Nanodimension artificial cells containing a nanobiotechnological complex of polyhemoglobin-tyrosinase for enzyme therapy of tyrosine-depending tumours

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Abstract

Artificial cells bioencapsulated enzymes have been developed for different medical applications. Research in this thesis is to further extend this using a novel combination of two nanobiotechnological methods. Biodegradable nanodimension artificial cells are used to enclose a nanobiotechnological complex of polyhemoglobin-tyrosinase. We then investigated this in melanoma and prostate cancer since both of these require higher level of tyrosine for growth and metastasis.

Biodegradable nanodimension artificial cell containing nanobiotechnological complex of polyhemoglobin-tyrosinase was first characterized including its ability to remove tyrosine. Then, we first analyzed its effect on melanoma using B16F10 murine cell line. Results indicated that it was effective in inhibiting tumor proliferation, attachment and colonization. To further understand the therapeutic mechanism, we studied and determined its effects on cell cycle, apoptosis and ROS generation. Furthermore, we developed a 3D cell culture system that is closer to the *in vivo* system, thus allowing us to confirm the effects and verify the suitable dose for the animal study. In the following *in vivo* research, the tumor-bearing mice model was established based on B16F10 cells injection, and results showed the artificial cell' effectiveness in suppressing the growth of melanoma.

Besides, we analyzed the effects of the different components of this system. By itself the soluble nanobiotechnological polyhemoglobin-tyrosinase was not effective because it could not be retained to stay longer locally. Similarly, the vehicle, biodegradable nanodimension artificial cell without any content, also did not show effect since its function is to act as a carrier to retain the complex locally. Another component, biodegradable nanodimension artificial cell containing only polyhemoglobin without tyrosinase, has some but less effect as shown in both *in vitro* and *in vivo* studies. Biodegradable nanodimension artificial cell with all its components was the most effective. Finally, we also verified its effect on the prostate cancer PC3 cell culture with similar results as for melanoma.

RÉSUMÉ

Les enzymes encapsulées dans les cellules artificielles furent initialement développées pour différentes applications médicales. Le travail effectué au cours de cette thèse a pour but d'élargir les connaissances actuelles, afin d'utiliser une nouvelle combinaison de deux méthodes de nano-biotechnologie. Les cellules artificielles biodégradables de taille nano sont d'abord utilisées pour se rapprocher d'un complexe nano-biotechnologique de poly-hémoglobine-tyrosinase. Ensuite, nous appliquerons cette méthode dans un modèle de cancer de la prostate et de mélanome, étant donné que ces deux cancers requièrent de haut niveau de tyrosine pour la croissance des tumeurs et la formation de métastases.

Les cellules artificielles biodégradable de taille nano contenant un complexe de poly-hémoglobine-tyrosinase ont d'abord été caractérisée pour leur capacité à ôter les résidus tyrosine. Ensuite, nous avons analysé leur effet sur le mélanome en utilisant la lignée cellulaire murine B16F10. Les résultats indiquent que ce complexe est efficace dans l'inhibition de la prolifération tumorale, l'adhésion et la colonisation. Afin de comprendre en détail le mécanisme thérapeutique sous-jacent, nous avons également étudié son effet sur le cycle cellulaire, l'apoptose et la formation de ROS. Par ailleurs, nous avons développé un modèle 3D de culture cellulaire, très proche du système *in vivo*, et permettant de confirmer l'efficacité du complexe ainsi que d'établir le dosage pour l'étude chez l'animal. Ensuite, dans ce modèle d'étude *in vivo*, nous avons établi un modèle murin basé sur l'injection de cellules B16F10 de mélanome murin, et le résultat a permis la mise en évidence de l'efficacité des cellules artificielles dans la suppression de la croissance tumorale. Nous avons ensuite analysé les effets des différents composants du système. Utilisée seule, la nano-biotechnologie de poly-hémoglobine-tyrosinase n'est pas suffisante pour être efficace, de par le fait qu'elle ne peut être retenue localement. De plus, le transporteur, c'est à dire les cellules artificielles biodégradable de taille nano, n'ont aucun effet utilisées seules, puisque leur fonction est d'agir en tant que transporteur afin que le complexe soit retenu et maintenu au niveau ciblé. De plus, si le complexe est utilisé seulement avec la poly-hémoglobine sans la tyrosinase, on observe un effet moindre comme montré à la fois *in vivo* et *in vitro*. Le complexe de nano-biotechnologie de poly-hémoglobine-tyrosinase utilisée avec tous ses composants est le plus efficace. Finalement, nous avons également vérifié ses effets sur la lignée

cellulaire de cancer de la prostate PC3, et nous avons observé les mêmes résultats que pour le mélanome.

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Contribution to publications

Chapter 2 to 5 are manuscripts

I am the first author in all manuscripts and other author is my Ph.D. thesis supervisor Dr. Thomas Ming Swi Chang. There is no other author involved in all manuscripts.

1. Wang, Y. and Chang, T.M., Nanobiotechnological Nanocapsules Containing Polyhemoglobin-Tyrosinase: Effects on Murine B16F10 Melanoma Cell Proliferation and Attachment. J Skin Cancer, 2012. 2012: p. 673291.

2. Wang, Y. and Chang, T.M., Biodegradable nanocapsules containing a nanobiotechnological polyhemoglobin-tyrosinase for the suppression of a skin cancer, melanoma in a mice model of melanoma (Submitted to Nanomedicine-Nanotechnology Biology and Medicine)

3. Wang, Y. and Chang, T.M., *In vitro* and *in vivo* antitumor effects of the different components of the PLA nanocapsule Polyhemoglobin-Tyrosinase on B16F10 murine melanoma. (To be submitted to Biomaterials)

4. Wang, Y. and Chang, T.M., PLA nanocapsules containing a nanobiotechnological complex of polyhemoglobin-tyrosinase inhibit the growth of prostate cancer PC3. (Manuscript being finalized)

Original Contribution

1. The main contribution is the design, preparation, development, therapeutic effect, *in vitro* and *in vivo* mechanisms of novel PLA nanocapsules containing a nanobiotechnological complex of polyhemoglobin-tyrosinase.

2. Successful preparation of a novel combination of two nanotechnological methods into one single therapeutic agent: (1) formation of a soluble nanobiotechnological complex polyHb-Tyrosinase; (2) further nanoencapsulation into PLA nanocapsules. The physical and physiological characteristics were evaluated. (Chapter 2)

3. PLA nanocapsules containing polyhemoglobin-tyrosinase are effective in the suppression of B16F10 murine melanoma growth and migration (metastasis) both in cell culture and in the mice melanoma model. The possible mechanisms involved were also confirmed by detailed analysis including Western blot methods. (Chapter 3)

4. The effects of the different components of PLA nanocapsules containing polyhemoglobin-tyrosinase were evaluated on the B16F10 murine melanoma mice model. (Chapter 4)

5. PLA nanocapsules containing polyhemoglobin-tyrosinase was shown to be effective for the suppression of the cell culture of PC3 prostate cancer. The effects and mechanism research were analyzed. (Chapter 5)

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Abbreviation

FDA	Food and Drug Administration
EPR	enhanced permeability and retention
Hb	Hemoglobin
PolyHb	Polyhemoglobin
Ncap	PLA nanocapsules vehicle
Ncap-PH	PLA nanocapsules containing polyHb without tyrosinase
Ncap-PH-TYR	PLA nanocapsules containing polyHb with tyrosinase
PEG	Polyethylene glycol
PEG-PLA	poly (ethylene glycol)-poly (lactic acid)
Phe	phenylalanine
PLA	Poly (lactide)
RES	reticuloendothelial system
ROS	Reactive oxygen species
Tyr	tyrosine

Chapter 1

Introduction and literature review

1.1. Tyrosine dependent tumours: melanoma and prostate cancer

1.1.1 Melanoma

1.1.1.1 General introduction of melanoma

Melanoma is the deadliest form of skin cancer that is responsible for 48,000 deaths per year in the world (Gray-Schopfer, Wellbrock et al. 2007). The incidence of melanoma has rapidly risen in developed countries during the past several decades (Berwick, Erdei et al. 2009). In 2010, cutaneous melanoma was estimated to be the fifth and seventh most common cancer diagnosed in men and women, respectively. It was estimated that more than 68,000 Americans (38,870 men and 29,260 women) were diagnosed with melanoma in 2010 (Jemal, Siegel et al. 2010).

The risk factors of melanoma can be divided into environmental factors and host related factors: On the environmental factors, it could be the exposure to UV radiation or to toxic chemicals. For the host factors, the family history of melanoma and immunosuppression is an important consideration of the occurrence of melanoma (Markovic, Erickson et al. 2007). The most important one is the UV radiation from sun exposure. Long time exposure to sun, exposure during childhood, sunburns and the using of tanning bed will potentially enhance the risk of melanoma (Markovic, Erickson et al. 2007).

Melanoma initially arises from the malignant transformation of melanocytes, the pigmented cells of the skin. Because the most striking feature of melanoma is its unsurpassed ability to metastasis (Fig1.1), it is important to apply new strategies and target on multiple melanoma pathways.

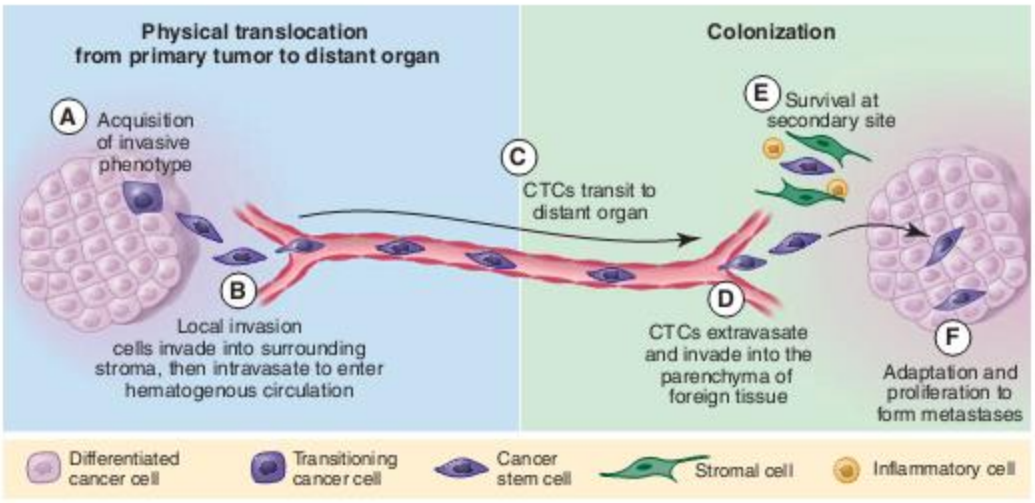

Figure 1.1 Metastatic cascade

Metastasis process can be divided into two major phases: cancer cells migrate from the primary tumor to a foreign organ, and then colonize in that organ. (A) In the beginning of the metastasis, cancer cells acquire an invasive phenotype. (B) Cancer cells migrate and invade into the surrounding matrix, blood vessel, and subsequently enter into the circulation. (C) Cancer cells travel through the circulation, and survive independent of anchorage. (D) Cancer cells exit the circulation and invade into the microenvironment of the foreign tissue. (E) In the foreign tissue, cancer cells are able to evade the innate immune response and also survive as a single cell or a small cell cluster. (F) Cancer cells adapt to the microenvironment, grow and proliferate (Chaffer and Weinberg 2011).

1.1.1.2 Surgery therapy on primary melanoma

Surgical excision is the corner stone of melanoma treatment (Tuong, Cheng et al. 2012). Adequate surgical management of primary melanoma and regional lymph node metastasis is the only established curative approach (Testori, Rutkowski et al. 2009). The five-year survival rate is about 97% after early remove of the nonulcerated, thin melanomas in the early stage (Balch, Gershenwald et al. 2009).

Once disseminated, melanoma becomes a clinical problem associated with a median survival time of 6 months. There are currently no FDA (the Food and Drug Administration) approved

agents available that have been shown to alter the natural history of metastatic disease in large randomized clinical trials. For the advanced melanoma, the 5-year survival rate will drop to 39% for ulcerated primary tumors (Mueller and Bosserhoff 2009). With this in mind, a huge amount of research has been undertaken to discover novel therapeutic interventions (Smalley 2010).

1.1.1.3 Other therapies on melanoma

Though the majority of melanomas can be detected early and surgically cured, melanomas diagnosed at late stages will have poor survival rates and are unaltered by available therapies (Berwick, Erdei et al. 2009). When it comes to the traditional chemotherapy, melanoma presents poor response. The alkylating agent dacarbazine (DTIC) is the only FDA approved single agent for malignant melanoma treatment, but only 5-10% patients show complete remission. Some present side effects, including allergic reactions, decreased bone marrow function, blood problems, nausea, vomiting, diarrhea, or loss of appetite (Serrone, Zeuli et al. 2000). An improved derivative of DTIC, Temozolomide can increase the drug permeability in brain metastasis, however, it still can not increase the overall survival (Soengas and Lowe 2003). The possible reasons are reduced levels of apoptosis induction in comparison to the other tumor cell types (Soengas and Lowe 2003).

Radiotherapy is another important strategy for cancer treatment. However, melanoma is comparatively radio-resistant. The response rate is typically dose dependent, and high doses of radiation required to eradicate tumors, will correspondingly generate adverse effects, thus it is less optimal as primary treatment for melanoma. Radiotherapy may be used as an adjuvant therapy when adequate surgical margins cannot be achieved, such as with lesions on the head and neck or bone and brain metastases (Testori, Rutkowski et al. 2009).

What's more, immunotherapy is another choice on the cancer therapy. High-dose of IL-2 was approved by FDA in 1998 (Garbe, Eigentler et al. 2011). However, research data showed no significant improvement in the overall survival and side effect induced was another consideration (Parkinson, Abrams et al. 1990). In 2011, the FDA approved ipilimumab for the treatment of patients in advanced-stage (Sondak, Smalley et al. 2011). Ipilimumab is a monoclonal antibody against cytotoxic T-lymphocyte-associated antigen-4, aiming to induce a T-cell mediated

response and inhibit tumor development. In clinical trials under ipilimumab treatment, survival was improved by 4 months in patients with stage III and IV melanoma (Hodi, O'Day et al. 2010).

1.1.2. Prostate cancer

Prostate cancer is dominant because of the high incidence and cancer death in men (Jemal, Siegel et al. 2009). In Europe and the United States, prostate cancer is one of the most frequently diagnosed malignancies, and 28,170 men could die from this disease in the United States alone and 241,740 new cases are diagnosed in 2012 (Siegel, Naishadham et al. 2012).

1.1.2.1 Current methods of prostate cancer treatment

Prostate cancer is divided into two types: androgen-dependent prostate cancer and androgen-independent prostate cancer. Up to now, effective treatments on prostate cancer are very limited and there is also problem related to compliance because of complications (Table 1.1). Generally, the primary option of hormone treatment can prolong the survival time to a certain degree on prostate cancer patients (Akaza, Homma et al. 2006; Antonarakis, Blackford et al. 2007). This anti-androgenic method is effective with the androgen-dependent prostate cancer. While once the prostate tumor has the metastasis characteristic, it usually loses androgen dependency, and develops into androgen-independent prostate cancer, which shows little sensitivity to anti-proliferation drugs and only modest survival can be expected (van Brussel and Mickisch 2003).

For the early stage (androgen-dependent) prostate cancer, localized prostate cancer can be treated by prostatectomy or radiation therapy. However, the problem with prostate cancer in the early stage is the absence of obvious symptom and when symptoms appear, the tumor has already developed into metastasis stage (Rennie 2008).

Once patients are diagnosed with metastatic androgen-independent prostate cancer, the disease is lethal and the median survival time is 10-12 months (Petrylak, Tangen et al. 2004). On this advanced prostate cancer, androgen ablation therapy eventually fails (Feldman and Feldman 2001). Many patients that receive androgen ablation become castration-resistant in a median of 18-24 months (Petrylak, Tangen et al. 2004). Other optional treatments are palliative. For example, through radiotherapy, 80% response rate is expected, however, no significant improvement in the overall survival of the patients (Droz, Flechon et al. 2002). In addition, to

deal with bone pain due to bone and lymph node is another palliative strategy (Droz, Flechon et al. 2002; Petrylak, Tangen et al. 2004).

Table 1.1 Limitation of common treatments on prostate cancers

Treatment	Complications
Surgery	Pain, urinary incontinence, and possible permanent impotence
Hormone therapy	Loss of sexual desire, impotence, and hot flashes
Radiation therapy	Tiredness, diarrhea, uncomfortable urination, and hair loss in the pelvic area
Chemotherapy	Hair loss, weakness, impotence, urinary retention, and erectile dysfunction

Conclusively, androgen-independent prostate cancer is still the fundamental problem to tackle and make breakthroughs. Novel strategies for the treatment of androgen-independent prostate cancer should be further investigated.

1.2. Nutrient deprivation strategies on tumor therapies

It has been found that some tumours appear to have specific nutrient dependency, such as selective amino acid dependency. Dietary factors can affect the process of cell cycle and cell fate (tumor growth, proliferation, metastasis or cell death) by modulating the expression and function of the relevant proteins.

Restriction of tyrosine and phenylalanine are widely researched in many tumor types, such as melanoma (Fu, Yu et al. 1999; Pelayo, Fu et al. 1999; Meadows, Zhang et al. 2001; Ge, Fu et al. 2002; Nunez, Liu et al. 2006; Fu and Meadows 2007), leukemia (Pine 1981), hepatocarcinoma (Abdallah, Starkey et al. 1987), lung carcinoma (Abdallah, Starkey et al. 1987) (Abdallah, Starkey et al. 1987; Elstad, Meadows et al. 1990) (Meadows, DiGiovanni et al. 1976; Meadows, Pierson et al. 1982; Pierson and Meadows 1983), prostate cancer (Fu, Yu et al. 2003; Nunez, Liu et al. 2006; Fu, Yu et al. 2008) and breast cancer(Nunez, Liu et al. 2006) (Table 1.2).

Table 1.2 Relative influence of specific amino acid deprivation on tumor invasion (Meadows, Ge et al. 2002)

Cell Line	Day[b]	Gln	Leu	Met	Tyr	Phe	Tyr/Phe
Breast Cancer							
MDA-231	1						↓↓↓↓
Melanoma							
A375	1						↓↓↓
	2						↓↓↓↓
	3	O	↑	↓↓	↓↓↓	↓	↓↓↓↓
B16BL6	3	O	↑	↓↓	↓↓↓	↓	↓↓↓↓
SK-MEL-28	1			↓↓			↓
	2			↓↓↓			↓↓
	3			↓↓↓			↓↓↓
Prostate							
DU-145	1	↓↓		↓↓			↓↓
	2	↓↓↓		↓↓↓			↓↓↓
	3	↓↓↓		↓↓↓			↓↓↓
PC-3	1	O		↓↓↓			↓↓
	2	O		↓↓↓			↓↓↓
	3	O		↓↓↓↓			↓↓↓

[a] ↑, denotes an increase in invasion; O, denotes no effect on invasion; ↓ to ↓↓↓↓, denotes 20% to 80% inhibition of invasion.
[b] number of days that cells were cultured in amino acid-deprived media ± 10% FBS.

1.2.1 Effects of specific amino acid restriction on Melanoma

The effects of tyrosine / phenylalanine deprivation are linked with inhibition of tumor growth and metastasis. In B16BL6 melanoma established mice model, researchers found that under the deprivation of tyrosine and phenylalanine culture condition, the tumor cells' growth and metastasis abilities were impaired. However, this treatment didn't induce toxicity to the normal

cells. The inhibition effect has been confirmed in both *in vitro* studies, the Matrigel ^{TM}, and *in vivo* studies of B16BL6 melanoma models (Elstad and Meadows 1993; Fu, Yu et al. 1997; Fu, Yu et al. 1999); (Uhlenkott, Huijzer et al. 1996; Pelayo, Fu et al. 1999). As one important evidence, the pulmonary tumor nodules were found to decrease (Elstad and Meadows 1993). Besides the effect on tumor growth and metastasis, the tyrosine and phenylalanine restriction can enhances the chemotherapeutic effect *in vivo* and the anti-melanoma activity of vitamin C (Pierson and Meadows 1983; Meadows, Abdallah et al. 1986; Elstad, Meadows et al. 1990). These effects are not due to decreased food intake, starvation, or the body weight loss, but a complex mechanism associated with the amino acid restriction. In further research, possible mechanisms involved in the molecular level were studied. The metastasis suppressor genes are strengthened, such as MKK4/SEK1, in tumors melanoma as well as breast cancer cells in the protein expression and/or phosphorylation by Tyr/Phe deprivation (Meadows, Ge et al. 2002).

1.2.2 Effect of specific amino acid restriction on prostate cancer

Specific amino acid restriction can induce apoptosis in invasive, androgen-independent prostate cancer cells, but not in normal human prostate epithelial cells or in non-invasive, androgen-dependent LNCaP prostate cancer cells (Fu, Yu et al. 2003; Fu, Yu et al. 2008). This can ensure the safety and target when applied on prostate cancer.

Moreover, selective amino acid restriction also affects mitochondria function. The mechanisms involved are the energy product, generation of ROS and the regulation of apoptosis (Fu, Zhang et al. 2006). What's more, the deprivation of amino acid is involved in multiple steps in the tumor growth and invasion, such as cell adhesion, spreading, motility and directionality, and the cell cycle is arrest at G0/G21 (Fu, Yu et al. 2003; Fu, Yu et al. 2008).

1.3. Further development based on nanomedicine

Based on the discussions above, more advanced strategies are in great need for the treatment of malignant melanoma.

1.3.1. Artificial cells

The concept of artificial cell was first shown by Dr. Chang. He described this idea as a specific physical entity prepared in the cellular dimension, and can be applied on the cells function deficient diseases as a replacement or supplement (Chang 1964; Chang and Poznansky 1968; Chang 1971; Chang 1971). The design and function of artificial cells are based on the living cells. Similarly, they consist of selectively permeable membrane encapsulating functional materials inside. The active materials can be microorganism, cells, enzymes, DNA/RNA, magnetic materials or drugs (Fig 1.2). Diffusion of smaller molecules is rapid across the artificial cell membrane due to the large surface area and ultrathin membrane. At the same time, the contents of artificial cells are protected from contacting with the extracellular space. The diameter can be varied ranging from macro to micro to nano to nanobiotechnological complexes (Figure 1.2). He and other groups around the world have expanded on this idea that is now used in various fields, such as drug delivery, blood substitutes, enzyme and gene therapy, cell and stem cell therapy, regenerative medicine, microdevice and nanodevice, biotechnology and nanobiotechnology, nanomedicine, nanotechnology and so forth (Chang 2007, 2013).

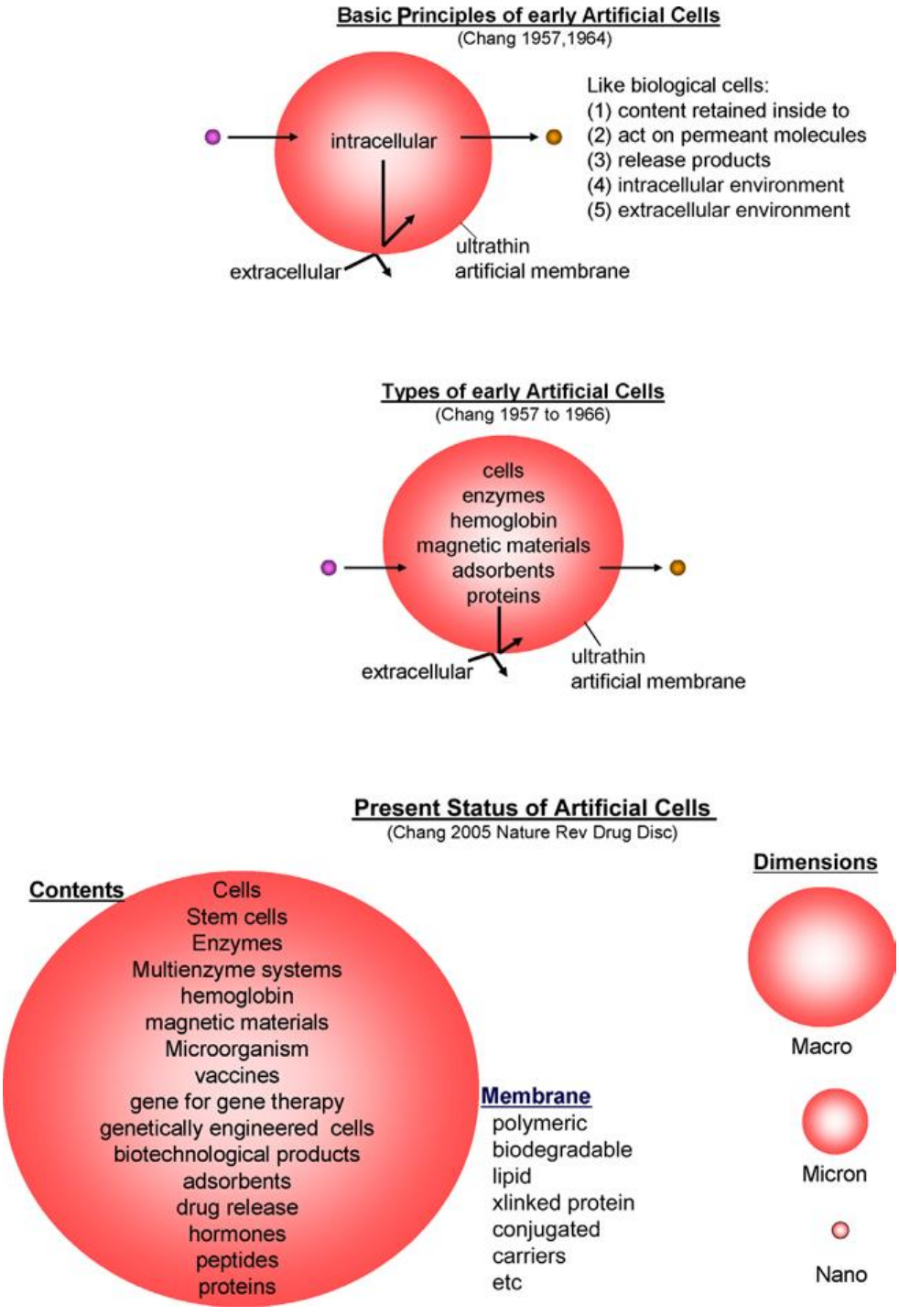

Figure 1.2 Basic principles of artificial cells (Chang 2007)

Upper: Early artificial cells

Middle: different types of earlier artificial cells

Lower: Present status of artificial cells

1.3.1.1 Application artificial cells in enzyme delivery

In the artificial cells-based enzyme delivery system, the enzymes are retained inside and substrates and products can diffuse in and out.

Artificial cells bioencapsulated enzymes have been prepared for enzyme therapies (Chang 1964), such as catalase for the depletion of hydrogen peroxide in acatalasemia (Chang and Poznansk.Mj 1968); asparaginase to remove asparagine in 6C3HED lymphosarcoma (Chang 1971); phenylalanine ammonia-lyase to remove phenylalanine in phenylketonuria (Bourget and Chang 1986) and xanthine oxidase to remove hypoxanthine in Lesch-Nyhan Disease (Palmour, Goodyer et al. 1989) (Fig 1.3). Detailed *in vitro* studies showed that enzymes in artificial cells no longer induce immunological problems (Poznansky and Chang 1974). More recent development is to apply nanobiotechnological approach and biodegradable polymeric membrane into artificial cells (Chang 2007, 2013).

The present thesis concentrates on a novel approach of combining (1) Nanodimension artificial cells and (2) Nanobiotechnological complex.

1.3.2. Nanodimension artificial cells

One major extension of the principle of artificial cells is to prepare these much smaller into nanodimension (Chang 2007, 2013; Bian, Wei & Chang. 2013) (Fig 1.2). These are commonly called nanocapsules or nanoparticles containing biologically active materials. The nanocapsules as a novel delivery system, a branch of nanomedicine, open a new way for cancer therapy and promise to overcome the drawbacks on the present traditional methods. Up to now, nanocapsules have been mostly developed as drug delivery systems.

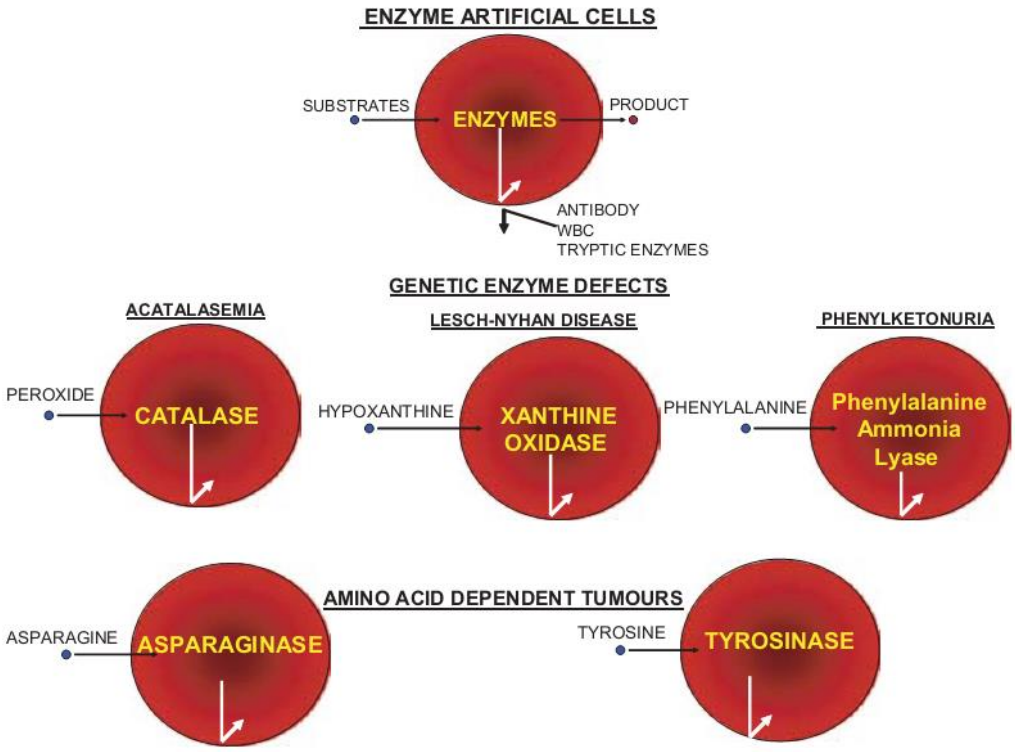

Figure 1.3 Examples of enzyme artificial cells (Chang 2007)

Nanocapsules are nano dimension artificial cells with a core-shell structure in which the drug or active substance can be entrapped inside the inner space core and the shell is normally formed by the polymeric materials and form the membrane structure (Quintanar-Guerrero, Allemann et al. 1998; Letchford and Burt 2007; Anton, Benoit et al. 2008). Depending on the specific application, some modifications can be performed inside or outside the membranes to make the nanocapsules multifunctional. The application of biodegradable polymers on the membrane preparation can avoid accumulation of the polymer matrix following repeat dosing (Yu and Chang 1994; Discher and Eisenberg 2002; Sheikh Hassan, Sapin et al. 2009).

Firstly, nanocapsules are subcellular in size which provides higher intracellular uptake than other drug systems (Mosqueira, Legrand et al. 2001; Mosqueira, Legrand et al. 2001).

Secondly, the half-life and stability of the enclosed drug are extended (Pinto Reis, Neufeld et al. 2006; Anton, Benoit et al. 2008; Ourique, Pohlmann et al. 2008). Some research has found that the half-life of drugs can be improved as much as 10 times (Maeda, Bharate et al. 2009). One

example is the encapsulated of L-asparaginase that has less antigenic reaction in addition to the lower dose and less frequency of injection required (Masurekar, Fong et al. 2014).

Thirdly, the physical and physiological characters can be easily modified and altered. This can help to improve the selective distribution when using nanocapsules as drug carriers. Nanocapsules can selectively and effectively deliver the contents (drug or enzyme) to the targeted site at therapeutic concentration, without accumulating in the non-targeted sites (Cummings, Allan et al. 1994).

Fourthly, toxicity can be lower. Benefit from improved delivery ability and also the protective membrane from the outside contact, lower drug dose can increase therapeutic effect. Furthermore, this could decrease side effects resulted from the drugs (Peer, Karp et al. 2007).

Other advantages include good biocompatibility when using biocompatible or biodegradable polymers, and the easy preparation of nanocapsules to contain different characters of drugs.

The nanocapsules can be captured by liver when the nanocapsules are larger than 100nm, or cleared by renal when they are smaller than 5nm (Das 2014)

1.3.2.1 Tumor target strategies

Overall, clinically most relevant drug targeting strategies are free drug delivery, passive drug delivery, active drug delivery and local drug delivery (Fig 1.4).

1.3.2.1.1 Passive targeting

Passive targeting is based on the specific pathophysiological features of the tumor vessels. In the normal situation, the vessels are properly organized. While in the tumor tissue, the vessels are disorganized with pores and gap junctions between endothelial cells and also compromised lymphatic drainage. The leaky vascularization structure, the enhanced permeability and retention effect (EPR), is one universal phenomenon in solid tumors. This means that nanoparticles of up to 400nm in diameter can get to the tumor area (Matsumura and Maeda 1986; Sinha, Kim et al. 2006). The exploitation of EPR effect is currently the most important strategy for improving the delivery of therapeutic agents to tumors (Matsumura and Maeda 1986; Sinha, Kim et al. 2006).

Up to now, FDA has approved some nano-delivery systems that utilize the passive tumor targeting strategy. What's more, various polymers based nano-delivery systems are in clinical evaluation. One successful case is paclitaxel albumin-bound nanoparticles of about 130 nm (Araxane®). It has been approved by the FDA in 2005 for patients with metastatic breast cancer and non-small cell lung cancer (NSCLC) (Ma and Mumper 2013). Another new design is to form large macromolecular conjugation of paclitaxel and poly-L-glutamic acid, showing improved therapeutic effect of paclitaxel in virtue of the enhanced permeability of tumor vasculature and the lack of lymphatic drainage (Singer, Shaffer et al. 2005). Also prolonged tumor exposure to active agents and at the meantime minimized systemic exposure lead to a better response. There were less side effects and better patient compliance (Singer 2005). Besides these two patterns mentioned above, other encapsulation approaches are PLA-PEO (Xiao, Xiong et al. 2011), PLA-PEO (Jie, Venkatraman et al. 2005), and PLA-PEG-PLA (Venkatraman, Jie et al. 2005; He, Ma et al. 2007). Many more are in clinical development (Ma and Mumper 2013).

1.3.2.1.2 Active targeting

Another approach is to make use of the ligand-receptor interaction to actively deliver drugs or proteins to the tumor area or cells. Some receptors or epitopes are considered to be specifically and highly expressed on the surface of certain tumors, but low or no expression on the normal cells. The nanocapsules can be modified on the surface membrane. Various conjugation chemistries could be used to bind the corresponding affinity ligands, which can be antibodies, peptides, aptamers or small molecules. Then by the active-receptor integration, the nanocapsules can transport the drugs or active agent to the target tumor area and internalized and function inside cells (Farokhzad and Langer 2009).

For example, human epidermal growth factor receptor-2 (HER2) is widely known as a biomarker owing to its overexpression on breast cancer cells. This is notably important for the pathogenic feature on this tumor, and it corresponding antibody, Trastuzumab, can be utilized to modify nanocapsules for targeted chemotherapy with anticancer drug, such as paclitaxel (Sun, Ranganathan et al. 2008). Another case is folic acid (FA), the receptor of which have been found to overexpress in some cancer cells, and thus FA can be incorporated to the surface of the polymers via coupling reaction to achieve tumor targeting characters (Chen, Zhang et al. 2008).

In addition, the specific maker in tumor lymphatics, LyP-1, has been recognized for its specific home to tumors and their lymphatics. The modification of nanocapsules with LyP-1 can be applied to target lymphatic metastases. *In vivo* test has confirmed that the uptake of LyP-1-NPs in metastasis lymph nodes was much higher than that of NPs (~8 times higher) (Luo, Yu et al. 2010).

1.3.2.1.3 Nanocapsules uptake and subcellular localization

The nanocapsules' reach and accumulation in tumor tissues are triggered by two main parameters: the particle's size and surface properties of the nanocapsules (Schadlich, Caysa et al. 2011) (Table 1.3).

(1) For the size: It has been shown that 400 nm is the upper size limit for extravasation into solid tumors. NP with diameter <200 nm can effectively accumulate (Moghimi, Porter et al. 1991; Hobbs, Monsky et al. 1998; Moghimi, Hunter et al. 2001) than bigger ones (150-300nm) which are uptaken by reticuloendothelial system (RES) (Moghimi 1995). A lower size limit is hard to define due to further influencing parameters like structure, surface charge, and molecular-flexibility (Nakaoka, Tabata et al. 1997). So the optimum NP size for tumor accumulation is between 70 and 200 nm (Storm, Belliot et al. 1995).

(2) For the surface properties: the polymer polyethylene glycol (PEG) can reduce opsonisation and "stealth " from the clearance by the mononuclear phagocytic system (or RES system) which has been considered as a main challenge for effective drug delivery by targeting strategy (Brigger, Dubernet et al. 2002). Consequently, the circulation time and half-life of nanocapsules can be considerably improved. The report also showed that PEG encapsulated nanocapsules was able to achieve and accumulate in the brain with a compromised blood-brain barrier (BBB). This is expected to facilitate the effective drug delivery within the brain and further apply on brain tumor, stroke and other brain diseases with compromised BBB (Nance, Woodworth et al. 2012).

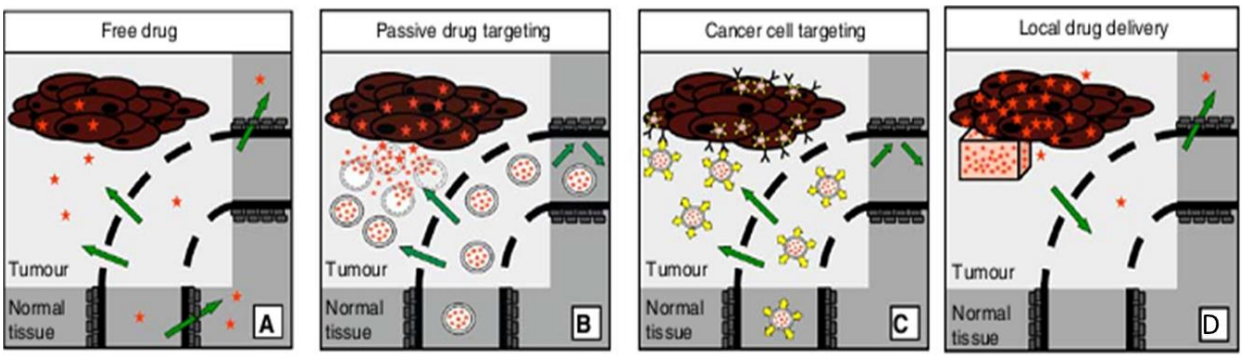

Figure 1.4 Drug targeting strategies. (A) Free drug delivery: the free therapeutic agent injection with intravenous administration is often impaired by the circulation system clearance. Only comparatively low level of the drug can reach and accumulate in the tumor areas, while the localization of certain healthy organs and tissues could be high. (B) Passive drug delivery: by virtue of the enhanced permeability and retention (EPR) effect, the drug can be delivered by passive targeting system. Higher accumulation of drug in cancer area or cancer cells can be obtained. (C) Active drug delivery: by virtue of the selectively and specifically high expression of cell surface receptors of cancer cells, the ability of binding these surface proteins can improve the drug delivery system on the cellular uptake. (D) Local drug delivery: during surgery, sustained-release delivery devices can be implanted or injected directly into tumours (Lammers, Hennink et al. 2008).

Table 1.3 various factors affect nanocapsules' uptake and subcellular localization (Thurn, Brown et al. 2007)

Nanoparticle	Cell type	Localization	Uptake mechanism	References
1. NP size				
50 nm silica magnetic NP	A549 lung cancer	Endosomal	CME	Kim et al. [12]
24 and 43 nm Polystyrene(PST)	HeLa	24 nm = perinuclear, 43 nm = lysosome	24 nm = CME independent, 43 nm = CME	Lai et al. [13]
100 nm PLGA	Primary RHEC	Membrane bound, intracellular	Clathrin?, Caveolin independent	Qaddoumi et al. [32]
78 nm–1 μm Microspheres	RBC	Cytoplasm	Passive uptake?	Rothen-Rutishauser et al. [14]
40 nm–4.5 μm Microshperes	Dendritic	Cytoplasm and membrane bound	No experimental evidence	Foged et al. [11]
2. NP charge				
PEG-PLA NP(+) and (−) charge	HeLa	Both types perinuclear	(+) NP = CME/macropinocytosis, (−) NP = CME/caveolin independent	Harush-Frenkel et al. [30]
100 nm MSN (uncoated, weak, moderate, and strong (+) charge)	hMSC and 3T3-L1	No experimental evidence	hMSC: uncoated, weak, mod. (+) = CME, strong (+) unknown. 3T3-L1 = All CME	Chung et al. [10]
3. Cell type				
78 nm–1 μm PST Microsphere	Macrophage vs. RBC	Intracellular, not membrane bound	Macrophage: 1 μm = phagocytosis, .078–0.2 μm = actin-independent; RBC: all actin-independent	Geiser et al. [33]
MSN strongly (+)	hMSC vs. 3T3-L1	No experimental evidence	hMSC = CME-independent, 3T3-L1 = CME	Chung et al. [10]
4. Surface modifications				
Folic acid-LDL NP	KB Cells (FR+)	Cytoplasm, not in nucleus	Receptor mediated endocytosis	Zheng et al. [38]
PVA and vitamin E TPGS coated PLGA NPs	Caco-2	Cytoplasm and nucleus	No experimental evidence	Win and Feng [108]
Trastuzumab—HSA NP	BT-474 and SK-BR-3	No experimental evidence	Receptor mediated endocytosis	Steinhauser et al. [40]
Tat peptide conjugated Gold NP	hTERT-BJ1 fibroblast	Nucleus	No experimental evidence	de la Fuente and Berry [73]

NP = nanoparticle, PEG = poly(ethylene glycol), CME = clathrin-mediated endocytosis, PLGA = Poly(D,L-lactic-co-glycolic acid), (+) = positively charged, (−) = negatively charged, MSN = mesoporous silica nanoparticle, hMSC = human mesenchymal stem cell, RBC = red blood cell, FR+ = folate receptor positive, LDL = low density lipoprotein, PVA = polyvinyl alcohol, TPGS = d-alpha-tocopheryl polyethylene glycol 1000 succinate, HSA = human serum albumin

1.3.2.2 Preparation of nanocapsules by nanoprecipitation

The method nanoprecipitation (Yu and Chang, 1994) is an extension of the interfacial precipitation method for preparing artificial cells (Chang 1964, 2007) that makes the preparation of nanocapsules simple, fast and economical (Fig 1.5). Two phases are necessary for this method, the aqueous phase and the organic phase. In this method, the organic phase can be prepared by acetone, ethanol, dimethylformatmide, and dimethylsulfoxide. The lipophilic components and polymers are able to dissolve in this phase. And the polymeric materials cannot be dissoluble in the aqueous phase. When the organic solvent is added to the aqueous phase, the nanocapsules will form instantaneously as a colloidal suspension owing to the fast diffusion of the solvent into the aqueous phase. The formed particles can fall within a narrow sizdimethye distribution (Saha, Vasanthakumar et al. 2010). Numerous factors can influence this process, among which the key steps are associated with the choosing of the organic phase, the injection rate of the organic phase into the aqueous phase, and the speed of stirring, and so on.

Popular polymers applied are biodegradable polyesters, such as poly-e-caprolactone (PCL), poly (lactide) (PLA) and poly (lactide-co-glicolide) (PLGA). In addition, some polymers, like PEG are also widely applied which has the ability to avoid mononuclear phagocyte system recognition (de Assis, Mosqueira et al. 2008).

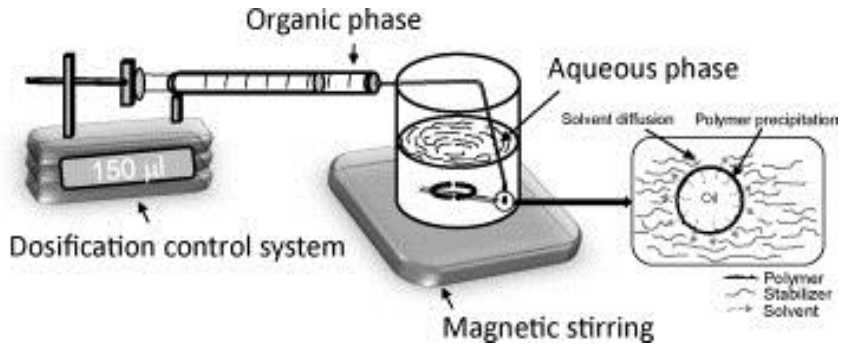

Figure 1.5 Preparation of nanocapsules by the nanoprecipitation method (Mora-Huertas, Fessi et al. 2010)

1.3.2.3 Characterization of nanocapsules

1.3.2.3.1 Particle size

The size of nanocapsules is a key parameter when design and prepare nanocapsules. Because it can directly decide the fate of the nanocapsules on whether they can be recognized by the mononuclear phagocyte system (MPS), and whether they will be cleaned by the renal and liver when administered by intravenous injection. The fate after the administration can finally decide whether they can reach the targeted tumor areas. Many factors can influence the size, including the feature and concentration of the polymer chosen, the two phases and surfactants (Santos-Magalhaes, Pontes et al. 2000; Zili, Sfar et al. 2005).

For the nanocapsules with the diameter less than 200 nm, it has been found that opsonin can't bind to the particles due to the high radius of curvature thus allowing for longer circulation time (Owens and Peppas 2006; Champion, Katare et al. 2007). On the opposite, for the large nanocapsules with the diameter larger than 200nm, they are difficult to avoid opsonisation, as a result instead of localizing on tumor, they tend to accumulate in other organs, like lung, liver, kidney and spleen. However, it doesn't mean the smaller of nanocapsules, the better circulation time can be obtained. Because when the size is smaller than 20-30nm, the nanocapsules can be cleaned by the kidney. In conclusion, the optimal size for the nanocapsule as a carrier is between 70 to 200 nm (Moghimi, Hunter et al. 2001).

1.3.2.3.2 Entrapment/encapsulation efficiency (EE)

Another vital parameter of nanocapsules is the entrapment/encapsulation efficiency. Ideally higher drug loading and entrapment efficiency can decrease the volume or frequency that is required for administration. When it comes to the nanocapsules containing active components, such as enzymes, the high EE is also important in having high protein entrapment.

The EE can be determined directly or indirectly.

In the direct method, the nanocapsules are dissolved in suitable solvent to allow the release of encapsulated component. The analysis is based on the amount of the component before and after encapsulation. (Snehalatha, Venugopal et al. 2008).

$$EE\ (\%) = \frac{\text{Amount of drug in nanocapsules}}{\text{Initial amount of drug taken}} \times 100$$

In the indirect method, the nanocapsules can be separated by centrifuging. The free unencapsulated content is in the supernatant, while the encapsulated content in the sediment, which can be calculated by minus the free component from the initial total amount.

$$EE\ (\%) = \frac{\text{Total amount of drug} - \text{Free drug}}{\text{Total amount of drug}} \times 100$$

For the calculation of the nanocapsules with active component, the activity is used instead of the amount in the above formulas.

1.3.2.4 Polylactic acid (PLA) nanocapsules

PLA polymer has been used to form PLA membrane microcapsules containing biologically active material as early as 1976 (Chang 1976), and later for the preparation of nanocapsules containing haemoglobin (Yu and Chang 1994). It is now one of the most widely used polymers, and has been extensively used as the matrix of nanocapsules for more than 20 years (Fig 1.4). It is approved by the FDA for a host of therapeutic devices owing to their biodegradability, biocompatibility, and non-toxic (Parveen and Sahoo 2008). In the human body, PLA can degrade into lactic acid and glycolic acid then become carbon dioxide. These are the by-products of various metabolic pathways and the intermediate of carbohydrate metabolism. Compared with the human body's degrade ability, the generated amount is much small. So there is very little systemic toxicity (Park 1995).

1.3.2.5 Polyethylene glycol (PEG)

PEG is another commonly used polymer due to its non-toxic, non-immunogenic, hydrophilic, uncharged and biodegradable characters. It also has been approved by FDA as a safe material on clinical application. As discussed below, it has been used to modify the surface of liposomes to increase its circulation time.

Table 1.4 Application of PLA nanocapsules

Payload	Proposed application	Reference
Cloricromene	Anti-ischemic	(Leo, Brina et al. 2004)
Neurotoxin-I (NT-I)	Transport into the brain after intranasal administration	(Cheng, Feng et al. 2008)
Cy5	*In vitro* and *in vivo* imaging applications	(Tong, Coyle et al. 2010)
Docetaxel	Breast cancer therapy	(Youm, Yang et al. 2011)
Oridonin	Anti-tumor, anti-bacteria, scavenging active oxygen free radicals and anti-inflammatory effects	(Xing, Zhang et al. 2007)
Savoxepine	Neuroleptic drug delivery	(Allemann, Leroux et al. 1993; Leroux, Allemann et al. 1996)

Firstly, PEG has good flexibility and can modify numerous water molecules, which process is called "PEGylation". PEGylated protein's hydrodynamic volume can be improved with an apparent molecular weight 5 to 10 times higher than that of a globular protein with the same molecular mass. This can degrade kidney filtration. Secondly, PEG chains when binding protein molecule show stealth properties. The bound protein can be protected from the environment, and then avoid the proteolytic degradation and immune system's recognition. Finally, the PEGylation not only can protect the proteins, but also can modify its charges and prevent connection with charged blood components. Conclusively, these features of PEG can increase the bound protein's half-life from 2 to 400 times longer in the circulation compared with the native protein(Caliceti and Veronese 2003) (van Vlerken, Vyas et al. 2007).

Besides PEG, many other polymers can also show the capability as stealth protection, such as polyvinyl pyrrolidone (Torchilin, Levchenko et al. 2001), polyvinyl alcohol (Mu and Zhong 2006), polyacril amide (Torchilin, Shtilman et al. 1994) and polymethyl and polyethyl oxazoline (Woodle, Engbers et al. 1994; Woodle 1998). These polymers appear to be attractive alternatives for designing long-circulation nanocapsules or drug carriers.

1.3.2.6 PEG-PLA Nanocapsules

A copolymer of PEG and PLA can be prepared for the preparation of nanocapsules biologically active materials like hemoglobin and multienzyme system (Chang 2003) (Fig 1.6). The multienzyme system can prevent methemoglobin formation and allowing the haemoglobin to function in the circulation. Furthermore, this increases the circulation time markedly as compared to the PLA nanocapsules. One example is PEG-PLA nanocapsule containing psychotic drug savoxepine to decrease the MPS uptake when intravenous administration is applied (Allemann, Leroux et al. 1993; Leroux, Allemann et al. 1996).

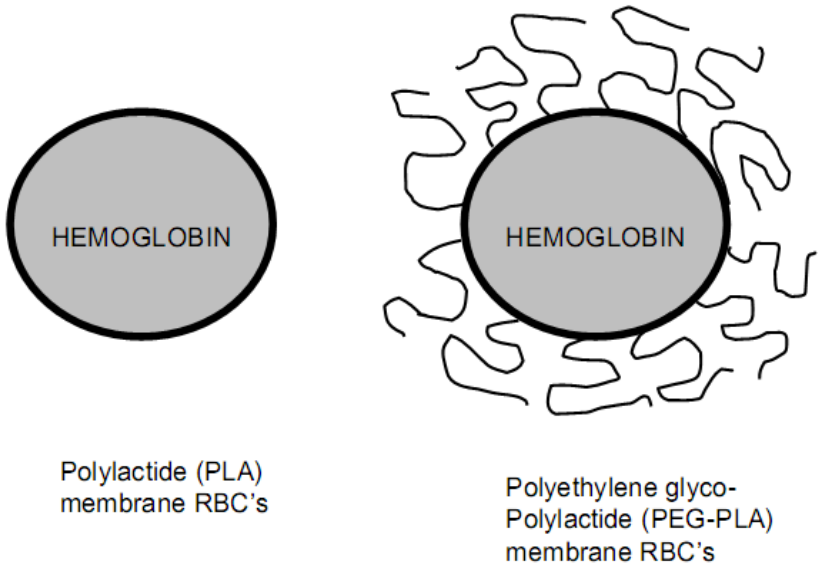

Figure 1.6 PLA nanocapules and PEG-PLA nanocapsules containing hemoglobin

Left: PLA membrane encapsulated hemoglobin. Right: Incorporation of polyethylene-glycol (PEG) in the PLA membrane results in marked improvement of circulation time of hemoglobin (Chang 2007).

1.3.3. Soluble Nanobiotechnological Complexs

Since artificial cell was firstly stated in 1964, it has elicited great interest, and various types have been developed with a large range of sizes. Polyhemoglobin (polyHb) is one excellent example of this advanced pattern of artificial cells. By nanobiotechnology, the bifunctional crosslinker glutaraldehyde can effectively bind the free hemoglobin molecules together and form a soluble complex polyhemoglobin (polyHb) (Chang 1971; Chang 2010).

1.3.3.1. PolyHemoglobin-enzymes

Due to the vital functional component hemoglobin, polyHb is designed and studied in the field of blood substitute as an oxygen carrier. PolyHb has now been approached for routine clinical application in Russia and South Africa. Furthermore, glutaraldehyde can also crosslink hemoglobin with other proteins, most potential ones are enzymes. It has been found that the oxygen therapeutic function can increase tissue oxygenation, improving the effects of radiation and chemotherapy in tumor therapy (Linberg, Conover et al. 1998; Han, Yu et al. 2012). PolyHb-enzyme system as a platform was developed by crosslinking haemoglobin to enzymes (Chang 1971; D'Agnillo and Chang 1998). The substrates can diffuse into the cells and are converted by the enzymes while the products of the enzymatic reaction leak out. In such a way, the artificial cells could extend the application on disease therapy.

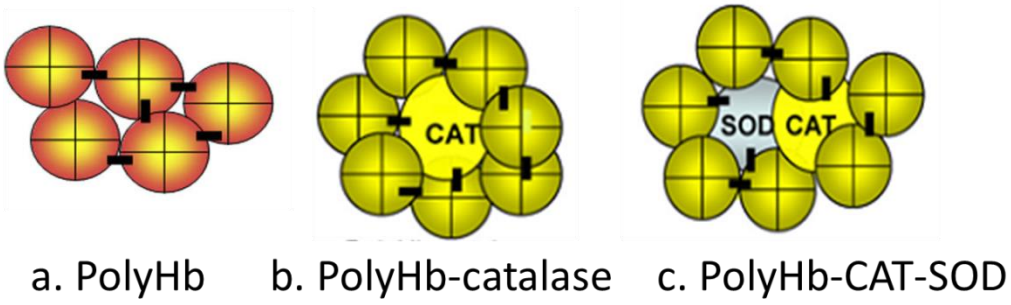

Figure 1.7 Soluble nanobiotechnological complexs (Chang 1964; Chang 1971; D'Agnillo and Chang 1998)

1.3.3.2 Polyhemoglobin-tyrosinase

Polyhemoglobin (polyHb) shows the benefit of preventing the adverse effects of free tyrosinase and gaining a longer circulation time (Yu and Chang 2004). Moreover, after crosslinking with tyrosinase, the hemoglobin component retains its ability to bind and release oxygen. Hemoglobin's ability to increase the levels of tissue oxygen tension and higher efficiency in tumor perfusion will improve the treatment effect of radiotherapy. In addition, crosslinking does not affect tyrosinase activity, and the hemoglobin component could stabilize its activity (Yu and Chang 2004).

PolyHb-tyrosinase added to B16F10 melanoma cell cultures prevented melanoma development, and the number of tumor cells decreased significantly during culture. *In vivo* studies on normal mice and rats also identified its efficiency to lower systemic tyrosine level and safety. Although polyHb-tyrosinase seems very efficient at quickly lowering (within 2 hours) tyrosine levels, it required daily intravenous infusions and after the injection, the tyrosine level increases back to normal. As a result, this strategy only slowed the growth of melanoma without inhibiting the growth (Yu and Chang 2004).

1.3.3.3 The strategy based on nanocapsules and tyrosine deprivation

One characteristic of melanoma cells is that they need a higher concentration of tyrosine to synthesize melanin and protein and sustain the cancer cells growth. A novel approach taken into account this theory is to utilize tyrosinase to degrade tyrosine. However, the injection of free enzyme can lead to immunological reactions. Dr. Chang's laboratory has shown that placing tyrosinase inside various nanocapsules would prevent the enzyme from contacting cells and macromolecules in the "extracellular environment" and meanwhile, tyrosine can enter the nanocapsules to be converted by tyrosinase. Thus, the application of encapsulated tyrosinase can circumvent the problems related to immunological rejection and tryptic enzyme destruction.

1.3.3.4 PEG-PLA nanocapsules containing polyhemoglobin-tyrosinase

This is the new generation of nanodimension artificial cells containing tyrosinase for the treatment of melanoma. The polymeric membrane is prepared with the polymer poly (ethylene glycol)-poly (lactic acid) (PEG-PLA). This strategy has several advantages: First of all, they can be used in intravenous administration, which means a quick response could be expected just as in

the case of polyHb-tyrosinase capsules; moreover, the tyrosinase is completed protected by the nanocapsules and a better enzyme function is expected; thirdly, with the function of PEG, it can be developed to a longer circulation time. Indeed, the circulation half-time is twice that of polyHb-tyrosinase. It was demonstrated that the enzyme activity was very much stable. In addition, animal experiments showed that tyrosinase nanocapsules were able to decrease systemic tyrosine levels as efficiently and quickly as PolyHb-tyrosinase.

Preliminary result showed that when the rats were given one-injection of PEG-PLA nanocapsules containing polyhemoglobin-tyrosinase intravenously, the systematic tyrosine level can be reduced to 10-13% after 5 minutes. However, this low tyrosine level could only be maintained for 4 to 5 hours (Fustier and Chang 2012).

1.3.3.5. Biodegradable nanodimension artificial cells containing a nanobiotechnological complex of polyhemoglobin-tyrosinase

Research in this thesis is to solve the above problems by using a novel combination of two nanobiotechnological methods. Biodegradable nanodimension artificial cells are used to enclose a nanobiotechnological complex of polyhemoglobin-tyrosinase. We then tested this in melanoma and prostate cancer since both of these require higher level of tyrosine for growth and metastasis.

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Chapter 2

Nanobiotechnological nanocapsules containing polyhemoglobin-tyrosinase: effects on murine B16F10 melanoma cell proliferation and attachment

Foreword

As mentioned in the Introduction in Chapter 1, the soluble PolyHb-Tyrosinase could only slow down the growth but could not completely suppression murine B16F10 melanoma *in vivo* even with daily intravenous injections. The aim of the present thesis research is to investigate a method to suppress the growth. We started this by designing two types of biodegradable nanocapsules containing the nanobiotechnological complex of polyHb-tyrosinase. One type is PLA nanocapsules and the other is the PEG-PLA nanocapsules. We mainly concentrated on the evaluation of PLA nanocapsules since we planned to apply it for local injection. The physical parameters and also the *in vitro* effects were tested on two B16F10 melanoma cell culture models: the proliferation model and the attachment model.

Nanobiotechnological nanocapsules containing polyhemoglobin-tyrosinase: effects on murine B16F10 melanoma cell proliferation and attachment

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Abstract

Previously we have reported that daily intravenous infusions of a soluble nanobiotechnological complex, polyhemoglobin-tyrosinase [polyHb-Tyr], can suppress the growth of a murine B16F10 melanoma in a mouse model. In order to avoid the need for daily intravenous injections, we have now extended this further as follows. We have prepared two types of biodegradable nanocapsules containing [polyHb-Tyr]. One type is to increase the circulation time and decrease the frequency of injection and is based on polyethyleneglycol–polylactic acid (PEG–PLA) nanocapsules containing [polyHb-Tyr]. The other type is to allow for intratumoural or local injection and is based on polylactic acid (PLA) nanocapsules containing [polyHb-Tyr]. Cell culture studies show that it can inhibit the proliferation of murine B16F10 melanoma cells in the "proliferation model". It can also inhibit the attachment of murine B16F10 melanoma cells in the "attachment model". This could be due to the action of tyrosinase on the depletion of tyrosine or the toxic effect of tyrosine metabolites. The other component, polyhemoglobin (polyHb), plays a smaller role in nanocapsules containing [polyHb-Tyr] and this is most likely by its depletion of nitric oxide needed for melanoma cell growth.

Keywords:

Melanoma; Nanocapsules; Tyrosinase; Tyrosine; B16F10 murine melanoma; Artificial cells; Nanomedicine; Nanobiotechnology; Polyhemoglobin, nitric oxide

1. Introduction

Deregulated proliferation and differentiation of melanocytes lead to the formation of melanoma [1]. Although not as common as the other skin basal cell skin cancer or skin squamous cell cancer, melanoma is far more dangerous. Surgical removal is effective in the early stage. However, once it has metastasized beyond the local lymph nodes, it is eventually fatal [2-4]. Chemotherapy, radiotherapy and other approaches in combination are being investigated [5-10].

Melanoma cells show specific amino acid-dependence for Tyrosine (Tyr) and phenylalanine (Phe) [11-14] and also arginine [15,16]. Tyr/Phe deprivation induces G0/G1 cell cycle arrest in murine melanoma [17], and induces apoptosis by inhibiting integrin / focal adhesion kinase (FAK) pathway and activating caspases [18-20]. Tyr/Phe deprivation induces apoptosis in murine and human melanoma cells but not in normal cells [21]. One method is to utilize the tyrosinase-dependent catalytic reaction to suppress Tyr level and also consume Phe [22,23]. In addition, the generated products of tyrosine metabolism, such as dopa, 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) and others are also toxic to melanoma cells [24]. Oxidation of these tyrosine metabolites can produce reactive oxygen species [24]. In the melanoma cells, the excessive reactive oxygen species will stimulate cell apoptosis by activating DNA damage-repair pathway and also opening mitochondrial pore [25]. Despite the potential of tyrosinase, injection of free enzymes has problems related to immunology, stability and duration of action.

Artificial cells bioencapsulated enzymes were first prepared for different medical applications [26]. This approach has shown potentials in catalase for the depletion of hydrogen peroxide in acatalasemia [27]; asparaginase for the depletion of asparagine for 6C3HED lymphosarcoma [28]; phenylalanine ammonia-lyase for the depletion of phenylalanine in phenylketonuria [29] and xanthine oxidase to remove hypoxanthine in Lesch-Nyhan Disease [30]. Detailed *in vitro* studies show that the enzyme in artificial cells no longer has

immunological problems [31]. Two further developments have led to the possible clinical applications of these animal studies. One is the development of nanobiotechnological approach for artificial cells [32]. Another development is the first artificial cells with biodegradable polymeric membrane [33]. These have now led to extensive developments in this area [34-36].

One area is the clinical applications of oxygen therapeutics using the basic nanobiotechnological procedure of crosslinking hemoglobin [32] to form soluble polyHb [37,38]. PolyHb has been tested in phase III clinical trials as blood substitutes [39,40] and has now been approved for routine clinical patient uses in Russia and South Africa. These oxygen carriers have also been tested in animal studies and found to increase tissue oxygenation and enhance radiation and chemotherapy in solid tumors [42,43]. We have methods for the crosslinking of enzymes to hemoglobin to form polyHb-enzyme systems [37,38,41]. We therefore studied the crosslinking of tyrosinase to hemoglobin to form polyHb-tyrosinase [44,45]. The increased tissue oxygenation could then be an additive effect on Tyr depletion for melanoma. Our studies show that this approach can significantly suppress the growth of murine B16F10 melanoma mice [44]. However, polyHb-tyrosinase requires daily intravenous infusions. Furthermore, polyHb-tyrosinase is a solution that does not stay at the site of intratumoural and local injection. This solution also cannot be located at the drainage lymphatic nodes or organs. We are in the process of improving this approach by combining the nanobiotechnological approach of polyHb-tyrosinase with biodegradable nano dimension artificial cells. We have developed the original biodegradable polymeric artificial cells [33] into a nano dimension system that can be given intravenously [46]. PolyHb in PEG-PLA membrane nano dimension artificial cells has a much longer circulation time than polyHb [47]. Infusion of 1/3 the total blood volume into rats does not cause long-term adverse effects on the histology and function of liver, spleen and kidney [48,49]. Other centers are now using this approach [50-53].

In order to avoid the need for daily intravenous injections, in the present study we have prepared two types of biodegradable nanocapsules containing [polyHb-Tyr]. One type is to increase the circulation time and decrease the frequency of injection and is based on PEG–PLA nanocapsules containing [polyHb-Tyr]. The other type is to allow for intratumoural or local injection and is based on PLA nanocapsules containing [polyHb-Tyr]. The cell culture study shows that it can inhibit the proliferation of murine B16F10 melanoma cells in the “proliferation

model". It can also inhibit the attachment of murine B16F10 melanoma cells in the "attachment model". The component analysis shows that the enzymatic action of tyrosinase on tyrosine plays the major role in nanocapsule-[polyHb-Tyr]. This could be due to the depletion of tyrosine or the toxic effects of the metabolites of tyrosine. The other component, polyHb, plays a smaller role in nanocapsule-[polyHb-Tyr] and this is most likely due to its depletion of nitric oxide needed for melanoma cell growth.

2. Materials and Methods

2.1. PolyHb and PolyHb-tyrosinase Preparations. This is as described in details elsewhere [36,45,45]. Briefly, stroma-free hemoglobin (SFHb) with a concentration of 7g/dL Hb with or without tyrosinase was dissolved in a 0.1 mol/L sodium phosphate buffer (pH=7.4). Lysine was added at a molar ratio of 7:1 lysine/hemoglobin. Glutaraldehyde as crosslinker was added at a molar ratio of 16:1 glutaraldehyde/hemoglobin. The process of crosslink continued for 24 hours. After that, lysine was added again to stop the crosslink reaction at a molar ratio of 200:1 lysine/hemoglobin. The sample was dialyzed overnight with a dialysis membrane (molecular weight cut off = 12-14 kDa) and then passed through sterile 0.45 μm syringe filters to remove impurities. All operations were conducted at 4ºC and under nitrogen in order to prevent the formation of methemoglobin and the degradation of enzyme.

2.2. Tyrosinase Nanocapsules Preparation. Nanocapsules were prepared as described [54]. Briefly, 50 mg PLA and 25 mg hydrogenated soybean phosphatidylcholine were firstly dissolved in 4 ml acetone and 2 ml ethanol by sonication for 30 min. The formed organic phase was dropwise added into 5 ml 7g/dl polyHb-Tyr solution with a syringe under moderate magnetic stirring. Then the suspension was stirred continually for 30 min. The organic solvent was removed using rotary evaporator under vacuum for about 1 hour. Then the resultant nanocapsules were separated by centrifugation at 20,000 rpm for 20 min to separate the supernatant and sediment. Then the suspension was concentrated with filtration.

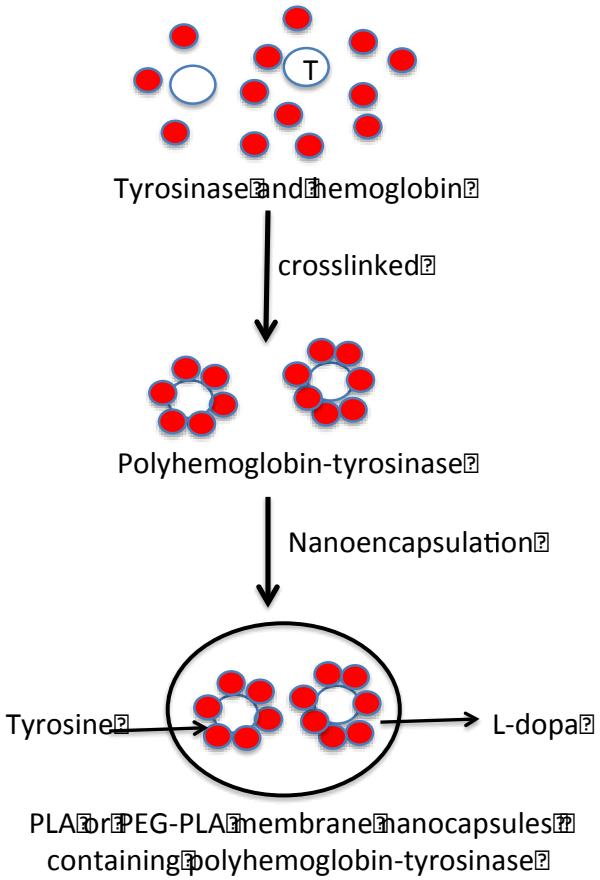

FIGURE 1: Schematic representation of the preparation of nanobiotechnological procedure for the PLA or PEG-PLA membrane nanocapsules containing polyhemoglobin-tyrosinase. Tyrosinase: Larger blue circles. Hemoglobin: smaller red circles.

2.3. Molecular Weight Distribution. Sephacryl-300 HR column (V total = 560 ml) was used for molecular weight distribution analysis of polyHb-Tyr. The elution buffer (0.1M Tris-HCl and 0.15M NaCl (pH 7.4) at a flow rate of 36 ml/hour) was monitored by a 280nm UV detector. Three molecular weight fractions were collected (1) > 450 kDa; (2) 100-450 kDa; and (3) >100 kDa.

2.4. Tyrosinase Activity Assay. In our assay, tyrosinase catalyzes the substrate tyrosine into L-dopa under O_2, and the formed L-dopa is then converted into L-dopa-quinone and H_2O. Tyrosinase activity was tracked by monitoring the production rate of the enzymatic product L-dopaquinone at 300 nm, as described previously [54]. Briefly, the reaction solution was prepared

with 0.34 mM L-tyrosine and 12.8 mM potassium phosphate buffer. This solution was adjusted to pH 6.5 and oxygenated by bubbling oxygen through for 10 minutes before usage. 10 to 100 µl of samples were mixed with the reaction solution 2.9 ml in a cuvette. Absorbance at 300 nm was monitored. The generated absorbance values were converted into enzyme activity unit by comparing with standard curves.

2.5. Tyrosinase Entrapment Efficiency. Drug entrapment efficiency of the nanocapsule was calculated using the following equation:

Entrapment efficiency (%, w/w) =

$$\frac{\text{Tyr activity in polyHb-Tyr} - \text{Tyr activity in nanocapsules' supernatant}}{\text{Tyr activity in the polyHb-Tyr}}$$

2.6. Physicochemical Characterization of Nanocapsules. The size and morphological examination of nanocapsules were performed by Transmission electron microscopy (TEM). Specifically, about 10 ul nanocapsule samples were placed on 200 mesh carbon-coated copper grids and then examined with a JEOL JEM-2000FX microscope (Jeol Ltd., Tokyo, Japan) and photographed with a Gatan Wide Angle Multiscan CCD Camera.

2.7. Tumour Cells and Culture Conditions. B16F10 murine melanoma cells (American Type Culture Collection, ATCC®, #CRL-6475) were cultured in standard Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37 °C and 5% CO_2, humidified atmosphere. Cells were passaged every 2–3 days. When the cells reached 90-100% confluence, they were washed with 0.25% trypsin/EDTA for about 1 min to be detached from the dish. Then cell suspensions were collected in a sterile 15 mL tube and centrifuged at 1,500 rpm for 5 min. Cells were counted by trypan blue exclusion and suspended in fresh medium to the desire density.

2.8. Cell Proliferation Assays. $5x10^4$ Melanoma cells in 2 ml complete DMEM were seeded in 6-well plates. When the tumor cells became 30–40% confluent, different nanocapsules or the same amount of saline were mixed with DMEM and added into each well. Cells were cultured for 0, 24, and 48 hours and collected by 0.25% trypsin/EDTA. The cell proliferative ability was then

determined by trypan blue exclusion. The data from triplicate wells were averaged, and each experiment was repeated three times.

2.9. Cell Attachment Assays. For the experiment, melanoma cells suspension was mixed with different nanocapsules formats or the same amount of saline in the complete DMEM. Then the cells together with nanocapsules were mixed well and seeded in 6-well plates for 0, 24, and 48 hours observation. Use PBS to wash the unattached tumor cells, and collect the attached tumor cells by 0.25% trypsin/EDTA. The cell attachment ability was then determined by trypan blue exclusion. The data from triplicate wells were averaged, and each experiment was repeated three times.

2.10. Data Analysis. Statistical analysis was performed using the Student's t -test within analysis of variance and was considered to be significant at $P < 0.05$.

3. Results

3.1. Physicochemical Characteristics of PLA Nanocapsule [polyHb-Tyr]. TEM image showed that the PLA nanocapsule [polyHb-Tyr] was spherical with smooth surface and freely dispersed (Figures 2 (a) and 2 (b)). The size distribution based on the TEM images showed a range from 88 to 267 nm (187 ± 35 nm), and 64 % of the nanocapsules are in the range of 120 to 200 nm (Figure 3).

3.2. Nanoencapsulation efficiency of PLA nanocapsule [polyHb-Tyr]. Sepacryl S-300 gel column chromatography was used to analyze the molecular weight distribution of polyHb-Tyr. The molecular weight distributions for polyHb-Tyr showed three types of molecular weight distributions: (1) low (< 100 kDa); (2) intermediate (100-450 kDa); and (3) high molecular weight (> 450 kDa). The low molecular weight fraction (< 100 kDa) (11.5% of the sample) was discarded. The fraction of > 100 kDa (88.5% of the samples) was used for nanoencapsulation. The encapsulation efficiency was 75.4% (Figure 4).

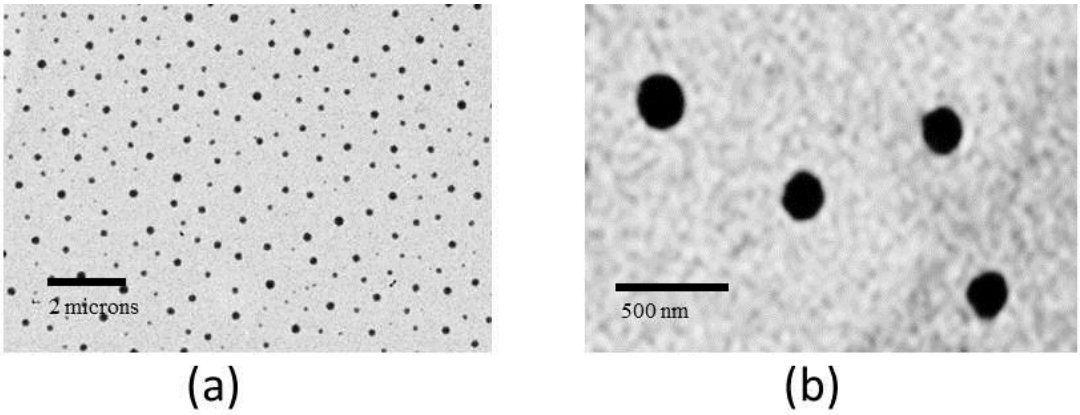

FIGURE 2: Physicochemical characteristics of PLA nanocapsule [polyHb-Tyr].

(a) TEM of PLA nanocapsule [polyHb-Tyr] at low magnification.

(b) TEM of PLA nanocapsule [polyHb-Tyr] at high magnification.

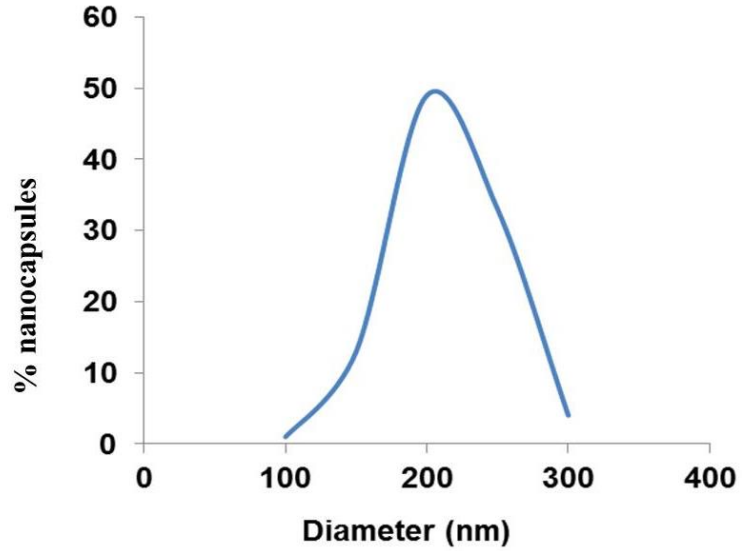

FIGURE 3: Size distribution of PLA nanocapsule [polyHb-Tyr]

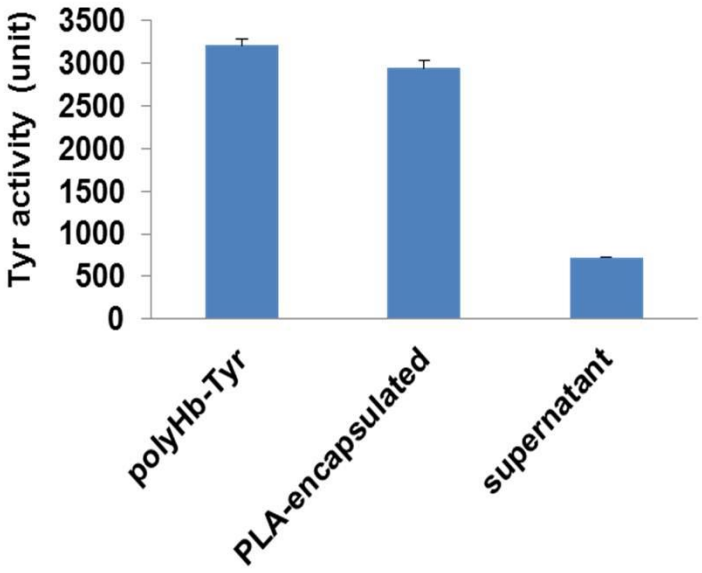

FIGURE 4: Entrapment efficiency of PLA nanocapsule [polyHb-Tyr].

3.3. Effects of PLA nanocapsule [polyHb-Tyr] on the Proliferation of B16F10 Cells. We treated B16F10 melanoma cells with PLA nanocapsules [polyHb-Tyr] containing a large range of enzyme activities, from 1.6 units to 200 units. The result showed that PLA nanocapsule [polyHb-Tyr] inhibited tumor proliferation and the effect was tyrosinase dose dependence. Compared with the control group, the higher the tyrosinase activity the greater was the inhibition of tumour proliferation as shown by the decrease in cell viability at 24 hours and 48 hours. At 48 hours, the tumour cells growth was most effectively inhibited in the groups of 40 units and 200 units of tyrosinase activity (Figure 5). The control group showed normal morphology with spindle-shaped and epithelial-like features (Figure 6 (a)); after the treatment with PLA nanocapsule [polyHb-Tyr], dead cell clusters were observed with more dead cell clusters in the higher tyrosinase groups (Figure 6 (b) to (e)).

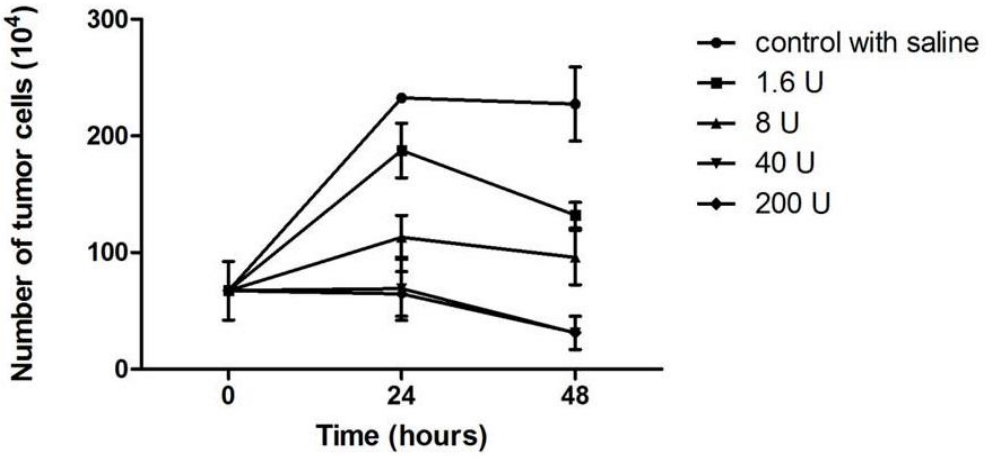

FIGURE 5: The effects of different amounts of PLA nanocapsule [polyHb-Tyr] on the proliferation of B16F10 melanoma cells.

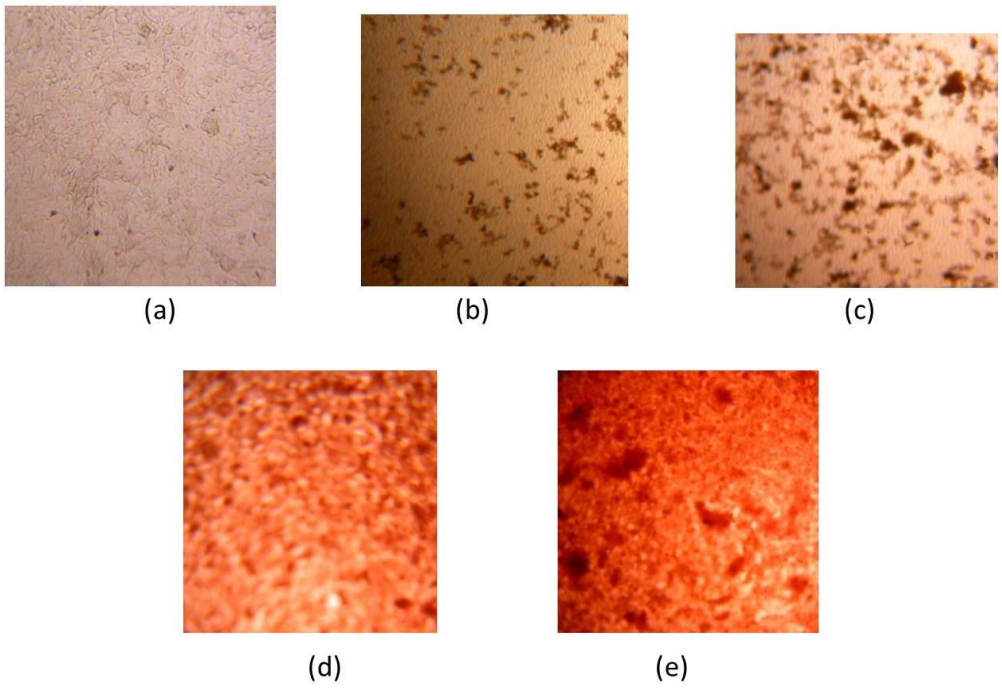

FIGURE 6: Effects of PLA nanocapsule [polyHb-Tyr] on the proliferation of B16F10 melanoma cells. The microscopy images of B16F10 melanoma cells treated with the nanocapsules containing enzyme activities increasing in enzyme activity from a to e: 0, 1, 6, 8, 40, 200 units.

We also carried out study to analyze the different PLA nanocapsule [polyHb-Tyr] components on the inhibition of cells proliferation (Figure 7). The components tested included the PLA nanocapsule [polyHb-Tyr] group; the PLA material; empty PLA nanocapsules; and PLA nanocapsule [polyHb] without tyrosinase. The result showed that PLA nanocapsule [polyHb-Tyr] was responsible for the major role in the inhibition of cells proliferation. PLA nanocapsule [polyHb] can also supress the tumor cells growth although not as much as that of the PLA nanocapsule [polyHb-Tyr]. In addition, the PLA material and empty PLA nanocapsule showed a very slight activity. Collectively, these results suggested that the PLA nanocapsule containing polyHb-Tyr had the ability to inhibit the tumor cells proliferation and the tyrosinase contributed to the major effect.

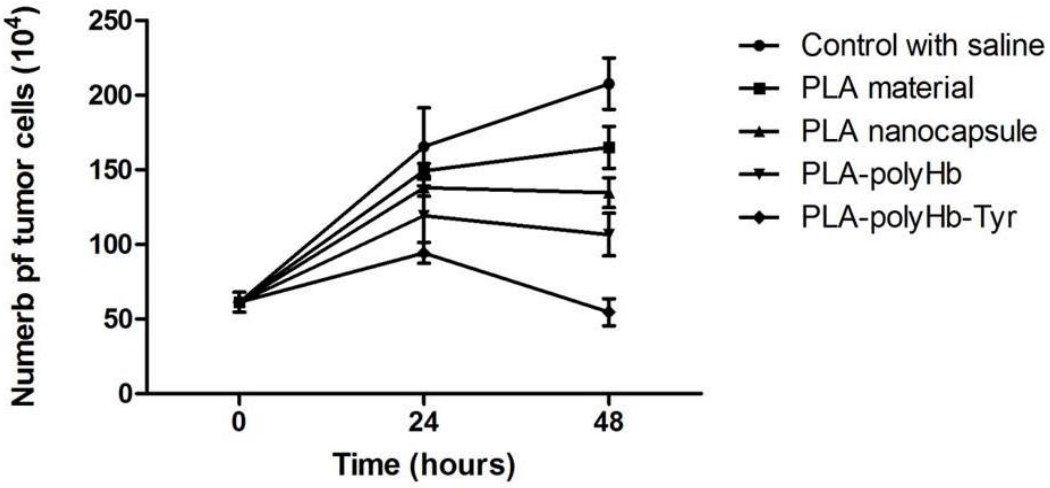

FIGURE 7: PLA nanocapsule [polyHb-Tyr] and the effects of its components on the proliferation of B16F10 melanoma cells.

3.4. Effects of PLA Nanocapsule polyHb-Tyr on the Attachment of B16F10 Cells. For the solid cancer melanoma, the metastatic dissemination from a primary lesion to a secondary site is believed to be the major reason leading to death [55,56]. The tumor cells attach to the secondary site is one of the vital steps of this metastatic process [57]. Thus, we carried out the following experiment to identify the effect of PLA nanocapsule [polyHb-Tyr] on melanoma cell attachment. We mixed melanoma cells and PLA nanocapsule [polyHb-Tyr] and co-cultured the suspension solution in 6 well plates to observe the cell viability. It was found PLA nanocapsule [polyHb-Tyr]

effectively inhibited tumor cells attachment to plastic plates. In 24 hours, most of the tumor cells died and by 48 hours of culture there were no viable cells (Figure 8).

We also tested the different components of PLA nanocapsule [polyHb-Tyr]. Saline was used as negative control and the free tyrosinase as positive control. Free tyrosinase reached its maximal effect at 24 hours. PolyHb-Tyr could inhibit tumor cells attachment in 24 hours culture and maintain this function to 48 hours. At 24 hour, PLA nanocapsule [polyHb-Tyr] was as effective as free enzyme and polyHb-Tyr and this was maintained for 48 hours. Empty PLA nanocapsules had a slight effect at 24 hours, but this effect was minimal by 48 hours (Figure 9).

PLA nanocapsule [polyHb-Tyr] is for the use in intratumoural and local injection. For intravenous injection, we have prepared PEG-PLA nanocapsule [polyHb-Tyr]. A comparison of PEG-PLA nanocapsule [polyHb-Tyr] and PLA-nanocapsule [polyHb-Tyr] showed that there were no significant differences in their effects on B16F10 melanoma cells (Figure 10).

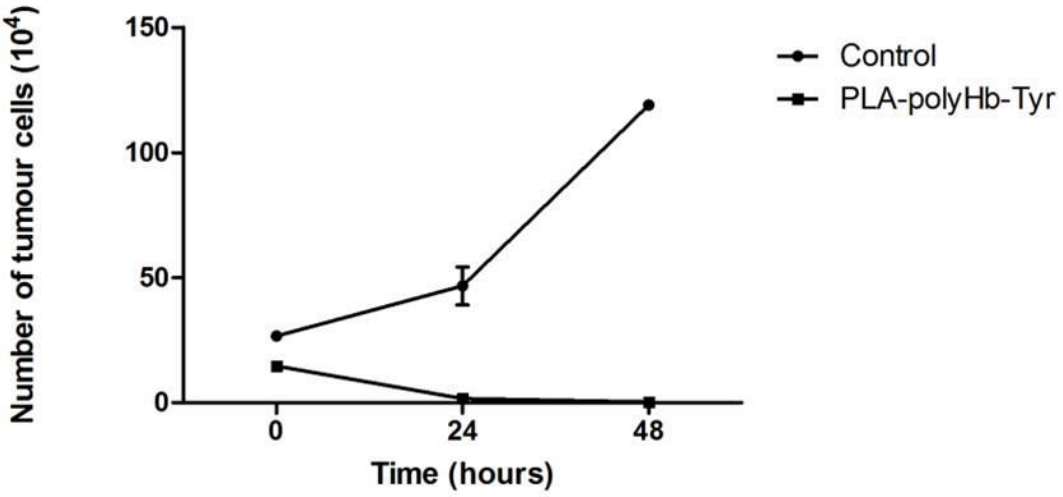

FIGURE 8: The effects of PLA nanocapsule [polyHb-Tyr] on the attachment ability of B16F10 melanoma cells.

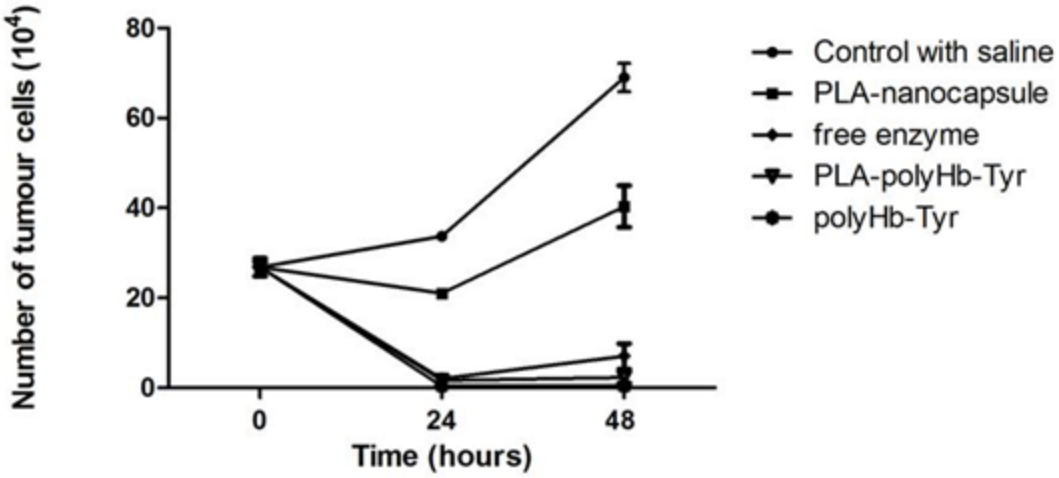

FIGURE 9: The effects of PLA nanocapsule [polyHb-tyr] and the functions of its components on the attachment of B16F10 melanoma cells.

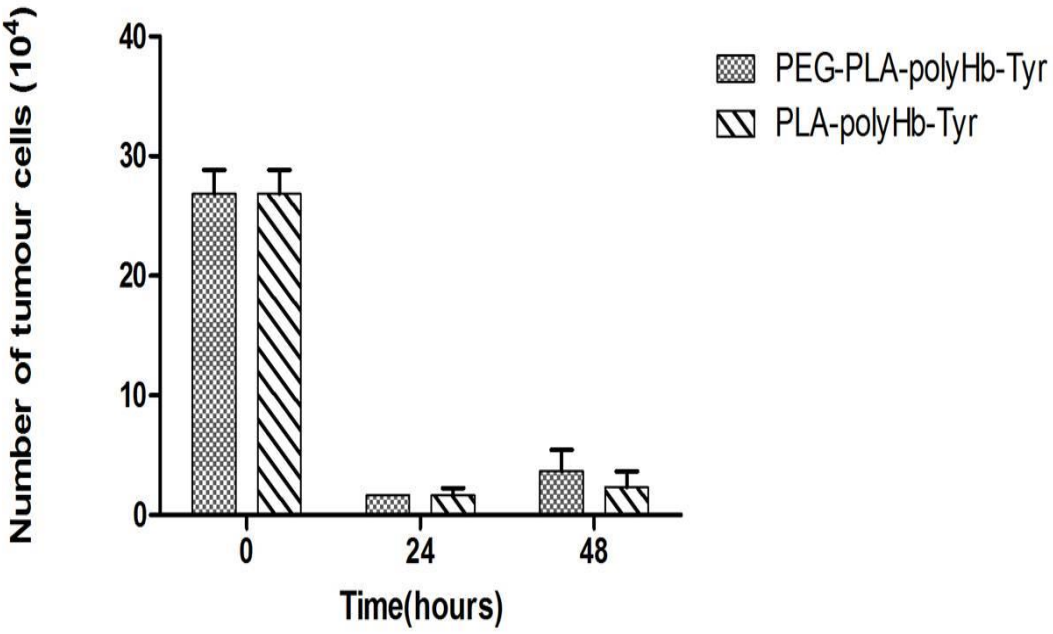

FIGURE 10: The functions of PEG-PLA and PLA nanocapsules on the attachment of B16F10 melanoma cells.

4. Discussion

Oxygen carriers increase tissue oxygenation in poorly perfused solid tumours and increase the sensitivity of the tumours to radiation and chemotherapy [42,43]. By crosslinking tyrosinase to hemoglobin to form polyhemoglobin-tyrosinase we can simultaneously act on tyrosine and increase oxygenation for melanoma cells to be more sensitive to the lowered tyrosine level or the toxic effects of tyrosine metabolites, resulting in the inhibition of murine B16F10 melanoma growth in mice [44,45]. To avoid the need for daily intravenous infusions we have prepared PEG-PLA nanocapsules containing the nanobiotechnological complex of polyhemoglobin-tyrosinase [54]. This has a much longer circulation time than the polyhemoglobin complex [47]. By using PLA instead of PEG-PLA we can prepare PLA nanocapsule [polyHb-Tyr] for intratumoral or local injection around the tumour sites or for locating at the lymph nodes that drain the area. The smaller nanocapsules can enter the melanoma cells to inhibit cell growth and attachment especially if the cells metastasize. The larger ones can act on tyrosine in the extracellular environment of the melanoma cells to convert extracellular tyrosine. They can also follow the same path as the metastasis of melanoma cells to the lymph nodes or organs to continue to act on tyrosine in the region. The diameters are in the range of 88 to 267 nm (187 ± 35 nm). This would have nanocapsules for both intracellular and extracellular functions. A range of 33 to 300 nm in diameter is also possible.

At present, two types of experiments on murine B16F10 melanoma cells were carried out. (1) Proliferation model: attached B16F10 melanoma cells were treated with PLA nanocapsules containing polyHb-Tyr or the different components. The effects on cell viability were followed. (2) Attachment model: melanoma cells were co-cultured with PLA nanocapsules containing polyHb-Tyr or the different components. The effects on cell attachment were followed. The data showed that PLA nanocapsule [polyHb-Tyr] could inhibit both the proliferation and attachment of melanoma cells. This is related to the action enzymatic action of tyrosinase on tyrosine. This could be due to the lowered tyrosine level [17-20] or the toxic effect of products tyrosine metabolism [24,25]. The results also showed that polyhemoglobin, one of the components of PLA nanocapsule [polyHb-Tyr], had by itself some ability to inhibit melanoma cells growth. Hemoglobin is a known nitric oxide scavenger [58] and the depletion of endogenously produced nitric oxide can inhibit melanoma proliferation and promote apoptosis [59]. As a result,

polyhemoglobin in the PLA nanocapsule [polyHb-Tyr] could have an additional effect on limiting the growth of melanoma cells. For intravenous use, PEG is added to PLA to form PEG-PLA nanocapsule [polyHb-Tyr] resulting in much longer circulation time. The present study showed that it was also as effective as PLA nanocapsule [polyHb-Tyr] in inhibiting melanoma cells attachment.

5. Conclusion

Previously we have reported that daily intravenous infusions of a soluble nanobiotechnological complex, polyhemoglobin-tyrosinase [polyHb-Tyr], can suppress the growth of a murine B16F10 melanoma in a mouse model [44]. In order to avoid the need for daily intravenous injections, we have now prepared two types of biodegradable nanocapsules containing [polyHb-Tyr] with diameters in the range of 88 to 267 nanometers. One type is to increase the circulation time and decrease the frequency of injection and is based on PEG–PLA nanocapsules containing [polyHb-Tyr]. The other type is to allow for intratumoural or local injection and is based on PLA nanocapsules containing [polyHb-Tyr]. The cell culture study shows that it can inhibit the proliferation of murine B16F10 melanoma cells in the "proliferation model". It can also inhibit the attachment of murine B16F10 melanoma cells in the "attachment model". The component analysis shows that the enzymatic action of tyrosinase on tyrosine plays the major role in the nanocapsule-[polyHb-Tyr]. The other component, polyHb, plays a smaller role in the nanocapsules containing [polyHb-Tyr] and this is most likely due to its depletion of nitric oxide needed for melanoma cell growth.

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Chapter 3

Biodegradable nanocapsules containing a nanobiotechnological polyhemoglobin-tyrosinase for the suppression of a skin cancer, melanoma

Foreword

In the last chapter, we have obtained preliminary results on the therapeutic effect of PLA nanocapsule on B16F10 cell culture and showed its feasibility. In chapter 3 we carried out further research on the possible mechanisms involved in the inhibition of tumor growth and metastasis in cell culture. This was followed by actual study in a melanoma mice model to analyze the feasibility of suppressing the growth of melanoma. The results showed that the inhibition of metastasis are associated with, but not limited to, the steps of proliferation, migration and colonization. The possible mechanisms are the influences on cell cycle, cell apoptosis and ROS generation.

Biodegradable nanocapsules containing a nanobiotechnological polyhemoglobin-tyrosinase for the suppression of a skin cancer, melanoma

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ABSTRACT

At present, there is no effective therapy for melanoma, a fatal skin cancer, once it has spread. Unlike normal cells, melanoma depends on a high supply of tyrosine for growth. Unfortunately, dietary restriction of tyrosine is not tolerated by the patients. In this paper, we report a nanobiotechnological approach for the specific depletion of tyrosine inside the melanoma cells and in the microenvironment. We combine two nanobiotechnological technologies into a single therapeutic agent. A soluble nanobiotechnological complex is first formed by crosslinking haemoglobin and tyrosinase into a soluble polyhemoglobin-tyrosinase complex. This is then nanoencapsulated into biodegradable polylactide (PLA) nanocapsules to form nanocapsules containing polyhemoglobin-tyrosinase. Our hypothesis is that this prevents the dispersion of the soluble polyhemoglobin-tyrosinase after injection and facilitates the entry of the nanocapsules into the melanoma cells. This would deplete tyrosine inside the melanoma cells and also in the microenvironment. Our results in this paper support this hypothesis. Cell culture showed the entry of the nanocapsules into the melanoma cells. This inhibited tumor growth, migration and colonization in a highly malignant melanoma cell line B16F10. We also analyzed possible mechanisms of action including ROS generation, apoptosis induction and effect on cell cycle. This was then tested in a B16F10 melanoma mice model where a single local injection was found to inhibit the growth of implanted melanoma.

KEYWORDS: Nanocapsules, enzyme therapy, melanoma, nanomedicine, nanobiotechnology, artificial cells

INTRODUCTION

The incidence of melanoma, a fatal skin cancer, has increased rapidly in the last few years. Once metastasized, melanoma is a challenge and only palliative therapies are available. Optional strategies include immunotherapy, photodynamic therapy, chemotherapy and radiation therapies, whereas the side effects must not be overlooked (Martinez and Otley 2001; Lopez, Lange et al. 2004; Bundscherer, Hafner et al. 2008). With increasing interest in the development of nanoscale delivery systems, various strategies have been developed to enhance drug delivery. However, the therapeutic effects for drugs are limited in the case of melanoma.

Meadow's group demonstrated for the first time that melanoma cells depend on tyrosine (Tyr) and phenylalanine (Phe) for growth (Meadows, Pierson et al. 1982; Lawson, Stockton et al. 1985; Norris, Meadows et al. 1990; Elstad, Meadows et al. 1994; Uhlenkott, Huijzer et al. 1996; Fu, Yu et al. 1997). Treatments focused on Tyrosine/ phenylalanine deprivation can remarkably impair tumor cells, but have little impact on the normal cells. This can ensure the safety of the specific amino acid strategy. Possible therapeutic mechanisms involve G0/G1 cell cycle arrest and cell death owing to apoptosis (Fu, Yu et al. 1997; Slominski, Tobin et al. 2004; Slominski, Zmijewski et al. 2012).

Based on the specific amino acid dependency of melanoma, the most direct application is to restrict dietary intake of Tyr and Phe (Meadows, Pierson et al. 1982), but this is not well tolerated. Another promising strategy is to use tyrosinase to deprive Tyr and thus inhibit melanoma growth. Unfortunately, problems relative to free enzymes injection appear, including immunology, stability and duration of action. One possible solution is enzyme encapsulation into micro-dimension structures for enzyme therapy (Chang 1964). These include catalase in acatalasemia(Chang 1968); asparaginase in 6C3HED lymphosarcoma(Chang 1971); phenylalanine ammonialyase in phenylketonuria (Bourget L 1986), and xanthine oxidase in Lesch-Nyhan Disease (Palmour RM1989). Recent interest in nanotechnology has resulted in the extension of this approach to prepare even more effective nano-dimension structures like nanocapsules and nanoparticles (Chang 2007, 2013). Another extension is the

nanobiotechnological approach of crosslinking hemoglobin or hemoglobin and enzymes to form soluble nano-dimension complexes (Chang 2007, 2013). For example, polyhemoglobin-tyrosinase (PH-TYR) can be constituted by the crosslinking of hemoglobin (Hb) and tyrosinase into a soluble nanobiotechnological complex for testing in melanoma mice (Yu and Chang 2004; Yu and Chang 2004). However, in the case of melanoma despite daily intravenous injections, it can only delay but not suppress the growth of melanoma in mice model (Yu and Chang 2004; Yu and Chang 2004).

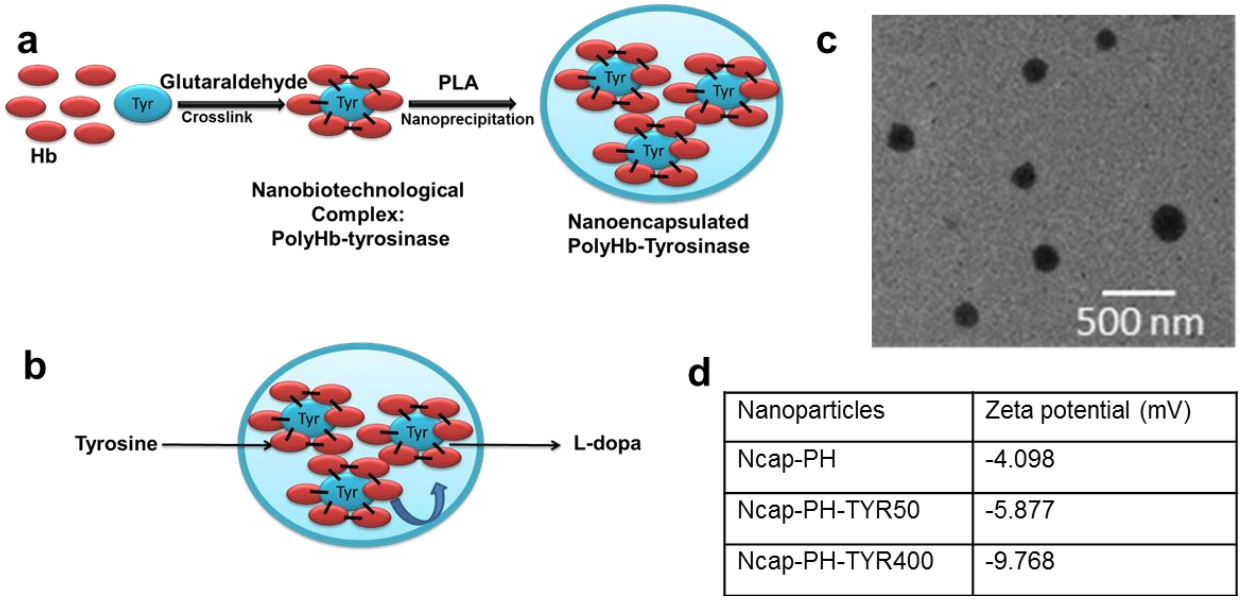
In this paper, we combine two nanobiotechnological technologies into a single therapeutic agent. A soluble nanobiotechnological complex is first formed by crosslinking haemoglobin and tyrosinase into a soluble polyhemoglobin-tyrosinase complex. This is then nanoencapsulated into biodegradable polylactide (PLA) nanocapsules to form nanocapsules containing polyhemoglobin-tyrosinase. We tested this in a highly malignant melanoma cell line B16F10 on inhibition of tumor growth, migration and colonization. We also analyzed possible mechanisms of action like ROS generation, apoptosis induction and effect on cell cycle. This was followed by studying its effect in a B16F10 mice model.

RESULTS AND DISCUSSION

Preparation and characterisation. The principle of the combination of these two nanotechnological methods into a single therapeutic agent is briefly summarized here (Fig. 1a). Details of the method can be found in the method section. The preparation involved two steps: (1) The use of gluataraldehye to covalently crosslink hemoglobin and tyrosinase into a soluble nanobiotechnological complex. This improves the stability of the enzyme tyrosinase. However, being a soluble complex it cannot accumulate at the site of injection nor enter the melanoma cells. (2) Thus the next step is to nanoencapsulate this soluble complex into PLA nanocapsules. In order to facilitate their entry into melanoma cells, we nanoencapsulated this inside PLA nanocapsules of 100 to 300 nm in diameter (Fig.1a, b, c).

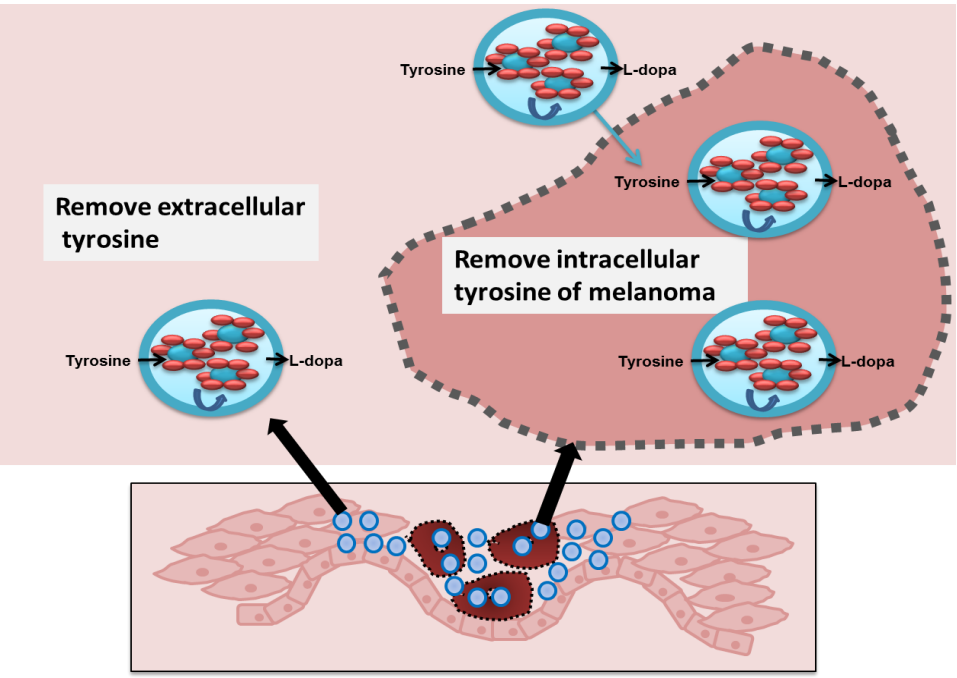
Transmission electron microscopy (TEM) images demonstrated the uniform spherical formation and the particle diameter range at 100 to 300 nm (Fig 1c). Zeta values were -4 to -10 (mV) (Fig.1d). The decrease of the zeta potential with the enzyme loading is because that the increased loading enzymes will increase the disctribution on the membrane, enhancing the negative charge.

The smaller diameter nanocapsules can enter and act inside the melanoma cells, while the larger ones can accumulate outside the melanoma cells. This can decrease the tyrosine level both inside the melanoma cells and also in the tumor microenvironment (Scheme 1).



Nanoparticles	Zeta potential (mV)
Ncap-PH	-4.098
Ncap-PH-TYR50	-5.877
Ncap-PH-TYR400	-9.768

Figure 1. PLA nanocapsules containing a nanobiotechnological complex of polyhemoglobin-tyrosinase: preparative procedure and physical characteristics. (a) Nanocapsules were formed by a functional nanocapsule core (polyhemoglobin-tyrosinase) and exterior PLA shell via a novel combination of two nanotechnological methods: (1) glutaraldehyde crosslinks hemoglobin and tyrosinase into a soluble nanobiotechnological complex of polyhemoglobin-tyrosinase. (2) The soluble polyhemoglobin-tyrosinase complex is nanoencapsulated into biodegradable polylactide (PLA) nanocapsules. (b) The PLA nanocapsules can modify tumor microenvironment by converting tyrosine into L-dopa and lowering tyrosine level locally. (c) TEM image of the PLA nanocapsules. (d) Zeta value of PLA nanocapsules containing different doses of tyrosinase. Ncap-PH : PLA nanocapsules containing polyHb without tyrosinase ; Ncap-PH-TYR#: PLA nanocapsules containing polyHb and # units of tyrosinase.



Scheme 1. PLA nanocapsules containing a nanobiotechnological complex of polyhemoglobin-tyrosinase to remove tyrosine needed for melanoma cell growth. After injection the PLA nanocapsules accumulate in the tumor microenvironment via the enhanced permeability and retention (EPR) effect. They can also enter the tumor cells. This results in the specific depletion of only tyrosine both inside the melanoma cell and also in the microenvironment. Depletion of tyrosine leads to the induction of apoptosis and suppression of growth and metastasis of melanoma cells that have a higher requirement for tyrosine. Normal cells are not affected because they can be maintained at a lower tyrosine concentration.

Penetration of nanocapsules into the melanoma cells and effect on tyrosine level. Thus, we further evaluated the detail penetration and distribution of PLA nanocapsules. To facilitate tracking the intracellular distribution, we engineered fluorescence labelled nanocapsules with coumarin-6 and studied by confocal florescence imaging. The results indicated PLA nanocapsules were able to enter the cells within 1 hour after co-culturing with melanoma cells and remained inside the cytoplasm for the whole 72h observation (Fig. 2a). This intracellular distribution is also consistent with the previous publish intracellular distribution of PLA-TPGS nanoparticles (Zhang, Lee et al. 2008).

The next question is to determine whether the PLA nanocapsules containing polyHb-tyrosinase can remove and lower tyrosine level. We tested the tyrosine changes before and after co-culturing nanocapsules and tumor cells. After treating melanoma cells for 48h, the tyrosine level significantly decreased to 1% compared to that in the control (Fig. 2b). These results implied that nanoencapsulated polyHb-tyrosinase can dramatically act to remove tyrosine.

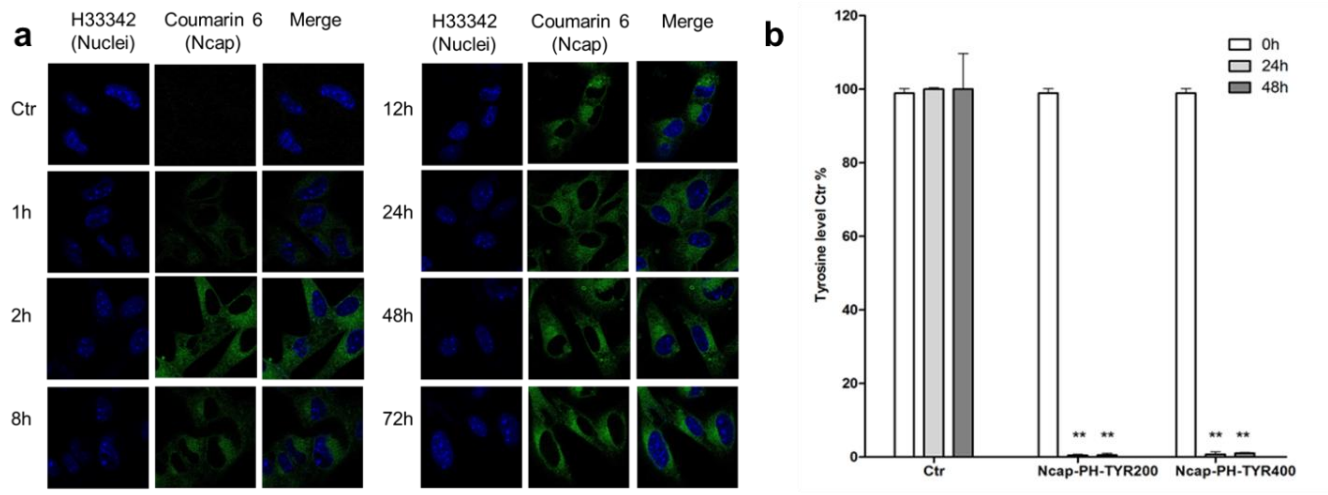


Figure 2. Penetration of PLA nanocapsules into to the cell cytoplasm and the intracellular removal of tyrosine. (a) Intracellular location and distribution of PLA nanocapsules observed for 72h. PLA nanocapsules (green) were labelled with coumarin-6. Nuclei (blue) were stained with H33342. The PLA nanocapsules mainly distribute in the cytoplasm of B16F10 murine melanoma cells. (b) Tyrosine levels at different time points. The tyrosine levels in the 0 time point were set as 100%. Ctr: control with saline; Ncap-PH-TYR200 (PLA nanocapsules containing polyHb and 200 units of tyrosinase); Ncap-PH-TYR400 (PLA nanocapsules containing polyHb and 400 units of tyrosinase). Results are mean+s.e.m., n= 3. Double asterisk indicates P <0.005 compared to control (saline).

***In vitro* effect on the proliferation, migration and colonization of B16F10 melanoma cells**. For the solid cancer melanoma, metastatic dissemination from a primary lesion to a secondary site is believed to be the major lethal reason (Gupta and Massague 2006). This process involves the tumor cells proliferating at the primary sites, migrate/invade the surround tissue then into blood vessels, circulate in the bloodstream, adhere to vascular endothelial cells, and exhibit

invasion, proliferation, angiogenesis and metastatic colonization at the secondary sites. Previous studies proved the deprivation of Tyr/Phe can significantly decrease the metastatic phenotype of B16BL6 melanoma cells *in vivo* and reduce the *in vitro* invasion (Fu, Yu et al. 1999). We therefore further assessed the impact of Ncap-PH-TYR on the proliferation, migration and colonization of B16F10 melanoma cells.

Firstly, MTT test was carried out to study tumor growth. The results exhibited that Ncap-PH-TYR significantly decreased the viability of melanoma cells in their proliferation phase when followed for 48 hours (Fig.3a). This was time and dose dependent.

Scratch test is commonly performed to study the invasive migration of tumour cells(Shin, Kim et al. 2008). We therefore used this to study the effect of PLA nanocapsules containing polyHb-tyrosinase (Ncap-PH-TYR). In the control group, B16F10 tumor cells migrated into the scratch area and after 48 h the scratch area is decreased to 1/3 of that at 0h time. Treatment with Ncap-PH-TYR impaired the migration ability during the 48h observation as shown by the lack of decrease in the scratched area (Fig 4b). It was enzyme activity dependent and the higher enzymatic dose (Ncap-PH-TYR100) the more effective in the prevention of migration of the tumour cells.

Colonization test is designed to assess the capability of tumour growth after it has metastasized. We treated tumor cells with different PLA nanocapsules and detected the colonization ability in soft agar assay. Colony counts were significantly decreased with increased tyrosinase enzyme activity (Fig.4cd). In the test group (Ncap-PH-TYR400), few visible colonies were formed.

The above results suggested implied PLA nanocapsules containing polyHb-tyrosinase have the potential to inhibit melanoma metastasis in proliferation, migration and colonization

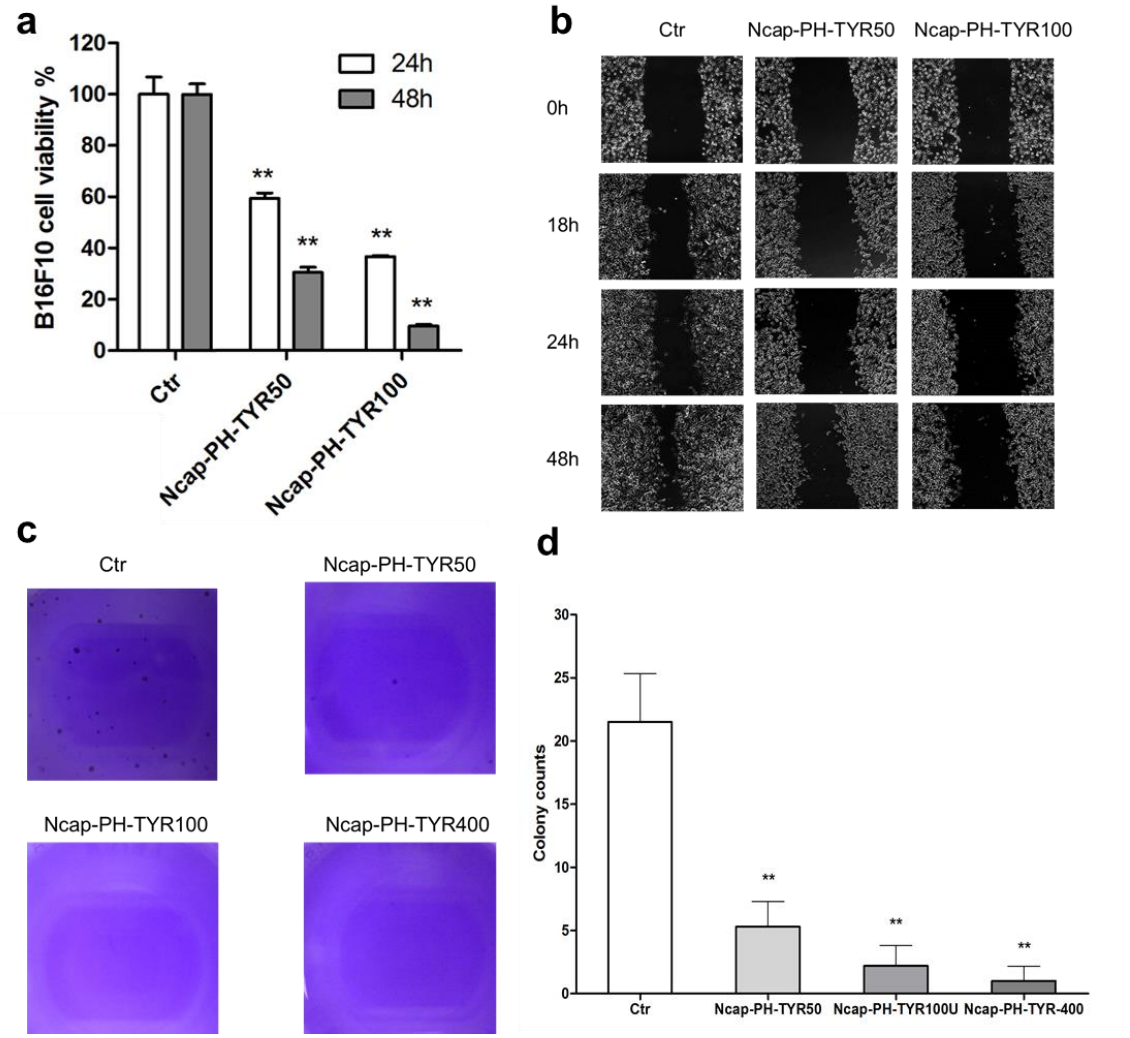


Figure 3. ***In vitro*** **effects on the growth and migriation (metastasis) of melanoma cells. (a) Cell viability was detected by MTT at the time points of 24h and 48h. Ncap-PH-TYR lowered the viability of melanoma cells in their proliferation phase when followed for 48h. (b) Scratch assay to test the ability of tumour to migrate (metastasize) into the cell free scratched area. A cell free zone was made by "scratching" with a sterile tip. Following this the migration of cells into the cell free scratch zone was followed over 48 hours. Unlike the control, Ncap-PH-TYR treated cells had decreased ability to migrate into the cell free zone. (c) Colonization test using soft agar assay. The soft agar was prepared with 1% base agar and 0.7% top agar containing tumor cells. Crystal violet staining was used for calculating visualized colonies. (d) Quantitative analysis of colonization test. Results are mean+s.e.m., n= 3. Double asterisk indicates P <0.005 compared to control.**

Possible Mechanism. We firstly performed the apoptosis analysis by flow cytometry. After incubating B16F10 tumor cells with PLA nanocapsules for 12h, they were stained with annexin V-FITC and PI. The result showed apoptosis of the Ncap-PH-TYR treated melanoma cells (Fig.4a). Another possible mechanism for the decreased cell viability is the tumor cells' arrest in G0/G1 stage owing to Tyr/Phe depletion(Fu, Yu et al. 1997). In our experiment, this mechanism was also confirmed and Ncap-PH-TYR resulted in 25% of G0/G1 arresting in melanoma cells (Fig.4b). It was dose and time dependent. Tyr/Phe limited culture(Fu, Yu et al. 1997) is less effective than PLA nanocapsules containing PolyHb-tyrosinase.

Oxidative stress is another possible cause of cell impairment. In the melanoma cells, oxidative stress stimulates cell apoptosis by activating DNA damage-repair pathway and also opening mitochondrial pore(Fruehauf and Trapp 2008). To analyze this mechanism for the reactive oxygen species (ROS) level was followed. CellROX reagent was applied to track the ROS generation on a short term (1h, 2h) and a long term (24h, 48h). ROS level was raised in 1h and reached its peak at 48 hours (Fig.4cd). In addition, the quantitative estimation of intracellular ROS generation was also further calculated. In the presence of ROS, fluorescent intensity of cells stained with dyes will increase, leading to a right shift of the emission maximum. The results approved that ROS generation increased after nanocapsules treatment for 24h and 48h when compared with the untreated control (Fig.4e). When tyrosinase is used to convert the tyrosine in the tumor microenvironment, the generated products of tyrosine metabolism, such as dopa, 5,6–dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) and others are also toxic to melanoma cells(Urabe, Aroca et al. 1994). Oxidation of these tyrosine metabolites can produce ROS (Urabe, Aroca et al. 1994).

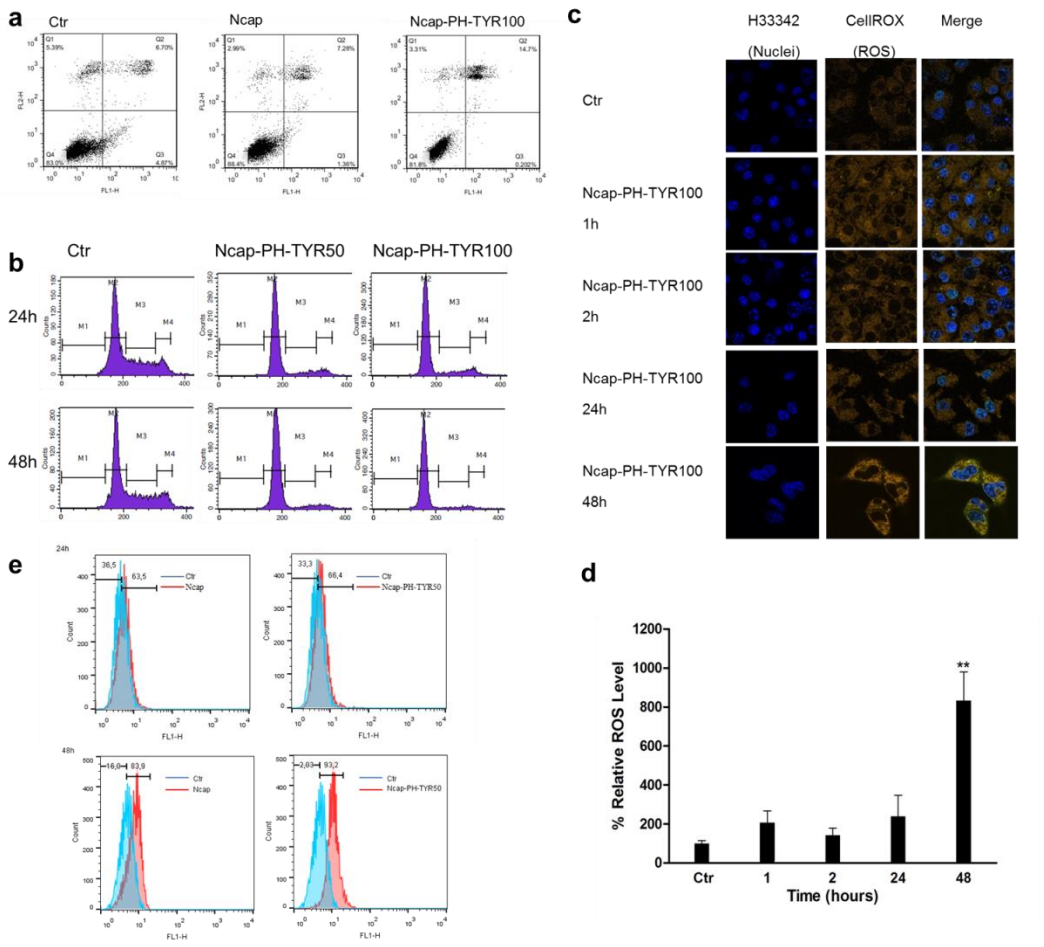


Figure 4. Effects on tumor apoptosis induction, growth inhibition and ROS generation. (a) Flow-cytometric analysis of PLA nanocapsules (Ncap-PH-TYR100) mediated apoptosis in B16F10 cells. Annexin V-FITC and PI staining showed apoptosis in the PLA nanocapsules (Ncap-PH-TYR100) treated group. (b) Cell cycle detection on melanoma cells treated with PLA-nanocapsules (Ncap-PH-TYR100). PI staining was applied for the cell cycle detection. The results showed G0/G1 arresting on the nanocapsules (Ncap-PH-TYR50 and Ncap-PH-TYR100) treated groups at M2: G0/G1 phase; M3: S phase; M4: G2/M phase. (c) Confocal fluorescence images of tumor cells incubated with Ncap-PH-TYR100. Nuclei (blue) were stained with H33342 and the generated ROS (orange) were stained by CellROX Orange. (d) Quantitative analysis of the ROS generation. Significant at 48 hours at $p<0.005$. (e) Flow-cytometric analysis showing a gradual increase in ROS generation when compared with control cells. Blue peaks are the control groups and the red peaks are the treatment groups (Ncap or Ncap-PH-TYR50) at 24h and 48h.

To further confirm the action of PLA nanocapsules on melanoma cells, we performed western blot to study the expression changes on relative molecular proteins (Fig.5). This includes the integrin receptors that are required for cell adhesion and attachment to the extracellular matrix. This attachment is needed for the survival, growth, and inhibition of cell apoptosis. FAK (focal adhesion kinase) is a major signaling mediator that influences both integrin attachment and cell spreading. The integrins are cell surface receptors for the components of extracellular matrix. They will be activated during cell adhesion and spreading, and initiate signaling pathways that control growth and invasion of tumor cells. In our study, the expressions of FAK and α4 integrin were inhibited in the B16F10 melanoma cells after treatment with PLA nanocapsule (Fig.5a). The present study indicated that Ncap-PH-TYR can inhibit B16F10 melanoma's attachment and spreading via inhibition of specific integrin expression and FAK expression. About the cell cycle of melanoma cells, the cyclin D and their attendant CDKs are key regulators of G1 progression, which is an important checkpoint of cell cycle. Our results showed that the expression of cyclin D1, cyclin D3and CDK2 are also decreased (Fig.5b).

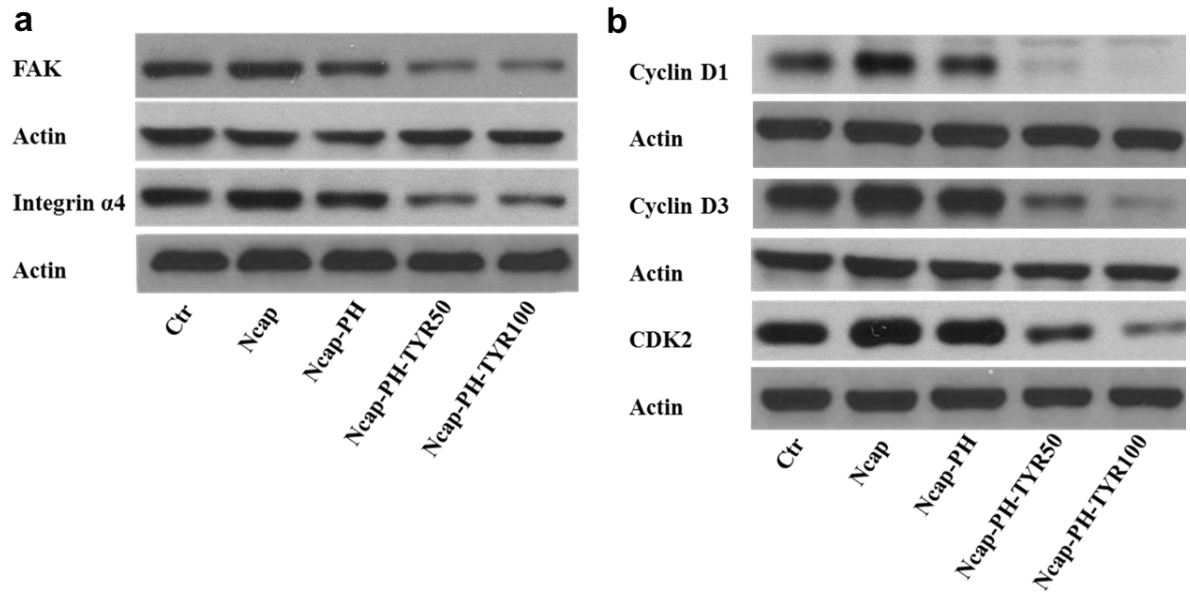
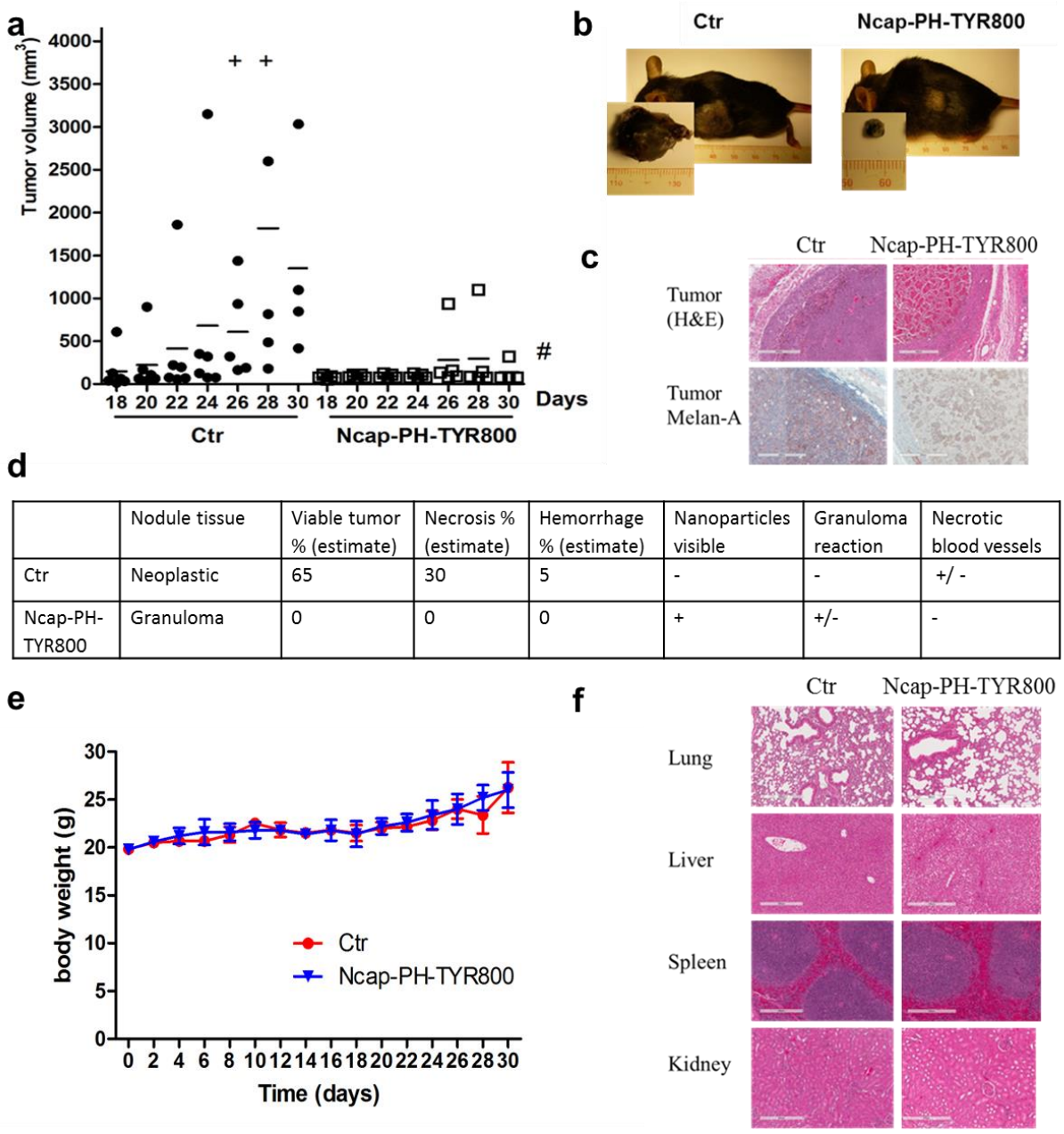


Figure 5. Western blot analysis of the expression of relative molecular proteins. (a) Western blot analysis of the expression of FAK and integrin-α4 that are involved in cell adhesion. The declined expression indicated impaired cell adhesion ability. (b) Western blot analysis of the expression of Cyclin D1, Cyclin D3 and CDK2, the cell cycle relative molecules. Declined expressions are consistent with the G0/G1 arresting.

***In vivo* effects.** Lastly, we performed animal experiment to determine PLA nanocapsule' suppressive effect on the growth of B16F10 melanoma cell. The control group of C57BL/6 mice received a subcutaneous injection of B16F10 melanoma cells. The test group received subcutaneous injection of Ncap-PH-TYR800 and the same amount of B16F10 melanoma cells. Tumor volumes were measured every 2 days after the tumor cells injection. Ncap-PH-TYR800 was effective in inhibiting tumor growth during the 30 days (Fig.6a,b). What's more, HE staining showed that unlike the control group, there was no observable neoplastic cell in the Ncap-PH-TYR800 group (Fig.6c,d). Melan-A staining also verified the inhibition effect of the Ncap-PH-TYR800 group (Fig.6c). Thus, one injection of this novel double nanotechnological preparation can inhibit the growth of the implanted melanoma. This novel double nanotechnological preparation is important since with free soluble polyHb-Tyrosinase daily injections for 30 days could only slow the growth of the melanoma (Yu and Swi Chang 2004). Injection of the Ncap-PH-TYR800 did not result in body weight changes when compared to the control (Fig.6e). In addition, there were no observable histological changes in spleen, kidney, lung and liver (Fig.6f). Theses verified the safety of the application of PLA nanocapsules. In addition, in term of the oxygen condition, 5% CO_2 was applied *in vitro* for general cell culture, and the tumor cells grew well. The oxygen tension will decrease in the tumor tissue, which should be different from that of the *in vivo* condition. However, both the *in vitro* and *in vivo* results confirmed the therapeutic effects.



	Nodule tissue	Viable tumor % (estimate)	Necrosis % (estimate)	Hemorrhage % (estimate)	Nanoparticles visible	Granuloma reaction	Necrotic blood vessels
Ctr	Neoplastic	65	30	5	-	-	+/ -
Ncap-PH-TYR800	Granuloma	0	0	0	+	+/-	-

Figure 6. The application of PLA nanocapsules containing polyhemoglobin-tyrosinase in B16F10 melanoma-established mice model.(a) $0.5x10^5$ B16F10 melanoma tumor cells and 0.075 ml PLA nanocapsuels (same volume of saline in the control group) were premixed and injected into the lateral flank of C57BL/6 mice subcutaneously. Tumor volumes were followed every 2 days. Ctr: control with saline; Ncap-PH-TYR 800: PLA nanocapsules containing polyhemoglobin crosslinked with tyrosinase 800 units. +means the tumor

volume has reached the ceiling which was set as 4000mm3, and the mice were euthanasia. #means the mouse was under bad condition and was stopped from experiment. (b) At the end of the 30 days, the tissues were separated. (c) Histological staining of melanoma tumor tissues (HE staining and Melan-A). (d) Histological analysis of the tumor tissues with H&E staining. (e) Body weights were measured every 2 days and no significant difference between groups. (f) Histological staining of other organs, lung, liver, spleen and kidney.

CONCLUSION

There is much potential for enzyme therapy. This includes the clinical use of galactosidase A in Fabry disease (Lidove, West et al. 2010). There is also ongoing development in other areas especially for cancer treatment (Xu and McLeod 2001). This includes p450 reductase for gliosarcoma (Chen, Yu et al. 1997) and nitroreductase for ovarian tumor (Weedon, Green et al. 2000). However, most enzyme therapies rely on electroporation and viral vectors of therapeutic enzyme genes into tumor cells. Safety concern of gene therapy includes potential toxicity or unwanted side effects. The delivery of active enzymes rather than genes into tumors may overcome some of the problems related to gene therapy. The principle of bioencapsulation of enzymes into micro-dimension microcapsules has been verified for experimental enzyme therapy (Bourget and Chang 1986; Palmour, Goodyer et al. 1989; Yu and Chang 2004; Chang 2013). In this paper, we applied a novel combination of two nanotechnological methods based on PLA nanocapsules containing a nanobiotechnological complex of polyhemoglobin-tyrosinase. The present study shows that it is effective for the suppression of a skin cancer, murine B16F10 melanoma *in-vitro* and *in vivo*.Further research may pave the way for another potential new approach for the application of enzyme therapy.

MATERIALS AND METHODS

Nanocapsules Preparation and Characterization. The first step was to prepare nanobiotechnological complexes of polyHb and polyHb-Tyrosinase. The stroma-free Hb was diluted to concentration of 7g/dL with or without tyrosinase in PBS (pH7.4). Lysine was added at a molar ratio of 7:1 lysine/Hb, followed by the glutaraldehyde, a crosslinker, at a molar ratio

of 16:1 glutaraldehyde/Hb. After 24hours, lysine was added again to stop the reaction at a molar ratio of 200:1 lysine/Hb. Dialysis membrane and sterile syringe filters were applied to purify the polyHb and polyHb-tyrosinase samples. To prevent the methemoglobin and to protect the enzymes activity, the operations were performed at 4℃ and under nitrogen filled.

Then, the next step was the PLA nanocapsulated polyHb-tyrosinase preparation by nanoprecipitation and solvent evaporation methods. PLA and hydrogenated soybean phosphatidylcholine dissolved in acetone and ethanol formed the organic phase. The organic phase was dropwise added into the prepared polyHb or polyHb-tyrosinase solution through a 26G needle at a rate of 3mL/min under moderate magnetic stirring with a speed set at 6 at 4℃. The organic solvent was removed by following evaporation and PLA nanoparticles were formulated in the form of colloidal dispersion in aqueous phase. Tween 20 was used to stabilize the nano formulation. To prepare fluorescence labeled PLA nanocapsules, additional coumarin-6 was added into the organic phase, and following the same procedure as above.

The size and morphological of PLA nanocapsules were studied by Transmission electron microscopy (TEM). Typically, 10uL samples were dropped on carbon-coated copper grids and observed and analysed by a JEOL JEM-2000FX microscope (Jeol Led., Tokyo, Japan) and images were taken by a Gatan Wide Angle Multiscan CCD camera. Zeta potential of the nanocapsules were analysed by dynamic light scattering (DLS) using a Malvern Zetasizer Nano series 3000 (Malvern Instruments, Worcestershire, UK)

Tyrosinase Activity Assay. Tyrosinase activity was analyzed by monitoring the production rate of the enzymatic product L-dopaquinone at 300nm, as described previously (Wang and Chang 2012).

Tyrosine detection. Yeast enzyme phenylalanine ammonia-lyase (PAL) can converse Tyr to trans-coumarate detectable at 315 nm (Wibrand 2004). [Tyr]= ΔA315/α315 (ΔA was determined as final absorbance minus initial one after subtraction of blank values; α315 is the slopes of the standard curves).

Tumour Cells and Culture Conditions. B16F10 murine melanoma cells (American Type Culture Collection, ATCC®, #CRL-6475) were cultured in standard Dulbecco's modified

Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37 °C and 5% CO_2, humidified atmosphere.

MTT assay. B16F10 melanoma cell proliferation was tested by MTT assay. 200uL of 5mg/mL MTT solution (prepared in DMEM medium without serum) was added to the PLA nanocapsules treated-cells and incubated at 37℃ in a CO_2 incubator for 4h. Dimethyl sulfoxide was then added to dissolve the formazan crystals, after which the absorbance was measured at 570nm using an ELISA plate reader.

Scratch assay for migration. $5x10^5$cells/well were seeded overnight. Wound culture was made by scraping cells with a sterile tip and maintained in 0.5% FBS medium with nanocapsule treatment. Scratch areas were calculated (Shin, Kim et al. 2008).

Colony assays in soft agar. The methods were carried out as previously described with some modification (Bisht, Feldmann et al. 2007). Briefly, 5 $x10^5$ cells were treated with PLA nanocapsules containing different concentrations of polyhemoglobin-tyrosinase for 24 hours and were collected. The base agar was prepared by 1% agar and top agar by 0.7% agar containing the treated tumor cells. The dishes were kept in incubator allow colonies growth for 2-3 weeks. The colonies were visualized by Crystal Violet staining.

Apoptosis detection by Annexin V-FITC and PI staining. In order to evaluate apoptosis and necrosis, Annexin V-FITC/PI (propidium iodide) dual staining was used. Cells were analyzed using a FACSCalibur flow cytometer.

Cell cycle analysis using flow cytometry. Cell cycle analysis was carried out by staining the DNA with PI following flow cytometric measurement on a FACSCalibur and CellQuest software (BD Biosciences).

ROS generation detection by confocal microscope and quantification by flow cytometry. The generation of ROS was tracked with fluorogenic probe, CellROX oxidative stress reagent. This cell-permeable reagent can exhibit strong fluoregonic signal upon oxidation that can be measured by fluorescence microscope. For the ROS quantification, cells treated with PLA nanocapsules were collected and incubated with CellROX reagent at 37℃ in dark. Samples were then analysed on FACSCalibur (BD Bioscience, USA).

Cellular distribution of PLA nanocapsule by confocal microscopy. To test cellular distribution, tumor cells were treated with coumarin-6-labeled nanocapsules for short term and long term and observed on a LSM 710 confocal microscope (Yu, Lu et al. 2010).

Western blot analysis. Antibody for integrin α4 was obtained from Millipore. Antibodies for FAK, Cyclin D1, Cyclin D3, and CDK2 were purchased from Cell Signaling. Antibody for actin was from Santa Cruz Biotechnology.

***In vivo* study.** Mix B16F10 murine melanoma cell suspension with different test solutions as shown below. and inoculate subcutaneously into the lateral flank of C57BL/6 mice. Measure the primary tumor size and body weight every 2 days. The tumor volume was calculated as $V= (A*B^2)/2$. V: volume (mm^3); A: the longest diameter (mm); B: the shortest diameter (mm). At the endpoint, the tumor tissues were collected for histological analysis.

Melanoma specimen analysis. Malignant melanoma specimens were collected. Tissues sections were stained with hematoxylin and eosin and the histological analysis were analyzed with the help of an expert pathologist. The expression profiles of Melan-A were analyzed by immunohistochemistry (IHC) methods.

Data analysis. Statistical analysis was performed using the Student's t-test or one-way ANOVA and for significant at $P < 0.05$ and $P < 0.005$.

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Chapter 4

Analysis of the effects of the different components of Polylactide nanocapsules-polyhemoglobin-tyrosinase on the *in vitro* and *in vivo* suppression of B16F10 murine melanoma

Foreword

We have showed that PLA-nanocapsules containing PolyHb-Tyrosinase could inhibit the growth and migration of melanoma cells in cell culture and in animal study in Chapter 3. In this chapter, we focused on the detailed roles of the different components of the PLA nanocapsules containing a nanobiotechnological polyhemoglobin-tyrosinase. Here we investigated each of these components in both cell culture and also in animal studies. It was shown PLA nanocapsules containing polyhemoglobin-Tyrosinase could effectively remove tyrosine and inhibit the growth and migration of melanoma cells. PLA nanocapsules containing only polyhemoglobin could not lower tyrosine but could inhibit tumor growth and migration but to a much less extent. PLA nanocapsule vehicle has no function. One injection of polyhemoglobin-Tyrosinase could not inhibit melanoma growth.

Analysis of the effects of the different components of Polylactide nanocapsules-polyhemoglobin-tyrosinase on the *in vitro* and *in vivo* suppression of B16F10 murine melanoma

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Abstract

Melanoma is a fatal skin cancer, and up to now, few treatments are effective for the advanced stage of melanoma to inhibit its growth and migration (metastasis). Melanoma requires a higher level of tyrosine for growth, thus one potential strategy is to lower the tyrosine level to inhibit its growth. We showed that PLA-nanocapsules containing PolyHb-Tyrosinase could inhibit the growth and migration of melanoma in cell culture and in animal studies. The present research is to analyze the role of different components of PLA-nanocapsules containing PolyHb-Tyrosinase in melanoma cell culture and also in animal studies: PLA nanocapsules with no contents (Ncap); free Polyhemoglobin-tyrosinase (PH-TYR); PLA-nanocapsules containing only PolyHb (Ncap-PH); PLA-nanocapsules containing PolyHb-Tyrosinase (Ncap-PH-TYR). Animal studies in B16F10 bearing melanoma mice were carried out. PLA nanocapsules containing polyhemoglobin-tyrosinase can effectively inhibit tumor growth. PLA nanocapsules containing only polyhemoglobin also have some inhibitory effects. The other components did not have any effects. *In vitro* studies showed that PLA nanocapsules containing polyhemoglobin-Tyrosinase could effectively remove tyrosine and inhibit tumor growth and migration. PLA-nanocapsules containing only polyhemoglobin could to a less extent inhibit tumour growth and migration.

Keywords:

Nanocapsule, Melanoma, Nanomedicine, enzyme therapy, artificial cells

1. Introduction

Melanoma is a malignant skin cancer due to the high ability of metastasis and resistance to therapy. The incidence has increased rapidly in the recent years. Surgical treatment is effective in the early stage of melanoma. However, nonsurgical strategies, like chemotherapy, immunotherapy and photodynamic treatment are limited in their effectiveness and have problem related to side effects and drug resistance (Martinez and Otley 2001; Lopez, Lange et al. 2004; Bundscherer, Hafner et al. 2008).

Melanoma cell has one metabolic characteristic, that is the specific dependency on a higher concentration of amino acid tyrosine (Tyr) and phenylalanine (Phe) (Lawson, Stockton et al. 1985; Norris, Meadows et al. 1990; Elstad, Meadows et al. 1994; Uhlenkott, Huijzer et al. 1996). Thus, one promising strategy is to remove the tyrosine systematically or locally, so that the growth and metastasis of melanoma can be impaired (Slominski, Tobin et al. 2004; Slominski, Zmijewski et al. 2012). However, dietary means to lower systemic tyrosine level were not clinically practical.

We expanded upon this approach and investigated a new nanobiotechnological approach based on polylactide (PLA) membrane nanoencapsulated polyhemoglobin-tyrosinase Intermolecular polymerization of Hb with tyrosinase can improve the stability of native tyrosinase. Nanoencapsulation would prevent the rapid dispersion of PolyHb-Tyrosinase after subcutaneous injection and allow them to enter the melanoma cells and also to act locally to lower the tyrosine level. PLA has been used for the encapsulation of biologically active materials (Chang 1976). Since then PLA has been extensively investigated as the matrix for nanocapsules as delivery system (Orozco, Kozlovskaya et al. 2010). PLA is a FDA approved polymer (Parveen and Sahoo 2008). It degrades in the body producing its original monomers of lactic acid and glycolic acid, and finally carbon dioxide which are the by-products of various metabolic pathways (Park 1995).

Our recent study showed that PLA-nanocapsules containing PolyHb-Tyrosinase could inhibit the growth and migration of melanoma cells in cell culture and in animal study (Wang and Chang Submitted). The present paper is to analyze the role of the different components of PLA-nanocapsules containing PolyHb-Tyrosinase: PLA nanocapsules with no contents (Ncap); free

Polyhemoglobin-tyrosinase (PH-TYR); PLA-nanocapsules containing only PolyHb (Ncap-PH); PLA-nanocapsules containing PolyHb-Tyrosinase (Ncap-PH-TYR). Here we test each of these components in cell culture and also in animal studies.

2. Methods and Materials

2.1 Preparation of polyHb and polyHb-tyrosinase

Details can be found in the publication (Wang and Chang 2012). For the preparation of polyHb, 7g/dL stroma-free Hb was first diluted in PBS (pH 7.4). Glutaraldehyde and lysine were added to crosslink the Hb. The formed polyHb was purified by dialysis and filters. For the preparation of polyHb-tyrosinase, tyrosinase was added to the stroma-free Hb solution at the very beginning and then followed the same steps.

2.2 Preparation of PLA nanocapsules containing polyHb-tyrosinase

The PLA nanocapsules were synthesis by nanoprecipitation and solvent evaporation. The organic phase consisting of PLA was added dropwise into the polyHb or polyHb-tyrosinase solution under moderate magnetic stirring, and was removed by evaporation. Tween 20 was used to stabilize the nanoformulation. Details are available (Wang and Chang 2012).

2.3 Tumour Cells and Culture Conditions

B16F10 murine melanoma cells (American Type Culture Collection, ATCC®, #CRL-6475) were cultured in standard Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37 °C and 5% CO_2, humidified atmosphere. Cells were washed with 0.25% trypsin/EDTA for about 1 min to be detached from the dish. Then cell suspensions were collected in a sterile 15 mL tube and centrifuged at 1,500 rpm for 5 min. Cells were counted by trypan blue exclusion.

2.4 *In vivo* study

Mix B16F10 murine melanoma cell suspension with different test solutions were used (Ncap: empty PLA nanocapsules without content: Ncap-PH: PLA nanocapsules containing polyhemoglobin without tyrosinase; Ncap-PH-TYR##: PLA nanocapsules containing

polyhemoglobin crosslinked with tyrosinase ## units). Each suspension was inoculated subcutaneously into the lateral flank of C57BL/6 mice. Measure the primary tumor size and body weight every 2 days. The tumor volume was calculated as $V= (A*B^2)/2$. V: volume (mm^3); A: the longest diameter (mm); B: the shortest diameter (mm). At the endpoint, the tumor tissues were collected for histological analysis.

2.5 Melanoma specimen analysis

Malignant melanoma specimens were collected. Tissues sections were stained with hematoxylin and eosin and the histological analysis were analyzed with the help of an expert pathologist. The expression profiles of Melan-A were analyzed by immunohistochemistry (IHC) methods.

2.6 Data analysis

Statistical analysis was performed using the Student's t -test or one-way ANOVA within analysis of variance and was considered to be significant at $P < 0.05$.

3. Results and discussion

Melanoma is a malignant skin cancer, although surgical removing is the priority strategy to remove the visible lesions, the small, invisible and metastasis masses are still a challenge upon presently available treatment. In our research, we have developed the new tyrosine deprivation strategy combining nanotechnology to enhance the therapeutic effect. Here, we focused on studies on the detail mechanisms and *in vivo* research on the PLA nanocapsules containing polyhemoglobin-Tyrosinase. We further analyzed the effect of the different components contained in the PLA nanocapsules containing polyhemoglobin-Tyrosinase, including free polyHb-tyrosinase, PLA nanocapsules containing only polyhemoglobin and the PLA nanocapsules vehicle.

3.1 *In vivo* effect of the different components of PLA nanoencapsulated polyhemoglobin-tyrosinase

Nanoparticles and tumor cells were mixed together and administered to tumor-bearing C57BL/6 mice subcutaneously. The growth of tumor mass following the injection of different components was measured every 2 days and histological analysis was performed at the endpoint.

3.1.1 Effect of PLA nanocapsules containing polyHb-tyrosinase.

We first followed the effect of PLA nanocapsules containing polyHb-tyrosinase on B16F10 melanoma cells bearing mice model. Soluble polyhemoglobin-tyrosinase inside the nanocapsules is prevented from being dispersed quickly after subcutaneous injection thus allowing it to accumulate longer in the injection sites. Furthermore, some nanocapsules can enter the melanoma cells to act inside the cells (Wang and Chang Submitted). This way it can lower the tyrosine level in both the tumor cells and in the tumor microenvironment (Fig 1a).

Unlike the control group, the two treatment groups all exhibited significant tumor inhibition as shown by the tumor volume when followed for 30 days (Fig 1b). Consistent with this, the changes in tumor weight were also confirmed at the endpoint of 30 days and tumor weights were <10% of that of control group (Fig 1c,d). We also carried out H&E staining for histological study by the animal pathologist. The large nodule specimen from the control group showed 75% visible neoplastic cells. There were no neoplastic tissues in small nodules of the two test groups, only foreign body reactions were found (Fig 1e). The body weight didn't display significantly differences between the groups (Fig 1f).

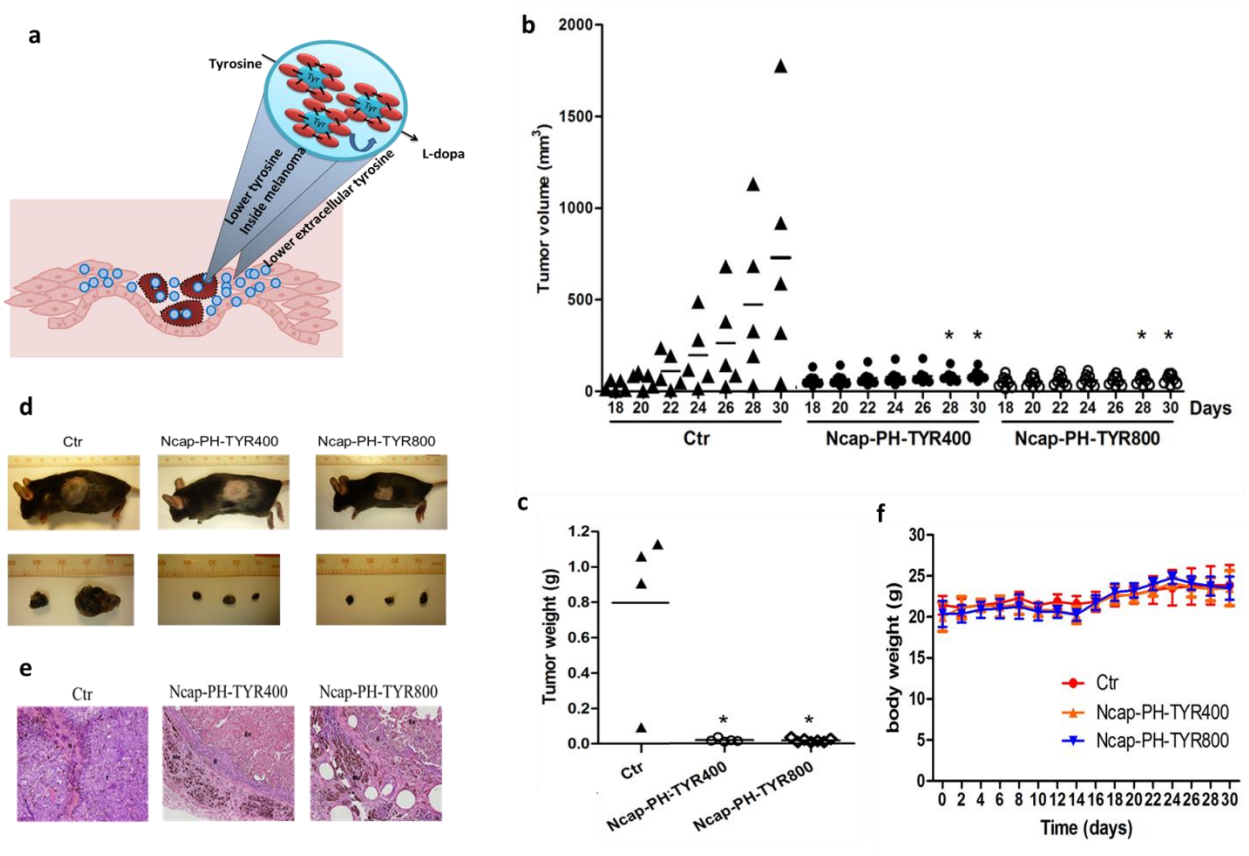


Figure 1. ***in vivo*** **effect of PLA nanoencapsulated polyhemoglobin-tyrosinase**

a. Model with PLA nanocapsules containing polyhemoglobin-tyrosinase.

b. 0.25x10^5 B16F10 melanoma tumor cells and 0.075 ml PLA nanocapsules (same volume of saline in the control group) were premixed and injected into the lateral flank of C57BL/6 mice subcutaneously. Tumor volumes were followed every 2 days.

(Ctr: control with saline; Ncap-PH-TYR400 and Ncap-PH-TYR 800: PLA nanocapsules containing polyhemoglobin crosslinked with tyrosinase 400 and 800 units, separately)

c,d. At the end of the 30 days, tissues were separated and weighted

e. HE staining was performed for the histological analysis.

f. Body weights were measured every 2 days and no significant difference between groups.

Single asterisk indicates P <0.05 compared with control (no treatment).

3.1.2 *In vivo* effect of the component soluble free polyhemoglobin-tyrosinase

To further investigate the effects of the PLA nanocapsules, we carried out detail experiments to test the component polyHb-tyrosinase, without nanoencapsulation with PLA (Figure 2a).

The results showed that compared with the control group, PH-TYR400 could only inhibit tumor up to Day 16. However, it did not have any inhibitory effects after 16 days (Fig. 2b). Possible reasons are: (1) The soluble polyHb-tyrosinase is quickly dispersed from the injection site. That's why, with the same PolyHb-tyrosinase dose, the PLA nanocapsules format can significantly improve the therapeutic effect (Fig 1). No body weight differences were found between groups (Fig. 2c).

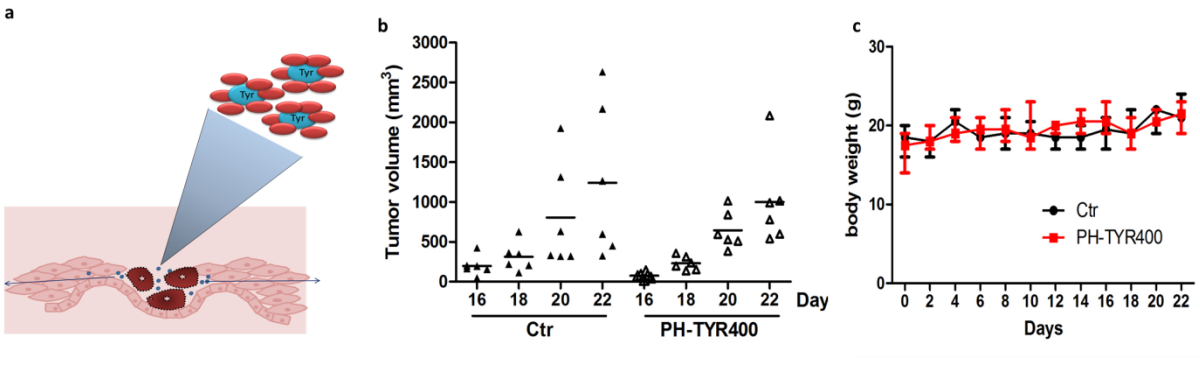


Figure 2. *In vivo* effects of soluble free polyhemoglobin-tyrosinase component

a. Model with polyhemoglobin-tyrosinase.

b.10^5 B16F10 melanoma tumor cells and 0.3ml PH-TYR400 (same volume of saline in the control group) were premixed and injected into the lateral flank of C57BL/6 mice subcutaneously. The tumor volumes were followed every 2 days.

(Ctr: control with saline; PH-TYR400: polyhemoglobin crosslinked with tyrosinase 400 units)

c. Body weights were measured every 2 days and no significant difference between groups.

3.1.3 *In vivo* effects of component PLA nanoencapsulated polyhemoglobin

The results showed that the Ncap-PH can also suppress the tumor growth for 30 days (Fig3abc) without any adverse effect in body weight (Fig 3d). Consistent with this, the HE staining and melanoma cells specific stainings, Melan-A, CD31 and Ki67, all shown significant differences in comparison with the control group (Fig5a). In addition, the histological analysis also verified that few remaining tumor cells were found (Fig5c). Analysis of the other organs of lung, liver, spleen and kidney didn't present side effect upon the administration of PLA vehicle (Fig5b).

3.1.4 *In vivo* effects of PLA nanocapsule vehicle

After confirming the two functional contents tyrosinase and polyHb, the next is the PLA polymer nanocapsules themselves (Fig4a). PLA polymer, as one of the mostly used polymers, has been widely used as the matrix of the nanocapsule for more than 20 years. It is approved by FDA to apply in a host of therapeutic devices owing to their biodegradability and biocompatibility, non-toxic (Parveen and Sahoo 2008). In human body, PLA can degrade in to original monomers of lactic acid and glycolic acid. These are the by-products of various metabolic pathways and the intermediate of carbohydrate metabolism. Compared with the human body's degrade ability, the generated amount is in a low level. So there is very little systemic toxicity (Park 1995).

Our result showed that the PLA nanocapsules vehicle group had no tumour suppressive effect when compared with the control group (Fig 4bc). There was also no body weight differences were found (Fig4d). The HE staining and me lanoma cells specific stainings, Melan-A, CD31 and Ki67, all showed no differences compared with the control group (Fig5a). In addition, the histological analysis showed no improvement on tumor cells killing (Fig5c). PLA did not have any adverse effects on the histology the lung, liver, spleen and kidney. (Fig5b). These evidences indicated that the PLA polymer itself has no impact on the inhibition of tumor growth. However, it plays an important role as a nanocapsule vehicle for the polyhemoglobin-tyrosinase. Without this, the soluble free polyhemoglobin-tyrosinase would not be able to stay at the site of the melanoma cells to carry out its function.

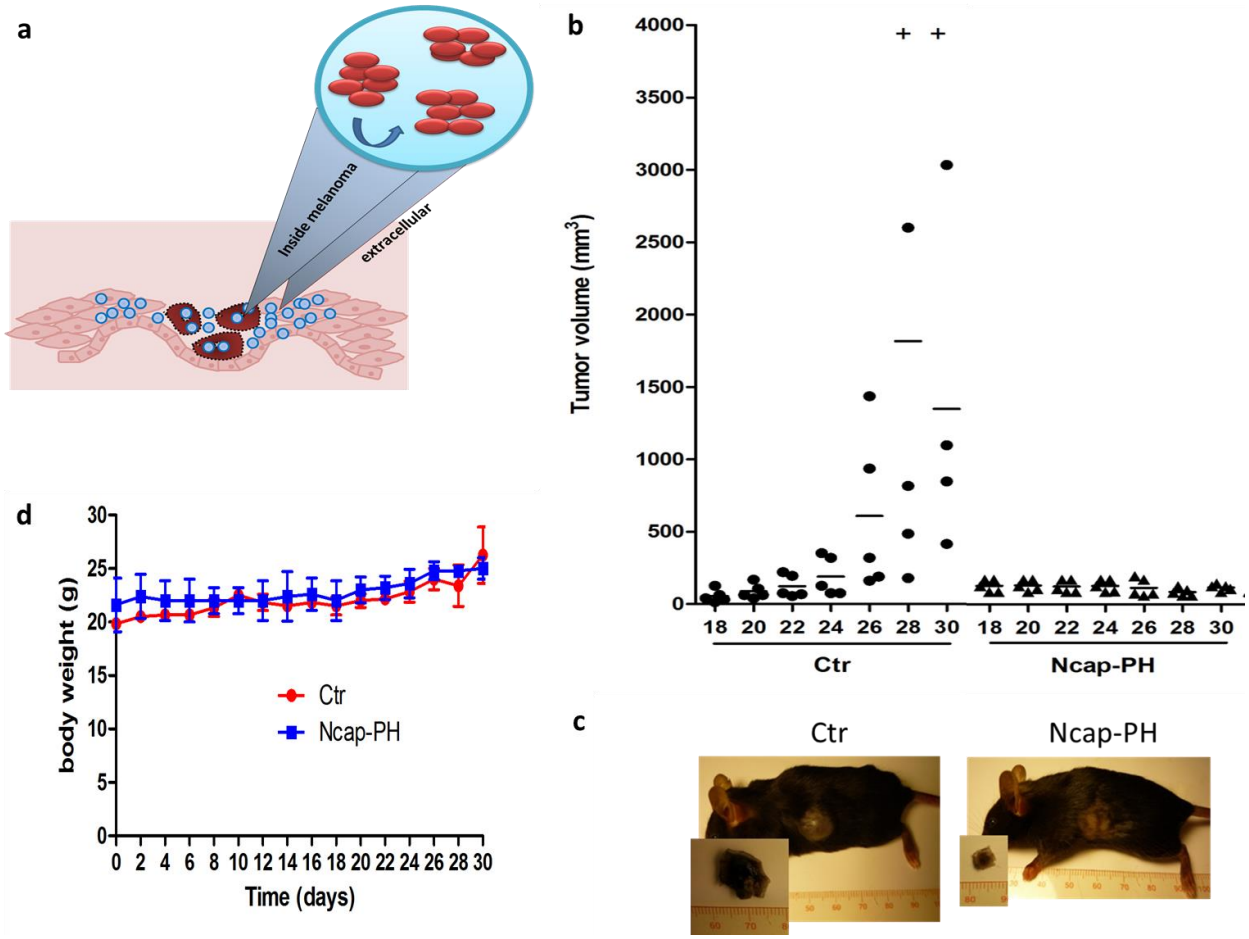


Figure 3. ***In vivo*** **effects of component PLA nanoencapsulated polyhemoglobin**

a. Model with PLA nanocapsules containing polyhemoglobin

b. 0.5x10^5 B16F10 melanoma tumor cells and 0.15ml PLA nanocapsules (same volume of saline in the control group) were premixed and injected into the lateral flank of C57BL/6 mice subcutaneously. Tumor volumes were followed every 2 days.

(Ctr: control with saline; Ncap-PH: PLA nanocapsules containing polyHb)

+means the tumor volume has reached the ceiling which was set as 4000mm^3, and the mice were euthanasia

c. At the end of the 30 days, the tissues were separated.

d. Body weights were measured every 2 days and no significant difference between groups.

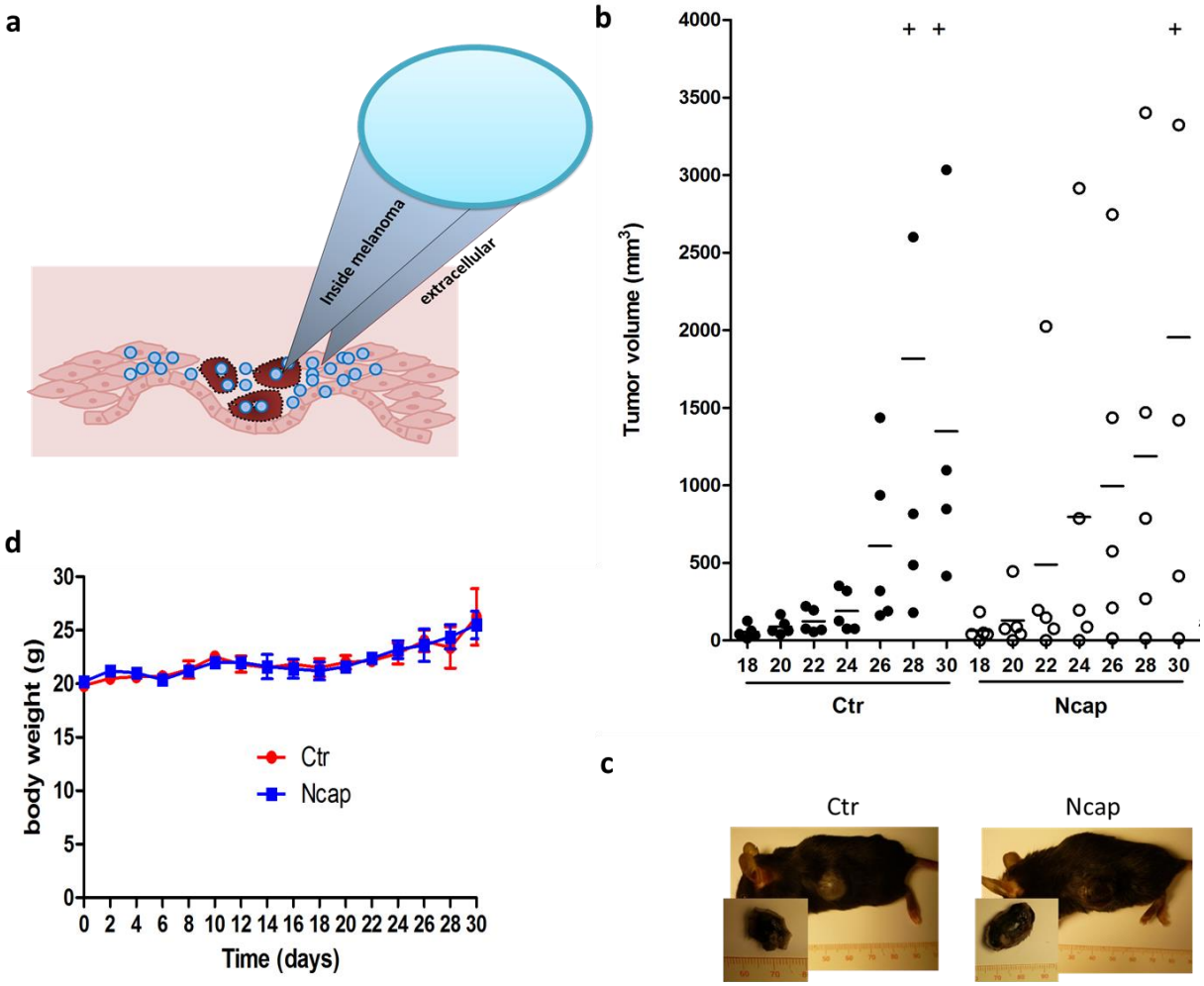


Figure 4. *In vivo* effect of component PLA nanocapsules vehicle

a. Model with PLA nanocapsules vehicle without polyHb-tyrosinase

b.0.5x10^5 B16F10 melanoma tumor cells and 0.15ml PLA nanocapsules (same volume of saline in the control group) were premixed and injected into the lateral flank of C57BL/6 mice subcutaneously. Tumor volumes were followed every 2 days.

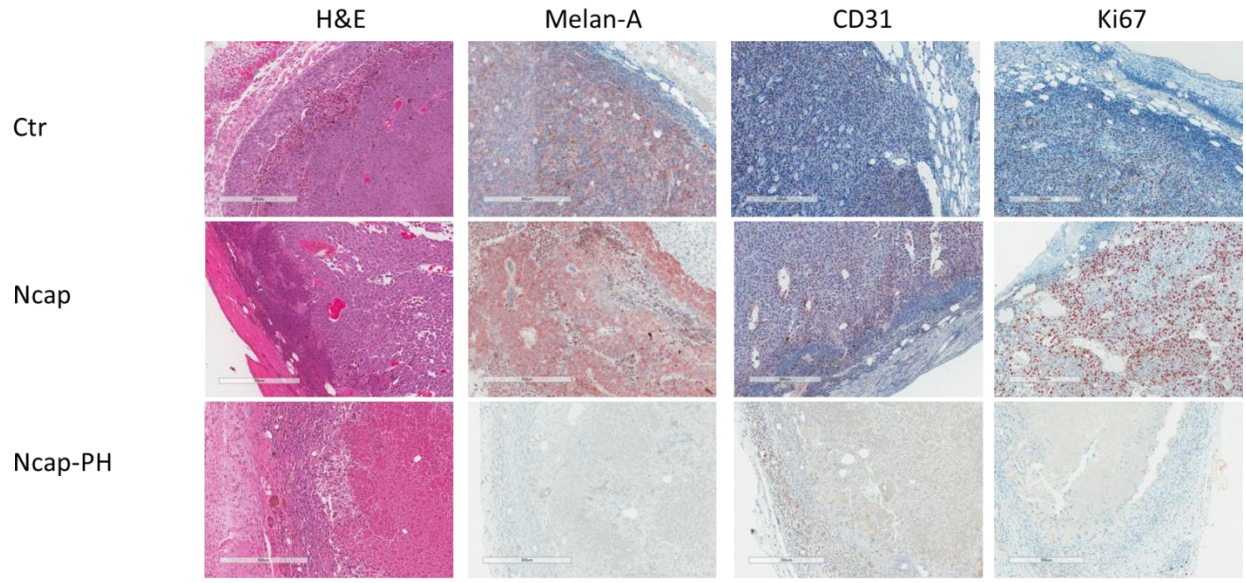
(Ctr: control with saline; Ncap: empty PLA nanocapsules without content)

+means the tumor volume has reached the ceiling which was set as 4000mm3, and the mice were euthanasia.

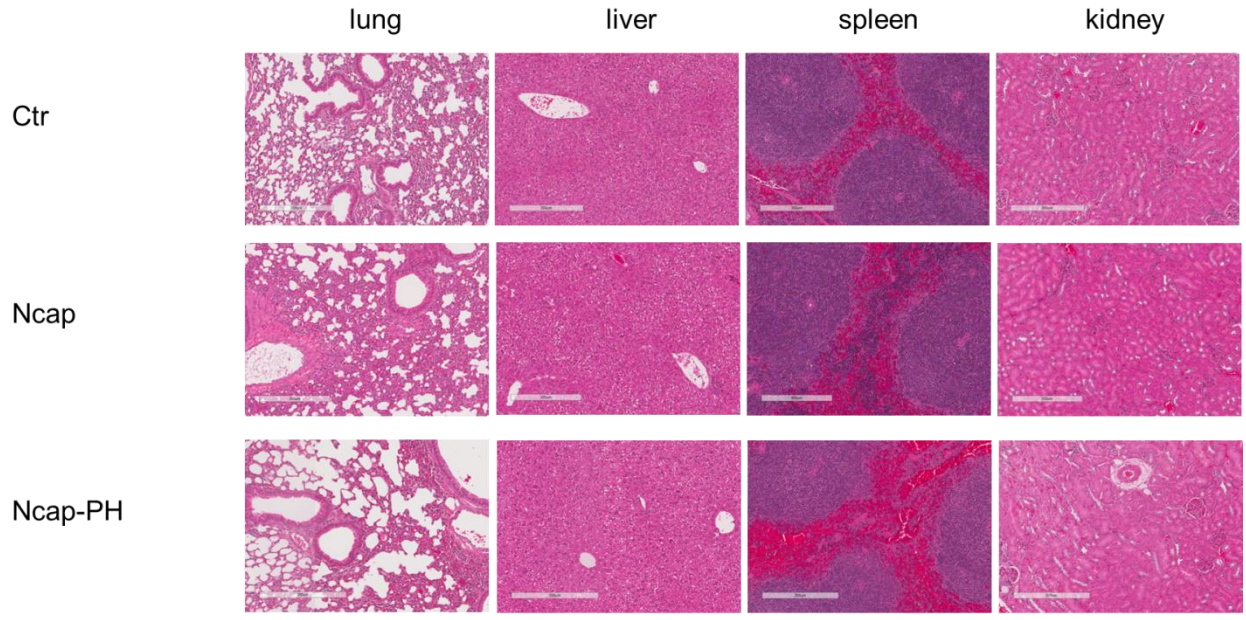
c. At the end of the 30 days, the tissues were separated.

d. Body weights were measured every 2 days and no significant difference between groups.

a.


H&E
Melan-A
CD31
Ki67
Ctr
Ncap
Ncap-PH

b.


lung
liver
spleen
kidney
Ctr
Ncap
Ncap-PH

c.

	Nodule tissue	Viable tumor % (estimate)	Necrosis % (estimate)	Hemorrhage % (estimate)	Nanoparticles visible	Granuloma reaction	Necrotic blood vessels
Ctr	Neoplastic	65	30	5	-	-	+/ -
Ncap	Neoplastic	65	32	2.5	-	-	+
Ncap-PH	Granuloma	0	0	0	+/-	+	-

Figure 5. Histological analysis of components Ncap and Ncap-PH

a. Histological analysis of the tumor tissues with H&E staining and Melan-A, CD31 and Ki67 stainings were performed to identify melanoma cells.
b. HE staining of lung, liver, spleen, and kidney. No observable changes were found in these organs.
c. Histological analysis of the tumor formed with different treatments.

3.1.5 Summary of animal studies

Animal studies in B16F10 bearing melanoma mice showed that PLA nanocapsules containing polyhemoglobin-tyrosinase could effectively inhibit melanoma growth. PLA nanocapsules containing only polyhemoglobin also have inhibitory effects. The other components did not have any effects.

We carried out preliminary studies and found that if the melanoma cells load was increased from 50,000 to 100,000, PLA nanocapsules containing polyHb-tyrosinase200 units could not inhibit melanoma growth unless the tyrosinase was increased to 400units. This showed that at higher melanoma cell loads, even PLA nanocapsules containing polyHb-tyrosinase200, could not inhibit melanoma growth. This also would indicate that PLA nanocapsules-PolyHb would act at

lower melanoma cells loads but tyrosinase would be needed when the melanoma cell load increases. In order to investigate this in more detail, we have carried out the following *in vitro* cell culture studies to compare the functions of these two components.

3.2. Cell culture study

3.2.1 PLA nanocapsules containing polyhemoglobin-Tyrosinase (Ncap-PH-TYR) for the removal of tyrosine

Previously, we have demonstrated that tyrosinase can maintain its enzymatic activity when crosslinked with hemoglobin. Here, we investigated the enzyme function in the new format, PLA nanocapsules containing polyHb-tyrosinase.

After treating the tumor cells with Ncap-PH-TYR for 48h, the tyrosine level can be decreased to as low as 1% compared to the control (Fig.6). Only PLA nanocapsules with the component of tyrosinase can lower tyrosine, since the vehicle (Ncap) or PLA nanocapsules without tyrosinase (Ncap-PH) were not able to lower the tyrosine level.

3.2.2 Therapeutic effect and dose in 3D model

To prolong the observation period on tumor cells viability is very pivotal. This can help to set a suitable nanocapsule dosage for *in vivo* study. Also this can test the PLA membrane's long term function. While the present 2D model presents some weaknesses: firstly, B16F10 cells have fast growth speed and observation term is short; secondly, it's not easy to change medium without removing Ncap. 3D culture should be an ideal approach to solve these problems. Tumor cells will grow slower with the growth speed closer to that in the body condition. Moreover, nanocapsules trapped in the 3D gel allows one to change and refresh the culture medium. Thus the information from the 3D culture can better guide further *in vivo* studies. The approach used was collagen which is a natural matrix component. Another advantage is that at the completion of the experiments, collagenase can be used to digest the gels and to release the cells for detailed analysis.

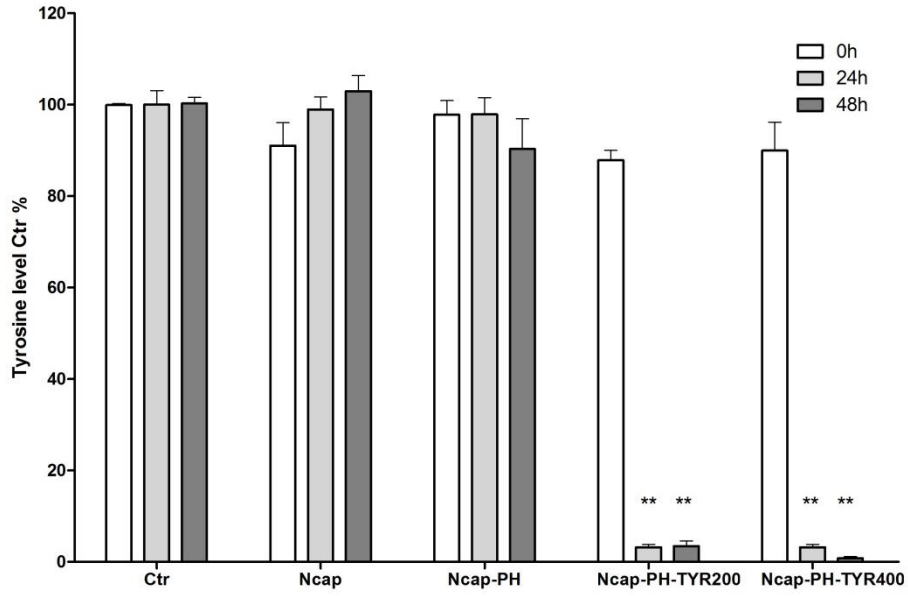


Figure 6. Effects on tyrosine level

Tyrosine levels with different time points were tested in the attachment model. The tyrosine levels in the 0 time point were set as 100%. Ctr : control with saline; Ncap: empty PLA nanocapsules; Ncap-PH : PLA nanocapsules containing polyHb without tyrosinase ; Ncap-PH-TYR200: PLA nanocapsules containing polyHb and 200 units of tyrosinase; Ncap-PH-TYR400: PLA nanocapsules containing polyHb and 400 units of tyrosinase. Results are mean+s.e.m., n= 3. Double asterisk indicates P <0.005 compared with control (no treatment).

In the established 3D culture model, tumor cells in the control group can grow well in collagen gel. The observation period can last for at least 24 days and the cell viability maintained. In the test groups (Ncap-PH-TYR200 and Ncap-PH-TYR400) the cell numbers have decreased to less than 10% (Fig.7). The Ncap and Ncap-PH only slower the tumor growth, but unlike the two test groups, they did not inhibit the growth. These data were consistent with the above evidence that tyrosine deprivation plays a major role in modulating the cellular viability.

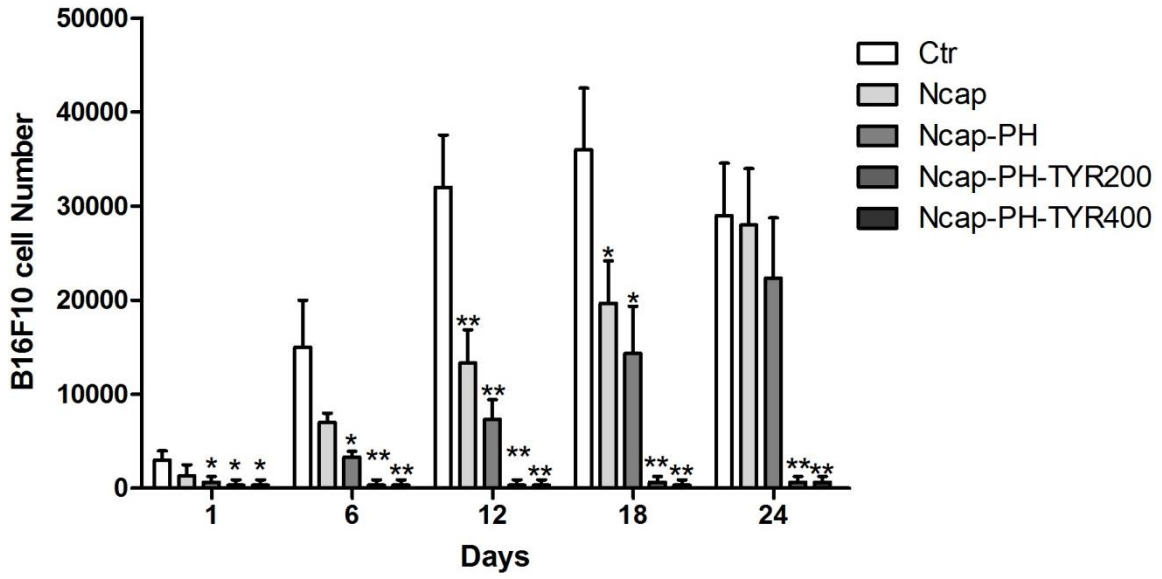


Figure 7. The effects of different components on cell growth in 3D cell culture

Tumor cell viability was followed for 24 days. The 3D cell culture model was established by collagen gel in 96-well plate. At the time point, the tumor cells were collected by collagenase digestion of the gel. Results are mean+s.e.m.. Single asterisk indicates P <0.05 compared with control (no treatment). Double asterisk indicates P< 0.005.

3.2.3 Effects on migration (metastasis).

One reason for the malignant feature of melanoma is its ability to metastasize. Thus we performed the scratch test, commonly used to study the invasive migration of tumour cells (Shin, Kim et al. 2008). Compared with the control group, the PLA nanocapsules containing polyHb-tyrosinase can inhibit tumor migration (Fig8). It was found that the PLA nanocapsules containing polyHb without enzymes can also decrease the scratch area but to a less extent. Again this seems to show that the tyrosinase component in the PLA nanocapsules containing polyHb-tyrosinase plays an important role.

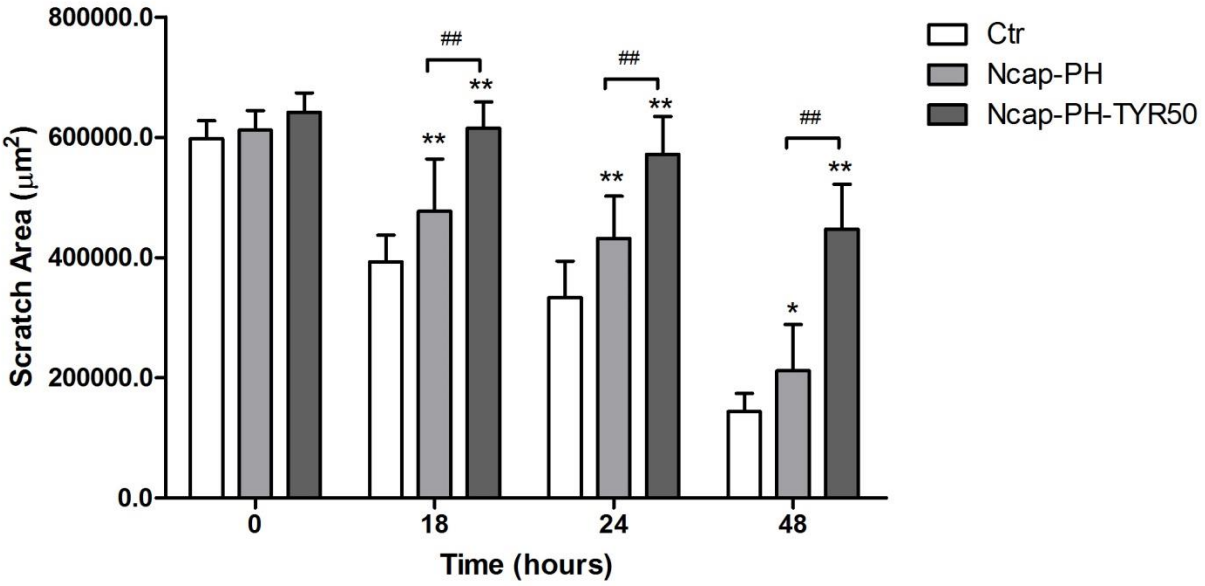


Figure 8. The effects of different components on cell migration (metastasis)

The ability of the tumour to metastasize was tested as its ability to migrate using the standard scratch assay. A bare area in the cell culture plate was made by scraping cells with a sterile tip. Quantitative analysis of the migration ability was conducted by calculating changes of the scratch area during treatment for 48h. The scratch area for the control group decreased quickly with time showing its migration ability. Treatment with Ncap-PH-TYR50 inhibited migration significantly ($p<0.005$), whereas Ncap-PH also resulted in some inhibition of migration, but not as effective as Ncap-PH-TYR50 ($P<0.005$). Single asterisk indicates $P <0.05$ and double asterisk indicates $P< 0.005$ compared with control. ## indicates $P<0.005$ compared between Ncap-PH and Ncap-PH-TYR.

3.2.4 Effects on tumor's cell cycle

Tyr/Phe depletion resulted in the arrest of cultured tumor cells in the G0/G1 stage (Fu, Yu et al. 1997). In our cell cycle experiment, the results also confirmed this. Majority of melanoma tumor cells were arrested in G0/G1 when treated with Ncap-PH and Ncap-PH-TYR50. It was time dependent (Fig9). About the cell cycle of melanoma cells, the cyclin D and their attendant CDKs

are key regulators of G1 progression, which is an important checkpoint of cell cycle. Our results showed that the expression of cyclin D1, cyclin D3and CDK2 are also decreased.

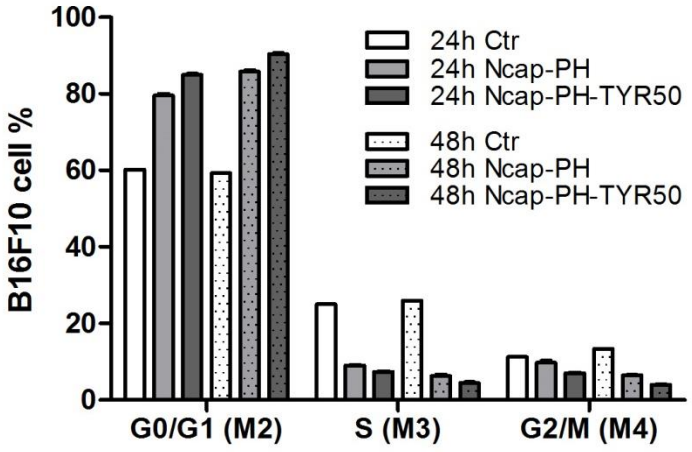


Figure 9. Different components' function on cell cycle

Cell cycle detection on melanoma cells treated with PLA-nanocapsules containing PolyHb (Ncap-PH) and PLA-nanocapsules containing PolyHb-Tyrosinase (Ncap-PH-TYR50). PI staining was applied for the cell cycle detection. M2: G0/G1 phase; M3: S phase; M4: G2/M phase.

3.3. Conclusion:

We showed that PLA-nanocapsules containing PolyHb-Tyrosinase could inhibit the growth and migration of melanoma cells in animal study. Analysis of the role of the different components of PLA-nanocapsules containing PolyHb-Tyrosinase showed the importance of nanoencapsulating Polyhemoglobin-tyrosinase within PLA nanocapsules to enhance its local retention and function. PLA-nanocapsules containing only PolyHb could also inhibit melanoma growth but only at lower melanoma cell load. The carrier PLA nanocapsules did not have a significant effect by itself on the inhibition of melanoma. Detail cell culture studies showed that PLA nanocapsules containing polyhemoglobin-Tyrosinase could effectively remove tyrosine and inhibit the growth and migration of melanoma cells. PLA nanocapsules containing only polyhemoglobin could not lower tyrosine but could inhibit tumor growth and migration but to a much less extent. These

results support the *in vivo* study of the effects of the different components of the PLA-nanocapsules PolyHb-Tyrosinase.

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Chapter 5

PLA nanocapsules containing a nanobiotechnological complex of polyhemoglobin-tyrosinase inhibit the growth of prostate cancer PC3 in cell culture

Foreword

In the Chapter 2 to Chapter 4, we have performed experiments on the B16F10 melanoma. In order to further test the PLA nanocapsules and observe the tyrosine deprivation effect on the wider application of tumor treatment, we chose the PC3 prostate cancer as the next model. The functions were investigated. Our study indicated that tumor growth was suppressed on the proliferation and attachment models, and the possible mechanisms are apoptosis and ROS generation.

PLA nanocapsules containing a nanobiotechnological complex of polyhemoglobin-tyrosinase inhibit the growth of prostate cancer PC3

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Abstract

Prostate cancer is one of the frequently diagnosed tumors among men in Europe and the United States. Clinically, many patients die from this disease every year owing to drug resistance and tumor metastasis. The recent investigations reveal prostate cancer has a specific metabolic abnormality that is the tyrosine dependence. Upon this characteristic, the strategy to deprive tyrosine in tumor microenvironment could provide an enhanced treatment for prostate cancer cells which are less responsive to present treatments. Herein, we applied PLA nanocapsules containing polyhemoglobin-tyrosinase to convert and consume tyrosine. The androgen-independent malignant prostate cell line, PC3, was used in this study as a model. The cell viability tests on the proliferation model and attachment model both confirmed the therapeutic effects on PC3. The possible mechanisms involved were also studied. One is the induction of apoptosis induced, and the other is the generation of reactive oxygen species (ROS). Our study provides a potential novel strategy on the treatment of prostate cancer, especially on the androgen-independent type.

Introduction

Prostate cancer is one of the most frequently diagnosed malignancies in men in Europe and the United States. For hormone-dependent prostate cancer, the anti-androgenic methods are effective. While, resistance and less responsive to chemotherapy often occurs in the androgen-independent

prostate cancers. So a novel strategy for the treatment of androgen-independent prostate cancer needs to be investigated.

Detailed tumor researches have verified that some cancers show specific amino acid dependency (Cantor and Sabatini 2012). Examples are melanoma and prostate cancers, possessing specific dependency for tyrosine (G. G. Meadows 2005). Thus, deprivation of this amino acid can starve cancers. By the combination of enzymatic therapy, tyrosinase as a tyrosine cleaner, can lower tyrosine level and significantly inhibit tumor growth and migration in melanoma (Yu and Swi Chang 2004; Wang and Chang 2012). Whereas the effects of this strategy on prostate cancer have not been fully studied.

In addition, to improve the efficacy with this enzymatic therapy, combination with nanotechnology is one promising strategy (Chang 2013). The nanocapsule structure can maintain the tyrosinase inside the nano frame and allow the tyrosinase to function for a longer time. Moreover, crosslinking with polyhemoglobin (PolyHb) can further enhance the function of the enzyme and decrease the immune response. Besides, polymer polylactic acid (PLA) is one of the most developed biodegradable materials that can be used on the nanocapsules preparation and form PLA nanocapsules. The encapsulation of tyrosinase into PLA nanocapsules not only improve delivery of this enzyme, but also prolong the duration of function and improve the therapeutic effect in melanoma by virtue of the size-dependent passive accumulation in tumor area (Wang and Chang 2012).

The objective of this research is to test the effect of PLA nanocapsules containing crosslinked PolyHb-tyrosinase on prostate cancer cell PC3, an androgen-independent type. We use this cell line because it is well established and detailed studies on its growth, proliferation, and mechanisms involved have been extensively studied (Zhang and Waxman 2010).

Materials and Methods

1. Preparation of PLA nanocapsules containing polyHb-tyrosinase

PolyHb and polyHb-tyrosinase were first prepared (Wang and Chang 2012). Briefly, stroma-free hemoglobin in the concentration of 7 g/dL Hb with or without tyrosinase was dissolved in PBS solution. Crosslinker glutaraldehyde was added and the crossing process continues for 24

hours. Then lysine was added to stop the reaction. Purification was performed by dialysis and syringe filters. Next, the PLA nanocapsules were formed by nanoprecipitation. PLA and hydrogenated soybean phosphatidylcholine were dissolved in organic phase containing ethanol and then added into polyHb-Tyrosinase or polyHb solution under magnetic stirring. The formed PLA-polyHb-tyrosinase or PLA-polyHb was purified by rotary evaporator, and then separated by centrifugation.

2. Cell culture

PC3 prostate cells (American Type Culture Collection, ATCC®, CRL-1435) were cultured in DMEM/F-12 (Invitrogen) supplemented with 10% fetal bovine serum at 37 °C and 5% CO_2, humidified atmosphere. Cells were passaged every 2–3 days. When the cells reached 90-100% confluence, they were washed with 0.25% trypsin/EDTA for about 1 min to be detached from the dish. Then cell suspensions were collected in a sterile 15 mL tube and centrifuged at 1,500 rpm for 5 min. Cells were counted by trypan blue exclusion and suspended in fresh medium to the desire density.

3. Cell dose test

$2.5x10^3$ PC3 cells were seeded on 24-well plate and cultured until 50-60% confluence. Treat cells with PLA nanocapsules for 24h, and collect cells by adding tryptic enzyme. Cell viability was measured by trypan blue staining.

4. Proliferation assay

$5x10^3$ PC3 cells were seeded on 12-well plate overnight and culture until cells cover 50-60%. Different nanocapsules or the same amount of saline were mixed with medium and added into each well. Tumor cells were cultured for 24 and 48 hours. By the end of the time points, cells were collected by 0.25% trypsin/EDTA. Trypan blue staining was performed on the cell viability measurement.

5. Attachment assay

For the attachment model, similar to proliferation assay, the difference is that the tumor cells suspension was mixed with PLA nanocapsules or the same amount of saline in the full medium,

and then seeded on 12-well plate for 24 h and 48 h together. Cell attachment ability was determined by trypan blue exclusion test.

6. IncuCyte cell growth measurement assay

PC3 tumor cells were treated with PLA nanocapsules for 24 hours, and then the cells were collected for the cell growth measurement assay. PC3 cells were seeded in 24-well plates. The plate was incubated in an IncuCyte (Essen BioScience) inside a cell culture incubator. Images were captured every day to monitor the cell growth. All samples consisted of a minimum of 4 replicates (Waaler, Machon et al. 2012).

7. Apoptosis detection with Annexin V-FITC and PI staining

In order to evaluate apoptosis and necrosis, Annexin V-FITC/PI (propidium iodide) dual staining was used to test the externalization of phosphotidylserine (PS) during apoptosis and leakage from necrosis cells. Cells were seeded onto a six-well plate at a density of $1x10^5$ cells per well and incubated at 37℃ in a CO_2 incubator. Treat the cells with different types of PLA nanocapsules. The staining steps were followed the manufacturer’s instructions. The samples were analyzed using a FACS Calibur flow cytometer.

8. Flow cytometry analysis of the ROS generation

The generation of ROS was tracked with fluorogenic probe, CellROX oxidative stress reagent. This cell-permeable reagent can exhibit strong fluoregenic signal upon oxidation that can be measured. PC3 cells treated with PLA nanocapsules were collected and incubated with CellROX reagent at 37℃ in dark. Samples were then analysed on FACS Calibur (BD Bioscience, USA).

9. Data analysis

Statistical analysis was performed using the Student’s t-test or one-way ANOVA and for significant at $P < 0.05$ and $P < 0.005$.

Results

1. Effects of PLA PolyHb-Tyrosinase nanocapsules (Ncap-PH-Tyr) dose on the cell viability of PC3.

First, we studied the suitable dose on the treatment of PC3 tumor cells and observed the therapeutic effect. PLA nanocapsules containing different tyrosinase doses were separately prepared, ranged from enzyme activity Ncap-PH-Tyr12.5 to Ncap-PH-Tyr400 (Fig1). The amount of polyHb (PH) remained the same. In the control group, same amount of saline was added. For 24 hours treatment on the PC3 tumor cells, significant difference was found between groups compared with control. The therapeutic effect displayed dose dependent. The medium dose of Ncap-PH-Tyr50 was capable to inhibit tumor cell growth, and the cell viability was lower than 30% compared with that of the control group. In the high dosage group, Ncap-PH-Tyr400, the cell viability can be suppressed to lower than 5% compared with the control group. The differences compared with the control were significant ($P<0.005$).

2. Effects of Ncap-PH-Tyr on the proliferation of PC3 cells.

We treated PC cells with PLA nanocapsules containing a large range of enzyme activities, from Ncap-PH-Tyr0 to Ncap-PH-Tyr100. The results indicated that PLA nanocapsules inhibited tumor proliferation, and the effect was tyrosinase dose and time dependent (Fig 2). Compared with the control group, the higher the tyrosinase activity, the greater the inhibition on tumor proliferation as shown by the declined cell viability at 24 hours and 48 hours. The greatest effect was achieved in the Ncap-PH-Tyr100 group at 48 hours. The differences compared with the control are significant ($P<0.005$).

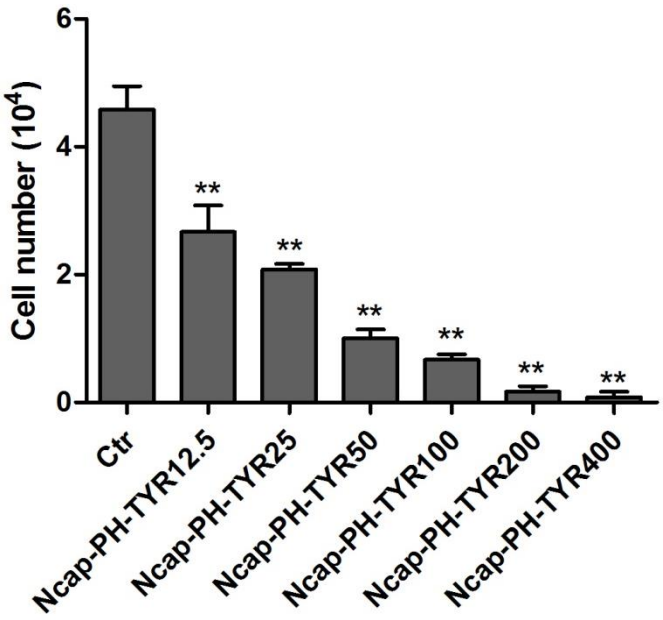

Figure 1. Effects of PLA nanocapsules containing polyhemoglobin-tyrosinase with different tyrosinase activities on the cell viability of PC3 followed for 24 hours. Ctr: control group treated with saline. Ncap-PH-TYR#: PLA nanocapsules containing PolyHb-tyrosinase with # units of tyrosinase. Results are expressed as mean+ s.e.m.. **double asterisk indicates P<0.005 compared with control.

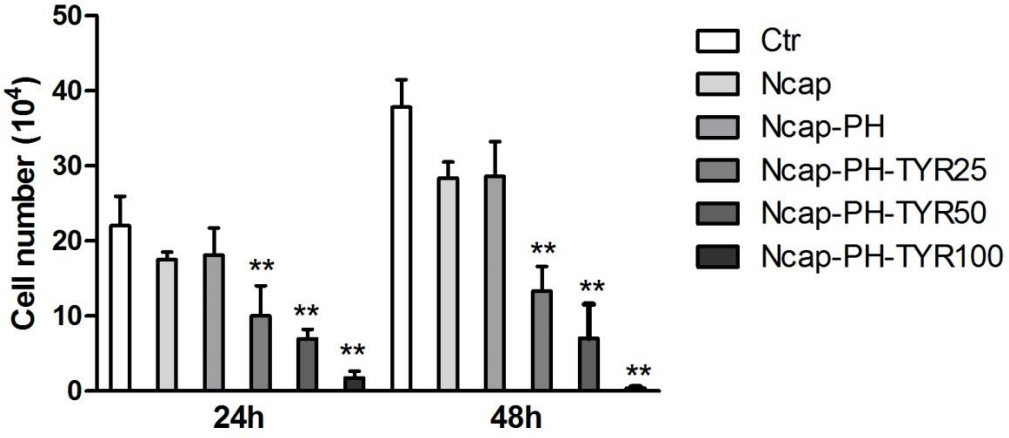

Figure 2. Effects of PLA nanocapsules containing polyhemoglobin-tyrosinase on the proliferation of PC3 cells. PC3 tumor cells treated as follows: Ctr: saline control group. Ncap: PLA nanocapsules without polyHb or tyrosinase. Ncap-PH: PLA nanocapsules containing polyHb but no tyrosinase. Ncap-PH-TYR#: PLA nanocapsules containing polyHb with # units of tyrosinase. Results are expressed as mean+ s.e.m.. **double asterisk indicates P<0.005 compared to control.

3. Effects of PLA nanocapsules on the attachment of PC3 cells.

For the solid cancer melanoma, the metastatic dissemination from a primary lesion to a secondary site is believed to be the major reason leading to death(Gupta and Massague 2006). The tumor cells attached to the secondary site is one of the vital steps of this metastatic process(Mehlen and Puisieux 2006). Thus, we carried out the following experiment to identify the effect of Ncap-PH-Tyr on PC3 tumor cells attachment. We mixed PC3 cells with Ncap-PH-Tyr and co-cultured the suspension in 6-well plates to observe the cell viability (Fig3). After 24h-culture, it was indicated that Ncap-PH-Tyr can effectively inhibit tumor cells attachment on plastic plates. It was time and dose dependent. After 48h, only a few viable cells were found in the test groups of Ncap-PH-Tyr50 and Ncap-PH-Tyr100 groups. This was significant to $P<0.005$. The Ncap-PH group with no enzymes can also suppress cell attachment to a certain degree, whereas the effect was not as obvious as that in the test groups (Ncap-PH-Tyr25, Ncap-PH-Tyr50 and Ncap-PH-Tyr100). There were no significant differences between empty PLA nanocapsules (Ncap) and the control at 24h and 48h ($P>0.05$).

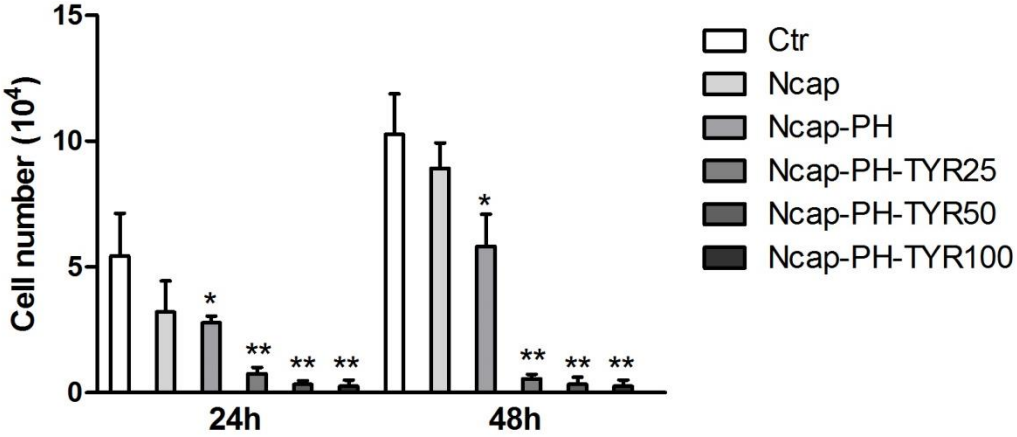

Figure 3. Effects of PLA nanocapsules containing polyhemoglobin-tyrosinase on the attachment of PC3 cells. Tumor cells suspension was mixed with PLA nanocapsules or the same amount of saline in the full medium, and then seeded on 12-well plate for 24 h and 48 h together. Cell attachment ability was determined by trypan blue exclusion test. Results are expressed as mean+ s.e.m.. *single asterisk indicates P<0.05 compared to control. **double asterisk indicates P<0.005 compared to control.

4. Growth curve of PC3 tumor cells after the treatment with Ncap-PH-Tyr.

The next is to test the growth rate of PC3 tumor cells treated with Ncap-PH-Tyr. Ncap-PH-Tyr was added and coculture with tumor cells for 24h. Subsequently, the nanocapsules were removed and tumor cells were allowed to grow in the normal full medium. The cell confluence was monitored every day. The results showed that the tumor cells growth curve was considerably slowed down in the test group of Ncap-PH-Tyr100, compared with the control group ($P<0.05$) (Fig4). On the 5th day, the control group was close to 95% confluence, whereas the Ncap-PH-Tyr50 group reached 50% and Ncap-PH-Tyr100 group reached 30% confluence. The results indicated that even if PC3 tumor cells were separated from the Ncap-PH-Tyr after 24 hour treatment, the therapeutic effect continued to inhibit tumor growth for at least 5 days.

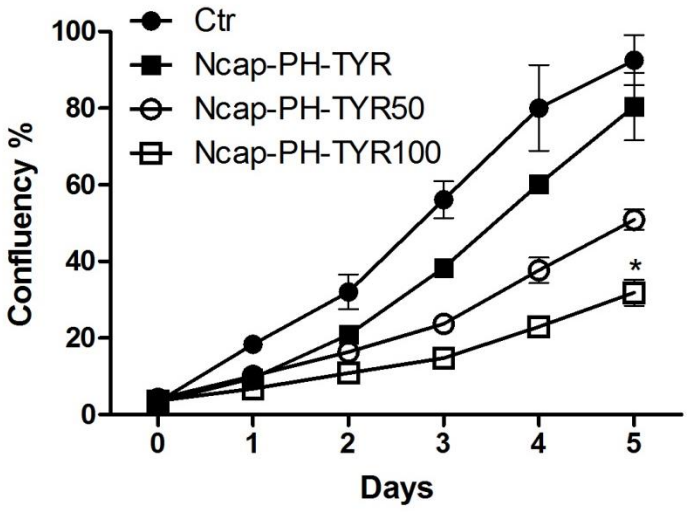

Figure 4. The growth curve of PC3 tumor cells after treatment with PLA nanocapsules containing polyhemoglobin-tyrosinase. PC3 tumor cells were treated with PLA nanocapsules for 24 hours, and then the cells were collected and seeded for the cell growth measurement assay in an IncuCyte. Data were recorded every day to monitor the cell growth. Results are expressed as mean+ s.e.m.. *single asterisk indicates $P<0.05$ compared with the control group for the 5 days.

5. Effects of Ncap-PH-Tyr in apoptosis induction on PC3.

The next question is to explain possible mechanisms involved in the impaired cell viability. We performed apoptosis analysis by flow cytometry in PC3 tumor cells after the treatment with Ncap-PH-Tyr for 24h. Obvious apoptosis was induced (Fig 5). Compared with the control group, both the early apoptosis and the late apoptosis were increased in the test group Ncap-PH-Tyr100 ($P<0.005$), and the final apoptosis (combining early apoptosis and late apoptosis) effect was mostly obvious with $P<0.005$.

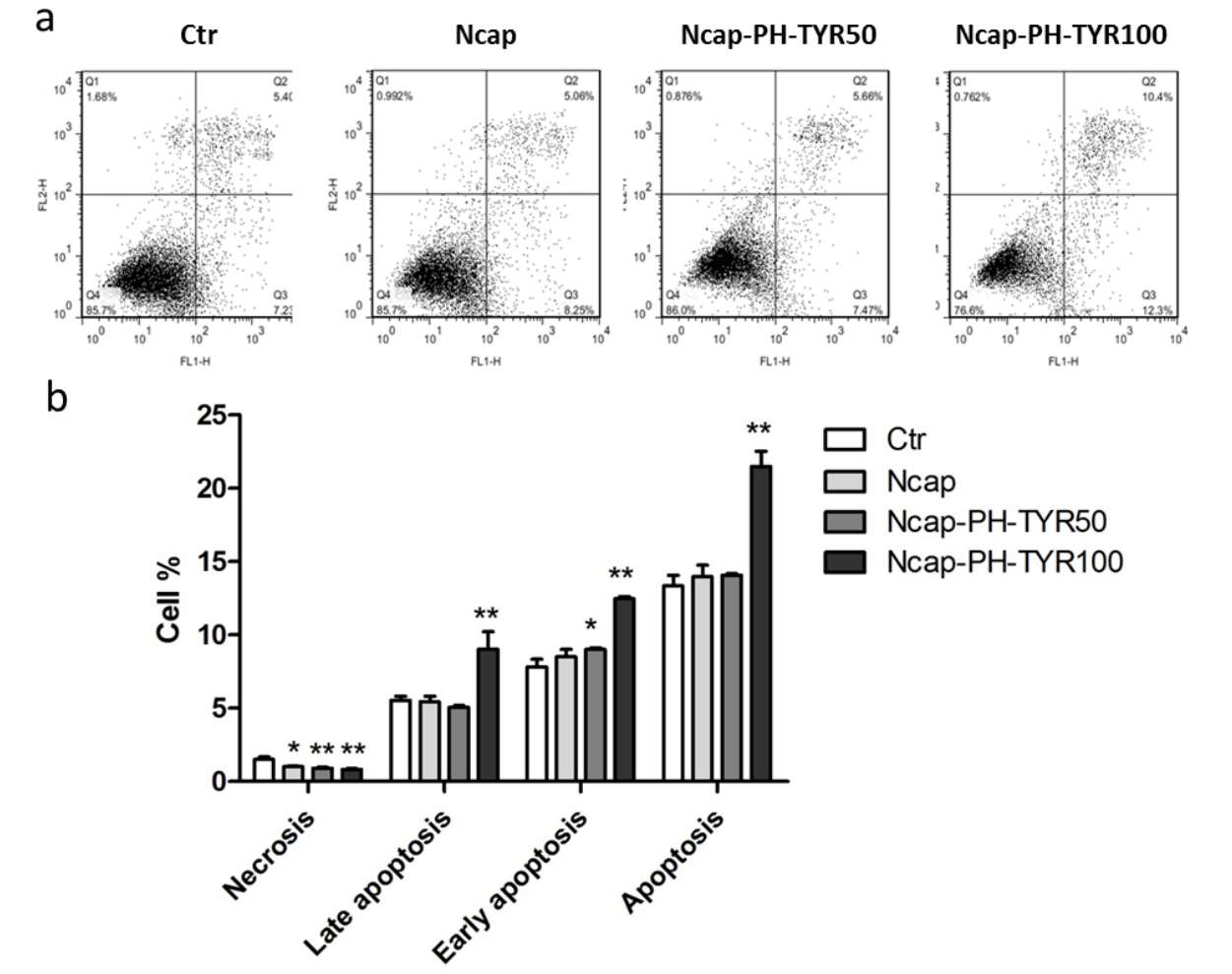

Figure 5. Flow cytometric analysis of apoptosis in PC3 cells. Annexin V-FITC/PI dual staining was utilized and samples were analyzed using a FACSCalibur flow cytometer. Results are expressed as mean+ s.e.m.. *single asterisk indicates $P<0.05$ compared to control. **double asterisk indicates $P<0.005$ compared to control.

6. Ncap-PH-Tyr can generate ROS.

Another possible mechanism is oxidative stress relative to Ncap-PH-Tyr. Oxidative stress will stimulate cell apoptosis by activating DNA damage-repair pathway and also opening mitochondrial pore. To confirm this mechanism for the Ncap-PH-Tyr treated PC3, we followed the ROS levels. CellROX reagent was applied to track the ROS generation for 12h and 24h, aiming to quantitatively estimate the intracellular ROS generation. In the presence of ROS, fluorescent intensity of the cells stains with dyes will increase, leading to a right shift of the emission maximum. The results displayed that the ROS generation increased after Ncap-PH-Tyr treatment for 12h and 24h when compared to the untreated control.

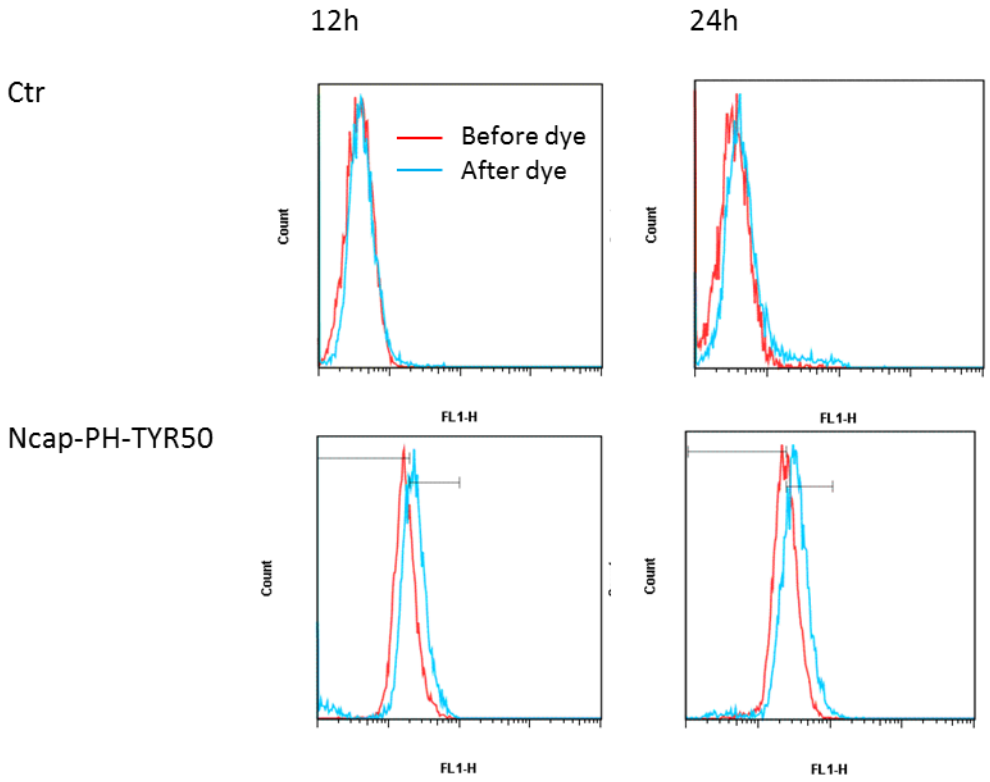

Figure 6. Generation of ROS from PC3 tumor cells treated for 12h and 24h with PLA nanocapsules containing polyhemoglobin-tyrosinase. In the presence of ROS, fluorescent intensity of the cells stained with dyes will increase, leading to a right shift of the emission maximum. Red peak is the cells without dye; Blue peak is the cells stained with the CellROX dye.

Discussion

Specific amino acid dependency, especially tyrosine dependency, is one of metabolic abnormalities of malignant tumors. In our previous studies, we proved that PLA nanocapsules containing polyhemoglobin-tyrosinase (Ncap-PH-Tyr) can inhibit melanoma growth, proliferation and migration (Wang & Chang, submitted). The effects were due to the arrest of G0/G1 phase, apoptosis induction, and ROS generation. Based on our results of melanoma, we further studied the effects of Ncap-PH-Tyr on an androgen-independent prostate cancer cell line, PC3.

There is a high incidence and death caused by prostate cancer in men (Jemal, Siegel et al. 2009). Up to now, the primary therapy optional for prostate cancer is hormone treatment in advance prostate cancer patients, and prolong survival time can be expected (Akaza, Homma et al. 2006; Antonarakis, Blackford et al. 2007). However, androgen-independent prostate cancers are poorly responsive to this therapy. This form of prostate cancer is high metastasis, poor prognosis, and only modest survival can be obtained owing to the drug-resistance (van Brussel and Mickisch 2003). PC3 is a human chemoresistant and hormone-refractory cell line of prostate cancer, commonly applied in the study of anti-proliferation and relative mechanism. Therefore, PC3 is employed in our current study.

PLA nanocapsules containing polyhemoglobin-tyrosinase were prepared by nanoprecipitation method. This involved two steps: (1) The use of gluataraldehye to covalently crosslink hemoglobin and tyrosinase into a soluble nanobiotechnological complex. (2) The next step is to nanoencapsulate this soluble complex into PLA nanocapsules of 100 to 300 nm in diameter (Wang and Chang 2012). This is to facilitate their entry into melanoma cells.

Two models were implemented to assess the effect of PLA nanocapsules on the PC3 growth, the proliferation model and attachment model. In the proliferation model, the PC3 cells with confluence of 50-60% were treated with Ncap-PH-Tyr. In the attachment model, the cells were co-cultured with Ncap-PH-Tyr and seeded on the culture plate together, so as to mimic the process that the metastasis tumor cells migration in the second site. The cell viabilities of the two models were followed. The results verified that PLA nanocapsules containing polyHb and tyrosinase Ncap-PH-Tyr can inhibit both the proliferation and attachment of prostate cells during

the 24h and 48 h observation. This implied that the removal of the specific amino acid of tyrosine can inhibit prostate tumor proliferation and attachment. The results were consistent with the previous observation, that the cell viability was significantly inhibited after 1 day of Tyr/Phe free treatment (Fu, Yu et al. 2003). Our present study with Ncap-PH-Tyr showed a much greater inhibition effect.

Free Tyr/Phe did not induce apoptosis in PC3 cell line (Fu, Yu et al. 2003). However, in our experiment using Ncap-PH-Tyr, apoptosis was also induced when comparing with that of the control group. The apoptosis effect induced involved both the early apoptosis and the late apoptosis. The combined effect, called the "apoptosis", increased to about 80% when compared with the control group. In addition, the apoptosis induction can only be observed when the dose reaches a high degree. No significant changes presented between the low dosage groups compared to the control group.

Another mechanism of the Ncap-PH-Tyr on melanoma is the generation of ROS in B16F10 melanoma (Wang and Chang, submitted). ROS have essential functions in living organisms, and its homeostasis is crucial for the normal cell's growth and survival. The increased level of ROS is associated with cancer cells' abnormal growth with disruption of redox status, a result from increased ROS generation or decline of ROS scavenging, which is called oxidative stress(Toyokuni, Okamoto et al. 1995; Trachootham, Alexandre et al. 2009).The increased oxidative stress due to the exogenous agents can damage the cancer cells (Pelicano, Carney et al. 2004). The increased ROS level to a certain threshold, which is incompatible with cell's survival, may cause a cytotoxic effect and lead to the death of malignant cells, and further limit cancer progression (Schumacker 2006) (Fruehauf and Meyskens 2007).

In the single Tyr/Phe restriction treatment, there was no increase of ROS in PC3 cells (Liu, Fu et al. 2011). On the other hand, our present study with Ncap-PH-Tyr showed ROS generation. This could be explained by the generated products of tyrosine metabolism in the enzymatic reaction of tyrosinase, such as dopa, 5,6-dihydroxy indole (DHI), 5,6-dihydroxy indole-2-carboxylic acid (DHICA) and others. Oxidation of these tyrosine metabolites can produce reactive oxygen species (Urabe, Aroca et al. 1994). The present study shows that Ncap-PH-Tyr

containing polyhemoglobin and tyrosinase can affect PC3 in multiple aspects, such as cell proliferation, attachment, oxidative stress and apoptosis.

Conclusion

Previously, we have reported that the subcutaneous injection of the nanobiotechnological complex, PLA nanocapsules containing polyHb and tyrosinase (Ncap-PH-Tyr), can suppress melanoma growth, proliferation, and migration by inducing apoptosis, cell cycle arrested on G0/G1, and ROS generation. To further test the PLA nanocapsules effects based on the mechanism of tyrosine restriction, we performed the test on another malignant cancer, prostate cancer, of which the tyrosine-dependency was also confirmed. We researched on the effects of cell proliferation, attachment, and the potential mechanisms involved. The *in vitro* results support the feasibility of vivo experiments.

Acknowledgements

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Chapter 6

General Discussion and Conclusion

We have included detailed discussion and conclusion in Chapter 2 to 5. These are now summarized together below.

6.1 General Conclusion

Earlier study by Yu and Chang was carried out using polyHb-tyrosinase for the treatment of murine B16F10 melanoma both *in vitro* and *in vivo* (Yu and Chang 2004). However, the results showed it can only slow the process but cannot suppress tumor growth, and daily intravenous injection is needed to maintain the tyrosine in a low level. To improve the tyrosinase delivery and also its therapeutic effect, the main purpose of this thesis is to design and develop a novel nano-artificial cells based on PLA nanocapsules containing a nanobiotechnological polyhemoglobin-tyrosinase and test this in B16F10 melanoma cell culture and melanoma mice model.

6.1.1 Preparation of novel PLA nanocapsule is based on the novel combination of two nanobiotechnological technologies, nanocapsules containing the polyHb-tyrosinase complex, so as to allow the nanocapsules containing the soluble nanobiotechnological complex to stay longer at the site of injection. The preparation involved two steps. (1) Formation of a soluble nanobiotechnological complex of polyHb-tyrosinase. Glutaraldehyde crosslinks hemoglobin and tyrosinase to form polyHb-tyrosinase. This reinforces the stability of the enzyme tyrosinase and also allows a longer *in-vivo* function. However, being a soluble complex it cannot stay at the site of injection. Furthermore, it is less likely to enter into the melanoma cells. (2) Thus the next step is to nanoencapsulate this soluble complex into PLA nanocapsules. In order to facilitate their entry into melanoma cells, we nanoencapsulated this inside PLA nanocapsules of 100 to 300 nm in diameter.

Physicochemical characterization confirmed that PLA nanocapsules containing polyHb-Tyrosinase has ideal surface feature and uniform diameters in the ideal size range in the range 88 to 267 nm with 64% in the range of 120 to 200nm.

6.1.2 The earlier studies showed the potential use of nutrient deprivation of tyrosine and phenylalanine on melanoma and prostate cancer (G. G. Meadows 2005; Zhang, Chen et al. 2005; Fu and Meadows 2007; Liu, Fu et al. 2011). However, nutrient deprivation of tyrosine and phenylalanine resulted in systemic lowering of these amino acids throughout the body. There were also side effects that the patients could not tolerate. Our research showed that local injection of nano-artificial cells based on PLA nanocapsules containing a nanobiotechnological polyhemoglobin-tyrosinase can enter the melanoma cell and the surrounding area to act more locally. This would prevent a systemic decrease of these amino acids, lessen possible side effect and improve patient compliance.

The therapeutic effects were verified. The therapeutic effect on inhibiting tumor growth and growth (metastasis) was first tested in two experimental tissue culture models: the proliferation model and the attachment model. (1) In the proliferation model, attached B16F10 melanoma cells were treated with PLA nanocapsules containing polyHb-tyrosinase. This model is used to imitate the treatment on the primary small melanoma cells. (2) In the attachment model: tumor cells were co-cultured with PLA nanocapsules containing polyHb-tyrosinase. The attachment model is to imitate the process that the nanocapsules will be used for metastatic cancer dissemination from a primary lesion to a secondary site. The tumor attached to the secondary site is one of the vital steps of the metastasis. These studies showed that PLA nanocapsules containing polyHb-tyrosinase act effective in both models to decrease tumor viability and decrease tyrosine level.

6.1.3 Mechanisms of the action of PLA nanocapsules containing polyHb-tyrosinase were also confirmed in our experiment, such as the G0/G1 arrest of the cell cycle and the changes of the relative molecular expression. Specifically, expressions of FAK and α4 integrin were inhibited in B16F10 melanoma cells indicating attachment and spreading impairment. The expressions of key regulators of G1 progression, such as cyclin D1, cyclin D3and CDK2, were also decreased. Moreover, the apoptosis and ROS generation phenomenon were also found in the B16F10 murine model. Further analysis of the action of the different components showed that the complete structure of PLA nanocapsules containing polyHb-tyrosinase was responsible for the therapeutic effect. The PLA nanoencapsulated polyHb component has some effect when the

tumour cell load was low. There was no effect from either one local inject of PolyHb-tyrosinase or the vehicle PLA nanocapsules with no content

6.1.4 The tumor model we chose is the malignant B16F10 murine melanoma. Because this cell line is mostly used for the metastasis of melanoma and also the mice model is easy to establish. To further test the effects of PLA nanocapsules containing polyHb-tyrosinase, more cells lines, especially human source malignant tumors, should be tested. In the prostate cancer, we applied PC3 cell line in cell culture and found that PLA nanocapsules containing polyHb-tyrosinase can inhibit tumour growth. PC3 cell line is androgen-independent. This form of prostate cancer has high metastasis and poor prognosis and only modest survival can be obtained due to drug-resistance (van Brussel and Mickisch 2003). However, considering the heterogenesis of tumors, different cell lines of prostate cancer should be tested.

6.2 Conclusion and future directions

Our detailed *in vitro* and *in vivo* analyses have shown that the PLA nanocapsules containing polyHb-tyrosinase is a promising strategy on tumor therapy. However, still some improvements are necessary before clinical application.

To further improve the targeting on tumor lesions, the next model of nanocapsules should be designed to actively target tumour cells. This would increase the therapeutic effects on tumor cells, and decrease the dose that needed for the therapeutic effects. Also, melanoma stem cells could also be targeted and killed.

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