Synthesis and characterization of thymol-loaded lauryl glycol chitosan for pesticide formulation

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Abstract. Thymol is a hydrophobic active ingredient and widely used as an alternative pesticide for pest control. However, due to its low solubility in water, a water-soluble carrier is required in its formulation. Therefore in this study, the potential of an amphiphilic chitosan, namely lauryl glycol chitosan (LGC) as a carrier to encapsulate and release thymol was evaluated. The physical and chemical properties of LGC were characterized using Fourier Transform Infrared (FTIR) Spectrometer, Proton Nuclear Magnetic Resonance (1H NMR) Spectrometer, UV-Visible Spectrophotometer, Thermogravimetric Analyser (TGA) and Transmission Electron Microscope (TEM). A Fluorescence Spectrometer was used to determine the critical micelle concentration (CMC) value of LGC in aqueous solution. The encapsulation efficiency of LGC for thymol was determined by using a High Performance Liquid Chromatography (HPLC). The FTIR and 1H NMR analyses confirmed the structure of the synthesized LGC. It was noted that the addition of lauryl and glycol groups to the chitosan backbone has enhanced the solubility properties of chitosan in neutral and basic media. TEM observation confirmed that LGC could form self-aggregates in the solution with a spherical shape. The CMC value determined for LGC was 0.008 mg/mL. LGC exhibited good affinity towards thymol with an encapsulation efficiency of 58%. The findings from in vitro release study showed that the LGC could prolong the release of thymol from its micelles. Results from this study highlight that LGC possesses great characteristics to be further developed as a promising carrier in the pesticide formulation.

1. Introduction
Thymol, a natural monoterpene phenol isolated from certain Lamiaceae families such as thyme and oregano has good acaricidal, antimicrobial, bactericidal and insecticidal properties [1]. Due to its low level of residue and risks in the environment, it has been employed as an alternative pesticide to fights insects, mites, ticks and bacteria [1,2]. However, the application of thymol as a pesticide has a drawback as it exhibits low water solubility (0.9 mg/mL) [3,4]. Therefore, in order to dissolve thymol, a large amount of organic solvents is frequently utilized in its current formulation [3]. Nevertheless, there are various negative side effects on the usage of organic solvents in pesticide production [5]. Organic solvents in the pesticide formulations can leach and volatile to the environment thus can end up in the atmosphere, surface water and adsorb into soil or sediment [5,6]. Consequently, it can contaminated the environment and caused health hazard to human and animals [5,6].

New trends of pesticides formulation have lean towards reducing the application of organic solvent, control the release of pesticide and improve the activity of pesticide active ingredients [7]. In recent years, the application of polymeric micelles particularly amphiphilic chitosan derivatives as a carrier
in controlled release formulation for hydrophobic pesticides has received a lot of attention from scientist [5,8]. By conjugating hydrophilic and hydrophobic segments on the polymer, the polymer will exhibits amphiphilic properties which can self-assemble in aqueous solution. The hydrophobic segment of the amphiphilic chitosan derivatives can encapsulate the hydrophobic pesticide in its core, while the hydrophilic segment can interact with water [8,9]. Therefore, its solubility in aqueous solution can be enhanced and the excessive use of organic can be reduced. In addition, the encapsulation of thymol in amphiphilic chitosan derivative could protect the pesticide from degradation and premature leakage before arrival at the target site [8,9]. The aims of the present study were to assess the ability of an amphiphilic chitosan derivative, lauryl glycol chitosan (LGC) to encapsulate thymol and its potential as a carrier for pesticide formulation.

2. Materials and methods

2.1. Preparation of LGC

Glycol chitosan (GC) was prepared following a procedure from our previous work [10]. Meanwhile, lauryl glycol chitosan (LGC) was synthesized using similar methods to that proposed by Mobarak and Abdullah [11] and Zhang et al. [12], with some modifications. First, 2 g of GC was dissolved in the 100 mL mixture of deionized water and methanol (1:1 v/v) to form GC solution. Then, about 2 g of lauric aldehyde was slowly added to the GC solution. The mixture was reacted under horizontal agitation (100 rpm) for 24 hours. After 24 hours, 0.5 g of sodium borohydride that was dissolved in 5 mL of water was slowly dropped to the solution. The solution was reacted for further 24 hours, before neutralized with HCl solution. The resulting solution was dialyzed against ethanol solution (25% v/v) for 3 days before lyophilized to produce solid LGC.

2.2. Preparation of thymol-loaded LGC

Thymol-loaded LGC was prepared by a modified version of the procedure suggested by Lao et al., using reverse micelles technique [13]. For this experiment, thymol solution (10 mg/mL) was prepared by dissolving an appropriate amount of thymol crystal in methanol. Briefly, the synthesized LGC was dissolved in the thymol solution at the weight ratio of 100 to 1 (w/w). Then, deionized water at the volume of five-folds of the initial volume of thymol solution was added slowly to the solution. The solution was gently agitated for 2 hours before centrifuged for 10 minutes at speed of 1,000 rpm. The resulting solution was then filtered with 0.45 μm pore-size membrane.

An Agilent 1200 Infinity High Performance Liquid Chromatography (HPLC) was employed to determine the amount of thymol that successfully encapsulated by LGC. The mobile phase used for the analysis was a mixture of acetonitrile (HPLC grade) and deionized water at a ratio of 70 to 30 (v/v). The column used for the analysis was an Agilent Poroshell 120 C18 (50 mm x 4.6 mm, 2.7 μm) and its temperature was set at 40 °C. The injection volume for the sample was set at 10.0 μL, while the flow rate of the system was set at 1.0 mL/min. The analysis was carried out at a wavelength of 204 nm. The percentage of encapsulation efficiency (%EE) of thymol by LGC was calculated following Equation 1 [14]:

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%EE = \frac{\text{Amount of thymol in LGC}}{\text{Total amount of thymol used}} \times 100
\]

2.3. Characterization study

Fourier Transform Infrared (FTIR) spectra of chitosan and LGC were acquired from a Thermo Nicolet 6700 ATR-FTIR Spectrometer. The scanning range was from 4000 to 400 cm\(^{-1}\), at 32 cumulative scans. A Jeol JNM-ECX-500 Nuclear Magnetic Resonance Spectrometer was utilized to record the Proton Nuclear Magnetic Resonance (\(^1\)H NMR) spectrum of LGC. For the \(^1\)H NMR analysis, acetic acid-d was used as a solvent to dissolve LGC. Thermogravimetry analysis (TGA) was carried out by using a TGA/DSC 1 Mettler Toledo Analyser to identify the thermal decomposition pattern of chitosan and LGC. The analysis was performed from 25 to 900 °C, at a heating rate of 10 °C/min under argon gas (flow rate of 20 mL/min). The solubility properties of chitosan and LGC in different pH was determined as a function of the percentage of transmittance according to a method proposed
by Kubota et al. [15], with some modification. For this analysis, the sample solution (2 mg/mL) was prepared by dissolving LGC in 2% (v/v) HCl. The analysis was conducted using an Agilent Cary 60 UV-Visible Spectrophotometer at a wavelength of 600 nm. The critical micelle concentration (CMC) of LGC was determined by using a spectrofluorometer (Agilent Cary Eclipse Fluorescence Spectrometer), with pyrene was utilized as a fluorescence probe [14]. For the CMC study, pyrene was first dissolved in the methanol to prepare a pyrene solution with a concentration of 1.25 x 10^{-3} mg/mL. Then, pyrene solution (1 mL) was transferred into a vial, before methanol was completely eliminated by evaporation. After that, 6 mL of LGC solution at concentration varied from 1.0 x 10^{-4} to 1.0 mg/mL was added individually to the vial. The mixture was then sonicated for 10 minutes. Lastly, the pyrene emission spectra of the samples were scanned from a wavelength of 344 to 700 nm, with excitation wavelength was set at 334 nm. For this experiment, both opening slits of the emission and excitation was set at 5 nm. Transmission electron microscopy (TEM) was used to examine internal morphology of LGC. The study was carried out using a Hitachi SU 8020 UHR High Resolution Field Emission Scanning Electron Microscope, operated at 20 kV.

2.4. In vitro release kinetics studies for thymol-loaded LGC micelles
The in vitro release of thymol from LGC micelles was studied through dialysis method in 0.1 M phosphate buffered saline (PBS) solution at pH 7.4. For this study, 5 mg of thymol-loaded LGC was dissolved in 3 mL PBS solution and placed in a dialysis bag that has molecular weight cut off 14,000 D. Then, it was introduced into a beaker containing 250 mL of PBS solution. The system was maintained at room temperature under continuous stirring (100 rpm). To determine the amount of thymol released from LGC micelles, 3 mL of PBS solution was periodically withdrawn from the beaker. After each withdrawal, 3 mL of fresh PBS was added into the beaker to maintain the volume of PBS solution at 250 mL. The thymol released was assayed by using an Agilent Cary 60 UV-Visible Spectrophotometer at 204 nm. The release study of thymol was carried out in triplicates.

The release data of thymol can be mathematically modeled to obtain information concerning the release mechanism. Four kinetic models, namely zero-order, first-order, Higuchi and Korsemeyer-Peppas kinetic models were applied to the thymol’s release data to deduce the mechanism involved in the release of thymol from LGC micelles [16,17].

3. Results and discussion
3.1. FTIR analysis
The FTIR analysis was used to confirm the chemical structure of chitosan and LGC. Figure 1 presents the FTIR spectra of chitosan and LGC. Figure 1(a) shows the basic characteristics of chitosan at 3464 cm^{-1} (OH and N-H stretches), 2951 and 2887 cm^{-1} (–C-H stretches), 1664 cm^{-1} (C=O stretch), 1596 cm^{-1} (N-H bend), 1438 and 1383 cm^{-1} (C-N stretches) and 1153 and 1081 cm^{-1} (C-O stretches) [14,18]. Meanwhile for LGC, when comparing to the FTIR spectrum of chitosan (figure 1(a)), the intensity of peaks related to –C-H stretching vibration (2923 and 2853 cm^{-1}) in the FTIR spectrum of LGC (figure 1(b)) were evidently increased, revealing the presence of the long alkyl chain from the hydrophobic lauryl group and methylene groups of glycol in the chitosan derivatives [11,19]. It was observed that the absorption bands related to hydroxyl groups (3308 and 1054 cm^{-1}) shifted and became more intense due to the conjugation of glycol groups [14]. It also can be noted that the intensity of absorption bands related to the amino group (3308, 1456 and 1416 cm^{-1}) became relatively weaker. It may be due to the formation of amide linkage between GC and lauryl group, which proved the substitution of lauryl to amino group of chitosan [13,19].
3.2. \(^1\text{H NMR analysis}\)

The formation of LGC was further confirmed by characterization with \(^1\text{H NMR analysis}.\) Figure 2 shows the \(^1\text{H NMR spectrum of LGC}.\) To interpret the spectrum, the solvent peak that appeared at 2.02 ppm was used as a reference. As presented in figure 2, the spectrum of LGC exhibited a signal at the chemical shift of 0.87 ppm, which attributed to the methyl hydrogen of lauryl (-NH(CH\(_2\))\(_{11}\)CH\(_3\)) \([11,20]\). The signals that detected at the chemical shifts of 1.52 to 1.57 ppm could be associated with methane hydrogen of lauryl, (-NH(CH\(_2\))\(_{11}\)CH\(_3\)) \([11,20]\). Besides that, the peaks observed at the chemical shifts of 3.63 to 3.99 ppm could be to overlap signals from proton related to glycol group and chitosan \([14]\).

3.3. \text{TGA analysis}\)

TGA analysis was conducted to study the thermal decomposition pattern of chitosan and LGC. The TGA thermograms of chitosan and LGC are displayed in figure 3. The thermal decomposition of chitosan progressed through two decomposition stages. The initial decomposition event was observed from 27 to 141 °C, due to vaporization of water bound to chitosan \([21]\). The second decomposition event occurred at the temperature between 235 to 449 °C, with 46% of the weight was a loss at this stage. This might due to the dehydration of the polysaccharide ring and decomposition of chitosan polymer \([21]\). Similar to chitosan, the thermal decomposition of LGC also takes place at two stages. At the initial decomposition stage that occurred at the temperature between 30 and 81 °C, LGC
showed a weight loss of 4% due loss of residue water in LGC [21,22]. Meanwhile, the LGC exhibits rapid weight loss (76%) at the second stage (165 to 560 °C) was due to the degradation of the polymer and glycosidic bond [21,22]. The results from TGA analysis demonstrate that the introduction of lauryl and glycol groups onto chitosan have enhanced its thermal stability.

3.4. Solubility study

The solubility of chitosan and LGC in acidic, neutral and basic media were assessed based on their light transmittance (%) at different pH. Figure 4 shows the solubility trends on chitosan and LGC at pH 1.0 to 13.0. Based on figure 4, the solubility of chitosan was notably depended on the pH of the dissolution media. For example, chitosan has good solubility in the acidic medium as the values of percentage transmittance (%T) at pH 1.0 to 6.0 are nearly 100%. However, chitosan showed poor solubility in both neutral and basic media, as %T at pH 7.0 to 13.0 varies from 29 to 48%. The poor solubility of chitosan at aforementioned media maybe due to the rigidity of the chitosan structure, which owed to the presence of strong intra- and/or intermolecular hydrogen bonds [23]. In contrast to chitosan, LGC exhibits excellent solubility in all media with %T ranged from 70 to 82%. The conjugation of the hydrophilic segment (glycol group) to chitosan has advocated the formation of hydrogen bonds between the polymer and hydrogen atom in water, thus subsequently leading to the enhancement of solubility for chitosan [23]. Nonetheless, slight solubility observed on LGC at pH 4.0. A probable reason for the observation is the formation of aggregation caused by highly acetylated chain segments in LGC solution [24]. This also could occur due to the formation of the amide bond between alkyl groups [24]. Overall, these results highlighted that the LGC endowed important characteristic as an ideal carrier for hydrophobic pesticide active ingredient in aqueous solution.

3.5. Critical micelles concentration

The ability of LGC to form micelles in aqueous solution was monitored by using fluorescence probe studies. Pyrene has a strong hydrophobic characteristic, thus resulted in poor solubility and self-quenching in an aqueous solution [25]. However, pyrene will emits strong fluorescence radiation if the hydrophobic micro-domains are a presence in aqueous solution as it has tendency to stay close to or lies inside these micro-domains [25]. In general, CMC is a threshold concentration when an amphiphilic molecule possibly undergoes self-aggregation by intra- and/or intermolecular association [25]. The intensity of the first and third peaks of pyrene emission spectra at 373 and 392 nm, respectively are highly sensitive to the change in polarity of its microenvironment [13,25]. Hence, the ratio of the I373/I392 versus logarithm concentrations of LGC in aqueous solution can be used to determine CMC value [13,25]. The CMC value of LGC that was determined by interception of two line from the aforementioned graph was 0.008 mg/mL. This value was lower than the CMC value of low molecular-weight surfactant such as sodium dodecyl sulfate (2.3 mg/mL) [26]. In addition, the CMC value of LGC is comparable or lower than some others polymeric micelles likes N-lauryl-N,O-
carboxymethyl chitosan (0.063 mg/mL) [27] and \(N\)-mercapto acetyl-\(N'\)-octyl-\(O,N'\)-glycol chitosan (0.03278 ± 0.00589 mg/mL) [28]. The result suggests that LGC micelles are stable in aqueous solution, even after dilution and might be able to retain thymol in its micelles for a longer period.

### 3.6. TEM analysis

TEM analysis was adapted in order to examine the internal morphology and directly measure the size of the LGC micelles. Figure 5 displays the TEM micrographs of LGC at different magnifications, namely 10,000x and 100,000x. From figure 5, the TEM images showed that LGC micelles have a core-shell configuration, with the shape of the micelles almost spherical. The size of the LGC micelles (dry state) are ranged from 39.7 to 53.6 nm.

[Figure 5. TEM images of LGC at (a) 10,000x and (b) 100,000x magnifications.]

### 3.7. Encapsulation efficiency of thymol into LGC micelles

The efficacy of LGC to encapsulate thymol in its micelles was evaluated in terms of percentage of encapsulation efficiency (%EE). In this study, thymol was successfully encapsulated in the LGC micelles using reverse micelle technique. The LGC shows good %EE as it can successfully encapsulated thymol in its micelles for about 58%. The good %EE value suggests that thymol has a good affinity towards hydrophobic segments (lauryl group) in LGC [14]. The encapsulation efficiency of LGC to encapsulate thymol was comparable with other polymers. For example, thymol encapsulated by polylactide microcapsules has an encapsulation efficiency of 55.02% [29]. Meanwhile, sodium caseinate has encapsulation efficiency of 72.1 to 92.3% [3].

### 3.8. In vitro release and kinetics studies of thymol-loaded LGC micelles

To evaluate the ability of LGC as a carrier for controlled release system, the release study of thymol was carried out. The in vitro release profile of free thymol and thymol-loaded LGC in PBS solution is presented in figure 6. Notably, the encapsulation of thymol by LGC can effectively improve the release profile of the hydrophobic pesticide. The LGC was able to prolong the release of thymol in PBS solution up to 90 hours. In contrast, free thymol solution showed complete release from dialysis bag in 38 hours. The controlled release properties of thymol from LGC micelle suggest that LGC might be able to protect and improve the pesticide availability on target for a longer period.

The mechanism for the release of a solute from polymeric matrices usually influenced by several factors such as the desorption of the solute from the surface, the diffusion of the solute through the pore or polymeric wall and dissolution of the polymeric wall [30]. The mathematical models can be applied to provide information on the release mechanism of pesticide from the polymer matrices [16,30]. In this study, the release data of thymol from LGC micelles were individually fitted to zero-order, first-order, Higuchi and Korsmeyer-Peppas kinetic models. The kinetic model with correlation coefficients closer to 1 was chosen as the best model to explain the mechanism of thymol from LGC. Table 1 displayed the correlation coefficients values obtained from the fitted kinetics models. Based on table 1, the thymol release data was correlated well to the Korsmeyer-Peppas kinetic model, as it gave the best correlation coefficient value \(R^2 = 0.9940\) than three other kinetic models.
Figure 6. In vitro release of thymol and thymol-loaded LGC in PBS solution at room temperature.

Table 1. The correlation coefficient ($R^2$) of the fitted kinetic models for the release of thymol.

| Model              | $R^2$ |
|--------------------|-------|
| Zero-order model   | 0.9160|
| First-order model  | 0.7271|
| Higuchi model      | 0.8255|
| Korsmeyer-Peppas   | 0.9940|

The Korsmeyer-Peppas kinetic model can be described as Equation 2 [17]:

$$\frac{M_t}{M_\infty} = k t^n$$

where $M_t$ represents the amount thymol release at time $t$ and $M_\infty$ is the maximum amount of thymol release. Meanwhile, $k$ is the kinetic constant that integrates the characteristic of LGC and thymol system, and $n$ is the release exponent. The application of the Korsmeyer-Peppas model to the thymol release data gave values of release constant ($k$) and release exponent ($n$) of 0.005 hr$^{-1}$ and 1.266, respectively. It was noted that the value of the release exponent ($n$) of thymol from LGC, which corresponds to the type of the release mechanism involved, was higher than 0.85. Thus, this finding suggests that the release of thymol from LGC micelles govern by the Case II type transport, which associates with stress and state transition of the hydrophilic group in polymer (relaxation of polymer chain) in water and biological fluids [17,30].

4. Conclusion
In the present work, an amphiphilic chitosan derivative (LGC) was successfully synthesized by conjugating glycol and lauryl groups onto chitosan backbone. The introduction of the glycol group as a hydrophilic segment and the lauryl group as hydrophobic segment have facilitated the self-assemble properties of LGC, thus was able to form micelles or self-aggregates in aqueous solution. The fluorescence and TEM analyses confirmed that LGC was able form stable core-shell self-aggregate in aqueous solution as LGC has low CMC value. TGA analysis showed that LGC is thermally stable, hence could protect thymol from thermal degradation. The LGC micelle showed good affinity with thymol and was able to enhance the solubility of thymol for about 6.48 times higher than its inherent water solubility (0.9 mg/mL). The release of thymol from LGC micelles was controlled by relaxation of the hydrophilic group in polymer chains in aqueous solution. From this research, it is concluded that LGC can offer promising applicability to be utilized as a carrier in pesticide formulation.

5. References
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