In situ precipitation: a novel approach for preparation of iron-oxide magnetoliposomes

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Background: Conventional methods of preparing magnetoliposomes are complicated and inefficient. A novel approach for magnetoliposomes preparation was investigated in the study reported here.

Methods: FeCl₃/FeCl₂ solutions were hydrated with lipid films to obtain liposome-encapsulated iron ions by ultrasonic dispersion. Non-encapsulated iron ions were removed by dialysis. NH₃·H₂O was added to the system to adjust the pH to a critical value. Four different systems were prepared. Each was incubated at a different temperature for a different length of time to facilitate the permeation of NH₃·H₂O into the inner phase of the liposomes and the in situ formation of magnetic iron-oxide cores in the liposomes. Single-factor analysis and orthogonal-design experiments were applied to determine the effects of alkalization pH, temperature, duration, and initial Fe concentration on encapsulation efficiency and drug loading.

Results: The magnetoliposomes prepared by in situ precipitation had an average particle size of 168±14 nm, zeta potential of −26.2±1.9 mV and polydispersity index of 0.23±0.06. The iron-oxide cores were confirmed as Fe₃O₄ by X-ray diffraction and demonstrated a superparamagnetic response. Encapsulation efficiency ranged from 3% to 22%, while drug loading ranged from 0.2 to 1.58 mol Fe/mol lipid. The optimal conditions for in situ precipitation were found to be an alkalization pH of 12, temperature of 60°C, time of 60 minutes, and initial Fe concentration of 100 mM Fe³⁺ + 50 mM Fe²⁺.

Conclusion: In situ precipitation could be a simple and efficient approach for the preparation of iron-oxide magnetoliposomes.

Keywords: magnetoliposomes, in situ precipitation, iron oxide, alkalization, permeability

Introduction

Magnetoliposomes have been widely used as targeted drug-delivery systems and highly efficient magnetic resonance imaging contrast agents. These biocompatible systems also offer the possibilities for targeting and delivering therapeutic agents for “theragnostics”, a therapy and diagnosis strategy.¹⁻³

Current ways of preparing magnetoliposomes usually involve two steps: 1) magnetofluid is prepared by surface modification of iron-oxide nanocrystals, usually requiring several cycles of heating, precipitation, and purification, and 2) magnetofluid is encapsulated into liposomes to form magnetoliposomes. However, this conventional approach has limitations, including the fact that the magnetofluid preparation procedure is very complex and laborious, and the encapsulation efficiency (EE) and drug-loading capacity of magnetofluid in liposomes are very low due to the instability of magnetofluid. The drug loading of magnetoliposomes reported so far has ranged from...
0.1 to 1.67 mol Fe/mol lipid. However, magnetoliposomes with a high drug-loading capacity (>0.5 mol Fe/mol lipid) were unstable in those studies.4–6

In this study, we developed a novel approach to magnetoliposome preparation that may be applied in targeted drug-delivery systems and which involves two steps. Step 1 involved hydrating FeCl₃/FeCl₂ solutions with lipid films to obtain liposomes by ultrasonic dispersion. Non-encapsulated FeCl₃/FeCl₂ was removed by dialysis. In Step 2, the liposomes were alkalized by adding NH₃ to raise the pH to a critical value and incubated at a certain temperature for a certain time. After centrifugation and dialysis, the purified magnetoliposomes were obtained.

The novel methodology is based on the principle of the different permeability of NH₃ and Fe³⁺/Fe²⁺ into the lipid bilayer of liposomes. As a small molecule, NH₃ has great permeability into lipid bilayer, with a permeability coefficient of nearly 10⁻² cm/s,7 while, as multivalent cations, Fe³⁺ and Fe²⁺ have poorer permeability into the lipid bilayer, with a permeability coefficient of about 10⁻¹³ cm/s.8–10 Thus, when NH₃ is added to the solution of encapsulated Fe³⁺/Fe²⁺ liposomes, NH₃ diffuses into the liposomes rapidly, but only a limited amount of Fe³⁺/Fe²⁺ leaks out from the liposomes. When the solution is heated, the difference is more significant. As NH₃ diffuses into the liposomes, the pH value of the internal phase increases and Fe³⁺/Fe²⁺ undergo a hydrolytic reaction (2 Fe³⁺ + Fe²⁺ + 8NH₃ + H₂O = FeO₂⁺ + 8NH₄⁺ + 4H₂O). In this way, the magnetic cores of the magnetoliposomes were formed in situ.

Materials and methods
Materials
The components of liposomes – 1, 2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC; molecular weight [MW] 734), cholesterol (CH, MW 387), and 1, 2-distearyl-sn-glycero-3-phosphoethanolamine-N-poly(ethylene glycol)2000 (PEG2000DSPE, MW 2, 805) – were purchased from Avanti Polar Lipids (Alabaster, AL, USA). FeCl₃ · 4H₂O (MW 201) and FeCl₂ · 6H₂O (MW 273.5) were used to prepare the solutions of FeCl₃ and FeCl₂ at various concentrations.

Preparation of magnetoliposomes
Preparation of liposome-encapsulated iron ions
FeCl₃/FeCl₂ solutions were hydrated with lipid films to obtain liposomes by ultrasonic dispersion. Non-encapsulated FeCl₃/FeCl₂ was removed by dialysis.

In detail, lipid mixtures of DPPC, CH, and PEG2000DSPE in 55:40:5 mole ratios were dissolved in chloroform in a round-bottom flask and formed into a thin film by removing chloroform under a nitrogen stream followed by evaporation under vacuum for 12 hours. Dry films were hydrated with various concentrations of FeCl₃/FeCl₂ (mole ratios =2:1) solutions to give a lipid concentration of 20 mM. The mixtures were then sonicated gently in a Transonic Digitals Bath Sonifier (Elma Hans Schmidbauer GmbH & Co. KG, Singen, Germany) within a 50°C water bath until the film totally detached from the flask (about 60 minutes). The mixtures were downsized by further agitated sonication using a 600 W ultrasound probe (Kejie Co. Ltd, Nanjing, People’s Republic of China) treated in a 50°C water bath for 2 minutes (work time 2 seconds, gap time 3 seconds). Then the mixtures were dialyzed overnight at room temperature in a 100,000-molecular-weight-cutoff dialysis bag against distilled water to remove non-encapsulated FeCl₃/FeCl₂. Thus, we obtained liposome-encapsulated iron ions.

Alkalization of liposome-encapsulated iron ions
The liposomes were alkalized by adding NH₃ to raise the pH to a critical value and then incubated at a certain temperature for a certain time. The pH value was chosen from 10 to 13, because a lower pH value would be unable to alkalize iron ions and a higher pH value would accelerate the hydrolysis of phospholipids incorrectly. After centrifugation and dialysis, the purified magnetoliposomes were obtained.

In detail, the pH values of the liposome-encapsulated iron ions were measured using a pH meter. To adjust the pH to the target value (pH 10, 11, 12, or 13), 1M NH₃ · H₂O was added to the liposome solutions. Following this, the mixtures were incubated at the target temperature (30°C, 40°C, 50°C, and 60°C) in a water bath for a certain time (15, 30, 60, and 120 minutes) with vigorous stirring. After that, the black sediment was removed by centrifugation for 15 minutes at 4,000 × g, and the brown supernatant was collected. The supernatant was placed in a 10,000-molecular-weight-cutoff dialysis bag suspended in a 50-fold volume of distilled water and stirred for 24 hours to remove the NH₃ · H₂O. Thus, we obtained magnetoliposomes with iron-oxide cores.

Characterization of magnetoliposomes
Characterization of the iron-oxide cores
Prior to characterization of the iron-oxide cores by X-ray diffraction (using a D8 Advance; Bruker, Billerica, MA, USA), 10 mL of magnetoliposomes was ruptured in a tenfold volume excess of ethanol. The mixture was centrifuged after which the liquid that had pooled at the top was poured off without
disturbing the sediment at the bottom. The sediment was dried under vacuum at room temperature for 24 hours. The X-ray patterns were taken using an X’Pert Powder system (PANalytical, Almelo, the Netherlands). The divergence slit was 1° and the receiving slit was 0.15°. The starting and final 20 angles were 20° and 80°, respectively. The step size was 0.05° 20 and the measuring time was 3 seconds per step. The result was compared with the Joint Committee on Powder Diffraction Standards powder-diffraction file card for iron oxide.

The magnetization curves of the iron-oxide cores were determined using a Model 7410 Vibrating Sample Magnetometer (Lake Shore Cryotronics, Westerville, OH, USA).

**Liposome size and morphology**

The mean particle size and particle size distribution of the magnetoliposomes were determined by dynamic light scattering at 25°C with a Zetasizer Nano (Malvern Instruments, Malvern, UK). To measure the particle size distribution of the dispersion, a polydispersity index ranging from 0.0 for an entirely monodisperse sample to 1.0 for a polydisperse sample was used.

Liposome morphology was studied by transmission electron microscopy (TEM) with a JEM-1200EX microscope (JEOL, Tokyo, Japan) operated at an acceleration voltage of 80 kV. The liposome sample was first diluted in distilled water and then mixed with phosphotungstic acid in a 1:1 volume ratio. The sample solutions were then deposited onto negatively charged copper grids that had been pre-coated with carbon.

**Determination of EE**

The initial concentration of iron ions added to the system was recorded as Fe\(_a\). The concentration of the iron ions entrapped in the liposomes before alkalization was recorded as Fe\(_b\). To measure Fe\(_b\), 0.1 mL of the liposomes was ruptured before alkalization by 0.9 mL of ethanol, and the concentration of the iron ions was measured by atomic absorption spectroscopy (3100 Atomic Absorption Spectrometer; PerkinElmer, Waltham, MA, USA).

The total amount of iron in the liposomes after alkalization was determined based on the concentration of iron ions. After alkalization, 0.1 mL of liposomes was ruptured by 0.9 mL of ethanol, and then 9 mL of concentrated HCl (37%) was added to the samples to ionize the iron-oxide crystal cores. The total iron-ion concentration of the mixture was measured by atomic absorption spectroscopy and recorded as Fe(t).

As some liposomes may not have been totally alkalized, they may have contained free iron ions. To determine the concentration of the free iron ions, after alkalization, 0.1 mL of liposomes was ruptured by 0.9 mL ethanol. The mixture was centrifuged at 3,000 rpm for 5 minutes. Thus, the iron-oxide cores would settle on the bottom, while the free iron ions would remain in the upper layer liquid. Then, 0.5 mL of the upper layer liquid was taken to determine the iron-ion concentration by adding 4.5 mL concentrated HCl (37%) and measurement by atomic absorption spectroscopy. It was recorded as Fe(f). The true amount of iron-oxide core – Fe(c) – was calculated by Fe(c) = Fe(t) – Fe(f). The EE of iron was expressed as EE% = Fe(c)/Fe\(_a\) = Fe(t) – Fe(f)/Fe\(_a\).

**Determination of drug loading**

Drug loading was expressed as the amount of encapsulated iron (mol) per mol of phospholipid. Drug loading = Fe(c) (mM)/lipid content (mM). The lipid content of the magnetoliposomes was determined based on the DPPC concentration, as measured by high-performance liquid chromatography (HPLC). The HPLC system consisted of a Waters 600 Solvent Delivery System (Waters Corporation, Milford, MA, USA), a Waters 712 WISP Autoinjector, a column heater, PerkinElmer Nelson Model 610 data acquisition system (PE Nelson System Inc., San Jose, CA, USA), an Astec 250x4.6 mm diol-bonded phase column (Sigma-Aldrich, St Louis, MO, USA), and an evaporative light scattering detector (SEDEX ELSD; SEDERE, Alfortville, France). The mobile phase consisted of chloroform, methanol, and water at a volume ratio of 65:25:4. A 50 μL aliquot of the sample was diluted to 10 mL with chloroform:methanol (85:15, volume/volume) and injected into the HPLC system. The sample was prepared in duplicate and analyzed at a flow rate of 1.3–1.5 mL/minute. The amount of DPPC in the formulation was quantitatively determined using external standards.

**Effects of alkalization pH, temperature, duration, and initial Fe concentration**

The effects of alkalization pH, temperature, duration, and initial iron-ion concentration on the EE and drug loading of magnetoliposomes were determined using single-factor analysis. Specifically, four levels of alkalization pH (pH\(_1\)=13, pH\(_2\)=12, pH\(_3\)=11, and pH\(_4\)=10), four levels of alkalization temperature (T\(_1\)=30°C, T\(_2\)=40°C, T\(_3\)=50°C, and T\(_4\)=60°C), four levels of alkalization duration (t\(_1\)=15 minutes, t\(_2\)=30 minutes, t\(_3\)=60 minutes, and t\(_4\)=120 minutes), four levels of initial iron-ion concentration (C\(_1\)=400 mM FeCl\(_3\)+200 mM FeCl\(_2\), C\(_2\)=200 mM FeCl\(_3\)+100 mM FeCl\(_2\), C\(_3\)=100 mM FeCl\(_3\)+50 mM FeCl\(_2\), and C\(_4\)=50 mM FeCl\(_3\) were tested. The effects of alkalization on the EE and drug loading were analyzed using ANOVA, and differences were considered significant at p < 0.05.
FeCl$_3$+25 mM FeCl$_2$ were studied individually. Then, an orthogonal experimental design – L$_{16}$($4^5$) – was applied to determine the best composition of those experimental conditions.

The pH range of 10–13 was chosen because moderate alkaline conditions would be suitable for iron-ion precipitation. High pH may cause vigorous lipid hydrolysis. The temperature range was chosen around the phase-transition temperature of DPPC, which might be the optimal temperature for getting a balance between lipid membrane permeability and liposome integrity. The alkalization duration range was chosen based on the common incubation time for drug loading by pH gradient. The iron-ion concentration range was chosen around 150 mM, which had a pH value of about 1, as a higher concentration with a lower pH value would greatly intensify lipid hydrolysis.

In vitro stability analysis
The in vitro stability of the magnetoliposomes was assessed when they were stored at 4°C or 37°C for 3 months. For every week in the first month and every two weeks in the following two months, a 1 mL sample of the liposomes was drawn for size determination by dynamic light scattering and EE determination using the method described earlier in the “Characterization of magnetoliposomes” section.

Results
Characterization of the iron-oxide cores
The iron-oxide cores of magnetoliposomes were obtained by the method described earlier in the “Characterization of magnetoliposomes” section. The X-ray diffraction patterns of the iron-oxide cores confirmed the particles were single-phase cubic magnetite (Fe$_3$O$_4$) (Figure 1). The room-temperature magnetization curves of the iron-oxide cores showed superparamagnetic response and the saturation magnetization value of 19.5 emu/g (Figure 2).

Liposome size and morphology
The magnetoliposomes were of brownish semitransparent appearance. The size, determined by dynamic light scattering and expressed as z-average, was 168±14 nm (polydispersity index =0.23±0.06; Figure 3) and the zeta potential was $-26.2\pm1.9$ mV (n=3).

TEM micrographs of magnetoliposomes are presented in Figure 4. Figure 4A shows the liposome-encapsulated FeCl$_3$/FeCl$_2$ solution before alkalization, while Figure 4B shows the liposomes after alkalization. Before alkalization, the liposomes were basically transparent. After alkalization, the liposomes became opaque with high-density areas scattered irregularly through them.
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EE and drug loading
The EE and drug loading were determined with varying initial iron-ion concentrations, alkalization pHs, temperatures, and durations. The initial lipid concentration was 20 mM.

The EE ranged from 3% to 22%, and the drug loading ranged from 0.2 to 1.58 mol Fe/mol lipid. Details are shown in Tables 1–4.

Effects of alkalization pH, temperature, duration, and initial Fe concentration
Single-factor analysis was performed to determine the effect of alkalization pH, temperature (T), duration (t), and initial iron-ion concentration (C) on the EE and drug loading of the magnetoliposomes. An orthogonal experimental design – L_{16}(4^5) – was applied to determine the best combination of experimental conditions.

Alkalization pH
Four levels of alkalization pH (pH_1=10, pH_2=11, pH_3=12, and pH_4=13) were applied to determine the effect of alkalization pH. The values of initial iron-ion concentration (C), alkalization temperature (T), and alkalization time (t) were fixed at C=150 mM, T=50°C, and t=30 minutes according to the results of the preliminary study. The initial lipid concentration was 20 mM.

The EE and drug loading reached their maximum at pH 12. The total Fe concentrations in liposomes after alkalization (Fe[t]) were very close at pH 10–12, but decreased significantly at pH 13. The free iron-ion concentrations in the liposomes (Fe[f]) decreased significantly as the pH increased (as shown in Table 1 and Figure 5).

Alkalization temperature
Four levels of alkalization temperature (T_1=30°C, T_2=40°C, T_3=50°C, and T_4=60°C) were applied to determine the effect of alkalization temperature. The values of initial iron-ion concentration (C), alkalization pH, and alkalization time (t) were fixed at C=150 mM, pH 12, t= 30 minutes according to the results of the preliminary study. The initial lipid concentration was 20 mM.

The drug loading increased as the alkalization temperature increased and reached its maximum at 60°C, while the EE was not augmented further beyond 50°C. Fe(t) decreased slightly as temperature increased, while Fe(f) decreased dramatically as the temperature increased (as shown in Table 2 and Figure 6).

Alkalization duration
Four levels of alkalization duration (t_1=15 minutes, t_2=30 minutes, t_3=60 minutes, and t_4=120 minutes) were
applied to determine the effect of alkalization duration. The values of initial iron-ion concentration (C), alkalization pH, and alkalization temperature (T) were fixed at C=150 mM, pH 12, and T=60°C according to the results of the preliminary study. The initial lipid concentration was 20 mM.

The EE and drug loading were increased as alkalization duration lengthened and reached a maximum at 60 minutes. The Fe(t) decreased slightly with longer alkalization duration, while the Fe(f) markedly decreased at 30 minutes, then remained at almost the same level after that (as shown in Table 3 and Figure 7).

Initial iron-ion concentration

Four levels of initial iron-ion concentration (C₁=400 mM FeCl₂+200mMFeCl₃, C₂=200mMFeCl₂+100mMFeCl₃, C₃=100 mMFeCl₂+50mMFeCl₃, and C₄=50 mMFeCl₂+25 mMFeCl₃) were applied to determine the effect of ionic concentration. The values of alkalization pH, alkalization temperature (T), and alkalization time (t) were fixed at pH 12, T=60°C, t=60 minutes, according to the results of the preliminary study. The initial lipid concentration was 20 mM.

With the decrease of the initial iron-ion concentration, the EE increased gradually, while the drug loading reached maximum at an initial Fe concentration of 150 mM (as shown in Table 4 and Figure 8).

Orthogonal experimental design

An orthogonal experiment – L_{16}(4³) – was applied to explore the optimal formulation with drug loading as the index. The detailed orthogonal experiment and related results are listed in Tables 5 and 6, respectively. Specifically, factor A, alkalization pH; factor B, initial Fe concentration; factor C, alkalization temperature; factor D, alkalization duration; factor E, random (control factor). Each factor had four levels as indicated in Table 5. As determined by variance R analysis, the order of influence for drug loading was found to be A>B>C>D. The best combination level of the four factors was A3B2C4D3. Therefore the optimum preparation conditions were: an initial Fe concentration of 150 mM, an alkalization pH of 12, a temperature of 60°C, and an alkalization duration of 60 minutes. The average drug loading of magnetoliposomes prepared with the optimal formulation was 1.58±0.04 mol Fe/mol lipid (n=3).

In vitro stability

The magnetoliposomes prepared at pH 12, T=60°C, t=60 minutes, and C=150 mM were stored at 4°C or 37°C for three months. The size of magnetoliposomes did not undergo significant changes for almost 4 weeks at 4°C and 3 weeks at 37°C. After that, the size of the magnetoliposomes decreased gradually and the appearance gradually changed...
in an alkaline environment. The schematic diagram of the
formation of crystal cores would be rather irregular,
and tends to aggregate and settle down during the preparation process of magnetoliposomes,
leading to the low EE of magnetofluid in liposomes. The EE of magnetoliposomes prepared by a conventional method ranges between 2% and 12%,6,15 while a high EE of 22% was achieved by our new method. Although the EE decreased with the increase of initial Fe concentration in our study, the maximal drug loading (1.58 mol Fe/mol lipid) achieved was close to the reported maximal value (1.67 mol Fe/mol lipid).4

Another potential advantage of this novel approach is that it may be useful in preparing magnetoliposomes
The effect of alkalization temperature

Figure 6 The effect of alkalization temperature.
Abbreviations: Fe(t), the amount of total iron in the liposomes after alkalization; Fe(f), the amount of free iron ions in the liposomes after alkalization.

The effect of alkalization duration

Figure 7 The effect of alkalization duration.
Abbreviations: Fe(t), the amount of total iron in the liposomes after alkalization; Fe(f), the amount of free iron ions in the liposomes after alkalization.

The effect of initial iron ions concentration

Figure 8 The effect of initial iron-ion concentration.
Abbreviations: Fe(t), the amount of total iron in the liposomes after alkalization; Fe(f), the amount of free iron ions in the liposomes after alkalization.

Table 5 Four levels of five factors in orthogonal experiments

| Level | Factor                          | A: initial Fe concentration (mM) | B: alkalization pH | C: alkalization temperature (°C) | D: alkalization duration (minutes) | E: random* |
|-------|---------------------------------|---------------------------------|--------------------|---------------------------------|----------------------------------|------------|
| 1     |                                 | 600                             | 13                 | 30                              | 15                               | 1          |
| 2     |                                 | 300                             | 12                 | 40                              | 30                               | 2          |
| 3     |                                 | 150                             | 11                 | 50                              | 60                               | 3          |
| 4     |                                 | 75                              | 10                 | 60                              | 120                              | 4          |

Note: *Random is the control factor of orthogonal experiment.
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for loading insoluble compounds such as 5-Fluorouracil (5-FU). 5-FU is almost insoluble in water but shows good solubility in acidic solution. With FeCl₃/FeCl₂ solutions as hydrating mediums for lipid-film hydration, the new approach could significantly increase the drug loading of 5-FU in magnetoliposomes. So, this new approach may provide a promising option to increase the drug loading of compounds with poor solubility in water but good solubility in acidic solutions.

Disadvantage of the novel approach

The disadvantage of this approach is the inevitable phospholipid hydrolysis that occurs during the preparation process. In an acidic or basic aqueous environment, phospholipid molecules are subjected to hydrolysis following pseudo first-order kinetics.¹¹ The rate of hydrolysis depends on temperature and pH, with a minimal rate at a pH of 6.5.¹²,¹³ Accumulation of hydrolyzed products in the liposome membrane has been shown to alter the integrity of the bilayer and to induce leakage, fusion, and the transformation of liposomes into alternative aggregate structures.¹⁴ The hydrolysis rate and the influence of the hydrolyzed products on the liposome structure were not directly examined in this study, but the lipid content was determined at various conditions, which could be treated as an indirect index of phospholipid hydrolysis.

The lipid content decreased with the increase of initial Fe concentration (Table 4), because the pH value of the initial liposome formulation decreased dramatically with the increase of initial Fe concentration (eg, pH 1.66 at Fe = 75 mM, pH 0.85 at Fe = 150 mM). The excess hydro-

Table 6 Results of orthogonal experiments

| Experiment number | A: initial Fe concentration (mM) | B: alkalinization pH | C: alkalinization temperature (°C) | D: alkalinization duration (minutes) | E: random* | Drug loading (mol Fe/mol lipid) |
|-------------------|---------------------------------|----------------------|-----------------------------------|-------------------------------------|-----------|-------------------------------|
| 1                 | 1                               | 1                    | 1                                 | 1                                   | 1         | 0.24                          |
| 2                 | 1                               | 2                    | 2                                 | 2                                   | 2         | 0.95                          |
| 3                 | 1                               | 3                    | 3                                 | 3                                   | 3         | 0.69                          |
| 4                 | 1                               | 4                    | 4                                 | 4                                   | 4         | 0.12                          |
| 5                 | 2                               | 1                    | 2                                 | 3                                   | 4         | 1.02                          |
| 6                 | 2                               | 2                    | 1                                 | 4                                   | 3         | 0.94                          |
| 7                 | 2                               | 2                    | 3                                 | 1                                   | 2         | 0.89                          |
| 8                 | 2                               | 4                    | 3                                 | 2                                   | 1         | 0.51                          |
| 9                 | 3                               | 1                    | 3                                 | 4                                   | 2         | 0.95                          |
| 10                | 3                               | 2                    | 4                                 | 3                                   | 1         | 1.58                          |
| 11                | 3                               | 3                    | 3                                 | 1                                   | 2         | 0.43                          |
| 12                | 3                               | 4                    | 2                                 | 1                                   | 3         | 0.58                          |
| 13                | 4                               | 1                    | 4                                 | 2                                   | 3         | 0.57                          |
| 14                | 4                               | 4                    | 3                                 | 1                                   | 4         | 0.77                          |
| 15                | 4                               | 1                    | 3                                 | 4                                   | 1         | 0.48                          |
| 16                | 4                               | 4                    | 1                                 | 3                                   | 2         | 0.23                          |

k₁ 0.500 0.695 0.460 0.620 0.703
k₂ 0.840 1.060 0.757 0.615 0.755
k₃ 0.885 0.623 0.730 0.880 0.695
k₄ 0.512 0.360 0.790 0.622 0.585
R 0.385 0.700 0.330 0.265 0.170

Note: *Random is the control factor of orthogonal experiment.

Abbreviations: k, the average drug-loading value of magnetoliposomes at different levels of every factor; R, the range of k at different levels of every factor.

Figure 9 In vitro stability of magnetoliposomes.
gen ions greatly catalyzed the lipid hydrolysis reaction and decreased the stability of the liposomes, therefore greatly decreased the EE of the liposomes (Table 4). In contrast, with the increase of the alkalization pH, the lipid content decreased slightly (Table 1). This indicates that an excess amount of hydroxyl ions also catalyzed the hydrolysis reaction, although the effect of the hydroxyl ions was less profound than that of the hydrogen ions.

With the increase of alkalization temperature (Table 2) and alkalization duration (Table 3), the lipid content decreased slightly. This indicates that high temperature and long reaction duration accelerated the lipid hydrolysis reaction, although the effects of these were less profound than that of pH value.

**Predominant factors influencing the EE and drug loading of the novel methodology**

Alkalization pH had the most profound effect on the results. As pH increased, the amount of free iron ions in liposomes decreased greatly (Figure 5). This indicates that the increase in pH gradient between the outer and inner phases of the liposomes facilitated the influx of NH₃. The critical pH point was 12; below this point, the majority of liposomes could not be alkalized. Increasing the pH value only slightly increased the extent of alkalization, but decreased the drug loading due to the increase in lipid hydrolysis.

Alkalization temperature and duration had similar influence on the results. Higher temperature led to a higher NH₃ permeability coefficient and higher permeability of the lipid bilayer, which, on the one hand, facilitated the process of alkalization but, on the other, also enhanced the lipid hydrolysis rate. The critical point was T=50°C; below this point, most liposomes could not be alkalized. Above this point, the EE and drug loading could not be enhanced further due to the increase in lipid hydrolysis (Figure 6). The critical point of alkalization duration was 30 minutes; below this point, most liposomes could not be alkalized. Above this point, the EE and drug loading increased slightly and reached maximum at 60 minutes (Figure 7).

**Effect of initial Fe concentration**

This has been discussed earlier. A higher initial Fe concentration led to stronger acidic conditions and more severe lipid hydrolysis. When the initial Fe concentration was greater than 150 mM, the EE decreased significantly.

**Other critical factors**

Aside from pH, temperature, duration, and initial Fe concentration, the traditional critical factors of liposome preparation such as lipid composition and ultrasound parameters were not evaluated in this study because these factors were less involved in the alkalization process and their effects on EE and drug loading have already been well elucidated in conventional liposome preparation.

Concerning the lipid composition, saturated phospholipids such as DPPC would be more suitable for this novel magnetoliposome-preparation approach than unsaturated phospholipids, since unsaturated phospholipids are more easily oxidized by iron ions. A high molar percent of CH (>30%) may be also critical to the success of in situ precipitation, since liposomes with a high CH percentage are more stable and have a broader range of liquid crystal transition temperatures.

**Future directions**

To increase the drug loading and stability of magnetoliposomes prepared by in situ precipitation, further research is needed on the prevention of lipid hydrolysis. The possible resolutions include switching FeCl₃/FeCl₂ solutions with a less acidic agent such as pure FeCl₂ solution or ferric ammonium citrate solution, adding a lipid-hydrolysis protective agent, and dissolving the FeCl₃/FeCl₂ compound in an amphiphilic solvent.

**Conclusion**

In this study, we developed a novel approach (in situ precipitation) for iron-oxide magnetoliposome preparation. We investigated the physical and chemical properties of magnetoliposomes prepared by this new method and also evaluated...
the critical factors influencing the EE and drug loading of magnetoliposomes. Taken together, our findings indicate we have established a simple and efficient approach for the preparation of iron-oxide magnetoliposomes.

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Disclosure
The authors declare no conflicts of interest in this work.

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