Construction and In Vitro Validation of Liposomes Loaded with ROS-Responsive Probe QM-CF

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Abstract. In this study, liposomes loaded with the reactive oxygen species-responsive probe QM-CF (Lipo@QM-CF) were prepared using a phospholipid composition consisting of dipalmitoylphosphatidylcholine (DPPC), sphingomyelin (SM), cholesterol (CHO), DSPE-PEG2000, and DSPE-PEG2000-maleimide, selected for their roles in membrane stability, fluidity, and potential for functional modification. The particle size, stability, encapsulation efficiency, and probe loading status of the liposomes were preliminarily investigated. The results showed that the prepared liposomes exhibited uniform particle size (approximately 160–170 nm, PdI < 0.15) and showed no significant changes in particle size during 21 days of storage at 4°C, indicating good in vitro stability. Elemental analysis revealed that chlorine signals colocalized with the liposome positions, suggesting the colocalization of QM-CF with liposomes. Chemiluminescence imaging further verified the probe loading status: free QM-CF exhibited luminescence only in methanol, while Lipo@QM-CF showed no luminescence but recovered upon methanol demulsification, with further enhancement in the presence of reactive oxygen species, confirming that QM-CF was successfully encapsulated within liposomes and possessed good ROS-responsive release capability. This study successfully demonstrates the construction of a liposome-based probe for ROS imaging, offering a novel strategy for detecting inflammatory microenvironments.

1 Introduction

1.1 Reactive oxygen species and the inflammatory microenvironment

Reactive oxygen species (ROS) are a class of chemically active oxygen-containing molecules with strong oxidizing properties, primarily including hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), hydroxyl radical (·OH), and singlet oxygen ($^1O_2$) [1]. Under physiological conditions, ROS serve as important signaling molecules involved in the regulation of various biological processes including cell proliferation, differentiation, apoptosis, and immune defense [2].

Under pathological conditions, excessive ROS production can induce oxidative stress, which is closely associated with inflammatory disorders, cancer, neurodegenerative diseases, and other conditions [3-4]. In inflammatory microenvironments, activated inflammatory cells (such as macrophages and neutrophils) generate large amounts of ROS through "respiratory burst", mediating tissue damage and amplification of inflammatory signals [5]. Therefore, detection of ROS levels offers valuable insights for studying the development and progression of inflammation-related diseases and for evaluating therapeutic effects.

1.2 Design strategy of liposomes loaded with a reactive oxygen species-responsive probe

QM-CF is a novel chemiluminescent probe that generates specific signals through ROS-mediated cleavage of chemical bonds, offering advantages such as high signal-to-noise ratio and rapid response [6]. However, the relatively strong lipophilicity of this probe poses challenges for direct in vivo application, including poor solubility and difficulties in administration. Liposomes, as classical drug delivery systems, exhibit excellent biocompatibility and the capacity to solubilize hydrophobic drugs, thereby improving probe solubility, reducing toxicity, and enabling sustained and targeted delivery [7-10]. Encapsulating QM-CF within liposomes is expected to enhance its stability while leveraging the protective effect of the lipid bilayer to maintain the probe in a signal "off" state at non-target sites, enabling specific activation and luminescence upon encountering ROS in the inflammatory microenvironment. This strategy provides a promising approach for designing probes for inflammation imaging.

Based on the above rationale, the main contents of this study include: (1) preparation of QM-CF-loaded liposomes with varying QM-CF concentration gradients (5, 10, and 25 μM) using the thin-film hydration method; (2) systematic characterization of the prepared liposomes

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including analysis of particle size, size distribution, Zeta potential, and morphology via dynamic light scattering (DLS) and transmission electron microscopy (TEM), evaluation of *in vitro* stability under 4°C, establishment of a UV-Vis spectrophotometry standard curve for QM-CF and determination of encapsulation efficiency at different concentrations, and verification of successful QM-CF loading through energy-dispersive X-ray spectroscopy (EDS) elemental mapping using chlorine (Cl) as the characteristic marker; (3) investigation of the luminescence behavior of QM-CF under different states via chemiluminescence imaging, along with analysis of signal changes in the presence of ROS, to confirm successful encapsulation of QM-CF within liposomes and its favorable ROS-responsive release capability.

In summary, this study successfully developed QM-CF-loaded liposomes with uniform particle size, good stability, and high encapsulation efficiency. These results show a novel liposomal probe platform for ROS imaging in inflammatory and related microenvironments, thereby establishing an experimental foundation for subsequent cellular-level and in vivo application applications.

2 Materials and methods

2.1 Materials

Sphingomyelin (SM, >99%) was purchased from Avanti Polar Lipids (USA). Cholesterol (CHO, >99%) was obtained from Beyotime Biotechnology Co., Ltd. (China). Dipalmitoylphosphatidylcholine (DPPC, >99%) was purchased from Avanti Polar Lipids (USA). DSPE-PEG2000 (>99%), DSPE-PEG2000-Maleimide (>99%), and phosphotungstic acid (1.0%, w/v) were obtained from Shanghai Yuanye Biotechnology Co., Ltd. (China). The ROS-responsive fluorescent probe QM-CF was synthesized in-house. Chloroform (analytical grade), methanol (chromatographic grade), and hydrogen peroxide (H_2O_2, 30% solution) were purchased from Sinopharm Chemical Reagent Co., Ltd. (China). Phosphate buffered saline (PBS, 0.01 M, pH 7.4) was obtained from KeyGen Biotech Co., Ltd. (China).

2.2 Preparation of QM-CF-loaded liposomes (Lipo@QM-CF)

As shown in **Figure 1**, QM-CF-loaded liposomes were prepared using the thin-film hydration method. Lipids were mixed at a molar ratio of DPPC:SM:CHO:DSPE-PEG2000:DSPE-PEG2000-Mal = 4:1:4:0.5:0.5. QM-CF was added to achieve final concentrations of 5, 10, and 25 μM. The mixture was rotary evaporated at 65°C to form a lipid film, which was dried overnight under vacuum. The film was hydrated with 5 mL of PBS (65°C) for 60 min to obtain a crude liposome suspension. The suspension was extruded through 400 nm and 200 nm polycarbonate membranes (10 cycles each) using a pneumatic extruder. Unencapsulated QM-CF was removed by ultrafiltration centrifugation with PBS washing (2-3 times). The purified liposomes were resuspended in PBS and stored at 4°C. Based on QM-CF concentrations (5, 10, 25 μM), the liposomes were named Lipo@QM-CF^5, Lipo@QM-CF^{10}, and Lipo@QM-CF^{25}, respectively.

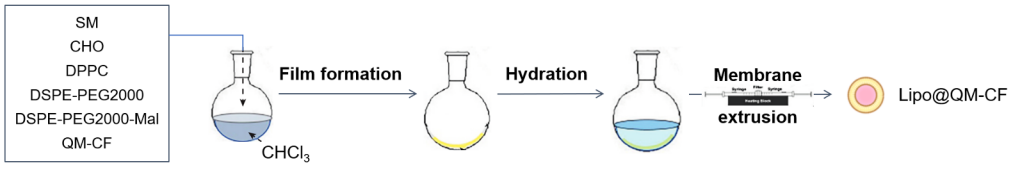

Fig. 1. Schematic illustration of the preparation process of Lipo@QM-CF.

2.3 Characterization and performance evaluation of Lipo@QM-CF

2.3.1 Particle size, size distribution, and Zeta potential measurement

The hydrodynamic diameter, polydispersity index (PdI), and Zeta potential of liposomes were determined using a Zetasizer Nano ZS90 (Malvern, UK). Measurements were performed on blank liposomes and Lipo@QM-CF^5, Lipo@QM-CF^{10}, and Lipo@QM-CF^{25} at 25°C. Each sample was measured in triplicate, and results were expressed as mean ± standard deviation.

2.3.2 In vitro stability evaluation

The colloidal stability of liposomes was evaluated by monitoring particle size changes during storage at 4°C. Blank liposomes and Lipo@QM-CF^5, Lipo@QM-CF^{10}, and Lipo@QM-CF^{25} were stored at 4°C protected from light. Particle size and PdI were measured on days 1, 2, 3, 7, 14, and 21 using DLS as described in 2.3.1. Each sample was measured in triplicate at each time point.

2.3.3 Morphological observation

The morphology of liposomes was observed using transmission electron microscopy (TEM) with negative staining.

Elemental distribution was analyzed using energy-dispersive X-ray spectroscopy (EDS) mapping. Chlorine (Cl) was used as a characteristic marker element for QM-CF, and phosphorus (P) was detected as a reference for the lipid bilayer.

2.4 Determination of QM-CF encapsulation efficiency

The encapsulation efficiency of QM-CF in liposomes was determined using UV-Vis spectrophotometry. QM-CF

was dissolved in methanol to prepare a series of standard solutions at concentrations of 1, 5, 10, and 25 μM. The absorbance at the maximum absorption wavelength (425 nm) was measured, and a standard curve was constructed by plotting absorbance against concentration, yielding the linear regression equation.

Freshly prepared liposome suspension was transferred to ultrafiltration centrifuge tubes and centrifuged at 4°C to separate unencapsulated free QM-CF. The filtrate containing free QM-CF was collected, and the retentate was demulsified with methanol to release encapsulated QM-CF. The absorbance of both fractions was measured at 425 nm. The concentrations of free and encapsulated QM-CF were calculated using the standard curve. The encapsulation efficiency was calculated as Equation (1):

$$EE\% = \frac{W_{\text{encapsulated}}}{W_{\text{total}}} \times 100\% = \frac{W_{\text{encapsulated}}}{W_{\text{encapsulated}} + W_{\text{free}}} \times 100\% \quad (1)$$

Where $W_{\text{encapsulated}}$ is the mass of QM-CF encapsulated in liposomes, W_{free} is the mass of unencapsulated free QM-CF, and W_{total} is the total mass of QM-CF added.

Three batches of each liposome formulation were measured, and results were expressed as mean ± standard deviation.

2.5 In vitro ROS response validation

To verify the successful encapsulation of QM-CF within liposomes and evaluate its ROS-responsive release behavior, chemiluminescence measurements were performed under different conditions. Samples including free QM-CF in methanol, free QM-CF in PBS, blank liposomes, Lipo@QM-CF, and methanol-demulsified Lipo@QM-CF were prepared. The samples were added to a black 96-well plate and incubated in the dark for 30 min. Chemiluminescence signals were then detected using a multifunctional microplate reader. Each sample was measured in triplicate, and results were expressed as mean ± standard deviation. Detailed grouping and treatments are described in the corresponding figure legends in the Results section.

2.6 Statistical analysis

All experiments were independently repeated three times, and the experimental results were expressed as mean ± standard deviation (Mean ± SD). GraphPad Prism software was used for data processing and statistical analysis.

3 Results and discussion

3.1 Preparation and characterization of Lipo@QM-CF

QM-CF-loaded liposomes with different probe concentrations (5, 10, and 25 μM) were successfully prepared using the thin-film hydration method combined with extrusion homogenization, with blank liposomes (Control) as comparison. The particle size, distribution, Zeta potential, and morphology of the liposomes were systematically characterized by dynamic light scattering (DLS) and transmission electron microscopy (TEM).

3.1.1 Particle size distribution and in vitro stability evaluation

The hydrodynamic diameter, polydispersity index (PdI), and Zeta potential of blank liposomes and Lipo@QM-CF with different QM-CF concentrations were determined by DLS, with results shown in **Figure 2**. The overlay of size distribution curves of all formulations is shown in **Figure 2a**, with all samples exhibiting monomodal peaks, indicating good uniformity of the prepared liposomes. All liposomes exhibited uniform particle sizes ranging from 160 to 170 nm (**Figure 2b**), meeting the general requirements for nanodelivery systems. The PdI values of all samples were between 0.05 and 0.15 (**Figure 2c**), far below 0.3, indicating excellent particle size uniformity and dispersity of the prepared liposomes. Zeta potential analysis showed that all liposomes exhibited negative Zeta potentials ranging from -23.0 to -27.2 mV (**Figure 2d**), with absolute values all greater than 20 mV, suggesting good colloidal stability under storage and physiological conditions. The negative potential slightly increased after QM-CF loading, which might be attributed to the contribution of QM-CF molecules or conformational changes in the lipid bilayer surface induced by probe loading.

Furthermore, the particle size changes of blank liposomes and Lipo@QM-CF at different concentrations (5, 10, 25 μM) during storage at 4°C were investigated by DLS on days 1, 2, 3, 7, 14, and 21, with results shown in **Figure 2e-f**. Throughout the entire observation period, no significant changes in particle size were observed for any samples (**Figure 2e**), and the PdI values of all groups remained consistently below 0.3 during storage (**Figure 2f**), further confirming the excellent dispersion stability of the system. These results demonstrate that the prepared liposomes possess good in vitro stability under 4°C storage conditions, which can be primarily attributed to the steric stabilization effect of the PEG layer on the liposome surface and the electrostatic repulsion provided by the high Zeta potential.

3.1.2 Morphological analysis

The morphology of liposomes with different QM-CF concentrations was observed using transmission electron microscopy (TEM) combined with negative staining, with results shown in **Figure 3**. TEM images revealed that both blank liposomes and Lipo@QM-CF with different QM-CF concentrations (5, 10, and 25 μM) exhibited regular spherical or spheroidal structures with clear and complete contours, uniform size, and good dispersity, without obvious aggregation or adhesion. All liposomes showed similar morphological features regardless of QM-CF loading concentration, indicating that probe encapsulation did not affect the basic structural integrity of the liposomes.

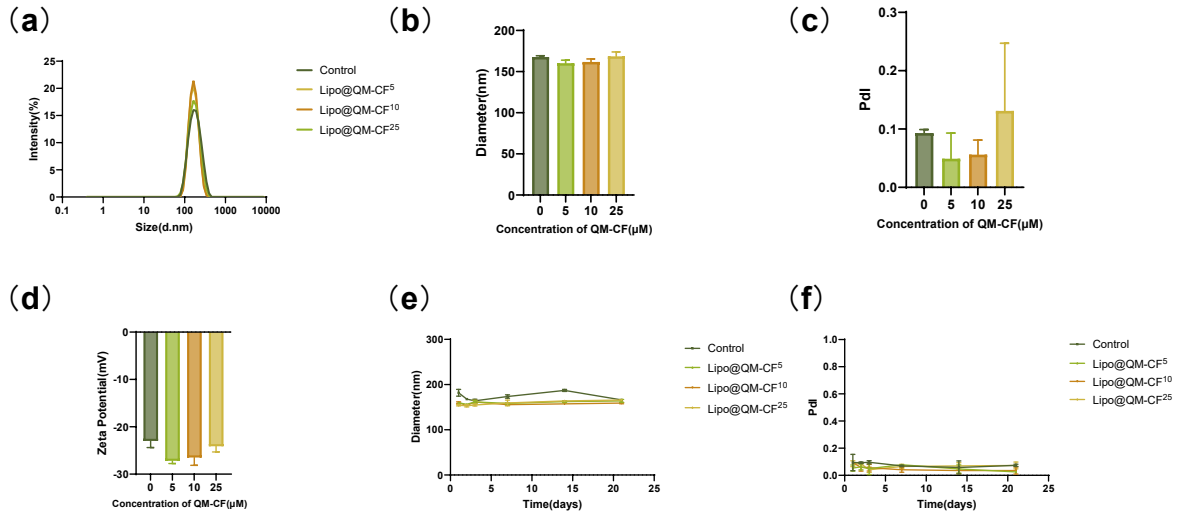

Fig. 2. Particle size distribution and in vitro stability evaluation of liposomes. (a) Overlay of size distribution curves of all liposome formulations. (b) Hydrodynamic diameter, (c) PdI, and (d) Zeta potential. Data are presented as mean ± SD (n=3). (e-f) Changes in hydrodynamic diameter (e) and PdI (f) of liposomes during storage at 4°C over 21 days.

To further verify the relationship between QM-CF and liposomes at the elemental level, energy-dispersive X-ray spectroscopy (EDS) mapping analysis was performed on blank liposomes and QM-CF-loaded liposomes, with results shown in **Figure 3**. Since the QM-CF molecular structure contains characteristic chlorine (Cl) elements, the distribution of Cl elements served as an indicator for QM-CF localization. Bright-field TEM images showed that both blank liposomes and QM-CF-loaded liposomes exhibited typical spherical structures. Elemental mapping analysis revealed that blank liposomes showed relatively dispersed Cl signals, whereas QM-CF-loaded liposomes displayed distinct Cl aggregation signals that highly coincided with the positions of the liposomes in bright-field images, suggesting colocalization of QM-CF with liposomes. The overlay of elemental maps with bright-field images further supported this observation.

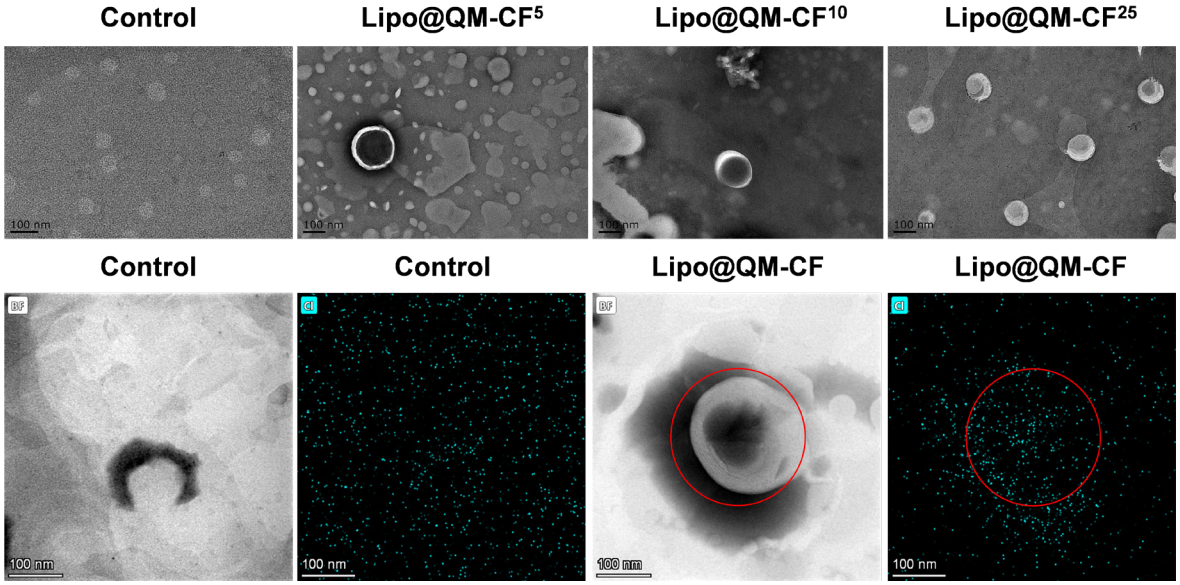

Fig. 3. TEM morphology and elemental distribution analysis of liposomes. Upper row: TEM images of blank liposomes and Lipo@QM-CF with QM-CF concentrations of 5, 10, and 25 μ M. Scale bar: 100 nm. Lower left two panels: Bright-field TEM image and corresponding Cl elemental mapping of blank liposomes. Lower right two panels: Bright-field TEM image and corresponding Cl elemental mapping of QM-CF-loaded liposomes. Scale bar: 100 nm.

3.2 Evaluation of QM-CF encapsulation characteristics

The absorption spectra of QM-CF in methanol were measured using UV-Vis spectrophotometry, and a standard curve was established, with results shown in **Figure 4**. The UV absorption spectra of QM-CF at different concentrations (1, 5, 10, and 25 μM) exhibited a characteristic absorption peak at 425 nm, with absorbance values increasing progressively with concentration (**Figure 4a**). A good linear relationship was observed

between absorbance and QM-CF concentration in the range of 1-25 μM ($R^2 = 0.9991$, $P < 0.0001$), as shown in **Figure 4b**.

The encapsulation efficiency of QM-CF in liposomes at different concentrations was determined by ultrafiltration centrifugation combined with UV-Vis spectrophotometry, with results shown in **Figure 4c**. Lipo@QM-CF^5 (5 μM) exhibited an encapsulation efficiency of 88.64%, Lipo@QM-CF^{10} (10 μM) showed an encapsulation efficiency of 73.46%, while Lipo@QM-CF^{25} (25 μM) displayed a significantly decreased encapsulation efficiency of 28.78%. When the probe concentration increased from 5 μM to 10 μM, the encapsulation efficiency decreased slightly; however, when the concentration further increased from 10 μM to 25 μM, the encapsulation efficiency dropped by approximately 60%, indicating a saturation effect in the drug loading capacity of liposomes for QM-CF. At concentrations of 5 μM and 10 μM, the lipid bilayer could effectively encapsulate most of the probe molecules; whereas at the higher concentration of 25 μM, the loading capacity of the lipid bilayer was exceeded, resulting in a large number of probe molecules failing to be effectively encapsulated.

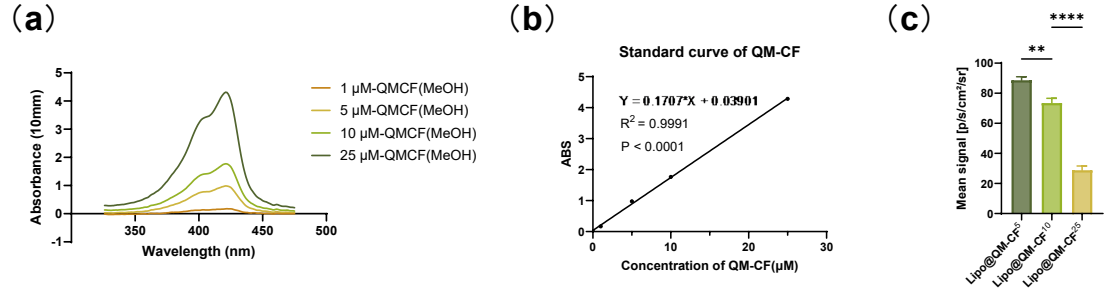

Fig. 4. UV-Vis absorption spectra and standard curve of QM-CF in methanol. (a) UV absorption spectra of QM-CF at different concentrations (1, 5, 10, and 25 μM). (b) Standard curve of QM-CF at 425 nm, showing a good linear relationship between absorbance and concentration ($R^2 = 0.9991$). (c) Encapsulation efficiency of Lipo@QM-CF at different concentrations (5, 10, and 25 μM). Data are presented as mean ± SD (n=3).

3.3 In vitro ROS response validation

To verify the successful encapsulation of QM-CF within liposomes and its ROS-responsive release behavior, a series of chemiluminescence experiments were designed, with results shown in **Figure 5**.

First, a positive control system was established. Free QM-CF dissolved in methanol at different concentrations (0, 5, 10, and 25 μM) exhibited concentration-dependent chemiluminescence signals, with signal intensity increasing progressively with concentration (**Figure 5a**), indicating that QM-CF possesses good luminescence properties under suitable conditions and can serve as a positive reference for subsequent experiments. In contrast, as-prepared Lipo@QM-CF at the same concentrations showed no obvious luminescence, preliminarily suggesting that QM-CF remained in a non-luminescent state after encapsulation within liposomes.

To further eliminate the influence of drug loading differences on experimental results, two concentrations corresponding to the actual loading amounts of Lipo@QM-CF (3.67 μM and 7.35 μM) were selected based on encapsulation efficiency measurements to compare the luminescence behavior of QM-CF under different states (**Figure 5b**). The results showed that under the same drug loading conditions, only the QM-CF(MeOH) sample group exhibited strong chemiluminescence signals, while Lipo, QM-CF(PBS), and Lipo@QM-CF showed no obvious luminescence. This result confirmed that QM-CF luminescence depends on its release from liposomes, and neither the encapsulated state nor the aqueous environment alone could induce luminescence.

To verify whether QM-CF was successfully encapsulated within liposomes, luminescence detection was performed on samples at different preparation stages (**Figure 5c**). Five sample groups were set up: QM-CF(PBS), QM-CF(MeOH), as-hydrated Lipo@QM-CF crude suspension, extruded Lipo@QM-CF, and blank liposomes (Lipo). The results showed that QM-CF(MeOH) produced the strongest luminescence signal; the as-hydrated crude suspension also exhibited obvious luminescence, though with lower intensity than QM-CF(MeOH); while QM-CF(PBS), extruded Lipo@QM-CF, and blank liposomes showed no obvious luminescence. The comparison between as-hydrated crude suspension and extruded Lipo@QM-CF indicated the presence of unencapsulated free QM-CF during the hydration stage, which was effectively removed after extrusion and purification, confirming successful encapsulation of QM-CF within liposomes.

Finally, the ROS responsiveness of Lipo@QM-CF was investigated. Hydrogen peroxide (H_2O_2), as an important member of the reactive oxygen species family, is commonly used to simulate ROS oxidative stress environments *in vitro*[11]. Based on this, H_2O_2 was selected to examine the responsive luminescence behavior of Lipo@QM-CF in the presence of ROS (**Figure 5d**). After methanol demulsification treatment of Lipo@QM-CF at different concentrations (5, 10, and 25 μM), the 25 μM sample group exhibited the strongest luminescence signal, followed by the 10 μM group, while the 5 μM group showed relatively weak luminescence. Upon subsequent addition of H_2O_2, the luminescence signals of all concentration groups were further enhanced, indicating that Lipo@QM-CF can effectively release QM-CF and generate luminescence signals in a ROS environment.

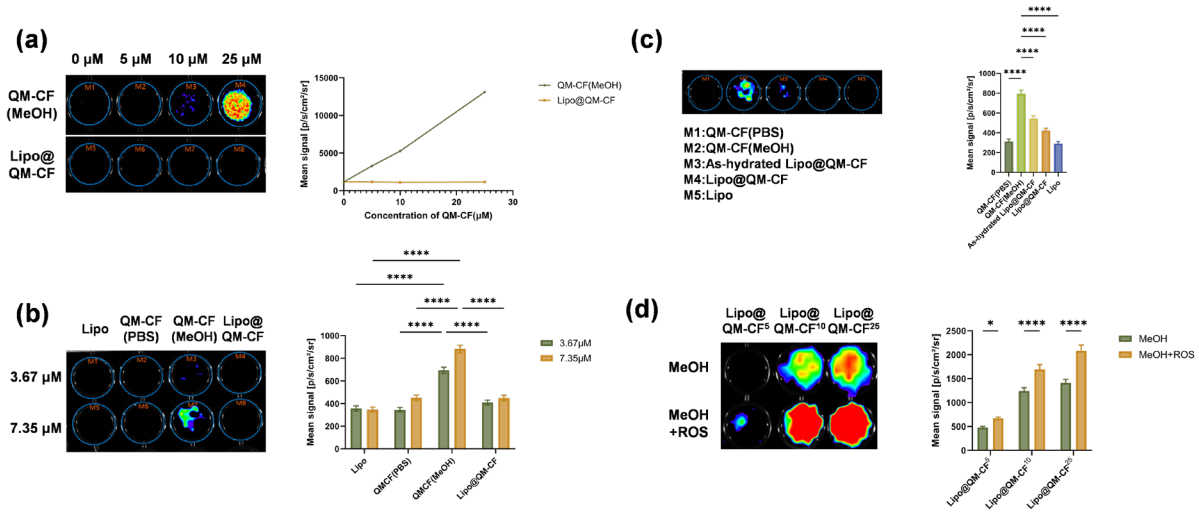

Fig. 5. In vitro ROS response validation. (a) Chemiluminescence intensity of free QM-CF in methanol and as-prepared Lipo@QM-CF at different concentrations (0, 5, 10, and 25 μM). (b) Chemiluminescence intensity comparison of Lipo, QM-CF(PBS), QM-CF(MeOH), and Lipo@QM-CF at actual loading concentrations of 3.67 μM and 7.35 μM. (c) Chemiluminescence intensity of samples at different preparation stages: QM-CF(PBS), QM-CF(MeOH), as-hydrated Lipo@QM-CF, extruded Lipo@QM-CF, and blank liposomes (Lipo). (d) Chemiluminescence intensity of Lipo@QM-CF at different concentrations (5, 10, and 25 μM) after methanol demulsification (upper panel) and after subsequent H_2O_2 treatment (lower panel). Data are presented as mean ± SD (n=3).

3.4 Conclusion

In this study, QM-CF-loaded liposomes (Lipo@QM-CF) were successfully constructed using the thin-film hydration method based on a phospholipid composition of dipalmitoylphosphatidylcholine (DPPC), sphingomyelin (SM), cholesterol (CHO), DSPE-PEG2000, and DSPE-PEG2000-maleimide. Their physicochemical properties, encapsulation characteristics, and in vitro ROS-responsive performance were systematically evaluated.

DLS and TEM characterization revealed that the prepared liposomes exhibited uniform particle size (approximately 160–170 nm, PdI < 0.15) and regular spherical morphology, with no significant particle size changes observed during 21 days of storage at 4°C, indicating good in vitro stability. Encapsulation efficiency measurements revealed a saturation effect in the drug loading capacity of liposomes for QM-CF: the encapsulation efficiency decreased from 88.64% at 5 μM to 73.46% at 10 μM, and further dropped to 28.78% at 25 μM. EDS elemental analysis further confirmed the colocalization of QM-CF with liposomes, consistent with the encapsulation efficiency results.

Chemiluminescence experiments systematically verified the encapsulation state and ROS-responsive performance of QM-CF. Free QM-CF exhibited luminescence only when dissolved in methanol, while remaining in a non-luminescent state after encapsulation within liposomes. Luminescence detection of samples at different preparation stages showed that the as-hydrated crude suspension exhibited detectable signals, whereas the extruded and purified Lipo@QM-CF showed no luminescence, confirming that QM-CF was successfully encapsulated within the liposomes rather than surface-adsorbed. In the H_2O_2-simulated ROS environment, the luminescence signal of Lipo@QM-CF was significantly enhanced, demonstrating its good ROS-responsive release capability.

In summary, this study explored the construction of a liposomal probe for ROS imaging. Based on the ROS-responsive mechanism of QM-CF, this probe offers advantages such as excitation-free luminescence, high intensity, and good stability, preliminarily validating its feasibility for specific imaging in inflammatory microenvironments. This study represents an attempt to combine a chemiluminescent probe with a liposomal delivery system, providing a new approach for the detection of inflammation-related microenvironments. However, certain limitations exist in this study: only H_2O_2 was used as a ROS model molecule for validation, while various ROS species exist in vivo, and the response efficiency to different types of ROS requires further investigation. Additionally, only *in vitro* validation has been completed, and subsequent cellular and animal experiments, as well as exploration of targeted modification for active delivery, are needed in future studies.

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