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The encapsulation rate and pH sensitivity of arsenic were improved in liposome nanoparticles by the calcium acetate gradient method

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Abstract: The study proposed improving the arsenic encapsulation efficiency (EE) in liposomes and make it pH responsive. Liposomes were prepared using the ethanol injection method (EIM), thin film dispersion method (TFM), and CAGM with sodium arsenite ($NaAsO_2$). The orthogonal experimental was used to optimize the preparation conditions of the CAGM. The arsenic-carrying liposomes were characterized by polydispersity index (PDI), transmission electron microscopy (TEM), *in vitro* release experiments, and inductively coupled plasma emission spectrum (ICP). The toxicity was investigated by rat glioma cells (C6) and human brain microvascular endothelial cells (HBMEC). The results indicated that the CAGM can effectively improve the EE of NaAsO2 and has a pH response compared with EIM and TFM. The size of nanoparticles prepared by CAGM was 118.8±56.67 nm, the arsenic EE was 54.3±9.82%, the drug loading rate was 7.13±0.72% (P<0.01), pH sensitivity was shown at pH 5.5. The optimal parameters of the CAGM were 3 mg NaAsO2, 5:1 egg phosphatidylcholine (EPC) to cholesterol (CHOL), and 240 mmol/L calcium acetate (CaAC2). The results showed that the CAGM has good biocompatibility and is one of the effective ways to improve the NaAsO2 encapsulation rate and pH response in liposome nanoparticles.

Keywords: Arsenic, liposome, calcium acetate, encapsulation rate, pH sensitivity.

INTRODUCTION

Liposome nanoparticles are emerging target drug carriers with good biocompatibility and amphiphilicity (Wang *et al.*, 2016). Some liposome-based products have been used to treat clinical diseases (Kraft *et al.*, 2014; Ahmed *et al.*, 2018). It shows a good development prospect.

Liposomes are mainly divided into three types according to relevant literature: small unilamellar liposomes (20-100 nm), giant unilamellar liposomes (>100nm) and multilamellar liposomes (>1µm) (Wang *et al.*, 2017). When the liposome size is smaller than 200 nm, it can penetrate the tumor tissue, giving play to enhanced permeability and retention effect (EPR) (La-Beck *et al.*, 2021; Yang *et al.*, 2021). In addition, pH in the solid tumor microenvironment is lower than other normal tissue at the same time, if the carrier has pH sensitivity, it can promote the release of the drug at the targeted tumor site and enhance the anti-tumor effect (Qin *et al.*, 2007; Bai *et al.*, 2018). In addition, liposomes have many functional phospholipid materials and the surface structure and particle size are easy to control. These characteristics make it a suitable target carrier to reduce toxicity and increase efficacy.

Liposomes prepared by traditional methods are usually suitable for carrying lipophilic drugs, but it is difficult to carry hydrophilic drugs. Many liposome studies have reported methods to improve hydrophilic drug encapsulation rates, such as ion or pH gradient drug loading (Hwang *et al.*, 1999), the use of a supersaturated solution of drugs (Modi *et al.*, 2012), the formation of complex precipitation (Li *et al.*, 2018), multivesicular liposome (Gorain *et al.*, 2021). However, most current studies and applications focus on liposoluble drugs and these methods are generally applicable to specific drugs or biomacromolecule drugs (Clerc *et al.*, 1995). Meanwhile, each of these approaches has flaws, such as severe toxicity problems and difficulty metabolizing (Khodayar *et al.*, 2018; Umar *et al.*, 2019; Umar *et al.*, 2023). There are few studies on water-soluble drugs carried by liposomes. The problem of low encapsulation and loading rate limits liposomes as carriers of water-soluble drugs (Tazina *et al.*, 2011; Guimarães *et al.*, 2021).

Arsenic, a traditional Chinese medicine, is the main chemical component of arsenic trioxide and $NaAsO_2$. On the one hand, it can treat some malignant tumors, such as leukemia and liver cancer. On the other hand, direct application is widely distributed in the body and will produce a wide range of toxicity. The ideal arsenic preparation should have suitable targeting properties, increased drug concentration in the tumor site, and distribution in other parts as little as possible, thereby reducing side or unexpected effects (Antman, 2001; Chen *et al.*, 2002; Evens *et al.*, 2004; Emadi *et al.*, 2010). $NaAsO_2$ is also water-soluble (Farzaneh *et al.*, 2018; Rao

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et al., 2018). Due to the low encapsulation rate of hydrophilic drugs in liposomes, liposomes that carry arsenic have been limited. Therefore, liposomes act as carriers for water-soluble drugs such as $NaAsO_2$. How to control the particle size so that it can play the EPR effect while increasing the hydrophilic drugs encapsulation rate of arsenic is a problem that needs to be solved. In this study, the encapsulation rate of arsenic in liposomes was successfully increased, and the particle size was controlled at about 100 nm using the CAGM combined with ultrafiltration technology. These results may expand the application of arsenic.

MATERIALS AND METHODS

Materials and Instrumentation

Egg phosphatidylcholine (EPC) was commercially obtained from LIPOID GMBH. FRIGENSTRASSE (Ludwigshafen, Germany), Cholesterol (CHOL) from A.V.T. Pharmaceutical Co., Ltd. (Shanghai, China). $NaAsO_2$ was provided by the pharmacy laboratory of the University of Traditional Chinese Medicine (Hangzhou, China). Sodium sulfate anhydrous was purchased from Aladdin Industrial Corporation (Shanghai, China). $CaAC_2$ was obtained from LingFeng Chemical Reagent CO.LTD (Shanghai, China). The dialysis bag (MD3500) was obtained from Yuan Ye Bio-Technology Co. Ltd (Shanghai, China). All other reagents are analytically pure.

Ultrasonic cell disrupter JY 88-IIN (SCIENTZ, China), High-Pressure Homogenizer AH100D (ATS, USA), ultrafiltration device MILLIPORE Labscale TFF System XX42LSS12 (Millipore, USA) were used in the experiments. Deionized water was from an EQ7000 Milli-Q deionization unit (Millipore, USA). Particle size was measured by Nano-ZS 90 Malvern Zetasizer (Malvern Instruments, UK). Arsenic content was determined using ICAP-7000 (Thermo Fisher, USA).

Preparation of arsenite liposomes

Ethanol Injection Method (EIM)

We used EPC, CHOL and $NaAsO_2$ as the primary materials. EPC 500mg and CHOL 100mg (5:1, W/W) dissolved in 10mL absolute ethyl alcohol (65°C by water bath), then added to 50mL of $NaAsO_2$ solution (3 mg/mL) at 45°C, rapidly mixed water phase under the condition of constant temperature magnetic stirring for 30 min, then transferring into rotary vacuum evaporator to remove the ethanol, then using ultrasound of 25% power for 10 minutes, interval of 1 second. 400 nm, 200 nm, 100 nm and 50 nm filter membranes were used for homogeneous film extrusion through the homogenizer and the collected liposome was kept at 4°C (Shaker *et al.*, 2017; Du *et al.*, 2023).

Thin-film lipid hydration dispersion method (TFM)

Dissolving EPC 500 mg and CHOL 100mg (5:1, W/W) in absolute ethyl alcohol 10mL (65°C by water bath), remained at 45°C. The solution was divided into five parts, 10mL at a time and treated with a rotary vacuum evaporator for 10 min. The dried thin film was dissolved in 10mL $NaAsO_2$ solution (3mg/mL) for 10 min, using 25% power ultrasound for 10 min (1s interval), then extruded through 400nm, 200nm, 100nm and 50 nm filter membrane with the homogenizer. The liposomes collected after preparation were kept at 4 degrees °C (Zhang, 2017).

Calcium acetate gradient method (CAGM)

Dissolving EPC 500 mg and CHOL 100mg (5:1, W/W) in absolute ethyl alcohol 10mL (65°C by water bath), remained at 45°C. The solution was divided into five parts, 10mL at a time and vacuum-rotary evaporation was applied for 10 min. The dried thin film was dissolved in 10mL $CaAC_2$ solution (120 mmol) for 10 min, followed by hydration and 25% power ultrasonic was treated for 10 min (1s interval). After combining the five liposomes, a homogenizer was used to extrude liposomes and the filter membrane (400nm, 200nm, 100 nm and 50nm) was used successively. All extrusions were collected for ultrafiltration. Precooled sodium sulfate solution (120 mmol/L, 4°C) was used as a washing solution, concentrated to about 50mL after washed 5 times. Then, the liposome was added with 50mL $NaAsO_2$ solution (3 mg/mL) and kept at room temperature overnight, avoiding light. Finally, Precooled sodium chloride solution (0.9% W/V, 4°C) was used as a washing solution, concentrated to about 50mL after washed 5 times (Clerc *et al.*, 1995).

Dynamic light scattering measurements

The Z-average scale and polydispersity index (PDI) of the distribution of liposomes were obtained through Malvern Zetasizer, which used dynamic light scattering (DLS). The zeta surface potential of arsenic-carrying liposomes was determined simultaneously. All measurements were performed three times to obtain the mean and standard deviation for each liposome (Wang *et al.*, 2017).

Transmission electron microscopy

We used transmission electron microscopy (TEM) to directly observe the morphology of liposomes (Khodayar *et al.*, 2018). First, dilute the liposome to a suitable concentration, and then take a small amount of specimen fluid and add it on the surface of a formvar carbon-coated copper grid, followed with a phosphotungstic acid solution (3%, W/V) by negative staining. Drying for 30 seconds under infrared rays and morphology was observed by TEM operated at 100 kV.

ICP analysis

Quantitative analyses of arsenic were performed by inductively coupled plasma emission spectrum (ICP). The analytical ICP equipment was an ICAP-7000 series.

The prepared liposome solution of 100 milliliters added to

the ultra filtration tube was centrifuged for 10 min at 12000 revolutions per minute (rpm). The centrifugal liquid was taken for free arsenic concentration after diluting with 5% nitric acid.

Taking another liposome solution of 100 milliliters, added a perchloric acid solution of 50μL, diluted with 5% nitric acid and volume to 10mL to measure the total arsenic concentration. Both were tested after treatment with a 0.22μm filter membrane (Velez-Quinones *et al.*, 2018).

Encapsulation efficiency (EE) and drug loading (DL)

The drug loading and encapsulation rate of arsenic in liposomes were determined by ICP detection of arsenic content. Calculating formula as follows:

$$\text{Encapsulation efficiency}(\%) = \frac{\text{Wtotal arsenic - Wfree arsenic}}{\text{Wtotal arsenic}} \times 100\%$$

$$\text{Drug Loading }(\%) = \frac{\text{Wtotal arsenic - Wfree arsenic}}{\text{Wtotal}} \times 100\%$$

Orthogonal optimization of conditions for CAGM

Orthogonal experiments were carried out on three key parameters (Dong *et al.*, 2012; Zheng *et al.*, 2013). The $C_9(3^4)$ orthogonal table was used to optimize the encapsulation rate of CAGM.

In vitro drug release experiment

The drug-loaded liposomes (1mL) were taken in a dialysis bag (3500 kD) and immersed into 100mL acetate buffer (pH 5.5, 6.5, 7.4). The beakers were kept in a water-bathing constant temperature vibrator at 37ºC with a proper shaking rate. Solution (1mL) was taken out at a scheduled time, and 1mL of buffer was replenished. Then, 1 mL solution from the beaker was diluted to 4mL with 5% nitric acid, filtering through 0.22μm film for ICP. The experiment was repeated three times, and the cumulative arsenic was calculated as formula (Zucker *et al.*, 2009; Fei *et al.*, 2017):

$$\text{Qn }(\%) = \frac{\sum CnVn}{CoVo} \times 100\%$$

Q_n (cumulative drug release rate at time point n), C_0 (concentration of test sample at each time point), V_0 (volume of the test sample), C_n (concentration in medium), Vn (volume of medium).

Blank vector cytotoxicity observation

The blank vector Cytotoxicity was determined by Methylthiazolyldiphenyl-tetrazolium bromide (MTT). They took a 96-well plate culture for 24h with 100μL cell suspension per well (3000-5000 cells). Drug concentrations of 150μL (serum-free medium dilution) were added and cultured for 48h. Add MTT solution 10 μL per well (5mg/mL, diluted in serum-free medium) and incubate for 4h (avoid light). Discard solution, washing 2 times with PBS, then adding 150μL DMSO, shaken for 10 min and detected at 570 nm wavelength using enzyme absorbance value, calculating cell viability as formula (Floris *et al.*, 2014; Zhang *et al.*, 2018):

$$\text{Cell Viability }(\%) = \frac{\text{Asample - Ablank}}{\text{Acontrol - Ablank}} \times 100\%$$

STATISTICAL ANALYSIS

All the experimental results were expressed as the mean ± SD after repeated three times. Experimental Data were analyzed using SPSS 26.0 and Graphpad Prism 8.0. Statistical methods used the T-test, one-way analysis, and Chi-square analysis. The level of $P<0.05$ was considered statistically significant.

RESULTS

Liposomes Characterization

Size of the liposomes

The results of particle size distribution, PDI and zeta surface potential values of different methods are given in table 1 and fig. 1. Data were assumed as mean ±SD. One-way ANOVA and Turkey's post hoc test for multiple group comparisons were performed as statistical analysis.

The results showed that the particle size of the liposomes could be controlled below 100 nm and there was no significant difference was observed between the particle size prepared by TFM and that prepared by EIM, but the particle size prepared by CAGM was enlarged compared with the other two preparation methods ($P<0.01$).

According to the data, there was only peak 1 in all preparations, indicating that the particle size distribution of preparations was concentrated and the preparation had no agglomeration of particles occurred, which was consistent with the relevant stability data of liposomes. The liposomes were stored in the refrigerator (2-8ºC) for three months and the particle size and PDI did not change significantly.

Zeta potential of the liposomes

Zeta potential is one of the essential factors affecting the stability of liposomes. Experimental data showed that the potentials of the three preparations were all negative, and no significant difference was found among the groups (fig. 1).

The negative zeta potential of liposomes prepared by TFM and CAGM was higher than that prepared by EIM, which may be related to the degree of dissociation of phosphates in lecithin at different pH. The conditions in preparing TFM and CAGM were almost neutral, while the pH of the ethanol solution was acidic.

Table 1: Particle Z-Average and PDI Values of the Prepared Liposomes (n = 3)

	Z-average (nm)	PDI	Peak 1 (nm)	Intensity (%)	Peak2 (nm)
TFM	85.21±0.23**	0.165±0.004	103.21±46.73	100	/
EIM	82.87±0.35**	0.175±0.026	104.03±51.68	100	/
CAGM	96.75±0.57	0.189±0.016	118.85±56.67	100	/

**P<0.01 when compared with CAGM

Table 2: Orthogonal experiment level and factor

Levels	EPC/CHOL (W/W)	$NaAsO_2$ (mg/mL)	$CaAC_2$ (mmol)	Null
1	3:1	0.5	60	1
2	5:1	3	120	2
3	7:1	5	240	3

Table 3: Orthogonal experimental results

Number	EPC/CHOL (W/W)	$NaAsO_2$ (mg/mL)	$CaAC_2$ (mmol)	Null	Encapsulation efficiency (%)
1	3:1	0.5	60	1	14.67
2	3:1	3	120	2	43.8
3	3:1	5	240	3	62.35
4	5:1	0.5	120	3	39.11
5	5:1	3	240	1	67.32
6	5:1	5	60	2	37.64
7	7:1	0.5	240	2	22.76
8	7:1	3	60	3	25.85
9	7:1	5	120	1	45.19
K1	48.94	26.11	35.39	43.73	
K2	51.62	53.32	44.97	46.07	
K3	31.93	53.06	52.14	42.70	
R	19.69	27.21	16.76	3.36	
F	38.36, P<0.05	82.23, P<0.05	23.78, P<0.05		
Influence factor	$NaAsO_2$ > EPC/CHOL> $CaAC_2$				
Optimal levels	$NaAsO_2$=3 mg/mL EPC/CHOL=5/1 $CaAC_2$ = 240 mmol				

Polydispersity index of the liposomes

Generally, PDI <0.2 indicates high homogeneity. Experimental results (table 1) showed that all DPI of liposomes prepared using different methods was less than 0.2. Therefore, the size of nanoliposomes prepared by the three methods was uniform, and the High-Pressure Homogenizer may play a significant role.

Transmission electron microscope

The morphology of liposomes prepared by the TFM, EIM, and CAGM was observed using a TEM (fig. 2). The experimental results show that all the samples exhibited spherical appearance characteristics. The liposome fragments produced by EIM had an apparent hollow structure consistent with the liposome structure.

Encapsulations efficiency

Our investigation showed that the encapsulation rate of arsenic was lower in the traditional liposome preparation method (fig. 3). The TFM and EIM were 10.44±2.52% and 9.89±1.86% (P>0.05) respectively. The encapsulation rate of CAGM was 54.30± 9.82%, which was significantly higher than the traditional liposome preparation method (P<0.01). TFM, EIM and CAGM drug loading were 2.14±0.62%, 1.37±0.81% and 7.13 ±0.72%, respectively. The statistical results of drug loading were similar to that of the encapsulation rate.

Orthogonal optimization of conditions for CAGM

According to the results of previous experiments, orthogonal tests were conducted to investigate three factors that may affect the encapsulation rate of $NaAsO_2$ in the preparation of CAGM, the ratio of EPC and CHOL, the concentration of $NaAsO_2$ and $CaAC_2$. Three levels of each factor were investigated, among which EPC/CHOL was 3:1, 5:1 and 7:1, the concentration of $NaAsO_2$ was 0.5mg/mL, 3mg/mL and 5mg/mL and the concentration of $CaAC_2$ was 60 mmol, 120 mol and 240 mol, respectively. From the experimental results, the effects on the encapsulation rate of arsenic, from the largest to the smallest, were the concentration of $NaAsO_2$, EPC/CHOL, and $CaAC_2$. The results demonstrated that sodium arsenite 3mg/mL, EPC/CHOL 5:1 and $CaAC_2$ concentration 240 mmol was the optimal combination of three factors and three levels.

In vitro drug release

We also experimented with the release of liposomes

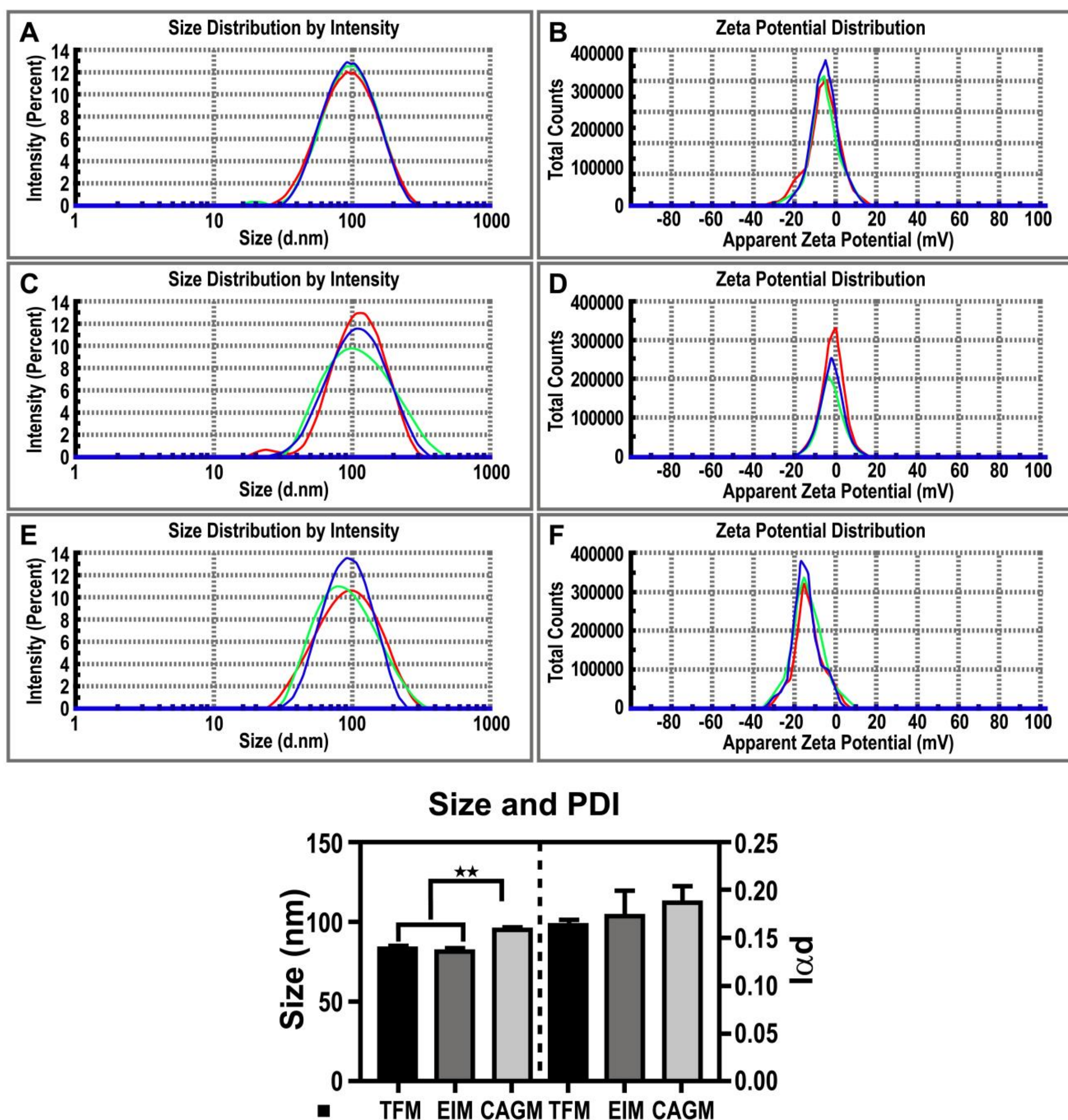

Fig. 1: Size and Zeta potential distribution of different methods. TFM (A-B), EIM (C-D), CAGM (E-F). **P<0.01

prepared by CAGM *in vitro*. The release of arsenic was scheduled and recorded for 12 h in acetate buffer (pH 7.4, 6.5, and 5.5, 0.1mol/L) at 37ºC with constant oscillation, and the *in vitro* cumulative release rate curve was shown in fig. 5. In the case of pH 5.5, free $NaAsO_2$ reached the maximum release rate at 2h, then gradually becomes stable (the cumulative release rate was 89.11-96.21%). Liposomes prepared by CAGM have a similar release at pH 5.5 (the cumulative release rate was 69.95-80.72%). However, in the case of pH 7.4 and pH 6.5, the peak time of the cumulative release rate was significantly prolonged, appearing at about 8 h (the cumulative release rate was 54.41- 60.41% and 60.51- 69.45%).

Blank vector cytotoxicity assay

We used C6 and vascular HBMEC to investigate the toxicity of blank liposomes prepared by CAGM to observe the effect of inorganic substances introduced in the preparation process on cell growth. The experimental results (fig. 6) showed that the blank vector promoted the growth of C6 cells at a low concentration. The growth promotion effect peaked when the concentration of the blank vector was 10ug/mL and then the effect became less noticeable when the concentration continued to increase. For HBMEC, the concentration of the blank vector peaked at 100ug/mL and then continued to increase, and the concentration began to show inhibition.

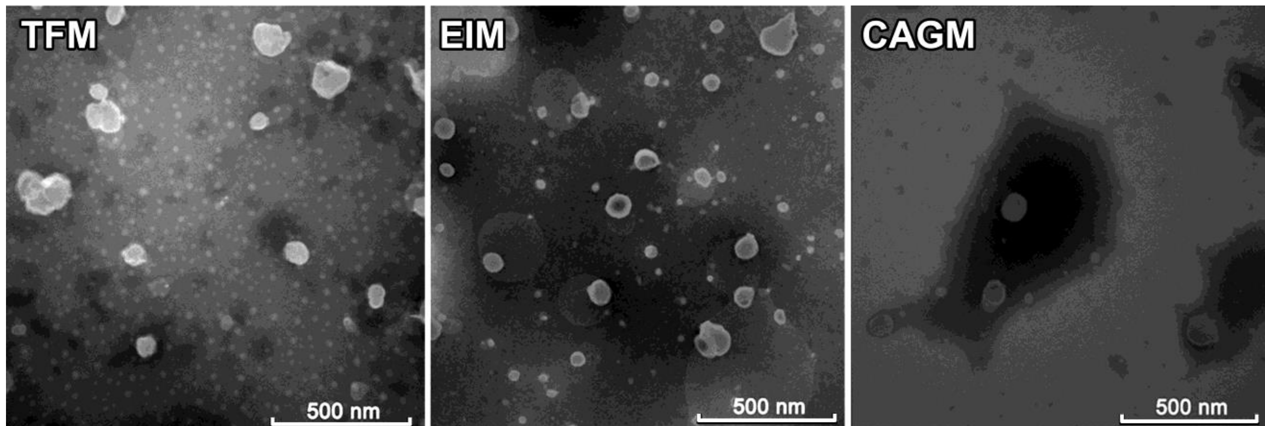

Fig. 2: Transmission electron microscope results

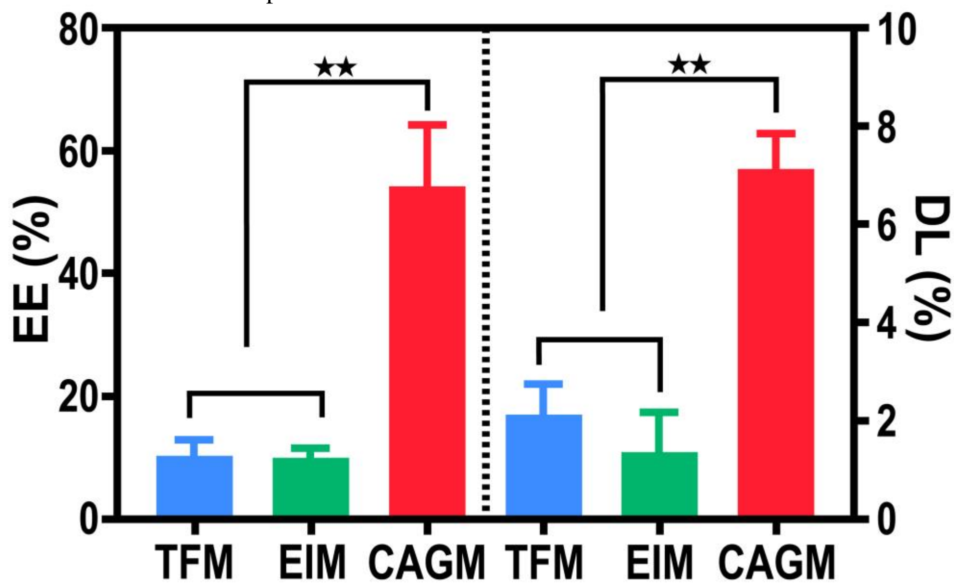

Fig. 3: Arsenic encapsulation and drug loading rates of the three preparation methods. ** $P < 0.01$

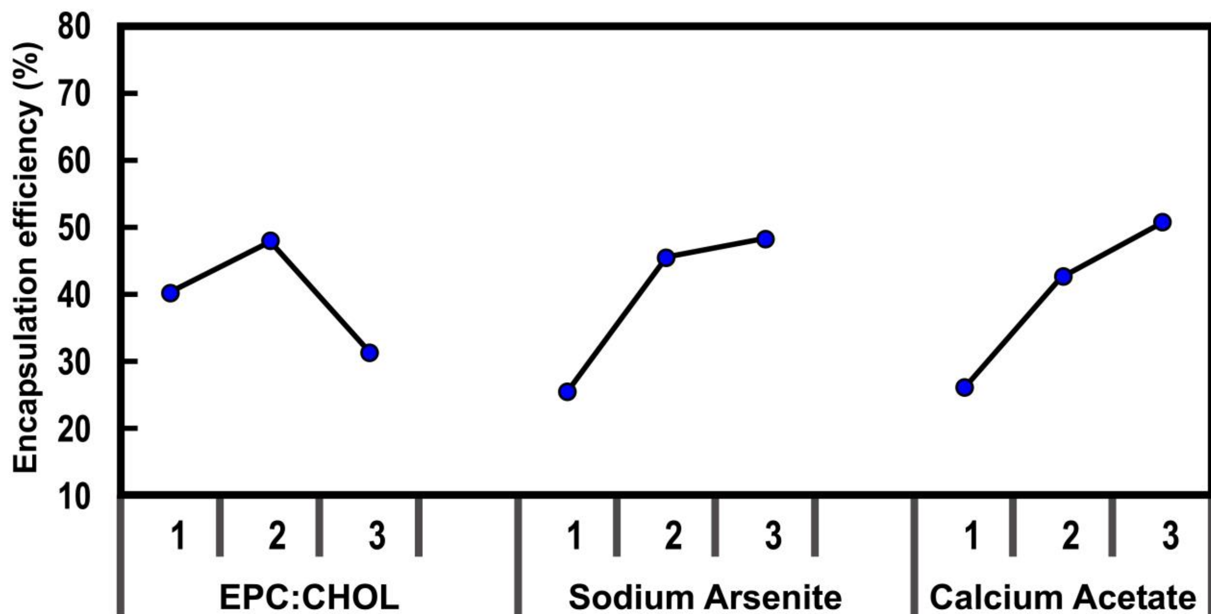

Fig. 4: Orthogonal experimental trend diagram

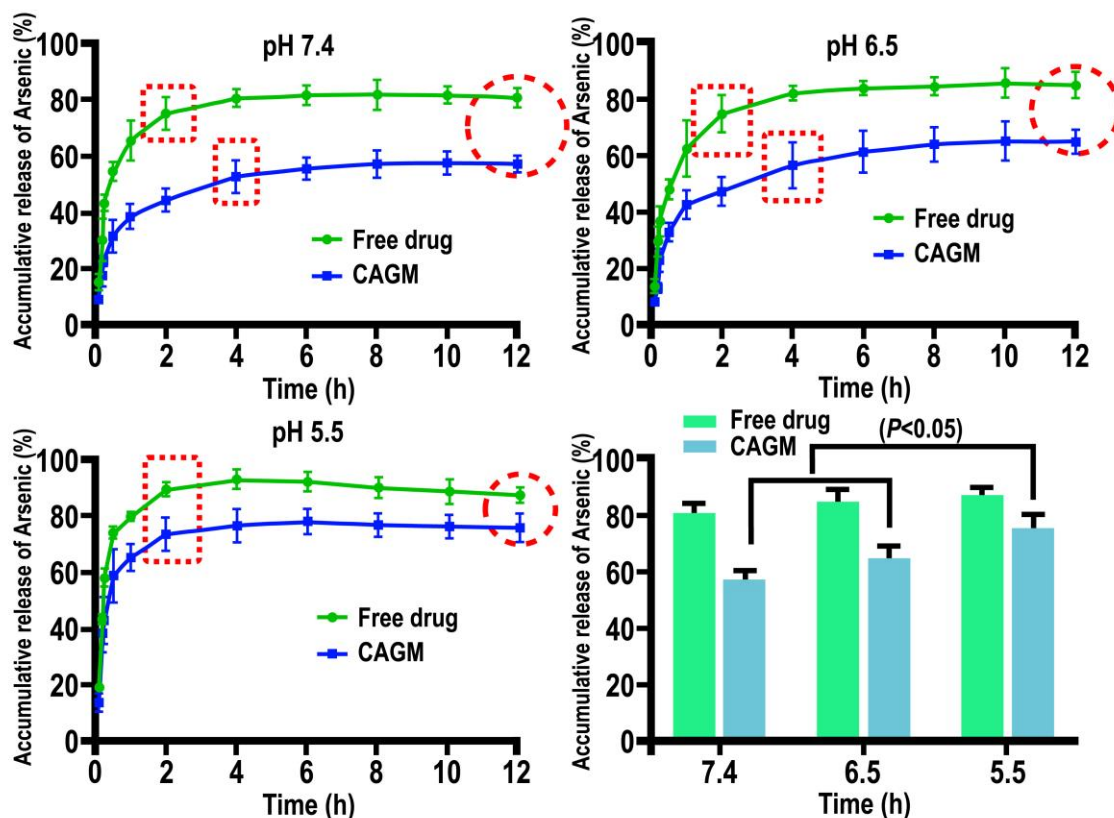

Fig. 5: Cumulative release profiles of arsenic in free $NaAsO_2$ and CAGM preparations

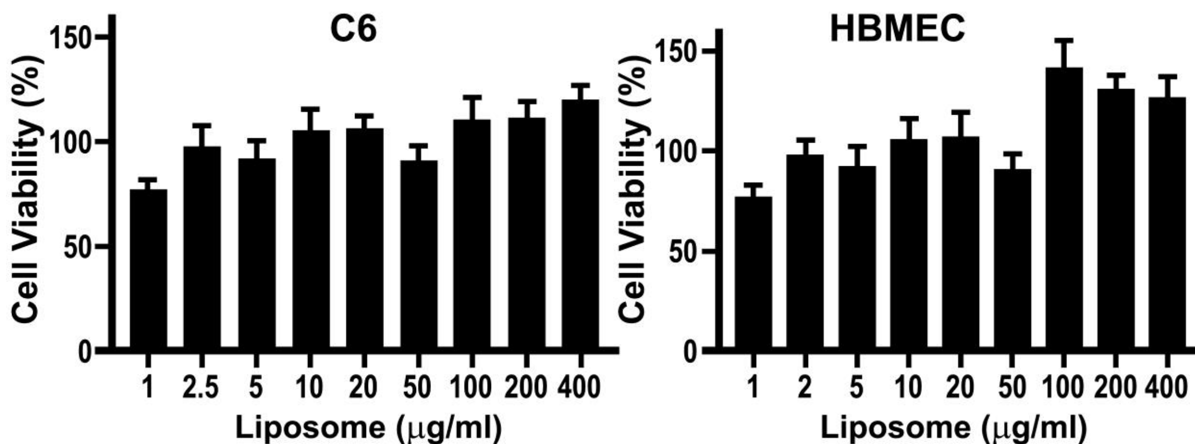

Fig. 6: Toxicity of blank vector to C6 and HBMEC

Overall, the blank carrier had a growth-promoting effect on both kinds of cells investigated, and no cytotoxicity was observed.

DISCUSSION

Arsenic is a traditional Chinese medicine and a water-soluble drug, but it is widely distributed in the body and has high toxicity. How to enrich it in the target site and achieve the effect of increasing efficiency and decreasing toxicity is one of the development directions of arsenic research.

Liposome has good biocompatibility and modifiable surfaces. It is an ideal carrier for targeting agents. It has developed rapidly in recent years and is widely used in the targeting research of various anti-tumor drugs. Although liposome drugs are amphiphilic, many studies have been used to encapsulate lipophilic drugs and the application of liposome-encapsulating hydrophilic drugs is limited due to their low encapsulation rate (Tazina *et al*., 2011). Improving the encapsulation rate of arsenic in liposomes is the key to its application. Researchers have adopted various methods to improve the encapsulation rate of arsenic, including the use of inorganic carriers

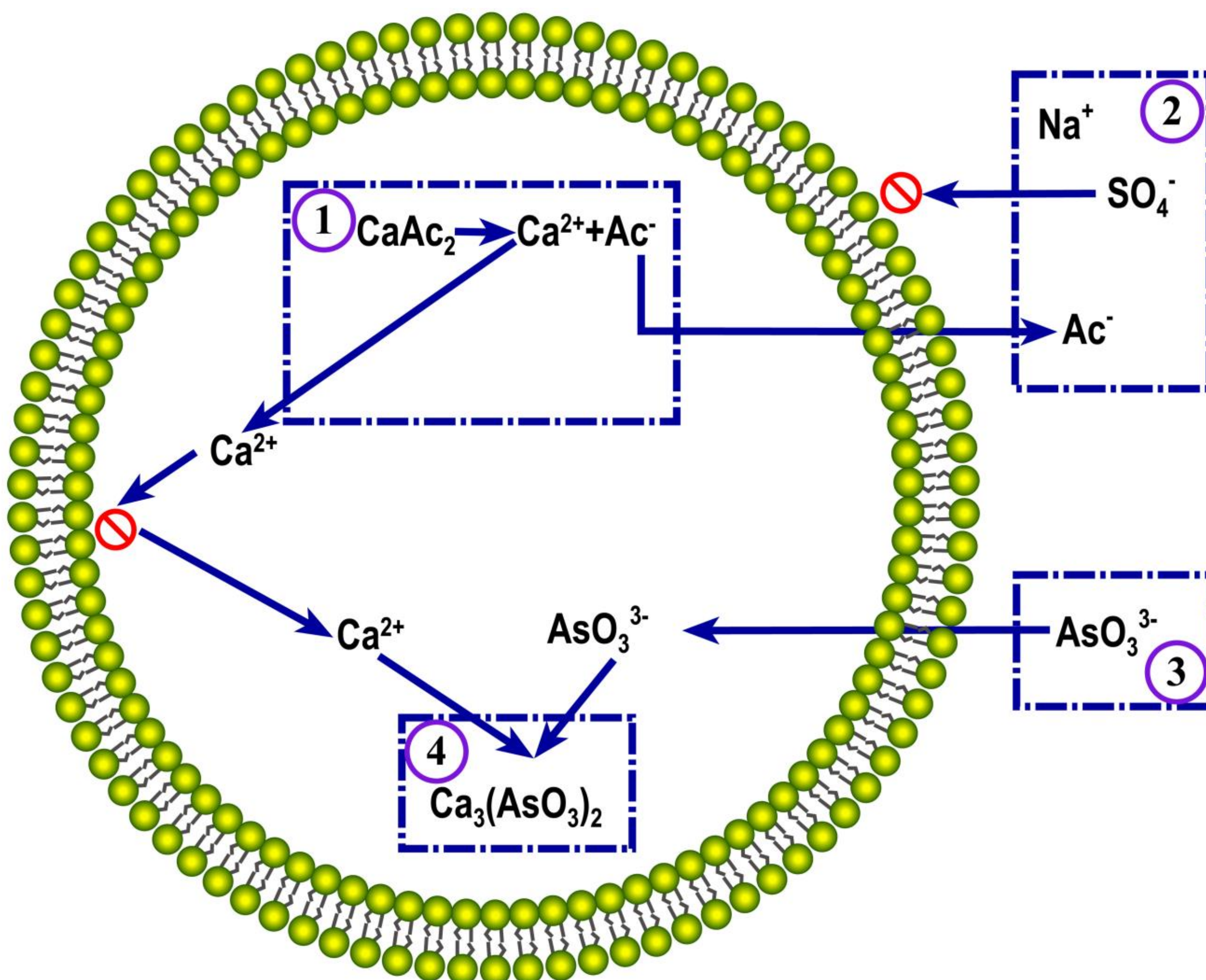

Fig. 7: Possible mechanism diagram of CAGM to increase arsenic encapsulation

such as silica or the formation of complex methods (Muhammad *et al.*, 2014; Xiao *et al.*, 2016), but inorganic carriers face the problem of difficult degradation *in vivo*, and the lack of targeting of the complex, the application is still limited.

This experiment used CAGM to prepare liposomes containing $NaAsO_2$ to improve arsenic encapsulation efficiency and drug loading capacity. Experimental results revealed that the encapsulation rate of arsenic increased by 4 times by CAGM. The cytotoxicity test of blank vector in vitro showed that the liposomes prepared by CAGM had good biocompatibility and no cytotoxicity was observed.

This research used a 5:1 ratio of EPC and CHOL to construct liposomes. Phospholipids are mainly divided into egg phosphatidylcholine and soybean phospholipids, and soybean phospholipids include hydrogenated soybean phospholipids, such as soybean phosphatidylcholine (SPC). Previous studies have found that the particle size of liposomes prepared using SPC materials is significantly larger than that of liposomes prepared by EPC. Although the particle size is more uniform, preparing liposomes below 100 nm is extremely difficult. The addition of surfactants can reduce particle size but lead to drug release. EPC is more elastic than soybean phospholipids; it is not easy to produce fragments when preparing liposomes through membrane extrusion, and the smaller particle size is conducive to further preparation of nano-targeting preparations. According to the results of the orthogonal tests, we use EPC/CHOL on a 5:1 basis. CHOL is the regulator of the fluidity of the lipid bilayer. Too little CHOL will cause insufficient membrane fluidity, resulting in poor deformability and fragmentation; too much CHOL will improve the lipid solubility, which is not conducive to the encapsulation of water-soluble drugs and easy to disintegrate and reduce the stability of the liposome (Almeida *et al.*, 2020; Nsairat *et al.*, 2024). In the choice of calcium reagent, when calcium chloride or calcium carbonate was used, the solution may form micron-scale precipitation, while no significant precipitation is observed with $CaAC_2$.

The possible mechanisms for CAGM are shown in fig. 7. The mechanism includes four steps. Step 1, $CaAC_2$ was first wrapped inside the liposome. $CaAC_2$ can be dissociated into calcium ions and acetate ions. Calcium ions cannot pass through the bilayer of the liposome and remain in the liposome, but acetate ions can pass through the bilayer. Step 2. Under the hyperosmotic pressure of sodium sulfate solution, the acetate ions diffuse into the solution outside the liposome. Step 3. After the external ions were washed by ultrafiltration and incubated with $NaAsO_2$ solution, arsenite ions were diffused into the lipid bilayer. Step 4. Calcium ions in the liposomes combine with arsenite ions to form complexes, thereby increasing the inclusion rate of arsenic.

The arsenic-carrying nanoliposomes prepared by CAGM have a higher arsenic encapsulation rate, 4 times higher than the other two methods. Therefore, it is concluded that higher osmotic pressure may be formed inside the liposomes prepared by CAGM and the particle size will increase after water absorption reaches equilibrium, which is consistent with the experimental results that arsenic-carrying liposomes prepared by CAGM are about 10 nm higher than those prepared by TFM and IEM. Nevertheless, there was no significant change in PDI. Meanwhile, the negative zeta surface potential of the liposomes prepared by CAGM was higher than that prepared by the other two methods, possibly due to arsenite adsorption. However, regarding stability, no significant difference was observed when the preparation was stored at 4°C.

The orthogonal test diagram showed that the influence of EPC/CHOL on the encapsulation rate of $NaAsO_2$ was first high and then low and too high proportion will lead to a decrease in the encapsulation rate. The arsenic encapsulation increased with the concentration of $NaAsO_2$, but as the concentration reached a certain level, the increase of the dosage of $NaAsO_2$ could not significantly increase the encapsulation rate. The concentration of $CaAC_2$ seemed to be positively correlated with the encapsulation rate, but the influence tends to decrease with the increase of concentration and considering the saturation of the solution, the range of increasing the concentration of $CaAC_2$ was limited.

The results of TEM showed that the liposomes morphology prepared by CAGM were denser, while the liposomes prepared by EIM appeared to have more fragments, which may be due to the presence of trace ethanol in the liposomes prepared by EIM, leading to the easy rupture of the liposomes when subjected to ultrasound and over-membrane extrusion.

Release results demonstrated that the drug release rate of liposomes prepared by CAGM increased company with the decreased pH *in vitro*, the peaking time of cumulative release changed from 8h to 2h and the cumulative arsenic release rate increased accordingly, the cumulative arsenic release rate reached 69.95-80.72% at pH5.5, while the cumulative release rate was only 54.41-60.41% at pH7.4. At pH5.5, the drug release rate of the arsenic-carrying liposomes was consistent with that of free drugs, showing pH responsiveness, which may be related to the solubility of the calcium arsenite complex formed at different pH.

Given that the tumor microenvironment is acidic, liposomes prepared by CAGM have certain advantages for drug aggregation in tumor tissues; at the same time, the nanoparticle less than 100 nm is conducive to tissue penetration in terms of liposomes. However, compared with the encapsulation rate of liposomes containing lipid-soluble drugs in related literature, the encapsulation rate of arsenic-carrying drugs in liposomes still needs to be explored and improved.

CONCLUSION

The traditional liposome preparation method is challenging to use as the carrier of arsenic because of its low encapsulation rate. Our study found that CAGM can significantly increase the encapsulation rate of water-soluble sodium arsenite and at the same time, the particle size can be controlled to below 100 nm. The liposome had a reasonable encapsulating rate of arsenic, biocompatibility and *in vitro* pH sensitivity. These characteristics make it more suitable for the anti-tumor effect, such as increasing tumor tissue penetration, rapid release in the tumor microenvironment and offering a new perspective for preparing and applying arsenic-containing nanoliposomes.

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