

Role of Ultrasound in Anti-Cancer Drug Delivery Loaded on Microspheres

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ABSTRACT

This study investigate the role of ultrasound in anti-cancer drug delivery loaded on microspheres The 5-fluorouracil (5-FU) chitosan microparticles was prepared by the chemical cross-linking method. A total of 80 male Swiss albino mice weighting (20-25 g) received subcutaneous injections of 2×10^6 (Ehrlich ascites carcinoma) cells mammary in origin. A week later, the tumor bearing mice were divided into four main groups: group of 20 mice serves as a control untreated group, group of 20 mice which were injected with 5-FU only, group of 20 mice which were injected with blank chitosan microspheres, and a group of 20 mice which were injected with 5-FU chitosan microspheres. Each group was divided into two subgroups each of 10 mice, where one group was treated with ultrasonic waves and the other subgroup received no treatment with ultrasonic waves. Calculations were made 14 days after treatment in order to compare the volumes, inhibition ratios of the tumors and survival curves of each group. Results obtained indicate that tumor volume delayed 28 days by combined treatment of 5-FU chitosan microparticles and ultrasound than that with 5-FU chitosan microparticles alone.

Keywords: drug delivery, cancer, 5-fluorouracil, chitosan microparticles.

1 INTRODUCTION

Targeting is a new therapeutic tool for malignant tumor as a result of combining micro and nanotechnology with chemotherapeutics. Ultrasound has been shown to enhance degradation and drug delivery from biodegradable and non biodegradable polymeric devices. If a microsphere is partially filled with an entrapped drug substance, it is then able to transport the drug through blood vessels and release its load upon being triggered by an ultrasound pulse, which cracks the shell [1]. Drug targeting by ultrasound requires the use of sharp ultrasound waves focused on the tumor and does not require ultra-high ultrasound energies. It has also been shown that at constant frequency; drug release increases with increasing power density. Optimal power density of ultrasound waves ranges from 1 to 5 W cm⁻², depending on the time, period of sonication which is typically 30 s up to a few minutes when continuous wave ultrasound is applied [2]. The present work aims to study the effect of ultrasonic waves and chitosan polymer microspheres

loaded with 5-fluorouracil (5-FU) in improving the tumoricidal effect on mice bearing Ehrlich ascites carcinoma.

2 MATERIALS AND METHODS

I- preparation and characterization of microspheres

The chitosan microspheres were prepared by chemical cross-linking method as described by Dubey and Parikh [3]. Briefly, 100 ml of paraffin oil was mixed with 1 ml of span 80. To this, 3 mL of chitosan solution was added dropwise with continuous stirring at 2000 rpm. After the complete addition of chitosan solution, 0.25 mL of glutaraldehyde was added to the mixture three times, twice after 1 hour and then after 2 hours, respectively. Suspension obtained was allowed to stand for 1 hour. Microspheres obtained as residue were washed 4 times with ether. After the final wash, microspheres were allowed to dry in air.

The loading of 5-Fluorouracil on prepared chitosan microspheres was carried out by keeping 100 mg chitosan microspheres in 20 ml phosphate buffer solution (pH 5) containing 10 mg of 5-fluorouracil for 48 h. The amount of 5-fluorouracil loaded on microspheres was determined by recording the absorbance of the loading solution at $\lambda = 257$ nm using spectrophotometer. The prepared chitosan microspheres were characterized by analysis of particle size, scanning electron microscope, Fourier-transform Infrared Spectroscopy (FTIR), X-ray Diffraction (XRD) and thermogravimetric Analysis (TGA). Encapsulation efficiency (EE) was calculated using the equation:

$$EE \% = \frac{\text{Actual drug content}}{\text{Theoretical drug content}} \times 100 \quad (1)$$

To investigate the drug release behavior of prepared microspheres at 25 °C, microspheres loaded with 100 mg 5-Fu were kept in 20 ml phosphate buffer solution (pH 7). The amount of 5-Fu released in solution at different time intervals with different ultrasound intensities (Watt/cm²) was estimated recording absorbance at $\lambda_{max} = 257$ nm of filtered release medium using Shimadzu UV-VIS-1601 PC spectrophotometer.

II- In vivo study

A total of 80 male Swiss albino mice 6 weeks age of mean weight 25±2 gm (purchased from Faculty of Medicine,

Alexandria University, animal house). 2×10^6 Ehrlich ascites carcinoma cells, mammary in origin, diluted approximately (1-4) in 0.9% saline were injected into the thigh of each mice. A week later, the tumor reached approximately a size of about 0.5-1 cm in diameter. The tumor bearing mice were divided into four main groups: group of 20 mice serves as a control untreated group, group of 20 mice which were injected with 5-FU only, group of 20 mice which were injected with blank chitosan microspheres, and a group of 20 mice which were injected with 5-FU chitosan microspheres. Each group was divided into two subgroups each of 10 mice, where one group was treated with ultrasonic waves and the other subgroup received no treatment with ultrasonic waves. To study the cytotoxic effect of 5-fluorouracil loaded chitosan microspheres irradiated with ultrasonic waves, tumor volume, tumor inhibition ratio, mice survival time and increased life span were calculated.

3 RESULTS AND DISCUSSION

The particle size distribution curve of prepared chitosan microspheres (Fig 1) shows sharp distribution range of microspheres, with 90% of spheres in size range of 280–560 nm with average particle size of 422 nm and only 10% were oversized. The shapes of the dried microspheres were completely spherical, and the surface was rough and unfolded as shown by SEM (Fig 2, a). Furthermore, the cross-section view showed that cavities inside the microspheres were hollow (Fig 2, b).

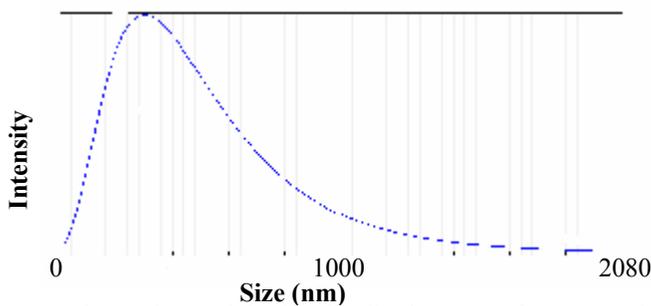


Fig 1: The particle size distribution curve for prepared hollow chitosan microspheres.

The FTIR spectra of chitosan and cross-linked chitosan (Fig 3) showed a characteristic band attributed to $-\text{NH}_2$ and $-\text{OH}$ groups stretching vibration and the band for amide I is seen in the infrared spectrum of chitosan. Whereas in the FTIR spectra of cross-linked chitosan two new peaks appear and others disappear. The disappearance of the band could be attributed to the cross-linking process [4].

XRD spectra of chitosan showed two prominent crystalline peaks (Fig 4). In the case of cross-linked chitosan there was significant decrease in the intensity of characteristic peaks of chitosan, which was in agreement with the study reported by Wan et al [5]. The distinct differences in the diffraction patterns of chitosan and cross-linked chitosan could be attributed to modification in the arrangement of

molecules in the crystal lattice, which might be due to amorphization.

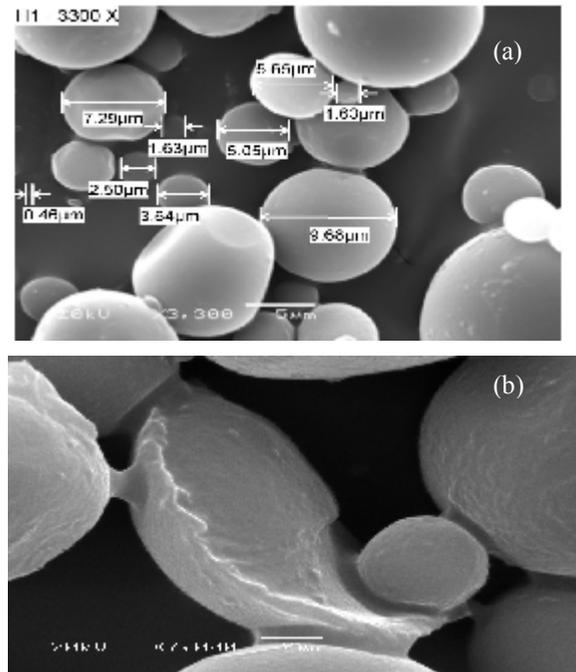


Fig 2: SEM image of chitosan microspheres (a) (X 3000) and Cross-section view of hollow microsphere showing cavity of chitosan microsphere (b) (X 7000).

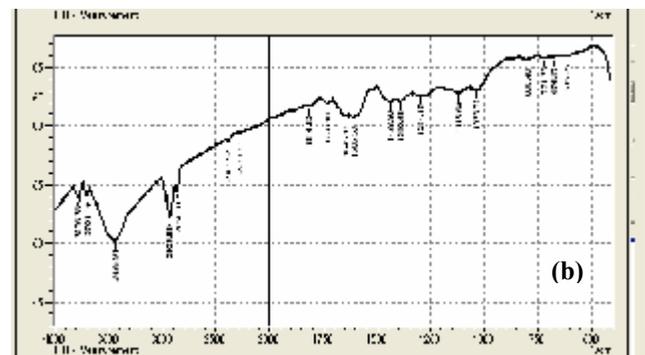
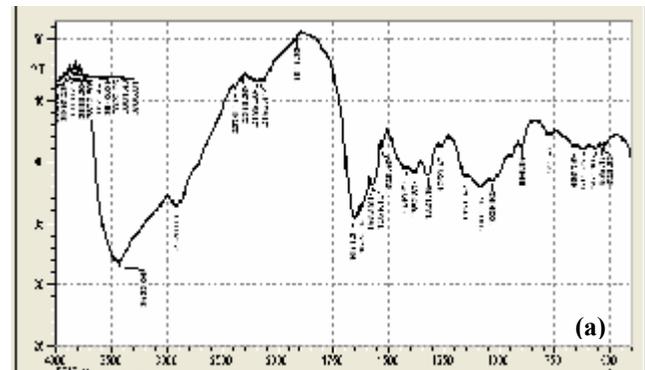


Fig 3: Fourier-transform Infrared spectra of chitosan powder (a) and crosslinked chitosan microspheres (b).

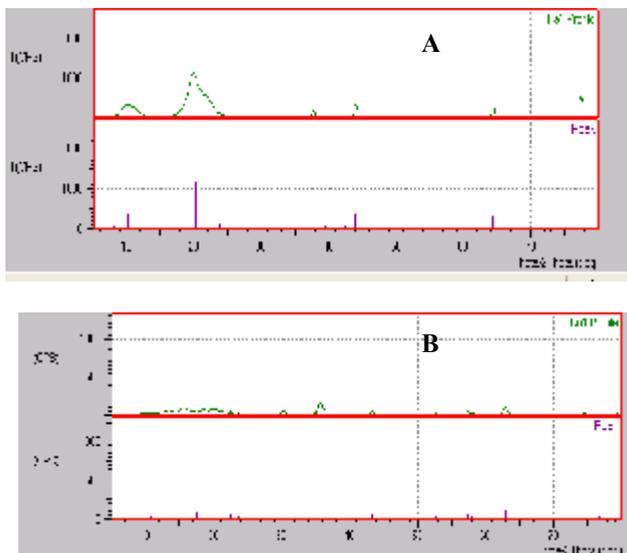


Fig 4: X-ray diffraction pattern of chitosan powder (A), cross-linked chitosan microspheres (B).

TGA (fig 5) showed that the chitosan powder and cross-linked microspheres showed strong transitions peaks were found at 210-230 °C and at 211-298 °C respectively. These results are in agreement with the work done by Piyakulawat et al [4].

The percentage drug entrapment (PDE) was 33.09%. The amount of drug load was 132 µg drug/mg microspheres. The percent of 5-fluorouracil released from chitosan microspheres was 97.73% after 30 min exposure for 0.8 MHz pulsed ultrasound waves at 1 W/cm². Similar results are described by Dubey and Parikh [3].

In vivo study

After 15 min sonication of pulsed ultrasound of 0.8 KHz at 1 W/cm², mice treated with 5-Fu microspheres showed a significant decrease in tumor volume, decreased tumor volume ratio and increased inhibition ratio of tumor in mice injected with 5-FU chitosan microparticles and ultrasound (Table 1). Tumor volume delayed 28 days by combined treatment of 5-FU chitosan microparticles and ultrasound than that with 5-FU chitosan microparticles alone (Fig 6). Significant increase in mean survival time in mice injected with 5-FU chitosan microparticles and ultrasound (Fig 7 & table 2).

These results are in accordance with that obtained by Marin et al [2]. The acoustically-enhanced drug up-take from microspheres may result not only from the ultrasound-triggered drug release from microspheres that results in the enhanced uptake of free drug but also from the perturbation of cell membranes that increases the uptake of the drug encapsulated in microspheres.

Ultrasound induces formation of cavitation regions in the cell membrane, which results in increased membrane permeability. This process, called sonoporation, is

responsible for enhanced intracellular uptake of both the released and microspheres-encapsulated drug [6].

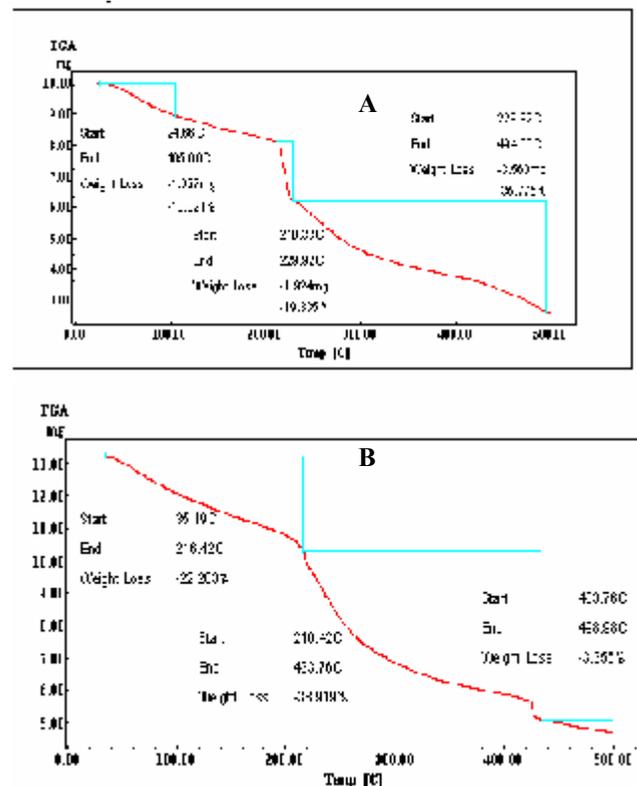


Fig 5: Thermogravimetric analysis of chitosan powder (left), cross-linked chitosan microspheres (right).

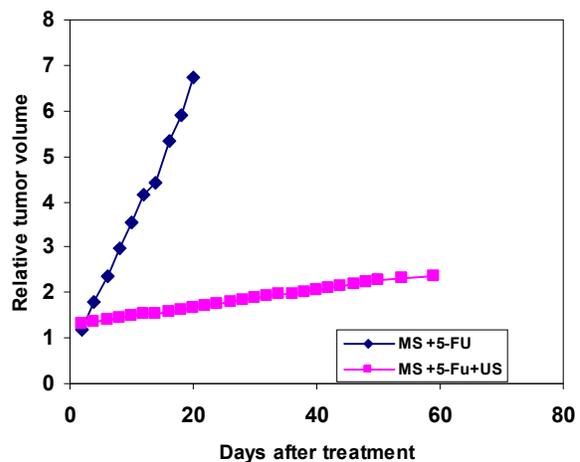


Fig 6: Tumor growth curves for the group injected with 5-FU loaded chitosan microspheres without and with ultrasound treatment.

Focusing ultrasound on the tumor provides three important advantages:

- I- Ultrasound increases the permeability of blood vessels [6], thus increasing microspheres extravasation at the tumor site.

II- Sonication enhances drug release from microspheres, and subsequently increases the concentration of the free (non-encapsulated) drug at the tumor site.

III-Ultrasound mediated perturbation of cell membrane and other cellular structures results in formation of cavities along the cell membrane (sonoporation), thereby increasing the uptake of microspheres-encapsulated drugs.

All three factors work in synergy to ensure localized and effective drug uptake at the tumor site.

Table 1: Tumor volume (V), tumor volume ratio (TVR), and tumor inhibition ratio (TIR) after treatment (mean±SD) without and with ultrasound treatment.

Groups	V (mm ³)		TVR	TIR
	Before treatment	After treatment		
Control	554± 68	2976±272	5.97	
MS	486 ± 44	2474±139	5.28	2.21
MS + 5-FU	477± 61	2105 ± 629	4.65	11.49
5-FU	468 ± 43	1537±94	3.28	35.69
Control +US	498±84	2406±336	4.83	
MS + US	468 5±43	2282±313	4.87	23.13
MS + 5-FU + US	452±49	873±69*☼	1.93	75.22
5-FU + US	449±48	1181±850*	2.63	50.92

* Significant compared with control group; ☼ Significant compared with group injected with 5-FU; ☼ Significant compared with with group injected with MS.

Table 2: Mean survival time and increased life span (%ILS) (mean±SD) without and with ultrasound exposure.

	Groups	Mean survival time (d)	ILS %
No US treatment	Control	12.80 ± 1.98	
	MS	9.40 ± 1.38	-26.56
	5-FU	21.60 ± 3.44	68.75
	MS+5-FU	12.60 ± 1.89	1.56
With US treatment	Control	17.0 ± 2.50	
	MS	12.20 ± 1.85	-28.24
	5-FU	23.0 ± 3.58	35.29
	MS+5-FU	35.60 ± 5.56	101.09

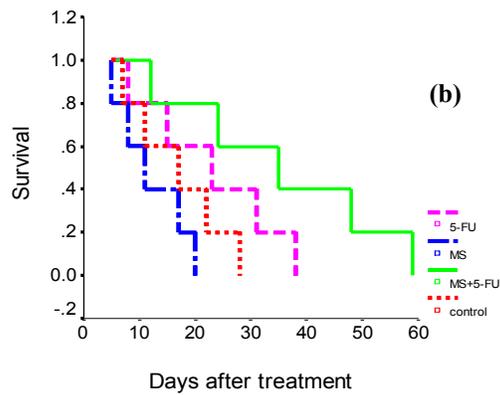
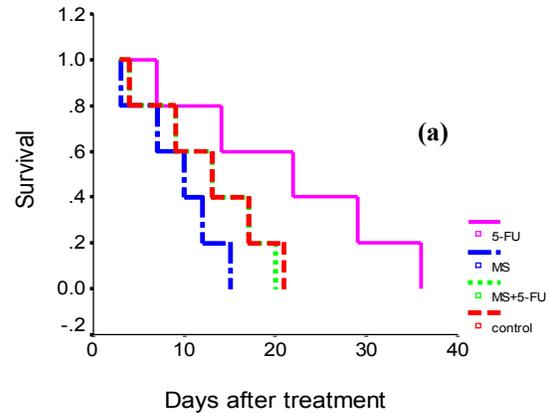


Fig 7: Kaplan–Meier survival curves for the four subgroups (a) without and (b) with ultrasound treatment.

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