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Biodegradable Nanocapsules Containing A Nanobiotechnological Complex for the *In-vitro* Suppression of A Melanoma Cell Line B16F10

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Abstract

Melanoma is a fatal skin cancer. In this paper, we design a nanobiotechnological approach for the specific depletion of tyrosine that is essential for melanoma growth. We combine two nanobiotechnological methods into a single therapeutic agent in the form of Polylactide (PLA) nanocapsules containing a nanobiotechnological complex of polyhemoglobin-tyrosinase. Our results show that nanocapsules can enter into the melanoma cells to deplete tyrosine. This leads to the inhibition of tumor growth, migration and colonization in a highly malignant melanoma cell line B16F10. We also analyzed possible mechanisms of action including ROS generation, apoptosis induction and effect on cell cycle.

Keywords: Nanocapsules; Enzyme therapy; Melanoma; Nanomedicine; Nanobiotechnology; Artificial cells

Introduction

The incidence of melanoma, a fatal skin cancer, has increased rapidly in the last few years. The American Cancer Society's estimates for melanoma in the United States for 2016 are 76,380 new cases of melanomas and about 10,130 people are expected to die of melanoma in 2016 [1]. Once metastasized only palliative therapies are available. Immunotherapy, photodynamic therapy, chemotherapy and radiation therapies with their adverse side effects cannot improve the survival rates in advance melanoma [1-3]. Thus, there is an urgent need for new therapeutic strategy.

Melanoma depends on tyrosine (Tyr) for growth [4]. Recent interest in nanomedicine has resulted in the extension of artificial cells [5-7] to prepare nano-dimension nanocapsules, nanoparticles and soluble nanobiotechnological complexes [8,9]. For example, polyhemoglobin-tyrosinase can be prepared by the crosslinking of hemoglobin (Hb) and tyrosinase into a soluble nanobiotechnological complex for testing in melanoma mice [10]. However, this can only delay but not suppress the growth of melanoma in mice model [10,11].

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

Methods

Nanocapsules preparation and characterization

The first step was to prepare nanobiotechnological complexes of PolyHb and PolyHb-Tyrosinase. The stroma-free Hb was diluted to a concentration of 7g/dL with or without tyrosinase in PBS (pH7.4). Lysine was added at a molar ratio of 7:1 lysine/Hb, followed by the glutaraldehyde, a crosslinker, at a molar ratio of 16:1 glutaraldehyde/Hb. After 24hours, lysine was added again to stop the reaction at a molar ratio of 200:1 lysine/Hb. Dialysis membrane and sterile syringe filters were applied to purify the polyHb and polyHb-tyrosinase samples. To prevent methemoglobin formation and to protect the enzymes activity, the operations were performed at 4°C and under nitrogen.

Then, the next step was the PLA nanocapsulated polyHb-tyrosinase preparation by nanoprecipitation and solvent evaporation methods. PLA (25 mg) and hydrogenated soybean phosphatidylcholine (12.5 mg) were dissolved in the organic phase (2 ml of acetone and 1 ml of ethanol and 8 ul Tween 20). The organic phase was added dropwise into 5 ml of polyHb or polyHb-tyrosinase solution at a rate of 3 ml/min and allow the magnetic stirring for 1 hour [12]. The organic solvent was removed by following evaporation and PLA nanoparticles were formulated in the form of colloidal dispersion in aqueous phase. Tween 20 was used to stabilize the nano formulation.) To prepare fluorescence labeled PLA nanocapsules, additional coumarin-6 was added into the organic phase, and following the same procedure as above.

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

Tyrosinase activity assay

Tyrosinase activity was analyzed by monitoring the production rate of the enzymatic product L-dopaquinone at 300nm, as described previously [13].

Tyrosine detection

Yeast enzyme phenylalanine ammonia-lyase (PAL) can converse Tyr to trans-coumarate detectable at 315 nm [14]. [Tyr]= A315/α315 (A

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was determined as final absorbance minus initial one after subtraction of blank values; α315 is the slopes of the standard curves).

Tumour cells and culture conditions

B16F10 murine melanoma cells (American Type Culture Collection, ATCC®, #CRL-6475) were cultured in standard Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37°C and 5% CO_2, humidified atmosphere.

MTT assay

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

Scratch assay for migration

5×10^5 cells/well were seeded overnight. Wound culture was made by scraping cells with a sterile tip and maintained in 0.5% FBS medium with nanocapsule treatment. Scratch areas were calculated [15].

Colony assays in soft agar

The methods were carried out as previously described with some modification [16]. Briefly, 5×10^5 cells were treated with PLA nanocapsules containing different concentrations of polyhemoglobin-tyrosinase for 24 hours and were collected. The base agar was prepared by 1% agar and top agar by 0.7% agar containing the treated tumour cells. The dishes were kept in incubator allow colonies growth for 3 weeks. The colonies were visualized by Crystal Violet staining.

Apoptosis detection by annexin V-FITC and PI staining

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

Cell cycle analysis using flow cytometry

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

ROS generation detection by confocal microscope and quantification by flow cytometry

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

Cellular distribution of PLA nanocapsule by confocal microscopy

To test cellular distribution, tumour cells were treated with coumarin-6-labeled nanocapsules for short term and long term and observed on a LSM 710 confocal microscope [17].

Western blot analysis

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

In vivo study

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

Melanoma specimen analysis

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

Data analysis

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

Results

Synthesis and characterisation of PLA nanocapsules encapsulating polyhemoglobin-tyrosinase

Nanocapsules encapsulating polyhemoglobin-tyrosinase (Ncap-PH-TYR) were synthesized via the technique combining of these two nanotechnological methods into a single therapeutic agent (Figure 1A). Details of the method can be found in the method section. The preparation involved two steps: (1) The use of glutaraldehye to covalently crosslink hemoglobin and tyrosinase into a soluble nanobiotechnological complex. This improves the stability of the enzyme tyrosinase. However, being a soluble complex it cannot accumulate at the site of injection nor enter the melanoma cells. (2) Thus the next step is to nanoencapsulate this soluble complex into PLA nanocapsules. The PLA nanocapsules can accumulate longer at the local injection site allowing them to enter the melanoma cells to lower tyrosine both in in melanoma cells and in the microenvironment (Figure 1B).

After synthesis, Transmission electron microscopy (TEM) was used to analyze the nanocapsules' characters and images demonstrated the uniform spherical formation and the particle diameter range at 100 to 300 nm (Figure 1C). Zeta values were -4 to -10 mV (Figure 1D). The smaller diameter nanocapsules can enter and act inside the melanoma cells, while the larger ones can accumulate outside the melanoma cells. This can decrease the tyrosine level both inside the melanoma cells and also in the tumour microenvironment.

Penetration of nanocapsules into the melanoma cells and effect on tyrosine level

We followed the penetration and distribution of PLA nanocapsules into the melanoma cells by engineering fluorescence labelled nanocapsules.

In the confocal imagining, the nanocapsules were stained by coumarin-6 (green dye) and the nucleus of the melanoma cells were stained with H33342 (blue dye). The result showed that the nanocapsules were able to enter the cells within 1 hour after co-

culturing with melanoma cells and remained inside the cytoplasm for the 72h of observation (Figure 2A). This intracellular distribution is also consistent with the previous published intracellular distribution of PLA-TPGS nanoparticles [18].

The next question is to determine whether the PLA nanocapsules containing polyHb-tyrosinase can remove and lower tyrosine level. After treating melanoma cells for 24 h, the tyrosine level significantly decreased to 1% compared to that in the untreated control (Figure 2B). The low tyrosine level can maintain at least 48 h. These results implied that the enzymatic activity of tyrosinase was well protected by the protection of PLA and nanoencapsulated polyHb-tyrosinase can dramatically act to remove tyrosine.

In vitro effect on the proliferation, migration and colonization of B16F10 melanoma cells

After the confirmed tyrosine deprivation induced by nanocapsules, we subsequently researched on the therapeutic function on melanoma growth and metastasis. MTT test was carried out to study tumour growth. The results exhibited that Ncap-PH-TYR significantly decreased the viability of melanoma cells in their proliferation phase at 24 hours, and the effect was enhanced and more than 80% tumour were inhibited in growth at 48 h (Figure 3A). This growth inhibition effect was in a time and dose dependent manner.

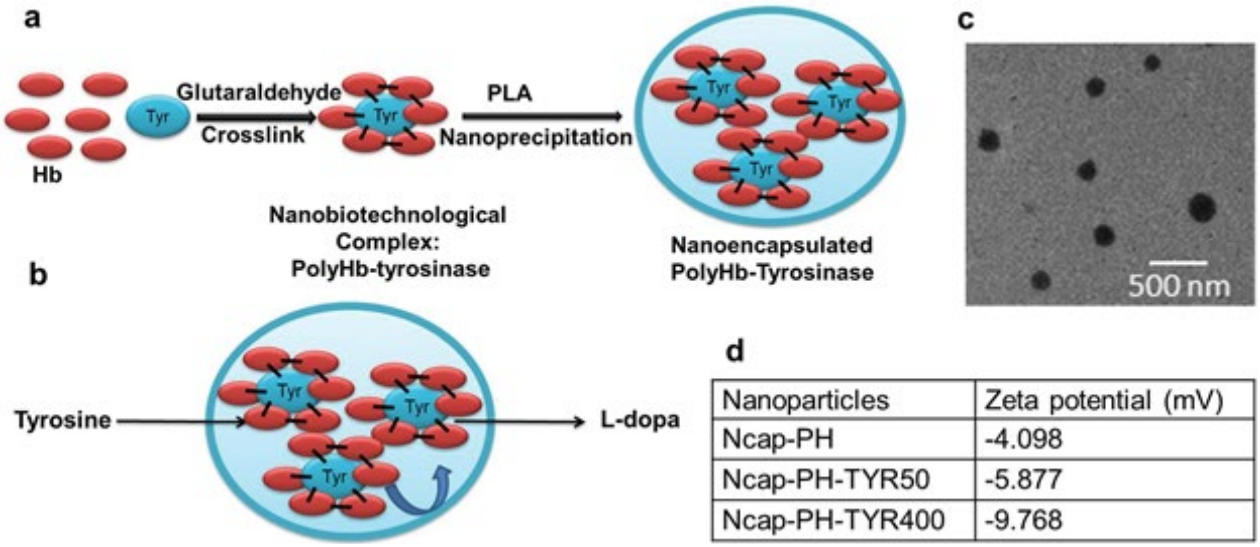
Scratch test is commonly performed to study the invasive migration of tumour cells [15]. We therefore used this method to study the effect of Ncap-PH-TYR. In the control group, B16F10 tumor cells migrated into the scratch area and after 48 h the scratch area is decreased to 1/3 of that at 0h time. Treatment with Ncap-PH-TYR impaired the migration ability during the 48h observation as shown by the lack of decrease in the scratched area (Figure 3B). It was enzyme activity dependent and the higher enzymatic dose (Ncap-PH-TYR100) can obtain higher prevention of migration of the tumor cells.

Next, colonization test was designed to assess the capability of tumour growth after it has metastasized. We treated tumour cells with different PLA nanocapsules and detected the colonization ability in soft agar assay. The colonies were visualized by Crystal Violet staining. Figure 3C and 3D showed that colony counts were significantly decreased with increased tyrosinase enzyme activity. Especially in the test group (Ncap-PH-TYR400), few visible colonies were formed after 3 weeks.

Possible mechanisms involved apoptosis induction, cell cycle arrest and ROS generation

The observed suppression effect on tumour growth and metastasis led us to explore potential mechanisms. Apoptosis analysis by flow cytometry showed apoptosis of melanoma cells treated with the Ncap-PH-TYR (Figure 4A). The impact on cell cycle is another possible reason attributed to the decreased cell viability. PI staining was applied on melanoma cells after 24h and 48h of treatment with Ncap-PH-TYR. In our experiment, Ncap-PH-TYR resulted in 25% of G0/G1 arresting in melanoma cells (Figure 4B). It was dose and time dependent. Oxidative stress is another potential cause of cell impairment. We analyzed this mechanism by following the reactive oxygen species (ROS) level. CellROX reagent was applied to track the short term (1h, 2h) and long term (24h, 48h) ROS generation. ROS level increased in 1h and reached its peak at 48 hours (Figure 4C and 4D). In addition, the quantitative estimation of intracellular ROS generation was also further calculated. In the presence of ROS, fluorescent intensity of cells stained with dyes will increase, leading to a right shift of the emission maximum. The results showed that ROS generation increased after nanocapsules treatment for 24h and 48h when compared with the untreated control (Figure 4E).

To further confirm these mechanisms involved, we explored



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

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

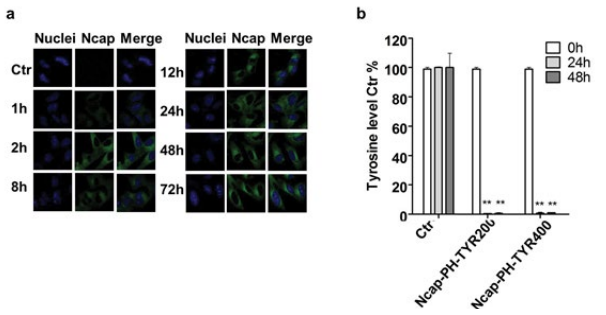


Figure 2: Penetration of PLA nanocapsules into to the cell cytoplasm and the intracellular removal of tyrosine.

(a) Intracellular location and distribution of PLA nanocapsules observed for 72h. PLA nanocapsules (green) were labelled with coumarin-6. Nuclei (blue) were stained with H33342. The PLA nanocapsules mainly distribute in the cytoplasm of B16F10 murine melanoma cells.

(b) Tyrosine levels at different time points. The tyrosine levels in the 0 time point were set as 100%. Ctr: control with saline; Ncap-PH-TYR200 (PLA nanocapsules containing polyHb and 200 units of tyrosinase) ; Ncap-PH-TYR400 (PLA nanocapsules containing polyHb and 400 units of tyrosinase). Results are mean+s.e.m., n= 3. Double asterisk indicates P <0.005 compared to control (saline).

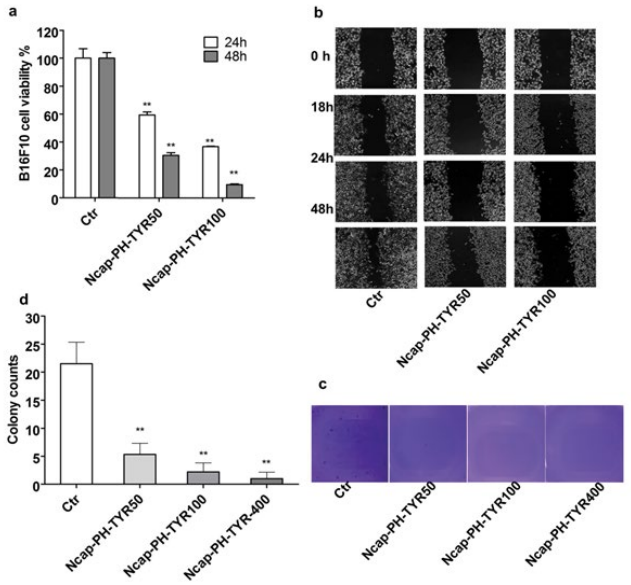


Figure 3: *In vitro* effects on the growth and metastasis of melanoma cells.

(a) Cell viability was detected by MTT at the time points of 24h and 48h. Ncap-PH-TYR lowered the viability of melanoma cells in their proliferation phase when followed for 48h.

(b) Scratch assay to test the ability of tumour to migrate (metastasize) into the cell free scratched area. A cell free zone was made by "scratching" with a sterile tip. Following this the migration of cells into the cell free scratch zone was followed over 48 hours. Unlike the control, Ncap-PH-TYR treated cells had decreased ability to migrate into the cell free zone.

(c) Colonization test using soft agar assay. The soft agar was prepared with 1% base agar and 0.7% top agar containing tumor cells. Crystal violet staining was used for calculating visualized colonies.

(d) Quantitative analysis of colonization test. Results are mean+s.e.m., n= 3. Double asterisk indicates P <0.005 compared to control.

the molecular changes. This includes the integrin receptors that are required for cell adhesion and attachment to the extracellular matrix. This attachment is needed for the survival, growth, and inhibition of cell

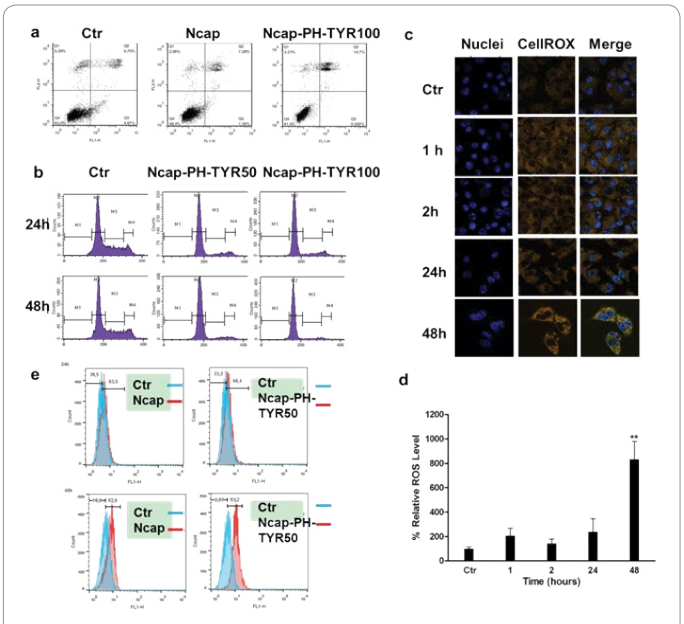


Figure 4: Effects on tumor apoptosis induction, growth inhibition and ROS generation.

(a) Flow-cytometric analysis of PLA nanocapsules (Ncap-PH-TYR100) mediated apoptosis in B16F10 cells. Annexin V-FITC and PI staining showed apoptosis in the PLA nanocapsules (Ncap-PH-TYR100) treated group.

(b) Cell cycle detection on melanoma cells treated with PLA-nanocapsules (Ncap-PH-TYR100). PI staining was applied for the cell cycle detection. The results showed G0/G1 arresting on the nanocapsules (Ncap-PH-TYR50 and Ncap-PH-TYR100) treated groups at M2: G0/G1 phase; M3: S phase; M4: G2/M phase.

(c) Confocal fluorescence images of tumor cells incubated with Ncap-PH-TYR100. Nuclei (blue) were stained with H33342 and the generated ROS (orange) were stained by CellROX Orange.

(d) Quantitative analysis of the ROS generation. Significant at 48 hours at p<0.005.

(e) Flow-cytometric analysis showing a gradual increase in ROS generation when compared with control cells. Blue peaks are the control groups and the red peaks are the treatment groups (Ncap or Ncap-PH-TYR50) at 24h and 48h

apoptosis. FAK (focal adhesion kinase) is a major signaling mediator that influences both integrin attachment and cell spreading. The integrins are cell surface receptors for the components of extracellular matrix. They will be activated during cell adhesion and spreading, and initiate signaling pathways that control growth and invasion of tumor cells. In our study, the expressions of FAK and α4 integrin were inhibited in the B16F10 melanoma cells after treatment with Ncap-PH-TYR (Figure 5A). The present study indicated that Ncap-PH-TYR can inhibit B16F10 melanoma's attachment and spreading via inhibition of specific integrin expression and FAK expression. About the cell cycle of melanoma cells, the cyclin D and their attendant CDKs are key regulators of G1 progression, which is an important checkpoint of cell cycle. Results showed that the expression of cyclin D1, cyclin D3and CDK2 were also downregulated (Figure 5B).

Preliminary results in a B16F10 melanoma mice model

We investigate the therapeutic effect in an *in vivo* B16F10 melanoma mice model. Preliminary result shows the following. In the control group: 0.5×10^5 B16F10 melanoma tumor cells and 0.075 ml of saline were mixed and injected into the lateral flank of C57BL/6 mice subcutaneously. The tumor volume has reached the maximal allowable volume of 4000 mm^3 within 30 days. In the test group 0.5×10^5 B16F10 melanoma tumor cells and 0.075 ml PLA nanocapsuels were mixed

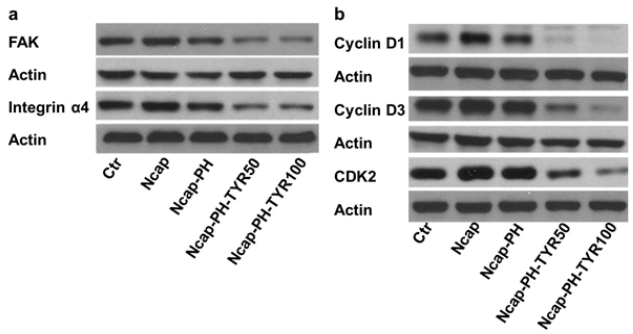


Figure 5: Western blot analysis of the expression of relative molecular proteins.

(a) Western blot analysis of the expression of FAK and integrin-α4 that are involved in cell adhesion. The declined expression indicated impaired cell adhesion ability.

(b) Western blot analysis of the expression of Cyclin D1, Cyclin D3 and CDK2, the cell cycle relative molecules. Declined expressions are consistent with the G0/G1 arresting.

and injected into the lateral flank of C57BL/6 mice subcutaneously. The result shows that Ncap-PH-TYR800 is effective in inhibiting tumor growth during the 30 days. What's more, HE staining showed that unlike the control group, there was no observable neoplastic cell in the Ncap-PH-TYR800 group. Melan-A staining also verified the inhibition effect of the Ncap-PH-TYR800 group. Injection of the Ncap-PH-TYR800 did not result in body weight changes when compared to the control. In addition, there were no observable histological changes in spleen, kidney, lung and liver.

Discussion

In this paper, we applied a novel combination of two nanotechnological methods (1) The use of glutaraldehye to covalently crosslink hemoglobin and tyrosinase into a soluble nanobiotechnological complex. This improves the stability of the enzyme tyrosinase. However, being a soluble complex it cannot accumulate at the site of injection nor enter the melanoma cells. (2) Thus the next step is to nanoencapsulate this soluble complex into PLA nanocapsules. The PLA nanocapsules can stay longer at the local injection site allowing them to enter the melanoma cells to lower tyrosine both in in melanoma cells and in the tumour microenvironment. PLA polymer is one of the most widely used polymers, and has been extensively used as the matrix of nanocapsules for more than 20 years [19-25]. The FDA has approved the use of PLA for a host of therapeutic devices owing to their biodegradability, biocompatibility, and non-toxic [26]. Unlike the traditional enzyme delivery methods that the prepared particles encapsulate enzymes and complete the catalyst reaction by releasing the enzymes, in our enzyme delivery system, the enzymes maintain inside and the substrate and products can diffuse in and out. As such a design, the problems of release profile optimization or the condition of drug release can be avoid.

In initial studies, we fabricated and characterized the physical properties of PLA-PH-TYR. Subsequently, we confirmed the penetration of nanocapsules into the melanoma cells and confirmed the tyrosine depletion effect. *In vitro* effect on the proliferation, migration and colonization were investigated and the potential mechanisms were also further studied.

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

For the solid cancer melanoma, metastatic dissemination from a primary lesion to a secondary site is believed to be the major lethal reason [27-30]. This process involves the tumor cells proliferating at the primary sites, migrate/invade the surround tissue then into blood vessels, circulate in the bloodstream, adhere to vascular endothelial cells, and exhibit invasion, proliferation, angiogenesis and metastatic colonization at the secondary sites. Previous studies proved the deprivation of Tyr/Phe can significantly decrease the metastatic phenotype of B16BL6 melanoma cells *in vivo* and reduce the *in vitro* invasion [31-33]. We therefore further assessed the impact of Ncap-PH-TYR on the proliferation, migration and colonization of B16F10 melanoma cells. Results suggested implied PLA nanocapsules containing polyHb-tyrosinase have the potential to inhibit melanoma metastasis in proliferation, migration and colonization [27-30].

In the possible mechanism research, we performed the detection on tumor apoptosis, cell cycle and ROS generation.

The possible mechanism for the decreased cell viability could be the tumor cells' arrest in G0/G1 stage owing to Tyr/Phe depletion [34]. In our experiment, this mechanism was also confirmed and Ncap-PH-TYR resulted in 25% of G0/G1 arresting in melanoma cells. Tyr/Phe limited culture [34] is less effective than PLA nanocapsules containing PolyHb-tyrosinase. We also tested the important cyclin D-CDKs which control critical cell cycle during G1 phase and S phase. In the present study, we found the treatment can inhibit the expression of Cyclin D1, Cyclin D3 and CDK2.

Oxidative stress is another possible cause of cell impairment. In the melanoma cells, oxidative stress stimulates cell apoptosis by activating DNA damage-repair pathway and also opening mitochondrial pore [35]. Breaking ROS tolerance of melanoma cells by either impairing their antioxidant system or further elevating their intracellular ROS level by new therapeutic might hold a future promise as an alternative therapeutic approach. This has been applied in the CeO_2 nanoparticles that producing significant oxidative stress in tumor cells, resulting in a decrease in cell viability and lowering of the invasive capacity of cancer cells [36]. We also confirmed the ROS generation. When tyrosinase acted on tyrosine the generated products of tyrosine metabolism, such as dopa, 5,6–dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) and others can produce ROS [37].

The results obtained also confirm earlier study that NP with diameter <200 nm can effectively accumulate in solid tumours [27-30]. Further influencing parameters include structure, surface charge, and molecular-flexibility [31]. The nanocapsules we fabricated are between 100 to 300 nm, which could be expected to accumulate in the tumour area with those <200 nm penetrate into tumour cells. These have negative zeta values of -4 to -10 (mV). This is important because nanoparticles with negative zeta potential can more easily penetrate and accumulate in cancer cells [38].

In conclusion, we presented a novel nanocapsules containing polyHb-tyrosinase. It represents a functional platform to encapsulate enzymes. The successful intracellular delivery and effectively deletion of tyrosine can suppress the growth and metastasis of a skin cancer, murine B16F10 melanoma *in-vitro*. Preliminary results in a B16F10 melanoma mice model shows that this approach can suppress the growth of melanoma. Further research may pave the way for another potential new approach for the treatment of melanoma. This is important because the incidence of melanoma, a fatal skin cancer, has increased rapidly in the last few years. The American Cancer Society's estimates

for melanoma in the United States for 2016 are 76,380 new cases of melanomas and about 10,130 people are expected to die of melanoma in 2016. This is a deadly cancer because it tends to metastasize and spread very widely. Once metastasized only palliative therapies like immunotherapy, photodynamic therapy, chemotherapy and radiation therapies with adverse side effects are available [1-3]. The availability of a new and effective method of treatment is urgently needed.

Acknowledgements

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References

1. American Cancer Society (2016) Melanoma Skin Cancer.
2. Lopez RF, Lange N, Guy R, Bentley MV (2004) Photodynamic therapy of skin cancer: controlled drug delivery of 5-ALA and its esters. Adv Drug Deliv Rev 56: 77-94.
3. Bundscherer A, Hafner C, Maisch T, Becker B, Landthaler M, et al. (2008) Antiproliferative and proapoptotic effects of rapamycin and celecoxib in malignant melanoma cell lines. Oncol Rep 19: 547-553.
4. Uhlenkott CE, Huijzer JC, Cardeiro DJ, Elstad CA, Meadows GG (1996) Attachment, invasion, chemotaxis, and proteinase expression of B16-BL6 melanoma cells exhibiting a low metastatic phenotype after exposure to dietary restriction of tyrosine and phenylalanine. Clin Exp Metastasis 14: 125-137.
5. Chang TM (1964) Semipermeable Microcapsules. Science 146: 524-525.
6. Chang TM, Poznansky MJ (1968) Semipermeable microcapsules containing catalase for enzyme replacement in acatalasaemic mice. Nature 218: 243-245.
7. Chang TM (1971) The in vivo effects of semipermeable microcapsules containing L-asparaginase on 6C3HED lymphosarcoma. Nature 229: 117-118.
8. Chang TMS (2007) Monograph on "Artificial Cells: Biotechnology, Nanotechnology, Blood Substitutes, Regenerative Medicine, Bioencapsulation, Cell/StemCell Therapy". World Science Publishers/Imperial College Press, Singapore, London, United Kingdom.
9. Chang TMS (2013) "Artificial Cells that started Nanomedicine" Opening Chapter in book on "Selected Topics in Nanomedicine". World Science Publisher/ Imperial College Press, Singapore, London, United Kingdom.
10. Yu B, Chang TM (2004) In vitro and in vivo enzyme studies of polyhemoglobin-tyrosinase. Biotechnol Bioeng 86: 835-841.
11. Yu B, Swi Chang TM (2004) In vitro and in vivo effects of polyhaemoglobin-tyrosinase on murine B16F10 melanoma. Melanoma Res 14: 197-202.
12. Fustier C, Chang TM (2012) PEG-PLA Nanocapsules Containing a Nanobiotechnological Complex of Polyhemoglobin-Tyrosinase for the Depletion of Tyrosine in Melanoma: Preparation and In Vitro Characterisation. J Nanomedic Biotherapeu Discover. 2: 1.
13. Wang Y, Chang TM (2012) Nanobiotechnological Nanocapsules Containing Polyhemoglobin- Tyrosinase: Effects on Murine B16F10 Melanoma Cell Proliferation and Attachment. J Skin Cancer 2012: 1-9.
14. Wibrand F (2004) A microplate-based enzymatic assay for the simultaneous determination of phenylalanine and tyrosine in serum. Clin Chim Acta 347: 89-96.
15. Shin DH, Kim OH, Jun HS, Kang MK (2008) Inhibitory effect of capsaicin on B16-F10 melanoma cell migration via the phosphatidylinositol 3-kinase/Akt/ Rac1 signal pathway. Exp Mol Med 40: 486-494.
16. Bisht S, Feldmann G, Soni S, Ravi R, Karikar C, et al. (2007). Polymeric nanoparticle- encapsulated curcumin ("nanocurcumin"): a novel strategy for human cancer therapy. J Nanobiotechnology 5: 3.
17. Yu DH, Lu Q, Xie J, Fang C, Chen HZ (2010) Peptide-conjugated biodegradable nanoparticles as a carrier to target paclitaxel to tumor neovasculature. Biomaterials 31: 2278-2292.
18. Zhang Z, Lee SH, Gan CW, Feng SS (2008) In vitro and in vivo investigation on PLA-TPGS nanoparticles for controlled and sustained small molecule chemotherapy. Pharm Res 25: 1925-1935.
19. Leo E, Brina B, Forni F, Vandelli MA (2004) In vitro evaluation of PLA nanoparticles containing a lipophilic drug in water-soluble or insoluble form. Int J Pharm 278: 133-141.
20. Cheng Q, Feng J, Chen J, Zhu X, Li F (2008) Brain transport of neurotoxin-I with PLA nanoparticles through intranasal administration in rats: a microdialysis study. Biopharm Drug Dispos. 29: 431-439.
21. Tong R, Coyle VJ, Tang L, Barger AM, Fan TM, et al. (2010) Polylactide nanoparticles containing stably incorporated cyanine dyes for in vitro and in vivo imaging applications. Microsc Res Tech 73: 901-909.
22. Youm I, Yang XY, Murowchick JB, Youan BB (2011) Encapsulation of docetaxel in oily core polyester nanocapsules intended for breast cancer therapy. Nanoscale Res Lett 6: 630.
23. Xing J, Zhang D, Tan T (2007) Studies on the oridonin-loaded poly(D,L-lactic acid) nanoparticles in vitro and in vivo. Int J Biol Macromol 40: 153-158.
24. Allémann E, Leroux JC, Gurny R, Doelker E (1993) In vitro extended-release properties of drug-loaded poly(DL-lactic acid) nanoparticles produced by a salting-out procedure. Pharm Res 10: 1732-1737.
25. Leroux JC, Allemann E, DeJaeghere F, Doelker E, Gurny R (1996) Biodegradable nanoparticles - From sustained release formulations to improved site specific drug delivery. J Control Release 39: 339-350.
26. Parveen S, Sahoo SK (2008) Polymeric nanoparticles for cancer therapy. J Drug Target 16: 108-123.
27. Moghimi SM, Hunter AC, Murray JC (2001) Long-circulating and target-specific nanoparticles: theory to practice. Pharmacol Rev 53: 283-318.
28. Hobbs SK, Monsky WL, Yuan F, Roberts WG, Griffith L, et al. (1998) Regulation of transport pathways in tumor vessels: role of tumor type and microenvironment. Proc Natl Acad Sci 95: 4607-4612.
29. Moghimi SM, Porter CJH, Muir IS, Illum L, Davis SS (1991) Non-Phagocytic Uptake of Intravenously Injected Microspheres in Rat Spleen - Influence of Particle-Size and Hydrophilic Coating. Biochem Biophys Res Commun 177: 861-866.
30. Moghimi SM (1995) Mechanisms of Splenic Clearance of Blood-Cells and Particles - Towards Development of New Splenotropic Agents Adv Drug Deliv Rev 17: 103-115.
31. Nakaoka R, Tabata Y, Yamaoka T, Ikada Y (1997) Prolongation of the serum half-life period of superoxide dismutase by poly(ethylene glycol) modification. J Control Release 46: 253-261.
32. Gupta GP, Massagué J (2006) Cancer metastasis: building a framework. Cell 127: 679-695.
33. Fu YM, Yu ZX, Pelayo BA, Ferrans VJ, Meadows GG (1999) Focal adhesion kinase-dependent apoptosis of melanoma induced by tyrosine and phenylalanine deficiency. Cancer Res 59: 758-765.
34. Fu YM, Yu ZX, Ferrans VJ, Meadows GG (1997) Tyrosine and phenylalanine restriction induces G0/G1 cell cycle arrest in murine melanoma in vitro and in vivo. Nutr Cancer 29: 104-113.
35. Fruehauf JP, Trapp V (2008) Reactive oxygen species: an Achilles' heel of melanoma? Expert Rev Anticancer Ther 8: 1751-1757.
36. Alili L, Sack M, Von Montfort C, Giri S, Das S, et al. (2013) Downregulation of tumor growth and invasion by redox-active nanoparticles. Antioxid Redox Signal 19: 765-778.
37. Urabe K, Aroca P, Tsukamoto K, Mascagna D, Palumbo A, et al. (1994) The inherent cytotoxicity of melanin precursors: a revision. Biochim Biophys Acta 1221: 272-278.
38. Patil S, Sandberg A, Heckert E, Self W, Seal S (2007) Protein adsorption and cellular uptake of cerium oxide nanoparticles as a function of zeta potential. Biomaterials 28: 4600-4607.