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Targeting effect of folate on cancer cell through curcumin carrier nano-system

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Abstract

Folate receptor (FR) is well known for its overexpression on surface of various cancer cell lines, which is identical to normal tissue. Folic-based targeting drug delivery systems, therefore, are one of the most effective targeting carriers that effectively bind to FR up-regulated cancer cells. Curcumin was used both for labeling and chemotherapy. The materials were characterized and structurally confirmed by FT-IR spectra, fluorescent images and FE-SEM images. Bioassays were conducted on *HeLa* and *HT29* cancer cell lines after 4 and 12 hours. Results show that folic acid significantly enhanced both targeting efficiency and internalization of curcumin to FR-expressing cancer cells.

Keywords: Drug delivery systems, Nanoparticle, Targeting effect, Curcumin, OCMCs, Folic acid.

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Introduction

Cancer is one of the most lethal diseases. Vast amount of investigation has been attracted for feasible treatment. For chemotherapy, large numbers of agents such as cisplatin, doxorubicin, paclitaxel or combination of drugs have been widely investigated [1]. However, unexpected toxicity to normal organs and serious side effects to the patients still hinder their applications. Therefore, it is crucial to develop a therapeutic model with no or minimal side effects to normal organs.

Curcumin (Cur), 1,7-bis(4-hydroxy-3-medithoxyphenyl)-1,6-heptadiene-3,5-dione, isolated from a herb *Curcuma longa*, has been received considerable attention, for it proves remarkable non-toxic and promising anti-cancer activities [2-4]. Although previous hindrance due to its low aqueous solubility has been enhanced [5-7], Cur still remains an enormous gap to be a perfect moiety in term of chemotherapy. To eradicate this, collaboration with novel biodegradable polymer that offers significant enhancement in bioavailability, solubility and retention time [6, 7] for the drug has been developed.

O-carboxylmethyl chitosan (OCMCs) is a derivative of a natural product named chitosan of which hydroxyl groups on C_6 along the chain is substituted by carboxymethyl groups via an ether bond. OCMCs is advanced not only for non-toxic, biocompatible, biodegradable, amphiprotic and strong bioactive properties [8] but also for high aqueous solubility. More impressively, it has a very large loading capacity to anticancer drugs [8, 9], which are water insoluble. Therefore, OCMCs-based nanoparticles not only protect the bioactive substances but also facilitate the control release of the materials during chemotherapeutic process.

Nonetheless, protecting and releasing drugs are not the only requirements for a perfect drug delivery system. All these advances might fall into a fallacy if the drug were delivered to liver, kidney or normal tissue instead of desired tumor [10,11]. Here emerges one of the most crucial factors determining the effectiveness of the delivery nanoparticles so-called targeting capacity or selectivity. To actively and effectively target to the cancer cells, folic acid (Fol) was corporated with OCMCs drug carriers. Folic acid has received enormous consideration for biocompatibility or no immunogenicity, high stability and economic cost [12]. By mean of receptor-mediated endocytosis, it has been reported that folate conjugates mediate faster internalization kinetics than other approaches through cellular membrane [12]. The fact is impressive because unlike normal tissues a various cancer cell lines, namely ovary, brain, breast, kidney, myeloid and lung cancer, over expresses folate receptor (FR) on their outer membrane therefore have special affinity to Fol [12-14]. Moreover, according to literature [15], Fol released from OCMCs-Fol nanocarrier behaved as an antidote, relieving healthy tissues from neurotoxicity caused by drugs at high dose.

This study is aimed to develop OCMCs-Fol nanoparticles containing and a targeting factor (Fol) on the purpose of increasing the active targeting effect.

Materials and methods

Materials

Curcumin and OCMCs were obtained from Institute of Chemistry (VAST). Folic acid, N-Hydroxysuccinimide (NHS), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), ethanol, ammoniac solution were purchased form Sigma-Aldrich. Human cervical carcinoma (*HeLa*) and Human colon adenocarcinoma (*HT-29*) cell lines were obtained from Lab of Department of Biology (HUS). Solvents and chemicals for bioassays were purchased from Invitrogen.

Preparation of Curcumin encapsulated OCMCs nanoparticles (Cur-OCMCs NPs)

Polymer-encapsulated curcumin was prepared by nanoprecipitation technique.

Firstly, 40 mg of OCMCs was disovled in 40 ml of double distilled water and stirred for 1 hour. Then 40 ml of 1.5 mg/ml curcumin solution in ethanol was added into OCMCs solution, stirred or ultrasonically vibrated this mixture for hours until optained a clear dispersion. The organic solvent was removed by vaccum evaporated. To remove the large aggregate and free polymer, the suspension after evaporated was centrifugated at 5600 rpm in 10 minutes. The supernatant containing Cur-OCMCs nanoparticles then was ultracentrifugated at 30000 rpm to optain Cur-OCMCs nanoparticles.

Preparation of Folate grafted Curcumin encapsulated OCMCs nanoparticles (Fol-Cur-OCMCs NPs)

To synthesize folate grapted OCMCs, folate was attached to the surface amino groups of OCMCs via a carbodiimide reaction [15, 16]. Briefly, 20 mg of folic acid was dissolved in 20 ml of distilled water at pH 8 and then was activated by adding 5.5 mg of NHS and 8.5 mg of EDC and stirred for 24 hours in dark. On the other hand, 40 mg of OCMCs and 8.5 mg of EDC were dissolved in 40 ml of distilled water and stirred for 1 hour. pH of OCMCs solution was adjusted to 8 by adding 2M NH_3 solution. The activated folic acid was added slowly to the OCMCs solution and stirred in dark for 24 hours. The resulting solution was filtered to remove unsoluble substance and pH was changed to 7 by adding 2M HCl solution. The solution was dialyzed against distilled water for 24 hours. The obtained yellow solid was redissolved into 40 ml of distilled water.

The Fol-Cur-OCMCs nanoparticles was optained by the process in the same with that of Cur-OCMCs NPs by replacing OCMCs with Fol-OCMCs.

Characterization methods

Molecular structure of synthesized copolymer was characterized by Fourier transform infrared spectroscopy (FTIR, SHIMADZU spectrophotometer) using KBr pellets in the wave number region of 400–4000 cm^{-1} and the Fluorescence spectra was recorded by using a Jobin-Yvon FL3-22 and taken with a 442 nm excitation line. Surface morphology of Cur-OCMCs and Fol-Cur-OCMCs nanoparticles were observed by a microscopy (FE-SEM) on a Hitachi S-4800 system.

Cellular uptake experiments

To investigate targeting efficiency of folic acid, two cancer cell lines *HeLa* and *HT29* were chosen. $2x10^5$ cells were seeded on a coverslip placed in 24-well plate and cultured for 24 hours. After that, the cells were treated with Cur-OCMCs or Fol-Cur-OCMCs at the concentration 10 $\mu g/ml^{-1}$ and incubated for different periods of time and then fixed with 4% PFA (Sigma). After icubating, the cells got fluorescent staining to label actins with Rhodamine-phalloidin and nuclei with Hoechhst (Invitrogen), then the presence of Curcumin inside *HeLa* and *HT29* cells were indicated with fluorescent images taken by LSM 510 microsope (Carl Zeiss). The green signal is due to the auto-fluorescence of Curcumin excited by Argon laser (488 nm).

Results and Discussion

Chemical structure of Cur-OCMCs NPs and Fol-Cur-OCMCs NPs

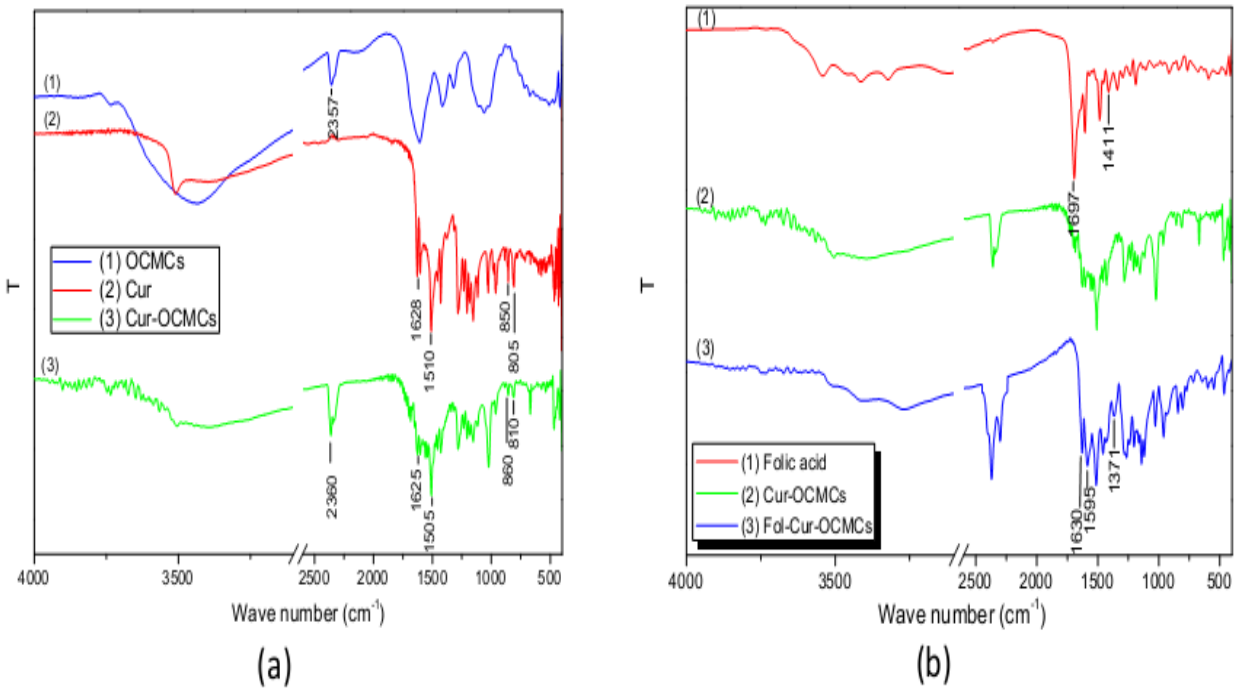
Chemical structures of Cur-OCMCs NPs and Fol-Cur-OCMCs NPs were investigated by FT-IR spectroscopy.

The characteristic peaks at 1628 cm^{-1} (C=O stretching), 1510 cm^{-1} (C=C olefienic stretching) and 850 cm^{-1}, 805 cm^{-1} (C=C-H aromatic stretching) [17] in the FT-IR spectra of Cur were shifted to 1625 cm^{-1}, 1505 cm^{-1} and 860 cm^{-1}, 810 cm^{-1} in the FT-IR spectra of Cur-OCMCs, respectively. Meanwhile the characteristic peak of OCMCs at 2357 cm^{-1} [18] also appeared at 2360 cm^{-1} in the FT-IR spectra of Cur-OCMCs. These changes were due to the success in encapsulating Cur by OCMCs (See Supporting information 1a [25]).

The characteristic peaks of folic acid at 1697 cm^{-1} (corresponds to the C=O stretching carboxyl group) and 1411 cm^{-1} (attributed to the OH deformation of phenyl skeleton) [19] were shifted to 1630 cm^{-1} and 1371 cm^{-1}, respectively. Especially, the appearance of peak at 1595 cm^{-1} were demonstrated the formation of amide bond (-CONH-) between amine group of OCMCs and carboxyl group of folic acid [20] (See Supporting information 1b [25]).

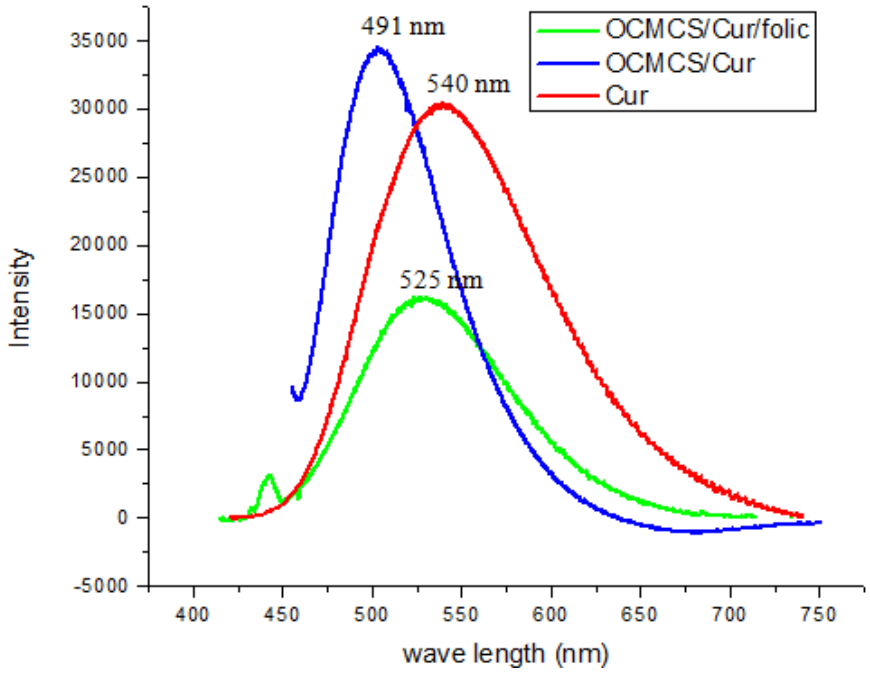
In the fluorescence spectra of Cur, Cur-OCMCs and Fol-Cur-OCMCs, curcumin in ethanolic solution exhibited an absorption peak at 540 nm, while the solutions of Cur-OCMCs and Fol-Cur-OCMCs had peaks at 491 and 525 nm, respectively. The blue-shifts in the fluorescence were likely due to the intermolecular hydrogen bonding between curcumin and polymers (See Supporting information 2 [25]).

Supporting information 1



FT-IR spectra of Cur-OCMCs and Fol-Cur-OCMCs.

Supporting information 2



Fluorescence spectra of Cur, Cur-OCMCs and Cur-OCMCs-Fol.

Morphology and size of Cur-OCMCs NPs and Fol-Cur-OCMCs NPs

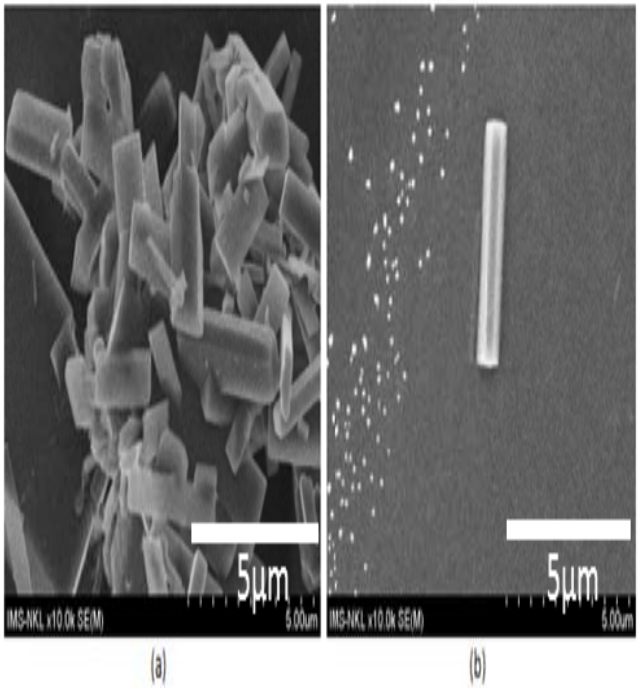


Figure 1. FE-SEM images of crystal curcumin (a) and Cur-OCMCs (b)

Figure 1a shows the crystal curcumin. It is clear from Figure 1b that, thanks to the stearic stability benefited from the polymers, solubility of curcumin nanoparticles was significant increased, compared to that of untreated curcumin.

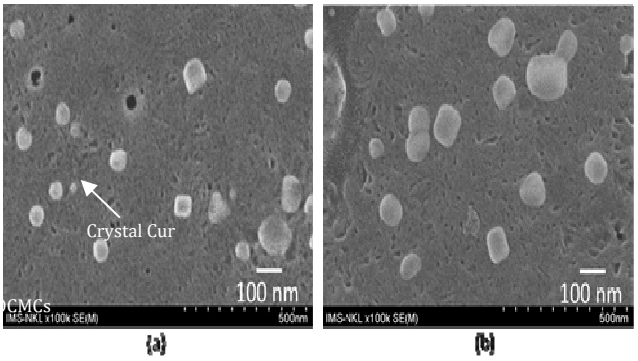


Figure 2. FE-SEM images of Cur-OCMCs (a) and Fol-Cur-OCMCs (b)

FE-SEM images in Figure 2 show the size and morphology of the curcumin encapsulated by OCMCs (a) and OCMCs-Fol (b). It is obvious that the particles have an average size of 50-100nm, which lies in the optimal size range (below 200 nm) for drug delivery applications.

In the free-surfactant environment, crystal will grow to become large particle which is in low energy state. In the cases of Cur-OCMCs and Fol-Cur-OCMCs, OCMCs and folate grafted OCMCs act as the surfactants which help to form Curcumin nanoparticles. Curcumin with the surface encapsulated by hydrophilic OCMCs polymer and the very small size will not only be able to well-disperse in aqueous environment but also increase its biocompatible and therefore promise the increase in therapeutic efficacy.

Cellular uptake of Cur-OCMCs NPs and Fol-Cur-OCMCs NPs

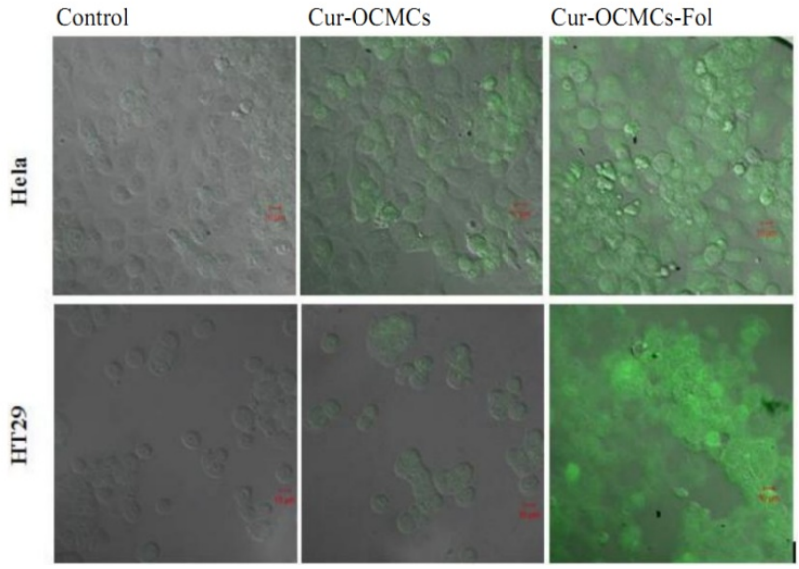


Figure 3. Fluorescent image of *HeLa* and *HT29* after 4h incubating with Cur-OCMCs and Fol-Cur-OCMCs.

After 4h of incubating with Fol-Cur-OCMCs, fluorescence intensity in *HeLa* and *HT29* is higher than that of cells incubated with Cur-OCMCs. Importantly, Fol-Cur-OCMCs nanoparticles were distributed in nuclei, indicate that the folate-conjungated nanoparticles were uptaken by the endocytosis mediated folate receptor on cell memberanes. This observation clearly infers that folate-conjugated OCMCs would be better effective carriers for targeting anticancer drugs.

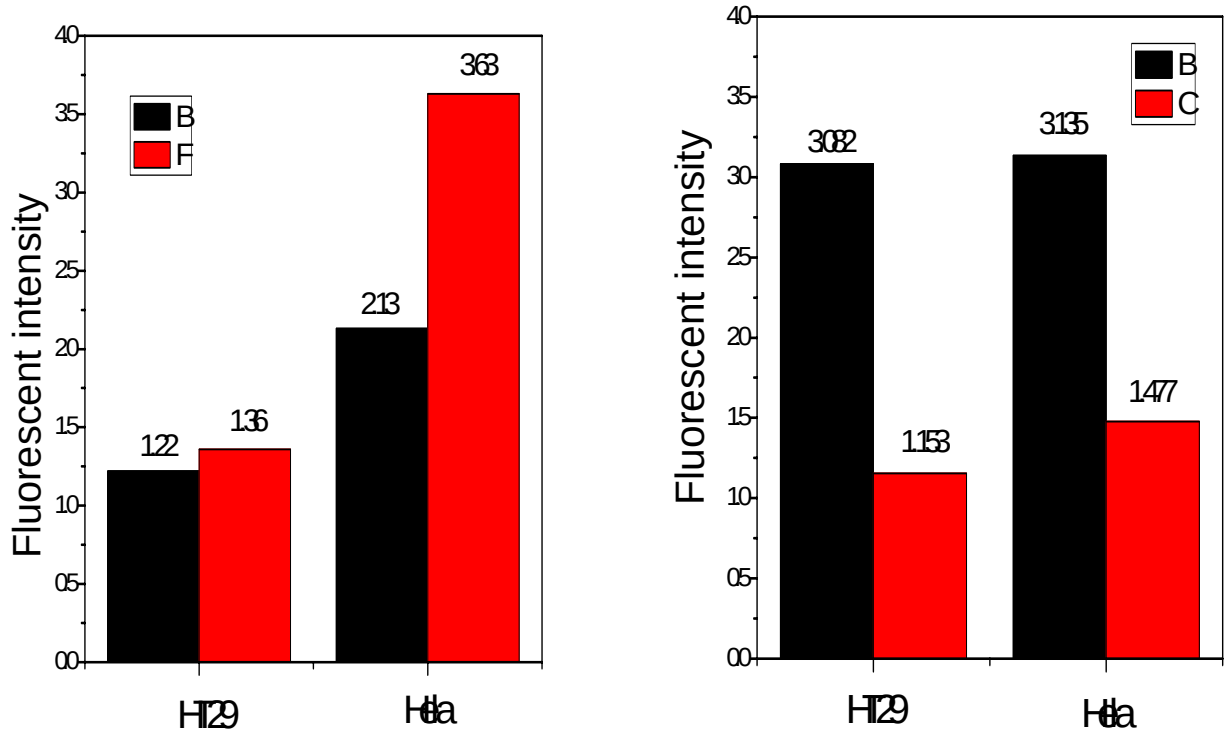
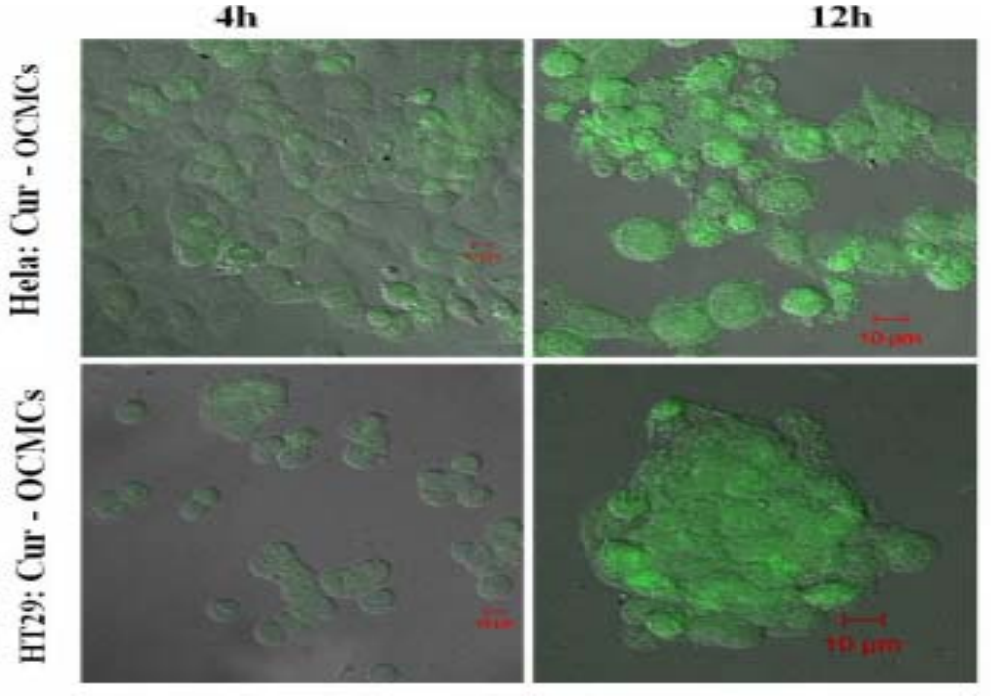


Figure 4. Comparison of cellular uptake between Cur-OCMCS (left) and Fol-Cur-OCMCs (right) on *HT29* and *HeLa* cell lines at 4 and 12 hours

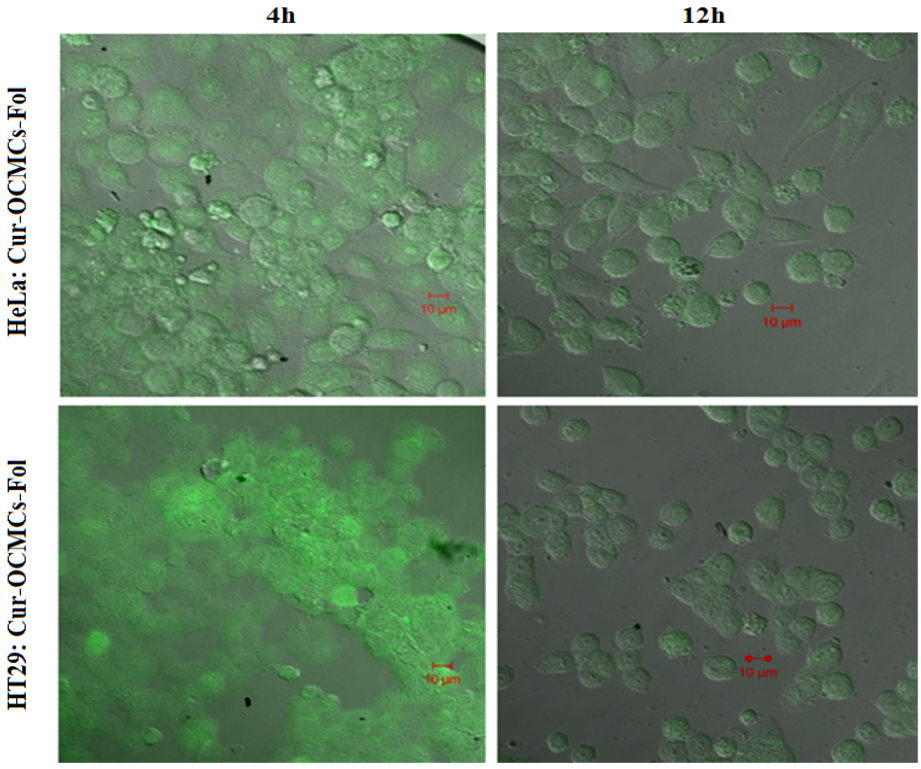
The expression of folate receptor in different cell lines are different, so that the internalization of folic acid depends on the cell lines. We observed that proportion of fluorescence intensity of Fol-Cur-OCMCs and Cur-OCMCs is 2.53 for *HT29* and 1.47 for *HeLa*. Because of the overexpression of
FR on *HT29*s cell surfaces, there are more folate-conjugated OCMCs uptaken by *HT29* than by *HeLa*, therefore *HT29* have stronger fluorescent intensity than *HeLa.*
The fluorescence intensity after 12 hours of incubation was higher than that of 4 hours for Cur-OCMCs (See Supporting information 3 [25]), while that intensity decreased from 4 hours to 12 hours for Fol-Cur-OCMCs (See Supporting information 4 [25]). It could be explained that folic acid inducts the internalization of Cur, so curcumin is internalizied into cancer cells more quickly and efficiently. When curcumin accumulates to a certain concentration, there are chemical reactions that produces by-product such as trans-6-(4-hydroxy-3-methoxyphenyl)-2,4-dioxo-5-hexenal,
vaniline, ferulic acid or feruloyl methane which do not auto-fluoresce [21,22].Eventually, fluorescent signal in cancer cells incubated with Fol-Cur-OCMCs after 12h is weaker than that with Cur-OCMCs.
Moreover, red signal of actins in cells after 12h incubated with Fol-Cur-OCMCs demonstrates that actins localization changes (Figure 5). It no longer formed the sharp and clear circle around the cell since actins was transformed from G structure into F structure with smaller molleculars [23,24].

Supporting information 3



Cur-OCMCs at 12h shows the higher fluorescent intensity than that at 4h.

Supporting information 4



Cur-OCMCs-Fol at 12h shows the lower fluorescent intensity than that at 4h.

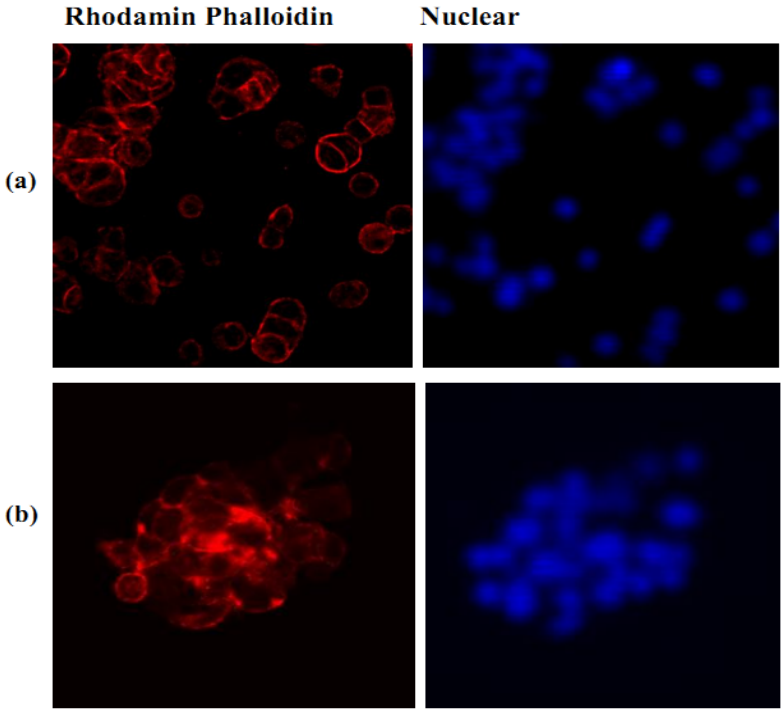


Figure 5. Fluorescent Image of *HT29* in control group (a) and after 12h incubating Fol-Cur-OCMCs (b).

Conclusion

The Cur-OCMCs and Fol-Cur-OCMCs nanosystems were successfully prepared. With an average size from 50 to 100nm, they are also believed to be suitable for drug delivery applications. The systems have been proved that successfully target curcumin to *HT29* and *Hela* cells. Most importantly, it was determined that folic acid substantially increases the internalization of the nanosystem into the cancer cells. The efficiency internalization of Fol-Cur-OCMCs system depends on level of FR expressions on the cell surface: the stronger affinity to folic acid, the more efficient the treatment was. In addition, folic acid facilitates impacts of nanosystem on cell's growth and structure after internalization. With all these good features of Fol-Cur-OCMCs, it is promising to be a new smart nano-material for drug delivery.

Acknowledgements

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