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International Journal of Pharmaceutics 307 (2006) 168–174
INTERNATIONAL JOURNAL OF PHARMACEUTICS
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Mitoxantrone-loaded BSA nanospheres and chitosan nanospheres for local injection against breast cancer and its lymph node metastases I: Formulation and in vitro characterization

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Received 21 June 2005; received in revised form 11 September 2005; accepted 24 September 2005
Available online 28 November 2005

Abstract

Positively charged mitoxantrone (MTO) was absorbed by negatively charged blank bovine serum albumin (BSA) and chitosan (CS) nanospheres to form MTO–BSA–NS and MTO–CS–NS, respectively. In addition to other conditions, values of pH of every step were optimized. On optimized conditions, MTO–BSA–NS of a mean size of 77 nm with an encapsulation yield (EY) of (98.86 ± 1.43)% [drug loading rate (DL) (19.82 ± 0.29)%] and MTO–CS–NS of a mean size of 75 nm with an EY of (97.57 ± 1.00)% [DL (9.78 ± 0.10)%] were obtained. After lyophilization and sterilization by ^{60}Co, the mean size increased about 10% but no significant change was observed in EY and DL. Tests for in vitro release in physiological saline or physiological saline containing 0.5% (w/v) ascorbic acid by a dialysis bag showed sustained release and little burst effect.

Keywords: Mitoxantrone; Bovine serum albumin; Chitosan; Nanospheres; Nanoprecipitation; Ion gelation; Lyophilization

1. Introduction

Breast cancer is one of the most frequently occurring cancers in women, and the second leading cause of cancer deaths in women. Even after the surgical operation, lymph node metastases are still the most serious problem that may cause secondary cancer (Li, 2000).

Mitoxantrone (MTO) is often used to treat breast cancer clinically. But its side-effects, such as heart toxicity and myelosupression, in particular leukopenia, are often a problem.

The lymphatic capillaries usually have open intercellular junctions with a size of 30–120 nm in the endothelium, which can act as channels for colloidal particles to pass into the lymphatic system. It has been demonstrated that particles less than 100 nm in size are preferable for lymph node targeting (Phillips et al., 2000).

BSA molecules are amphoteric and chitosan (CS) molecules contain amine and imine groups. BSA itself can form negatively charged nanospheres, and CS, though it cannot be negatively charged itself, can be transformed into negatively charged nanospheres by ion gelation method. MTO can be positively charged. A method was designed and optimized to prepare MTO–BSA–NS and MTO–CS–NS with high encapsulation yield (EY) by mixing the nanospheres with MTO.

In an attempt to inhibit the primary breast cancer and to cure and prevent its lymph node metastases, MTO–BSA–NS and MTO–CS–NS with a mean size of less than 100 nm for local injection were prepared, characterized and comparatively investigated.

2. Materials and methods

2.1. Instruments, reagents and animals

Cryogenic ultracentrifuge (L8-50M, Beckmann, USA), thermostatic magnetic stirrer (model 85-2, Sile Instrument Co., Shanghai, China) were used for preparation of nanospheres. Lazer particle sizer (Mastersizer 2000, Malvern, UK) and a transmission electron microscope (JEM-100SX, Electron Co., Japan) were used to characterize the nanospheres. A spectrophotometer (UV-2201, Shimazu, Japan) was used to determine the drug contents in solutions. Freeze-dryer (type 2040, Snijfers Sci-

DOI of original article:10.1016/j.ijpharm.2005.09.038.
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doi:10.1016/j.ijpharm.2005.09.037

entific Ltd., Holland) and ^{60}Co-γ radiator (Agricultural Institute of Sichuan, China) were also used. Mitoxantrone (Batch no. 20001009) was a gift from Sichuan Shenghe Pharmacy, China. Bovine serum albumin (BSA, Batch no. 980407, Lizhudongfeng Biotech Co. Ltd., Shanghai, China), chitosan (CS, Sigma), sodium tripolyphosphate (STPP) and other reagents of reagent grade were used.

2.2. Preparation of nanospheres

2.2.1. MTO–BSA–NS

A two-step method was used to prepare MTO–BSA–NS. Firstly, the blank BSA–NS were prepared by nanoprecipitation/solvent diffusion method. The basic procedures were as follows. To 1% (w/v) BSA was added 1% (w/v) NaOH to adjust pH to 7.0. Then acetone of four times the volume was added under stirring to form a light blue opalescent colloidal solution of BSA. BSA–NS were formed after being stirred at (90 ± 2) °C for 30 min.

The BSA–NS colloidal solution was cooled down to room temperature and adjusted to pH 8.0. MTO was dissolved in pure acetic acid and water added. The solution was adjusted to pH 8.0 by 3 M NaOH and mixed with BSA–NS colloidal solution (weight ratio BSA/MTO = 4/1) and stirred for 10 min.

2.2.2. MTO–CS–NS

Blank CS–NS were prepared by ion gelation method first. CS was dissolved in 0.05 M acetic acid to make a 0.2% (w/v) solution and 1 M NaOH added to adjust the pH to 4.6. 0.4% (w/v) STPP (gelation agent) solution was added (weight ratio CS/STPP = 10/3) to the mixture under stirring to produce a stable colloidal solution of CS–NS.

MTO–CS–NS were prepared by mixing CS–NS with MTO solution. The CS–NS colloidal solution was first concentrated by addition of sucrose to make a 55% (w/v) sucrose solution and centrifuging at 9000 × *g* using Cryogenic ultracentrifuge for 30 min. The precipitate was redispersed in water to obtain CS–NS colloidal solution [0.5% (w/v) of CS]. The colloidal solution was adjusted to pH 5.5, then mixed thoroughly with a MTO solution of pH 5.5 at a weight ratio MTO/CS of 1/10.

2.3. Quantification of MTO in solutions

P_2O_5-dried MTO was used to prepare the stock solution, which was diluted with 0.5% (w/v) ascorbic acid in physiological saline (designated Vc-saline) to prepare the standard solutions of different concentrations. Absorbance at 609 nm of the standard solutions was measured and the regression equation (A) obtained in the MTO concentration range of 2.72–24.52 μg/ml was:

$$A_{609\,\mathrm{nm}} = 3.5815 \times 10^{-3} + 0.0402C, \quad r = 0.9999 \qquad \text{(A)}$$

When ethanol with 0.1 M HCl (3:1, v/v), designated ethanol–HCl, was used as the solvent to prepare the MTO solution, $A_{611\,\mathrm{nm}}$ was measured in the MTO concentration range of 2.02–20.2 μg/ml and the regression equation (B) was also obtained.

$$A_{611\,\mathrm{nm}} = 5.1378 \times 10^{-3} + 0.04762C, \quad r > 0.9999 \qquad \text{(B)}$$

For in vitro release tests, MTO samples were diluted with Vc-saline, and determined spectrophotometrically at 609 nm using equation (A).

For determination of EY of MTO–BSA–NS and MTO–CS–NS, samples (supernatant after centrifugation) were diluted with ethanol–HCl and determined spectrophotometrically at 611 nm using equation (B). Six hundred and nine and 611 nm were the maximum absorption wavelengths in the corresponding medium, respectively.

2.4. Determination of nanosphere yield of BSA–NS and removal of acetone residue

Different concentrations (C = 0.3–1.6 mg/ml) of BSA were used to measure the absorbance at 278 nm (the maximum absorption wavelength of BSA). The standard regression equation was obtained:

$$A_{278\,\mathrm{nm}} = 0.0102 + 0.5881C, \quad r > 0.9999.$$

The amount of W_{total} was used to produce BSA–NS, and the BSA–NS were dialyzed (3 × 500 ml water, each 1 h) to separate small molecules. Then the BSA–NS were centrifuged at 90,000 × *g* for 30 min, and the supernatant used for determination of $A_{278\,\mathrm{nm}}$ to calculate the weight of free BSA, W_{free}. The nanosphere yield was calculated by

$$\text{yield of BSA-NS} = \frac{W_{\mathrm{total}} - W_{\mathrm{free}}}{W_{\mathrm{total}}} \times 100\%$$

The boiling point of acetone is 56.5 °C. After thermal cross-linking at 90 °C for 30 min, almost no acetone was left. Acetone residue was further removed by dialysis. BSA–NS were put in a Visking bag and dialyzed for 1 h against 150 ml of water. After three times of dialysis, the absorbance at 264 nm (absorption peak of acetone) was below 0.004, which indicated the removal of acetone was almost complete.

2.5. Determination of the cross-linking rate of BSA–NS and encapsulation yield

Two portions of the same volume of BSA–NS were taken. To one portion was added guanidine hydrochloride until its concentration was 6 M (solution a), another portion was diluted by water to the same volume (solution b). After standing in the room temperature for 1 h, the turbidity at 540 nm of the two solutions was measured and the ratio of T_a/T_b used as a measure of cross-linking rate.

5.0 ml MTO–BSA–NS was centrifuged at 90,000 × *g* for 30 min. The supernatant was diluted with ethanol–HCl and determined spectrophotometrically at 611 nm, and the amount of MTO in the supernatant, $W_{\mathrm{free\,MTO}}$, was obtained. The total amount of MTO in the 5.0 ml added during the preparation of MTO–BSA–NS was designated $W_{\mathrm{total\,MTO}}$. EY was calculated

according to the following equation:

$$EY = \frac{W_{\text{total MTO}} - W_{\text{free MTO}}}{W_{\text{total MTO}}} \times 100\%$$

2.6. Lyophilization and sterilization

Fifty milligram of lactose was dissolved in 1 ml MTO–BAS–NS and 100 mg of lactose dissolved in 1 ml MTO–CS–NS. The mixtures was stored at −50 °C for 5 h, and then put into the cryogenic freeze-dryer for lyophilization under vacuum for 36 h.

The lyophilized MTO–BAS–NS or MTO–CS–NS were radiated with 10 kGy ^{60}Co for sterilization, and then examined for asepsis (Pharmacopoeia of PR China, 2000, Appendix XI H) and particle size.

2.7. Tests for in vitro release

Visking bag method was used to investigate in vitro release. Free MTO solution was tested first. The solution was transferred into the dialysis bag, and dialyzed in physiological saline or Vc-saline, which was thermostated at (37 ± 0.5) °C and stirred at 75 rpm. After designated time periods, a portion of the dialysis solution was taken for measurement of MTO at 609 nm and the same volume of fresh medium added.

Sterilized lyophilized MTO–BSA–NS or MTO–CS–NS were redispersed in water and tested the same way.

3. Results and discussion

3.1. Preparative technology

3.1.1. MTO–BSA–NS

3.1.1.1. Blank BSA–NS. For nanoprecipitation/solvent diffusion method, ethanol, acetone and ethyl acetate were reported as the organic solvent in literature (Chen et al., 1994). BSA has a molecular weight of 66,000 with an isoelectric point of 4.77. Taking the amphoteric characteristic of BSA into consideration, we adjusted the pH of BSA solution to form negatively charged BSA, then the formed BSA–NS of small size could hardly coagulate when the organic solvent was added because of the electrostatic repulsion force between the particles. We tested the nanoprecipitation/solvent diffusion method using ethanol, ethyl acetate and acetone, respectively, and found that only acetone could form nanospheres with small size and high nanosphere yield on the same conditions. In our experiment, acetone was used as the organic solvent.

After addition of acetone, BSA is isolated from the solution due to decrease of solubility. But BSA can dissolve again when acetone is evaporated or diluted. So cross-linking is necessary for preparation of stable BSA–NS.

Thermal cross-linking of BSA might be an amide-formation reaction between the amino group and adjacent carboxyl group (Esposito et al., 1996). Weber et al. (2000) reported that colloidal particles of albumin could not be stabilized at 50 °C for 48 h or 60 °C for less than 24 h, and BSA microspheres were reported to be stabilized at over 100 °C (Wu et al., 1999). Sugibayashi et al. (1979) reported that 5-fluorouracil-loaded albumin microspheres thermally cross-linked at 100, 150 and 180 °C, respectively, for 10 min showed different in vitro release behavior: the higher the temperature, the slower the release rate.

Preliminary tests showed that when BSA ≥ 2% (w/v), very viscous solution was formed after thermal cross-linking. Hence, 1% (w/v) BSA was used for thermal cross-linking.

The conditions for preparation of BSA–NS by thermal cross-linking were optimized by single factor method. pH value was optimized first. One percent (w/v) BSA solutions of pH 3.0, 5.5, 6.0, 7.0 and 9.0 were tested, respectively. $2V_0$ (V_0 being the volume of BSA solution) acetone was added before thermal cross-linking at (80 ± 2) °C for 30 min. Solutions at pH 3.0 or 9.0 resulted in low nanosphere yield due to hydrolysis of BSA. At pH 5.5, the resulted solution looked like milk and coagulated slices could be observed under microscope and precipitates formed at the bottom of the container after standing. Only BSA–NS formed at pH 6.0 and 7.0 were stable, but the turbidity at 540 nm of the BSA–NS formed at pH 7.0 was significantly smaller (0.844 ± 0.043 compared to 1.692 ± 0.262 at pH 6.0, $P < 0.032$), which indicated the particle size was smaller, and its cross-linking rate significantly higher [(65.67 ± 0.66)% compared to (44.59 ± 2.07)%, $P < 0.0036$]. Theoretically, BSA at pH 7.0 should be more negative than at pH 6.0. Hence, pH 7.0 was used for the following experiments.

The influence of the amount of the acetone showed a saturation effect as shown in Table 1: nanosphere yield, cross-linking rate (T_a/T_b) and turbidity at 540 nm all increased as the increasing volume of acetone. After $V = 4V_0$, they had no significant change (compared $V = 5V_0$ with $V = 4V_0$, $P > 0.05$). $4V_0$ was used for the following experiments.

At (80 ± 2) °C for 15, 30 and 60 min, respectively, nanosphere yield, cross-linking rate and turbidity all increased with increasing time period. But when time >30 min, coagulated pieces of BSA were observed. Hence, 30 min was used for the following experiments.

In 30 min time period, the results of BSA–NS formed at (80 ± 2), (90 ± 2), (100 ± 2) and (120 ± 2) °C showed insignificant difference ($P > 0.05$) in terms of cross-linking rate and turbidity. (90 ± 2) °C was used for the following experiments because of the highest nanosphere yield [94%, compared to 91.6, 91.8 and 92.6% for (80 ± 2), (100 ± 2) and (120 ± 2) °C, respectively].

The optimized conditions for preparation of BSA–NS by thermal cross-linking based on the above experiments were as described in Section 2.2.1.

Table 1
Influence of acetone volume on the preparation of BSA–NS

V_{acetone}	Nanosphere yield (%)	T_a/T_b (%)	T_{540}
V_0[a]	67.19 ± 4.23	38.60 ± 2.04	0.375 ± 0.025
$2V_0$	89.68 ± 8.17	49.07 ± 5.05	0.508 ± 0.032
$3V_0$	91.03 ± 4.52	62.36 ± 2.09	0.819 ± 0.025
$4V_0$	91.60 ± 1.23	65.67 ± 0.66	0.844 ± 0.043
$5V_0$	91.62 ± 1.32	65.48 ± 0.75	0.906 ± 0.038

[a] V_0 standing for the volume of BSA.

3.1.1.2. MTO–BSA–NS. Aqueous solution of MTO was mixed with BSA–NS to produce MTO–BSA–NS. MTO hydrochloride ($C_{22}H_{28}N_4O_6 \cdot 2HCl$) is a polyprotonic acid, with $pK_{a1} = 5.99$ and $pK_{a2} = 8.6$. MTO appears as positive ions at pH < 8.6, which could be easily absorbed by negatively charged and cross-linked BSA–NS to form MTO–BSA–NS with high EY.

Aqueous solution of MTO was prepared by dissolving MTO in a small amount of pure acetic acid and adjusting pH to 7.0 after addition of water. The MTO solution was mixed with BSA–NS colloidal solution (pH 8.0) and stirred for 10 min. The mixture was used as the MTO–BSA–NS. 1/6, 1/4, 1/3, 1/2 and 1/1 of weight ratio of MTO/BSA were tested, respectively. EY was determined as the evaluation index. It was found that, when the weight ratio was larger than 1/4, EY significantly decreased as the weight ratio increased ($P < 0.0072$). Hence, weight ratio of 1/4 was used in the following tests.

The aqueous solution of MTO was adjusted to pH values of 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0, respectively. After mixing with BSA–NS, EY was found to increase as the pH values increased until a plateau came after pH 8.0. Why was pH 8.0 better than 7.0 or 6.0? In the pH range of 4.77 < pH < 8.6, BSA would be negatively charged and MTO positively charged. According to the equation $pH = pK_a + \log([\text{basic form}]/[\text{acidic form}])$, for MTO ($pK_{a2} = 8.6$), the acidic form (positively charged MTO) would be about 80% of its total amount at pH 8.0. After the acidic form was absorbed by BSA–NS, more acidic form would be quickly transferred from the basic form. For cross-linked BSA–NS, pH 8.0 gave more negatively charged particles than pH 7.0 or 6.0. This may explain why pH 8.0 gave higher EY. That pH 8.0 and 9.0 gave almost the same EY indicated that the negative charge of BSA–NS became saturate after pH 8.0. At pH 9.0, though the acidic form of MTO was only about 29% of its total amount (calculated from the above equation), more acidic form could be quickly transformed from its basic form when it was absorbed by BSA–NS.

The ionic strength of the MTO solution was optimized using water or NaCl solutions of different concentrations. EY was found to decrease with increasing ionic strength. Based on the above tests, the optimized conditions for preparation of MTO–BSA–NS were as described in Section 2.2.1.

The EY of MTO–BSA–NS prepared under the optimized conditions was (98.86 ± 1.43)% [DL (19.82 ± 0.29)%] with a mean diameter of 77 nm and polydispersity index (PDI) of 0.3162. Particle size and its distribution of BSA–NS thus prepared are shown in Fig. 1.

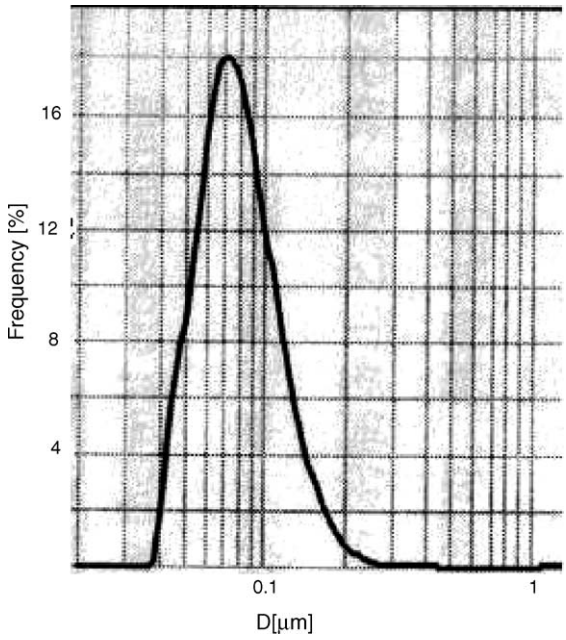

Fig. 1. Particle size and its distribution of MTO–BSA–NS by thermal cross-linking.

3.1.2. MTO–CS–NS

3.1.2.1. Blank CS–NS. The conditions for preparation of CS–NS were optimized by single factor method.

Various gelation agents were compared, including NaOH, sodium carboxylmethylcellulose (CMC-Na) and STPP. Gradual addition of NaOH solution decreased the solubility of CS and slice precipitates formed when pH > 6.0, which gradually dissolved without formation of opal colloidal solution when an acid was added dropwise. CMC-Na could form CS–NS when few drops of 1% (w/v) CMC-Na solution were added to a CS solution of pH 2.7. Unfortunately, these CS–NS were very unstable and changed into flocculent precipitate when NaOH was added to raise the pH value. When 1% (w/v) STPP solution was used as the gelation agent, 2% (w/v) CS solution could form stable colloidal solution. Then pH value of 2% (w/v) CS solution was optimized. Two percent (w/v) CS solutions of pH 3.5, 4.0, 4.6, 5.0 and 5.5 were added dropwise various amounts of 1% (w/v) STPP solution under stirring, respectively. It was found that slices of CS precipitated when pH ≥ 5.0. Stable CS–NS could be formed only on the following conditions: pH 3.5–4.0, $W_{CS}:W_{STPP} = 10{:}1–10{:}2$; or pH 4.6, $W_{CS}:W_{STPP} = 10{:}1–10{:}3$. Addition of STPP solution to a CS solution of pH 4.0 gave a stable opal colloidal solution, of which pH only raised to about 4.2, and the opal appearance remained unchanged even when pH was adjusted to 5.5.

The weight ratio of CS to STPP affected the stability and EY of CS–NS. When W_{CS}/W_{STPP} weight ratio was 10/1 or 10/2, STPP was insufficient and precipitates formed after pH was adjusted to 5.5. But when $W_{CS}/W_{STPP} > 10/3$, flocculent precipitates of CS were formed and EY decreased. Hence, $W_{CS}/W_{STPP} = 10/3$ was used.

From a comparison of the results of CMC-Na and STPP, one may predict that, since the CS–NS with STPP as the gelation agent had extraordinary stability, the mechanism of its gelation (and even the structure of the CS–NS) might be unique. CS, a positive polyelectrolyte, has a pK_a value of 6.5, so it mainly exists as positive ions at pH < 6.5. The possible explanation for STPP gelation is that the negatively charged polyvalent polyphosphate ions as the counter ion not only showed a gelation effect, but also entered into the adsorbed layer of CS particles and made the CS–NS charged negatively thus still stable when NaOH is added. A test of electrophoresis evidenced that, after gelation with STPP and pH being adjusted to 5.5, the formed CS–NS migrated towards the anode, which was visible based

on its pale blue opalescence. The formed negatively charged CS–NS might favor the high EY of MTO–CS–NS when positively charged MTO was added.

3.1.2.2. MTO–CS–NS. CS–NS formed at pH 3.5 (a), 4.0 (b) and 4.6 (c) by addition of STPP were tested, respectively, to mix with MTO (weight ratio MTO/CS = 1/10). When the obtained CS–NS were directly mixed with neutral MTO solution, no MTO could be encapsulated, i.e. EY = 0. When the three CS–NS colloidal solutions were adjusted to pH 5.5, respectively, and then neutral MTO solution added. The EY obtained was (a) (34 ± 1.45)% (n = 3), (b) (56 ± 2.40)% (n = 3) and (c) (59.59 ± 2.61)% (n = 3), respectively. Hence, pH 4.6 was thus used in the following tests.

When CS–NS of pH 5.5 and MTO solution of pH 5.5 were mixed, a weight ratio of MTO/CS = 1/10 gave EY = (72.0 ± 2.1)%, and a weight ratio of 1/7 gave a significantly smaller EY [(66.2 ± 1.9)%, $P < 0.0034$]. Hence, weight ratio of 1/10 was used.

CS content in the CS–NS colloidal solution was only about 0.12% (w/v). To concentrate the CS–NS, the colloidal solution was added sucrose to form a 55% (w/v) sucrose solution and centrifuged at 9000 × g for 30 min. The precipitate was redispersed in water to obtain 0.5% (w/v) CS–NS and adjusted to pH 5.5, which was mixed with MTO solution of pH 5.5 (weight ratio MTO/CS = 1/10) to obtain MTO–CS–NS. EY increased to (97.57 ± 1.00)% [compared to (72.0 ± 2.1)% before centrifugation, $P < 0.00001$].

Based on the above results, the optimized conditions for preparation of MTO–CS–NS were as described in Section 2.2.2. MTO–CS–NS was obtained (shown in Fig. 2) with EY of (97.57 ± 1.00)% [DL (9.78 ± 0.10)%] and the average particle size was 75 nm with a PDI of 0.1779.

3.2. Quantification of MTO in solutions

3.2.1. Recovery, precision and stability for assay of MTO in Vc-saline

Three levels of MTO solutions (high, medium and low concentrations, three samples each) were prepared and diluted with Vc-saline to 10 ml. The recovery results of the three solutions were (99.43 ± 0.69), (101.40 ± 0.28) and (99.49 ± 0.35)% (mean ± S.D., n = 3). Two Vc-saline solutions of MTO (stored in the dark at 4 °C) were tested for stability. In the time range of 0–144 h, the concentrations obtained were 10.621 ± 0.180 and 20.180 ± 0.100 μg/ml (mean ± S.D., n = 9), respectively, R.S.D. of both being less than 2%.

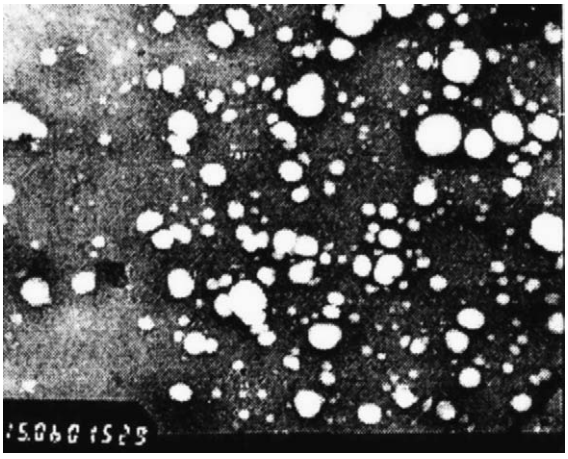

Fig. 2. TEM photo of MTO–CS–NS (×15,000).

3.2.2. Recovery, precision and stability for assay of MTO in ethanol–HCl

Three levels of MTO solutions (high, medium and low concentrations, three samples each) were prepared and diluted with ethanol–HCl to 10 ml. The recovery results of the three level solutions were (98.69 ± 2.23), (100.25 ± 1.21) and (100.57 ± 0.88)% (mean ± S.D., n = 3). R.S.D. were all within 3%. Three levels of ethanol–HCl solution of MTO (three samples each, stored in the dark at 4 °C) were tested for stability. In the time range of 0–172 h, the concentrations obtained were 4.635 ± 0.044, 9.293 ± 0.131 and 14.008 ± 0.139 μg/ml (mean ± S.D., n = 6), respectively, of which R.S.D. being all less than 2%.

3.3. Determination of cross-linking rate of BSA–NS

Up to now there is no perfect method to determine the cross-linking rate of protein particles.

The turbidity of a colored colloidal solution can be determined by turbidimetry, in which the transmitted light after a beam of incident light of a certain wavelength passing through the solution of light path length l is measured. Turbidity T can be calculated from the following equation

$$\frac{I_t}{I_0} = e^{-Tl}$$

in which I_t and I_0 are the strength of the transmitted and the incident light, respectively. For our light blue opalescent colloidal solution, light of wavelengths 540, 630 and 650 nm were tested and the light of wavelength 540 nm gave the best repeatable results of turbidity, thus in our experiments, incident light of 540 nm was used for turbidity measurement. Cross-linking rate of protein particles can be determined by a measurement of turbidity (van Kleef, 1986). Briefly, after reaction with guanidine hydrochloride, the non-covalent bonds in protein particles (hydrogen bonds, electrostatic force, hydrophobic force, etc.) are broken and only covalent bonds remained. The turbidity of this solution (T_a) divided by that of the same solution before reaction with guanidine (T_b) could be regarded as the cross-linking rate of the particles, provided that cross-linking results only in the formation of covalent bonds. But if BSA–NS were overreacted with guanidine hydrochloride, denaturation and precipitation might happen. Preliminary tests showed that 6 M guanidine hydrochloride reacting for 1 h at room temperature was suitable.

3.4. Lyophilization and sterilization

MTO–BSA–NS or MTO–CS–NS can be stored in refrigerator at 4 °C for 6 months without significant change. But

coagulation occurred over 6 months. So freeze-drying was investigated for longer storage.

Glucose, mannitol, lactose, sorbitol, sucrose and trehalose were used, respectively, as the cryoprotector. One milliliter of MTO–BSA–NS or MTO–CS–NS was mixed with 50, 100 or 150 mg of the cryoprotector, and shaked to make the cryoprotector completely dissolved. The mixture was stored at −50 °C for 5 h, and then put into the cryogenic freeze-dryer for lyophilization under vacuum for 36 h. MTO–BSA–NS or MTO–CS–NS without the cryoprotector were used as the control. The appearance, color and redistribution ability were used to evaluate the cryoprotector.

All lyophilized MTO–BSA–NS and MTO–CS–NS looked blue in color. Free MTO–BSA–NS and MTO–CS–NS without the cryoprotector looked like loose powders. When mannitol or sorbitol used as the cryoprotector, the lyophilized product of MTO–BSA–NS showed spots or design and gave slice precipitates after redispersed in 1 ml of water. When glucose, mannitol or sorbitol used as the cryoprotector, the lyophilized product of MTO–CS–NS resulted in gelation after redispersed in water.

The lyophilized products of MTO–BSA–NS with 5% (w/v) lactose and those of MTO–CS–MS with 10% (w/v) lactose showed the best result of redispersion without formation of precipitation. The transmission electron microscopic photo of redispersed MTO–BSA–MS is shown in Fig. 3.

After radiation with 10 kGy ^{60}Co, MTO–BSA–NS and MTO–CS–NS met the requirements of asepsis. The mean diameter was 85 nm with a PDI of 0.3066 for MTO–BSA–NS (77 nm with a PDI of 0.3162 before lyophilization), and the mean diameter of 83 nm with a PDI of 0.2726 for MTO–CS–NS (75 nm with a PDI of 0.1779 before lyophilization). The EY

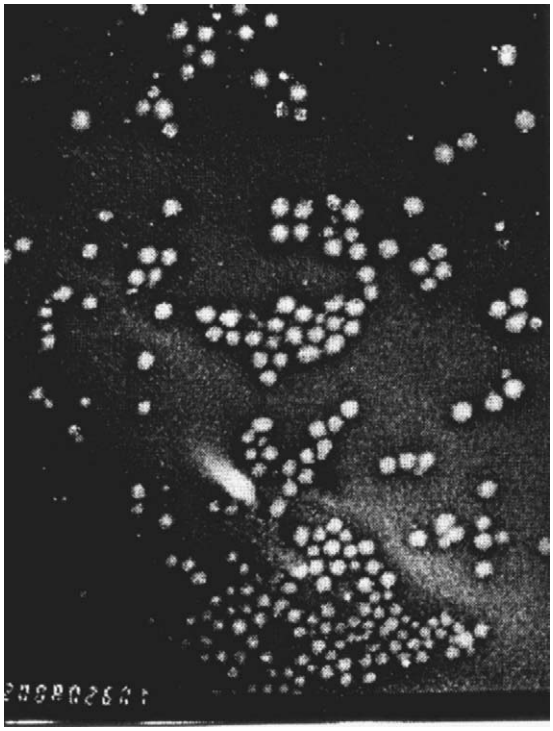

Fig. 3. TEM photo of redispersed MTO–BSA–NS (×20,000).

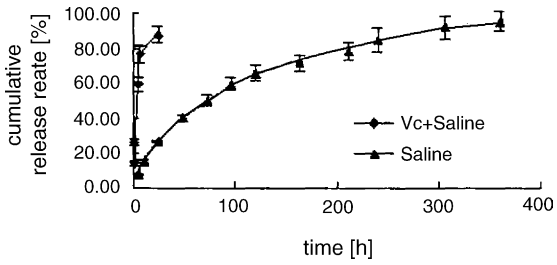

Fig. 4. In vitro release profile of MTO–BSA–NS and fitted smooth lines.

was (98.54 ± 1.03)% (n = 3) and DL (19.77 ± 0.17)% (n = 3) for MTO–BSA–NS, and EY was (97.61 ± 1.17)% (n = 3) and DL (9.78 ± 0.11)% (n = 3) for MTO–CS–NS, both of which showed no significant change compared to those before freeze-dying (P > 0.6). The pH value of redispersed MTO–BSA–NS and MTO–CS–NS was 7.0 and 5.6, respectively.

3.5. Tests for in vitro release

In the literature (Zhang and Liao, 1997), 1.0% (w/v) ascorbic acid was applied to make MTO solution stable, since MTO was not stable enough in a saline solution. We used 0.5% (w/v) ascorbic acid, which has a higher pH value closer to physiological fluids and proved very stable for in vitro release tests and assay. MTO, a base with limited solubility in water, was tested in physiological saline and in Vc-saline, respectively, for in vitro release behavior. Since Vc-saline was acidic, MTO might show faster release rate in it. Anyway, physiological saline was also used for comparison, though the data were not so reliable (MTO was not so stable in it)), because it might show closer property with physiological fluids.

Release $t_{1/2}$ of free MTO by Visking bag method was 2.0 and 7.4 h in Vc-saline and physiological saline, respectively.

Release $t_{1/2}$ of MTO–BSA–NS was 2.8 and 74.0 h in Vc-saline and physiological saline, respectively. The corresponding $t_{1/2}$ for MTO–CS–NS was 4.3 and 11.2 h, respectively. The two nanospheres showed little burst effect, which amounted to 5.5–6.3% in physiological saline and 14.9–21.7% in Vc-saline in the first hour. But the reason why MTO–BSA–NS released faster than MTO–CS–NS in Vc-saline and MTO–CS–NS released faster than MTO–BSA–NS in physiological saline remained unclear.

The best fitted regression equation with the largest r (correlation coefficient) and smallest SUM [=$\sum$(Obs − Pred)^2] are:

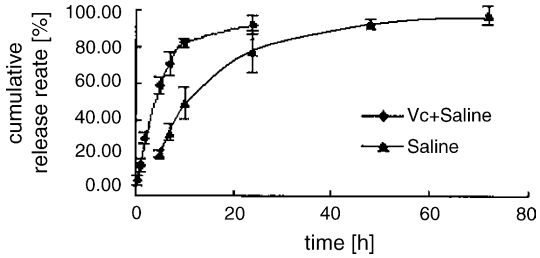

Fig. 5. In vitro release profile of MTO–CS–NS and fitted smooth lines.

for in vitro release of MTO–BSA–NS

in Vc-saline $Q = 23.9728t - 2.3104t^2 + 0.06098t^3$, $r = 0.9951$, SUM $= 39.03$

in physiological saline $Q = 6.3773t^{1/2} - 0.06488t$, $r = 0.9947$, SUM $= 102.59$

for MTO–CS–NS

in Vc-saline $Q = 16.0629t - 1.1204t^2 + 0.0239t^3$, $r = 0.9997$, SUM $= 4.54$

in physiological saline $Q = 5.6174t - 0.1134t^2 + 0.000753t^3$, $r = 0.9969$, SUM $= 30.19$

where Q stands for the cumulative release rate at time t. The in vitro release data and the fitted lines from the above equations (smooth lines in the figure) are shown in Figs. 4 and 5.

4. Conclusions

The major features of our MTO nanospheres were: (1) pretty uniform with a mean size of less than 100 nm and little burst release and (2) simple preparative procedures to give nanospheres with high EY and almost no organic solvent residue.

MTO is not stable enough in physiological saline or normal tissue homogenates. The feasibility of utilization of physiological saline containing 0.5% (w/v) ascorbic acid or ethanol–0.1 M HCl (3:1, v/v) as the medium for assay of MTO was approved by a careful evaluation of accuracy, precision and stability for spectrophotometrical determination.

References

Chen, G.Q., Lin, W., Combes, A.G.A., 1994. Preparation of human serum albumin microspheres by a novel acetone-heat denaturation method. J. Microencapsul. 11, 395–407.

Esposito, E., Cortesi, R., Nastruzzi, C., 1996. Gelatin microspheres: influence of preparation parameters and thermal treatment on chemico-physical and biopharmaceutical properties. Biomaterials 17, 2009–2020.

Li, S.L. (Ed.), 2000. Oncology of Mammary Gland. Publishing House of Science and Technology, Beijing, pp. 1–134.

Phillips, W.T., lipper, R.K., Goins, B., 2000. Novel method of greatly enhanced delivery of liposomes to lymph nodes. J. Parmacol. Exp. Ther. 295, 309–313.

Sugibayashi, K., Morimoto, Y., Nadai, T., Kato, Y., Hasegawa, A., Arita, T., 1979. Drug-carrier property of albumin microspheres in chemotherapy. II. Preparation and tissue distribution in mice of microsphere-entrapped 5-fluorouracil. Chem. Parm. Bull. 27, 204–210.

van Kleef, F.S.M., 1986. Thermally induced protein gelation: gelation and rheological characterization of highly concentrated ovalbumin and soybean protein gels. Biopolymers 25, 31–59.

Weber, C., Coester, C., Kreuter, J., 2000. Desolvation process and surface characterization of protein nanoparticles. Int. J. Pharm. 194, 91–102.

Wu, W., Lu, B., Xiong, S.B., 1999. Influence of temperature and time of thermal denaturation on the amount of active surface amino group of BSA microspheres prepared by spray-drying. Chin. Pharm. J. 34, 752–754.

Zhang, Z.R., Liao, G.T., 1997. Studies on mitoxantrone nanospheres for hepatic targeting. In: Liao, G.T. (Ed.), Targeted Drug Delivery Systems. Publishing House of Science and Technology, Sichuan, pp. 230–231.