Preparation and Evaluation of Chrysin Encapsulated in PLGA-PEG Nanoparticles in the T47-D Breast Cancer Cell Line

Sina Mohammadinejad\textsuperscript{2,3}, Abolfazl Akbarzadeh\textsuperscript{2}, Mohammad Rahmati-Yamchi\textsuperscript{2}, Saeid Hatam\textsuperscript{5}, Saeed Kachalaki\textsuperscript{4}, Sanaat Zohreh\textsuperscript{1}, Nosratollah Zarghami\textsuperscript{1,2}*

Abstract

Background: Polymeric nanoparticles are attractive materials that have been widely used in medicine for drug delivery, with therapeutic applications. In our study, polymeric nanoparticles and the anticancer drug, chrysin, were encapsulated into poly (D, L-lactic-co-glycolic acid) poly (ethylene glycol) (PLGA-PEG) nanoparticles for local treatment. Materials and Methods: PLGA: PEG triblock copolymers were synthesized by ring-opening polymerization of D, L-lactide and glycolide as an initiator. The bulk properties of these copolymers were characterized using 1H nuclear magnetic resonance spectroscopy and Fourier transform infrared spectroscopy. In addition, the resulting particles were characterized by scanning electron microscopy. Results: The chrysin encapsulation efficiency achieved for polymeric nanoparticles was 70% control of release kinetics. The cytotoxicity of different concentration of pure chrysin and chrysin loaded in PLGA-PEG (5-640\textmu M) on T47-D breast cancer cell line was analyzed by MTT-assay. Conclusions: There is potential for use of these nanoparticles for biomedical applications. Future work should include in vivo investigation of the targeting capability and effectiveness of these nanoparticles in the treatment of breast cancer.

Keywords: Triblock copolymer - chrysin - encapsulation - drug encapsulation efficiency

Introduction

Cancer is becoming one of the most public health problems in the world; in 2008, Ferlay et al. estimated the occurrence of new cancer cases about 12.7 million and cancer related death nearly 7.6 million. The most commonly diagnosed cancers in wild world are lung cancer (12.7% of the total), breast cancer (10.9%) and colorectal cancer (9.7%), while the most common cancer related to death are lung cancer (18.2% of the total), stomach cancer (9.7%) and liver cancer (9.2%) (Ferlay et al., 2010). In United States, occurrence of new breast cancer case alone is 29% (Siegel et al., 2012).

Common cancer therapies including surgery, chemotherapy and radiation are often insufficient in treating of cancer diseases, though developing new methods of treatment or cancer therapy are necessary. Some plant medicines have been widely consumed for treatment of various diseases including cancer (Butler, 2004; Koehn and Carter, 2005). Polyphenols account for an important group of plant constituents which contain up to 8,000 different well-known structures (Bravo, 1998). These polyphenolic components produced by plants are used as secondary metabolites. These components, upon their structure are classified into 10 different types: flavonoids, simple phenols, coumarins, phenolic acids, naphthoquinones, anaquaquinones, lignins, isocoumarins, stilbenes and xanthones (Cook and Samman, 1996). The most important class of these polyphenolic constituents is flavonoids, which is generally safe and shows low toxicity (Samarghandian et al., 2011; Khacha-Ananda et al., 2013). Chrysin (5,7-dihydroxyflavone) is a biologically active flavonoid extracted from plants, propolis and honey (Suganya et al., 2014). Chrysin possesses anti-inflammatory, anti-oxidant and anti-allergic properties (Kadir et al., 2013). Many studies in recent years revealed that chrysin is candidate for cancer therapy in various cancer cell lines by inhibition of cell proliferation and induction of apoptosis (Monasterio et al., 2004; Khoo et al., 2010; Sak, 2014). Chrysin treatment lead to a strong
increase in p21 and accumulation of cells in G1 phase 
(Pal-Bhadra et al., 2012). Activate P21 is a cdk (Cyclin-
dependent kinases) inhibitor, which lead to apoptosis or 
cell cycle arrest by reducing cyclin and cdk(Cai and 
Dynlacht, 1998).

Cell proliferation is strongly regulated by enormous 
interactions between molecules (Zhou et al., 2011). The 
regulatory protein called cyclin when joined toCDKs, 
promote the cell through the cell cycle (Dickson and 
Schwartz, 2009; Zeybek et al., 2013). Cyclin D1/ CDK2 
and CDK4 transit cell from G1 to S phase, while cyclin 
E/ CDK2 and cyclin A/ CDK2 respectively, controls 
entry to S phase and controls S phase progression (Sherr 
and Roberts, 1999; Zhang et al., 2012). Overexpression 
of cyclin D1 has been reported to be directly associated 
with tumorigenicity, poor prognosis and resistance to 
therapy in several cancer cell lines including breast 
cancer (Hosokawa and Arnold, 1998; Yu et al., 2001; 
Fu et al., 2004). Several studies show that cyclin D1 is 
overexpressed in breast cancer up to 50% (Gillett et al., 
1994; Ishii et al., 2006; Sui et al., 2014). Thus, inhibition 
of cyclin D1 provides an approach to treatment of breast 
cancer.

Although chrysin possesses anti-cancer properties 
but poor bioavailability of this phenolic compound is an 
important obstacle for cancer treatment (CAO et al., 2014), 
which can be due to low absorption, quick metabolism 
and rapid systemic elimination. One of the approaches 
to overcome this issue is use of nanoparticles (Yin et 
al., 2013). Biodegradable polymeric nanocapsules are 
massively in use in recent years to maintain the quality 
of many drugs and biologically active compounds. 
Nanoparticle encapsulation helps to protect therapeutic 
molecules from being prematurely degraded, enhances 
their solubility, and provides controlled drug targeting 
condition (Khalil et al., 2013). PLGA (poly lactic-co-
glycolic acid) is polymeric nanoparticle that shows high 
degree of biocompatibility and Biodegradability as well 
as safety for human, which is approved by U.S. Food 
and Drug Administration (FDA) (Alimohammadi and 
Joo, 2014). Surface modification of PLGA with PEG 
(polyethylene glycol) could improve permeability and 
half-life of circulation (Dwivedi et al., 2014). Chrysin to 
be loaded in PLGA_PEG nanoparticles could increase 
its bioavailability and efficiency of anti-cancer properties 
(Braden et al., 2008).

In this study we hypothesize that encapsulation of 
chrysin with PLGA-PEG have strong effect in cell cycle 
arrest compared to the effect of pure chrysin and we 
investigate the efficacy of chrysin loaded in PLGA-PEG 
inhibition of cell proliferation and reduction of cyclinD1.

Materials and Methods

Materials
Chrysin, penicillin G, streptomycin, glycolide, PEG 
(6000), 3(4, 5-dimethylthiazol-2-yi) 2, 5-diphenyl-
tetrazolium bromide (MTT), stannous octoate [Sn (Oct) 2], 
dimethyl sulphoxide (DMSO), dichloromethane (DCM), 
polyvinyl alcohol (PVA) and D, L-lactide were purchased 
from Sigma-Aldrich (USA). T47-D breast cancer cell line 
(code: c203) was obtained from Pasteur Institute of Iran. 
Trypsin-EDTA, Fetal bovine serum (FBS) and RPMI-
1640 were from Gibco, Invitrogen (UK). Primers were 
purchased from Takapouzist. 2-step RT-PCR kit obtained 
from vivantis while, Hot TaqEvaGreenPCR Mix and 
RNX-Plus kit purchased from fromCinnaGen (Iran) and 
used for cDNA synthesis, real time PCR and total RNA 
 extraction respectively. Real-time PCR was done using 
Corbett (Rotor Gene 6000). Nanodrop spectrophotometer 
was Bio Spectrophotometer. KYKY model EM3200 and 
Fourier transform infrared spectroscopy (FTIR) Perkin Elmer 
Series was used for Scanning electron microscopy (SEM) 
and Infrared spectra respectively.

Synthesis of PLGA-PEG

Synthesis of PLGA-PEG was done through ring open 
polymerization of glycolide and DL lactide followed by 
addition of PEG6000. As stated in melt polymerization 
procedure under vacuum, PEG 6000 and PLGA were co-
 polymerized in presence of stannous octoate [Sn (Oct) 2] 
as the catalyst. DL-lactide (2.882g), PEG6000 1.54 g (45% 
w/w) and glycolide (0.570 g) were melted in bottleneck 
flask in 140 Celsius degrees under a nitrogen atmosphere. 
Reaction mixture comprising a 3: 1 proportion of DL-
lactide to glycolide and 0.05% (w/w) stannous octoate 
was prepared and heated to 180° C and maintained for 
four hours.

Chrysin encapsulation with PLGA-PEG

Chrysin were loaded in PLGA-PEG nanoparticle by 
using s/o/w technique. Briefly, PLGA-PEG (200 mg) 
was dissolved in dichloromethane (DCM) while pure 
chrysin (20 mg) was added to this solution and sonicated 
for 1 minute to yield the s/o primary emulsion. Dimethyl 
sulphoxide (DMSO) and polyvinyl alcohol (PVA) 1% 
(1:1) was added to s/o emulsion then sonicated for 1 
minute to produces/o/w emulsion. Subsequently solvents 
of this emulsion were evaporated with rotary evaporator 
then this emulsion was centrifuged 30 minutes at 10000xg. 
The drug encapsulation efficiency of chrysin loaded in 
PLGA-PEG was measured by assessing the supernatant 
of centrifuged emulsion at 348 nm with spectrophotometer 
(Braden et al., 2008). Encapsulation efficiency measured 
by this formula:

\[
\text{Encapsulation } \% = \left( \frac{\text{Drug}_{\text{encapsulated}}}{\text{Drug}_{\text{total}} - \text{Drug}_{\text{filtrate}}} \right) \times 100
\]

FTIR analysis

Functional groups were characterized using FTIR 
analysis. Figure 1 shows that absorption band at 3509.9 
cm\(^{-1}\) is assigned to terminal hydroxyl groups in the 
copolymer from which PEG homopolymer has been 
removed. The bands at 3010 cm\(^{-1}\) and 2955 cm\(^{-1}\) are due 
to C-H stretch of CH, and 2885 cm\(^{-1}\) due to C-H stretch 
of CH. A strong band at 1762.6 cm\(^{-1}\) is assigned to C=O 
stretch. Absorption at 1186-1089.6 cm\(^{-1}\) is due to C-H 
stretch. Absorption at 1186-1089.6 cm\(^{-1}\) is due to C=O 
stretch.
**SEM analysis**

Measurement of size and checking the morphology of nanoparticles were performed by scanning electron microscopy (SEM). The nanographs of PLGA-PEG polymeric nanoparticles (Figure 2A), and Chrysin-loaded PLGA-PEG copolymers nanoparticles are shown. Observing the photograph, it can be seen that the nanoparticles were well aggregated, which was due to the Nano-size of the polymeric nanoparticles about 15 nm. After encapsulation, the size of Chrysin-loaded PLGA-PEG copolymers nanoparticles with PLGA-PEG copolymers, changed to 20-75 nm and dispersion of the particles was greatly improved (Figure 2B), which can be explained by the electrostatic repulsion force and steric hindrance between the copolymer chains on the encapsulated polymeric nanoparticles. The samples were coated with gold particles.

**Cell culture**

T47-D breast cancer cell line were cultured in RPMI-1640 medium with 10% FBS and incubated at 37°C and 5% CO₂.

The RPMI-1640 also has streptomycin (50 μg/mL), penicillin G (100U/mL) and NaHCO₃ (2mg/mL).

**Cell viability analysis**

MTT assay was done to measure T47-D cell viability. 10000 cell/well were seeded on 96-well plate and allowed them to attach for 24 h. Afterward cells were treated with different concentration of pure and Nano chrysin (5-640 μM) for 24, 48 and 72h. For each concentration triplicate were chosen and control received same amount of solvent (DMSO). After 24, 48 and 72 h medium were removed from all well while, 200 μL medium and 50 μL MTT solution were added to each wells and incubated for 4 h at 37°C. Then mixture of MTT solution and medium discarded from all wells and MTT crystals were dissolved by adding 200 μL DMSO and 50 μL Sorenson’s buffer. The viability of the cells was calculated by this formula:

$$\text{cell viability} = \frac{\text{absorbance of experimental wells}}{\text{absorbance of control wells}} \times 100$$

**RNA extraction, cDNA synthesis and real-time PCR**

T47-D cells were treated with different concentrations of pure and Nano chrysin (34, 54 and 74 μM) for 24 h. After drug exposure time, according to the instructions of the RNX-Plus manufacturer total RNA were extracted. Nanodrop was proved the purity and quantity of total RNA. After the integrity of total RNA was examined by using electrophoresis, complementary DNA (cDNA) was synthesis by 2-step RT-PCR kit according to the instructions of the manufacturer. Next Quantitative real-time PCR technique was used to determine cyclin D1 expression levels and Hot Taq EvaGreen PCR Mix used following the instructions of the manufacturer. Sequence of forward and reverse primers for cyclin D1 were, F: 5’-TGCCCTCTGTGCCACAGATG-3’, R: 5’-TCTGGAGAGGAAGCGTGTTGA-3’ and primers for β-actin, F: 5’-TCTGGAGAGGAAGCGTGTTGA-3’, R: 5’-GTAGTTTCGTGGATGCCACA-3’. The samples were incubated in following order (Table 1). Relative cyclin D1 expression levels was normalized by housekeeping gene (β-actin) and relative expression of cyclin D1 calculated by this formula:

$$\text{Relative gene expression} = \frac{E_{\text{target}}}{E_{\text{reference}}} = \frac{E_{\text{CP(target)}}}{E_{\text{CP(reference)}}}$$

E_{$\text{target}$} is referred to real-time efficiency of target gene transcript while E_{$\text{reference}$} is referred to real-time efficiency of reference gene transcript. ΔCP_{target} is referred to CP deviation of (control - sample) of the target gene transcript and ΔCP_{reference} is referred to CP deviation of (control - sample) of the reference gene transcript.
Data analysis and statistics

GraphPad.Prism.6.01. was employed for all data analysis and plotting graphs. Statistical analysis was performed by ANOVA test (by one-way analysis of variance). The result was assumed of statistical importance when/if pvalue was smaller than 0.05.

Results

Encapsulation efficiency

Analysis of supernatant of chrysin loaded in PLGA-PEG showed that chrysin were encapsulated via PLGA-PEG nanoparticle in 98.6% encapsulation efficiency. Success in PLGA-PEG synthesis by open ring copolymerization was confirmed by SEM (Figure2). The results of FTIR verified present of chrysin in PLGA-PEG.

Table 2. IC₅₀ Value of Pure and Nano Chrysin

<table>
<thead>
<tr>
<th>Different concentrations of chrysin (μM)</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free chrysin</td>
<td>54.56</td>
<td>46.74</td>
<td>40.28</td>
</tr>
<tr>
<td>Nano chrysin</td>
<td>48.72</td>
<td>44.78</td>
<td>37.54</td>
</tr>
</tbody>
</table>

Figure 3. Compared Different Concentration of Pure and Nano Chrysin for 24, 48 and 72 Hours. A: Related to 24 Hours Treatment, B and C Also Related to Chrysin Treatment for 48 and 72 hours, Respectively

Cell viability assay

The cytotoxicity of different concentration of pure chrysin and chrysin loaded in PLGA-PEG (5-640μM) on T47-D breast cancer cell line were analyzed by MTT-assay technique. These serial dilution of pure and Nano chrysin were treated on T47-D for 24, 48 and 72 h and results suggested that pure chrysin and chrysin loaded in PLGA-PEG could inhibit cell proliferation by dose dependent manner. The IC₅₀ value for 24 h treatments were 54.56 for pure chrysin and 48.72 for chrysin loaded in PLGA-PEG (Figure 3A). IC₅₀ value of 48 h were 46.74 and 44.78 respectively for pure and Nano chrysin (Figure 3B) while for 72 h treatment of pure chrysin and Nano chrysin IC₅₀ value were 40.28 and 37.54 respectively (Figure 3C). These results showed that PLGA-PEG nanoparticle could improve the efficiency of chrysin in cell growth inhibition (Table 2).

Quantitative real-time PCR

The levels of cyclin D1 expression was measured by real-time PCR. Cyclin D1 mRNA levels were normalized by β-actin (housekeeping gene). Different concentration of pure chrysin (34,54 and 74 μM) for 24 treatment decreased relative cyclin D1 expression in .78±.04, .37±.03 and 24±.02 respectively. Whereas chrysin loaded in PLGA-PEG nanoparticle in same concentration reduced further relative mRNA expression of cyclin D1 in .56±.04, 23±.03 and .16±.03 for 34, 54 and 74 μM respectively (Figure 4). The results suggested that chrysin loaded in PLGA-PEG had better effect in decline cyclin D1 expression rather than pure chrysin.

Discussion

Breast cancer is one of the most common types of cancer with >1,300,000 cases and 450,000 mortalities
annually worldwide (27-1). Although fatalities due to breast cancer are decreasing, breast cancer remains the second leading cause of cancer related mortality among females (27-2, 3). Surgical removal of the primary tumor, chemotherapy and radiotherapy are inadequate for cancer treatment. This high-lights the requirement for therapies that are able to treat the advanced stages of the disease. Improvement in the understanding of the molecular mechanisms involved in cancer has led to the identification of novel targets and the development of specific therapies, which are referred to as targeted therapies. The cell cycle is a promising target involved in cancer growth. Cyclin D1 is proto-oncogenes that over express in many cancers, in which drive the cells from G1 to S phase also play important role in tumorigenicity and cancer progression (6, 7). Medicinal plants have been used worldwide and have been demonstrated to be a source of effective anticancer agents (8). Chrysin is phenolic compound that extract from honey and shows anti-cancer properties and down regulated cyclin D1 expression in many cancer cell line. Due to poor oral bioavailability, chrysin may not be successfully used as a dietary flavonoid for breast cancer therapy. Obviously, our results are limited than chrysin alone and PLGA-PEG is promising approach to maintenance the anti-cancer effects of this dietary flavonoids. In parallel to our works Manika Pal-Bhadra and colleague showed that chrysin arrest the cell cycle and reduce cyclin D1 expression in skin cancer. Whereas we showed that chrysin could decreased cyclin D1 levels but Nano chrysin (74μM) down regulated cyclin D1 mRNA up to 80%(Pal-Bhadra et al., 2012). In conclusion: chrysin encapsulated with PLGA-PEG show significant repression effect in cell growth rather than chrysin alone and PLGA-PEG is promising approach for breast cancer therapy. Obviously, our results are limited to a number of cell lines in vitro which should be further accredited by testing the drug on live animal models(with enough replicates).

Acknowledgements

Department of Medical Biotechnology, Faculty of Advanced Medical Sciences, Tabriz University of Medical Sciences, for all support provided. This work is funded by 92/20 grant of Hematology and Oncology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.

References


Chang H, Mi M, Ling W, et al (2004). Other studies shown that, the cytotoxicity of chrysin more than other analogs and these properties come from two hydroxyl groupin AC rings of flavone were responsible for effective cytotoxicity and modification of -OH groups in flavone could harm potential of cytotoxicity (Chang et al., 2008). While we used chrysin loaded in PLGA-PEG nanoparticle to maintenance the anti-cancer effects of this dietary flavonoids. PLGA-PEG had greater bioavailability and effectiveness of chrysin in cell growth inhibition was enhanced. Boon Yin Khoo et al. used phosphorylated chrysin to develop the effectiveness of anti-cancer properties, although chrysin could inhibit proliferation and induced apoptosis but diethylyl chrysin-7-yl phosphate (CPE) and tetraethyl bis-phosphoric ester of chrysin (CP) have more potent have more affect in cell progression(Zhang et al., 2004). Other studies shown that, the cytotoxicity of chrysin more than other analogs and these properties come from two hydroxyl groupin AC rings of flavone were responsible for effective cytotoxicity and modification of -OH groups in flavone could harm potential of cytotoxicity (Chang et al., 2008). While we used chrysin loaded in PLGA-PEG nanoparticle to maintenance the anti-cancer effects of this dietary flavonoids. In parallel to our works Manika Pal-Bhadra and colleague showed that chrysin arrest the cell cycle and reduce cyclin D1 expression in skin cancer. Whereas we showed that chrysin could decreased cyclin D1 levels but Nano chrysin (74μM) down regulated cyclin D1 mRNA up to 80%(Pal-Bhadra et al., 2012).

In conclusion: chrysin encapsulated with PLGA-PEG show significant repression effect in cell growth rather than chrysin alone and PLGA-PEG is promising approach for breast cancer therapy. Obviously, our results are limited to a number of cell lines in vitro which should be further accredited by testing the drug on live animal models(with enough replicates).


