國立台灣大學醫學院暨工學院

醫學工程學研究所

博士論文

Institute of Biomedical Engineering

College of Medicine and College of Engineering

National Taiwan University

Doctoral Dissertation

合成磷酸鈣磁性奈米粒子於癌症熱療之應用

Calcium Phosphate-Based Magnetic Nanoparticles as

Thermoseeds for Cancer Hyperthermia Therapy

侯君翰

Chun-han Hou

指導教授：林峯輝 博士

Advisor: Feng-huei Lin　Ph.D.

中華民國九十八年七月

July, 2009

國立臺灣大學博士學位論文
口試委員會審定書

合成磁性磷酸鈣奈米粒子於癌症熱治療之應用
Calcium Phosphate-Based Magnetic Nanoparticles as Thermoseeds for Cancer Hyperthermia Therapy

本論文係侯君翰君（學號：D95548016）在國立臺灣大學醫學工程學研究所完成之博士學位論文，於民國 98 年 07 月 17 日承下列考試委員審查通過及口試及格，特此證明

口試委員：

　　　　　　（指導教授）

所長：

致謝

　　三年博士班一眨眼就過去了，這三年當中的後兩年，我奉派到雲林分院服務。鄉下地方人力不足，從急診、住院值班、開刀、術後照顧都一手包，然後假日又要北上兼顧課業、做實驗、寫論文，還要提心吊膽祈禱病人不要突然出狀況，這些甘苦沒有親身經歷過是不會瞭解的。這當中我第一個要感謝的是我的指導教授— 林峯輝老師。老師在這段期間為了配合我的時間（因為我常常不在台北），不惜犧牲假日，在星期六日或週一很晚的晚上，和我及學弟妹、助理們討論研究進度和方向，也幫我找了許多學弟妹來幫忙我一起作實驗，減輕我的負擔。我還記得有一天週日遇到颱風天，老師穿著涼鞋、親自和我及學弟一起到總區工學院的地下室作實驗。作為一位教授，實在是對學生太好了，甚至我常常跟他說：「您對學生太好了，以致於學生都爬到您頭上」。爬到頭上的學生們，很遺憾的，我也是其中之一，不過我既不是第一個，也不是最後一個。第二個要感謝骨科 林晉教授在研究經費（助理費用）上面的大力幫忙、不求回報，讓我有很大的空間可以嘗試錯誤，自由發揮。另外要感謝口試委員們在百忙之中撥空參加，提供論文上的一些指導和意見，讓論文可以更加地完整。

　　實驗室的同學們在我三年的研究生活中，給我許多幫助。大頭、文熙、蘇 P、靖孋、老周、家菁、郁君、安寶，都是我請益的對象。薛育昇和李玉婷同學分別擔任我一段時間的助理，在實驗上幫忙許多，我們也歷經許多失敗、結果不如預期等等的挑戰，但我們最後都一起克服了。我表弟楊政達（現為台大醫院影像醫學部住院醫師）利用服完兵役、上班前的空檔幫我作 in vitro biocompatibility test，在此感謝。還有功力高強的凱強在最後一年的幫忙，可以用兩肋插刀來形容，我們也常常週六（甚至週日）一起悶在暗無天日、沒有目擊者的實驗室和動

I

物中心裡，一起「男男」…ㄟ…「喃喃自語」的做著實驗。助理呂岱樺小姐在最後半年加入，也在文獻編排、資料整理、動物實驗及行政事物上幫忙許多。

最後感謝我的父母和家人，沒有你們在求學和日常生活上的栽培和支持，很難想像有現在的我。還有林鳳玲--我的太太，能夠在研究的日子中陪伴我，我雖然沒有像牛頓一樣生活白癡，把手錶放進大同電鍋裡煮，但的確家裡食衣住行（不含育樂）的大小事，都是她一手包辦，讓我可以放手追求我學術上的夢想。衷心希望你們都能健康快樂。
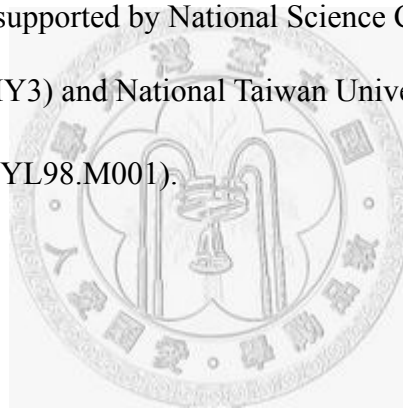
Abstract

Hyperthermia therapy for cancer has drawn more and more attention these days. Many different types of magnetic particles have been developed for the purpose of hyperthermia cancer therapy. In the first part, we conducted an *in vivo* cancer hyperthermia study of the new magnetic hydroxyapatite nanoparticles (mHAP) on a mouse model. Only the mice which were injected with mHAP and had been treated inside the magnetic field showed dramatic reduction of tumor volume, in the 15-day observation period. No local recurrence was noted. Therefore, our new magnetic hydroxyapatite nanoparticles have demonstrated therapeutic effect in a mouse model with little toxicity.

In the second part, a magnetic nanoparticle based on dicalcium phosphate dihydrate (DCPD) was formed by co-precipitation method. Addition of different concentrations of ferrous chloride to DCPD can alter its material properties. Various physical, chemical and magnetic tests of the magnetic DCPD nanoparticles (mDCPD) were performed, including x-ray diffraction (XRD), inductively coupled plasma-optical emission spectrometer (ICP-OES), superconducting quantum interference device (SQUID), and transmission electron microscopy (TEM). The heating efficiency of mDCPD in alternating magnetic field was proved to be suitable for hyperthermia. The results of cytotoxicity tests (WST-1 and LDH assay) showed no harmful effect. The mDCPD showed relative cancer-killing ability without damaging normal cells *in vitro*.

Keywords: magnetic nanoparticles, cancer, hyperthermia, hydroxyapatite, dicalcium phosphate dehydrate.

摘要

　　腫瘤熱治療是目前非常熱門的研究題目，而許多新型的磁性奈米粒子也被研發出來，作爲熱治療的熱種子（thermal seeds）。在本論文的第一部份，我們嘗試以氫氧基磷灰石（hydroxyapatite）爲基底之磁性奈米粒子（magnetic hydroxyapatite nanoparticles，簡稱 mHAP）做爲熱種子，注射入小鼠皮下腫瘤附近。結果於 15 天的觀察期內，只有注射 mHAP 及置入高頻交互磁場之小鼠，其腫瘤會在五天內迅速縮小。並且老鼠犧牲後之血清測試證明 mHAP 之毒性甚低。第二部分則是以雙水雙鈣磷酸鹽 dicalcium phosphate dihydrate (DCPD)爲基底合成新的磁性奈米粒子（mDCPD），隨著不同的鐵含量被加入，其所表現的物理化學性質皆有顯著的不同，可以用 X 光散射 x-ray diffraction (XRD), 感應耦合電漿發射光譜分析儀 inductively coupled plasma-optical emission spectrometer (ICP-OES), 超導量子干涉儀 superconducting quantum interference device (SQUID), 和穿透式電子顯微鏡 transmission electron microscopy (TEM)來檢查。加熱效率也在可接受之範圍內。細胞毒性測試(WST-1 and LDH assay)亦顯示其無毒性。在體外試驗中，mDCPD 能顯著地殺死癌細胞而不損傷正常細胞。
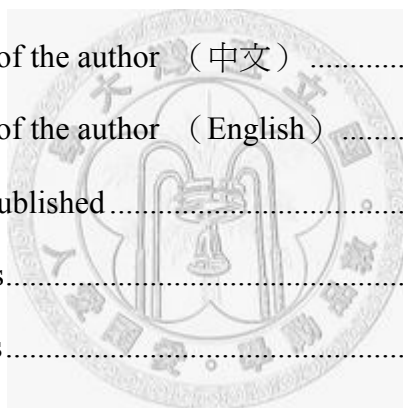

關鍵字: 磁性奈米粒子、癌症、腫瘤熱治療、氫氧基燐灰石、雙水雙鈣磷酸鹽

Contents

List of tables

List of figures

Chapter 1   Introduction

1.1 Prologue

According to the health record of Year 2009 published by Department of Health, Executive Yuan, cancer is the leading cause of death in Taiwan for straight 27 years (Table 1-1). It is also the second leading cause of death in United States in 2003 [1]. Cancer has become a serious threat to human life. Comparing with the mortality rates of United States in 1950 and 2003, we can discover the mortality rates of heart diseases and cerebrovascular disease have declined significantly after 50 years. (Fig. 1-1) [2]. However, the death rate of cancer does not decline significantly and maintains almost the same, despite of many technologic advances in the 50 years.

Table 1-1   Ten leading cause of death, Taiwan, 2009

Rank	Cause of death	% of all death
1	Cancer	27.3
2	Heart Diseases	11.1
3	Cerebrovascular diseases	7.5
4	Pneumonia	6.1
5	Diabetes mellitus	5.6
6	Accidents	5.0
7	Chronic lower respiratory diseases	3.8

8	Chronic hepatitis and liver cirrhosis	3.5
9	Suicide	2.9
10	Nephritis and kidney disease	2.8

Hyperthermia, with or without other concurrent cancer therapies, remains a promising option of cancer treatment.[3] Hyperthermia can induce the death of cancer cells by simply raising temperature and can minimize complications of harming normal cells. The mechanism of hyperthermia and the purpose of this study will be introduced in the following sections.
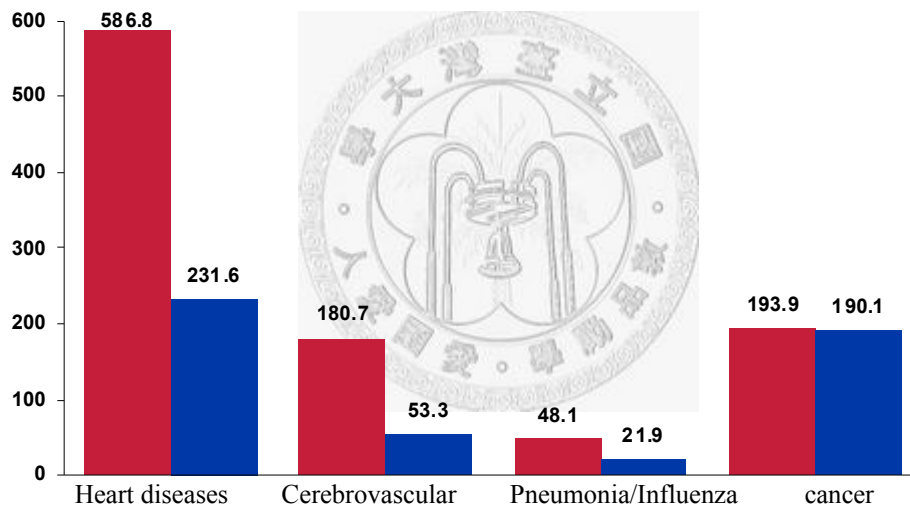
Fig. 1-1 Change in the US Death Rates (death per 100,000) by cause, 1950(red) & 2003(blue) [2]

1.2 Cancer

The definition of tumor and cancer remains uncertain until 1930. Jamie Ewing, who was a pathologist, gave a definition of neoplasm: a neoplasm is relatively autonomous growth of tissue [4]. So called "autonomy" means the cells insides a tumor are out of control. However, the autonomy of cancer is relative, not absolute [5]. Actually, the growth rate of cancer can be as slow as normal cells in an extreme situation, or as fast as embryo tissues in another extreme situation.

All abnormal mass or abnormal growth of tissue are called tumor (or neoplasm) , such as swellings, bruises, scars, or lumps. They might be a simple aggregation of normal tissue. Cancer can be named as malignant neoplasm by scientists and physicians. Broadly speaking, cancer is an abnormal cell, a cell transformed by virus, chemical matters or other things, a kind of tumor, and a kind of malignant neoplasm [6].

Cancer cells are different from normal cells because of their special growth behavior. It includes five parts: proliferation, metaplasia, dysplasia, anaplasia, and metastasis [6].

1. **Proliferation**: the basic characteristic of cancer cells. The cell division is unstoppable if not intervened. But we cannot differentiate cancer and normal cells simply by proliferation itself, because some normal cells in human body also carry this character. [7].

2.   **Metaplasia**: In a specific organ or tissue, adult cells can be substituted by another type of adult cells. It is usually reversible and can be a pre-clinical stage of tumor or cancer.

3.   **Dysplasia**: It means that sizes, shapes and compositions of mature cells change from original morphology. Dysplasia is a common character of cancer cells, but it also happens in normal cells. However, dysphasia is reversible in normal cells [8].

4.   **Anaplasia**: A reversion of differentiation in cells that is characteristic of malignancy in tumors. Sometimes, the term also includes an increased capacity for multiplication.[1] Lack of differentiation is considered a hallmark of malignancy. The term anaplasia literally means "to form backward." It implies dedifferentiation, or loss of structural and functional differentiation of normal cells. Normal cells seldom have the phenomenon of anaplasia.

5.   **Metastasis**: Metastatic disease, sometimes abbreviated mets, is the spread of a disease from one organ or part to another non-adjacent organ or part. Cancer cells can peel off from its primary tumor, enter lymphatic and blood vessels, circulate through the bloodstream, and settle down to grow within normal tissues elsewhere in the body. Generally speaking, the major difference between malignant tumor and benign tumor is that malignant tumor would metastasize benign tumor not.

1.3 Cancer therapy

1.3.1 Surgery

Surgical excision is the earliest and most common therapeutic method for cancer treatment. It is usually the first line of cancer treatment. Therefore, the importance of surgical excision is higher than radiation and chemotherapy because it has more opportunities to remove the whole tumor. Some cancer has no definite "tumor mass", such as leukemia or myeloma and hence surgery is not possible. However, for most of the cancers, surgery is the most effective way for curing the cancer. Generally speaking, cancer surgery is operated on the primary cancer site [9]. During surgery, doctors would excise the adjacent normal tissues or lymph node next to the tumor tissue, so called as a safety "margin", in order to decrease the possibility of metastasis or local recurrence. Sometimes surgeon will perform metastectomy -- excision of the metastatic lesion. However, this procedure is often a salvage one.

Before a definite surgical excision, or even chemotherapy and radiotherapy can begin, a procedure called "biopsy" is mandatory to performed. It is a procedure in diagnose of malignant disease that provides doctors and pathologists with adequate samples of tumor. It is very crucial to have a correct tissue proof of cancer, since complication rates of cancer treatment are high. Several biopsy techniques are available, including exfoliative cytology, punch biopsy, incisional biopsy, excisional biopsy, shave biopsy, and needle aspiration biopsy [10].

Although cancer surgery is the most direct treatment, there are still many shortcomings. Cancer cells carries the ability to metastasize, which makes them possible for speading to other parts of human body. Local recurrence is another bothersome complication after surgery. Therefore, radiation therapy or chemotherapy, as adjunct therapies before or after surgery, are usually mandatory to eradicate the cancer [11, 12]. Other complications of surgical site include pain, disability after amputation or excision, and infection. In summary, cancer surgery is one of the most prevailing cancer therapies, but there are still many drawbacks and rooms for breakthrough.

1.3.2 Radiation

Radiation therapy (also radiotherapy or radiation oncology, sometimes abbreviated to XRT) is the medical use of ionizing radiation as part of cancer treatment to control malignant cells. Radiation oncology is an effective and convenient clinical therapy[13, 14]. Ionizing radiation usually comes from X-ray or gamma rays from natural radioisotope, because these rays possess a specific frequency or wavelength and radiate in form of quantum or photons. By absorbing directly high energy of particles, it can generate free radicals to disturb cancer cell division and destroy them. Because the free radicals in a serial of reactions act on organic compounds such as DNAs, RNAs, enzymes or other proteins, the change of original structure and the loss or function can be predicted.

Radiotherapy may be used for curative or adjuvant cancer treatment. It is used as palliative treatment (where cure is not possible and the aim is for local disease control or symptomatic relief) or as therapeutic treatment (where the therapy has survival benefit and it can be curative).

There are many advantages of radiation therapy. First, It dose not rely on the transportation of drugs by blood vessels or diffusion into target tissues. Thus it is free of systemic toxicities and anatomical restrictions. Second, the preservation of the structure and function of normal tissue can be ideally ensured. Finally, anesthesia is usually not needed. Therefore, compared with surgery, radiation therapy has less impact on patients' medical status.

However, there are still shortcomings and side effects of radiation therapy. Potential radiation injury still can be found in normal tissues or organs easily. For example, radiation therapy may damage the following organs: liver for hepatitis, lung for acute and chronic pneumonitis, heart for pericarditis and lens for cataract. The detail information can be seen in Table 1-2 [12]. The possibility of occurrence of undesirable sequela can been reduced by better equipment, more precise dosimetry, more careful computer-assisted treatment planning. Those are the recent advances in radiation therapy. Moreover, radiation therapy have no systemic effect on multiple metastasis of cancer cells. Therefore radiation, same as surgery, needs to combine with other therapies like chemotherapy as an auxiliary treatment.

Table 1-2 Radiation complications [12]

	Organ	Injury	$CD_{5/5}$* (cGy)	$CD_{5/50}$† (cGy)	Portion of Organ
Potentially severe or fatal radiation injury	Bone marrow	Pancytopenia, aplasia	250	450	Whole
			3,000	4,000	Segmental
	Liver	Hepatitis	2,500	4,000	Whole
	Stomach	Ulcer, hemorrhage	4,500	5,500	100 sq cm
	Intestine	Ulcer, perforation	4,500	5,500	400 sq cm
	Rectum	Stricture, ulcer	6,000	8,000	100 sq cm
	Brain	Infarct, necrosis	6,000	7,000	Whole
			7,000	8,000	25%
	Spinal cord	Infarct, myelitis, necrosis	4,500	5,500	10 cm
	Heart	Pericarditis	4,500	5,500	60%
	Lung	Acute and chronic pneumonitis	3,000	3,500	100 sq cm
	Kidney	Acute and chronic nephrosclerosis	1,500	2,500	Whole
	Fetus	Death	200	400	Whole
Potentially mild or moderately severe radiation injury	Bladder	Contracture	6,000	8,000	Whole
	Testes	Sterilization	100	200	Whole
	Ovary	Sterilization	200–300	625–1,200	Whole
	Lens	Cataract	500	1,200	Whole/part
	Vagina	Ulcer, fistula	9,000	10,000	Whole/part
	Breast (child)	No development	1,000	2,500	Whole
	Breast (adult)	Atrophy, necrosis	>5,000	>10,000	Whole

*$CD_{5/5}$, dose to which a given population of patients is exposed at standard time-dose fractionation resulting in a 5 percent complication rate within five years after treatment.
†$CD_{5/50}$, dose to which a given population of patients is exposed at standard time-dose fractionation resulting in a 50 percent complication rate within five years after treatment.
From Moosa AR, et al: Comprehensive Textbook of Oncology. Baltimore, Williams & Wilkins Co., p 267, 1985. Copyright, Williams & Wilkins, 1985. Modified from Rubin : Clinical Oncology. Rochester, American Cancer Society, 1978.

1.3.3 Chemotherapy

Chemotherapy, in its most general sense, refers to treatment of disease by chemicals that kill cells, both good and bad, but specifically those of micro-organisms or cancer. Surgery and radiation therapy are only effective in early stage of cancer occurrence. Once metastatic (systematic) cancer starts, nowadays, one of the most popular feasible therapies is chemotherapy. Chemotherapy has a long history since 1865. The development of chemotherapy agents was listed in Table 1-3.

Drugs for chemotherapy can be classified for several groups such as alkylating agents [15], antimetabolites, antibiotics, plant alkaloids, hormones, miscellaneous agent and others. The mechanism differs from each other. The first category is alkylating agents such as nitrogen mustards. They can act on DNA directly and combine DNA by covalent bonding to disturb the synthesis of DNA [16]. Second,

antimetabolites such as 5-FU [17], 6-TG [18, 19]and MTX [20] are with similar structures to normal metabolites that are required for synthesizing DNA [21]. Third, antibiotic such as bleomycin [22] can insert the DNA base pair, change the stereo structure, and ultimately disturb the synthesis of DNA. Fourth, plant alkaloids [23] can be divided into several subgroups and each one has its own unique mechanism. Fifth, hormone therapy has two subtypes [24]: ablative therapy (eg.excision of the ovary) and additive therapy (eg.adding estrogens to cure breast cancer) [25]. Of course there are still many different drugs with their unique methods to kill cancer cells.

However, chemotherapy has many side effects, such as hematologic toxicity, gastrointestinal toxicity, immunosuppression, dermatologic reactions, vascular and hypersensitivity reactions, hepatic toxicity, pancreatic toxicity, pulmonary toxicity, cardiac toxicity, genitourinary toxicity, neurotoxicity, impaired sexually and gonadal function, and ocular toxicity [12]. Therefore, in clinical settings, the physicians should control the dosage and frequency of chemotherapy very carefully and precisely. They have to monitor the patient's general condition and decide whether to proceed rest of the courses. In addition, secondary malignancies after chemotherapy are also one of the concerns of physicians.

Table 1-3   Development of chemotherapeutic agents

Approximate Date	Agent	Diseases Treated
1865	Potassium arsenite	Leukemias, various malignancies
1893	Colcy's toxins	various malignancies
1941	Estrogens	Prostate and breast carcinomas

	Androgens	Breast cancer
1945	Nitrogens	Lymphomas, solid tumors
1948-1950	Andrenocorticosteroids	Leukemias, lymphomas, multiple myeloma
	Methotrexate	Acute leukemia, choriocarcinoma
1950-1955	Busulfan	Chronic granulocytic leukemia
	6-Mercaptopurine	Acute leukemia
	Actinomycin D	Wilms' tumor, testicular tumor, choriocarcinoma
1955-1960	5-Fluorouracil	Carcinomas of breast gastrointestinal tract
	Progestins	Endometrial carcinoma
	Cyclophosphamide	Lymphomas, solid tumors
	Mitotane	Adrenal carcinoma
	Vinca alkaloids	Lymphomas, acute leukemia, miscellaneous tumors
	Mitomycin C	Gastrointestinal tumors
1960-1965	Hydroxyurea	Chronic granulocytic leukemia
	Procarbazine	Hodgkin's disease
	Cytarabine	Acute leukemia
	Mithramycin	Testicular tumors
	Nitrosoureas	Lymphomas, brain tumors, solid tumors
	Daunorubicin	Acute leukemia
1965-1970	L-Asparaginase	Acute leukemia
	Dacarbazine	Melanoma
	Cisplatin	Testicular and ovarian tumors
1970-1980	Doxorubicin	Sacromas and awide spectrum of other
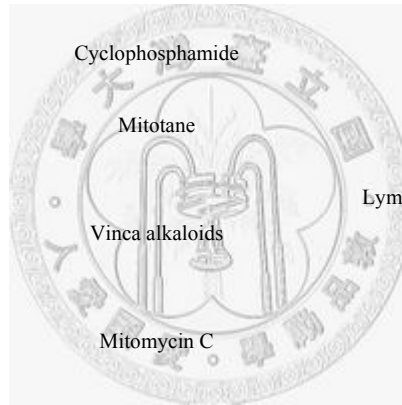
		tumors
	Bleomycin	Lymphomas, head and neck cancer
1980-1990	Tamoxifen	Breast cancer
	Leuprolide	Prostate cancer
	Flutamide	Prostate cancer
	Etoposite	Germ cell tumors, small cell lung tumor
	Streptozocin	Islet cell carcinoma
	Interferons	Hairy cell leukemia, Kaposi's sarcoma
	Mitoxantrone	Acute leukemia, some solid tumors
	Octreotide acctate	Carcinoid and islet cell carcinomas
	Ifosfamide	Refractory germ cell tumors
	Carboplatin	Refractory ovarian carcinoma
1990-	Fludarabine	Chronic lymphocytic leukemia
	Pentostatin	Hairy cell leukemia
	Cladribine	Hairy cell leukemia
	Paclitaxel	Refractory ovarian carcinoma, breast cancer
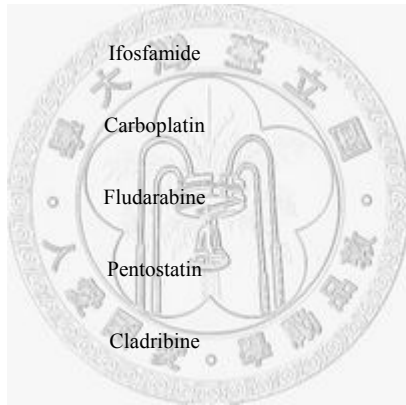
1.3.4 Immunotherapy

It is a strategy to treat cancer by enhancing immune activity directed at tumor cells or neoplasms. Immunotherapy is one of biologic therapies that change the relationships of host and cancer cells. In 1867, Busch and Coley were the first scientists to announce that the infection of Streptococcal could cause the necrosis of

human cancer[26]. By their research and experiment, the famous Coley's mixed bacterial toxins or Coley's toxins were made [27]. However, the mechanism remained unclear. Not until twentieth century, immunotherapy has not become a popular, promosing and less toxic cancer treatment.

The approach of immunotherapy is to utilize techniques that can simulate a person's immune system to seek out and destroy cancer cells. It is a systemic effect and hence metastatsis is within its indications. The other advantage of immunotherapy is that theoretically it only attacks cancer cells without harming normal cells, because of the activation of immune system on recognizing specific tumor antigens. There are three types of immunotherapy [28]: 1. Passive immunity: introducing lymphatic cells, immune serums, and transfer factors that have immunity to a specific cancer. 2. Active specific immunotherapy: utilizing killed cancer cells or antigens with ability to cause active immunity for therapy.3. Active non-specific immunotherapy. using non-specific drugs such as BCG [29] (Bacillus Calmette-Guerin) to strength immune system for resistance of cancer. However, the development of immunotherapy is just in the early stage. More studies should be done.

1.3.5 Gene therapy

Gene therapy is the insertion of genes into an individual's cells and tissues to treat a disease. Although the technology is still in its infancy, it has been used with some success. Generally it was used in hereditary disease, rather than cancer. The discovery of oncogenes and tumor suppressor genes makes gene therapy a potential

cancer therapy. The other theoretical type of gene therapy for cancer is that scientists can transfer genes of cytokine or immune mediators into cancer cells, in order to enhance the host immune response against tumor [30].

Three potential strategies of cancer gene therapy were introduced [12]. First, to replace a missing or defective gene (such as a mutated tumor suppressor gene) with a normal gene. Second, to inhibit over-expressed genes. Third, to introduce a fragment of functional gene into a cell to express or not to express for desired treatment. For example, the insertion of a normal fragment of *p*53 gene into cancer cells by a virus draws lots of attention [31]. It is based on the fact that more than half of all human cancers are with *p*53 mutations.

However, there are some factors that keep cancer gene therapy from an effective treatment[32]. The gene introduced into targeting cells has to be stable and long-lasting, but cancer cells are notoriously rapid-dividing. Thus patients should probably need several rounds of gene therapy. Immune response is also a risk when a foreign gene is introduced into human cells. Moreover, the vectors of gene delivery are usually virus which carries certain risks to patients. Although non-virus vector is found for gene delivery recently, the efficiency of delivery still remains unsatisfactory. Gene therapy for single mutations is the most common treatment. But most of cancer cells carries more than two mutant genes (the "double-hit" theory). Therefore, cancer gene therapy is not as good as other current treatments nowadays.

1.3.6 Hyperthermia

Hyperthermia has a long history. It is combined by two words -- **hyper**, from the Greek, and **therme**. *Hyper* means beyond, above, or over and *therme* means heat. So hyperthermia can be described as kinds of heat treatment, thermal therapy, or thermotherapy. Hippocrates (400 B.C.) has said that *fire will succeed when all other methods fail* [33]. He and Galen also used red-hot irons and chemical caustic to treat small and unknown cancers. Around last nineteenth and early twentieth centuries, many reports proved that the elevated temperature might be effective to cancer cells. For example, Selawry in 1957 discovered that there are one third of 450 cancer patients with spontaneous remission owing to high fever caused by malaria and typhoid [34]. Huth also found that one third of children with lymphatic cancer had spontaneous remission related to fevers [35]. Therefore, these results encourage scientists and doctors to induce patient's fever intentionally for curing cancer. Gradually hyperthermia has become one of potential cancer therapies [36].

In brief, hyperthermia is the therapy of raising tumor or cancer cell temperature to 42-47℃ [37]. Within this temperature range, cancer cells or tumor would tend to die while normal cells would not. However, nowadays the inevitable technical problem of hyperthermia is controlling temperature in the effective and safe range and keeping the normal tissues or cells from overheating at the same time.

Nowadays, hyperthermia is often applied as an adjunctive therapy with various established cancer therapy, like radiotherapy and chemotherapy [3]. If local

hyperthermia has conspicuous improved, the development of hyperthermia combined with other auxiliary treatment will be anticipated. For example, the combination of adjunctive chemotherapy can alleviate the pain caused by local tumor or remove the lethal tumor. A synergistic interaction [38, 39] of heat and radiation has been validated in preclinical studies. Moreover, hyperthermia can treat different types of cancers and has little side effect. If the feasible modality for hyperthermia was developed, it would be one of the most promising therapies in the future.

1.4 Comparisons of cancer therapies

The comparison of various cancer treatments is shown in Table 1-4. It can be known that hyperthermia has a lot of advantages and fewer side effects . Although hyperthermia is with some risks and drawbacks so far, these problems are believed to be solved in the future. Therefore, hyperthermia is one of the most potential therapies nowadays [12, 21, 28, 40] [3, 32, 36, 38, 39].

Table 1-4 Comparisons of cancer therapies

	Benefits and advantages	Disadvantages and risks
Surgery	1. the most direct way 2. fast 3. biopsy is mandatory to have "tissue proof"	1. possible local recurrence and metastasis 2. loss of normal tissue and function 3. wound complications
Radiation	1. effective and convenient 2. preserve organs and tissues 3. alleviate and mitigate symptom 4. free of systemic toxicities and anatomical restrictions	1. free radicals damaging normal tissues 2. various side effects 3. limited cure effect after metastasizing
Chemotherapy	1. suitable for treating metastasis 2. no wound complications 3. alleviate and mitigate symptom	1. cannot eradicate 2. drug toxicity and side effects 3. susceptible to second malignancy

	Advantages	Disadvantages
		4. need different drugs for different cancers
Immunotherapy	1. less side effects 2. suitable for treating metastasis 3. without injuring normal tissues	1. risk of causing serious immune response, even death 2. need different drugs for different cancers 3. hard to control and evaluate
Gene therapy	1. possible to eradicate cancer 2. less side effects	1. several rounds needed 2. Immune response 3. viral vector may cause problems 4. lower efficiency for non-viral vector 5. multigene disorder
Hyperthermia	1. easy and fast 2. fewer side effects 3. suitable for treating metastasis by drug targeting method 4. same treatments can apply for different cancers 5. few normal tissues and organs can be damaged by exact control 6. can combine with other therapies	1. hard to control temperature 2. hard to eradicate irregular shape of cancer 3. hard to determine the dosage and timing
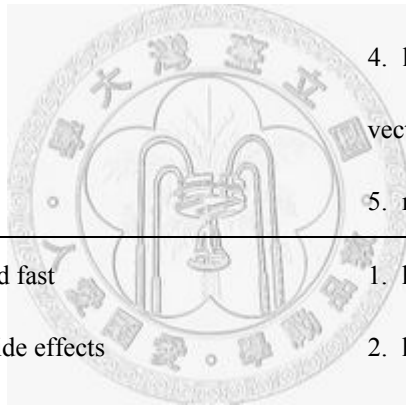
1.5 Mechanism of hyperthermia

Many reports regarding relationship of temperature and cell survival in hyperthermia have been done [41-44], and there is an optimal temperature range to kill cancer cells without damaging normal cells [45, 46]. However, the temperature range depends on different tissues and animals, even each individual in the same kind. Generally speaking, the temperature for hyperthermia treatment is about 42-47℃ [37]. A study done by Song et al [47] demonstrated that the survival of Chinese hamster ovary cells (CHO) sharply declined above 42.5℃ (Fig. 1-2). Another study done by Li GC et al [45] compared the change of blood flow when applied with hyperthermia, in normal tissues and tumor (Fig. 1-3). When applied temperature rises to 44 to 46℃, the blood flow of normal tissues, like skin and muscle, also increase. But the blood flow of tumor seems not to increase as the temperature rises. When the temperature continues to rises more than 47℃, the blood flow of normal tissues and tumors both decline significantly. Therefore, in order to kill cancer cells and not to destroy normal tissue, the most appropriate temperature for hyperthermia is about 42-47℃.
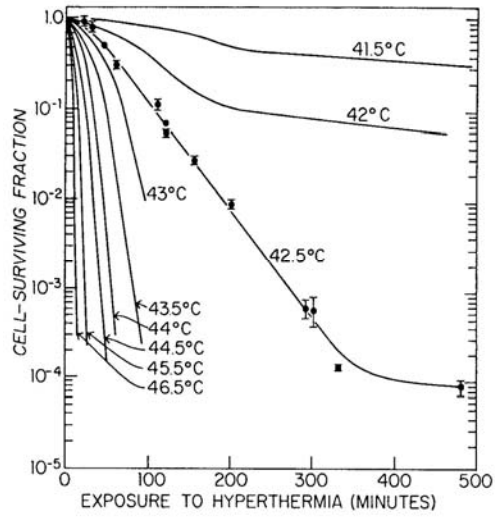
Fig. 1-2 Survival curve of asynchronous CH cell sheated in different temperature[47]
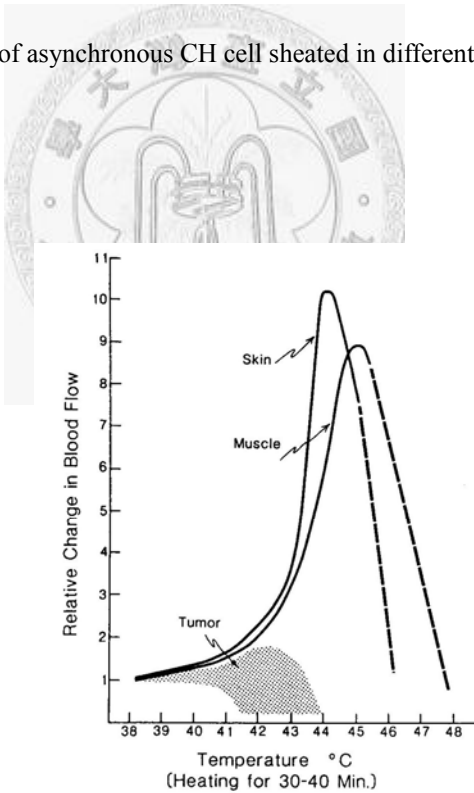
Fig. 1-3 Blood flow change in different tissues when temperature rises [45]

As the phenomenon and feature of hyperthermia has been illustrated, the following introduction would explain the cellular and molecular basis of cell killing by hyperthermia [48].
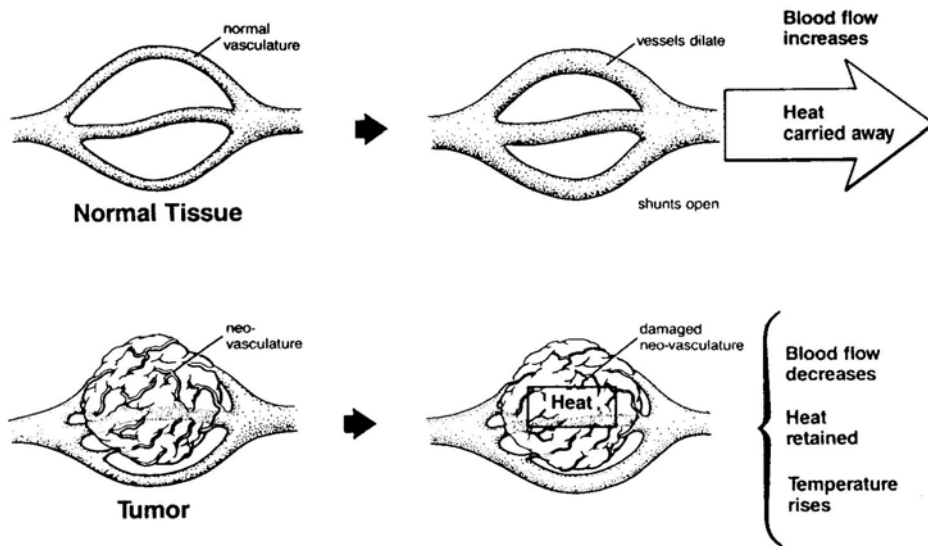
Fig. 1-4 Comparison the vessels structure of normal tissues and tumors when temperature rises [49]
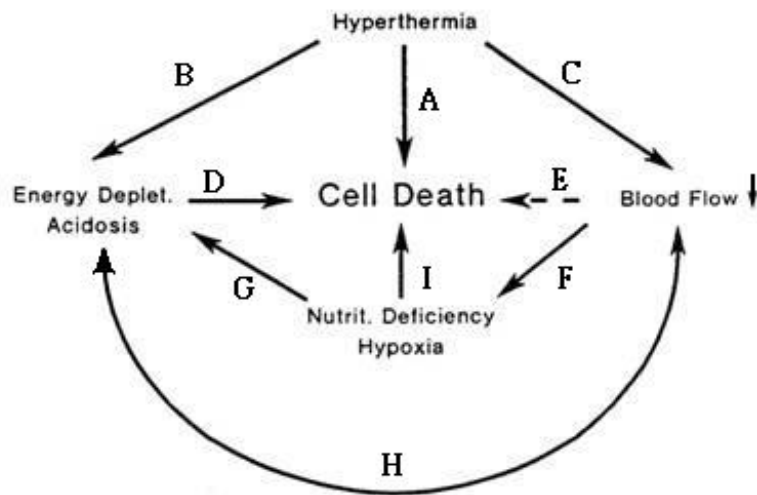
Fig. 1-5 Schematic for the introduction of cell death by hyperthermia and interaction with other physiological as well as metabolic factors [50]

The micromilieu of cells has high thermosensitivity [49-54] and it is mainly dominated by two physiological factors: blood flow and energy metabolism. Due to poorer function of blood vessels in tumors than in normal tissues, the oxygen pressure and nutrition is often lower in tumors than in normal tissues (Fig. 1-4) [49]. Therefore heat tends to be retented inside tumors, which leads to conformational changes of proteins (denaturation)[55]. Crucial proteins inside the cells such as structure of the cytoskeleton and enzymes complexes for DNA synthesis may be damaged. Then the cell will die easily (Path A in Fig. 1-5 [50]). It is known that high temperature affects the function of the membrane-based receptors. The change of sodium and potassium ion concentration alters membrane permeability that affects the conformation of intracellular ion channels [56-60].

Heat shock proteins (HSPs) [61-63] are produced for stabilizing cytoskeleton structure during hyperthermia[64]. Although heat shock proteins are made to protect cells under high temperature, there are also side factors that could make situation worse (Path B in Fig.1-5). Once excessive shock proteins are generated, it means that there are more and more energy needed to be used and more carbon dioxide produced. The increasing demands of ATP turns into depletion [65-68] and excessive carbon dioxide dissolved in water results in acidosis [69].

Moreover, it is possible for hyperthermia to induce the breakdown of blood flow, which was shown by Path C in Fig. 1-5. Furthermore, complete mechanism can be seen in Fig. 1-6 [70]. Hypoxia and nutrient deficiency caused by decreasing blood flow make cell death [45, 61, 70-73] (Path F, H and I in Fig. 1-5). In addition, when

more heat shock proteins are produced, more oxygen is required for metabolizing

glucose to water, carbon dioxide and energy (ATP) [69]. However, under hypoxic

conditions, the stimulation of glycolysis increases the amounts of lactoses [45],[72],

[51, 74-77], especially in tumors. It will also leads to acidosis (Path G and D in Fig.

1-5). Furthermore, protein denaturation may occur owing to low pH value.
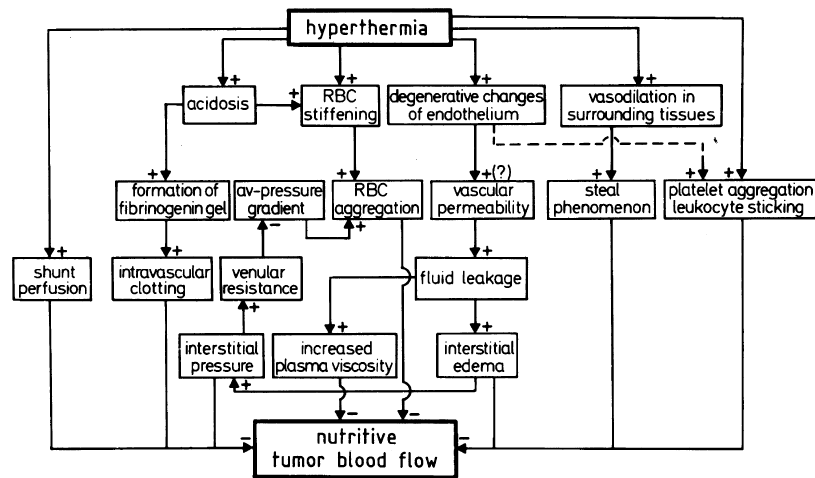
Fig. 1-6 Possible mechanisms involved in hyperthermia-induced breakdown of blood flow [70]

1.6 Classification of hyperthermia by heating method

In terms of heating methods, hyperthermia can be classified into local hyperthermia and systematic hyperthermia[50, 78]. Local hyperthermia means that heat treatment only is carried on targeting site, instead of the whole body. There are two ways for local hyperthermia: 1. External heating source is outside the body and heat has to penetrate through skin to curial site. 2. Heating source is set in intracavitary or interstitial for internal heat treatment. However, systematic hyperthermia treatment makes the temperature of the whole body increase. Owing to non-equal temperature increment, mild temperature is adequate for systematic hyperthermia. The comparisons of different heating methods of hyperthermia can be seen in Table 1-5.

Table 1-5 Comparisons of various heating methods of hyperthermia

Hyperthermia types	Sub-types and Characters

Microwave
  a. Frequency between 50-240 μ Hz
  b. Transmit fast (velocity of light)
	1. Single or multiple external devices: superficial heating
	
	2. Phased array devices: regional deep heating
	
	3. Interstitial or intracavitary devices such as antenna

Radiofrequency	1. Inductive or capacitive external device such as loop
a. frequency between 0.5-27 MHz	
b. wave transmit fast (velocity of light)	2. Inductive or capacitive for intracavitary or interstitial
Ultrasound	1. Single or multiple external devices: superficial
a. Frequency between 0.5-5 MHz	
b. Wave transmit slow (mechanic waves)	2. External focused array devices: regional deep
	3. Interstitial or intracavitary
Systematic heating	1. Induction of fever (immunological aspects)
a. Suitable to metastatic advanced cancer	
	2. Intravascular blood perfusion
b. Various modalities has been developed	3. External heating technology (such as special magnetically mediated hyperthermia)
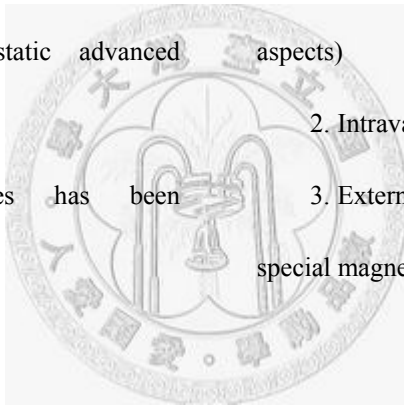
1.7 Magnetically mediated hyperthermia (MMH)

1.7.1 Background

Hyperthermia for human body can be divided into three separate domains in terms of the heating region: whole body hyperthermia (WBH), regional hyperthermia (RHT), and local hyperthermia (including superficial local and interstitial local hyperthermia) (LHT). Lately, non-invasive WBH introduced by Robins *et al*.[79]can be administered with a radiant heat device and successfully achieved temperature raising. Currently LHT is most commonly performed using single microwave or ultrasound applicators, whereas deep RHT is performed using arrays of multiple applicators for deep heating[80] (BSD 2000). Urano *et al*. [81] has reviewed isolated limb perfusion and other perfusion techniques. On the other hand, Seegenschmiedt *et al*. [82] also reviewed on local interstitial hyperthermia.

Hyperthermia with temperature above 42 ° C has been proven leading to cell necrosis[37, 83]. The mechanism is complicated, but briefly, the hyperthermia status hinder the function of many structural and enzymatic proteins within cells, which in turn alters cell growth and differentiation and can induce apoptosis[84-86]. Hyperthermia therapy for cancer is drawing more and more attention due to its fewer side effects. Moreover, it will attenuate the therapeutic effect of radiotherapy[87-89]and chemotherapy [88, 90].

There are two main categories of hyperthermia in terms of the means of heating: Invasive means and non-invasive means. The invasive hyperthermia include microwave antenna, radiofrequency electrode, and laser fiber. On the other hand, the

noninvasive methods include high-energy ultrasound and magnetically mediated hyperthermia (MMH).

1.7.2 Definition

The definition of magnetically mediated hyperthermia (MMH) is: by allocating magnetic particles or seeds within or around tumor tissue under an external alternating magnetic field to cause them to heat by hysteresis loss, Neel relaxation or induced eddy currents. It has the possible advantages of targeting tumor, comparing of other general hyperthermia modalities. The concept of MMH was first described in an in vitro study done by Gilchrist *et al*.[91] in 1957. They injected magnetite particles (diameter=0.02±0.1 mm) into the sub-serosa of the intestinal wall of dogs. The dissected regional lymph nodes with 5mg of magnetite per gram were then exposed to an alternating magnetic field of strength 200±240 Oersted (Oe). It could induce a temperature increase of 14 $^\circ$ C in 3 min. Two years later, another *in vivo* study was conducted by the same group. They used rabbits with inguinal lymph nodes successfully targeted with heat[92]. Total necrosis of the nodes was reported after 3 min of heating at 470 Oe. In 1965, this group demonstrated that 30 min of heating at 50 $^\circ$ C was necessary to kill all cells within a mesenteric lymph node of a dog *in vivo*[93]. This lymphatic uptake of microscopic ferromagnetic particles which was done around 60 years ago, lighten the path of further breakthroughs in selectively embolize and heat tissue *in vivo* using MMH.

The magnetically mediated hyperthermia(MMH) can be further divided into four

types[94]: arterial embolization hyperthermia (AEH), direct injection hyperthermia (DIH), interstitial implant hyperthermia (IIH), and intracellular hyperthermia (IH).

1.7.3 Arterial embolization hyperthermia (AEH)

The application of AEH depends on the embolization with magnetic particles of the arterial supply of a tumor. In the presence of an external alternating magnetic field heat was generated by hysteresis or Neel relaxation. The arterial supply of tumors such as hepatic and kidney tumor is suitable to utilize AEH in human. The blood supply of liver tumor usually drains from the hepatic arterial system. Thus the magnetic particles will have better targeting toward liver tumors simply by infusing them into the arterial system will have[95-98].

There are also similar current treatment modalities such as selective internal radiation therapy (SIRT), hepatic arterial chemotherapy (HAC) and transarterial chemoembolization (TACE). The successful tumor embolization was usually verified radiologically.

In 1976, the earliest reports done by Rand *et al.* [99] showed a dog kidney model on AEH by liquid silicone with finely powdered iron oxide particles. He injected the suspension transfemorally into the renal artery of dogs. Sako *et al.*[100] performed the same dog kidney model with 5mL of ferropolysaccharid e containing 10% of 30 mm iron particles. The same model was also tested by Matsuki and Yanada[101] , with different material[101] or animals[102] (such as rabbit). Magnetic fluid, defined as suspensions that contain particles less than 100nm in diameter, was also developed later. It can be heated by weaker magnetic fields and hence it generally less likely

causes tissue eddy current heating or peripheral nerve and muscle stimulation. On the other hand, stronger magnetic field was need for heating larger multi-domain particles. It is the advantage of magnetic fluid[103, 104]. Therefore, a rat liver tumor model was recently introduced by Minamimura *et al*.[105] in employing magnetic fluids for AEH. Furthermore, the rabbit liver tumor model with AEH was also demonstrated by Moroz *et al*.[106, 107] .

AEH has its own advantages theoretically, particularly in the treatment of hepatic or kidney tumors. Several small animal models have demonstrated encouraging results; however, many safety issues regarding particle clearance and toxicity remained unknown. Lots of work is still needed before a human trial of AEH could be conducted.
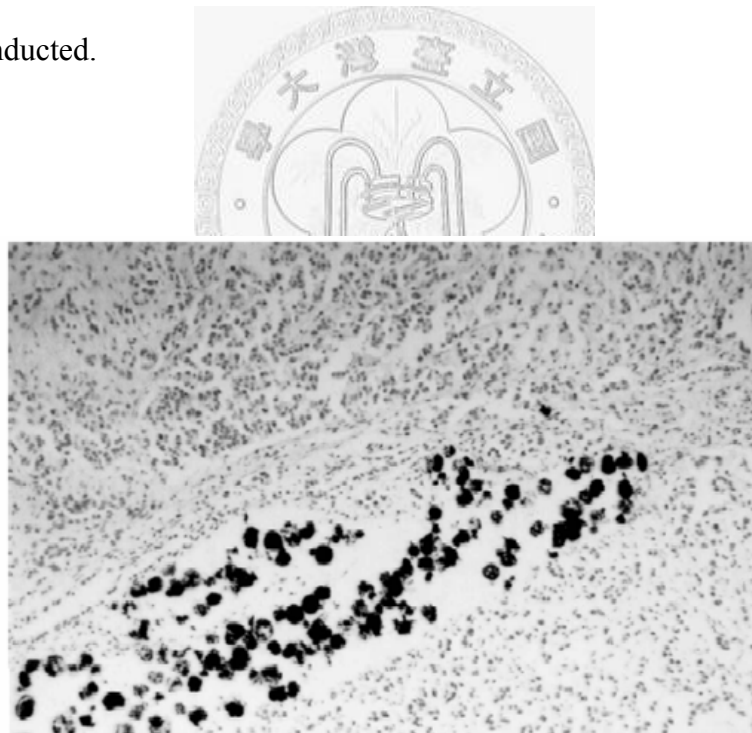
Fig. 1-7 A micrograph of treated tumors on 14 days and some micorspheres inside a blood vessel surrounded by necrotic tissue[106].

1.7.4 Direct injection hyperthermia (DIH)

The definition of DIH is to inject the magnetic fluid into tumor sites directly. The subsequent alternative magnetic field causes the heating of deposited particles. In contrast to AEH, the magnetic particles of DIH are deposited extracellularly. The AEH relies on the embolization of magnetic particles within blood vessels. Shinkai *et al*. reported his serious of experiments of magnetite cationic liposomes (MCLs) as the thermoseed for DIH [108, 109].

Rand *et al*.[110, 111] applied ferromagnetic particles which were suspended in normal saline in a rabbit model. He injected the suspension directly into the renal pelvis for curing unilateral implanted renal VX2 carcinomas. Luderer *et al*.[112] injected glass-ceramic coated magnetic particles subcutaneously around the implanted murine breast adenocarcinoma in mice. Similar material was also chosen by Borrelli *et al*.[113] Hase *et al*.[114, 115] combined DIH and AEH in a rabbit hepatic tumor model by direct injection of ferromagnetic particles via hepatic arterial embolization. In 1993, an in vitro study was conducted by Chan *et al*.[116] He used colloidal magnetic iron oxide particles in a medium containing cultured human lung cancer and breast cancer cells. In 1997, Jordan *et al*.[117] injected ferrite particles (dextran ferrofluid containing magnetite particles 3nm in size) into mammary carcinomas in mice directly. In 1999, Shinkai *et al*.[109] injected carboxymethylcellulose solution with colloidal magnetite material to femur of a pig. Local tissue temperature of 43℃ was achieved in 7 mins inside a 8MHz radiofrequency capacitative heating device. In 2000, Hilger *et al*.[118] also reported an in vitro study of cow muscle while applying magnetic thermoablation. Furthermore, Hilger *et al*.[119] have also recently tested DIH with direct injection of ferrofluid containing 10 nm magnetite particles into the

implanted human breast adenocarcinoma tumors on immunosuppressed mice. They were then exposed to a 6.5 kA/m magnetic field alternating at 400 kHz for 2-3 mins. Mean tumor temperatures of 63℃ were recorded and all tumors showed necrosis histologically.
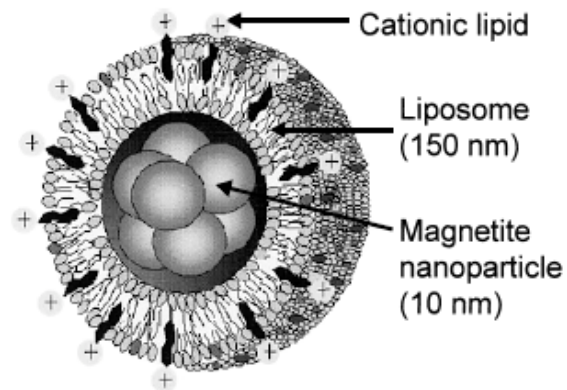
Fig. 1-8 Structure of MCLs: the core is magnetite for heating and the outer shell is lipsosome carried negative charge for better target [108].

A   B
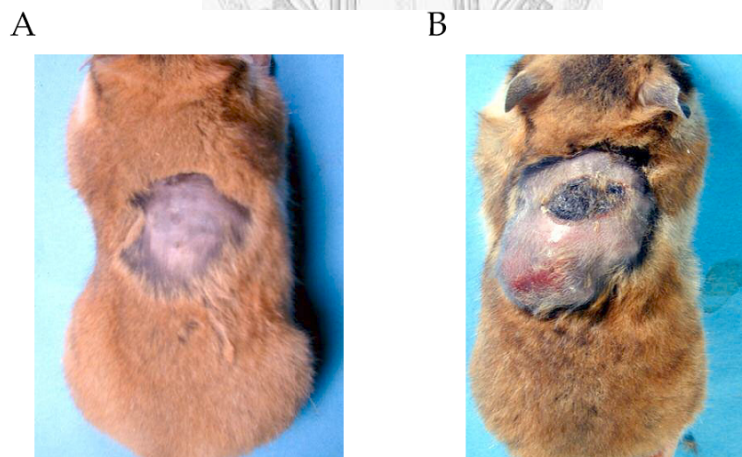
Fig. 1-9 A. Typical photographs of hamsters on day 20 after the MCLs injection. B. Control group [109].

1.7.5 Interstitial implant hyperthermia (IIH)

The first step of IIH is the implantation of magnetic rods or seeds directly into the site of tumor. The second step is the exposure of external alternating magnetic field. Different alloys including Ni-Cu, Fe-Pt and Pd-Co have been used. The seeds are implanted percutaneously under radiologic guidance or via direct vision in the operation, with a separated space of 1 cm from each other within tumor tissue. The seeds are manufactured to have a desired Curie point to prevent local tissue overheating and reduce the need for invasive tissue thermometry. In order to avoid neuromuscular stimulation and minimize tissue eddy current heating, magnetic field frequencies of 10-100 kHz are typically selected. In a clinical setting, heating is repeated for three or four sessions. Microwave antennae with cables are the other option to achieve interstitial hyperthermia besides implanted seeds[120, 121].

In 1993, Mack et al.[122] reported a complete response rate of 61% and a partial response rate of 31.7% in 44 patients with head and neck, pelvic and chest wall tumors. The material he inserted is the Ni-Si seed of Curie points ranging from 50-80 ℃.However, almost 50% of the patients experienced some form of toxicity. In 1996, Tohnai et al.[123] reported a 100% histologic response rate in treating eight patients with oral cavity cancers. He used Fe-Pt seeds (Curie point = 68℃) for 45 min once a week with pre-operative chemotherapy. Besides human trials, Akagi et al.[124] and Takegami et al.[125] also conducted animal studies in treating bone maligancies with IIH.

IIH has a long history in treating intracranial tumors. Burton et al.[126] was the first one who apply ferromagnetic thermoseeds to ablate tissue of brain tumors.

Following his report, Kida *et al.*[127] implanted Fe-Pt alloy seeds with a Curie point of 68-69℃ into the brain metastases of seven patients. While exposing to a 240 kHz magnetic field, the tissue temperature of 44-46℃ was maintained for 30-60 mins. The hyperthermia treatment was done two-to-three times a week. IIH has evolved into clinical use for a variety of human tumors. Kobayashi *et al.*[128] reported a response rate of 35% among 23 patients with brain tumors after using thermoseeds with a Curie point of 68℃. Stea conducted two studies in 1992 respectively by applying ferromagnetic seeds in the supratentorial gliomas[129]. Later, in 1994, he also compared the synergic effect of external beam radiotherapy with or without hyperthermia. The mortality rate was almost halved in the group "with hyperthermia"[130]

Ocular tumors are currently treated with IIH in animal models. Steeves *et al.*[131] implanted ferromagnetic seeds in sub-retinal ocular melanomas of rabbits. He found that hyperthermia should be given during irradiation. Murray *et al.*[132, 133] compared the therapeutic effect of external beam radiotherapy, IIH or the combination treatments in a transgenic retinoblastoma murine model. A 100% cure rate among tumors that had been treated with hyperthermia at 54℃ was shown. And the dose of radiation is largely reduced in the combination group. It proved a synergistic effect between radiation and IIH.

In 1997, Loening and Tucker[134] conducted a phase I trial on 10 patients with prostate cancer. The treatment was well-tolerated with minimal side effects of urinary retention, pelvic pressure, urge to urinate and tingling in the thighs and penis. Histologic examination of the prostate revealed necrosis up to the capsule, with very little necrosis beyond the capsule after 90 days
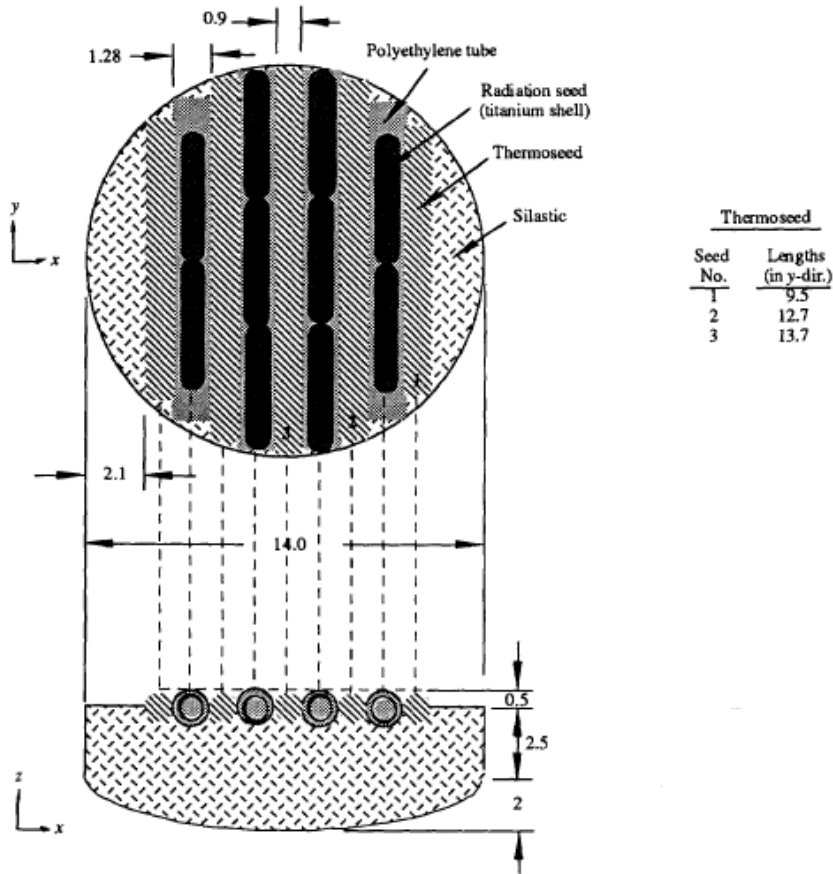
Fig. 1-10 Description of ferromagnetic thermoseed [131]

1.7.6 Intracellular hyperthermia (IH)

IH is a modality of magnetically mediated hyperthermia (MMH) that employs more intricate magnetic particles. The particles in this category usually have some kind of coating with or without tumor specific antibodies, in order to achieve differential endocytosis-- selective ingestion by the tumor cells with minimal uptake by normal cells. The delivery to the tumor can be done by either arterial embolization or direct injection. Extracellular deposits of particles may remain and make some

contribution to the heating of the tumor and surrounding tissue. Differential heating can be expected while exposure to an alternating magnetic field, because normal cells will ingest relatively fewer particles. Neel and Brownian relaxation is the basic mechanism of heat generation due to the fact that the ferromagnetic component of the particles used in IH is typically less than 100nm in diameter (sub-domain).

In 1979, Gordon et al.[135] proved intracellular hyperthermia was applicable because tumors may ingest very small magnetic iron oxide particles and generate heat under an alternating magnetic field. Finite magnetic iron oxide particles (100 mg) suspended in a sucrose solution were injected into the tail vein of 26 Sprague Dawley rats implanted with mammary tumors. The Suzuki's study[136] in 1999 demonstrated the hyperthermia therapy on VX2 carcinomas implanted in the thighs of rabbits. The particles they used consisted of ferrosoferric oxide (Fe_3O_4) coated with a liposomal membrane containing hematoporphyrin with neoplastic affinity. Furthermore, in 1995, Suzuki et al.[137] labeled magnetite particles with tumor specific monoclonal antibodies via polyethylene glycol with terminal carboxy or amino groups. After incubation, 90 pg of magnetite had been adsorbed per BM314 tumor cell, four times the amount adsorbed per control cell. Shinkai et al.[138] applied cationic liposomes containing magnetite on rat glioma cells in vitro. The cationic liposomes were electrostatically attracted to the negatively charged glioma cells. A maximum magnetite concentration of 55 pg/cell was obtained in the cationic liposomes group, which was 10 times greater than the intracellular concentration of neutral liposomes group. The same group also conducted an in vivo study using F344 rats containing implanted femoral subcutaneous gliomas in 1999[109]. In this study, magnetite cationic liposomes were injected subcutaneously into the tumors. Tumor temperatures over 43℃ were recorded and 90% of tumors regressed completely on histological

examination. In 2001, Le *et al*.[139] developed a new antibody fragment coated magnetoliposomes, which posses a 7- times greater tumor affinity compared to those used by Shinkai *et al*. In the past ten years, Jordan *et al*.[140-143] have also conducted several studies on IH for human colonic adenocarcinoma, human mammary carcinoma, and solid tumors (neurologic and prostate tumors).
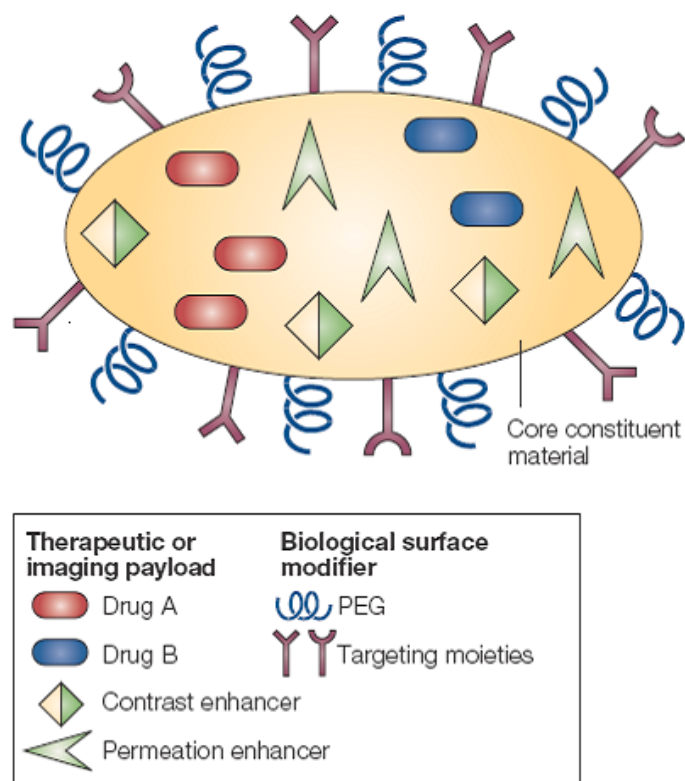
Fig. 1-11 The basic concept of structure of multifunctional nanoparticles for intracellular hyperthermia [144].

1.7.7 The future - magnetic nanoparticles

Along with the great advances in nano-technology recently, various magnetic nanoparticles were invented for different medical purposes [108, 145]. Among them, Superparamagnetic iron oxide nanoparticles (SPION) with appropriate surface chemistry have its potentials in magnetic resonance imaging, hyperthermia, drug delivery, tissue repair, cell and tissue targeting and transfection[146, 147]. Magnetic nanoparticles are used alone or in combination with other treatment modalities, such as surgery, chemotherapy and hyperthermia. There are already several reports proving their effect in treating different cancers, such as breast cancer[148]. While applying alternating magnetic fields on these magnetic nanoparticles, phase I clinical trials reports the safety for clinical application, although optimization is still unknown and needed to be discovered[149, 150]. Magnetite cationic liposomes (MCLs) is a newly-invented magnetic nanoparticles, which are easily internalized by target cells. Therefore intracellular hyperthermia is applicable under alternating magnetic field. Furthermore, they could be loaded with chemotherapeutic agents so that synergistic effect of chemotherapy and hyperthermia is achieved. A study done by Ito *et al.*[151] compared the therapeutic effect of 4-S-cysteaminylphenol (4-S-CAP) incorporated in MCLs alone with or without combination of hyperthermia against malignant melanoma. The in vitro study of Ito proved that the cytotoxic effect has the following effects: 4-S-CAP < hyperthermia < 4-S-CAP + hyperthermia. He chose the agent 4-S-CAP because it can selectively kill melanocytes and melanoma cells. It is a substrate of melanoma tyrosinase[152]. Danamudi *et al.* also reviewed the fact that by loading both magnetite and chemotherapeutic agents simultaneously in liposomes, chemotherapy can be administered in combination with hyperthermia. [153]

1.7.8Comparison of magnetically mediated

hyperthermia

MMH has a very long history as a treatment modality for cancer. However, the progress was very slow. The limitation of its development relies much on the advancement of technologies. In the past decade, several breakthrough of the biomagnetic materials have been made and hence a great advance of MMH was achieved. To date, IIH, currently the most advanced modality of MMH, has been used to treat human tumors successfully for years. IH is a very hot topic due to the recent advances in magnetic fluids, which contain sub-domain sized magnetic particles that generate heat via Neel relaxation. Several human trials are currently undergoing. By comparing different modalities of magnetically mediated hyperthermia, IH is the most potential modality by combining drug delivery[94].

In terms of AEH and DIH which is based on hysteretic heating of multidomain ferromagnetic particles, the progress still stopped at the stage of laboratory experiments. The advantages and limitations of the four categories of MMH was described in Table 1-6. Due to the rapid developments in magnetic materials, we believe MMH will have great potentials in this century.

Table 1-6 Comparisons of magnetically mediated hyperthermia

	Advantages	**Disadvantages**
AEH	1. more effective tissue temperature distribution [68] 2. No sharp drop in temperature at the tumor edge (due to particle gradient) 3. Repeat treatment without further dosing and invasion (the particles remained *in situ* after AEH) 4. No major inflammatory response (apoptosis)	1. Not applicable to tumors without a good arterial supply and tumor outside liver 2. Risk of embolization and then subsequent ischemic necrosis of normal tissues, which was worsen by subsequent heating 3. The need for pretreatment radiological determination of particles distribution
DIH	1. Good control over the deposition of ferromagnetic particles 2. Not dependent on an arterial pathway to the tumor (a wider range of tumor is applicable) 3. It can be done percutaneously under radiological guidance	1. Necessity of repeated injections for large or irregularly shaped tumors 2. Cannot apply for miliary disease 3. Very localized deposition leads to possible neglecting on micro-extension or micro-metastasis 4. Increased risk of needle track implantation or local tumor spread

IIH	1. Self-regulating thermal seeds with reduced invasive thermometry and higher safety. 2. Rapid initial heating results in rapid tumor reduction 3. Biocompatible seeds can be permanently left in situ for repeated treatments 4. already works in a wide variety of human tumor types in vivo, concurrent with chemo- and radio-therapy	1. Thermoseed migration or contact allergies (Nickel) 2. The seeds may interfere with MRI 3. Difficult to treat irregular shaped tumors completely 4. Complications of implantation procedure and the risk of infection 5. Non-uniform cancer cell temperatures may result in cold spots
IH	1. With potential advantages that accompany AEH and DIH 2. potential focus heat on tumor and hence better efficacy 3. Able to treat metastasis and scattered cancer cells 4. The particles are often sub-domain (magnetic fluids) and required only moderate magnetic fields.	1. Relative contributions of intracellular heating and extracellular heating has to further evaluate in a live tumor model 2. Need to specific target and/or ingest a sufficient quantity of particles

1.8 Magnetic nanoparticles for biomedical applications

1.8.1 Particle size

Intravenous administration (IV) injection of nanoparticles is one of the most effective ways to reach targeting cells or tissues. Therefore, particle size of drug is an important factor and so are the sizes and types of vascular capillaries.

First, it is necessary to define the particle size. It is defined as the total diameter of the particles with main inner core and outer surface coating. Because the smallest diameter of vascular capillaries in body is 4 μ m [154], magnetic particles that tend to aggregate for their magnetic force and larger surface energy of particles may be over this range. These particles will be dangerous for being retained and caught by capillary bed of the lungs, which may result in lung embolism[155, 156]. Moreover, MPS (Macrophages of the mononuclear phagocytosis system) can eliminate these IV applied particles immediately owing to their ability in removing foreign bodies [157].

If the particles are smaller than 4 μ m, they are eliminated quickly by cells of reticuloendothelial system (RES) [155, 157]. However, part of these particles can reach the target cells and tissues by blood circulation. The permeability of vascular capillary wall that drugs go through becomes a decisive factor. There are mainly four types of blood capillaries (Fig. 2-9) [158].

Tight-junction capillaries (blood-brain barrier, BBB) with connection of their endothelial lining cells, in central nervous system, are usually hard to be penetrated. Thus particles can not diffuse out of capillaries. Continuous capillaries, in major tissue like muscle, skin, lung, and connective tissue, have limited particles with size over 6 nm from diffusion. Therefore larger particles(6~50 nm) can be excreted from fenestrated capillaries in kidney. If particles are larger than 60 nm, they only can be cleared by the RES which has sinusoid capillaries. RES can capture particles, like 60~90% particles taken up by Kupffer cells in liver and 3~10 % particles by macrophages in spleen [155, 157, 158].
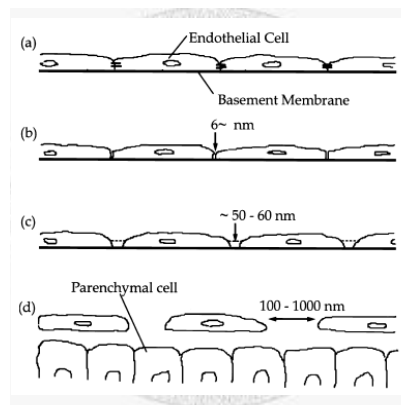
Fig. 1-12 The four types of capillaries: (a) tight junction capillaries (b) continuous capillaries (c) fenestrated capillaries (d) sinusoid capillaries[158]

Particles up to 100 nm would be phagocytosed by Kupffer cells and particles larger than 200 nm tend to be filtered by spleen [159]. When particles between 30 and 100 nm, the liver would eliminate larger particles compared to smaller ones. Besides by dividing the particle size, there are two types of uptakes: one is phagocytosis for all sizes and another is pinocytosis for particles size less than 150 nm [157, 160]. In another words, uptake of larger particles can only proceed by cells capable of phagocytosis and uptake of smaller ones by all kinds of cells through pinocytosis (all of cells are able to pinocytosis).

Therefore we can conclude that particle size above 10 nm cannot pass through the endothelium. However when under inflammation, when tumor infiltration in body, or with help of medication, heat and radiation, the increased permeability can allow particles over 700 nm to penetrate [159].

1.8.2 Biomedical applications of magnetic particles

In the last decade, magnetic particles for biomedical applications have been developing dramatically due to the advances of nanotechnology. Although magnetic particles like iron oxide have been used for many decades, nano-sized magnetic particles did not draw scientists' the attention until nowadays. Applying iron oxide nanoparticles (superparamagnetic particles are preferable) is the mainstay of treatment for its easy production [161-163] and proven biocompatibility [164]. The following

description would show their several applications [146].

Cell label and separation

Stem cell tracking is one of the hottest topic recently. Labeling cells by magnetic particles gives a chance for cell separation [165]. First, the target cells should be labeled by magnetic particles. There are two common methods for labeling: 1. attaching magnetic particles to the surface of targeting cells [166] 2. internalizing magnetic particles by endocytosis [167], phagocytosis [168], and receptor-mediated endocytosis [169]. After labeling magnetic particles, the targeting cells can be detected by MRI [170].

MRI (magnetic resonance imaging) contrast enhancement

MRI is one of the most important invention in medicine (Nobel Prize) for its non-invasive and precise imaging. One of potential materials for contrast agents is SPION (superparamagnetic iron oxide nanoparticles). Due to the different relaxation times (T1 and T2) of hydrogen atoms[158], these contract agents can enhance the sensitively and specifically [171-173]. In the past decade, more and more various type of surface modification of the magnetic particles have been applied as specific contrast agents for targeting cells [168]. It opens a door to molecular imaging.

Tissue repair

By means of the heat generated by magnetic particles, two tissue surfaces can be

joined together. The applied temperature is often greater than 50℃ [174]. It is because the denaturation of proteins can entangle together after temperature decrease [174, 175].

Immunoassay

Magnetic particles have been used in many immunoassays, like fluoroimmunoassays [176], enzyme immunoassays [177] and radioimmunoassay. Primary or secondary antibodies can be conjugated on the surfaces of magnetic particles. In this way, they can separate and quantify antigens quickly and precisely [178].
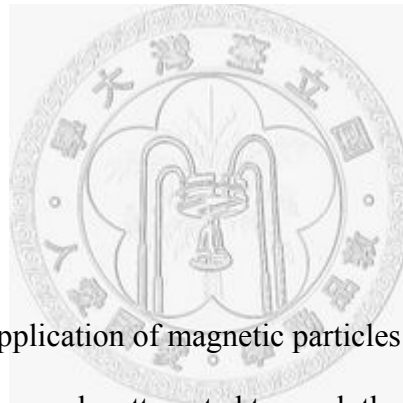
Drug delivery

Another biomedical application of magnetic particles is as drug carriers. When guided by magnetism, drugs can be attempted to reach the tatget site efficiently. Magnetic Targeted Carriers (called MTCs), designed by FeRx Inc. of San Diego, can be used for many aspects, such as site-specific targeting, tissue retention and sustained-release drugs (Fig. 2-10) [178]. Moreover, magnetoliposomes also have been used as drug delivery carriers[179-181].
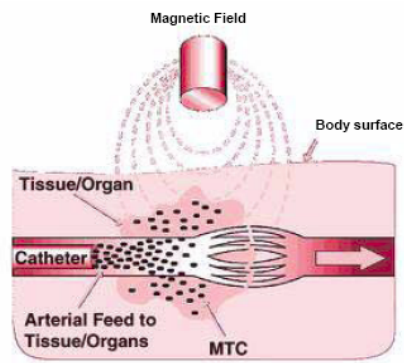
Fig. 1-13 Drug Targeting using MTCs [178]

Hyperthermia

Magnetic particles for hyperthermia have been mentioned in the previous section.

1.9 Drug delivery system for hyperthermia

Drug delivery system of hyperthermia is getting more and more attention. It is why the study focuses on intracellular hyperthermia by injecting magnetic particles into body (IV injection) or by other modalities that deliver medicine via blood circulation.

The diagnosis and treatment of cancer is very crucial for the survival of patients. Magnetic nanoparticles has the potential characters to combine diagnosis with treatment at the same time. Fig. 1-14 shows that magnetic nanoparticles can be targeted to cancer cells and kill them. Magnetic nanoparticles also can be used as cancer diagnosis by magnetic resonance imaging (MRI) or magnetoimpedance (MI) sensor [108]. After detecting cancer cells, applying an alternative magnetic field can induce heat generation of magnetic nanoparticles for hyperthermia. In this way, magnetic nanoparticles can be used as diagnosis and treatment tool at the same time. It is relatively time-saving for patients and increases the compliance of patients.
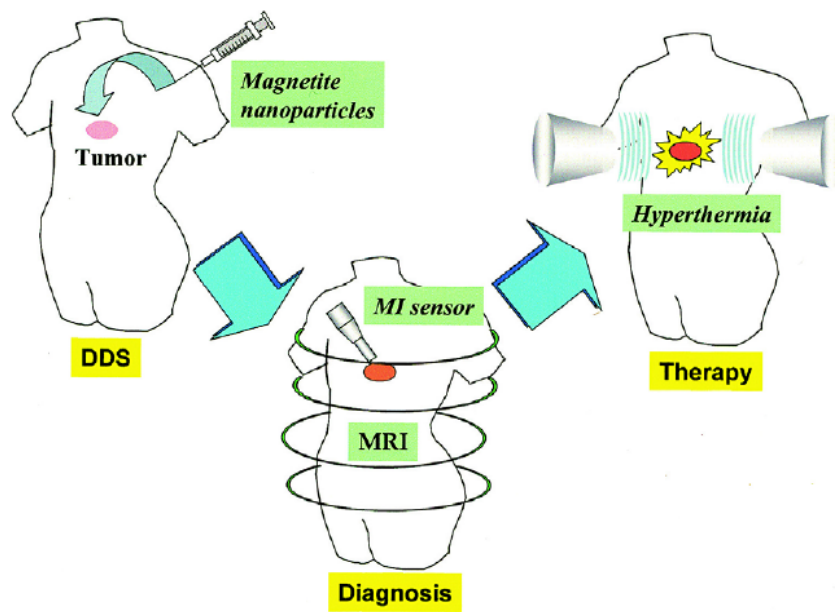
Fig. 1-14 The therapeutic strategy by using magnetic nanoparticles [108]

1.10 Purpose of the study

Nowadays, iron oxides (magnetite Fe_3O_4 and maghemite γ-Fe_2O_3) are the most popular thermoseeds for intracellular hyperthermia. Various magnetic nanoparticles of ferrimagnetic bioglass ceramics (FBCs) were developed recently, and aimed to provide magnetic properties for MRI and hyperthermia purposes. Nevertheless, during the preparation of FBC, it had to heat up to 800 ∘C and then quenched to room temperature. Particle size, especially after further milling, is difficult to control at the nanoscale, and quasi-crystallisation may occur during the thermal processing. This has made it difficult to synthesize magnetic bioglass nanoparticles, which has limited their potential clinical applications.
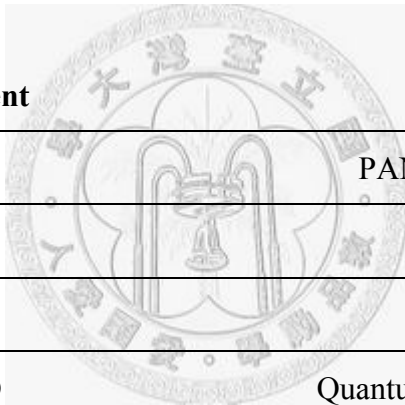
Therefore, in this thesis, we would like to introduce two new magnetic particles synthesized by co-precipitation: magnetic hydroxyapatite nanoparticles (mHAP) and magnetic DCPD nanoparticles (mDCPD). Various physical, chemical, magnetic, *in vitro* and *in vivo* biocompatibility tests were performed to exam its properties and prove its safety. Furthermore, in vitro and in vivo cancer hyperthermia experiment were performed to evaluate its therapeutic effect.

Chapter 2   Experiment settings

2.1 Experiment equipments

The equipments used in the study are listed in Table 2-1.

Table 2-1 Equipments list

Equipment	Manufacturer
XRD	PANalytical X'Pert Pro
SEM	Philips XL30
EDS	EDAX DX4
SQUID	Quantum Design MPMS-XL7
TEM	Hitachi TEM H-7500
Electronic balance	Mettler Toledo
Ultrasonic	Branson 3510
Stirring/Hot plate	Dataplate
FTIR	JASCO FTIR 410 Series
UV-Vis absorption spectroscopy	JASCO V-550
Microscope	Nikon Eclipse TS100
Centrifuge	KUBOTA 5010
Autoclave	Tomin medical equipment

pH meter	Microcomputer Meter 6171
Heating bath	TUNGTEC INSTRUMENT
ELISA reader	Tecan
Cell culture incubator	Revco
Laminar flow	TSAO HSIN KM420
Chemical hood	TSAO HSIN

2.2 Reagents

The raw materials used in the study are listed in Table 2-2.

Table 2-2 Reagents list

Chemical Name	Manufacturer
Polyoxyethylene-bis-amine	Sigma-Aldrich
Folic acid	Sigma-Aldrich
N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC)	Sigma-Aldrich
N-hydroxysuccinimide (NHS)	Sigma-Aldrich
Hydrochloric acid (HCl)	Riedel-de Haën
Vivaspin 20	Vivascience
Cellulose tubular membrane (Nominal MWCO:6000-8000) (Part No. 8030-32)	Membrane Filtration Product, Inc.
Cellulose tubular membrane (Nominal MWCO:3500) (Part No. 5030-46)	Membrane Filtration Product, Inc.
Dulbecco's Modified Eagle's Medium (DMEM)	Sigma-Aldrich
Minimum essential medium eagle (MEME)	Sigma-Aldrich
Thiazolyl blue tetrazolium bromide (MTT)	Sigma
WST-1 Kit	BioVision

Sodium bicarbonate	Sigma-Aldrich
Fetal bovine serum (FBS)	Gemini Bio-Products
0.22 μm, GP express plus membrane, 500 ml funnel, 45 mm neck size	Millipore
Sodium chloride	Riedel-de Haën
Potassium duhydrogen phosphate	Riedel-de Haën
Potassium chloride	Riedel-de Haën
Penicillin − Streptomycin − Neomycin Solution (100x)	Sigma-Aldrich
90 mm Petri Dish	Greiner bio-one
15 ml centrifuged tube	Greiner bio-one
50 ml centrifuged tube	Greiner bio-one
70 % HNO_3	J. T. Baker
Potassium hexacyanoferrate (II) trihydrate	Sigma
Iron (III) chloride	Sigma
Trypsin-EDTA solution (10×)	Sigma
Trypan blue solution (0.4%)	Sigma-Aldrich
96-well plate	Greiner bio-one
12-well plate	Greiner bio-one
CytoTox 96 Non-Radioactive Cytotoxicity Assay	Promega

2.3 X-ray diffractometer (XRD)

XRD (x-ray diffractometer) is a powerful device to determine the compositions of crystallized material by measuring the intensity of the reflected x-ray and Bragg angles from a crystal structure. The basic theory of XRD is established by Bragg's law [182]. Since the wavelengths of some x-rays are approximately equal to the distance between planes of atoms in crystalline materials, the intensity and angles of constructive waves can be measured when x-rays strike the materials. An x-ray diffraction pattern shows the intensity of diffracted beams vs. the diffraction angles 2θ. By Bragg's law: $2d\sin\theta = n\lambda$, the diffracted angles (θ) and wavelength (λ) are known, so the distance of planes can be obtained. Moreover, the equation: $d = a/(h^2+k^2+l^2)^{\wedge}(1/2)$ (for cubic) can determine the value of lattice constant when h, k, l are well-known by specific diffracted angles [183]. The compositions of materials can then be acquired from the lattice constant. However, now it is convenient to know the result of XRD directly without calculating by means of the construction of data base (JCPDS or PCPDF). In the study, the composition of nanoparticles was checked by XRD (x-ray powder diffraction, PANalytical X'Pert Pro) (Fig. 2-2) .

$$n(\lambda) = 2d\sin(\theta)$$

$AB = d\sin(\theta) = BC \quad \therefore \quad ABC = 2d\sin(\theta)$

$A'B' = 2d\sin(\theta) = B'C' \quad \therefore \quad A'B'C' = 2ABC = 4d\sin(\theta)$
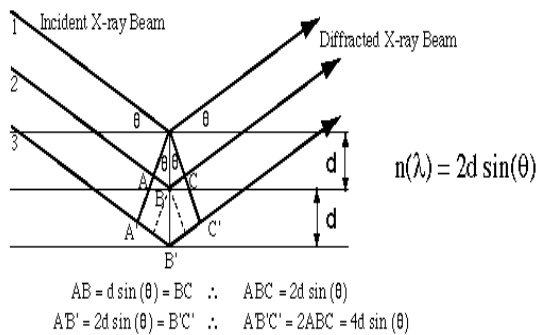
Fig. 2-1 Bragg's law [182]

Fig. 2-2 PANalytical X'Pert pro diffractometer

2.4 Scanning electron microscopy (SEM) and energy-dispersive x-ray spectroscopy (EDS)

SEM (scanning electron microscopy) is commonly utilized for observation of surface morphology of the materials, and EDS (energy-dispersive x-ray spectroscopy or called EDX) is often attached on SEM for analyzing chemical compositions. Comparing to optical microscope, the resolution of SEM is much higher due of the much smaller wavelength of electron than visible light. The most ordinary images of SEM come from detecting the secondary electrons ejected from the surface of the sample [183]. EDS is a device that detects characteristic x-ray emitted from the excitation of electron beam. When characteristic x-ray ejects into the detected

semiconductor, it generates the electrons and electron holes. By transforming the number of electrons and electron holes to the magnitude of energy, the compositions of materials can be determined easily [184]. In Fig. 2-3, it shows the equipments of SEM (Philips XL30), which is located in the right of the figure. The red circle in the figure is EDS (EDAX DX4) attached on SEM.

Fig. 2-3 SEM with EDS

2.5 Superconducting quantum interference device (SQUID)

SQUID (superconducting quantum interference device) is a promising magnetometer for its sensitive detector for magnetic flux (Fig. 2-4). It consists of two Josephson junctions (two superconductors linked by an insulated barrier [185]) that behavior tunneling effect [186]. When a current in one end of semiconductor is larger

than a critical value or the thickness of insulated barrier is less than a critical value,

the current can tunnel to the other end of semiconductor. Moreover, the current that

follows the Josephson formulas would radiate electromagnetic waves. When applying

SQUID for measure, pick-up and input coil form a superconducting loop. This loop

interacts with magnetic sample and generates inductive current by Lenz's law. The

inductive current has magnetic flux that can be detected by Josephson junctions of

SQUID, and then the following calculation gives the result of magnetic field.
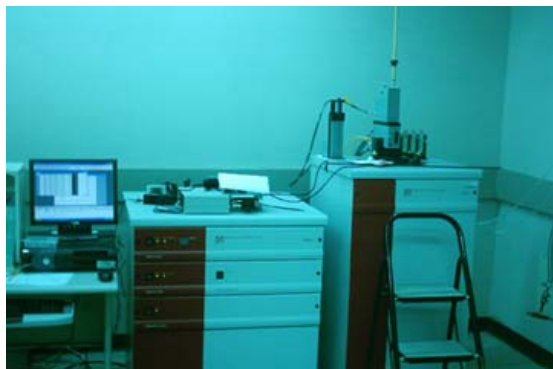
Fig. 2-4 SQUID
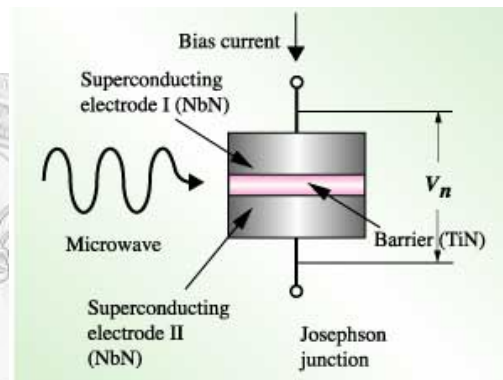
Fig. 2-5 Josephson junction [187]

2.6 Transmission electron microscopy (TEM)

TEM (transmission electron microscopy) is an useful equipment for materials

analysis, because it can acquire the morphology and composition of material at the

same time. The basic theory of TEM is that a beam of electrons is transmitted through a thin specimen and generates electron diffraction. There are many types of analysis method of TEM, including bright field, dark field, diffraction pattern and high-resolution TEM imaging. Bright and dark field is the standard method to obtain the image of material morphology. The structure composition and crystallization of material can be known by calculation of diffraction pattern. High-resolution TEM imaging can show atom image or arrange stripe of atoms by phase contrast.

There are four parts of TEM: column, electric system, vacuum system and panel. The column is the main body of TEM contains of magnetic lens, illumination system (electron gun, condenser lens, specimen chamber, image forming system, objective lens, intermediate lens and projective lens). When an electron beam emits from the electron gun by thermionic emission or field emission, it can go through the condenser lens and objective lens. If the electron beam focuses on focal plane and passes through intermediate and projective lens, the diffraction pattern can be formed (selected-area diffraction, SAD). If there is only one electron beam (transmitted beam) filtered out by an aperture and focuses on image plane, the bright or dark field can be formed. The difference between bright field and dark field: the former is only the direct electron beam allowed to pass when an aperture placed in the back focal plane of the objective lens, and the later is one or more diffracted beams allowed to pass when the direct electron beam blocked by aperture. Moreover, if there are one more electron beams go through the aperture, the high-resolution TEM imaging can be formed [188, 189]. (Fig. 2-6)

However, there are some limitations for TEM, although TEM is a powerful

device for material analysis. Sample preparation of many materials is time-consuming

and complicated because it has to produce extreme thin sample for transparent of

electrons. Moreover, sample with polymers, cells, tissues, and nanoparticles may

pollute the vacuum system. It is also difficult for TEM to analyze magnetic materials

owing to the aberration and damage of magnetic lens.
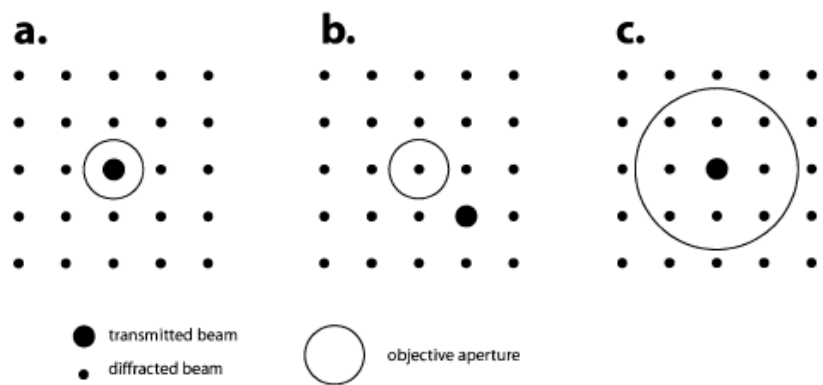
Fig. 2-6 Three observation types in electron microscopy using an aperture and the center of the aperture

is on the optical axis. (a) Bright field (b) Dark field (c) High-resolution TEM imaging[189]
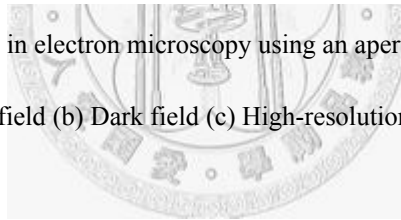
2.7 Biocompatibility

2.7.1 Lactate dehydrogenase (LDH) assay

Lactate dehydrogenase (LDH) is an enzyme present in the cytoplasm of cells and rapidly released into the cell culture supernatant upon damage of the plasma membrane. Therefore, cell death or cytotoxicity is evaluated by the quantification of plasma membrane damage, based on the measurement of activity of LDH released from damaged cells. LDH activity can be determined by a coupled enzymatic reaction. LDH oxidizes lactact to pyruvate which than reacts with tetrazolium salt to form formazan. The increase in the amount of formazan produced in culture supernatant directly related to the increase in the number of lysed cells. The formazan dye is water-soluble and can be detected by ELISA reader at 490 nm. [190, 191]. Therefore, LDH activity released from the cells into the medium was measured with a commercially available assay kit. The commercialized assay kit (CytoTox 96® Assay, Promega) can be used for this assay. The concept is shown in Fig. 2-7 [192]. The ELISA reader can be used to measure absorbance at 490 nm.
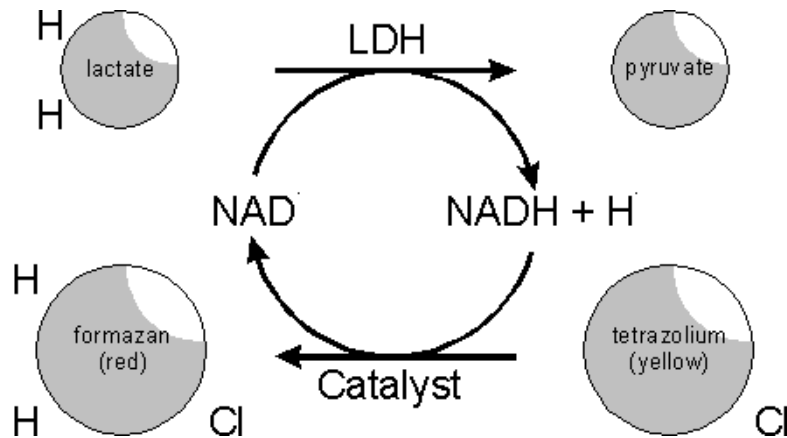
Fig. 2-7 The process of formazan generation from LDH [192]

2.7.2 Water-soluble tetrazolium salt-1 (WST-1)

assay

Dehydrogenase in the mitochondria of living cells is capable of cutting

tetrazolium ring. Hence tetrazolium salts are often used to test for cell proliferation.

WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl) -2H-5-tetrazolio]-1, 3-benzene

disulfonate) is one of tetrazolium salts. After dehydrogenase cut its tetrazolium ring,

red WST-1 tends to generate deep red Formazan. [190] The WST-1 reagent (10%

WST-1 in culture medium) is directly measured for its absorbance value at 420 nm by

ELISA reader. Higher value indicates better cell viability and proliferation. (Fig. 2-8)

[193]
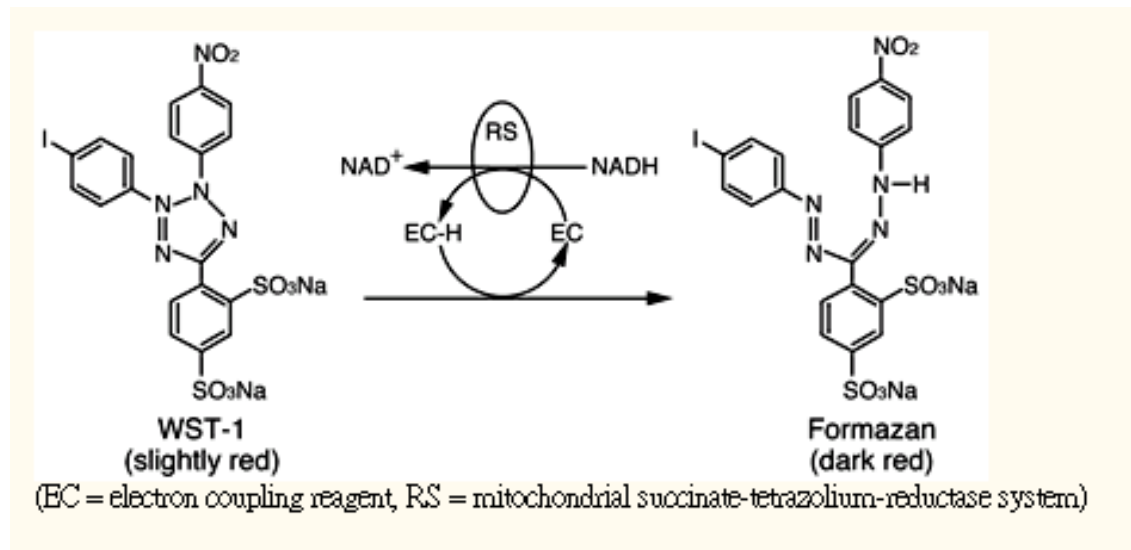
Fig. 2-8 Reaction of WST-1 and dehydrogenase [193]

Chapter 3   Hydroxyapatite -modified

biomagnetic nanoparticles

3.1 Introduction

Magnetic nanoparticles have been used commonly for various biomedical circumstances [162, 178, 194]. Recently, the functional properties of the magnetic nanoparticles have been modified individually so that they could be used in different biological applications, such as cell label and separation [166, 167, 195, 196], immunoassay [177, 197], drug delivery [179-181], MRI contrast agents [158, 168, 198-201], and hyperthermia [94, 108, 109]. Iron oxide (maghemite γ-Fe$_2$O$_3$ or magnetite Fe$_3$O$_4$) is one of the most popular magnetic nanoparticles in medicine and biotechnology [164, 202]. Recently, many breakthrough of synthesis and surface engineering of iron oxide nanoparticles have been made among several magnetic particles [146]. Moreover, there are still newly-formed magnetic nanoparticles proposed (bioglass ceramics [125, 203] and hydroxyapatite [204]).

Many researchers have developed different magnetic nanoparticles of ferrimagnetic bioglass ceramics (FBCs) which provide magnetic properties for MRI and hyperthermia purposes [125]. Nevertheless, during the preparation of FBC, it has been put in an oven at temperature higher than 800℃. Then it was quenched to room temperature. This procedure would probably cause crystal growth, which made it

difficult to synthesize nano-sized particles [205]. Therefore its huge size limited its own application in biomedical settings, such as injection into venous system and reaching the tumor tissue inside the animal body.

One of the mineral components inside the bone and teeth of the human body is Calcium hydroxyapatite (HAP), with a chemical formula of $Ca(2)_6Ca(1)_4(PO4)_6(OH)_2$ ($P6_3/m$). Therefore it possessed good biocompatibility property and adequate biodegradation rate that not only has been widely used in orthopaedics as bone grafts but also in drug delivery systems for controlled release [206-208]. The physicochemical and biological properties of apatite can be dominated by different compositions and crystal structures [209]. By particular coprecipitation with various amounts of metal ions, such as Ni(II), Co(II), Al(III), and La(III), scientists could substitute the calcium ions (Ca^{2+}) in the HAP. The magnetic nanoparticles would have different surface configurations, morphologies, and crystal architectures [210-212].

In our previous study [204], magnetic hydroxyapatite nanoparticles were fabricated by the co-precipitation process with different concentration of Fe^{2+} added. The characteristics and biocompatibility of this newly developed magnetic nanoparticle were analysed in each sample. The particle size was around 20-50nm with a shape of short-rod or sphere. The magnetization was around 3.42-20.92 emu/g. Among all different nanoparticles proposed, the sample with molar ratio of Fe/Ca as

1($X_{Fe/Ca}$=1) was chosen for this *in vivo* study. The ratio showed optimum crystal size with better heating rate when magnetic field applied on. It should have a better performance as a thermoseed for hyperthermia (as shown in table 2 and Fig. 9 of reference 91).

In this study, we try to apply the magnetic biomaterial for hyperthermia therapy in an animal model. We would like to evaluate its *in vivo* heating efficiency and tumor curing ability.

3.2　Materials & Methods

3.2.1　Preparation of hydroxyapatite (HAP) and

magnetic-HAP(m-HAP) nanoparticles

The synthesis of Hydroxyapatite (HAP) is referred to [213, 214]. The reaction is as follows:

$10Ca(OH)_2 + 6H_3PO_4 \rightarrow Ca(2)_6Ca(1)_4(PO_4)_6(OH)_2 + 18H_2O$.
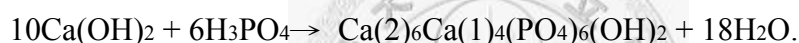
The magnetic- HAP powders were synthesized by a similar method, and it was introduced by our previous paper [204]. In brief, the steps contained addition of iron (II) chloride tetrahydrate ($FeCl_2\cdot4H_2O$, Fluka, USA) , adjusting pH to 8.5, stirring for 2 h followed by ageing for another 10 h, washing, and freeze-drying. The final product of a dispersible apatite powder was made. As mentioned before in the "Introduction" section, nanoparticles with molar ratio of Fe/Ca as 1($X_{Fe/Ca}=1$) were chosen for the following experiments in this study.

3.2.2　Heating efficiency (*in vivo*)

In the pilot study we have tested *in vivo* heating efficiency of the material. The 0.8g magnetic-HAP powder (mHAP) or pure HAP powder (HAP) were mixed with 5ml phosphate buffer solution (PBS), respectively. An amount of 0.5c.c mixture was

injected subcutaneously around the tumor of mice with no.21 syringe.

In order to achieve hyperthermia, the mouse were placed into a 3cm diameter coil of the inductive heater (Power cube 64-power cube HF2; AREZZO, PRESIDENT HONOR IND CO.,LTD., Taiwan). Then the heater was turned on and alternating magnetic field (60Hz, 110V) was generated inside the coil (47.75G). An optic fiber was inserted inside the tumor in order to monitor temperature. The temperature was recorded by a thermometer connected to the optic fiber (Luxtron One, Lambda Photometrics, United Kingdom).(Fig 3-1, Fig 3-2)
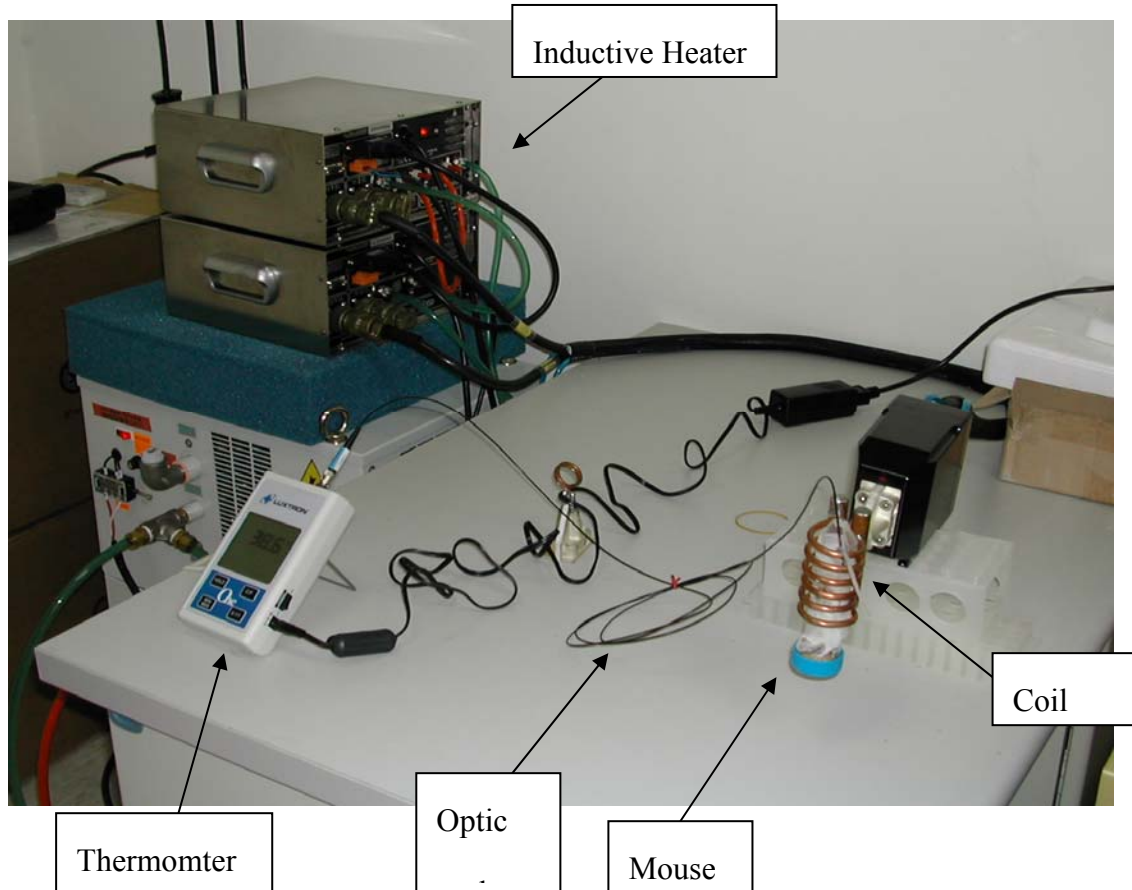
Fig. 3-1 The photo of the animal study.
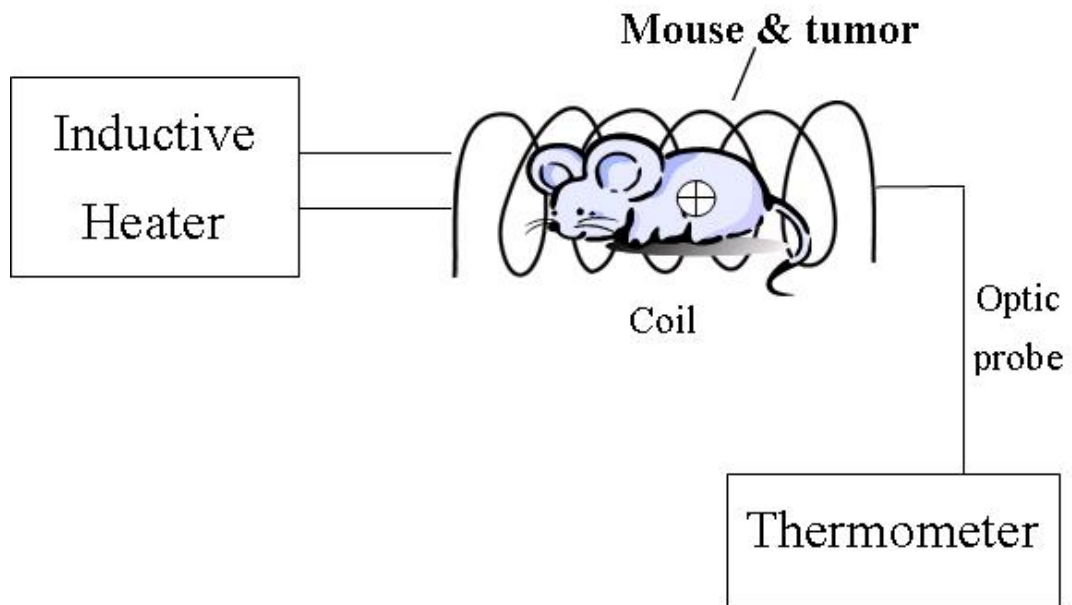
Fig. 3-2 The line-drawing setting of the experiment.

The heating efficiency was tested with different concentration of mHAP and the optimal concentration of mHAP and the optimal concentration (0.8g/5ml) was selected. Only the body temperature of mHAP-injected mice with magnetic field raised. The mHAP injected mice without magnetic field did not show hyperthermia effect. In the mHAP + magnetic field group, the temperature was raised to the desired temperature (45~46 °C) within 15 minutes. A pedal was connected to the inductive heater. Only when the pedal is pressed, electric power will be supplied to the coil. By stepping on and off the pedal intermittently, we were able to switch the magnetic field on and off. Therefore we could keep the temperature between 45~46 °C for 20 mins without overheating the animal. In addition, we also put another optic fiber into the rectum of mice and recorded the core body temperature. During the 20 minutes of the hyperthermia, the core body temperature slightly raised from 38°C to 40°C. (Fig. 3-3)
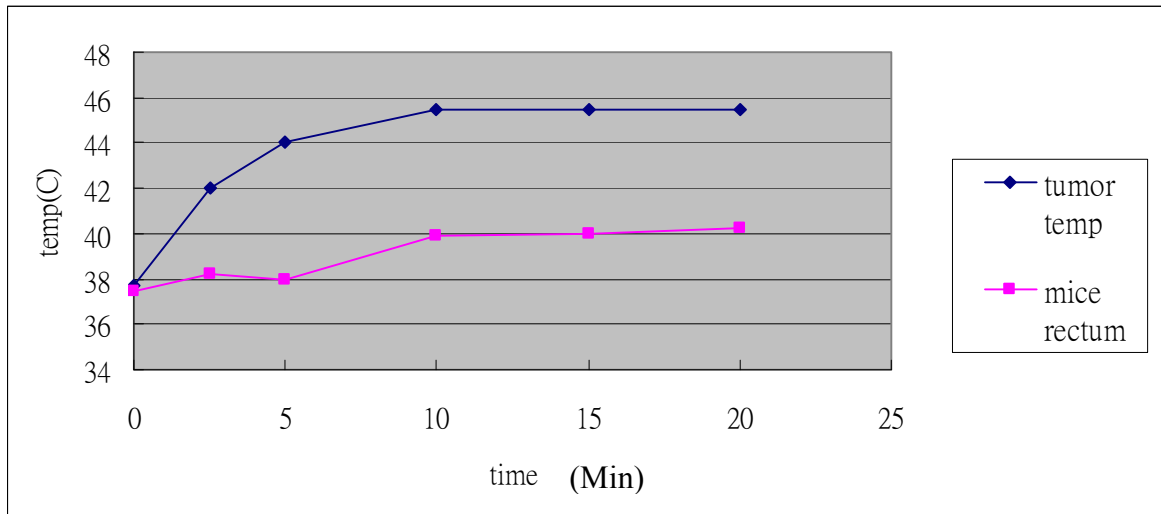
Fig. 3-3   The heating efficiency of mHAP. During the 20 minutes of the hyperthermia, the core body temperature slightly raised from 38°C to 40°C in all six groups.

3.2.3 Animal study

A total number of 37 six-week old balb/c mice were prepared. Each mouse was inoculated with 5×10^6 murine colorectal cancer cells (CT26 cell line). Then, we waited for another 7-10 days and allowed tumor to grow as big as its diameter achieved 0.7cm~1.2cm.

These 37 mice were divided into two categories and six groups. Mice in the first category did not receive any magnetic field; whereas those in the second category were put into the coil of the heater. The first category contained three groups: Group 1 (control group, n=6), the mice were not injected with any material. Group 2 (n=6), the mice were injected with HAP. Group 3 (n=6), the mice were injected with mHAP.

On the other hand, the second category (with magnetic filed) also contained three groups: Group 4 (control group, n=6), the mice were not injected with any

material. Group 5 (n=6), the mice were injected with HAP. Group 6 (n=7), the mice were injected with mHAP. In this category, all mice were treated with hyperthermia each day during the first three day of the experiment. Then they were put into the coil on the other day (Day 5,7,9,11,13,15).

All 36 mice were sacrificed on Day 15 and blood test was performed to check the liver and renal function.

3.3 Results

During the 20 minutes of the hyperthermia, the core body temperature slightly raised from 38°C to 40°C in all six groups. (Fig. 3-4) The core body temperature was not effected by the focal hyperthermia effect of mHAP-injected site on the back. All mice sweated during these 20 minutes. But they did not have symptoms of dehydration and all survived the whole experiment of two weeks.

Among the six groups, only the tumors in Group 6 (mHAP with magnetic field) shrinked significantly. The tumors in Group 1(control group without magnetic field) growed faster than any other groups; except on Day 13&15, the size of tumor in Group 2 (HAP without magnetic field) was larger than Group 1. (Fig. 3-5)
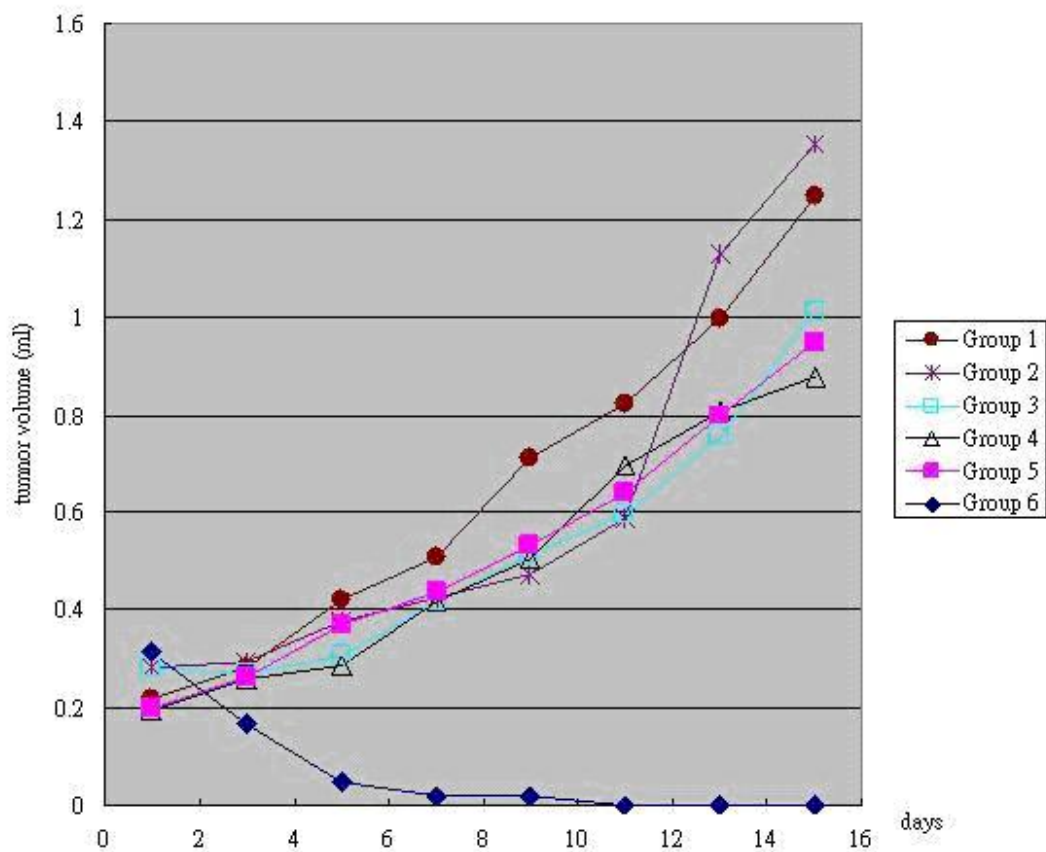
Fig. 3-4 The tumor size of different groups in the experiment period of 15 days. Among the six groups, only the

tumors in Group 6 (mHAP with magnetic field) shrinked significantly. The tumors in Group 1(control group

without magnetic field) growed faster than any other groups; except on Day 13&15, the size of tumor in Group 2

(HAP without magnetic field) was larger than Group 1.

The tumors not only shrinked rapidly in Group 6(mHAP with magnetic field),

but they became black, flat and hard in day3-5. The black area also disseminated

across the back of the mouse. No skin necrosis was noted in the observation period of

15 days (Fig. 3-5). The tumor surface did not turn black in Group 3 (mHAP without

magnetic field), suggesting the Ferrous ion deposition is not the only cause of the dark

tumor surface (Fig. 3-6). Furthermore, in Group 4 (control group with magnetic field),

central necrotic area of the tumor was noted in day 5 and enlarging till day 14

(Fig.3-7). The same phenomenon was also noted in Group 1 (control group without

magnetic field)(Fig. 3-8).

(A)   (B)

(C)
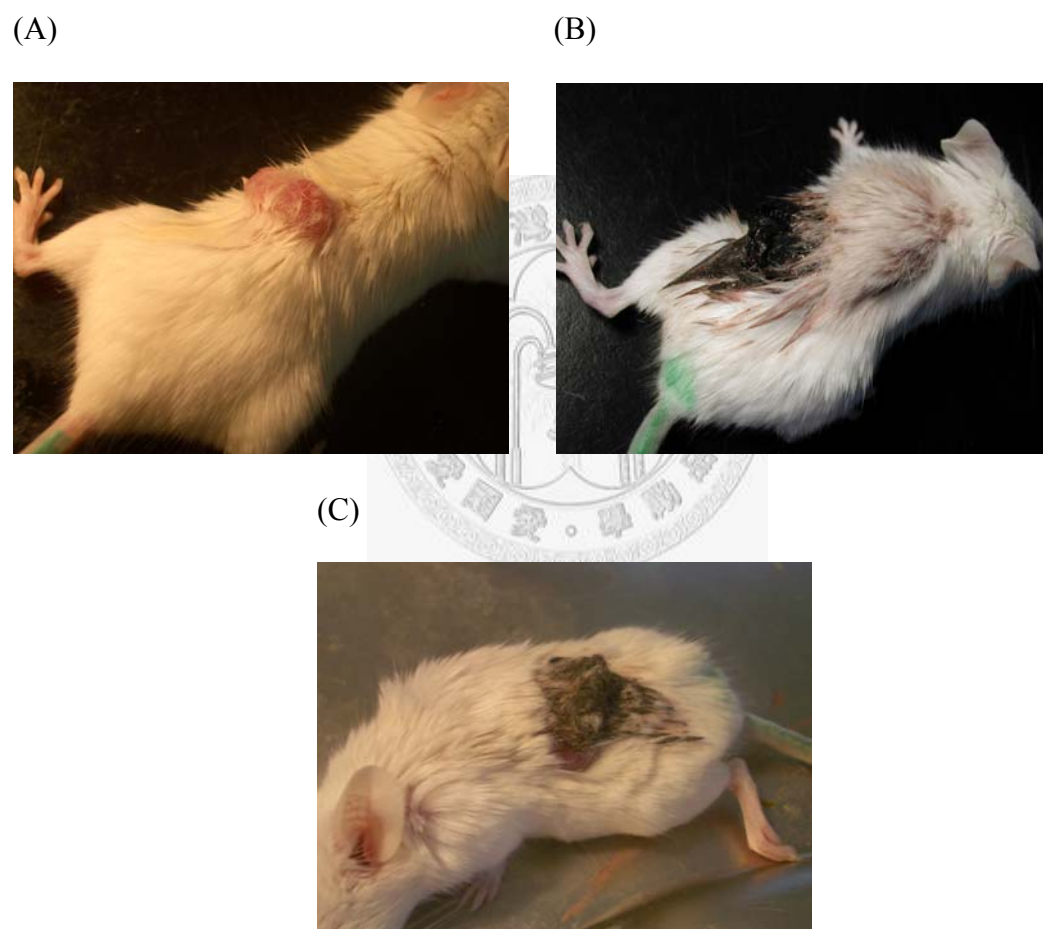
Fig. 3-5 The clinical photographs of the tumor in Group 6 (mHAP with magnetic field). The tumor in

day1 (A), day5 (B), and day 14 (C).

(A)　　　　　　　　　　　　　　(B)

(C)

Fig. 3-6 The clinical photographs of the tumor in Group 3 (mHAP with magnetic field). The tumor in day1 (A),

day5 (B), and day 14 (C).

(A)
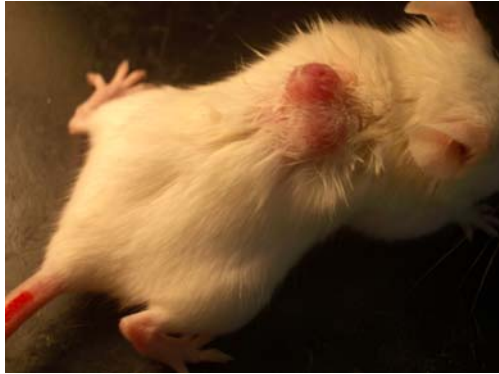
(B)

(C)
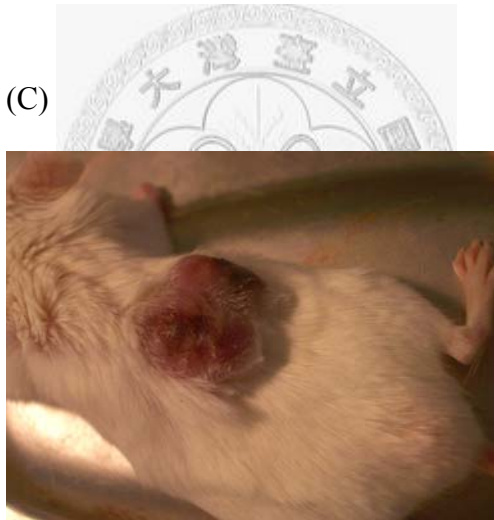
Fig. 3-7 The clinical photographs of the tumor in Group 4 (control group with magnetic field). The tumor in day1 (A), day5 (B), and day 14 (C).
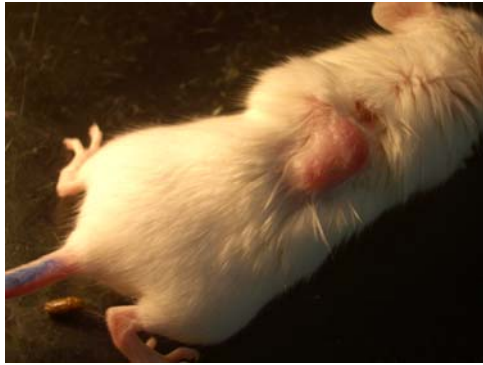
(A) (B)
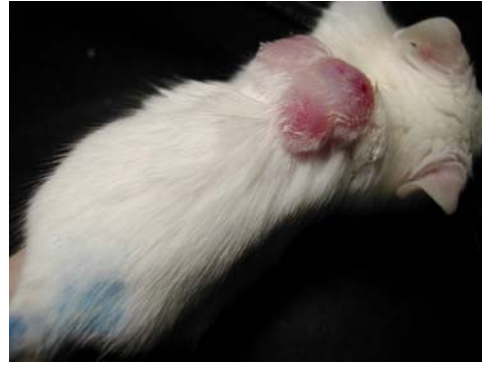
(C)

Fig. 3-8 The clinical photographs of the tumor in Group 1 (control group without magnetic field). The tumor in day1 (A), day5 (B), and day 14 (C).

The blood test result of liver and renal function was listed in Table 3-1. All the animals in six groups showed normal renal function due to they have normal BUN and Creatinine levels. However, all the animal have abnormal liver function due to elevated ALT and AST levels. All the animals have normal ALP level.

Table 3-1 The blood test result of liver and kidney function after the animal was sacrificed.

	BUN (mg/dL)	Creatinine (mg/dL)	Bilirubin (mg/dL)	ALT* (units/L)	AST* (units/L)	ALP* (units/L)
Group 1	20.67±3.54	0.43±0.15	10.37±5.13	1626.33±849.36	2289.33±1755.62	20.67±3.54
Group 2	28.50±11.15	0.22±0.08	4.18±4.83	1452.00±598.38	2050.80±778.28	62.00±38.86
Group 3	28.33±16.35	0.18±0.08	2.90±1.31	943.50±750.06	1756.83±704.33	43.00±28.04
Group 4	21.00±5.22	0.32±0.12	7.25±4.82	1542.67±1023.08	1694.80±181.16	71.67±57.03
Group 5	23.5±8.78	0.25±0.10	3.42±3.77	710.00±564.23	1390.8±358.74	34.40±12.97
Group 6	23.29±15.82	0.34±0.28	1.5±1.26	2268.86±1708.68	2442.67±603.49	74.71±43.73
Normal Range	18-29	0.2-0.8	0.1-0.9	28-132	15-247	62-209

All the animal in six groups have normal kidney function due to normal BUN and Creatinine levels.

However, all the animal have abnormal liver function due to elevated ALT and AST levels. All the

animals have normal ALP level.

*AST= Aspartate aminotransferase (AST). AST formerly was called serum glutamic oxaloacetic

transaminase (SGOT). ALT= Alanine aminotransferase (ALT). ALT formerly was called serum

glutamic pyruvic transaminase (SGPT). ALP= Alkaline phosphatase (ALP)

3.4 Discussion

The body temperature of the testing animals in the magnetic groups was higher than the non-magnetic groups. We though it might be due to direct thermal conduction from the heated, working coil. And the ferrous material inside the mice, such as the hemoglobulin circulating in blood vessels, might also play a role in this phenomenon.

As mentioned before, the tumors shrinked and became black, flat and hard in day3-5 (Fig. 3-5). The black area also disseminated across the back of the mouse. The black color was only partially contributed by repeated injection of Ferrous-containing HAP and subsequent local deposition of Ferrous ion, because we did not observe the same finding in Group 3 (mHAP without magnetic field) (Fig 3-5). Our explanation of the disseminated black area in Group 6 is: the hyperthermia killed the tumor and created a void space. Then, the mHAP particles flowed out of the injection area and filled the new space all over the back.

In Group 4 (control group with magnetic field), central necrotic area of the tumor was noted in day 5 and enlarging till day 14 (Fig. 3-7). The same phenomenon was also noted in Group 1 (control group without magnetic field)(Fig. 3-8). Because there was no mHAP particle injected, the tumor itself should not generate heat. The central necrosis should be merely due to poor nutrition supply in a rapidly-growing tumor.

Among the six groups, only the tumors in Group 6 (mHAP with magnetic field) shrinked significantly. The tumors in Group3 (mHAP without magnetic field) still growed rapidly, which indicating that the mHAP along could not reduce the tumor

volume. In other words, the shrinkage of the tumor in Group 6 was solely due to the hyperthermia effect caused by mHAP , and the mHAP along did not have the ability of killing tumor cells.

The tumor in Group 1(control group without magnetic field) grew faster than any other groups; except on Day 13&15, the size of tumor in Group 2 (HAP without magnetic field) was larger than Group 1. It suggested that the HAP itself may help the colorectal tumor growth after 2 weeks. However, we did not observe the same phenomenon in Group 5(HAP with magnetic field). We thought that the elevated body temperature in the magnetic field could kill part of the tumor cells, and it neutralized the anabolic effect of HAP on tumor cells.

The blood test result of liver and kidney function contained a lot of important information. All the animals in six groups have normal kidney function due to normal BUN and Creatinine levels. However, all the animals have abnormal liver function due to elevated ALT and AST levels. It indicated that the metabolism of HAP and mHAP was mainly through liver. However, the elevated AST and ALP levels only indicated hepatitis in this point. Because all the animals survived through the whole experiment. It indicated that the liver was not seriously damaged and it was not fatal.

Moreover, the animals in the magnetic groups did not show consistent elevated liver and kidney function, comparing with the animals in the non-magnetic groups. Therefore we could conclude that the high frequency reciprocal magnetic field we applied on the animals did not show significant impact on the liver and kidney function.
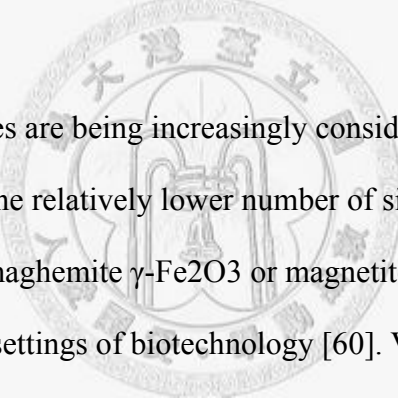
All the animals have normal ALP levels, indicating that bone metabolism was not disturbed with the application of HAP. As stated before, the HAP was one of the

important constitution of bone and teeth. Thus we are concerned about its possible

effect on the bone absorption and metabolism in the beginning.

Chapter 4　Dicalcium phosphate dihydrate (DCPD)- modified biomagnetic nanoparticles

4.1 Introduction

Magnetic nanoparticles are being increasingly considered for hyperthermia cancer therapy because of the relatively lower number of side effects associated with them [69-71]. Iron oxide (maghemite γ-Fe2O3 or magnetite Fe3O4) is frequently used in clinical or experimental settings of biotechnology [60]. Various magnetic nanoparticles of ferrimagnetic bioglass ceramics (FBCs) were developed recently, and aimed to provide magnetic properties for MRI and hyperthermia purposes[45]. Nevertheless, during the preparation of FBC, it had to heat up to 800 °C and then quenched to room temperature. Particle size, especially after further milling, is difficult to control at the nanoscale, and quasi-crystallisation may occur during the thermal processing. This has made it difficult to synthesis magnetic bioglass nanoparticles[61], which has limited their potential clinical applications. This has led to a need for different forms of magnetic nanoparticles for clinical use. Furthermore, magnetic nanoparticles have also been proven useful in other applications, such as

cell labeling [70].

Calcium phosphate ceramic (CPC) has good biocompatibility, little toxicity and an adequate biodegradable rate. Therefore, many studies have proved its efficacy as a bone substitute [72-74]. Its adequate biodegradable rate make it a good drug delivery vehicle [75, 76]. It has also been used quite often by dentists [51], as root canal fillers, drug-delivery vehicles, or scaffolds in pulp tissue engineering [77]. It was also been modified to be used in vertebroplasy as potentially valuable alternative to polymethylmethacrylate (PMMA) [78].

There are different phases in the CPC system, such as: dicalcium phsosphate dihydrate (DCPD, $CaHPO_4.2H_2O$), also called brushite; hydroxyapatite (HAP, $Ca_{10}(PO_4)_6(OH)_2$) and tricalcium phosphate (TCP, $Ca_3(PO_4)_2$). Among them, DCPD is the most stable phase in the acid environment [50]. DCPD is not a magnetic ceramic, but the iron ions can substitute the calcium ions of DCPD and form a magnetic DCPD (mDCPD), which is a possible candidate for cancer hyperthermia application.

Thus, in this study, we synthesized mDCPD with different concentration of iron and evaluated their physical properties and cytotoxicty. In vitro tests to assess their potential for killing cancer cells during hyperthermia were then carried out.

4.2 Material & Methods

4.2.1 DCPD synthesis

DCPD was prepared by co-precipitation method: mixing equal Molality (0.2M) of calcium hydroxide (Riedel-de Haen) aqueous solution and orthophosphoric acid (Riedel-de Haen,85%) solution. The calcium hydroxide solution was slowly dropped into the orthophosphoric acid solution until the pH level reached 5.2. During this titration process, the rotation speed of stirrer was kept at 800 rpm with a constant temperature of $37°C$ overnight. Then the DCPD powders were centrifuged, washed with alcohol, and dried at $65°C$ in oven for another night.

4.2.2 mDCPD synthesis
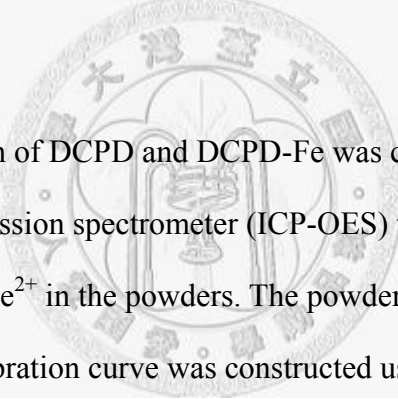
Magnetic dicalcium phosphate dihydrate (mDCPD)particles was synthesized by mixing different concentration (10%, 20%, 30%, 40%, and 60%) of iron chloride tetrahydrate (Fluka) with calcium hydroxide aqueous solution. Briefly, the orthophosphoric acid was slowly titrated into the solution containing calcium and iron. The rotation speed of stirrer was kept at 800 rpm. Temperature was fixed at $37°C$ and ph level was maintained around 5-5.2. Magnetic dicalcium phosphate dihydrate (mDCPD) powders were centrifuged, washed with alcohol, and dried at $65°C$ in oven

overnight.

4.2.3 X-ray diffraction (XRD)

The composition of DCPD and mDCPD were tested by X-ray diffraction meter (Rigaku Geigerflex DMX-2200). The XRD was operated at 40KV, 30mA , $2°$per min scanning speed and the 2θ range for $10\text{-}60°$.

4.2.4 Chemical composition (ICP-OES)

Chemical composition of DCPD and DCPD-Fe was checked by inductively coupled plasma-optical emission spectrometer (ICP-OES) to determine the concentration of Ca^{2+} and Fe^{2+} in the powders. The powders were dissolved in 1M HCL for analyses. The calibration curve was constructed using standard solutions in the range of 0.1, 0.5, 1 and 10 ppm.

4.2.5 Magnetic property

The magnetic property of DCPD-Fe was measured by Superconducting quantum interference device (SQUID) in 300K and at alternating ±10000oe magnetic field.

4.2.6 Inductive heater and temperature recording

The 50 mg mDCPD powder was dissolved in a test tube with 3 ml D.I water. It was then placed into 1cm diameter coil of the inductive heater (Power cube 64-power cube HF2; Arezzo, President Honor Ind Co.,LTD., Taiwan). Then the heater was turned on and alternating magnetic field with high frequency was generated inside the coil. The temperature was recorded by a thermometer (Luxtron One, Lambda Photometrics, United Kingdom) every 30 seconds, with an optic fiber inserted inside the test tube. (Fig 4-1, 4-2)

Fig. 4-1 The vials placed in the induction coil
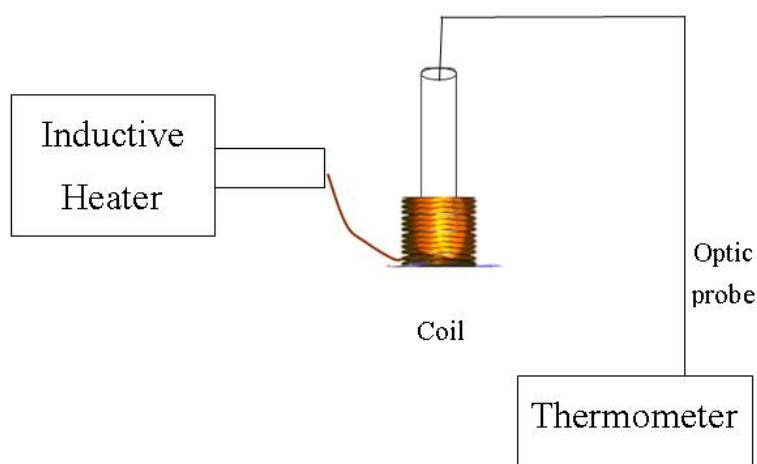
Fig. 4-2 The setting of the experiment for hyperthermia

4.2.7 Particle size

The particle size and morphology of DCPD-20%Fe was analysed by Transmission Electron Microscopy (TEM, JOEL-100CXⅡ 100kV). The sample was prepared according to standard procedure: DCPD-20%Fe particles were dispersed in the 99.5% ethanol. The solution with DCPD-20%Fe particles were dropped on the Cu-grid and dried in room temperature for later examination.

4.2.8 The WST-1 assay for mitochondrial function

DCPD-10%Fe and DCPD-20%Fe particles were immersed in the culture medium. They were kept in an environment of 5% carbon dioxide, 95% relative humidity, and 37°C for 3 days. The 3T3 (mouse fibroblast) were seeded in 96-well

culture plates at the cell density of 10^4 cells/well and cultured for one day. The extract of mDCPD-containing medium with different concentration (1mg/ml, 5mg/ml and 10mg/ml) was then added into the culture plate of 3T3 cells. It was then cultured for another three days. On Day 1 and Day 3, we collected 100 μ l medium of each well and the WST-1 assay was done. The WST-1 reagent (10% WST-1 in culture medium) is directly measured for its absorbance value at 420 nm by the ELISA reader(Tecan). Higher value indicates better cell viability and proliferation.

4.2.9 Lactate dehydrogenase (LDH) assay for cell lysis

The 3T3 (mouse fibroblast) were seeded in 96wells culture plates at a cell density of 104 cells/well. After 1 day culture, the extracts of DCPD-Fe were added into 96 wells culture plates and incubated for another 3 days. A "total lysis" positive control group was prepared by mixing lysis solution for 45 minutes. Another negative control group was also prepared by culture medium only (without any mDCPD particles). On Day 1 and Day 3, we collected 50 μ l medium of each well and the LDH assay was done. LDH released from the cells into the medium was measured by a commercially available assay kit. The absorbance was recorded at 490nm in the ELISA reader.

4.2.10 In-vitro test for cancer hyperthermia

A549(lung carcinoma) and HFL-1(human lung fibroblast) cell lines were seeded at a density of 5×10^4 cells/vials. They were incubated for 1 day, allowing cells

to be attached to the bottom of vials. In the experiment group, DCPD-20%Fe (10mg/ml) was added into the culture medium. The vials were then placed inside the induction coil and the temperature was maintained around 43-46°C for 30 minutes. 50 μl medium of each vials was collected and transferred to a 96-well culture plate. The absorbance was recorded at 490nm in ELISA reader (LDH assay). Positive control (OD "total lysis") and negative control (OD "medium") were also prepared. The percentage of cytotoxicity was then calculated by the following equation:

$$\text{Cytotoxicity (\%)} = \frac{OD_{exp} - OD_{medium}}{OD_{total\ lysis} - OD_{medium}} \times 100$$

4.3 Results

4.3.1 XRD pattern

The XRD patterns of synthesized DCPD are shown in Fig. 4-3. All the corresponding peaks are matched to the standard pattern of DCPD from JCPDS NO. 72-0713. The pure DCPD was successfully synthesized in the study that would be used as the base for mDCPD synthesis. The results are shown as follows.

The XRD patterns of Fe-doped DCPD are shown in Fig. 4-4. The XRD results for DCPD-10%Fe and DCPD-20%Fe are similar to pure DCPD. In the XRD analysis DCPD-10%Fe and DCPD-20%Fe showed no iron formation. In the XRD results of DCPD-30%Fe, DCPD-40%Fe and DCPD-60%Fe, there were some extra peaks visible in smaller angles.
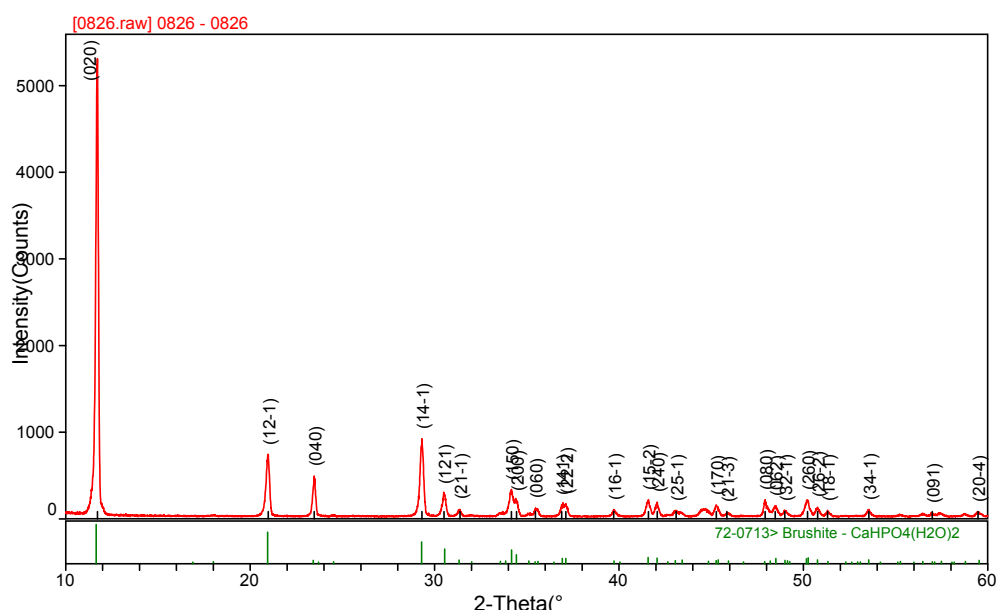
Fig. 4-3 XRD pattern of DCPD.
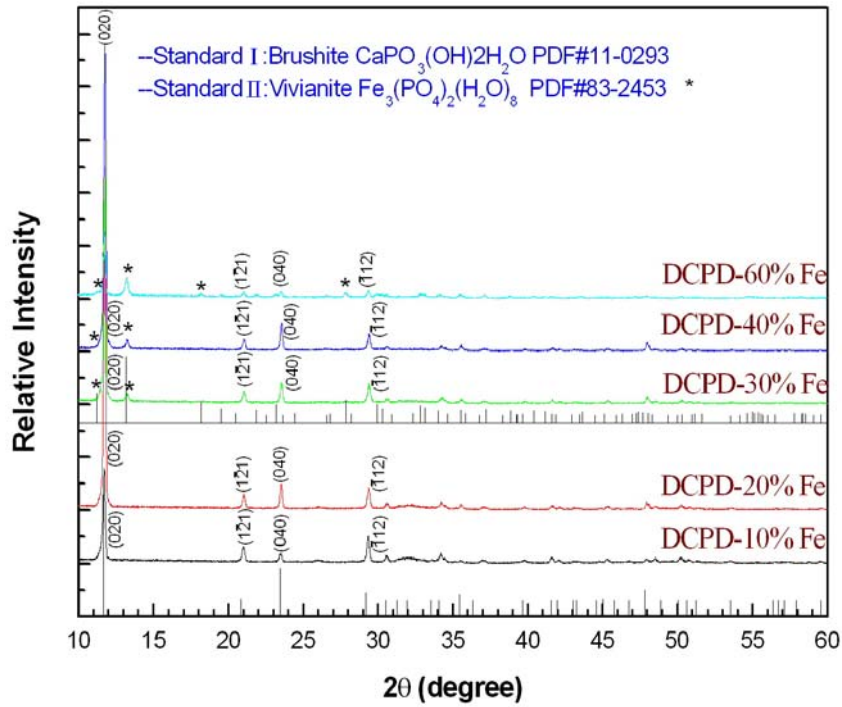
Fig. 4-4 XRD pattern of DCPD-Fe.

4.3.2 Lattice parameters

The lattice parameters of DCPD and DCPD-Fe can be calculated by the

following equation $\dfrac{1}{d^2} = \dfrac{1}{\sin^2 \beta} \left(\dfrac{h^2}{a^2} + \dfrac{k^2 \sin^2 \beta}{b^2} + \dfrac{l^2}{c^2} - \dfrac{2hl\cos\beta}{ac} \right)$, where d is the

interplanar distance. The results are shown in Fig. 4-5. It revealed that more Ca^{2+} are

substituted by Fe^{2+}, smaller lattice parameter was formed. Thus the value of b-axis is

decreased from DCPD-10%Fe to DCPD-40%Fe, but increased thereafter. The volume

of DCPD-Fe with different iron concentrations is shown in Fig. 4-6.
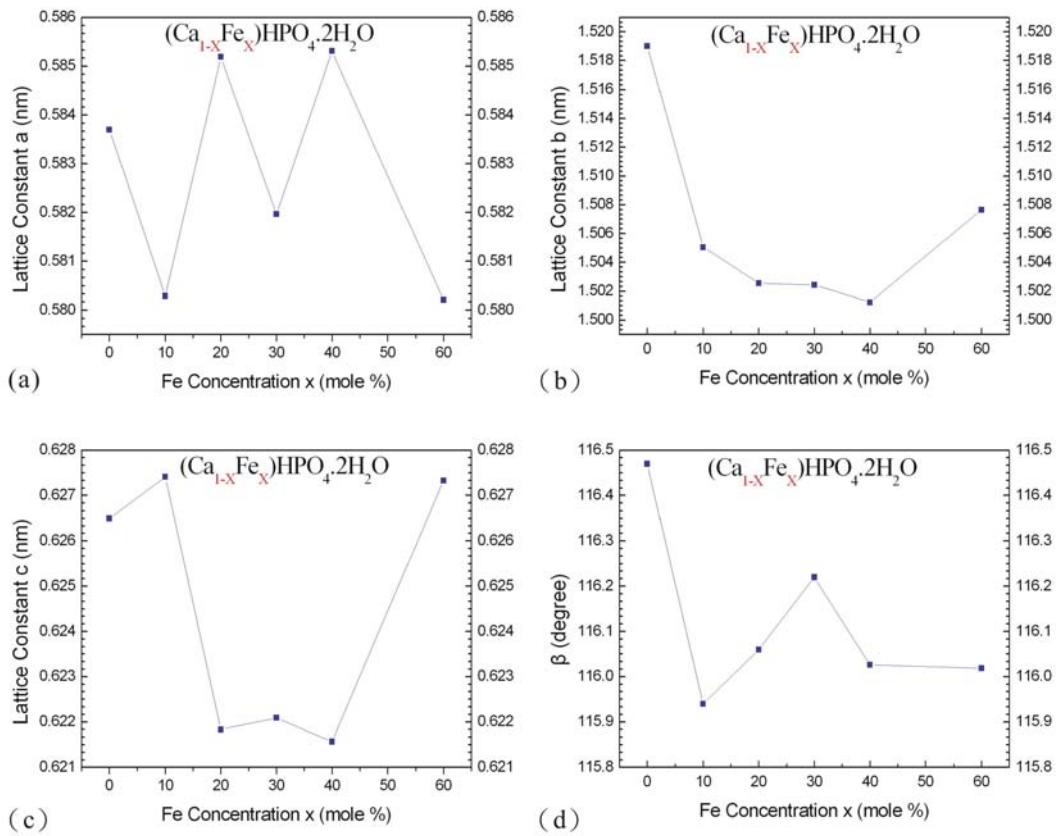
Fig. 4-5 The lattice parameters of DCPD and DCPD-Fe (a) a (b) b (c) c (d) β .
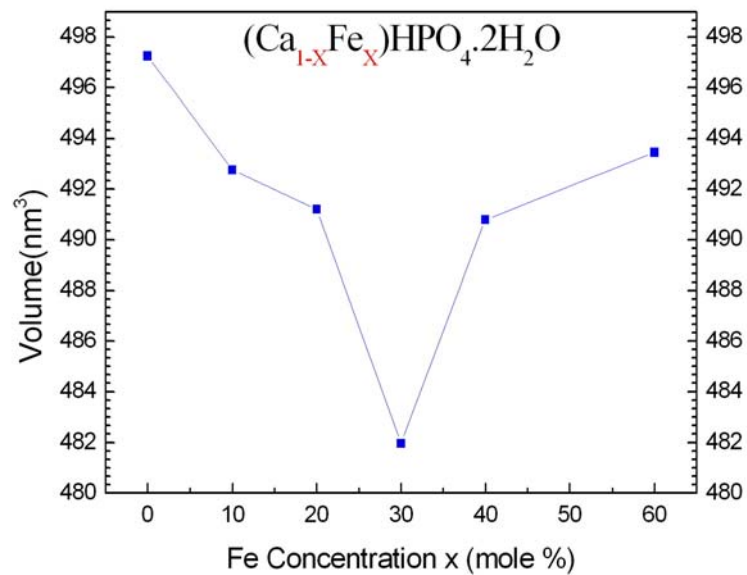
Fig. 4-6 The volume of DCPD and DCPD-Fe

4.3.3 Chemical composition (ICP-OES)

The results of elemental analysis are shown in Fig. 4-7. In DCPD-10%Fe and DCPD-20%Fe, the observed concentrations of calcium ions were decreasing with increased iron ions. The DCPD-30%Fe, DCPD-40%Fe and DCPD-60%Fe followed the same condition.

	DCPD	DCPD-10%Fe	DCPD-20%Fe	DCPD-30%Fe	DCPD-40%Fe	DCPD-60%Fe
☐ Ca	100	91.19	81.64	71	58	38
▩ Fe	0	8.81	18.36	28	42	62
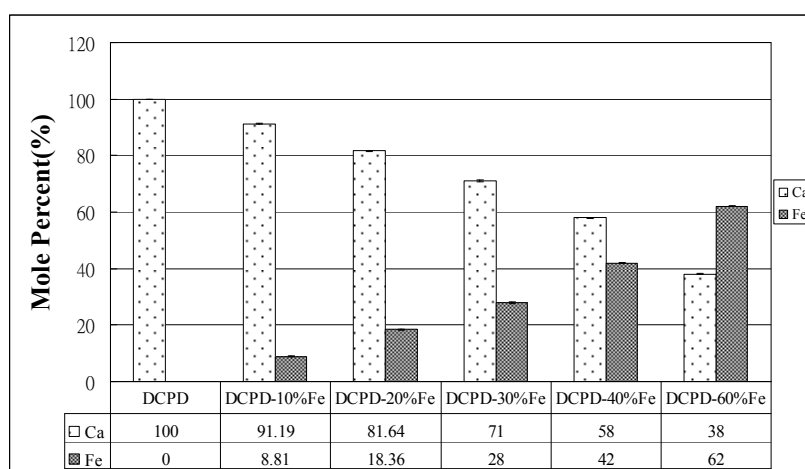
Fig. 4-7 The chemical composition of DCPD and DCPD-Fe

4.3.4 Magnetic property

Hysteresis loop of DCPD-Fe can be measured by SQUID in 300K (Fig. 4-8). Hysteresis loop was observed in DCPD-10%Fe and DCPD-20%Fe. But from DCPD-30%Fe to DCPD-60%Fe, the hysteresis loop became less and less prominent with increasing iron percentage.

By integration, the area of hysteresis loop of DCPD-10%Fe and DCPD-20%Fe

was 168.7($\frac{emu}{g} \times Oe$) and 552.9($\frac{emu}{g} \times Oe$) respectively. It is supposed that 1mg

DCPD-10%Fe and DCPD-20%Fe under 5000Oe alternative magnetic field of 10^6HZ,

the energy generation was 0.004038 cal/sec and 0.01328 cal/sec respectively.
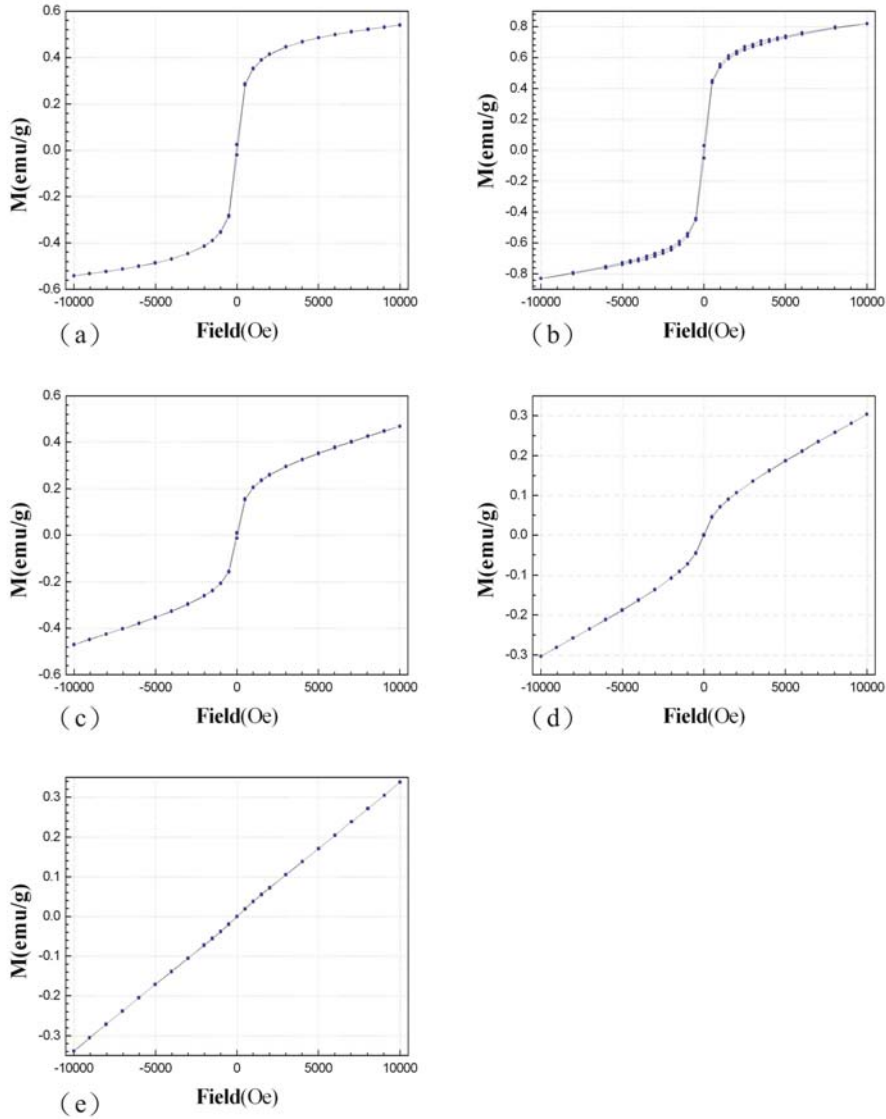
Fig. 4-8 The hysteresis loop of DCPD and DCPD-Fe (a)DCPD-10%Fe (b)DCPD-20%Fe

(c)DCPD-30%Fe (d)DCPD-40%Fe (e)DCPD-60%Fe.

4.3.5 Heating efficiency

The temperature of mDCPD inside the coil was recorded per 30 second (Fig. 4-9, Fig. 4-10). The normal human body temperature was 37°C, and the temperature scope needed by hyperthermia was 43-46°C. Therefore, mDCPD had achieved the temperature of hyperthermia within 10 mins.
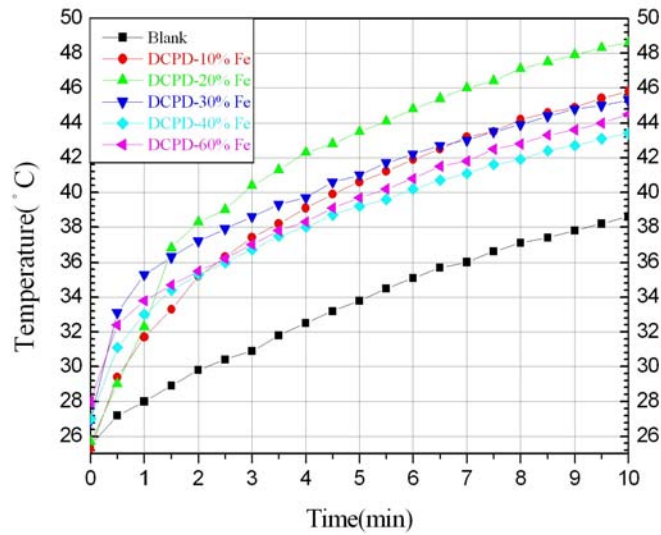
Fig. 4-9 The raised temperature of DCPD-Fe under an alternating magnetic field.
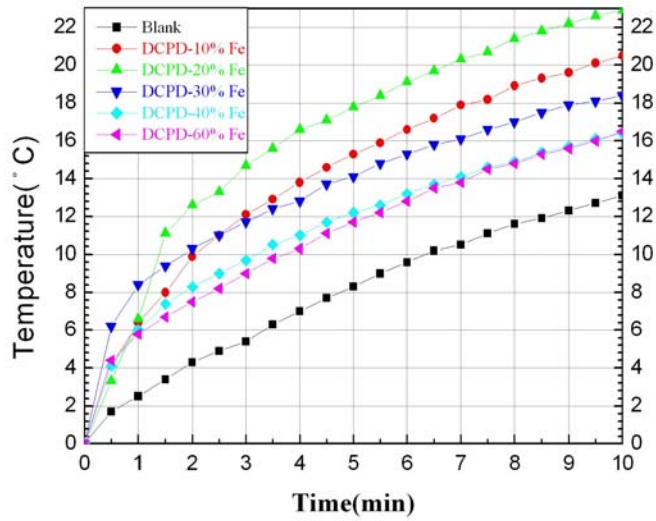
Fig. 4-10 The difference raised temperature of DCPD-Fe under an alternating magnetic field.

4.3.6 Particle size (TEM)

The TEM images of DCPD-20%Fe is shown in Fig. 4-11. The results revealed that particle size of DCPD-20%Fe was around 130nm to 200nm.
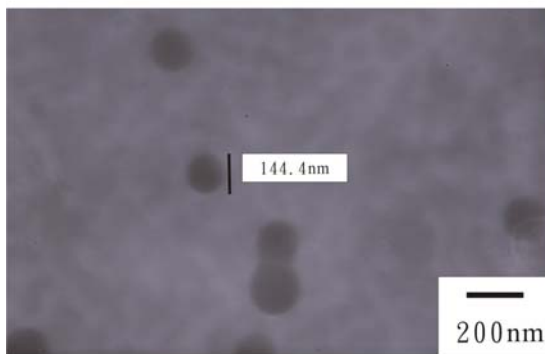
(a) TEM image of DCPD-20%Fe Hv=50kv   (b) TEM image of DCPD-20%Fe Hv=100kv
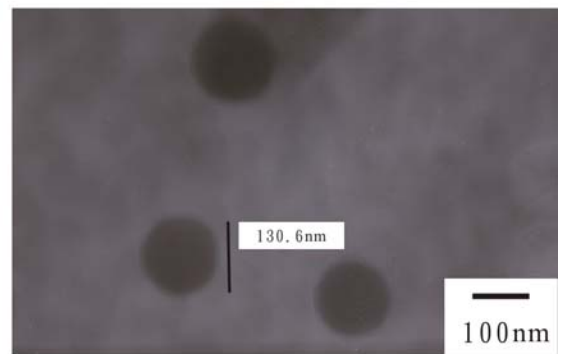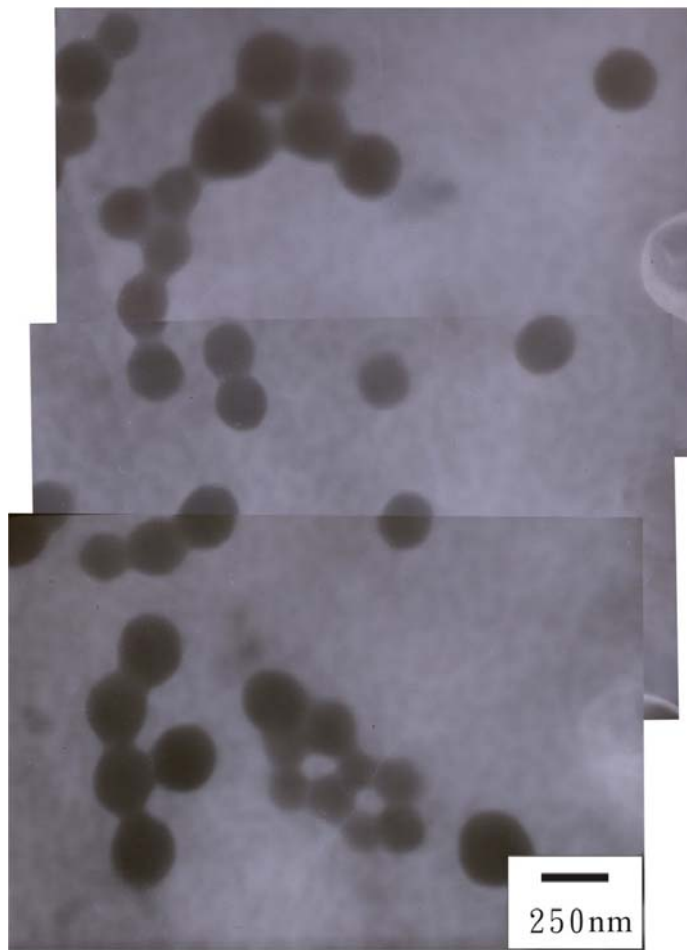
(c) TEM image of DCPD-20%Fe Hv=40kv

Fig. 4-11 The TEM images of DCPD-20%Fe(a)Hv =50kv(b)Hv=100kv (c)Hv=40kv

4.3.7 WST-1 assay for mitochondrial function

The absorbance value at 420nm of WST-1 reagent (Roche) was measured by ELISA reader (Fig. 4-12). Higher value indicates better cell viability and proliferation. There was no statistic significant difference of OD values observed between control group and any of the experimental groups (1mg/ml, 5mg/ml and 10mg/ml), on Day 1

and Day 3.

Fig. 4-12 WST-1 as a function of time after the addition of 1, 5 and 10mg/ml concentration of

DCPD-10%Fe and DCPD20%Fe.

4.3.8 LDH assay for cell lysis

The LDH assay of 3T3 cells is shown in Fig. 4-13. On Day 1, no statistics

significant difference in OD values between control group and experimental groups

(1mg/ml, 5mg/ml and 10mg/ml) were observed. The same results were observed on

all concentrations of DCPD-20%Fe on Day 3. However, the OD values of

DCPD-10%Fe were lower than the control group on Day 3 and it was the only group

which showed significant difference. ($p < 0.05$)
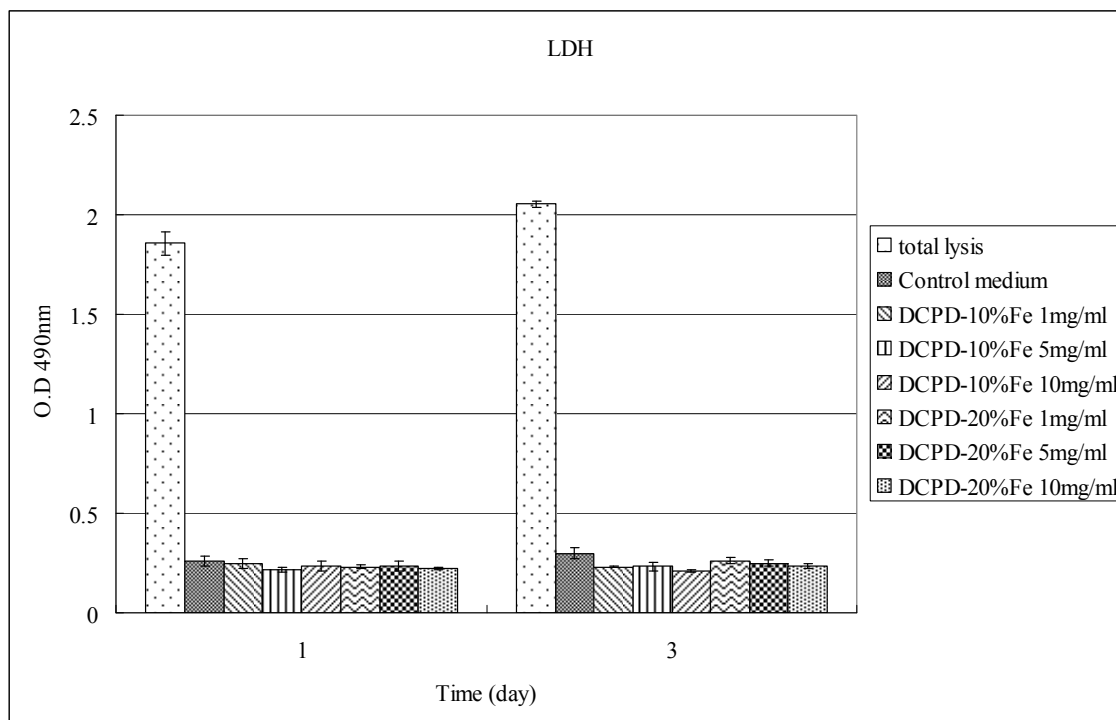
Fig. 4-13 LDH as a function of time after the addition of 1, 5 and 10mg/ml concentration of DCPD-10%Fe and DCPD20%Fe.

4.3.9 In-vitro test for cancer hyperthermia

The cytotoxicity(%) of HFL-1(human lung fibroblast) showed no significant difference observed on the percentage of cytotoxicity between control and experiment group.(Fig. 4-14) On the other hand, the cytotoxicity(%) of A549 (human lung carcinoma) with DCPD-20%Fe under magnetic field was significant higher than the control group (A549 cells with medium only). ($p < 0.05$) (Fig. 4-15)
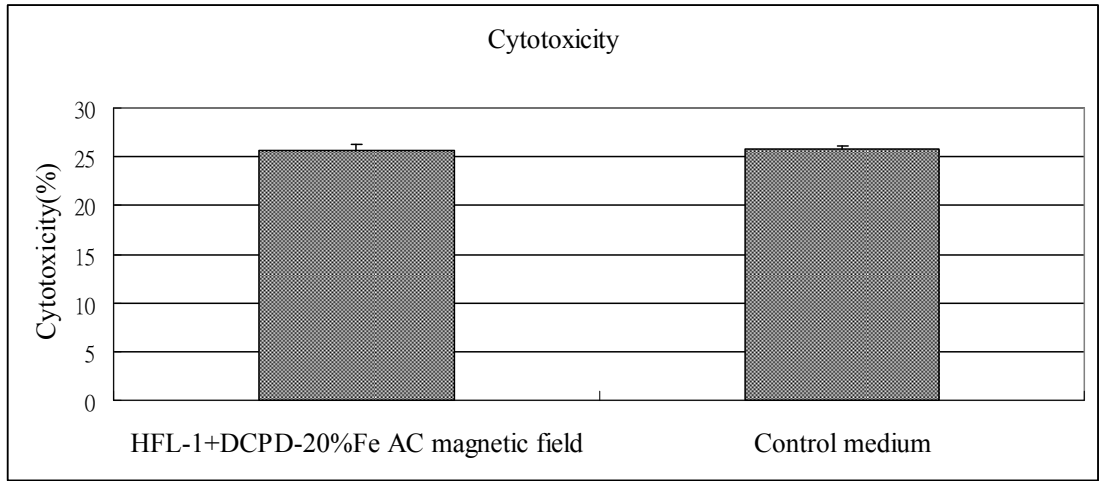
Fig. 4-14 The percentage of cytotoxicity of HFL-1.
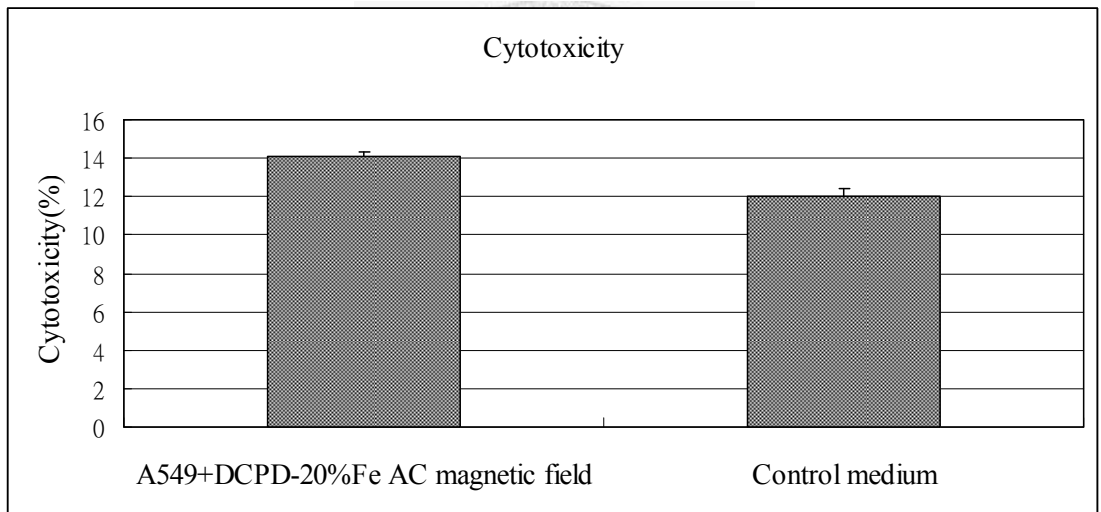
Fig. 4-15 The percentage of cytotoxicity of A549.

4.4 Discussion

The XRD results for DCPD-10%Fe and DCPD-20%Fe are similar to pure DCPD (Fig. 5-4). We can tell that all the Fe^{2+} replace Ca^{2+} in the DCPC crystal lattice and cause a major peak shift to larger angle, which is partly due to the ionic radius of Fe^{2+} smaller than Ca^{2+} to shorten the b-axis. In the XRD pattern, some of extra peaks could be traced in the sample of DCPD-30%Fe, DCPD-40%Fe and DCPD-60%Fe. It was corresponding to the presence of second phase, vivianite ($Fe_3(PO_4)_2.8H_2O$), which would be crystallized in the sample of high Fe^{2+} addition. The structure of vivianite is monoclinic (same as DCPD) and its magnetic property was antiferromagnetic [94, 106].

The results of lattice parameters are shown in Fig. 5-5. It revealed that more Ca^{2+} are substituted by Fe^{2+}, smaller lattice parameter was formed. Thus the value of b-axis is decreased from DCPD-10%Fe to DCPD-40%Fe, but the increased thereafter due to second phase vivianite formation. The other lattice parameters of DCPD-Fe with different concentration of Fe^{2+} showed no significant difference. It means that the Fe^{2+} substituted randomly in others axes.

In DCPD-10%Fe and DCPD-20%Fe, the observed concentrations of Ca^{2+} were decreasing with increased Fe^{2+} (by ICP-OES, Fig. 5-7). It may be resulted from the substitution of Fe^{2+} to Ca^{2+}. The ionic radius of calcium ion is larger than Fe2+. Therefore, Ca^{2+} can be easily substituted by Fe^{2+} in the DCPD structure. In the sample of high Fe^{2+} addition, such as, DCPD-30%Fe, DCPD-40%Fe and DCPD-60%Fe, the solubility of Fe^{2+} in DCPC crystal lattice was over limitation that was resulted in the

second phase formation (vivianite). Once vivianite nucleation and crystallization in the solution, Fe^{2+} would be preferentially to attract to the new phase and then lower down the Fe^{2+} substitution to Ca^{2+} in the DCPD lattice.

In the SQUID test, hysteresis loop was observed in DCPD-10%Fe and DCPD-20%Fe (Fig. 5-8). But from DCPD-30%Fe to DCPD-60%Fe, the hysteresis loop became less and less prominent with increasing iron percentage. This was due to the appearance of the second phase, vivianitite. The magnetic property of vivianite was antiferromagnetism, so the magnetism descends after the formation of vivianite.

In WST-1 assay (Fig. 5-12), no statistic significant difference of OD values was observed between control group and any of the experimental groups (1mg/ml, 5mg/ml and 10mg/ml), on Day 1 and Day 3. It indicated that addition of DCPD-Fe10%Fe and DCPD-Fe20%Fe did not harm the cell viability in an acute phase.

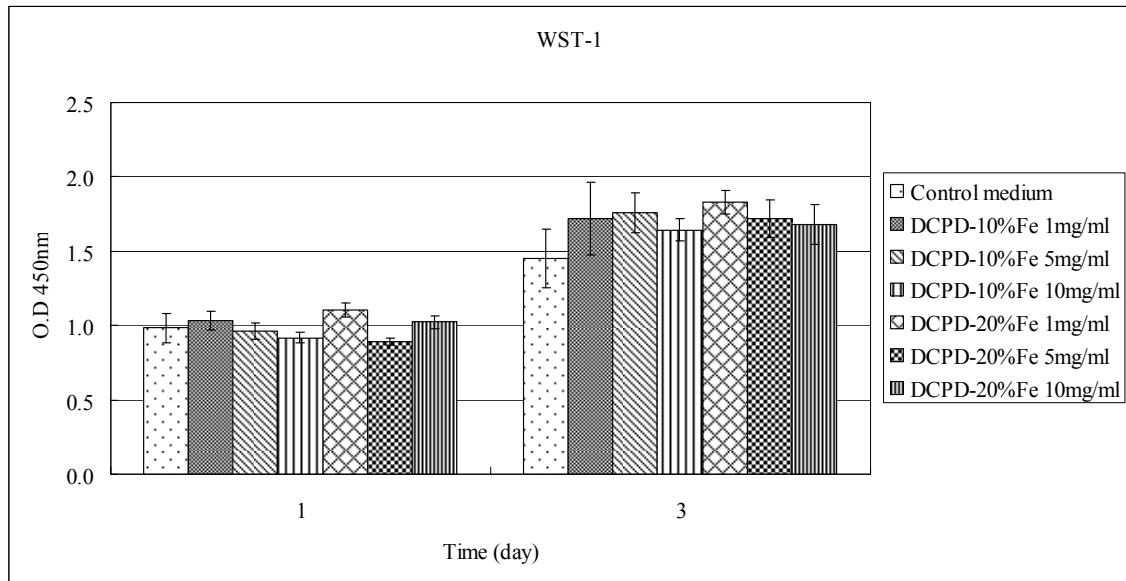In the LDH assay, no significant differences were observed between experiment group and control group, except for the DCPD-10%Fe group on Day 3. Moreover, the OD value of DCPD-10%Fe was even lower than control group. It might be due to the anabolic effect of DPCD with lower concentration of iron. Chai *et al* [215] reported the importance of preculture conditioning treatment of calcium phosphate hydraulic cement (CPHC) samples. Their results showed that the cement material will dissolve and release calcium and phosphate in the liquid cell culture environment continuously. Excessive acid was produced in this process and it still has some influence on cell growth after 24 h. Only the group with longer pre-culture conditioning will have better cell adaption and osteoblast growing. Therefore, we thought our DCPD-10%Fe material might have the same phenomenon of dissolving and producing excessive acid. The fibroblast still needed time to adapt the acidic environment. Hence on Day 3,

after the 3T3 fibroblast was adapted to the environment, the OD value was decreased in the DCPD-10%Fe group. As for the DCPD-20%Fe group, the iron might still have some negative impacts of fibroblasts and neutralize the anabolic effect of DCPD. In conclusion, DCPD-10%Fe and DCPD-20%Fe have relative low cytotoxicity to the 3T3cells.

The in-vitro test of hyperthermia therapy showed encouraging results. There was no significant difference observed on cytototxicity(%) of HFL-1 cells between control and experiment group. It proved that DCPD-20%Fe particles and high temperature for 30 mins did not harm the normal HFL-1 cells. On the other hand, the cytotoxicity(%) of the experiment group of A549 cancer cells was statistically higher than control group. It revealed that DCPD-20%Fe particles and high temperature for 30 mins could kill the A549 cells successfully. Therefore, we can conclude that the hyperthermia therapy conducted by DCPD-20%Fe could kill the cancer cells without harming the normal cells.
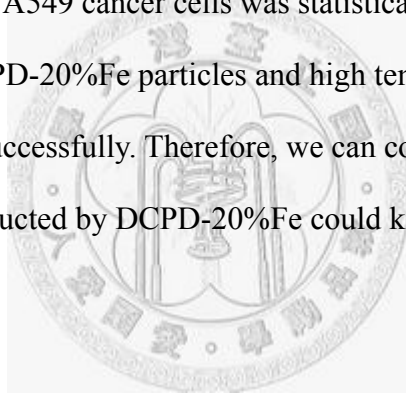
Chapter 5   Conclusions

Hyperthermia therapy for cancer has drawn more and more attention these days. In this study, we conducted an *in vivo* cancer hyperthermia study of the new magnetic hydroxyapatite nanoparticles by a mouse model. The magnetic hydroxyapatite nanoparticles were first made by co-precipitation method with the addition of Fe^{2+}. Then, magnetic-HAP powder (mHAP) or pure HAP powder (HAP) were mixed with phosphate buffer solution (PBS), respectively. The mixture was injected around the tumor. In order to achieve hyperthermia, the mice were placed into an inductive heater with high frequency and alternating magnetic field. Only the mice which were injected with mHAP and had been treated inside the magnetic field showed dramatic reduction of tumor volume, in the 15-day observation period. No local recurrence was noted. The blood test of mice proved that mHAP powders possessed good biocompatibility and little toxicity when injected subcutaneously. Therefore, our new magnetic hydroxyapatite nanoparticles demonstrated a possible therapeutic effect in a mouse model with little toxicity. Further study should be done before its application inside the human body.

By using co-precipitation method, DCPD was synthesized successfully at 37℃, pH 5-5.2. Addition of different concentrations of ferrous chloride to DCPD powders increased the property of magnetic. From the ICP-OES, the observed concentrations of calcium ions were decreased and iron ions were increased. It might be due to the

replacement of calcium ions with iron ions. The magnetism of DCPD-30%Fe ~ 60%Fe was decreased, due to the formation of the second phase, vivianite. DCPD-Fe had significant ability to raise temperature. The results of LDH and WST-1 assay revealed that DCPD-10%Fe and DCPD-20%Fe were non-toxic. The particle size of DCPD-20%Fe was around 130nm to 200nm. The results of in-vitro cancer hyperthermia test proved its ability to kill the cancer cells without harming the normal cells. Thus it is a good candidate for hyperthermia therapy with good heating efficiency and little toxicity.

Future work

We will continue our study in several ways:

1. The animal study of mDCPD will be performed. However, the pilot study showed severe skin necrosis after hyperthermia. DCPD is part of the chemical composition of bone and the mechanism of this skin necrosis remained unknown. We will overcome this technical problem by examing the pH value or other possible harming effect of DCPD inside the animal body.

2. We will try to increase the tumor-targeting ability of these nanoparticles. Theoretically there are several ways to accomplish that: by folate side chain connecting, by specific tumor antibody, or liposomes. Each has its own advantages and drawbacks.

3. Currently in this thesis the injection of nanoparticles into animals was too frequent, and it will significantly decrease patients' compliance when applied in the human body in the future. We will test the optimal strategy

of nanoparticles injections and try to lower the number of injections.

4.   The animal model we used here is merely subcutaneous injection. Due to the profession of the author (Orthopedic surgeon), I would like to test it in an osteosarcoma or bone malignancy model. The size of tumor is obvious more difficult to be measured and it probably needs x-ray examinations.

Chapter 6   References

1.   US mortality public use data type 2003,   national center for health statistics, centers of disease control and prevention
2006.

2.   1950 mortality data – CDC/NCHS, NVSS, Mortality revised. And 2003 mortality data: US mortality public use data tape, 2003, NCHS, Centers of disease control and prevention. 2006.

3.   Wust P, Hildebrandt B, Sreenivasa G, Rau B, Gellermann J, Riess H, et al. Hyperthermia in combined treatment of cancer. Lancet Oncol 2002 Aug;3(8):487-497.

4.   Ewing J. "Neoplastic Diseases: A Treaties on Tumors", 4th ed. Philadelphia: W. B. Saunders, 1940.

5.   Lynch RG. Differentiation and cancer: the conditional autonomy of phenotype. Proc Natl Acad Sci U S A 1995 Jan 31;92(3):647-648.

6.   李門輝. "癌的歷史記載和定義", 癌的基礎科學: 合記圖書出版社, 1987.

7.   Marshall CJ, Nigg EA. Oncogenes and cell proliferation. Cancer genes: lessons from genetics and biochemistry. Curr Opin Genet Dev 1998 Feb;8(1):11-13.

8.   Webb DD. Introduction. GERD warrants increased physician appreciation and improved treatment. Postgrad Med 2001 Oct;Spec No:5-10.

9.   Holmes EC, Morton DL. Pulmonary resection for sarcoma metastases. Orthop Clin North Am 1977 Oct;8(4):805-810.

10.   黃學進, 王躬仁. "癌症": 茂昌圖書有限公司, 1985.

11.   Abeloff M, Armitage J, Niederhuber J, Kastan M, WG. M. Clinical Oncology. 3rd ed: Elsevier, 2004.

12.   Haskell CM. F.A.C.P.,"Cancer Treatment ". 4th ed. Philadelphia: W.B. Saunders, 1995.

13.   Buschke F. Radiation Therapy in Cancer Management. New York: Grune & Stratton, 1972.

14.   Kramer S. The patterns of care study: a nationwide evaluation of the practice of radiation therapy in cancer management. Int J Rad Oncol Biol Phys 1976;1:1231.

15.   Farmer PB. Metabolism and reactions of alkylating agents. Pharmacol Ther 1987;35(3):301-358.

16.   Tan KB, Per SR, Boyce RA, Mirabelli CK, Crooke ST. Altered expression and transcription of the topoisomerase II gene in nitrogen mustard-resistant human cells.

Biochem Pharmacol 1988 Nov 15;37(22):4413-4416.

17. Pinedo HM, Peters GF. Fluorouracil: biochemistry and pharmacology. J Clin Oncol 1988 Oct;6(10):1653-1664.

18. Ling YH, Chan JY, Beattie KL, Nelson JA. Consequences of 6-thioguanine incorporation into DNA on polymerase, ligase, and endonuclease reactions. Mol Pharmacol 1992 Nov;42(5):802-807.

19. Carrico CK, Sartorelli AC. Effects of 6-thioguanine on RNA biosynthesis in regenerating rat liver. Cancer Res 1977 Jun;37(6):1876-1882.

20. Baram J. J Clin Invest 1987;79:692.

21. Haskell CM. F.A.C.P.,"Cancer Treatment". 4th ed. Philadelphia: W.B. Saunders, 1995.

22. Levi JA, Raghavan D, Harvey V, Thompson D, Sandeman T, Gill G, et al. The importance of bleomycin in combination chemotherapy for good-prognosis germ cell carcinoma. Australasian Germ Cell Trial Group. J Clin Oncol 1993 Jul;11(7):1300-1305.

23. Facon T, Caulier MT, Wattel E, Jouet JP, Bauters F, Fenaux P. A randomized trial comparing vinblastine in slow infusion and by bolus i.v. injection in idiopathic thrombocytopenic purpura: a report on 42 patients. Br J Haematol 1994 Mar;86(3):678-680.

24. Epstein RJ. The clinical biology of hormone-responsive breast cancer. Cancer Treat Rev 1988 Mar;15(1):33-51.

25. 李門輝. "化學藥物療法", 癌的基礎科學: 合記圖書出版社, 1987.

26. Busch W. "Aus der Sitzung der medicinischen Section vom 13 ". Wochenschr: Berl Klin 1867.

27. Bickels J, Kollender Y, Merinsky O, Meller I. Coley's toxin: historical perspective. Isr Med Assoc J 2002 Jun;4(6):471-472.

28. 李門輝. "免疫療法", 癌的基礎科學,: 合記圖書出版社, 1987.

29. Steinman RM. The dendritic cell system and its role in immunogenicity. Annu Rev Immunol 1991;9:271-296.

30. Roth JA, Cristiano RJ. Gene therapy for cancer: what have we done and where are we going? J Natl Cancer Inst 1997 Jan 1;89(1):21-39.

31. Nielsen LL, Maneval DC. P53 tumor suppressor gene therapy for cancer. Cancer Gene Ther 1998 Jan-Feb;5(1):52-63.

32. Gene therapy. [cited; Available from: http://www.ornl.gov/sci/techresources/Human_Genome/medicine/geerapy.shtml

33. Storm FK. Hyperthermia in cancer therapy. Boston: G.K. Hall Medical

Publishers, 1983.

34.　Selawry OS, Goldstein MN, Mc CT. Hyperthermia in tissue-cultured cells of malignant origin. Cancer Res 1957 Sep;17(8):785-791.

35.　Huth E. Die Relle der bakteriellen infection bei spontanremission maligner tumoren und leukosen. Geschwultsts: Korpereigene Abwehr und bosartige 1957.

36.　Nielsen OS, Horsman M, Overgaard J. A future for hyperthermia in cancer treatment? Eur J Cancer 2001 Sep;37(13):1587-1589.

37.　Dewey WC. Arrhenius relationships from the molecule and cell to the clinic. Int J Hyperthermia 1994 Jul-Aug;10(4):457-483.

38.　Bull JM. An update on the anticancer effects of a combination of chemotherapy and hyperthermia. Cancer Res 1984 Oct;44(10 Suppl):4853s-4856s.

39.　Dahl O. Interaction of hyperthermia and chemotherapy. Recent Results Cancer Res 1988;107:157-169.

40.　李門輝. "外科手術與輻射療法", 癌的基礎科學: 合記圖書出版社, 1987.

41.　Gerner EW, Schneider MJ. Induced thermal resistance in HeLa cells. Nature 1975 Aug 7;256(5517):500-502.

42.　Henle KJ, Leeper DB. Interaction of hyperthermia and radiation in CHO cells: recovery kinetics. Radiat Res 1976 Jun;66(3):505-518.

43.　Li GC, Hahn GM. A proposed operational model of thermotolerance based on effects of nutrients and the initial treatment temperature. Cancer Res 1980 Dec;40(12):4501-4508.

44.　Li GC, Fisher GA, Hahn GM. Induction of thermotolerance and evidence for a well-defined, thermotropic cooperative process. Radiat Res 1982 Feb;89(2):361-368.

45.　Song CW. Effect of local hyperthermia on blood flow and microenvironment: a review. Cancer Res 1984 Oct;44(10 Suppl):4721s-4730s.

46.　Gerweck LE. Hyperthermia in cancer therapy: the biological basis and unresolved questions. Cancer Res 1985 Aug;45(8):3408-3414.

47.　Dewey WC, Hopwood LE, Sapareto SA, Gerweck LE. Cellular responses to combinations of hyperthermia and radiation. Radiology 1977 May;123(2):463-474.

48.　Hildebrandt B, Wust P, Ahlers O, Dieing A, Sreenivasa G, Kerner T, et al. The cellular and molecular basis of hyperthermia. Crit Rev Oncol Hematol 2002 Jul;43(1):33-56.

49.　Hall EJ. "Hyperthermia", Radiobiology for the radiologist. 5th ed. Philadelphia: Lippincott Williams & Wilkins, 2000.

50.　Streffer C. "Molecular and cellular mechanisms of hyperthermia", Hyperthermia and the therapy of malignant tumors. Berlin; New York: Springer-Verlag,, 1987.

51.   Vaupel P, Kallinowski F. Physiological effects of hyperthermia, In: Streffer (ed) Hyperthermia and the therapy of malignant tumors. Berlin Heidelberg, New York: Springer, 1987.

52.   Gerweck LE. Environment and vascular effect, In: Overgaard J (ed) Hyperthermia oncology. London: Taylor & Francis, 1985.

53.   Reinhold HS, Wike-Hooley JL, van den Berg AP, van den Berg-Blok A. Environmental factors, blood flow and microcirculation, In: Overgaard J (ed) Hyperthermia oncology. London: Taylor & Francis, 1985.

54.   Debye P. Polar Molecules. New York: The Chemical Catalog Company, 1929.

55.   Kampinga HH. Thermotolerance in mammalian cells. Protein denaturation and aggregation, and stress proteins. J Cell Sci 1993 Jan;104 (Pt 1):11-17.

56.   Calderwood SK, Hahn GM. Thermal sensitivity and resistance of insulin-receptor binding. Biochim Biophys Acta 1983 Mar 15;756(1):1-8.

57.   Coss RA, Linnemans WA. The effects of hyperthermia on the cytoskeleton: a review. Int J Hyperthermia 1996 Mar-Apr;12(2):173-196.

58.   Konings AW, Ruifrok AC. Role of membrane lipids and membrane fluidity in thermosensitivity and thermotolerance of mammalian cells. Radiat Res 1985 Apr;102(1):86-98.

59.   Majda JA, Gerner EW, Vanlandingham B, Gehlsen KR, Cress AE. Heat shock-induced shedding of cell surface integrins in A549 human lung tumor cells in culture. Exp Cell Res 1994 Jan;210(1):46-51.

60.   Stevenson MA, Minton KW, Hahn GM. Survival and concanavalin-A-induced capping in CHO fibroblasts after exposure to hyperthermia, ethanol, and X irradiation. Radiat Res 1981 Jun;86(3):467-478.

61.   Streffer C. Aspects of metabolic change after hyperthermia. Recent Results Cancer Res 1988;107:7-16.

62.   Henle KJ, Leeper DB. Effects of hyperthermia (45 degrees) on macromolecular synthesis in Chinese hamster ovary cells. Cancer Res 1979 Jul;39(7 Pt 1):2665-2674.

63.   Hahn GM. Hyperthermia and Cancer. New York: Plenum Press 1982.

64.   Burdon RH. "Heat shock proteins", In: Overgaard J (ed) Hyperthermia oncology,. London: Taylor & Francis, 1985.

65.   Francesconi R, Mager M. Heat- and exercise-induced hyperthermia: effects on high-energy phosphates. Aviat Space Environ Med 1979 Aug;50(8):799-802.

66.   Ohyana H, Tamada T. Reduction of rat thermocyte interphase death by hyperthermia. Radiat Res 1980;82:342-351.

67. Lunec J, Cresswell SR. Heat-induced thermotolerance expressed in the energy metabolism of mammalian cells. Radiat Res 1983 Mar;93(3):588-597.

68. Mirtsch S, Streffer C, van Beuningen D, Rebmann A. ATP metabolism in human melanoma cells after treatment with hyperthermia (42 °C), In: Overgaard J (ed) Hyperthermia oncology, . London: Taylor & Francis, 1984.

69. Streffer C. Metabolic changes during and after hyperthermia. Int J Hyperthermia 1985 Oct-Dec;1(4):305-319.

70. Vaupel P, Kallinowski F, Kluge M, Egelhof E, Fortmeyer HP. Microcirculatory and pH alterations in isotransplanted rat and xenotransplanted human tumors associated with hyperthermia. Recent Results Cancer Res 1988;109:173-182.

71. Folkman J. What is the evidence that tumors are angiogenesis dependent? J Natl Cancer Inst 1990 Jan 3;82(1):4-6.

72. Vaupel P, Kallinowski F, Okunieff P. Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: a review. Cancer Res 1989 Dec 1;49(23):6449-6465.

73. von Ardenne M, Reitnauer PG. [Manipulated selective inhibition of microcirculation in cancer tissues]. J Cancer Res Clin Oncol 1982;103(3):269-279.

74. Hiraoka M, Hahn GM. Comparison between tumor pH and cell sensitivity to heat in RIF-1 tumors. Cancer Res 1989 Jul 15;49(14):3734-3736.

75. Overgaard J. Effect of misonidazole and hyperthermia on the radiosensitivity of a C3H mouse mammary carcinoma and its surrounding normal tissue. Br J Cancer 1980 Jan;41(1):10-21.

76. Reinhold HS, Endrich B. Tumour microcirculation as a target for hyperthermia. Int J Hyperthermia 1986 Apr-Jun;2(2):111-137.

77. Vaupel P, Okunieff P, Kluge M. Response of tumour red blood cell flux to hyperthermia and/or hyperglycaemia. Int J Hyperthermia 1989 Mar-Apr;5(2):199-210.

78. Paliwal R, Hetzel FW, Dewhirst MW. Biological, physical, and clinical aspects of hyperthermia. New York: Publication Billing Division, 1988.

79. Robins HI, Woods JP, Schmitt CL, Cohen JD. A new technological approach to radiant heat whole body hyperthermia. Cancer Lett 1994 May 16;79(2):137-145.

80. Hand JW, Hind AJ. *A review of microwave and RF applicators for localised hyperthermia, . Physical techniques in clinical hyperthermia* Letchworth: Research Studies Press, 1986. p. pp. 98-148,.

81. Urano M, Kuroda M, Nishimura Y. For the clinical application of thermochemotherapy given at mild temperatures. International Journal of

Hyperthermia 1999 Mar-Apr;15(2):79-107.

82. Seegenschmiedt M.H. KG, Seidel R., Stauffer P.R. *Clinical practice of interstitial thermoradiotherapy. Thermoradiotherapy and Thermochemotherapy: Clinical Applications with Contributions by Numerous Experts v 2, .* Berlin: Springer Press, 1995. p. pp. 207-252,.

83. Steeves RA. Hyperthermia in cancer therapy: where are we today and where are we going? Bull NY Acad Med 1992 Mar-Apr;68(2):341-350.

84. Fairbairn JJ, Khan MW, Ward KJ, Loveridge BW, Fairbairn DW, O'Neill KL. Induction of apoptotic cell DNA fragmentation in human cells after treatment with hyperthermia. Cancer Lett 1995 Mar 2;89(2):183-188.

85. Bertone V, Barni S, Silvotti MG, Freitas I, Mathe G, Pontiggia P. Hyperthermic effects on the human metastatic liver: a TEM study. Anticancer Res 1997 Nov-Dec;17(6D):4713-4716.

86. Christophi C, Winkworth A, Muralihdaran V, Evans P. The treatment of malignancy by hyperthermia. Surgical Oncology 1998 Jul-Aug;7(1-2):83-90.

87. Overgaard J. The current and potential role of hyperthermia in radiotherapy. Int J Radiat Oncol Biol Phys 1989 Mar;16(3):535-549.

88. Anderson RL, Kapp DS. Hyperthermia in cancer therapy: current status. Med J Aust 1990 Mar 19;152(6):310-315.

89. Overgaard J, Gonzalez D, Hulshof MC, Arcangeli G, Dahl O, Mella O, et al. Randomised trial of hyperthermia as adjuvant to radiotherapy for recurrent or metastatic malignant melanoma. Lancet 1995 Mar 4;345(8949):540-543.

90. Engelhardt R. Hyperthermia and drugs. Recent Results Cancer Res 1987;104:136-203.

91. Gilchrist RK, Medal R, Shorey WD, Hanselman RC, Parrott JC, Taylor CB. Selective inductive heating of lymph nodes. Ann Surg 1957 Oct;146(4):596-606.

92. Medal R, Shorey W, Gilchrist RK, Barker W, Hanselman R. Controlled radio-frequency generator for production of localized heat in intact animal. Archives of Surgery 1959;79(3):427-431.

93. Gilchrist RK, Shorey WD, Hanselman RC, Depeyster FA, Yang J, Medal R. Effects of Electromagnetic Heating on Internal Viscera: A Preliminary to the Treatment of Human Tumors. Ann Surg 1965 Jun;161:890-896.

94. Moroz P, Jones SK, Gray BN. Magnetically mediated hyperthermia: current status and future directions. Int J Hyperthermia 2002 Jul-Aug;18(4):267-284.

95. Breedis C, Young G. The blood supply of neoplasms in the liver. Am J Pathol 1954 Sep-Oct;30(5):969-977.

96.   Stribley KV, Gray BN, Chmiel RL, Heggie JC, Bennett RC. Internal radiotherapy for hepatic metastases II: The blood supply to hepatic metastases. J Surg Res 1983 Jan;34(1):25-32.

97.   Archer SG, Gray BN. Vascularization of small liver metastases. Br J Surg 1989 Jun;76(6):545-548.

98.   Archer SG, Gray BN. Comparison of portal vein chemotherapy with hepatic artery chemotherapy in the treatment of liver micrometastases. Am J Surg 1990 Mar;159(3):325-329.

99.   Rand RW, Snyder M, Elliott D, Snow H. Selective radiofrequency heating of ferrosilicone occluded tissue: a preliminary report. Bull Los Angeles Neurol Soc 1976 Oct;41(4):154-159.

100. Sako M, Morita M, Ohtsuki S, Adachi S, Watanabe H, Hasegawa M, et al. Studies on selective embolo-hyperthermic therapy of tumors by using ferromagnetic particles. Nippon Gan Chiryo Gakkai Shi 1984 Oct 20;19(9):2168-2171.

101. Matsuki H, Yanada T, Sato T, Murakami K, Minakawa S. Temperature-sensitive amorphous magnetic flakes for intratissue hyperthermia. Mat Sci Eng 1994;182:1366-1368.

102. Mitsumori M, Hiraoka M, Shibata T, Okuno Y, Nagata Y, Nishimura Y, et al. Targeted hyperthermia using dextran magnetite complex: A new treatment modality for liver tumors. Hepato-Gastroenterology 1996 Nov-Dec;43(12):1431-1437.

103. Fannin PC, Charles SW. Measurement of the Neel relaxation of magnetic particles in the frequency range 1 kHz to 160MHz. Journal of Physics D-Applied Physics 1991 Jan;24(1):76-77.

104. Jordan A, Wust P, Scholz R, Faehling H, Krause J, R. F. Magnetic Fluid hyperthermia. *Scientific and Clinical Applications of Magnetic Carriers, .* New York: Plenum Press, 1997.

105. Minamimura T, Sato H, Kasaoka S, Saito T, Ishizawa S, Takemori S, et al. Tumor regression by inductive hyperthermia combined with hepatic embolization using dextran magnetite-incorporated microspheres in rats. International Journal of Oncology 2000 Jun;16(6):1153-1158.

106. Moroz P, Jones SK, Winter J, Gray BN. Targeting liver tumors with hyperthermia: Ferromagnetic embolization in a rabbit liver tumor model. J Surg Oncol 2001 Sep;78(1):22-29.

107. Moroz P, Jones SK, Gray BN. The effect of tumour size on ferromagnetic embolization hyperthermia in a rabbit liver tumour model. International Journal of Hyperthermia 2002 Mar;18(2):129-140.

108. Ito A, Shinkai M, Honda H, Kobayashi T. Medical application of functionalized magnetic nanoparticles. J Biosci Bioeng 2005 Jul;100(1):1-11.

109. Shinkai M, Yanase M, Suzuki M, Honda H, Wakabayashi T, Yoshida J, et al. Intracellular hyperthermia for cancer using magnetite cationic liposomes. J Magn Magn Mat 1999;194:176-184.

110. Rand RW, Snow HD, Elliott DG, Snyder M. Thermomagnetic surgery for cancer. App Biochem Biotech 1981 June;6:265-272.

111. Rand RW, Snow HD, Brown WJ. Thermomagnetic surgery for cancer. J Surg Res 1982 Sep;33(3):177-183.

112. Luderer AA, Borrelli NF, Panzarino JN, Mansfield GR, Hess DM, Brown JL, et al. Glass-ceramic-mediated, magnetic-field-induced localized hyperthermia: response of a murine mammary carcinoma. Radiat Res 1983 Apr;94(1):190-198.

113. Borrelli NF, Luderer AA, Panzarino JN. Hysteresis heating for the treatment of tumours. Phys Med Biol 1984 May;29(5):487-494.

114. Hase M, Sako M, Fujii M, Ueda E, Nagae T, Shimizu T, et al. Experimental study of embolo-hyperthermia for treatment of liver tumor--induction heating to ferromagnetic particles injected into tumor tissue. Nippon Igaku Hoshasen Gakkai Zasshi 1989 Sep 25;49(9):1171-1173.

115. Hase M, Sako M, Hirota S. Experimental study of ferromagnetic induction heating combined with hepatic arterial embolization for treatment of liver tumors. Nippon Igaku Hoshasen Gakkai Zasshi 1990 Nov 25;50(11):1402-1414.

116. Chan DCF, Kirpotin DB, Bunn PA. Synthesis and evaluation of colloidal magnetic iron oxides for the site specific radiofrequency induced hyperthermia of cancer. J Magn Magn Mat 1993;122:374-378.

117. Jordan A, Scholz R, Wust P, Fahling H, Krause J, Wlodarczyk W, et al. Effects of magnetic fluid hyperthermia (MFH) on C3H mammary carcinoma in vivo. International Journal of Hyperthermia 1997 Nov-Dec;13(6):587-605.

118. Hilger I, Hergt R, Kaiser WA. Effects of magnetic thermoablation in muscle tissue using iron oxide particles: an in vitro study. Investigative Radiology 2000 Mar;35(3):170-179.

119. Hilger I, Andra W, Hergt R, Hiergeist R, Schubert H, Kaiser WA. Electromagnetic heating of breast tumors in interventional radiology: in vitro and in vivo studies in human cadavers and mice. Radiology 2001 Feb;218(2):570-575.

120. Nakajima T, Roberts DW, Ryan TP, Hoopes PJ, Coughlin CT, Trembly BS, et al. Pattern of response to interstitial hyperthermia and brachytherapy for malignant intracranial tumour: a CT analysis. Int J Hyperthermia 1993 Jul-Aug;9(4):491-502.

121. Emami B, Scott C, Perez CA, Asbell S, Swift P, Grigsby P, et al. Phase III study of interstitial thermoradiotherapy compared with interstitial radiotherapy alone in the treatment of recurrent or persistent human tumors. A prospectively controlled randomized study by the Radiation Therapy Group. Int J Radiat Oncol Biol Phys 1996 Mar 15;34(5):1097-1104.

122. Mack CF, Stea B, Kittelson JM, Shimm DS, Sneed PK, Phillips TL, et al. Interstitial thermoradiotherapy with ferromagnetic implants for locally advanced and recurrent neoplasms. Int J Radiat Oncol Biol Phys 1993 Sep;27(1):109-115.

123. Tohnai I, Goto Y, Hayashi Y, Ueda M, Kobayashi T, Matsui M. Preoperative thermochemotherapy of oral cancer using magnetic induction hyperthermia (Implant Heating System: IHS). Int J Hyperthermia 1996 Jan-Feb;12(1):37-47.

124. Akagi M, Tsuboyama T, Ikenaga M, Matsusue Y, Hiraoka M, Nakamura T. Anti-tumour effects of localized hyperthermia on an experimental bone tumour using an intramedullary nail. Int J Hyperthermia 1997 Jul-Aug;13(4):387-400.

125. Takegami K, Sano T, Wakabayashi H, Sonoda J, Yamazaki T, Morita S, et al. New ferromagnetic bone cement for local hyperthermia. J Biomed Mater Res 1998 Summer;43(2):210-214.

126. Burton C, Hill M, Walker E. The RF thermoseed- a thermally self-regulating implant for the production of brain lesions. Ieee Transactions on Biomedical Engineering 1971;18(2):104.

127. Kida Y, Ishiguri H, Ichimi K, Kobayashi T. Hyperthermia of metastatic brain tumor with implant heating system: a preliminary clinical results. No Shinkei Geka 1990 Jun;18(6):521-526.

128. Kobayashi T, Kida Y, Tanaka T, Hattori K, Matsui M, Amemiya Y. Interstitial hyperthermia of malignant brain tumors by implant heating system: clinical experience. J Neurooncol 1991 Apr;10(2):153-163.

129. Stea B, Kittelson J, Cassady JR, Hamilton A, Guthkelch N, Lulu B, et al. Treatment of malignant gliomas with interstitial irradiation and hyperthermia. Int J Radiat Oncol Biol Phys 1992;24(4):657-667.

130. Stea B, Rossman K, Kittelson J, Shetter A, Hamilton A, Cassady JR. Interstitial irradiation versus interstitial thermoradiotherapy for supratentorial malignant gliomas: a comparative survival analysis. Int J Radiat Oncol Biol Phys 1994 Oct 15;30(3):591-600.

131. Steeves RA, Tompkins DT, Nash RN, Blair JR, Gentry LL, Paliwal BR, et al. Thermoradiotherapy of intraocular tumors in an animal model: concurrent vs. sequential brachytherapy and ferromagnetic hyperthermia. Int J Radiat Oncol Biol

Phys 1995 Oct 15;33(3):659-662.

132. Murray TG, O'Brien JM, Steeves RA, Smith BJ, Albert DM, Cicciarelli N, et al. Radiation therapy and ferromagnetic hyperthermia in the treatment of murine transgenic retinoblastoma. Arch Ophthalmol 1996 Nov;114(11):1376-1381.

133. Murray TG, Steeves RA, Gentry L, Bresnick G, Boldt HC, Mieler WF, et al. Ferromagnetic hyperthermia: functional and histopathologic effects on normal rabbit ocular tissue. Int J Hyperthermia 1997 Jul-Aug;13(4):423-436.

134. Loening SA, Tucker RD. Treatment of prostate cancer by thermal ablation using interstitial temperature self-regulating seeds. *Poster Presentation, 15th World Congress on Endourology* 1997.

135. Gordon RT, Hines JR, Gordon D. Intracellular hyperthermia. A biophysical approach to cancer treatment via intracellular temperature and biophysical alterations. Med Hypotheses 1979 Jan;5(1):83-102.

136. Suzuki S, Arai K, Koike T, Oguchi K. Studies on liposomal ferromagnetic particles and a technique of high frequency inductive heating--in vivo studies of rabbits. Nippon Gan Chiryo Gakkai Shi 1990 Nov 20;25(11):2649-2658.

137. Suzuki M, Shinkai M, Kamihira M, Kobayashi T. Preparation and characteristics of magnetite-labelled antibody with the use of polyethylene glycol derivatives. Biotechnol Appl Biochem 1995 Jun;21:335-345.

138. Shinkai M, Yanase M, Honda H, Wakabayashi T, Yoshida J, Kobayashi T. Intracellular hyperthermia for cancer using magnetite cationic liposomes: in vitro study. Jpn J Cancer Res 1996 Nov;87(11):1179-1183.

139. Le B, Shinkai M, Kitade T, Honda H, Yoshida J, Wakabayashi T, et al. Preparation of tumor-specific magnetoliposomes and their application for hyperthermia. Journal of Chemical Engineering of Japan 2001 Jan;34(1):66-72.

140. Jordan A, Wust P, Fahling H, John W, Hinz A, Felix R. Inductive heating of ferromagnetic particles and magnetic fluids: physical evaluation of their potential for hyperthermia. Int J Hyperthermia 1993 Jan-Feb;9(1):51-68.

141. Jordan A, Wust P, Scholz R, Tesche B, Fahling H, Mitrovics T, et al. Cellular uptake of magnetic fluid particles and their effects on human adenocarcinoma cells exposed to AC magnetic fields in vitro. Int J Hyperthermia 1996 Nov-Dec;12(6):705-722.

142. Jordan A, Scholz R, Wust P, Schirra H, Schiestel T, Schmidt H, et al. Endocytosis of dextran and silan-coated magnetite nanoparticles and the effect of intracellular hyperthermia on human mammary carcinoma cells in vitro. J Magn Magn Mat 1999;194(1-3):185-196.

143. Jordan A, Scholz R, Maier-Hauff K, Johannsen M, Wust P, Nadobny J, et al. Presentation of a new magnetic field therapy system for the treatment of human solid tumors with magnetic fluid hyperthermia. J Magn Magn Mat 2001;225(1-2):118-126.

144. Ferrari M. Cancer nanotechnology: opportunities and challenges. Nat Rev Cancer 2005 Mar;5(3):161-171.

145. Duguet E, Vasseur S, Mornet S, Devoisselle JM. Magnetic nanoparticles and their applications in medicine. Nanomed 2006 Aug;1(2):157-168.

146. Gupta AK, Gupta M. Synthesis and surface engineering of iron oxide nanoparticles for biomedical applications. Biomaterials 2005 Jun;26(18):3995-4021.

147. Gupta AK, Naregalkar RR, Vaidya VD, Gupta M. Recent advances on surface engineering of magnetic iron oxide nanoparticles and their biomedical applications. Nanomedicine 2007 Feb;2(1):23-39.

148. Hilger I, Hergt R, Kaiser WA. Use of magnetic nanoparticle heating in the treatment of breast cancer. IEE Proceedings-Nanobiotechnnology 2005 Feb;152(1):33-39.

149.Campbell RB. Battling tumors with magnetic nanotherapeutics and hyperthermia: turning up the heat. Nanomed 2007 Oct;2(5):649-652.

150. Gazeau F, Levy M, Wilhelm C. Optimizing magnetic nanoparticle design for nanothermotherapy. Nanomedicine 2008 Dec;3(6):831-844.

151. Ito A, Fujioka M, Yoshida T, Wakamatsu K, Ito S, Yamashita T, et al. 4-S-Cysteaminylphenol-loaded magnetite cationic liposomes for combination therapy of hyperthermia with chemotherapy against malignant melanoma. Cancer Sci 2007 Mar;98(3):424-430.

152. Minamitsuji Y, Toyofuku K, Sugiyama S, Yamada K, Jimbow K. Sulfur containing tyrosine analogs can cause selective melanocytotoxicity involving tyrosinase-mediated apoptosis. J Investig Dermatol Symp Proc 1999 Sep;4(2):130-136.

153. Dandamudi S, Campbell RB. The drug loading, cytotoxicty and tumor vascular targeting characteristics of magnetite in magnetic drug targeting. Biomaterials 2007 Nov;28(31):4673-4683.

154. Schmidt RF, Thews G. Physiologie des Menschen. 26th ed: Springer, Berlin, 1995.

155. Kreuter J. Pharm. Acta Helv 1983;58:196.

156. Kreuter J. Factors influencing the body distribution of polyacrylic nanoparticles, Drug Targeting. Amsterdam: Elsevier, 1985.

157. Müller RH, Lück M, Harnisch S. Scientific and Clinical Applications of

Magnetic Carriers. New York: Plenum Press, 1997.

158. Okuhata Y. Delivery of diagnostic agents for magnetic resonance imaging. Adv Drug Deliv Rev 1999 Apr 5;37(1-3):121-137.

159. Moghimi M, Hunter AC, Murray JC. Long-circulating and target-specific nanoparticles: theory to practice. Pharmacol Rev 2001;53:283.

160. Maßen S, Fattal E, Müller RH. STP Pharm Sci 1993;3:11.

161. Gilchrist RK, Medal R, Shorey WD, Hanselman RC, Parrot JC, Taylor CB. Selective inductive heating of lymph nodes. Ann Surg 1957;146:596-606.

162. Gupta AK, Gupta M. Synthesis and surface engineering of iron oxide nanoparticles for biomedical applications. Synthesis and surface engineering of iron oxide nanoparticles for biomedical applications Biomaterials 26 3995-4021 2005 26(18):3995.

163. Charles SW, Dormann JL, Fiorani D. Magneticproperties of fine particles. North-Holland: Elsevier, 1992.

164. Schwertmann U, RM. C. Iron oxides in the laboratory: preparation and characterization. Weinheim: Cambridge: VCH, 1991.

165. Olsvik O, Popovic T, Skjerve E, Cudjoe KS, Hornes E, Ugelstad J, et al. Magneticseparati on techniques in diagnostic microbiology. Clin Microbiol Rev 1994;7:43–54.

166. Handgretinger R, Lang P, Schumm M, Taylor G, Neu S, Koscielnak E, et al. Isolation and transplantation of autologous peripheral CD34+ progenitor cells highly purified by magnetic-activated cell sorting. Bone Marrow Transplant 1998 May;21(10):987-993.

167. Schoepf U, Marecos EM, Melder RJ, Jain RK, Weissleder R. Intracellular magnetic labeling of lymphocytes for in vivo trafficking studies. Biotechniques 1998 Apr;24(4):642-646, 648-651.

168. Weissleder R, Heautot JF, Schaffer BK, Nossiff N, Papisov MI, Bogdanov A, Jr., et al. MR lymphography: study of a high-efficiency lymphotrophic agent. Radiology 1994 Apr;191(1):225-230.

169. Cuatrecasas P, Roth TF. Receptor-mediated endocytosis: Dordrecht: Kluwer Academic Publishers, 1983.

170. Yeh TC, Zhang W, Ldstad ST, Ho C. Intracellular labeling of T cells with superparamagnetic contrast agents. Magn Reson Med 1993;30:617–625.

171. Bulte JWM, Books RA. Scientific and Clinical Applications of Magnetic Carriers. New York, London: Plenum Press, 1997.

172. Chambon C, Clement O, Le Blanche A. Magn Reson Imaging 1993;11:509

173. Nunn AVW, Barnard ML, Bhakoo K, Murray J, Chilverrs EJ, Bell JD. Characterisation of secondary metabolites associated with neutrophil apoptosis. FEBS Lett 1996;392:295-298.

174. Lobel B, Eyal O, Kariv N, Katzir A. Temperature controlled CO2 laser welding of soft tissues: Urinary bladder welding in different animal models (rats, rabbits, and cats). Lasers Surg Med 2000;26:4–12.

175. Fried NM. Radiometric surface temperature measurements during dye-assisted laser skin closure: in vitro and in vivo results. Lasers Surg Med 1999;25:291–303.

176. Fried NM, Choi B, Welch AJ, Walsh JT, Jr. Radiometric surface temperature measurements during dye-assisted laser skin closure: in vitro and in vivo results. Lasers Surg Med 1999;25(4):291-303.

177. Nakamura N, Burgess JG, Yagiuda K, Kudo S, Sakaguchi T, Matsunaga T. Detection and removal of Escherichia coli using fluorescein isothiocyanate conjugated monoclonal antibody immobilized on bacterial magnetic particles. Anal Chem 1993 Aug 1;65(15):2036-2039.

178. Saiyed Z, Telang S, Ramchand C. Application of magnetic techniques in the field of drug discovery and biomedicine. Biomagn Res Technol 2003 Sep 18;1(1):2.

179. Babincova M, Cicmanec P, Altanerova V, Altaner C, Babinec P. AC-magnetic field controlled drug release from magnetoliposomes: design of a method for site-specific chemotherapy. Bioelectrochemistry 2002 Jan;55(1-2):17-19.

180. Kubo T, Sugita T, Shimose S, Nitta Y, Ikuta Y, Murakami T. Targeted systemic chemotherapy using magnetic liposomes with incorporated adriamycin for osteosarcoma in hamsters. Int J Oncol 2001 Jan;18(1):121-125.

181. Kubo T, Sugita T, Shimose S, Nitta Y, Ikuta Y, Murakami T. Targeted delivery of anticancer drugs with intravenously administered magnetic liposomes in osteosarcoma-bearing hamsters. Int J Oncol 2000 Aug;17(2):309-315.

182. Geophysics G. 13th, X-ray crystallography. 2007 [cited; Available from: http://www.geology.wisc.edu/courses/g360/xray992.html

183. Shackelford JF. Magnetic materials, Introduction to Materials Science for Engineerings. 5th ed: Prentice Hall International, 2001.

184. Williams DB, Carter CB. The energy-dispersive spectrometer", Transmission electron microscopy. New York: Plenum Press, 1996.

185. Advanced industrial science and technology. Development of Josephson Junctions for Liquid-Helium Free Voltage Standard System, http://www.aist.go.jp/aist_e/aist_today/2001_02/hot_line/hot_line_44.html. 2007, 13th Apr.; 2007, 13th Apr.

186. Josephson BD. The discovery of tunnelling supercurrents. Rev Mod Phys 1947;46(2):251-254.

187. Advanced industrial science and technology tA. Development of Josephson Junctions for Liquid-Helium Free Voltage Standard System, http://www.aist.go.jp/aist_e/aist_today/2001_02/hot_line/hot_line_44.html. 2007, . 2007, .

188. Williams DB, Carter CB. Forming diffraction patterns and images: the TEM imaging system", Transmission electron microscopy. New York: Plenum Press, 1996.

189. Bendersky LA, Gayle FW. Electron diffraction using transmission electron microscopy. Journal of research of the national institute of standards and technology 2001;106:997-1012.

190. Denizot F, Lang R. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J Immunol Methods 1986 May 22;89(2):271-277.

191. Nachlas MM, Margulies SI, Goldberg JD, Seligman AM. The determination of lactic dehydrogenase with a tetrazolium salt. Anal Biochem 1960 Dec 10;1:317-326.

192. BioVision, LDH-Cytotoxicity Assay Kit, Montain View: BioVision Research Products, Catalog # K311-400.

193. Takara, Premix WST-1 Cell Proliferation Assay System, Takara, Catalog # MK400.

194. Pankhurst Q A, Connolly J, Jones S K, Dobson J. Applications of magnetic nanoparticles in biomedicine. Applications of magnetic nanoparticles in biomedicine J Phys D: Appl Phys 36 167-81 2003 36:167.

195. Olsvik O, Popovic T, Skjerve E, Cudjoe KS, Hornes E, Ugelstad J, et al. Magnetic separation techniques in diagnostic microbiology. Clin Microbiol Rev 1994 Jan;7(1):43-54.

196. Weissleder R, Cheng HC, Bogdanova A, Bogdanov A, Jr. Magnetically labeled cells can be detected by MR imaging. J Magn Reson Imaging 1997 Jan-Feb;7(1):258-263.

197. Matsunaga T, Kawasaki M, Yu X, Tsujimura N, Nakamura N. Chemiluminescence enzyme immunoassay using bacterial magnetic particles. Anal Chem 1996 Oct 15;68(20):3551-3554.

198. Chambon C, Clement O, Le Blanche A, Schouman-Claeys E, Frija G. Superparamagnetic iron oxides as positive MR contrast agents: in vitro and in vivo evidence. Magn Reson Imaging 1993;11(4):509-519.

199. Nunn AV, Barnard ML, Bhakoo K, Murray J, Chilvers EJ, Bell JD.

Characterisation of secondary metabolites associated with neutrophil apoptosis. FEBS Lett 1996 Sep 2;392(3):295-298.

200. Tanoura T, Bernas M, Darkazanli A, Elam E, Unger E, Witte MH, et al. MR lymphography with iron oxide compound AMI-227: studies in ferrets with filariasis. AJR Am J Roentgenol 1992 Oct;159(4):875-881.

201. Kawamura Y, Endo K, Watanabe Y, Saga T, Nakai T, Hikita H, et al. Use of magnetite particles as a contrast agent for MR imaging of the liver. Radiology 1990 Feb;174(2):357-360.

202. Ramchand CN, Priyadarshini P, Kopcansky P, RV. M. Applications of magnetic fluids in medicine and biotechnology. Indian J Pure Appl Phys 2001;39:683-689.

203. Chen MM, Hsu   KC, Lin F, Stobinski L, J P. Folic Acid Immobilized Ferrimagnetic DP-Bioglass to Target Tumor Cell for Cancer Hyperthermia Treatment. Advances in science and technology 2006;53:50-57.

204. Wu HC, Wang TW, Sun JS, Wang WH, Lin FH. A novel biomagnetic nanoparticle based on hydroxyapatite. Nanotechnology 2007;18(16):165601.

205. Arcos D, del Real RP, Vallet-Regi M. Biphasic materials for bone grafting and hyperthermia treatment of cancer. J Biomed Mater Res A 2003 Apr 1;65(1):71-78.

206. Kumta   PN, Sfeir C, Lee D H, Olton D, Choi D. Nanostructured calcium phosphates for biomedical applications: novel synthesis and characterization. Nanostructured calcium phosphates for biomedical applications: novel synthesis and characterization Acta Biomater 1 65-83 2005 1(1):65.

207.Matsumoto T, Okazaki M, Inoue M, Yamaguchi S, Kusunose T, Toyonaga T, et al. Hydroxyapatite particles as a controlled release carrier of protein. Hydroxyapatite particles as a controlled release carrier of protein Biomaterials 25 2807-12 2004 25(17):3807.

208. Mizushima Y, Ikoma T, Tanaka J, Hoshi K, Ishihara T, Ogawa Y, et al. Injectable porous hydroxyapatite microparticles as a new carrier for protein and lipophilic drugs. Injectable porous hydroxyapatite microparticles as a new carrier for protein and lipophilic drugs J Control Release 110 260-5 2006 110(2):260.

209. Morrissey R, Rodriguez-Lorenzo L M, Gross KA. Influence of ferrous iron incorporation on the structure of hydroxyapatite. Influence of ferrous iron incorporation on the structure of hydroxyapatite J Mater Sci Mater Med 16 387-92 2005 16(5):387.

210. Misono M, Hall WK. Oxidation-reduction properties of copper and nickel-substituted hydroxyapatites. Oxidation-reduction properties of copper-and nickel-substituted hydroxyapatites J Phys Chem 77 791-800 1973 77(6):791.

211. Wakamura M, Kandori K, Ishikawa T. Surface structure and composition of calcium hydroxyapatites substituted with Al(III), La(III) and Fe(III) ions. Surface structure and composition of calcium hydroxyapatites substituted with Al(III), La(III) and Fe(III) ions Colloids Surf A 164 297-305 2000 164(2-3):297.

212. Wakamura M, Kandori K, Ishikawa T. Surface composition of calcium hydroxyapatite modified with metal ions. Surface composition of calcium hydroxyapatite modified with metal ions Colloids Surf A 142 107-16 1998 142(1):107.

213. Tanaka Y, Hirata Y, Yoshinaka R. Synthesis and characteriastics of ultrafine hydroxyapatite particles. Synthesis and characteriastics of ultrafine hydroxyapatite particles J Ceram Process Res 4 197-201 2003 4:197.

214. Aoki H. What is hydroxyapatite? What is hydroxyapatite? Medical Applications of Hydroxyapatite (Tokyo: Takayama Press) 1994:1.

215. Chai F, Blanchemain N, Lefevre A, Hildebrand HF. In vitro studies on the influence of precultural conditioning method on osteoblast reactions of a new type of injectable calcium cement material. J Biomed Mater Res B Appl Biomater 2006 Apr;77(1):104-113.
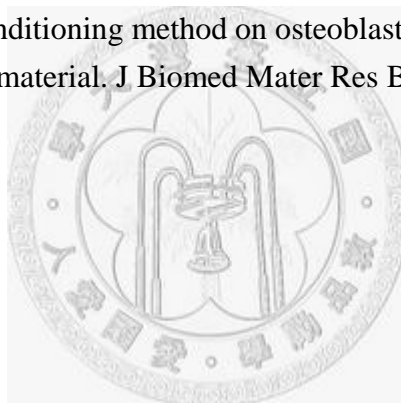
Chapter 7   Appendix

7.1 Curriculum Vitae of the author （中文）

- ➤ 姓名：侯君翰　醫師
- ➤ 出生年月日： 民國六十五年七月三日

- ➤ 主要學歷：
  1. 台灣大學醫學院醫學系　醫學士
     - 民國九十年六月畢業
  2. 台灣大學醫學院臨床醫學研究所　碩士
     - 民國九十五年七月畢業
  3. 台灣大學醫學院暨工學院醫學工程學研究所　博士候選人
     - 民國九十五年七月入學

- ➤ 主要經歷：
  1. 台大醫院實習醫師　　民國八十九年七月至民國九十年六月
  2. 台大醫院骨科部住院醫師　　民國九十年七月至民國九十三年十二月
  3. 台大醫院骨科部總住院醫師　　民國九十四年一月至十二月
  4. 台大醫院骨科部臨床研究員　　民國九十五年一月至民國九十六年六月
  5. 台大醫院雲林分院骨科部主治醫師　　民國九十六年七月至今　（現職）

- ➤ 獲獎記錄：
  1. 大學五年級時以 ”Ultrasound Examination of Patellar Tendon after Harvest for Anterior Cruciate Ligament Reconstruction” 論文榮獲台大醫學院青年學者優等獎
     - 民國八十八年五月
  2. 於台大醫院骨科部實習時榮獲「最佳實習醫師獎」
     - 民國八十九年
  3. 骨科醫學會優秀論文競賽，以” Hospital-Based Allogenic Bone Bank – Ten-Year Experience” 論文榮獲中華民國骨科醫學會優秀論文獎佳作
     - 民國九十一年十月

4. 台大骨科年度最佳住院醫師
   - 民國九十一年、九十二年、九十三年、九十四年
5. 骨科醫學會優秀論文競賽,以" Clinical Application of Proton MR Spectroscopy of the Femoral Head on Patients of Osteonecrosis" 論文榮獲中華民國骨科醫學會優秀論文獎第一名
   - 民國九十三年十月
6. 救國團社會優秀青年--台北市代表
   -民國九十五年四月
7. 國家衛生研究院「臨床醫師攻讀臨床醫學碩士在職進修專班獎助」
   -民國九十五年十二月

➢ 國外進修資歷:
1. 英國倫敦 King's College 暑期英文學校進修及遊學一個月
   - 民國八十六年七月
2. 美國哈佛大學醫學院交換學生,於波士頓之 Brigham & Women's Hospital 及 Beth Israel Hospital 各見習一個月
   - 民國八十九年三到五月
3. 獲選亞太骨科醫學會(APOA)每年提供兩位「日本交流學者」名額,至日本國立九州大學骨科部研習兩星期,參訪實驗室、骨骼肌肉系統腫瘤及脊椎手術
   - 民國九十六年七月

➢ 領導統馭經驗:
1. 創立台大醫學院羽球隊,並在畢業前率隊榮獲醫學杯冠軍及季軍各一次、大醫杯亞軍一次、台大杯冠軍一次

➢ 教學相關經驗:
1. 第一屆楓城醫學營,教學對象為高中一二年級有志從醫之學生。負責擔任醫院內病歷室、手術室之教學參觀流程。
   - 民國八十八年七月
2. 骨科學術總醫師,負責邀請外賓擔任科部之學術演講,安排科內學術活動,以及住院醫師、實習醫師及見習醫師之教育
   - 民國九十四年一月年十二月

7.2 Curriculum Vitae of the author （English）

Name: Chun-han Hou M.D. MS

Birth: July 3rd, 1976.

Education:

1. High school: Taipei Municipal Jeng-Kuo High School
   1991-1994.
2. Medical school: National Taiwan University School of Medicine, MD Degree
   1994-2001.
3. Master, Graduate Institute of Clinical Medicine, Medical College, National
   Taiwan University
   Sep. 2004~July 2006.
4. PhD candidate, Institute of Biomedical Engineering, National Taiwan University
   July 2006~now.

Position:

1. Internship, National Taiwan University Hospital
   July, 2000 ~ June, 2001.
2. Residency, Department of Orthopedic Surgery of National Taiwan University
   Hospital.
   July, 2001 ~ Dec. 2005.
3. Fellow, Department of Orthopedic Surgery of National Taiwan University
   Hospital.
   Jan. 2006 ~ July 2007.
4. Attending Physician, Department of Orthopedic Surgery of National Taiwan
   University Hospital, Yun-Lin Branch.
   July 2007~ Now.   (CURRENT POSITION)

Honors and Awards:

1. Young Investigator's Award in School of Medicine, National Taiwan University –
   May 1999.

" Ultrasound Examination of Patellar Tendon after Harvest for Anterior Cruciate Ligament Reconstruction"

2. Best Intern Award- Oct., 2000.
   By Department of Orthopedic Surgery, National Taiwan University Hospital.

3. Excellent Paper Award of Taiwan Orthopedic Association- Oct. 2002.
   "Hospital – Based Allogenic Bone Bank- Ten Year Experience".

4. Best Orthopedic Resident Award of the Year
   National Taiwan University Hospital, Year 2002, 2003, 2004, and 2005.

5. Best Paper Award of Taiwan Orthopedic Association- Oct. 2004.
   "Clinical Application of Proton MR Spectroscopy of the Femoral Head on Patients of Osteonecrosis

6. Elite Youth Award of Taipei City
   April 2006.

7. "Scholarship for Medical and Master Degree" in Academic Sinica, Taiwan
   December 2006.

Fellowship & Exchange programs:

1. English training course July 1997 in King's College, UK

2. Exchange student of Harvard Medical School, USA, March-May 2000
   -One-month clerkship in Brigham & Women's Hospital
   -One-month clerkship in Beth Israel Hospital

3. Japan visiting fellow of Year 2007, Asia Pacific Orthopedic Association (APOA), Fukuoka, Japan, July 2007
   - visiting lab
   - studying Musculoskeletal oncology and spine surgery

7.3 Refereed papers published

Items No.6 ~ No.15 were published during the PhD semesters.

1. **Hou CH**, Wang CL, Lin CC. Ultrasound examination of patellar tendon after harvest for anterior cruciate ligament reconstruction. J Formos Med Assoc 100(8): 315-318, 2001 May (SCI)

2. **Hou CH**, Chang CH. Neuropathy in Elbow Arthroplasty. J Orthop Surg Taiwan 20(2): 58-63, 2003

3. **Hou CH**, Hou SM, Yang RS. Clinical application of proton MR spectroscopy of the femoral head in patients with osteonecrosis. J Orthop Surg Taiwan 21:153-159, 2004.

4. **Hou CH,** Yang RS, Hou SM. Hospital-based allogenic bone bank--10-year experience. J Hosp Infect. 2005 Jan;59(1):41-5. (SCI)

5. Chen PY, Wu CT, **Hou CH**, Hou SM. Loosening of total arthroplasty with a prosthesis employing a skirted femoral head. J Formos Med Assoc. 2005 May;104(5):370-3. (SCI)

6. **Hou CH**, Liu CY, Li YD, Enright T, Shih TTF. Proton MR Spectroscopy of the Femoral Head ─ Evaluation of Patients at Risk for Avascular Necrosis. J Magn Reson Imaging. 2006 Aug;24(2):409-17. (SCI)

7. **Hou CH**, Wang WD, Leung KK, Hou SM. An Analysis of Practice Outcome and Opinions of physicians from the Government-Sponsored General Physician Training Program in the Last Ten Years. J Medical Education 2007; 11:47-57.

8. **Hou CH**, Tan TW, Tang CH. AMP-activated protein kinase is involved in COX-2 expression in response to ultrasound in cultured osteoblasts. Cell Signal. 2008 May;20(5):978-88 (SCI) IF: 4.887 (Cell biology: 37/156)

9. Huang YM, **Hou CH**, Hou SM, Yang RS. The Metastasectomy and Timing of Pulmonary Metastases on the Outcome of Osteosarcoma Patients. Clinical Medicine: Oncology 2008:2 1–8

10. **Hou CH**, Fong YC, Chen JT, Liu JF, Lin MS, Chang CS, Tang CH. The novel isoflavone 7-hydroxy-3',4'-benzoisoflavone induces cell apoptosis in

human osteosarcoma cells. Cancer letters 2008; 271(1):117-28 (SCI) IF: 3.277 (Oncology: 48/127)

11. **Hou CH**, Hsiao YC, Fong YC, Tang CH. Bone morphogenetic protein-2 enhances the motility of chondrosarcoma cells via activation of matrix metalloproteinase-13. Bone 2009 Feb;44(2):233-42 (SCI) IF: 3.966 (Endocrine & Metabolism: 22/92)

12. **Hou CH**, Hou SM, Tang CH. Ultrasound increased BMP-2 expression via PI3K, Akt, c-Fos/c-Jun and AP-1 pathways in cultured osteoblasts. Journal of Cellular Biochemistry 2009 Jan; 106(1):7-15 (SCI) IF: 3.381 (Cell biology: 67/156)

13. **Hou CH**, Lin J, Huang SC, Hou SM, Tang CH. Ultrasound stimulates NF-κB activation and iNOS expression via the Ras/Raf/MEK/ERK signaling pathway in cultured preosteoblasts. Journal of Cellular Physiology 2009 Jul;220(1):196-203. (SCI) IF: 3.643 (Physiology: 22/78=28.2%)

14. **Hou CH**, Hou SM, Hsueh YS, Wu HC, Lin FH. The in vivo performance of biomagnetic hydroxyapatite nanoparticles in cancer hyperthermia therapy. Biomaterials. 2009 Aug;30(23-24):3956-60. Epub 2009 May 14. IF: 6.262 (MATERIALS SCIENCE, BIOMATERIALS: 1/16= 6.25%)

15. **Hou CH**, Chen CW, Hou SM, Li YT, Lin FH. The fabrication, characterization, and in vitro hyperthermia test of the new dicalcium phosphate dihydrate-modified biomagnetic nanoparticles (mDCPD). Biomaterials. 2009 Jun 5. [Epub ahead of print] IF: 6.262 (MATERIALS SCIENCE, BIOMATERIALS: 1/16= 6.25%)

7.4 Conference papers

Items No.12 ~ No.18 were published during the PhD semesters.

1. Hou SM, **Hou CH**: Prefabricated vascularized bone graft on rats
    6th Congress of the International Federation of Societies for Surgery of the Hand(IFSSH), Helsinki (Finland), 1995
2. **Hou CH**, Wang CL, Lin CC: Ultrasound examination of patellar tendon after harvest for anterior cruciate ligament reconstruction.
    42nd Annual meeting, Taiwan Orthopedic Association, 2001.
3. **Hou CH**, Yang RS, Hou SM: Hospital –Based Allogenic bone bank-ten-year experience.
    43rd Annual meeting, Taiwan Orthopedic Association, 2002.
4. **Hou CH,** Hou SM: Shoulder arthrodesis following brachial plexus injury.
    45th Annual meeting, Taiwan Orthopedic Association, 2003.
5. **Hou CH**, Wu CD, Hou SM: Scaphoid cyst caused by crystal-induced tenosynovitis- a case report.
    45th Annual meeting, Taiwan Orthopedic Association, 2003.
6. Hou SM, Yeh LS, **Hou CH**, Yang LS: Allograft Transplantation of Extremities.
    96th Annual meeting of Formosan Medical Association, 2003.
7. **Hou CH**, Yang RS, Hou SM: Hospital –Based Allogenic bone bank-ten-year experience.
    71st Annual meeting of American Academy of Orthopedic Surgery, 2004.
8. **Hou CH**, Yang RS, Hou SM: A ten-year retrospective study of allogenic bone bank at a medical center in Taiwan.
    77th Annual meeting of Japanese Orthopedics Association, 2004.
9. **Hou CH**, Shih TF: Clinical Application of Proton MR Spectroscopy of the Femoral Head on Patients of Osteonecrosis.
    47th Annual meeting, Taiwan Orthopedic Association, 2004.
10. SM Hou**, Hou CH**, TK Liu, DH Lin: Clinical outcome of minimally-invasive hip arthroplasty.

78th Annual meeting of Japanese Orthopedics Association, 2005.

11. **Hou CH**, Yang RS**,** Mau TJ, Lung CY, Chen CC, Hou SM Comparison of the augmentation effects of polymethyl methacrylate and cortical bone allograft on the screw fixation strength.
13th International Symposium on Limb Salvage (ISOLS), Korea, 2005

12. Huang YM, **Hou CH**, Yang RS, Hou SM: The therapeutic value of thoracotomy and timing of pulmonary metastases identification for osteosarcoma patients.
73rd Annual meeting of American Academy of Orthopedic Surgery, 2006.

13. **Hou CH**, Ku YF, Yeh LS, Yang RS, Hou SM: Study of End-to-Side Neurorrhaphy in Rat Myocutaneous Iso- and Allo-transplantation.
73rd Annual meeting of American Academy of Orthopedic Surgery, 2006.

14. **Hou CH**, Li YD, Liu CY, Hou SM, Yang RS, Chiang CC, Hu MH, Shih TTF,: Proton magnetic resonance spectroscopy as a prognostic factor of femoral head avascular necrosis
74[rth] Annual meeting of American Academy of Orthopedic Surgery, 2007.

15. **Hou CH**, Li YD, Liu CY, Hou SM, Shih TTF,: FAT COMPOSITION CHANGE AS A PROGNOSTIC FACTOR OF FEMORAL HEAD AVASCULAR NECROSIS？ THE CLINICAL VALUE OF PROTON MAGNETIC RESONANCE SPECTROSCOPY
6th Annual meeting of Combined Orthopedic Research Society, 2007.

16. Yang CT, **Hou CH**, LinJ, Huang SC, Hou SM. Balloon kyphoplasty and vertebroplasty in the management of vertebral compression fracture- a systemic review
55th Annual meeting, Taiwan Orthopedic Association, 2008.

17. **Hou CH**, Hou SM, Shieh YS, Lin J, Wu SC, Lin FH. The application of a novel biomagnetic nanoparticle based on hydroxyapatite for cancer hyperthermia: an in vivo study
55th Annual meeting, Taiwan Orthopedic Association, 2008

18. **Hou CH**, Fong YC, Chen JT, Liu JF, Lin MS, Chang CS, Tang CH. The new isoflavone derived, HBI, induces cell apoptosis in human osteosarcoma cells.
55th Annual meeting, Taiwan Orthopedic Association, 2008

Invited Speeches (Special or Keynote Lectures):

1. **Hou CH**, Yang RS. Bone bank: our ten-year experience.
   Taiwan Regenerative Medicine Society, 2009 Annual meeting
2. **Hou CH.** The animal model of cancer hyperthermia with magnetic nanoparticles.
   Animal oncology and pathology meeting, Taiwan, 2009.

7.5 Other publications

1. **Hou CH**：The past, present and future of proton magnetic resonance spectroscopy- and its application on femoral head avascular necrosis. Taiwan Orthopedic Research Society Communication, July 2006

2. **Hou CH**：Proton Magnetic Resonance Spectroscopy ─ Evaluation of lipid Composition and Assessment of Avascular Necrosis of the Femoral Head. Master thesis, Graduate Institute of Clinical Medicine, Medical College, National Taiwan University, 2006