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اندازه‌گیری خواص ضدسرطانی نانوذره پلی (D,L-لاکتید-کو-گلیکولید)-بلاک-پلی اتیلن گلیکول بارگذاری‌شده با نارنجنین

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Determination of Anti-Cancer Effects of Poly (L,D-Lactide-co-Glycolide)-Block-Poly (Ethylene Glycol) Nanocarrier Loaded with Naringenin

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چکیده
در این کار تحقیقاتی نانوذرات پلی (D,L-لاکتید- کو- گلیکولید)- بلاک- پلی اتیلن گلیکول با قطر کمتر از ۱۹۵ نانومتر بارگذاری شده با فلاونویید نارنجنین سنتز شده است. فرم کپسوله شده نارنجنین باعث افزایش حلالیت و خواص درمانی آن می‌گردد. خاصیت درمانی نارنجنین آزاد و نارنجنین کپسوله‌شده بر تکثیر سلول‌های سرطانی اپیتلیال ریه و پستان انسان و موش آزمایشگاهی با تست MTT انجام و معلوم شد که سمیت سلولی نانوذرات نانجنین از نارنجنین آزاد به مراتب بیشتر است. خواص آنتی اکسیدانی نانوذرات نارنجنین و نارنجنین آزاد به روش FRAP اندازه‌گیری شد و نتایج حاصل نشان می‌دهد که خاصیت ضدسرطانی دارو به علت خاصیت آنتی اکسیدانی و کیلیت شوندگی آن با یون‌های آهن است.

واژه‌های کلیدی
سمیت سلولی؛ آنتی‌اکسیدانی؛ خط سلولی؛ نارنجنین؛ نانوذرات.

Abstract
In this research, poly ($_{DL}$-lactide-co-glycolide)-block-poly (ethylene glycol), (PLGA-PEG) nanoparticles (NPs) of less than 195 nm in diameter containing of Naringenin (NRG) a naturally flavonoid were synthesized. Encapsulated form NRG improves its medical properties and solubility. The therapeutic efficacy of the encapsulated naringenin (NRG-NPs) and NRG on human lung epithelial (A549) and mouse mammary (4T1) carcinoma cells proliferation was determined by MTT assays. The cytotoxicity potency was rated as follows: NRG-NPs > NRG. The antioxidant effects of the NRG and NRG-NPs were also determined by FRAP method. Our results show that NRG-NPs are cytotoxic compounds for cancer cells and anti-cancer effect can be attributed to the presence of Fe chelatory and antioxidant effects of NRG-NPs.

Keywords
Cytotoxicity; Antioxidant; Cell Line; Naringenin; Nanoparticles.

1. INTRODUCTION

Anti-cancer drugs are used to induce controlled cell death and because of that stop cancer cells from growing or multiplying. Once the cancer had spread, surgery and radiotherapy may become ineffective and the systemic delivery of chemotherapeutics provides the anti-cancer treatment. Nanocarriers can bring about several improvements in cancer therapy. They can stabilize lipophilic drugs in circulation or increase the circulatory duration of drugs by controlled release. Therefore, the toxicity related to the initial high concentrations in periodic doses can be prevented.

Naringenin (NRG, 4', 5, 7- trihydroxy flavanone, (Fig. 1a), a naturally occurring flavonoid and aglycone of naringin, is widely present in citrus fruits, tomatoes, cherries, grapefruit and cocoa [1]. It is well known for various biological actions, such as antioxidant, anti- inflammatory and anti-carcinogenic effects. In the best-case scenario, only 15% of ingested NRG will get absorbed in the human gastrointestinal tract. Yet

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it suffers from biopharmaceutical restrictions due to its poor water solubility which results in poor absorption and short half-life about 2 hours [2-3]. Encapsulated form flavonoid improves its medical properties and solubility. PEG and PLGA polymers are biodegradable polyesters that degrade in the body by simple hydrolysis of the ester backbone to non-harmful and non-toxic compounds. The degradation products are either excreted by the kidneys or eliminated as carbon dioxide and water through well-known biochemical pathways. Current applications of the polymers include surgical sutures and implants, with significant interest to further expand the use of these materials to drug encapsulation and delivery applications [4-5].

For nanoparticles to work as intended, they must be internalized by the target cells in significant quantity. Among the many factors mentioned above, surface charge also has an important effect on particle internalization. For example, circulating nanoparticles with little or no surface charge are easily trapped by the mononuclear phagocytes of the reticulo-endothelial system (RES), primarily in the liver and spleen. One approach in overcoming this problem is to increase the hydrophilicity of the particle surface. The presence of hydrophilic polymer on the surface can protect nanoparticles from capture by macrophages.Recent reports demonstrate that the rapid RES uptake of PLGA nanoparticles could be significantly reduced by modifying their surface with polyethylene glycol (PLGA-PEG, Fig. 1b) [6-7].

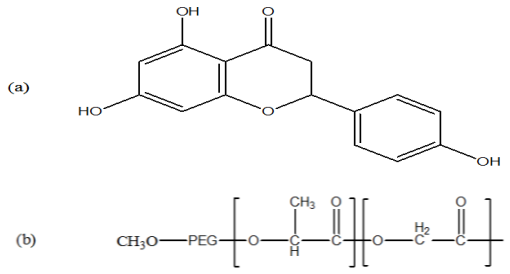

Fig. 1. Chemical structure of (a): Naringenin and (b): PLGA-PEG.

2. EXPERIMENTAL

2.1. Materials

PLGA-PEG di-block (15% PEG with 5 kDa) was obtained from Behringer Intgeihim (Germany). meso Naringenin (99% purity), sodium cholate, Butylated hydroxytoluene (BHT), MTT (3-[4,5-dimethylthiazol-2-yl] 2, 5-diphenyltetrazolium bromide) and other materials were obtained from Sigma-Aldrich (GmbH, Sternheim, Germany). The human lung epithelial (A549) and mouse mammary (4T1) carcinoma cell lines were procured from Pasteur Institute of Iran (Pasteur Institute, Tehran, Iran).

2.2 Methods

2.2.1 NRG-NPs Preparation

PLGA– PEG nanoparticles loaded with NRG were prepared using the double emulsion method [8], with minor modifications. An aqueous solution of NRG (5ml, 2.5 mg / ml) was emulsified in 2 ml dichloromethane, in which 100 mg of the copolymer had been dissolved, using probe sonication Scientific; model (Hielscher, UP 4000 S, Germany) at 10 W for 45 s. This w / o emulsion was transferred to an aqueous solution sodium cholate (6 ml, 12mM) and the mixture was probe sonicated at 18 W for 1 min. The w/o/w emulsion formed was gently stirred at room temperature until the evaporation of the organic phase was complete. The nanoparticles were purified by applying two cycles of centrifugation (28 000 rpm for1h in a Microelite RF, USA) and reconstitution with deionized and distilled water. Finally the nanoparticles were filtered using 0.22 μm syringe filter to remove non encapsulated NRG. The complex was then lyophilized and drug loading and encapsulation efficiency of NRG-NPs micelles were quantified.

2.2.2. The Total Antioxidant Capacity Assay

The total antioxidant capacity (TAC) of the NRG and NRG-NPs were determined by FRAP (Ferric Reducing Antioxidant Power) method, a simple, speedy and repeatable method, which can be used to the assay of antioxidants in plasma or botanicals [9]. The FRAP assay based on the ability of antioxidant compounds to reduce complex [Fe(III)–TPTZ)] to [Fe(II)–TPTZ] which gives a blue color with an absorbance maximum at 593 nm. The FRAP reagent composed of 10 mM TPTZ solution in 40 mM HCl, 20 mM $FeCl_3$ solution and 300 mM acetate buffer (pH= 3.6) in a ratio of 1:1:10 (v/v). 50 µl of NRG-NPs and NRG (100 µg/ml) were added to 3 ml of freshly prepared FRAP reagent and reaction mixtures incubated at 37 °C for 30 min. Aqueous solutions of ferrous sulfate were used to construct standard curve. Absorbance was determined at 593 nm. Triplicate measurements were taken and the FRAP values were expressed as mmol of Fe (II)/ g dry weight of NRG and NRG-NPs.

2. 2. 3. Cell Culture and Cytotoxicity Assay

A549 and 4T1 were grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO, USA) containing FBS (10%, v/v) and antibiotics [penicillin (80 units/mL) and streptomycin (80 µg/mL)] at 37 °C in a CO_2 incubator (5% CO_2 and 95% relative humidity). In order to evaluate the

cytotoxic effect of NRG, NPS and NRG-NPs, Cell viability measured by MTT (3-[4,5-dimethylthiazol-2-yl] 2, 5-diphenyltetrazolium bromide) assay. The MTT assay measures the reduction of a tetrazolium component into an insoluble formazan product by the mitochondria of viable cells. Identical cell numbers (1×10^5 cells) in 200 μL DMEM containing 10% FBS were seeded in triplicate on 96-well plates and incubated overnight. Cells were subsequently treated with various concentrations of NRG-NPs, NPs and NRG for 48 h and then 20 μL of MTT (5 mg/mL) was added to each well and incubated for an additional 4 h followed by adding 200 μL of dimethyl sulfoxide (DMSO). The color intensity generated is directly proportional to the number of viable cells. Relative cell viability was then determined using a 96-well plate reader (TECAN, Switzerland) at 540 nm. All experiments were performed in triplicates, standard deviations were calculated.

3. RESULTS AND DISCUSSIONS

Nanoparticles were characterized by scanning Electron Microscopy. NRG-NPs had a small size (<195 nm), good encapsulation efficiency (91.26 ± 3.15%) and high drug loading (10.49 ± 0.25%) (Fig. 2, 3). Antioxidants protects biological systems from oxidative damage produced by reactive oxygen species (ROS) are therefore considered as health-promoting compounds in nutrition. The NRG-NPs also showed antioxidant activity with the greater activity of the NRG (Table 1).

The antioxidant activity of NRG has been reported by other authors [10-11]. There is a mutual relation between Fe chelatory and antioxidant properties of the compounds. Chelation of iron and quenching of singlet oxygen are the major characteristics of antioxidant activity. NRG-NPs significantly suppressed the proliferation of A549 and 4T1 cancerous cells in a dose and time dependent manner in comparison with NRG and NPs.

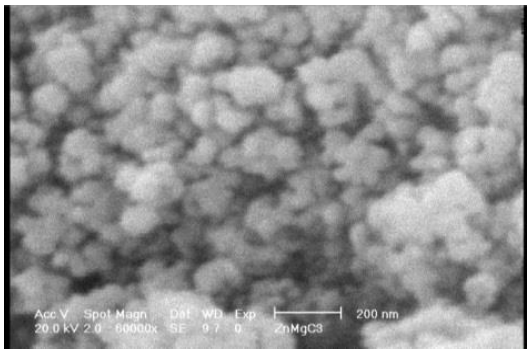

Fig. 2. Scanning electron microscope (SEM) of NRG-NPs.

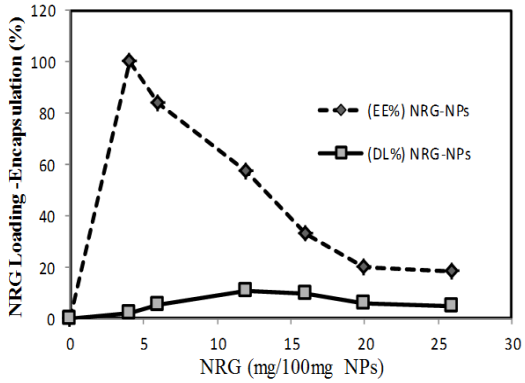

Fig. 3. Drug loading and Encapsulation efficiency of NRG-NPs.

Table 1. Total Antioxidant Capacity (TAC) of NRG and NRG-NPs.

Samples	TAC (mmol Fe^{+2}/g sample)
NRG	3.87± 0.09*
NRG-NPs	5.11 ± 0.12*

*Values expressed are means ± SD

Therefore, we showed that IC_{50} value (the concentration of material required to achieve 50% reduction in cell viability in comparison to untreated controls) of the NRG-NPs is significantly lower than NRG. In addition, no significant toxicity was observed for NRG-NPs. NRG-NPs IC50 for 4T1 cells was 25 μM within 48 h and for A549 cells was 18 μM (Fig. 4).

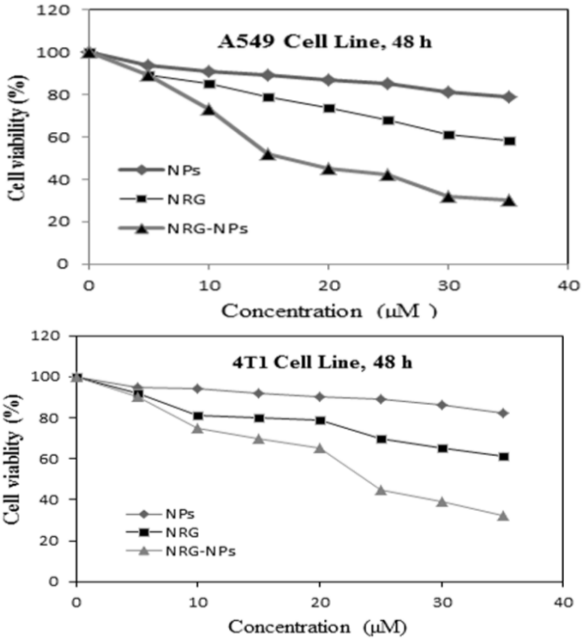

Fig. 4. Cytotoxic effects of NRG, NPs and NRG-NPs on 4T1 and A549 cell lines.

We showed that IC50 of the free NRG is significantly higher than NRG-NPs, and NRG-

NPs significantly suppressed cell growth compared to free NRG. In addition, no significant toxicity was observed for NPs. The cytotoxicity potency was rated as follows: NRG-NPs > NRG. There was a correlation between Fe-chelatory and cytotoxicity activities of the NRG-NPs.

In our study, the NRG-NPs significantly suppressed proliferation of human and mouse carcinoma cells *in vitro* and not only boost NRG solubility and uptake in cell lines but also increase its toxicity on cancer cells. Because of cancer cells rapidly proliferate, these cells have higher requirement for iron than normal cells [12-13]. The increased requirement of cancer cells to iron has led to sensitivity of these cells to cytotoxic effects of Fe. Considering the vital role of iron in cellular proliferation and its potential to mediate deleterious oxidative damage when in excess, Fe-chelating agents provide a promising form of treatment for both iron overload disease and cancer therapy [14]. Due to the problems with the current synthetic drugs, finding natural products with iron-chelating activity could be a good approach for treating cancer.

4. CONCLUSIONS

Our results show that NRG-NPs are cytotoxic compounds for A549 and 4T1 cancer cell lines and anti-cancer effect can be attributed to the presence of Fe chelatory and antioxidant effects of NRG-NPs. These nanocarriers are effective in suppressing tumor growth in vitro and reducing anticancer drug side effects.

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REFERENCES

[1] N. Suwannateep, S. Wanichwecharungruang and S. F. Haag, Encapsulated curcumin results in prolonged curcumin activity in vitro and radical scavenging activity ex vivo on skin after UVB-irradiation, *Eur. J. Pharm. Biopharm.* 82 (2012) 485–490.

[2] Sahu, U. Bora, N. Kasoju and P. Goswami, Synthesis of novel biodegradable and self-assembling methoxy poly (ethylene glycol)-palmitate nanocarrier for curcumin delivery to cancer cells, *Acta Biomaterialia* 4 (2008) 1752–1761.

[3] S. Bisht, G. Feldmann, S. Soni, R. Ravi, C. Karikar and A. Maitra, Polymeric nanoparticle-encapsulated curcumin (nanocurcumin): a novel strategy for human cancer therapy, *J. Nanobiotech.* 5 (2007) 276-282.

[4] M. Gou, K. Men and H. Shi, Curcumin-loaded biodegradable polymeric micelles for colon cancer therapy in vitro and in vivo, *Nanoscale* 3 (2011) 1558–1567.

[5] M. Mohsenikia, A.M. Alizadeh and S. Khodayari, The protective and therapeutic effects of alpha-solanine on mice breast cancer, *Eur. J. Pharm.* 7 (2013) 1–9.

[6] J. Heyes, K. Hall, V. Tailor, R. Lenzand and G. Mac Lachlan, Synthesis and characterization of novel poly (ethylene glycol)-lipid conjugates suitable for use in drug delivery, *J. Control. Release.* 112 (2006) 280–290.

[7] R.L. Prior, X. Wu and K. Schaich, Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements, *J. Agric. Food Chem.* 53 (2005) 4290-4302.

[8] Felgines, O. Texier, C. Morand, C. Manach, A. Scalbert, F. Régerat and C. Rémésy, Bioavailability of the flavanone naringenin and its glycosides in rats, *Am. J. Physiol. Gastrointest. Liver Physiol.* 279 (2000)1148–54.

[9] A. Amiri, S.J. Fatemi and S.N. Fatemi, Removal of thallium by combining desferrioxamine and deferiprone chelators in rats, *Biometals* 20 (2007) 159- 163.

[10] R. Langer and J. Folkman, Polymers for the sustained release of proteins and other macromolecules, *Nature* 263 (1976) 797–800.

[11] Peer, J.M. Karp, S. Hong, O.C. Farokhzad, R. Margalit and R. Langer, Nanocarriers as an emerging platform for cancer therapy, *Nat Nanotech.* 2 (2007) 751–760.

[12] Duncan, R., Polymer conjugates as anticancer nanomedicines, *Nat Rev. Cancer* 6 (2006) 688–701.

[13] L.E. Pelzer, T. Guardia, A.O. Juarez and E. Guerreiro, Acute and chronic anti-inflammatory effects of plant flavonoids, *Farmaco.* 53 (1998) 421–424.