Int. J. Bio-Inorg. Hybr. Nanomater., 7(4): 297-303, Winter 2018

Investigation of cytotoxicity properties of zinc oxide nanoparticles in spherical and rod shaped on leukemia cells

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Received: 22 July 2018; Accepted: 25 September 2018

ABSTRACT: In this study, we reported a method to associate doxorubicin drug on folic acid functionalized SiO_2/ZnO nanoparticles (NPs) in rod and spherical shapes. The clinical usage of the anticancer drug, doxorubicin (DOX), is limited by severe side effects and cell resistance. Targeted drug delivery by binding DOX to nanoparticles could overcome these limitations. The surface functionalization of the ZnO nanoparticles with silica and folic acid (FA) was confirmed by fourier transform infra-red spectra (FTIR) and Ultraviolet-visible spectroscopy. Drug is bound to the nanoparticle surface through electrostatic interactions. The potential of as-prepared anticancer drug loaded nanoparticles against the leukemia cancer cells, K562, was evaluated using the MTT assay and its anti tumor efficacy was clearly enhanced compared with free drug. Cell viability reached nearly 21% and 25% by drug loaded nanoparticles, whereas DOX itself led to only 40% viability at a concentration of 5 mg/ml. Moreover, the results showed that the rod- shaped particles have better cytotoxicity against leukemia cells than spherical particles because of their samller sizes.

Keywords: *Drug delivery; Doxorubicin; Folic acid; In-vitro cytotoxicity; Leukemia cells; ZnO nanoparticles.*

INTRODUCTION

Nanoparticles introduce a new platform that provide a wide range of novel applications and improved technologies for biomedical usages. These small size particles are comparable to naturally biomolecules in the cells and are notably smaller than the typical diameters of many human cells (McNeil, 2005). Nanoparticles typically possess a higher number of atoms at their surface which lead to improved surface reactivity (Nel, *et al.*, 2006) and increase their ability to load therapeutic agents for targeted drug delivery application (Rasmussen, *et al.*, 2010). The anthracycline doxorubicin (DOX) is a highly efficient antineoplastic agent commonly used in the treatment of a variety of cancers like leukemia, ovarian cancer, and especially late-stage breast cancer. The clinical usage of DOX is limited by the resistance arised from cancer cells and also by severe side effects, namely, a dose-dependent and cumulative cardiotoxicity (Leonard, *et al.*, 2009). Drug targeting helps to prevent side effects and to increase cytotoxicity of anticancer drugs by delivering the drug directly to the pathological site; thus, leading to increased drug concentration at the tumor site (Munnier, *et al.*, 2011). Various targeting ligands against tumor-cell-specific receptors have been immobilized

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on the surface of nano-particulate carriers to deliver them within cells via receptor-mediated endocytosis. Among them, folic acid (folate) vitamin has been widely employed as a targeting moiety for various anti-cancer drugs (Reddy and Low, 2000; Horowitz, *et al.*, 1999; Guo, *et al.*, 2000; Lu and Low, 2002; Lee and Low, 1995). Folate binding protein, a glycosylphosphatidylinositol (GPI) anchored cell surface receptor for folate, has been known to be overexpressed in several human tumors including ovarian and breast cancers, while it is highly restricted in normal tissues (Cummings and McArdle, 1986). More recently, we developed folate-targeted biodegradable nanoparticle system for conjugation of doxorubicin. ZnO NPs were synthesized by hydrothermal method and coated by silica for high biocompatibility (Zare, *et al.*, 2013). The potential of DOX against the resistant leukemia cancer cells, K562, was evaluated using the MTT assay. The DOX loaded FA/SiO_2@ZnO NPs present interesting properties that are favorable for use in drug targeting incompared to conventional DOX. This gain in antineoplastic activity could be due to the vectorization of the drug. Indeed, when DOX is brought to the cell by a vector, this may change its route of internalization and zones of accumulation in the cell. It was reported that DOX can change its intracellular distribution when it is bound to an entity, for example as conjugated to polymers (Hovorka, *et al.*, 2002) or cell penetrating peptides (Aroui, *et al.*, 2009). These distribution changes are most of the time correlated to differences in cytotoxicity between conventional and vectorized DOX.

MATERIALS and METHODS

Tri ethanol amine (TEA), Cetyl trimethylammonium bromide (CTAB), $ZnCl_2$, KOH, absolute ethanol, Dimethyl formamide (DMF), Dimethyl sulfoxide (DMSO) Tetraethylorthosilicate (TEOS), Aminopropyltri-methoxysilane (APTMS), Dicyclohexylcarbodiimide (DCC) and folic acid were received from Merck chemical company and used without further purification. Deionized and distilled water was used for the preparation of solutions. Doxorubicin hydrochloride (DOX) was used for the drug loading and it was purchased from farmacia Italia Company.

Synthesis of nano ZnO in rod and spherical shapes

In a typical synthesis for rod nanoparticles, $ZnCl_2$ and KOH in 1:2 molar ratios were dissolved in distilled water. After stirring at room temperature for a few minutes, appropriate amount of CTAB, as a surfactant, was added. The pH was maintained at about 8-9 during the reaction by adding KOH. The mixture was transferred into a Teflon-lined stainless steel autoclave and hydrothermal treatment was carried out at 120°C for 5 h. Then, the autoclave was allowed to cool down naturally. White precipitate was collected and washed with distilled water and ethanol several times to remove impurities. Finally, the precipitate was dried at 50°C for 5 h in an oven. For spherical ZnO nanoparticles, this procedure was repeated by different surfactant tri-ethanol amine (TEA) and other steps were exactly the same.

Synthesis of SiO_2@ZnO core-shell NPs

In a typical procedure for the preparation of SiO_2@ ZnO NPs, 30 mL of absolute alcohol, 2.5 mL of deionized water, 1.5 mL of aqueous ammonia, 1200 µL of TEOS, and 600 µL of APTMS were purred into a conical flask; this solution was stirred for 5 min at room temperature. After that, ZnO nanoparticles (0.11 g) were added to the solution under stirring. The mixture was continuously stirred for 3 h at the same conditions. The resulting ternary nanocomposite was then isolated by centrifugation and washed with ethanol and deionized water several times.

Synthesis of folic acid/SiO_2@ZnO conjugates

The assembling of folic acid to silica coated ZnO nanoparticles were carried out by adding 0.12 g of folic acid and 0.06 g of dicyclohexylcarbodiimide (DCC) into 30 ml DMF under ultrasonic irradiation. The sonicating was continued for 1 hour. The obtained solution and 0.06 g of nanoparticles was stirred at room temperature for about 15 hours. Finally, the resultant powder (FA/SiO_2@ZnO conjugate) was washed with deionized water and dried during 2 days in the labrotary at room tempreture.

Preparation of Doxorubicin (DOX) loaded ZnO NPs

The water-soluble anti-cancer drug doxorubicin was chosen as a model drug. The DOX loading was carried out by dispersing 0.06 g of FA/SiO_2@ZnO conju-

gate and DOX (3 mg) in 30 ml DMF. The mixture was stirred for 24 hours to facilitate DOX uptake. The optical density of residual DOX in the supernatant was measured at 480 nm by UV–Vis spectrophotometer. The drug loaded nanoparticles were then separated, washed several times with distilled water and dried at room temperature.

Cytotoxicity assays (MTT assay)

Prior to the experiment, the leukemia cancer cells, K562, were seeded in standard 96-well plates at 2×10^4 cells per well and grown for 24 h. The culture medium was then discarded and cells were treated for 24 and 48 hours with 0.0, 0.65, 1.25, 2.5, and 5 mg/mL of nanopaticles containing doxorubicin drug. The effect of DOX and DOX-loaded nanoparticles on the cell viability were assessed using a tetrazolium dye, (3-(4, 5-dimethy-lthiazol-2-yl)-2,5-diphenyltetrazolium bromide, named MTT assay. The medium containing DOX was discarded and cells were rinsed thrice with phosphate buffered saline (PBS). They were incubated during 4 h with 0.5 mg/mL MTT solution in culture medium. Then, the medium was replaced by 200 µL DMSO to dissolve the formazan crystals formed by viable cells.The cell viability percentage (%), compared to vehicle-treated control cells which arbitrary assigned 100% viability, was determined by measuring absorbance at 480 nm.

RESULTS and dISCUTION

Field emission scanning electron microscopy (FE-SEM)

Fig. 1 represents FESEM images of the prepared ZnO and silica coated nanoparticles. In these images (a, b) well-distributed nanorod aggregates of ZnO and silica coated nanocrystals with a diameters in the range of 50-200 nm can be observed. These particles were prepared by CTAB as a surfactant. Spherical shaped nanoparticles with diameters in the range of 38-50 nm are seen for ZnO nanoparticles synthesized by TEA as a surfactant. Elemental analysis using energy dispersive X-ray analysis (EDX) of the samples are shown in Table 1 giving the elemental and atomic weight percentage of ZnO and SiO_2 coated nanoparticles.

XRD charactrization

Fig. 2 shows powder X-ray diffraction patterns of uncoated and SiO_2 coated nanoparticles. The diffraction peaks in both patterns can be indexed to hexagonal wurtzite structure of ZnO (JCPD36-1451). The

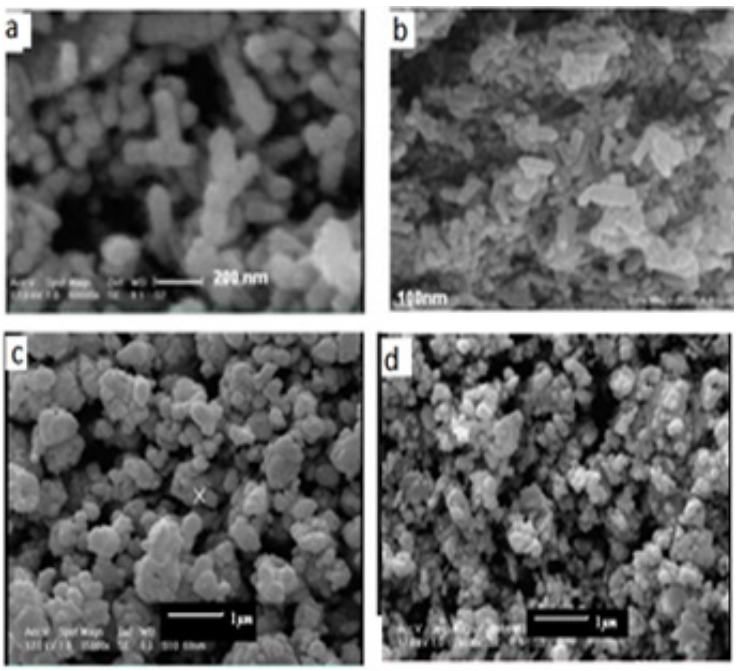


Fig. 1. FESEM images of (a) and (b): ZnO; (c) and (d): SiO_2@ZnO

Table 1. EDX analysis results

Sample	Element	Wt%	At%
ZnO rod	Zn	89.56	67.86
	O	10.44	32.14
SiO_2@ZnO rod	Zn	41.12	18.83
	Si	36.05	38.44
	O	22.83	42.73
ZnO spherical	Zn	89.36	67.56
	O	10.64	32.44
SiO_2@ZnO spherical	Zn	42.73	19.88
	Si	38.12	37.05
	O	19.15	43.07

broad peak around 2θ= 23° in the coated samples (rod and spherical) shows typically the presence of amorphous silica in the samples. The mean crystallite sizes, calculated using Scherrer's formula, are 38 and 30 nm for rod and spherical NPs and 21 and 29 nm for silica coated NPs, respectively (Bhatti, *et al.*, 2005). The sharp peaks in the patterns exhibit high degree of crystallinity and large crystalline domains. There are no unexpected diffraction peaks that demonstrate the absence of other impurities.

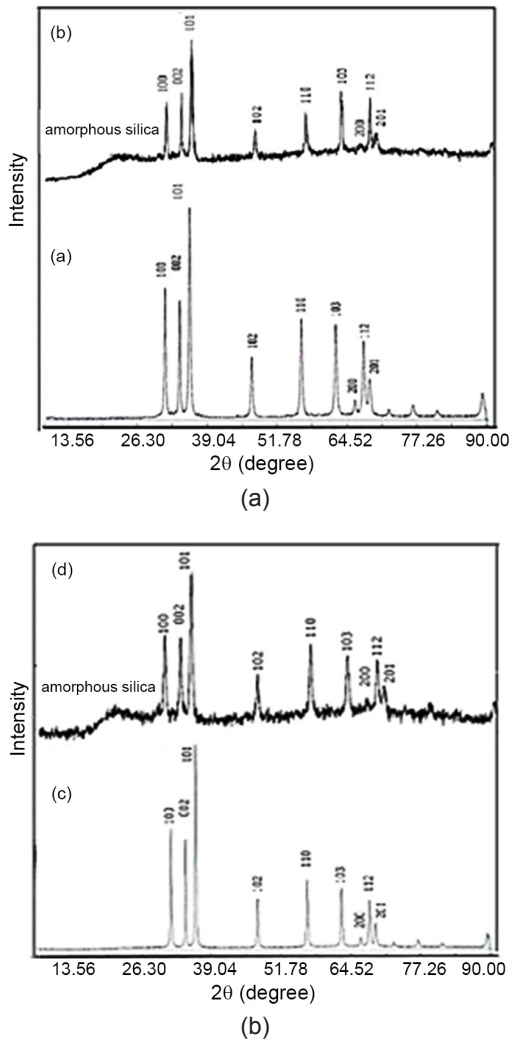


Fig. 2. a. ZnO, b. SiO_2@ZnO rod / c. ZnO, d. SiO_2@ZnO spherical.

Fourier transmission infrared spectroscopy (FTIR)

Fig. 3 shows bonding between carboxyl group of FA (folic acid) and amino group (NH_2 groups of APTMS) on the surface of nanopaticles by respective peaks lookated at 1680-1700 cm^{-1} (C=O stretching of amide bond) and 1570-1630 cm^{-1} (N-H stretching of amid bond). The characteristic peaks of folic acid at 1483, 1605 and 1696 cm^{-1} in Fig. 3 c also exist in the FTIR spectrum of the FA conjugated NPs (Fig. 3a and 3b). FTIR spectra of pure DOX and DOX loaded silica coated nanoparticles are presented in Fig. (4a-c). The spectrum of pure DOX shows peaks at 3382 cm^{-1} due to N–H and O-H stretching vibrations for the primary amine structure. The bands observed at 891 cm^{-1} and 782 cm^{-1} due to N–H wagging in pure DOX appear in the FTIR spectrum of DOX-conjugated nanoparticles, too. From this FTIR results, it can be interpreted that attachment of DOX to the FA/SiO_2/ZnO NPs occurs by

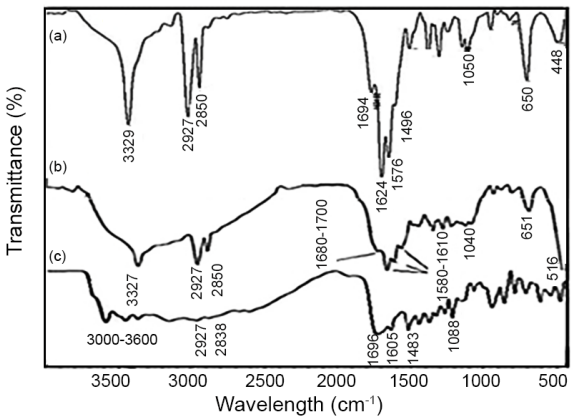
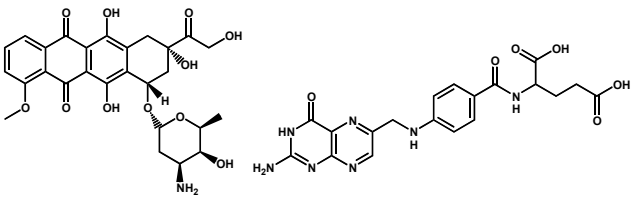


Fig. 3. FTIR spectra of (a) FA/SiO_2@ZnO spherical (b) FA/SiO_2@ZnO rod (b) pure FA.



Scheme 1. a) Doxorubicin chemical structure; b) folic acid structure.

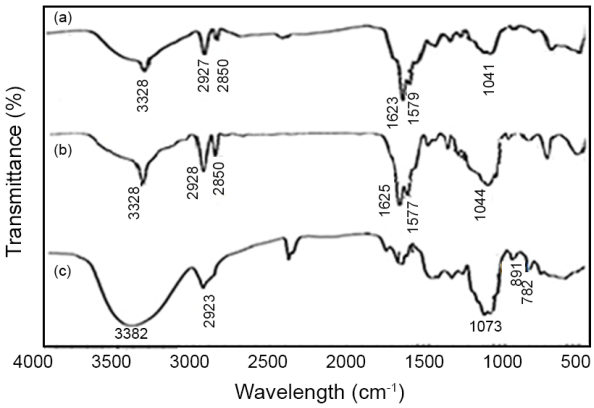


Fig. 4. FTIR spectra of (a) DOX loded FA/SiO_2/ZnO rod; (b) DOX loded FA/SiO_2/ZnO spherical; (c) pure DOX.

electrostatic intractions because N-H and O-H bonding in about 3300-500 cm^{-1} are not changed (Zhang and Zhang, 2005; Moussodia, *et al.*, 2010; Muhummadh and Kaiser, 2008; Huang, 2011).The structures of doxorubicin and folic acid are shown in scheme 1.

Ultraviolet/visible study

The prepared conjugated nanoparticles were first dispersed in distilled water by ultrasonication and then the Ultraviolet-visible (UV-Vis) optical absorption characteristics of the specimens were measured. The

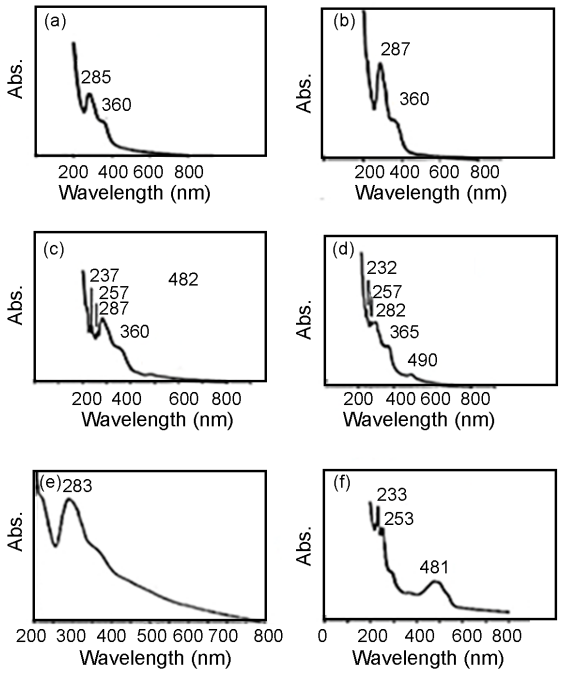


Fig. 5. UV-Vis spectra of (a) FA/SiO_2@ZnO rod; (b) FA/SiO_2@ZnO spherical; (c) DOX loaded FA/SiO_2@ZnO rod; (d) DOX loaded FA/SiO_2@ZnO spherical; (e) pure FA; (f) pure DOX.

peaks lookated at 285 and 287 nm in Fig. 5 a and b are due to the assembling of folic acid on NPs surface by the formation of amide bonding between them. On the other hand, pure folic acid has an absorbtion peak at 283 nm. After the loading of DOX on NPs, the UV-Vis peaks were observed around 237 and 482 nm (232 and 490 nm for spherical ZnO) attributing to the loaded DOX molecules (Fig. 5c and 5d), which were slightly shifted from the corresponding absorption peaks of free DOX molecules at 233, 253 and 481 nm, respectively. The slight shifts of the spectra may be originated from the interaction of the loaded DOX drugs and the conjugated components. Fig. 5e and 5f represent the UV-Vis spectra of pure FA and DOX, respectively. All these results demonstrated that DOX molecules were successfully loaded onto NPs via the efficient interaction between the drug molecules and drug-carriers.

Cytotoxicity study of DOX-loaded NPs on leukemia cells

We studied the cytotoxicity of our DOX-FA/SiO_2@ZnO NPs and pure DOX suspensions at different drug concentrations. The results presented in Fig. 6 show that, within 24 h, the DOX- FA/SiO_2@ZnO NPs (both rod and spherical) suspension was significantly more active against K562 cells than pure DOX solution, with enhanced cell mortality compared to conventional drug. In addition, cell viability reached nearly 21 and 25% by drug loded nanoparticles, whereas DOX in solution led to only 40% viability at the concentration of 5 mg/ml. On the other hand, rod shaped particles have better cytotoxicity against leukemia cells

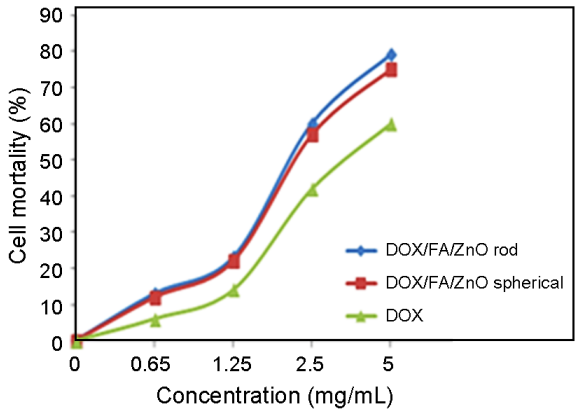


Fig. 6. Compare effect of DOX- FA/SiO_2@ZnO Nps and DOX at different concentration for 24 h on k562 cells.

Table 2. Cytotoxicity at different concentrations for 24h

Concentration (mg/ml)	Cell mortality (%)		
	DOX- FA/SiO_2 @ZnO rod	DOX- FA/SiO_2 @ZnO spherical	DOX
0	0	0	0
0.65	13	12	6
1.25	23	22	14
2.5	60	57	42
5	79	75	60

than spherical particles. These data indicate that the intracellular action of internalized DOX-FA/SiO_2@ ZnO NPs plays an important role in the gain of cytotoxicity. Table 2 shows the results of dose-dependence cytotoxicity of DOX and DOX-FA/SiO_2@ZnO NPs against leukemia cancer cells, K562 line, within 24 h. The NPs were deposited at time duration greater than 24 hours so the experiments were carried out during this time.

The morphology and behavior of DOX-FA/SiO_2@ ZnO NPs against leukemia cancer cells, K562 cultured in vitro was observed under phase-contrast microscope and evaluated by MTT assays. The phase-contrast micrographs represented in Fig. 7 a and b show cell attachments on the DOX-FA /SiO_2@ZnO NPs after culture for 24 h at a dose of 5 mg/ml. After the treatment with nanocarriers, the cell morphology was changed spherical shape from the primary spindle shape, the intercellular connections was disappeared and the cell density was reduced.

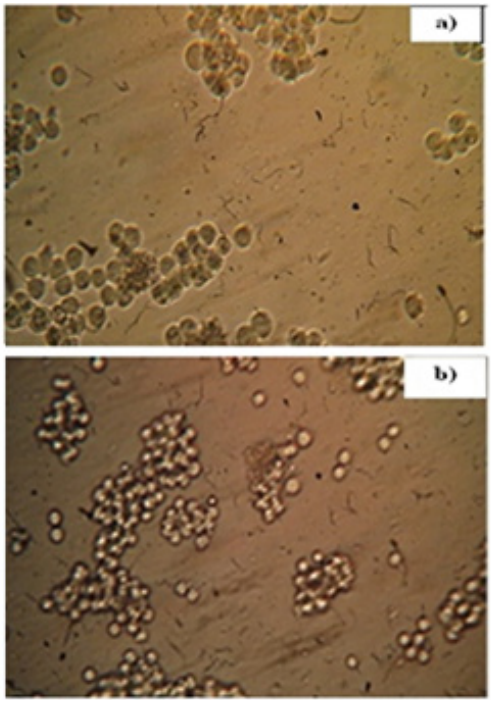


Fig. 7. Phase contrast micrographs of K562 cells, (a) before and (b) after incubation with DOX loaded NPs for 24h at concentration 5 mg/ml.

CONCLUSIONS

In this study, we explored the potential of ZnO nanoparticles along with anticancer drug, doxorubicin, for cancer therapy application. Nanocarriers consisting of ZnO nanoparticles in rod and spherical shapes were synthesized by hydrothermal method, and subsequently coated with SiO_2 (TEOS and APTMS) to reach high biocompatibility. Folic acid molecules were attached on nanoparticles by the amide bonding approach to gain more internalization into cancer cells and high efficiency of the nanocarriers. Characterization of the prepared nanocarriers was performed using FTIR and UV-Vis techniques. The attachment of the DOX molecules to the NPs via electrostatic interaction was confirmed by FTIR analysis. DOX loaded FA/SiO_2@ZnO NPs induced higher cell mortality than conventional drug. The cytotoxicity findings by MTT assay indicated that the intracellular action of internalized DOX-FA/SiO_2@ZnO NPs plays an important role to reach high level of cytotoxicity. Moreover, rod shaped nanoparticles have more cytotoxicity against leukemia cells than spherical that seems to be due to their smaller diameter. The results show that DOX loaded NPs are promising candidate for targeted drug delivery.

ACKNOWLEGEMENT

This work has been supported by east Tehran Branch of Islamic Azad University.

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