Research Article

Study of the Dynamic Uptake of Free Drug and Nanostructures for Drug Delivery Based on Bioluminescence Measurements

Zhongjian Fang,^{1,2,3} Houchao Xu,⁴ Xiangjun Ji,⁵ Congbiao Liu,¹ Kai Wang,⁶ Xiaoqing Qian,⁷ and Wencong Zhou¹

¹School of Pharmacy, Shanghai University of Medicine & Health Sciences, Shanghai 200237, China

²China Pharmaceutical University, Nanjing 211198, China

³Health School Attached to Shanghai University of Medicine & Health Sciences, Shanghai 200237, China

⁴State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, China

⁵Center for Bioinformatics and Computational Biology and the Institute of Biomedical Sciences, School of Life Science, East China Normal University, Shanghai 200241, China

⁶School of Agriculture and Biology, Shanghai Jiao Tong University, Shanghai 200240, China

⁷Institute of Nano Biomedicine and Engineering, School of Biomedical Engineering, Shanghai Jiao Tong University, Shanghai 200240, China

Correspondence should be addressed to Zhongjian Fang; shilyvip@sina.com

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The past two decades have witnessed the great growth of the development of novel drug carriers. However, the releasing dynamics of drug from drug carriers in vivo and the interactions between cells and drug carriers remain unclear. In this paper, liposomes were prepared to encapsulate D-luciferin, which was the substrate of luciferase and served as a model drug. Based on the theoretical calculation of active loading, methods of preparation for liposomes were optimized. Only when D-luciferin was released from liposomes or taken in by the cells could bioluminescence be produced under the catalysis of luciferase. Models of multicellular tumor spheroid (MCTS) were built with 4T1-luc cells that expressed luciferase stably. The kinetic processes of uptake and distribution of free drugs and liposomal drugs were determined with models of cell suspension, monolayer cells, MCTS, and tumor-bearing nude mice. The technology platform has been demonstrated to be effective for the study of the distribution and kinetic profiles of various liposomes as drug delivery systems.

1. Introduction

Since Bangham A. D. and Standish M. M. found liposomes and put forward the concept of liposomes in the 1960s, the technology of liposomes has developed greatly [1]. Various preparation technologies are constantly emerging and gradually applied into clinical use from the laboratory. Among all sorts of research and practice of bioluminescent technology, the firefly luciferase system research is the most thorough and mature one. However, many unsolved problems exist in liposome. For example, the method for dynamically monitoring liposomes and study platform for linking the models in vivo and in vitro are still far from mature.

With the gradual development in D-luciferin research, a growing number of D-luciferin derivatives have been synthesized. These derivatives have various characteristics in molecular weight, solubility, hydrophilicity, and other physicochemical characteristics, which means that they can serve as good model drugs for small molecules [2].

According to the chemical structure and spectral characteristics, D-luciferin has an ultraviolet absorption at 333 nm, and its emission ray peak is at a wavelength of 526 nm [3]. The quantitative detection of D-luciferin can be achieved by both its spectral characteristics and enzymatic reaction with luciferase. These methods provide standards for assays of encapsulation and drug loading of liposomes, which may

also be applied to D-aminoluciferin and other D-luciferin derivatives [4].

Compared with two-dimensional models, multicellular tumor spheroids (MCTS) could serve as models which can bridge monolayer cells in vitro and tumor models in vivo [5]. MCTS are composed of core layers (necrotic layer and layer gangrene), transition layers, and surface layers. Polarity makes MCTS more similar to tumor models in vivo. Besides, since MCTS can be obtained by the method of cell culture, it is more convenient and economical than building tumor models with nude mice [6].

Methods of preparing MCTS include cell suspension dispersive cultivation, tissue engineering method, the microfluidic system, and three-dimensional printing technology. Among all the above methods, cell suspension dispersive cultivation and tissue engineering method are most commonly used. On the other hand, in order to meet the requirements to model a wide variety of tumor cells, a combination of various preparation methods is also a recent trend [7].

The main cell models are two-dimensional models with cell culture plates. It is popular for its short training period and high repeatability. However, two-dimensional plane of monolayer cells has significant limitations. For example, the basal side of cells is contracted to culture plate, and the upper side is exposed to nutrition solution. Although uniformity exists, a good simulation of tumor stroma cannot be achieved.

Therefore, we introduce a liposome encapsulation technology platform for studying the dynamic uptake of nanoparticles delivery system in vitro and in vivo with D-luciferin as a model. We plan to study the preparation of liposomes to encapsulate D-luciferin with pH gradient both theoretically and practically. We also want to investigate the kinetic uptake of free drugs and drug-loaded nanostructures in cell suspension, monolayer cells, MCTS, and tumor-bearing mice. A three-dimensional model is expected to be built to bridge tumor cells in vitro and tumor models in vivo.

2. Materials and Methods

2.1. Materials. EPC and HSPC were purchased from Lipids Corp (Japan). Cholesterol, DPPC, DSPE-PEG2k, and DPPC were purchased from Avanti Polar Lipids Inc. (US). Triton X-100, ATP, Coenzyme A, and agarose were obtained from Sigma-Aldrich (US). D-luciferin and $5 \times$ cell lysis buffer were purchased from Promega Corp (US). Sephadex G-75 was purchased from GE Corp (US). RPMI 1640 cell culture fluid, FBS, trypsin, penicillin/streptomycin solution, and microdialysis tubes were purchased from Thermo Fisher (US). 4T-luc was purchased from Shanghai Science Light Corp (China). DSPE-PEG2000-Mal-GE11 and H1299-pGL3 (C11) were obtained from School of Pharmacy, Shanghai Jiao Tong University. Nude mice (BALB/C, female, 4-week-old, 12.5~17.5 g) were purchased from Slac Laboratory Animal Corp (China). Dialysis bags (MWCO 3.5 KD) were purchased from Sangon Biotech Corp (China). Other chemical reagents for common use were purchased from Shanghai Chemical Reagent Corp (China).

2.2. Methods

2.2.1. Preparation of Liposomes. Most liposomes prepared with the method of passive loading had encapsulation efficiency lower than 15% [8]. In order to prepare liposomes that could carry enough D-luciferin for imaging in vivo and to reduce lipid costs, theoretical calculation has been studied to identify the feasibility of active loading with pH gradient.

The theoretical calculation was based on the following physicochemical parameters and hypothetical conditions:

- The surface of the head of EPC was $70 \text{ \AA}^2$ [8–12]. The pKa of D-luciferin was 8.6.
- The thickness of the lipid bilayer of the liposome models was uniformly $40 \text{ \AA}$ [9–12].
- In order to obtain the oil-water partition coefficient of D-luciferin, the software of Marvin-Sketch has been applied with the computed result of $\log P = 2.24$.
- All the liposomes prepared were homogeneous SUV with diameter of 200 nm.

In this hypothetical case, liposomes were composed of EPC: cholesterol: DSPE-PEG2000 with molar ratio of 56:39:5. During the process of drug loading, 0.05 mL D-luciferin (0.725 mg, pH 5.0) was added to liposomes (2.9 mg lipid) with formed pH gradient (pH 5.0 in intrawater phase and pH 10.0 in interwater phase) [13, 14].

$1 \mu\text{mol}$ EPC can be dispersed into 4200 cm^2 lipid monolayer or 2100 cm^2 lipid bilayer.

$$V = S * h. \quad (1)$$

The volume of the oil phase of liposomes composed of $1 \mu\text{mol}$ EPC was $8.4e - 10 \text{ m}^3$. The volume of the oil phase of liposomes composed of 1 mol EPC was $8.4e - 4 \text{ m}^3$.

As shown in Figure 1, in a model of a liposome with diameter of 200 nm, the thickness of lipid bilayer is 4 nm.

$$V_{\text{water-phase}} = \frac{4}{3} \pi r^3 \quad (2)$$

$$V_{\text{oil-phase}} = \frac{4}{3} \pi (R^3 - r^3) \quad (3)$$

$$v_{(\text{water-phase})} = 3.7041e - 21 \text{ m}^3 \quad (\text{Data 1})$$

$$v_{(\text{oil-phase})} = 4.8257e - 22 \text{ m}^3. \quad (\text{Data 2})$$

In the case of liposomes composed of EPC:cholesterol: DSPE-PEG2000 with molar ratio of 56:39:5 [15], when drug: lipid (w/w) = 1: 4, liposomes composed of 2.9 mg total lipid contained $2.24309e - 6 \text{ mol}$ EPC and $2.00325e - 7 \text{ mol}$ DSPE-PEG2000.

Hypothesis Condition A. The addition of cholesterol to liposomes only increases the density of membrane but makes no difference on the volume of oil phase. The volume of the oil phase is the sum of volume of EPC and DSPE-PEG2000.

Hypothesis Condition B. When 1 mol EPC and 1 mol DSPE-PEG2000 are forming liposomes, they occupy the same

FIGURE 1: Structure schematic of liposome with diameter of 200 nm.

volume. To be specific, 1 mol EPC or 1 mol DSPE-PEG2000 forms an oil phase of $8.4e - 4 \text{ m}^3$.

Based on the hypotheses stated above, in the liposomes composed of 2.9 mg lipid

$$V_{(\text{EPC})} = 1.8842e - 9 \text{ m}^3 \quad (\text{Data 3})$$

$$V_{(\text{DSPE-PEG2000})} = 1.6827e - 10 \text{ m}^3 \quad (\text{Data 4})$$

$$V_{(\text{oil-phase})} = 2.0525e - 9 \text{ m}^3. \quad (\text{Data 5})$$

Hypothesis Condition C. All 2.9 mg lipid forms homogeneous liposomes with diameters of 200 nm.

As shown in (Data 2), in a liposomal model of SUV with diameter of 200 nm, $V_{(\text{oil-phase})} = 4.8257e - 22 \text{ m}^3$. Based on the hypothesis above, 2.9 mg lipid can form $N = 4.253269e12$ liposomes of homogeneous SUV with a diameter of 200 nm. Accordingly, as shown in (Data 1), the total volume of the water phase formed with the 2.9 mg lipid was $1.57545e - 8 \text{ m}^3$.

$$\begin{aligned} V_{(\text{water-phase})} &= N * v_{(\text{water-phase})} \\ &= 1.57545e - 8 \text{ m}^3. \end{aligned} \quad (\text{Data 6})$$

In this case [16], the total volume of liposomal solution was 0.35 mL.

$$V_{(\text{inter-water})} = V - V_{(\text{intra-water})} - V_{(\text{oil-phase})}. \quad (4)$$

Based on the above equation,

$$V_{(\text{inter-water})} = 3.3404e - 7 \text{ m}^3. \quad (\text{Data 7})$$

In order to make sure the calculation for the volume of intra-water phase, interwater phase, and oil phase was reliable and objective enough, a second computing method was applied.

In the model shown in Figure 1, the hydrophilic radicals (polar head) of lipid bilayer faced the intrawater phase

and interwater phase. The inside diameter and the outside diameter of the liposomal model were 186 nm and 200 nm, respectively.

$$S = 4\pi r^2. \quad (5)$$

Based on (5), the sum area of the inner surface and the outside surface was $S = 2.4135e - 13 \text{ m}^2$. Since the area of the polar head of EPC was $70 \text{ \AA}^2$ [9, 10, 17], in order to form a liposomal model with diameter of 200 nm, the number of EPC molecules needed was $3.44786e5$.

$$n_{200 \text{ nm}} = 3.44786e5. \quad (\text{Data 8})$$

Accordingly, 1 mol EPC can form $1.7466e18$ liposomal models with a diameter of 200 nm.

$$N_{200 \text{ nm}} = 1.7466e18. \quad (\text{Data 9})$$

In the case of liposomes composed of 2.9 mg lipids with molar ratio of EPC : chol : SPE-PEG2000 = 56 : 39 : 5,

$$n_{(\text{EPC})} = 2.24309e - 6 \text{ mol} \quad (\text{Data 10})$$

$$n_{(\text{DSPE-PEG2000})} = 2.00325e - 7 \text{ mol}. \quad (\text{Data 11})$$

Hypothesis Condition D. Liposomes are composed of EPC and DSPE-PEG2000 solely. Cholesterol makes no difference on forming liposomes.

Hypothesis Condition E. The head of DSPE-PEG2000 occupies an area of $70 \text{ \AA}^2$ when it is forming liposomes.

Based on the two hypothesis conditions stated above,

$$\begin{aligned} n_{(\text{total lipid})} &= n_{(\text{EPC})} + n_{(\text{DSPE-PEG2000})} \\ &= 2.443415e - 6 \text{ mol}. \end{aligned} \quad (\text{Data 12})$$

Based on (Data 9) and (Data 12), 2.9 mg lipid can form $4.2677e12$ liposomal models of SUV with diameter of 200 nm.

$$N = 4.2677e12. \quad (\text{Data 13})$$

Based on (Data 1) and (Data 2), in 0.35 mL liposomal solution containing 2.9 mg lipids,

$$V_{(\text{oil-phase})} = 2.0710e - 9 \text{ m}^3 \quad (\text{Data 14})$$

$$V_{(\text{intra-water phase})} = 1.5808e - 8 \text{ m}^3. \quad (\text{Data 15})$$

Based on (4)

$$V_{(\text{inter-water phase})} = 3.3212e - 7 \text{ m}^3. \quad (\text{Data 16})$$

Based on the above data, in order to identify the feasibility of active loading of pH gradient, the balanced concentration of D-luciferin in different phases was calculated.

Hypothesis Condition F. When the active loading of pH gradient finished, the pH of intrawater phase was 5.0 and the pH of interwater phase was 10.0.

FIGURE 2: Schematic diagram of the concentration equilibrium of D-luciferin in the internal water phase, external water phase, and oil phase.

TABLE 1: Concentration of molecular and ionized D-luciferin in different phases in the model of liposomes.

Molecular or ionized D-luciferin	Concentration
Ionized D-luciferin in internal water phase (Luc-COO ⁻)	a_1
Molecular D-luciferin in internal water phase (Luc-COOH)	a_2
Molecular D-luciferin in oil phase (Luc-COOH)	a_3
Molecular D-luciferin in external water phase (Luc-COOH)	a_4
Ionized D-luciferin in external water phase (Luc-COO ⁻)	a_5
Total concentration of ionized and molecular D-luciferin	$a_{(\text{total})}$

As shown in the liposome model in Figure 2, the distribution of D-luciferin in each phase was mainly determined by the balance of oil-water partition and molecular dissociation.

According to the definition of oil-water partition coefficient of D-luciferin and the concentration set in Table 1,

$$P = \frac{C_{\text{oil}}}{C_{\text{water}}} \quad (6)$$

$$P = \frac{a_3}{a_2} = \frac{a_3}{a_4} \quad (7)$$

$$a_2 = a_4 = \frac{a_3}{P}. \quad (8)$$

According to the definition of dissociation constant,

$$K_a = \frac{C_{(\text{Luc-coo}^-)}C_{(\text{H}^+)}}{C_{(\text{Luc-COOH})}} \quad (9)$$

$$a_5 = \frac{K_a \cdot P \cdot a_3}{C_{(\text{H}^+ \text{ external-water-phase})}} \quad (10)$$

$$\text{p}K_a = -\lg K_a. \quad (11)$$

As the physiochemical parameters of D-luciferin, $\text{p}K_a = 8.6$ [18],

$$\begin{aligned} K_a &= 10^{-8.6} \text{ (mol/L)} \\ &= 2.5119 \times 10^{-9} \text{ (mol/L)}. \end{aligned} \quad (\text{Data 17})$$

Based on hypothesis condition F and $\log P = 2.24$,

$$a_1 = 4.3652 \times 10^3 \cdot a_3 \quad (12)$$

$$a_2 = 173.7801 \cdot a_3 \quad (13)$$

$$a_4 = 173.7801 \cdot a_3 \quad (14)$$

$$a_5 = 4.3652 \times 10^{-2} \cdot a_3. \quad (15)$$

From the above equations, we can conclude that $a_1 > a_2 = a_4 \gg a_5$.

$$\begin{aligned} a_1 \cdot V_{\text{inner-water}} + a_2 \cdot V_{\text{inner-water}} + a_3 \cdot V_{\text{oil}} + a_4 \\ \cdot V_{\text{outer-water}} + a_5 \cdot V_{\text{outer-water}} = a \cdot V \end{aligned} \quad (16)$$

$$\log P = \log \frac{C_{\text{oil-phase}}}{C_{\text{water-phase}}}. \quad (17)$$

Based on the above equations, 0.725 mg D-luciferin is equal to 2.2767×10^{-6} mol [19], and the concentration of D-luciferin in different phases can be calculated.

According to the above theoretical calculation, both pH gradient and the volume of external water phase affected the encapsulation efficiency. The following experiments were conducted to identify whether the effect of pH gradient on encapsulation efficiency in practical conditions was consistent with theoretical calculation.

In this study, lipid films composed of EPC:chol:DSPE-PEG2k with molar ratio of 56:39:5 were prepared in 4 eggplant-shaped flasks. The 4 samples of lipid films were labeled as ①, ②, ③, and ④, respectively. TRIS buffer (300 mL, pH

10.0) was added to the flasks with lipid membranes, and the final concentration of lipid was adjusted to 10 mg/mL. After manual extrusion with polycarbonate membranes with pore size of 400 nm and 200 nm, liposomes of ① and ③ were dialyzed with dialysate of 20 mL citric acid solution (150 mL NaCl, pH 6.0). While liposomes of ② and ④ were dialyzed with dialysate of 20 mL citric acid solution (150 mL NaCl, pH 3.0). The process of dialysis lasted for 12 h with exchange of fresh dialysate every 4 h. 50 μ L purified liposomes of ①, ②, ③, and ④ with lipid concentration of 10 mg/mL was added to 950 μ L 10% Triton solution. The mixtures were kept static for 30 min after being vortexed for 30 s. 50 μ L liquid was transferred to 96-well plates in triplicate. Microplate reader was used to detect the absorption with wavelength set at 330 nm. By comparing with the standard curve, the amount of D-luciferin and the entrapment efficiency of liposomes could be calculated.

In order to further optimize the method of preparing liposomes, the relationship between pH gradient and encapsulation efficiency has been studied based on the method designed by Kheirrolomoom et al. [20].

2.2.2. Assays with Cell Suspensions

- (1) SMMC-7721-pGL3 cells were cultured according to conventional procedures (cell culture was carried out using RPMI 1640 cell culture medium containing 10% fetal bovine serum). The cells in the logarithmic growth phase of a flask (T25-sized cell culture flask) were collected using trypsin digestion.
- (2) The cells were counted by a hemocytometer, and the density of the cell suspension was adjusted to 4.0×10^5 cells/mL.
- (3) The cells were added to 24-well plates at 1.0 mL per well. Namely, the inoculation density per hole was 4.0×10^5 cells/mL.
- (4) The cells were placed in a cell incubator and incubated in a 5% CO₂ incubator at 37°C for 48 h.
- (5) The cells were cultured for 48 h. The complete medium was removed in a biological clean bench and washed twice with a PBS (pH 7.4) solution. The complete medium was removed in a biological clean bench and washed twice with a PBS (pH 7.4) solution. After digestion with trypsin for 2 min, RPMI 1640 cell culture medium containing 10% fetal bovine serum (FBS) was added to stop digestion. After centrifugation (300 $\times$ g, 5 min for each time), the cells were washed 3 times with PBS (pH 7.4) to completely remove trypsin.
- (6) The cells were counted with a hemocytometer. And the cell density was adjusted to 1.0×10^5 cells/mL with PBS solution.
- (7) Liposomes composed of DPPC : chol : DSPE-PEG2k with molar ratio of 86 : 10 : 4 and 86 : 10 : 10 were prepared with the method of thin-film dispersion. They were purified with dialysis to separate the unloaded

D-luciferin. The concentration of encapsulated D-luciferin was diluted to 0.3 mg/mL. And free D-luciferin with the same concentration was for control.

- (8) 10 μ L cell suspension was mixed with 10 μ L 0.3 mg/mL free D-luciferin in a tube. Bioluminescence was measured with illuminance meter (FB12/Sirius Illuminance meter, Berthold Science and Technology Company, Germany) immediately after mixing. The detection mode of the illuminometer was set as single kinetics. The delay time and the measure time were set as 2.0 s and 12.0 s, respectively. The measurement was repeated for three times.

2.2.3. Assays with Monolayer Cells

- (1) Cells of H1299-pGL3 were cultured according to routine operation (the cell culture fluid of RPMI 1640 which contained 10% fetal bovine serum was used). Trypsin was used to digest and a bottle of cultured cells (T25 specification) in logarithmic growth phase was collected.
- (2) The number of the cells was counted by the blood cell counting plate, and the density of cell suspension was adjusted to 8.0×10^4 /mL.
- (3) 0.1 mL cell suspension was added to each well in 96-well plates (Corning 3610, whiteboard). In order to avoid the difference of the volume between the wells caused by the evaporation of the outer ring of the well plate of culture medium, the outermost one was not inoculated with cells; only 0.1 mL PBS was added to it. Therefore, only $10 \times 6 = 60$ wells are inoculated in every 96-well plate.
- (4) Cell culture medium was taken out after the incubation of 48 h in the incubator of 5% CO₂ at 37°C. The culture medium was absorbed by a straw and then discarded at bioclean working platform, washing them up 3 times with PBS (pH 7.4) and making every well left 100 μ L RPMI 1640 without fetal bovine serum. Three wells of them were digested trypsin, and the cell number was also counted by the blood cell counting plate. According to the calculation, the number of cells was 5.334×10^4 /mL in every well.
- (5) PBS (pH 7.4) was used to prepare the solution of D-luciferin with the concentration of 0.05 mg/mL.
- (6) 96-well plates were moved to the Multimode Detection Platform, and the automatic injector was also used to add 100 μ L of different concentrations of D-luciferin to the plates; the measure time was set as 10 ms, 100 ms, 1000 ms, and 4000 ms; then continuous determinations of biological luminescence signal were made. 100 μ L PBS (pH 7.4) was added to groups of blank control. Every measurement was repeated 3 times.
- (7) Microsoft Excel was applied to sort the data, and the figure was generated by setting time as the horizontal coordinate and setting signal intensity of bioluminescence as the vertical coordinate.

In order to elaborate the mechanism of the uptake of D-luciferin, endocytosis inhibitors have been added to the monolayer cells of H1299-pGL3. The only difference compared with the method above was that the D-luciferin solution was mixed with 50 μ L 2 μ g/mL methyl- β -cyclodextrin, 25 μ g/mL cytochalasin B, 25 μ g/mL cytochalasin B, 1 μ g/mL Filipin III, or Omeprazole, respectively.

2.2.4. Assays with MCTS. The preparation of MCTS models referred to the method used by Juergen Friedrich. Specific operations are listed as follows [14]:

- (1) Weigh 0.75 g of agarose into a 250 mL beaker and add RPMI 1640 to 1.5% (w/v) agarose. 50 mL of agarose solution was generally sufficient for about 10 plates of 96-well plates.
- (2) The beaker was sealed with aluminum foil and put into an autoclave and autoclaved according to conventional procedures.
- (3) After autoclaving, the agarose was removed from the autoclave immediately before the agarose solution had been solidified (about 90°C) and placed in a 60°C water bath prepared in a biological clean bench device in advance.
- (4) The agarose solution was added to a flat-bottomed 96-well plate. 50 L of solution was added to each well.

(Note: common transparent cell culture plate can be used for the preparation of MCTS models for routine experiments; to increase the sensitivity of bioluminescence detection with Multimode Detection Platform, 96-well plates with white wall and transparent bottom were recommended (such as Corning #3610); a transparent bottom facilitated the observation of inverted microscopes and the use of phase contrast microscopy, and a white wall can decrease the interference from adjacent wells.)

In this experiment, Eppendorf 96-well plate was chosen.

- (5) The 96-well plates were placed horizontally and allowed to stand for cell inoculation after the agarose solution was cooled and solidified. To reserve pre-coated 96-well plates for future use, they should be wrapped with plastic and aluminum foil in a sterile environment.
- (6) To investigate the effect of cell seeding density on the MCTS model, 4T1-luc cell suspension was collected and cultured according to the conventional method in the previous section. And the density of the 4T1-luc cell suspension was adjusted to 2.5×10^3 cells/mL, 3.75×10^3 cells/mL, 5.0×10^3 cells/mL, 7.5×10^3 cells/mL, 1.0×10^4 cells/mL, 1.5×10^4 cells/mL, 2.0×10^4 cells/mL, 3.0×10^4 cells/mL, 4.0×10^4 cells/mL, and 6.0×10^4 cells/mL.
- (7) The cell suspensions of each concentration were inoculated into 96-well plates and the volume of each well was 0.2 mL. Cultured for 48 h, the formed cell aggregates or MCTS were observed using a phase

contrast microscope. Cell aggregates or MCTS were photographed using phase contrast microscopy and the diameter of multicellular tumor spheres was measured using matching image processing software. Then the volume of MCTS was calculated.

- (8) The above method of agarose-coating was also applied to H1299-pGL3 (C11), A549-luc, and SMMC-7721-pGL3 to identify whether they could form MCTS.

In order to detect the dynamic uptake of nanostructures for drug delivery in MCTS, the bioluminescence produced from the reaction of liposomes encapsulating D-luciferin with MCTS models of 4T1-luc was detected.

- (1) Models of 4T1-luc were built with seeding density of 1.0×10^4 cells/mL (8.0×10^3 cells per well) following the above method.
- (2) After culture for 3 d, models of homogeneous MCTS with diameter of 776.61 ± 22.12 μ m were prepared.
- (3) Liposomes composed of HSPC:chol = 70:30 and HSPC:chol:DSPE-PEG2k-Mal-GE11 = 68.6:29.4:2 were prepared with the above method and were purified with dialysis to eliminate the D-luciferin that had not been encapsulated.
- (4) The concentration of encapsulated D-luciferin was adjusted to 6.0 mg/mL.
- (5) After 50 μ L of liposomes was added to each well containing MCTS, the final concentration of D-luciferin in each well was 1.2 mg/mL. The same volume of free D-luciferin with the same concentration was added to the control groups.
- (6) Bioluminescence was detected with small animal imaging system at 5 min, 15 min, 25 min, 40 min, 50 min, 75 min, and 720 min. The exposure time was set at 5 min. And the bioluminescence was calculated with the software attached to the instrument.
- (7) The dynamic curve was generated with Microsoft Excel.

2.2.5. Assays with Tumor-Bearing Nude Mice

- (1) 4T1-luc cells were cultured and collected. 5 μ g/mL of Blasticidin S (sterilized fungus) was added in the cell culture to obtain 4T1-luc cells which were with higher purity and could express luciferase stably.
- (2) The density of 4T1-luc cell suspension was adjusted to 1.0×10^7 cells/mL. Cancer cells were cultured under the armpit skin folds of 12 nude mice (BALB/C nude mice) with the inoculation amount being 1.0×10^6 cells each nude mouse.
- (3) The subcutaneous tumors formed after 10 days. The length and width of subcutaneous tumors were measured by Vernier calipers.
- (4) Liposomes composed of HSPC:chol = 70:30 and EPC:chol = 70 were prepared.
- (5) In order to study the dynamic uptake of nanostructures for drug delivery in tumor, the liposomes were

TABLE 2: Distribution of D-luciferin in different phases.

Molecular or ionized D-luciferin in different phases	Concentration (mol/m ³)	Amount of substance (mol)	Distribution of D-luciferin
a_1	76.7533	1.2133×10^{-6}	53.2920%
a_2	3.0556	4.8303×10^{-8} mol	2.1216%
a_3	1.7583×10^{-2}	3.6414×10^{-11} mol	0.0016%
a_4	3.0556	1.0148×10^{-6} mol	44.5146%
a_5	7.6753×10^{-4}	2.5491×10^{-10} mol	0.0112%

TABLE 3: Fraction of the amount of substance of D-luciferin in intraliposomal, extraliposomal, and oil phase.

Phase	Fraction of the amount of D-luciferin in different phases
Internal water phase	53.7969%
Oil phase	0.0029%
External water phase	46.2013%

administered by tail vein injection and intratumoral injection, respectively.

For intravenous injection, the concentration of encapsulated D-luciferin was adjusted to 15 mg/mL, and the dose was 0.2 mg/g body weight. The control group was injected with the same volume of saline. Bioluminescence was detected with ZKKS-MI-II small animal imaging system (Guangzhou Zhongke Kaisheng Medical Technology Co., Ltd). The exposure time was set as 1 min. And the images were captured continuously for 100 min after injection.

For intratumoral injection, the concentration of encapsulated D-luciferin was adjusted to 10 mg/mL, and the dose was 0.1 mg/g body weight. The control group was injected with the same volume of saline. The exposure time was set 4 min.

- (6) The data of bioluminescence was processed with the software attached to the instrument. And the dynamic curve was generated with Excel (Microsoft Office).

Animal welfare and experimental procedures were approved by the animal ethics committee of Shanghai University of Medicine and Health Sciences and Shanghai Jiao Tong University.

3. Results and Discussion

3.1. Preparation of Liposomes

3.1.1. Theoretical Calculation. The results of the theoretical calculation indicated that the active loading with pH gradient was feasible. As is shown in Tables 2 and 3, in the case of active loading designed by Kheiolomoom et al. [20], the total amount of ionized and molecular D-luciferin in internal water phase could reach 55.4136% in ideal conditions. It

should be noted that, in the above theoretical calculation, all D-luciferin in oil phase was considered to be molecular forms that did not dissociate.

The result demonstrated that, in order to achieve active loading and to sustain high concentration of D-luciferin in interwater phase, at least two factors were related. One was the pH gradient between internal and external water phase, and the other was the volume of external water phase.

It must be noted that the above theoretical calculation was based on the balance of oil-water partition and molecular dissociation. In practice, the distribution is related to time and the diffusion rate is associated with lipid components. For example, it takes time for molecular D-luciferin to pass from external water phase to oil phase, or to pass from oil phase to intrawater phase.

Practical experience and long-term exploration indicated that the leakage of encapsulate D-luciferin from liposomes could be a significant factor, especially when the process of dialysis lasted long. Compared with dialysis, glucan gel column chromatography (Sephadex G-75) was generally a better choice for the purification of liposomes.

In vivo imaging tended to cost a big amount of D-luciferin; thus column chromatography (Sephadex G-75) was recommended. While for in vitro test with cells or MCTS, dialysis was recommended because the separation of free D-luciferin was more thorough; thus the interference effect resulting from incomplete purification could be decreased.

Column chromatography often resulted in the dilution of liposomes. Three practical approaches could be applied to condense the liposomes. Firstly, ultrafiltration tubes (MWCO 3 KD) were strongly recommended. It should be noticed that liposomes with diameters less than 100 nm could not be cut off completely by ultrafiltration tubes (MWCO 10 KD). The second approach was to employ frozen centrifugation technology to evaporate water. The third approach was to put liposomes into dialysis bag (MWCO 3 KD) with PEG2k coated outside. After putting the bag into a drying oven for 24 h, liposomes could be condensed. Since the last two approaches only eliminated water, the ionic concentration tended to increase. In brief, ultrafiltration was the best choice.

3.1.2. Encapsulation Efficiency by pH Gradient Method. In Figure 3, under the condition that pH = 10.2 in internal water phase and pH = 6.0 in external water phase, the encapsulation efficiency of liposome was $5.29 \pm 1.20\%$. While in conditions that pH = 10.2 in the interwater phase and pH = 3.0 in external

FIGURE 3: Effect of pH gradient on encapsulation efficiency of liposomes.

water phase, the encapsulation efficiency was $22.04 \pm 0.52\%$. This indicated that the increasing pH gradient was favorable for pH gradient active drug loading, which was consistent with the theoretical calculation results mentioned before.

3.2. Assays with Cell Suspensions. Figure 4 showed kinetic curves of bioluminescence produced from the reaction of cells suspensions with liposomal and free D-luciferin with concentration of 0.3 mg/mL. The bioluminescence of free D-luciferin peaked at 105 s. The curve of the liposomes with molar ratio of DPPC : chol : DSPE-PEG2k = 86 : 10 : 4 had two gentle peaks, with peaking time at 119 s and 273 s, respectively. The first peak might be resulting from the same mechanism of free D-luciferin which was released from liposomes, which needed further study. The releasing process might happen outside or near the surface of cell membrane. The second peak might be caused by the transfer of liposomes to the cells and the later reaction of released D-luciferin with cellular lysate.

The kinetic curve of liposomes with molar ratio of DPPC : chol : DSPE-PEG2k = 86 : 10 : 10 had the highest bioluminescence at 189 s, which was almost at the midpoint of the 119 s and 273 s. In other words, this curve might be the overlap of 2 peaks resulting from free D-luciferin and liposomal D-luciferin. The kinetic curve was totally lower than that of liposomes of DPPC : chol : DSPE-PEG2k = 86 : 10 : 4. It might be explained that DSPE-PEG2k increased the hydrophobicity of liposomes and thus decreased the attachment of liposomes to cell membrane.

3.3. Assays with Monolayer Cells. Figure 5 shows the kinetic curve of bioluminescence produced from the reaction of free D-luciferin and liposomal D-luciferin in different formulations with monolayer cells. The peaking value of the bioluminescence of liposomes of EPC : chol = 70 : 30 was found to be higher than that of liposomes in other formulations. It could be explained that the phase transition temperature of EPC was lower than other lipids. Thus the liposomes composed majorly of EPC would be “softer,” which made the leakage and release of D-luciferin easier than other liposomes.

Compared to the liposomes of EPC : chol = 70 : 30, both the value of bioluminescence reaching peak and the value remaining stable were higher than that of the liposomes of EPC : chol : DSPE-PEG2000 = 56 : 39 : 5. It could be explained

FIGURE 4: Dynamic curves of bioluminescence signal produced from the reaction of cell suspension with liposomal and free D-luciferin. (Liposomes composed of DPPC : chol : DSPE-PEG2k with molar ratio of 86 : 10 : 4 and 86 : 10 : 10 were prepared with the method of thin-film dispersion. They were purified with dialysis to separate the unloaded D-luciferin. The concentration of encapsulated D-luciferin was diluted to 0.3 mg/mL. And free D-luciferin with the same concentration was for control. The density of cell suspension was adjusted to 1.0×10^5 cells/mL. The testing mode of the illumination meter was set as single kinetic. After mixing the cell suspension with liposomal or free D-luciferin, bioluminescence was detected immediately. The delay time for each measurement was 2.0 s, and the measurement time was 10.0 s. RLU is short for relative light unit.)

that the addition of DSPE-PEG2000 increased the hydrophobicity of the liposomes, which decreased the adhesion of liposomes to cells. Similarly, the bioluminescence of liposomes of HSPC : chol = 70 : 30 was found to be lower than that of liposomes of EPC : chol = 70 : 30. It could be explained that the liposomes were composed majorly of HSPC, which had a higher phase transition temperature. Due to the increase of the phase transition temperature, the barrier effect of the “hard” liposomes could decrease the leakage and release of the encapsulated D-luciferin.

Figure 6 shows the kinetic curve of bioluminescence produced from the reaction of liposomes of EPC : chol = 70 : 30 with monolayer cells within 10,000 s. The curve had two overlapped peaks. The first peak was sharper with a value of 1,000 at 300 s, while the second peak had a value of 1200 at about 6,700 s.

The first hypothesis of the overlapped peaks was that the uptake of liposomes was via two routes. The first route of cellular uptake happened earlier with liposomes fused with cellular membranes after absorption. Thus higher concentration of D-luciferin was formed near the area of inner surface of cellular membranes. In this route, encapsulated drugs were transported into the cells in forms of free D-luciferin. In the kinetic curve, the second peak was wide with gentle slope.

FIGURE 5: Kinetic curves of bioluminescence produced from the reaction of monolayer cells with free D-luciferin and luciferin-loaded liposomes of different formulations.

FIGURE 6: Kinetic curve of bioluminescence produced from the reaction of liposomes of EPC:chol = 70:30 and monolayer cells of H1299-pGL3 within 10,000 s. (Luciferin-loaded liposomes were separated from nonencapsulated luciferin by passing the extruded liposomal solution through a column of Sephadex G-75. To further prepare the liposomes, the released luciferin from the liposomes was separated using centrifugal ultrafiltration units with a molecular cutoff of 3 KD at 2000 $\times$ g, 15°C for 20 min prior to detection. Interval of the measure time was set at 1000 ms.)

The uptake of liposomes might be in form of endosomes. Bioluminescence was produced after the reaction of released D-luciferin with cellular luciferase following endocytosis. However, this was only a hypothesis on the mechanism of explaining the cellular uptake of liposomes. To verify this hypothesis, further study with confocal microscope, radioactive labeling, and imaging techniques was needed.

FIGURE 7: Effect of endocytosis inhibitors to the bioluminescence produced after free D-luciferin was added to monolayer cells of H1299-pGL3. (The 96-well plates had white walls with clear bottoms. Each well was seeded with 3000 cells. Bioluminescence was detected with multifunctional microplate reader. The unit of measurement time was set at 1000 ms.)

FIGURE 8: Multicellular tumor spheroid of 4T1-luc. (The picture was taken with a phase contrast microscope. The seeding density was 8.0×10^3 cells in each well. The pink background shows the color of cell culture medium of RPMI 1640.)

As shown in Figure 7, endocytosis inhibitor group and the control group did not show significant difference. It demonstrated that the uptake of D-luciferin was not related to caveolin, clathrin, or micropinocytosis.

3.4. Assays with MCTS. As shown in Figure 8, a model of MCTS (4T1-luc) was composed of marginal layer, transition layer, and a necrotic core. Table 4 showed the morphological parameters of MCTS with different seeding densities. Practical experience indicated that MCTS with a seeding density of 8,000 cells per well showed the best morphology and stable repetition in different wells and different batch.

TABLE 4: Morphological parameters of cell aggregates and MCTS of 4T1-luc with different seeding density.

Initial seeding density (cell number in each well)	Longest diameter (μm)	Shortest diameter (μm)	Diameter (μm)	Volume (mm^3)
500	411.15	319.90	365.52 ± 73.23	27.73 ± 13.05
750	475.67	418.24	446.96 ± 35.11	47.38 ± 11.13
1000	543.38	514.05	526.43 ± 30.37	76.85 ± 12.92
2000	595.81	587.67	591.74 ± 5.76	108.45 ± 3.16
3000	680.18	647.61	663.90 ± 23.03	153.41 ± 15.94
6000	757.28	706.00	736.49 ± 53.97	211.33 ± 47.64
8000	792.25	760.97	776.61 ± 22.12	245.42 ± 20.95
10000	884.82	853.48	869.15 ± 22.16	343.94 ± 262.86

FIGURE 9: Kinetic curve of bioluminescence of luciferin-loaded liposomes of HSPC:chol = 70:30 and HSPC:chol:GE11 = 68.6:29.4:2 added to MCTS.

Except for 4T1-luc, the other three cell lines have been studied for building models of MCTS with the method of loading agarose on the bottom of wells. Cells of H1299-pGL3 could only form loose aggregates that could not bear pipetting. Cells of SMMC-7721-pGL3 and A549-luc could not form aggregates with this method.

In Figure 9, the kinetic curve of bioluminescence of liposomes modified with GE11 was totally above the curve of the control group without modification. It was consistent with the fact that the modification with GE11, a new peptide ligand, promoted the delivery of nanoparticles to cells that had epidermal growth factor receptor (EGFR) [9, 12].

3.5. Assays with Tumor-Bearing Nude Mice. Figure 10 shows the kinetic curve of bioluminescence on the tumor area captured by the in vivo imaging system after tail vein injection of free or liposomal D-luciferin. The bioluminescence of liposomes of HSPC:chol = 70:30 peaked to a photon number of 4.14×10^7 at 41 min. Compared to free D-luciferin, the kinetic curve of the liposomes shows a certain degree of sustained releasing, which could be explained by the barrier effect of the liposomal membranes to D-luciferin.

FIGURE 10: Kinetic detection of bioluminescence of the tumor region in tumor-bearing nude mice following tail vein injection of luciferin-loaded liposomes and free D-luciferin. (The volume of the subcutaneous tumor of 4T1-luc was $0.440 \pm 0.022 \text{ cm}^3$. Each mouse was injected with either liposomal or free D-luciferin with dosage of 0.2 mg/g body weight. The exposure time of the in vivo image system was set at 1 min.)

Figure 11 shows the kinetic curve of bioluminescence of the tumor region in tumor-bearing nude mice after intratumoral injection of luciferin-loaded liposomes (EPC:chol = 70:30). Compared with free D-luciferin, the kinetic curve of liposomes shows a sustained releasing effect.

4. Conclusion

Focused on the dynamic uptake of nanostructures for drug delivery based on bioluminescence measurements, this study included three parts. The first part was the preparation of liposomes to encapsulate D-luciferin, which served as a model drug. The distribution and the active loading of D-luciferin with pH gradient have been studied. Based on the theoretical calculation, the feasibility was demonstrated by experiments. The second part of this study was building

FIGURE 11: Kinetic detection of bioluminescence of the tumor region in tumor-bearing nude mice following intratumoral injection of luciferin-loaded liposomes and free D-luciferin. (The volume of the subcutaneous tumor of 4T1-luc was $0.440 \pm 0.022 \text{ cm}^3$. Each mouse was injected with either liposomal or free D-luciferin with dosage of 0.1 mg/g body weight. The exposure time of the in vivo image system was set to 4 min.)

three-dimensional models to bridge tumor cells in vitro to tumor models in vivo. The third part of this study focused on kinetic uptake on free drugs and drug-loaded nanostructures in cell suspension, monolayer cells, MCTS and tumor-bearing mice. This paper laid a solid foundation for establishing a technology platform for tracking and analyzing the dynamic uptake of luciferin and luciferin-loaded nanoparticles.

5. Prospective

This study offered a technology platform for studying the dynamic uptake of luciferin and luciferin-loaded nanostructures in vitro and in vivo.

First of all, scientists have synthesized dozens of derivatives of D-luciferin and D-aminoluciferin [2, 14, 15, 21, 22]. Due to the various groups modified to the core structure, these derivatives could have vastly different characteristics on molecular weight, solubility, hydrophobicity, hydrophilicity, and so on. The differences on their characteristics make them quite suitable to serve as model drugs for small molecules. By modifying D-luciferin with grand organs like peptides or polysaccharide, Mäger et al. have tried to study the uptake mechanism of small molecules and to identify the effect of cell penetrating peptides on cellular endocytosis [22]. Besides, radioactively labeled D-luciferin is also popular for studying the mechanism of cellular uptake [23].

Except for liposomes, the development of other novel nanostructures has attracted more and more scientists. Li et al. have tried to prepare nanostructures by caging luciferin and to apply ultrasound to promote the release of caged luciferin [24, 25].

Targeting modification has been applied to the preparation of nanostructures for loading D-luciferin. The nanostructures prepared in this study could also be applied to screen membrane-targeting peptides and cell penetrating peptide. Luciferin-loaded nanostructures might also play a role in explaining their mechanism on promoting membrane targeting and cell penetrating [20]. In order to increase the mechanical strength of MCTS and to maintain their morphology during pipetting, combination of Matrigel, centrifuge tube culture, cell suspension, and agarose-coating was expected [17, 18].

Building postoperation models for the study of mutual reaction between nanostructures and tumor stroma is expected to be a research hotspot. Thermal therapy and the digestion of collagenase to collagen fibers are two good tools. The luciferin-loaded nanostructures in this study have great potential for studying the difference of nanostructures and tumor stroma after operation, thermal therapy, or treatment with collagenase [26, 27].

Nanostructures that load two or more kinds of drugs are shown to be a novel direction. These findings are expected to have a great potential in therapies of drug combinations. The extension of this study also includes the fluorescence modification of nanostructures encapsulating D-luciferin or D-aminoluciferin. By detecting both fluorescence and bioluminescence, further study of their dynamic uptake and distribution with models both in vitro and in vivo is expected. The tracing of these dual labeled nanostructures may yield more interesting results in the near future [28–30].

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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