Preparation and properties evaluation of a novel pH-sensitive liposomes based on imidazole-modified cholesterol derivatives

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A B S T R A C T

As a new kind of drug carrier, pH-sensitive liposomes have been widely studied in tumor therapy for their advantages of target ability and sustained-release. Here, we synthesized a pH-sensitive material, N-(3-Aminopropyl)imidazole-cholesterol (IM-Chol) and prepared a novel pH-sensitive liposomes using IM-Chol and phosphatidylcholine. IM-Chol was synthesized through amidation reaction between the amino group of N-(3-Aminopropyl)imidazole and acyl chloride group of cholesteryl chloroformate in a weak base solution. Optimal conditions to prepare liposomes were obtained by the orthogonal experiment with the higher encapsulation efficiency as the evaluation indicator. The properties of liposomes, such as particle size, zeta potential, morphology, encapsulation efficiency, drug release behavior and in vitro cell toxicity were evaluated by transmission electron microscopy (TEM), dynamic light scattering (DLS) and MTT assay respectively. The results showed that the average particle size of IM-Chol liposomes was 141 nm (PDI 0.323). Liposomes can assemble into uniform spheres at pH 7.4, but under the condition of pH 5.0, the spherical structure of IM-Chol liposomes was broken, exhibiting pH-sensitive property. In vitro drug releasing studies demonstrated the controlled-release behavior of the curcumin (CUR) in the IM-Chol liposomes. The cumulative release of CUR reached to 72.5% in the first 24 h at pH 5.0, faster than that at pH 7.4, which confirmed that the drug carrier displayed pH-sensitive release behaviors. In addition, the MTT assay was employed to test the cytotoxicity of IM-Chol liposomes and CUR-IM-Chol liposomes. All cell viabilities were greater than 80% after incubating for 24 h, even up to the highest dose of 500 mg/L, indicating that IM-Chol liposomes had good biocompatibility. The tumor inhibitory results towards EC109 cells of free CUR and CUR-loaded IM-Chol liposomes indicated that IM-Chol liposomes indeed enhanced the cell killing effect of CUR. These results showed that the novel IM-Chol liposomes prepared in this paper had pH-sensitive property and were expected to play a huge potential in tumor treatment.

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1. Introduction

As drug carriers, liposomes possess the capability of target and sustained-release, to a certain extent, making the drug efficacy enhanced and drug toxicity extenuated (Hardiansyah et al., 2015). However, the traditional liposomes still have quite a lot of shortages in clinical application compared with the new type of liposomes. In the areas of the tumor stroma, ischemia and infection or inflammation, the pH is quite lower than the surrounding normal tissues (Brown, 2002; Hede, 2004). Based on the change of pH in diseased tissue, researchers developed a new type of liposome, pH-sensitive liposome, which has a huge advantage in intracellular targeting and controlled drug release (Moku et al., 2016; Paliwal et al., 2016). The membrane structure of pH-sensitive liposomes changed together with the adjustment of pH from 7.4 to 5.3 ~ 6.3, which prompted the fusion of liposome membrane and endosome membrane, leading to the release of their aqueous contents (Paliwal et al., 2015).

Different classes of pH-sensitive liposomes have been proposed in the literatures according to the mechanism triggering pH-sensitivity (Pacheco-Torres et al., 2015; Xu et al., 2015; Chen et al., 2016). The most commonly recognized concept involves the combination of phosphatidylethanolamine (PE) (Sánchez et al., 2011) or its derivatives with compounds containing an acidic group (e.g. Carboxylic group) (Collins et al., 1990) that act as a stabilizer at neutral pH. Recently, the use of novel pH-sensitive lipids (Mevel et al., 2011; Gujrati et al., 2015), synthetic fusogenic peptides/
proteins (Kakudo et al., 2004; Yamada et al., 2005), and association of pH-sensitive polymers (Kyriakides et al., 2002; Attama and Ezeamama, 2005) with liposomes either encapsulated or incorporated in the lipid bilayer have been reported. For practical use of liposomes as drug delivery systems, there are two conflicting requirements that must be simultaneously fulfilled: high stability and high pH sensitivity of liposomal structures before and after administration. Researchers introduced charged lipid (Asami Aoki et al., 2015), ionized groups (Chang et al., 2015) and pH-sensitive chemical bonds (Qing Chen et al., 2016) to achieve this purpose.

In this work, we designed an imidazolyl-modifie pH-sensitive material N-(3-Aminopropyl)imidazole-cholesterol (IM-Chol). The compound IM-Chol was synthesized by using cholesteryl chloroformate and N-(3-Aminopropyl)imidazole (Fig. 1) (Chiu et al., 1999). Since the pKa of imidazole group was about 6.0, the protonation of imidazole under slightly acid environment would vanish and destroy the liposomes, leading to the decreased stability of liposomes and rapid release of drugs at low pH. The liposomes were characterized in detail, including particle size, zeta potential, surface morphology and cell cytotoxicity. Moreover, we also investigated the drug release of curcumin (CUR) loaded pH-sensitive liposomes in vitro.

2. Materials and methods

2.1. Materials

Phosphatidylcholine (PC>90%) was purchased from Shanghai JinSui Biological Technology Co., Ltd. (Shanghai, China). Cholesterol was purchased from Xi’an Chemical Reagent Company (Xi’an, China). Cholesteryl chloroformate and N-(3-Aminopropyl)imidazole were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Curcumin was purchased from Aladdin Industries Corporation (Shanghai, China). Ethyl alcohol, sodium chloride, potassium chloride, disodium hydrogen phosphate, sodium dihydrogen phosphate dihydrate were purchased from Tianjin Chemical Reagent Company (Tianjin, China).

2.2. Preparation of N-(3-Aminopropyl)imidazole-cholesterol (IM-Chol)

Cholesteryl chloroformate (0.45 g, 1 mmol) was dissolved in dichloromethane (10 mL). N-(3-Aminopropyl)imidazole (0.6 mL, 4 mmol) was added dropwise, then 200 μL triethylamine was added and the mixture was stirred under ice bath for 10 h. After reaction, the solvent was removed by vacuum distillation. The obtained solid was washed with deionized water (15 mL × 3) and extracted with dichloromethane (15 mL × 3). The product was purified by column chromatography (silica, dichloromethane/ Methanol, 8:1) to get target product IM-Chol as light yellow viscous liquid (0.497 g, 92.3%).

2.3. Preparation of liposomes

Liposomes were prepared by thin-film dispersion method (Moghimipour and Handali, 2012). IM-Chol, cholesterol and phosphatidylcholine were dissolved in 10 mL of chloroform solution in a 50 mL round-bottom flask. After dissolving, the solvent was evaporated in a totally evaporator operated at 90 rpm under reduced pressure. The mixture was vacuum dried overnight to remove residual solvent. Further, a thin dry lipid film would form on the wall of the round bottom flask. Hydration process of the dry lipid film was accomplished by adding PBS buffer (pH 7.4, 20 mM), which resulted in liposomes suspension. Afterwards, the liposomes were homogenized using an ultrasonicator at 24 W for 10 min. The suspension was extruded through a 0.22 μM filter for sterilization. Curcumin encapsulated liposomes were prepared using the same procedures as those mentioned above.

2.4. Orthogonal experiment

The orthogonal design (4 factors and 3 levels) was adopted to optimize preparation conditions of liposomes (Table 1). The four

![Fig 1. Synthetic route of N-(3-Aminopropyl)imidazole-cholesterol (IM-Chol).](image-url)
factors are the proportion of phosphatidylcholine and cholesterol, the amount of pH-sensitive material (IM-Chol), CUR and PBS.

2.5. Particle size and zeta potential measurement

The particle size and zeta potential of the liposomes were measured by Nano-ZS (Malvern, ZEN3600, United Kingdom). All IM-Chol liposome dispersions in 50 mM PBS buffer (pH 5.0 or pH 7.4) were loaded into the cuvette and determined.

2.6. TEM observation

The morphology of IM-Chol liposomes was investigated using TEM system (Jeol, JEM-2100, Japan) at an acceleration voltage of 100 kV. The sample was prepared by immersing a copper grid into the solution. A few minutes after the deposition, the copper grid dried in air before measurement.

2.7. Encapsulating efficiency

To determine the entrapment efficiency (EE) of liposomes, 1 mL CUR liposomes samples was placed in a dialysis bag (MWCO 1000). Then the dialysis bag was suspended in 100 mL PBS (pH 7.4) which was incubated at 37°C under constant rotation at 500 rpm for 24 h. After dialysis, 9 mL demulsifier (ethanol:ethyl ether = 7:2) was added to the liposomes for demulsification. Then we calculated the entrapment efficiency (EE) by measuring fluorescence value. The EE was calculated according to the following equation:

\[ EE = \frac{\text{mass of loaded drug}}{\text{mass of feed drug}} \times 100\% \]

2.8. In vitro drug release study

To investigate the release behavior of CUR from the liposomes, CUR-loaded liposomes were transferred into a dialysis bag (MWCO 1000). Then the dialysis bags were introduced into the release medium containing 10 mL PBS buffer solution (50 mM, pH 5.0 or pH 7.4), and were gently shaken in a water bath at a constant temperature (37°C). In all cases, the conditions were maintained by replacing 0.5 mL of the release medium with fresh medium at defined time intervals. To estimate the amount of drug release, the drug in the release medium at each sampling point was measured by fluorescence analysis.

2.9. Cell cytotoxicity study

The MTT assay was employed to test the cytotoxicity of liposomes against EC109 cells. After seeded at a density of 6000 cells/well, cells were allowed to grow on a 96-well plate for 24 h in 100 μL DMEM containing 10% FBS. After that, samples with different concentrations were added to each well. 48 h later, the medium was replaced with 200 μL fresh medium. Subsequently, 20 μL MTT (5 mg/mL in PBS buffer) solution was added into each well and incubated for another 4 h. Finally, the medium was replaced with 200 μL DMSO. The absorbance was measured at 570 nm by a microplate reader (Bio-Rad, Model 550, USA). The relative cell viability was assessed as follow, where OD_{570}(samples) was obtained in the presence of samples and OD_{570}(control) was obtained in the absence of samples.

\[ \text{cell viability} (\%) = \frac{OD_{570} \text{(samples)}}{OD_{570} \text{(control)}} \times 100 \]

3. Results and discussion

3.1. Characterization of N-(3-Aminopropyl)imidazole-cholesterol (IM-Chol)

The structure of IM-Chol was confirmed by NMR. The results were shown in Figs. 2 and 3. \(^1\)H NMR (300 MHz, CDCl3): δ = 7.497 (imidazole ring), 7.282 (imidazole ring), 7.582, 5.032, 4.492, 4.018, 3.471, 3.181, 3.169, 2.373 ~ 0.675 (cholesterol) ppm. \(^13\)C NMR (75 MHz, CDCl3): δ = 156.62 (carbonyl carbon), 139.66 (imidazole ring), 137.09, 129.07 (imidazole ring).

![Fig. 2. The \(^1\)H NMR of IM-Chol.](image-url)
122.49 (imidazole ring), 118.89, 74.27, 56.60, 56.11, 49.90, 44.23, 42.23, 39.67, 39.46, 38.58, 37.65, 36.93, 36.46, 36.14, 35.76, 31.81, 31.78, 31.40, 28.19, 28.16, 27.92, 24.23, 23.83, 22.81, 22.55, 20.99, 19.30, 18.69, 11.82 ppm. $^1$H NMR and $^{13}$C NMR results proved that N-(3-Aminopropyl)-imidazole was connected to the cholesterol successfully.

3.2. Orthogonal experiment analysis

By orthogonal designs and range analysis, the main results were as follows (Table 2): (1) The major-minor order that effected on entrapment efficiency were D>C>A>B, namely the amount of curcumin>the proportion of phosphatidylcholine and cholesterol>the volume of PBS>the amount of pH-sensitive material IM-Chol. (2) The optimal level of phosphatidylcholine:cholesterol was 10:1. The EE increased with the increase of the proportion of phosphatidylcholine and cholesterol. As another important material of liposomes, cholesterol could adjust membrane fluidity and influence the EE. (3) With the increase of the volume of PBS, the entrapment efficiency increased. The optimal level of the PBS volume was 30 mL. Liposomes were prone to adhere when the volume of PBS decreased. (4) With the increase of pH-sensitive material IM-Chol, the envelopment rate may increase or reduce. It could be due to pH-sensitive materials can be part of the double

![Diagram of IM-Chol structure](image)

**Fig. 3.** The $^{13}$C NMR of IM-Chol.

<table>
<thead>
<tr>
<th>No.</th>
<th>Phosphatidylcholine: cholesterol</th>
<th>IM-Chol (mg)</th>
<th>PBS (mL)</th>
<th>CUR (mg)</th>
<th>F</th>
<th>EE%</th>
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<tr>
<td>1</td>
<td>A1 (2:1)</td>
<td>B1 (1)</td>
<td>C1 (10)</td>
<td>D1 (0.1)</td>
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<td>30.14</td>
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<tr>
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<tr>
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<td>T1</td>
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<td>1.31</td>
<td>37.97</td>
<td>45.82</td>
<td>K = 3T</td>
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<tr>
<td>Optimal levels</td>
<td>A3</td>
<td>B3</td>
<td>C3</td>
<td>D1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2 Orthogonal experimental results of IM-Chol liposomes preparation.
molecular film and will be affected by the proportion of phosphatidylcholine and cholesterol. Taken together, the optimum amount of IM-Chol was 10 mg. (5) The optimal conditions of preparing IM-Chol liposomes were as follows: phosphatidylcholine: cholesterol = 10:1, CUR 0.1 mg, PBS 30 mL, IM-Chol 10 mg.

### 3.3. Particle size, zeta potential and morphology measurement

The particle size distribution of liposomes was detected by DLS. As shown in Fig. 4, the average particle size of IM-Chol liposomes was 141 nm (PDI 0.323). Further observed by TEM (Fig. 5), we found that IM-Chol liposomes can form uniform spheres, but the uniform spherical structure of IM-Chol liposomes was broken under the condition of pH 5.0. The result of zeta potential was shown in Table 3. The zeta potential of IM-Chol liposomes were −15.1 mV (pH 7.4) and 9.85 mV (pH 5.0) respectively. Under neutral conditions, IM-Chol liposomes had a negative potential because of phosphatidylcholine containing a small part of negative charge. However, the zeta potential of IM-Chol liposomes increased to positive with the decrease of pH value. These findings could be attributed to the protonation of imidazole ring at low pH, resulting in the swelling of liposomes and increase of zeta potential (Lee et al., 2011).

### 3.4. Drug loading and release behavior in vitro

CUR was chosen as the model drug to assess the drug loading behavior. The liposomes could efficiently envelop the CUR due to the hydrophobic interaction between the hydrophobic tails of phosphatidylcholine and CUR. To demonstrate the pH sensitivity, the drug release behavior under different pH was also evaluated, and pH 5.0 was chosen to imitate the tumor pH. As shown in Fig. 6, the CUR release rate was relatively slow in neutral medium, just reaching to 35.25% within 24 h. However, the cumulative release of CUR in IM-Chol liposomes at pH 5.0 reached to 72.5% in the first 24 h, presenting a burst release phenomenon. Since the pKa of imidazole group was about 6.0, the protonation of imidazole under slightly acid environment would vanish and destroy the structure of liposomes, leading to rapid release of drugs at pH 5.0. With the extension of time, the release of CUR presented a gradually increasing trend. The schematic was shown in Fig. 7. These results strongly demonstrated that the IM-Chol liposomes presented a pH responsive drug release behavior.

### 3.5. In vitro cytotoxicity

Usually the survival rate of cells under the effect of carrier material was chosen to measure the toxicity. In this study, the toxicity of IM-Chol liposomes were evaluated with MTT method in EC109 cells. As shown in Fig. 8A, the results displayed that IM-Chol liposomes did not cause significant cytotoxicity against EC109 cells.

---

**Table 3**

<table>
<thead>
<tr>
<th>pH value</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IM-Chol LPs</td>
<td>7.4</td>
</tr>
<tr>
<td>IM-Chol LPs</td>
<td>5.0</td>
</tr>
</tbody>
</table>

---

![Fig. 4](image1.png)

**Fig. 4.** Size distribution of IM-Chol liposomes in pH 7.4.

![Fig. 5](image2.png)

**Fig. 5.** TEM images of IM-Chol liposomes in pH 7.0 (A) and pH 5.0 (B).

![Fig. 6](image3.png)

**Fig. 6.** In vitro drug release behavior of IM-Chol liposomes at different pHs (pH 7.4 and pH 5.0).
even up to the highest dose of 500 mg/L. All cell viabilities were greater than 80% after incubating liposomes for 24 h, indicating that IM-Chol liposomes had good biocompatibility and suitable for a potential drug delivery system.

The cell toxicity of free CUR and CUR-loaded IM-Chol liposomes were also measured by MTT method. From Fig. 8B, we found that the inhibitory effect of free CUR and CUR-loaded IM-Chol liposomes against EC109 cell had obvious concentration-dependent manners and CUR-loaded IM-Chol liposomes showed higher cytotoxicity than that free CUR. The toxicity of active materials embedded into the delivery system depends on the uptake ability of cell and release property of materials. Here, the cycocoxicity of CUR increased after coating by IM-Chol liposomes. It may be due to cell had higher uptake capacity because the bilayer membrane structure of liposomes was similar to cell membrane. Thus it can be seen that the pH-sensitive IM-Chol liposomes we prepared can be used as antitumor drug carriers to improve biocompatibility, reduce drug dosage and extenuate side effect.

4. Conclusion

In this report, a novel pH-sensitive material IM-Chol was synthesized successfully and IM-Chol liposomes were prepared by film dispersion method. Here, imidazolyl group plays the role of pH-sensitive effect. Imidazole group had an electron lone pair on the unsaturated nitrogen and was easily protonated to a pKa value of 6.5, thus giving liposomes obvious acid-sensitive feature. The synthetic strategy of IM-Chol had the advantages of simple operation, mild reaction conditions and high yield. By comparing the result of TEM, zeta-potential and in vitro drug release in different pH environment, it can be found that the IM-Chol liposomes had obvious pH-sensitivity. Combined with the fact that the pH value of tumor site was lower than that of normal tissue, it can improve targeting property of drugs. The tumor inhibitory results towards EC109 cells of free CUR and CUR-loaded IM-Chol liposomes indicated that IM-Chol liposomes indeed enhanced the cell killing effect of CUR. Thus it can be seen that the pH-sensitive IM-Chol liposomes we prepared can be used as antitumor drug carriers to improve biocompatibility, achieve controlled release, reduce drug dosage, providing theoretical basis for the development and application of CUR in tumor target treatment.

Acknowledgement

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