Food Hydrocolloids for Health 1 (2021) 100023

Contents lists available at ScienceDirect

Food Hydrocolloids for Health

journal homepage: www.elsevier.com/locate/fhfh

Tuning mechanical properties of seaweeds for hard capsules: A step forward for a sustainable drug delivery medium

Mohd Aiman Hamdan[a], Mohd Amin Khairatun Najwa[a], Rajan Jose[b], Darren Martin[c], Fatmawati Adam[a,d,*]

[a] Faculty of Chemical and Process Engineering Technology, Universiti Malaysia Pahang, Lebuhraya Tun Razak, Kuantan, Pahang 26300, Malaysia
[b] Nanostructured Renewable Energy Materials Laboratory, Faculty of Industrial Sciences and Technology, Universiti Malaysia Pahang, Lebuhraya Tun Razak, Kuantan, Pahang 26300, Malaysia
[c] Australian Institute for Bioengineering and Nanotechnology (AIBN), The University of Queensland, Brisbane Qld 4072, Australia
[d] Center of Excellence for Advanced Research in Fluid Flow (CARIFF), Universiti Malaysia Pahang, Lebuhraya Tun Razak, Kuantan, Pahang 2630, Malaysia

ARTICLE INFO

Keywords:
Renewable biocomposite
Drug delivery
Tensile strength
Disintegration test
Antibiotic stability

ABSTRACT

At present, vegetarian hard capsules from hydroxypropyl methyl cellulose is highly attractive to consumer market. However, the production volume is still low due to expensive raw polymer materials. Most of the renewable polymers do not meet sufficient mechanical strength for developing hard capsules. This work demonstrates that the mechanical properties of carrageenan, a sea-based edible material known as seaweeds, could be enhanced by the addition of small amounts (<2 wt./v%) of cellulose nanocrystals (CNC) for developing hard capsules. The microstructure and mechanical properties of the cellulose toughened carrageenan films are systematically correlated to the rheology of the solution from which the films were prepared. Carrageenan films containing 1.6 wt./v% of CNC showed an optimum viscosity of 1.17 mPas with 58% higher tensile strength than films without CNC. The hard capsules thereby developed are stable to three types of antibiotics, which are amoxycillin 500 mg, ampicillin 500 mg, and doxycycline 100 mg. The carrageenan biocomposite hard capsule is suitable for human consumption based on the cytotoxicity test on human body. All the hard capsule samples showed excellent disintegration behavior in physiological conditions. These achievements pose promising directions for sustainable materials supply for pharmaceutical industries.

1. Introduction

Plant-based drug delivery material systems offer numerous advantages over its animal protein-based counterparts including renewability and environmental cleanliness; a recent study emphasize that a widespread switch to vegetarianism would cut the carbon dioxide (CO_2) emission by nearly two-thirds thereby significantly contributing to a green environment (Springmann, Godfray, Rayner & Scarborough, 2016). Beside, drug delivery is a $980 billion industry, therefore, finding renewable and clean resources are extremely important for sustainability and healthy life (Aitken, 2014; Tibbitt, Dahlman & Langer, 2016). Currently, gelatine, a constitute of protein produced by partial hydrolysis of collagen extracted from animal organs, skins and bones is widely utilized as a drug delivery system because of its unique properties as gelling and foaming agents, an emulsifier, a stabilizer, a thickener and a water binder (Azilawati, Hashim, Jamilah & Amin, 2015). Beside, gelatine is biodegradable, biocompatible and has hydrophilic properties in amino acid and offer numerous oxygen containing functional groups for enhancing adsorption in composites (Zhou et al., 2017). Due to these reasons, gelatine is widely used in drug, protein and cell delivery applications particularly in the form of hard or soft capsules (Xue et al., 2014) and expects that the global gelatine market would be ~$2.8 billion by 2021 (Sandesara & Sandesara, 2016). However, beside being a global-warming-contributing animal protein, gelatine has low barrier against water vapor, poor thermal and mechanical stability, contribute to outbreak of Bovine Spongiform Encephalopathy (BSE), incorporated allergen, etc. These have motivated researchers to investigate new alternatives for gelatine (Barham, Tewes & Healy, 2015; Karim & Bhat, 2009).

Many plant-based polymers such as dialdehyde carboxymethyl cellulose, hydroxypropyl methyl cellulose (HPMC), starch, pullulan, yeast biomass, and carrageenan have been proposed as alternatives for gelatine (Barham, Tewes & Healy, 2015; Delgado, Sceni, Peltzer, Salvay, De La Osa & Wagner, 2016; Fakharian et al., 2015; Mu, Guo, Li, Lin & Li, 2012; Rabadiya & Rabadiya, 2013). Among them, carrageenan is particularly interesting as they are widely used in the food industry as a gelling

* Corresponding author at: Faculty of Chemical and Process Engineering Technology, Universiti Malaysia Pahang, Lebuhraya Tun Razak, Kuantan, Pahang 26300, Malaysia.
E-mail address: fatmawati@ump.edu.my (F. Adam).

https://doi.org/10.1016/j.fhfh.2021.100023
Received 29 December 2020; Received in revised form 10 August 2021; Accepted 18 August 2021

and stabilizing agent (Dewi, Darmanto, & Ambariyanto, 2012). Carrageenan has valuable biological functions, due to the superior gelling and high viscosity properties (Abad et al., 2010; Elsupikhe, Ahmad, Shameli, Ibrahim & Zainuddin, 2016). Oligomers from carrageenan contains antiherpetic, anti-HIV (human immunodeficiency virus), antitumor, and anticoagulant properties (Prajapati, Maheriya, Jani & Solanki, 2014; Yamada, Ogamo, Saito, Uchiyama & Nakagawa, 2000). However, after drying process, carrageenan tends to become brittle due to formation of double helices upon heating (Farahnaky, Azizi, Majzoobi, Mesbahi & Maftoonazad, 2013; Zarina & Ahmad, 2015). This phenomenon caused the carrageenan film to have low mechanical stability, thereby imposing significant challenges in removing carrageenan films from the capsule mold without breaking. One of the solutions to solve the brittleness issue is by incorporating plasticizer and crosslinker in the product formulation. Plasticizers such as glycerol and polyethylene glycol (PEG) have been incorporated in the carrageenan biocomposite formulation and successfully reduced the brittleness and improve the flexibility of the films (Phan The, Debeaufort, Voilley & Luu, 2009). Meanwhile the incorporation of crosslinker improved the mechanical properties and stability of carrageenan biocomposite film (Adam, Hamdan & Abu Bakar, 2020a).

Renewable resource and plant-based materials have been investigated for fabricating hard capsules. Hydroxypropyl cellulose (HPC) and polyethylene oxide (PEO) based hard capsule achieved hardness of 8 N and 6.5 N, respectively (Lee, Chen, Sheu & Liu, 2006). However, formation of HPC and PEO based hard capsules is rather cumbersome; mold and pestle capsule-forming device should be adapted to form the capsule shell from these materials. In another report, Mung bean starch added with kappa and iota carrageenan produced a significant tensile strength of 19.0 MPa and could be dissolved in water and HCl solution at a pH of ~1.2 in less than 10 min (Bae, Cha, Whiteside & Park, 2008). However, a higher processing temperature (90 °C) is desired for preparing the above mixture. Kappa-carrageenan was incorporated into acid hydrolyzed and hydroxy propylated sago starch based capsule film (Fakharian et al., 2015); however, the storage modulus of resulting film is order of magnitude (10^3 Pa) lower than the gelatine films (10^4 Pa) despite their comparable viscosities (sago starch ~0.8 to 1.1 Pa.s; gelatine viscosity ~1.15 Pa.s). Interestingly, the tensile strength reduced to ~13 MPa after the incorporation of iota and kappa carrageenan into the native sago starch (Poeloengasih, Pranoto, Anggraheni & Marseno, 2017). Formulation of gum Arabic increased the carrageenan biocomposite film tensile strength up to 36.21 MPa and capsule loop up to 34.11 MPa (Adam, Jamaludin, Abu Bakar, Rasid & Hassan, 2020c). This increase in the mechanical strength was due to van der Waals forces and hydrogen bonding. Thus, to improve the mechanical properties of carrageenan based biocomposite film and hard capsule, reinforcing filler such as cellulose nanocrystals (CNC) is proposed in this work.

Recently, CNC has gained considerable attention because of its functional properties and renewability; widening the application domain of CNC has become an active area of research. The CNC crystalizes in the form of whiskers, rod shape and needle-like structure (Dufresne, 2012) and could be an attractive polysaccharide-based nanofiller material due to their biodegradability and biocompatibility (Chirayil, Mathew & Thomas, 2014; Samir, Alloin & Dufresne, 2005). Beside, CNC have excellent physical properties such as high specific strength and stiffness, reasonably high aspect ratio, low density (1.6 g/cm) and reactive surfaces with –OH functional groups (Lee, Aitomäki, Berglund, Oksman & Bismarck, 2014; Mohd Amin, Amiralian, Annamalai, Edwards, Chaleat & Martin, 2016; Moon, Martini, Nairn, Simonsen & Youngblood, 2011). Additionally, incorporation of whiskers as reinforcement materials is proven to increase the tensile and mechanical strength in composites (Miranda et al., 2015; Moon et al., 2011); i.e., they can act as a filler and reinforcement material in many applications such as gelatine hydrogels, construction cement paste, polyvinyl alcohol (PVA), and polyurethane composites (Cao, Zavaterri, Youngblood, Moon & Weiss, 2015; Mohd Amin et al., 2016; Ooi, Ahmad & Mohd Amin, 2015; Roohani, Habibi, Belgacem,Ebrahim, Karimi & Dufresne, 2008) and degradable in the human body (Gupta, 2012).

In this article, we show that small amounts of CNC (<2 wt./v%) would act as an excellent reinforcing filler in the carrageenan matrix and would enhance the mechanical properties of the resulting carrageenan film. An optimized composition has a tensile strength of ~33 MPa, tensile strain of 43% and Young's modulus of 1.3 GPa and relatively lower moisture content (~15%) whereas a control sample without CNC has >30% lower tensile strength and tensile strain. The microstructure and mechanical properties of the cellulose toughened carrageenan films are systematically correlated to the rheology of the forming solution. Hard capsules were fabricated using an indigenous machine and examined their usefulness as a drug delivery medium. The hard capsules thereby developed are stable to amoxycillin, ampicillin, and doxycycline antibiotics in 15–17% moisture and showed excellent disintegration behavior in physiological conditions. The capsules were disintegrated in <15 min. These achievements pose promising directions for sustainable materials supply for food and pharmaceutical industries.

2. Materials and methods

2.1. Materials

Semi refined carrageenan (SRC) was purchased from Tacara Sdn. Bhd., Sabah, Malaysia. Food grade crosslinker (CL) 4-methoxybenzyl alcohol, microcrystalline cellulose (MCC), and alginic acid, were obtained from Sigma-Aldrich, USA. The plasticizer polyethylene glycol (PEG) was bought from Merck, Germany. Semi refined carrageenan was analyzed by gel permeation chromatography (GPC) and elemental analysis for molecular weight and sulphur content, respectively. The Active Pharmaceutical Ingredients (API) antibiotics of amoxycillin (500 mg) and ampicillin (500 mg) were purchased from Chemical Company of Malaysia Bhd. (CCM) and doxycycline (100 mg) from Hovid, Malaysia.

2.2. Cellulose nanocrystals (CNC) filler preparation

Approximately 5.0 g of MCC was dispersed in 500 mL of deionized water and stirred overnight. Then, the MCC dispersion was sonicated using a QSonica (Q700, USA) ultrasonicator at an amplitude of 20% for 50 min (Pulse-ON time is 8 s and Pulse-OFF time is 2 s) (Mohd Amin, Annamalai, Morrow & Martin, 2015). After rested for 10 min, three layers of solution could be observed. The bottom layer of the solution was removed, and the solution was left to rest overnight. The top clear layer of the solution was removed until it was reduced to 300 mL to increase the filler concentration. The remaining solution was designated as CNC solution.

2.3. Preparation of carrageenan biocomposite film and hard capsule

Semi refined carrageenan (SRC) and crosslinker (CL) 4-methoxybenzyl alcohol were mechanically stirred with distilled water in a double jacketed glass reactor at 60 °C. Then, CNC solution (0 to 2.0 wt./v%), PEG, and alginic acid were added and mixed for a total of 3 h in order to obtain a homogeneous solution. The samples were labeled as Control, Carra-CNC0.4, Carra-CNC0.8, Carra-CNC1.2, Carra-CNC1.6, and Carra-CNC2.0 where the number represents the wt./v.% of CNC. Next, the solution was casted on a stainless steel tray and evaporated in an oven at 40 °C for 24 h. The dried film was then employed for characterization and analysis. Meanwhile, the carrageenan based hard capsules were fabricated via dipping process using self-fabricated capsule dipping machine with size "1″ capsule shell.

2.4. Molecular weight determination

Molecular weights of SRC were determined by gel-permeation chromatography (GPC) on Agilent 1260 Infinity (Agilent, USA) instrument

and PL aquagel-OH MIXED-H column. Water was used as the eluent for analysis. Approximately 5 mg of SRC was dissolved in 100 mL ultrapure water and the mixture was stirred overnight. Approximately 50 μL of sample solution was injected into GPC column with flow rate of 1.0 mL/min. The refractive index (RI) was analyzed using Agilent PL-LS 15/90 Light Scattering Detector.

2.5. CNC morphology

The CNC were suspended in deionized water at a concentration of 0.1 mg/mL (Mohd Amin et al., 2015). The CNC powder was examined for morphology analysis using field emission scanning electron microscope (FESEM) (Jeol JSM-7800F, Japan). The solution was dropped and dried on the carbon tape overnight. The sample was coated with platinum under vacuum before observation. The sample morphology was studied on FESEM at an accelerating voltage of 5 kV and different magnifications. The carrageenan biocomposite surface film and particle distribution were also observed via the FESEM method.

2.6. Crystallinity analysis

A Rigaku Miniflex X-Ray Diffractometer (Miniflex II, USA) employing Ni filtered Cukα was used to study the degree of crystallinity of CNC. The crystallinity index (CrI) was calculated based on the XRD peak height method, using the following equation, Eq. (1) (El Achaby, Kassab, Barakat & Aboulkas, 2018):

$$CrI = \frac{I_{002} - I_{am}}{I_{002}} \times 100 \quad (1)$$

where I_{002} refers to the maximum intensity of the peak corresponding to plane having the miller indices 002 (2θ= 23°), while I_{am} is the minimal intensity of diffraction of the amorphous phase at $2\theta = 18^{\circ}$.

2.7. Functional group analysis

Fourier transform infrared spectroscopy (FTIR) was conducted to examine chemical bonding occurred in the carrageenan biocomposite film. A Perkin Elmer ATR-FTIR spectrometer (Frontier) was used with the spectra range of 400 to 4000 cm^{-1}. A total of 32 scans were acquired at 0.15 s/scan and with a spectral resolution of 8 cm^{-1}. The spectra were analyzed using OMNIC software.

2.8. Viscosity analysis

The viscosity of carrageenan formulation solutions was analyzed using a Rheometer (Brookfield, Rheo 3000, USA) equipped with LCT 25 4000010 geometry. Approximately 16.5 mL of the sample solution was programmed at speed of 300 rpm and at a constant temperature of 60°C in triplicate.

2.9. Mechanical properties analysis

The tensile strength and elongation at break of 2 cm x 10 cm strips of carrageenan control and composite films were determined using an electronic Universal Testing Machine (VEW 260E, Victor, Malaysia) fitted with a 5 kN load cell. The method was in accordance with ASTM D882-12 and a crosshead speed of 50 mm/min was employed. The tensile machine was operated with an initial grip separation of 50 mm and a crosshead speed of 50 mm/min (Rhim & Wang, 2014).

2.10. Hard capsule disintegration test using basket method

Disintegration test of carrageenan hard capsules was carried out using Pharma Test, Dist. 3, Serial (Germany) at temperature of 37 ± 2 °C, in 600 mL distilled water. This test was conducted in accordance with the U.S. Pharmacopeia (USP) standard (World Health Organization, 2011). The hard capsule was filled with lactose as placebo. One hard capsule was placed in each tube (disintegration tester contains 6 tubes) and the basket rack was positioned in a 1 L beaker of distilled water as immersion fluid which simulates the intestinal fluid (pH ~6–7) (Fallingborg, 1999; World Health Organization, 2011). The hard capsule remained 2.5 cm below the surface of liquid on their upward movement and not closer than 2.5 cm from the bottom of the beaker in their downward movement. To prevent floating hard capsule, a perforated plastic disc was placed on the hard capsule. Disintegration time measurement was started when the hard capsule got into the water and stopped when the placebo was released from the hard capsule and passed the 10-mesh screen. If one or two hard capsules failed to disintegrate completely, test was repeated for additional 12 hard capsules.

2.11. Hard capsule stability test by using antibiotic

Stability test was conducted to study for any potential reaction of antibiotics on the physical stability of carrageenan hard capsule sample. Three active pharmaceutical ingredients (API) antibiotics- amoxycillin 500 mg, ampicillin 500 mg, and doxycycline 100 mg were used for stability test. Each antibiotic was mixed with lactose (placebo). Certain amount of antibiotic-placebo mixture was fully filled into the hard capsule body and closed with the cap. The sample with lactose filled was used as a blank sample. The sample were dusted, cleaned and the physical image was recorded. Then, the samples were stored in amber and screw cap bottle. The bottles were kept for 336 h (14 weeks) in a humidity chamber at temperature of 30 °C and relative humidity (RH) at 75% (ICH Climatic Zone IV) (WHO, 2009). At every 24 h, the capsules were analyzed for any physical observation changes (Vaksman, Daniels, Boyd, Crady, Putcha & Du, 2011).

2.12. Hard capsule cytotoxicity test

The safety of hard capsule samples can be determined by cytotoxicity test (IC 50). The IC 50 test was carried out through measured cell viability using MTT assay after 24 and 48 h of cell treatment with solution that have been exposed with the capsule sample. The cytotoxicity test followed the USP standard. Higher mitochondrial activity shows higher number of viable cells which reflects the conversion of MTT into formazan crystals by living cells. Thus, this assay was used to measure the *in vitro* cytotoxic effects of interest chemical on the cell line.

In this work, HDF cell line was seeded in 96-well plates with a cell density of 0.8×10^5 cells/cm^3 for 48 h or until confluence. The cells were treated with 150 μL/well of:

i Carrageenan biocomposite hard capsule;
ii Gelatine hard capsule (control); and
iii Untreated cells (cells incubated with complete media only).

At 24 and 48 h of post-incubation, 20 μL of MTT solution (5 mg/mL) was added into each well with test samples and cultures continue to incubate for four hours. The medium then was carefully removed and 100 μL of DMSO added to each well to dissolve the formazan crystals formed. The colorimetric changes were quantified by using Infinite M200 NanoQuant (Tecan, Switzerland) microplate reader. The absorbence was measured at the wavelength of 570 nm with a reference wavelength of 630 nm. MTT assay was done in triplicate. The percentage of viable cells was calculated using following equation, Eq. (2):

$$Percentage\ of\ viable\ cells = \frac{Sample\ Absorbance - Blank\ Absorbance}{Solvent\ Control\ Absorbance - Blank\ Absorbance} \times 100 \quad (2)$$

2.13. Statistical analysis

Analysis of variance (ANOVA) was conducted to calculate the confidence level (p-value) for tensile strength, tensile strain, Young's modulus, moisture content, and disintegration time of Control, Carra-CNC0.4,

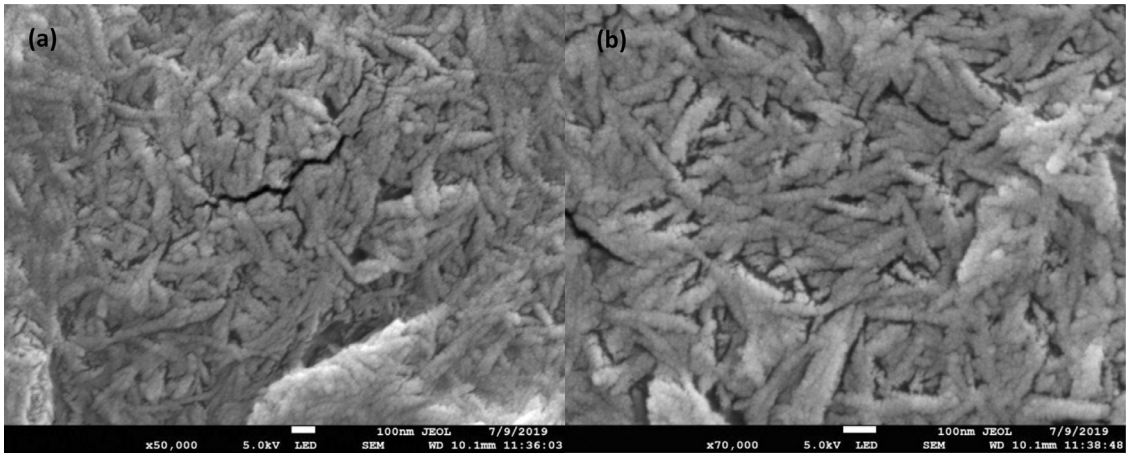

Fig. 1. FESEM images of CNC at magnification of (a) 50,000 × and (b) 70,000 ×.

Carra-CNC0.8, Carra-CNC1.2, Carra-CNC1.6, and Carra-CNC2.0 samples. The level of significance $\alpha = 0.05$ was set for comparison of rejection value. Duncan's post-hoc test using IBM SPSS Version 20.0 software (SPSS Inc., Chicago, USA) was conducted to compare the means value and significant level.

3. Results and discussion

3.1. Semi refined carrageenan (SRC) characterization

The molecular weight of pure SRC, which was characterized using gel permeation chromatography (GPC), is in the 139–236 kDa range. The GPC graph of SRC showed a broad peak at reflective index (RI) of ~5.8. The RI between 5.5 and 6.8 indicates the carrageenan molecular weight (Uno, Omoto, Goto, Asai, Nakamura & Maitani, 2001). The sulphur content of the SRC sample is 1.84%. Sulphur in carrageenan has an important role in physical crosslinking between carrageenan and crosslinker (CL) 4-methoxybenzyl alcohol. This is because the physical crosslinking through hydrogen bonding is suggested to occur at the sulphate region in carrageenan with the hydroxyl group in the CL (Adam, Hamdan, Hana, Bakar, Yusoff & Jose, 2020b).

3.2. CNC characterization

Cellulose nanocrystals (CNC) were prepared via a cleaner approach using a high intensity ultrasonication method from an MCC precursor (Mohd Amin et al., 2015). This solvent free method is suitable for the reinforcement of the carrageenan matrix for potential pharmaceutical and food applications. Fig. 1 shows the FESEM images of the CNC at two typical magnifications. The rod-like or needle-like shape of CNC can be clearly seen with dimensions ranging from ~52 to ~265 nm in length and ~19 to ~71 nm in width, as measured from 200 different particles. The average aspect ratio of the CNC is ~2.7. These results are in agreement with the previous studies reporting CNC preparation via acid hydrolysis and ultrasonication method (Chan, Chia, Zakaria, Ahmad & Dufresne, 2013; Mohd Amin et al., 2015; Tan et al., 2015).

Fig. 2(a) shows the XRD pattern of the CNC; three clear diffraction peaks are observed at 2θ ~15.6°, ~22.7° and ~34.7°, which corresponds to (011), (020), and (030) planes, respectively (Johar, Ahmad & Dufresne, 2012). The crystallinity index (CrI) of cellulose was determined from the XRD peaks employing. The CrI of CNC was ~81.9% whereas that of MCC was ~95% (Mohd Amin et al., 2015). The value was higher than that achieved by acid hydrolysis method in the earlier studies, 75% (Chan, Chia, Zakaria, Ahmad & Dufresne, 2013), 65% (Sulaiman, Chan, Chia, Zakaria, & Jaafar, 2015), and 59% (Johar et al., 2012); and, which employed high pressure homogenization method (36%) (Li et al., 2012). This result indicates that the ultrasonication method adopted here retain the crystal structure of CNC as compared to the acid hydrolysis and other mechanical methods.

3.3. Fourier transforms infrared spectroscopy (FTIR)

The presence of carrageenan in the biocomposite film was analyzed by the FTIR spectra, Fig. 2(b). The peak in Fig. 2(b) at ~925 cm^{-1} is assigned to C-O of 3,6-anhydrogalactose and that at ~842 cm^{-1} refers to C-O-SO_4 on galactose-4-sulphate in carrageenan (Adam et al., 2020b; Dewi, Darmanto, & Ambariyanto, 2012). Peaks at ~1033 and ~1219 cm^{-1} are attributed to glycosidic linkage and ester sulphate stretching of carrageenan, respectively (Hamdan, Lakashmi, Mohd Amin & Adam, 2020; Hosseinzadeh, 2009). Meanwhile, the absorbence peaks at ~892 cm^{-1} refers to the glycosidic C-H deformation with ring vibration and OH bending, which are characteristic to cellulose (Ooi et al., 2015). Thus, the cellulose peaks around ~3496 cm^{-1} (O-H), ~1110 cm^{-1} (C-O of secondary alcohol), and ~2868 and ~2970 cm^{-1} (C-H from CH_2) were detected.

Absorption peaks at ~3331, ~2917 and ~1150 cm^{-1}, represents the functional groups belong to the OH stretching, vibration C-H stretching groups, and C-O of secondary alcohol of the carrageenan-CNC biocomposite mixture, respectively (Missoum, Martoïa, Belgacem & Bras, 2013). The intensity of these functional groups increased with the increase in the CNC fraction in the composite. Increase in the OH stretching suggested that the physical crosslinking through hydrogen bonding occurred in the carrageenan biocomposite film, which in turn is due to CNC filler incorporation. This physical crosslinking is expected to increase in mechanical properties of the sample.

The FTIR spectra of the Carra-CNC1.6 composite film showed absorbence peaks from both carrageenan and CNC; no new peaks other than those of carrageenan and CNC were observed. This result implies that the crosslinker used in this study crosslinked the carrageenan chains without disrupting the CNC structure. The peaks belong to CNC at ~1150, ~1033 and ~892 cm^{-1} could also be observed in the FTIR spectrum for the Carra-CNC1.6 film. This demonstrates that the CNC and carrageenan retain their individual chemical structures during the various stages of materials preparation and that a composite is formed.

3.4. Film surface morphology analysis

The FESEM images displaying the morphology and dispersion of CNC in carrageenan matrix are shown in Fig. 3. The results showed that in the Carra-CNC1.6 film (Fig. 3a and b), there are fine distribution of CNC in the carrageenan matrix, which resulted in a smooth film. While, a

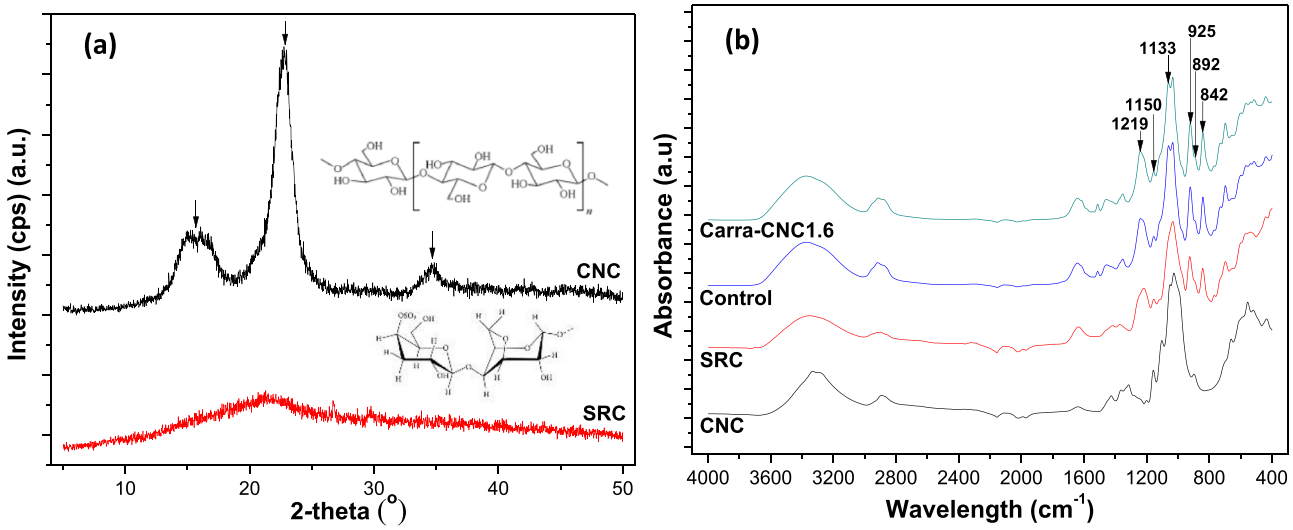

Fig. 2. (a) XRD pattern of semi refined carrageenan (SRC) and CNC obtained via ultrasonication (Börjesson & Westman, 2016; Hezaveh & Muhamad, 2012) and (b) FTIR spectrum for CNC, semi refined carrageenan (SRC), Control film and Carra-CNC1.6 film.

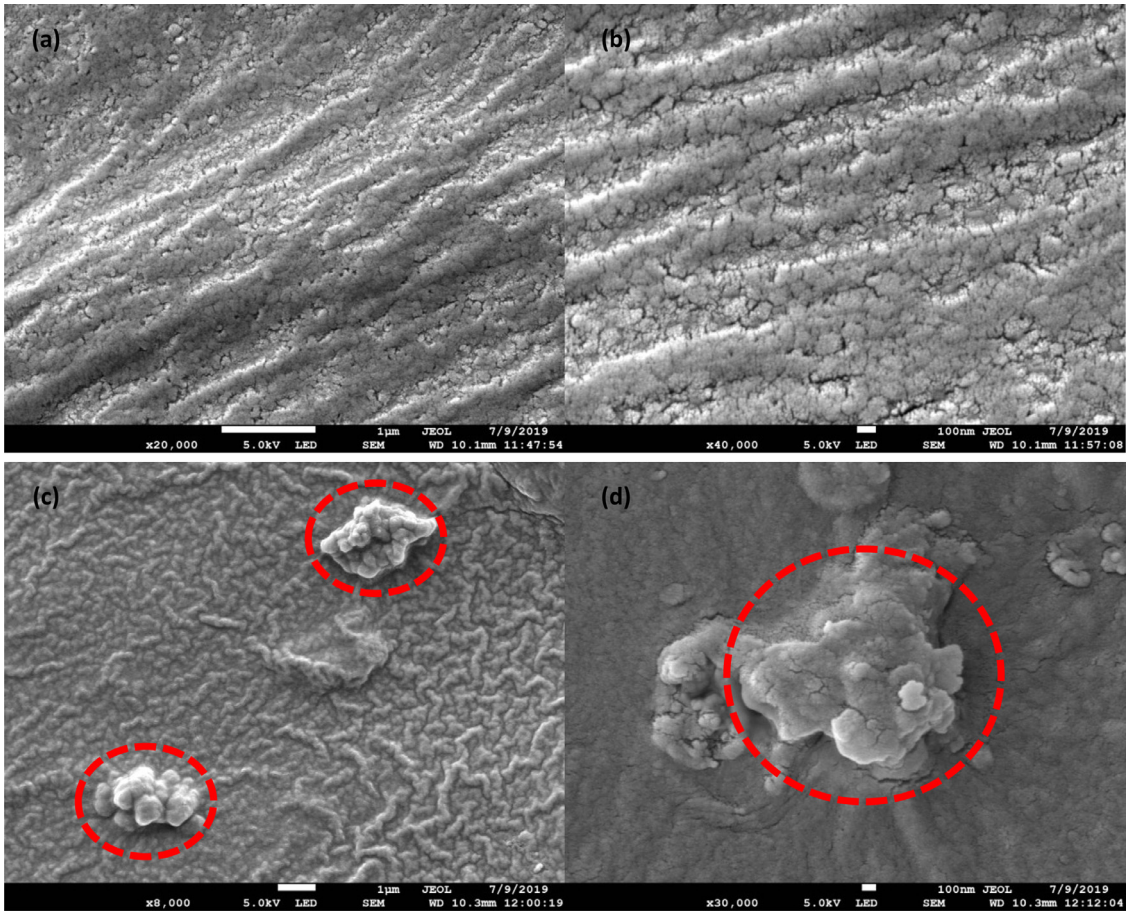

Fig. 3. FESEM images of Carra-CNC1.6 (a and b) and Carra-CNC2.0 (c and d) at different magnification. Circle indicates the agglomeration of CNC.

rough film surface may be linked to a greater progress of aggregation and creaming process in the carrageenan matrix (Annisa, Satriana, Supardan, Wan Mustapha & Arpi, 2016). Aggregation and creaming occur during the drying step, thereby causing irregularities on the film surface. The results show that CNC was not agglomerated in the matrix, thereby proving that Carra-CNC1.6 is the optimum concentration of CNC for carrageenan-CNC hard capsule development.

Upon analysis, some agglomerations were observed in the film solution suspension because of self-association of CNC via hydrogen bonding of the filler (Flauzino Neto, Silvério, Dantas, & Pasquini, 2013; Zarina & Ahmad, 2015). Aggregation occurs due to inter-particle interaction between the CNC particles thus limit the potential of mechanical reinforcement properties (Dufresne, 2013). When the acid hydrolysis is employed, the CNC end product contains sulphate group in the result-

Table 1
Mechanical properties and moisture content of carrageenan film and its nanocomposite.

Sample	Tensile Strength (MPa)	Tensile Strain (%)	Young's Modulus (GPa)	Moisture Content (%)
Control	20.95 ± 1.607 [a]	35.93 ± 7.60 [a]	1.89 ± 0.41 [a]	14.77 ± 1.2 [a]
Carra-CNC0.4	24.67 ± 0.825 [b]	34.46 ± 4.45 [a]	1.50 ± 0.71 [a]	17.03 ± 0.76 [a,b]
Carra-CNC0.8	26.44 ± 1.214 [b,c]	19.13 ± 6.13 [b]	1.75 ± 0.60 [a]	17.45 ± 1.73 [b]
Carra-CNC1.2	28.15 ± 0.562 [c]	53.81 ± 6.38 [c]	1.12 ± 0.55 [a]	16.25 ± 0.88 [a,b]
Carra-CNC1.6	33.08 ± 1.186 [d]	41.33 ± 9.71 [c]	1.29 ± 0.06 [a]	15.49 ± 1.03 [a,b]
Carra-CNC2.0	11.82 ± 1.287 [e]	12.70 ± 4.38 [b]	1.60 ± 1.13 [a]	16.00 ± 1.85 [a,b]

*Data was statistically analyzed using Duncan post-hoc test and Single Factor ANOVA, α<0.05. Different letters represent significant difference between the group and vice versa. While, letters a,b or b,c represent no significant different between the group.

ing structure, which in turn increase the stability of the CNC in water (Brinchi, Cotana, Fortunati & Kenny, 2013).

In Carra-CNC2.0 shown in Fig. 3 (c & d), there were large agglomerates of CNC (in dotted circle) in the matrix, forming strong bonding among themselves (Cao, Zavattieri, Youngblood, Moon & Weiss, 2016). Agglomeration could increase the size of nanoparticles such as CNC and led to non-uniform distribution of particles and phase separation in the matrix (Dufresne, 2012; Samir et al., 2005). As a result, it intensify brittleness in the film due to stress concentration, thereby leading to poor mechanical properties as proven by a decrease in tensile strength (Khoshkava & Kamal, 2014).

3.5. Mechanical and rheological properties

Mechanical properties of biocomposite films are highly associated with the nature of film-forming materials, functional group interaction, and the processing methods (Galus, Mathieu, Lenart & Debeaufort, 2012; Tian, Xu, Yang & Guo, 2011).

Table 1 shows the tensile properties of carrageenan film and its nanocomposites. The addition of CNC significantly increased the tensile strength and the tensile strain at probability of rejection *p*-value of 9.47×10^{-10}, which is less than 0.05. The addition of 1.6 wt./v% of CNC increased the tensile strength from ~20.95 MPa to ~33.08 MPa and tensile strain from ~35.9% to ~41.3%. These enhancements certainly suggest good CNC dispersion in the carrageenan matrix and also good adhesion at the filler-matrix interface, (Frone et al., 2011) as proven by FESEM (Fig. 3) which reflects that CNC is acting as a load bearing components in carrageenan matrix.

Mechanical properties of a composite depend on the interfacial interactions between composite matrix and reinforcing filler (Qian et al., 2017). Due to high interfacial area and energy, the CNC most likely interacted each other throughout the matrix, thus increased the reinforcing effect (Abdollahi, Alboofetileh, Rezaei & Behrooz, 2013). The result may also be due to high surface area of CNC. The high surface area may induce a strong interfacial interaction through hydrogen or ionic bonds between the nanomaterial and the composite matrix (Dan et al., 2016). As a result, changes in the intra and intermolecular interactions between molecules in the carrageenan matrix affect the mechanical properties of carrageenan biocomposite (Kaya et al., 2018).

On the other hand, addition of CNC beyond 1.6 wt./v% reduced the mechanical properties of the films due to formation of aggregates of CNC in the composite matrix, as observed in the FESEM analysis. Larger amounts of CNC in the composite matrix is likely to reduce the interaction between CNC and the matrix, without penetrating the double helix structure of the carrageenan. This is due to the non-uniform stress distribution in the film, leading to reduction of strength of the composite film (Qian et al., 2017; Siqueira, Bras & Dufresne, 2010). This fact is also proven in the previous literature, with 3 wt.% of CNC addition has decreased the mechanical properties of polymer matrix due to CNC agglomeration (Pinheiro et al., 2017).

The Young's modulus of nanocomposite films was reduced from ~1.9 GPa to ~1.3 GPa as shown in Table 1 with increase in the CNC content. However, the increment of CNC in the carrageenan matrix did not affect the biocomposite elongation significantly, as shown in the Table 1. It is expected that plasticization was occurred due to hygroscopic properties of CNC (Dufresne, 2012; Savadekar, Karande, Vigneshwaran, Bharimalla & Mhaske, 2012). This result suggested that the occurrence of plasticization process enhanced the carrageenan biocomposite network via hydrogen bonding, which in turn led to the increase in the flexibility of the biocomposite film (Farhan & Hani, 2017). Hygroscopic properties of CNC were evaluated by moisture content determination. The CNC has increased the ability of carrageenan biocomposite film to absorb or release more water. Moisture content determination result in Table 1 proves that the addition of CNC in carrageenan matrix significantly increased the water content (Samir et al., 2005). The decrease in modulus may be caused by the destruction of the fine network structure in carrageenan itself.

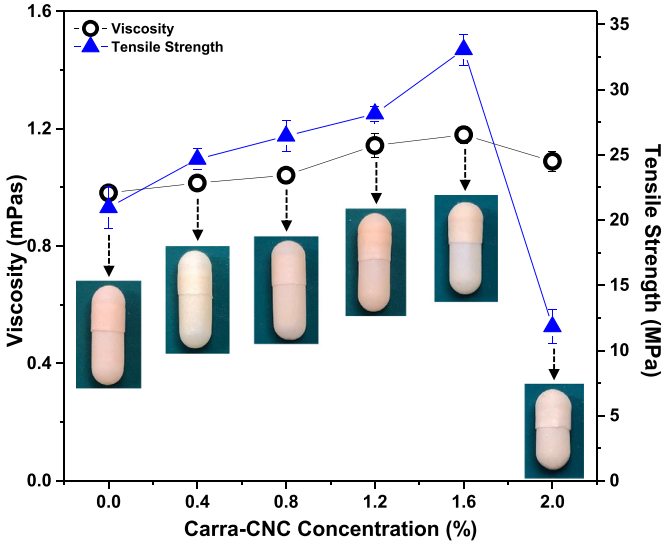

Fig. 4. Viscosity of carrageenan solution, tensile strength of sample film, and physical appearance of carrageenan hard capsule.

The rheology of carrageenan matrix by addition of CNC also can be observed through the viscosity of the solution; the viscosity is an important parameter to determine the thickness of dried materials in a processing line (Fakharian et al., 2015). As shown in Fig. 4, the trends for solution viscosity and tensile strength of films increased as the concentration of CNC increased from 0 to 1.6 wt./v.%, which is in accordance with changes in tensile properties. Fig. 4 shows the physical appearance of hard capsules produced from the solution viscosity ranges of ~0.9 mPas to ~1.2 mPas.

Without the CNC addition, the viscosity and tensile strength were ~0.98 mPas and ~20.95 MPa, respectively, and these values were increased to ~1.17 mPas and ~33.08 MPa at 1.6 wt./v.% addition of CNC. The percentage of increase in strength from Control sample to 1.6

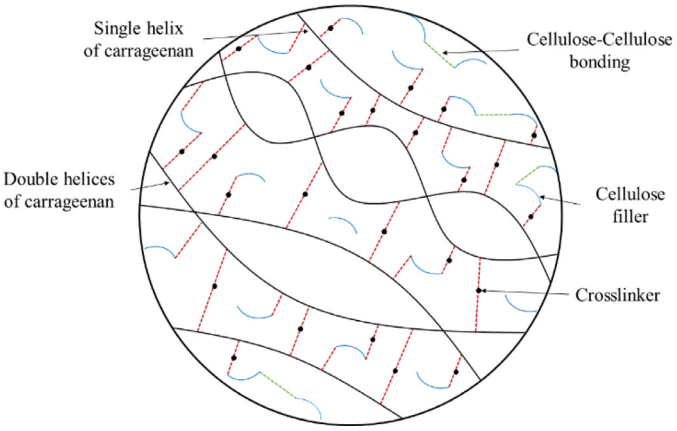

Fig. 5. Illustration showing the mechanism of CNC as a filler in the carrageenan matrix.

Table 2
Hard capsule disintegration time of carrageenan film and its nanocomposites.

Sample	Disintegration Time (min)
Control	11.96 ± 0.50 [a,b]
Carra-CNC0.4	12.38 ± 0.55 [b,d]
Carra-CNC0.8	11.85 ± 0.58 [a,b]
Carra-CNC1.2	8.39 ± 0.53 [c]
Carra-CNC1.6	11.27 ± 0.15 [a]
Carra-CNC2.0	13.12 ± 0.16 [d]

*Data was statistically analyzed using Duncan post-hoc test and Single Factor ANOVA, α<0.05. Different letters represent significant difference and vice versa. While, letters a,b or b,d represent no significant different between the group.

wt./v.% loading is ~58%. These results indicate that even at relatively low volume percentages of CNC, it could significantly increase the tensile strength of the carrageenan film. However, at Carra-CNC2.0, the viscosity and tensile strength decreased to ~1.08 mPas and ~11.82 MPa, respectively, due to agglomeration of CNC in the carrageenan matrix. Due to the aggregation, the area for water molecule to access and disperse in the carrageenan biocomposite decreases (Wang, Rademacher, Sedlmeyer & Kulozik, 2005). Therefore, the formulation solution was not homogenous and decreased the tensile strength.

Based on all these characterizations of the carrageenan film and its nanocomposites, a mechanism for carrageenan, CNC, and crosslinker dispersion is proposed and illustrated in Fig. 5. The CNC act as filler in the carrageenan matrix, which fill the void space between single and double helix of carrageenan. The 4-methoxybenzyl alcohol, which acts as a crosslinker used in this study, crosslinked carrageenan and CNC and it is proven by the FTIR analysis (Fig. 2b). A physical crosslinking has occurred between the glyoxylic acid crosslinker and carrageenan through the hydrogen bond interaction to produce a stable film, which also can promote disintegration in physiological conditions (Abu Bakar & Adam, 2017).

3.6. Hard capsule disintegration and stability test by using antibiotic

All the carrageenan biocomposite hard capsule disintegrated in less than 15 min in water at pH 7 as summarized in Table 2. A disintegration time of less than 15 min is required for a hard capsule for drug delivery carrier application (World Health Organization, 2011). Previous research by Hamdan, Adam, & Mohd Amin, 2018 showed that modification of mixing time in the carrageenan formulation solution increased the hard capsule disintegration time, but still within the acceptance range as regulated by the international standard.

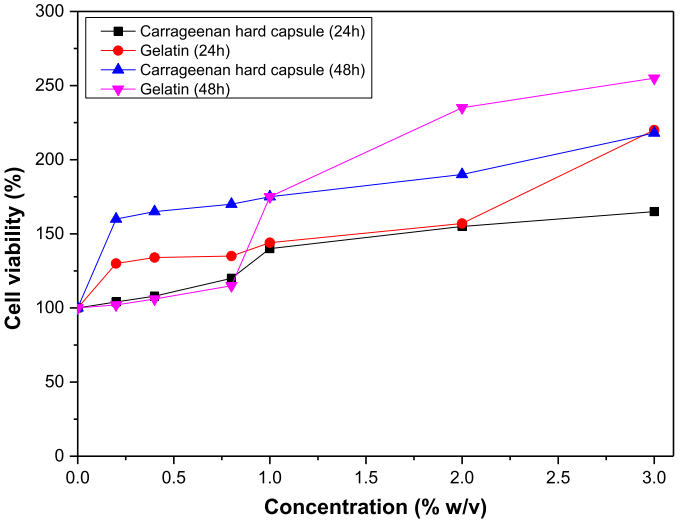

Fig. 6. Cell viability was measured using MTT assay at 24 and 48 h of cell treatment for control and anisyl crosslinked sample.

In addition, the hard capsule should comply a few physical properties such as hardness, remains intact, no changes in color and odor, and not leaking during the stability test (Tingstad, 1964). Table 3 shows the physical properties of carrageenan hard capsule stored for 14 days in a humidity chamber. After 14 days of storage in humidity chamber, all the hard capsules showed no physical changes in color and odor, remained intact, and did not stick. Furthermore, the antibiotic powders did not leak out of the hard capsule. The results suggested that the application of acidic antibiotics did not affect the physical properties of carrageenan hard capsule.

3.7. Cytotoxicity study of carrageenan hard capsule

The cytotoxicity studies were been carried out to measure the toxicity of hard capsule application for the carrageenan biocomposite which was crosslinked with anisyl crosslinker. The anisyl crosslinker is a compound extracted from Genus Acacallis which is normally used as essential oil in the cosmetic industry. As shown in Fig. 6, the MTT assay carrageenan hard capsule shows an increase of cell viability by 50% at 24 h and 100% cell viability at 48 h, which represents no toxic chemical released to the cell structure. It is quite similar to commercial gelatine hard capsule which is used for a comparison purpose (Fig. 6). Total mitochondrial activity is related to the number of viable cells and this assay can measure the *in vitro* cytotoxic effects of drugs or chemical on the cell line (Van Meerloo, Kaspers & Cloos, 2011). The MTT assay results show that the carrageenan biocomposite hard capsule is safe for consumption as it does not give negative impact to HeLa cell by releasing any toxic chemical.

4. Conclusion

We have developed a renewable plant-based material system to be used as a viable alternative for animal protein-based gelatine as a drug delivery material system. In this work, we have shown that cellulose nanocrystals (CNC) could be a viable filler to significantly increase the mechanical strength of carrageenan film for their application as a hard capsule. The CNC was used as a filler, they are rod shaped with length in the ~52–265 nm and diameter in the ~19–71 nm ranges and had an average aspect ratio of ~2.7. The CNC toughened carrageenan had a tensile strength up to 58% compared to the films without the filler. Furthermore, addition of CNC favorably decreased the Young's modulus for the successful fabrication of hard capsules. Among the many compositions investigated, carrageenan films containing 1.6 wt./v% of CNC

Table 3
Antibiotic stability test of carrageenan hard capsule.

Antibiotic name	pKa of antibiotics	Physical properties observation after 14 days	Hard capsule sample image
Blank	–	No changes observed in hard capsule and powder color and odor	
Amoxycillin 500 mg	2.4	No changes observed in hard capsule and powder color and odor	
Ampicillin 500 mg	2.5	No changes observed in hard capsule and powder color and odor	
Doxycycline 100 mg	3.5	No changes observed in hard capsule and powder color and odor	

is optimum for producing stable films. This composition also had the favorable rheological properties for formation of stable hard capsules. Field emission scanning micrographs showed that further increase of the CNC content in the carrageenan decreased the tensile strength due to agglomeration of CNC in the film matrix. A disintegration test conducted on the stability of the hard capsule at physiological conditions showed complete disintegration in less than 15 min. The hard capsule passed the stability test with three types of antibiotics, which suggested that it is suitable for application as a drug delivery system. This study also indicated that this solvent free CNC is suitable for food and pharmaceutical application especially incorporation with carrageenan.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

The authors would like to thank the Center of Excellence for Advanced Research in Fluid Flow (CARIFF), Universiti Malaysia Pahang for providing facilities for this study.

Funding

This work was supported by Fundamental Research Grant Scheme (RDU1901111 and FRGS/1/2019/TK05/UMP/02/2), UMP-Community Technology Solution Platform Fund (UIC160906), and eScience Fund (02-01-16-SF0086).

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