Gelatin nanoparticles produced by a simple W/O emulsion as delivery system for methotrexate

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Biodegradable hydrophilic gelatin nanoparticles, containing different initial amounts of methotrexate (MTX), were prepared using a simple solvent evaporation technique based on a single water-in-oil emulsion and stabilized by the use of glutaraldehyde as cross-linking agent.

The effects of several parameters on particle size, drug encapsulation efficiency and drug release were investigated. Size and shape of the nanoparticles were examined by scanning electron microscopy.

The release of MTX was monitored *in vitro* and the mechanism of release was studied.

Particles with a mean diameter of 100–200 nm were produced, which were able to release MTX following a diffusion-controlled mechanism of release. It was observed that the initial amount of MTX used for sample loading did not have any effect on the pattern of release, while it affected the amount of drug entrapped into the nanoparticles and also both the release rate and the total amount of drug released.

Introduction

Many efforts made to develop more rational approaches to specific cancer therapy are based on the concept of drug targeting [1].

Because of their submicron sizes, colloidal drug carriers such as liposomes and nanoparticles facilitate the transport of a drug from injection site to the target tissues via the vascular system [2]. In particular these microcarriers could be useful for the treatment of some forms of cancer when the tumor cells have become resistant to the uptake of free drugs. Poor stability during storage, exchange of phospholipids with certain blood components and difficulties in obtaining a reproducible preparation on a large scale [1] are some of the problems associated to the use of liposomes [3–6]. Nanoparticles represent novel dosage forms: these particles, less than 1 μm in size [7, 8], allows to reduce associated adverse effects of various drugs [1, 8–10].

Methods that can be used in preparing nanoparticles from biodegradable polymers include: solvent evaporation [11], monomer polymerization [12], and salting out procedure [13, 14]. However, these techniques result efficient only for lipophilic drugs; they show a poor encapsulation efficiency of water soluble drugs and methods to encapsulate water-soluble active agents are still scarce.

The aim of the present study was the production of gelatin nanoparticles able to release the anticancer drug methotrexate (MTX), using a simple procedure, based on a single water-in-oil emulsion.

Gelatin was selected because it is a natural biodegradable biocompatible polymer [1] non-toxic, readily available, and widely used in parenteral formulations [15].

MTX is an antimetabolite of folic acid which acts inhibiting cellular proliferation. It is widely employed in the treatment of pathologies such as: neoplastic disorders, autoimmune disorders, restenosis caused by neointimal hyperplasia, etc. Gelatin particles able to release MTX have been produced by a polymer dispersion technique [15, 16], but these particles were more than 1 μm in size.

On the other side, gelatin particles in the nanometer range have been obtained by a process deriving from the coacervation method of microencapsulation [17], but this method does not allow a good incorporation of drugs such as MTX (unpublished data).

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In the present work, gelatin nanoparticles containing different initial amounts of MTX were produced. The effect of several parameters on particle size, drug encapsulation efficiency, and drug release were investigated. The release of MTX was monitored *in vitro* and the mechanism of release was studied.

Materials and methods

Materials

Gelatin (type B, from bovine skin), poly(methylmethacrylate) (PMMA) of average molecular weight M_w 120 000, MTX and glutaraldehyde (GTA) (25% and 8% aqueous solutions, respectively) were supplied by Sigma Aldrich; chloroform, toluene, and acetone were supplied by Carlo Erba Reagenti, Italy.

Preparation of gelatin nanoparticles loaded with methotrexate

Gelatin nanoparticles were prepared by a solvent evaporation procedure based on a single water-in-oil (W/O) emulsion. MTX was dissolved in a 1.5% (w/v) sodium hydroxide solution. A 40% (w/v) gelatin solution was prepared in phosphate buffered saline solution (PBS) at 40 °C under stirring. This solution was mixed with the MTX solution to obtain the ''water phase''.

A 25% (w/v) PMMA solution was prepared by dissolving the polymer in a chloroform/toluene (1/1 v/v) mixture to obtain the ''oil phase''.

The ''water phase'' was added to the ''oil phase'' while being mixed with a high speed homogenizer (Art. Miccra-D8, Falc Instruments) at a speed of 23 500 rpm. The whole process was carried out in a beaker cooled in an ice bath. Mixing was continued for 8 min at 23 500 rpm and then for 30 min at 400 rpm. Then cross-linking was performed by the addition dropwise of a GTA/toluene (1/1 v/v) solution (previously prepared by mixing a GTA aqueous solution with toluene in a test tube and shaking for 2 h), in order to produce nanoparticles. The system was maintained at low temperature (4 °C) overnight under magnetic stirring in order to allow the completion of the gelatin cross-linking reaction. Nanoparticles were cleaned by centrifuging and resuspending in toluene three times and then twice in acetone. The final product was dried at room temperature to obtain a fine yellow powder.

Scanning electron microscopy (SEM)

The morphological characteristics of the MTX loaded gelatin nanoparticles, were observed using SEM (Jeol T300). The samples were prepared on aluminum stubs and coated with gold prior to examination.

Percentage entrapment of MTX in gelatin nanoparticles

The total amount of MTX entrapped in the nanoparticles was determined by the following direct method: 10 mg of nanoparticles were added with 20 ml of dichloromethane and after complete dissolution the amount of MTX was measured spectrophotometrically at $\lambda = 303$ nm. An indirect method was used to calculate the amount of MTX absorbed onto the surface of the nanoparticles. 10 mg of nanoparticles were suspended in 10 ml of 95% ethanol and after 10 min stirring the supernatant was analyzed spectrophotometrically at $\lambda = 303$ nm. The 95% ethanol solution is a poor solvent for gelatin whilst it is a good solvent for MTX. It was assumed that only the drug absorbed onto the surface of the nanoparticles passed into the supernatant after 10 min.

In vitro release test

The release of MTX from the gelatin nanoparticles was evaluated using a side by side diffusion chamber (Crown Glass, Somerville, NJ, USA). This chamber is constituted of two identical glass cells separated by a Millipore membrane LPVP 0.1 μm thick. The volume of each cell is 1.5 cm^3 and the surface area for material exchange is 0.64 cm^2. A nanoparticles suspension in PBS was placed in one of the two cells (donor side) while PBS was placed in the other cell (acceptor side). The apparatus was maintained at 37 °C under stirring at 70 rpm.

At regular time intervals an aliquot of solution was removed from the acceptor side of the chamber and stored. The eluate was replaced with fresh PBS. The amount of drug contained in the eluates was measured spectrophotometrically at $\lambda = 303$ nm.

Results and discussion

The ability of nanoparticles to reduce the adverse effects associated to the administration of various drugs and in particular of anticancer agents, has been widely pointed out [1, 7–10]. A nanoparticle system with a high drug loading and a high entrapment efficiency allows to reduce the quantity of carrier required for the administration of a sufficient amount of pharmacologically active agent to the target site. In this work gelatin nanoparticles, containing MTX, were prepared by a simple method based on a single water in oil emulsion. The emulsion was produced by dispersing an aqueous phase into a high concentrated PMMA organic solution.

The usage of high molecular weight PMMA as dispersant prevented the coagulation of nanoparticles before and during GTA cross-linking without the need for surfactants.

Although the technique is conceptually simple, it was observed that many variables can influence the final product.

Particle size

Preparation parameters such as concentration and M_w of PMMA, and GTA concentration were investigated in order to elucidate the conditions for the production of a stable emulsion and therefore of MTX loaded particles in the 100–200 nm range.

With regard to the influence of PMMA concentration, it was found that a concentration equal to or higher than 25% (w/v) had to be employed in order to avoid the

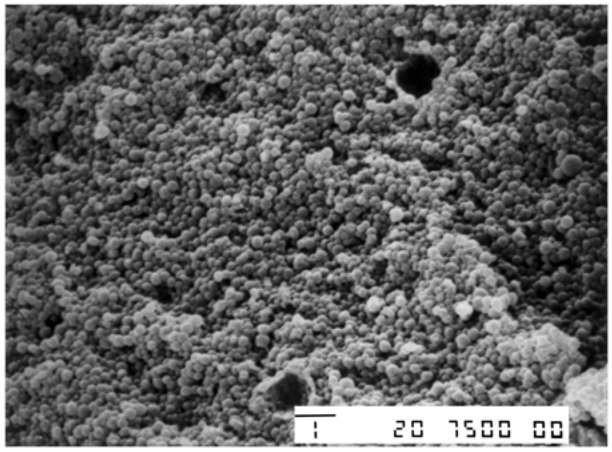

Figure 1 SEM image of gelatin nanoparticles loaded with MTX (bar = 1 μm).

aggregation of the particles that remained entrapped into the highly viscous organic phase. On the other side PMMA concentration had to be not higher than 30% in order to allow the total removal of the polymer during the washing phase.

Another factor able to affect the emulsion stability was the molecular weight of PMMA. Particle aggregation resulted hindered by increasing M_w. However increasing M_w, the viscosity of the organic phase increased and therefore a PMMA with a M_w of 120 000 was selected as the optimal.

For the production of gelatin nanoparticles an emulsification temperature as low as 4 °C was selected to achieve gelation. Since the gelation process is reversible with the temperature [18], cross-linking by GTA was perfomed to stabilize the gelatin nanoparticles. The cross-linking degree of the gelatin matrix represents an important parameter in controlling the shape and dimension of the particles. It was observed that increasing GTA concentration, nanoparticle size decreased because the cross-linking degree was increased and thus gelatin particles had a higher dense structure.

The results of morphological analysis performed by SEM (Fig. 1) showed that smooth and solid nanoparticles with an average diameter of 100–200 nm were obtained using the above mentioned parameters.

Drug loading determination

Both a direct and an indirect method [16] were used in order to calculate the efficiency of MTX entrapment in the nanoparticles. The effect of the initial MTX/gelatin (w/w) ratio on the ratio (D_e/D_t) between the amount of drug entrapped into the nanoparticles and the total amount of drug contained in the nanoparticles was also analyzed (i.e. drug entrapped into the nanoparticles plus drug adsorbed onto the surface of the nanoparticles). It was found that nanoencapsulation efficiency varied from 5.6 to 15.6% depending on the initial MTX content (Table I); in addition it was observed that increasing the initial MTX/gelatin ratio, the (D_e/D_t) ratio incresed (Table I). These data are confirmed in the literature: by increasing the MTX/gelatin ratio the D_e/D_t ratio

TABLE I

MTX: Gelatin ratio	Nanoencapsulation efficiency (%)	D_e/D_t ratio (%)
1:20	5.6	30
1:8	10	54
1:4	15.92	86

increases until a maximum corresponding to a MTX/gelatin ratio of 1 : 2 is reached [16].

Drug release

Fig. 2 shows the release curve for MTX from nanoparticles prepared using an initial MTX/gelatin ratio of 1 : 8. A burst phase can be observed during the first 10 h, releasing about 40% of the loaded drug. This massive initial release can be related to the drug adsorbed onto the external surface of the particles that rapidly comes out. After this initial phase a decrease in releasing rate can be observed followed by a new increase after about 30 h.

It can be supposed that the external aqueous phase diffuses into the polymeric matrix inducing it to swell and favoring the subsequent release of the drug entrapped inside the matrix itself. Approximately 97% of the loaded MTX was released in 10 days.

Fig. 3 shows the release curves for MTX from nanoparticles prepared using different initial MTX/gelatin ratios: 1 : 20, 1 : 8, 1 : 4. It can be observed that all the curves have the same shape, but the release rate and the total amount of drug released increase by increasing the amount of drug used for sample's loading. Therefore the initial MTX concentration did not affect the pattern of release, but did, however, affect the total amount released.

The mechanism of MTX release was also investigated. It is known that drug release may be diffusion controlled or dissolution controlled, depending on parameters such as the permeability of the polymer to water, the solubility

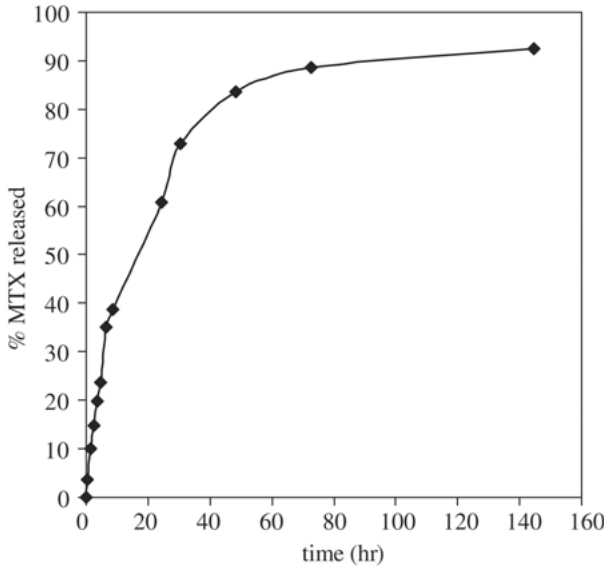

Figure 2 In vitro release of MTX, from gelatin nanoparticles prepared using an initial MTX/gelatin ratio of 1 : 8.

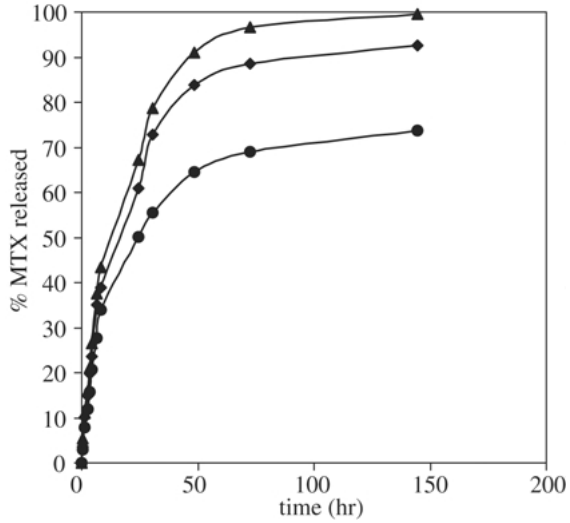

Figure 3 In vitro release of MTX, from gelatin nanoparticles prepared using different initial MTX/gelatin ratios: 1 : 4 (▲), 1 : 8 (◆), 1 : 20 (●).

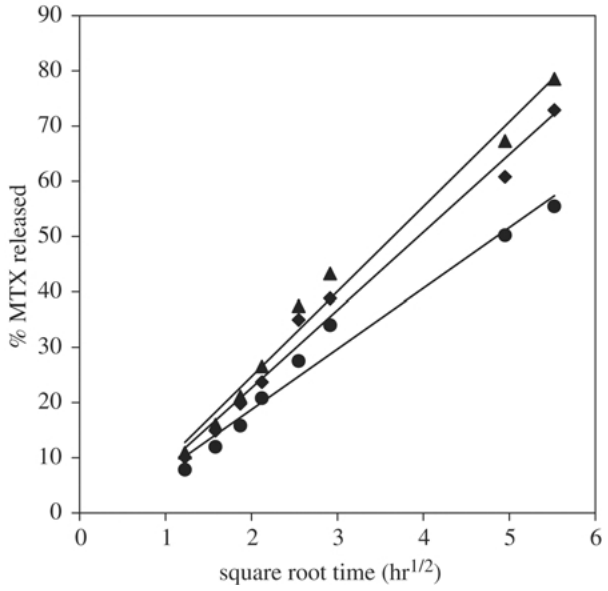

Figure 4 Apparent diffusion-controlled release profiles, according to Higuchi's model for MTX. Initial MTX/gelatin ratio of: 1 : 4 (▲), 1 : 8 (◆), 1 : 20 (●).

of the drug in the polymer and in water and the size of the drug.

In our case the dissolution model is not applicable because MTX is not present in a solid form, but is completely dissolved into the gelatin matrix in the form of sodium salt. In addition a drug release due to the erosion of the matrix can also be excluded because it was observed (unpublished data) that the biodegradation of cross-linked gelatin starts at a time longer than that taken into consideration in the evaluation of drug release. Therefore a diffusion controlled mechanism seemed to be the more suitable.

Higuchi [19] developed an equation (Higuchi square root of time equation) for diffusion-controlled release of drugs from solid matrices. Plotting the amount of drug released, during the first 36 h, against the square root of time, straight lines were obtained with good approximation (Fig. 4). These linear plots appear to indicate that drug release in these systems is diffusion controlled in accordance with Higuchi's equation.

Conclusions

It can be concluded from this study that biodegradable gelatin nanoparticles, containing MTX, can be prepared using a simple solvent evaporation process based on a single water-in-oil emulsion. These nanoparticles showed to be able to release the drug by a diffusion-controlled mechanism.

It was observed that the initial amount of MTX used for sample loading did not have any effect on the pattern of release, while it affected the amount of drug entrapped into the nanoparticles and also both the release rate and the total amount of MTX released. Therefore, it seems that this parameter could be used as a tool in controlling the release depending on the type of disorder that is being treated by the administration of this drug.

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