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Preparation of curcumin-loaded PLGA nanoparticles and investigation of its cytotoxicity effects on human glioblastoma U87MG cells

Hossein Arzani1, 2, Mahdi Adabi1, Jafar Mosafer2, 3, Farid Dorkoosh4, Masood Khosravani1, *, Hasan Maleki1, Houra Nekounam1, Morteza Kamali1

1 Department of Medical Nanotechnology, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, Iran
2 Research Center of Advanced Technologies in Medicine, Torbat Heydariyeh University of Medical Sciences, Torbat Heydariyeh, Iran
3 Nanotechnology Research Center, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran
4 Department of Pharmaceutics, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran

*corresponding author e-mail address: drkhosravani@tums.ac.ir

ABSTRACT
Curcumin, an herbal derivative, could be used as anticancer agent in drug delivery. Due to hydrophobic properties of curcumin, its bioavailability decreases to cancer cells. The aim of this study was to prepare curcumin loaded poly (lactic-co-glycolic acid) nanoparticles (CUR-PLGA-NPs) as a drug delivery system to overcome the limitation of hydrophobicity of CUR as well as to investigate its toxicity effect on human glioblastoma U87MG cells compared to free CUR. Single emulsion method was used to prepare CUR-PLGA-NPs. Under optimum conditions, the size of nanoparticles was characterized by dynamic light scattering (DLS) and scanning electron microscopy (SEM). Encapsulation efficiency and loading content were 89.77% and 9.06%, respectively; with a biphasic release behavior. Differential scanning calorimetric (DSC) thermograph of CUR-PLGA-NPs exhibited that CUR was dispersed in amorphous phase. The cytotoxicity studies on U87MG cell line indicated that CUR-PLGA-NPs had high cytotoxicity effects compared to free CUR. The half maximal inhibitory concentration (IC50) after cell incubation for 72 h was 57.99 μg/mL and 32.90 μg/mL for free CUR and CUR-PLGA-NPs, respectively. In conclusion, it is suggested that CUR-PLGA-NPs can be used for drug delivery to cancer cells.

Keywords: Curcumin, PLGA, Nanoparticle, Glioblastoma.

1. INTRODUCTION
Bioactive plant compounds have been recently applied for the treatment of many diseases such as diabet, skin, inflammatory and cardiovascular diseases and cancers [1-3]. In addition, many species of the plants have the anti-cancer property [4] such as polyphenols [5, 6]. One of the most widely used polyurethanes in the research is CUR (diferuloylmethane) which is derived from curcuma longa planet. It has high potential to be used as anticancer agent in drug delivery[7-9] due to several properties including anti-cancer effects, antioxidant and anti-inflammatory activity that affect cell cycle regulation and apoptosis pathways[10-12]. The effects of CUR as a treatment agent on different types of cancers such as prostate, colon, liver, breast and glioma have been performed by many studies[11, 13, 14]. In spite of all these desired properties, the application of CUR as anti-cancer agent is limited due to its highly hydrophobic nature and low bioavailability [15, 16]. To overcome this problem and sufficient delivery of therapeutic agents to the tumors, the design of new drug and delivery system is needed. Nanocarriers have created new opportunities to enhance the efficacy of drug delivery especially for small hydrophobic molecules in tumors cells via decreasing in drug side effect and increasing in bioavailability [17, 18]. The most notable nanostructures used in drug delivery are liposomes and nanostructures consist of nanopore or biocompatible polymeric nanoparticles [19-21]. The clinical application of PLGA, as a safe, biocompatible and biodegradable controlled release polymer system, has been well demonstrated [22-24]. The preparation of PLGA nanoparticles can have several advantages including drug protection, drug delivery of hydrophobic compounds, control of its release profiles and faciliation of the penetration of the drug into the tissue [25-27]. In our previous works, we prepared methotrexate loaded PLGA nanoparticles and imatinib base-loaded human serum albumin [28, 29]. In this study, we prepared the CUR-PLGA-NPs using solid-in-oil-in-water (S/O/W) emulsion method [30, 31] and investigate the toxicity effect of CUR on the U87 cancer cell line.

2. MATERIALS AND METHODS
2.1. Material.
Curcumin (purity > 80%, Cas No: 458-37-7), Poly (vinyl alcohol) with molecular weight 30000-70000, Rhodamin G6, phosphate-buffered saline (PBS) and tetrazolium dye 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were purchased from Sigma-Aldrich. PLGA polymer (lactic/glycolic acid ratio 50:50, Resomer® 503H) was bought from Boehringer Ingelheim (Germany). Other solvents and reagents were in analytical grade. Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were received from Gibco® (Germany).

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2.2. Preparation of nanoparticles.
CUR-PLGA-NPs were prepared using S/O/W method through 10 steps [31]. Briefly, 50 mg PLGA to 4 mL chloroform was added and mixed with the 5 mg CUR and then sonicated to prepare the organic phase. The organic phase was added dropwise into PVA 1% as an aqueous phase to form a suspension. Sonication and magnetic stirrer was also used to produce the CUR-PLGA-NPs. Finally, the sedimentary components of centrifugation were freeze dried for 8 h at -80 °C. Blank PLGA nanoparticles were prepared according to the same procedure. Applying One-Factor Experiment, effect of PVA and CUR concentration were evaluated on size distribution and encapsulation efficiency. Cellular uptake ability of CUR-PLGA-NPs was examined by adding rhodamin to organic phase (0.3 mg/ml).

2.3. Particle size, size distribution and morphology.
DLS (Malvern Instruments, model Zetasizer® 3000-HS, UK) was used to investigate the size of nanoparticles, polydispersity index (PDI) and zeta potential. Furthermore, the size and morphology of nanoparticles were also characterized using SEM. Nanoparticles were placed on sample holder and sputter coated with gold (180 s at 14 mA under argon atmosphere). Then, the average particle size and morphology of the nanoparticles were characterized.

2.4. Drug loading and encapsulation efficiency.
After the centrifugation of produced suspension in the preparation stage, CUR-PLGA-NPs were separated from supernatant and then the concentration of free CUR were measured in the supernatant by UV-VIS spectrophotometry (Optizier 2120 UV plus) at λmax = 425 nm. The drug entrapment efficiency (EE) and loading capacity (LC) were quantified by the following equation :[32]

\[
LC \% = \frac{\text{weight of encapsulated CUR in NPs}}{\text{weight of CUR ~loaded NPs}} \times 100 \quad (\text{Eq. 1})
\]

\[
EE \% = \frac{\text{weight of CUR loaded in NPs}}{\text{Weight of CUR initially used}} \times 100 \quad (\text{Eq. 2})
\]

2.5. DSC analysis.
DSC analysis of the samples were performed to the physical state of CUR in polymeric nanoparticles and interaction between CUR and polymer in nanocarriers. To record DSC curve (TGA Q50, Thermal Analysis –TA, USA) a small amount of CUR, PLGA polymer, PLGA/CUR mixture and CUR-PLGA-NPs samples were placed in aluminum pan and heated from 0 to 300 °C under inert nitrogen atmosphere at a heating rate of 10°C/min while the instrument was calibrated with the alumina powder.

2.6. In vitro drug release.
In our study, we used the dialysis bag method to evaluate the in vitro release of CUR. The release of CUR-PLGA-NPs was determined using an aqueous buffer that contains 100 mL PBS solution and 3% v/v ploronic F-68. Then, 20 mg of CUR-PLGA-NPs were suspended to the aqueous buffer. The samples were shacked at constant shaking of 50 rpm at temperature condition of 37 °C. At selected time duration (0, 0.5, 1, 2, 3, 6, 12, 24, 48 and 72 h), 3 mL of solution were removed and then centrifuged. The amount of free CUR concentration was determined by spectrophotometry at λmax = 425 nm using calibration curve of CUR in buffer phosphate-methanol (1:9) media. To compare the effects of nanoparticles in drug release profile, free drug release curve was obtained in the same condition and method.

2.7. Cellular uptake and viability assay of nanoparticles.
Cell growth inhibition assay was measured by MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) test. CUR, PLGA, and CUR-PLGA-NPs were used for cell viability assay on U87 glioblastoma cell line. The cells were cultured by routine procedure (DMEM environment, 10% FBS, 100 µg /mL penicillin-streptomycin at 37°C and 5% CO2). For this investigation, 2 × 103 cell/well were cultured and then the cells treated with 0, 5, 10, 30 and 50 µg/mL of CUR, PLGA and PLGA-CUR-NPs for 24, 48 and 72 h. The absorbance of the wells was recorded by a microplate reader (Biotec ELx808 Absorbance Microplate Reader) compared to the control cells line according the following formulation:

\[
\text{Cytotoxicity (}) = 1-\text{(Abs test cells/Abs control cells)} \times 100 \quad (\text{Eq. 3})
\]

To investigate the intracellular uptake of nanoparticles, the cultivated cells were incubated with rhodamin G6-CUR-PLGA-NPs for 4 h and were investigated by fluorescence microcopy (Oxion fluorescence, Euromex Company). IC50 that represents the concentration of a substance required for 50% inhibition in vitro, was calculated with GraphPad Prism software (v6.07). All assessments were repeated three times for the statically analysis. Data were displayed along with mean ±SD (standard deviation).

3. RESULTS
In medical applications, polymeric nanoparticles can be used to reduce the hydrophobicity and to improve efficient delivery of the CUR. According to this object, S/O/W method was applied to prepare of the CUR-PLGA-NPs to reduce the CUR hydrophobicity and increase bioavailability.

Physicochemical properties of nanoparticles such as particle size and size distribution depend on several parameters such as polymer types, molecular weight of polymer, emulsifier type, emulsifier and drug concentration, sonication power, evaporation time, rotational speed on stirrer, etc. [33, 34]. Emulsifier and therapeutic agent concentration had great impact on particles size, EE and DL. Therefore, we decided to investigate the effects of two main factors including PVA and CUR concentration on the physicochemical properties of prepared CUR-PLGA-NPs.

3.1. Effects of CUR concentration on particle size, EE and LC.
It has been showed that the drug concentration could affect the size of nanocarrier, LC, therapeutic efficacy, pharmacokinetics and toxicity of drug [35, 36]. As showed in table 1, when the amount of CUR increased from 0 to 10 mg, the size of nanoparticles significantly increased from about 114 to 206 nm (P < 0.05). It may be due to more content of CUR available in the emulsion droplets or adsorption of drug on surface of nanoparticles[37].

| CURcumin(mg) | Main Size (nm) | PDI | ZP (mv) | EE (%) | DL (%) |
|-------------|----------------|-----|--------|--------|--------|
| 0           | 114 ± 8        | 0.029 | -6.39 | -      | -      |
| 3           | 119 ± 9        | 0.220 | -4.38 | 77.81  | 7.86   |
The obtained results of EE (89.77%) and LC (9.06%) were similar to the results of Anindita Mukerjee et al.[30]. The EE and LC of CUR-PLGA-NPs enhanced by an increased amount of CUR and the increase were not remarkable (P > 0.05). Therefore, by increasing the amount of CUR from 3 to 10 mg in organic phase an increase in EE and LC occurred from 77.81 to 89.87 % and 7.86 to 10.53 %, respectively. The prepared nanoparticles with the amount of 4-5 mg of CUR were desirable in the results of EE, LC and particles size. By increasing the concentration of CUR, the amount of EE and LC did not increased significantly, but the size of nanoparticles increased. The reduced cellular uptake capacity of nanoparticles is due to the increase in the particle size. Hence, the mean size of nanoparticles is particularly one of the limiting factors in the delivery of the CUR to the tissue such as brain [6, 38, 39] and should be optimized. On the other hand, by adding CUR concentration in the organic phase, presumably, CUR places on the outer surface of the nanoparticles and result in an unsuitable EE, LC, release profile and therapeutic efficacy. As a final result, using low concentrations of CUR is more appropriate to obtain smaller particle size with an optimum value of drug entrapment.

3.2. The effects of PVA concentration on particle size.

Due to the influence of PVA on the particle size, polydispersity index, zeta potential, surface properties of NPs such as hydrophobicity and other pharmaceutical properties, PVA as emulsifiers in the preparation of nanoparticles has been used [40]. PVA concentration affects the stability of emulsion. In our study, typically the NPs size increased at low and high PVA concentration in the aqueous phase. In some studies, contradictory data have been reported, because many paprameters such asorganic phase, molecular weight of polyvinyl alcohol, speed and time of homogenizer and sonication affect particles size diameter [30, 41].

| PVA con. (%) | Mean size of NPs (nm ± SD) |
|-------------|---------------------------|
| 3           | 294±17                    |
| 2           | 237± 21                   |
| 1           | 114± 8                    |
| 0.5         | 354 ±13                   |

At low PVA concentrations (0.5%) and high PVA concentrations (2 and 3%), chains of emulsifier may have unsuitable chain orientation [42, 43]. As indicated in the table 2, based on the other experimental condition, PVA with 1 % concentration was chosen.

3.3. Physicochemical properties of nanocarriers.

The optimized amount of CUR-PLGA-NPs includes 5 mg CUR, 50 mg PLGA, 10 mL PVA 1 %, 2 min sonication and magnetic stirrer for 8-10 h. Base on the result of SEM and DLS (as seen in figure 1), CUR-PLGA-NPs were monodispersed (PDI<0.27) with the mean size of 123 nm and according to the SEM image, CUR-PLGA-NPs have a spherical morphology.

PLGA nanoparticles can have high negative zeta potential due to carboxyl groups (about -40 mV). However, because of the presence of PVA, the zeta potential was less (-7 mV) [44-46].

3.4. Curcumin-polymer interaction study.

The obtained results of DSC graph is useful to understand the drug release profile [47]. The DSC thermogram of free CUR (curve A), PLGA (curve B), CUR-PLGA-NPs (curve C) and mixture of PLGA and free CUR (curve D) are shown in figure 2. According to DSC curve, endothermic peak of CUR was shown about 180 °C whereas PLGA had an amorphous state with a peak at the 45 °C which indicates glass to rubbery transition temperature (Tg). Exothermic curve of free CUR and PLGA mixture consist of two peak at the 45 °C and 180 °C while these peaks were not observed in CUR-PLGA-NPs sample. Base on the obtained result, in this preparation, no reaction occurred between free CUR and PLGA while CUR was trapped inside PLGA NPs in an amorphous state. Additionally, CUR-PLGA-NPs have structural rigidity at physiological condition. It can be due to the higher Tg relative to the physiological temperature. However, with the water penetration into the CUR-PLGA-NPs, the Tg decreases and chain mobility increases. These changes increase the drug release [48, 49].

3.5. In vitro CUR release.

The CUR release profile from nanoparticles was studied in PBS solution of 3% ploronic F-68 at physiological pH. As seen in figure 3, two-phase behavior of the CUR release from nanoparticles is observed. In a burst phase during the first 12 h, the release percentage of CUR from polymeric NPs were about 50%. At the sustain release phase, the release rate of CUR after 72 h was less than 80%. However, about 80% free CUR was released into the medium in the first 12 h. These results indicate that the release of the CUR from nanoparticles is suitable for drug delivery into the tumor tissue.

3.6. Uptake and cytotoxicity effects.

In order to evaluate the toxicity of nanoparticles, we should ensure from its cellular uptake of CUR-PLGA-NPs. In this work, 100 (μg/ml) rhodamin G6-CUR-PLGA-NPs were exposed to the
cultivated cell line and then investigated by fluorescence microscopy. Rhodamin G6-CUR-PLGA-NPs were prepared using the nanoprecipitation. After exposure the cells with the rhodamine G6-CUR-PLGA-NPs, red fluorescence of rhodamine G6 was observed in the cell line. The results exhibited that rhodamine G6-CUR-PLGA-NPs is able to pass through the cell membrane and accumulate inside the cells. With the increase of the exposure time of rhodamine G6-CUR-PLGA-NPs on the cell line, florescent intensity enhanced. According to the cellular uptake studies, NPs in the size of 100-200 nm can be internalized by receptor-mediated endocytosis mechanism[50, 51].

After incubation of U87 cells with CUR-PLGA-NPs, it is observed that both exposure time and CUR-PLGA-NPs concentration are effective in toxicity. However, concentration has a greater effect on toxicity. On the other hand, toxicity of free CUR depends on concentration and time (figure 5 and 6). According to our results, it was observed that the effect of PLGA nanoparticles on cytotoxicity can be negligible. Additionally, the toxicity of PLGA nanoparticles increased over time. This can be due to the acidification of the environment due to the decomposition of nanoparticles or the presence of PVA remaining on the surface of nanoparticles.

**Figure 4.** The internalization of CUR-PLGA-NPs into the U87 cell line. A) Represents the nucleus of U87 cells painted by DAPI dye. B) U87 Cells exposed to the fluoresce CUR-PLGA-NPs.

**Figure 5.** Cytotoxicity effects of PLGA, free CUR and CUR-PLGA-NPs on U87 cells after A) 24 h, B) 48 h and C) 72 h.

**Figure 6.** The toxicity correlation of free CUR and CUR-PLGA-NPs with time and concentration. A) free CUR and B) CUR-PLGA-NPs toxicity at times 24 h, 48h and 72 h. C) free CUR and D) CUR-PLGA-NPs toxicity at the concentrations of 10, 30, 50 and 100 μg/mL.
As shown in figure 5, it is observed that at low concentrations toxicity of CUR-PLGA-NPs decrease over time. Especially, this decrease is observed after 48 hours. Two main reasons for this decline can be related to the growth pattern of glioblastoma cells and release profile of CUR-PLGA-NPs. Based on cell culture conditions, glioblastoma cells replicate every about 48 hours. Therefore, the number of live glioblastoma cells doubles. As a result, this leads to decrease in toxicity levels after 48 hours exposure,[52]. On the other hand, CUR release from PLGA nanoparticles in the first 24 hours is more than 48 hours. This can also be effective in reducing toxicity after 48 hours.

**Figure 7.** Comparison of free CUR and CUR-PLGA-NP toxicity at a concentration of 10 µg/mL.

4. CONCLUSIONS

The aim of our investigation was to prepare of CUR-PLGA-NPs and study the release profile of PLGA-CUR-NPs and their toxicity. In this study, S/O/W method was used to prepare CUR-PLGA-NPs. In addition, the size of the prepared nanoparticles was about 123 nm with high EE (89.77%) and LC (9.06%) in optimized conditions. The toxicity studies of CUR-PLGA-NPs by the MTT assay showed that their toxicity depends on concentration and time. Due to the biphasic release profile and toxicity results, CUR-PLGA-NPs are suggested as an effective treatment agent in the drug delivery application to cancer tissues.

Although the toxicity of free CUR seems to be greater than CUR-PLGA-NPs at the same concentration and time, application of free CUR is limited due to hydrophobic properties. Also, it should be noted that the toxicity of CUR-PLGA-NPs at a concentration of 100 µg/ml (containing approximately 10 µg/ml of CUR) has the toxicity equal with the amount of 100 µg/ml of free CUR at 72 h (figure 7).

At the last result, IC<sub>50</sub> values were used to compare the cytotoxicity of CUR-PLGA-NPs and free CUR. According to the table 3, as the IC<sub>50</sub> of CUR-PLGA-NPs is less than free CUR, it seems that CUR-PLGA-NPs is more effective than free CUR.

**Table 3.** IC<sub>50</sub> values of free CUR and CUR-PLGA-NPs after the incubation time of 24 h, 48 h, and 72 h via MTT test.

| time  | Free CUR | CUR-PLGA-NPs |
|-------|----------|--------------|
| 24 h  | 26.65    | 30.13        |
| 48 h  | 49.33    | 30.40        |
| 72 h  | 57.99    | 32.91        |

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