行政院原子能委員會

委託研究計畫研究報告

轉移性腫瘤動物模型建立及體內放射治療應用研究

Establishment of metastatic animal tumor models and their application in internal radiotherapy

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目　錄

中文摘要

微脂質體通過一個所謂"增強通透性和保留(Enhanced Permeation Retention, EPR)"的過程而達到藥物累積至腫瘤的優勢，脂質體作為化療藥物的輸送系統較易分布於具滲漏性的腫瘤相關血管，如此可改善常規化療藥物的藥理學特性。核研所發展之脂質體包覆銖-188 藥物(Re^{188}-liposome)已在皮下及肺部轉移之大腸癌動物模型中顯現療效。一個探索性新藥研究以評估脂質體包覆銖-188 體內分佈、藥物動力學及安全性藥物的人體臨床試驗近日將開始招募病人。為探索脂質體包覆銖-188 藥物抗癌作用以發揮這種新治療的最大潛力，我們持續建立各種腫瘤模型 以協助測試脂質體包覆銖-188 藥物療效。此外，我們持續進行改良脂質體包覆銖-188 藥物(Re^{188}-liposome) 的工作，我們成功製備了上皮細胞生長素標記的 EGF-LipoDox，並已證實其對許多上皮細胞生長素受體過度表達的腫瘤細胞有較佳的殺傷力，EGF-LipoDox 相較於 LipoDox 並沒有更明顯的毒性，我們正嘗試純化更多改良的 EGF 重組蛋白,希望新的 EGF 重組蛋白可具有對上皮細胞生長素受體具更佳的結合能力。

關鍵字: 微脂體; 銖-188;上皮細胞生長素; 上皮細胞生長素受體

Abstract

Through a process so-called "enhanced permeation retention" (EPR), liposome is more easily distributed into leaky tumor-associated blood vessels, leading to preferable accumulation within tumor microenvironment, thereby improving pharmacological properties of conventional chemotherapy, such as pharmacokinetic and biodistribution. Re^{188}-liposome developed by Institute of Nuclear Energy Research (INER) had shown significant efficacy in subcutaneous and lung metastasis of colorectal cancer in animal models. An exploratory clinical trial for evaluation of biodistribution, pharmacokinetics and safety of Re^{188}-liposome will start recruiting patients shortly. To demonstrate the greatest potential of this new treatment, we continuously explore the potential indications of Re^{188}-liposome by establishing various animal tumor models for efficacy studies. Additionally, we are improving Re^{188}-liposome by inserting epidermal growth factor (EGF) to a liposomal drug, LipoDox. The resultant EGF-LipoDox displayed a superior killing effect on EGF receptor-overexpressing cancer cells. In preliminary animal study, EGF-LipoDox did not show increased toxicity as compared with LipoDox. We are working on several modified recombinant EGF

proteins which will be incorporated into liposomal drug with better affinity to EGFR.

Key words: liposome; Re^{188}; epidermal growth factor (EGF); epidermal growth factor receptor (EGFR)

壹、計畫緣起與目的

Nanoscale liposomes as drug delivery systems containing chemotherapy drugs have been widely used for treatment of cancer (1, 2). Many of the pharmacological properties of conventional chemotherapy drugs can be improved using this drug delivery system, which composed primarily of lipids and/or polymers. These novel therapeutic complex are designed to improve the pharmacokinetics (PK) and biodistribution (BD) of the coupled chemotherapy drugs (3). As compared with conventional chemotherapy, circulation of liposome coupled chemodrugs could be prolonged. Moreover, the liposome coupled drugs could be redirected to relatively leaky tumor-associated blood vessels, leading to superior accumulation in tumors via a process often referred to as the "enhanced permeability and retention" (EPR) effect (4, 5).

Although liposomal doxorubicin displayed superior localization of doxorubicin in relatively leaky tumor microenvironment, killing of tumor cells required release of this chemodrug and the coupling to its target, DNA. To take advantage of the EPR effect of liposomal drug and the cytotoxic effect of radiation even in the absence of internalization of

liposome by cancer cells, Institute of Nuclear Energy Research (INER) had successfully developed a liposomal therapeutics, ^{188}Re-BMEDA-labelled pegylated liposome (^{188}Re-liposome), and examined its biodistribution, pharmacokinetics and cytotoxic effects, compared with unencapsulated ^{188}Re-BMEDA control in a subcutaneous murine C26-colon tumor model (6, 7). Series of preclinical efficacy and toxicity studies had shown encouraging results and investigation of new drug (IND) is currently under preparation.

The goals of this proposal are to broaden the indications of ^{188}Re-liposome for future clinical trials and to generate an active targeting liposomal drug specific for EGFR-expressing cancer cells. Most of the data generated from INER support a potential role of ^{188}Re-liposome in the treatment of metastatic colon cancer. This project is aiming to establish various animal models related to human clinical condition, such as orthotopic renal cancer and liver metastasis of colon cancer, etc. Our previous experiment had demonstrated significant cytotoxic effect of EGF-LipoDox, which is 10-100 folds more potent than LipoDox for killing of EGFR-overexpressing tumor cells, such as A431, BT474, and MDA-468, etc. EGF-LipoDox is internalized into EGFR-overexpressing BT474 to a much greater extent as compared

with LipoDox in the absence of EGF-tag. Based on the preliminary success of EGF-LipoDox, we continue working on cloning, expression and purification of various modified forms of EGF for better coupling of liposomal drug with enhanced affinity to EGFR.

The rationale for choosing EGF as the targeting agent is based on the realization of the important role of EGF-EGFR signaling pathway in cancer development (8-10). In the recent 20 years, antagonists targeting this pathway have been developed for cancer treatment. Two monoclonal antibodies, Cetuximab and panitumumab, and two small molecules, genitinib and erlotinib, had been approved for cancer treatment (11, 12). The goal of this proposal is to improve a therapeutic efficacy of a widely used chemodrug, LipoDox by (1) addition of radioisotope, Re^{188}, to this agent, and (2) making it more specific to EGFR expressing tumor cells. Additionally, Immunoliposome, in which tumor targeting peptide or monoclonal antibody (mAb) or fragments are conjugated to liposomes, represent the next generation of molecularly targeted drug delivery systems (13-15). By combining the tumor targeting properties of peptide or mAbs with the pharmacokinetics and drug delivery advantages of liposomes,

immunoliposomes offer the promise of selective drug delivery to tumor cells, thereby enhancing the cytotoxic effect toward tumor cells, while sparing normal tissues (16, 17). Additionally, radiation combined with chemotherapy could significantly increase the cytotoxic effect against tumor cells. Therefore, immunoliposome coupled with chemodrug and radioisotope should precisely deliver both treatment modalities of chemotherapy and radiation to the tumor site.

貳、研究方法與過程

(1) Colon cancer with liver metastasis model: CT26 stably expressing luciferase gene (CT26-luc) will be cultured in RPMI 1640 with 10% fetal bovine serum (FBS). The cells will be harvested with trypsin–EDTA, washed twice, and resuspended in serum free RPMI. Female BALB/c mice aged 5-7 weeks will be used for establishment of colon cancer liver metastasis model. The mice will be anesthetized by intraperitoneal injection of 80 mg/kg Ketamine and 10 mg/kg Xylazine. A small left subcostal incision will be carried out in these mice under anesthesia and $1x10^5$ CT26-luc in 50 l of phosphate-buffer ed saline (PBS) will be injected into the spleen. After 3 to 5 min, the spleen vessels will be ligated and the spleen will be surgically removed. Incidence of liver metastasis will be examined by autopsy on days 15 and 30 after tumor cell inoculation. The livers will be removed and weighed, and the number of visible tumor nodules on the liver will be counted. Liver samples will be fixed overnight in AFA (5% acetic acid, 75% ethyl alcohol, 2% formalin, 18% water), transferred to 100% ethanol, and embedded in paraffin, and 5- m sections will be prepared. The number of metastatic nodules will be quantified on the liver

sections.

(2) Orthotopic renal cancer with spontaneous lung metastasis: Renca-luc cells (1×10^5 stable Renca cells expressing luciferase) will be mixed with Matrigel and orthotopically injected into the subcapsular space of the left kidney of BALB/c female mice. Similar to the orthotopic liver cancer model, light pressure will be applied for 1 min using a cotton swab placed on the injection site to prevent bleeding after injection. The surgical wounds of the mice will be closed with a 6-0 silk suture. In vivo tumor growth will be monitored as described previously using bioluminescence technology. The lungs of experimental mice will be removed and weighed, and the number of visible tumor nodules on the lung will be counted. Moreover, lung samples will be fixed and embedded in paraffin and previously described. The 5- m sections will be prepared and stained with HE, followed by quantification of number of metastatic nodules under microscope.

(3) Cloning, expression and purification of hEGF-his_6 with various number of Lysine at N-terminus: We had adopted PCR method using oligonucleotides encoding from three to six multiple Lysine residues to build in potential pegylation site over N-terminus of hEGF. The

resultant yeast expression pPICZ-α constructs were transformed into wild-type X-33 *Pichia pastoris*, plated on zeocin-containing (200 μg/mL) agar plates and incubated for 3-4 days until appearance of colonies. Individual colonies were screened for high expression of individual proteins detected with antibody against c-myc. For large-scale expression, 0.5 liter of BMD medium in shake flasks was inoculated with the selected colony and grown up to an OD_{600} of 8-10 and protein expression was induced with daily addition of methanol. Three days after induction, the protein-containing culture medium was then collected and subjected to filtering before being loaded onto a nickel-resin column (Qiagen). The column was washed with 10 column volumes of PBS buffer containing 5 mM imidazole, and the bound proteins were eluted with increasing concentration of imidazole using an ÄKTAprime plus purification system.

(4) Preparation of EGF-LipoDox: The process adopted for generation of EGF-LipoDox was previously described by Chen etl al. (7). For the synthesis of hEGF-his6-PEG–DSPE molecule, hEGF-his6 was dissolved in PBS–EDTA and mixed at 1:1.2 molar ratio with *N*-succinimidyl 3-(2-pyridyldithio) propionate dissolved in DMSO (18).

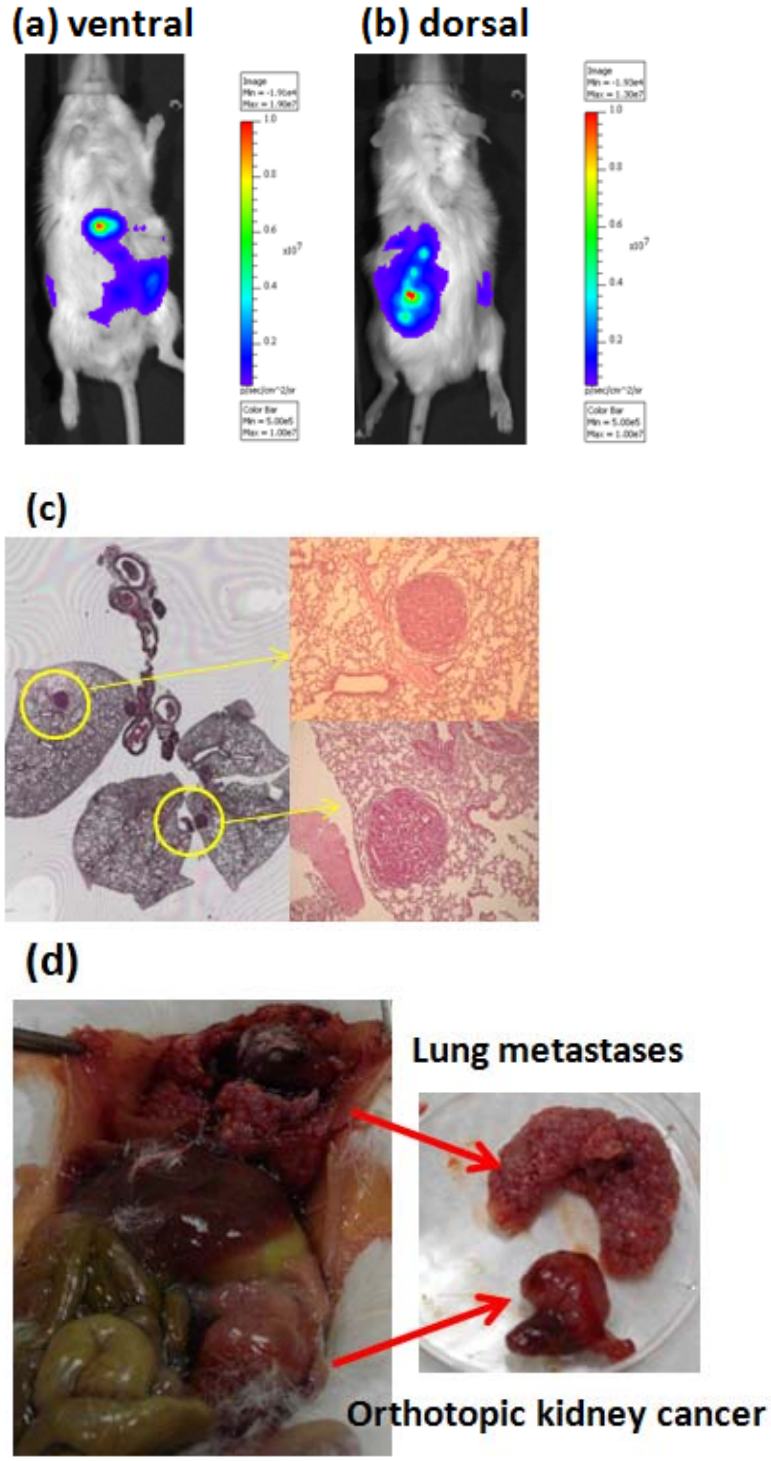
After 1 h in room temperature, the mixture will be lyophilized, and dissolved in the solution containing tris(2-carboxyethyl) phosphine under nitrogen to expose the –SH group. The thiolated protein or peptide will be then added to the MAL–PEG2000–DSPE micelle solution at 5:1 molar ratio while maintaining mixing under nitrogen at 10 ◦C overnight. HPLC analysis will be used to confirm that most of MAL–PEG–DSPE molecules were conjugated with the hEGF-his6 after such reactions. Ligand-conjugated lipids will be transferred into preformed 188Re-Dox-liposome based on the procedure developed by Ishida et al. (19) with minor modifications. hEGF-his6–PEG–DSPE solution will be added into the preformed liposome solution at 9:100 molar ratio and incubated at 50 ◦C for 30 min. The solutions will be then dialyzed against PBS for 4 h to remove unconjugated hEGF-his6. Non-targeted liposomes were prepared similarly by substituting hEGF-his6-conjugated lipid with mPEG2000–DSPE.

(5) Detection of doxorubicin uptake by cancer cells: EGFR-overexpressing MDA-MB-468 and EGFR low expresser, MDA-MB-231, cancer cells were plated in 6-well plate ($5x10^5$ cells/well) one day before experiment. The cells were incubated with 10mM of either LipoDox or EGF-LipoDox at 37 ^oC for 30 minutes. The cells

were later washed 3 times with PBS followed by lysis. The amounts of doxorubicin were detected and quantified using the intrinsic fluorescence of doxorubicin (excitation wavelength, 480 nm; emission wavelength, 580 nm).

參、主要發現與結論

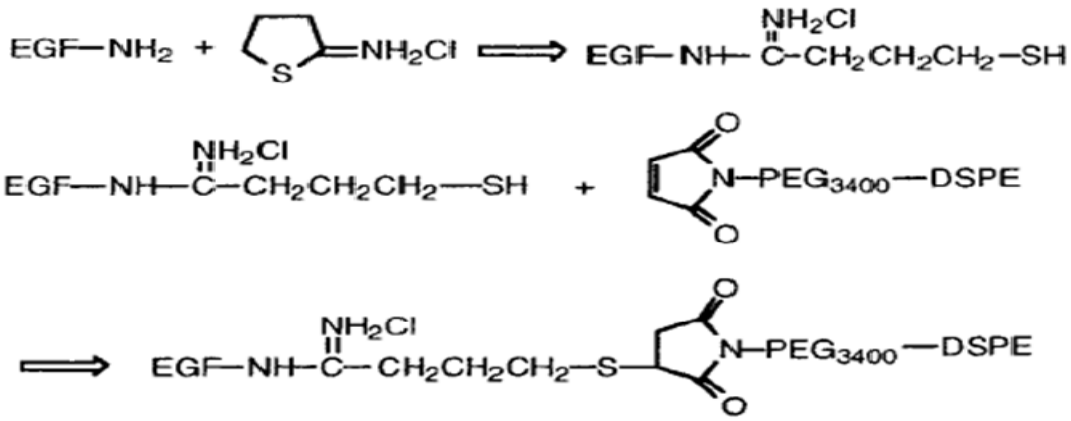
(1)



(Fig. 1) Model of orthotopic renal cell carcinoma and spontaneous lung

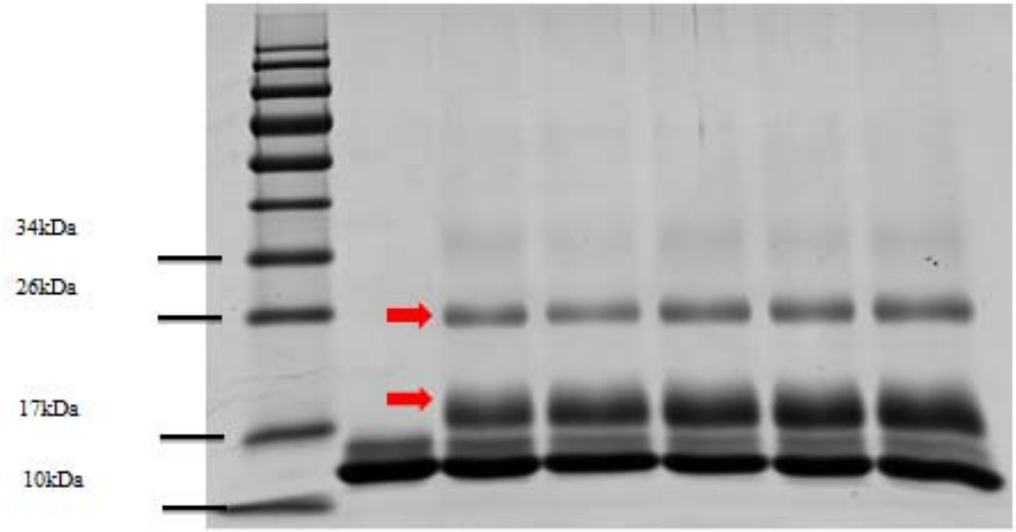
metastasis models (a & b) Ventral and dorsal views of bioluminescent intensity resulted from activities of luciferase stably expressed by murine renal cancer cells, RENCA, which were orthotopically inoculated under kidney capsule. (c) Microscopic pictures of spontaneous lung metastases from orthotopic renal cell cancer. (d) Gross view of a experimental mouse dissected five weeks after orthotopic inoculation of RENCA (10^5 cells/mouse).

(2)



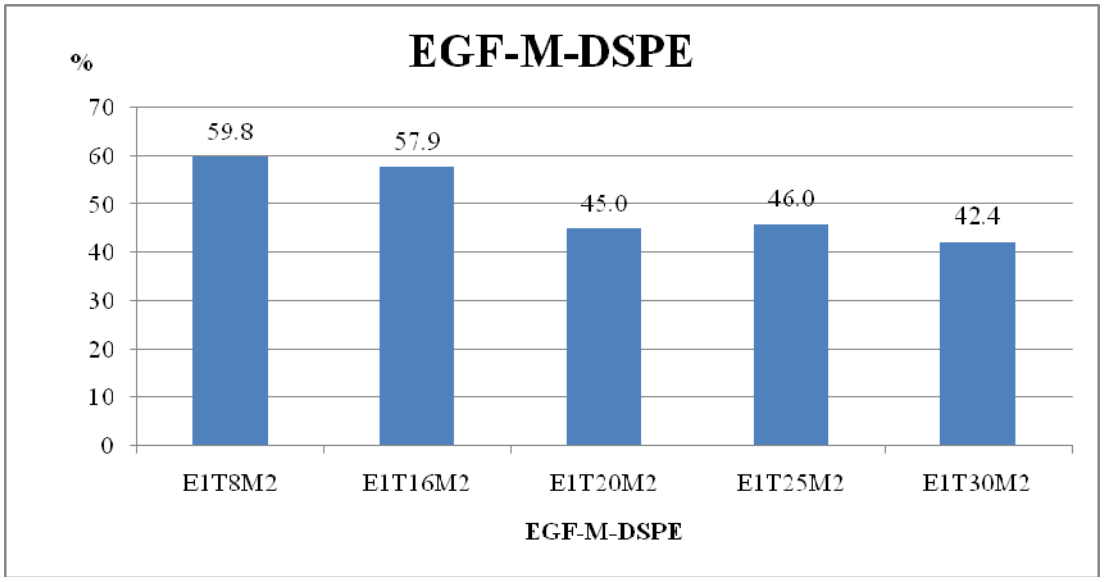
(Fig.2) Human EGF was coupled with Traut's reagent to form hEGF-Traut followed by attachment to Maleimide-PEG-DSPE (M-DSPE) and became Maleimide-PEG-DSPE (EGF-M-DSPE)

(3)



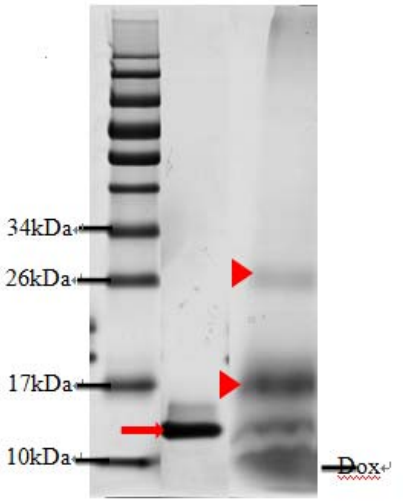
(Fig.3) SDS-PAGE gel of EGF-M-DSPE: Shown are the resultant products with different ratios of EGF:Trout:M-DSPE (lanes from left to right: Marker、EGF、$E_1T_8M_2$、$E_1T_{16}M_2$、$E_1T_{20}M_2$、$E_1T_{25}M_2$、$E_1T_{30}M_2$)

(4)



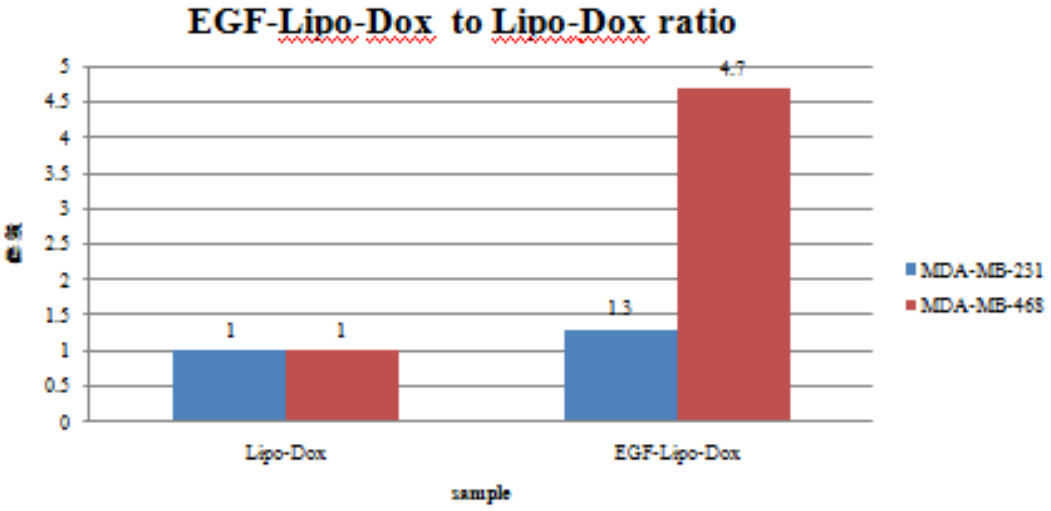
(Fig. 4) SDS-PAGE analysis and quantitation of EGF-M-DSPE under different ratios of EGF and Traut's reagent.

(5)



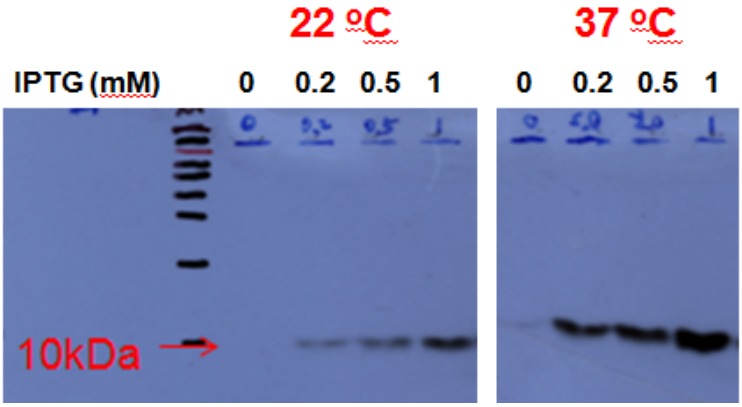
(Fig. 5) SDS-PAGE gel analysis of amount of EGF in EGF-LipoDox. From left to right: Marker、EGF、EGF-Lipo-Dox. Arrow head represents EGF-Lipo-Dox

(6)



(Fig. 6) Uptake of doxorubicin by MDA-MB-468 and MDA-MB-231: The cells were inducted with either EGF-LIpoDox or LipoDox and the amounts of doxorubicin were measured at emission wavelength of 580 nm after the samples were excited with wavelength of 480 nm.

(7)



(Fig. 7) Expression condition of human EGF with multiple Lys residues: The E. coli. transformed with construct encoding –K_3hEGF were incubated at 22 or 37 ^{o}C and protein productions were induced with increasing concentrations of IPTG (from 0 -1 mM)

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