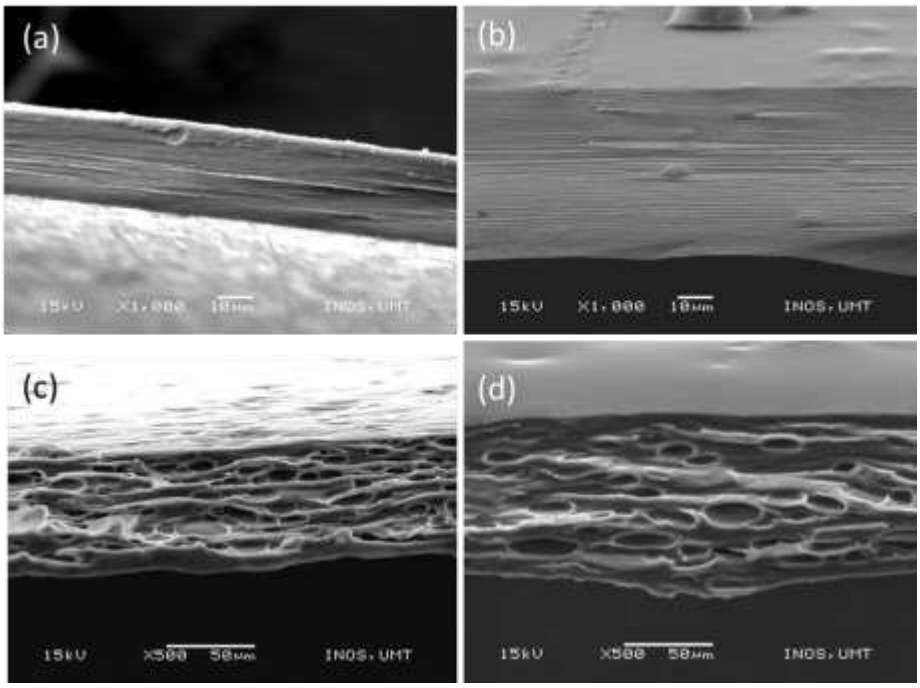

Figure 4. Scanning electron microscopy images of cross-sectional area of (a) gellan gum film, (b) gellan gum film containing norfloxacin (GG-NOR), (c) gellan gum film with virgin coconut oil (GG-VCO) and (d) gellan gum film containing virgin coconut oil and norfloxacin (GG-VCO-NOR).

The antibacterial activity of GG, GG-VCO, GG-NOR and GG-VCO-NOR films were measured by qualitative (disk method) as shown Figure 5. The inhibition zone was recorded after 24 h against *Staphylococcus aureus* and *Escherichia coli* executed by virgin coconut oil and norfloxacin (Table 2). The GG and GG-VCO film shows no antibacterial activities. It has been elaborated that the VCO does not possess antibacterial activity on its own, but rather is induced by its free fatty acids, particularly lauric acid (C12), and small extent of capric acid (C10) and caprylic acid (C8) [31]. Or, in other words, the VCO must be metabolized to release those components and exert its antimicrobial effects [32]. On the other hand, the inhibition zone of GG-NOR about 5.3 mm and 5.0 mm resisted *Staphylococcus aureus* and *Escherichia coli*, respectively (Table 2). The effectiveness antibacterial activity of norfloxacin against Gram-positive and Gram negative bacterial is well known by inhibits deoxyribonucleic acid synthesis intracellularly in bacteria [33-39]. It alters the structure of the enzyme resulting in abnormal change in polypeptide production. Norfloxacin differs from other quinolones, which include nalidixic acid and cinoxacin, by having a fluorine atom at the six position and a piperazine moiety at the seven position. The fluorine atom at the six position provides for increased potency against gram-negative organisms, while the piperazine moiety at the seven position provides for antipseudomonal activity [33]. Inhibition zone of GG-VCO-NOR films incorporated with both virgin coconut oil and norfloxacin improved the antibacterial properties are shown in Figure 5 and Table 2. This is due to the antibacterial properties of virgin coconut oil was enhanced and supported to inhibit the bacterial growth. The inhibition zone of GG-VCO-NOR films (5.7±0.06 and 6.3±0.06 against *S. aureus* and *E. coli*; respectively) were larger compared to GG-NOR001 (5.3±0.06 and 5.0±0.06 against *S. aureus* and *E. coli*; respectively).

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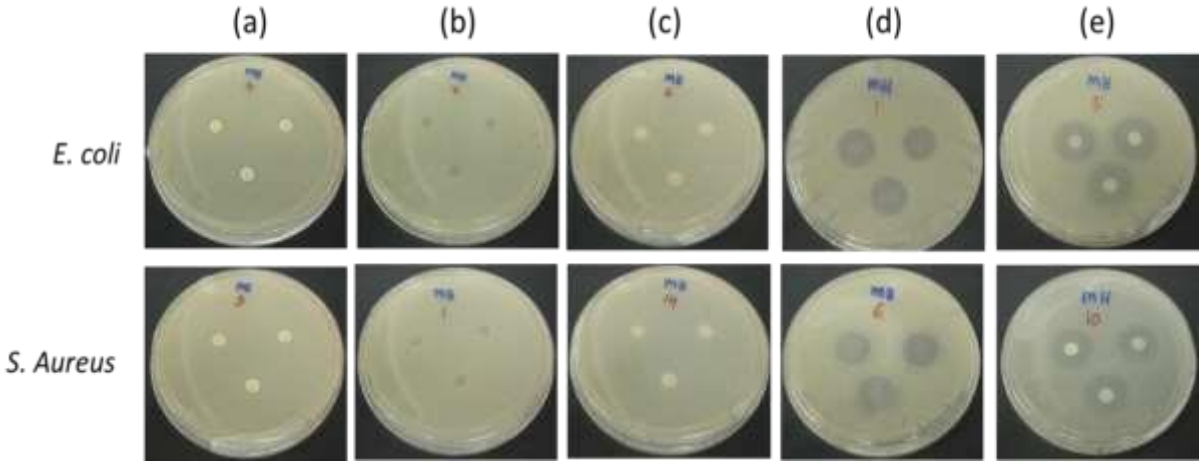

Figure 5. Disk diffusion results of (a) penicillin (positive control), (b) gellan gum film (c) gellan gum film with virgin coconut oil (GG-VCO) (d) gellan gum film containing norfloxacin (GG-NOR), and (e) gellan gum film containing virgin coconut oil and norfloxacin (GG-VCO-NOR) after incubated for 24 h against Escherichia coli and Staphylococcus aureus bacteria.

Material	Inhibition zone (mm)	
	S. aureus	*E.coli*
GG	-	-
Penicillin	-	-
GG-VCO	-	-
GG-NOR	5.3±0.06	5.0±0.01
GG-VCO-NOR	5.7±0.06	6.3±0.06

Table 2. Qualitative results of gellan gum containing different concentrations of norfloxacin (GG-NOR) films, gellan gum with virgin coconut oil (GG-VCO) and gellan gum containing virgin coconut oil and norfloxacin (GG-VCO-NOR) film against the Staphylococcus aureus (S. aureus) and Escherichia coli (E. coli) bacteria as indicated by the zone of inhibition (mm) (mean±SD) (n=3)

4. Conclusion

This work successfully explored the formation of composite films through an evaporative casting technique by using gellan gum (GG), virgin coconut oil (VCO), and norfloxacin (NOR). The mechanical properties of GG films were improved by incorporated of VCO and NOR. The morphological appearance of the GG-VCO films shows the micelles formation due to the presence of surfactant. For the qualitative antibacterial studies, the incorporating of NOR into GG contained VCO was increased the inhibition zone to 5.7±0.06 mm and 6.3±.0.06 mm against *Staphylococcus aureus* and *Escherichia coli*, respectively.

5. References

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