Alzheimer disease (AD) is the most common disease among the elderly people and a major social and medical problem. Amaryllidaceous alkaloids, acting as acetylcholinesterase inhibitors, represent a potential treatment of AD. However, they also have some deficiencies, such as extensive toxicity and widespread side effects. In order to improve the bioavailability and reduce the toxic and side effects, brain targeting of amaryllidaceous alkaloids was enhanced by considering low density lipoprotein (LDL) receptors of blood-brain barrier (BBB) endothelial cells as therapeutic targets. Amaryllidaceous alkaloids were highly selectively and quantitatively riveted to the surface of low density lipoproteins by using a new method - mild click chemistry. The structure of products has been characterized by NMR, FT-IR, and other methods. In addition, drug loading rate, encapsulation rate, and drug release by the nanoparticles were determined to assess the quality of the nanoparticles.

**1 Introduction**

Alzheimer’s disease (AD) is a form of senile dementia, a common central nervous system degenerative disease in the elderly people [1]. The major symptoms of AD patients are progressive cognitive impairment, memory loss, mental and behavior disorders, and loss of life ability [2-4]. With the coming of an increasingly aging society, the incidence of AD is increasing, and AD became the third leading cause of death, exceeded only by cardio-cerebrovascular diseases and cancer [5-7]. There are probably over 4 million dementia patients in our country, which puts a serious burden on the family and society. Therefore, research on the treatment of AD is not only a medical issue to be resolved, but also a serious social problem [8].

One of the first theories on AD pathogenesis was reduction of the function of cholinergic synaptic transmission in the central nervous system, the so-called cholinergic hypothesis [9]. The hypothesis provides the theoretical basis for treatment of AD. Acetylcholinesterase inhibitors were first approved by FDA. Acetylcholinesterase inhibitors are first level drugs, which were the earliest and most successfully used to treat AD in clinics, and deemed as the standard treatment of mild-to-moderate AD patients [10].

*Lycoris radiata* is a kind of Chinese herb, and is resourceful in our country. A certain alkaloid, named galantamine, which is extracted from *Lycoris*, has been proved as reversible acetylcholinesterase inhibitor and registered for the treatment of AD in Europe [11]. Furthermore, other *Lycoris* alkaloids have been proved for inhibiting acetylcholinesterase [12-14], but have been limited in clinical application because of great toxicity [15] and lack of penetrance through the blood-brain barrier (BBB). Therefore, the quickest and most effective
way to solve these clinical problems of alkaloid use is by preparing brain-targeted and sustained-release carriers.

Based on preliminary results of amaryllidaceous alkaloids in treatment of AD, this study aimed to design a series of nano-drug carriers to improve the targeting properties and slow-releasing potential, and reduce their toxicity, by considering low-density lipoproteins (LDL) as a targeting factor [16]. The effects of components and structures of drug carriers on the loading efficiency and the sustained-release rate were studied. The morphology and topography of nanoparticles were characterized by transmission electron microscopy and laser diffraction particle size analyzer.

2 Materials and methods

2.1 Reagents

Chitosan (CS, deacetylation degree 88% (elementary analysis)), potassium periodate (KIO₄), glacial acetic acid, sodium acetate, sodium chloride, palmitic acid, 2-(2-aminoethoxy)ethanol (purity: 98%), tosyl chloride, propargylamine (purity:98%, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, 2-8 °C), N-hydroxybenzotriazole (HOBt), sodium bicarbonate, sodium sulfate, hydrochloric acid (36%~38%), citric acid, acetic ether, N,N-dimethylformamide, N-hydroxysuccinimide (NHS), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC·HCl) were purchased from Sinopharm Chemical Reagent Co.,Ltd; sodium azide (Chengdu Gray Asia Chemical Co. Ltd), trichloromethane (Tianjin Dongli District Tianda chemical reagent factory), absolute ethyl ether (Tianjin Fu Yu Chemical Co., Ltd.), trichloromethane (Tianjin Dongli District Tianda chemical reagent factory), absolute ethyl ether (Tianjin Fu Yu Chemical Co., Ltd.), silica gel for column chromatography (Reagent, Kiselgel A, 100–200-mesh sieve, Shanghai five four Chemical Reagent Co., Ltd.), silica gel plate (GF254, Qingdao Ocean Chemical Factory) were also used. All other chemicals used in this study were of analytical grade.

2.2 Experimental methods

2.2.1 Synthesis and characterization of periodate oxidated derivatives of chitosan (CS-CHO)

Different aldehyde degree of CS-CHO was obtained by adjusting the molar ratio of periodate and CS (Figure 1). The effects of components and structures of drug carriers on the loading efficiency and the sustained-release rate were studied. The morphology and topography of nanoparticles were characterized by transmission electron microscopy and laser diffraction particle size analyzer.

![Figure 1](synthesis_route_of_CS_CHO.png)

**Figure 1.** Synthesis route of CS-CHO

After periodate oxidation, water-solubility of CS-CHO has been remarkably improved, and then it could be easily dissolved in deionized water and be decorated in homogeneous medium. One-pot reaction was carried out in aqueous medium by using N-hydroxysuccinimide (NHS) to activate carboxyl and N-(3-Dimethylaminopropyl)-N’-ethlycarbodiimide hydrochloride (EDC·HCl) as condensation agent. All reactants and products were dissolved in water. Low molecular weight impurities could be removed by dialysis. Figure 2 shows the synthesis route. Under the protection of N₂, NHS was dissolved in propiolic acid aqueous solution. After that, CS-CHO aqueous solution was added into the solution drop by drop, stirred evenly, and EDC·HCL was added. After a certain period of time, the mixture was migrated to dialyze against NaCl (0.1 M) aqueous solution and deionized water to remove the small molecules which have not reacted. The freeze-dried product was spared. FT-IR, ¹H NMR and UV were applied to identify the structure of the products, and to measure the molecular weight and its distribution. ¹H NMR was performed on a Mercury-Plus 300 MHz spectrometer at room temperature. Tetramethylsilane (TMS) was used as internal standard. PA-CS-CHO was dissolved in 2% (v/v) CD₃COOD (D₂O as solvent). FT-IR spectra were recorded on a Fourier-transform infrared (FT-IR) spectrometer (Nicolet/ Nexus 670, USA). PA-CS-CHO was mixed with KBr and pressed into a plate for measurement.
2.2.3 Extraction of LDL and synthesis of azide probe

LDL was extracted from human plasma by density gradient method. Before use, LDL was diluted with phosphate buffer solution (PBS), sterilized by filtering through a filter with a pore size of 0.45 μm, and preserved at 4 °C. The “probe” could be fixed in phospholipids layer of LDL through palmitic acid with hexadecyl anchors. In order to prevent reactivity loss of functional group - azide end group - because of being embedded in the LDL, the single diethylene glycol unit was introduced as “spacer” to synthesize N-(2-(2 azidoethoxy)ethyl)palmitamide (NAEP) (Figure 3).

Synthesis of C15H31CONHCH2CH2OCH2CH2OH (N-(2-(2-hydroxyethoxy)ethyl)palmitamide, NHEP)

5.129 g of hexadecanoic acid (20 mmol), 2.103 g of hydroxyethoxy ethylamine (20 mmol), and 2.8 g of triethylamine (20 mmol) were dissolved in 200 ml DMF under N₂ atmosphere. The mixture was cooled in ice-bath. Then, 7.585 g of pre-blended 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (20 mmol) and 2.702 g of N-hydroxybenzotriazole (HOBt) (20 mmol) were added. The mixture was let to react for 24 h at room temperature. End point of the reaction was determined by TLC. Then, the reactant solution was added into a double volume of cold water drop by drop, and extracted 3 times with 50 ml chloroform. The collected chloroform solution was scrubbed by 10 mM citric acid solution, 4 % NaHCO₃ solution, and saturated sodium chloride solution successively, and dried with anhydrous sodium sulfate. The crude product was obtained by using rotary evaporation to remove the chloroform. The crude product was purified by recrystallization from ethanol, dried in vacuum for 48 h, and stored in dryer.

Synthesis of C15H31CONHCH2CH2OCH2CH2Ots (2-(2-palmitamidoethoxy)ethyl) methylbenzene sulfonate, PEMBS):

2.58 g of NHEP were dissolved in 30 ml chloroform. 20 ml pyridine was added into the chloroform solution (about 4 times dosage of p-toluenesulfonyl chloride). After cooling to 0 °C, 2.85 g of p-toluenesulfonyl chloride were added. The mixture was stirred overnight after returning to room temperature.
Synthesis of C15H31CONHCH2CH2OCH2CH2N3 (NAEP): 2.58 g of PEMBS were dissolved in 20 ml DMF under N₂ atmosphere. 1.2 N NaN₃ was added into the solution. The solution was slowly heated to 50 °C, refluxed for 42 h, and cooled to room temperature. Then, the product solution was dropped into ice water of three-fold volume during stirring. Filter cake was obtained by filtration and washing with water repeatedly. After that, filter cake was dried in vacuum at room temperature for 48 h. The reaction was sheltered from light during the whole course. The purified final product was obtained by column chromatography at RF = 0.309 by using ethyl acetate-n-hexane as eluent. Their volume ratio was 5:5. NAEP-LDL (N₃-LDL) was obtained by a self-assembly reaction where LDL and NAEP were mixed in co-solvent. The structures of the obtained products were characterized by FT-IR, ¹H NMR and ¹³C NMR.

2.2.4 Encapsulation of amaryllidaceous alkaloids using interface reaction between PA-CS-CHO and N₃-LDL

The alkynyl group of PA-CS-CHO and the nitrine group of N₃-LDL reacted by click chemistry reaction under mild conditions to synthesize chitosan-conjugated low density lipoprotein (CS-LDL), which had the LDL targeted group as hydrophobic end group. The solution of CS-LDL and galantamine hydrobromide or lycoramine hydrobromide solution were mixed in co-solvent to prepare nanoparticles (named CS-G or CS-L) through self-assembly reaction by dialysis. The morphology and topography of nanoparticles were characterized by transmission electron microscopy and laser diffraction particle size analyzer.

2.2.5 Drug encapsulation efficiency and in vitro release

Encapsulation efficiency (EE) and drug loading rate (DL) of drugs encapsulated with the nanoparticles were calculated by the following equation:

\[ EE = \frac{A}{B} \times 100\% \]
\[ DL = \frac{A}{(B+C)} \times 100\% \]

where A represents the amount of drug retained in nanoparticles, B is the initial amount of drug fed for encapsulation, and C is the amount of carrier material. The amount of galantamine hydrobromide or lycoramine hydrobromide in the nanoparticles was determined by using a Beckman Coulter (Indianapolis, IN, USA) DU 800 UV-Vis spectrophotometer based on their absorbance at 289 or 290 nm, respectively. The standard curves of galantamine hydrobromide and lycoramine hydrobromide were C (mg/L) = 120.86A-0.14 (r = 0.999, n = 5) and C(mg/L) = 135.73A-0.16 (r = 0.999, n = 5), respectively. For in vitro drug release study, drug-laden nanoparticles (20–30 mg) were reconstituted in PBS (5 ml, pH 5 or 7.4) and transferred into dialysis bags (MWCO: 10 kDa) that were placed in 30 ml of the same PBS at 37 °C and stirred. At appropriate time points, 100 µl of the dialysate was collected and the dialysate replenished with the same amount of fresh PBS. The concentration of the released galantamine hydrobromide or lycoramine hydrobromide in the removed dialysate was determined by using UV-Vis spectrophotometer based on their absorbance.

Informed consent: Informed consent has been obtained from all individuals included in this study.

Ethical approval: The research related to human use has been complied with all the relevant national regulations, institutional policies and in accordance the tenets of the Helsinki Declaration, and has been approved by the authors’ institutional review board or equivalent committee.

3 Results and discussion

3.1 Synthesis and characterization of PA-CS-CHO

As shown in Figure 4, after the occurrence of propargyl acylation reaction, the maximum absorption wavelength of the product shifted from the 325 nm to 275 nm and the absorption wavelength of 325 ~ 350 nm disappeared. Due to the amino groups that were acylated, the lone pair electrons reacted with propargyl acyl to form conjugate micelles which reduced electron transition of amino of sugar ring and this led to the wavelength blue shift, while for the propargyl group the substituent caused a red shift.
The common results led to the change of UV absorption wavelength.

There were also differences in the FT-IR spectra of CS-CHO and PA-CS-CHO (Figure 5). At 2200 cm\(^{-1}\) (see the circle), there was a weak stretching vibration peak of acetylene, which proved the existence of alkyne. In addition, the C-N stretching vibration of free amino of CS-CHO at \(-1500\) cm\(^{-1}\) (see the small arrow) was lost, being replaced by the C-N stretching vibration of amide at \(-1400\) cm\(^{-1}\). \(^1\)H NMR proved the occurrence of propargyl acylation reaction (Figure 6). Absorption peaks of H-2 proton moved from 3.0 ppm to 2.85 ppm. The reason was that the amino groups were acylated and hydrogen bonds formed with solvent were weakened. Meanwhile, the absorption of H-a appeared at \(-2.75\) ppm.

### 3.2 Synthesis and characterization of N-(2-(2-azido Ethoxy) ethyl) Palm amide (NAEP) probe

In this study, we introduced a single shrinkage diethylene glycol unit as “spacer” to avoid inactivation of nitrine group. A three-step reaction was used to synthesize the final product, N-(2-azidoethoxy ethyl) palmitamide (NAEP). The final yield was about 36%. The synthesis route is shown in Figure 3. A hydrophilic unit was first introduced into the palmitic acid chain as a spacer. In the study, 2-(2-aminoethoxy) ethanol reacted with palmitic acid through amidation reaction, which avoided the low
yield of esterification, harsh conditions, by-products, and other shortcomings. The carboxyl group was activated by HOBt and HBTU, which were used as dehydration agents. With the “one-pot” method to complete the reaction, the processing method was simple and mild. The purity and yield of the product were satisfactory.

$^1$H NMR proved the completeness of amidation reaction (Figure 7 a, b). Absorption peaks of four methylene protons appeared at 3.0-4.0ppm. Due to the decrease in electronegativity of the carbonyl carbon, the absorption peak of the methylene proton near the carboxyl group moved to the high field (from d to d'). The integral ratio of the absorption peak was that $a':b':c':d': (e+f+g)=1.54:11.48:1.08:1.00:4.25$, which was in accordance with the theoretical value. From $^1$H NMR results of Figure 7c, characteristic absorption of the benzene ring appeared between 7.0-8.0ppm after sulfonic acid esterification reaction, and absorption peak of the methylene proton adjacent to the terminal hydroxyl moved from 3.9ppm (d) to 4.1ppm (d'); at the same time, absorption peak of the originally active hydroxyl proton (2.4ppm, e) disappeared, which confirmed the occurrence of esterification reaction. Figure 7d shows that the characteristic absorption of methyl-phenyl-sulfonic group (7.0-8.0ppm:e, f; 2.4ppm, g) was lost after the substitution reaction. Due to the electronic effect of the nitrine group, the absorption peak of the methylene proton adjacent to the terminal greatly moved from 4.1ppm to 3.85ppm (d'). The absorption peaks of three sub-methyl protons a, b, c also moved to high field in different degrees.

The FT-IR spectra clearly showed the presence of nitrine group (Figure 8). Finally, the structure of NAEP was confirmed by the $^{13}$C NMR spectrum (Figure 9).

### 3.3 Properties of nanoparticles loaded with amaryllidaceous alkaloids

Transmission electron microscopy (TEM) images of CS-G nanoparticles or CS-L nanoparticles are shown in Figure 10. One can notice that the drug-loaded particles were spherical and their appearance was round. The particle size was uniformly distributed and there was a clear core shell structure. The average particle sizes of CS-G nanoparticles and CS-L nanoparticles were 26 nm and 25 nm, respectively (Table 1), and the polydispersity coefficients (PDI) were smaller.

![Figure 7. $^1$H NMR spectra of palmitic acid (a), C$_{15}$H$_{31}$CONHCH$_2$CH$_2$OCH$_2$CH$_2$OH (b), C$_{15}$H$_{31}$CONHCH$_2$CH$_2$OCH$_2$CH$_2$Ots (c), and NAEP (d) (Figure 7. $^1$H NMR spectra of palmitic acid (a), C$_{15}$H$_{31}$CONHCH$_2$CH$_2$OCH$_2$CH$_2$OH (b), C$_{15}$H$_{31}$CONHCH$_2$CH$_2$OCH$_2$CH$_2$Ots (c), and NAEP (d))](image-url)
Figure 8. FT-IR spectra of NAEP and PEMBS (C_{n}H_{2n+1}CONHCH_{2}OCH_{2}CH_{2}OTs)

Figure 9. $^{13}$C NMR spectrum of NAEP

Figure 10. TEM images of CS-G nanoparticles (left) and CS-L nanoparticles (right)
3.4 Drug encapsulation efficiency and in vitro release

Results of EE and DL of CS-G nanoparticles and CS-L nanoparticles are shown in Table 2. EE gradually increased with the increase of the dosage. The highest EE of CS-G and CS-L were 26% and 25%, respectively. The DL did not change significantly, DL of CS-G was about 65%~80% and DL of CS-L was about 65%~80%, proving good compatibility between copolymer and galantamine or lycoramine.

Table 1. Particle size and polydispersity coefficient (PDI) of CS-G and CS-L nanoparticles

<table>
<thead>
<tr>
<th>Initial amount of drug (mg)</th>
<th>Particle size (nm)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>26.0±1.2</td>
<td>0.161±0.017</td>
</tr>
<tr>
<td>2</td>
<td>25.1±0.4</td>
<td>0.179±0.012</td>
</tr>
<tr>
<td>3</td>
<td>24.2±1.0</td>
<td>0.219±0.007</td>
</tr>
<tr>
<td>4</td>
<td>26.0±1.1</td>
<td>0.178±0.021</td>
</tr>
<tr>
<td>5</td>
<td>27.0±0.7</td>
<td>0.168±0.004</td>
</tr>
<tr>
<td>6</td>
<td>28.0±1.6</td>
<td>0.175±0.041</td>
</tr>
</tbody>
</table>

Table 2. Encapsulation efficiency (EE) and drug loading rate (DL) of CS-G and CS-L nanoparticles

<table>
<thead>
<tr>
<th>Initial amount of drug (mg)</th>
<th>EE (%)</th>
<th>DL (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.54±0.14</td>
<td>65.26±0.34</td>
</tr>
<tr>
<td>2</td>
<td>9.83±0.09</td>
<td>79.84±0.04</td>
</tr>
<tr>
<td>3</td>
<td>13.25±0.04</td>
<td>78.75±0.13</td>
</tr>
<tr>
<td>4</td>
<td>17.34±0.23</td>
<td>77.34±1.05</td>
</tr>
<tr>
<td>5</td>
<td>20.16±0.27</td>
<td>75.35±1.39</td>
</tr>
<tr>
<td>6</td>
<td>26.05±0.37</td>
<td>78.38±1.26</td>
</tr>
</tbody>
</table>

In vitro release profiles of CS-G and CS-L nanoparticles are shown in Figure 11. Galantamine hydrobromide solution (GHS) and lycoramine hydrobromide solution (LHS) were quickly released within 12h, and the cumulative release percentage was about 87% and 89%, respectively. Compared with the solutions, the release of nanoparticles was relatively slower. The cumulative release percentages during 120 h of CS-G and CS-L nanoparticles were 70.4% and 65.2%, respectively. The results show that CS-G and CS-L nanoparticles have obviously sustained release effect.
4 Conclusion

Nanoparticles loaded with galantamine hydrobromide and lycoramine hydrobromide solution were synthesized. These particles were uniformly distributed, and the average particle sizes of CS-G nanoparticles and CS-L nanoparticles were 26 nm and 25 nm, respectively. The results of release profiles of CS-G and CS-L nanoparticles in vitro proved that these nanoparticles have obviously sustained release.

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Conflict of interest: Authors state no conflict of interest.

References


Figure 11. Release profiles of CS-G and CS-L nanoparticles in vitro