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碩士論文

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兼具熱敏感性及標靶功能之藥物載體在癌症治療及生
醫影像之應用

Thermo-sensitive, targeted delivery nanoshuttle with
dual-function in improved chemotherapy and MRI
imaging

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中文摘要



多數的抗癌藥物，由於其化學結構之疏水性導致生物體的吸收不佳，造成患者在接受癌症化學治療時，藥物達到病灶的有效劑量偏低。為了使累積在病灶處的藥物劑量具實際療效，目前臨床醫師的做法是讓病患提高服用藥物的濃度，但此舉卻造成不可避免的化療副作用。近年來，眾多研究都希望能夠利用藥物傳遞系統的設計，發展出多功能、智慧型的奈米藥物載體，其目的即在解決現今臨床醫學所遇到的上述難題。

有鑑於此，本研究的動機與目的即在設計出以蓖麻油及 12-羥基硬脂酸為主成份，利用自組裝方式，形成具生物相容性的膠態三維纖維結構，並以磷脂質修飾構成穩定的膠體溶液系統。該奈米三維纖維結構可同時包覆抗癌藥物(喜樹鹼)及磁性氧化鐵奈米粒子於其中，形成熱敏感性的藥物載體；若對其施以高頻變化的磁場，可使磁性氧化鐵奈米粒子產生磁熱效應釋放出熱能使局部溫度上升，當溫度達到 44 °C 時，此熱敏感載體會產生相轉變成溶膠態，即可快速並有效地釋放出藥物。此外，由於溫熱療法對癌細胞的殺傷力較正常細胞大，故此熱敏感載體亦可同時抑制癌細胞生長的效率。

本研究亦對該熱敏感載體進行相關定性分析；以動態光散射儀測得其粒徑大小約 260 nm，界面電位為 -55 mV，相變溫度為 44°C，輔助磁場 33.9 kA/m 及頻率 33.9 kHz 條件下其損耗功率值為 369 W/g_{Fe}；此外，該載體亦具磁振造影 T2 顯影劑之功能。初步 *in vitro* 實驗證實，此熱敏感載體不具生物毒性，且表面修飾具有標靶特性的分子(如葉酸)能成功的對葉酸過量表現的癌細胞(HeLa)進行選擇性的靶向傳遞。此熱敏感載體應可在 *in vivo* 模式中被再次證實其化療及溫熱治療之協同效應。

關鍵詞：藥物傳遞系統、靶向傳遞系統、熱敏感性藥物載體、膠態三維纖維結構、磁場誘導磁熱效應、溫熱療法、磷脂質



Abstract



More than 50% of anti-cancer drugs obtained directly from synthesis have poor water solubility. Loading of the hydrophobic drug into nanocarriers, followed by delivery of the carriers to the site of desired action and subsequent release of the encapsulated drugs *in situ* is one common approach to circumvent the aforementioned problem. Passive targeted delivery system keeps circulating in the blood stream and allows itself to be taken to the target receptor; passive release of drug payload, however, is commonly observed with conventional drug delivery nanosystems, restraining their capability of discharging drugs on an effective concentration at a desired time window. Compared to passive targeting/passive releasing strategy, active targeting (such as receptor-mediated approach)/controllable release mechanism may further enhance the efficacy of drug delivered by nanovehicles.

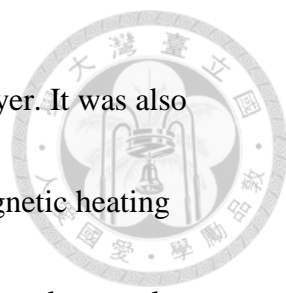
Hyperthermia is one clinical protocol used as co-adjuvant therapy for cancer treatment. A clear synergistic effect was described previously when combined with radiotherapy, as well as increase efficacy of chemotherapy. Magnetic hyperthermia using ferrite nanoparticles has recently emerged as a promising approach for cancer therapy. Magnetic nanoparticles, serving as a therapeutic agent, a heat nanomediator or

a MRI contrast agent, gained significant popularity in recent years due to their superparamagnetic properties.



Taking advantage of the potential benefits of nanotechnology, a biocompatible nanocarrier for controlled releasing entrapped hydrophobic drugs was designed and developed herein. The novel nanocarrier was prepared via nanoemulsion method by sonicating 12-hydroxystearic acid (12-HSA)/castor oil/phospholipid (DPPC, DPPG, DSPE-PEG₂₀₀₀-folic acid) mixture in a phosphate buffered saline solution (PBS), followed by the formation of a nanoshuttle composed of an inner gel core and an outer phospholipid shell. Camptothecin (CPT, selected herein as a model drug) or superparamagnetic iron oxide nanoparticles (SPIONs, a heat generator), could be loaded either individually or concurrently into the core of the nanoshuttle by dissolving either/both hydrophobic CPT or/and oleic acid-coated SPIONs in the oil phase before nanoemulsion.

Results showed that the as-prepared nanoshuttles were stable up to 7 days with an average hydrodynamic of 260 nm, surface charge of -55 mV and a phase transition temperature of 44°C. Transmission electron microscopic analyses revealed that our (phospholipid-gel-SPIONs)PLG-SPION nanoshuttles contained multiple 10-nm-sized



SPIONs encapsulated in gel network, surrounded by phospholipid layer. It was also confirmed that the PLG-SPIONs nanoshuttles exhibited superior magnetic heating ability with specific loss power (SLP) value 369 W/g_{Fe}. Cytotoxicity study was also carried out to verify the biocompatibility of the PLG-SPIONs nanoshuttles. In addition, our PLG-CPT/SPIONs nanoshuttles (both CPT and SPIONs were loaded into PLG particles) demonstrated excellent efficacy in inhibiting the proliferation of HeLa cells. Taken all together, our PLG-CPT/SPIONs nanoshuttles hold the great potential for the development of innovative biomedical applications such as targeted drug delivery, tumor heating and *in vivo* contrast agents, and eventual expansion into possible alternative approach for cancer treatment.

Key word: thermo-sensitive nanocarrier, targeted drug delivery, phospholipid-capped nanosystem, AC magnetic hyperthermia

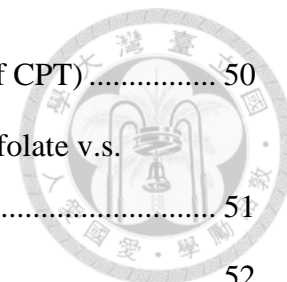
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第一章 緒論

根據行政院衛生署至民國 100 年的資料統計顯示，國人的第一大死因乃癌症所致¹。儘管快速的醫療發展下，始終無單一治療癌症的方法。臨床上，患者需同時接受外科手術以及化學藥物治療或放射治療…等等才能有效的控制病情。化學藥物治療所遇到的難題是，多半抗癌藥物的水溶性不佳、抵達病灶處的濃度隨時間衰減且比例低於 3% (Figure 1²)，所以需要連續服藥達才可達到有效劑量範圍。^{3,4} 因此，若能提高化療藥物的藥效，必定能減輕患者的身體負擔，並降低副作用的發生。

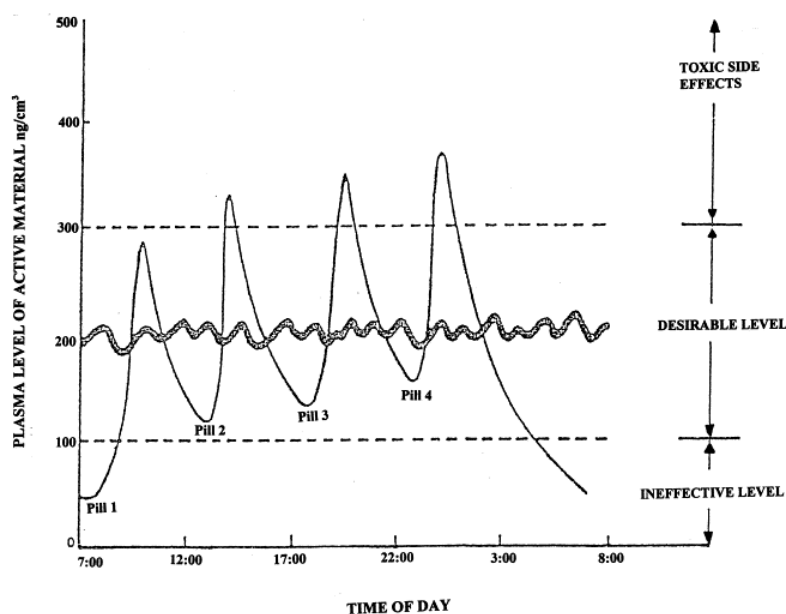


Figure 1²：血漿中傳統口服藥物隨時間測得藥劑濃度變化圖

1986 年兩位日本學者 Yasuhiro Matsumura 及 Hiroshi Maeda 證實，在硬塊腫瘤組織中，癌細胞為了得到養分出現異常的血管新生，其血管壁表皮細胞間的孔隙 (~400 nm) 較正常血管壁大，使得通透性上升；且腫瘤組織周圍缺乏正常組織所具

有的淋巴系統，其清除外來物的能力下降，以上種種現象稱之為增強通透性和保留效應 (enhanced permeability and retention, EPR effect)(Figure 2⁵)^{5,6}。在發現 EPR effect 後，有更多研究團隊致力發展出小於 200 nm 的藥物載體，並結合靶向藥物傳輸系統及控制釋放系統…等等，以降低藥物的副作用及提高藥物到病灶處之比例(Figure 3⁷、Figure 4⁸)。⁷⁻¹¹

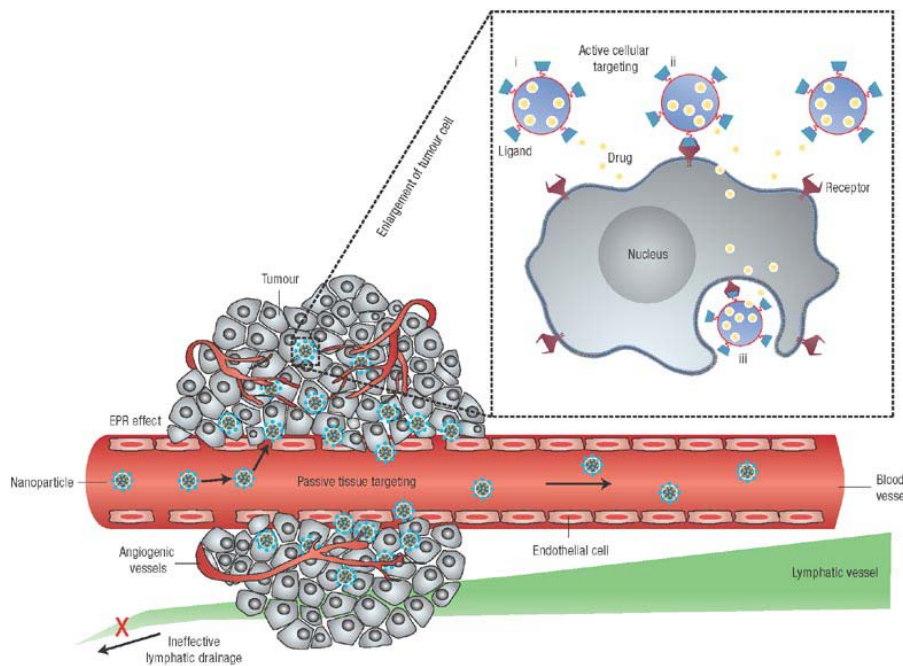


Figure 2⁵：增強通透性和保留效應

除了發展多功能的藥物載體，如何搭配非侵入性的輔助工具(例如：光、微波、電磁場…等等)以增強癌症治療效果也逐漸受到重視。溫熱治療(hyperthermia therapy)是被廣泛研究的選項之一，因為癌細胞對溫度的耐受性較正常細胞低，42 – 45 °C 的溫熱即可促使癌細胞凋亡。¹² 而熱能的提供，以電磁感應生熱(AC magnetic hyperthermia)最被廣泛應用，因為沒有穿透組織的深度限制，所以病灶處若有磁性奈米顆粒的存在，外部施予高頻率電磁場後，只會在局部產熱，並能控制在溫熱治療的溫度範圍。

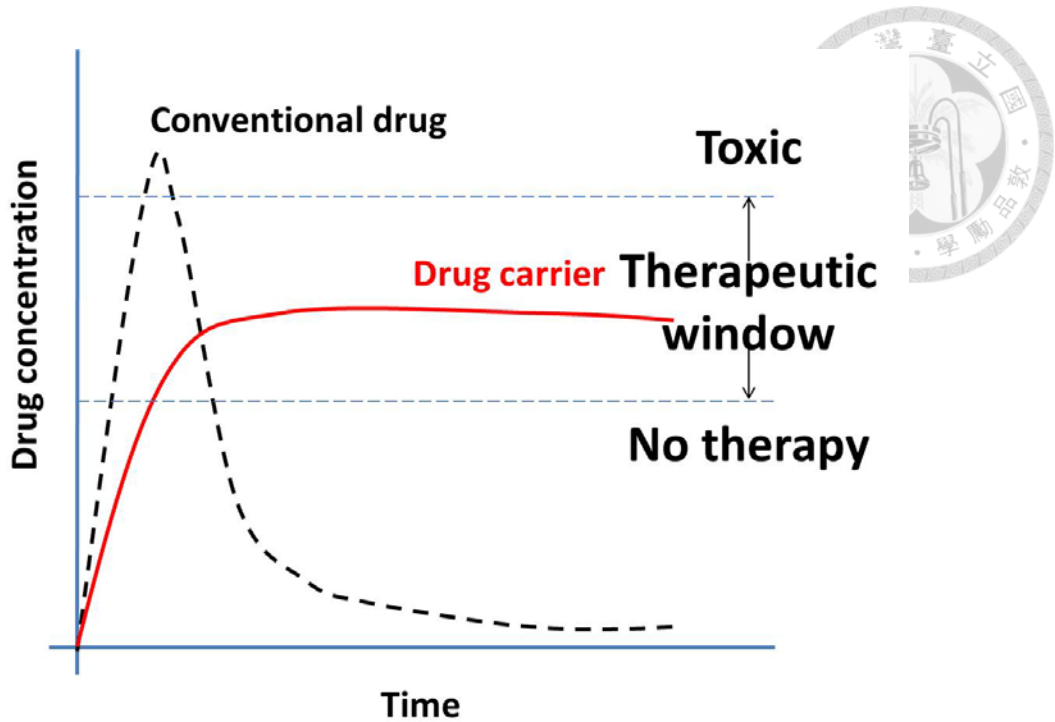


Figure 3⁷: 傳統藥物與藥物載體其藥物釋放對隨時間變化圖 (由參考文獻 7 做修改)

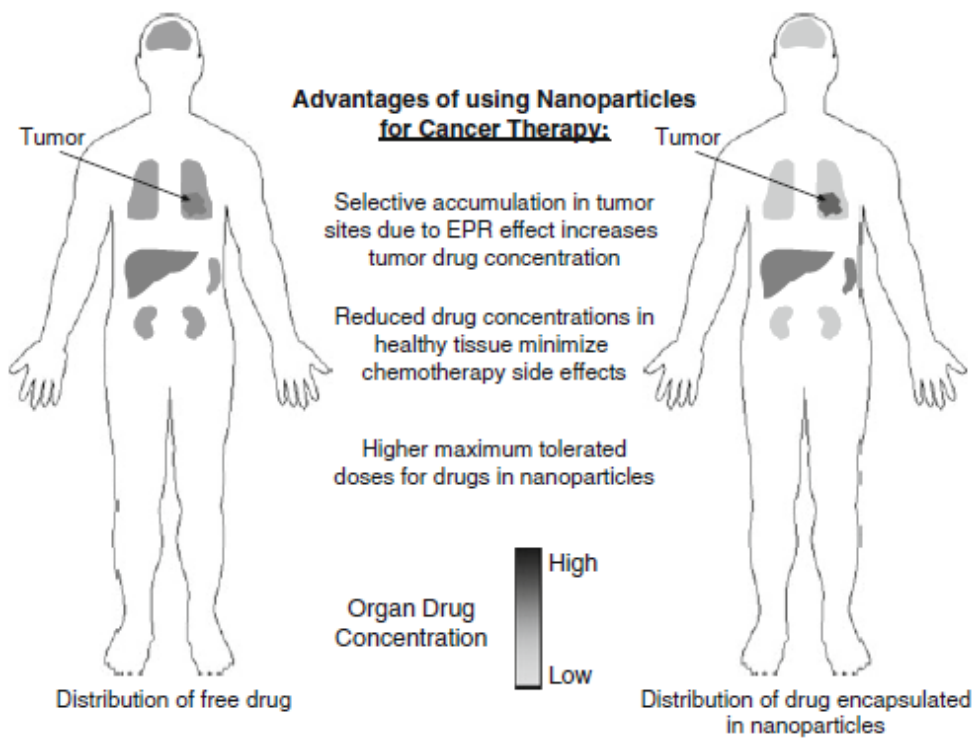



Figure 4⁸: 藥物載體與一般藥物在體內累積量比較圖



基於此，本研究的動機與目的即在解決抗癌藥物的疏水性結構導致水溶性差，造成口服或靜脈注射達病灶處的藥劑量很低，為了達到有效劑量，短時間內連續的服藥隨之伴隨著高副作用的發生；為了強化疏水性的抗癌藥物(camptothecin, CPT)之功效，將抗癌藥物包埋在疏水性膠態(hydrophobic gel core)的熱敏感藥物載體(thermo-sensitive nanoshuttles)內，並於載體表面上修飾葉酸(DSPE-PEG₂₀₀₀-folate)分子當作標靶分子，再利用包埋超順磁氧化鐵奈米粒子(superparamagnetic nanoparticles, SPIONs) 於疏水性膠態艙室內，當作啟動電磁生熱之開關形成 phospholipid-gel-superparamagnetic nanoparticles nanoshuttles (PLG-CPT/SPIONs nanoshuttles)，不但讓熱敏感藥物載體受熱並釋放藥物之化學藥物治療，同時，電磁生熱所提供之熱能也落在溫熱治療之溫度範圍，以協同效應增之方式強化毒殺癌細胞之效果，其研究設計與解決方案架構圖如 Figure 5。

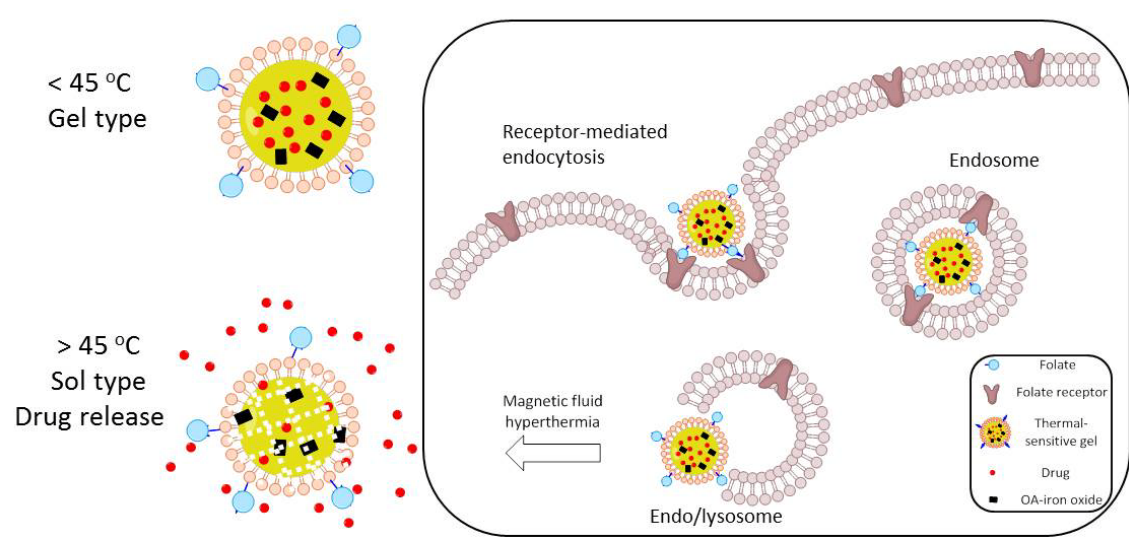
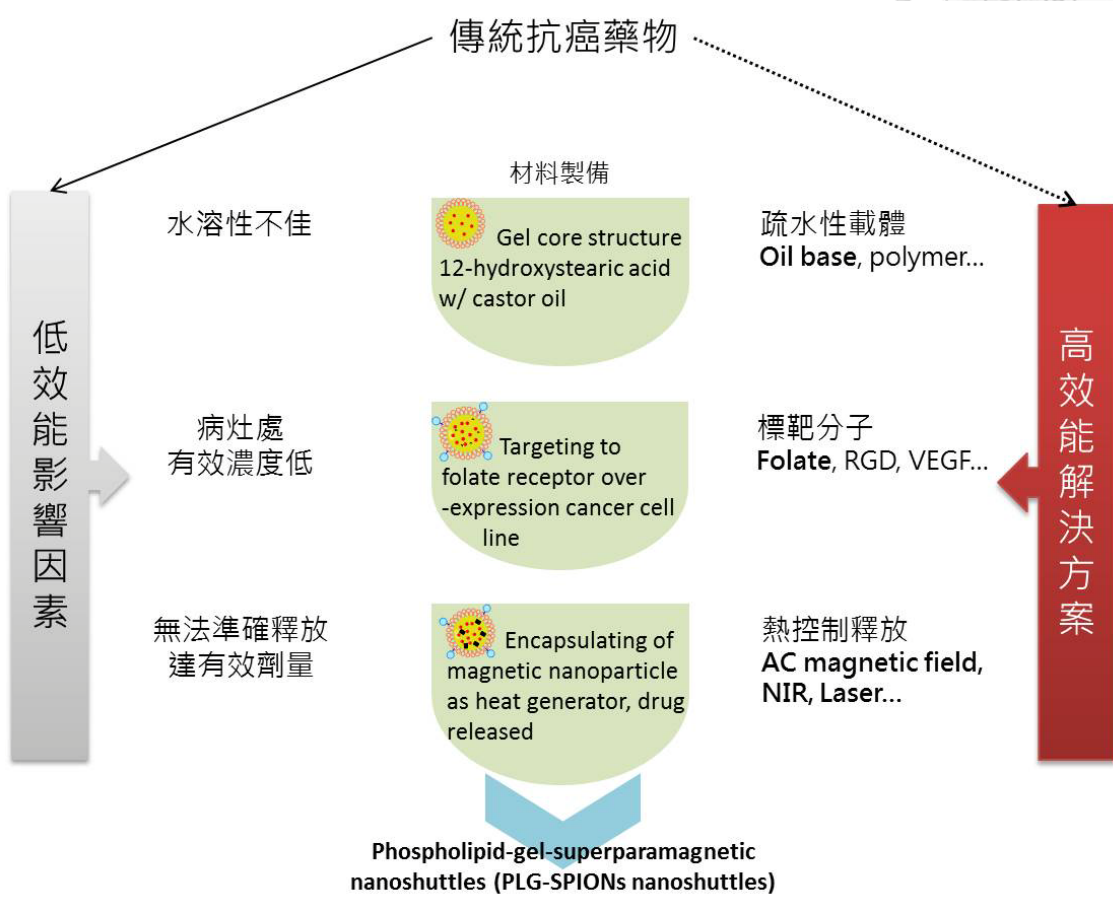


Figure 5 : 研究設計與解決方案架構圖

第二章 文獻回顧



為了強化傳統抗癌藥物的藥效，研究文獻上所使用的解決方法及材料於本章做簡單的介紹。

2.1 藥物傳遞系統 (Drug delivery system, DDS)

藥物傳遞系統(drug delivery system)係將藥物裝載於奈米藥物載體內，利用主動傳遞(active targeting)或被動傳遞(passive targeting)^{13,14}(Figure 6¹⁴)方式被細胞攝入，藥物包覆在載體內也改變了原本藥物釋放的藥物動力學(absorption, distribution, metabolism, and excretion, ADME)，使得藥效更加長久、有效，且降低副作用之發生。^{8,10,15}

具醫療用途的奈米顆粒，廣義定義其直徑介於 1-1000 nm 之範圍，最早於 1955 年德國研究學者 Jatzkewitz 利用 polymer 共軛藥物以形成奈米載體，1965 年 Bangham 發現微脂體(liposome)結構，接著之後有更多突破性的發展，Figure 7¹⁰ 為藥物奈米載體重要發展年代圖。^{10,16}

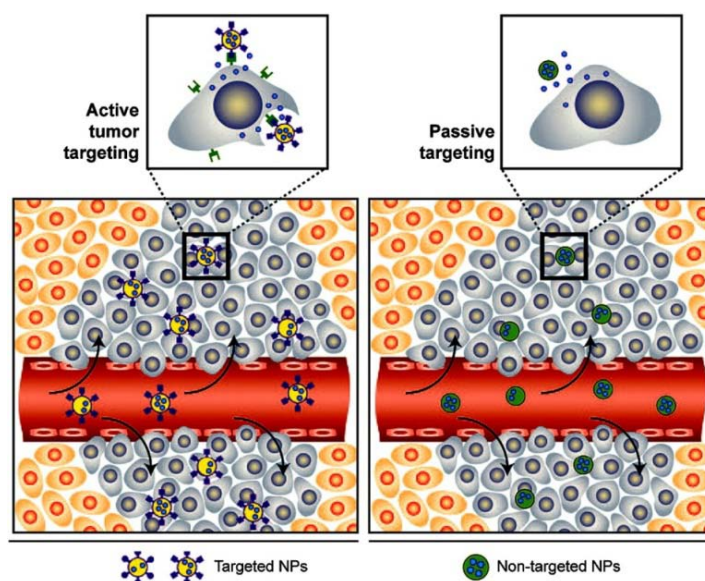


Figure 6¹⁴：藥物傳遞系統以主動或被動傳遞達腫瘤組織之示意圖

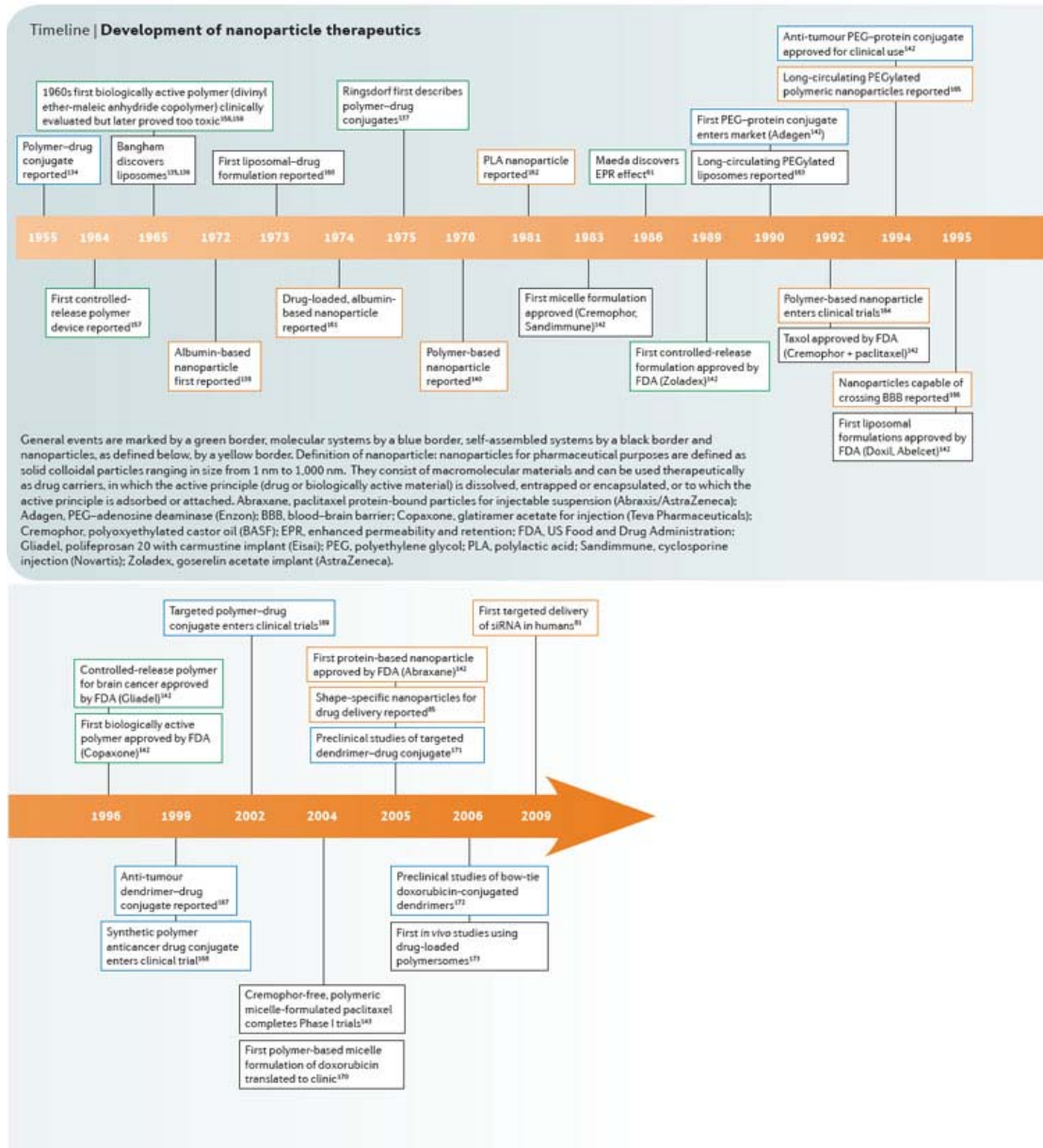


Figure 7¹⁰ : 奈米藥物載體重大發展年代圖



2.2.1 奈米藥物 (Nano-drug, Doxil[®])

由於科學家們發現傳統藥物的缺點後，1960 年代開始，科學家們紛紛致力開發新的藥物載體，並且發展以奈米尺度的材料。^{10,16}

舉例來說，Alberto Gabizon、Yechezkel (Chezy) Barenholz 與 Terry Allen，利用微脂體與包覆抗癌藥物 Doxorubicin(Dox)結合成 liposomal Doxorubicin 命名其載體為 Doxil[®](Figure 8¹⁷)，經過調整磷脂質(phospholipid)配方及比例後，經過多次的細胞及動物實驗數據證明 Doxil[®]優於 free Dox，¹⁷ 1994 年(耶路撒冷，以色列)完成人體臨床試驗後，也證實有顯著降低副作用的發生及增加達病灶處的比例^{7,18}，於 1993、1994 年在以色列提出專利申請，並於 1995 年向美國食品及藥物許可局(FDA)申請為第一個被允許上市的”奈米藥物(nano-drug)”¹⁷。

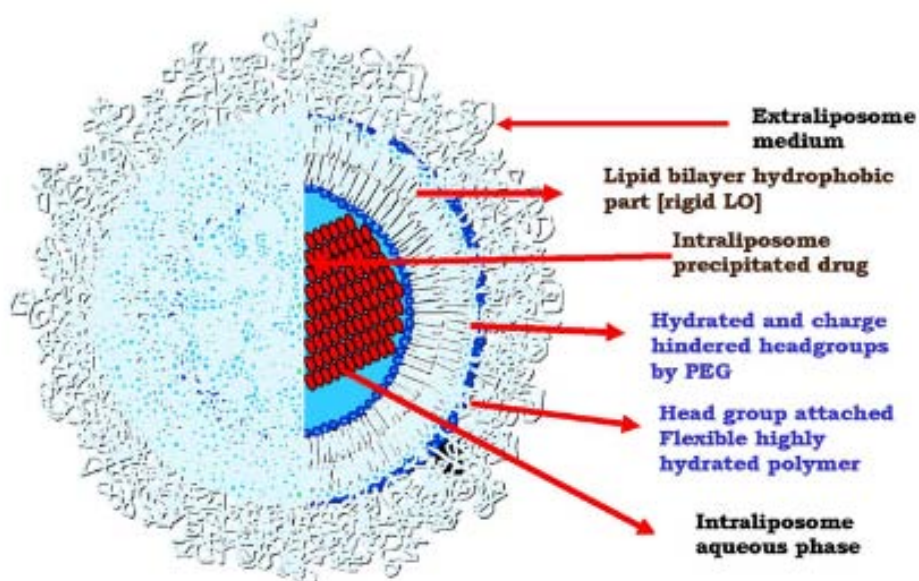


Figure 8¹⁷ : Doxil[®] 示意圖

2.2.2 抗癌藥物-喜樹鹼 (Anticancer drug - camptothecin, CPT)

屬紫樹科(Nyssaceae)的喜樹(*Camptotheca acuminata*)在 1960 年代由三位研究學者，分別從事研究抗癌植物的 Jonathan Hartwell、天然物萃取的 Monroe E. Wall 及化學結構鑑定的 Mansukh C. Wani，共同開啟了研究喜樹鹼之路。¹⁹

喜樹鹼(Figure 9)為五環結構(A 及 B 環屬於 quinoline ring、C 環屬於 pyrrole ring、D 環屬於 lactam ring、E 環屬於 lactone ring)，結構上相當疏水，所以應用在人體靜脈注射勢必做結構上之修飾以增加其水溶性，有研究指出在 20 號碳上的 α -hydroxy 官能基其 20-(S)構型被認為是具活性的構型且 E 環的內酯結構不能形成開環結構，其中在 AB 環上分別做不同修飾的喜樹鹼類似物 Topotecan(Hycamtin, GlaxoSmithKline)及 Irinotecan(Camptosar, Pfizer; Campto, Yakult Honsha)為最成功的例子，同時也是被美國 FDA 允許上市之抗癌藥物。²⁰

1985 年 Leroy F. Liu 研究團隊發現 CPT 的抗癌機制作用於細胞週期的 S phase，抑制 DNA topoisomerase I 之功能，使之形成 CPT-DNA-Topo I ternary 結構讓 DNA 無法繼續被複製，最後使細胞死亡，常用於治療卵巢癌、小細胞肺癌及直腸結腸癌。²¹

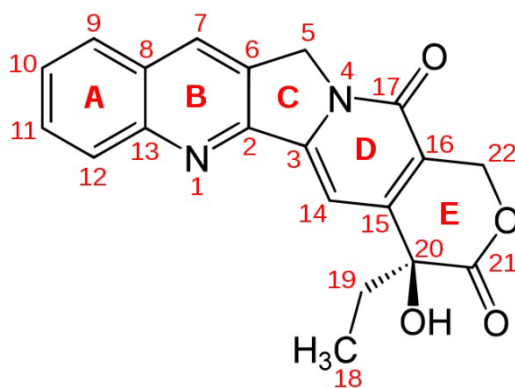


Figure 9 : Camptothecin 化學結構



2.2 靶向藥物傳遞系統 (Target drug delivery system)

雖然 Doxil[®] 成功運用藥物傳遞系統也改變了當時使用抗癌藥物治療的新思維，但仍有不足之處；此時，標靶治療的概念也紛紛出籠並套用在奈米藥物載體上，好比裝了偵測雷達帶著彈藥的轟炸機，只會攻擊鎖定目標，然而靶向藥物傳遞系統這個名詞在也隨之被創造出來。1986 年 Rober Langer 的研究團隊利用 polymer 為材料，發展出第一個利用靶向藥物傳遞系統，將藥物送至病灶處。²²

靶向運輸系統之所以蓬勃發展是因為癌細胞為了快速生長，其細胞膜上會表現特有或大量表現的分子或受體(receptor)，利用這些標的物當作目標，將奈米藥物載體表面修飾，例如：葉酸(folate)^{23,24}、Arginine-glycine-aspartic (RGD) 胺基酸片段²⁵、運鐵蛋白(transferrin)^{26,27} 或是抗體(antibody)²⁸...等等，經由受體與配體(ligand)的特異性結合(specific binding)後，由受體媒介細胞吞噬作用(receptor-mediated endocytosis)方式進入細胞或是其他內吞作用(Figure 10)²⁹，進而將包覆在奈米藥物載體內的藥物於進入細胞後才釋放使細胞凋亡(apoptosis)。^{30,31}

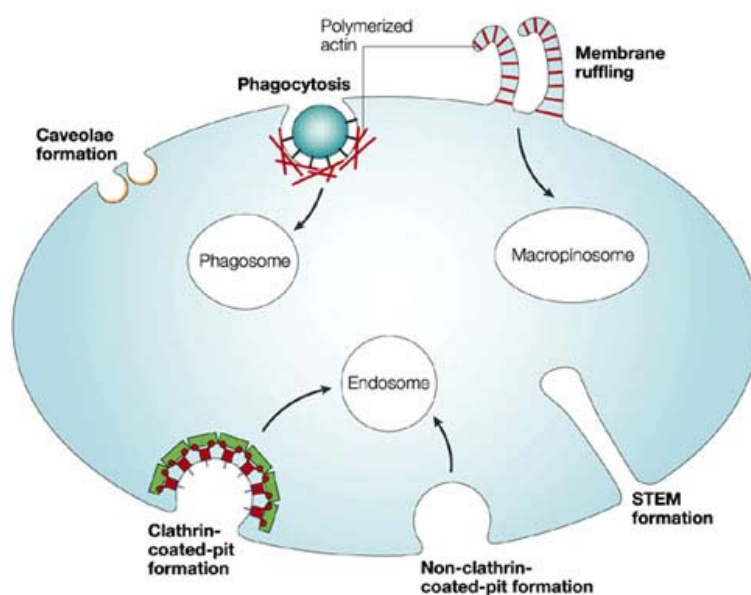


Figure 10²⁹：細胞內吞作用途徑

2.2.1 葉酸與葉酸受體 (Folate and folate receptor)

葉酸又稱維他命 B₉，是由蝶啶環(pteridine ring)、對氨基苯甲酸(para-aminobenzoic acid)及穀胺酸(glutamate)所組成(Figure 11)。葉酸是細胞內重要的輔酶(coenzyme)參與了許多細胞內一個碳的轉移反應也是合成核酸的重要起始物，由於人體無法自己合成出葉酸分子，若細胞缺乏葉酸的攝取，會造成細胞無法繼續複製生長。^{32,33}

正常細胞透過葉酸受體或還原葉酸載體(reduced folate carrier)將葉酸攝入細胞，前者多出現在活化的巨噬細胞及極化的上皮細胞(polarized epithelial cells)表面上，後者則多參與在攝取一般生理代謝上所需的葉酸。正常細胞表面低濃度的還原葉酸載體就足以攝取生理需求的葉酸，相反的，癌細胞為了得到更多生長養分，其細胞表面則會過量表現的葉酸受體。³⁴

人類的葉酸受體家族有三種 isoforms(FR- α , - β & - γ/γ')，有 70~80% 其胺基酸序列皆相同，其中 FR- α 及 FR- β 是 glycosylphosphatidylinositol (GPI) anchor 膜蛋白，其中 FR- α 對葉酸的親和力(binding affinity)($K_D \sim 0.1$ nM)比 FR- β ($K_D \sim 1$ nM)好。³³

然而，界定是否為葉酸受體過量表現之細胞株，將特定組織經過處理後其溶解性膜蛋白(solubilized membrane protein)與 [³H]葉酸反應經同位素分析後，若超過 6 pmol FR/mg protein 則視為高度表現(high positive)，例如：L1210³⁵, KB³⁶, M109³⁷, 及 HeLa；若低於 2.5 pmol FR/mg protein 視為不表現(negligible)，例如：(4T1, 24JK, 及 A549)；若介於 6~2.5 pmol FR/mg protein 視為低度表現(low positive)，但某些正常細胞組織也會有相對高度表現的葉酸受體(Table 1³²：人類腫瘤組織與正常組織之 FR 表現量)。³² 然而，葉酸受體在卵巢、肺、乳腺、腎、腦、子宮內膜、結腸癌... 等等細胞株中皆發現其細胞表面都有 FR 高度表現的現象發生，³² 其中卵巢癌中大約有 90% 的細胞株都有 FR 過量表現的現象。³⁸⁻⁴⁰

1991 年 FR 胺基酸被定序完成後，很多研究利用藥物載體修飾上葉酸分子，原因是葉酸分子具有眾多優點如：低免疫原性(low immunogenicity)、修飾方法容易、分子小(Mr 441.4 Da)、穩定性高、溶劑相容性高、價格便宜...等等⁴¹，經過修飾葉酸分子的藥物載體，再經由受體媒介細胞內吞作用進到細胞內的 endo/lysosome 內(Figure 12)³³，至今有相當多篇的研究應用葉酸分子當作標靶分子，且發現與未修飾的藥物載體相較下，都有更佳的抑制癌細胞生長效果。⁴²⁻⁴⁴

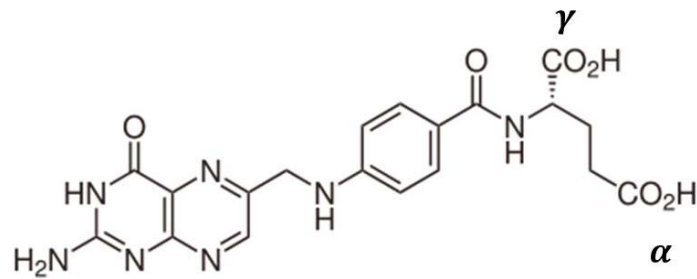


Figure 11：葉酸化學結構

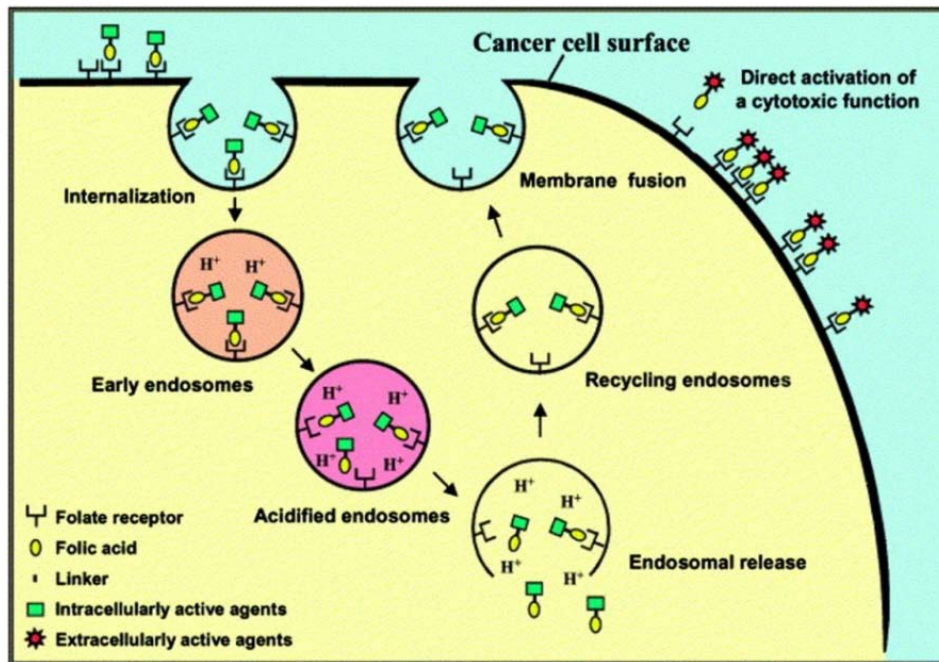
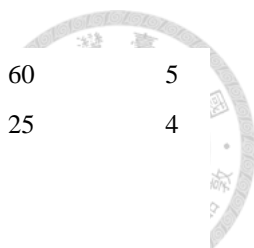


Figure 12³³：葉酸與葉酸受體經由受體媒介細胞內吞作用進入細胞之路徑

Table 1³² : 人類腫瘤組織與正常組織之 FR 表現量

FR levels in various human cancer and normal tissues

Type of tissue	Average picomoles FR/milligram protein	High positive (%)	Low positive (%)	Negligible (%)	<i>n</i>
Ovarian					
Serous carcinoma	34.31 ± 22.87	100	—	—	7
Endometrioid carcinoma	15.66 ± 9.26	100	—	—	4
Mucinous carcinoma	1.83 ± 1.19	—	36	64	14
Metastatic	46.36 ± 49.68	100	—	—	4
Normal	1.54 ± 1.00	—	25	75	12
Kidney					
Primary carcinoma	12.42 ± 6.90	86	—	14	7
Metastatic	4.30 ± 4.15	50	—	50	4
Normal	14.40 ± 6.70	100	—	—	8
Endometrial					
Primary carcinoma	9.32 ± 18.10	20	20	60	10
Metastatic	8.17	100	—	—	1
Normal	0.95 ± 0.50	—	—	100	7
Lung					
Primary carcinoma	6.11 ± 5.71	36	36	27	11
Adenocarcinoma	6.85 ± 3.22	43	57	—	7
Mesothelioma	7.60 ± 4.21	67	33	—	3
Metastatic	5.92 ± 4.34	50	25	25	4
Normal	7.79 ± 2.99	75	17	8	12
Breast					
Primary carcinoma	7.44 ± 5.83	43	43	14	7
Normal	3.99 ± 1.68	20	60	20	5
Bladder					



Primary carcinoma	3.49 ± 3.95	20	20	60	5
Normal	5.20 ± 3.96	50	25	25	4
Pancreas					
Primary carcinoma	3.56 ± 2.28	10	60	30	10
Metastatic	8.78	100	—	—	1
Normal	2.43 ± 1.80	—	40	60	5
Liver					
Primary carcinoma	1.59 ± 0.09	—	—	100	3
Normal	1.23 ± 0.42	—	—	100	4
Colorectal					
Primary carcinoma	0.49 ± 0.37	—	—	100	3
Metastatic	5.21 ± 7.86	25	—	75	4
Normal	6.75 ± 3.10	50	50	—	4
Prostate					
Primary carcinoma	2.84 ± 1.36	—	67	33	3
Normal	2.3 ± 1.04	—	40	60	5
Lymphoma					
Primary	1.94 ± 1.77	—	12	88	8
Metastatic	4.16 ± 2.64	50	—	50	2
Brain					
Primary carcinoma	4.51 ± 6.15	25	25	50	4
Normal	0.32 ± 0.30	—	—	100	3
Head and neck					
Primary carcinoma	1.30 ± 0.81	—	—	100	3

2.2.2 控制釋放 (Controlled release)

控制釋放係指利用環境 pH 值的不同、提供光照或熱能將藥物載體的結構改變導致藥物釋放；例如：對 pH-sensitive 的藥物載體，在進入細胞的內體(endosome)、溶酶體(lysosome)後才釋放藥物，或是腫瘤組織微環境的偏酸性使得載體結構被破壞⁴⁵⁻⁵¹；熱敏感(thermo-sensitive)的藥物載體，施予外加熱源或電磁場達相變化溫度^{7,45,52-58}；光敏感(photo-sensitive)藥物載體，在經過短時間特定波長的照射下，載體修飾的化學鍵斷裂⁵⁹⁻⁶⁴...等等方法都能使藥物從載體內釋放，並且在相對短時間內讓藥物達到有效劑量免除不斷服藥的不便。⁶⁵⁻⁶⁸

以熱敏感藥物載體來說，其額外使用熱源不但能使藥物釋放，也能搭配溫熱治療使治療效果增強，因為癌細胞對溫度的耐受性差；而熱能的生成可以用近紅外線或是電磁感應生熱之方法，但由於紅外光穿透組織的能力只有幾公厘而已，若癌組織非表層就難以達到熱治療之效果，相對的，電磁生熱沒有穿透組織的限制，將會是一個很好的產熱工具。¹²



2.3 藥物載體使用之材料 (Materials)

在研究領域上所使用的藥物載體種類相當多元，且基本上都要符合生物相容性(biocompatible)、生物可降解性(biodegradable)、生物穩定性(biostability)及無毒性(non-toxicity)這些大條件。^{46,69}

簡單來說大部分的藥物載體都屬於膠體溶液(colloidal solution)的範疇。以軟物質(soft materials)來說，微脂體(liposome)^{47,70-75}、高分子材料形成的聚合物(polymer)⁷⁶⁻⁸¹、微包(micelle)^{54,57,82-86}、樹枝狀聚合物(dendrimer)⁸⁷、奈米乳化油滴(organogel emulsion nanoparticle)^{66,88}...等等；以無機材料為例，金奈米粒子(gold nanoparticle)⁸⁹⁻⁹⁵、氧化鐵奈米粒子(iron oxide nanoparticle)^{12,13,96-104}、多孔洞矽材奈米顆粒(mesoporous silica nanoparticle, MSN)^{68,105-108}、奈米碳管(carbon nanotube)¹⁰⁹⁻¹¹³ ... 等等。

2.3.1 磷脂質修飾奈米藥物載體 (Phospholipid-capped nano drug carrier)

真核細胞的細胞膜主要構成成分是磷脂質具介面活性劑的特性，一端是親水端，另一端是疏水端的結構(Figure 13¹¹⁴)，不同結構組成帶有不同電性其應用也有所不同，例如：帶負電的 1,2-dihexadecanoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (DPPG)(Figure 14)及具兩性的 1,2-dihexadecanoyl-*sn*-glycero-3-phosphocholine (DPPC)(Figure 15)。若以磷脂質當作藥物載體的表面修飾材料不但具有生物相容性及生物可降解性的特性，且磷脂質的親水端可以額外修飾上其他具標靶性、螢光分子...等等，使藥物載體具專一性或具光學追蹤功能。

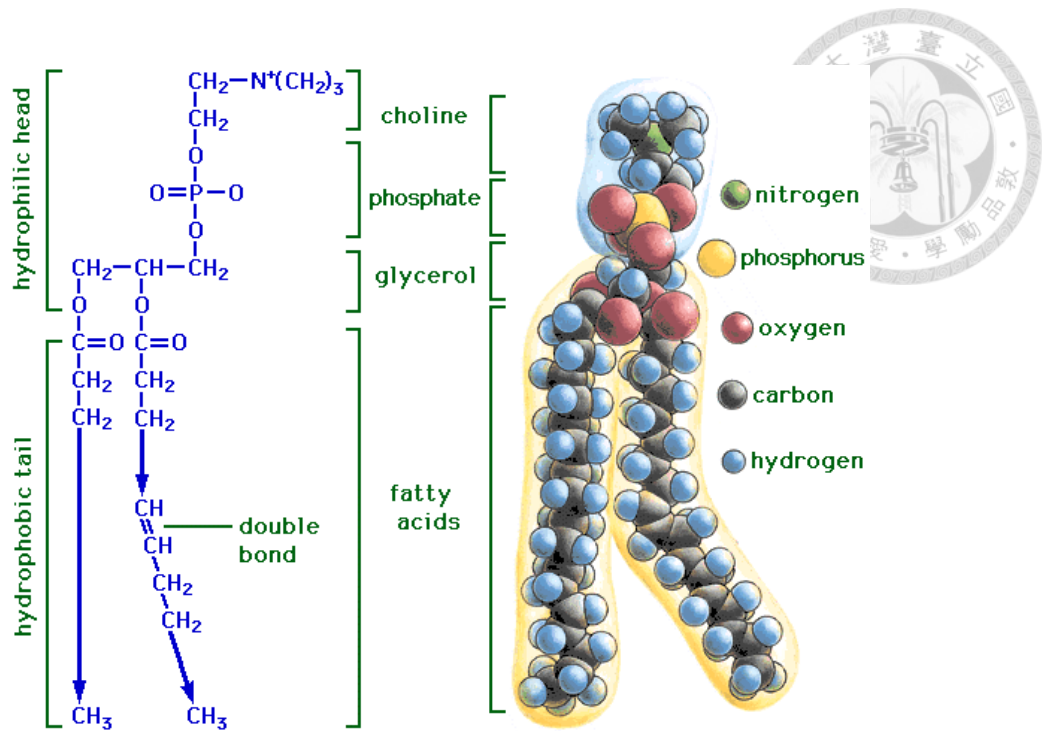


Figure 13¹²⁸ : 磷脂質組成結構

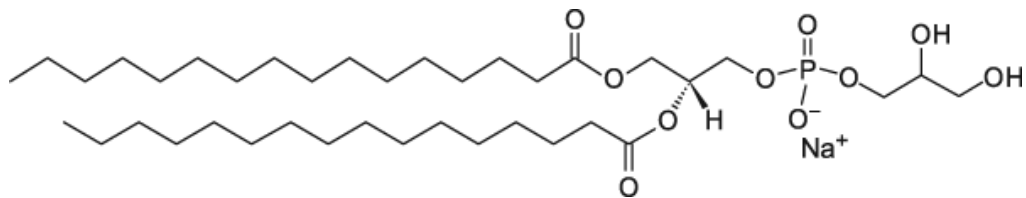


Figure 14 : DPPG 化學結構

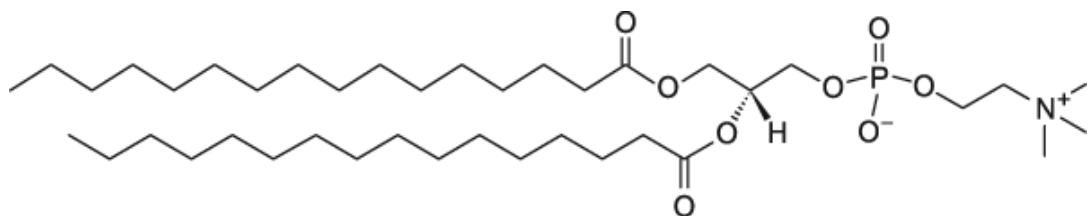


Figure 15 : DPPC 化學結構



2.3.1.1 DSPE-PEG₂₀₀₀-Folate

由於眾多癌細胞的表面會高度表現葉酸受體，利用藥物載體修飾上葉酸分子的文獻報導相當多(如 2.2.1 葉酸與葉酸受體 (Folate and folate receptor) 章節)，由於葉酸分子與葉酸受體的特異性結合位在 glutamate 上的 α -COOH，由於 γ -COOH 的化學合成反應性也較佳，所以偏好的修飾位為 γ -COOH，且避免無法與葉酸受體有特異性的結合¹¹⁵⁻¹¹⁷，例如以磷脂質作為包覆藥物載體的材料會利用 DSPE-PEG₂₀₀₀-amine 修飾上葉酸分子^{106,118,119}，其合成方法如 Figure 16¹¹⁸。

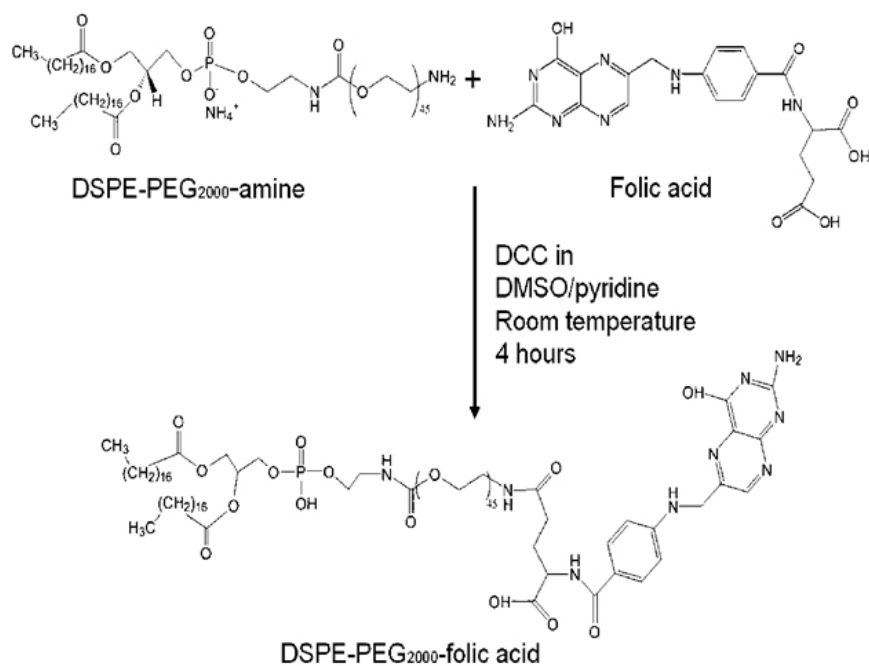


Figure 16¹¹⁸ : 合成 DSPE-PEG₂₀₀₀-Folate scheme

2.3.2 熱敏感膠體奈米粒子 (Thermo-sensitive nanoparticle)

溫度敏感性的熱敏感載體的共同特色是在特定溫度有相變(phase transition)的現象，從膠態(gel type)到溶膠態(sol type)，或是從溶膠態到膠態。其製備材料大致可分為兩大類組成，(一) polymer⁴⁸、(二)小分子有機成膠劑(low molecular-mass organic gelators, LOMGs)所構成的結構¹²⁰。

(一) Polymer 組成的熱敏感載體可以是單一組成或是共聚物(copolymer, diblock copolymer, triblock polymer)，例如：
poly(N-isopropylacrylamide)(PNIPAm)^{121,122}， polycaprolactone^{123,124}，
polycarbonate¹²⁵， poly(lactic acid-co-glycolic acid)/poly(ethylene glycol)
(PLGA/PEG)^{126,127}， multiblock poly(ethylene glycol)/poly(propylene glycol) (PEG/
PPG)^{128,129} ... 等等。這些材料會因為溫度的變化改變其構型，而此溫度點稱為最低溶解溫度(lower critical solution temperature, LCST)；低於 LCST 時，其構型為似微包結構，所以藥物可以被完好的包埋其中，高於 LCST 時改變材料在水中的溶解度，進而脹破(swelling)或皺縮(shrinkage)讓藥物釋放，例如 Figure 17¹³⁰ 之 diblock copolymer 超過臨界微包濃度(critical micelle concentration, CMC)形成微包後，將抗癌藥物包覆在疏水結構內，當溫度高於 LCST 點此時親水性結構的溶解度改變，使得結構改變將藥物釋放。^{130,131}

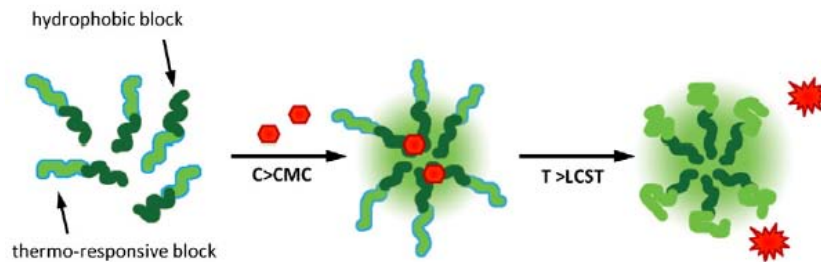
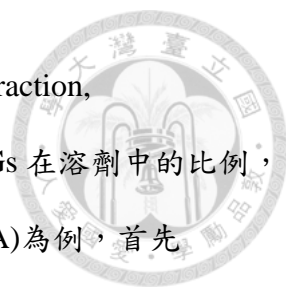


Figure 17¹³⁰：以 polymer 形成的熱敏感載體其藥物釋放圖



(二) LOMGs 在溶劑中彼此以非共價鍵(H-bonding, π - π interaction, hydrophobic interaction...etc)形成鍵結進而成膠，藉由改變 LOMGs 在溶劑中的比例，改變相變溫度。以 12-羥基硬脂酸(12-hydroxystearic acid, 12-HSA)為例，首先 12-HSA 在油中環境下先聚集(nucleation)，接著 12-HSA 的羧酸分子彼此形成環狀或非環狀的分子間氫鍵(Figure 18¹³² & Figure 19¹³²)後形成二維的自主裝網狀結構(self-assembled fibrillar networks, SAFiNs)，最後形成三維的網狀結構將油包埋其中(Figure 20¹³³)^{120,132-134}。

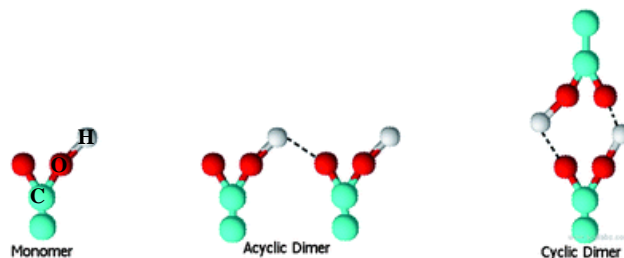


Figure 18¹³²：12-羥基硬脂酸其羧酸單體，分子間形成氫鍵的非環狀二聚體及環狀二聚體

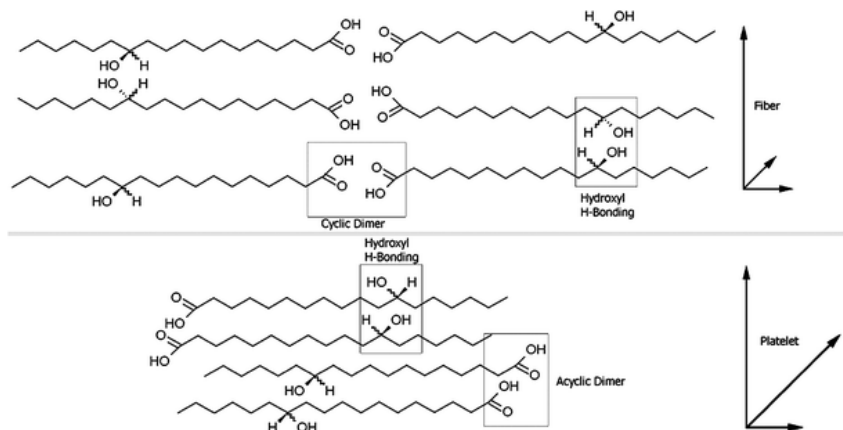


Figure 19¹³²：12-羥基硬脂酸分子間形成自主裝網狀結構的堆疊型態

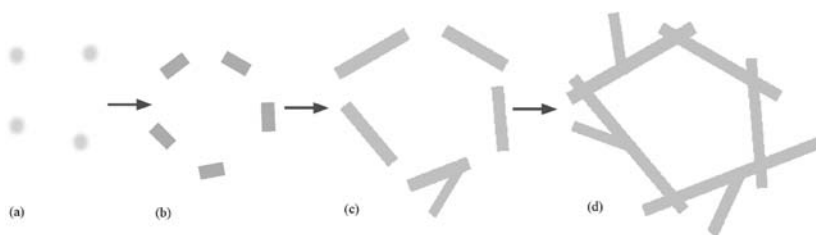


Figure 20¹³³：12-羥基硬脂酸成膠示意圖

2.3.3 磁性奈米粒子 (Magnetic nanoparticle)

磁性奈米粒子的應用已超過幾十年載，不論已經在臨床醫療當作磁振造影 (MRI)的 T2-weighted 顯影劑⁴⁷、實驗研究上純化分離的媒介，或是電磁感應生熱都是利用具磁性的特質，而且以氧化鐵為主成分的磁性奈米粒子具低生物毒性、細胞可分解性，而現今的商品化的應用產品也相當多例如 Table 2¹³：商業化磁性奈米粒子之應用。¹³

Table 2¹³：商業化磁性奈米粒子之應用

公司	應用	參考網站
European Institute of Science	Hyperthermia	www.biotechniques.com
Magforce	Hyperthermia	www.magforce.de
AMAG Pharmaceuticals (Advanced Magnetics)	MRI contrast agents; Diagnosis of cardiovascular disease and cancer	www.amagpharma.com
Diagnostics Biosensors	Diagnostics biosensors	www.diagnosticbiosensors.com
Invitrogen and Dynal Biotech (bought by Invitrogen in 2005)	In vitro diagnostics, protein, cell and biomolecular purification and separation	www.invitrogen.com
Micromod	Drug delivery, biomagnetic separation, nucleic acid purification and protein separation	www.micromod.de
Magnisense	Bioassays for human and animal diagnostics, food safety and environmental protection	www.magnisense.com

大部分的磁性奈米粒子的材料可取於 (一)亞鐵磁性的磁鐵礦(magnetite, Fe_3O_4)、磁赤鐵礦(maghemite, Fe_2O_3 & $\gamma\text{-Fe}_2\text{O}_3$)；(二)反鐵磁性赤鐵礦(hematite,



$\alpha\text{-Fe}_2\text{O}_3$);^{16,103}(三)以 MFe_2O_4 為主材料，摻雜(doping)帶正二價的陽離子金屬如錳、鐵、鈷或鎳。

利用超導量子干涉磁量儀(superconducting quantum interference device magnetometer, SQUID magnetometer)在固定絕對溫度(5K ~ 300K)下，可得其 $M-H$ 曲線圖(Figure 21¹³⁵)，可得 Y 軸為磁化飽和量(saturation magnetization, M_s)係指磁場由零點(A)開始增加後，磁性物質之感應磁性也隨之增加(A→B)，但無論施予再大的磁場，其磁場變化量都不會再改變即達飽和點(B)，反之，隨著磁場強度逐漸減小，磁性物質之感應磁性也隨之降低(B→C)至反向磁性飽和點(D)；而 X 軸為矯頑磁力(coercivity, H_c)係指需要額外施予多大的磁場強度(A~ E)使其去磁化為零，而測得此封閉曲線稱為磁滯曲線圖(hysteresis loop)。

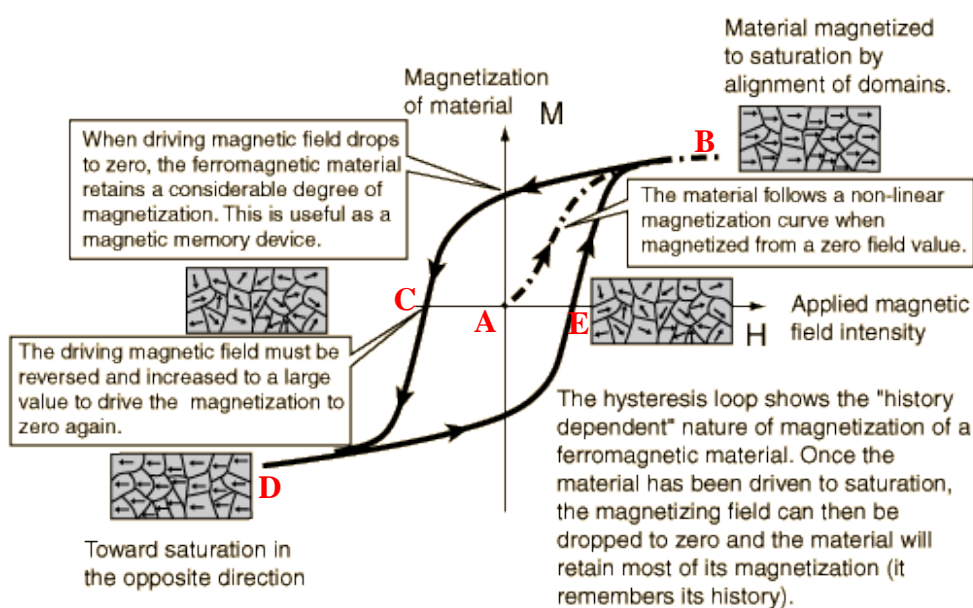


Figure 21¹³⁵ : 磁滯曲線圖

磁性奈米粒子依尺度可分成三大類如 Figure 22¹³⁶：

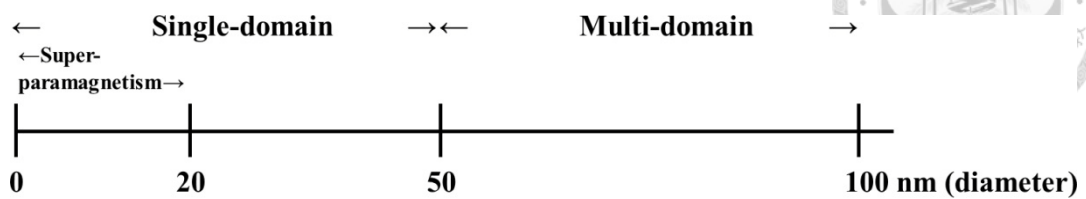


Figure 22¹³⁶：磁性奈米粒子尺度上的分類 (由參考文獻 136 做修改)

(一)超順磁性奈米粒子(superparamagnetism nanoparticle)：矯頑磁力趨近於零，在室溫下不具磁性，施予外加磁場後才擁有磁性，所以其穩定性是最佳的。

(二)單磁域磁性奈米粒子(single-domain magnetic nanoparticle)：鐵原子的電子自旋(electro spin)方向聚集形成的磁域(domain)，進而形成排列的晶格結構，使得磁域內的淨磁矩會朝向單一的方向，所以在無外加磁場下本身就具磁性。

(三)多磁域磁性奈米粒子(multi-domain magnetic nanoparticle)：為多個單磁性磁域所構成。



2.4 電磁感應溫熱療法 (AC magnetic hyperthermia)

廣義來說，溫熱療法係利用非侵入性的方法(光、微波、電磁波或超聲波)，將熱能(42–45 °C)導入患部的組織或器官中，使血管通透性提高及血流速度增加，以達到舒緩的目的。以癌症治療來說，有研究指出相同單位時間內，癌細胞組織所能承受的溫度(42–45 °C)相對正常細胞組織(60 °C)來的低，由於腫瘤組織的異常血管新生造成血液循環不佳，所以受熱後熱量不易散溢，使癌細胞的蛋白質變性，而且血管壁的孔隙也會變大，增加奈米藥物載體滲入腫瘤組織當中，最後驅使癌細胞走向細胞凋亡的路徑。¹²

熱量的產生方式相當多種，電磁感應生熱具最大的優勢就是沒有穿透組織的深度限制，若患部已累積大量磁性奈米粒子，只有磁性奈米粒子存在的區域會因為電磁感應生熱，其他正常組織並不會受到影響減少相關副作用的發生。

根據美國國家癌症研究所(National Cancer Institute)的規範，可將熱療法(hyperthermia)區分成三大類：^{137,138}

(一) 局部性熱療法(local hyperthermia)又稱磁流體熱療(magnetic fluid

hyperthermia)：由於癌組織有 EPR effect 的特性，若利用標靶性的磁性奈米粒子到達特定範圍的腫瘤組織，給予適當磁場之後，藉由磁性奈米粒子以電磁生熱的方式釋放熱能，使癌細胞凋亡。

(二) 區域性熱療法(regional hyperthermia)：癌細胞遍布整個器官，其熱治療的範圍更大。

(三) 全身性熱療法(whole body hyperthermia)：若癌細胞已經轉移至其他臟器，全身性的方式治療效果較佳。

2.4.1 磁流體熱療 (Magnetic fluid hyperthermia)

磁感應生熱可依磁性奈米顆粒大小的差異，以不同方式釋放熱能；顆粒大小大於 20 nm 的磁性奈米粒子本身具磁性，若感應到電磁場會以磁滯損失(magnetic hysteresis loss)的方式釋放熱能(Figure 23)¹³⁶；小於 20 nm 則以 Néel-relaxation 及 Brown-relaxation 方式產生熱能，當額外施予高頻率電磁場後，磁奈米粒子因受到交流電不斷改變的磁場方向，使磁自旋(magnetic spins)方向不斷的重新排列中產生熱量稱 Néel-relaxation(spin rotation)；另外在環境溶液中，磁性奈米粒子本身就行布朗運動，加上外部電磁場的影響造成不斷地擾動進而產生熱能稱 Brown-relaxation(particle rotation) (Figure 24)¹³⁷。¹³⁷

Hysteresis loss (P_h)

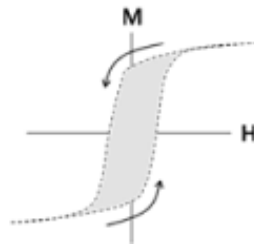


Figure 23¹³⁶：磁滯損失

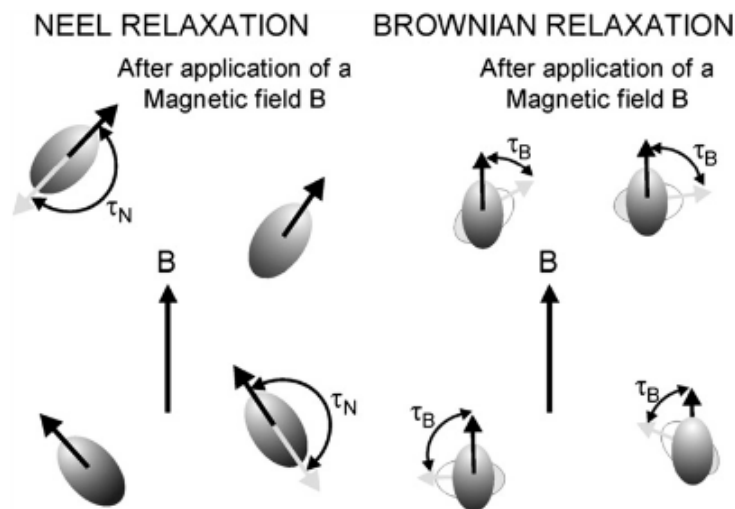
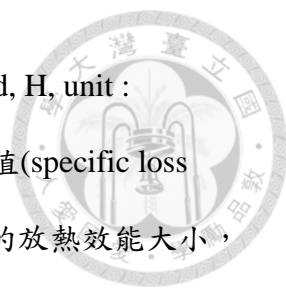


Figure 24¹³⁷：施加外部交流電場後，小於 20 nm 的磁性奈米粒子以鬆弛效應釋放熱能



施予不同交流電頻率及不同磁輔助場(magnetic auxiliary field, H, unit : mA/meter)條件下，藉由 Equation 1 可以計算出不同的損耗功率值(specific loss power, SLP or specific absorption rate, SAR)，可得磁性奈米粒子的放熱效能大小，一般磁性奈米粒子的損耗功率值會藉於 100~500 W/g_{Fe}。⁴⁷

$$SLP = \frac{CV_s}{m} \cdot \frac{dT}{dt} \dots\dots\dots \text{Equation 1}$$

- C : Volumetric specific heat capacity of the sample solution (J/L·K)
- V_s : Sample volume (L)
- m : Mass of magnetic material in the sample (g)
- $\frac{dT}{dt}$: Initial slope of the change in temperature versus time curve (K/s)

2.5 結語

利用靶向藥物傳遞系統攜帶抗癌藥物能解決傳統藥物的缺點，其必要條件是藥物載體所使用的材料需符合高生物相容性、生物可降解性及無細胞毒性，再搭配更有效的控制釋放之方法(例如：電磁生熱)，使藥物能在短時間內達到有效劑量，必能提升其毒殺癌細胞之效應並減少副作用之發生。



第三章 實驗藥品與儀器



3.1 實驗藥品 (Chemicals and reagents)

Table 3 : 實驗藥品表單

藥品名稱, 縮寫	分子式	製造廠商
1,2-dipalmitoyl-sn-glycerol-3-phosphocholine, DPPC	$C_{40}H_{80}NO_8P$	Avanti Polar Lipids (Alabaster, AL, USA)
1,2-dipalmitoyl-sn-glycerol-3-phospho-(1'-rac-glycerol) (sodium salt), DPPG	$C_{38}H_{74}O_{10}PNa$	Avanti Polar Lipids (Alabaster, AL, USA)
1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (ammonium salt), DSPE-PEG ₂₀₀₀ -Amine	$C_{132}H_{266}N_3O_{54}P$	Avanti Polar Lipids (Alabaster, AL, USA)
Folic acid, FA	$C_{19}H_{19}N_7O_6$	Sigma (St. Louis, MO, USA)
Dicyclohexylcarbodiimide, DCC	$C_{13}H_{22}N_2$	Sigma (St. Louis, MO, USA)
Pyridine	C_5H_5N	Sigma (St. Louis, MO, USA)
10 nm Oleic acid-coated superparamagnetic iron oxide nanoparticles, SIONPs	-	Provide by NCKU Prof. Yeh (Tainan, Taiwan)
Castor oil	-	Sigma (St. Louis, MO, USA)
12-hydroxyoctadecanoic acid (12-hydroxystearic acid), 12-HSA	$C_{18}H_{36}O_3$	Sigma (St. Louis, MO, USA)
Coumarin-6	$C_{20}H_{18}N_2O_2S$	Sigma (St. Louis, MO, USA)
Nile red	$C_{20}H_{18}N_2O_2$	Sigma (St. Louis, MO, USA)
Chloroform	$CHCl_3$	Sigma

		(St. Louis, MO, USA)
Methanol	CH ₃ OH	Sigma (St. Louis, MO, USA)
Sodium chloride	NaCl	J.T Baker (PA, USA)
Potassium chloride	KCl	J.T Baker (PA, USA)
Sodium phosphate dibasic	Na ₂ HPO ₄	Riedel-deHaën (Seelze, Germany)
Potassium phosphate monobasic	KH ₂ PO ₄	J.T Baker (St. Louis, MO, USA)
Sodium chloride	NaCl	Sigma (St. Louis, MO, USA)
Potassium chloride	KCl	Sigma (St. Louis, MO, USA)
Sodium phosphate dibasic	Na ₂ HPO ₄	Sigma (St. Louis, MO, USA)
Potassium phosphate monobasic	KH ₂ PO ₄	Sigma (St. Louis, MO, USA)
Penicillin/streptomycin	-	HyClone (Logan, UT, USA)
Dulbecco's modified Eagle's medium, DMEM	-	HyClone (Logan, UT, USA)
Fetal bovine serum, FBS	-	Biological Industries (Kibbutz Beit Haemek, Israel)
Trypsin-EDTA	-	HyClone (Logan, UT, USA)
Methylthiazolyldiphenyl-tetraz oilum bromide, MTT	C ₁₈ H ₁₆ BrN ₅ S	Sigma (St. Louis, MO, USA)

3.2 實驗儀器 (Apparatus)



Table 4 : 實驗儀器表單

儀器名稱, (英文, 縮寫)	廠牌型號
超聲波液體震盪器 (Ultrasonic Cleaner)	VWR scientific products, Aquasonic 75D (NY, USA)
針筒注射泵 (Syringe pump)	New Era Pump Systems, NE-1000 (NY, USA)
動態光散射儀 (Dynamic light scattering, DLS)	Malvern Nano ZS (Worcestershire, UK)
穿透式電子顯微鏡 (Transmission electron microscopy, TEM)	Hitachi, H-7500 (Tokyo, Japan)
冷凍乾燥機 (Freeze dryer)	Thermo Electron Corporation Modulyod-115 (MA, USA)
示差掃描熱卡計 (Differential scanning calorimeter, DSC)	PerkinElmer Pyris (CA, USA)
螢光光譜儀 (Fluorescence spectrophotometer)	Varian Cary Eclipse (Palo, VIC, Australia)
微孔盤分析儀 (ELISA reader)	Tecan, Sunrise (Männedorf, Switzerland)
熱電偶感測棒 K-type (Thermocouple)	TES, TES-1311A (Taipei, Taiwan)
電晶體式高週波金屬加熱機 (Induction heating machine)	Lantech Industrial Co., Ltd LT-35-80 (Taichung, Taiwan)
超導量子干涉磁量儀 (Superconducting quantum interference device magnetometer, SQUID magnetometer)	Quantum Design MPMS-7 (CA, USA)

3.3 癌細胞株 (Cancer cell lines)



Table 5：實驗細胞株表單

癌細胞株	所屬種類	取得來源
HeLa	Human cervical adenocarcinoma	BCRC No.60005 (Hsinchu, Taiwan)
A-549	Human lung carcinoma	BCRC No.60074 (Hsinchu, Taiwan)

第四章 製備方法與鑑定



4.1 數學計算蓖麻油與磷脂質之重量比值 (Mathematical calculation the mass ratio of castor oil and phospholipid)

假設形成直徑為 100 nm 的蓖麻油滴顆粒($D = 9.6 \cdot 10^5 \text{ g/m}^3$)，已知 DPPC (M.W. $\cong 734.0 \text{ g/mol}$)的親水端表面積(大約 70\AA^2)¹³⁹，利用計算油滴表面積反推需要多少重量的 DPPC？

$$1 \cdot 4\pi \cdot (500\text{\AA})^2 = X \cdot 70\text{\AA}^2$$

$$X \cong 44,880 \text{ 個 DPPC} \cong 7.455 \cdot 10^{-20} \text{ mole} \cong 5.472 \cdot 10^{-17} \text{ g}$$

\therefore 1 個直徑為 100 nm 的蓖麻油滴需要 $5.472 \cdot 10^{-17} \text{ g}$ 的 DPPC ①

$$D = V/M$$

$$M = 960,000 \text{ g/m}^3 \cdot \frac{4}{3} \cdot \pi (5 \cdot 10^{-8} \text{ m})^3 = 5.027 \cdot 10^{-16} \text{ g}$$

\therefore 1 個直徑為 100 nm 的蓖麻油滴重量為 $5.027 \cdot 10^{-16} \text{ g}$ ②

若使用 10 mg 的蓖麻油($1.989 \cdot 10^{13}$ 個 100 nm 蓖麻油滴)，需要 y 克的 DPPC？

由 ① & ② 係是可得知：

$$1/5.472 \cdot 10^{-17} \text{ g} = 1.989 \cdot 10^{13} / y$$

$$y \cong 1.089 \text{ mg DPPC 分子}$$

\therefore 10 mg 蓖麻油需要 1.089 mg DPPC 分子 ③



4.2 熱敏感藥物載體之製備 (Preparation of phospholipid-gel-superparamagnetic iron oxide nanoparticles, PLG-SPIONs nanoshuttle)

4.2.1 實驗材料

- 1) 親脂性(lipophilic)溶劑
：CHCl₃/MeOH 體積比 = 9 : 1
- 2) 水相溶液
：Phosphate buffered saline, PBS (137 mM NaCl, 2.70 mM KCl, 10.0 mM Na₂HPO₄, 1.80 mM KH₂PO₄)
- 3) DPPC, DPPG, DSPE-PEG₂₀₀₀-Folate (mass ratio = 14.00 : 1.000 : 0.278)
：1.089 mg DPPC/ 4 mL PBS
- 4) 2.5 wt% 12-hydroxystearic acid w/ castor oil
：10 mg/ 4mL PBS
- 5) 10 nm oleic-capped iron oxide nanoparticles(SPIONs) (5.670mg/mL)
：80 μL/ 4mL PBS
- 6) 5 mM CPT or 2.5 mM coumarin-6
：150 μL/ 4mL PBS

4.2.2 使用儀器

- 1) 超音波震盪器，水浴溫度 45 °C
- 2) 針筒注射泵
- 3) 動態光散射儀(DLS)，其 Z-Ave (d. nm)及 PDI (polydispersity index)當作判斷合成 PLG-SPIONs 的粒徑及勻散性



4.3 合成控制變因及應變變因 (Variable factors of synthetic process)

使用 nanoemulsion 一步(one-pot)合成法合成 PLG-SPIONs nanoshuttle。

- 1) 4.2.1 之實驗材料 3), 4), 5), 6)溶於親脂性溶劑中。
- 2) 由 4.1 計算結果可得知 2.5 wt% 12-HSA w/castor oil : DPPC 重量比
= 10 mg : 1.089 mg。

以上 1)及 2)條件為控制變因，應變變因為 a) 親脂性溶劑：水相溶液體積比例
b)超音波震盪時間 c)針筒注射泵流速。

初步以 DLS 之 Z-Ave (d. nm)及粒徑勻散性 PDI (polydispersity index)當作判定之指標，一般來說 PDI(weight average molecular weight/number average molecular weight)是指聚合物分子量的分佈狀況；然而在光散射儀應用當中，PDI 表示粒徑的勻散性。假設粒徑是單一分佈並符合高斯分佈(Gaussian distribution)，Malvern 的動態光散射儀其 PDI 可表示為 Equation 2，其中 σ 為標準差(standard deviation)， Z_D 為平均粒徑的散射光強度(the intensity weighted Z average mean size)。

$$PDI = \sigma^2 / Z_D^2 = \text{Relative variance} \dots\dots\dots \text{Equation 2}$$

Polydispersity (Pd) = Standard deviation or width (absolute polydispersity)

%Polydispersity (%Pd) = Coefficient of variation (relative polydispersity)

$$= (PDI)^{1/2} \cdot 100$$

一般的經驗法則來說，若 %Pd < ~20%則可視為單一分散(monodisperse)的粒徑分布。¹⁴⁰

4.3.1 調整親脂性溶劑：水相溶液體積比

Figure 25 結果圖分別為 1 : 5/1 : 20/1 : 40 之比例，左側 Y 軸為 Z-Ave (d. nm)



及右側 Y 軸為 PDI 值，以 1:20 之比例可得其粒徑大小與 PDI 值的標準誤差最小，故選之為最佳條件。

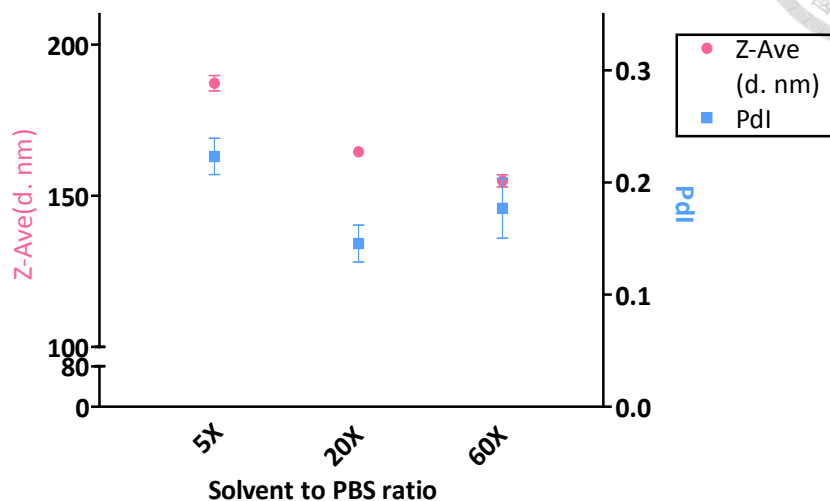


Figure 25：不同油水體積比其粒徑及 PDI 值

4.3.2 超音波震盪時間

維持反應溫度為 45°C(親脂性物質其相轉變溫度接近 45°C)，改變超音波震盪時間分別為 1/2.5/3.5/4 小時。Figure 26 為其結果圖，可得知反應時間為 3.5 小時的 PDI 最好其粒徑大小與 4 小時的差不多，所以此項實驗的最佳化時間為 3.5 小時。

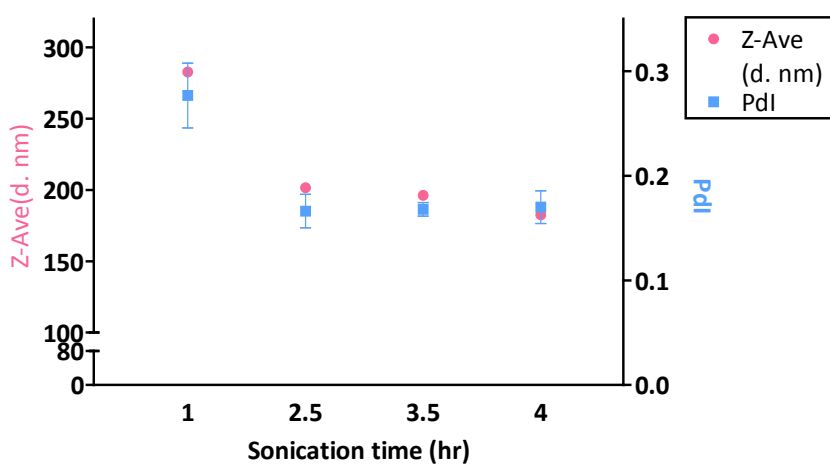


Figure 26：不同超音波震盪反應時間其粒徑及 PDI 值

4.3.3 改變針筒注射泵之流速

親脂性溶劑滴入 PBS 之流速會影響合成所得到的粒徑大小，分別使用 50/125 $\mu\text{L}/\text{min}$ 之流速，或不使用注射泵直接加到 PBS 溶液中。由 Figure 27 之結果得知以 50 $\mu\text{L}/\text{min}$ 之流速其粒徑及 PdI 值最佳，故此項實驗選定最佳化流速為 50 $\mu\text{L}/\text{min}$ ，其可能是因為滴入 PBS 內的液滴與下個液滴間隔較久，超音波持續震盪的條件下，在下個液滴滴入前就能有效地把前一個液滴震散變更小顆，不會使他們凝聚變成大液滴，所以得到得粒徑分佈較小。

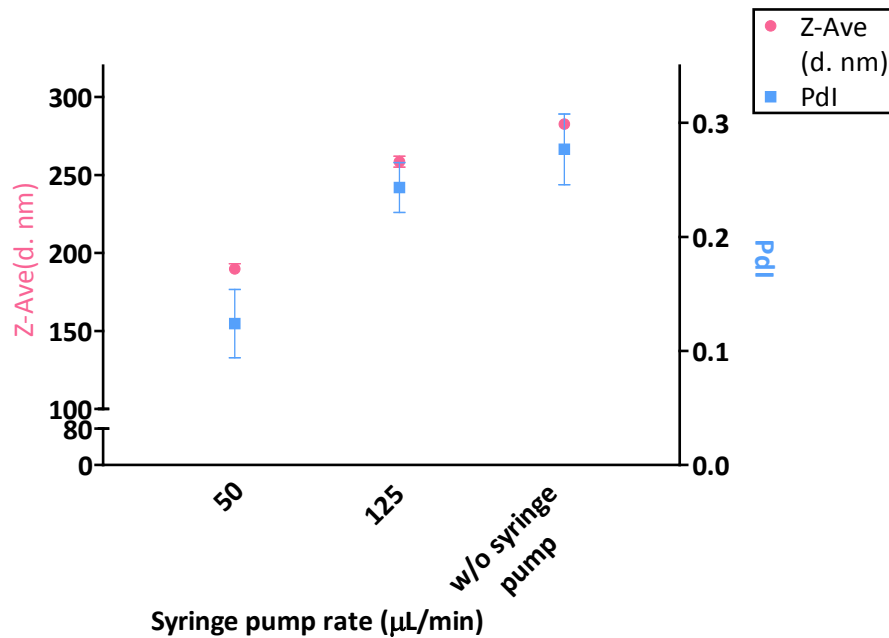


Figure 27：不同針筒注射泵流速其粒徑及 PdI 值

4.3.4 最佳化合成方法與條件

由 4.3.1、4.3.2 及 4.3.3 的調整結果可歸納出，油水體積比為 20 倍，超音波震盪水域溫度為 45°C ，反應時間為 3.5 小時，針筒注射泵的流速為 50 $\mu\text{L}/\text{min}$ ，所做出來的 PLG-SPIONs nanoshuttles 可得最佳的粒徑大小及 PdI 值。



4.4 純化分離 (Purification and separation)

4.4.1 離心方法一

4.3.4 最佳化實驗方法之溶液，以 500 x g 離心 5 分鐘(初步離心先去除沒有被包到可能已經聚集的 SPIONs)，取之上清液，接者以 5000 x g 離心 10 分鐘(去除沒有包覆到 SPIONs 的空包彈熱敏感載體)，得到的沉澱物推測是含有 SPIONs 的 PLG-SIPONs，最後再以 PBS 回溶。得到不同階段的分離溶液以 DLS 做初步判斷。

4.4.2 離心方法二

500 x g 離心 5 分鐘，取之上清液，接者以 8000 x g 離心 10 分鐘，取其沉澱物以 PBS 回溶，以 DLS 鑑定。

4.4.3 離心方法三

500 x g 離心 5 分鐘，取之上清液，接著強力磁鐵磁吸 5 分鐘，移除上清液，得到的沉澱物再以 PBS 回溶，以 DLS 鑑定。

Figure 28 為各種分離方法所得到 DLS 粒徑大小及 PDI 值。

Original 表示尚未純化分離之原液。

500 x g pellet 表示初步去除之沉澱物，很明顯得知其粒徑分布偏大，原因是以油酸修飾的 SPIONs 在水溶液當中很容易聚集沉降。

5000 x g pellet 表示經過 500 x g 初步離心後的上清液，以 5000 x g 所得到的沉澱物粒徑大小及分佈。

8000 x g pellet 表示經過 500 x g 初步離心後的上清液，以 8000 x g 所得到的沉澱物粒徑大小及分佈。



500 x g pellet, magnetic pellet 表示經過 500 x g 初步離心後的上清液，以強力磁鐵磁吸後沉澱物的粒徑大小及分佈。

Magnetic pellet 表示未經純化分離的原液，直接以強力磁鐵磁吸 5 分鐘後，所得到的粒徑大小及分佈。

由 Figure 28 可以得到已 5000 x g 的分離效果相對最佳，因為其粒徑大小跟原液最接近且 PDI 的標準差最小，而直接以磁吸分離的結果最差，推斷是沒有被包到的 SPIONs 及包覆在 PLG-SIPONs 的奈米粒子都被吸下來了，而且強力磁鐵的磁力太強可能會破壞 PLG-SPIONs nanoshuttles 的結構所致。

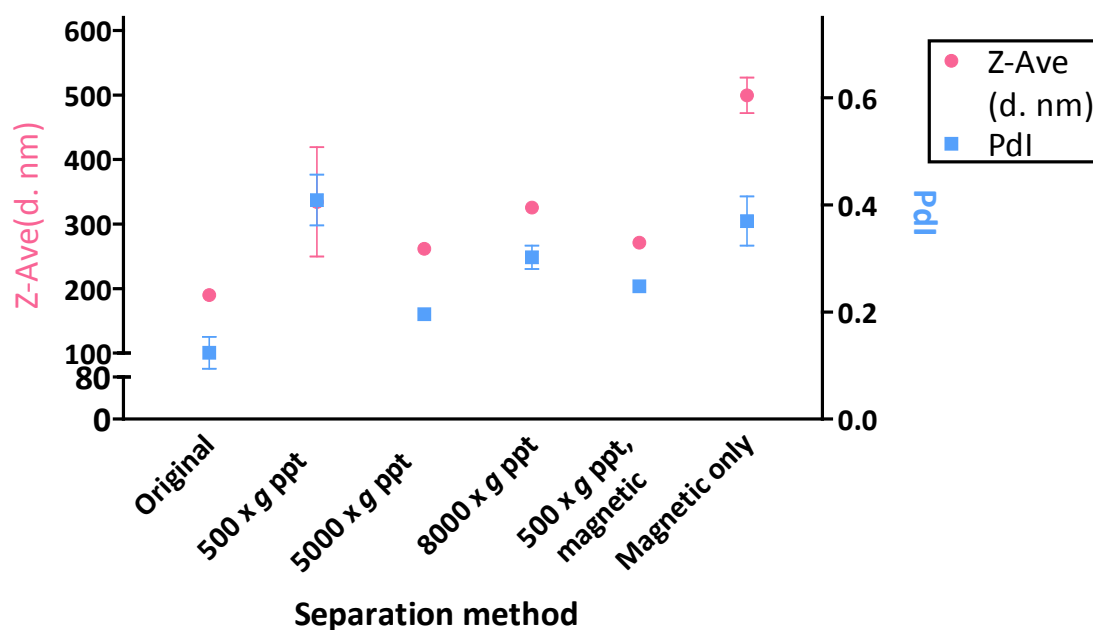


Figure 28：不同純化分離之方法，以 DLS 鑑定其粒徑及 PDI



4.5 界面電位值 (Zeta-potential)

若要判斷膠體溶液(colloidal solution)的穩定性，可以 zeta-potential 的靜電位當作判斷的指標。假設奈米顆粒帶負電性如 Figure 29，其表面會吸附帶正電之離子形成緊密的固定層(Stern layer, also called fixed layer)，然而固定層外又會被帶異電性的離子所吸至電性中和的範圍稱擴散層(diffuse layer)，此二層所構成的電雙層(double layer)與溶液(bulk fluid)之間的剪力面(slipping plane)的靜電能稱 zeta-potential。一般來說界面電位值大於 30 mV 或小於-30 mV 可視為此奈米顆粒帶有電性，彼此有靜電排斥力，不會因為受到凡德瓦力的吸引造成膠凝(flocculation)的狀況發生。¹⁴¹

經過 4.4.1 離心方法一之溶液，如 Figure 30 之結果可得 PLG-SPIONs nanoshuttles 其界面電位值為-55.4±1.47 mV，表示 PLG-SPIONs nanoshuttles 帶有電性，彼此有靜電排斥所以不會聚集沉降，其穩定性佳。

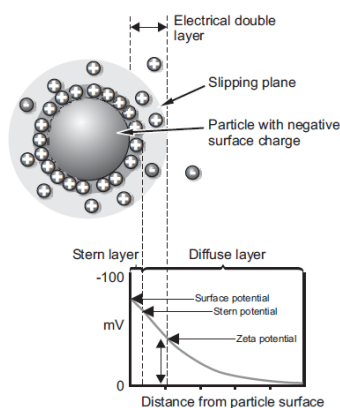


Figure 29¹⁴²：膠體溶液系統中，帶負電性的顆粒其周圍電性分布圖

Zeta potential (mV) : -55.4 ± 1.47

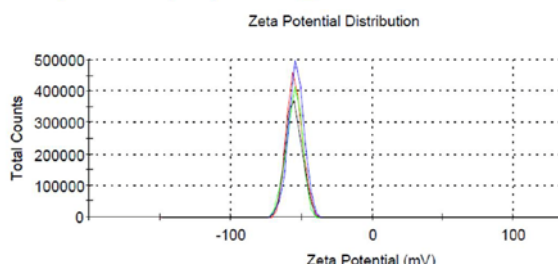


Figure 30：PLG-SPIONs nanoshuttles 之界面電位值



4.6 穿透式電子顯微鏡圖 (TEM image)

經過直接磁吸純化分離及 4.4.1 離心純化分離之樣品，以 100 倍稀釋於 ddH₂O，取 5 μ L 滴在 200 mesh 之鍍碳銅網上，未經負染得到以下的 TEM 圖；由於 PLG 為有機物所組成，使用電子束最大加速電壓為 80.0 kV。

以直接磁吸純化分離法在 Figure 31 黃色方框內可以觀察到 PLG-SPIONs nanoshuttles，但由於直接磁吸的純化方法也把沒被包覆到的 SPIONs 也一並吸附下來了，所以此視野下可觀察到聚集之 SPIONs。Figure 32 可以看到小視野下，PLG-SPIONs nanoshuttles 包埋有多顆 SPIONs。由此兩張 TEM 結果圖可以推斷，直接磁吸純化的分離方法不佳。

Figure 33-35 為 4.4.1 離心純化分離之樣品，可以觀察到 PLG-SPIONs 其形態 (morphology) 為一致的球形 (spherical) 並且包埋多顆的 SPIONs。

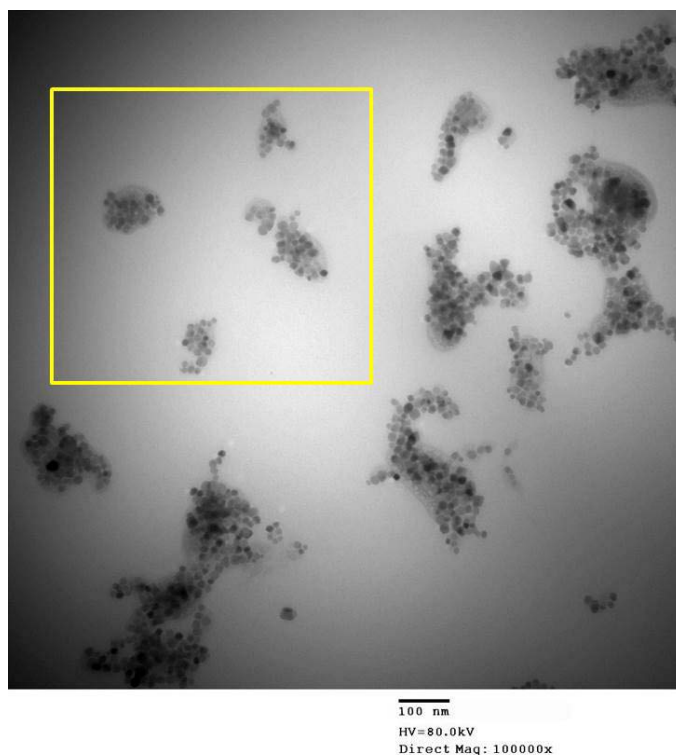


Figure 31：以 TEM 鑑定直接磁吸分離方法於大視野下之 PLG-SPIONs nanoshuttles

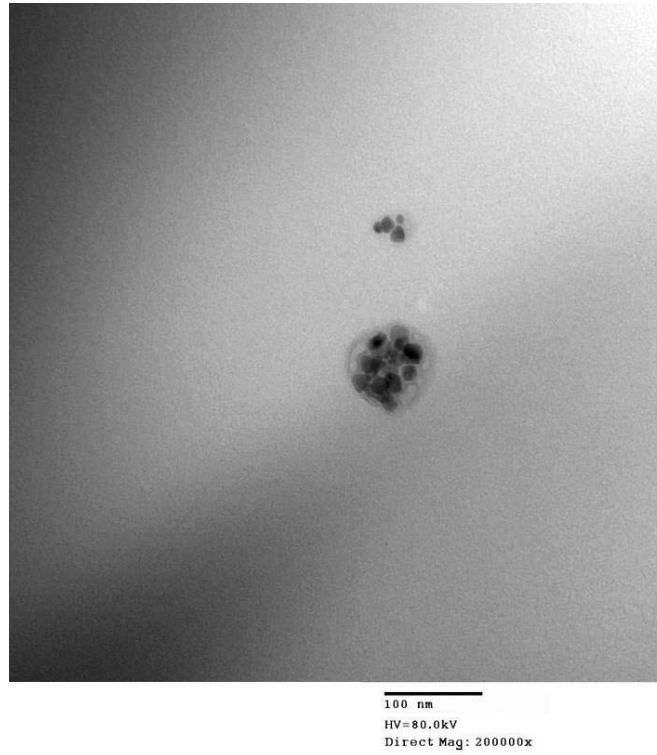


Figure 32：以 TEM 鑑定直接磁吸分離之方法，單顆 PLG-SPIONs nanoshuttles

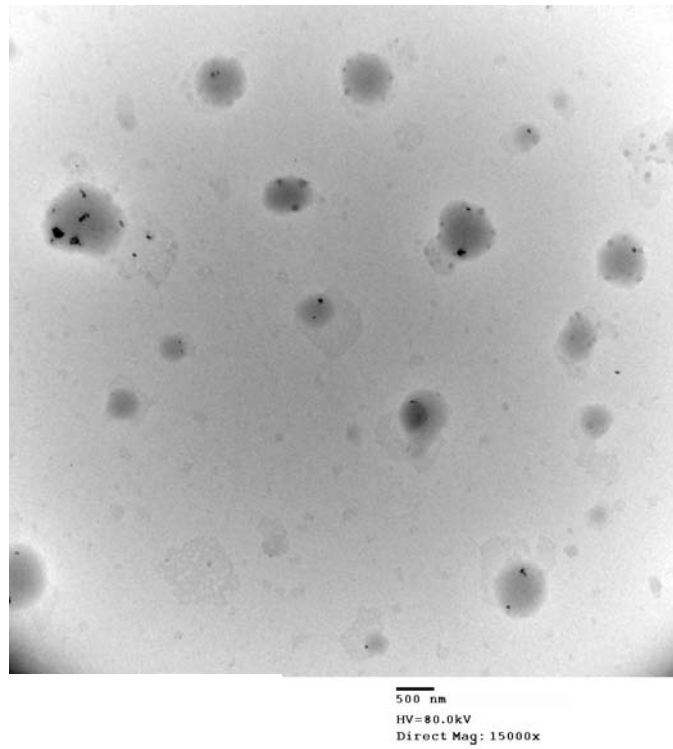


Figure 33：4.4.1 離心純化分離之樣品，大範圍下 PLG-SPIONs nanoshuttles 之 TEM

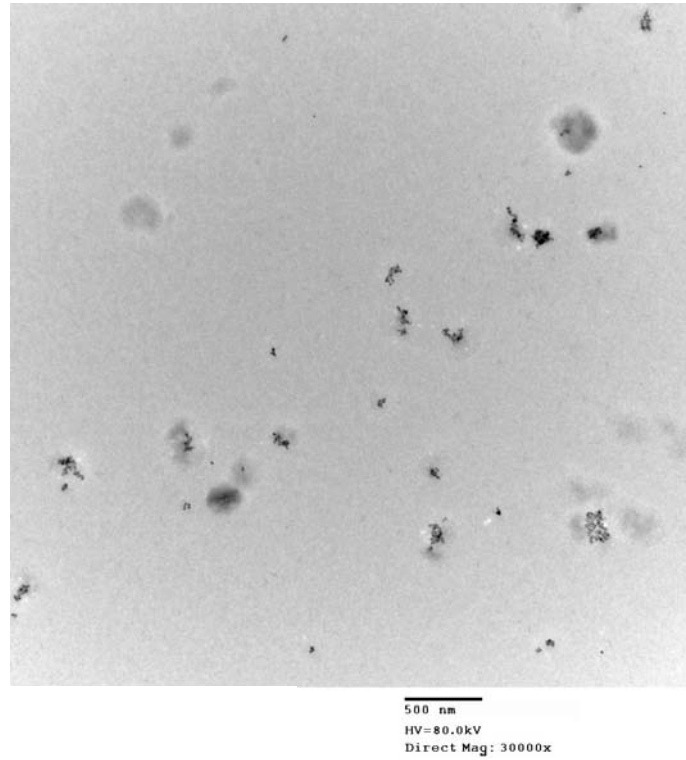


Figure 34: 4.4.1 離心純化分離之樣品，大範圍下 PLG-SPIONs nanoshuttles 之 TEM

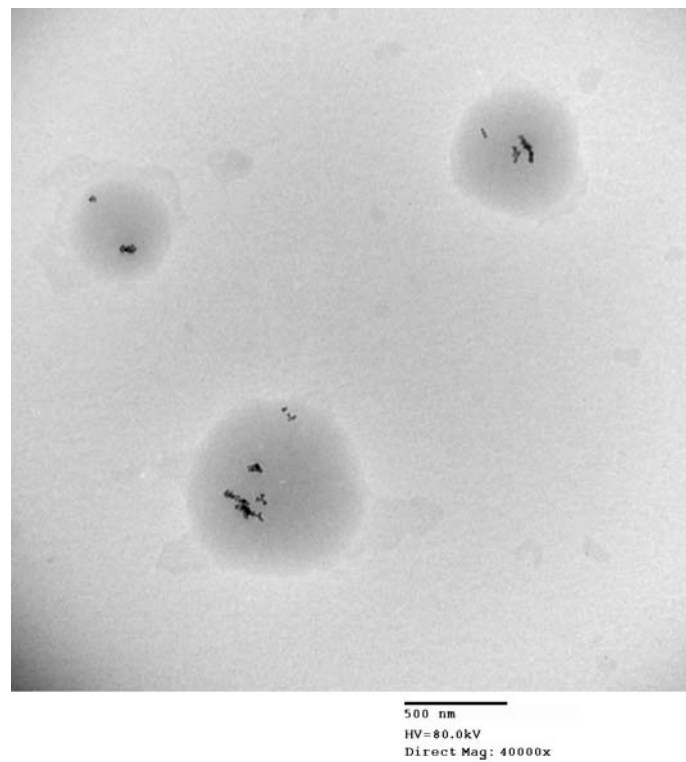


Figure 35: 4.4.1 離心純化分離之樣品，zoom-in 之 PLG-SPIONs nanoshuttles

4.7 塊材相變溫度圖 (Phase transition of bulk material)

將 2.5 wt% 12-羥基硬脂酸混合蓖麻油之塊材(bulk material)裝入樣品瓶內。

Figure 36 為倒立樣品瓶其膠態及溶膠態之圖像。

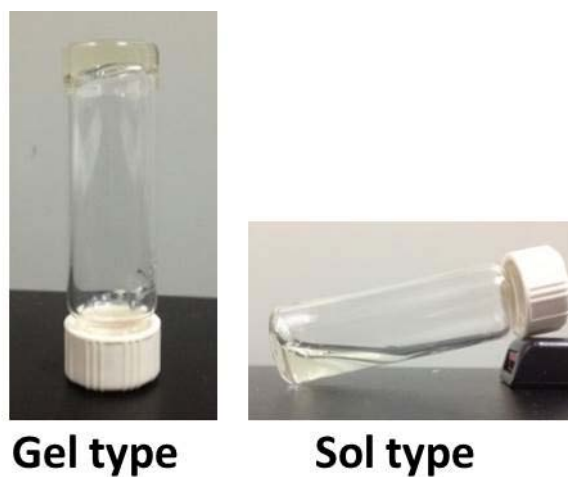


Figure 36 : 2.5 wt% 12-HSA w/ castor oil 之塊材膠態與溶膠態

4.8 示差掃描熱卡計圖 (Differential scanning calorimetry, DSC profiles)

調整 12-HSA 在 castor oil 中的重量百分比會改變其相變溫度。分別使用 2.0, 2.5, 3.0 wt% 12-HSA w/castor oil 所形成的 PLG-SPIONs nanoshuttles，經過冷凍乾燥後除去水份，取 5~10 mg 待測，升溫速率為 20 °C/min。

Figure 37 可得 PLG-SPIONs nanoshuttles 的 gel core 使用 2.0 wt% 12-HSA w/ castor oil 之相變溫度圖大約為 41 °C。Figure 38 使用 3.0 wt% 12-HSA w/ castor oil 得變溫度圖大約為 49 °C。Figure 39 使用 2.5 wt% 12-HSA w/ castor oil 得變溫度圖大約為 44 °C，確實落在溫熱治療(42 - 45 °C)的溫度範圍內。

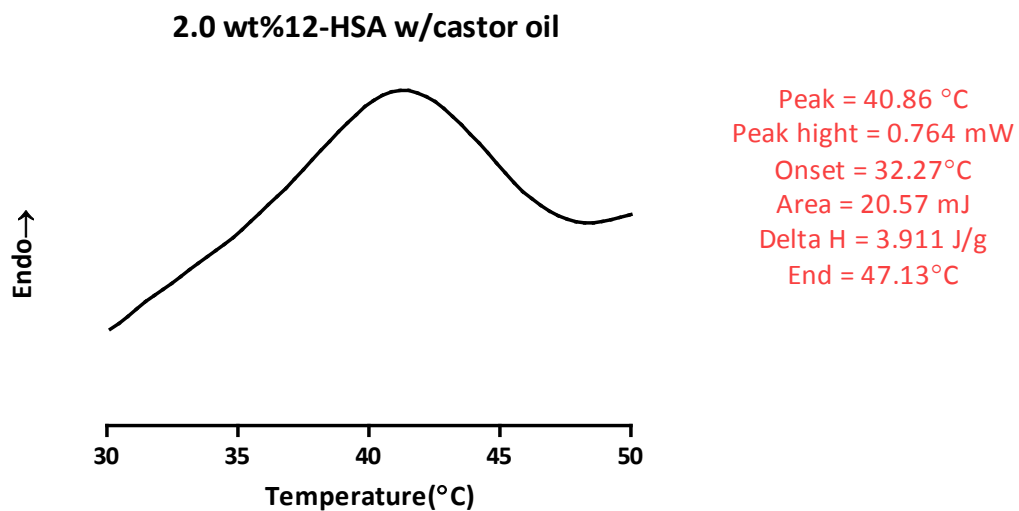


Figure 37：使用 2.0 wt% 12-HSA w/ castor oil 之 PLG-SPIONs nanoshuttles 相變溫度

圖



3.0 wt% 12-HSA w/castor oil

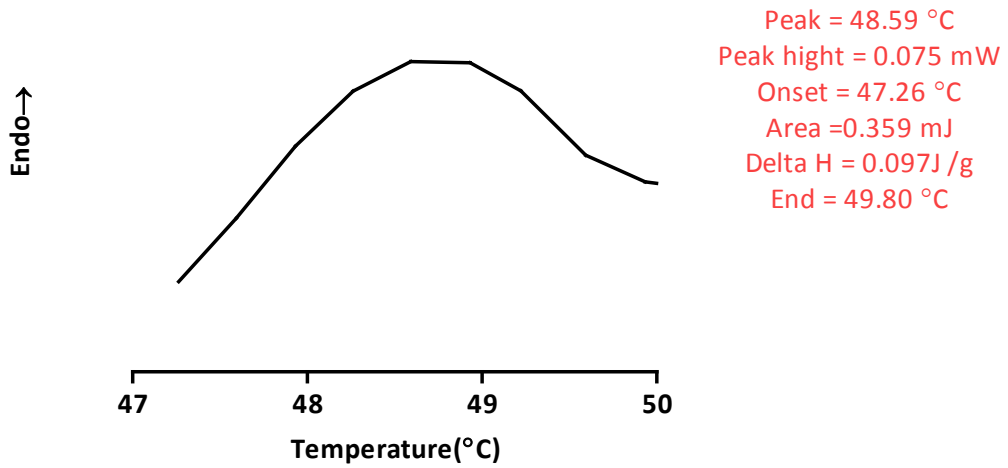


Figure 38 : 使用 3.0 wt% 12-HSA w/ castor oil 之 PLG-SPIONs nanoshuttles 相變溫度

圖

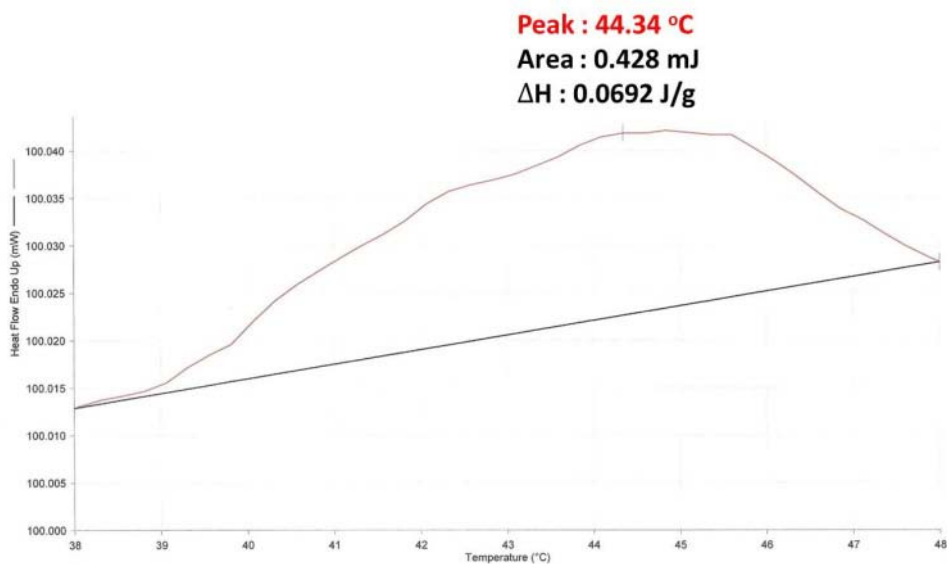


Figure 39 : 使用 2.5 wt% 12-HSA w/ castor oil 之 PLG-SPIONs nanoshuttles 相變溫度

圖



4.9 加熱曲線圖 (Heating profile)

磁吸純化分離之溶液以 5 倍稀釋於 ddH₂O 中，每管 eppendorf 250 μ L 含有 18.9 μ g_{Fe}，將 eppendorf 放置在絕熱保麗龍內再放進電磁生熱線圈內，在固定時間內取出樣品，以 k-type 熱電偶感測棒(thermocouple)偵測溫度。

使用儀器參數設定如下：交流電頻率：33.9 kHz，磁輔助場(H)：33.9/239.4/339 kA/m，功率：1.05/6.3/10.5 kW

在固定 PLG-SPIONs nanoshuttles 下，由 Table 6 及 Figure 40 之結果可得知隨著輔助磁場增加與 dT/dt 的斜率變化有正相關之現象。

Table 6：不同功率下，PLG-SPIONs nanoshuttles 其加熱曲線之 dT/dt 斜率值

H (kA/m)	Power (kW)	Slope : dT/dt (K/s)
33.9	1.05	1/150
239.4	6.3	7/375
399	10.5	41/1000

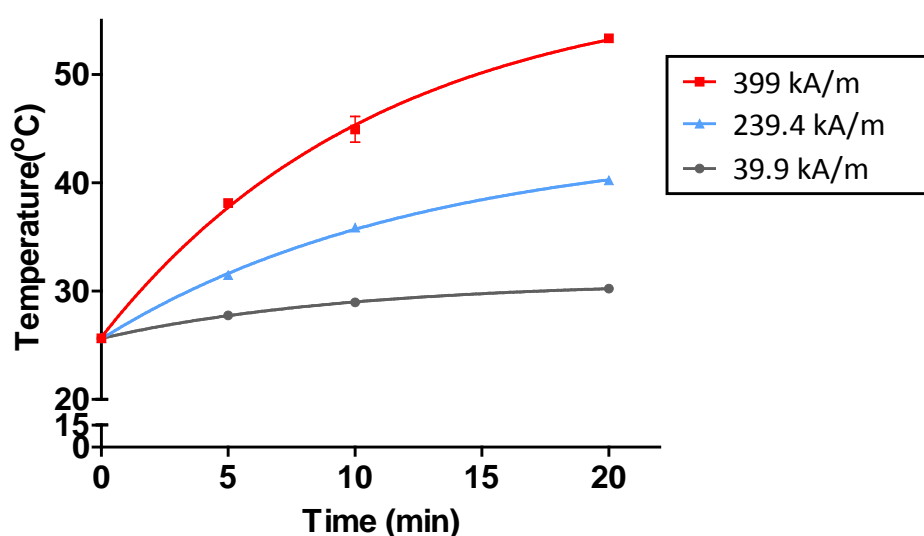


Figure 40：PLG-SPIONs nanoshuttles 加熱曲線圖



4.10 損耗功率值 (Specific loss power, SLP value)

$$SLP = CV_s/m \cdot dT/dt$$

C : Volumetric specific heat capacity of the sample solution (J/L·K)

V_s : Sample volume (L)

m : Mass of magnetic material in the sample (g)

dT/dt : Initial slope of the change in temperature versus time curve (K/s)

已知 H₂O 在 1 atm, 25 °C 下的比熱為 4179.6 J/L·K，由 Table 6 可推算出 SLP 值(Table 7)。

Table 7 : 固定 PLG-SPIONs nanoshuttles 之濃度其損耗功率值

H (kA/m)	功率 (kW)	Slope : dT/dt (K/s)	SLP (W/g _{Fe})
33.9	1.05	1/150	369
239.4	6.3	7/375	1032
399	10.5	41/1000	2267

$$(SLP_{1.05 kW} = \frac{4179.6 \text{ J/L} \cdot \text{K} \cdot 250 \mu\text{L}}{18.9 \mu\text{g}_{Fe}} \cdot \frac{1 \text{ K}}{150 \text{ s}} = 369 \text{ W/g}_{Fe})$$



4.11 T2 顯影圖 (In tube T2-weighted imaging)

1x 的樣品為 PLG-SPIONs nanoshuttles 原液，並以 PBS 為稀釋溶液。由於 SPIONs 是 MRI T2 顯影劑，所以 Figure 41 之 T1-weighted imaging 無變白之影像屬合理狀況。由 Figure 42 隨著稀釋倍率降低其影像確實有變黑，但沒有非常顯著其原因從 TEM Figure 33 圖可得知 PLG-SPIONs nanoshuttles 中所包覆的 SPIONs 數量可能略少，所以變黑的效果有限；另外對照組為無包覆 SPIONs 的 PLG nanoshuttles 熱敏感載體其 T2-weighted 影像是無變化的。

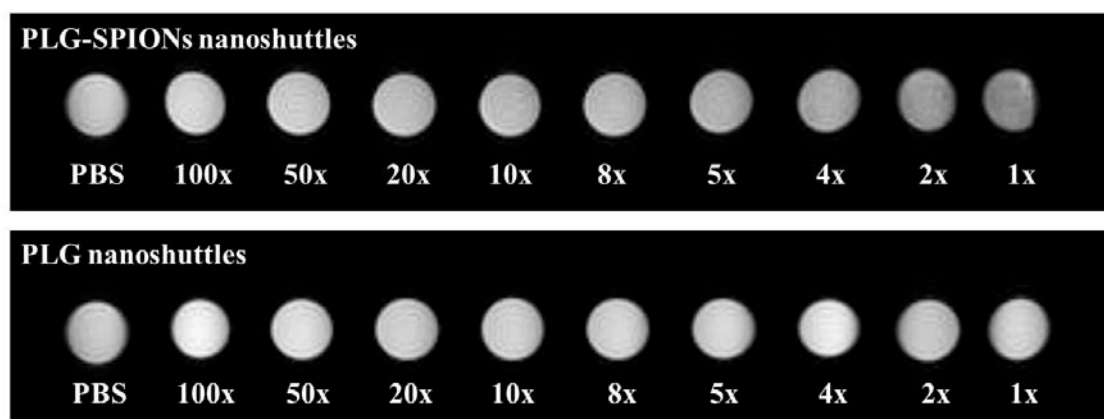


Figure 41 : PLG-SPIONs nanoshuttles in tube T1-weighted imaging

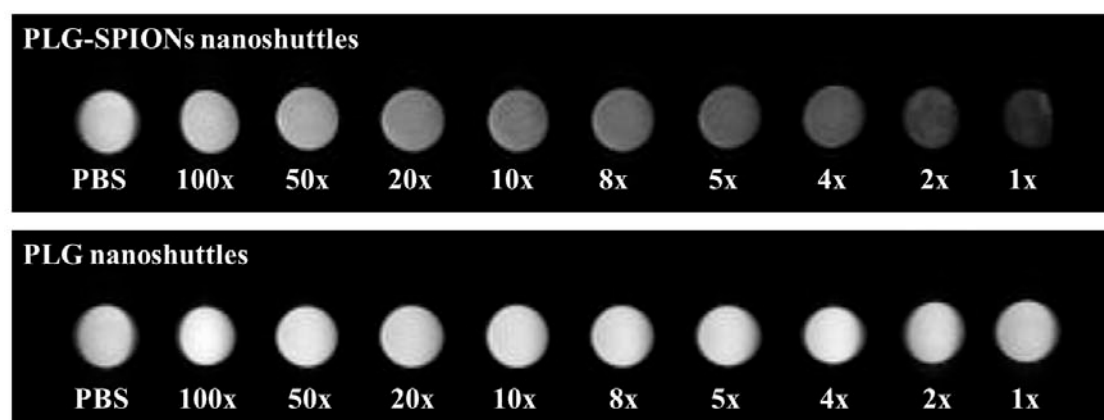


Figure 42 : PLG-SPIONs nanoshuttles in tube T2-weighted imaging

4.12 藥物釋放曲線圖 (In tube drug release profile via direct heating)

包覆 coumarin-6 螢光染劑的 PLG-SPIONs nanoshuttles，以直接加熱方法在不同時間點取樣，經過磁吸純化分離後的沉澱物以 DMSO 回溶，取其上清液測螢光強度。

以室溫下時間為零點所測得的 coumarin-6 螢光強度視為 100 % 釋放。

(Cumulative release % = $(1 - \text{intensity(a.u.)}_{\text{different time}} / \text{intensity(a.u.)}_{\text{initial}} \cdot 100\%$) Figure 43 為乾裕槽內直接加熱的釋放曲線圖，由此可顯觀察出加熱至 45°C 的組別會隨著溫度上升，coumarin-6 所釋放的量有上升的趨勢。

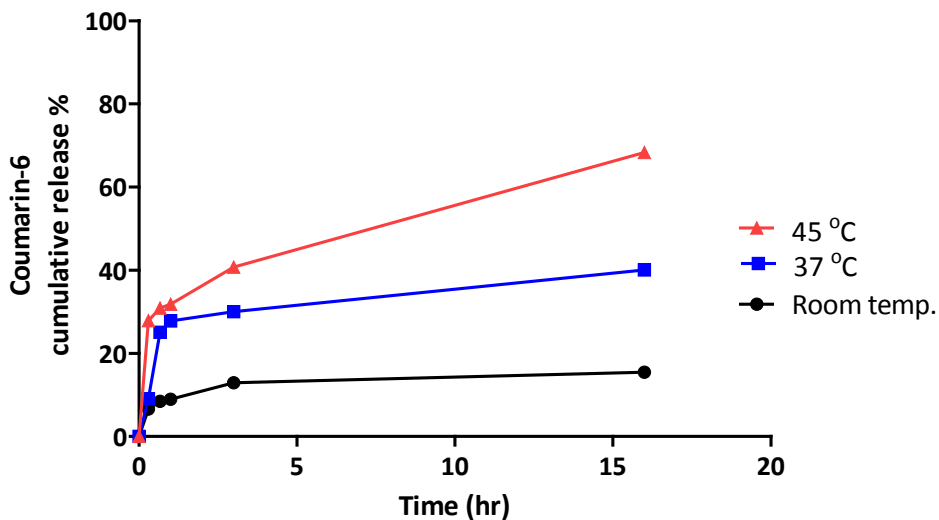


Figure 43：以直接加熱其 PLG-SPIONs nanoshuttles in tube 之 coumarin-6 累積釋放

圖

4.13 藥物包覆率之計算 (Encapsulation efficiency percentage of CPT)

將 CPT 溶於 DMSO 中($\lambda_{ex} = 370 \text{ nm}$, $\lambda_{em} = 428 \text{ nm}$)得一檢量曲線(Figure 44)。使用 4.4.1 離心純化分離後之 PLG-SPIONs nanoshuttles 溶液取 200 μL ，磁吸 5 分鐘移除上清液，加入 200 μL DMSO 回溶 shaker 上震盪 5 分鐘將 PLG-SPIONs nanoshuttles 破壞，再磁吸 5 分鐘，取上清液測其螢光強度，並回推藥物包覆率為 5.15%。

$$\text{Encapsulation efficiency \%} = (\text{detected concentration}/\text{initial concentration}) \cdot 100\%$$

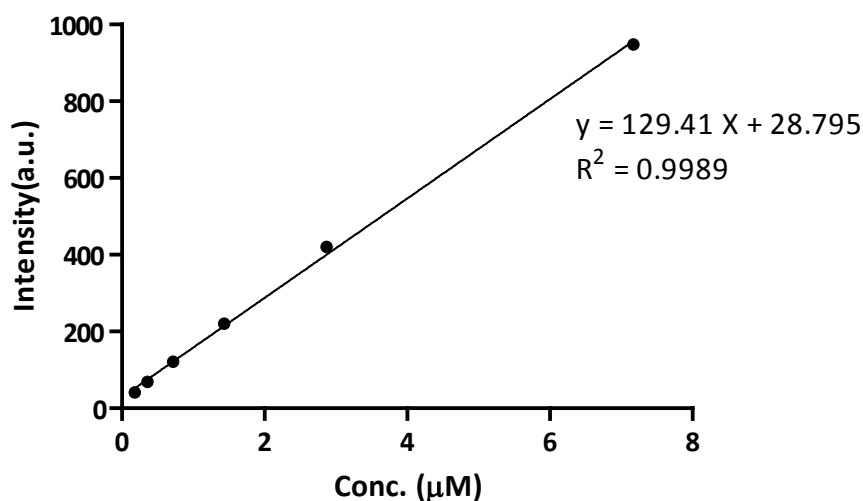
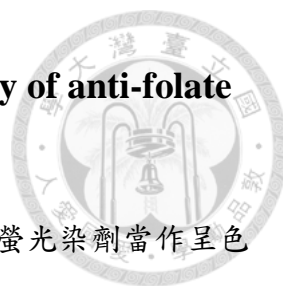


Figure 44 : CPT 溶於 DMSO 之檢量曲線







4.14 免疫試片呈色反應 (Membrane strip immunoassay of anti-folate v.s. anti-biotin)

表面修飾葉酸之 PLG-SPIONs nanoshuttles，包覆 Nile red 之螢光染劑當作呈色之指標。待測樣品溶液以 2 倍稀釋於 PBS 溶液中。

使用 pre-wet buffer (10 % MeOH in 10 mM phosphate buffer, 0.15 M NaCl and 0.01 % NaN₃, pH= 7.0) 前處理過的 strip 試片乾燥後，使用濃度為 0.1 mg/mL 的抗體 (anti-folate and anti-biotin)，每次取 1 μL 點樣於 1 cm x 1 cm 之 strip 上，並重複點樣 5 次，室溫真空箱抽乾 30 分鐘後，將 strip 放入 blocking buffer (0.02 M Tris-HCl, 0.15 M NaCl, 0.5 % PVP, 0.03 % casein, 0.01 % NaN₃, pH= 7.0) 於室溫反應 30 分鐘，完成 blocking 的 strip 與待測樣品溶液於 4 °C 環境下反應 over-night，待反應完成後，移除待測樣品溶液以 PBS 潤洗 5 分鐘重複 2 次。最後將反應完成的 strip 於室溫真空箱抽乾 30 分鐘後，於 UV 燈下檢視實驗結果。

Table 8 是實驗二重複反應之結果圖，很明顯在 anti-folate 的組別有明顯的螢光亮點，表示有特異性吸附，然而控制組的 anti-biotin 則是沒有反應，表示 PLG-SPIONs nanoshuttles 上確實有修飾帶有葉酸修飾的磷脂質分子。

Table 8：免疫試片呈色反應

Anti-folate (positive-control)		
Anti-biotin (negative-control)		



4.15 *In vitro* 實驗 (*In vitro* experiments)

4.15.1 實驗材料

培養條件：

培養液為 DMEM(10% FBS, 1% P/S)。培養箱條件為 37°C, 5 % CO₂

細胞密度：

$5 \cdot 10^4$ cell/0.5 mL (24-well plate)

實驗方法：

細胞貼盤 24 小時後，下藥與細胞共培養 3.5 小時，除去 DMEM 以 PBS 沖洗 2 次去除未被細胞攝入的 PLG-(CPT)/SPIONs nanoshuttles，並更換新的 DMEM，額外培養 48 小時後移除 DMEM 以 PBS 沖洗 2 次，更換新的 DMEM 並加入 0.5 mg MTT/mL 反應 1 小時。以 MTT assay 測細胞存活率，其原理為 MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole) 與活細胞內粒線體的脫氫酶(dehydrogenases)反應，tetrazolium 被反應還原成 MTT formazan 之藍紫色非水溶性的結晶，最後以 DMSO 將結晶溶解於波長 570 nm 下測得吸收值。

4.15.2 實驗組別

PLG-CPT/SPIONs nanoshuttles 或 PLG-SPIONs nanoshuttles 序列稀釋 5 組如 Table 9，並與對照組比較其細胞存活率(每組實驗三重複)。

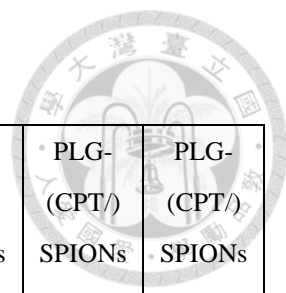


Table 9 : *In vitro* 實驗設計組別

實驗組別 細胞株	No drug	Free drug CPT	PLG-(CPT)/SPIONs conc. 1	PLG-(CPT)/SPIONs conc. 2	PLG-(CPT)/SPIONs conc. 3	PLG-(CPT)/SPIONs conc. 4	PLG-(CPT)/SPIONs conc. 5
HeLa (positive CTRL) (FR-overexpression)	Medium only	20 ng/mL	1.25 ng/mL	2.5 ng/mL	5.0 ng/mL	10 ng/mL	20 ng/mL
A549 (negative CTRL) (Non-FR-overexpression)							

4.15.3 熱敏感載體對細胞之毒性

PLG-SPIONs nanoshuttles 與 PLG-CPT/SPIONs nanoshuttles 取同體積的量如 Table 9，以 MTT assay 測細胞存活率。由 Figure 45 之結果可以得知 PLG-SPIONs nanoshuttles 其材料組成對細胞是不具毒性的，所以安全性高。

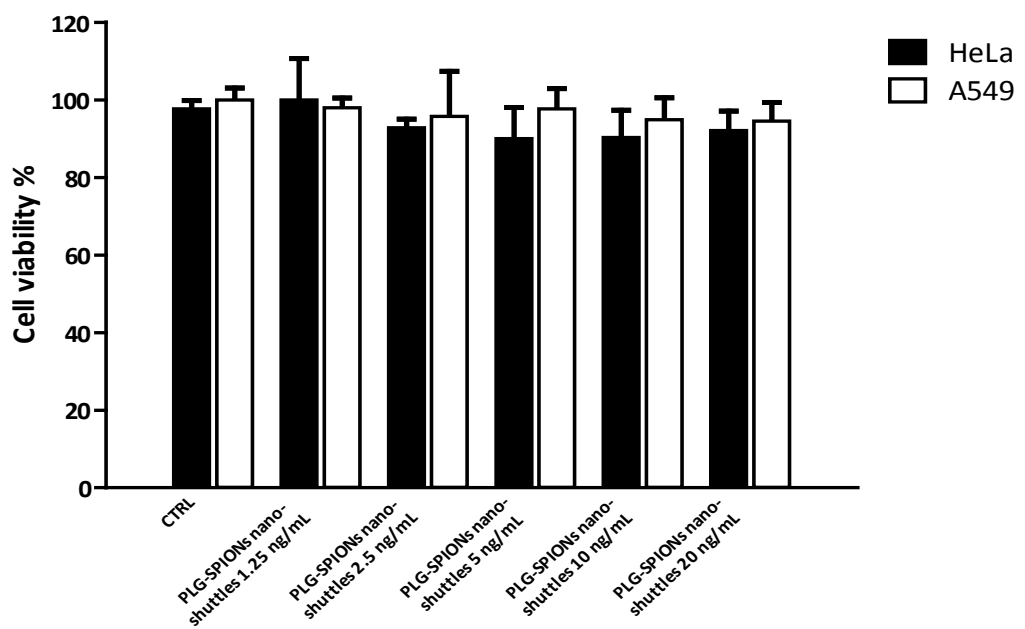


Figure 45 : PLG-SPIONs nanoshuttles 的細胞毒性存活率

4.15.4 包覆喜樹鹼之熱敏感載體其細胞存活率

如 Table 9 的實驗組別。Figure 46 之細胞存活率可得知，直接使用喜樹鹼(free CPT)與 PLG-CPT/SPIONs nanoshuttles 對細胞的毒殺能力是有差異的，而 HeLa cell 是葉酸受體高度表現的癌細胞株，對照組 A549 為葉酸受體不表現之癌細胞株，由此結果可歸論出，帶有葉酸修飾的 PLG-CPT/SPIONs nanoshuttles 確實對葉酸受體高度表現的癌細胞株有較強的毒殺能力。

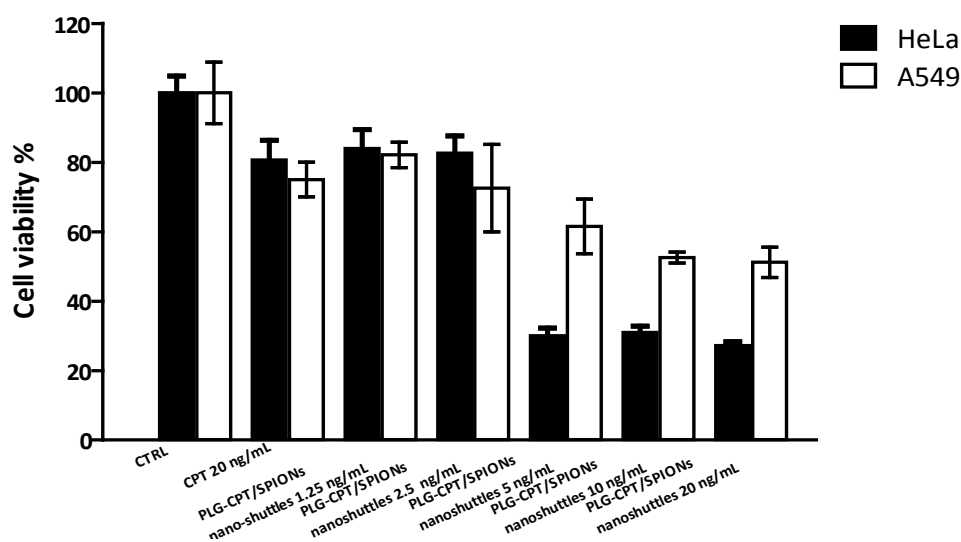


Figure 46：無外加磁場下 PLG-CPT/SPIONs nanoshuttles 之細胞存活率

第五章 結果與討論



以材料製備鑑定、in tube 實驗及初步 *in vitro* 實驗分別進行探討。

此熱敏感性的 PLG-SPIONs nanoshuttles 其粒徑大小約為 260 nm 及界達電為值約為 -55 mV，確實符合藥物傳遞系統的尺度且是個穩定的膠體溶液系統，然而 TEM 結果圖可以發現 PLG-SPIONs nanoshuttles 內所包覆的 SPIONs 數量可能略少，導致 MRI T2 顯影效果不夠顯著，所以這部分的實驗方法有待未來調整至最佳化。既然 PLG-SPIONs nanoshuttles 的 gel core 是熱敏感性材料所組成，由 DSC 測得相變溫度得約為 44°C，確實落在溫熱治療溫度範圍內。

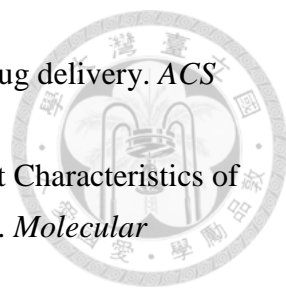
以直接加熱的釋放曲線圖可以觀察出，在 45°C 能有效地釋放藥物，但由於實驗時間不夠久，所以有待未來做更長的時間，去探究之後的釋放狀況是不是會到達飽和點。初步實驗證實，施予高頻率電磁場 (frequency : 33.9 kHz, H : 33.9 kA/m) 後，PLG-SPIONs nanoshuttles 確實有優良的升溫效果其 SLP 值為 369 W/g_{Fe}，所以未來也需要做一組以電磁生熱的方式其藥物釋放曲線圖，去探討其釋放模式與效率是否比直接加熱法更有效也有待確認。

經過初步 *in vitro* 實驗證實，PLG-SPIONs nanoshuttles 無細胞毒性，且包覆喜樹鹼之 PLG-CPT/SPIONs nanoshuttles 對癌細胞的毒殺能力較 free drug CPT 強，未來需證明經過啟動外部電磁場後，PLG-CPT/SPIONs nanoshuttles 確實結合化學藥物治療及溫熱治療強化了毒殺癌細胞的有效性，並且此熱敏感載體應可在 *in vivo* 模式中被再次證實其化療及溫熱治療之協同效應。

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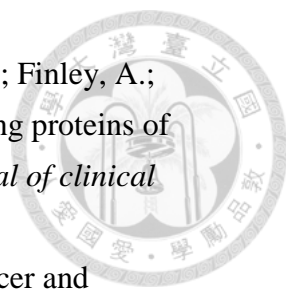
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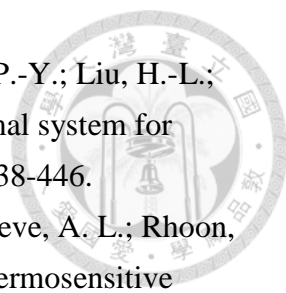
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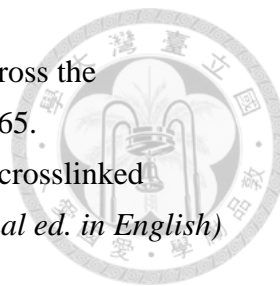
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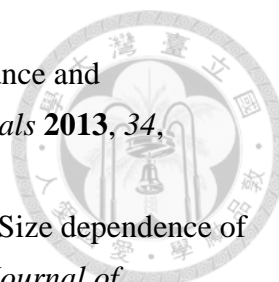
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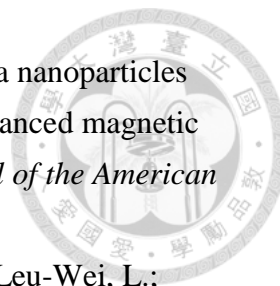
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