

Poly (ethylene-co-vinyl alcohol)-based polymeric thermo-responsive nanocarriers for controlled delivery of epirubicin to hepatocellular carcinoma

Farshid Hassanzadeh¹, Maryam Farzan², Jaleh Varshosaz³, Ghadam Ali Khodarahmi², Sahar Maaleki², and Mahboubeh Rostami^{1,*}

¹Department of Medicinal Chemistry and Isfahan Pharmaceutical Sciences Research Center, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, I.R. Iran.

²Department of Medicinal Chemistry, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, I.R. Iran.

³Department of Pharmaceutics, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, I.R. Iran.

Abstract

In this study, poly(ethylene-co-vinyl alcohol) (EVOH) as a novel biocompatible polymeric scaffold was surface modified by succinylation to get EVOHS and further pegylated to improve structural properties using methoxypolyethylene glycol (5000 Da) succinate (PEGS) along with targeting with retinoic acid (RA) to get final modified active and passive targeted conjugate (PEGS-EVOHS-RA) to evaluate its ability in carrying and delivery of epirubicin to hepatocellular carcinoma cell lines in response to varying temperatures. In this regard, the PEGS-EVOHS-RA conjugate was prepared through the desired chemical reactions and its structure was confirmed using ¹H-NMR and FT-IR spectra. The micelles were prepared from PEGS-EVOHS-RA by dialysis method. The Particle size and zeta potential were measured, and entrapment efficacy along with *in vitro* release efficiency in different temperatures were also studied. The structural morphology of optimized nanomicelle was studied by transmission electron microscopy micrographs. The desired final micelles were evaluated for their toxicity using MTT assay on HepG2 human hepatocellular carcinoma cell lines at normal (37 °C) and elevated temperature (45 °C). The results revealed that, as the hydrophilicity of micelles increased, all characteristic properties improved. Then, these micelles can be considered as potentially effective thermo responsive delivery systems for targeted delivery of cytotoxic agents to hepatocellular carcinoma.

Keywords: Nanomicelles; PEGylation; Thermo-responsive; Poly(ethylene-co-vinyl alcohol) (EVOH); Cytotoxicity

INTRODUCTION

Polymeric micelles (PMs) due to their suitable characteristic features could improve the pharmacokinetic and pharmacodynamic properties such as increased plasma half-life, protection from enzymes, enhanced permeation and solubility (1).

Bader, *et al* in 1984 (2) started the use of PMs as drug delivery carrier and later improved by Yokoyama, *et al.* in the early 1990s through the preparing doxorubicin-conjugated block copolymer micelles (3).

PMs keep a promise to get fascinating biopharmaceutical and pharmacokinetic

profile of drugs (4) and enhance their bioavailability due to their size, high and excellent capacity to solubilize and load the hydrophobic drug and good site-specific delivery.

Enhanced permeation and retention (EPR) is a strategy in which the therapeutic agent incorporated into a carrier passively reaches the target organ, harness the leaky vasculature of cancer tissue and owing to the lack of a well-defined lymphatic system their permeation and retention is enhanced.

*Corresponding author: M. Rostami
Tel: 0098 3137927107, Fax: 0098 3137927096
Email: m.rostami@pharm.mui.ac.ir

Access this article online



Website: <http://rps.mui.ac.ir>

DOI: 10.4103/1735-5362.202449

However, the therapeutics cannot be effectively delivered to target organs by the EPR effect due to difficulties reaching the cells positioned deep into the malignant tissues; this makes the synergistic passive and active targeting strategies more important (5).

The surface modification of polymeric carrier, usually with polyethylene glycol (PEG) is the traditional approach for synthesizing polymeric conjugates that could be delivered via passive targeting using a stealth circulation. PEGylation can reduce serum protein adherence by creating a stealth surface that results in avoiding uptake by the reticuloendothelial system (RES) and prolonging circulation time (5). It has shown promising results for enhanced *in vivo* stability (6).

Despite the advantages of biodegradable polymeric nanocarriers, an obvious downside to using these systems is their early hydrolysis in a site far from the intended organ. In this sense, the nonbiodegradable systems are less likely to release their therapeutic content before reaching the target organ because of hydrolysis stability and hence, could lead to reduced side effects, longer circulation and controlled release. Use of nondegradable biocompatible polymers is a new and promising strategy for controlling the release of therapeutics to target organs (7).

In this research, we have studied the feasibility of using polyethylene-co-vinyl alcohol (EVOH) in the manufacturing of novel nanocarriers. EVOH is a copolymer of vinyl alcohol and polyethylene with excellent mechanical and gas barrier properties (8). We utilized succinate and PEG succinate modified EVOH (PEGS-EVOHS) conjugate targeted by retinoic acid (RA) to manufacture biocompatible thermoresponsive polymeric nanocarriers of epirubicin (EPI) for the treatment of hepatocellular carcinoma. The toxicity of PEGS-EVOHS-RA micelles were tested on HepG2 human hepatocellular carcinoma cell line using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay at temperature higher than 37 °C (45 °C). Using the synergistic effect of active and passive targeting strategies, these nanomicelles are predicted to

show a long circulation and accumulation in the target site. The results obtained from this study are being further used to investigate the drug release efficacy under the temperature increment condition using a magnetic field in an ongoing project.

MATERIALS AND METHODS

Materials

EPI HCl (PIRUCIN[®]) was provided by Deva Holding A.S. (Istanbul, Turkey). Retinoic acid was purchased from the Solmag Chemical Company (Italy). EVOH (44% ethylene), dicyclohexylcarbodiimide (DCC), 4-dimethylamino pyridine (DMAP), and MTT were supplied by Sigma Aldrich (USA). Anhydrous dimethylsulfoxide (DMSO), methoxy(poly ethylene glycol (MeO-PEG) 5000, succinic anhydride (SA), and ethanol were purchased from Merck Chemical Company (Germany). RPMI-1640 culture medium, fetal bovine serum (FBS), and antibiotic mixture were supplied by Biosera (USA). HepG2 cell line was supplied by the Pasteur Institute of Iran (Iran).

Methods

Preparation of PEGS-EVOHS-RA

We prepared the EVOHS by the reaction of EVOH (2 g in 10 mL DMSO) and SA (1.1 g, 11 mmol), the PEGS by the reaction of PEG (6 g, 1.2 mmol) and SA (0.24 g, 2.4 mmol) in the presence of DMAP (0.146 g, 1.2 mmol), activated PEGS through the reaction of PEGS with DCC (0.29 g, 1.4 mmol) and DMAP (0.015 g, 1.2 mmol) in DMSO and activated RA by the reaction of RA (0.20 g, 0.67 mmol), DCC (0.29 g, 1.40 mmol) and DMAP (0.11 g, 0.67 mmol) in DMSO. Finally, we added the activated PEGS and RA respectively to a solution of EVOHS to get the last conjugate PEGS-EVOHS-RA, crude powder which further purified by dialysis (cut off 10 KDa). We checked the final pure conjugates for their structure accuracy using FT-IR and ¹H-NMR techniques, compared to its components. ¹H-NMR spectrum was used to determine the degree of substitution (DS) of both RA and PEG. For this purpose, the ratio of RA peak at 5.8 ppm or PEG peak at 3.5 ppm as indicator

peaks to the 4.2 ppm peak of EVOH was calculated according to the following equations. This is because we used the EVOH with 56% of vinyl alcohol then the number of protons at 4.2 ppm assigned to EVOH is 56 per each 100 repeating units.

$$DS (RA) (\%) = \frac{\text{Integral of signal at } 5.8 \times 56}{\text{Integral of signal at } 4.2 \times 1}$$

$$DS (PEG) (\%) = \frac{\text{Integral of signal at } 3.5 \times 56}{\text{Integral of signal at } 4.2 \times 3}$$

Preparation of nanomicelles

We dissolved 20 mg of PEGS-EVOHS-RA sample and 3.5 mg of EPI in 2 mL of DMSO, the mixture was dialyzed against water for 24 h using dialysis membrane (cut off 12 KDa). The resulting micelle dispersion was then sonicated in a bath sonication (Misonix, S3000, USA) for 5 min.

Determination of critical micelle concentration (CMC) of PEGS-EVOHS-RA

The CMC of PEGS-EVOHS-RA conjugate was measured by fluorescence measurement using pyrene as a hydrophobic fluorescence probe. The fluorescence emission spectrum of pyrene in different concentrations (varying from 1 to 250 $\mu\text{g/mL}$) of PEGS-EVOHS-RA conjugate (containing 6×10^{-7} M pyrene), were recorded using a spectrofluorometer (LS-3, PerkinElmer, USA) with the excitation wavelength set at 390 nm. From the pyrene emission spectra, the intensity ratio of the first peak (I_1 , 373 nm) to the third peak (I_3 , 389 nm) was plotted against the logarithm of polymer concentration. Two tangents were then drawn and the CMC values were taken from the intersection between the two tangents. (9).

Entrapment efficiency of EPI

Nanomicelle dispersions (500 μL) were centrifuged (MIKRO200, Hettich, Germany) at 10,000 rpm for 5 min and the supernatants were analyzed for free EPI using a UV spectrophotometer at 233 nm. The following equation was used to calculate the entrapment efficiency (EE) of EPI:

$$EE (\%) = \frac{\text{The amount of the entrapped drug}}{\text{The total amount of the added drug}} \times 100$$

In vitro drug release studies

We placed three dialysis membranes (cut off 10 KDa), each containing 1 mL of the drug-loaded micelle dispersion in 20 mL of phosphate buffer (pH = 7.4) while being stirred at the temperatures of 25 ± 2 , 35 ± 2 , 45 ± 2 , and 55 ± 2 °C respectively. Aliquot of 100 μL was taken from each phosphate buffer at specified time intervals and the EPI content was determined at 233 nm. The release efficiency was then calculated using the following formula:

$$RE (\%) = \frac{\int_0^t y \cdot dt}{y_{100} \times t}$$

where, y is the released percent at time t .

Transmission electron microscope analysis

Samples of well-dispersed PEGS-EVOHS-RA nanomicelles were further sonicated for 3 min using bath sonication (Misonix, S3000, USA). The samples were then placed on a 300 mesh formvar carbon coated copper grid, and the grid was left to dry at room temperature. Micrographs were taken with different levels of magnification with an accelerating voltage of 80 kV using a transmission electron microscope (TEM) (Zeiss, EM10C, Germany).

Cell viability assay

We studied the adopted micelles for their cytotoxicity in comparison with controls (free EPI and bare non-targeted nanoparticles PEGS-EVOHS). In this regards, we maintained the HepG2 cells in 5% CO_2 , 95% humidified atmosphere at 37 °C in RPMI-1640 medium containing 10% (v/v) of FBS, and 1% of the antibiotic mixture (penicillin/streptomycin 50 IU/mL). We transferred the HepG2 cells in two different 96-well plates at 5×10^4 cells/mL density and incubated for 24 h. The cells in two plates simultaneously were then treated with EPI-loaded PEGS-EVOHS-RA nanomicelles, bare PEGS-EVOHS nanomicelles, EPI loaded non-targeted nanomicelles PEGS-EVOHS, and free EPI all at 0.25, 0.50, 1, and 2 $\mu\text{g/mL}$ concentrations at 37 °C or 45 °C for 24 h. To study the temperature effect on the result of cell culture study, the heat-treated suspension of EPI-PEGS-EVOHS-RA nanomicelles at

45 °C was decanted on the cells. We used the blank nanomicelles with the same concentration for comparison. After this time, 20 μ L of MTT added to plates and the plates were further incubated for 3 h. After the removal of the culture medium, we dissolved the violet formazan crystals by adding 150 μ L of DMSO. In each well, we measured the absorbance at 570 nm using an ELISA plate reader (Awareness, USA). Untreated cells were taken as the negative control with 100% viability and the blank culture medium was used as a blank control Cell viability for each sample was calculated using following equation:

$$\text{Cell viability (\%)} = \frac{\text{Mean of each group} - \text{mean of blank}}{\text{mean of negative control} - \text{mean of blank}} \times 100$$

We used the SPSS software (version 20, IBM, USA) for statistical analysis of cell culture data.

The cell culture data were reported as mean \pm SD and were reasonably compared by analysis of variance (ANOVA) followed by the post hoc test of LSD. A meaningful level of $P < 0.05$ was considered significant in all cases.

RESULTS

Synthesis of PEGS-EVOHS-RA

The PEGS-EVOHS-RA conjugate was synthesized through the chemical pathway shown in Fig. 1. The structure of the PEGS-EVOHS-RA conjugate was confirmed by $^1\text{H-NMR}$ (Fig. 2).

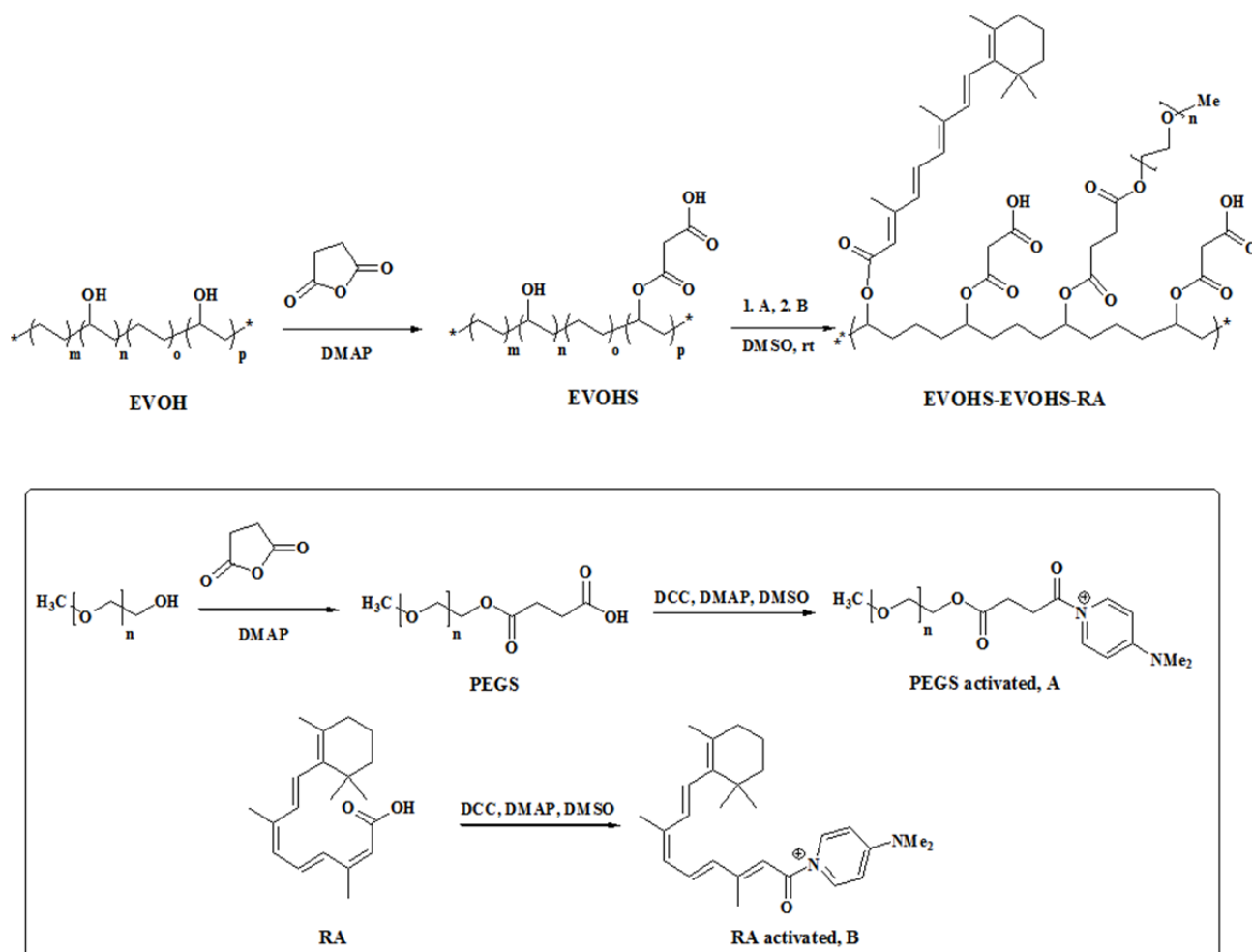


Fig. 1. Synthesis of PEGS-EVOHS-RA conjugate.

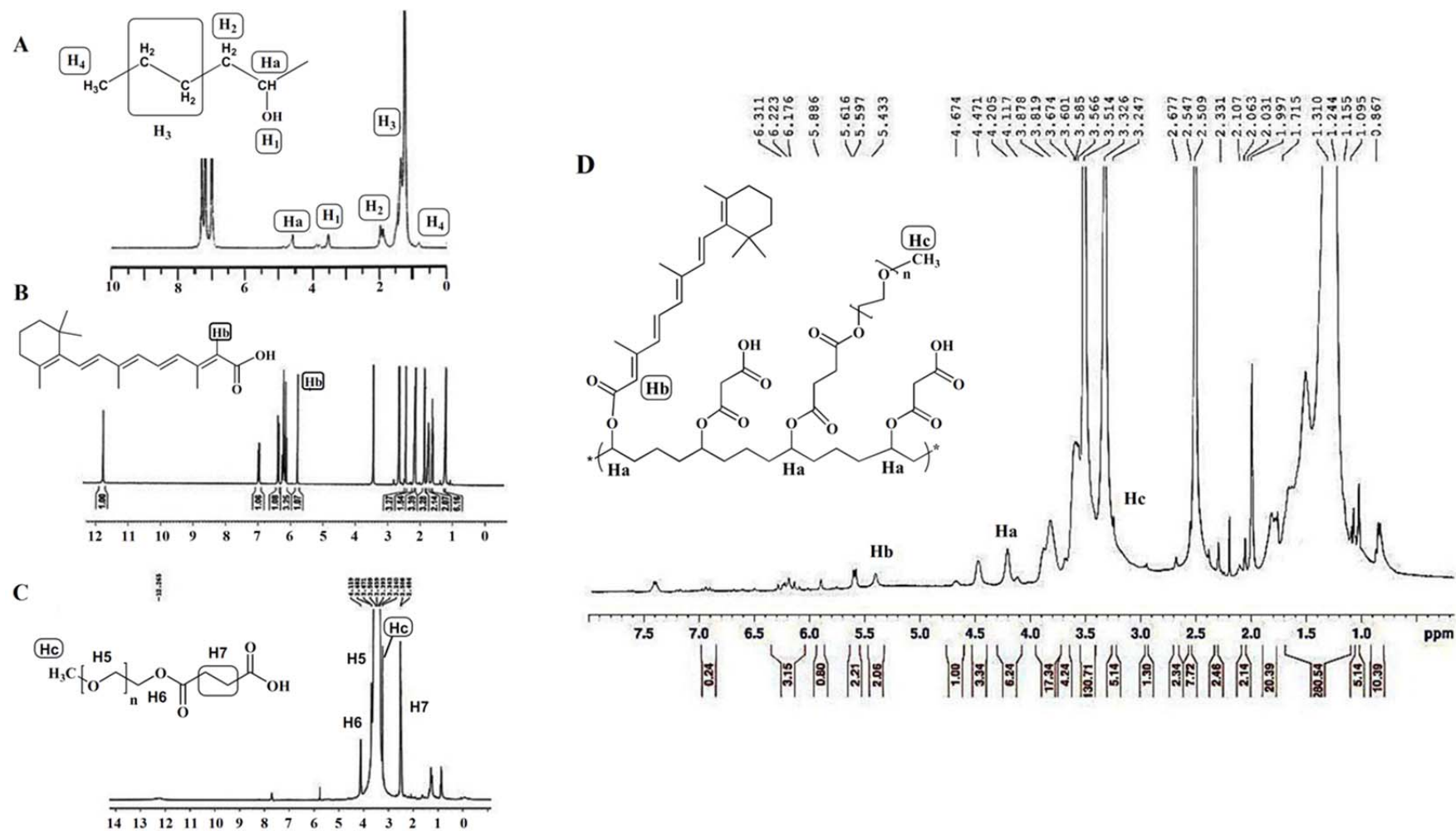


Fig. 2. ¹H-NMR spectra of (A) EVOH, (B) RA, (C) PEGS, and (D) PEGS-EVOHS-RA.

The reference peaks of components comprising RA (around 1.00 (s, 6H, $-(CH_3)_2$ geminal groups on ring), 1.40-1.50 (m, 4H, $-CH_2-CH_2-$ in ring), 1.70 (s, 3H, $-CH_3$ group on cyclohexene ring bonded to $-C=C-$), 2.00 (m, 5H, $-CH_2-$ and $-CH_3$ groups), 2.30 (s, 3H, $-CH_3$ group next to the carboxylic moiety), 5.80 (s, 1H, proton next to the $-(C=O)O-$ group), 6.00-6.50 (m, 4H, vinylic protons), and 7.00 (m, 1H (vinylic proton) ppm) (10), succinate subunit (2.50 ppm ($-CH_2-CH_2-$ moiety)), EVOH (1.5 (bs, 3H, $-CH_3$), 2.05 (m,

$-CH_2-CH_2-$ (ethylenic moiety), around 3.70 (m, $-CH_2-CH(OH)-$ and 4.20 (s, 1H, $-CH_2-CH(OH)-$ ppm) (11,12) and PEGS (3.2 ppm, (OCH_3) , 3.5 ppm, $(-CH_2CH_2-$ of PEG moiety) and 2.50 (CH_2CH_2 of succinate moiety)) were clearly visible in the final conjugates spectra at the right places with the accurate intensity as predicted. Similarly, the IR bands related to conjugate components were clearly seen in spectral regions for EVOHS, RA, and PEG respectively around 3370, 2929, 1727, 1673, 1109 cm^{-1} (Fig. 3).

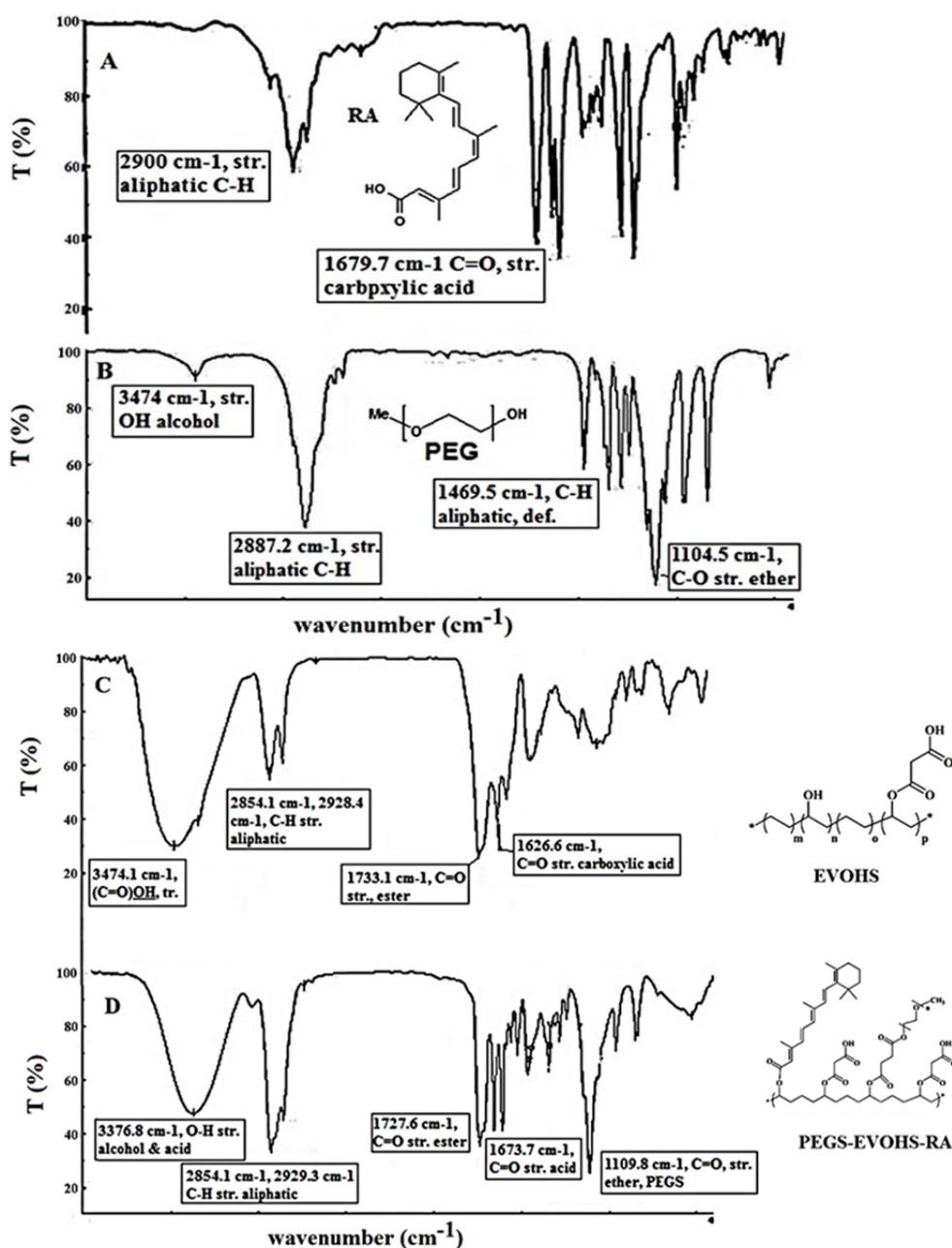


Fig. 3. FT-IR spectra of (A) RA, (B) PEG, (C) EVOHS, and (D) PEGS-EVOHS-RA.

These absorption bands respectively related to O–H stretching vibration of carboxylic acid and alcohol functionality, C–H stretching vibration of aliphatic backbone, C=O stretching mode vibration of ester bonds, C=O stretching mode of vibration for carboxylic acid moieties, and C–O stretching vibration of PEG moieties.

Characteristics of nanomicelles

Fig. 4 shows the variation of fluorescence intensity ratio (I_{1}/I_{3}) against the logarithm of PEGS-EVOHS-RA concentration. The estimated CMC value for prepared PEGS-EVOHS-RA micelles was 8.43 $\mu\text{g}/\text{mL}$, Table 1 listed particle size, zeta potential,

polydispersity index (PDI) (determined by Zetasizer-ZEN 3600 Malvern Instrument Ltd., Worchestershire, UK), entrapment efficiency, and release efficiency of drug loaded micelles. Furthermore the results for *in vitro* drug release in response to different temperatures have provided in Fig. 5.

As Table 1 shows, the particle size of the designed micelles are about 212.30 ± 20.24 , the PDI as a factor that represents the dispersion homogeneity is around 0.22 ± 0.05 , the zeta potential of the designed micelles is -2.40 mV, entrapment efficiency of EPI in the micelles is about $88.00 \pm 8.6\%$ and release efficiency of EPI from the micelles is 32.20 ± 0.75 .

Table 1. Results for particle size, zeta potential, PDI, CMC, loading efficacy, and release efficacy of micelles (mean \pm SD, n = 3)

Formulation	Particle size (nm)	PDI	Zeta potential (mV)	Loading efficiency (%)	RE ₄₀₀ % at 45 °C	CMC ($\mu\text{g}/\text{mL}$)
PEGS-EVOHS-RA	212.30 ± 20	0.22 ± 0.05	-2.40	88.00 ± 8.6	32.20 ± 0.75	8.43

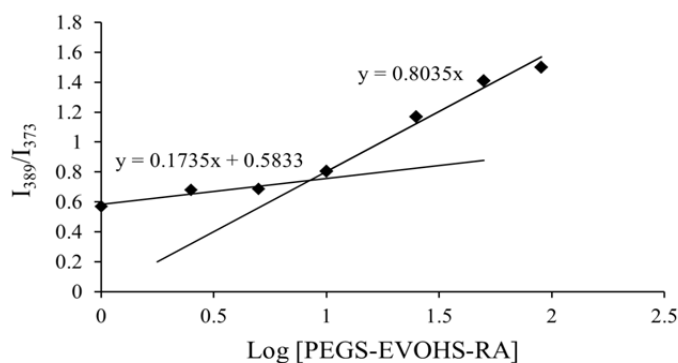


Fig. 4. Changes in fluorescence intensity ratio in I_{389}/I_{373} versus the log of concentration of PEGS-EVOHS-RA macromolecule to determine the CMC.

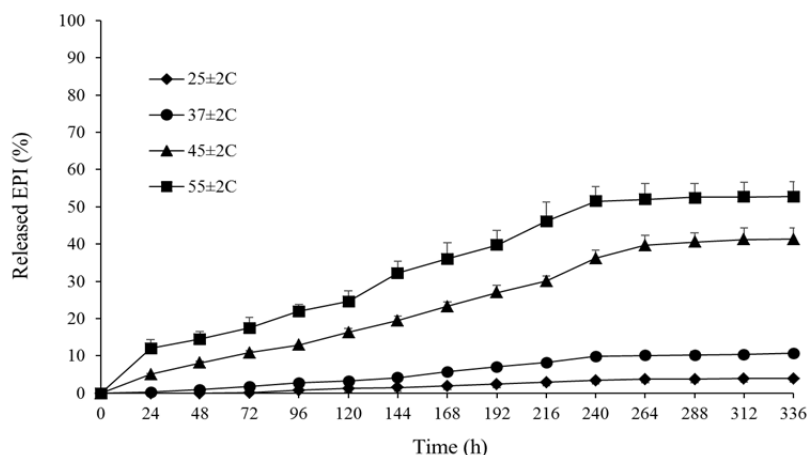


Fig. 5. Release profile of EPI from PEGS-EVOHS-RA nanomicelles at different temperatures.

Fig. 5 presents the results for drug release from PEGS-EVOHS-RA sample in the temperatures of 25, 35, 45, and 55 °C. The temperature of 45 °C was chosen as the best release temperature since the release efficacy was around 40% which was significantly higher than the release in lower temperatures (25 °C, 37 °C) ($P < 0.05$) ($< 10\%$).

TEM study

Fig. 6 shows the TEM images of adopted nanocarriers. The nanomicelles are spherical and have conformity in shape and size. The scale bars show that the particle sizes are smaller than the results obtained by DLS method using zetasizer (Table 1).

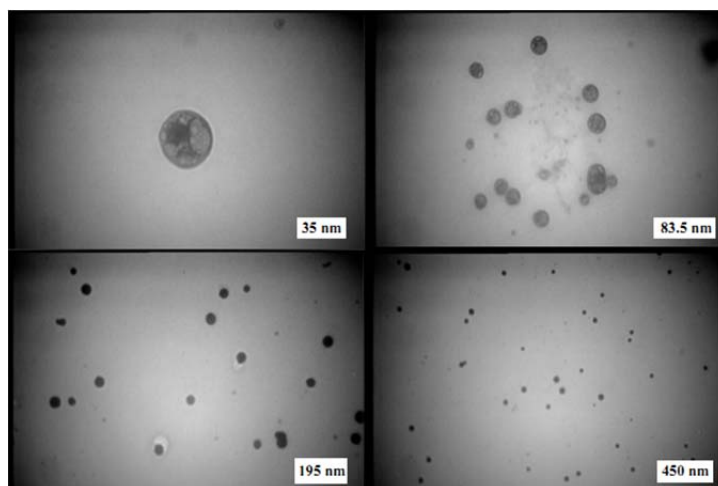


Fig.6. TEM micrographs of PEGS-EVOH-RA conjugate.

Cell viability assay

Fig. 7 shows the results for inhibition of HepG2 cells proliferation using MTT assay. As observed, the viability of the HepG2 cells was about 75% after 24 h of incubation at 37 °C with the EPI-loaded PEGS-EVOHS-RA micelles at highest concentration, whereas no reduction in cell viability was observed after incubation under the same conditions with blank or non-targeted nanoparticles. Heat-treated EPI-loaded PEGS-EVOHS-RA micelles were more cytotoxic than micelles at 37 °C in the same condition.

The IC₅₀ for (EPI-loaded PEGS-EVOHS-RA) targeted nanomicelles at 45 °C was 0.51 μM.

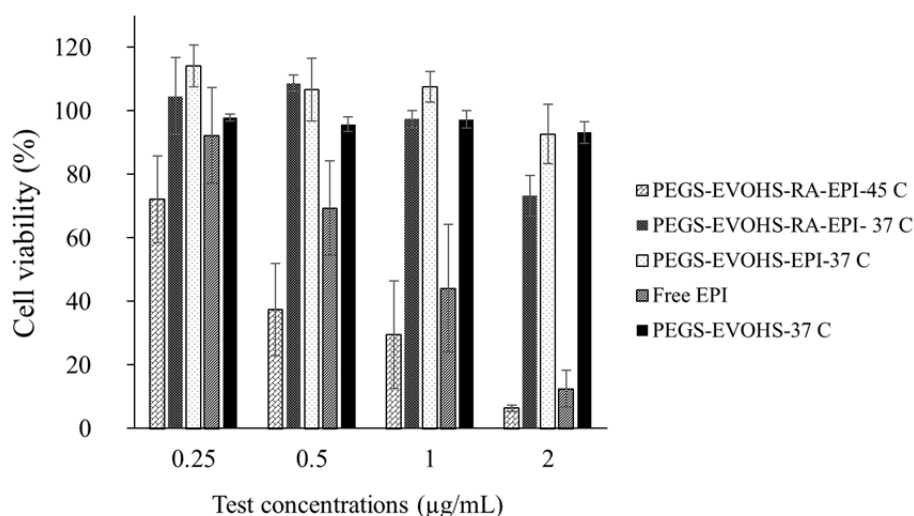


Fig. 7. Viability of HepG2 cells after treatment with different concentrations of PEGS-EVOHS-RA-EPI-45°C, PEGS-EVOHS-RA-EPI-37°C, PEGS-EVOHS-EPI-37°C, and free EPI, PEGS-EVOHS-37°C .

DISCUSSION

EVOH consists of hydrophobic ethylene and hydrophilic vinyl alcohol segments, has good solubility in DMSO. It has also been shown that EVOH is only soluble in solvent mixtures such as THF/methanol and DMSO/n-propanol (13). PEG is a hydrophilic linker that through the conjugation to a molecule could impact on the structural characters and hence enhances their water solubility, these PEGylated systems can further lead to the formation of the drug delivery systems with a stealth circulation property. This strategy has formerly been successfully used for solubility enhancement of protein and polymeric compounds (14,15).

EVOH itself has been PEGylated by Pacetti, *et al.* (16) to get a biocompatible coating for drug delivery stents. Succinylation is also another process widely used for the proteins improvement and enhance the water solubility of other molecules (17). In this regards, we succinylated EVOH in a theoretical ratio of 80 percentage of the total OH groups on the polymer backbone in a simple reaction with SA to enhance its water miscibility.

Fig. 2 shows the $^1\text{H-NMR}$ spectra (400 MHz, DMSO-d_6) of RA, EVOHS, PEGS, and conjugates. As it is observed in the spectra, absence of the carboxylic OH proton of RA in 12 ppm in the final products accompanied by the presence of reference peaks of components comprising RA, succinate subunit, EVOH and PEGS in the spectra of final PEGS-EVOHS-RA conjugate could motivate us to the precise synthesis of desired conjugates. The degree of substitution for RA and PEG connections were calculated and respectively were 18.5 and 15.4 per each 100 vinyl alcohol repeating sections. Fig. 3 presents the FT-IR spectra, along with the $^1\text{H-NMR}$ results, the characteristic absorption bands of EVOHS, RA, and PEG at the right places are the best proofs to the synthesis of the conjugate.

As discussed earlier, the size of polymeric micelles has a crucial role in the EPR effect-mediated accumulation. Tumor vasculature cutoff sizes can vary between tumors (200-800 nm), and determine the diffusion and

accumulation of molecules within the tumor interstitium (18). The benefit of small-sized polymeric micelles, which are well below the cutoff limit for most tumors, ensures that they stay in circulation for longer intervals without being taken up by mononuclear phagocytic system and eventually enter the tumor vasculature through the EPR effect. In this study, the proper particle size of micelles (212.3 nm) was the major advantages obtained by surface modification using PEG.

The entrapment efficacy of EPI in PEGS-EVOHS-RA nanomicelles was 88% which increased with the improvement in the amphiphilic trait in the micellar formulations. Vivek, *et al.* (19) have studied the entrapment efficiency of a hydrophobic drug in lipid particles and found that the more hydrophobic the matrices, the more hydrophobic drug could be efficiently entrapped. Furthermore, Jeong, *et al.* reported the embedment of doxorubicin as a hydrophobic drug in the core of the micelles of dextran/poly(lactic-co-glycolic acid) which exhibited a slow release to more than 4 days after the first eruption release (20).

As Fig.5 illustrated, the release studies confirmed that the prepared micelles are temperature sensitive and as shown in Fig. 4, with the increase in the induced temperature, the release rate of EPI increased and this is fully consistent with the assumption of thermosensitivity of the synthesized conjugates. In fact, EVOH is thermally sensitive flexible thermoplastic material and its structure changes in response to a temperature around its T_g . Two fundamental changes can cause drug release from this PMs, sheath swelling, and re-orientation of chains around the T_g temperature (21).

The estimated CMC value for PEGS-EVOHS-RA micelles was 8.43 $\mu\text{g/mL}$ (Fig. 4). In the aqueous environments, the hydrophobic moieties of macromolecules orient toward the hydrophobic cores, while the hydrophilic parts and PEG moieties surround these cores, to reach the lowest Gibbs free energy level. Micelle solutions are diluted in the body fluids and they are expected to be disassociated, so, the lower the CMC value, the more micelle stability in the body fluids (22). In this study, PEGS-EVOHS-RA

macromolecule had the CMC value (8.43 $\mu\text{g}/\text{mL}$), around 270 times lower than the value for the low-molecular weight surfactants, e.g., sodium dodecyl sulfate (SDS) whose CMC is around 2.3 mg/mL (23). This result indicates that the PEGS-EVOHS-RA conjugate can form micelles even in highly diluted solutions.

Regarding the zeta potential value (-2.4 mV) in this case, the relatively small value may be related to one of the especial conformation of polymeric backbone or in another view due to the low water affinity which causes lower hydrodynamic interactions with aqueous environment in the restricted time of measurement. Also it has to be kept in mind that adsorbed layers of polymers (large molecules) shift the plane of shear to a farther distance from the particle surface. This leads to a reduction of the measured zeta potential. That means even in the case of highly charged particle surfaces, a relatively low zeta potential will be measured (24). Despite the low zeta potential, the suspensions are stable because colloids can be stabilized by two different mechanisms (or a combination thereof) including electrostatic stabilization and steric stabilization. If steric stabilization of the particles is the main driving force, then there is no rule of the thumb for zeta potential. With these systems, even a formulation of near zero zeta potential can be stable, because it is not the charge of the particles, but rather the excluded volume interaction that keeps particles from sticking to each other. Of course even then, additional charge (i.e. slightly stronger zeta potential) will help contribute to the stability of the dispersion (25).

The TEM picture of optimized PEGS-EVOHS-RA particles has shown in Fig. 6, as you see in this image, compared to the DLS results, the particles partially have smaller sizes in the solid state. The data obtained from TEM is direct structural and size information from individual particles while DLS method gives an average hydrodynamic particle diameter by measuring diffusion in particle dispersion and interpreting them with Einstein-Stokes equation. Therefore there are often some differences between sizes obtained from DLS and TEM which could be attributed to the electrokinetic effects and the hydrodynamic of

the particle in DLS and the vacuum condition of TEM (26,27). Thus the main criterion contributing to the difference between DLS and TEM results for small-sized particles is the radius of curvature effect (28).

As Fig. 7 reveals the non-targeted, non-loaded particles did not affect the viability of HepG2 cells (survival rate 94.19%) at the highest concentration while targeted micelles loaded with equal EPI concentrations at 45 °C resulted in higher decline in cell viability compared to free EPI as expected from the vinyl alcohol copolymers (29). Meanwhile, even though the targeted micelles loaded with EPI applied to cells at 37 °C did not inhibit cell viability significantly when compared to free EPI due to their small release fraction, these targeted micelles still demonstrated a larger proliferation decline compared to the untargeted micelles of the same concentration. The micelles, which released their content at 45 °C showed a much higher proliferation inhibition compared to micelles at 37 °C ($P < 0.05$). This result demonstrates that temperature is, in fact, an effective tool for control of drug release and hence, the efficacy of micelles. Even though a difference is seen between the targeted and non-targeted polymers, this difference is not statistically significant which may be due to the fact that the main drug release from micelles was thermoresponsive and the substitution rate of RA has not been enough to create a remarkable effect (as you see this difference is more considerable at higher concentration). Certainly, *in vivo* studies should be undertaken where hyperthermia could, in fact, be applied at the location of the tumor. But yet, the RA moiety of targeted conjugate in the heated suspension can bind to its dedicated receptors and shows its activity in cell culture study.

Since there is no significant difference between cells treated with bare micelles and the EPI loaded micelles at 37 °C temperature, we conclude that the designed micelles have no significant drug release at temperature lower 45 °C which results in a nanosized drug delivery system that could effectively inhibit hepatocellular carcinoma growth in response to the temperature applied. By applying the temperature to carriers, due to the structural

changes, drug release and cell adhesion improved, then the cell viability decreased efficiently. Varshosaz, *et al.* have previously formulated retinoic acid decorated albumin chitosan nanoparticles which showed increased uptake of particles by HepG2 cells and increased cell proliferation inhibition for targeted nanoparticles compared to free doxorubicin (30). Here we found that IC₅₀ for targeted nanomicelles at 45 °C (0.51 μM) was almost half that of free EPI (1.04 μM) which can be attributed to the more released EPI from micelles in response to heating.

CONCLUSION

Herein, we prepared retinoic acid grafted PEGS-EVOHS-RA micelles for effective delivery of EPI. It was indicated that the release efficiency of the micelles increased linearly with temperature. The optimum formulation released around 40% of its drug content within 400 h. The growth of HepG2 cells was significantly inhibited at higher temperature. This temperature depending cytotoxicity reveals the potential usefulness of the PEGS-EVOHS-RA nanomicelles as thermo-sensitive anticancer drug delivery systems. These observations need confirmation with local hyperthermia before using this system for anticancer drug delivery to hepatocellular carcinoma.

ACKNOWLEDGEMENTS

The content of this paper is extracted from the Pharm.D thesis of Maryam Farzan (No. 291217) which was financially supported by the Pharmaceutical Sciences Research Center of Faculty of Pharmacy, Isfahan University of Medical Sciences, Isfahan, I.R. Iran.

REFERENCES

1. Xu W, Ling P, Zhang T. Polymeric micelles, a promising drug delivery system to enhance bioavailability of poorly water-soluble drugs. *J Drug Deliv.* 2013;2013:340315..
2. Bader H, Ringsdorf H, Schmidt B. Watersoluble polymers in medicine. *Macromol Mater Eng.* 1984;123(1),457-485.
3. Yokoyama M, Kwon GS, Okano T, Sakurai Y, Seto T, Kataoka K. Preparation of micelle-forming

- polymer-drug conjugates. *Bioconjug Chem.* 1992;3(4):295-301.
4. Kalal J, Drobnik J, Kopecek J, Exner J. Water soluble polymers for medicine. *Polym Int.* 1978;10(2):111-114.
5. Wong C, Stylianopoulos T, Cui J, Martin J, Chauhan VP, Jiang W, Popovic Z, *et al.* Multistage nanoparticle delivery system for deep penetration into tumor tissue. *Proc Natl Acad Sci U S A.* 2011;108(6):2426-2431.
6. Mishra S, Webster P, Davis ME. PEGylation significantly affects cellular uptake and intracellular trafficking of non-viral gene delivery particles. *Eur J Cell Biol.* 2004;83(3):97-111.
7. Pillai O, Panchagnula R. Polymers in drug delivery. *Curr Opin Chem Biol.* 2001;5(4):447-451.
8. Mokwena KK, Tang J. Ethylene vinyl alcohol: a review of barrier properties for packaging shelf stable foods. *Crit Rev Food Sci Nutr.* 2012;52(7):640-650.
9. Aguiar J, Carpena P, Molina-Bolivar JA, Carnero Ruiz C. On the determination of the critical micelle concentration by the pyrene 1:3 ratio method. *J Colloid Interface Sci.* 2003;258(1):116-122.
10. Varshosaz J, Hassanzadeh F, Sadeghi Aliabadi H, Banitalebi M, Rostami M, Nayebsadrian M. Novel worm-like amphiphilic micelles of folate-targeted cyclodextrin/retinoic acid for delivery of doxorubicin in KG-1 cells. *Colloid Polym Sci.* 2014;292(10):2647-2662.
11. Jiang H, He J, Liu J, Yang Y. Synthesis and characterization of poly(ethylene-co-vinyl alcohol)-graft-poly(epsilon-caprolactone). *Polym J.* 2002;34(9):682-686.
12. Brandolini AJ, Deborah DH. NMR spectra of polymers and polymer additives. New Jersey: Mobil Chemical Company, Marcel Dekker; 2000. 104-106.
13. Tylkowski B, Peris S, Giamberini M, Garcia-Valls R, Reina JA, Ronda JC. Light-induced switching of the wettability of novel asymmetrical poly(vinyl alcohol)-co-ethylene membranes blended with azobenzene polymers. *Langmuir.* 2010;26(18):14821-14829.
14. Sherman MR, Saifer MG, Perez-Ruiz F. PEG-uricase in the management of treatment-resistant gout and hyperuricemia. *Adv Drug Deliv Rev.* 2008;60(1):59-68.
15. Zhao RY, Wilhelm SD, Audette C, Jones G, Leece BA, Lazar AC, *et al.* Synthesis and evaluation of hydrophilic linkers for antibody-maytansinoid conjugates. *J Med Chem.* 2011;54(10):3606-3623.
16. Pacetti SD, inventor; Coating for drug delivery devices comprising modified poly(ethylene-co-vinyl alcohol). US patent 0054104 A1, 2004.
17. Lawal OS, Adebawale KO. Effect of acetylation and succinylation on solubility profile, water absorption capacity, oil absorption capacity and emulsifying properties of mucuna bean (*Mucuna pruriens*) protein concentrate. *Nahrung.* 2004;48(2):129-136.
18. Yuan F, Dellian M, Fukumura D, Leunig M, Berk DA, Torchilin VP, *et al.* Vascular permeability in a human tumor xenograft: molecular size dependence

- and cutoff size. *Cancer Res.* 1995;55(17):3752-3756.
19. Vivek K, Reddy H, Murthy RSR. Investigations of the effect of the lipid matrix on drug entrapment, *in vitro* release, and physical stability of olanzapine-loaded solid lipid nanoparticles. *AAPS Pharm Sci Tech.* 2007;8(4):16-24.
 20. Jeong YII, Kim DH, Chung CW, Yoo JJ, Choi KH, Kim CH, et al. Doxorubicin-incorporated polymeric micelles composed of dextran-b-poly(DL-lactide-co-glycolide) copolymer. *Int J Nanomedicine.* 2011;6:1415-1427.
 21. Congdon T, Shaw P, Gibson MI. Thermoresponsive, well-defined, poly(vinyl alcohol) co-polymers. *Polym Chem.* 2015;6:4749-4757.
 22. Bian F, Jia L, Yu W, Liu M. Self-assembled micelles of N-phthaloylchitosan-g-polyvinylpyrrolidone for drug delivery. *Carbohydr Polym.* 2009;76(3):454-459.
 23. Shirahama K, Kashiwabara T. The CMC-decreasing effects of some added alcohols on the aqueous sodium dodecyl sulfate solutions. *J Colloid Interface Sci.* 1971;36(1):65-70.
 24. Quaglia F, Ostacolo L, Mazzaglia A, Villari V, Zaccaria D, Sciortino MT. The intracellular effects of non-ionic amphiphilic cyclodextrin nanoparticles in the delivery of anticancer drugs. *Biomaterials.* 2009;30(3):374-382.
 25. Honary S, Zahir F. Effect of zeta potential on the properties of nano-drug delivery systems - A review (part 2). *Trop J Pharm Res.* 2013;12(2):265-273.
 26. Ito T, Sun L, Bevan MA, Crooks RM. Comparison of nanoparticle size and electrophoretic mobility measurements using a carbon-nanotube-based coulter counter, dynamic light scattering, transmission electron microscopy, and phase analysis light scattering. *Langmuir.* 2004;20(16):6940-6945.
 27. Pabisch S, Feichtenschlager B, Kickelbick G, Peterlik H. Effect of interparticle interactions on size determination of zirconia and silica based systems – A comparison of SAXS, DLS, BET, XRD and TEM. *Chem Phys Lett.* 2012;521:91-97.
 28. Lim J, Yeap SP, Che HX, Low SC. Characterization of magnetic nanoparticle by dynamic light scattering. *Nanoscale Res Lett.* 2013;8(1):381.
 29. Congdon T, Shaw P, Gibson MI. Thermoresponsive, well-defined, poly(vinyl alcohol) co-polymers. *Polym Chem.* 2015;6:4749-4757.
 30. Varshosaz J, Hassanzadeh F, Sadeghi H, Ghelich Khan Z, Rostami M. Retinoic acid decorated albumin-chitosan nanoparticles for targeted delivery of doxorubicin hydrochloride in hepatocellular carcinoma. *J Nanomat.* 2013;2013: Article ID 254127, 12 pages.