

Preparation and evaluation of liver targeting nano device based on Graphene Oxide mediated Miriplatin delivery

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Abstract: To prepare and evaluate a novel liver targeting nano device which based on graphene oxide (GO) loading miriplatin (MP). Glycyrrhetic acid (GA) was linked to polyethylene glycol (PEG) via an amide linkage forming GA-PEG; then GA-PEG and GO were coupled by an ester linkage to form a triblock copolymer GA-PEG-GO (GPG). MP was packaged in liposome which conjugated to GO by means of modifying 1-Pyrenebutyric acid (PBA) on dioleoyl phosphoethanolamine (DOPE). Free MP was separated from liposome by 0.22 μ m filter membrane and determined by HPLC. The average size of nano device was 125.6 nm and average potential was +35.7 mV. The encapsulation efficiency (EE) of MP was 87.62%.

Keywords: Liver targeting; Miriplatin; Graphene oxide; Liposome

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1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant cancers in the world which has pretty high recurrence rate, ranking fifth in the global incidence of malignant tumors, the third cause of death that increases year by year. Miriplatin (MP) is a new fat-soluble platinum antitumor drugs developed by Sumitomo Pharmaceutical Co. Ltd, it is of good efficacy and safety in treating HCC [1]. The regular dosage form of MP is dissolved in meters iodized poppy seed oil to make it suspend, then drug is given to hepatic artery by transcatheter arterial chemoembolization (TACE), which is a kind of interventional therapy. Interventional therapy has severe side effects, such as chemoembolization can cause persistent pain in the patients' liver, fever, appetite loss, etc. [2]. In addition, it is difficult and infeasible for the liver cancer issue with multiple vascular blood supply. Other more, the interventional treatment must use angiography, fluoroscopy guided machines, Computed Tomography (CT) and other imaging equipments, which will cause some damages to the health of patients and medical personnel. This experiment uses Graphene Oxide (GO) as the basic carrier to load MP for the treatment to prepare a new compound targeting liver cells to relieve the pain of interventional treatment for patients and maintain the medical staff's health, while furtherly improves the targeting of drugs to reduce the side effects.

GO is a two-dimensional hexagonal plane consists of a single layer of sp² hybridized carbon atoms [3], containing carboxyl group, hydroxyl group and epoxy group, etc. on the plane of carbon atoms [4]. Unique two-dimensional plane make GO has large surface area that up to 2600m²/g [5], so it has higher drug loading capacity comparing to other vector systems. GO can be modified easily as a result of rich surface functional

groups [6]. Covalent attaching PEG or ligand to the surface of GO [7] endow GO with the characteristics of long circulating and targeting. Studying found that the chemical drugs, DNA, RNA, etc. can be effectively fixed on the GO via π - π conjugate [8], hydrogen bonds [9], and other non-covalent bond. Moreover, GO has good water dispersity [10] and biocompatibility. All these features make GO an extremely promising drug delivery device.

Studies have shown that liver is composed of parenchyma cells and non-parenchyma cells. Liver cancer occurs more easily in parenchymal cells than in non-parenchyma cells. In 1991, Negishi had confirmed that the rat's liver parenchyma cell membrane contains a large number of glycyrrhetic acid (GA) specific binding sites [11], the combination of GA and the sites is saturated and highly specific. In recent years, studies have also confirmed that the liver parenchyma cell surface do exists a large number of GA receptors [12], thus connecting GA on carrier can realize active targeting to the liver parenchyma cells.

GA is bridged to the surface of GO [13] via terminal chemical modified polyethylene glycol (NH₂-PEG-COOH) as targeting ligand. GA and PEG endow our delivery system with the characteristics of targeting and long circulating. We use Dioleoyl Phosphoethanolamine (DOPE) and (2, 3-Dioleoyloxy-propyl)-trimethylammonium (DOTAP) to prepare liposome and package water to insoluble drug MP. Amino group of DOPE and carboxy group of 1-Pyrenebutyric acid (PBA) are coupled by an amide linkage. The π - π interaction will be formed between phenanthrene of PBA and carbon ring of GO so that liposome can combine with GO. At the same time, we have established a HPLC method [14] for the

quantitative evaluation of MP to measure the encapsulation efficiency (EE).

2. Materials and instruments

Mass spectrometer (LTQ Orbitrap XL), Thermo Fisher Scientific Inc.; Nuclear Magnetic Resonance Spectrometer (JNM-ECP600), JEOL; Fourier Transform Infrared Spectrometer (Spectrum One), PerkinElmer Inc.; Transmission electronic microscope (JEM2010), JEOL; Varian Cary Eclipse Fluorescence Spectrophotometer, Varian Inc.; Zetasizer Nano instrument (Zetasizer Nano-ZS90), Malvern Instruments Ltd.; High Performance Liquid Chromatography (LC-2010C), Shimadzu; Electronic balance (AL204), Mettler Toledo; Vacuum freeze drying (FD-1D-50) and vacuum drying oven (VO-30E), Biocool Instruments Ltd.; Ultrasonic cell disruptor (BILON92-IID), Shanghai Bilon Instruments Ltd.; Ultrasonic cleaner (SK5210HP), Kedao corporation; Rotary Evaporator, Shanghai Ailang Instruments Ltd.; Centrifugal Machine (TDL-40C), Anke corporation; Dialysis bag (MW cut-off 1,000), Shanghai Yuanye Biotech, Inc..

Graphene Oxide (GO), provide by Liu Jingquan Lab; Glycyrrhetic acid (GA, 98%), Zelang Medical Technology Co., Ltd.; NH₂-PEG-COOH 2000 (PEG, 90%), Seebio Biotech, Inc.; 1-Pyrenebutyric acid (PBA, 99%), Aladdin Industrial Corporation; Dioleoyl Phosphoethanolamine (DOPE, 98%) and (2,3-Dioleoyloxy-propyl)-trimethylammonium (DOTAP, 98%), Shanghai Advanced Vehicle Technology Pharmaceutical, Ltd.; N-Hydroxysuccinimide (NHS, 98%), N,N'-Dicyclohexylcarbodiimide (DCC, 98%), 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC, 99%) and 4-dimethylaminopyridine (DMAP, 99%), J&K Scientific Ltd.; Tert-butyl alcohol, China Sinopharm International (Shanghai) Co., Ltd., CP; Tetrahydrofuran (THF), Dichloromethane (DCM), Dimethyl sulfoxide (DMSO) and N,N-Dimethylformamide (DMF), China Sinopharm International (Shanghai) Co., Ltd., AR; Acetonitrile and Ethyl alcohol absolute, China Sinopharm International (Shanghai) Co., Ltd., HPLC; Methanol, Anaqua Chemicals Supply, HPLC.

3. Methods

3.1. Synthesis of GA-PEG-GO (GPG)

3.1.1. Synthesis of GA-NHS

GA (0.5 mmol), DCC (0.75mmol) and NHS (0.75mmol) were dissolved in 5 ml of THF. GA was activated by NHS forming GA-NHS after the solution was stirred for 24h at room temperature. The resulting mixture was filtrated by 0.45 μm filter membrane and the filtrate was precipitated and washed with excess glacial diethyl ether for 3 times. The obtained precipitate was dried in vacuum drying oven after diethyl ether volatile completely.

3.1.2. Synthesis of GA-PEG

GA and terminal chemical modified polyethylene glycol (NH₂-PEG-COOH) were connected via amidation reaction. GA-NHS (0.075 mmol) and NH₂-PEG-COOH (0.05 mmol) were dissolved in 10 ml of DCM. The solution was filtrated by 0.45 μm filter membrane after stirred at 35 °C for 48h. Then the filtrate was precipitated and washed with excess glacial diethyl ether for 3 times. The precipitate was dried in vacuum drying oven after diethyl ether volatile completely, obtaining GA-PEG.

3.1.3. Synthesis of GA-PEG-GO (GPG)

0.01 g of GO was dispersed into 10 ml of DMF under ultrasonic to achieve GO/DMF dispersion with mass concentration of 1 mg/ml. GA-PEG (0.1 mmol), EDC (0.1 mmol) and DMAP (0.02 mmol) were dissolved in 10 ml of GO/DMF dispersion and stirred at 60 °C for 24h. The solution was placed into the dialysis bag (MW cut-off 1,000) with ultrapure water as dialysis medium. The ultrapure water was refreshed at 1, 2, 6, 12, 24 and 48h. Then solution that left in the dialysis bag was lyophilized to get GA-PEG-GO (GPG).

3.2. Preparation of PBA-PEG-DOPE/DOTAP/MP liposome (PPDDM liposome)

3.2.1. Synthesis of PBA-PEG

PBA (0.05 mmol), EDC (0.075 mmol) and NHS (0.075 mmol) were dissolved in 5 ml of DMSO, stirred at 25 °C for 2h. Then added NH₂-PEG-COOH (0.05 mmol) to this solution, continued stirring for 48h. After reaction finished, the solution was put into the dialysis bag (MW cut-off 1,000), dialyzed against ultrapure water for 48h. Then solution that left in the dialysis bag was lyophilized to get crude PBA-PEG.

The crude PBA-PEG was dissolved in 0.2 ml of DCM. Dropwise added this solution into excess glacial diethyl ether with ultrasonic to precipitate and wash for 3 times to remove free PBA. The precipitate was dried in vacuum drying oven after diethyl ether volatile completely to obtain pure PBA-PEG.

3.2.2. Synthesis of PBA-PEG-DOPE (PPD)

PBA-PEG (0.05mmol), EDC (0.075mmol) and NHS (0.075mmol) were dissolved in 10 ml of trichloromethane. Added DOPE (0.05mmol) to this solution after stirred at 25 °C for 2h, keep stirring for 48h. Then the reaction mixture was put into the dialysis bag (MW cut-off 1,000) for 48h with ultrapure water as dialysis medium. Then solution that left in the dialysis bag was lyophilized to get PBA-PEG-DOPE.

3.2.3. Preparation of PPDDM liposome

PPDDM liposome was prepared by the rotary-film evaporation method. MP (2.5 mg), PPD (1 mg) and DOTAP (4 mg) were added into a round-bottom flask and dissolved by 3.5 ml of trichloromethane. The solution was rotated and

evaporated until trichloromethane was completely removed. The phospholipids film was hydrated by 5 ml of PBS (pH 7.4). Then the resulting hydrate was treated with a probe-type ultrasonicator, obtaining PPDDM liposome solution (mass concentration of MP was about 500 µg/ml).

3.3. Combination of PPDD liposome and GPG

0.002 g of GPG was dispersed into 10 ml of ultrapure water under ultrasonic to achieve GPG dispersion. Then GPG dispersion (2 ml) and PPDD liposome (1 ml) were mixed at 25 °C for 24h to obtain PPDD/GPG compound.

3.4. Determination of the encapsulation efficiency (EE)

3.4.1. Chromatographic conditions

Chromatographic column, XSELECT CSH C18 (4.6×250 mm, 5µm); Mobil phase, Methanol-Ethanol (90:10); Flow rate, 1.0 ml/min; UV detection wavelength, 220 nm; Column temperature, 25 °C; Injection volume, 20µl.

3.4.2 Specificity

MP (4.0 mg) was dissolved and diluted with Tert-butyl alcohol-Acetonitrile (9:1) to 100 ml to obtain MP solution with mass concentration of 40 µg/ml. Preparing and demulsifying liposome without MP (PPDD liposome) and liposome with MP (PPDDM liposome) according to Methods 2.2.3. MP solution, PPDD liposome and PPDDM liposome were analyzed by HPLC.

3.4.3. Calibration curve

MP (0.01 g) was dissolved and diluted with Tert-butyl alcohol-Acetonitrile (9:1) to 10 ml to obtain stock solution (1 mg/ml). Appropriate volumes of stock solution were respectively transferred and diluted with Tert-butyl alcohol-Acetonitrile (9:1) to obtain standard solutions with concentration of 1.0, 5.0, 10.0, 20.0, 40.0, 80.0 µg/ml. The standard solutions were injected to HPLC for analysis.

3.4.4. Precision test

Appropriate volumes of stock solution were respectively transferred and diluted with Tert-butyl alcohol-Acetonitrile (9:1) to obtain solutions with concentration of 5.0, 40.0, 80.0 µg/ml. Analyzing for 5 times in a single day.

3.4.5. Recovery test

50, 400, 800 µl of stock solutions were respectively transferred into 0.1 ml PPDD liposome solutions. The mixed solutions were demulsified and diluted with Tert-butyl alcohol-Acetonitrile (9:1) to obtain solutions with concentration of 5.0, 40.0, 80.0 µg/ml, analyzed with HPLC.

3.4.6. Separation efficiency of filter membrane

Separation efficiency was investigated to estimate the effectiveness of 0.22 µm filter membrane separating free MP from liposome. Preparing PPDDM liposome solution and dividing into two equal parts. One part was added formula dosage of MP and another wasn't. Both of two parts were filtrated by 0.22 µm filter membrane. Then 0.1 ml of the two filtrates were demulsified and diluted with Tert-butyl alcohol-Acetonitrile (9:1) to 10 ml, analyzed with HPLC.

3.4.7. Solubility of MP

Formula dosage of MP, PPD, and DOTAP were added into PBS (pH 7.4) and treated with a probe-type ultrasonicator. The mixture was filtrated by 0.22 µm filter membrane. Then 0.2 ml of filtrate was diluted with Tert-butyl alcohol-Acetonitrile (9:1) to 10 ml. Repeating analysis for 3 times. The solubility was marked as C1.

3.4.8. Encapsulation efficiency (EE)

PPDDM liposome solution was filtrated by 0.22 µm filter membrane. Then 0.1ml of filtrate was demulsified and diluted with Tert-butyl alcohol-Acetonitrile (9:1) to 10 ml. The solution was injected to HPLC for analysis. The obtained concentration was marked as C.

In addition, 0.1 ml of PPDDM liposome solution was demulsified and diluted with Tert-butyl alcohol-Acetonitrile (9:1) to 10 ml. Analysis obtained concentration was marked as C₀. EE was calculated as follow formula.

$$EE (\%) = (C - C_1)/C_0 \times 100\%$$

4. Results

4.1. Synthesis of GA-PEG-GO (GPG)

4.1.1. Synthesis of GA-NHS

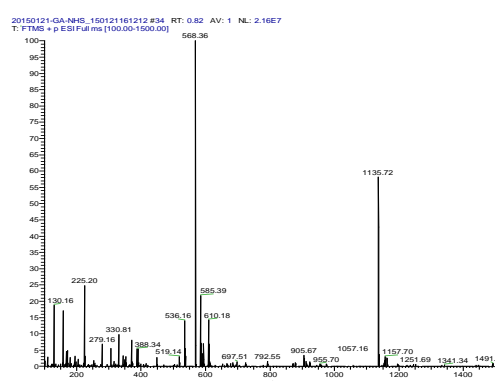


Figure 1. Mass Spectrometry analysis of GA-NHS.

Molecular weight of GA-NHS is about 567 (M_w (GA-NHS) = M_w (GA) + M_w (NHS) - M_w (H_2O)), which was showed in mass spectra.

4.1.2. Synthesis of GA-PEG

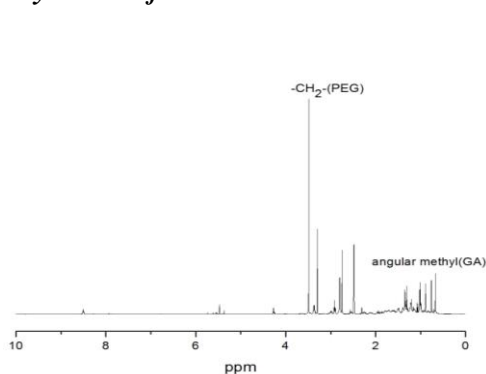


Figure 2. ^1H NMR spectra of GA-PEG.

In ^1H NMR spectra of GA-PEG, 7 signals between 0.67-1.37 ppm are attributed to the angular methyl group of GA. A sharp signal at 3.52 ppm belong to the $-\text{CH}_2-$ of $\text{NH}_2\text{-PEG-COOH}$, suggested that GA has already been linked to $\text{NH}_2\text{-PEG-COOH}$.

4.1.3. Synthesis of GA-PEG-GO (GPG)

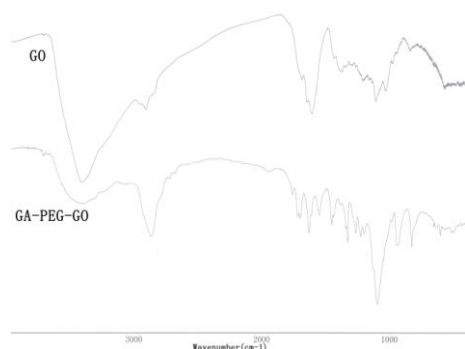


Figure 3. FT-IR spectra of GO and GA-PEG-GO measured in KBr pellets.

As showed in FT-IR spectra of GA-PEG-GO, O-H in GO stretching vibration absorption at 3427 cm^{-1} disappears. Peak shifts at 2886 cm^{-1} and 1467 cm^{-1} are attributed to the absorption of C-H in PEG. Peak shift at 1115 cm^{-1} belong to stretching vibration absorption of C-O in PEG and GO. Peak shifts at 1715 cm^{-1} and 1215 cm^{-1} are respectively the characteristic signals of C=O and C-O-C in ester bond. Successful GA attachment to the surface of GO was confirmed.

4.2. Preparation of PBA-PEG-DOPE/DOTAP/MP liposome (PPDD liposome)

4.2.1. Synthesis of PBA-PEG-DOPE (PPD)

In ^1H NMR spectra of PBA-PEG-DOPE, the characteristic signals of $=\text{CH}-$ in PBA between 7.70-8.31 ppm and $-\text{CH}_2-$ in PEG at 3.52 ppm can be expressly distinguished. Peak shifts at 0.88 ppm, 1.26-1.33 ppm and 5.34 ppm which represent $-\text{CH}_3$, $-\text{CH}_2-$ and $=\text{CH}-$ in aliphatic chain of DOPE. These evidences indicate that we have successfully synthesized target polymer.

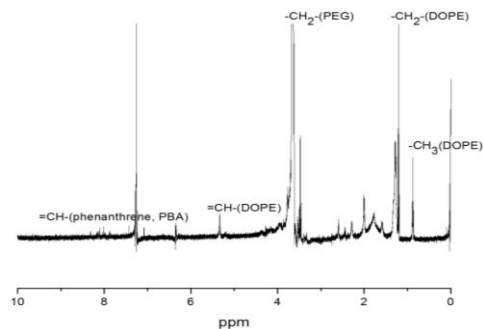


Figure 4. ^1H NMR spectra of PBA-PEG-DOPE.

4.2.2. Characterization of PPDD liposome

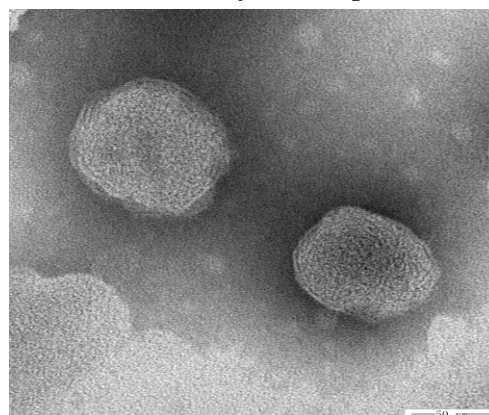


Figure 5. TEM image of PPDD liposome.

From TEM image of PPDD liposome it can be seen that liposome present spherical structure and fingerprint structure.

4.3. Combination of PPDD liposome and GPG

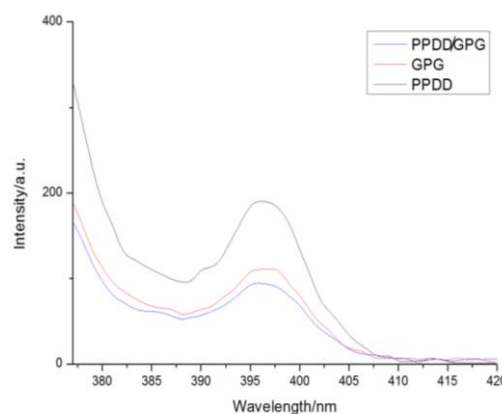


Figure 6. Fluorescence intensity of PPDD, GPG and PPDD/GPG (Excitation wavelength is 222 nm).

PPDD liposome has fluorescence because of containing PBA. Fluorescence intensity has decreased when PPDD liposome coupled with GO via $\pi\text{-}\pi$ interactions due to the fluorescence quenching effects of GO.

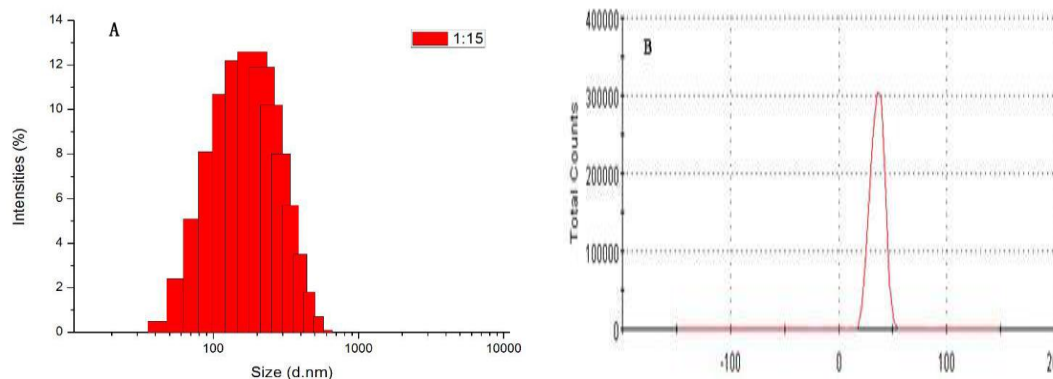


Figure 7. Size (A) distribution and zeta potential (B) distribution.

The particle sizes and zeta potentials of PPDD/GPG were determined with a Zetasizer Nano instrument,

obtaining its average size is 125.6 nm (PDI=0.457) and average potential is +35.7 mV.

4.4. Determination of the encapsulation efficiency (EE)

4.4.1. Specificity

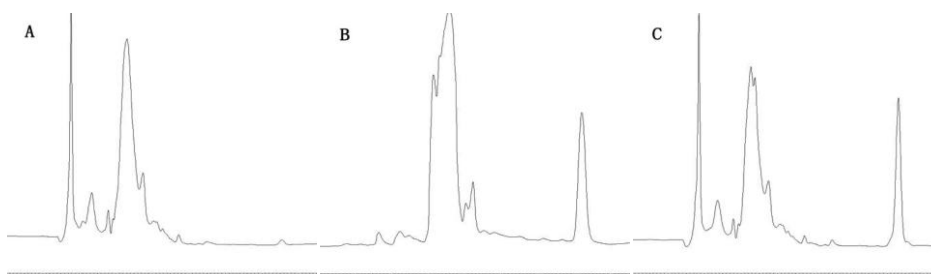


Figure 8. HPLC Chromatogram of PPDD liposome (A), MP (B) and PPDDM liposome (C).

The specificity study showed that the drug peak and impurity peaks are well detached. Excipients and reagents are no interference for drug determination.

4.4.2. Calibration curve

The calibration curve was built by plotting the peak area (A) versus the concentrations of MP (ρ). The regression equation was $A=6222.1 \rho + 2134.8$ ($r=0.9999$). The result suggested that there was a good linear relation between the peak area and the concentration of MP within the range of 1.0~80.0 μg/ml.

4.4.3. Precision text

Table 1 Intra-day precision of HPLC determination for MP (n=5).

Theoretical amount/μg·ml ⁻¹	Found amount/μg·ml ⁻¹	RSD/%
5	5.07	0.51
40	40.14	0.12
80	80.05	0.23

The precision experiment showed that the developed method is precise enough for the content determination of MP.

4.4.4. Recovery text

Table 2 Result of recovery text (n=5).

Add amount/μg·ml ⁻¹	Found amount/μg·ml ⁻¹	Recovery/%	RSD/%
5	5.03	100.60	0.40
40	39.82	99.55	0.29
80	79.00	98.75	0.03

The results of recovery experiment indicated that the method is exact and feasible for the measurement of MP.

4.4.5. Separation efficiency of filter membrane

Table 3 Separation efficiency of filter membrane (n=3).

	Add amount/ $\mu\text{g}\cdot\text{ml}^{-1}$	Found amount/ $\mu\text{g}\cdot\text{ml}^{-1}$	Recovery/%
PPDDM	\	441.12	3.29
PPDDM+MP	500	457.56	

The average recovery of free MP is just 3.29 % which demonstrated that the filter membrane can separate free MP from liposome effectively.

4.4.6. Solubility of MP

Through determination, the solubility of MP (C1) in PBS (pH 7.4) is $2.21\mu\text{g}\cdot\text{ml}^{-1}$ (n=3). It accounts for about 0.4% of MP concentration in liposome.

4.4.7. Encapsulation efficiency (EE)

Table 4 Encapsulation efficiency of MP liposome (n=5).

C/ $\mu\text{g}\cdot\text{ml}^{-1}$	C ₀ / $\mu\text{g}\cdot\text{ml}^{-1}$	EE/%
436.61	495.80	87.62

5. Conclusions

This study has tried to combine liposome with GO that joints as carrier loading water insoluble drug MP. Physicochemical properties of this complex are characterized through means such as MS, ¹H NMR, FTIR and TEM. Various evidences have proved that all parts of this complex are coupled together via covalent or non-covalent bond. The HPLC- filter membrane method to determinate the encapsulation efficiency of MP has been established and validated. Results indicate that the solubility of MP in aqueous medium is low enough that 0.22 μm filter membrane can separate free MP from liposome effectively and this method is accurate and feasible in determining content of MP.

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