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Research paper

Dextran cross-linked gelatin microspheres as a drug delivery system

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Abstract

This paper describes the use of oxidized dextran as a cross-linker for the preparation of gelatin microspheres. Microspheres were obtained by a thermal gelation method and their dissolution kinetic was examined. In order to find evidence of sugar mediated cross-linking, swelling tests and gelatin microspheres dissolution experiments were performed. The obtained results indicated that oxidized dextran can form a cross-linked gelatin network which can reduce the dissolution of gelatin. More interestingly, gelatin microspheres treated by both native and oxidized dextran slow down, even if to a different extent, the release of the antitumor drug TAPP-Br used as a model compound. Taken together, our results suggest that oxidized dextran could be an interesting means to cross-link gelatin microspheres allowing the use of this delivery formulation for controlled release of drugs. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Gelatin; Dextran; Microspheres; Cross-linking; Drug delivery

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1. Introduction

In recent years, microparticles have been proposed for the treatment of many diseases needing constant drug concentration in the blood or drug targeting to specific cells or organs [1–3]. For instance, the microencapsulation of antineoplastic drugs has been realized with the aim of: (a) maintaining constant drug levels in the blood possibly reducing the needing for multiple administrations [1]; (b) obtaining higher drug concentrations at the tumor site [2]; and (c) possibly targeting the antitumor agents to the lesion site [3].

Microparticulate delivery systems are generally produced with polymeric materials of synthetic or natural origin. Among natural polymers (preferred for their low toxicity and biocompatibility), gelatin represents a good raw material since it easily forms films and particles. In addition, gelatin is available in a pyrogen-free form and it can be used for the production of delivery systems requiring bioadhesion to mucosal tissues [4].

In spite of these possible advantages, gelatin presents the main drawbacks of a rapid solubilization in aqueous environments, thus resulting in fast drug release profiles. In this respect, it should be emphasized that many factors can influence the rate of cross-linking of gelatin [5,6]. These factors are typified by the structure parameters of gelatin (molecular weight and isoelectric point) and the reaction conditions (temperature and pH). The problem related to the rapid solubilization of gelatin was mainly solved by chemical cross-linking procedures [6]. The treatment of gelatin by di- or polyaldehydes gives rise to the formation of scarcely or non-soluble products which can slow down the release of the encapsulated drug. However, the presence of residual cross-linking agents could lead to toxic side effects, in addition, unwanted reactions between the drug and cross-linker could result in the formation of toxic or inactivated derivatives. In this view, thermal hardening treatments [7] or 'natural' cross-linking agents, such as sugars or sugar-derived compounds [8] have been proposed for the production of

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gelatin devices to achieve long term release of the drug. Particularly, the use of oxidized polysaccharides, such as dextran, has been described as a cross-linking agent for gelatin beads [9].

In the present paper we further investigated the influence of dextran both in its native or oxidized form as a possible cross-linking agent for gelatin microspheres. To this aim gelatin microspheres (GMs) treated by native or oxidized dextran were produced and characterized; in addition, the physicochemical and release characteristics of the GMs after dextran cross-linking was evaluated.

As model compound we considered the polyamidine TAPP-Br [10], which belongs to a class of compounds proposed in the experimental and clinical treatment of a large variety of pathologies, including *Pneumocistic carinii* pneumonia in AIDS patients [11,12] and cancer treatment [13,14]. Among aromatic polyamidines, the bromo-derivative of the *p*-amidino phenoxy neo-pentane (TAPP-Br) was found to be a potent antitumor agent, both in vitro [13] and in vivo [14], on a number of cell lines of different histotype. On the other hand, the effects of TAPP-Br, as in the case of other antineoplastic drugs, are not restricted to the tumor cells, since this compound binds A–T rich DNA sequences in a unspecific manner [12]. In this view, gelatin microspheres could be valuable drug carriers for TAPP-Br and other polyamidines, since after administration by subcutaneous injection they could assure a slow and gradual release the incorporated drug. Moreover, chemoembolization administration procedures could be useful to target the microencapsulated drug towards a specific organ.

2. Materials and methods

2.1. Chemicals

TAPP-Br, the 2′ bromo derivative of 1,3 di-(*p*-amidinophenoxy)-bis-2,2(*p*-amidinophenoxymethyl)propane, was a generous gift from Prof. Ferroni (University of Ferrara). Synthesis, melting point, recovery yield, crystallization solvents and analytical data of TAPP-Br have been reported elsewhere [15].

Microspheres were prepared using gelatin Type B 250 Bloom and pH_i (1% in water) 4.0–5.0, from Fluka (Buchs, Switzerland). Dextran T70 (mol. wt. 65 500) was purchased from Pharmacia (Uppsala, Sweden) all other materials and solvents were from Fluka.

2.2. Preparation of oxidized dextran

Two grams of dextran were dissolved in 30 ml of water, then an excess of sodium *m*-periodate was added. The solution was stirred at room temperature in the dark for 24 h. In order to eliminate the unreacted iodate excess, 3 ml of barium acetate (1.5 mM) were added. After 20 min, the solution was filtered through 0.45 μm pores GWVP filters (Millipore, Bedford, MA) to remove the precipitated barium complex. The excess of barium ions was then eliminated by the addition of sodium sulfate followed by a second filtration to remove the precipitated barium sulfate. The final supernatant containing the oxidized dextran (ox-dextran) was subsequently subjected to gel permeation chromatography on a Sepharose 48 column (Pharmacia, Uppsala, Sweden) (6.2 cm diameter, 10 cm length) pre-equilibrated and eluted with formic acid 1% w/v at 1 ml/min. In order to separate the resulting salts and formic acid that could alter the biocompatibility of the product, the fraction corresponding to the elution peak of oxidized dextran (ox-dextran) was collected and subjected to dialysis (dialysis tube with molecular weight cut-off 10 000–12 000; Medi Cell International. England), versus a high volume of distilled water for 24 h. Afterwards, the ox-dextran solution was lyophilized and the obtained dried powder was maintained at room temperature in a dessicator until used.

2.3. Evaluation of dextran oxidation

Since oxidized dextran reacts with hydroxylamine hydrochloride forming the corresponding oxime and liberating hydrogen chloride [16], the presence of aldehydic groups in the oxidized dextran can be evaluated by sodium hydroxide titration. A 25-ml sample of 0.4 M hydroxylamine hydrochloride was added to 10 ml containing 150 mg ox-dextran. The obtained solution was stirred for 24 h in the dark at room temperature. Afterwards, the solution was potentiometrically titrated with 0.1 N NaOH.

2.4. Preparation of microspheres

Five milliliters of a 15% (w/v) aqueous gelatin solution containing 25 mg of TAPP-Br preheated at 80ºC were added to 20 g of paraffin oil at 80ºC. The mixture was stirred under laminar flow conditions to form a w/o emulsion, using a mechanical stirrer (model IKA RW 20 DZM equipped with a 35 mm diameter rotor). After 5 min of continuous stirring, the solution was rapidly cooled at 15°C and then, 30 ml acetone were added in order to dehydrate and flocculate the coacervated droplets. Gelatin microspheres containing TAPP-Br were then recovered from the suspension mixture by filtration through a sintered glass filter. The removal of residual oil was performed by washing the microspheres with 3 × 80 ml aliquots of acetone. Then, GMs were maintained at room temperature in a dessicator at 8% relative humidity until used.

2.5. Cross-linking of gelatin microspheres

The treatment of gelatin with native or oxidized dextran was performed as follows.

One milliliter of water containing 75 mg/ml of dextran were left to react for 5 min at 80ºC with 750 mg of gelatin in 3 ml of water. Afterwards, 1 ml of water containing 25 mg of TAPP-Br pre-heated at 80ºC was added; then, the reaction mixture was used to prepare microspheres as above described (see Section 2.4).

2.6. Microsphere morphological and size analysis

The morphology of gelatin microspheres was evaluated by optical microscopy (Nikon Diaphot inverted microscope, Tokyo, Japan) and scanning electron microscopy (SEM) observations (360 Stereoscan Cambridge Instruments, Cambridge, UK). Microsphere size, and size and volume distributions were determined by a laser granulometer (Cilas model 715, Marcoussis, France).

2.7. Evaluation of the cross-linking treatment

The in vitro gelatin 'release' from untreated or dextran treated microspheres was determined as follows. Dissolution experiments were conducted at 37ºC by a flow through cell method [17]. Typically, 50 mg of microspheres were placed into a 45 × 18 mm glass column filled with 6 ml of isotonic borate buffer (pH 7.4). A polyethylene filter was placed at the bottom of the column in order to prevent microsphere leakage; both ends were fitted with Teflon tubes. Microspheres were quantitatively transferred into the column and the release buffer was pumped through the column at a flow rate of 0.2 ml/min using a peristaltic pump. Fractions were collected and analyzed for gelatin content using the method described below [18].

2.8. Gelatin determination

Gelatin concentration in the release buffer was determined by colorimetric assay, using a bicinchoninic acid protein assay kit [18] (Sigma, St. Louis, MO). The working solution was prepared by mixing a 4% copper (II) sulfate pentahydrate solution with an excess of bicinchoninic acid at a final ratio of 1:50 v/v. Release buffer (100 μl) were added to 2 ml of the working reagent in a test tube The mix was then incubated at 37ºC for 30 min. After incubation, all tubes were cooled at room temperature and the absorbance of each solution at 562 nm was measured using an UV spectrophotometer (Biochrom 4060, Pharmacia LKE Biochrom Ltd). The gelatin concentration was calculated from a referenced previously constructed calibration curve determined each time with freshly prepared solution as stated by the BCA protocol [18].

2.9. Drug content of microspheres

The amount of encapsulated TAPP-Br/mg of dried microsphere, was determined by two different methods.

(a) By difference, evaluating the concentration of unentrapped drug in the filtrate obtained after microspheres isolation. The filtrate, which consisted of water, acetone and paraffin oil was evaporated under vacuum in a rotary evaporator (Buchi, Germany) until the acetone was completely evaporated. At the remaining two-phase system composed of water and paraffin oil 50 ml of ethyl acetate were added. The resulting organic layer was extracted twice with 20 ml of distilled water. Since TAPP-Br is almost insoluble in organic solvent ($K_{o/w}$ 0.079), the total concentration of TAPP-Br was determined from the analysis of the aqueous phase by UV spectroscopy at 260 nm.

(b) By GMs dissolution; 5 mg of microparticles were dissolved in 1 ml of distilled water for 72 h. After filtration of the obtained solution through VVLP Millipore filters, the concentration of TAPP-Br in the filtered aqueous phase was determined by UV spectrophotometric analysis.

2.10. In vitro TAPP-Br release

In vitro TAPP-Br release profiles were obtained by a dialysis method [17]. The release experiments were conducted at 37ºC. Typically, 100 mg of microspheres were placed into a dialysis tube (molecular weight cut-off 10 000–12 000; Medi Cell International, UK), then placed into 100 ml of 0.1 M phosphate buffer (pH 7.4) with magnetic stirring.

Samples (200 ml) were withdrawn at regular time intervals from 1 to 48 h and analyzed by UV spectroscopy for TAPP-Br content.

2.11. Swelling tests

The swelling characteristics of untreated or dextran-treated GMs were determined in hydrophilic gel prepared by solubilizing poloxamer 407 (20%, w/v) in water at 0ºC [7]. The kinetic of the increase of the initial diameter of particles was performed at 37ºC using an optical microscope equipped with a micrometric device considering at least 500 particles/sample.

The swelling ratio q was calculated according to the equation below measuring the diameter of gelatin microsphere, assuming a reported spherical geometry for particles.

$$q = \frac{V_s}{V_d}$$

where V_s and V_d are the volume of the swollen microspheres and of the dried microspheres, respectively.

3. Results and discussion

In previous studies we investigated the possible use of 'biocompatible approaches' for the cross-linking of gelatin-based drug delivery systems [7]. Particularly, sugar such as mono- and di-saccharides have been considered as 'biocompatible' cross-linkers [8,9]. Sugars could in principle be used both in their native or oxidized form. In the case of native sugars, two main alternative reactions may occur as reported by Digenis et al. [6]. The first possibility, the Amadori rearrangement (Fig. 1a), involves the reaction of a gelatin amino group (i.e. lysyl ϵ-amino group) with the aldehydic group of the sugar giving a cationic imine. This imine, after reaction with another lysine amino-residue and subsequent rearrangement, leads to the formation of a methylene link between two ϵ-amino groups of lysine thus resulting in a cross-linked structure [6].

In the second type of reaction (Fig. 1b) the carbonyl group of the sugar open-chain form reacts with free amino groups of the gelatin molecule (i.e. lysine side chain group), resulting, after a number of tautomerizations, in the formation of a ketose sugar [8]. This carbonyl adduct can further react with another amine group (again from gelatin) forming a cross-linked structure [6].

However, since these two reactions are usually slow, probably due to the low concentration of the sugar in the open-chain form (usually less than 1% in the case of glucose), a more effective way to obtain gelatin cross-linking could be typified by oxidized sugars (ox-sugars) [18]. This approach is based on the previous *m*-periodate oxidation of sugars leading to the formation of a dialdehyde for each carbohydrate unit. After isolation and purification, the ox-sugar could be utilized for a stable and extensive cross-linking of gelatin based devices.

To this aim, we tested the influence of oxidized dextran on gelatin microspheres. The periodate oxidation of dextran is a complicated two-step reaction [18]. Firstly, the periodate oxidation of an α-1,6-linked anhydroglucoside unit leads to the formation of an α-hydroxy aldehyde group. In the second step, the α-hydroxy aldehyde group can be further oxidized generating formic acid and a dialdehyde group [18].

Dextran was treated with an excess of sodium *m*-periodate and after an overnight incubation at room temperature, the excess of untreated periodate was removed by addition of barium acetate and sodium sulfate. After a further purification step based on a gel permeation chromatography followed by a dialysis aimed to separate the sulfate salts and formic acid, the ox-dextran was lyophilized resulting in a manageable water soluble reagent. For the determination of the aldehyde content in the ox-dextran, the hydroxylamine hydrochloride titration method was used. Hydroxylamine hydrochloride reacts with aldehydes to form oximes and the hydrochloride liberated can be titrated with an alkali solution. After 24 h of periodate treatment the mean aldehyde content in completely oxidized dextran as determined by hydrochloride titration was found to be 81.7%.

In order to evaluate the influence and the possible cross-linking capacity of both native and ox-dextran on gelatin microspheres (GMs), particles with an average diameter comprised between 80 and 120 μm were considered. Microspheres were prepared by a thermal gelation method as reported in the experimental section [17]. The morphology and size distribution of GMs produced in the presence of native and ox-dextran were studied by SEM analysis and laser sizing (see Figs. 2 and 3). All GMs showed a good spherical geometry. Untreated microspheres (Fig. 2a) and GMs treated by native dextran (Fig. 2b) showed a wavy surface with the presence of small plaques; while ox-dextran treated microspheres showed a moon-like surface (Fig. 2c). In no case were macroscopic pores detectable on the surface of GMs.

The microsphere color, did not change appreciably when they are prepared in the presence of native dextran, whilst it has to be stressed that microsphere prepared from ox-dextran were darker in color than untreated microspheres or those treated with native dextran. This can be tentatively attribute to an increase in the cross-linking density (as it was previously demonstrated for thermal hardened particles) [7]. When native or ox-dextran are employed for gelatin cross-linking, a reduction of the mean diameter of the GMs can be observed if compared to plain gelatin microspheres (see particle size distribution data reported in Table 1 and Fig. 3).

In order to assess if dextran mediates hardening of GMs which results in an effective reduction of gelatin solubility, dissolution experiments were conducted. The dissolution of

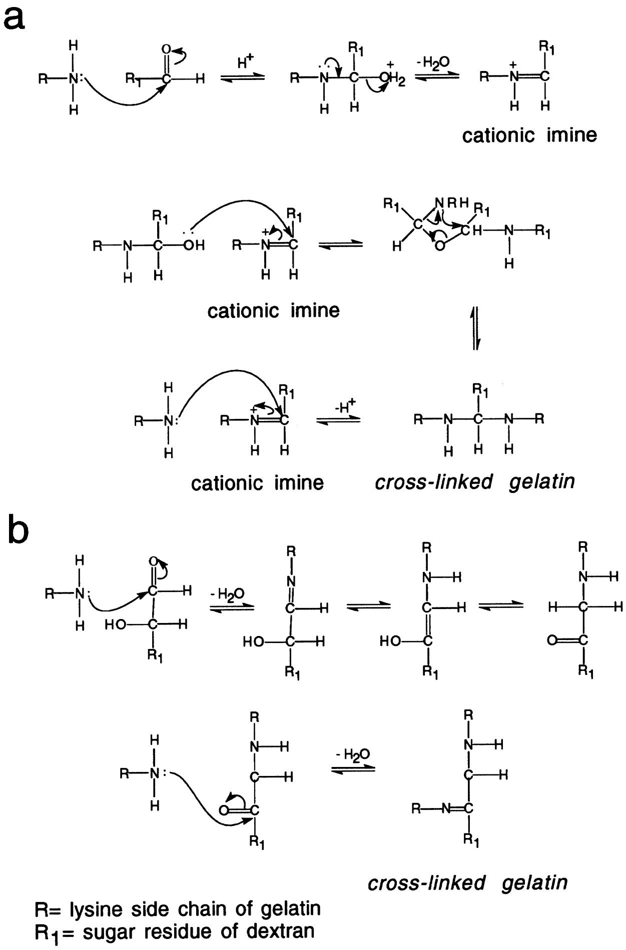

Fig. 1. Possible cross-linking pathways for native sugars. See text for details.

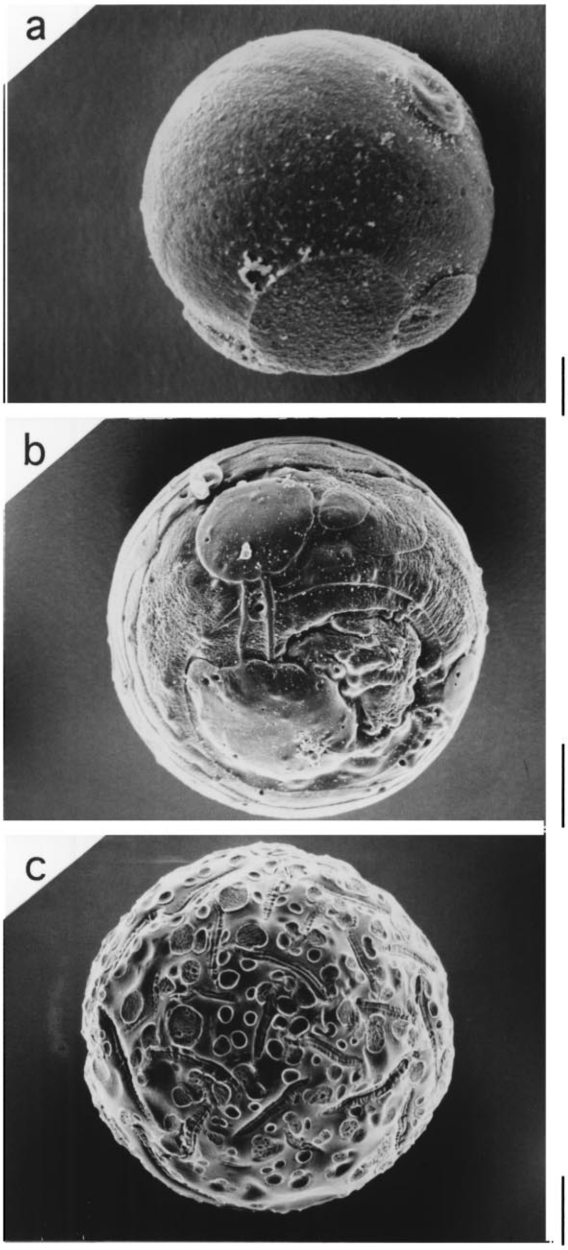

Fig. 2. Scanning electron micrographs of gelatin microspheres. (a) Untreated GMs. (b) GMs treated with native dextran. (c) GMs treated with ox-dextran. Bars represent 20 μm.

both treated and untreated GMs was analyzed either at room temperature and at 37ºC.

In Fig. 4 the dissolution of GMs prepared using native and ox-dextran performed at 37°C is reported. From an analysis of the release kinetics, the dissolution kinetics of GMs treated by ox-dextran is reduced with respect to untreated microspheres. Particularly, the dissolution of ox-dextran GMs was reduced to 40% if compared with that of sole gelatin microspheres. Conversely, GMs treated by native dextran displayed a dissolution rate comparable with that of untreated microspheres.

With the aim of evaluating if the reduced solubility of ox-dextran-treated microspheres could be ascribed to a reduced swelling of the particle, we analyzed the swelling characteristics of GMs at 37ºC [7]. Particles were dispersed in a 20% w/v Poloxamer 407 gel. The choice of this particular swelling medium was made since its viscosity enables to maintain the microspheres in fixed positions during microscopic observation. This method permits not only study of the average swelling of a microsphere population, but also the swelling of individual particles, thus allowing the analysis of the size influence on swelling. Fig. 5 reports the microphotographs of untreated microspheres (Fig. 5a) or those treated by native (Fig. 5b) and oxidized dextran (Fig. 5c). Pictures refer to microspheres incubated for 1 h in poloxamer gel at 37ºC, in addition the entire swelling kinetic is reported in Fig. 5d. From the analysis of data, it arises that GMs treated by ox-dextran show the lowest extent of swelling.

Table 1

Particle size distribution of plain native and ox- dextran-treated gelatin microspheres

Microsphere composition	Mean diameter (μm) ±SD	TAPP-Br encapsulation yield (%)
Gelatin	120 ± 14.2	94.92
Gelatin/native dextran	88 ± 9.8	94.31

The influence of dextran was in addition tested on microparticles containing the antitumor drug TAPP-Br, used as a

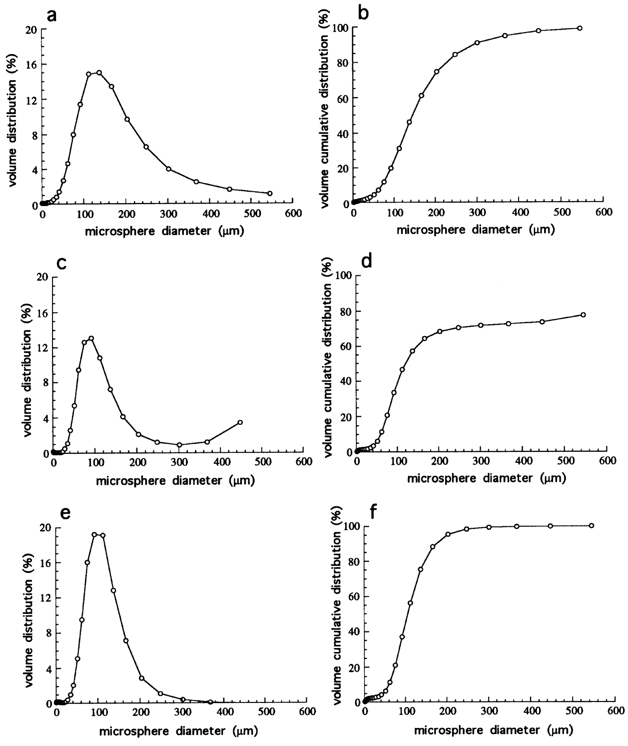

Fig. 3. Frequency and cumulative size distribution plots of gelatin microspheres. (a,b) Untreated gelatin microspheres. (c,d) Gelatin microspheres treated with native dextran. (e,f) Gelatin microspheres treated with ox-dextran.

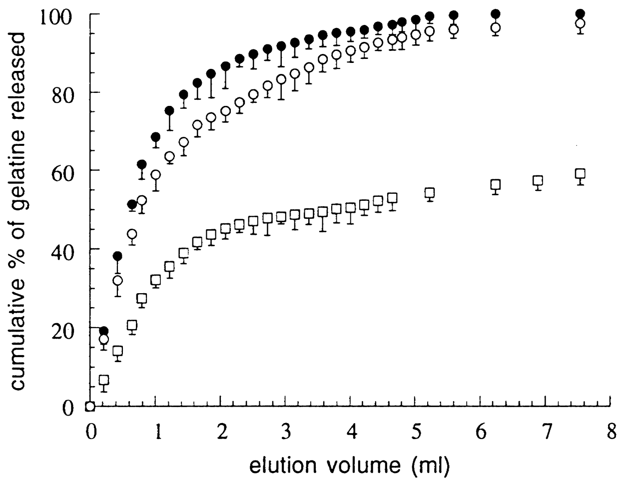

Fig. 4. Dissolution profiles of gelatin microspheres. Experiments were performed at 37ºC using a flow-through cell method. (●) Untreated microspheres; (○) native dextran-treated microspheres; (□) ox-dextran-treated microspheres. Data represent the average of three independent experiments.

model compound. The encapsulation yield of TAPP-Br was almost quantitative for untreated, native- and ox-dextran treated microspheres. All microspheres showed in fact a very high percentage of encapsulation ranging from 95 to 97% (Table 1), allowing the preparation of GMs with an high specific content of TAPP-Br. This is an important parameter to be considered in order to reduce the amount of microspheres to be injected to obtain in vivo pharmacological activity (milligrams of microspheres per kilogram of body weight). This could minimize possible host immunoresponses to the microsphere surface [19]. In order to characterize the release properties of microparticles, the release profile of TAPP-Br from microspheres was determined using a dialysis method [17]. This method was chosen for its simple experimental procedure and high degree of reproducibility. When other experimental protocols were used to determine TAPP-Br release kinetics, such as the column elution method, the results showed a higher degree of fluctuation [20]. After 48 h at 37ºC, the release of TAPP-Br from untreated and native dextran-treated microspheres was about 90% of the total amount of the encapsulated drug; whilst after the same length of time, the drug released from ox-dextran cross-linked microspheres was reduced to 20% of the total amount of the encapsulated drug (Fig. 6a). However, in all cases the drug release profiles showed a biphasic modulation characterized by an initial relatively rapid release period (a 'burst effect') followed by a slower release phase ('lag time'). It is to be emphasized that after drug release from microsphere, a control of polyamidine integrity by HPLC analysis was performed.

The reduction of drug release was tentatively attributed to the cooperative action of mainly two factors: (a) the decrease of the particle solubility and swellability; and (b) the increase of the density of the gelatin matrix. Neverthe-

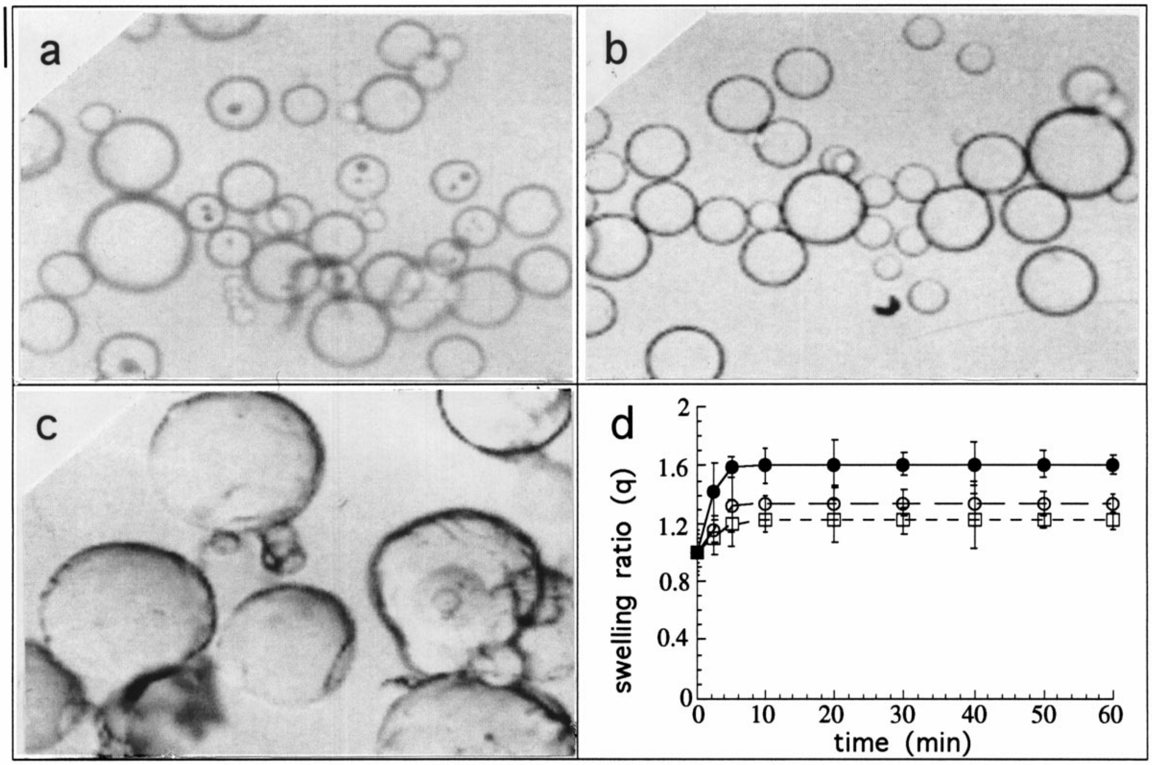

Fig. 5. Effects of dextran treatment on swelling of gelatin microspheres. Photomicrographs refer to untreated (a), native dextran (b) and ox-dextran-treated (c) GMs. Photographs were taken after 1 h of swelling. Bar represents 120, 90 and 50 μm for panels a, b and c, respectively. (d) Swelling profile of untreated microspheres (●) and native (○) or ox-dextran-treated (□) GMs. Data represent the average of three independent experiments on different microsphere batches ± SD.

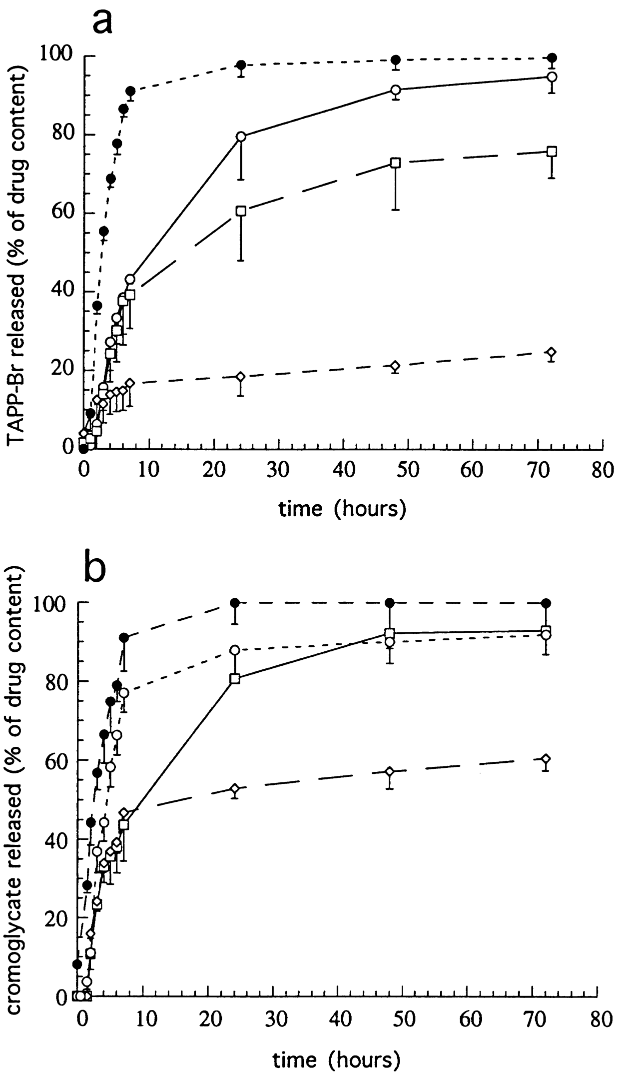

Fig. 6. Drug release profiles from untreated (○) and native (□) or ox-dextran-treated (◇) gelatin microspheres at 37°C. The reported values represent the average of four independent experiments ± SD. As a comparison the release of free drug from dialysis tube (●) is reported. (A) TAPP-Br containing microspheres. (B) Sodium cromoglycate containing microspheres.

less, our data cannot exclude that part of the polyamidine used as model drug can react with ox-dextran. In spite of this possibility, it should to be mentioned that enzymes present in vivo can hydrolyze the linkage which occurs between the drug and ox-dextran generating the free-acting drug. The use of drug-loaded dextran was, in fact, recently proposed as a polymeric pro-drug after in vivo administration [8].

In addition, in order to obtain more general results, a second model drug, namely sodium cromoglycate, was encapsulated in gelatin microspheres. The encapsulation yield of sodium cromoglycate for untreated, native dextran and ox-dextran treated microspheres was found to be 59, 60 and 66.5% of the total drug utilized, respectively. Fig. 6b reports the release profile obtained in the case of cromoglycate containing microspheres. Also for cromoglycate, the release kinetic from gelatin microspheres was found more or less in agreement, even in different extent, with that of TAPP-Br. After 48 h at 37°C in fact, the release of sodium cromoglycate from untreated and native dextran treated microspheres was about 90% of the total amount of the encapsulated drug; whilst after the same length of time, the drug released from ox-dextran treated microspheres was reduced to 55% of the total amount of the encapsulated drug.

4. Conclusions

With the aim of obtaining gelatin-based microparticles cross-linked by compounds, the cross-linking ability of native and oxidized dextran was described. GMs cross-linked by native or oxidized dextran were produced and characterized. Both native and oxidized dextran showed their capacity to reduce the dissolution of gelatin and, more interestingly, to slow down the release of both TAPP-Br and cromoglycate used as model drugs. Taken together our results suggest that oxidized dextran could be an interesting alternative method to cross-link GMs. This specialized delivery system could be potentially useful for the in vivo administration of antitumor drugs by subcutaneous injection or chemoembolization procedures.

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