Bioorganic Chemistry 88 (2019) 102959

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

Multifunctional nanoparticles from albumin for stimuli-responsive efficient dual drug delivery

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ARTICLE INFO

Keywords:
BSA
Albumin
Methotrexate
Curcumin
Co-delivery
Simultaneous delivery

ABSTRACT

In this project methotrexate (MTX) conjugated albumin based nanoparticles (MTX-BSA) loaded with curcumin (CUR) drug (CUR-MTX-BSA) for simultaneous delivery of multi-chemotherapeutic drugs and combination cancer therapy were designed. Co-delivery is a new strategy which minimize the amount of each drug, reduce of side effects and also to achieve the synergistic effect for cancer therapies. The MTX was conjugated to albumin via covalent bond. Next, this synthesized prodrug loaded with CUR. Afterward, the formulations were evaluated for physical and chemical properties by DLS, TEM, FTIR, UV/Vis, DSC analysis, in vitro cytotoxicity and *in vivo* biocompatibility studies. Furthermore, the drug loading and release study were evaluated. Proteinase K enzyme was used to break amid bond between MTX and BSA and also amidic bonds in BSA structure. Administration of up to 2000 mg/kg of BSA to healthy animals was non-toxic and all treated mice were still alive after 24 h. The result of this study proved that CUR-MTX-BSA can be used as a proficient vehicle for effective co-delivery of CUR and MTX in the treatment of cancer.

1. Introduction

Cancer with more than 10 million new cases every year, remains one of the world's most devastating illnesses. Surgical intervention, radiation and chemotherapeutic drugs are current cancer treatments [1]. Chemotherapeutic drugs often also kill healthy cells and cause toxicity to the patient. One of the promising strategies to overcome undesirable toxicity and other side effects is combination of two or more drugs in treatment plan which reduced dosage of each agent.

Methotrexate (MTX) is clinically applied for the cure of cancer. MTX inhibit the production of thymidine by competitively blocking the dihydrofolate reductase (DHFR) enzyme [2]. MTX acts specifically with mitotic cell division. Meanwhile, MTX shows dose- and duration-dependent teratogenicity [3]. Though conventional dosage forms are not as effective system due to limit of maximum therapeutic outcomes with lesser side effects.

Although interest of using natural products for therapeutic applications seems recent, researchers frequently used natural compounds to fight disease [4]. Curcumin (CUR) is derived from Curcuma longa, a plant of the ginger family. CUR exhibit vast biological features which is needed for the cure of most illnesses, and it is inexpensive and has been found to be safe in human clinical trials. Despite these hopeful properties extremely low solubility in aqueous solutions is a major drawback with CUR, which limits its bioavailability and clinical efficacy [5].

The efficacy of CUR in combination with gemcitabine against advanced pancreatic cancer was also recently evaluated in two different trials [6,7]. Kanai et al. found that CUR at a 8 g/day in combination with gemcitabine was safe and well-tolerated [7]. However, Epelbaum and coworkers found that some patients experienced abdominal pain at this dose, and did not obtain a highly promising therapeutic response [6]. CUR in combination with docetaxel in patients with advanced and metastatic breast cancer was also recently studied in a Phase 1 trial [8].

Recently, many multifunctional delivery systems have been designed for codelivery of different therapeutic agents [9–11].

Among the carriers have been used for drug delivery, biological carriers such as bovine serum albumin (BSA) have been of particular interest because of their unique properties [12]. BSA was selected from among several types of carriers for simultaneous delivery of MTX and CUR. BSA as a macromolecular carrier for MTX and CUR has many advantages such follows as: no toxicity or immunogenicity;

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https://doi.org/10.1016/j.bioorg.2019.102959
Received 1 January 2019; Received in revised form 17 April 2019; Accepted 28 April 2019
Available online 29 April 2019
0045-2068/

biodegradable; stable in circulation; has a long half-life; easy access to all tumor sites via the blood circulation; high chemical stability; and convenient to administer [13,14].

In this work, we developed a CUR loaded MTX coupled albumin (CUR-MTX-BSA) for efficient dual drug delivery.

In this way, firstly, we have prepared MTX-BSA by covalently binding the MTX with BSA. Secondly, CUR loaded on MTX-BSA with physical encapsulation and the loading capacity and release profile of the drug from the NPs was evaluated. Thirdly, *in vivo* biocompatibility study and *in vitro* anticancer effect of these nanoparticles was investigated.

2. Materials and methods

2.1. Materials

BSA, CUR, N-(3-Dimethylaminopropyl)-N0-ethyl-Carbodiimide hydrochloride (EDC) and N-Hydroxy Succinimide (NHS), and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), were achieved from Sigma Aldrich Chemicals, (St. Louis, MO, USA). Methotrexate sodium (MTX) was purchased from Zahravi Company, Iran. All other solvents were purchased from Emertat Chimi Company (Tehran, Iran).

2.2. Conjugation of MTX on BSA (MTX-BSA)

MTX-BSA was synthesized by conjugation of MTX (100 mg) to BSA (200 mg) using EDC (183 mg) and NHS (21.85 mg) for activation and linking MTX to BSA into deionized water (20 mL) as solvent in basic medium. This reaction was kept for overnight (24 h) at room temperature. For separation of the MTX-BSA, dialysis process (12 kDa) were used.

2.3. Physically loading of CUR on MTX-BSA

CUR (20 mg) was dissolved in 4 mL of acetone and added dropwise to the 10 mL of MTX-BSA with concentration of 6 mg/mL. The mixture was shacked for 24 h under the dark condition at room temperature. Final formulation CUR-MTX-BSA were purified by centrifugation at 18,000 rpm. The resulting CUR loaded MTX coupled BSA were labeled as CUR-MTX-BSA.

2.4. Characterization

The samples were characterized by DSC, FTIR, TEM, UV/Vis and DLS techniques.

The particle size and morphology of the CUR-MTX-BSA was studied by TEM measurements. The sample was imaged by a transmission electron microscope (TEM; Cambridge 360–1990 Stereo Scan Instrument-EDS).

FTIR spectra were measured by a FTIR spectrophotometer (Bruker, Tensor 27).

Furthermore, DSC (Mettler Toledo, model Star SW 9.30, Schwerzenbach, Switzerland) were applied for thermal analysis of the NPs. Samples were heated at a rate of 15 °C min^{-1}.

The ζ-potential and hydrodynamic size were measured using a nano/zetasizer (Malvern Instruments, Worcestershire, UK, model Nano ZS).

The absorption spectrum of the samples were documented by UV/vis spectrophotometer (Thermo Fisher Scientific, USA, Madison, model GENESYS-TM 10S) to determine the components of BSA, CUR and MTX in the final formulation CUR-MTX-BSA.

2.5. In vitro study

2.5.1. Drug release study

To evaluation the release behavior of MTX, we incubated CUR-MTX-BSA in PBS (pH 7.4) at 37 °C in a dialysis bag (molecular weight cutoff 12,000 Da) and next immersed this bag into 45 mL of PBS. At selected time intervals, the exterior solution (2 mL) was withdrawn and analyzed by spectrophotometer at 304 nm and returned back to the medium to keep the volume of the drug release test constant. The same method was done in the presence of Proteinase K enzyme to break amid bond between MTX and BSA and also amidic bonds in BSA structure. Also, to evaluation the release behavior of CUR, we incubated CUR-MTX-BSA in PBS containing 2% (v/v) Tween 80 (pH 7.4 and pH 5.5) at 37 °C in a dialysis bag (molecular weight cutoff 12,000 Da) and next immersed this bag into 45 mL of PBS.

2.5.2. Hemolysis assay

In order to confirm the hemo-compatibility, the in vitro hemolysis test was done. The hemolysis test was completed following the methods defined in the literature [15].

2.5.3. Cell culture

The mouse breast carcinoma cell line (4T1) were proliferated in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS) and 100 mg/ml penicillin G and 100 mg/ml streptomycin (Gibco, Germany) at 37 °C in 5% CO_2 atmosphere.

2.5.4. In vitro anti-tumor activity study

MTT-based in vitro cytotoxicity assay was performed to compare anti-tumor effects of CUR-MTX-BSA and free MTX against 4T1. The cells were seeded on a 96 well plate at a cell density of 3×10^4 cells/well. Every drug was tested in 5 wells.

After incubating the cells in a logarithmic phase with medium containing free MTX, and CUR-MTX-BSA at different concentrations from 200 to 1600 nM for 72 h, MTT dye (20 mL of 5 mg/mL) was added to each well. After incubation for additional 4 h, the percentage of cell viability was determined at 570 nm relative to non-treated cells.

2.6. In vivo acute toxicity

BALB/C mice were maintained as per standard protocol. Zanjan University of medical science Research and Ethics Committee confirmed the experimental protocol. The BSA dissolved in PBS was administered intravenously in graded doses of 500–2000 mg/kg body weight to experimental animals. Animals were observed continuously at regular intervals for 24 h and the mortality was recorded. Enough replicates for each dose were done. The dose lethal to fifty percent of experimental mice is considered as LD50. All animals were euthanized upon end of the experiment.

3. Results and discussion

Nowadays, combination therapy of anti-cancer genes and drugs has become a new method for cancer treatment. This strategy has synergistic effect to reduce drug resistance and reduce toxicity. Many researchers reported such synergies. In this work we employed the BSA molecules as a smart and biocompatible vehicle for combination therapy. In detail, the carboxyl groups of MTX was activated with EDC/NHS and reacted with BSA for preparation efficient smart and tumor targeted biomacromolecule carrier. Next, The CUR molecules were loaded physically by π-π interaction and also by hydrogen bonding and led to the formation of final formulation.

MTX can induce cellular internalization when it binds to its receptor on the cell surface through the endocytosis pathway. The folate receptor is up-regulated in various cancer types, such as cervical, ovarian, breast, lung, and brain tumors. But, the expression of folate receptor is

limited in normal cells. MTX shows a targeting role and also a therapeutic effect to many types of cancer. Hence, our group suggested that MTX modification could endow the BSA with more precise tumor targeting property.

Covalent bonding of drugs on carriers is favored since this type of bonds is highly stable and therefore is most likely to be disrupted only under harsh environments inside lysosomes.

3.1. Synthesis of CUR-MTX-BSA

BSA has been extensively used as a biocompatible delivery carrier. Biodegradability, stability in blood circulation and a long half-life are the some of advantages albumin. Abraxane®, is the best example of clinical applications of albumin as carrier [16]. In this work we used the albumin functional groups for conjugation of targeting and therapeutic agents (MTX). The MTX-BSA was synthesized by conjugation of MTX to BSA using EDC and NHS for activation and linking MTX to BSA molecules at room temperature. Afterward, the CUR as anticancer agent helper, antioxidant and also as a protective agent of normal cells was loaded physically and led to the formation of CUR-MTX-BSA. In this work we do not used any method or procedures for preparation of nanoparticles. The CUR-MTX-BSA prepared by simple method with a narrow particle size distribution and poly dispersity index (PDI) about 0.36.

The MTX content of the conjugated nanoparticles was evaluated utilizing ultraviolet spectrophotometry. A calibration curve of MTX with concentrations was recorded at 304 nm. According to the obtained optimal prescription, 8.60 ± 1.63 mg MTX was linked per 100 mg BSA.

A mean particle size of around 140 nm with a PDI of 0.366, and a zeta potential of −23.42 mV for the optimal CUR-MTX-BSA has been observed, respectively. The loading efficiency of CUR was 3.61 ± 0.16%, respectively.

3.2. Characterization

Optical properties and evidence for MTX conjugation and CUR loading was evaluated with UV–Vis absorption spectrophotometer. As shown in Fig. 1 the conjugation of MTX to the BSA molecules was studied by UV–Vis absorption. As shown in Fig. 1 the UV–Vis spectrum peak of the BSA is around 279 nm, while in the UV–Vis absorption spectrum of MTX we can see that three characteristic absorption peaks at 260, 305, 374 nm. Fig. 1 displays the UV–Vis absorption spectrum of the MTX-BSA, the peak in the UV–Vis absorption spectrum around 286 nm and 263, 308, 377 nm corresponds to the BSA and MTX, respectively. Four obvious peaks around 286, 263, 308 and 377 nm in MTX-BSA UV–Vis absorption spectrum indicate that the MTX was successfully conjugated to the BSA.

Also, as shown in Fig. 1 the loading of CUR to the MTX-BSA carrier was studied by UV–Vis absorption. As can be seen in the UV–Vis absorption spectrum of CUR a characteristic absorption centered at 428 nm and a sharp peak at 270 nm. As illustrated in Fig. 1, CUR-MTX-BSA have all of the characteristic peaks (285, 260, 308, 377 and 428 nm) which demonstrated the successful conjugation of MTX with BSA and loading of CUR to the MTX-BSA carrier, respectively.

The FTIR analysis can be give us some visions about the compositions of MTX, CUR, BSA, MTX-BSA and CUR-MTX-BSA. The characteristic adsorption peaks of BSA at 1644 and 1525 cm^{-1} were attributed to flexural vibration adsorption of Amide I and Amide II, respectively [17]. The FTIR spectrum of MTX-BSA shows appearance of a new peaks at 830, 1111 and 1687 cm^{-1} indicative of the conjugation of MTX on the BSA through amide bonds.

As shown in Fig. 2, all the characteristic absorption peaks of MTX-BSA and CUR could be found in the FTIR spectra of the CUR-MTX-BSA.

DSC was used to further study the conjugation and existing of the drugs in the formulation. As shown in Fig. 3 we can see that the melting points of MTX and BSA were placed at 154.27 °C, and 171.03 °C, respectively. While we can see the DSC thermogram of MTX-BSA demonstrated a single endothermic peak at 214.30 °C, which confirms the conjugate of MTX to the BSA molecules.

Also, as shown in Fig. 3, the CUR powders showed a sharp single endothermic melting peak at 170.83 °C. Whereas the DSC thermogram of CUR loaded MTX-BSA nanoparticles (BSA-MTX-CUR) exhibited a single endothermic peak at 247.83 °C. However, the CUR characteristic peak disappeared in the thermogram of CUR-MTX-BSA, revealing that CUR was not in the crystalline state after loading into the MTX- BSA nanoparticles. This result indicated the successful conjugation and loading of MTX and CUR on BSA, respectively.

The particle size is an important aspect for *in vivo* behavior and application, many studies have shown that particles with a size of less than 300 nm have an enhanced permeability and retention (EPR) effect,

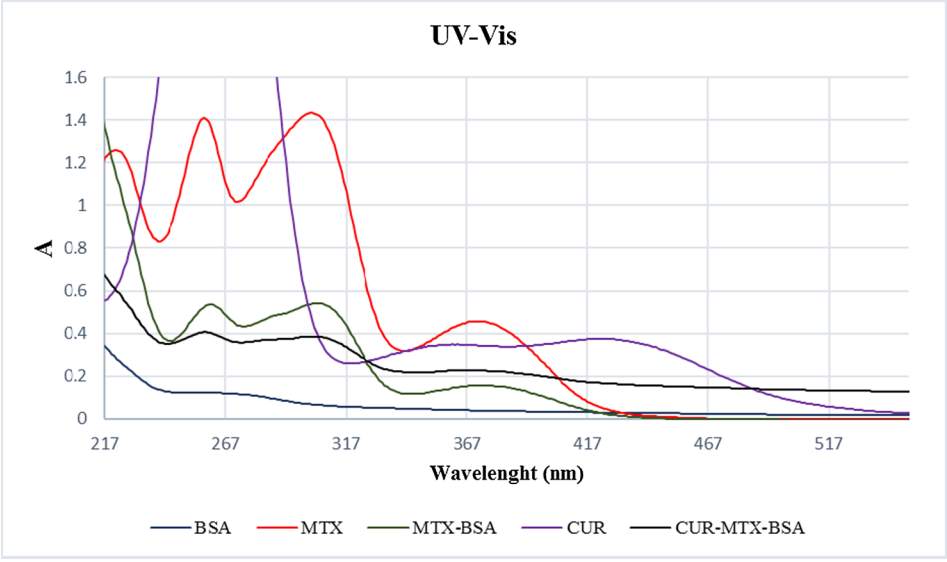

Fig. 1. UV–Vis spectra of CUR, MTX, BSA, MTX-BSA and CUR-MTX-BSA in PBS.

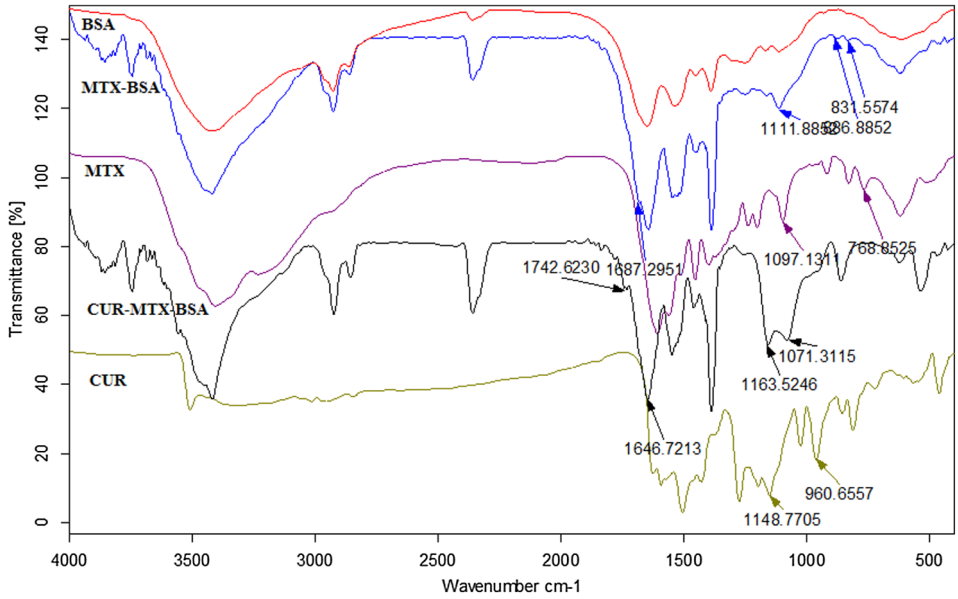

Fig. 2. FTIR spectrum of CUR, MTX, BSA, MTX-BSA and CUR-MTX-BSA from 400 to 4000 cm^{-1}.

which reveals desirable uptake compared to microparticles. As shown in Fig. 4, DLS was used to determine the hydrodynamic molecular size of CUR loaded MTX-BSA nanoparticles (CUR-MTX-BSA) and the result showed that this nanoparticle exhibited a diameter of about 140 nm with a PDI of 0.366. Therefore, it is predictable that the nanoparticles in size of less than 300 nm range are suitable for desirable drug delivery.

The CUR-MTX-BSA was negatively charged with a zeta potential of −23.42 mV. Thus, it can be decided that the particles were negatively charged, which provided good physical stability for the as prepared formulations.

The TEM analysis was conducted to detect the morphology and size. The results showed that CUR-MTX-BSA existed in a spherical structure (Fig. 5). The prepared nanoparticles Show a spherical morphology with average diameters 39.34 ± 6.52 nm (Mean ± SD, n = 22).

3.3. Drug release study

Previously, some of research groups have demonstrated that the covalent bond between an amine-modified nanoparticle and MTX was readily hydrolysable under intracellular conditions. Results of MTX release are shown in Fig. 6a the result data suggest that MTX release occurred prior to the 12 h interval, indicating that the protease readily cleaves the peptide bond. As we know, the enzymatic release of MTX is faster than hydrolytic release. It was also observed that compared against without proteinase K enzyme the MTX was released faster in the presence of mentioned enzyme. For example, about 80% of MTX was released from CUR-MTX-BSA in the presence of enzyme after 12 h whereas only 40% MTX was released from the nanoparticles without enzyme. Science the optimum activity of used enzyme in this work is

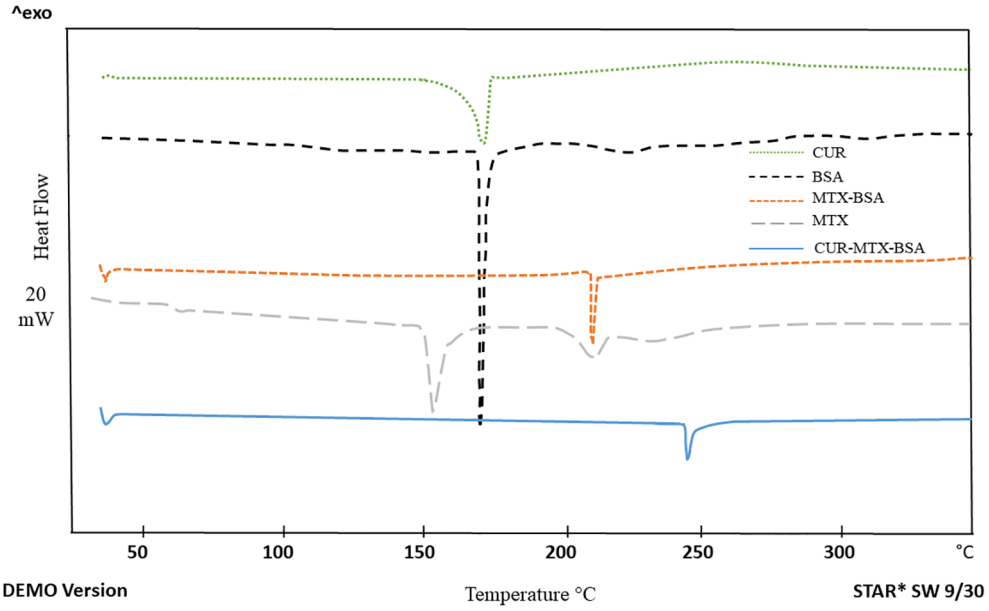

Fig. 3. DSC thermograms of CUR, MTX, BSA, MTX-BSA and CUR-MTX-BSA in the temperature range of 30–300 °C.

		Size (d.nm):	% Intensity:	St Dev (d.nm):
Z-Average (d.nm): 140.9	Peak 1:	188.7	95.7	111.1
PdI: 0.366	Peak 2:	4543	4.3	859.5
Intercept: 0.939	Peak 3:	0.000	0.0	0.000
Result quality : Good				

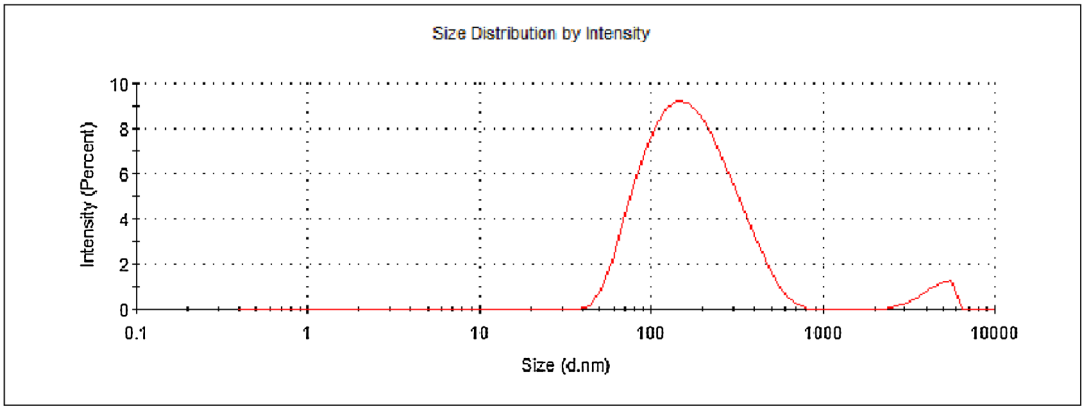

Fig. 4. The hydrodynamic size of final formulation CUR-MTX-BSA.

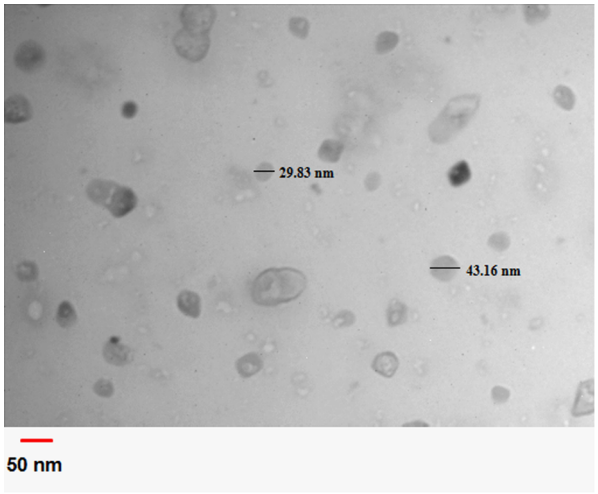

Fig. 5. The TEM image of CUR-MTX-BSA.

about pH = 7–8, this test was done in physiological pH. This test was used to evaluation of capability of enzymatic degradation of amidic bond and carrier. Besides, do not used to evaluation of pH value. It was obviously clear that the lysosomal compartment is best place for degradation and drug release.

Also, evidently, we can see that more CUR can be released with the decrease of pH. The nanoparticles in physiologic and acidic pH showed different drug release patterns and achieved comparable cumulative CUR release rates of 35 and 61% at 166 h, respectively.

Since the amidic bonds of carrier were cleavage in acidic medium the drug release from the as prepared nanoparticles was pH sensitive. As shown in Fig. 6b we can see that the cumulative release of CUR from CUR-MTX-BSA was very small at neutral pH (pH 7.4). This property is desirable in anticancer drug delivery due to the micro-environments of tumors area, lysosomes and endosomes are acidic, which can facilitate the drug release to suitable site.

3.4. Cytotoxicity assay

Hemolysis test was used to investigate the hemo-compatibility study. Fig. 7b shows comparison between samples and hemolytic assay results, this result showed and discovered that the hemolysis values are lower than 3% in experimental concentration (10 mg/ml). Based on ASTM to ASTMF 756-08 (ASTMF-756, 2009), Hemolytic values less than 3% produced by any material are mentioned as non-hemolytic.

To examine the inhibitory effect of BSA, MTX, MTX-BSA and CUR-MTX-BSA on 4T1 cells was studied as shown in Fig. 7a.

Untreated cells compared with exposed cells with BSA remained unchanged owing to their outstanding biocompatibility of BSA.

In contrast, when incubated with MTX-BSA, the number of living cells was markedly decreased with increasing of MTX concentration, and also when incubated with CUR-MTX-BSA the number of living cells was many significantly decreased with increasing of CUR concentration which is attributed to the released MTX and CUR from the nanoparticles.

As can be seen CUR-MTX-BSA resulted in a stronger tumor cell inhibition compared with free MTX. However, they all exhibited significantly higher cytotoxicity than BSA.

These results show that MTX-BSA and CUR-MTX-BSA nanoparticles reduced the IC50 value of free MTX on 4T1 breast cancer cells in comparison with free MTX.

3.5. Acute toxicity

Intravenous administration of varying doses of BSA (500 mg/kg, 1000 mg/kg and 2000 mg/kg) did not induce any toxic effect as evidenced by absence of animal death or deterioration of animal health.

4. Conclusion

In conclusion, a biodegradable and stealthy tumor targeted nanostructure has been successfully developed through a simple method for MTX and CUR delivery in living cell. As intrinsic structure of BSA, the reported tumor targeted nanocarrier in our work shows high biocompatibility and stability. This albumin based drug delivery system is dependent on the release of the MTX within the lysosomal compartment. Also, CUR loaded MTX-BSA exhibited attractive controlled release ability for CUR because of their pH responsiveness. These results show that MTX-BSA and CUR-MTX-BSA reduced the IC50 value of free CUR on 4T1 breast cancer cells in comparison with free MTX. This BSA-

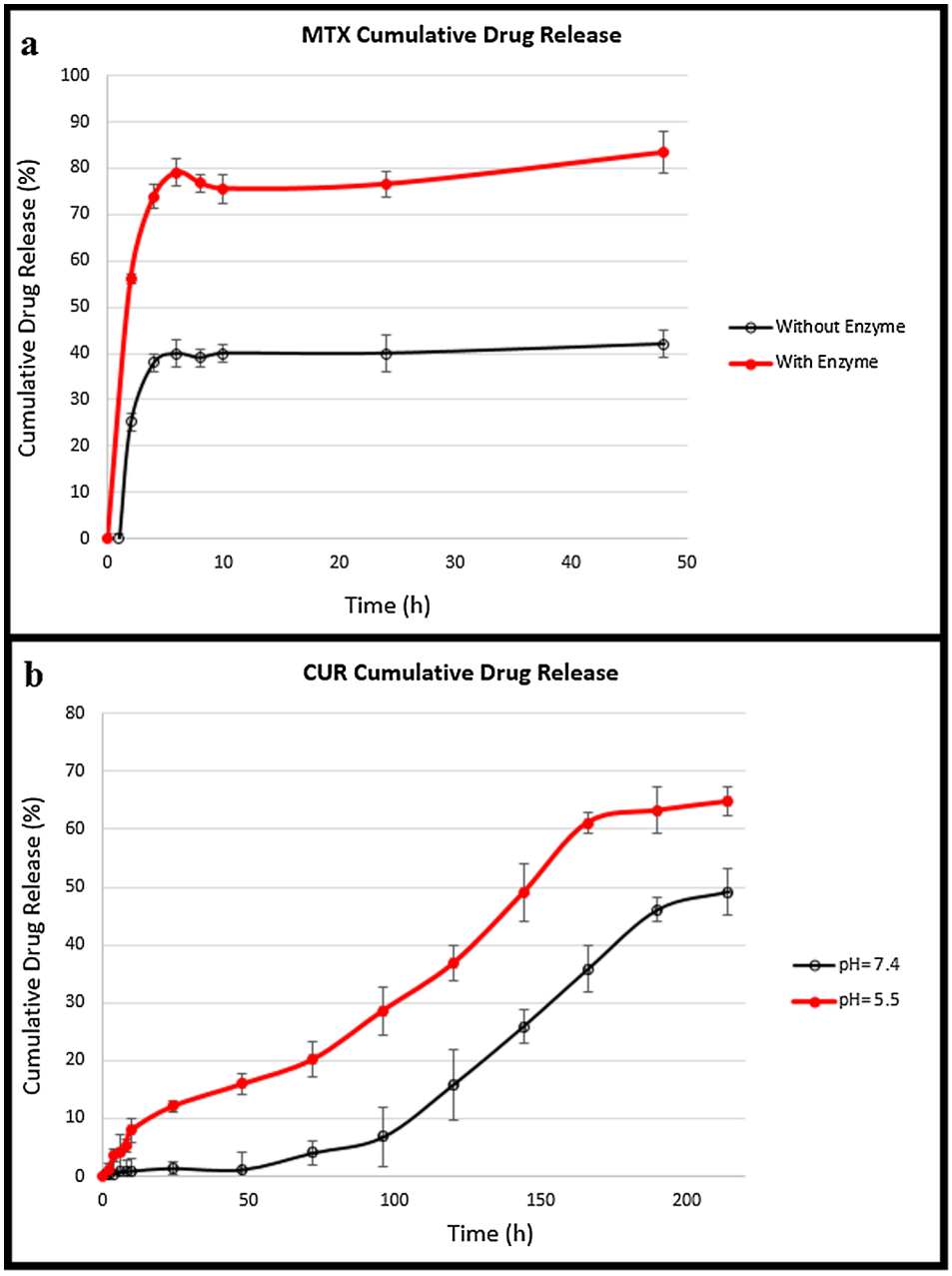

Fig. 6. The release profiles of the MTX from CUR-MTX-BSA with and without Proteinase K enzyme at pH 7.4 (a), and the release profiles of the CUR from CUR-MTX-BSA in different pH (b).

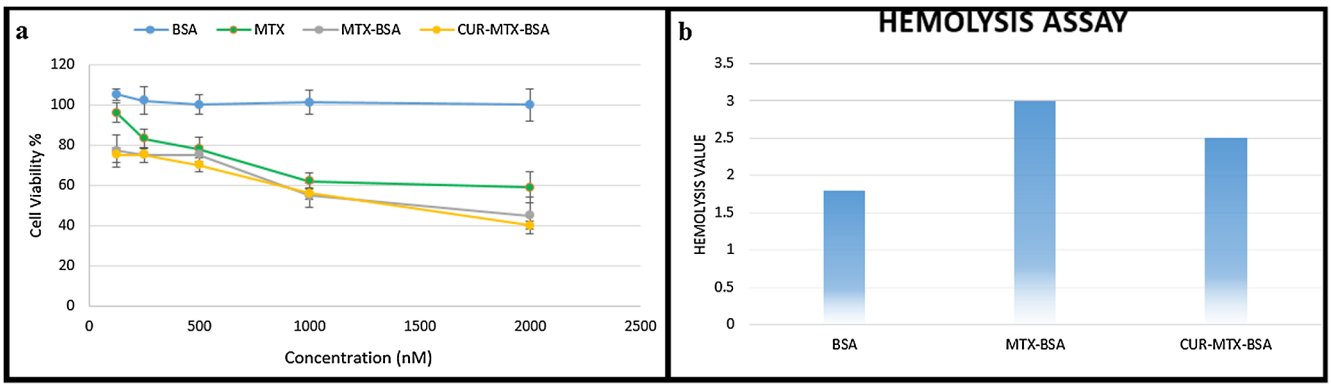

Fig. 7. Cytotoxicity analysis of BSA, free MTX, MTX-BSA and CUR-MTX-BSA against 4T1 cell line after incubated for 72 h (a), and hemolysis value of BSA, MTX-BSA and CUR-MTX-BSA (b).

F.A-CUR nanosystem represents an essential approach for efficiently drug delivery.

Acknowledgment

This work was supported by the deputy of research of Zanjan University of Medical Sciences (Grant no: A-12-430-20).

Ethical considerations

This study was approved by the Ethics Committee of the Zanjan University of Medical Sciences, and the study participants signed an informed consent.

Competing interests

The authors declare no competing interests.

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