POLYHEMOGLOBIN-TYROSINASE AND ARTIFICIAL CELLS MICROENCAPSULATED TYROSINASE FOR THE REMOVAL OF SYSTEMIC TYROSINE — A POTENTIAL NOVEL THERAPY FOR MELANOMA

by

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To my husband Jianguo Ma and my parents.

Abstract

Artificial cells microencapsulation have a number of potential areas of application. Studies showed that lowering of tyrosine level could inhibit the growth of melanoma. However, at present there is no practical method to lower the tyrosine level in humans. We have therefore devised novel methods as follows. (1) Microencapsulation of tyrosinase for the removal of systemic tyrosine by oral administration. Characterization, optimization, and feasibility studies were carried out to test the therapeutic potentials. In temperature and pH studies, the encapsulated tyrosinase maintained higher enzyme activity than the free enzyme in solution. The *in vivo* studies showed that daily oral administration of encapsulated tyrosinase by itself for about 3-5 days could lower the body tyrosine level. (2) A novel polyhemoglobin-tyrosinase preparation for intravenous injection can rapidly lower the body tyrosine level after one intravenous injection. In this form, the enzyme is covered by hemoglobin molecules and therefore has less immunological properties. Furthermore, polyhemoglobin is an oxygen carrier and being in a solution, it can more readily reach the narrower capillaries of the melanoma cancer cells than red blood cells and can therefore bring more oxygen for radiation therapy. Our *in vitro* studies showed that this novel polyhemoglobin-tyrosinase preparation inhibited the growth of melanoma cells in culture. (3) A combination of two intravenous injections of polyhemoglobin-tyrosinase with 3 times a day oral administration of encapsulated tyrosinase could immediately lower the body tyrosine and maintained this low level as long as the oral administration was continued.

Résumé

La micro-encapsulation dans les cellules artificielles a un grand nombre de domaines d'application potentiels. Les études montrent que l'abaissement du niveau de tyrosine peut empêcher la croissance de melanome. Cependant, actuellement il n'y a aucune méthode pratique pour abaisser le niveau de tyrosine chez l'humain. Nous avons donc conçu de nouvelles méthodes comme suit. (1) La micro-encapsulation de la tyrosinase pour la supression de la tyrosine systémique par administration orale. La caractérisation, l'optimisation, et les études de faisabilité ont été effectuées pour tester les potentiels thérapeutiques. Dans des études sur la température et le pH, la tyrosinase encapsulée a conservé une activité enzymatique plus élevée que l'enzyme libre en solution. Les résultats des études *in vivo* montrent que l'administration orale quotidienne de tyrosinase encapsulée pendant environ 3-5 jours peut abaisser par elle-même le niveau de tyrosine dans le corps. (2) Une nouvelle préparation de polyhémoglobine-tyrosinase pour l'injection intraveineuse peut rapidement abaisser le niveau de tyrosine corporelle après une seule injection intraveineuse. Sous cette forme, l'enzyme est couverte de molécules d'hémoglobine et a donc des caractéristiques moins immunogéniques. En outre, la polyhémoglobine est un transporteur d'oxygène. En solution, elle peut plus facilement atteindre les capillaires étroits des cellules cancéreuses de mélanome que les globules rouges et peut donc apporter plus d'oxygène pour les radio-thérapies. Nos études *in vitro* montrent que cette nouvelle préparation de polyhémoglobine-tyrosinase empêche la croissance des cellules de mélanome en culture. (3) La combinaison de deux injections intraveineux de polyhémoglobine-

tyrosinase et de l'administration orale 3 fois par jour de tyrosinase encapsulée peut immédiatement abaisser le taux de tyrosine dans le corps et maintenir ce niveau bas aussi longtemps que l'administration orale est continuée.

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I am very pleased to present this thesis to my parents. I am completely indebted to them for their love, encouragement, endless patience, and undeniable understanding. My family has been an important source of inspiration and motivation throughout my entire life. I am fortunate to have parents who instilled in me the qualities of self-respect, perseverance, determination and hard work.

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List of Abbreviations

kg	kilogram
gm	gram
g/L	grams per liter
mg/dl	milligrams per deciliter
ml	milliliter
µl	microliter
hr	hour
min	minute
s	second(s)
M	molar
mM	millimolar
µM	micromolar
µmol	micromole
mm	millimeter
nm	nanometer
°C	degree of centigrade
%	percent
E	enzyme
MW	molecular weight
No.	number
pp	pages
SD	standard deviation
SE	standard error
U	unit
UV	ultraviolet
v	velocity

>	greater than
<	less than
rpm	revolutions per minute
kDa	kilo Daltons
Fig.	figure
vs	versus
p	probability
vol	volume(s)
Tris	tris(hydroxymethyl)aminomethane
HCl	hydrochloric acid
PPB	potassium phosphate buffer
PBS	phosphate buffer saline
Hb	hemoglobin
PolyHb	polymerized hemoglobin
Tyr	tyrosine
Phe	phenylalanine
DOPA	3,4-dihydroxyphenylalanine
AC	artificial cells
GI	gastrointestinal tract
PF	proliferation factor or factors
DF	differentiation factors
K_m	Michaelis-Menten Constant
V_{max}	maximum velocity
V_d	volume of distribution
V_e	elution volume
V_o	void volume
V_t	total bed volume

Preface

The thesis is composed of six chapters. Chapter 1 is a general literature review that provides background information pertaining to the thesis. Chapter 6 includes general conclusions and claims to original research. For chapters 2,3,4, and 5, I have taken advantage of the option provided by section B.2 of the "Guidelines Concerning Thesis Preparation" which states:

> Candidates have the option of including, as part of the thesis, the text of one or more papers submitted or to be submitted for publication, or the clearly-duplicated text of one or more published papers. These texts must be bound as an integral part of the thesis.
>
> If this option is chosen, **connecting texts that provide logical bridges between the different papers are mandatory.** The thesis must be written in such a way that is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.
>
> The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". **The thesis must include:** A Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a comprehensive review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.
>
> Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.
>
> In the case of manuscripts co-authored by the candidate and others, **the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent.** Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers. **Under no circumstances can a co-author of any component of such a thesis serve as an examiner for that thesis.**

In this thesis, manuscripts of original papers are presented in **Chapter 2 to 5**. Each paper has its own Abstract, Introduction, Materials and Methods, Results, Discussion, and References. The results which are not included in the papers are described under appendix after each paper in the concerned chapter. A common Abstract, Introduction, a final overall Conclusion, Summary, and Claims to Original Contributions are also included.

Contribution of authors of manuscripts to be published:

Chapter 2 to 5 are manuscripts to be published. I am the first author in all manuscripts, the other author is my Ph.D. Thesis supervisor Dr. T.M.S. Chang. There is no other author involved in all manuscripts.

CHAPTER 1

Introduction

1.1 General Introduction

Melanoma, a fatal skin cancer, now represents the fifth most common type of cancer in North America. The incidence of melanoma has risen dramatically in the last century, doubling every 10 years in many countries [1]. At least 20% of people diagnosed with melanoma will experience advanced disease and die within 5 years of diagnosis [2]. At present there is no optimal treatment for this cancer except for adjuvant therapy with varying clinical results [3-5]. These include immunotherapy, such as interferon α–2b levamisole, vaccines, chemotherapy, autologous bone marrow transplantation, biochemotherapy and chemoimmunotherapy [3-5]. Lowering of tyrosine needed by melanoma can inhibit the growth of melanoma. However, there are no practical methods to lower tyrosine level in human. Since the first report of artificial cells [6], the principle of preparing artificial cells has been developed and investigated by numerous centers around the world (www.artcell.mcgill.ca). This thesis investigates the use of this principle to devise novel methods to lower systemic tyrosine. These consist of

(1) The use of daily oral administration of artificial cells containing encapsulated tyrosinase to enzymatically decrease systemic tyrosine level.

(2) A novel polyhemoglobin-tyrosinase preparation for intravenous injection to lower systemic tyrosine level.

(3) Combination of two intravenous injections of the novel polyhemoglobin-tyrosinase preparation with daily oral administration of encapsulated tyrosinase to

quickly lower tyrosine level and maintained this low level as long as the oral administration of artificial cells was continued.

These three approaches are novel. They demonstrate the first successful use of artificial cells containing tyrosinase to remove systemic tyrosine level required by melanoma growth. The concept investigated in this thesis, therefore, if successful, may establish a precedent for potential use of artificial cells for enzyme therapy and other applications.

In the following sections I will review the relevant literature to date. The concise statement of the objectives of the thesis is summarized at the end.

1.2 Melanoma

1.2.1 History and General

Most skin cancers are not generally considered very dangerous, since they are easily cured by surgery or medicine applied to the skin. The malignant mole called melanoma, which means black tumor, is a notable exception. Not only is melanoma the most malignant of all skin cancers, but if it spreads to other organs, it is among the most malignant of all cancers. In fact, melanoma can spread to nearly every organ and tissue in the body and can lead to death within a year after it recurs in distant sites. Melanoma is also one of the most common tumors to spread to the brain and spinal cord. Therefore, malignant melanoma belongs to the few cancers whose incidence and mortality is increasing every year. Metastatic melanoma represents one of the most difficult tumors to treat, the actual objective response rate of melanoma to

chemotherapy being less than 25% and the cure rate being virtually 0%, while the treatment of early melanoma results in a better prognosis rate which is higher than 25% [7-8]. Accurate early diagnosis of precursor lesions and of primary tumors is therefore of crucial importance. Melanomas are most commonly found on the skin, but 10% arise in the eye. Malignant melanoma of skin has a different type of distribution, 50% or so being found on the lower limbs, less frequently on the trunk, and less frequently again on the arms and skin of the head and neck [9]. There is only one type of malignant cell comprising all melanomas — the malignant pigment-producing cell called a melanocyte.

The symptoms are invariably of a visible lesion on the skin, often painless, which may have been present for some considerable time and is refractory to healing. Ulceration often leads to secondary infection with chronic discharge and occasionally bleeding. Early primary melanomas characterized by radial growth and with limited vertical thickness (< 0.75 mm) show a small tendency to metastasize, while the potential for metastasis formation is associated with the onset of a vertical growth phase and correlates with increasing vertical thickness [10]. One classification categorizes primary melanomas into five levels of invasion that a pathologist can easily recognize under the microscope (Clark's levels) [11]. Level 1 is melanoma at the place of origin, in the basal layer of the epidermis at the epidermal-dermal junction, where the outer and underlying layers of the skin meet. Melanomas in this level are almost always curable by surgical removal of the lesion. Level 2 is extension to the upper third of the dermis, the papillary dermis. Level 3 melanoma extends to the border of the papillary

and reticular dermis. About 20% of patients with melanoma in this level recurrence. Level 4 involves the reticular dermis. Level 5 invades subcutaneous tissue, such as fat. Fifty percent of cases in level 4 and 5 will recur. Melanoma less than 1.5 mm are usually considered highly curable by surgery alone, although some people with such "thin" lesions have had recurrences. Tumors between 1.6 mm and 4 mm are considered to have a moderately deep invasion and have a worse prognosis than thin lesions. Melanomas larger than 4 mm recur in 50% of individuals.

1.2.2 Etiology of Melanoma

The cause of melanoma is multifactorial. Although the exact etiology of melanoma is still unknown, sunlight has been implicated as one of the major factors [12]. Sun exposure may serve as both an initiating factor and a promoting factor in multistage carcinogenesis in the skin. Melanin pigment, which acts as an effective sunscreen, is derived from the melanocytes of the basal layer of the skin [13-14]. Skin tumors are more common in outdoor workers, such as farmers, road workers, etc than in indoor workers. The incidence of skin cancer is much greater in Australia's white skinned populations and Caucasian populations than other general populations due to unfiltered ultraviolet irradiation by their fair complexion. Skin cancer is also common in people who have worked or served in tropical climates during their early life, thus demonstrating the latent interval between exposure to a carcinogenic factor and the subsequent development of cancer. Ultraviolet radiation has a plethora of effects on the skin (immunological, pharmacological, etc.), and experimentally, ultraviolet radiations

B (UVB) are able to trigger cutaneous malignant lesions [15-16]. However, other studies found a lower risk for outdoor workers than for indoor workers [17-18], suggesting that chronic exposure to sunlight may induce deep tanned skin, which are enable to filter the UVB better, and therefore confer a certain degree of protection against their detrimental effects. These puzzling observations indicate that the relationship of cutaneous melanoma to the sun is more complex than one of total exposure. Based on other researches and studies, it appeared that intermittent overexposure to sunlight and sunburns could be the critical factors for cutaneous melanoma, rather than total lifetime exposure to sunlight [19-20].

Certain families have a high incidence of melanoma. The incidence of familial melanoma is 5 to 10%, and for a person with a family history of melanoma, the probability of developing the disease is three to four times the expected sporadic incidence in the general population [21]. Multiple primary melanomas are more frequent (11 to 27%) and arise at an earlier age in members of melanoma families. Dysplastic nevi can apparently serve as melanoma precursors and as indicators of susceptibility to the development of cutaneous malignant melanoma. At present, the presence of multiple large, ugly nevi or of the characteristic histological findings of melanocytic dysplasia in a patient with family history of melanoma requires consideration of dysplastic nevus syndrome in the differential diagnosis [22].

Changes in clothing styles and materials and in recreational habits resulting in increased exposure to sunlight is another factor. Tanning under sunlamps or sunbeds is becoming more prevalent, especially among young people. Modern sunbeds, which

generate only ultraviolet A radiation (UVA), are considered to be harmless. However, recent publications start to question the innocence of UVA, urging the development of sunscreens able to protect against them [23]. Two epidemiologic studies found a significant relationship between cutaneous melanoma and exposure to sunlamps and sunbeds [24].

Many studies have reported an increasing cutaneous melanoma incidence with the increase of socioeconomic status, indicating high socioeconomic status is associated with more time spent in sunny recreational activities [25-26]. Biological research has indicated a possible link of cutaneous melanoma with hormonal and reproductive factors. Certain chemicals like arsenic previously used in treatment of syphilis, are known to be carcinogenic to skin. In this case, an arsenical pigmentation of the skin was produced with an increased incidence of malignancy [9]. The possible role of trauma as a causative factor for melanoma has been controversial. Other factors, such as oral contraceptive use, tobacco use, alcohol consumption, food habits and infectious diseases do not appear to be related to frequency of cutaneous melanoma.

1.2.3 Differentiation and Progression of Melanoma

Malignant melanoma can be considered as a disorder of cell differentiation and proliferation (Figure 1.1). Normal melanocytes, stemming from precursor melanoblasts, usually undergo a series of differentiation events before reaching the final end-cell differentiation state. They can be arrested in their differentiation process at any given state of maturation, without loss of their proliferation capacity [27]. On the other hand,

neoplastic cells can be frozen in their proliferation mode by the accumulation of a proliferation factor or factors (PF). Such an over accumulation of PF completely inhibits the differentiation program of the target cells. However, this arrest at a given immature stage of differentiation can be reversed. A number of substances, such as retinoic acid, corticosteroids, prednisolone, some prostaglandins, phorbol esters, DMSO, and cyclic AMP [28-32], have been reported to induce differentiation. At sufficiently high concentration, differentiation factors (DF) are capable of interrupting the proliferation cycle by curtailing the transcription of mRNA required for the synthesis of PF and by reinducing the differentiation program. The deactivated neoplastic cells have then the possibility to mature to a nonproliferative end state of differentiation.

Histological as well as clinical studies have shown that the evolution of melanomas proceeds through sequential steps of local invasion, and that their malignancy is linked to the invasion level in the skin or the thickness of the primary tumor [10]. On purely histological grounds, one can distinguish melanocytic lesions corresponding to five stages of tumor progression in human melanoma [33]. The first two stages correspond to benign melanocytic lesions, while the three further stages correspond to the development of melanoma of increasing malignancy and to the final step of metastasis. The normal melanocyte in the skin usually shows little tendency to divide. However, primary melanoma cell can grow autonomously *in vitro* and transfer from radial growth phase to vertical growth phase, which is similar to cells in melanoma metastases. At the metastasis stage, melanoma cells are much less dependent

on growth factors and their lifespan is unlimited. The biochemical mechanisms of tumor progression have been studied for many years. A three-step hypothesis has been proposed to describe the invasion of the extracellular matrix [34]. The first step involves attachment of tumor cells through specific surface receptors to components of the matrix such as laminin or fibronectin. The tumor cell then secretes proteolytic enzymes, which degrade the matrix in the near vicinity of tumor cells. The final step is the metastasis of tumor cell, for example, extravasating to reach the organ parenchyma and resulting in a metastasis. The cyclic repetition of these steps leads to continuous invasion of the matrix.

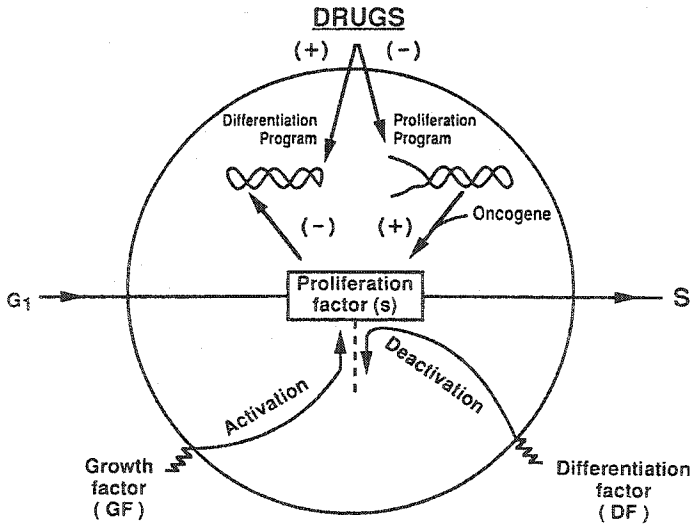

Figure 1.1: Schematic outline of cellular events considered in melanoma differentiation.

1.2.4 Current Approaches for the Treatment of Melanoma

At present, surgical resection is the only effective therapy for primary melanoma. Because melanomas are notorious for early lymphatic spread beyond their clinically detectable borders, the treatment of choice is wide excision. Based on the work of Handley, a 5-cm surgical margin for primary cutaneous melanoma is recommended [35]. Nearby lymph nodes may also be removed if they are involved. However, a major controversy exists over whether or not early lymph node dissection in patients with clinically negative lymph nodes offers a therapeutic advantage over later dissection of obvious nodal metastases. Studies showed that patients in Clinical stage I with micrometastases have a better chance of long-term survival if the nodes are removed before metastases grow to clinically detectable size [36-37]. Surgery is not generally indicated for the treatment of disseminated melanoma, but there are occasional, highly selective indications in which surgery can offer relief of symptoms and improvement in quality of life. For instance, melanoma frequently metastasizes to the gastrointestinal tract and produces bleeding, obstruction, or intussusception. In this case, patients having significant gastrointestinal symptoms may often be symptomatically palliated by resection of the metastatic lesions [38].

Radiotherapy is the application of ionizing radiation to the treatment of malignant disease. The effect of ionizing radiation is to deliver energy, measuring in photons, to the tissues under treatment [9]. Radiation is a particularly useful treatment for lesions at sites in which destruction of tissue is undesirable for functional or cosmetic reasons [39]. Radiation therapy can help shrink isolated large lesions,

particularly nodules, under the skin, or relieve pain, but high individual doses of at least 500 cGy are usually required to overcome the resistance of melanoma cells to radiation. Another important role for radiation therapy in the management of melanoma is in the treatment of brain metastases. A new method of focused radiation (stereotactic radiotherapy) is now replacing neurosurgery in some centers for treatment of one or a low number of small brain metastases. Radiotherapy can combine with other modalities, such as chemotherapy [40], hypoxic cell sensitizers [41] and hyperthermia [42]. It is well established that hypoxic tumor cells are more radioresistant than their oxygenated counterparts, therefore, this forms the rationale for the use of hyperbaric oxygen as a radiosensitizer. A report on this type of treatment in malignant melanoma gave a response rate of 57% [43]. Hyperthermia in conjunction with radiation may improve local control of large lesions because radioresistant hypoxic tumor cells remain sensitive to heat, and there is also evidence that heat has radiosensitizing effects.

Single-drug chemotherapy is effective in no more than 20% of patients with advanced disease. Recent reports suggest that drug combinations can cause responses of more than 50% shrinkage of all tumor masses for at least four weeks in 40 to 64% of cases. Dearbazine (DTIC, dimethyl triazeno imidazole carboxamide) has had the most extensive clinical trials of any single agent in the treatment of melanoma [44]. Nitrosoureas are the next most active single agents. The nitrosoureas are no better than DTIC as single-agent chemotherapy for metastatic melanoma, even at very high doses [44]. Cisplatin is another single agent that has undergone adequate clinical trials [45]. Several observations also suggest that melanomas may be influenced by hormones [46].

Many combination chemotherapy regimens have been tried in phase II and phase III clinical trials in patients with metastatic melanoma to improve response rates. Tamoxifen, DTIC, BCNU (carmustine), cisplatin have been included in some chemotherapeutic regimens with apparent improvement in response rates [47]. However, combination chemotherapy is usually more toxic. Chemotherapeutic regional perfusion was first reported in 1958 for the control of locally recurrent disease [48]. Melphalan has been used most commonly, and it is infused with or without heat. However, hyperthermic regional perfusion has local, regional, and systemic side effects and complications [49]. In addition, high-dose chemotherapy with drugs such as thiotepa or melphalan, followed by replacement of the patient's own stored bone marrow — autologous bone marrow transplantation — has high response rates, but the short duration of remission (about four months) does not justify the considerable risk of mortality (15 to 25%) [50]. This approach to treatment needs further investigation at this time.

Passive immunotherapy employs tumor-directed antibodies in an attempt to induce antibody-dependent cellular cytotoxicity. Melanoma antigens are among the best-characterized human tumor antigens, for instance, direct intralesional injections of bacilli Calmette-Guerin (BCG) resulted in greater than 90% of cutaneous melanoma nodules regressing [51]. In the last 10 years, biologic therapy of cancer using recombinant cytokines has been best studied. These include interferons α-2 (IFN α-2), interleukin-2 (IL-2), tumor infiltering lymphocytes (TIL), tumor necrosis factor (TNF) [52-55]. However, side effects experienced with interferon include fever, chills and

fatigue. The high cost of interferon is also a drawback due to the costly treatment and low response rate. Many other biologic agents are on the horizon for employment in melanoma clinical trials, including IL-1, IL-4, IL-6. Active specific immunotherapy (melanoma vaccines) has also shown considerable promise in treating advanced melanoma and theoretically should be even more effective in early stages of disease. There are no side effects from the vaccine, thus this makes it one of the easiest cancer treatments to tolerate.

The lack of effective treatment for advanced disease and the well-documented reports of occasional spontaneous remissions in patients with malignant melanoma have made this disease a common target for experimental gene therapy protocols. The strategy for gene therapy is attempt to immunize patients with the gene coding for a melanoma antigen to stimulate an antitumor immune response, with special emphasis on effective means to target these melanoma antigens for presentation by host antigen presenting cells. Other areas of research in gene therapy for melanoma include the use of specifically targeted replication-defective viral vectors, intended to deliver a toxic gene directly into the tumor cell by virtue of targeting a specific surface marker present on melanoma cells but not normal cells [56].

Other adjuvant treatments for melanoma have also been investigated, such as adjuvant cytostatic therapy, adjuvant combined cytostatic and immunotherapy, and secondary adjuvant indicator therapy [57-60].

1.2.5 Metabolism of Tyrosine in Melanoma

The initial, rate-limiting step in pigment production is the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA), which is catalyzed by tyrosinase (monophenol monooxygenase EC 1.14.18.1) [61-62]. The melanin biosynthetic pathway diverges into the production of eumelanins (brown to black pigments), or pheomelanins (yellow to red pigments) (Figure 1.2). Melanin is a sunscreen. Its formation in the melanocyte is enhanced upon exposure to ionizing and ultraviolet radiation. Melanin may protect cells from such radiation by its function as a conductor and a repository of electrons produced by photoenergy [63-64]. Therefore, melanin is used commercially as a component of photoprotective creams, mainly for its free radical scavenging [65]. Regardless of the type of pigment formed, tyrosine is an absolute requirement for the initial step. However, this tyrosinase-mediated melanin biosynthesis is highly elevated in malignant melanoma cells when compared to normal melanocytes [66].

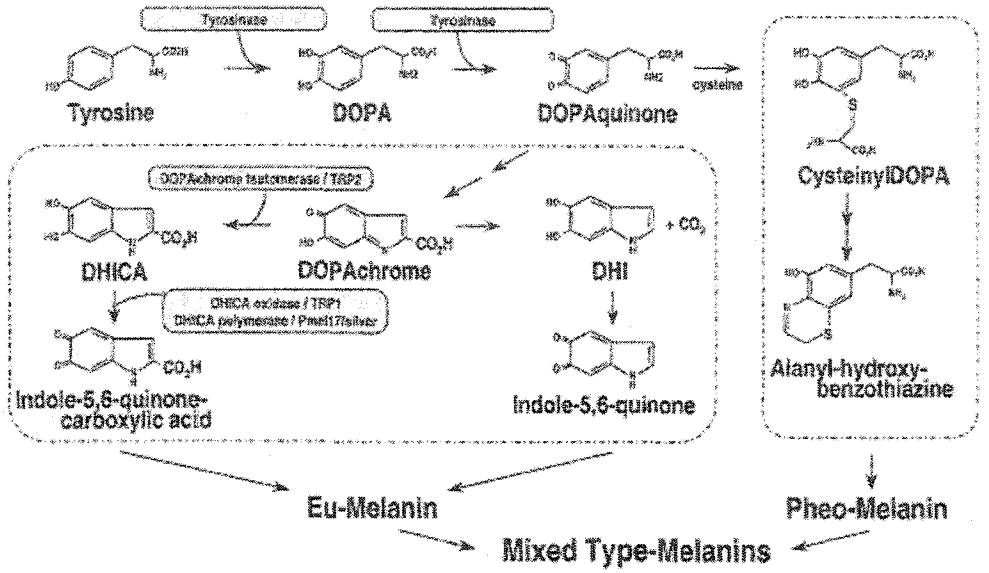

Figure 1.2: Metabolism of tyrosine and biosynthesis of melanin.

1.2.6 Dietary Therapy with Restriction in Tyrosine

Tyrosine plays a key role in the growth of melanoma cells because it is used for both melanin and protein synthesis. Researches found that malignant melanoma has a higher tyrosine requirement than normal cells, and dietary restriction in tyrosine intake can inhibit the growth of melanoma [67-75]. The rationale was that the large quantities of active tyrosinase in these tumors play a vital respiratory role in pigmented melanoma [76]. Therefore, the limitation of substrate, i.e. tyrosine, might result in selective energy deprivation. In order to decrease nutrient availability to the tumor, plasma phenylalanine and tyrosine concentration must be constantly kept at below normal level. Although this regimen is of benefit to some metastases, it is cumbersome, complex and unpalatable, thus make its application difficult in human [76-77]. On the

other hand, in animal studies Lorincz *et al* found that significant inhibition of tumor growth by amino acid deficiency, the host animals suffered severe body weight loss. The clinical application of such diets to human patients was thus contraindicated [78]. Later, Meadows *et al* suggested that dietary phenylalanine and tyrosine could be increased to a level that would maintain host weight and increase survival of tumor-bearing mice [79].

1.3 Artificial Cells

1.3.1 The Concept of Artificial Cells

The cell, a basic unit of all living animals and plants, consists of an aqueous interior surrounded by an ultrathin membrane. Chang [6,80-83] first stated the concept of artificial cells. He emphasized in his 1972 monograph [83] that "Artificial cell is not a specific physical entity. It is an idea involving the preparation of artificial structures of cellular dimensions for possible replacement or supplement of deficient cell functions. Many approaches can be used to demonstrate this idea." Since then Chang and many groups have developed and extended this idea of artificial cells [81-224].

1.3.2 General Principles of Artificial Cells

Like all living cells, each artificial cell consists of a selectively permeable membrane of cellular dimensions, enveloping biologically active materials. Therefore, the ultrathin membranes can retain macromolecules such as cells, proteins, enzymes and other synthetic materials. Simultaneously the large surface area and ultrathin

membrane allow the rapid diffusion of smaller molecules, such as substrates, products, and cofactors. In this way, each artificial cell retains the enclosed materials and separates them from undesirable external environment. A schematic representation of artificial cell is shown in Figure 1.3.

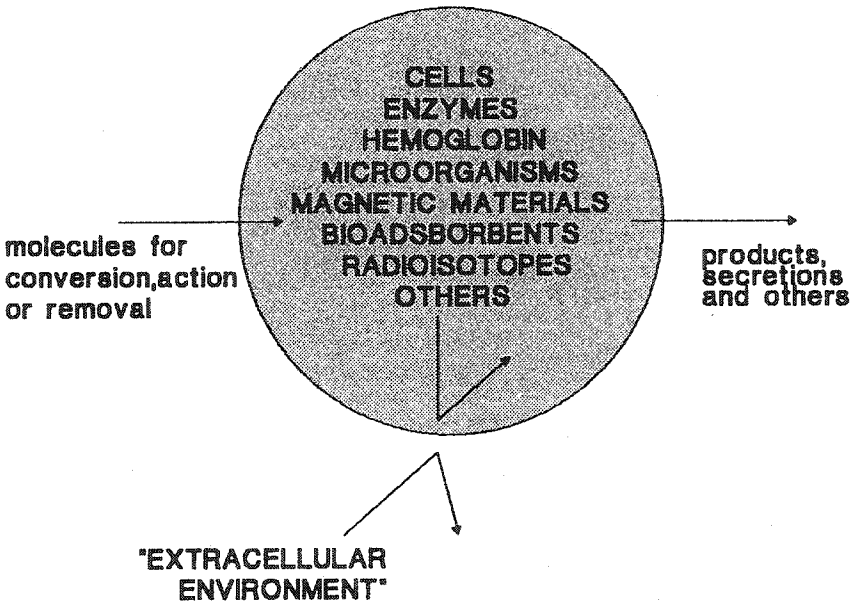

Figure 1.3: A schematic illustration of the artificial cell.

1.3.3 Properties of Artificial Cells

1.3.3.1 Contents

Like biological cells, artificial cells contain many biologically active materials. The content of artificial cells can be more varied than biological cells. Theoretically, an artificial cell can contain virtually anything [6,80-224]. Some of the materials encapsulated are enzymes, proteins and cell extracts [6,81-224]. Multienzyme systems and a combination of enzyme and adsorbent have also been encapsulated [6,123-128].

Enzymes have also been encapsulated together with a magnetic material. An external magnetic field therefore can be used to direct artificial cells to some specific location [94,123]. Other materials which have been encapsulated include radioisotope-labeled materials [94,123], insolubilized enzymes [83,129], coenzyme recycling multienzyme systems [96,130-142], antigens, antibodies, vaccines, hormones and pharmaceuticals [94,143-144].

As will be described in more detail later, microencapsulation of living cells and microorganisms has also been developed for possible clinical applications [83,114,116,123,145-146]. Microencapsulation of other mammalian cells, such as human erythrocytes and human diploid fibroblasts, has also been investigated and accomplished [158-161]. More recently, Huang *et al* found that co-immobilization of algae with bacterium increased the anabolic activity of algae, thus increasing the decolorization capacity of algae for dye [162].

1.3.3.2 Membrane

Each artificial cell has a spherical ultrathin selectively permeable membrane. The membranes of artificial cells can be extensively varied using many different types of synthetic or biological materials. Chang made the first artificial cells by interfacial precipitation of cellulose nitrate [83]. Since then, about 30 different polymer membranes and several protein membranes have been created, such as collodion membranes, nylon membranes, complex lipid membranes, silicone rubber membranes, and alginate-polylysine-alginate membrane [83,94,110,123,153-154]. Numerous

chemical reactions are presently being used for making artificial cells by other interfacial polymerization methods [6,83,123,126-127,136,163-165]. Chang also developed multiple-compartmental membrane systems to envelop small artificial cells within larger artificial cells [83,114]. A similar double-emulsion approach was evolved using a special hydrocarbon liquid to form a liquid-surfactant membrane [166-167].

Biological and biodegradable membranes are also available for encapsulating cells, enzymes and other biologically active materials. These membranes include cross-linked protein membranes [6,83,94,123], heparin-complexed polymer membranes [168], lipid membranes [169], lipid-polymer membranes [83,137,170-172], liposomes [173-178] and erythrocytes [179-180]. Biodegradable polymer membranes, such as polylactic acid membrane or polyglycolic acid membrane, have also been used to encapsulate cells, hemoglobin, enzymes, antibiotics or hormones [143,181-183]. In addition, Chang further improved the method for making blood compatible membranes by showing the required permeability, surface properties and blood compatibility [83,94,143]. Other groups have also developed artificial cells with liquid membrane [166-167], fiber extrusion [184-185], and hollow-fibers [186-189].

In systems employing artificial cells, the semipermeable membrane prevents encapsulated macromolecules from leaking out and thus separates them from the undesirable external environment [6,83,114]. Artificial cells therefore can reduce immunological problems arising in the host. However, small permeant molecules such as amino acids can equilibrate rapidly across the membrane and contact the encapsulated materials. Furthermore, proteolytic enzymes existing in the surrounding

environment cannot cross the membrane to deactivate the encapsulated enzymes. These considerations are the rationale for using artificial cells in enzyme therapy.

One of the most important characteristics of artificial cell is that it has a large surface area to volume ratio. For example, 10 milliliters of 20 μm diameter artificial cells have a total surface area of 2.5 M^2 [124]. This area is more than twice that of the membrane present in the standard hemodialysis machine (1 M^2). Furthermore, each artificial cell has an ultrathin membrane of 20 nm thickness, which is at least 100 times thinner than that of the standard hemodialysis machines [83,123]. Thus, artificial cells allow external permeant molecules to equilibrate much faster than in standard hemodialysis machines.

1.3.3.3 Permeability

Artificial cells will vary in shape depending on the suspending medium. For example, artificial cells suspended in distilled water are smooth and round in shape, while in hypertonic solution they quickly shrink and show folds in their membrane. The permeability can be controlled over a wide range, thus permitting the selective permeation of various types of molecules. The pore radius can vary depending on its preparation. These include variations in polymers, proteins, lipids, polysaccharides, charge, pores, thickness and surface properties [83,170]. Ultrathin membranes can retain macromolecules, such as proteins and enzymes. The enclosed impermeant materials are therefore prevented from escaping into the extracellular environment where they might be excreted, metabolized, destroyed or develop harmful effects. At

the same time, by simple diffusion or special carrier mechanisms, permeant molecules, like products and secretions, can equilibrate rapidly across the membranes to be acted upon by the cell contents. This way, each artificial cell retains the enclosed materials and separates them from undesirable external impermeant materials, while the large surface area and the ultrathin membrane allow permeant substrates and products to diffuse rapidly [83,99,190].

1.3.3.4 Dimension

Variations in dimensions are also possible. Dimensions depend on the type of use and contents, and it can be varied from millimeters to nanometers [83,126-127,152-155,182-183]. For instance, the dimension for encapsulated cell cultures, microorganisms, tissues and others is in millimeters. Microencapsulated enzymes, proteins, organelles, cells can be in micrometers [191-193]. For nanoencapsulated of enzymes, proteins, peptides, antibodies, hemoglobin, its dimension is in nanometers. For further smaller dimension, it will be the crosslinked hemoglobin as red blood cell substitutes in angstroms [205].

1.3.4 Applications of Artificial Cells

Artificial cells also have many areas of research and clinical applications. Artificial cells containing adsorbents have been routinely used as a form of treatment in hemoperfusion for patients with some types of poisoning. This makes use of on ultrathin membrane coating of sorbent granules [83-84,195-198]. Sorbents like

activated charcoal, resins and immunosorbents cannot be used in direct blood perfusion since they release fine particles into the blood and damaging blood cells by these biosorbents. However, these sorbents once encapsulated into the artificial cells, they did not release particles or damage blood cells [123,199-202].

Red blood cell substitutes based on modified hemoglobin are already in Phase I to Phase III clinical trials in patients. The microencapsulated hemoglobin not only carries oxygen, but also stays long in the circulating time after modification of surface properties and diameter [6,80,83,114,123,200-201,203-205]. More recently, Chang's group crosslinked superoxide dismutase and catalase to polyhemoglobin to add antioxidant properties in blood substitutes in conditions of ischemia reperfusion [194,206-207].

Encapsulated cell cultures are being studied for the treatment of diabetes, liver failure, gene therapy and other conditions. This procedure was proposed for use in the encapsulation of islets for the treatment of diabetes and hepatocytes for the treatment of liver failure [83,114,123]. Lim and Sun [102] microencapsulated rat islet cells, which were then implanted intraperitoneally into diabetic rats. These encapsulated islet cells were able to maintain normal glucose levels in the diabetic animals for up to one year. Artificial cells containing fibroblasts or hybridomas have also been used in biotechnology for the production of interferon and monoclonal antibodies [147]. Ergan *et al* [148] immobilized a bacterium containing NADH-oxidase by microencapsulation. This bacterium could recycle NADH back to NAD^+ within artificial cells in the presence of oxygen. Rat hepatocytes were also encapsulated within alginate-polylysine

membranes and served as a liver support system [149-151]. The encapsulated hepatocytes remained viable and were able to synthesize protein for up to 3 weeks in culture [149]. Wong and Chang's studies indicated that intraperitoneally implanted artificial cells containing hepatocytes increased the survival time of fulminant hepatic failure rats [150]. When co-encapsulated with bone marrow cells, the viability of hepatocytes in culture medium is prolonged to 28 days [151]. Further animal studies demonstrated that co-encapsulation of hepatocytes and bone marrow stem cells could improve hepatocyte viability when implanted into normal rats [153]. This new approach may provide the potential feasibility of using co-encapsulation of hepatocytes and stem cells in bio-artificial liver support for the treatment of liver failure. Recently, Prakash and Chang found that encapsulated genetically engineered *E. coli* DH5 cells, containing the *Klebsiella aerogens* urease gene, can efficiently remove urea from uremic rats with induced renal failure [154-157]. Artificial cells containing encapsulated cells and tissues were also used for biotechnological, food technological, biomedical applications.

Artificial cells containing enzymes used for treatment in hereditary enzyme deficiency diseases and other diseases have been extensively explored for many years [81-83,98-99,208]. The use of artificial cells containing catalase is the first model of the use of artificial cells for experimental enzyme therapy [81]. Oral administration of artificial cells containing urease has been found to efficiently remove the urea in the intestinal tract of rats [83-85,124]. Furthermore, enzymes in artificial cells do not leak out and the artificial cells remain intact. This did not cause hypersensitivity or

immunological reactions even with repeated injection [209]. The applications of microencapsulated urease have paved the way for other types of enzyme replacement therapies. Microencapsulated catalase by collodion membrane was studied by intraperitoneal administration into mice with a hereditary catalase deficiency, i.e. acatalasemic mice [81,209]. The results showed that artificial cells containing catalase are efficient in replacing the function of the deficient catalase enzyme. Later, Bourget and Chang developed artificial cells containing phenylalanine ammonia-lyase for the depletion of systemic L-phenylalanine in phenylketonuric rats [111-113,210]. Oral administration of high dosages not only lowered the plasma L-phenylalanine level to normal, but also returned behavior patterns to normal [211]. Oral administration of xanthine-oxidase artificial cells was used to lower systemic hypoxanthine in Lesch-Nyhan disease, which is an inborn error of metabolism due to hypoxanthine phosphoribosyltransferase deficiency [212-213]. Within one week there was a fall in plasma hypoxanthine level by administration of artificial cells containing xanthine-oxidase. L-asparaginase has also been microencapsulated into artificial cells to study the effect on mice with substrate-dependent tumors. It has been found that artificial cells containing L-asparaginase suppress the growth of implanted lymphosarcoma cells more efficiently than L-asparaginase given in free solution form [82,91,121,163,214-215]. Another example of the biomedical uses of artificial cells is the use of microencapsulated tyrosinase to remove L-tyrosine in a perfusion system in fulminant hepatic failure rats or for tyrosinemia animals [118-120,216]. Other conditions include encapsulated histidinase for histidinemia [217]. As far as specificity is concerned,

artificial cells containing enzymes are superior to ordinary hemoadsorbents. A summary of these and other biomedical and biotechnological applications of artificial cells is given below. (Table 1.1).

Acute poisoning	Routine clinical treatment
Aluminum and iron overload	Routine clinical treatment
End-stage kidney failure	Routine clinical treatment as supplement to dialysis
Liver failure	Routine clinical application for limited types of acute liver failure
Red blood cell substitutes for transfusion	Advanced phase III clinical trial
Enzyme defects in inborn errors of metabolism	Clinical trial
Monoclonal antibodies production from hybridomas	Experimental and production
Diabetic mellitus (bioencapsulated islets)	Phase I clinical trial
Bioartificial liver (bioencapsulated hepatocytes)	Experimental
Gene therapy using bioencapsulated cells or microorganisms	Experimental
Drug delivery systems	Experimental and routine clinical use
Blood group antibodies removal	Clinical trial
Clinical laboratory analysis	Experimental
Conversion of cholesterol into carbon dioxide	Experimental
Bilirubin removal	Experimental
Production of fine biochemical	Industrial application
Aquatic culture	Industrial application
Conversion of wastes (e.g. urea and ammonium) into useful products (e.g. essential amino acids)	Experimental
Other biotechnological and medical applications in progress	

Table 1.1: Summary of biomedical and biotechnological applications of artificial cells.

1.3.5 Routes of Administration of Artificial Cells Microencapsulated Enzymes

In order for the immobilized enzyme to have any therapeutic value, the artificial cells have to be in a location where they come in contact with a readily available supply of substrate. Chang's group and other groups have studied a number of routes of administration [6,83,86,114,123,218-219].

1.3.5.1 Local Implantation by Injection

Artificial cells prepared as a suspension can be injected intramuscularly, subcutaneously or intraperitoneally [6,81,123,209]. With these injections, substrates in the blood can diffuse through the artificial membrane to reach the immobilized enzyme. Injections are an easy way to introduce large amounts of enzyme, which are able to come in contact with substrate equilibrating across the peritoneal membrane. However, if repeated injections over a long period are required, the injected materials will accumulate in the body. This is especially imperfect because unlike encapsulated cells enzymes have a short duration of action in the body.

1.3.5.2 Intravenous Injection

For this route, artificial cells have to be prepared in the nanometer dimension as nanocapsules. They can also be prepared as a soluble polyhemoglobin-enzyme macromolecule.

1.3.5.3 Extracorporeal Shunt System

Taking advantage of the fact that the substrate to be acted on is in the bloodstream or in the body fluid, one may not necessarily have to inject the immobilized enzyme inside the body, since immobilized enzymes can be used in an extracorporeal shunt. This shunt, connected arterio-venously, would allow blood to recirculate through the system. Three examples of this system include immobilized urease used to remove urea [6,83,94,114,123,129,213], extracorporeal shunts containing microencapsulated catalase in the removal of perborate in the peritoneal fluid of acatalasemic mice [81,209], and artificial cells containing tyrosinase to remove systemic tyrosine for hemoperfusion in galactosamine-induced fulminant hepatic failure rats [118-120]. However, this requires the use of extracorporeal circulation.

1.3.5.4 Oral Administration into the Gastrointestinal Tract

Microencapsulated enzymes may be given orally to act in the intestine. However, this can only be used if the substrates can equilibrate across the gastrointestinal membrane to reach the artificial cells in the intestine. It has been found by Chang's group that there is an enterorecirculation of amino acids [210]. As a result, amino acids can reach the intestine in high concentration [81,83,87,111-113,124-125,129,131,210-211,223].

1.3.6 Methods of Preparation of Artificial Cells Containing Enzymes

Methods have been developed for depositing thin stable semipermeable polymer membranes around aqueous microdroplets by interfacial polymerization (nylon membrane) or interfacial coacervation (collodion membrane). The enclosed aqueous phase may contain enzymes or other proteins, cell fragments or intact cells. The successful procedures have so far involved three main steps (Figure 1.4). (A) The aqueous protein solution is emulsified in an organic liquid with the aid of a suitable detergent Span 85. (B) By the addition of a suitable material to the continuous phase, a polymer membrane is formed around each microdroplet. Membrane formation may be either interfacial polymerization or by interfacial coacervation. (C) With the aid of suitable solvents and detergents Tween 20, the newly formed microcapsules are removed from the organic phase and suspended in an aqueous medium.

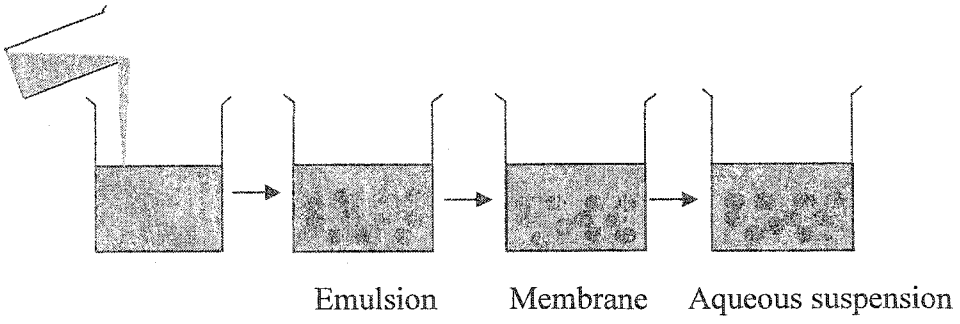

Figure 1.4: Preparation of small artificial cells.

Interfacial coacervation (collodion membrane) is a physical process depending on the lower solubility of the polymer at the interface. In this procedure, the polymer used to form the microcapsule membrane is the familiar mixture of cellulose nitrate known as collodion. Cellulose nitrate membrane microcapsules are prepared using an updated procedure based on previous publications [6,83,157,203,224]. Multienzymes and other materials to be microencapsulated are dissolved or suspended in hemoglobin solution. Water saturated ether is used as the organic phase. After first emulsifying, the collodion solution is added, and allowed to stand for 45 minutes. During this period, the cellulose ester gradually precipitates at the interface of each microdroplet. Then these microcapsules are transferred into another medium — n-butyl benzoate, which can help complete the formation of the membranes. At last, Tween 20 is used to transfer the microcapsules from the organic-liquid phase into an aqueous phase.

Hemoglobin plays an important role in the aqueous phase during microencapsulation. The presence of proteins decrease the surface tension and this facilitates the formation of the membrane. It was well known that a high concentration of protein solutions would increase the stability of enzymes. Also, the proteins within the artificial cells prevent the collapse of the artificial cells due to a colloid osmotic pressure gradient. Furthermore, proteins are polyamines containing many amino groups, which may participate in the formation of membrane by crosslinking with diacids. Therefore, the presence of proteins greatly strengthens the artificial cell membranes [6,114,164,203].

In the case of enzymes, once microencapsulated they stay inside the artificial cells. The enzymes are separated from external macromolecules like tryptic enzymes, cells and antibodies. The ultrathin polymer membranes allow substrate molecules to rapidly diffuse into the artificial cells. The enzyme in the artificial cells converts the substrate into its products which then diffuses out.

1.4 Aims and Objectives

My present study is a detailed analysis of a novel therapeutic approach using microencapsulated tyrosinase and polyhemoglobin-tyrosinase to lower systemic tyrosine in animals for the potential use in the treatment of melanoma. To reach these aims and objectives, the following 3 phases of research were carried out. In the first phase, I work with the procedure for microencapsulation of tyrosinase. Then I microencapsulate tyrosinase and analyze the enzyme activity at different concentrations of encapsulated tyrosinase. Before performing any studies in animals, I carry out detailed studies on enzyme kinetics for free and encapsulated tyrosinase, and analyze the effects of pH, temperature stability on enzyme activity. I also assess *in vitro* the action of encapsulated tyrosinase on rat intestine contents. Finally, *in vivo* animal experiments, I study the efficiency of encapsulated tyrosinase by oral administration.

The second phase involved the preparation, determining of the structural and functional properties of crosslinked hemoglobin tyrosinase (PolyHb-tyrosinase). Glutaraldehyde, a non-specific dialdehyde crosslinker, is used to polymerize hemoglobin and tyrosinase. This process produces a heterogeneous mixture of

molecules varying in size and protein composition. I, therefore, analyze the molecular weight distribution of PolyHb-tyrosinase and tyrosinase activity corresponding to the molecular weight. Oxygen carrying and releasing characteristics of PolyHb-tyrosinase and temperature stability of PolyHb-tyrosinase are also studied. In detailed animal studies, I test and optimize the feasibility of PolyHb-tyrosinase samples at different crosslinking times, or by intravenous injection at different volumes or at different enzyme concentrations.

In the third phase, to design a method which can quickly decrease systemic tyrosine level and keep it at that low level as long as it needs, I combine the oral administration of encapsulated tyrosinase method with the intravenous injection method. This novel biotechnological approach opens the potential for lowering tyrosine in human for melanoma therapy.

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CHAPTER 2

In-Vitro Enzyme Kinetics of Microencapsulated Tyrosinase

2.1 Abstract

In this study, we prepared microencapsulated tyrosinase and studied its enzyme kinetics and its stability at various pHs and temperatures. The K_m for both free and microencapsulated tyrosinase is 465 μM. The V_{max} for the microencapsulated tyrosinase is 49.02 mg/dl·min, whereas that of tyrosinase in free solution is 114.95 mg/dl·min. At the extreme pHs of pH 2 and pH 10, free tyrosinase lost all its enzyme activity, whereas microencapsulated tyrosinase retained 14% of its original activity at pH 2 and 17% of its original activity at pH 10. Further *in vitro* studies were carried out to study the pH optimum of the microencapsulated enzyme at different pH values corresponding to those along the gastrointestinal tract. In temperature stability studies at 4°C and 37°C, enzyme activity remained higher in microencapsulated tyrosinase solution than that of free enzyme solution. Therefore, our results show that microencapsulated tyrosinase is significantly more stable than free tyrosinase.

2.2 Introduction

More than four million people will be diagnosed with melanoma in the first decade of the 21st century. Half of those who will die will be individuals who would otherwise have had a life expectancy of another 25 years or more [1]. One unique characteristic is that the tyrosine requirement for malignant melanoma is much higher than for normal cells since tyrosine is needed for both protein and melanin synthesis [2]. Tyrosine is a semi-essential amino acid, derived from the liberation of tyrosine by hydrolysis of dietary or tissue protein. Although at present there is no optimal treatment for this cancer, earlier studies showed that lowering tyrosine level in experimental animals would inhibit the growth of established melanoma tumors [3-6]. Meadows *et al* [3,7] used tyrosine phenol-lyase (TPL) to lower tyrosine level in order to retard the growth of melanoma. However, tyrosine phenol-lyase needs pyridoxal phosphate (PLP) as a coenzyme to catalyze the conversion of L-tyrosine to phenol, pyruvate and ammonia [8]. Due to the extremely short intravenous half-life (about 15 min) of this cofactor and problems related to the large-scale isolation and purification of the enzyme TPL from *Erwinia herbicola*, it made this enzyme inapplicable. The enzyme tyrosinase is another enzyme which converts tyrosine to L-dopa (L-3,4-dihydroxyphenylalanine), and then further oxidizes L-dopa to L-dopaquinone. This enzyme requires no co-factors and can be easily obtained from mushrooms. The L-dopaquinone product is one of the intermediates in melanin synthesis [9]. The melanin biosynthetic pathway diverges into the production of eumelanins (brown to black pigment), or pheomelanins (red or yellow) pigment. Although in normal human skin eumelanin is the major pigment,

small amounts of phenomelanin can be seen in certain ethnic groups [10]. Melanin is a powerful cation chelator and may act as a free radical sink [11].

We, therefore, choose tyrosinase from mushroom to lower systemic tyrosine level. Previous studies showed that the high concentration of blood tyrosine can be decreased by charcoal perfusion or tyrosinase artificial cells, but charcoal adsorption is non-specific and removes both unwanted and essential substances [12-15]. Tyrosinase artificial cells can degrade specific amino acid, tyrosine, and therefore remove this unwanted amino acid. Based on the theory of enterorecirculation of amino acid [16], the specific amino acid is therefore removed and not reabsorbed into the body. If we can remove tyrosine from rat's intestine, it will not be reabsorbed into the body. Then, tyrosine concentration in blood will be kept in a certain low level. Furthermore, it would be more convenient if tyrosinase artificial cells could be administrated by the oral route. The present study is to microencapsulate tyrosinase inside artificial cells and to study the *in vitro* enzyme kinetics and the enzyme stabilities at different pH and temperatures. This is the first part of the study to see if microencapsulated tyrosinase is potentially suitable for potential oral administration in later studies.

2.3 Materials and Methods

Materials

L-tyrosine (98% TLC), L-tyrosine disodium salt, tyrosinase from mushroom (EC. 1.14.18.1, 3400 units/mg stated activity), hemoglobin from bovine (lyophilized powder) were purchased from Sigma Company. Collodion was purchased from Fisher Scientific Company. All other reagents were of analytical grade.

Preparation of tyrosinase loaded microencapsules

Various quantities (1.5 mg, 3 mg, 6 mg, 9 mg) of tyrosinase were dissolved in 2.5 ml of 10% hemoglobin solution respectively, then follow the methods described [17-22] to immobilize tyrosinase in collodion membrane microcapsules. Microcapsules prepared were analyzed as a 50% suspension. For tyrosinase in free solution, the same quantities of tyrosinase as loaded in artificial cells were dissolved in 0.05 M pH 6.5 potassium phosphate buffer.

Assessment of free and microencapsulated tyrosinase activity

Tyrosinase activity was assessed by measuring the formation of enzymatic products, at 300 nm [23]. All other substances present in the reaction mixture, i.e. tyrosine, tyrosinase, and hemoglobin had no significant adsorption at this wavelength. All reactions were carried out at 23°C. The total volume of tyrosine solution in each case was 3 ml, with 0.25 ml of artificial cells suspension added. The adsorbance at 300 nm was followed continuously for 8-14 minutes using Perkin Elmer Lambda 4B spectrophotometer, and changes in O.D./min were used to analyze the activity of the

enzyme. Then, change those data to product concentration mg/dl per minute. Therefore, the changing in product concentration (mg/dl) per minute is used for tyrosinase reaction velocity.

Studies of V_{max} and K_m in free tyrosinase and apparent V_{max}, apparent K_m in microencapsulated tyrosinase

For studies of V_{max} and K_m value, 1020 units/3ml were chosen for this assessment for free tyrosinase and microencapsulated tyrosinase. To test whether there is leakage of the enzyme from the microcapsules, at regular intervals an aliquot of the supernatant was tested for enzyme activity. The concentration of L-tyrosine used was 5 mg%.

pH studies in free and microencapsulated tyrosinase

The activities of microencapsulated tyrosinase at different pHs were measured and compared with tyrosinase in free solution.

Temperature studies in free and microencapsulated tyrosinase

The activities of microencapsulated tyrosinase after incubating at 4°C and 37°C were measured and compared with tyrosinase in free solution.

2.4 Results

Enzyme activity in free and microencapsulated tyrosinase

This is to optimize the preparation of microencapsulated tyrosinase for later use in oral administration. Figure 2.1 shows the activity of free tyrosinase and microencapsulated tyrosinase. For free enzyme, increases in tyrosinase concentration resulted in increase in reaction velocity. We assessed the enzyme activity for free tyrosinase and free tyrosinase in hemoglobin solution and found that there was no significant difference between free enzyme activity and enzyme activity in hemoglobin solution. Therefore, the interference due to the present of hemoglobin in the reaction mix can be ruled out. For microencapsulated enzyme, the reaction activity is increased when the concentration of tyrosinase inside the microcapsules is kept constant, but the volume of microcapsules is increased. However, the activity obtained from microcapsules is lower than enzyme in free solution. To analyze the reason for this, we broke the membrane of microcapsules to release the enzyme and tested the activity of enzyme. The results show that the activity of enzymes extracted from the microcapsules is only very slightly higher than that of the intact microcapsules. This shows that the decrease in enzyme activity after encapsulation is due to the inactivity of some enzyme during the preparation procedure and not due to mass transfer restrictions. This is only true when the enzyme concentration inside the microcapsules is as stated above. Since if the enzyme concentration inside the microcapsules is increased stepwise but the volume of microcapsules was kept constant, then the reaction activity is not increased corresponding to the increasing enzyme concentration. With the increase in enzyme

concentration, the rate of enzyme reaction inside the microcapsules is much faster than the mass transfer of the substrate. This important observation allows us to design tyrosinase microcapsules containing the optimal amount of enzyme.

V_{max} and K_m

This is to characterize the enzyme kinetics of the preparation and comparing this microencapsulated tyrosinase preparation to free tyrosinase. Figure 2.2 shows the activity of free tyrosinase in different concentration of tyrosine. The V_{max} for free tyrosinase is 114.94 mg/dl·min, and K_m is 4.65×10^{-4} M. Then, we studied the apparent V_{max} and the apparent K_m value for microencapsulated tyrosinase (1020 U/3ml). The leakage of microencapsulated tyrosinase is also measured. Figure 2.3 gives the apparent V_{max} is 49.02 mg/dl·min, and its apparent K_m is 4.65×10^{-4} M. There was no detectable tyrosinase activity in the supernatant and therefore no leakage of the enzyme through the collodion cell membranes.

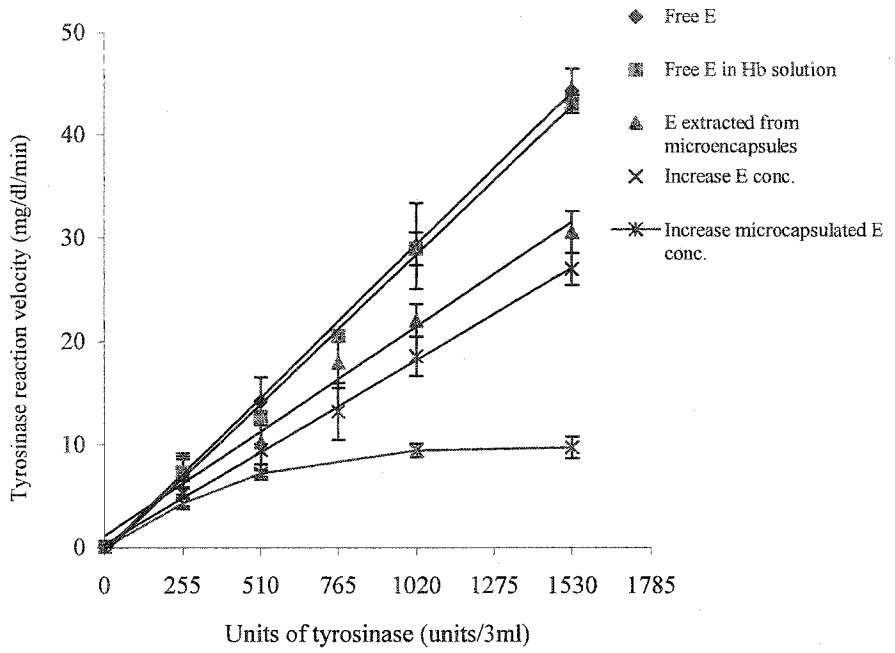

Figure 2.1 Tyrosinase activity in artificial cells at different volumes and enzyme concentrations when compared with activity of free tyrosinase. The lowest curve (Increase microencapsulated E conc.) represents increasing the internal concentration of enzyme inside the microcapsules, but the volume of microcapsules was kept constant. The second lowest curve (Increase E conc.) represents keeping the microcapsule internal enzyme constant, but increasing the number of microcapsules. The third curve (E extracted from microencapsules) represents the tyrosinase activity extracted from microcapsules. The first two curves represent the free tyrosinase activity in buffer and hemoglobin solution respectively. Curves represent the mean of three determinations for each sample.

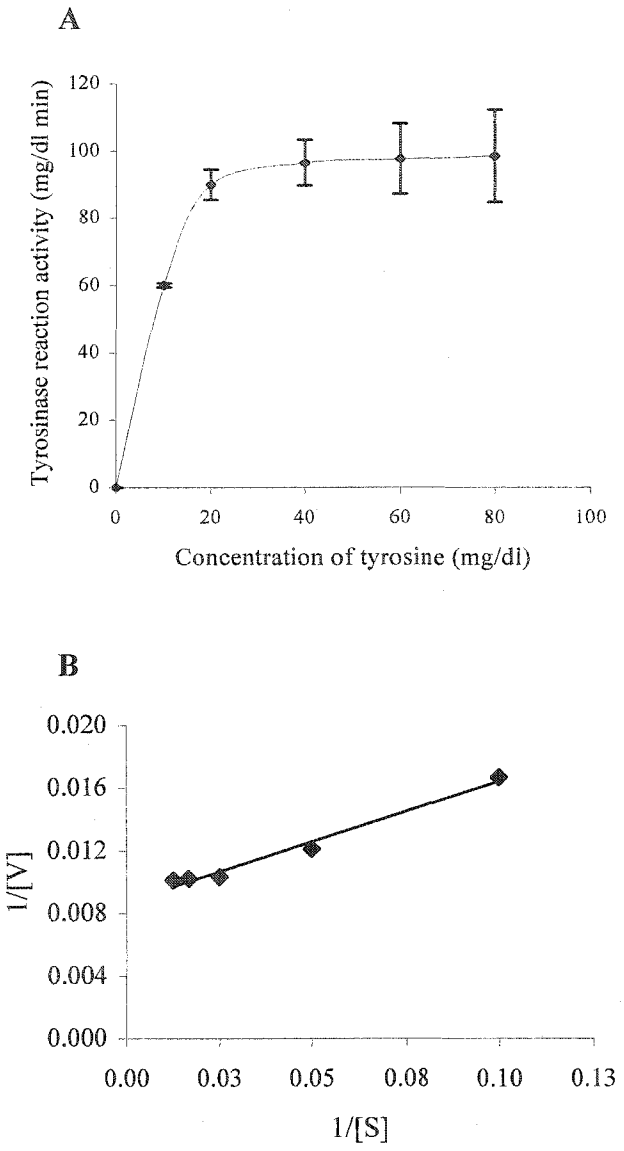

Figure 2.2 (A) The activity of free tyrosinase (1020 U/3ml) in different concentrations of tyrosine. (B) A double-reciprocal plot of activity of free tyrosinase (1020 U/3ml).

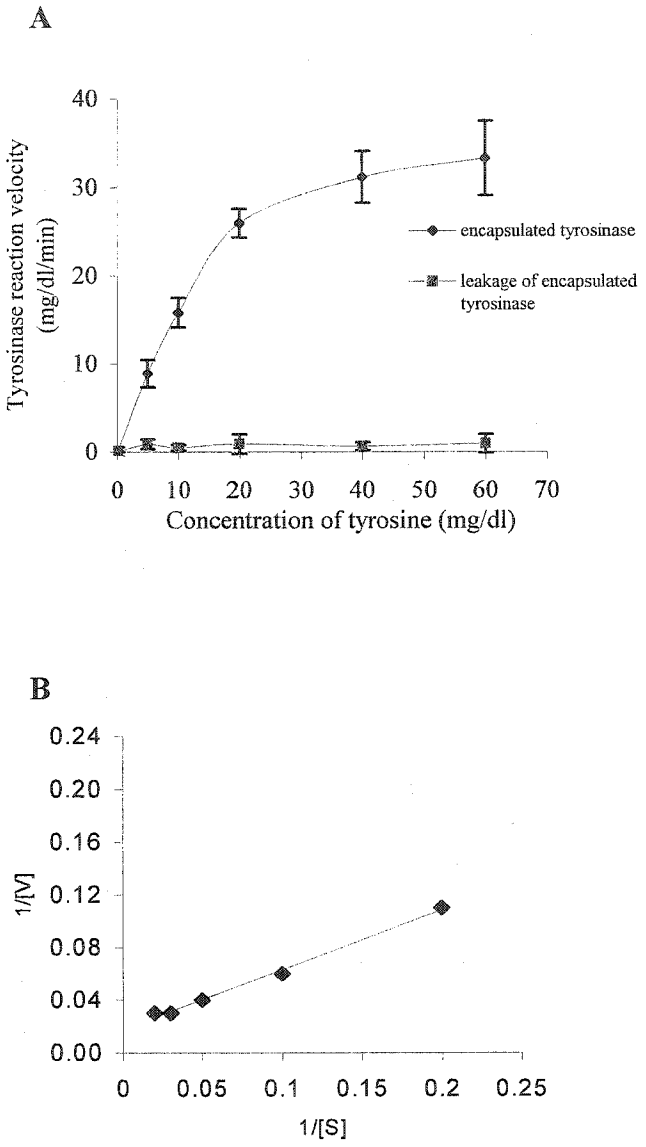

Figure 2.3 (A) The activity of microencapsulated tyrosinase (1020 U/3ml) in different concentrations of tyrosine. (B) A double-reciprocal plot of activity of microencapsulated tyrosinase (1020 U/3ml).

From our results (Table 2.1), the value of apparent K_m for microencapsulated tyrosinase is the same as that of free tyrosinase because the K_m value for one enzyme depends on the particular substrate and also environment conditions and ionic. V_{max} of the microencapsulated tyrosinase is 49.02 mg/dl·min, while that of tyrosinase in free solution is 114.94 mg/dl·min. The higher V_{max} value in free tyrosinase than that in microencapsulated tyrosinase is due to the inactivation of the enzyme activity during the preparation procedures of microencapsules.

	V_{max} (mg/dl·min)	**K_m (M)**
Free tyrosinase	114.94	4.65×10^{-4}
	Apparent V_{max} (mg/dl·min)	**Apparent K_m (M)**
Microencapsulated tyrosinase	49.02	4.65×10^{-4}

Table 2.1 Summary of studies for V_{max} and K_m in free and microencapsulated tyrosinase.

pH studies *in vitro*

Orally administered microencapsulated tyrosinase passes through the stomach and intestine having different pH conditions. Thus it is very important to analyze the effects of pH corresponding to those along the gastrointestinal tract. Figure 2.4 provides a comparison of free tyrosinase and microencapsulated tyrosinase at different pH from 2 to 10. The enzyme activity at pH 7 were taken as 100% original activity, the other data were expressed as percentage of the original activity. At pH 6, microencapsulated tyrosinase and free tyrosinase have 97% and 87% of the original activity respectively. At pH 4, microencapsulated tyrosinase has 21.0 % activity, while only 2.3% activity was detected in free tyrosinase solution. When pH decreases to 2, no activity was tested in free enzyme solution, but for microencapsulated enzyme, 14% activity still remained. Only 0.4-0.6% activity can be tested in free tyrosinase when pH goes up from 8 to 10. For microencapsulated tyrosinase, 49% activity remained at pH 8, even at pH 10, 17% activity can be measured. Therefore, the microencapsulated enzyme was more active than the free enzyme at lower or higher pH values. This is probably due to: first, artificial membranes protect enzyme inside, it separates macromolecules, such as proteins, enzyme from the external undesirable environment. Second, high concentration of hemoglobin (10gm/dl) inside the microcapsules has a buffering capacity to stabilize and protect the enzyme inside artificial cells. For free tyrosinase, its activity is easily affected by changing pH.

To further simulate the physiological conditions, microencapsulated tyrosinase was incubated at 37°C for one hour at different pH, then used buffer to wash the

microcapsules twice and tested for enzyme activity at its optimal pH of 7 (Figure 2.5). The results showed that at pH 6 to 9, the activity of microencapsulated tyrosinase had more than 57% of original activity after one hour of incubation at 37°C. Even at pH 2, about 30% activity can be tested for microencapsulated tyrosinase.

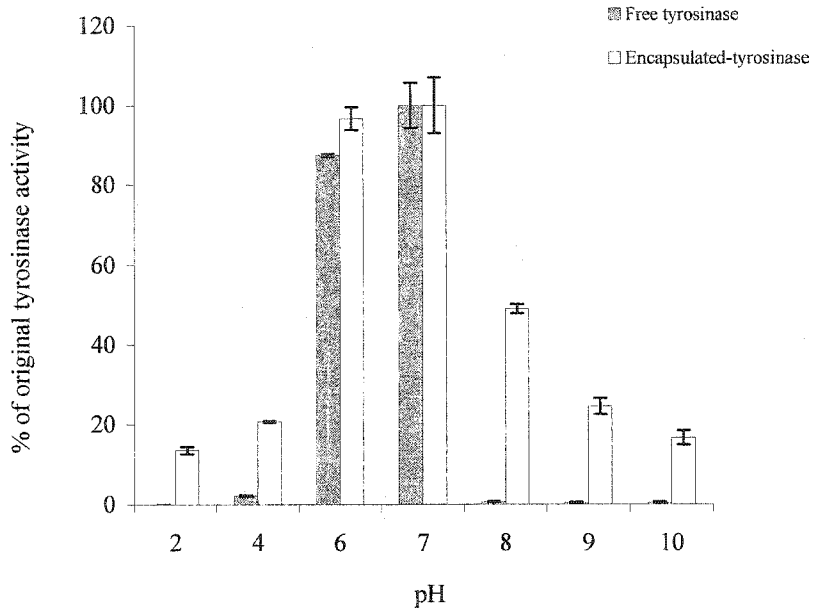

Figure 2.4 The activity of free tyrosinase and encapsulated tyrosinase at different pH.

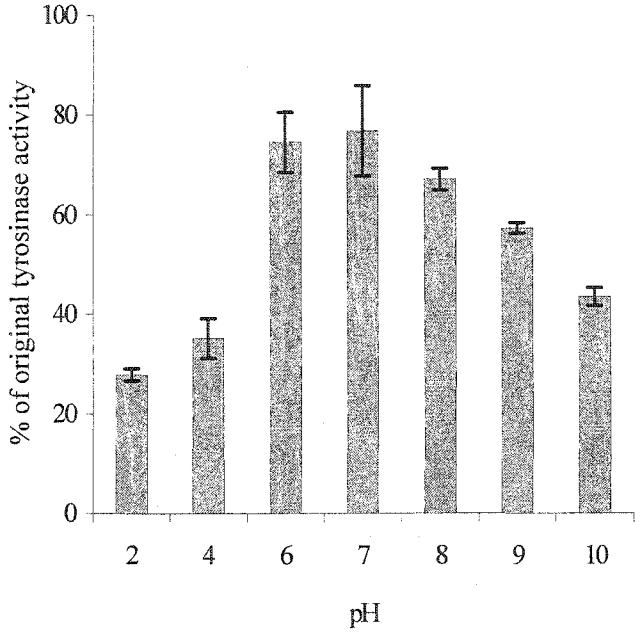

Figure 2.5 Microencapsulated tyrosinase activity after incubated at 37°C for 1 hour at different pH.

Temperature studies *in vitro*

It is important to know the temperature stability of the preparation before carrying out studies in the animal. Figure 2.6 and 2.7 show the difference for storage stability of free and microencapsulated tyrosinase at 4°C and 37°C. At 4°C (Figure 2.6), microencapsulated tyrosinase maintained full activity in the first three days and after 15 days it still had 68% of the original activity. On the other hand, the activity of free tyrosinase went down from the beginning and only had 28% of the original activity after 15 days.

At 37°C (Figure 2.7), microencapsulated tyrosinase has 61% of original activity after 10 hours and 28% after 24 hours when incubated in water bath at 37°C. This would allow the microencapsulated enzyme sufficient stability to carry out its function in the intestine after oral administration since the microcapsules stay in the small intestine for about 10-12 hours. For free tyrosinase, it only has 36% of original activity after 10 hours incubation and 7% after 24 hours. The activity of free enzyme decreases faster than that of microencapsulated enzyme. Therefore, microencapsulated tyrosinase is much more stable than free one under 4°C and 37°C.

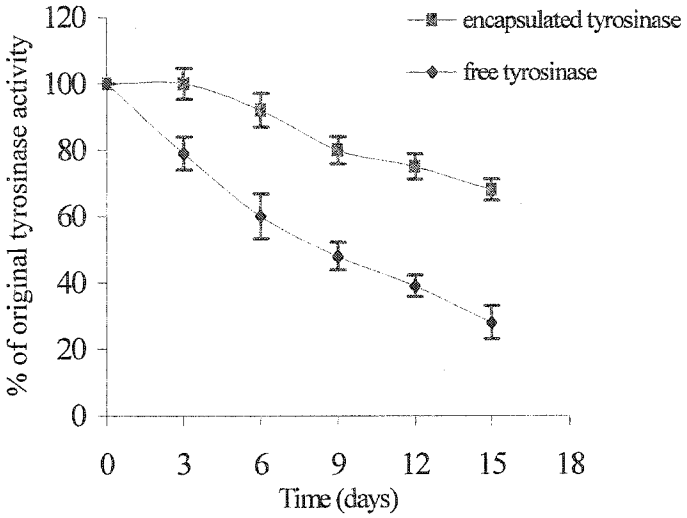

Figure 2.6 Storage stability at 4°C.

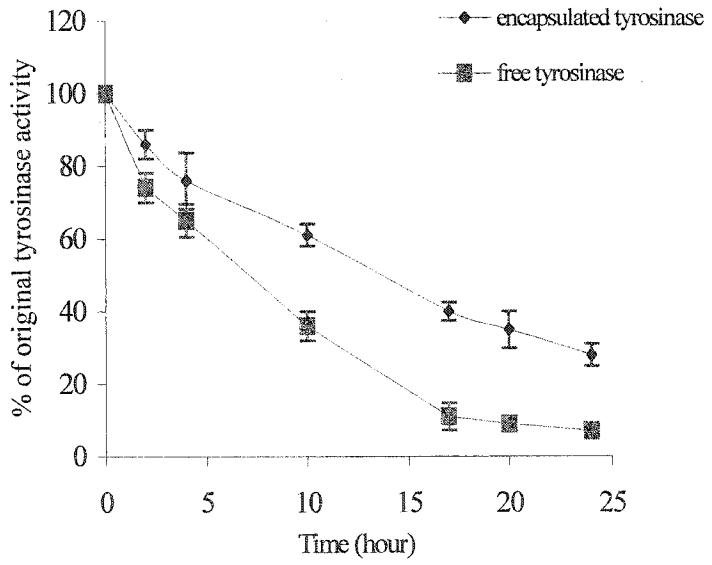

Figure 2.7 Storage stability at 37°C

2.5 Discussions

Treatment with a diet low in tyrosine and phenylalanine have been used as an adjuvant to reduce tyrosine level in blood and urine in melanoma bearing animals [4,6]. However, the diet restriction did not alleviate the burdens of diseases. This approach was not successful in human because it resulted in malnutrition in the severely sick cancer patients, and its unpalatable nature also made human compliance difficult. Besides being unpalatable, it is difficult to sustain this diet over long periods [24]. On the other hand, it was well documented that artificial cells containing enzymes can selectively remove amino acids when use this specific amino acid as substrate in enzyme reactions [13-15]. Thus, by oral administration of those artificial cells loading with enzymes, we can decrease or remove a specific amino acid from body system.

In this chapter, we studied kinetics of microencapsulated tyrosinase, and compared it to free tyrosinase. The results showed that microencapsulated tyrosinase is much more stable than free tyrosinase at different pH and temperature. Microencapsulated tyrosinase can catalyze tyrosine reaction as effectively as the free enzyme.

2.6 Acknowledgements

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Linkage to Chapter 3

Our *in vitro* results in Chapter 2 show that encapsulated tyrosinase is much more stable than the free tyrosinase solution at different pH's and storage temperatures. In addition, microencapsulated tyrosinase can catalyze tyrosine reaction as effectively as the free enzyme. The other enzyme kinetic properties have also been analyzed. We then can therefore proceed to the next step of carrying out *in vivo* studies to test whether encapsulated tyrosinase can lower the systemic tyrosine level. Thus in the next Chapter 3, we first study the action of encapsulated tyrosinase on rat intestine contents *in vitro*. Then, we carry out detailed animal studies on oral administrations of encapsulated tyrosinase at different doses.

CHAPTER 3

Effects of Oral Administration of Microencapsulated Tyrosinase on Systemic Tyrosine Levels in Rats

3.1 Abstract

In the present study, we analyze the ability of orally administered microencapsulated tyrosinase to remove and lower the systemic tyrosine level in the rat. We first carried out studies in which microencapsulated tyrosinase were incubated with intestinal contents of rats at 37°C *in vitro*. The results show that tyrosine level in the test group which incubated with microencapsulated tyrosinase decreased by 63% within half an hour, which is significantly lower than that of the control group which incubated without enzyme loaded microcapsules. We also carried out the study of oral administration of microencapsulated tyrosinase *in vivo*. One-dose a day was not effective in lowering the systemic tyrosine level. With two-dose a day, it took 7 days to lower the tyrosine level in the test group to 85% of that of the control group. The levels continue to decrease very slowly reaching 62.9% ($p<0.005$) after 11 days and 55.8% ($p<0.0005$) after 21 days. With three-dose a day, the tyrosine level in the test group decreased to 68.8% of the control group in 4 days. Then decrease to 52.6% after this and remain at this throughout the 22 days test period. In addition, our result shows that there is no abnormal effect in either the control group or test group and there are no significant differences in growth (gain weight) between the two groups.

3.2 Introduction

The incidence rate of melanoma has risen dramatically in the last century in all countries with a white-skinned population, doubling every 10 years in many countries, and is now approximately 10 per 100,000 per annum in Europe, giving an approximate lifetime risk of 1 in 200 [1]. At present, there is no optimal treatment for this cancer. Adjuvant therapy with varying clinical results includes immunotherapy, such as interferon α–2b levamisole [2], vaccines, chemotherapy, autologous bone marrow transplantation, biochemotherapy and chemoimmunotherapy [3-4]. Thus, there is at present no conclusive method for the treatment of melanoma.

One unique characteristic is that neoplastic cells need higher concentration of tyrosine for growth than that for normal cells [5]. L-tyrosine is a nutritionally dispensable amino acid, derived from protein degradation, dietary intake and phenylalanine hydroxylation [6]. The high tyrosine level can be reduced by the use of a low tyrosine diet. However, this diet is difficult to follow and it takes a long time to lower systemic tyrosine. The injection of the enzyme, tyrosinase, which catalyzes the conversion of L-tyrosine into L-dopaquinone, by itself was also not practical because the short half-life of a few minutes after intravenous injection required repeated injection resulting in immunological problems. In the present study we analyze the possible use of oral administration of microencapsulated tyrosinase to avoid these problems. Artificial cells containing enzymes have been successfully used for experimental enzyme replacement in acatalasemia and in phenylketonuria, two diseases caused by inborn error of metabolism [7-9]. Thus, artificial cells containing tyrosinase

might act on tyrosine from endogenous source, as well as tyrosine diffusing from the blood into the gastrointestinal tract. This approach has potential advantages in enzyme therapy when compared with earlier approaches in experimental enzyme therapy, based on parenteral injections or extracorporeal circulation of blood [10]. These include the absence of parental or surgical intervention, a large membrane surface area, and the improvement in enzyme stability. Artificial cells can be administered orally for substrate reduction in the gastrointestinal tract. Once microencapsulated within the artificial cells, the enzyme is protected from proteolytic enzymes in the intestinal tract. Our previous *in vitro* studies have shown that microencapsulated tyrosinase acts effectively in the conversion of tyrosine into L-dopaquinone. The activity of microencapsulated tyrosinase is much higher than that of free tyrosinase solution at different pH and temperature. In this chapter, these artificial cells were tested for their efficiency in decreasing tyrosine level in rat's intestine contents at 37°C *in vitro*. Having established some idea of the dosage needed from this *in vitro* study, we carried out oral administration study of these artificial cells *in vivo*.

3.3 Materials and Methods

Materials

Tyrosinase from mushroom (EC. 1.14.18.1, 5350 units/mg stated activity), hemoglobin from bovine (lyophilized powder) were purchased from Sigma Company. Collodion was purchased from Fisher Scientific Company. All other reagents were of analytical grade.

Preparation of control artificial cells

Control artificial cells were prepared by the standard published method [11-13]. Briefly, 1 g hemoglobin and 200 mg Tris was dissolved in 10 ml double distilled deionized water. Stir with a metal rod until everything is dissolved. Gravity filters the solution through a Waterman #42 filter into an Erlenmeyer flask. Take 2.5 ml of this 10 g/dl hemoglobin solution and was microencapsulated within spherical, ultrathin, cellulose nitrate membrane. Without tyrosinase loaded microcapsules were administered orally to control group. All control artificial cells were prepared daily and stored in 1% v/v Tween 20 solution at 4°C until use.

Preparation of tyrosinase loaded microencapsules

1.907 mg of 5350 units/mg tyrosinase was dissolved in 5 ml 10% hemoglobin solution, then followed the methods described above to immobilize tyrosinase in collodion membrane microcapsules. Microcapsules prepared as a 50% suspension for

later feeding. Tyrosinase loaded microencapsules were administered orally to test group.

Before oral administration for both control group and test group, artificial cells suspended in Tween 20 were washed and resuspended in 0.1 M Tris·HCl buffer (pH 8.5). The total volume of artificial cells for feeding was 2.5 ml (1 ml artificial cells plus 1.5 ml Tris·HCl buffer). This buffer is enough to protect the microencapsulated tyrosinase during its passage through the stomach with its acidic medium.

***In vitro* studies using rat intestinal juice**

Fasted male Sprague-Dawley rats (245-260 g) were anesthetized with intraperitoneal injection of pentobarbital (Somnotol, 65 mg/kg). Then, fresh intestine juice was extracted for the following experiments. We incubated microencapsulated tyrosinase with rat's intestine juice at 37°C, then took samples at different time intervals. After this we added 10% trichloroacetic acid (TCA) to stop the reaction. After centrifuging the sample, we measured the tyrosine level in the intestinal juice by fluorometric method using Perkin Elmer Luminescence Spectrometer LS50B [14-15].

Animal studies *in vivo*

Fasted male Sprague-Dawley rats (130-150 g) were used in this study. They were kept in a controlled 12 hr light/dark environment with food and water ad libitum. Two groups of rats were studied: (1) control group: each rat received oral administration of artificial cells containing no enzyme; (2) test group: each rat received

oral administration of artificial cells loaded with tyrosinase. The plasma in each blood sample was separated from the blood and placed in a 1.5 ml plastic tube, then stored at -80°C until analyzed. The tyrosine concentration in plasma was analyzed by fluorometric method using Perkin Elmer Luminescence Spectrometer LS50B.

One-dose animal experiment

On day 0, we took blood sample at 4:00 pm. No artificial cells were administrated on the first day. From this day on, and every subsequent day for 10 days, artificial cells were administrated orally at 10:00 am using stomach feeding tubes. Six hours after oral administration of the artificial cells we took blood samples from the lateral saphenous vein every two days and analyze the concentration of tyrosine in plasma.

Two-dose animal experiment

On day 0, we took blood samples at 4:00 pm. No artificial cells were administrated on this day. From this day on, and every subsequent day for 21 days, artificial cells were administrated orally at 10:00 am and 4:00 pm. We take blood samples every week (day 7, 14, 21) just before the second feeding.

Three-dose animal experiment

On day 0, we took blood samples at 4:00 pm. No artificial cells were administrated on this day. From this day on, and every subsequent day for 21 days,

artificial cells were administrated orally at 10:00 am, 2:00 pm and 6:00 pm. We take blood samples on day 4, 8, 11, 15, 18, 22 just after the second feeding.

Statistical analysis

Data are expressed as mean ± S.D. The differences of tyrosine concentration in rat's plasma between the two groups (control group and test group) at the same time point were determined by using Student's t-test within ANOVA and considered significant at $p< 0.05$ [16].

3.4 Results

Incubation with rat's intestine juice *in vitro*

This is to see the ability of the microencapsulated tyrosinase to lower tyrosine in intestinal juice before using this for oral administration. We take fresh intestine juice from anesthetized rat, then incubate this with artificial cells at 37°C in a shaker.

Our results (Figure 3.1 A) show a significant different between the control group and the test group. The intestinal juice contains high concentrations of proteins, enzymes, polypeptides, and peptides [17]. Tryptic enzymes in the intestine break these down into amino acids. Therefore, tyrosine level in control group after an initial decrease due to equilibrate of tyrosine in the intestine juice and the microcapsules increased with time from 177.58 ± 29.92 mg/dl at the beginning to 219.76 ± 15.21 mg/dl at 33 minutes. For the test group, tyrosine concentration in rat's intestine fell from the original concentration of 200.25 ± 10.16 mg/dl to 73.34 ± 14.72 mg/dl at 33 minutes. With increase in the volume of microencapsulated tyrosinase (Figure 3.1 B & C), tyrosine concentration in the test group can be maintained at the low level during the length of the experiment. For instance, in Figure 3.1B, tyrosine level in the control group was from 103.07 ± 6.83 mg/dl at the beginning to 201.62 ± 11.03 mg/dl at 33 minutes. For the test group the tyrosine level fell from 118.98 ± 16.47 mg/dl to 56.55 ± 8.21 mg/dl. Figure 3.1C shows that in the control group, the tyrosine level increased to 1.5 times of the original level. In the test group, because of the increase in the dosage of tyrosinase microcapsules, the tyrosine level decreased to even lower level when compared to that in Figure 3.1A & B. These results allow us to establish a suitable

dosage for the next study where we administer the tyrosinase microcapsules orally to rats.

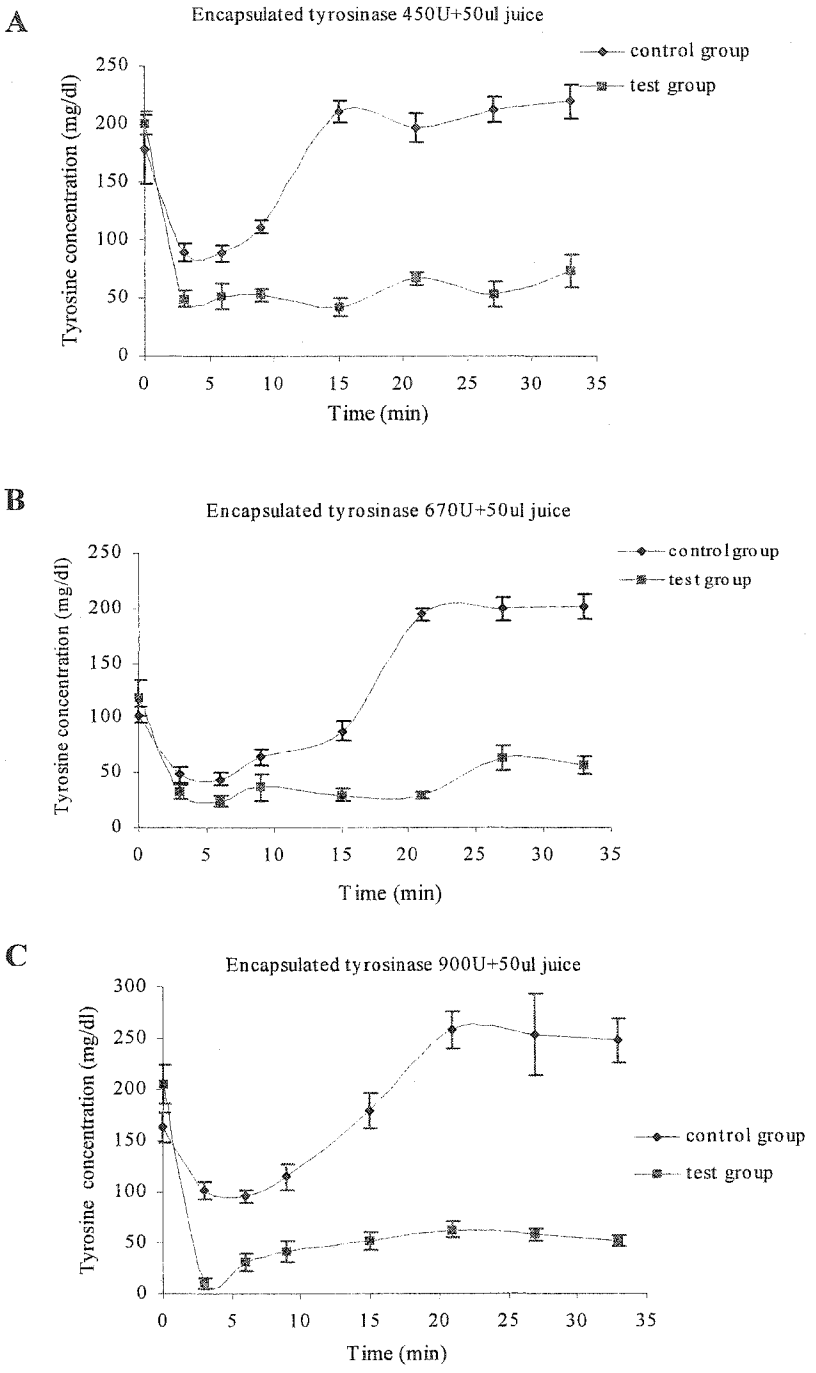

Figure 3.1 Tyrosine concentration in rat intestine juice after incubated with microencapsulated tyrosinase *in vitro*. (A) Encapsulated tyrosinase 450 U + 50 µl intestine juice. (B) Encapsulated tyrosinase 670 U + 50 µl intestine juice. (C) Encapsulated tyrosinase 900 U + 50 µl intestine juice.

One-dose animal experiment

The growth (body weight) of normal rats fed on regular rat chow and administered artificial cells once a day has not been disturbed over the 10-day study (Figure 3.2).

However, there was no significant change in tyrosine concentration in both groups during the 10-day study (Figure 3.3). Since the tyrosine concentration in plasma, as all other amino acids, tends to fluctuate even though the time of feeding and plasma collection are the same. This fluctuation is a normal phenomenon in all rat studies from other centers also. Thus, we take tyrosine level in control group as 100%, the other data are expressed as percentage of the original activity. Figure 3.3 shows plasma tyrosine concentration (%) 6 hours after oral administration of control microcapsules for the control group and microencapsulated tyrosinase for the test group once a day for 10 days. Statistical analysis (Table 3.1) shows that there are no significant differences between the two groups. Thus, one dose a day was not effective in lowering the plasma tyrosine levels.

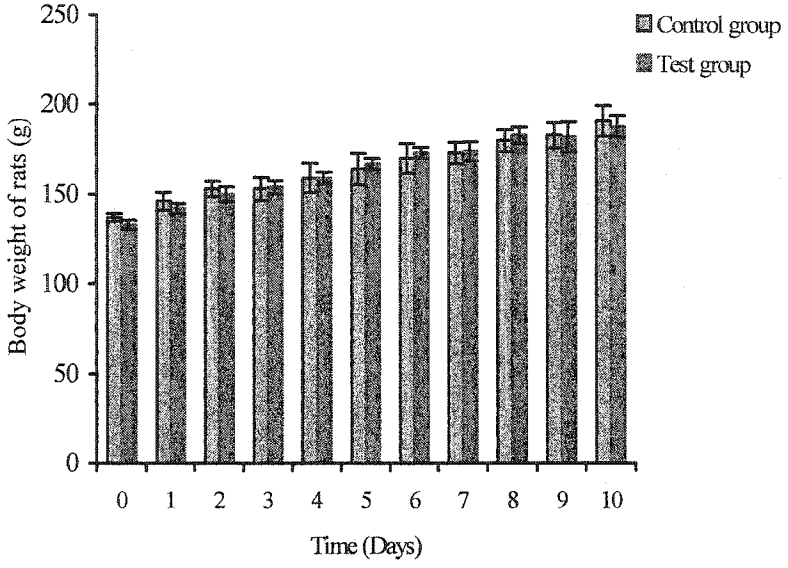

Figure 3.2 Body weight (g) of rats for control group fed with microencapsulated hemoglobin (10 g/dl) and for test group fed with microencapsulated hemoglobin with tyrosinase (255 U/0.25ml) once a day for 10 days.

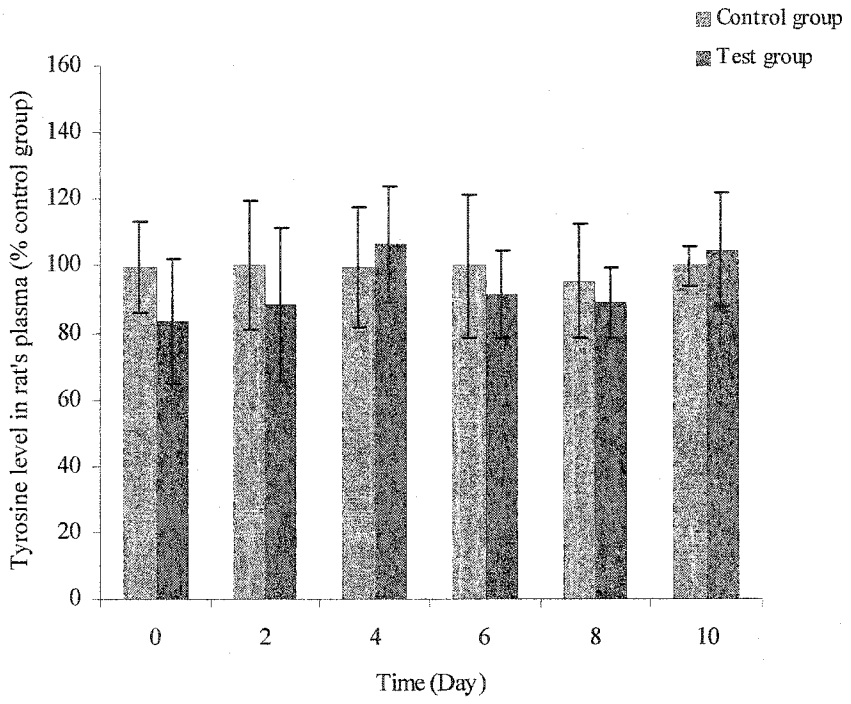

Figure 3.3 Tyrosine concentration (%) in rat plasma 6 hours after oral administration of microencapsulated hemoglobin (10 g/dl) for control group and microencapsulated hemoglobin with tyrosinase (255 U/0.25ml) for test group once a day for 10 days.

Comparing	P-value
C day 0 / T day 0	<0.15
C day 2 / T day 2	< 0.25
C day 4 / T day 4	NS
C day 6 / T day 6	NS
C day 8 / T day 8	<0.15
C day 10 / T day 10	NS

Table 3.1 P-value in tyrosine concentration between control group and test group in studying using one daily oral administration of artificial cells for 10 days. C stands for control group, which is fed with microencapsulated hemoglobin, T stands for test group, which is fed with microencapsulated tyrosinase.

Two-dose animal experiment

Since a single dose did not result in any significant decreases in plasma tyrosine levels, we studied the use of two-dose per day. Rats fed on regular rat food and administered with artificial cells twice a day gained weight with time during the 21-day experiment period. (Figure 3.4).

In this study, there was significant difference in tyrosine level in test group starting from the first week (Figure 3.5). Plotting the results as percent of control group, at day 7 the tyrosine concentration in the test group is decreased to 85% of that in control group. At day 14 and day 21, tyrosine concentration is further decreased in test group to 62.9% and 55.8% respectively. Our results showed that microencapsulated tyrosinase is effective to lower tyrosine level in the body but it required much time to reach this level. The reason is that once the tyrosine concentration is decreased in plasma, intracellular tyrosine will come out to equilibrate the imbalance of tyrosine in plasma. The basic theory for our experiments is that artificial cells loaded tyrosinase can remove tyrosine from amino acid pool in the intestinal tract and prevent its reabsorb into the body amino acids pool, thereby lowering circulatory tyrosine level. Table 3.2 shows the summary of statistical analysis for tyrosine concentration in the two groups. After one week of oral administration, there is significant decrease ($p<0.005$) in tyrosine level in the test group compared to the control group. Continuing this treatment until day 21, there is further increase in significance ($p<0.0005$).

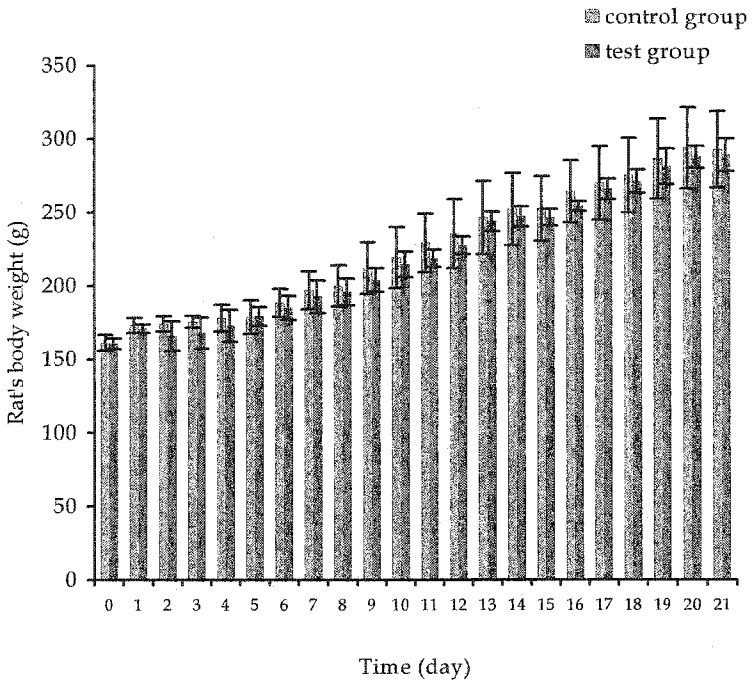

Figure 3.4 Body weight (g) of rats for control group fed with microencapsulated hemoglobin and for test group fed with microencapsulated hemoglobin with tyrosinase twice a day for 21 days.

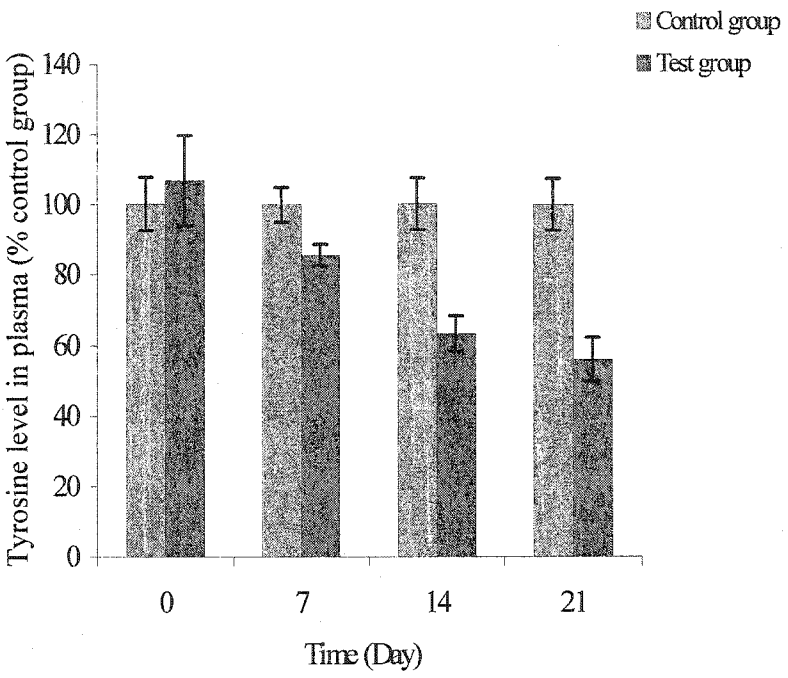

Figure 3.5 Tyrosine level in test group in rat's plasma expressed as percentage of those in control group. Control group: oral administration of microencapsulated hemoglobin twice a day for 21 days. Test group: oral administration of microencapsulated hemoglobin with tyrosinase twice a day for 21 days.

Comparing	P-value
C day 0 / T day 0	NS
C day 7 / T day 7	< 0.005
C day 14 / T day 14	< 0.005
C day 21 / T day 21	< 0.0005

Table 3.2 P-value in tyrosine concentration between control group and test group in two-dose daily oral administration of artificial cells for 21 days. C stands for control group, which is fed with microencapsulated hemoglobin, T stands for test group, which is fed with microencapsulated tyrosinase.

Three-dose animal experiment

To optimize and increase the rate of removal of systemic tyrosine, we fed rats artificial cells orally three-dose a day. The test group showed a weight gain curve identical to that of control group (Figure 3.6). No abnormal effect or behavior was observed in both groups. This again shows that at this increased dosage, there are no gross adverse effects when compared to the control. Figure 3.7 shows that there is much faster decrease in plasma tyrosine level in the test group when compared to that given 2 doses daily. Thus, on day 4 tyrosine levels in the test group decreased to 68.8%. By the 18^{th} day and 22^{nd} day of the treatment, the systemic tyrosine level decreased to 56.8% and 52.6% respectively. Our results show that 3 doses per day of oral administration can lower tyrosine concentration in rat's plasma from day 4. Table 3.3 shows the statistical analysis for tyrosine concentration. At day 4, there was already a significant decrease ($p<0.05$) in tyrosine level in the test group.

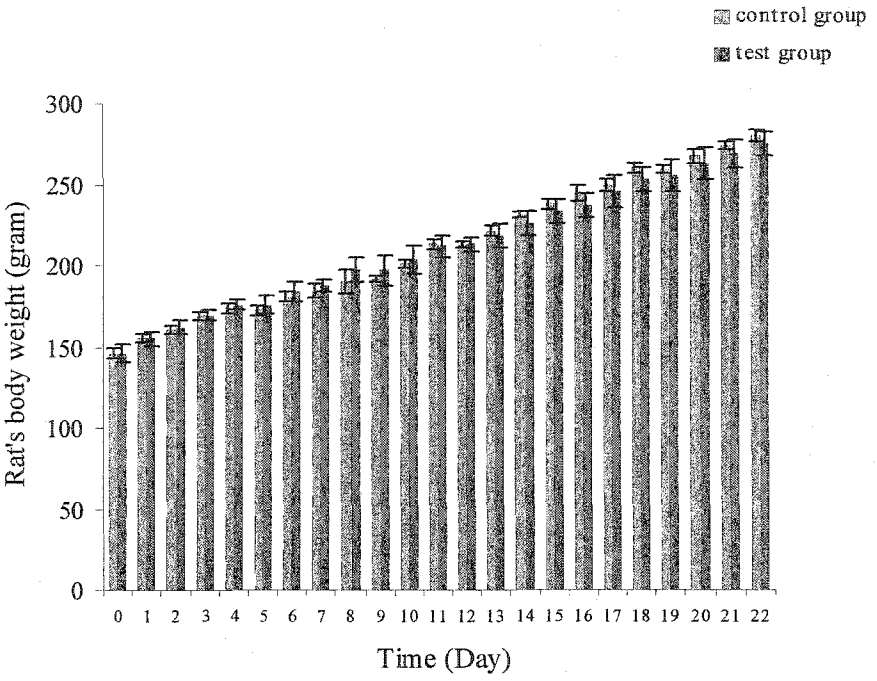

Figure 3.6 The body weight (g) of rats for 22 days experiments. For control group, the rats were given artificial cells orally three times a day. For test group, the rats were given artificial cells encapsulated tyrosinase orally three times a day.

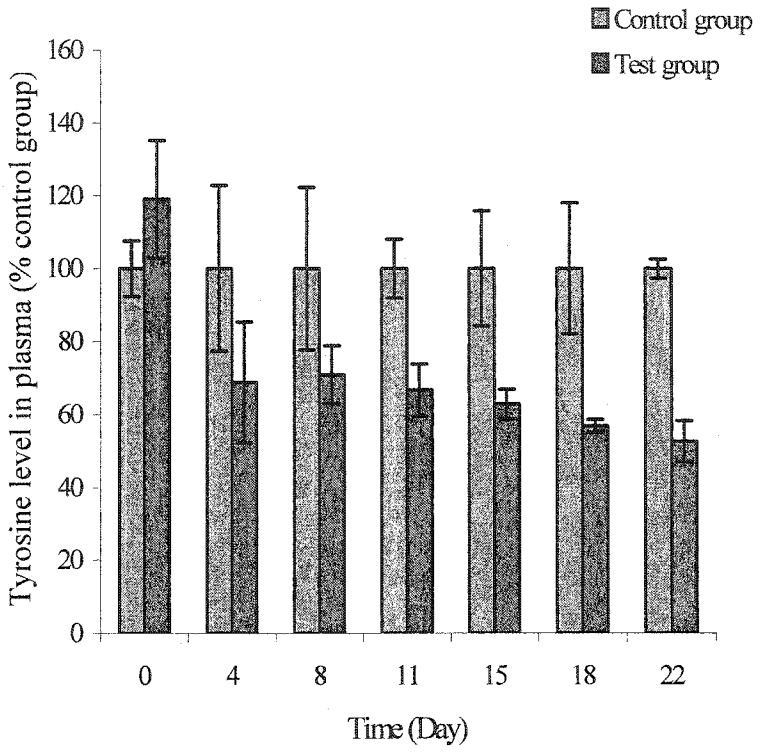

Figure 3.7 Tyrosine level in test group in rat's plasma expressed as percentage of those in control group. Control group: oral administration of microencapsulated hemoglobin three times a day for 22 days. Test group: oral administration of microencapsulated hemoglobin with tyrosinase three times a day for 22 days.

Comparing	P-value
C day 0 / T day 0	NS
C day 4 / T day 4	< 0.05
C day 8 / T day 8	< 0.05
C day 11 / T day 11	< 0.005
C day 15 / T day 15	< 0.005
C day 18 / T day 18	< 0.005
C day 22 / T day 22	< 0.0005

Table 3.3 P-value in tyrosine concentration between control group and test group in three-dose daily oral administration of artificial cells for 22 days. C stands for control group, which is fed with microencapsulated hemoglobin, T stands for test group, which is fed with microencapsulated tyrosinase.

3.5 Discussions

Previous studies indicate that tyrosine and phenylalanine restrictive diets decrease tumor growth and metastasis and increase the survival of B16 melanoma-bearing mice [18-20]. One of the main problems with these low amino acids diet is that it is difficult to sustain in human. Furthermore, these restrictive diets cause malnutrition in the severely ill patients. The exploitation of melanogenesis in the development of antitumor agents specific for malignant melanoma has been studied by many research groups. Inoue *et al* demonstrate that 4-S-Cysteaminylphenol (4-S-CAP) is one of the most promising antimelanoma agents. As 4-S-CAP is a good substrate for tyrosinase and produce ultimate toxic metabolite by tyrosinase oxidation, 4-S-CAP exerts the melanocytotoxicity and inhibits the growth of B16 melanoma [21]. However, one problem with 4-S-CAP is its strong hypotensive effect which limits the dose that can be administered *in vivo*. Jordan *et al* use prodrug, nitrogen mustard, to incorporate into tyrosinase substrates for the treatment of malignant melanoma, but still have some limitations in drug selective liberation [22]. In clinical, Castelli *et al* use melanoma-associated antigens (MAA) recognized by T-cell for boosting natural immune recognition of tumor cells [23]. The therapeutic potential of MAA still needs to be fully exploited to reach an effective and long-lasting *in vivo* immune control of melanoma growth and progression.

In our study, we used artificial cells containing tyrosinase to lower tyrosine level in the body system. Oral uses of artificial cells containing other enzymes have already been demonstrated in this laboratory [9] and offer several advantages over other

methods [10]. Since artificial cells pass through the intestine and are excreted once they have carried out their functions, it is easier to ensure their safety in patients. Furthermore, earlier clinical results of oral administration of other enzyme microcapsules have already shown their safety. Our present studies show that microencapsulated tyrosinase can effectively lower tyrosine level in rat's intestine contents *in vitro*. We use this *in vitro* result to establish an approximate dose for use in the animal studies. *In vivo* studies, we compared the results for one dose, two doses, and three doses of oral administration of tyrosinase artificial cells. Our results show that 2 or 3 dosages per day oral administered of microencapsulated tyrosinase to animals can effectively reduce the systemic blood tyrosine level throughout the intestinal tract and keep it at that low level as long as the oral administration is continued.

3.6 Acknowledgements

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3.8 Appendix: Tyrosine concentration measurement

The reaction between 1-nitroso-2-naphthol and tyrosine, under suitable conditions, yields a yellow product which fluoresces [1]. The fluorescence of the tyrosine derivative, resulting from its activation at 450 nm, is measured at 550 nm. This method has been utilized for the colorimetric estimation of tyrosine in biologic material. The reaction solution (4 ml in total) contained 1 ml of deproteinized plasma (0.1 ml of plasma is deproteinized by 1.6 ml of 10% trichloroacetic acid (TCA), then centrifuged and take the supernatant), 1 ml of 10% TCA, 1 ml of 0.05% nitrite acid, and 1 ml of 0.01% 1-nitroso-2-naphthol. The tube containing the above reaction solution is stoppered, shaken and placed in a water bath at 55°C for thirty minutes. After cooling, 10 ml of ethylene dichloride is added, and the tube is shaken to extract the unchanged nitrosonaphthol reagent. After the tube is centrifuged, the supernatant aqueous layer is transferred to a cuvette and read in the spectrophotofluorometer. The Perkin Elmer Luminescence Spectrometer LS50B was used for measurement of fluorescence and for recording of activation and fluorescence spectra.

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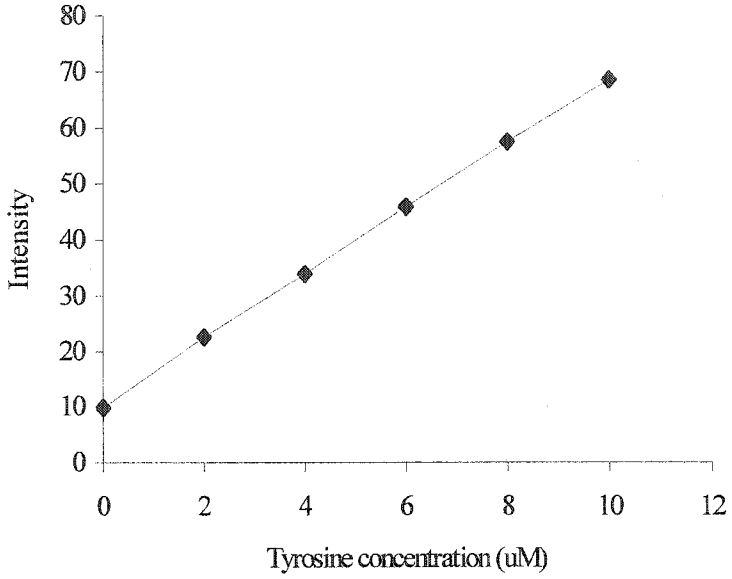

Figure 3.8 Calibration curve of tyrosine concentration.

3.9 Appendix: pH data in rat's stomach

To investigate whether 0.1 M Tris·HCl at pH 8.75 can neutralize stomach acid when given with microcapsules, we test stomach pH of rats after oral administration of microcapsules. Results show that the stomach pH in rats after administration of 1 ml of artificial cells with Tris·HCl buffer is 5.7±0.1. On the other hand, the stomach pH is around 2 in rats group without giving Tris·HCl buffer. Therefore, the use of this buffer is very important for the oral administration of encapsulated tyrosinase since this can neutralize the extremely low pH in stomach and protect the encapsulated enzyme.

Linkage to Chapter 4

In Chapter 3, our results show that oral administration of encapsulated tyrosinase 2- or 3-dose a day can lower systemic tyrosine level. However, this method needs at least 3 days to reach the significantly low level in tyrosine needed for the suppression of melanoma. Thus, the next step is to look at another method that can lower tyrosine level faster using polyhemoglobin-tyrosinase. Thus, chapter 4 describes detailed studies in the structural and functional properties of PolyHb-tyrosinase. This is followed by detailed *in vivo* studies to analyze the ability of PolyHb-tyrosinase to quickly lower systemic tyrosine level.

CHAPTER 4

In Vitro and In Vivo Enzyme Studies of Polyhemoglobin-tyrosinase

4.1 Abstract

In this study, we prepared Polyhemoglobin-tyrosinase (intermolecularly cross-linked hemoglobin and tyrosinase). We carried out the studies of the structural and functional properties of PolyHb-tyrosinase. Our *in vitro* studies show that longer crosslinking time results in higher molecular weight PolyHb-tyrosinase. We also evaluate the effects of varying the crosslinking time and the ratio of glutaraldehyde on the enzyme activity of PolyHb-tyrosinase. The stability of PolyHb-tyrosinase at 37°C is much more stable when compared to non-crosslinked tyrosinase solution. We found *in vivo* studies that the higher degree of polymerization correlated with a longer circulation time of PolyHb-tyrosinase. Our results show that one intravenous injection of PolyHb-tyrosinase lowers the plasma tyrosine to 10% of its original level within one hour. In cell culture studies, adding PolyHb-tyrosinase solution to the medium inhibit the growth of B16F10 melanoma cell line. These preliminary results show that intravenous injection of crosslinked hemoglobin with tyrosinase is a promising method to lower systemic tyrosine level.

4.2 Introduction

Melanoma, a fatal skin cancer in its many forms is a common tumor which accounts for an incidence of around 10% of all malignancies. Melanoma is most commonly found on the skin, but 10% arise in the eye [1]. Exposure to sunlight is a common causative factor. An ethnic factor is also important, for example Caucasians, red-haired people with very fair complexions are at the highest risk. Malignancy produced by exposure to low doses of irradiation over a prolonged period of time is well documented. Certain chemicals, such as arsenic, are known to be carcinogenic to skin. People with a strong family history (familial dysplastic nevus syndrome) have an increased tendency to develop melanoma [2].

The most important characteristic of differentiation in melanocyte is the presence of melanin pigment. Melanin pigment is a heterogeneous biopolymer formed from various intermediate products. Tyrosinase (EC 1.14.18.1) is the rate-limiting enzyme in melanin synthesis. This enzyme converts L-tyrosine as its substrate to L-dopa, and then further oxidizes L-dopa to L-dopaquinone and finally to melanin by different metabolic pathways [3-5]. Under normal conditions, proliferation and differentiation are regulated by specific growth factors and differentiation factors respectively. In neoplastic cells, over accumulation of proliferation factors inhibits the differentiation program of the target cells. Thus, malignant melanoma can be considered as a disorder of cell proliferation [6]. However, a number of substances, such as retinoic acid, corticosteroids, prednisolone, some prostaglandins, phorbol esters, DMSO, and cyclic AMP, have been reported to induce differentiation program

[7-13]. Bhatnagar *et al* found that citrate stimulated melanin synthesis and retarded cellular proliferation since citrate is an activator of cresolase activity of tyrosinase [14]. Investigators [15] are looking into the effects of the use of phenylalanine and tyrosine restricted diets to inhibit the growth and metastasis of B16BL6 murine melanoma since a malignant melanoma requires higher cellular concentration of tyrosine for its growth than that for normal melanocytes.

We therefore introduced the concept of chemically crosslinking tyrosinase with hemoglobin (PolyHb-tyrosinase) using a dialdehyde agent, glutaraldehyde. In this form, the enzyme is covered by hemoglobin molecules and therefore has less immunological properties [16-17]. Intermolecular polymerization overcomes potential therapeutic restrictions associated with the short circulation time of native tyrosinase. Furthermore, polyhemoglobin is an oxygen carrier [18-20] and being a solution, it can more readily reach the narrower capillaries of the melanoma cancer cells than red blood cells and can therefore bring more oxygen. The presence of a high concentration of oxygen is important in radiotherapy for cancer cells. It is well documented that well oxygenated cells, i.e. most normal tissue cells, are two to three times more sensitive to irradiation than hypoxic cells [$pO_2 < 10$ mmHg]. Even the smallest of clinically detectable tumors probably contains 10% to 15% or more of hypoxic cells which reduce markedly the effectiveness of radiation therapy. Three alternative methods dealing with hypoxic cells are undergoing clinical trials. In one method, patients breathe pure oxygen at increased pressure when they are given radiotherapy. Recently special electron affinic drugs have been developed which increase preferentially, the radiosensitivity of hypoxic cells. The

use of high energy neutrons instead of χ- or γ-rays are also undergoing clinical evaluation, since the former are less dependent during irradiation therapy upon the presence of oxygen to produce cell sterilization [2,21].

In this chapter, we investigate the structural and functional properties of this novel PolyHb-tyrosinase *in vitro* and we also test whether this novel PolyHb-tyrosinase can be given intravenously to lower the systemic tyrosine level in animals. We also study the effect of PolyHb-tyrosinase on the growth of B16F10 melanoma cell line in cell culture.

4.3 Materials and Methods

Materials

Purified bovine hemoglobin was purchased from Biopure Corporation, Boston, MA. Glutaraldehyde (25%) was obtained from Polysciences, Warrington, PA. Hemoglobin assay kit, molecular distribution standard kit, L-lysine (monohydrochloride, SigmaUltra > 99%), L-tyrosine (98% TLC), tyrosinase from mushroom (EC 1.14.18.1, 3000 units/mg stated activity) were purchased from Sigma-Aldrich (Ontario, Canada). All other reagents were of analytical grade.

Preparation of Polyhemoglobin and Polyhemoglobin-tyrosinase

Reaction mixtures were prepared containing hemoglobin (10 g/dl), tyrosinase (6000 U/ml) in 0.1 M potassium phosphate buffer, pH 7.6. In Polyhemoglobin mixtures, an equivalent volume of buffer replaced enzyme condition. Prior to the start of crosslinking, 1.3 M lysine was added at a molar ratio of 7:1 lysine/hemoglobin. Crosslinking reaction was started with the addition of glutaraldehyde (5%) at molar ratio of 8:1 or 16:1 glutaraldehyde/hemoglobin. Glutaraldehyde was added in four equal aliquots over a period of 15 minutes. After certain hours crosslinking (depend on different experiments) under aerobic conditions with constant stirring at 4°C, reaction was stopped with 2.0 M lysine at a molar ratio of 200:1 lysine/hemoglobin. Solutions were dialyzed using molecular porous dialysis membrane (MWCO: 12-14,000) against physiological saline solution over night and pass through sterile 0.45 μM filter. Aliquots (500 μl) of the 16:1 crosslinked preparation were concentrated using 100 KD

microconcentrators (Amicon, Beverly, MA). Samples were centrifuged at 2500 g for 55 minutes at 23°C. Then, retentate was collected. Hemoglobin concentration was determined by cyanomethemoglobin at 540 nm. Final retentates were diluted to desired concentration of 7 g/dl and store in 4°C fridge for later use.

Determination of molecular weight of PolyHb and PolyHb-tyrosinase

Molecular weight distribution analysis was performed using size-exclusion gel filtration chromatography on a Sephadex G-200 column (1.6 cm × 70 cm, V_{total} = 102 ml) equilibrated with 0.1 M Tris·HCl, pH 7.5. Samples (1 ml of 10 × dilution) were passed through the column at a rate of 12 ml/hour. The elutioning fractions were collected every 15 minutes for a period of 8 hours. The elution profiles were monitored at 280 nm.

Determination of hemoglobin concentration

Hemoglobin concentration was determined by spectrophotometric analysis with "Total Hemoglobin Kit" from Sigma-Aldrich.

Determination of oxygen affinity of hemoglobin and PolyHb-tyrosinase

The oxygen absorption curve for hemoglobin and PolyHb-tyrosinase were determined by the TCS Hemoxanalyser (TCS Medical Products Co., U.S.A.). Samples (5 ml) containing 0.3 g/dl of hemoglobin or 24 hrs crosslinked PolyHb-tyrosinase in 37°C 0.1 M PBS at pH 7.4 were used to obtain hemoglobin oxygen dissociation curves.

Determination of tyrosinase activity

Tyrosinase activity was assessed by measuring the formation of enzymatic products at 300 nm [22]. All other substances present in the reaction mixture, i.e. tyrosine, tyrosinase, and hemoglobin had no significant adsorption at this wavelength. All reactions were carried out at 23°C. The adsorbance at 300 nm were followed continuously for 8-14 minutes using Perkin Elmer Lambda 4B spectrophotometer, and changes in O.D./min were used to analyze the activity of the enzyme. Then, convert those O.D. data to the product concentration mg/dl per minute. Therefore, the changing in product concentration (mg/dl) per minutes is used for tyrosinase reaction velocity.

Determination of tyrosine concentration in rat's plasma

Tyrosine concentration in plasma was analyzed by fluorometric method using Perkin Elmer Luminescence Spectrometer LS50B [23]. One unit of enzyme activity was defined as the amount of enzyme which decreased 1 mg/dl of L-tyrosine per minute under the assay conditions described.

Animal experiments

Fasted male Sprague-Dawley rats (245-260 g) were obtained from Charles River Canada, St-Constant, QC. Animals were anaesthetized with intraperitoneal injection of 65 mg/kg pentobarbital (Somnotol, Decton Dickinson, NJ). Body temperature was maintained by a warming blanket. Incisions were carefully performed in one side of hindlimbs below the inguinal ligament, and femoral vessels were

carefully isolated. Polyethylene cannulae were inserted and secured distal to the superficial epigastric branches in the femoral veins (PE-10, PE-50 Clay Adams). Proper vessel access was tested with a small volume injection of heparinized saline (50 IU/ml). Blood samples were taken from each group at the beginning, then different samples were injected through the femoral vein. The femoral artery cannulae was connected to the venous cannulae for blood to circulate thoroughly for a short interval. Then blood samples were taken from the femoral artery at different time intervals. The plasma in each blood sample was separated from the blood and placed in a 1.5 ml plastic tube, then stored at -80°C until analyzed.

Tumor cells and culture conditions

B16-F10 murine melanoma cells were obtained from American Type Tissue Collection, Manassas VA. The tumor cells were routinely cultured in DMEM (Life Technologies, Invitrogen Canada) supplemented with 10% heat-inactivated FBS, sodium pyruvate, nonessential amino acids, 2-fold vitamin solution, L-glutamine, 100 IU/ml penicillin, and 100 IU/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2. For passage, cells were detached with 0.05% Trypsin-EDTA and transferred to fresh medium every 3 days. The cells were used *in vitro* between passage 5 and passage 10. For experiment, melanoma cells were cultured in complete DMEM until they became 30-40% confluent. Then, appropriate aliquots of different samples (0.57 ml sample per 10 ml medium) were added to the medium. The cell viability was followed up to 4 days thereafter [24-28]. Tumor cells were routinely monitored by

phase microscopy. Cell counts were obtained daily with a hemacytometer. Cell viability was determined by trypan blue exclusion.

4.4 Results

4.4.1 Preliminary in vitro studies of properties of PolyHb and PolyHb-tyrosinase

The effect of glutaraldehyde : hemoglobin ratio on tyrosinase activity

This experiment is designed to find out the effect on tyrosinase activity with the addition of glutaraldehyde at different molar ratio of 8:1 and 16:1 glutaraldehyde/hemoglobin. In Table 4.1, we consider hemoglobin with tyrosinase activity without the addition of glutaraldehyde as 100% original activity. After crosslinking, 99% of tyrosinase activity remained in PolyHb-tyrosinase when crosslinked using a glutaraldehyde molar ratio of 8:1. Ninety-five percent of tyrosinase activity remained for PolyHb-tyrosinase crosslinked using a glutaraldehyde molar ratio of 16:1.

Samples	% Tyrosinase retained
Hemoglobin + tyrosinase	100
PolyHb-tyrosinase at 8:1 Glut:Hb	99
PolyHb-tyrosinase at 16:1 Glut:Hb	95

Table 4.1 Tyrosinase activity after crosslinking to form PolyHb-tyrosinase.

The effect of crosslinking time and enzyme concentration on tyrosinase activity

To determine the effect of crosslinking time on tyrosinase activity, we crosslinked hemoglobin with tyrosinase from 3 hrs up to 11 hrs, then measured tyrosinase activity and tyrosine concentration. We consider tyrosinase activity before crosslinking as 100% of original activity, other data are expressed as the percentage of the original activity. From our results in Figure 4.1, there was only a slight decrease in enzyme activity up to 11 hrs. No significant difference in tyrosinase activity was found among these periods. The result shows that longer crosslinking time does not decrease enzyme activity significantly.

To further investigate the effect of crosslinking reaction on enzyme activity, we compare enzyme activity before and after crosslinking. Our results showed that there was no significant change in enzyme activity before or after crosslinking (Figure 4.2). Before crosslinking, the rate of tyrosine conversion was 2.63±0.11 mg/dl·min. After crosslinking the rate of conversion was 2.75±0.35 mg/dl·min, and for the non-crosslinked group, the rate of conversion was 2.65±0.38 mg/dl·min. This further confirms that crosslinking reaction does not affect enzyme activity significantly.

We then tested the enzyme activity at different enzyme concentration after crosslinking of 3.5 hrs. Crosslinking hemoglobin added with equal volume of buffer instead of enzyme solution served as control. Figure 4.3 shows the effects of increasing the concentration of PolyHb-tyrosinase on the rate of conversion of tyrosinase.

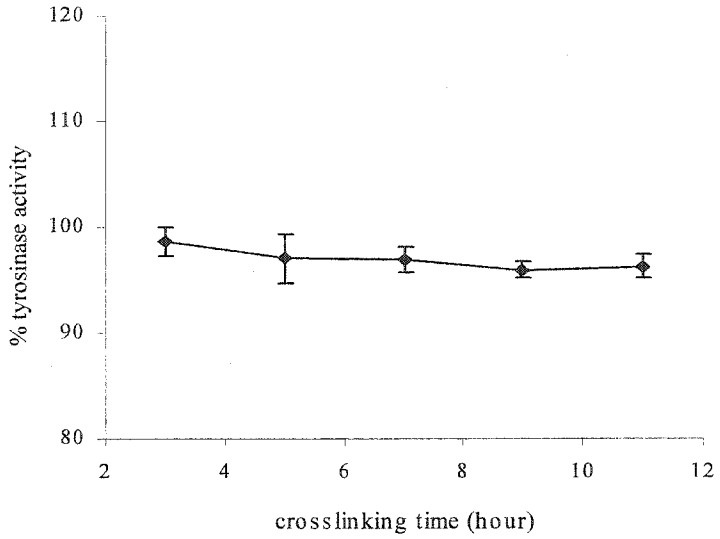

Figure 4.1 The activity of tyrosinase after different crosslinking times.

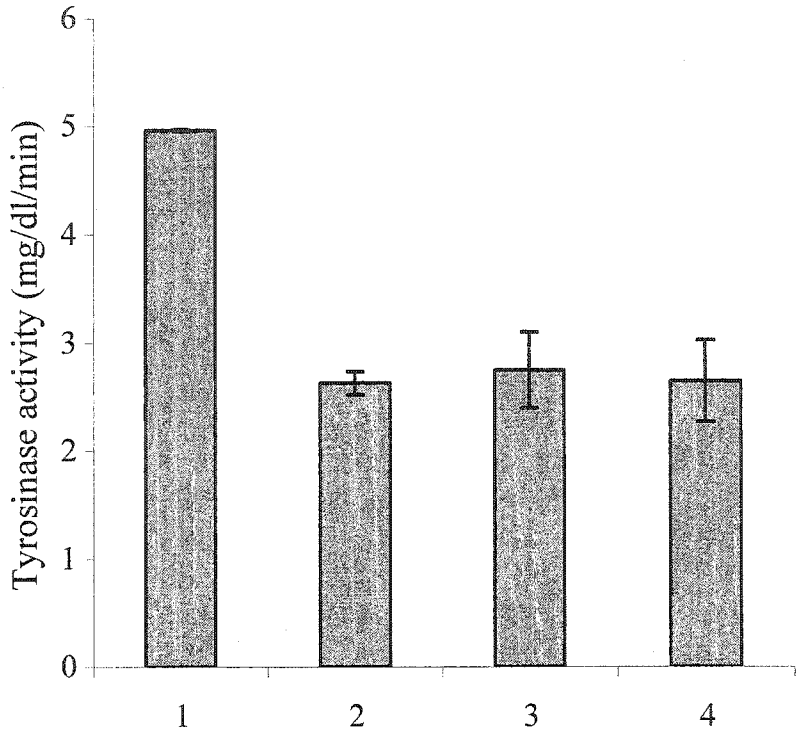

Figure 4.2 Tyrosinase activity before and after crosslinking and also in non-crosslinking solution.

Group1: PolyHb: 2.5 ml Hb+ 0.1 ml Buffer

Group2:Before crosslinking: 2.5 ml Hb + 0.1 ml tyrosinase solution (3000 U/0.1ml)

Group3:After crosslinking: 2.5 ml Hb + 0.1 ml tyrosinase solution (3000 U/0.1ml)

Group4:Non-crosslinking: Buffer instead of glutaraldehyde

Reaction mixture: 2.9 ml of 5 mg/dl tyrosine + 0.1 ml test sample= total volume 3 ml

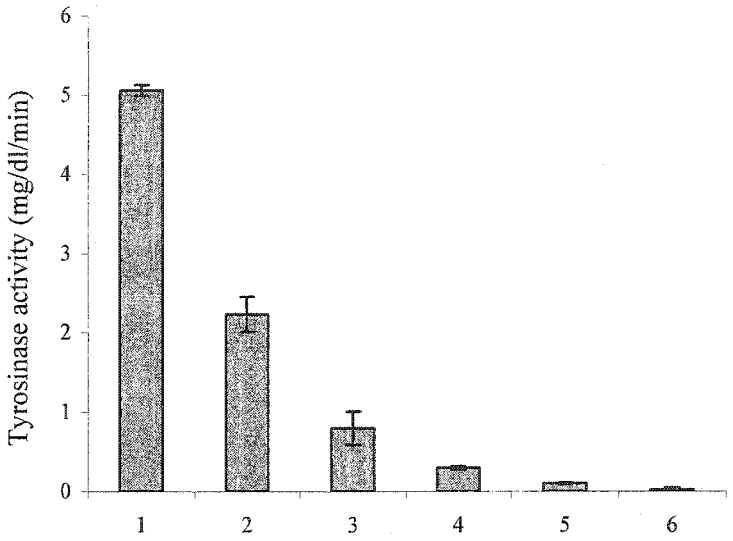

Figure 4.3 PolyHb-tyrosinase activity at different enzyme concentrations.

(1) Control: PolyHb (2.5ml 7g/dl Hb crosslinked with 100 μl Buffer)

(2) PolyHb-E: 2.5 ml Hb crosslinked with 3000 U of tyrosinase (3000U/0.1ml)

(3) PolyHb-E: 2.5 ml Hb crosslinked with 6000 U of tyrosinase (6000U/0.1ml)

(4) PolyHb-E: 2.5 ml Hb crosslinked with 9000 U of tyrosinase (9000U/0.1ml)

(5) PolyHb-E: 2.5 ml Hb crosslinked with 12000 U of tyrosinase (12000U/0.1ml)

(6) PolyHb-E: 2.5 ml Hb crosslinked with 15000 U of tyrosinase (15000U/0.1ml)

4.4.2 Molecular weight distribution of PolyHb and PolyHb-tyrosinase

To determine the degree of polymerization, samples were analyzed by gel filtration chromatography using Sephadex G-200 1.6 cm x 70 cm column at different crosslinking time, i.e. 3.5 hrs, 10 hrs, 24 hrs, 30 hrs and 48 hrs. Figure 4.4 and Table 4.2 show the increase in the higher molecular weight peaks with increase in the time of crosslinking. Also there was no significant difference in the molecular weight distribution between polyhemoglobin and polyhemoglobin-tyrosinase. This is because the ratio of hemoglobin to tyrosinase is 100 : 2 and therefore the added tyrosinase is not expected to significantly change the molecular weight distribution after being crosslinked with hemoglobin.

4.4.3 Oxygen affinity of hemoglobin and PolyHb-tyrosinase

Oxygen dissociation curves of the preparations were made by the TCS Hemoxanalyser. Purified bovine hemoglobin served as the control group. The curve of the preparation containing 7 g/dl hemoglobin is shown in Figure 4.5. There was no significant difference between polyhemoglobin-tyrosinase and free hemoglobin solution. Based on the analysis of oxygen saturation curves, the P_{50} values for non-crosslinked hemoglobin and PolyHb-tyrosinase were 23 mmHg and 21 mmHg respectively. They possess oxygen transport characteristics similar to other crosslinked hemoglobins [29-32]. Thus, polymerization process did not alter oxygenation characteristics of these modified hemoglobin.

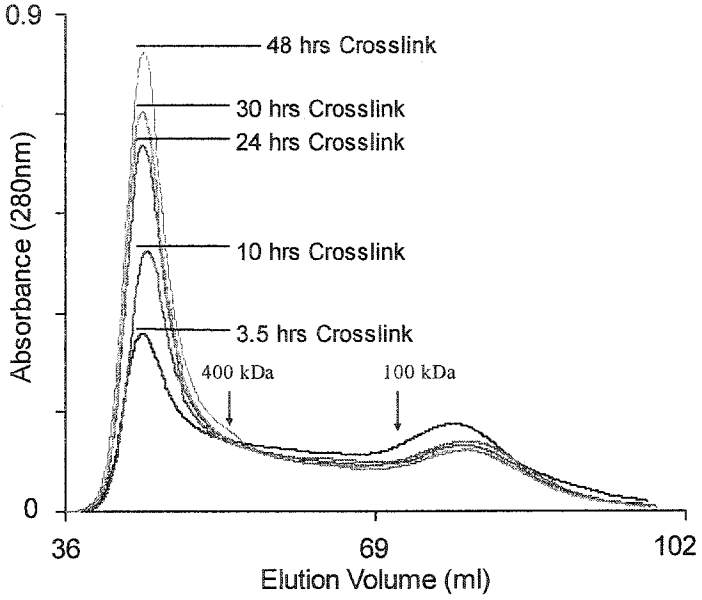

Figure 4.4 Typical elution profiles of 3.5-, 10-, 24-, 30-, 48-hour crosslinked PolyHb or PolyHb-tyrosinase (1 ml sample) were obtained by running on a Sephadex G-200 1.6 cm x 70 cm column, V_T=102 ml , equilibrated with 0.1 M Tris-HCl, pH 7.5, and eluted at 12 ml/hr.

Crosslinking Time (hours)	Samples	Percentage of Molecular Weight Distribution (KD)		
		> 400 kDa	Between 100 kDa and 400 kDa	< 100 kDa
3.5 hours	PolyHb	39%	31%	30%
	PolyHb-tyrosinase	39%	31%	30%
10 hours	PolyHb	58%	23%	19%
	PolyHb-tyrosinase	58%	23%	19%
24 hours	PolyHb	74%	14%	12%
	PolyHb-tyrosinase	74%	14%	12%
30 hours	PolyHb	76%	13%	11%
	PolyHb-tyrosinase	76%	13%	11%
48 hours	PolyHb	77%	12%	11%
	PolyHb-tyrosinase	77%	12%	11%

Table 4.2 Percentage of area under molecular weight distribution profiles.

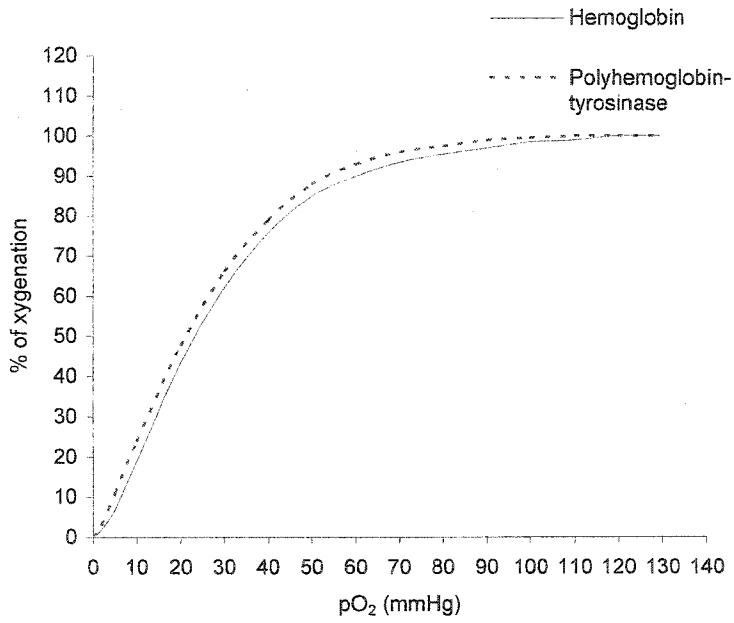

Figure 4.5 Oxygen dissociation curve of pure bovine hemoglobin in the free form and polyhemoglobin-tyrosinase form (crosslinking time 24 hrs). All oxygen-hemoglobin dissociation curves are presented as average of 3 trials.

4.4.4 Preliminary animal studies on the effect of PolyHb-tyrosinase

Intravenous injection of PolyHb, PolyHb-tyrosinase, free tyrosinase

The experiments are designed to find out if PolyHb-tyrosinase can lower tyrosine level in rat plasma. In this study, the crosslinking time was 3.5 hrs. We injected 1 ml of PolyHb, 1ml of free tyrosinase solution, and 1ml of PolyHb-tyrosinase to 3 groups of rats respectively. Figure 4.6 showed that the activity of free enzyme was less than 40% half an hour after injection and rapidly reaches zero by 3 hours. On the other hand, the enzyme activity of PolyHb-tyrosinase was 75±12% half an hour after injection and decreased at a much slower rate compared to the free enzyme. However, since the circulation time of PolyHb itself is much longer, it will be possible to optimize the procedure to increase the circulation time of the enzyme further. This could be done by further crosslinking to increase the degree of linkage of the enzyme or to increase the amount of enzyme. However, the decrease of tyrosinase activity could also be due to the decrease in enzyme activity at a body temperature of 37°C. We therefore carried out the following studies. (1) Stability of tyrosinase activity in PolyHb-tyrosinase at 37°C. (2) Effects of increasing the enzyme concentration in PolyHb-tyrosinase. (3) Effects of increasing the crosslinking by increase in time of crosslinking.

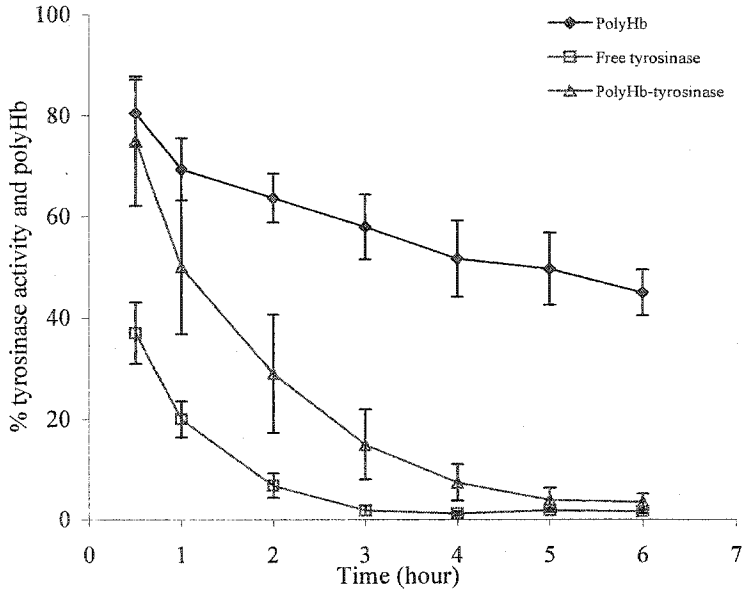

Figure 4.6 Plasma tyrosinase activity and PolyHb concentration after intravenous injection of tyrosinase and PolyHb-tyrosinase.

4.4.5 The stability of PolyHb-tyrosinase at 37°C

To determine whether body temperature is one of the major factors which affect tyrosinase activity, we test the enzyme activity at 37°C for up to 6 hrs using PolyHb-tyrosinase, free tyrosinase solution and PolyHb solution without enzyme as control (Figure 4.7). Our results showed that enzyme activity in free tyrosinase solution decreased faster than PolyHb-tyrosinase at 37°C. At six hrs, 79% activity remained in PolyHb-tyrosinase compared to the activity at time 0. On the other hand, only 60% of activity was found in free tyrosinase solution after 6 hrs incubation. Previous animal studies showed that at 6 hrs after injection of PolyHb-tyrosinase, the enzyme activity was zero. Thus, the decrease in circulatory tyrosinase activity is not due to inactivation of the PolyHb-tyrosinase at body temperature.

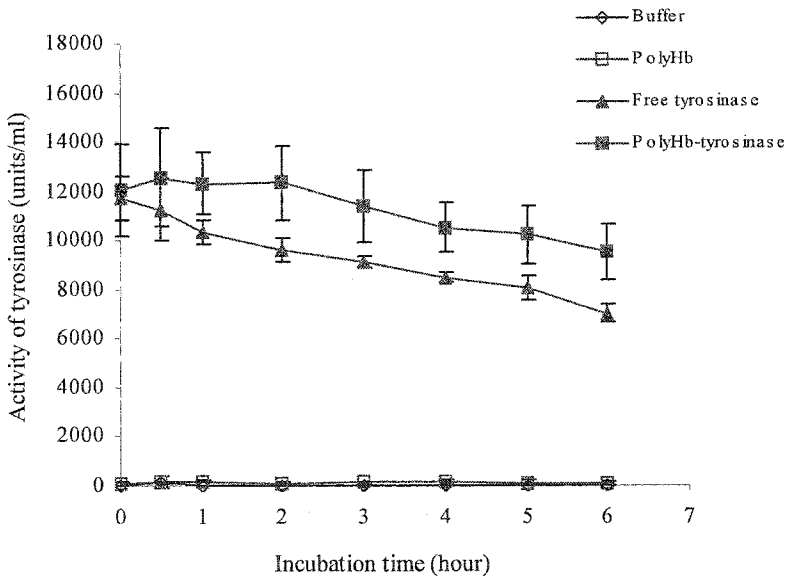

Figure 4.7 The activity of tyrosinase in free form and in PolyHb-tyrosinase solution at 37°C *in vitro*.

4.4.6 Optimization of tyrosinase concentration in PolyHb-tyrosinase

Figure 4.8 shows the result of increasing the tyrosinase concentration in the PolyHb-tyrosinase preparation. It shows that we have to double the tyrosinase in order for PolyHb-tyrosinase to lower plasma tyrosine to less than 15% of the original level within one hour and maintained the lowered level at 37% for up to 6 hours.

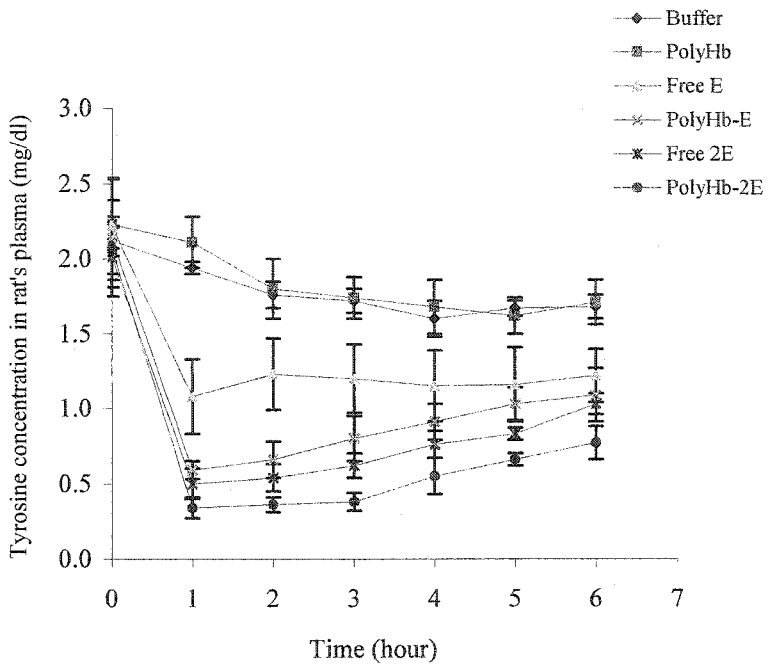

Figure 4.8 Tyrosine concentration in rat plasma (mg/dl) at different time intervals after i.v. injection of free tyrosinase or PolyHb-tyrosinase. Buffer, PolyHb solution served as control group, Free E: tyrosinase (6000 U/ml) in free solution, PolyHb-E: crosslinked hemoglobin with tyrosinase (6000 U/ml), Free 2E: double the amount of tyrosinase concentration, PolyHb-2E: double tyrosinase concentration when crosslinked to PolyHb.

4.4.7 Effects of increase in time of crosslinking

4.4.7.1 Effects of increase in time of crosslinking on degree of polymerization of tyrosinase to PolyHb

This study is to analyze whether all the tyrosinase are crosslinked to PolyHb. We crosslinked hemoglobin with tyrosinase at 3.5 hrs, 24 hrs, 30 hrs and 48 hrs. Then, take 1 ml of PolyHb-tyrosinase sample at different time intervals, run these through Sephadex G-200 1.6 cm x 70 cm column, equilibrated with 0.1 M Tris·HCl, and eluted at 12 ml/hr. Figure 4.9 shows when crosslinked for only 3.5 hours, a significant amount of the tyrosinase remained in free solution and not crosslinked. When the crosslinking time was increased to 24 hours, the uncrosslinked fraction significantly decreased. There was no significant difference in enzyme activity at the crosslinking time of 24 hrs, 30 hrs, and 48 hrs. Our results therefore show that 24 hours or more is needed to optimally crosslinked tyrosinase to hemoglobin during the polymerization. The incomplete crosslink may explain the rapid decrease in tyrosinase circulation time using PolyHb-tyrosinase crosslinked for only 3.5 hours.

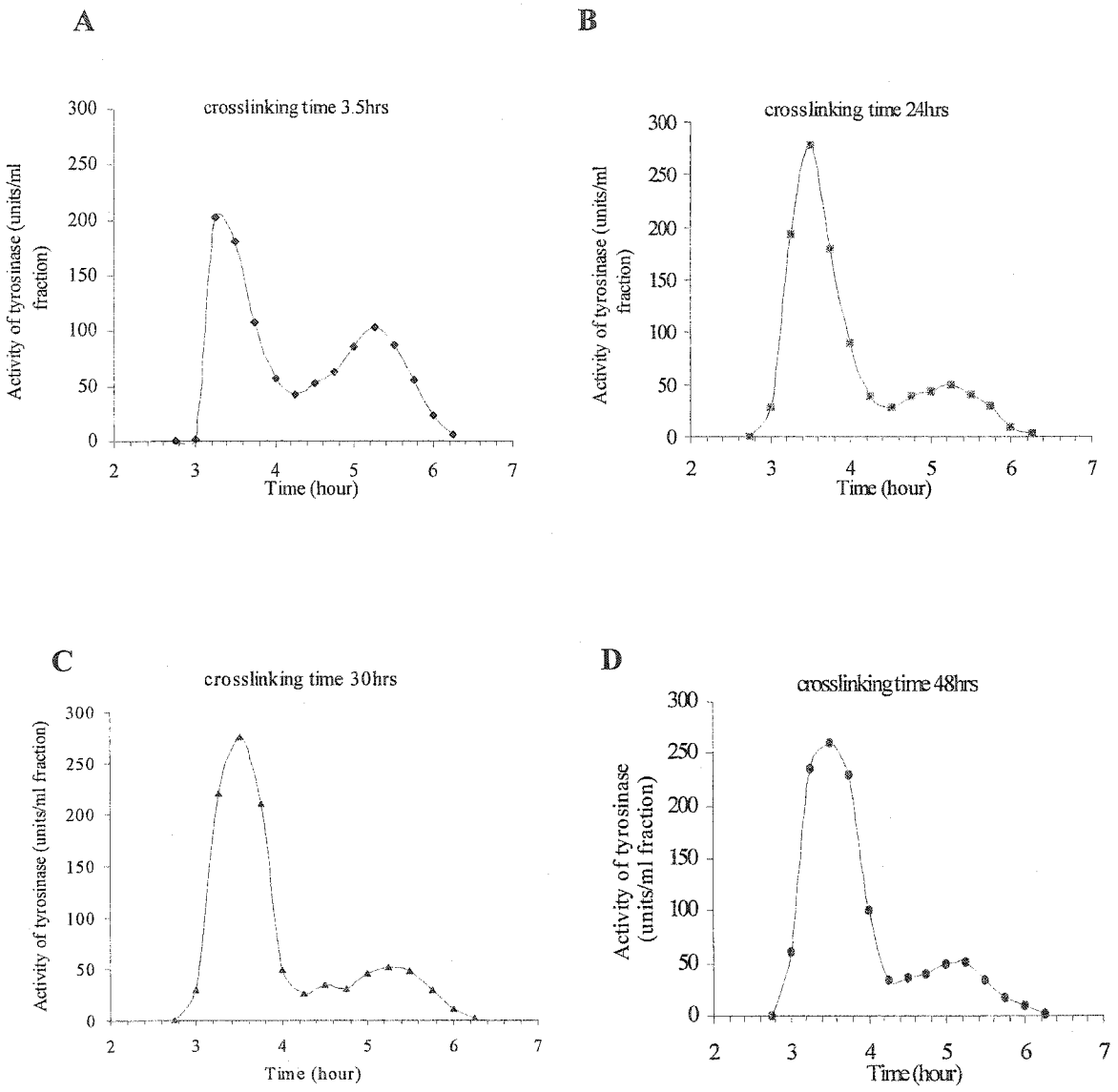

Figure 4.9 The activity of tyrosinase separated through Sephadex G-200 1.6 cm x 70 cm column, equilibrated with 0.1M Tris·HCl, and eluted at 12 ml/hr. (A) PolyHb-tyrosinase crosslinked of 3.5 hrs. (B) PolyHb-tyrosinase crosslinked of 24 hrs. (C) PolyHb-tyrosinase crosslinked of 30 hrs. (D) PolyHb-tyrosinase crosslinked of 48 hrs.

4.4.7.2 Animal studies in intravenous injection of PolyHb-tyrosinase crosslinked for efficient length of time

The above *in-vitro* study suggests that more complete crosslinking may increase the circulating tyrosinase activity of PolyHb-tyrosinase. The following study is to look at the effect of degree of crosslinking and its effects in animal after intravenous injection. We followed (1) changes in circulating tyrosinase activity, (2) changes in plasma tyrosine concentration and (3) changes in plasma polyhemoglobin concentration.

PolyHb-tyrosinase — 3.5 hours crosslinking

The reaction time was allowed to proceed for 3.5 hrs before being stopped by addition of excess lysine and one ml was injected per 250 g body weight per rat. Tyrosine concentration and tyrosinase activity were measured to investigate if PolyHb-tyrosinase is efficient on removing tyrosine in plasma. PolyHb concentration was also measured to test how fast the sample is being removed from the system. From our results, plasma tyrosine level markedly decreased after injection. At the first hour (Figure 4.10), tyrosine level went down quickly to 16% of the concentration before injection. In 6 hours, tyrosine concentration still stayed at 37% of that before injection. This indicated that PolyHb-tyrosinase could remove tyrosine efficiently. However, in 24 hours, tyrosine level went up to 83%. We measured the tyrosinase activity in plasma. Our results showed that the activity of tyrosinase also decreased with time. At the first hour, the activity of tyrosinase reached to the highest level at 1303 ±411 U/ml,

then went down gradually with time reaching 61±49 U/ml by 6 hours. For PolyHb concentration, at the first hour, PolyHb concentration reached the highest level, then decreased with time. However, by 24 hours, 31% of the PolyHb still remained in the circulation.

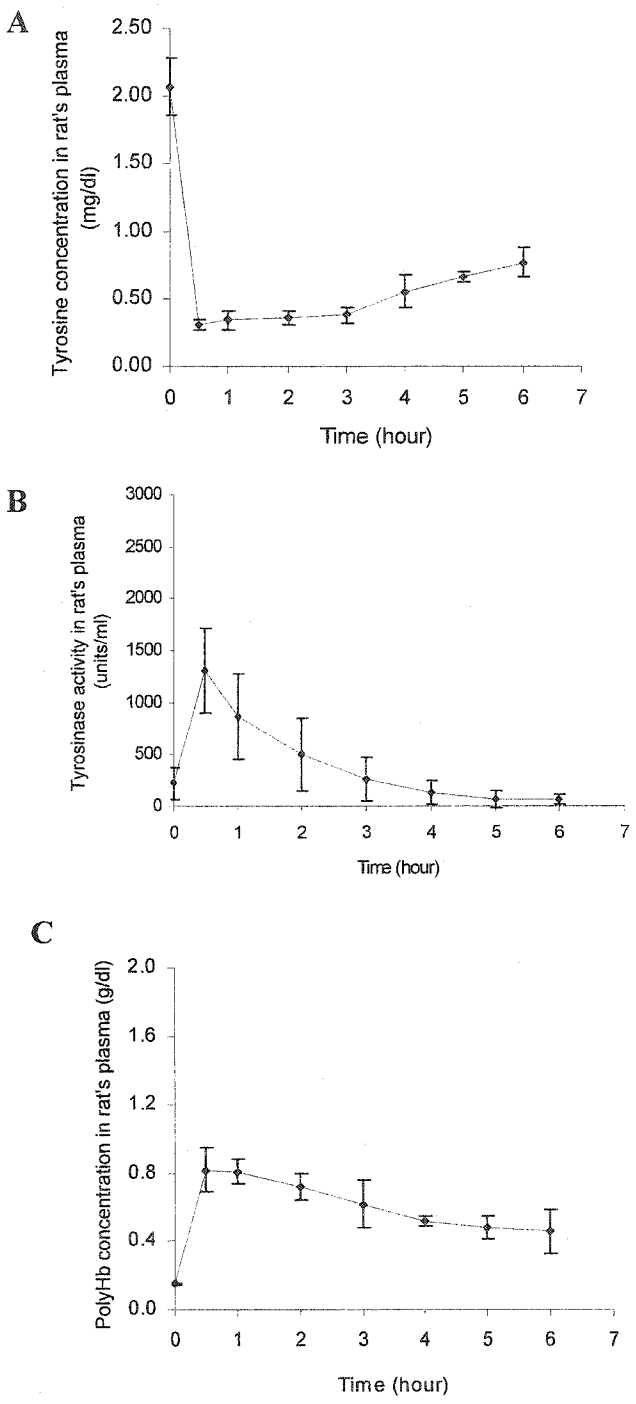

Figure 4.10: (A) tyrosine concentration, (B) tyrosinase activity and (C) PolyHb concentration in rat plasma after injecting 1 ml of PolyHb-tyrosinase (3.5 hrs crosslinked) to rats.

PolyHb-tyrosinase —10 hours crosslinking

As shown in our *in vitro* study, the longer the reaction time, the more efficient the crosslinking of tyrosinase to PolyHb. We therefore extend the crosslinking time to 10 hours. The same procedures were followed as above, and the same parameters were measured. Figure 4.11 showed that at the first hour, plasma tyrosine level went down quickly to 16% of the concentration before injection. In 24 hours, tyrosine level went up to 86% of that before injection. Tyrosinase activity was 1408±90 U/ml in 30 minutes after injection. This was only very slightly higher than that of PolyHb-tyrosinase crosslinked for 3.5 hrs. At 6 hrs after injection, the enzyme activity was 170±53 U/ml, which also slightly higher. During the first 6 hrs, PolyHb concentration was also slightly higher than that for in crosslinking time of 3.5 hrs. In 24 hrs, PolyHb level remained 33% more than the level before injection.

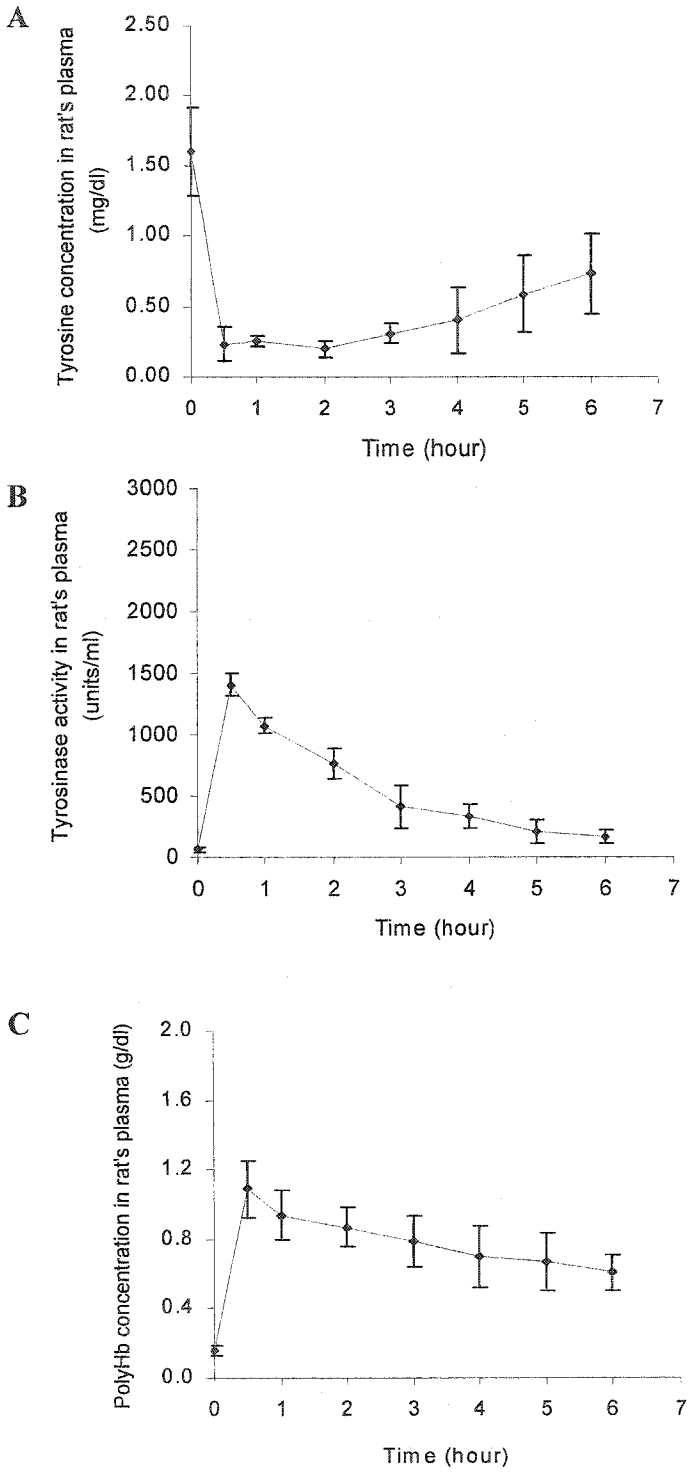

Figure 4.11: (A) tyrosine concentration, (B) tyrosinase activity and (C) PolyHb concentration in rat plasma after injecting 1 ml of PolyHb-tyrosinase (10 hrs crosslinked) to rats.

PolyHb-tyrosinase— 16 hours crosslinking with double the amount infused

From the above results, extending reaction time from 3.5 hours to 10 hours resulted in a slightly improved effect on plasma tyrosine level, tyrosinase activity, and PolyHb concentration. We therefore increased the crosslinking time to 16 hrs, and injected 2 ml samples per rat. Plasma tyrosine went down to 11% of the original activity at the first hour (Figure 4.12). In 24 hrs, tyrosine level reached to 78% of original level. For tyrosinase activity, 1.3 times higher activity was found in this group compared to that of in crosslinking of 10 hrs. PolyHb concentration was 1.5 times higher when compared to the group in which crosslinking time is 10 hrs. In 24 hrs, 50% of the PolyHb still remained in the plasma. Our results showed that longer reaction time and a larger volume of PolyHb-tyrosinase resulted in further improvement.

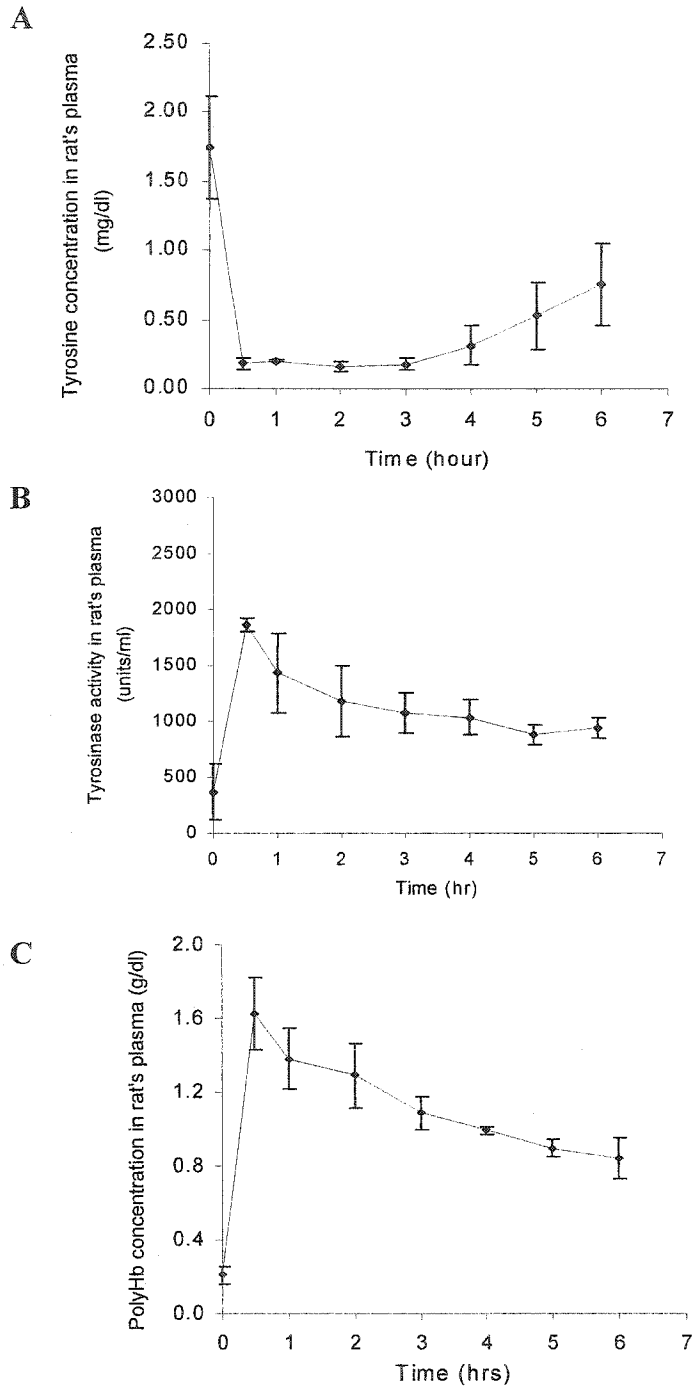

Figure 4.12: (A) tyrosine concentration, (B) tyrosinase activity and (C) PolyHb concentration in rat plasma after injecting 2 ml of PolyHb-tyrosinase (16 hrs crosslinked) to rats.

PolyHb-tyrosinase — 24 hours crosslinking and injection volumes of 1 ml, 2 ml, or 3 ml

In the previous preliminary study it was found that extended reaction time and larger injection volume of PolyHb-tyrosinase solution brought significant changes in tyrosine level, tyrosinase activity and PolyHb concentration. We therefore further extended the reaction time to 24 hrs, and inject 1 ml, 2 ml or 3 ml of PolyHb-tyrosinase solution per rat respectively (Figure 4.13-15).

From our result it would appear that crosslinking for 24 hours and injecting 1 ml PolyHb-tyrosinase per 250 gm body weight in rats has the same effectiveness as the injection of 2 ml or 3 ml. The high volume of 2 ml or 3 ml resulted in adverse effects in the animal — weight loss and inactivity. Thus we arrive at a crosslinking time of 24 hours and giving the animal a PolyHb-tyrosinase dose of 1 ml per 250 gm body weight.

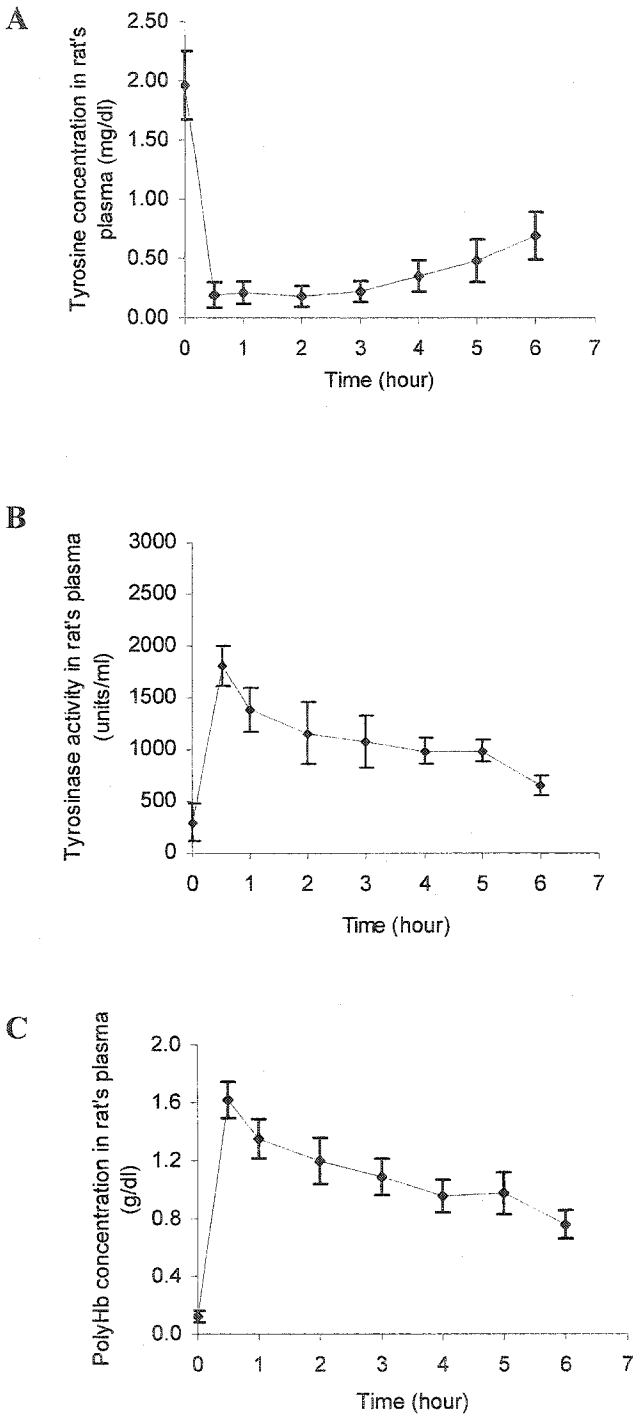

Figure 4.13: (A) tyrosine concentration, (B) tyrosinase activity and (C) PolyHb concentration in rat plasma after injecting 1 ml of PolyHb-tyrosinase (24 hrs crosslinked) to rats.

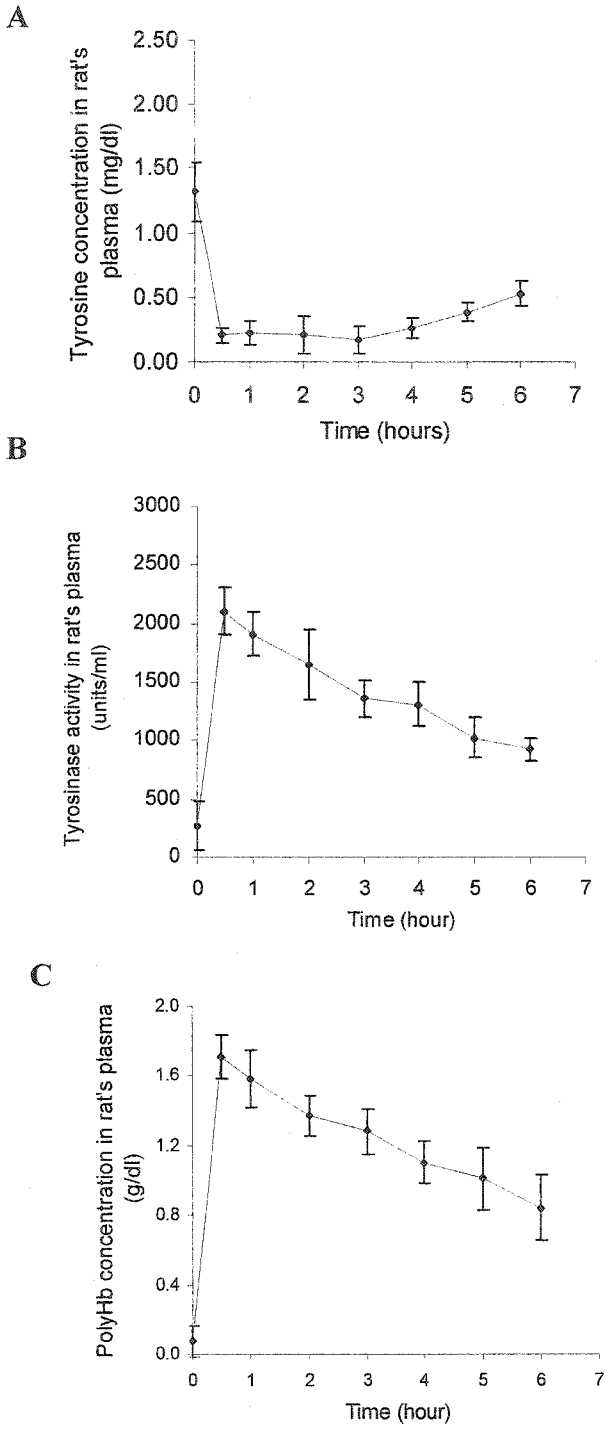

Figure 4.14: (A) tyrosine concentration, (B) tyrosinase activity and (C) PolyHb concentration in rat plasma after injecting 2 ml of PolyHb-tyrosinase (24 hrs crosslinked) to rats.

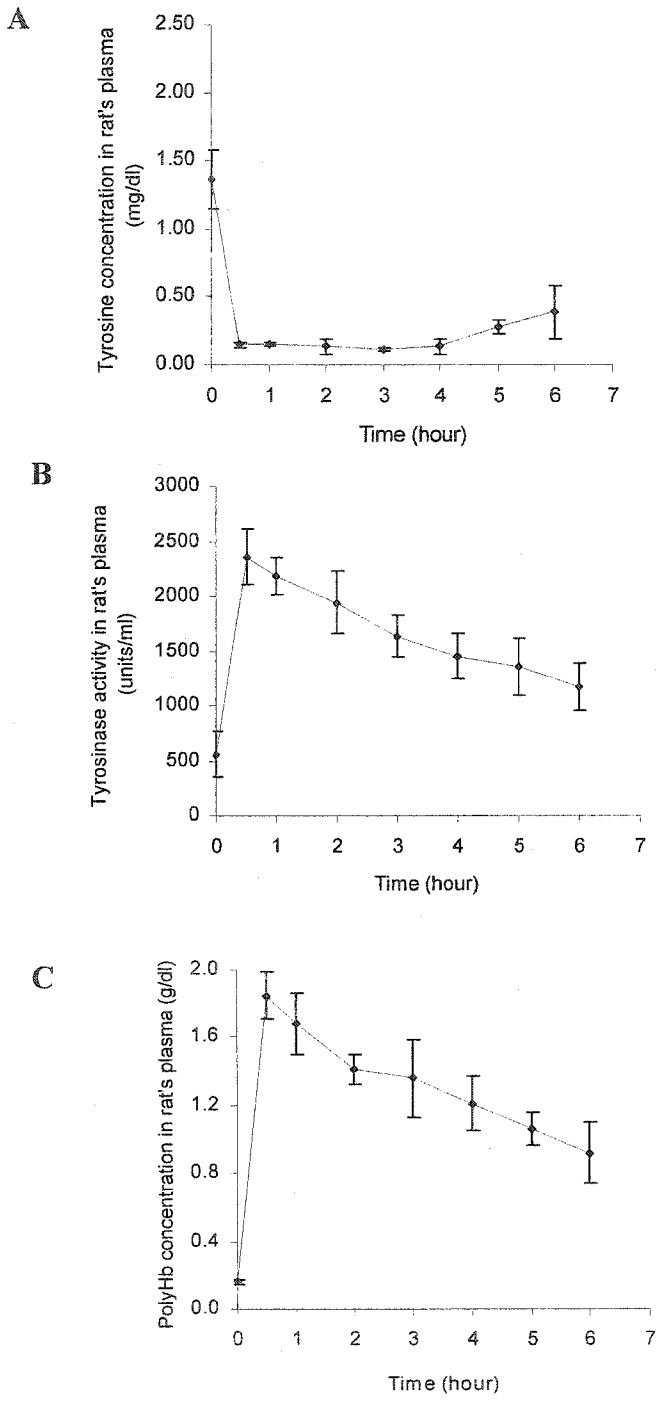

Figure 4.15: (A) tyrosine concentration, (B) tyrosinase activity and (C) PolyHb concentration in rat plasma after injecting 3 ml of PolyHb-tyrosinase (24 hrs crosslinked) to rats.

4.4.7.3 Changes in tyrosinase concentration in PolyHb-tyrosinase

We have estimated that 24 hours crosslinking is the optimal time of crosslinking to give the optimal *in vivo* effect. Since tyrosinase is expensive, the next step is to see the effect of using lower amount of tyrosinase in PolyHb-tyrosinase formed by crosslinking for 24 hours.

PolyHb-tyrosinase with 1/8 of the original tyrosinase

Figure 4.16 showed that at the first hour after injection plasma tyrosine level only decreased to 60% of the original level before injection. This is corresponding to the low plasma tyrosinase activity.

PolyHb-tyrosinase with 1/4 of the original tyrosinase

Figure 4.17 showed that at the first hour, plasma tyrosine level only decreased to 22% of that concentration before injection, and this was reflected by a corresponding lower level of plasma tyrosinase activity.

PolyHb-tyrosinase with 1/2 of the original tyrosinase

One ml of PolyHb-tyrosinase with half the concentration of tyrosinase was injected intravenously to rats (Figure 4.18). Tyrosine concentration decreased to 18% of the level before injection at the first hour. However, the plasma tyrosine increased rapidly by 3 hrs. Lower tyrosinase activity was observed in this group.

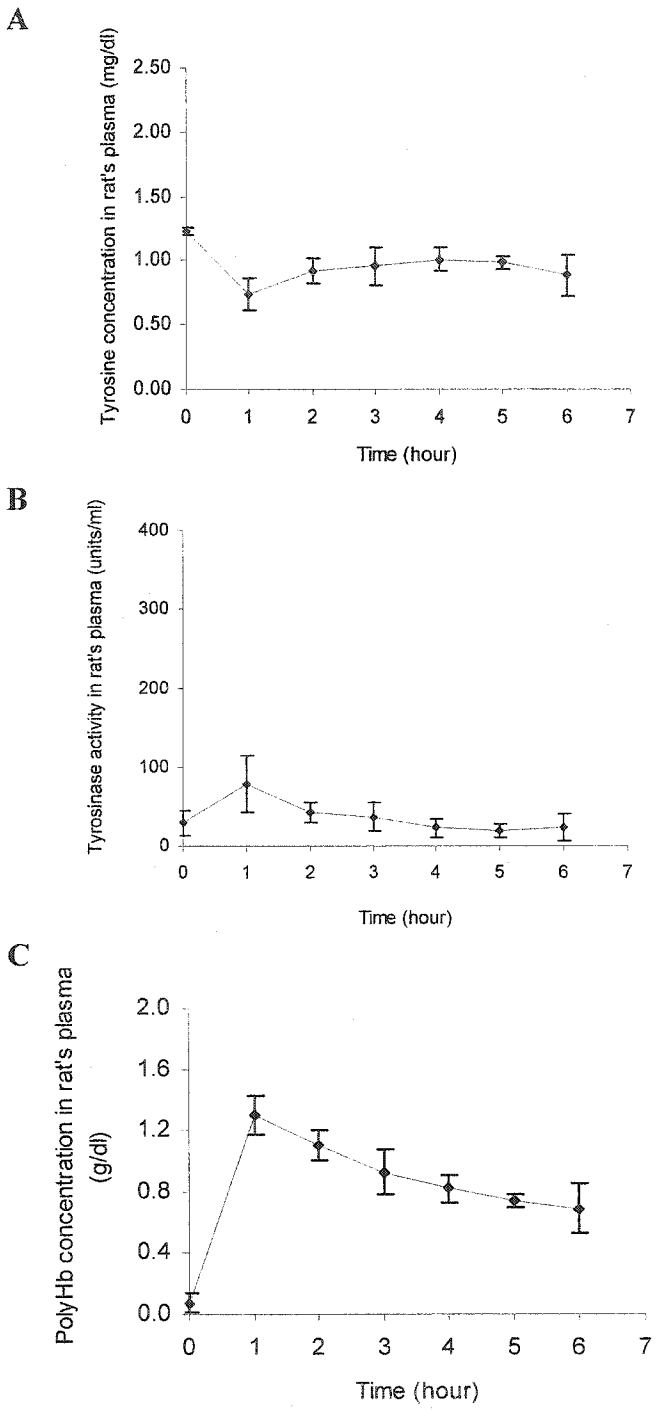

Figure 4.16: (A) tyrosine concentration, (B) tyrosinase activity and (C) PolyHb concentration in rat plasma after injecting 1 ml of PolyHb-1/8tyrosinase to rats.

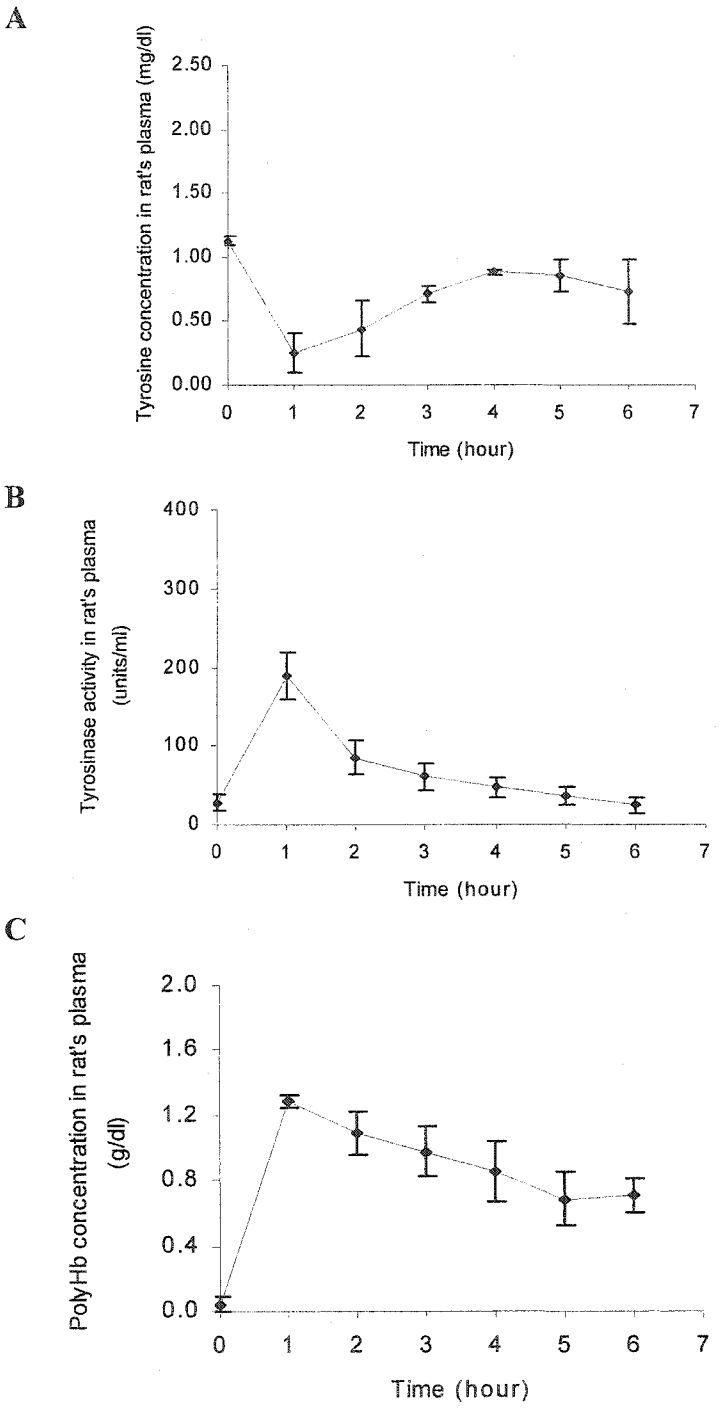

Figure 4.17: (A) tyrosine concentration, (B) tyrosinase activity and (C) PolyHb concentration in rat plasma after injecting 1 ml of PolyHb-1/4tyrosinase to rats.

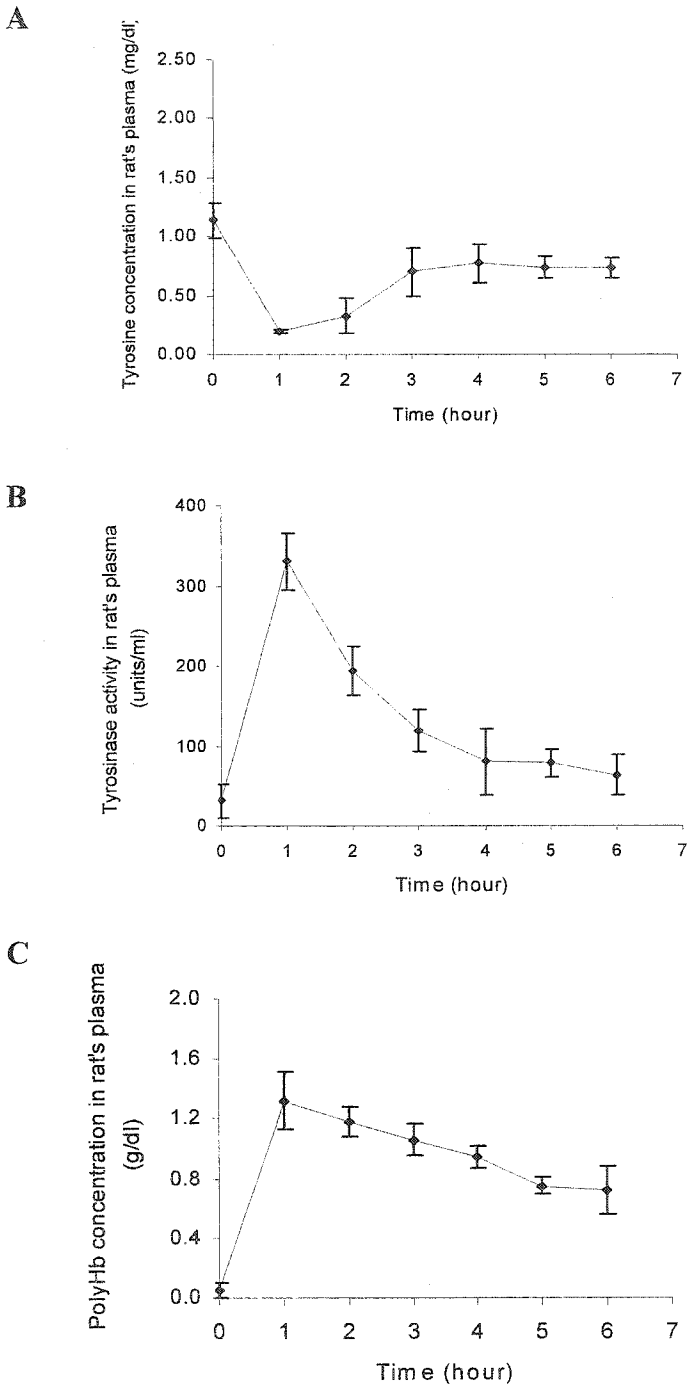

Figure 4.18: (A) tyrosine concentration, (B) tyrosinase activity and (C) PolyHb concentration in rat plasma after injecting 1 ml of PolyHb-1/2tyrosinase to rats.

The above results show that the optimal procedure would be to use the original tyrosinase concentration and carry out the reaction for 24 hours. The optimal dose of intravenous injection is also established to be 1 ml. This is therefore used in the next animal study in the next chapter.

4.4.8 Studies of melanoma cell culture.

B16-F10 is one of the variant cell lines of the B16 melanoma. To study whether PolyHb-tyrosinase can inhibit the growth of melanoma cells, we cultured B16F10 melanoma cells in DMEM with adding appropriate aliquots of saline solution, PolyHb solution, PolyHb-tyrosinase solution and free tyrosinase solution respectively. Cell growth was determined by trypan blue exclusion every day. Cells were counted at day 0 before the medium was added samples and on each day following. From our results, after day 1, melanoma cells in saline solution and PolyHb solution were growing up. On the contrary, the cell growth in PolyHb-tyrosinase solution and free tyrosinase solution was decreasing because tyrosinase inhibits the growth of melanoma cells by removing tyrosine from the medium (Figure 4.19).

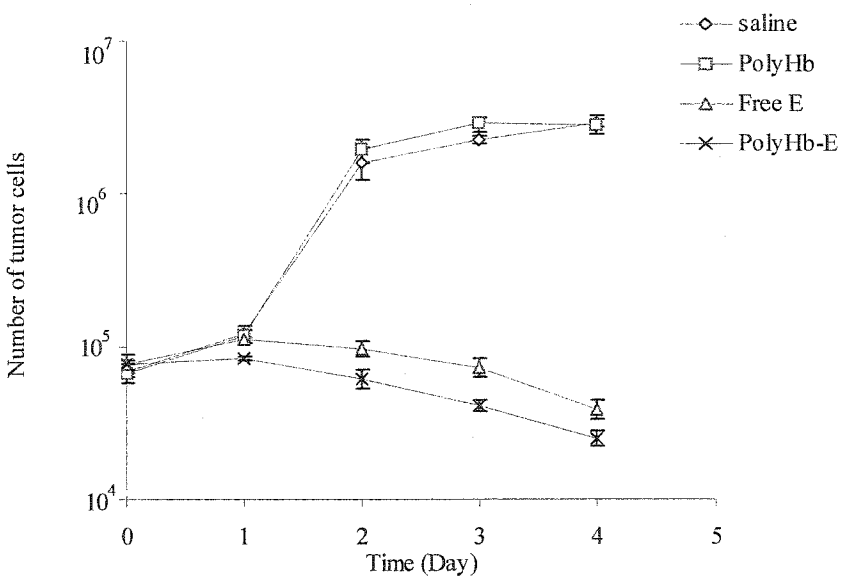

Figure 4.19 *In vitro* growth curves of B16F10 cell lines after adding saline, PolyHb solution, free tyrosinase solution and PolyHb-tyrosinase solution to the medium.

4.5 Discussions

In this report, we first studied the effect of varying glutaraldehyde ratio, crosslinking times and enzyme concentrations on PolyHb-tyrosinase activity. Our results in this study show that the potential usefulness of glutaraldehyde in polymerizing hemoglobin and tyrosinase for the development of a modified hemoglobin oxygen carrier with antitumor properties. Glutaraldehyde polymerization of hemoglobin has been extensively studied [33-35]. Glutaraldehyde preferentially reacts with ε-amino groups of lysine residues of proteins. The non-specific nature of this polymerization produces monomers, oligomers, and polymers differing in size, enzymatic activity, and protein composition. Our measurements of tyrosinase activity do not differentiate between the enzymatic activities of bound and unbound enzyme fractions. The increased circulation half-life of tyrosinase in the highly polymerized solution suggests that with increase in time of crosslinking more tyrosinase molecules crosslinked to PolyHb resulting in increasing in retention time. We speculate that the increased fraction of bound enzymes have largely retained their activity. Detailed analysis and optimization of enzymatic activity contained in individual molecular weight fractions is also investigated in the following experiments.

We next studied the molecular weight distribution of PolyHb-tyrosinase at different crosslinking hours. We found that the longer the crosslinking time, the more PolyHb-tyrosinase we can obtain in high molecular weight. In the study of oxygen affinity of free hemoglobin and PolyHb-tyrosinase, we found out that PolyHb-tyrosinase possess oxygen transport characteristics similar to the non-crosslinked

hemoglobin. We then carried out a preliminary study in animal by intravenous injection of PolyHb-tyrosinase. As tyrosinase activity decreased quickly at the end of experiment, we analyzed the possible reasons related to this decreasing. One could be enzyme concentration is not high enough. Second of all, it might be the body temperature decreased the enzyme activity inside the body. Another reason could be too little enzyme crosslinked to hemoglobin or crosslinking time is not long enough. We designed several experiments to find out the reason for this decreased enzyme activity. Our results showed that incubation at 37°C, PolyHb-tyrosinase remained 79% activity after 6 hours. We also tested the enzyme activity by passing Sephadex G-200 column and found that longer crosslinking time is necessary to get high activity of PolyHb-tyrosinase. We then further investigate the effects of varying crosslinking time from 3.5 hrs up to 24 hrs and also the use of different injection volumes and various enzyme concentrations on lowering systemic tyrosine level. We found that the optimal preparation is prepared from a crosslinking time of 24 hrs using an injection volume of 1 ml of PolyHb-tyrosinase. This way, it decreases the systemic tyrosine level, but also has no adverse effect and can maintain body weight. Higher volumes of PolyHb-tyrosinase injection yield the lowest level of systemic tyrosine, however, the animals dramatically lose body weight due to severe starvation for tyrosine [36]. Our cell culture studies confirm that lower tyrosine level retard the growth of B16F10 melanoma cell line.

4.6 Acknowledgements

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4.8 Appendix: Hemoglobin measurement

Drabkin's method was used to measure hemoglobin concentration [1]. Hemoglobin and its derivatives, except sulfhemoglobin, are oxidized to methemoglobin by ferricyanide. Methemoglobin is then converted to cyanomethemoglobin by its reaction with cyanide (Drabkin's reagent). The red colored complex that is produced has a peak absorbance at 540 nm, and its proportional to hemoglobin concentration. Drabkin's reagent contains sodium bicarbonate (1 g/l), potassium cyanide (0.052 g/l) and potassium ferricyanide (0.13 g/l). Cyanomethemoglobin standard (0.08 g/dl) is supplied in kit. Standard and reagent solution are combined to give working standards with hemoglobin equivalency (g/dl) based on dilution sample factor of 1:251. The procedure is performed as follows. Zero the spectrophotometer with 1 ml cuvettes containing reagent solution. Add 20 µl of sample to 5 ml reagent solution. After mixing, wait 15 min, transfer to cuvettes, then measure absorbance at 540 nm. Determine concentration of sample from standard curve.

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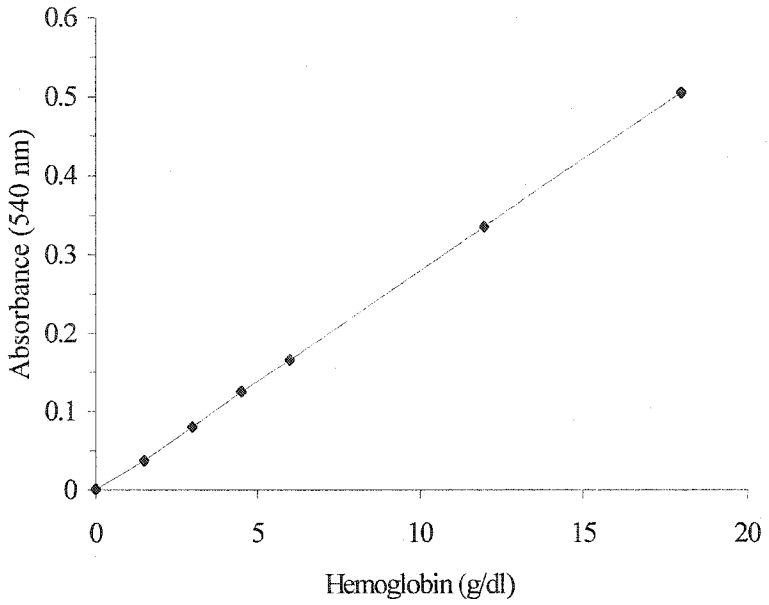

Figure 4.20 Standard curve for hemoglobin.

4.9 Appendix: Sephadex G-200: calibration curve

The molecular weight exclusion limits of the Sephadex G-200 column were 5-600,000 Daltons. The void volume (V_o) of the Sephadex G-200 column was found to be 40 ml determined by the initial elution peak of a sample of Blue Dextran 2000. K_{av} values were calculated from the equation, V_E-V_o/V_T-V_o. Where V_T represents effective bed volume (radius$^2\pi$ × gel height), V_E represents elution volume of sample. Calibration curve was plotted as Log molecular weight versus K_{av} values. The calibration proteins were thyroglobulin (669 kD), apoferritin (443 kD), β-amylase (200 kD), alcohol dehydrogenase (150 kD), albumin (66 kD), carbonic anhydrase (29 kD).

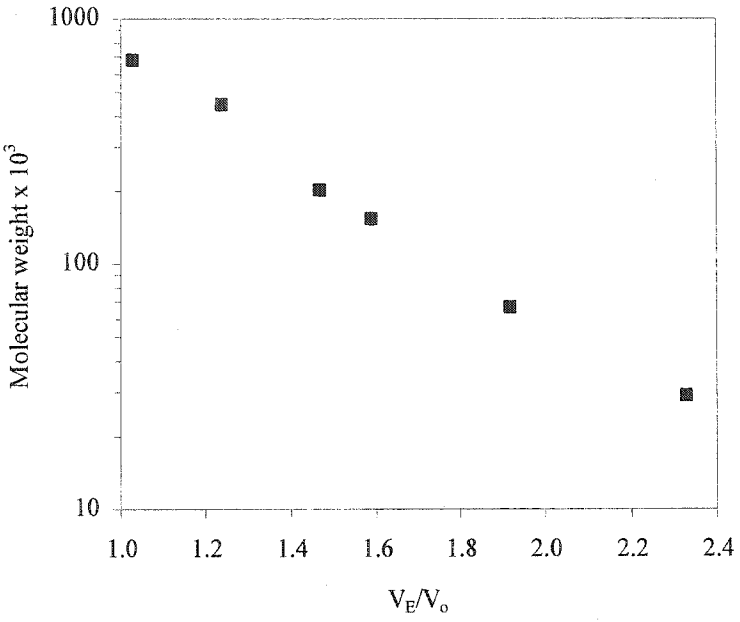

Figure 4.21: Molecular weight calibration curve for Sephadex G-200 column.

Linkage to Chapter 5

In the previous chapters, our studies show that oral encapsulated tyrosinase or intravenous PolyHb-tyrosinase can decrease systemic tyrosine level significantly in rats. However, oral administration of encapsulated tyrosinase can only lower the systemic tyrosine level usefully after 3-4 days. Although intravenous injection of PolyHb-tyrosinase can lower the systemic tyrosine to 10% in one hour, it only lasts for 24 hours. We therefore need to investigate the effects of intravenous injection of PolyHb-tyrosinase combined with oral administration of encapsulated tyrosinase on quickly lower the systemic tyrosine level and maintain at this low level.

CHAPTER 5

Effects of Combined Oral Administration and Intravenous Injection on Maintaining Decreased Systemic Tyrosine Levels in Rats

5.1 Abstract

Our previous studies indicated that encapsulated tyrosinase and crosslinked hemoglobin with tyrosinase (PolyHb-tyrosinase) decreased systemic tyrosine level significantly in rats. However, we needed a few days of oral administration of encapsulated tyrosinase before the systemic tyrosine level started to decrease. Although intravenous injection of polyhemoglobin-tyrosinase can lower the systemic tyrosine to 10%, the level increases towards normal after 24 hours. We therefore investigated the effects of intravenous injection of PolyHb-tyrosinase combined with oral administration of encapsulated tyrosinase on lowering the systemic tyrosine level. We also optimized this combined method for lowering systemic tyrosine in animal studies and found out that two intravenous injections of PolyHb-tyrosinase followed by three times a day oral administration of encapsulated tyrosinase could immediately lower the body tyrosine and maintained this low level as long as the oral administration was continued.

5.2 Introduction

Melanoma, a malignant neoplasm derived from melanocytes of the skin and other sites, continues to increase in frequency worldwide. In the United States and Canada, the rate of increase of melanoma is greater than for any other tumors except lung cancer in women [1]. The increase in melanoma frequency is greatest on body sites where sun exposure has increased because of changes in clothing styles and materials and in recreational habits. Although the exact cause of melanoma is unknown, sunlight has been implicated as a major factor [2]. Chronic long-term exposure to sun may develop lentigo maligna melanoma, while short high-intensity exposure to the sun (inducing sunburn) may lead the malignant transformation of melanocytes in the case of nodular melanoma or superficial spreading melanoma. Those with family history of melanoma have three to four times higher chance than the general population in the probability of developing the disease [3]. The possible role of trauma in causing of melanoma is one of the other etiology factors. Artificial sources of ultraviolet radiation as in tanning beds may be associated with an increased risk of melanoma [4]. On the cellular basis, there is a comparable low degree of cell differentiation in fish and human melanoma [5-6]. Furthermore, consistent chromosomal aberrations have been detected in melanoma, chromosomes 1, 6, and 7 being the most frequently involved [7].

Many different methods are explored for the treatment of melanoma. Surgery to remove the tumor plus regional lymphadenectomy is at present an effective therapy for primary melanoma [8-9]. In occasional case of disseminated melanoma, surgery can offer relief of symptoms and improvement in quality of life [10]. Radiation therapy

plays an important role in the management of brain metastases and following therapeutic lymph node dissection for bulky disease [11]. In chemotherapy, Dacabazine (DTIC) has been the most extensive clinical trials of any single agent with a reported response rate of 22% [12]. Nitrosoureas are the next most active single agents [13]. Cisplatin is another single agent that has undergone adequate clinical trials. Hormones have also been suggested [14]. Others include combination chemotherapy, adjuvant chemotherapy, regional perfusion and high-dose chemotherapy with autologous bone marrow transplantation although most combination chemotherapy is usually more toxic [15-19]. Immunotherapy including interferons, interleukin-2, TNF and many other biologic agents have been studied extensively in the treatment of melanoma [20-22]. Recently, as the lack of effective treatment for this advanced disease, gene therapy of melanoma has been investigated in the treatment of melanoma [23].

Another approach is to lower systemic tyrosine by dietary restriction of tyrosine because the growth of melanoma cells needs tyrosine. In fact, high cellular intake of tyrosine is increased further by the requirement of this amino acid for protein synthesis in the malignant melanoma cells. The rationale for dietary restriction is to limit tyrosine availability for protein synthesis and tumor growth. As early as in 1964, Halvorsen and Gjessing started to use dietary restriction in tyrosine and phenylalanine for the treatment of the acute and chronic forms in the diseases of the disorders of tyrosine oxidation [24]. It benefited to most patients and associated with dramatic clinical improvement. Demopoulos [25] later showed that this approach can be useful for patients with advanced malignant melanoma. However, this regimen is cumbersome,

complex. It is appetite depression due to not palatable and causes nausea and vomiting, thus making it not practical. Another disadvantage for this nutritional control of tumor growth is severe body weight loss [26].

We previously showed that oral administration of artificial cells encapsulated with tyrosinase could decrease tyrosine level although it took a few days to reach this low level. We also showed that intravenous injection of PolyHb-tyrosinase could lower systemic tyrosine level within one hour. However, the lowered tyrosine level increased back towards normal level after 24 hours. We therefore try to combine the oral administration method with intravenous injection to lower systemic tyrosine level and keep it at that low level as long as the oral administration is continued. We further optimize this combined method for its efficiency in animal experiments.

5.3 Materials and Methods

Materials

L-tyrosine (98% TLC), tyrosinase from mushroom (EC. 1.14.18.1, 5350 units/mg stated activity), hemoglobin from bovine (lyophilized powder), L-lysine (monohydrochloride, SigmaUltra > 99%) were purchased from Sigma-Aldrich Company. Glutaraldehyde (25%) was obtained from BDH. Collodion was purchased from Fisher Scientific Company. Purified bovine hemoglobin (10 g/dl) was purchased from Biopure Corporation, Boston, MA. All other reagents were of analytical grade.

Preparation of Polyhemoglobin and Polyhemoglobin-tyrosinase

Reaction mixtures were prepared containing hemoglobin (10 g/dl), tyrosinase (12000 U/ml) in 0.1 M potassium phosphate buffer, pH 7.6. In Polyhemoglobin mixtures, an equivalent volume of buffer replaced enzyme condition. Prior to the start of crosslinking, 1.3 M lysine was added at a molar ratio of 7:1 lysine/hemoglobin. Crosslinking reaction was started with the addition of glutaraldehyde at molar ration of 16:1 glutaraldehyde/hemoglobin. Glutaraldehyde was added in four equal aliquots over a period of 15 minutes. After 24 hours under aerobic conditions with constant stirring at 4°C, reaction was stopped with 2.0 M lysine at a molar ratio of 200:1 lysine/hemoglobin. Solutions were dialyzed in physiological saline solution overnight and pass through sterile 0.45 μM filter. Aliquots (500 μl) of the 16:1 crosslinked preparation were concentrated using 100 KD microconcentrators (Amicon, Beverly, MA). Samples were centrifuged at 2500 g for 55 minutes at 23°C. Then, retentate was

collected. Hemoglobin concentration was determined by cyanomethemoglobin at 540 nm [27]. Final retentates were diluted to desired concentration 7 g/dl and stored in 4°C fridge for later use.

Preparation of control artificial cells

Control artificial cells were prepared by the standard published method [28-30]. Briefly, 1 g hemoglobin and 200 mg Tris were dissolved in 10 ml double distilled deionized water. Stir with a metal rod until everything is dissolved. Gravity filters the solution through a Waterman #42 filter into an Erlenmeyer flask. Take 2.5 ml of this 10 g/dl hemoglobin solution and was encapsulated within spherical, ultrathin, cellulose nitrate membrane. Without tyrosinase loaded microcapsules were administrated orally to control group. All control artificial cells were prepared daily and stored in 1% v/v Tween 20 solution at 4°C until use.

Preparation of tyrosinase loaded microencapsules

1.907 mg of 5350 units/mg tyrosinase was dissolved in 5 ml of 10% hemoglobin solution, and then followed the methods described to immobilize tyrosinase in collodion membrane microcapsules. Microcapsules prepared as a 50% suspension for later feeding. Tyrosinase loaded microencapsules were administered orally to test group. All artificial cells were prepared daily and stored in 1% v/v Tween 20 solution at 4°C until use.

Before oral administration for both control group and test group, artificial cells suspended in Tween 20 were washed and resuspended in 0.1 M Tris·HCl buffer (pH 8.5). The total volume of artificial cells for feeding was 2.5 ml (1 ml artificial cells plus 1.5 ml Tris·HCl buffer). This buffer is enough to protect the microencapsulated tyrosinase activity during its passage through the stomach with its acidic medium.

Animal studies *in vivo*

Fasted male Sprague-Dawley rats (130-150 g) were used in this study. They were kept in a controlled 12-hr light/dark environment with food and water ad libitum. Two groups were studied: (1) control group: feed with artificial cells without enzyme; (2) test group: feed with artificial cells loaded with tyrosinase. Each experiment began on day 0 with blood taken, and no artificial cells were administered or intravenous injection was done on that day. The rats were anaesthetized with intraperitoneal injection of pentobarbital (Somnotol, 65 mg/kg). Polyethylene cannulae were inserted and secured distal to the superficial epigastric branches in the femoral veins (PE-10, PE-50 Clay Adams). Samples were injected through femoral vein, and blood was taken from femoral artery. The plasma in each blood sample was separated from the blood by centrifuge and placed in a 1.5 ml plastic tube, then stored at -80°C until analyzed. The tyrosine concentration in plasma was analyzed by fluorometric method using Perkin Elmer Luminescence Spectrometer LS50B [31].

Three-dose/day combined with one injection on day 1 animal experiment

On day 0, took blood at 4:00 pm. No artificial cells were administrated on this day. From that day on, and every subsequent day for 4 days, artificial cells were administrated orally at 10:00 am, 2:00 pm and 6:00 pm, took blood samples every day just after the second feeding. On day 1, injected PolyHb to control group or PolyHb-tyrosinase sample to test group at volume of 1 ml per 250 g body weight.

Three-dose/day combined with two injections on day 1 and day 2 animal experiment

On day 0, took blood at 4:00 pm. No artificial cells were administrated on this day. From that day on, and every subsequent day for 4 days, artificial cells were administrated orally at 10:00 am, 2:00 pm and 6:00 pm, and took blood samples every day just after the second feeding. On day 1, injected PolyHb to control group or PolyHb-tyrosinase sample to test group at volume of 1 ml per 250 g body weight. On day 2, inject half volume of PolyHb or PolyHb-tyrosinase sample to the control group and the test group respectively.

Statistical analysis

The differences of tyrosine concentration in rat's plasma between two groups (control group and test group) at the same time point were determined by using Student's t-test within ANOVA and considered significant at $p< 0.05$.

5.4 Results

Three-dose/day combined with one injection on day 1 animal experiment

Since intravenous injection of PolyHb-tyrosinase decreased tyrosine level quickly, we investigate whether we can decrease tyrosine to a low level at the beginning of our experiment and keep this low level with oral administration of encapsulated tyrosinase. Body weight and tyrosine level were measured.

There was no significant difference in body weight between the control group and test group (Figure 5.1).

On day 1, after intravenous injection, tyrosine level decreased rapidly to 53±4% of the control group. This is consistent to the results we obtained in our previously intravenous studies. On day 2, tyrosine level increased up to 73±6% of the control level. After day 2, tyrosine level started to decrease further reaching 59±3 % on day 4 (Figure 5.2). Our previous results indicated that oral administration of artificial cells alone cannot lower tyrosine level significantly or keep it at a very low level during the first 2 days of treatment. However, the addition of one intravenous injection of polyhemoglobin-tyrosinase to oral encapsulated tyrosinase was only able to maintain a sufficiently low tyrosine level one day after. The level two days later was significantly lower than control, but it is not sufficiently low. Table 5.1 shows that the statistical studies for tyrosine concentrations in the control group and test group.

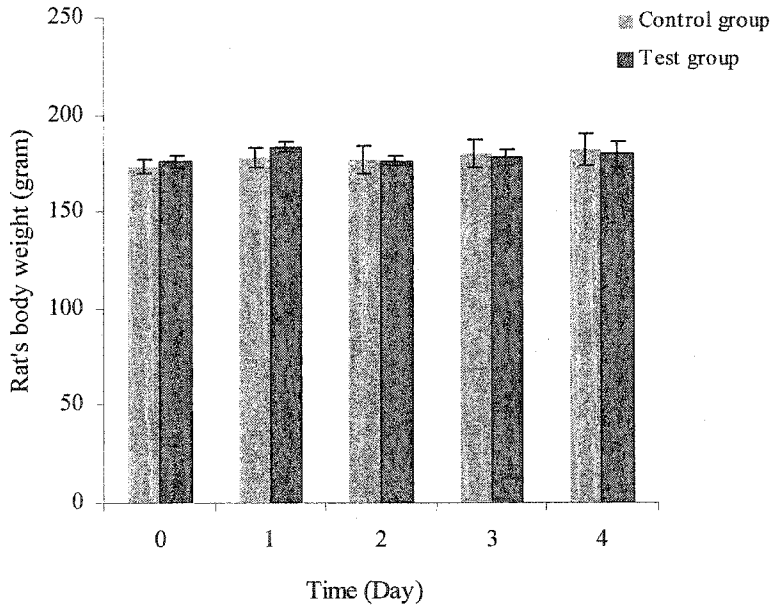

Figure 5.1 Body weight (gram) of rats for 4 days experiments. For control group: oral administration of artificial cells three times a day with injection of 1 ml of PolyHb solution per 250 g body weight on day 1. For test group: oral administration of artificial cells encapsulated tyrosinase three times a day with injection of 1ml of PolyHb-tyrosinase sample per 250 g body weight on day 1.

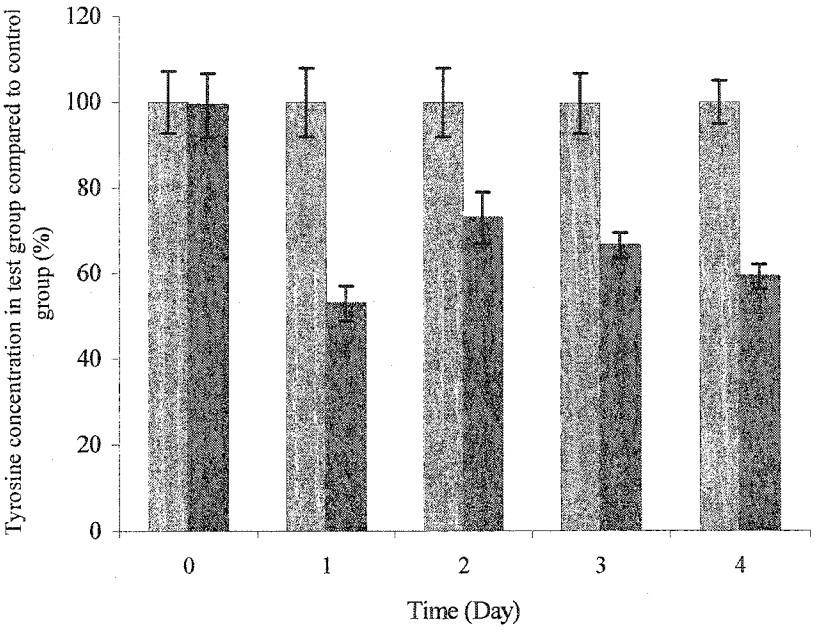

Figure 5.2 Tyrosine concentration in rat's plasma (%). For control group: oral administration of artificial cells three times a day with injection of 1 ml of PolyHb solution per 250 g body weight on day 1. For test group: oral administration of artificial cells encapsulated tyrosinase three times a day with injection of 1ml of PolyHb-tyrosinase sample per 250 g body weight on day 1.

Comparing	P-value
C day 0 / T day 0	NS
C day 1 / T day 1	NS
C day 2 / T day 2	NS
C day 3 / T day 3	< 0.0005
C day 4 / T day 4	< 0.005
C day 5 / T day 5	< 0.005
C day 6 / T day 6	< 0.0005
C day 7 / T day 7	< 0.0005

Table 5.1 P-value in plasma tyrosine concentration between control group and test group. C stands for control group receiving oral artificial cells three times a day and 1 ml intravenous injection of PolyHb solution on day 1. T stands for test group receiving oral artificial cells encapsulated tyrosinase three times a day and 1 ml intravenous injection of PolyHb-tyrosinase solution on day 1.

Three-dose/day combined with two injections on day 1 and day 2 animal experiments

The following experiment is designed to investigate if it is possible to maintain a sufficiently lower tyrosine level on the second day after the first intravenous injection. This is by giving a second intravenous injection on the 2nd day that is half the volume of the first injection.

As in previous studies, the body weight of rats was monitored during the experiment. Our results showed in Figure 5.3 that there was no significant difference in the body weight between the control group and the test group.

In this experiment, we followed tyrosine levels in three groups including the control group, the second group in which only orally administrated encapsulated tyrosinase and the third group of 2 intravenous injections combined with oral administration. In the third group, on day 1 tyrosine level decreased to 54±6% after the first injection. On day 2, another half volume of injection was given and the tyrosine level was maintained at a low level of 61±7%. The difference between the two days after the first injection and the second injection is 7%, which is much lower than that of after one injection. On day 3, tyrosine level was 70±7% and on day 4 tyrosine level was 61±8% (Figure 5.4). In the second group in which oral administration of encapsulated tyrosinase with no intravenous injection, there were no significant changes in tyrosine concentration until day 3. Starting at day 3, tyrosine level in this only oral feeding group was decreased to 79±5%, and as this feeding continued the tyrosine level was further decreased to 70±4% on day 4. There were no significant changes in tyrosine

level for the control group. Taken together, comparing results from these three groups, we found that in the third group which has been given combined method intravenous and oral treatment, the tyrosine level rapidly decreased starting on the first day and the level was maintained at 61±7% on day 4. The second group which was only given by oral administration, the tyrosine level did not change during the first day and only reach of 70±4% on day 4. Although intravenous injections could rapidly decrease the tyrosine level to below the normal concentration, it could not maintain a low level for more than one day. On the other hand, the much more convenient method of oral administration of artificial cells containing enzyme can bring down the tyrosine level in the body except it took more than 3 days to be effective. Therefore, we combine these two methods. Our data showed that the combined method could lower tyrosine level and maintain this low level. Furthermore this novel approach is unlike the tyrosine restricted diet that causes nausea, vomiting and severe weight loss. In the combined method there was no significant difference in body weight changes during the study (Figure 5.3). Table 5.2 shows the statistical analysis comparing tyrosine concentrations in the control group and the test group.

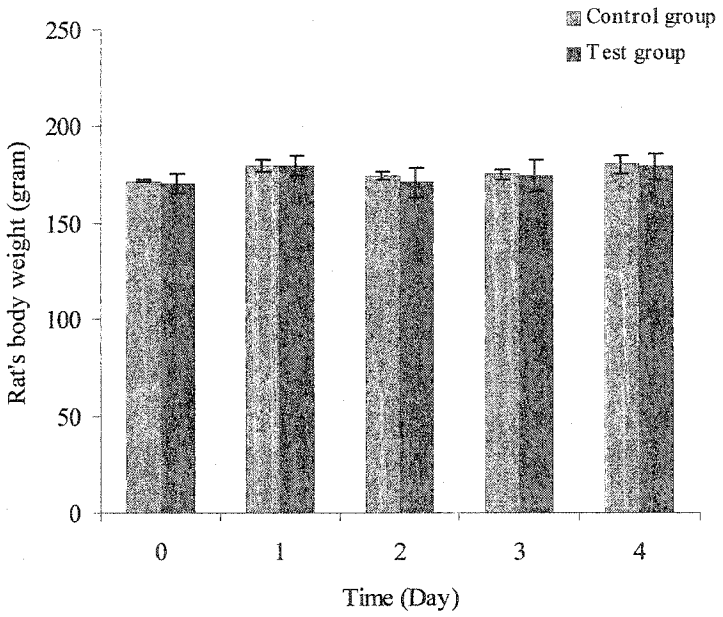

Figure 5.3 Body weight (gram) of rats for 4 days experiments. For control group: oral administration of artificial cells three times a day with injection of 1 ml of PolyHb solution per 250 g body weight on day 1 and another injection of half volume of the same sample on day 2. For test group: oral administration of artificial cells encapsulated tyrosinase three times a day with injection of 1 ml of PolyHb-tyrosinase solution per 250 g body weight on day 1 and another injection of half volume of the same sample on day 2.

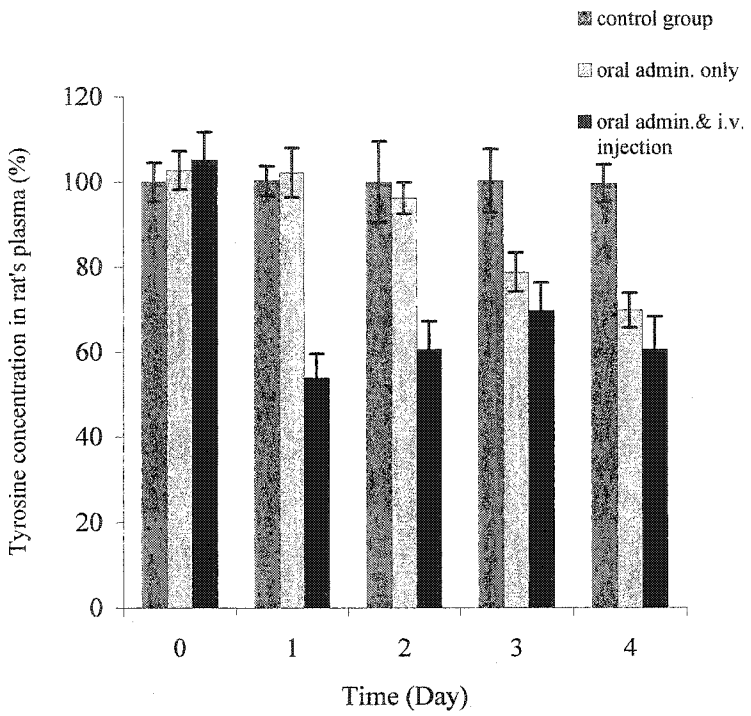

Figure 5.4 Tyrosine concentration in rat plasma (%). For control group: oral administration of artificial cells three times a day with injection of 1 ml of PolyHb solution per 250 g body weight on day 1 and another injection of half volume of the same sample on day 2. For the group of oral admin. only: oral administration of artificial cells encapsulated tyrosinase three times a day. For test group of oral admin. & i.v. injection: For test group: oral administration of artificial cells encapsulated tyrosinase three times a day with injection of 1 ml of PolyHb-tyrosinase solution per 250 g body weight on day 1 and another injection of half volume of the same sample on day 2.

Comparing	P-value
C day 0 / T day 0	NS
C day 1 / T day 1	<0.0005
C day 2 / T day 2	<0.05
C day 3 / T day 3	< 0.0005
C day 4 / T day 4	< 0.0005

Table 5.2 P-value in plasma tyrosine concentration between control group and test group. C stands for control group receiving oral artificial cells three times a day and 1 ml intravenous injection of PolyHb solution on day 1 and another half volume of the same sample on day 2. T stands for test group receiving oral artificial cells encapsulated tyrosinase three times a day and 1 ml intravenous injection of PolyHb-tyrosinase solution on day 1 and another half volume of the same sample on day 2.

5.5 Discussions

The rationale for decreasing systemic tyrosine level to retard melanoma growth is that melanoma requires higher level of tyrosine than normal tissues for growth. If normal tissues are able to retain their capacity to function, they will be able to compete successfully with the tumor for the limited amount of the essential nutrient supplied. This nutritional situation will slow the growth of the tumor without damaging normal tissues and causing catabolic wasting. We therefore realize that the basis of the management in lowering systemic tyrosine level is to starve the fast growing tumor by limiting tyrosine availability to it without simultaneously producing severe catabolic wasting of host tissues.

Previous studies by Lawson and his colleges indicated that a low level of plasma phenylalanine and tyrosine is important to decrease nutrient availability to the melanoma [32]. However, in patients, adherence to this tyrosine restricted diet had been very difficult due to fatigue, irritability, hunger, nausea and abdominal discomfort and severe wasting and weight loss. The tyrosine restriction diet is also a diet very low in protein. We therefore investigated a novel approach of lowing systemic tyrosine level by oral administration of encapsulated tyrosinase and intravenous injection of PolyHb-tyrosinase while a normal diet was continued. Our results showed that oral administration of encapsulated tyrosinase at three doses a day resulted the lowest level of tyrosine among all other doses experiments, but it took 3 days for this to happen. Intravenous injection of PolyHb-tyrosinase can rapidly decrease tyrosine level in the first hour and maintain a low level for 24 hours. In this study, we thus combined these

two methods to lower tyrosine level. Our results show that systemic tyrosine level can be lowered immediately by the intravenous injection of PolyHb-tyrosinase and keep at this low level by continuing oral administration of artificial cells encapsulated tyrosinase. This combined method did not cause body weight loss in animals, which is a main problem for dietary regimen in restriction of tyrosine and phenylalanine. During our experiments, a normal diet was given and there was no restriction in food intake for animals, which made our experiments easy to apply. In summary, our combined method efficiently decreases systemic tyrosine level without disrupting normal diet intake or causing any weight loss in the animals. However, further studies need to be carried out on the effects of encapsulated tyrosinase and PolyHb-tyrosinase on lowering tyrosine level in melanoma animal models.

5.6 Acknowledgements

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CHAPTER 6

General Discussion and Conclusions

6.1 General Conclusions

This thesis introduces a new biotechnological approach for the possible treatment of melanoma. Researches found that melanoma cells require higher tyrosine level for growth than normal cells. Therefore, if we can limit the availability of tyrosine to the tumor cells, we will inhibit the growth of these malignant tumor cells. The strategy in this Ph.D. research is to study a novel approach involving encapsulated tyrosinase and crosslinked hemoglobin with tyrosinase to lower tyrosine level from the animal amino acid body pool. The first phase of this research is to prepare microencapsulated tyrosinase and analyze its enzyme kinetics and its stability at different pH and temperature. The K_m for free and microencapsulated tyrosinase is identical (465 μM). The V_{max} for the microencapsulated tyrosinase is 49.02 mg/dl·min, whereas that of tyrosinase in free solution is 114.95 mg/dl·min. Microencapsulated tyrosinase was found to be more active at low and high pHs, when compared to the free enzyme solution. In temperature stability studies at 4°C and 37°C, higher enzyme activity was remained in microencapsulated tyrosinase solution than that of free enzyme solution. Further *in vitro* study shows that encapsulated tyrosinase can efficiently decrease the tyrosine level in rat's intestine contents. This was followed by studies on the oral administration of microencapsulated tyrosinase for the removal of systemic tyrosine level *in vivo*. Results show that oral administration of encapsulated tyrosinase three times a day can significantly decrease systemic tyrosine level after the third day. Furthermore, the treated rats showed no signs of abnormal behavior or weight loss throughout the 22 days of three times a day oral administration.

The second phase of this research involves the crosslinking of hemoglobin with tyrosinase using the dialdehyde agent, glutaraldehyde. Polymerization with glutaraldehyde has a minimal effect on the enzymatic activity of tyrosinase. The stability of PolyHb-tyrosinase at 37°C is much more stable when compared to the non-crosslinked tyrosinase solution. PolyHb-tyrosinase has similar oxygen carrying and releasing properties to the free hemoglobin solution. *In vivo* results show that intravenous injection of PolyHb-tyrosinase solution can immediately decrease systemic tyrosine level within one hour after injection. In the B16F10 melanoma cell culture study, PolyHb-tyrosinase can inhibit the growth of melanoma cell line. These results show that intravenous injection of PolyHb-tyrosinase can lower systemic tyrosine level.

The third phase of this research is to study the novel combined intravenous injection of PolyHb-tyrosinase method with oral administration of encapsulated tyrosinase. The results show that two intravenous injections of PolyHb-tyrosinase followed by three times a day oral administration of encapsulated tyrosinase can immediately lower the body tyrosine level and maintain this low level as long as the oral administration is continued. In summary, we have reported here a possible biotechnological solution for the sustained lowering of systemic tyrosine based on intravenous injection of PolyHb-tyrosinase followed by daily oral administration of encapsulated tyrosinase. This could solve the problem of how to safely lower systemic tyrosine levels for the treatment of melanoma.

6.2 Claims to Originality

The novelty of this research is such that McGill University has submitted an "U.S. priority patent application" in March 2002.

1. A novel approach was proposed and developed using oral artificial cells containing tyrosinase to remove systemic tyrosine level for potential use in the treatment of melanoma.

2. Microencapsulated tyrosinase was found to be more active at low and high pH, compared to the free enzyme in solution.

3. The storage stability profiles and temperature profiles of the microencapsulated tyrosinase were compared to the free form of the enzyme. Higher activity remained in microencapsulated tyrosinase than that of in the free form.

4. Oral administration of encapsulated tyrosinase at three doses a day can significantly decrease systemic tyrosine level in animals and maintain this low level with the animals continue to grow and gain weight.

5. The concept of chemically crosslinking hemoglobin and tyrosinase to form polyhemoglobin-tyrosinase.

6. Crosslinking hemoglobin with tyrosinase is feasible using glutaraldehyde. The resultant polyhemoglobin-tyrosinase retains enzymatic activity and oxygen carrying and releasing properties.

7. PolyHb-tyrosinase lowers systemic tyrosine level within one hour after one intravenous injection.

8. PolyHb-tyrosinase retains the same oxygen carrying and releasing characteristics as polyhemoglobin. Polyhemoglobin has already been used to more easily supply oxygen to narrower capillaries of tumor than red blood cells and increase the sensitivity to radiation therapy. Thus, the present novel polyhemoglobin-tyrosinase has the added function of increasing the effectiveness of radiotherapy.

9. Two intravenous injections of PolyHb-tyrosinase followed by daily oral administration of polymeric tyrosinase microcapsules maintain a low tyrosine level for the duration of the 22 days experiment.

10. Based on available theoretical and experimental knowledge, these results suggest that the method of intravenous injection combined with oral administration can be used as a novel potential biotechnological approach for the removal of systemic tyrosine level in the treatment of melanoma.