N-hexanoyl-O-glycol chitosan as a carrier agent for water-insoluble herbicide

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Abstract. The objective of this study was to assess the potential of an amphiphilic chitosan derivative, namely N-hexanoyl-O-glycol chitosan (HGC) as a carrier agent for herbicide atrazine. The physicochemical properties of HGC were characterised using several analytical instruments. The critical micelle concentration (CMC) of HGC was determined using a Fluorescence Spectrometer. A High Performance Liquid Chromatography (HPLC) was used to determine encapsulation efficiency and loading capacity of atrazine in the amphiphilic chitosan derivative. Research findings found that the addition of hexanoyl and glycol groups to the chitosan backbone has improved the thermal stability of chitosan. TEM observation confirmed that HGC can form self-aggregation in the solution with spherical shape. The CMC values determined for HGC was 0.008 mg/mL. HGC also exhibited excellent encapsulation efficiency and loading capacity. The release profiles of atrazine loaded in HGC showed that it has different release behaviour than the pure herbicide in solution. In conclusion, the results from the study suggest that the amphiphilic chitosan derivative is applicable to be utilised as carrier agent for herbicide atrazine in pesticide formulation.

1. Introduction
Atrazine (2-chloro-4-ethylamino-6-isopropylamine-1,3,5-triazine) is a selective pre- or post-emergence herbicide that widely applied, especially in United States to control invasive weeds on various crops such as maize, sorghum and sugarcane [1-3]. The herbicide kills the weeds by inhibits the photosynthesis activity in chloroplast membrane [4]. It is mostly used in agriculture settings due its moderate toxicity and persistence in environment [1,5]. However, atrazine has low solubility in water (0.028 mg/mL) [6]. Thus, it usually formulated by dissolving in large amount of organic solvent which could be source for volatile organic compounds (VOCs) contamination [7]. Moreover, the herbicide also has high mobility in soils which could lead to groundwater contamination [8].

Good carrier system that could enhance the solubility of hydrophobic pesticide and reduce the risk to environment has become main focus in current pesticide formulation [9]. In recent years, polymeric micelles particularly amphiphilic chitosan derivatives have attract tremendous attention as carrier agents in pesticide formulations [10-12]. The unique properties of amphiphilic chitosan derivatives that contain hydrophobic moieties that can interact with hydrophobic pesticide to form inner core, and hydrophilic moieties that will create outer shell in aqueous solution, can enhance the solubility and control the release behaviour of the pesticide [13].

In the present study, N-hexanoyl-O-glycol chitosan (HGC) was synthesised and herbicide atrazine was loaded in the HGC micelles. The amphiphilic chitosan derivative was characterised using several analytical instruments, and the encapsulation efficiency and in vitro release study were investigated.
2. Materials and methods

2.1. Preparation of HGC

The glycol chitosan (GC) was synthesised according to a modified method outline by Shen et al. [14]. Briefly, 1 g of chitosan was alkalised with 20 mL of 42% (w/v) NaOH for 24 hours in the freezer. Then, the mixture was diluted with deionised water until the concentration of NaOH in the mixture became 14% (w/v). Approximately 2 mL of 2-chloroethanol was added to the mixture and stirred for 24 hours. After 24 hours, the mixture was filtered and collected solid was then redispersed in deionised water. The dispersion was dialysed (MWCO 14,000 Da) against deionised water and lyophilised. N-hexanoyl-O-glycol chitosan (HGC) was prepared according to a modified procedure proposed by Cho et al. [15]. GC (0.2 g) was dissolved in 50 mL of deionised water and 50 mL of methanol. Then, 0.2 mL of hexanoic anhydride was added to the glycol chitosan solution. The solution was reacted under continuous stirring for 24 hours at room temperature. After 24 hours, the solution was dialysed against 25% (v/v) for 3 days followed by lyophilisation.

2.2. Preparation of atrazine-loaded HGC

Atrazine-loaded HGC micelles were prepared using a technique suggested by Lao et al., with some modification [9]. Atrazine solution with concentration of 100 mg/L was prepared by dissolving appropriate amount of atrazine in methanol. Different amount of HGC (atrazine:HGC ratios; 1:5, 1:50 and 1:100) was added separately into atrazine solution. Deionised water at five-times higher than amount of atrazine solution was added dropwise to the solution under constant stirring at room temperature. After 2 hours, the solution was centrifuged at 1,000 rpm for 10 minutes and filtered with 0.45 μm pore-size membrane.

The amount of atrazine that successfully encapsulated was determined using an Agilent 1200 Infinity High Performance Liquid Chromatography (HPLC). A mixture of acetonitrile (HPLC grade) and water with a ratio of 70 to 30 (v/v) was used as mobile phase. An Agilent Poroshell 120 C18 (50 mm x 4.6 mm, 2.7 μm) was used as a column. The injection volume, detection wavelength, flow rate and column temperature was set at 15.0 µL, 220 nm, 1.0 mL/min and 40 ºC, respectively. Equation 1 and 2 was used to calculate the encapsulation efficiency (EE) and loading capacity (LC) of atrazine in HGC, respectively [16]:

\[
EE = \frac{\text{Amount of atrazine in HGC}}{\text{Total amount of atrazine}} \times 100
\]

\[
LC = \frac{\text{Weight of atrazine in HGC}}{\text{Weight of HGC}} \times 100
\]

2.3. Characterisation study

Fourier Transform Infrared (FTIR) spectra of chitosan and HGC were recorded using a Thermo Nicolet 6700 ATR-FTIR Spectrometer. The spectrum was scanned at wavenumbers range between 4000 and 400 cm\(^{-1}\), for over 32 times. Proton Nuclear Magnetic Resonance (\(^1\)H NMR) spectrum of HGC (dissolved in acetic acid–d) was obtained by using a Jeol JNM-ECX-500 Nuclear Magnetic Resonance Spectrometer, operated at 500 MHz. The percentage of composition for carbon, hydrogen, nitrogen and oxygen in chitosan and HGC was determined by using a CHNS-O Flash EA 1112 Series Elemental Analyser. Thermal properties of chitosan and HGC were studied under argon gas (flow rate of 20ml/min) from temperature range of 20 to 900 ºC using a TGA/DSC 1 Mettler Toledo Analyser. The internal morphology of HGC was with a Hitachi SU 8020 UHR High Resolution Field Emission Scanning Electron Microscope, at acceleration voltage of 20 kv. The solubility at chitosan and HGC at various pH (1.0-13.0) was recorded as a function of percentage of transmittance using an Agilent Cary 60 UV-Visible Spectrophotometer. The samples were dissolved in 2% (v/v) HCl to give a solution with concentration of 2 mg/mL and the transmittance was recorded at 600 nm [17].
2.4. Critical micelles concentration (CMC) study
The critical micelles concentration (CMC) of HGC was determined by using pyrene as a hydrophobic probe. Approximately, 1 mL of $1.25 \times 10^{-3}$ mg/mL pyrene solution (in methanol) was added into a beaker, and then methanol was removed from pyrene solution by evaporation. HGC solution (6 mL) at concentration of $1.0 \times 10^{-4}$ to 1.0 mg/mL was added separately in beaker contained methanol-free pyrene. The solution was sonicated for 10 minutes and the pyrene emission spectra were recorded starting from 344 to 700 nm by using an Agilent Cary Eclipse Fluorescence Spectrometer. Pyrene probe was excited at 334 nm, and both of the excitation and emission slit opening was set at 5 nm.

2.5. In vitro release study
To perform in vitro release study, an exact amount (5 mg) of atrazine-loaded HGC was dissolved in 3 mL phosphate buffered saline (PBS) solution (0.1 M, pH 7.4). The resulting solution or pure atrazine solution was placed in a dialysis bag (MW cut off: 14,000 Da) and immersed in a beaker containing 250 mL of PBS solution under continuous agitation of 100 rpm at room temperature. The release of atrazine from HGC micelles was measure at predetermined time by collecting 3 mL of PBS solution from the beaker and replaced with equal amount of fresh PBS. The measurement was carried out by using an Agilent Cary 60 UV-Visible Spectrometer at 220 nm. The in vitro release study was conducted in three replicates.

3. Results and discussion

3.1. FTIR analysis
The chemical structure of chitosan and HGC was confirmed by FTIR analysis. Figure 1 shows the FTIR spectra of chitosan and HGC. As shown in figure 1(a), FTIR spectrum of chitosan exhibits a broad absorption band at 3440 cm$^{-1}$ that can be attributed to stretching vibration of OH group and N-H. Two absorption peaks at 2919 and 2870 cm$^{-1}$ are corresponded to –C-H stretch of methyl and methylene groups in chitosan. The characteristics of C=O stretch of NHCO- and N-H bend of amine group can be observed at 1646 and 1596 cm$^{-1}$, respectively. The peaks observed at 1421 and 1383 cm$^{-1}$ can represent C-N stretching vibration. Meanwhile, the absorption bands at 1153 and 1081 cm$^{-1}$ can be assigned to C-O stretch of secondary and primary alcohol groups [18,19]. Following the reaction with 2-chloroethanol and hexanoic anhydride (figure 1(b)), the wavenumber of the absorption band at 3440 cm$^{-1}$ shifted to 3356 cm$^{-1}$ and the absorption intensity decreased. In addition, the intensity of absorption bands corresponded to aliphatic C-H stretch, C=O stretch of NHCO- and N-H bend of amine group, and C-O stretching vibration of secondary and primary alcohol groups became stronger and sharper, and shifted to new wavenumbers when compared to FTIR spectrum of chitosan. These might be due to large incorporation of methylene groups from glycol and hexanoyl groups [15,19,20]. The results confirmed that the glycol and hexanoyl groups were successfully incorporated to the chitosan backbone.

3.2. $^1$H NMR analysis
The chemical structure of HGC was further confirmed by $^1$H NMR analysis. The $^1$H NMR spectrum of HGC is shown in figure 2. To interpret the $^1$H NMR spectrum of HGC, the acetic acid-d peak at chemical shift 2.02 ppm was used as a reference. From figure 2, the overlapped peaks from 3.66 to 3.90 ppm may be attributed to proton signals of the chitosan and glycol group [15,19]. Meanwhile, the peaks corresponded to the methyl, methylene and tertiary carbon hydrogen of hexanoyl group can be observed at chemical shift of 0.89, 1.27, 1.61 and 2.34 ppm [15,20,21]. The result from $^1$H NMR analysis ascertained that the HGC was successfully synthesised.
3.3. CHNO analysis
The composition of carbon, hydrogen, nitrogen and oxygen in chitosan and HGC was analysed through CHN-O elemental analysis. Based on elemental analysis, chitosan has molar percentage of C, H, N and O atoms as $44.08 \pm 0.2$, $7.94 \pm 0.8$, $6.92 \pm 0.1$ and $41.06 \pm 0.5$ %, respectively. Meanwhile, HGC has $51.44 \pm 1.1$, $8.12 \pm 0.5$, $5.66 \pm 0.4$ and $34.78 \pm 0.6$ % of C, H, N and O, respectively. The experimental results for elemental composition of chitosan and HGC were close to the theoretical values. Therefore, elemental analysis results suggest that the HGC was successfully synthesised.

3.4. TGA analysis
The thermal decomposition pattern of chitosan and HGC was evaluated by TGA analysis. Figure 3 presents the TGA thermograms of chitosan and HGC. Two weight loss steps were observed on the thermogravimetric curve of chitosan. The first weight loss observed at 54 ºC, due to removal of water content and second weight loss observed at 328 ºC, due to dehydration of polysaccharide ring and decomposition of chitosan polymer [12]. Meanwhile, three decomposition stages of HGC can be observed at 58, 216 and 335 ºC, with a weight loss of around 7.0, 10.2 and 67.7 %, respectively. The first weight loss was due loss of residue water in HGC. The second stage was due to decomposition of
hexanoyl side group. The third weight loss occurred may be due to dissociation of polysaccharide ring and glycolsidic bonds [22-24]. The total weight loss for chitosan as temperature reached 900 ºC was around 99.2 % while for HGC was around 86.5 %. Overall, thermal analysis shows that the modification of chitosan with hexanoyl and glycol groups improved the thermal stability of the chitosan.

![Figure 3. TGA thermograms of chitosan and HGC.](image)

3.5. TEM analysis
In order to observe the internal morphology and directly determine the size of the HGC micelles, the TEM analysis was performed. The TEM images of HGC at 10,000x and 100,000x magnifications are presented in figure 4. Based on figure 4, HGC micelles could form self-aggregate in aqueous solution. The HGC displayed nearly spherical shape micelles with the size at dry state ranged from 79.4 to 103.0 nm.

![Figure 4. TEM images of HGC at 10,000x and 100,000x magnification.](image)

3.6. Solubility study
The influenced of pH on solubility properties of chitosan and HGC was evaluated based on their percentage of light transmittance. The trends on the solubility of chitosan and HGC at different pH are displayed in figure 5. It was observed that the solubility of chitosan was significantly influenced by the pH of dissolution media. Chitosan shows good solubility in acidic medium. However, chitosan was insoluble in both neutral and basic media. This scenario could be due to the presence of strong hydrogen bonds which caused the structure of chitosan turned rigid, [25]. Meanwhile, the solubility study shows that HGC was soluble in all acidic, neutral and basic media (slightly soluble at pH 4.0) with percentage of transmittance (%T) around 70 to 83%. The addition of glycol group in the chitosan backbone has promoted the formation hydrogen bonds between the polymer and hydrogen atom in water which contribute to the increment in the HGC solubility. However, slight solubility of HGC at
pH 4.0 might due to the formations of aggregation by highly acetylated chain segments or amide bond between alkyl groups in HGC, and also could be due to the presence of electrostatic interaction that highly influenced by solution pH [25]. The data from solubility study shows that the HGC possess essential characteristic as a good carrier agent for hydrophobic herbicide in aqueous solution.

3.7. **Critical micelles concentration**

The critical micelle concentration (CMC) can be defined as the threshold concentration in which amphiphilic polymer can undergoes self-aggregation by intra- and/or intermolecular association [21]. To determine the CMC value of HGC, pyrene was used as hydrophobic probe. Pyrene, due to its hydrophobic nature, will involuntarily stay close or lies inside hydrophobic domain of micelle if present in aqueous solution [26]. The intensity of first peak (I<sub>373</sub>) and third peak (I<sub>392</sub>) of pyrene are highly sensitive to its surrounding environment [27]. Therefore, the CMC value can be determined by plotting the ratio of I<sub>373</sub>/I<sub>392</sub> against logarithm concentrations of HGC in aqueous solution [26]. The CMC value of HGC was determined as 0.008 mg/mL. The HGC could form stable self-aggregation in aqueous solution as its CMC value is lower than low molecular-weight surfactant, SDS (2.3 mg/mL) [28] and deoxycholic acid (1.0 mg/mL) [29]. The CMC value of HGC also comparable or lower than some polymeric micelles such as deoxycholic acid-O-carboxymethyl chitosan-folic acid conjugates (0.027-0.065 mg/mL) [26] and N-(octadecanol-1-glycidyl ether)-O-sulphate chitosan (0.00355-0.0055 mg/mL) [9].

3.8. **Encapsulation efficiency and loading capacity of atrazine into HGC micelles**

Atrazine was loaded into HGC micelles using reverse micelle method. Table 1 presents the encapsulation efficiency and loading capacity of atrazine into HGC micelles at different weight ratios (atrazine:HGC). The HGC shows good encapsulation efficiency to herbicide atrazine. When the amount of HGC was increased from 5 to 100, the encapsulation efficiency of HGC increased from 86.91 to 92.39%. This suggests that the hydrophobic herbicide has good affinity with hydrophobic moieties in HGC [19]. Loading capacity was determined to estimate the amount of atrazine can be loaded in micelles. From table 1, it is noted that the trend of loading capacity (%) decreased as the amount of HGC added to atrazine increased. This may be due to the amount of the atrazine added in this study remain constant. Hence, when the amount of HGC was increased from 5 to 100, much more HGC micelles available than the amount of atrazine that have to be loaded in micelles, thus reducing the loading capacity. Huo et al. [19] observed similar trend in loading capacity for paclitaxel loading on N-octyl-O-glycol chitosan.

![Figure 5. Solubility of chitosan and HGC at pH 1.0 to 13.0.](image-url)
Table 1. The encapsulation efficiency and loading capacity of atrazine-loaded HGC.

| Atrazine:HGC | Encapsulation efficiency (%) | Loading capacity (%) |
|--------------|-----------------------------|----------------------|
| 1:5          | 86.91 ± 0.20                | 1.74 ± 0.17          |
| 1:50         | 89.09 ± 0.41                | 0.18 ± 0.03          |
| 1:100        | 92.39 ± 0.47                | 0.09 ± 0.01          |

3.9. In vitro release studies of atrazine-loaded HGC

In vitro release study of pure atrazine and atrazine-loaded HGC was carried out using dialysis technique under sink condition. Figure 6 shows the release profile of pure atrazine and atrazine-loaded HGC at different weight ratios. All atrazine-loaded HGC shows similar release pattern, with each underwent initial burst release effect at the first 10 hours of in vitro study, followed by controlled release behaviour. Based on in vitro release data, HGC was able to control the release of atrazine for longer time. The total hour needed to release all atrazine was 58, 63 and 65 hours, for atrazine-loaded HGC with weight ratios of 1:5, 1:50 and 1:100, respectively. In contrast, the time taken for free atrazine to completely release was 28 hours. It was observed that the rate of atrazine from HGC micelles was greatly influenced by the total amount atrazine loaded in the micelles. The control release properties of atrazine from HGC micelle could protect and enhance the availability of the herbicide for longer period of time. These results highlighted that HGC can be suitable carrier agent for the hydrophobic pesticide.

![Graph showing in vitro release of pure atrazine and atrazine-loaded HGC with different weight ratios in PBS solution at room temperature.](image)

Figure 6. In vitro release of pure atrazine and atrazine-loaded HGC with different weight ratios (1:5, 1:50 and 1:100) in PBS solution at room temperature.

4. Conclusion

In this research, amphiphilic chitosan derivative, HGC was synthesised and characterised. The presence of glycol group as hydrophilic chain and hexanoyl group as hydrophobic chain is essential in the formation of micelle. The amphiphilic chitosan derivative was found to have low CMC value and therefore could form stable self-aggregate. TEM analysis confirmed that HGC can form self-aggregation with spherical shape. Thermal analysis show that HGC are thermally stable thus can protect the herbicide from degradation. The HGC micelle shows good affinity with atrazine. HGC was capable to enhance solubility of atrazine in water around 3 times higher than pure atrazine (0.028 mg/mL). The ability of HGC to encapsulate and control the release of atrazine efficiently highlights its potential as carrier agents for pesticide formulations.
References

[1] Kah M, Machinski P, Koerner P, Tiede K, Grillo R, Fraceto L F and Hofmann T 2014 *Environ. Sci Pollut Res.* 21 11699-11707

[2] Maruteascu L and Chifiriuc M C 2017 Molecular mechanisms of pesticides toxicity *New Pesticides and Soil Sensors* ed A M Grumezescu (Academic Press) chapter 11 pp 393-435

[3] Mitchell P D, 2014 *Pest Manag. Sci.* 70 1684-1696

[4] Venceslau A d F A, dos Santos F E, de Fátima Silva A, Rocha D A, de Abreu A J, Jaime C, Andrade-Vieira L F and Pinto L d M A 2018 *Energ. Ecol. Environ.* 3 81-86

[5] Lobo F A, de Aguirre C L, Silva M S, Grillo R, de Melo N F S, de Oliveira L K, de Morais L C, Campos V, Rosa A H and Fraceto L F 2011 *Polym. Bull.* 67 479-495

[6] Brand R M and Mueller C 2002 *Toxicol. Sci.* 68 18-23

[7] Jessop P G, Ahmadpour F, Buczynski M A, Burns T J, Green li N B, Korwin R, Long D, Massad S K, Manley J B and Omidbakhsh N 2015 *Green Chem.* 17 2664-2678

[8] Grillo R, de Melo N F S, de Lima R, Lourenço R W, Rosa A H and Fraceto L F 2010 *J. Polym. Environ.* 18 26-32

[9] Lao S-B, Zhang Z-X, Xu H-H and Jiang G-B 2010 *Carbohydr. Polym.* 82 1136-1142

[10] Feng B, Ashraf Muhammad A and Peng L 2016 *Open Life Sci.* 11 380-386

[11] Mei X D, Liang Y H, Zhang T, Ning J and Wang Z Y 2014 *Adv. Mater. Res.* 1051 21-28

[12] Liu Y, Sun Y, He S, Zhu Y, Ao M, Li J and Cao Y 2013 *Int. J. Biol. Macromol.* 57 213-217

[13] Kuskov A N, Kulikov P P, Goryachaya A V, Tzatzarakis M N, Tsatsakis A M, Velonia K and Shtilman M I 2017 *J. Appl. Polym. Sci.* 135 45637

[14] Shen C-R, Liu C-L, Lee H-P and Chen J-K 2013 *Molecules* 18 2978-2987

[15] Cho I S, Park C G, Huh B K, Cho M O, Khatun Z, Li Z, Kang S-W, Choy Y B and Huh K M 2016 *Acta Biomater.* 39 124-132

[16] Zhang J, Li M, Fan T, Xu Q, Wu Y, Chen C and Huang Q 2013 *J. Polym. Res.* 20 1-11

[17] Kubota N, Tatsumoto N, Sano T and Toya K 2000 *Carbohydr. Res.* 324 268-274

[18] He L, Wang H, Xia G, Sun J and Song R 2014 *Appl. Surf. Sci.* 314 510-515

[19] Huo M, Zhang Y, Zhou J, Zou A, Yu D, Wu Y, Li J and Li H 2010 *Int. J. Pharm.* 394 162-173

[20] Desai K G and Park H J 2006 *Drug Delivery* 13 375-381

[21] Hsiao M-H, Lin K-H and Liu D-M 2013 *Soft Matter* 9 2458-66

[22] Bhattacharai S R, Bahadur K C R, Aryal S, Khil M S and Kim H Y 2007 *Carbohydr. Polym.* 69 467-477

[23] Neamnark A, Sanchavanakit N, Pavasant P, Bunaprasert T, Supaphol P and Rujiravanit R 2007 *Carbohydr. Polym.* 68 166-172

[24] Mishra S K and Kannan S 2014 *J. Mech. Behav. Biomed. Mater.* 40 314-324

[25] Mourya V K, Inamdar N N and Tiwari A 2010 *Adv. Mater. Lett.* 1 11-33

[26] Wang F, Zhang D, Duan C, Jia L, Feng F, Liu Y, Wang Y, Hao L and Zhang Q 2011 *Carbohydr. Polym.* 84 1192-1200

[27] Liu K-H, Chen S-Y, Liu C-M and Liu T-Y 2008 *Macromolecules* 41 6511-6516

[28] Rahman A and Brown C W 2003 *J. Appl. Polym. Sci.* 28 1331–1334

[29] Kratohvil J P, Hsu W P and Kwok V 1986 *Langmuir* 2 256–258

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