Original Article

Preparation and assessment of chitosan-coated superparamagnetic Fe₃O₄ nanoparticles for controlled delivery of methotrexate

S. Mohammadi-Samani[1,*], R. Miri[1,2], M. Salmanpour[1], N. Khalighian[2], S. Sotoudeh[1] and N. Erfani[3]

[1]*Pharmaceutical Sciences Research Centre, Faculty of Pharmacy, Shiraz University of Medical Sciences, Shiraz, I.R. Iran.*
[2]*Medicinal and Natural Products Chemistry Research Center, Shiraz University of Medical Sciences, Shiraz, I.R. Iran.*
[3]*Institute for Cancer Research, School of Medicine, Shiraz University of Medical Sciences, Shiraz, I.R Iran.*

Abstract

In this study, Fe₃O₄ superparamagnetic nanoparticles were synthesized and stabilized by chitosan. Then the nanoparticles were characterized by Fourier transform infrared spectroscopy and transmission electron microscopy (TEM). Particle size distribution and Zeta potential of the particles also was assessed using Malvern Zetasizer. The paramagnetic behaviors of the uncoated and chitosan coated nanoparticles were measured using vibrating scanning magnetometry Particles morphology and size ranges of uncoated iron oxide nanoparticles were evaluated by TEM, showing uniform and narrow size distribution about 10 nm. After coating nanoparticles with chitosan and loading of methotrexate (MTX), the change in size was assessed using Zetasizer. Considerable increase in size was observed following the coating of the particles with chitosan and loading with MTX (the average size was 152 nm). Paramagnetic properties of the uncoated and chitosan-coated particles were assessed showing significant decrease in paramagnetic behavior after coating with chitosan, but it was enough to respond to the magnetic field. Finally loading efficiency, release rate and cytotoxicity of MTX were assessed indicating slow release behavior with the same levels of cell toxicity in SK-BR-3 cell lines, suggesting this formulation as a good candidate for the controlled delivery of MTX.

Keywords: Superparamagnetic; Fe₃O₄; Nanoparticles; Chitosan; Magnetic targeting drug delivery; Methotrexate

INTRODUCTION

Methotrexate (MTX) is a folate anti-metabolite with antineoplastic, antirheumatic and disease modifying properties. It has been used to treat trophoblastic neoplasms, leukemias, psoriasis, rheumatoid arthritis, various carcinomas of breast, head, neck and lung. It also has indications in osteosarcoma, soft tissue sarcoma, carcinoma of gasterointestinal tract, esophagus, testicle and lymphoma (1,2). Despite the extensive clinical uses, various side effects of MTX should be considered (3-5) which range from malaise and asthenia to cirrhosis, pneumonitis or pancytopenia, that can be life threatening and fatal (5). In spite of relatively short half-life of MTX in low dose administration, taking MTX weekly rather than daily dose can diminish the risk of toxic effects of the drug in rheumatoid arthritis (6). During the last decade numerous publications in the literature appeared focusing on controlled and sustained delivery of MTX to enhance efficacy and diminish the respective side effects (7-10).

Superparamagnetic iron oxide nanoparticles (SPION) have gained much attraction during the past decade because of its potential applications in biology, medicine and physics due to multifunctional properties, including small size, superparamagnetic behaviors, low toxicity, etc. (11). SPION tend to aggregate in aqueous medium despite of having hydrophilic nature, due to their high specific surface area and high levels of surface free energy,. Consequently, it is needed to use stabilizers

*Corresponding author: S. Mohamadi-Samani
Tel. 0098 711 2424127, Fax. 0098 711 2424126
Email: smsamani@sums.ac.ir

such as surfactants and hydrophilic polymeric compounds to coat the surface of the nanoparticles (11-13). On the other hand, the tendency of SPION to act directly with active pharmaceutical is low, so surface modification of these nanoparticles is needed.

Chitosan being deacetylated chitin is currently obtained from the outer shell of crustaceans. The positive charge of chitosan provides various and distinctive physiological and biological properties with great potential applications in a wide range of industries including agricultural, food, cosmetic and pharmaceutical (14,15). The cationic nature of chitosan has been considered for the develop-ment of particulate drug delivery systems. In addition to its ability to complex with negatively charged polymers, another interes-ting property of chitosan is its ability to form a gel on contact with specific polyanions (16). Chitosan has many significant biological properties including biocompatibility, bioacti-vity and biodegradability with reactive chemical group including OH and NH_2. Therefore, chitosan and its derivatives have been widely used in the fields of pharmacy and biotechnology (11,17,18).

MATERIALS AND METHODS

Materials

High molecular weight chitosan was from Fluka (USA). Ferric chloride hexahydrate ($FeCl_3 . 6H_2O$), ferrous chloride tetrahydrate ($FeCl_2.4H_2O$), MTX, acetic acid, potassium dihydrogen phosphate, disodium hydrogen phosphate, sodium chloride, potassium chloride and ammonium hydroxide were from Merck (Germany). Acetonitrile and methanol were purchased from domestic supplier of Caledon (Canada) in Iran. All other chemicals in this research were analytical grade and used without any further modification. Double distilled water was used during this research wherever needed.

Method
Preparation of superparamagnetic iron oxide (Fe_3O_4) nanoparticles

SPION were prepared by the co-precipitation of ferric and ferrous chloride in anaerobic condition at room temperature. In this regard 1.62 g of ferric chloride was dissolved in 60 ml distilled water (0.1 M), deoxygenated with continuous purging of nitrogen gas. Then, 0.6 g of ferrous chloride was dissolved in 30 ml of distilled water (0.1 M), deoxygenated by nitrogen purging. After mixing of the solution within three necks, round-bottom flask with magnetic stirrer and purging the nitrogen gas for 15 min, 4 ml of ammonium hydroxide solution diluted by 6 ml distilled water and after purging with nitrogen was added dropwise to the previous solution of iron salts while mixing the solution and purging the nitrogen gas was continued. Changing of the color of the solution to dark black due to the precipitation of Fe_3O_4 was considered as the end point of SPION formation within the flask (12). The resulting precipitate was collected with strong magnet and the supernatant solution was discarded and rinsed triplicate with distilled water to remove excess ammonia from remaining precipitate.

X-ray diffraction (XRD)

To confirm the crystalline structure of the Fe_3O_4, X-ray diffractogram of SPION was assessed using Philips XRD instrument (D/Max-2500). A monochromatized X-ray beam with nickel filtered Cu Kα radiation with 4° min^{-1} scan rate. In this regard a continuous scan mode was used to collect 2θ data from 10° to 90°.

Chitosan coating of the SPION

First, 20 mg of high molecular weight chitosan was dissolved in 1 M acetic acid solution with final volume of 100 ml. Then 70 mg of SPION was added to the previous solution and the mixture was mixed over night for 18 h. During this process chitosan molecules were adsorbed on the surface of the nanoparticles and a homogeneous dark brown suspension was obtained.

MTX loading on chitosan-coated SPION

To do so, 70 ml of suspension containing 14 mg of chitosan adsorbed on the surface of SPION was mixed with 4mg of MTX solution and was mixed for 48 h to ensure adequate interaction between negatively charged MTX

and positively charged chitosan molecules which were immobilized on the surface of SPION.

MTX quantitation

MTX was quantified in unknown samples throughout the study by reversed phase HPLC method developed and validated in the initial phase of the study. Briefly, 2.722 g mono potassium phosphate was dissolved in 100 ml distilled water to obtain 0.2 M solution. Then 50 ml of this solution was mixed with 3.6 ml of sodium hydroxide 0.2 M solution and the final volume of the solution was corrected to 200 ml and the pH of the solution was adjusted to 5.8 by dropwise addition of NaOH, 0.2 M or HCl, 0.2 M, if necessary. The mobile phase consisted of ternary mixtures of phosphate buffer: acetonitrile: methanol in proportion of 80:10:10. The chromatographic system consisted of a C18 column (250 × 4.6 mm; Knauer) filled with Eurosphere 5 μm particle and a precolumn guard with the same packing. A pump-controller unit (Knauer, Smartline, model 1000, Berlin, Germany) and a Rheodyne injection device equipped with a 50 μl loop were used for solvent delivery (flow rate 1 ml/min) and sample injection, respectively. The analyte detection was made by a UV detector (Knauer, model 2500) at 302 nm. Chromatograms were processed using compatible software (Knauer, Eurochrom). MTX peak eluted at 3 min after sample injection.

MTX loading and release assessment

Different amounts of MTX with predetermined amount of chitosan-coated SPION are mixed for specified time and then nanoparticles were separated from the supernatant with the help of strong magnet inserted below the nanoparticles containing vessel. Supernatant solution was removed and the amount of unloaded MTX was assessed using HPLC method. The release experiments were performed in100 ml PBS solution with pH 7.4 and stirring at 120 rpm with magnet stirrer at 37°C. In doing so, at predetermined intervals 1 ml of release medium was taken and aliquot volume of fresh PBS solution was replaced. The concentration of MTX in each sample was quantified by HPLC method.

FTIR assessment of the nanoparticles

The Fourier transform infrared spectroscopy (FTIR) spectra on SPION, chitosan-coated SPION, MTX loaded chitosan-coated SPION, Pure MTX and pure chitosan were assessed from 500-2500 cm^{-1}. In order to evaluate the effect of MTX concentration on FTIR spectra, three different concentrations were considered in these experiments.

In vitro cytotoxicity study

MTT cell viability test was performed on human mammary breast adenocarcinoma cells, SK-BR-3 cell line. Cells were cultivated in RPMI with 10% fetal bovine serum and 1% penicillin-streptomycin at 37ºC in humidified environment containing of 5% CO$_2$.

SK-BR-3 cells were seeded in 96-well plates at the concentration of 5000 cells per well. After 48 incubation and cell attachment, Cells were incubated with MTX, chitosan-coated magnetic nanoparticles and methotrexate-loaded chitosan-coated magnetic nanoparticles and cell culture without any other additive as blank for 24 h. After 24 h, supernatant fluid layer of cell cultures was replaced by media containing MTT (5 mg/ml) and cells were incubated for 4 h. MTT was removed and DMSO was added to lysed cells. Absorbance of the samples was assessed using microplate reader at 492 nm. Blank cells assumed to have 100% viability and other treated wells were compared with blank to compute cell toxicity in other wells treated with MTX or nano-particles.

Statistics

All the data collected in this study were repeated at least in triplicate and whenever needed standard deviation was reported. ANOVA test was used to compare the different samples and P value less than 0.05 was chosen as differences criteria.

RESULTS

XRD

To confirm the crystalline structure of Fe$_3$O$_4$, XRD diffractogram was assessed (Fig. 1). There are six diffraction peaks in this diffractogram which is the standard pattern for
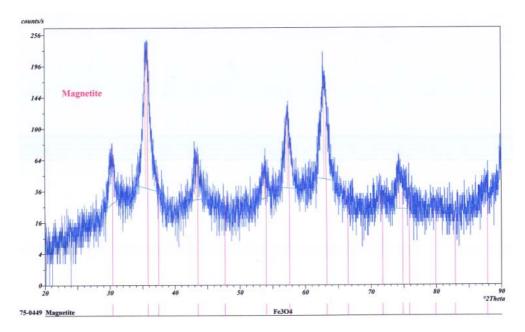
Fig. 1. XRD diffractogram of superparamagnetic iron oxide nanoparticles
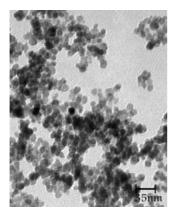

Fig. 2. TEM of superparamagnetic iron oxide nanoparticles

crystalline magnetite with spinal structure and it is in agreement with data published elsewhere for Fe_3O_4 (11,19).

Particle size assessment

The size of SPION was measured using transmission electron microscopy (TEM); Philips CM 10, Netherlands. The results were shown in Fig. 2, according to this figure the SPION have uniform particle with an average size of 10 nm. The particle size of MTX loaded and chitosan-coated nanoparticles were measured using laser light scattering technique by Malvern Zetasizer; Malvern instruments, 300-HS, UK. The results showed considerable increase in size because of the surface adsorption of chitosan molecules (Fig. 3). The z-average of particles was 159 nm with polydispersity index about 0.152, showing uniform distribution of the nanoparticles. The zeta potential of the MTX loaded chitosan-coated SPION also was measured by the same instrument and the average value was about +32 mv.

FTIR

The FTIR spectra of nude SPION, chitosan, chitosan coated SPION and MTX loaded chitosan-coated SPION are shown in Fig. 4. In these spectra a typical peak at 575 cm^{-1} can be seen which is characteristic of Fe-O-Fe bound in Fe_3O_4. Chitosan coating of the SPION quenches this peak in chitosan-coated SPION. Pure chitosan has typical spectrum at 1654 cm^{-1} and 1083 cm^{-1} which is clear and amplified in the presence of MTX in MTX loaded chitosan-coated nanoparticles. Also a considerable amplification in spectrum at 3162 cm^{-1} in the presence of the chitosan is observable in Fig. 4. In this regard we concluded that final particles were MTX loaded and chitosan-coated nanoparticles.

Vibrating scanning magnetometry

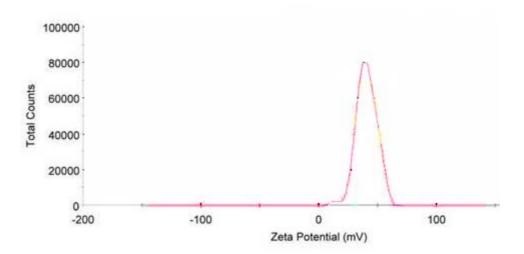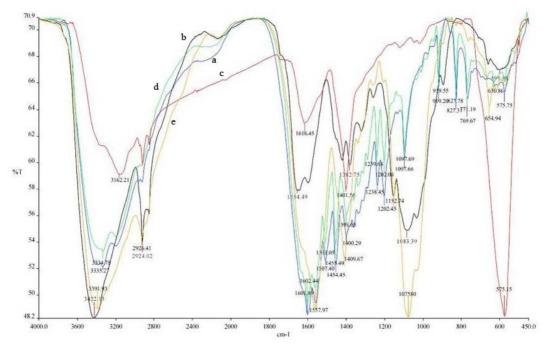Vibrating scanning magnetometry (VSM) was performed by MDK Magnetics (Iran) to

28

Fig. 3. The average size and size distribution curve of chitosan coated SPION assessed by laser light scattering method

Fig 4. The FTIR spectra of nude SPION, chitosan, chitosan-coated SPION and MTX loaded chitosan-coated SPION.
a. Pure MTX; b. Pure chitosan; c. Pure Fe$_3$O$_4$; d. MTX loaded and Chitosan coated Fe$_3$O$_4$; e. Mixture of chitosan MTX

confirm superparamagnetic structures of the synthesized nanoparticles. The magnetization of ferromagnetic Fe$_3$O$_4$ nanoparticles is very sensitive to the microstructure of the sample and super paramagnetism occurs when the particles are small enough so that thermal fluctuations can conquer the magnetic anisotropy. The lack of hysteresis loop in VSM profile is an important criterion required for the superparamagnetism behavior of the nanoparticles. VSM graphs of nude SPION and chitosan-coated SPION are presented in Fig. 5. As it is clear from this figure, nude SPION and chitosan-coated SPION have strong superparamagnetic behaviors, and although coating of the SPION with chitosan show decreasing pattern, it is enough for magnetic separation of the nanoparticles and also target the nanoparticles with the help of an external magnet.
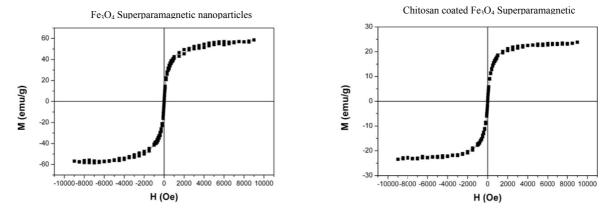
Fig. 5. VSM graphs of nude SPION and chitosan-coated SPION

Table 1. The relationship between MTX loading efficiency and amounts of incorporated MTX. In each experiment the amount of chitosan coated SPION was constant (70 mg)

Incorporated MTX(mg)	Loading (%)	SD
4	94.5	7.0
10	89.4	1.8
25	87.6	2.6
50	56	3.1
100	28.5	0.5

MTX loading and release assessment

The suitability of a carrier system for a specific drug can be determined by the ability of the carrier system to load sufficient quantity of drug. On the other hand the releasing behavior of the drug from the carrier system is another important criterion, because the therapeutic effects of the drug is directly related to the concentration of the drug on the receptor site. In this regard loading efficiency of the chitosan-coated SPION was determined according to the following equation:

$$\%LE = \frac{nominal\ added\ MTX - unloaded\ MTX}{nominal\ added\ MTX}$$

To estimate the ability of chitosan-coated SPION, 4 mg of MTX solution was mixed with 70 mg of chitosan-coated SPION and after 48 h exposure time, the content of unloaded MTX was determined after separation of the SPION, using strong external magnet. According to HPLC assay the loading efficiency was 94.5 ± 0.4 %. To assess the binding capacity of the nude SPION, the same experiment was also carried out with uncoated nanoparticles at the same concentrations and after 48 h of mixing, the loading efficiency was 2.78 ± 0.3 % confirming the adequate binding site on the chitosan molecules which coated the surface of the SPION. To determine the effect of the drug/carrier ratios on loading efficiency, different amounts of MTX with the same amount of chitosan-coated SPION were mixed and the loading efficiency was assessed in each time. The results showed that by increasing the ratio of MTX/ chitosan-coated SPION, loading efficiency strongly diminished, probably because of the saturation of binding sites for drug on chitosan molecule backbone (the data were presented in Table 1). As it is seen in this table, by increasing the ratio of MTX/ chitosan-coated SPION, loading efficiency was decreased from 94.5% for 4 mg MTX to 28.5 % for 100 mg MTX, indicating that the surface of chitosan-coated nanoparticles were saturated by MTX. But it is obvious that this delivery system is able to load sufficient amounts of MTX needed for sustain delivery of MTX.

The release profile of MTX from chitosan-coated SPION was studied in100 ml PBS medium, pH 7.4 at 37°C. At predetermined time interval a strong magnet was placed under the releasing medium containers and after precipitation of the chitosan-coated SPION, 1 ml samples from each vessels were withdrawn and the content of MTX in supernatant samples were quantified. The
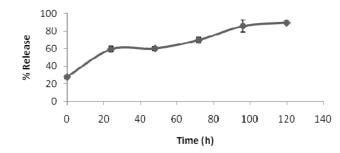
Fig. 6. The release profile of MTX from chitosan-coated SPION in PBS buffer, pH 7.4 at 37°C

■ MTX

■ MTX loaded chitosan coated nanoparticles
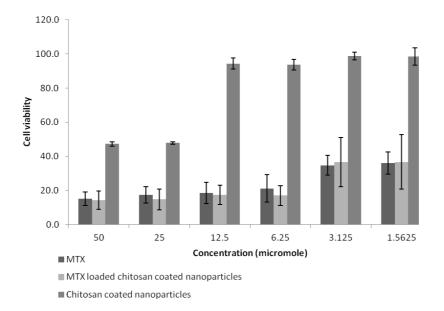
■ Chitosan coated nanoparticles

Fig. 7. MTT assay test to assess cell toxicity of MTX loaded chitosan-coated SPION in comparison to MTX solution and Chitosan-coated SPION.

results of release experiment are presented in Fig. 6. According to this profile, 30% of the drug was initially released indicating that 30 % of the drug molecules were loosely attached to the surface of the polymer. Since the remaining of the drug molecules were adhered to chitosan with stronger attraction forces a release profile for more than 120 h were observed, confirming a prolonged release behavior of MTX.

In vitro cell toxicity

According to MTT assay test, cell toxicity in SK-BR-3 cell lines in MTX loaded chitosan-coated SPION was similar to MTX solution in the same concentrations (Fig. 7). The *in vitro* cell toxicity assay confirmed the appropriate release rate which was able to kill SK-BR-3 cell in culture.

DISCUSSION

According to Fig. 1 and comparing the results of XRD diffractogram crystalline structure of the magnetite nanoparticles was confirmed (11,19).

Particle size assessment of the magnetite nanoparticles indicated uniform particles with average size range about 10 nm. The suspension of uncoated nanoparticles in water was unstable and precipitated completely in a short time since the mixing of the nanosuspension was stopped. Coating of SPION by chitosan, stabilized the nanosuspension for a long period of time and exhibited a well dispersed appearance. Surface adsorption of chitosan molecules is able to induce electrostatic repulsion between the SPION and because of the formation of similar surface

charge; this approach is a practical method to stabilize of the nanosuspension which is the result of the formation of an electrical double layer around the particles. The small particle size in such nanosuspension support Brownian motion in the medium and because of electrical repulsion on the particles having the same charge the uniformity of the chitosan-coated SPION remains for a long period of time and same results have been published by Zhu and coworkers (19). Low zeta potential in nude SPION (-13 mv) and high specific surface area due to the small size probably are the main reasons for rapid flocculation and precipitation in aqueous medium. Increase in particle size was observed after chitosan coating of the nanoparticles due to the surface adsorption of chitosan macromolecules. Surface modification of Fe_3O_4 nanoparticles using chitosan was reported previously by Li and coworkers. In this report covalent binding of the chitosan onto the surface of nano-particles was considered (11). The increase in size also reported by these authors, but considerable increase in nanosuspension stability is the direct effect of the surface modification by chitosan coating which is in agreement with the results obtained in this study and also the results which was published by Zhu and coworkers (19).

FTIR spectrum of magnetite nanoparticles, chitosan coated SPION and MTX loaded chitosan-coated SPION also revealed that in each step of coating and loading by chitosan and MTX respectively have some charac-teristic peaks confirming on surface coating the nanoparticles and loading with MTX.

Based on the VSM data which are presented in Fig. 5, chitosan coating the SPION nanoparticles reduced the super-paramagnetic behaviors of the nanoparticles. The same results also reported by other scientific groups for SPION after surface modifications of the particles which is the result of low Fe_3O_4 content because of the presence of surface modifying agent such as chitosan (13,19). Although the superparamag-netic behaviors of the SPION showed decreasing manner after coating with chitosan, but superparamagnetic properties of the nano-particles were high enough to facilitate the

separation of the chitosan-coated particles from the media and probably is enough to target the chitosan coated SPION in the body using an external strong magnet.

The release data revealed that approxi-mately 30 % of the MTX was loosely bound to the surface of the chitosan and this part was released at the early time, but 70 % of the remaining drug was released during 120 h indicating that this delivery system is able to deliver the MTX in a controlled manner.

In vitro cell toxicity data showed compar-able toxicity in MTX loaded chitosan-coated SPION with MTX solution at the same concentrations. However, the direct inter-nalization of the MTX loaded chitosan-coated SPION should be confirmed in future studies. These data showed promising results on the controlled delivery of MTX via this delivery system. The carrier potential of magnetic microparticles coated with MTX and conjugated with chitosan by peptide bonds also was evaluated before and confirmed the possibility of target delivery of MTX with such delivery system (20).

CONCLUSION

Chitosan-coated SPION are a suitable carrier system for MTX delivery, not only because of biocompatible and biodegradable nature of the chitosan, but also due to the need for modification of the surface characteristics of the SPION and control of the release rate of the MTX from this nano delivery system. Although in this delivery system 30 % of MTX was released at the beginning of the release study, 70 % of the MTX had a slower release pattern. The coating of the SPION although diminished the paramagnetic behavior of the SPION, but superparamagnetic property of the coated particle is strong enough to separate the chitosan-coated SPION from the medium and also it seems that target delivery of the MTX loaded chitosan-coated SPION by use of an external magnet is possible. Because of sustained releasing characteristic of MTX from this delivery system, higher concentrations of the MTX in specified organ are possible to improve the efficacy and reduce the drug adverse reaction

on the other tissues. The loading efficiency of drug/ chitosan coated SPION are appropriate for IV administration of MTX, but the effectiveness of this delivery system should be established through further *in vivo* studies.

ACKNOWLEDGMENT

REFERENCES

1. Lacy CF, Armstrong LL, Goldman MP, Lance LL. Drug Information Handbook. 18 ed: Lexi-Comp; 2010.
2. Lee SW, Kim JH, Park MC, Park YB, Chae WJ, Morio T, et al. Alleviation of rheumatoid arthritis by cell-transducible methotrexate upon transcutaneous delivery. Biomaterials. 2012;33:1563-1572.
3. Moura JA, Valduga CJ, Tavares ER, Kretzer IF, Maria DA, Maranhao RC. Novel formulation of a methotrexate derivative with a lipid nanoemulsion. Int J Nanomedicine. 2011;6:2285-2295.
4. Visser K, van der Heijde DM. Risk and management of liver toxicity during methotrexate treatment in rheumatoid and psoriatic arthritis: a systematic review of the literature. Clin Exp Rheumatol. 2009;27:1017-1025.
5. Neves C, Jorge R, Barcelos A. The network of methotrexate toxicity. Acta Reumatol Port. 2009;34:11-34.
6. Benedek TG. Methotrexate: from its introduction to non-oncologic therapeutics to anti-TNF-alpha. Clin Exp Rheumatol. 2010;28:S3-8.
7. Taheri A, Dinarvand R, Nouri FS, Khorramizadeh MR, Borougeni AT, Mansoori P, et al. Use of biotin targeted methotrexate-human serum albumin conjugated nanoparticles to enhance methotrexate antitumor efficacy. Int J Nanomedicine. 2011;6:1863-1874.
8. Yousefi G, Foroutan SM, Zarghi A, Shafaati A. Synthesis and characterization of methotrexate polyethylene glycol esters as a drug delivery system. Chem Pharm Bull (Tokyo). 2010;58:147-153.
9. Paliwal R, Rai S, Vyas SP. Lipid drug conjugate (LDC) nanoparticles as autolymphotrophs for oral delivery of methotrexate. J Biomed Nanotechnol. 2011;7:130-131.
10. Hu J, Su Y, Zhang H, Xu T, Cheng Y. Design of interior-functionalized fully acetylated dendrimers for anticancer drug delivery. Biomaterials. 2011;32:9950-9959.
11. Li G, Jiang Y, Huang K, Ding P, Chen J. Preparation and properties of magnetic Fe3O4-chitosan nanoparticles. Journal of Alloys and Compounds. 2008;466:451-456.
12. Jain TK, Morales MA, Sahoo SK, Leslie-Pelecky DL, Labhasetwar V. Iron oxide nanoparticles for sustained delivery of anticancer agents. Mol Pharm. 2005;2:194-205.
13. Li GY, Jiang YR, Huang KL, Ding P, Yao LL. Kinetics of adsorption of Saccharomyces cerevisiae mandelated dehydrogenase on magnetic Fe3O4-chitosan nanoparticles. Colloids and Surfaces A: Physicochemical and Engineering Aspects. 2008;320:11-18.
14. Bautista-Banosa S, Hernandez-Lauzardoa AN, Vallea MGV-d, Hernandez-Lopeza M, Barkab EA, Bosquez-Molinac E, et al. Chitosan as a potential natural compound to control pre and postharvest diseases of horticultural commodities. Crop Protection. 2006;25:108-118.
15. Hamidi M, Rafiei P, Azadi A, Mohammadi-Samani S. Encapsulation of valproate-loaded hydrogel nanoparticles in intact human erythrocytes: a novel nano-cell composite for drug delivery. J Pharm Sci. 2011;100:1702-1711.
16. Danesh-Bahreini MA, Shokri J, Samiei A, Kamali-Sarvestani E, Barzegar-Jalali M, Mohammadi-Samani S. Nanovaccine for leishmaniasis: preparation of chitosan nanoparticles containing Leishmania superoxide dismutase and evaluation of its immunogenicity in BALB/c mice. Int J Nanomedicine. 2011;6:835-842.
17. Wu P, He X, Wang K, Tan W, He C, Zheng M. A novel methotrexate delivery system based on chitosan-methotrexate covalently conjugated nanoparticles. J Biomed Nanotechnol. 2009;5:557-564.
18. Trapani A, Denora N, Iacobellis G, Sitterberg J, Bakowsky U, Kissel T. Methotrexate-loaded chitosan- and glycolchitosan-based nanoparticles: a promising strategy for the administration of the anticancer drug to brain tumors. AAPS Pharm Sci Tech. 2011;12:1302-1311.
19. Zhu A, Yuan L, Liao T. Suspension of Fe3O4 nanoparticles stabilized by chitosan and o-carboxymethylchitosan. Int J Pharm. 2008;350:361-368.
20. Zhang X, Chen F, Ni J. A novel method to prepare magnetite chitosan microspheres conjugated with methotrexate (MTX) for the controlled release of MTX as a magnetic targeting drug delivery system. Drug Deliv. 2009;16:280-288.