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RESEARCH PAPER

# Microfluidic synthesis of pH-sensitive nanoparticles containing curcumin against breast cancer cell

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## ABSTRACT

***Objective(s):*** Scientists have focused on the development of new drug delivery systems including pH-sensitive nanomaterials adaptive to tumor microenvironments. We aimed to fabricate a microfluidic system to synthesize and characterize curcumin (Cur)-containing PCL and Chitosan (CSN) polymeric nanoparticles against MCF7 breast cancer cells.

***Materials and Methods:*** The microfluidic chip was fabricated by photolithography and polydimethylsiloxane (PDMS) molding procedure. The chip was Y-shaped and equipped with two inlets and one outlet. PCL and Chitosan (CSN) were dissolved in acetic acid overnight and mixed with Cur for three hours. The prepared solution was injected from one inlet and a solution of tween 80 in distilled water was injected from the other inlet. The nanoparticles were characterized in size, electrical charge, structure, drug loading, and drug release efficiency. Finally, the cytotoxicity was assessed using the MTT assay at specific concentrations after 24 and 48 hr.

***Results:*** The mean diameter/zeta potentials of spherical-shaped nanoparticles with and without Cur were 209 ±2 nm / +15 and 219 ± 4 nm /+3 , respectively. FTIR results confirmed the presence of all components in the nanoparticles. The Cur loading rate was 1.5%, and Cur represented a sustained release manner. Also, the release profile showed faster release in a low-pH medium. MTT assay results showed that Cur-containing nanoparticles exerted a significant effect on cell viability.

***Conclusion:*** It can be concluded that microfluidic systems can pave the way for nanoparticle synthesis easily rapidly and cost-effectively for cancer agent delivery. Based on our observations, PCL-CSN-loaded Cur nanoparticles represent appropriate characteristics and suitable anti-cancer effects.

***Keywords:*** *Cancer cells, Chitosan microfluidics, Nanoparticles, PCL*

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## INTRODUCTION

Today, engineered nanoparticles have raised hopes to develop new drug delivery systems to target cancer cells, either active or passive targeting [1, 2]. On the other hand, creating new methods for the synthesis of tailor-made nanoparticles applies to the diagnosis and treatment of cancers. Researchers apply different ways to encapsulate drugs into nanoparticles effectively. However, several disadvantages are attributed with conventional encapsulation techniques, including non-uniformity in size, complex processes, and operative dependence [3]. Deposition, sol-gel, ultrasonic, microemulsion, and reduction are the most utilized mechanisms in conventional nanoparticle synthesis techniques [4]. In these methods, due to the lack of control during synthesis, leading to non-uniformity particle size [5], researchers are continuously looking for innovative ways to produce and fabricate nanoparticles. During the last two decades, the microfluidic approaches have been at the center of attention for fabricating nanoparticles to overcome the limitations of conventional methods [6, 7]. It

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has been demonstrated that microfluidic chips can be developed to fabricate monodispersed nanoparticles with a uniform reaction medium and to improve reproducibility. In microfluidic methods, the non-solvent and solution (solvent + solute) media are injected into different inlet microchannels and mixed via lateral diffusion in a laminar flow regime while flowing along the outlet microchannel. Microfluidic systems enable us to control better the process at micro/nanoscales and even offer real-time interference with changing the reaction conditions. That way, microfluidics turn into an ideal method for making nanoparticles[8, 9]. In a microfluidic system with a laminar flow, the molecules of solution and non-solvent media diffuse into each other, followed by nucleation, growth, and aggregation processes [10]. Different polymers can be used to fabricate nanoparticles using microfluidic systems depending on the purpose of the study and the characteristics of the polymers. In the present work, mixtures of polycaprolactone (PCL) and chitosan (CSN) polymers were used. PCL is a relatively inexpensive biodegradable synthetic aliphatic semi-crystalline polyester, used in many medical applications. PCL is resistant to degradation in body fluids *(*the total degradation time is up to 4 years) [11, 12]. To overcome that, we used PCL's blend solution with CSN. CSN is a natural linear polymer formed by the degradation and N-deacetylation of chitin[13]. CSN has fascinating biological effects such as biocompatibility, biodegradability, antimicrobial activity, and accelerated wound healing. For developing drug delivery systems, CSN cannot be used solely; hence it is suggested to use it in combination with a hydrophobic polymer[14] [15, 16]. So far, various chemical drugs have been used for therapeutic applications, especially cancer treatment. Recently, natural compounds have been considered as anticancer agents. Curcumin is the main component in the rhizome of the turmeric plant, which has anti-cancer, anti-fungal, and anti-inflammatory activity. Despite its excellent properties, the consumption of curcumin is limited since curcumin has slight solubility in aqueous solvents. It is also rapidly degraded at body pH, which means that curcumin has low bioavailability and poor pharmacokinetics [17]. Therefore, to address these issues, various formulations have been performed based on encapsulation of curcumin in the form of polymer nanoparticles, nanogels, liposomes, etc. [18]. One of the outstanding features of tumors is attributed to their high metabolism rate, which subsequently leads to forming a different microenvironment distinctive from normal tissues[19]. Due to the glycolytic cell metabolism in cancer cells, leading to the production of a large amount of lactate along with excess protons and carbon dioxide, the pH of the tumor microenvironment is low i.e. in the range of 6.5–6.8. This distinctive property of tumor tissue can be used in the active targeting approach using optimized nanoparticles[20-22]. Therefore, by designing pH-sensitive nanoparticles, drug delivery systems can be delineated to specifically degrade in tumor pH and release the loaded drug specifically into tumor tissue. This type of promising targeting has advantages over other methods of ligand and antibody because it is an ordinary quality among all solid tumors [23].

Due to the importance of nanoparticles in developing new drug delivery systems and the relatively low efficiency of conventional fabrication methods, the present study aimed to create a PCL-CSN nanoparticle-containing Cur using a microfluidic system. The fabricated nanoparticles were then characterized, and their anti-proliferative effects were investigated in vitro on MCF-7 breast cancer cells.

## MATERIALS AND METHODS

### *Microfluidic chip fabrication*

First, SU8 negative photoresist was poured onto the wafer. Afterwards, the wafer was placed in a spin coater (1500 rpm for 60 seconds), then it was placed on the prebake part (65°C). Then, the SU8-coated wafer was aligned under a mask, containing the chip pattern, and radiated with UV for 1 min. Thereafter, post-bake was performed at 95 °C for 10 min. The wafer was then placed in the developer solution for 10 min so that the solution covered the wafer. At this stage, the desired design mask remained prominent on the wafer, and the master made. The next step was the molding step, which was done by soft lithography using polydimethylsiloxane polymer (PDMS). The polymer was poured into the master and heated in an oven at 80 °C for 4 hr. The baked PDMS was then pulled off from the wafer surface. Two inlets and one outlet hole were punched. After treatment of the PDMS layer under $O_2$ plasma, it was bonded on a glass substrate. As the last step of preparing the experimental setup, chip fluid connections, tubings, syringes, syringe pumps, and sample

microtube were installed.

***Nanoparticle synthesis***

60 mg of PCL ( Sigma ) and 20 mg of CSN( Sigma) were dissolved in 10 ml of 100% acetic acid and 10 ml of 70% acetic acid, respectively, and mixed overnight with a steerer at room temperature. Then, the equal volumes of polymer solutions were mixed for 3 hr. Also, a mixture of tween 80 solution/ distilled water (with a volume ratio of 1:100) was prepared. The aqueous and polymer solutions were injected into microchip inlets and their flow rates were applied through an infusion pump. The flow rates were adjusted for polymer and aqueous solutions as 1 ml/h and 20 ml/hr, respectively. The synthesized nanoparticles were harvested from the system outlet and collected in a microtube. The Cur-loaded nanoparticle was fabricated by adding 8 mg of Cur to 5 ml of PCL-CSN solution.

***Nanoparticles characterization***

Different properties of NPs such as size/ diameter, shape, charge, structure, and stability were investigated using the following methods. The transmission electron microscope (TEM, Zeiss EMEMC, Germany) was used to determine the size and shape of nanoparticles, and the stability of nano-suspensions was evaluated by dynamic light scattering (DLS, Malvern Instruments nano/ zeta sizer, Worcestershire, UK, Model Nano ZS). Fourier-transform infrared (FTIR) spectroscopy was used to identify functional groups related to PCL, CSN, Cur, and functional groups in nanoparticles and nanoparticles containing Cur to evaluate the structurally successful production of nanoparticles. For this analysis, a small amount of each piece was mixed with KBr powder(1:100). The tablets were placed in the FTIR device, and then spectroscopy was performed for each sample separately.

***Drug loading, encapsulation efficiency and release***

This assay was done to evaluate the amount of Cur loaded on nanoparticles. 10 ml of nanoparticle suspension and nanoparticle suspension containing Cur were filtered and concentrated through centrifuging at 4000 rpm at 25 ° C for 30 min and transported to 1.5 ml microtubes and centrifuged at 18,000 rpm at 25 ° C for 1 hr. Then, the supernatant layer was removed and the microtubes were placed and kept in an oven at 38 °C for 48 hr. Pure nanoparticles (used as blank) and nanoparticles containing dried Cur were suspended in acetic acid. Their optical density was read using a spectroscope at a wavelength of 420 nm to calculate the mass of loaded Cur. Using the following equation, the drug loading percentage in nanoparticles and Encapsulation Efficiency(EE) were calculated.

$$\%\ loading = \frac{mass\ of\ drug}{mass\ of\ drug + mass\ of\ NPs} \times 100$$

$$\text{Encapsulation efficiency (\%)} = \frac{mass\ of\ drug}{total\ mass\ drug} \times 100$$

For evaluation of the amount of drug release a certain amount of nanoparticles and nanoparticles containing Cur were synthesized and transferred to a dialysis tube and placed in a 15 ml tube containing 5 ml of 1% Tween-PBS solution at pH of 7.4 and 5.5. The tubes were placed on an incubator shaker at 38°C. Sampling was performed at times intervals of 0, 0.5, 1, 2, 4, 8, 16, 24, 48 and 72 hr and then at intervals of seven days for four weeks. The Optical Density (OD) was read at 420 nm and the cumulative release diagram was calculated.

***Hemolysis***

To evaluate the toxicity of nanoparticles on red blood cells, a hemolysis test was performed. 5 ml of human blood was mixed with 0.1 ml of 0.5 M EDTA and centrifuged (2000 rpm for 15 min). By removing the supernatant layer, PBS was added equally and repeated three times. The blood was then diluted 10-folds using PBS, and then, serial dilutions of nanoparticles (from 13.3 to 400 µM) were prepared, added to the microtubes, and placed in an incubator shaker at 37 °C for 4 hr. The microtubes were centrifuged (for 15 min at 13000 rpm), the supernatant layer was separated and the Optical Density (OD) was read at 540 nm.

***Cancer cell viability***

Cell survival was evaluated through MTT assay. 1500 MCF-7 breast cancer cell lines were cultured in each well of a 96-well plate and placed in an incubator for 24 hr at 90% humidity, 37 °C, and 5% $CO_2$. After reaching the suitable confluence, DMEM+10% FBS containing specific concentrations (0.25, 0.5, 1, and 2 µM) of nanoparticles, nanoparticles-Cur, and free Cur were replaced with cells medium. Survival of MCF-7 cells was investigated 24 and 48 hr after treatment. The culture medium was removed, and 100 µl of the

prepared solution (90% DMEM and 10% MTT (3-(4,5-dimethylthiazoyl-2,5-diphenyltetrazolium bromide) was added and incubated. After 4 hr, the culture medium was removed from each well, and the DMSO solution. Finally, the optical density(OD) was determined at 570 nm.

### *Statistical analysis*

One-way analysis of variance was used to analyze data via GraphPad Prism 6 software (Graphpad Software Inc., La Jolla, CA). Data were represented as mean ± standard deviation (SD) with statistically significant level at *P*-value 0.05.

## RESULTS

### *Microfluidic chip fabrication*

A microfluidic chip has been fabricated using soft lithography technique. A schematic of nanoparticle formation has been shown in Fig. 1. In the Y-shaped microfluidic chip (with two inlets and one outlet) ,the width and depth of the channels were 500 m and 80 um, respectively.

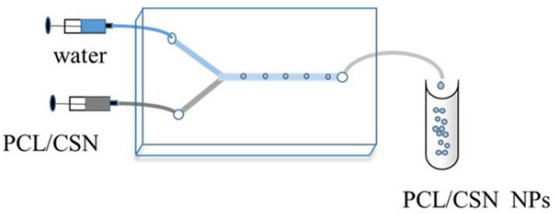


Fig. 1. The diagram of PCL-CSN nanoparticle synthesize using the microfluidic system

### *Synthesis of nanoparticles*

The diameter and surface charge of the nanoparticles were evaluated using DLS. Due to the effect of flow rates on the size of nanoparticles, different flow rates were used. The results of the size and size distribution of nanoparticles for each flow rate are shown in Table 1.

According to the results, the most appropriate ratio of flow rats was 1:20. According to the obtained results, the diameter, PDI and ζ-potential of PCL-CSN and PCL-CSN-Cur nanoparticles were (D: 209±2, PDI:0.2, ZP: +15) and (D:219±4, PDI:

Table 1. The effect of aqueous flow rated on the diameter of nanoparticles

| Polymers flow rate (ml/hr) | Aqueous flow rate (ml/hr) | Diameter (nm) | Polydispersity index (PDI) |
|---|---|---|---|
| 1 | 5 | 403.33±8.6 | 0.23±0.047 |
| | 10 | 318±8.2 | 0.36±0.047 |
| | 15 | 244.33±1.2 | 0.33±0.047 |
| | 20 | 203.66±1.6 | 0.3 |

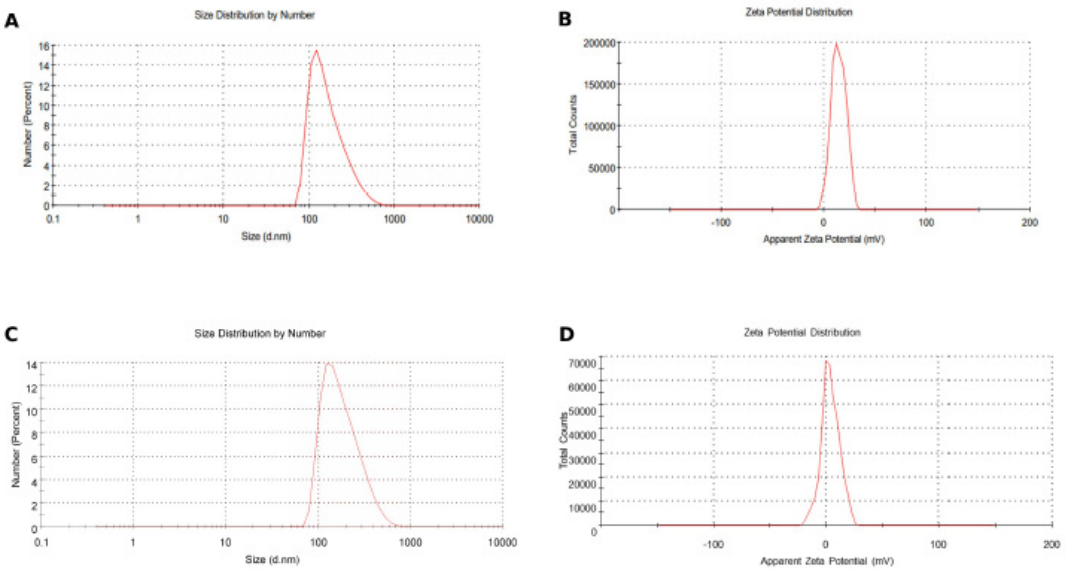


Fig. 2. Particle size and zeta potential of pure nanoparticles (A – B) and nanoparticles loaded with curcumin (C – D) was obtained by the relative proportion of 1:20.

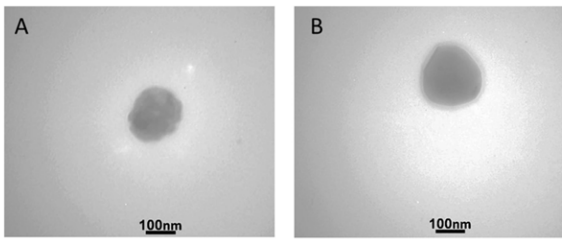


Fig. 3. TEM images of (A) nanoparticles and (B) nanoparticles loaded with Cur

0.3, ZP: +3), respectively (Fig. 2).

### *TEM Imaging*

TEM images show the successful synthesis of nanoparticles and nanoparticles containing curcumin and represented spherical shape and diameter of 154 and 179 nm, respectively (Fig. 3).

### *FTIR*

FTIR was used to ensure the synthesis of optimal nanoparticles and to study the chemical structure of nanoparticles (Fig. 4). The peaks of 1500 $cm^{-1}$ and 1300 $cm^{-1}$ indicate the C=C bond and the C-C bond in the phenol group of curcumin, respectively. In chitosan, the peaks at 1110 $cm^{-1}$ -1068 $cm^{-1}$ are related to the C-O bond; 1545 and 1630 are coupled to amines and amine bonds, respectively. In PCL, the peak at 2940 $cm^{-1}$ is related to the C = O bond, and the C-O-C bond stretch is attributed to the peak at 1175 $cm^{-1}$, while the C-H bond flexion is associated with the peaks at 1440 $cm^{-1}$-1390 $cm^{-1}$.

### *Nanoparticle stability*

The nanoparticle's diameter and PDI were examined for three months, and the results are represented in Fig. 5. Statistical analysis showed that the difference in the size of pure nanoparticles and their PDI was not significantly different during the follow-up period. Regarding drug-loaded nanoparticles, the statistical analysis also showed no significant difference in the size of drug-loaded nanoparticles and their PDI during the follow-up period.

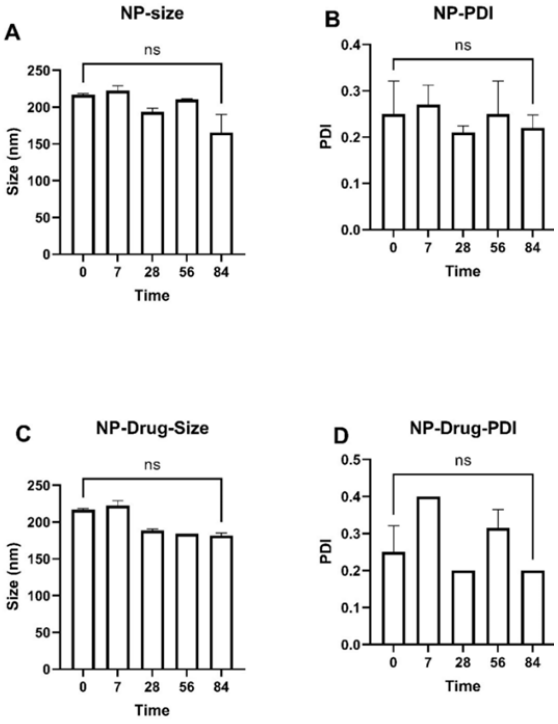


Fig. 5. Stability diagrams of nanoparticles based on size and PDI in pure nanoparticles (A and B) and nanoparticles loaded with Cur (C and D)

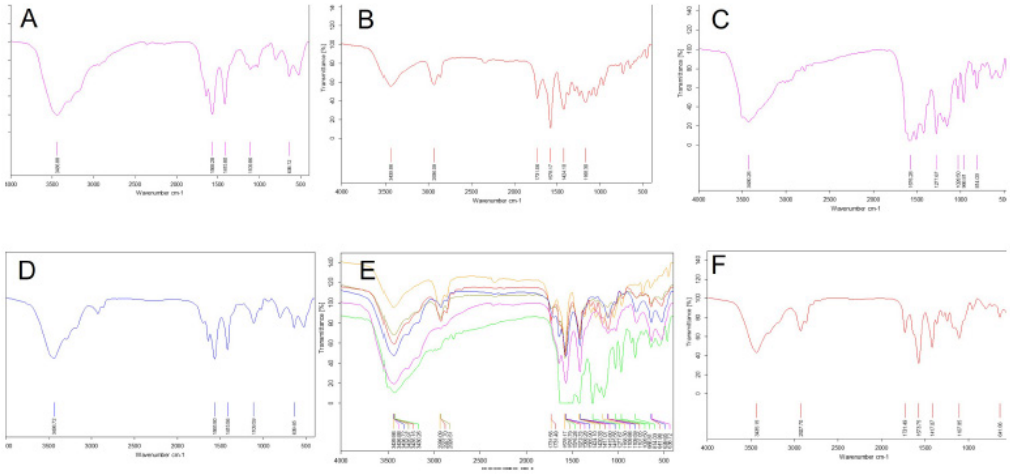


Fig. 4. The FTIR peaks for (A) Chitosan, (B) PCL, (C) Cur, (D) PCL-CSN, (E) All components, and (F) PCL-CSN-Cur

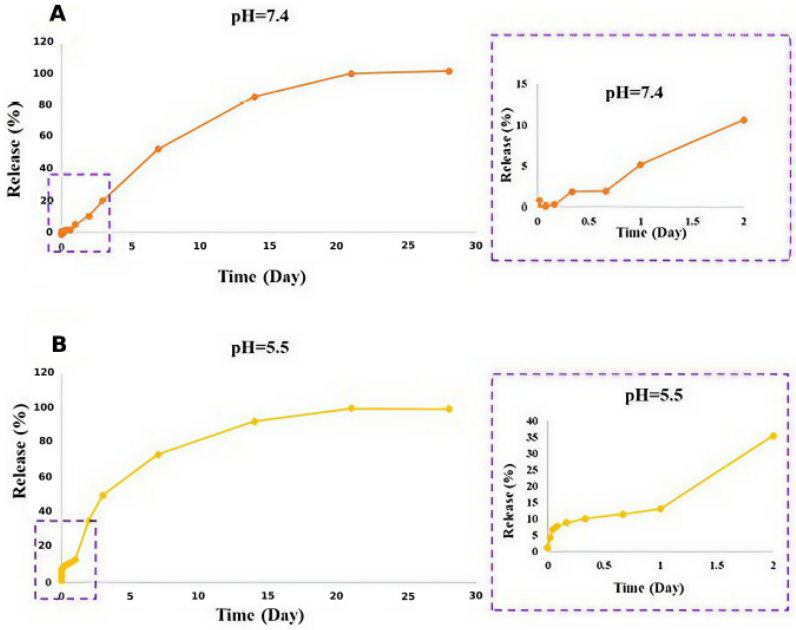


Fig. 6. Results of Cur release from PCL-CSN nanoparticles (A) at pH 7.4 and (B) pH 5.5

### *Release profile*

The obtained results indicate that the curcumin loaded in PCL-CSN nanoparticles and encapsulation effiency were 1.5% and 38%, respectively. Also, the release profile of Cur from PCL-CSN nanoparticles is shown in Fig. 6 at pH 7.4 and 5.5. The profiles indicate a sustained release manner for both environments. In an acidic medium, the Cur release was performed more rapidly than the normal pH at the same time duration of the experiment. On the second day, drug release in an acidic environment reached more than 30%, while the normal pH drug release rate was less than 15%.

### *Hemolysis*

The hemolysis results indicated that all nanoparticles had a higher hemolysis index (p <0.0001). Also, it was found that the higher the concentration of nanoparticles, the higher the toxicity (Fig. 7).

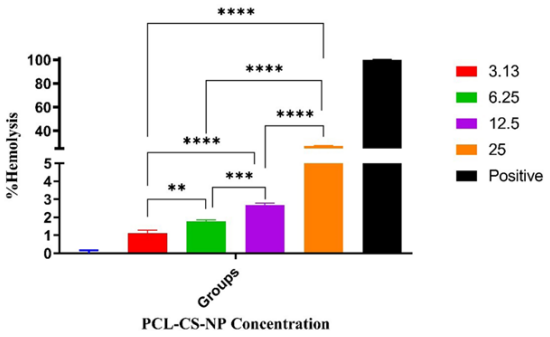


Fig. 7. Results of PCL-CSN nanoparticle hemolysis evaluation. (**: P<0.01, ***:P<0.001, ****:P<0.0001)

### *MTT assay*

MTT assay was performed at 24- and 48-hr intervals for evaluation of PCL-CSN-Cur

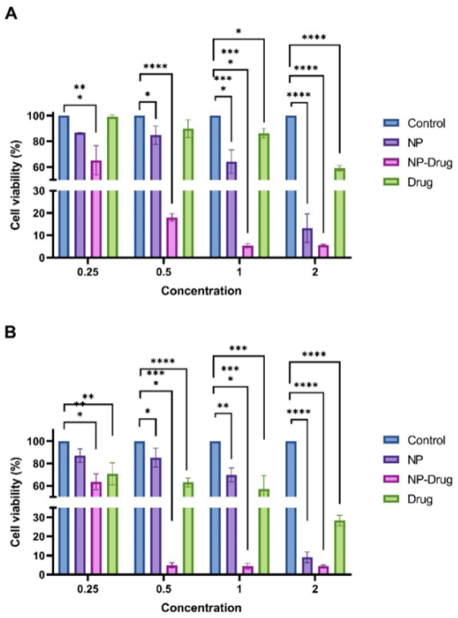


Fig. 8. Toxicity of PCL-CSN nanoparticles and PCL-CSN-Cur nanoparticles on MCF7 breast cancer cells at (A) 24 hr and (B) 48 hr. (*: P<0.05, **: P<0.01, ***: P<0.001, ****: P<0.0001)

nanoparticles effects on MCF7 cells. The results showed less survival in PCL-CSN-Cur nanoparticles than in free curcumin and PCL-CSN nanoparticles (Fig. 8). As the drug concentration increased, the survival rate of MCF7 cells treated with the PCL-CSN-Cur nanoparticle decreased more sharply than other groups. At 24 hr and a concentration of 0.25, pure nanoparticles and Cur had no significant effect on cell viability, while PCL-CSN-Cur nanoparticles significantly reduced cell viability compared to the control group ($P<0.001$).

At 48 hr, the results showed that the concentration of 0.25 of pure PCL-CSN nanoparticles did not affect cell viability ($P>0.05$), however, in other groups and concentrations, cell viability was significantly reduced.

**DISCUSSION**

A conventional nanoparticle fabrication method is based on adding polymer solution to a large amount of non-solvent, e.g. water, leading to a moderate and uncontrolled mixing. Microfluidics provides a relatively uniform and continuous reaction medium with a more controllable process, turning it into a promising technology [24]. By using a microfluidic chip, it is possible to synthesize more monodispersed nanoparticles and better control their properties, including diameter, surface properties, and drug loading[3]. In this study, a Y-shaped microfluidic chip was used to synthesize PCL-CSN nanoparticles to deliver curcumin to MCF7 breast cancer cells. After evaluating the properties of the produced nanoparticles, such as diameter, PDI, stability, shape, structure, loading, and drug release, we examined the blood toxicity of nanoparticles and cancer cell survival.

Using the microfluidic system was to produce uniform particles sharing the same characteristics, high reproducibility, and cost-effectiveness. It has been reported that the Y-shaped microfluidic system provides smaller nanoparticles than the T-shaped obtained [25, 26]. In the present study the PCL-CSN nanopartilces with and without Cur with sizes of 209 nm and 219 nm, respectively, were produced by Y-shaped microfluidic systems. In a study, Rachmawati et al. used solvent evaporation to fabricate curcumin-loaded polymeric nanoparticles and reported a diameter of 290 nm with the same curcumin ratio as what we used [27]. The surface charges of pure and Cur-loaded nanoparticles were + 15 and + 3 mV, respectively. These positive charges indicate that chitosan coated the surface of the nanoparticles. In a study by Jesus et al., the fabricated PCL-CSN nanoparticles were loaded with a hepatitis B vaccine. They have reported the positive charge of PCL-CSN while it was neutral and negative to some extent for PCL nanoparticles [28]. Generally, positively charged nanoparticles are expected to represent more cytotoxicity due to their interference with the cell membrane. However, Wang et al. stated that the toxicity effect is insignificant in some nanoparticles, especially those coated with CSN [29].

We also investigated the stability of nanoparticles for three months and the results confirmed their stability in terms of size and PDI, which is in excellent agreement with Prado et al. results [30]. However, the results indicate a little decrease in nanoparticle size, which is attributed to the biodegradation process. A previous study showed that the size of polymeric nanoparticles tended to decrease due to the degradation. That was associated with the high surface area exposed to the environment [31].

Nanoparticles have elevated hopes to develop a new approach to cancer drug delivery operating as a carrier to target cancer cells [1, 2]. Different ways have been used to encapsulate drugs into nanoparticles. Studies conducted by Chen et al. showed that the loading percent of Cur in polymeric nanocarriers was dependent on compounds, molecular weights, and fabrication methods [32]. In the present study, curcumin was loaded on PCL/CSN nanoparticles (approximately 1.5%) during nanoparticle synthesis by microfluidic systems.

As mentioned, the design of pH-sensitive nanoparticles is one of the strategies for targeting tumors. The utilization of pH-sensitive nanoparticles seemed to increase the accumulation of nanoparticles in tumor tissues compared to conventional nanoparticles, which can have more therapeutic effects and, at the same time low side effects. Here, PCL-CNS polymers were used to fabricate nanoparticles and the release profiles of Cur at two different pHs 7.4 and 5.5, were investigated. At both pHs, the release profile of Cur from PCL-CSN nanoparticles represented two distinct stages, the first as the burst phase followed by a sustained phase. It seems that, rapid release in the early hours was due to the presence and adsorption of Cur on the surface of nanoparticles [33]. Furthermore,

it has been shown a rapid release in low pH (5.5) than normal pH, which can be attributed to the pH sensitivity of the developed delivery systems essential for cancer drug delivery systems.

Various studies have shown that CNS could be used to fabricate pH-sensitive drug delivery systems. Popat et al. reported that chitosan formed a gel-like structure at pH 7.4, leading to the preventing of ibuprofen at the same time. The drug was released at a pH lower than 6.3 due to the protonation of the amino group on chitosan [34]. In another study, Omer et al. showed that chitosan increased drug release at low pH due to its pH-sensitive properties [35]. In another study, Javaid et al. showed that the release of Cefotaxime in PCL nanoparticles was higher at pH 7.4 than at pH 5.5 [36]. It does not seem that the designed system's pH sensitivity in our study is related to PCL. It can be concluded that the pH-sensitive property of the designed PCL-CNS delivery system in our study is associated with CNS presence.

In the present study, the effect of the Cur loaded in PCL-CNS nanoparticles on the killing and survival of tumor cell lines with specific concentrations of Cur and equivalent concentrations of pure nanoparticles and nanoparticles containing Cur at 24 and 48 hr was investigated using MTT assays. The results showed that the viability of cells treated with Cur-loaded nanoparticles at specific concentrations was lower than those exposed to the same concentration of free Cur and pure nanoparticles. It could be concluded that the inhibitory effect of Cur could be promoted by incorporating PCL-CSN nanoparticles compared to free Cur, which was in agreement with the results reported by Zeighamian et al. [37]. The present study also showed that pure nanoparticles were less toxic than free Cur, indicating low systemic toxicity of nanoparticles. With increasing concentration, the survival rate of cells treated with Cur nanoparticles decreased more sharply than other groups, indicating the positive effect of nanocarriers on the successful delivery of Cur.

## CONCLUSION

It can be concluded that microfluidic systems (Y-shaped) can pave the way for nanoparticle synthesis easily rapidly, and cost-effectively for cancer agent delivery. The results show that the constant phase flow has affected on the size of nanoparticles and we achieved a carrier that which releaases Cur more rapidly in an acidic environment than in a normal pH. Based on our observations, PCL-CSN-loaded Cur nanoparticles represent appropriate characteristics and suitable anti-cancer effects.

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## CONFLICT OF INTEREST

No conflict of interest.

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