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Doxorubicin-loaded gelatin nanoparticles stabilized by glutaraldehyde: Involvement of the drug in the cross-linking process

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Abstract

The possible involvement of the primary amino group of doxorubicin (DXR) in the cross-linking process of gelatin nanoparticles stabilized by glutaraldehyde was investigated. Nanoparticles were characterized with regard to particle size, drug content, enzymatic degradation and cross-linking degree. The size of nanoparticles was around 100–200 nm and DXR was loaded with an entrapment efficiency of 42%. Upon the study of crosslinking rate, DXR-loaded nanoparticles showed a greater number of free amino groups than the unloaded ones. This should be due to a competition between the amino group of DXR and the amino groups of the gelatin chains during the cross-linking process. Hence, a binding of a drug fraction to the protein matrix via glutaraldehyde was hypothesized and confirmed by the results of a thin-layer chromatography (TLC) analysis. According to the in vitro study only a little fraction of DXR was released as free drug (8%) when the nanoparticles were put in saline solution. The addition of proteolytic enzymes disrupts the matrix structure producing the release of a further 10–15% of the drug loading which was entrapped in the nanoparticle matrix. The remaining part of the drug corresponds to DXR covalently linked to peptide residues produced by nanoparticle digestion. © 1997 Elsevier Science B.V.

Keywords: Doxorubicin hydrochloride; Gelatin; Nanoparticles; Glutaraldehyde; Cross-linking degree; Drug release

1. Introduction

During the last decade various attempts have been performed to reduce the toxicity of some anticancer agents using polymeric nanoparticles in view of their ability to alter the biodistribution profile of drugs (Couvreur et al., 1990).

Among the polymers proposed to produce nano- and microparticles, gelatin, a natural

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macromolecule commonly employed as a pharmaceutical adjuvant and an encapsulating drug material, has focused a great interest for its biocompatibility and biodegradability (Jones, 1987). It was found that gelatin nano- and microparticles, prepared by means of different processes and hardened by a suitable cross-linking agent as glutaraldehyde, enhance tumoral cell phagocytosis (Oppenheim and Stewart, 1979; Tabata and Ikada, 1994). Hence, these systems have been widely studied in parenteral formulations as carriers of cytostatic drugs such as interferon, 5-fluorouracil, methotrexate, or doxorubicin (Narayami and Panduranga Rao, 1994; Oppenheim et al., 1984; Tabata and Ikada, 1994). Although the exact mechanism of protein cross-linking with glutaraldehyde is not yet clearly defined, certainly the ε-amino groups of the lysine residues and the N-terminal amino groups of the proteins are involved (Sokoloski and Royer, 1984). The primary amino group of doxorubicin should be suitable for a linkage with glutaraldehyde during the reticulation process of nanoparticles. Chen et al. (1988) found that in albumin microspheres cross-linked by glutaraldehyde a fraction of drug was covalently bound to the protein. Thus, the aim of this work was to investigate the possible interference of doxorubicin on the cross-linking process (i.e. on the cross-linking degree) of gelatin nanoparticles stabilized by glutaraldehyde.

Currently, there is not an absolute method to determine the degree of cross-linking in systems stabilized by glutaraldehyde. Various methods have been proposed as swelling (Longo and Goldberg, 1985), or the use of agents that disrupt only non covalent bounds in the particle matrix (Rubino et al., 1993). To achieve our aim, since the amino groups are implicated in the reticulation process, we have chosen to evaluate the number of free amino groups of loaded and empty nanoparticles, as indicators of their cross-linking degree. To determine the free amino group content, the method proposed by Edwards-Levy et al. (1993) was applied.

2. Materials and methods

2.1. Materials

Gelatin type A from porcine skin (250 Bloom, pH of 1% w/v water solution: 3.5–5; Fluka, Buchs, Switzerland) was used for the preparation of the nanoparticles; glutaraldehyde (25% w/w water solution, Fluka) was the cross-linking agent; doxorubicin hydrochloride (DXR) (ADRIBLASINA® Farmitalia-Carlo Erba, Milan, Italy) was the drug; polyoxyethylene sorbitanmonolaurate (polysorbate 20), sodium sulphate, sodium metabisulphite and isopropanol, used in the preparation of the nanoparticles, were purchased by Fluka. Sephadex G-50m (Sigma, St. Louis, MO) was used as column packing in the gel filtration purification procedure. Trypsin (from bovin pancreas; 8 units/mg solid), protease (from bovin pancreas; 10 units/mg solid) and α-chymotrypsin (from bovin pancreas; 42 units/mg solid) were supplied by Sigma. 2,4,6-trinitrobenzenesulfonic acid (10% w/w water solution, Fluka) was used for the determination of the cross-linking degree. All the products were used as received from the manufacturers.

2.2. Preparation of gelatin nanoparticles

Gelatin nanoparticles were prepared according to the method of Oppenheim and Stewart (1979), based on the coacervation-phase separation technique.

A water gelatin solution (1% w/v; 10 ml), containing polysorbate 20 as surfactant (0.5% w/v) and the drug (DXR) (5 mg), was stirred at 37°C and 200 rpm with a magnetic stirring bead. To induce the desolvation process of gelatin sodium sulphate (20% w/v in water; 7 ml) was used obtaining a permanent faint turbidity due to coacervate phase. Isopropanol (1.2 ml) was then added until the turbidity disappeared. The magnetic stirring was replaced with an Ultra Turrax® (Janke and Kunkel-Ika-Laboratortechnick, Staufen, Germany) and 0.4 ml of the 25% glutaraldehyde water solution were added. The crosslinking process was completed after 11 min by the addition of 5 ml of a water solution of sodium metabisul-

phite (12% w/v). After 1 h, excess of salts and unloaded drug were removed by passing the crude nanoparticle suspension (crude system) through a glass column (diameter 25 mm; height 50 cm) containing 120 ml of Sephadex G-50m. The colloidal system was shell frozen at −18°C (Shell-Freezer; Edwards, Crawley, UK) and lyophilized at 2 mbar and −40°C for 24 h (Lyovac GT 2; Leybold-Heraeus, Hanau, Germany). Nanoparticles were stored as freeze-dried powders under vacuum (2 mm Hg) at 25°C.

Unloaded nanoparticles were obtained as described above, but without adding the drug to the gelatin solution.

2.3. Drug content

The drug content in the DXR-loaded nanoparticles was calculated by difference between the total amount added in the gelatin solution and the amount of free drug present in the crude system. The free drug amount in the crude system was determined by spectrophotometric assay at 480 nm (Lambda 3B, Perkin Elmer, Norwalk, USA) in the clear supernatant obtained by separation of the nanoparticles with a combined ultrafiltration/centrifugation technique (Centrisart I cut-off 10 000; Sartorius, Göttingen, Germany).

2.4. Morphological analysis

The size and the morphology of nanoparticles were examined using a scanning electron microscope (SEM) (XL-40; Philips, Eindhoven, The Netherlands).

2.5. Enzymatic degradation

The enzymatic degradation of both empty and loaded nanoparticles was carried out according to the method proposed by Roser and Kissel (1993). Briefly, 10 ml of deionized water containing 0.4 mg/ml of trypsin, α-chymotrypsin, or protease was added to 15 mg of empty nanoparticles. The turbidity was determined at room temperature by the absorbance at 546 nm using a spectrophotometer (Lambda 3B, Perkin-Elmer). In a preliminary study it had been shown that at 546 nm the absorbance was a linear function of the nanoparticle concentration in the range 0.5–3 mg/ml.

2.6. Determination of cross-linking degree

Free amino group content in native gelatin, empty and loaded nanoparticles was measured using the 2,4,6-trinitrobenzensulfonic acid (TNBS) method (Satake et al., 1960) according to the procedure adapted by Edwards-Levy et al. (1993). This procedure consists in the incubation of the material with an excess of TNBS and the back titration of the unreacted amount of the reagent.

2.6.1. Determination of free amino group content in gelatin and in nanoparticles

In order to avoid interference due to the amino groups of free DXR molecules, the free drug in the loaded-nanoparticles was eliminated before the determination. Thus, 0.15 g of polysorbate 20 were added to 10 ml of ethyl ether suspension of loaded nanoparticles (30 mg/ml) the suspension was vortexing (ZX^3, Velp Scientifica, Carnate, Italy) for 5 min. Ethyl ether was then evaporated under oxygen-free nitrogen. The nanoparticle residue was washed with normal saline using Centrisart I (cut-off 10 000; Sartorius) to eliminate all the free DXR. Nanoparticles, recovered after lyophilization, were used to determine free amino group content.

The substrate (5 mg) was dissolved (gelatin) or suspended (empty and the treated loaded nanoparticles) in 3 ml of borate buffer (pH 8.0, 0.2 M) and incubed at 40°C in the dark for 1 h with 4 ml of TNBS (4 μmol/ml in pH 8.0 borate buffer). Then, the suspension or the solution was filtered by a combined ultrafiltration/centrifugation technique (Centrisart I cut-off 5 000 for gelatin and cut-off 10 000 for nanoparticles; Sartorius). In order to measure the excess of TNBS, 0.4 ml of ultrafiltrate was added to 5 ml of pH 8.0 borate buffer containing 0.1 ml of valine water solution (20 μmol/ml in 1% w/v trichloroacetic acid) and incubed at 40°C in the dark for 1 h. Then, 5 ml of HCl (0.5 N) were added and the absorbance of solution was measured at 340 nm against a blank prepared as described above, but

containing 0.1 ml of trichloroacetic acid instead of valine solution.

2.7. Thin-layer chromatography (TLC) experiment

TLC determination was carried out on glass sheets (2 mm) precoated with Silica gel 60 G (Merck, Darmstadt, Germany). A mixture of butanol/acetic acid/water (4/1/5, v/v/v) was used as mobile phase. The samples examined were: (a) free DXR; (b) loaded nanoparticles; (c) loaded nanoparticles after enzymatic degradation (degraded-loaded nanoparticles); (d) physical mixture empty nanoparticles/DXR (empty nanoparticles/DXR); and (e) physical mixture empty nanoparticles/DXR after enzymatic degradation (degraded-empty nanoparticles/DXR). The spots were detected at UV light ($\lambda = 245$ nm).

To prepare the empty nanoparticles/DXR (d), empty nanoparticles (100 mg) were dispersed in 10 ml of deionized water using a Vortex (Zx^3; Velp Scientifica). Then, DXR (2 mg) was dissolved in the dispersion. The physical mixture was then recovered after lyophilization. For the TLC analysis of both the degraded-loaded nanoparticles (c) and the degraded-empty nanoparticles/DXR (e), the degradation products obtained after 5 h of incubation in 10 ml of deionized water containing 0.4 mg/ml of trypsin were utilized.

2.8. Determination of free DXR content in nanoparticles

A weighed amount of loaded nanoparticles (20 mg) was dispersed in normal saline (25 ml) and kept in the dark at 37°C under magnetic stirring for 48 h. After this period, nanoparticles were separated from suspension using Centrisart I (cut-off 10 000; Sartorius) and the ultrafiltrate was analysed spectrophotometrically (Lambda 3B, Perkin-Elmer) at 480 nm for drug content. The nanoparticles were treated with the same procedure three more times, each time analysing DXR concentration in the ultrafiltrate.

Under the same experimental conditions, the same amount of empty nanoparticles produced no remarkable changes ($E < 0.005$) of the absorbance value.

2.9. Release of DXR from nanoparticles

A weighed amount of loaded nanoparticles (40 mg) was dispersed in 50 ml of normal saline at 37°C under magnetic stirring (800 rpm) in the dark. At predetermined time intervals (30 min, 4, 8, 12, 24, 48, and 72 h) an aliquot of suspension (2 ml) was withdrawn and nanoparticles were separated using Centrisart I (cut-off 10 000; Sartorius). The drug concentration was determined spectrophotometrically (Lambda 3B, Perkin Elmer) at 480 nm. Then, the ultrafiltrate and the nanoparticles were mixed and reintroduced in the releasing medium. After 72 h, 10 mg of one of the proteolytic enzymes (trypsin, α-chymotrypsin, or protease) were added. At predetermined time intervals (10 and 20 min, 1, 4, 12 and 24 h) an aliquot of suspension (2 ml) was withdrawn and the drug concentration was determined spectrophotometrically in the ultrafiltrate.

Under the same experimental conditions, empty nanoparticles produced no remarkable changes ($E < 0.005$) of the absorbance value.

3. Results and discussion

To produce loaded and empty nanoparticles an excess amount of glutaraldehyde (10 μmol of glutaraldehyde/mg of gelatin) was used in order to obtain a rapid and reproducible hardening (Oppenheim, 1981). Hardening process was carried out for 11 min since a shorter period was not sufficient for particle reticulation, and a longer period produced too many large particles. At the end of hardening process, unreacted glutaraldehyde was neutralized by the addition of sodium metabisulphite, that reacts with the excess of glutaraldehyde avoiding the formation of large particles or aggregates. After separation of salts, free drug and other low molecular weight species by gel chromatography, a colloidal suspension with a faintly turbidity was obtained. Following freeze drying, a spongy mass was recovered with a yield of $90\% \pm 5$. The spongy mass was readily dispersible in water forming a colloidal dispersion stable more than 24 h without visible precipitation. As shown by SEM analysis (Fig. 1),

nanoparticles have a size range of 100–200 nm and a spherical morphology. No differences in size and morphology were observed between loaded and empty nanoparticles (data not shown).

Actual loading of DXR in nanoparticles resulted in 21 μg of drug/mg of nanoparticles while the theoretical loading was 50 μg/mg of gelatin. The low entrapment efficiency was probably due to the high water solubility of DXR (100 mg/ml) (Marty et al., 1978).

In order to investigate the involvement of the drug in the crosslinking process, the amount of free amino groups in both empty and loaded nanoparticles was measured using the TNBS method. We used the procedure adapted to insoluble material not only for nanoparticles, but also for native gelatin. However, after the first incubation, gelatin was totally eliminated from the solution applying the combined ultrafiltration/centrifugation technique. According to gelatin identification test reported by British Pharmacopeia (1993), no trace of gelatin was found in the ultrafiltrate solution.

Owing to the reticulation process, the number of free amino groups in empty and loaded nanoparticles were less than those in native gelatin. Assuming the amino groups of gelatin as 100%, the results showed that the cross-linking process involved about 38% of the amino groups in the case of empty nanoparticles, whereas about 30% of amino groups were involved in the case of loaded nanoparticles (Table 1). Therefore, loaded nanoparticles resulted in less cross-linking processes than empty ones. Since the manufacture process of empty and loaded nanoparticles was the same and in both cases an excess of glutaraldehyde was used, the different crosslinking degrees could be due to the presence of the drug. In our opinion the amino group of DXR may interfere in the crosslinking process in two ways. Firstly, glutaraldehyde could link each of the two molecules of DXR, allowing less of the cross-linking agent to be available for gelatin reticulation. Since an excess of gelatin was used (DXR/gelatin ratio was 5:100), the extent of this process should be very weak. On the other hand, as reticulation was carried out in the presence of DXR, a fraction of glutaraldehyde could form a bridge between gelatin and DXR rather than between two protein chains. As a consequence, the number of free amino groups in loaded nanoparticles is more important (Fig. 2). The binding between DXR and gelatin via glutaraldehyde is possible according to the data reported in the literature. In fact, DXR was covalently linked to antibodies using glurataldehyde, (Hurwitz et al., 1975) and to polygluratadehyde microspheres amino group (Tokes et al., 1982) via the amino group. However, to demonstrate that in our case DXR was covalently linked to protein matrix, a TLC analysis was performed on enzyme degraded nanoparticles.

Table 1
Free amino group content present in gelatin, empty nanoparticles and DXR-loaded nanoparticles

Substrate	Free-NH_2/g of substrate (μmol)	Free-NH_2 (%)
Gelatin	1376 ± 46	100
Empty nanoparticles	857 ± 28	62.3
Loaded nanoparticles	958 ± 21	69.6

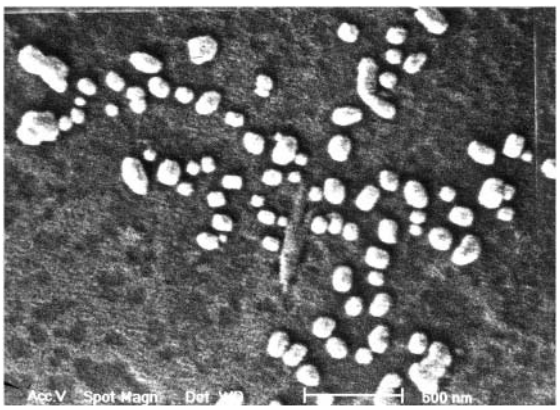

Fig. 1. Scanning electron microphotograph of empty gelatin nanoparticles.

Before carrying out TLC analysis, a study of nanoparticle in vitro degradation was accomplished. This study provides useful indications both on the efficiency of proteolytic enzymes in crosslinked nanoparticle degradation and on the

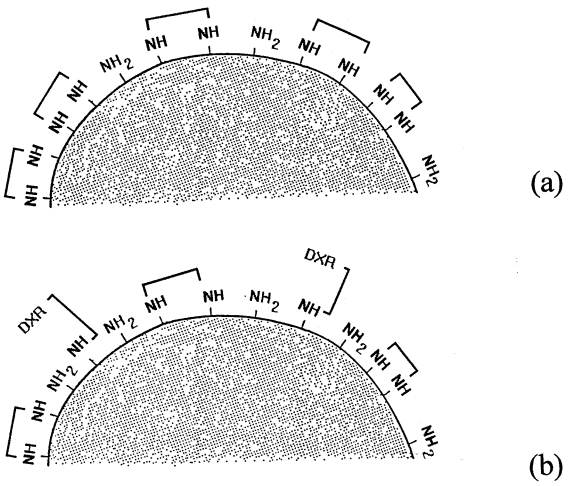

Fig. 2. Schematic representation of the cross-linking process via glutaraldehyde during the preparation of empty (a) and loaded (b) nanoparticles. (⊓): glutaraldehyde molecule; (DXR): Doxorubicin molecule

feasibility of the therapeutic use of the carrier system, as a carrier has to be easily degraded for the in vivo utilisation. Three different enzymes (protease, α-chymotrypsin and trypsin) were assayed on loaded and empty nanoparticles. As expected, no difference was observed in the degradation process between loaded and empty nanoparticles (Fig. 3). The tested enzymes were able to degrade, in 2 min, 50% of loaded and empty nanoparticles. Nevertheless, the degradation process of nanoparticles by protease or α-chymotrypsin stopped after 30 min and an incomplete degradation resulted (70% and 80% of nanoparticles, respectively). On the contrary, trypsin worked for a longer time (300 min) degrading about 100% of nanoparticles. Owing to the ease of nanoparticle degradation, it may be hypothesized that the carrier will be completely biodegradable in vivo. Furthermore, the difference in the degradation rate among the three enzymes suggests a degree of enzyme specificity. Trypsin appears the most suitable enzyme for gelatin nanoparticle degradation. These results agree with data reported in the literature for collagen nanoparticles (El Samaligy and Rohdewald, 1983). In this case trypsin appeared more specific than collagenase. Therefore, trypsin was used to digest the protein matrix of nanoparticles which were submitted to the TLC analysis.

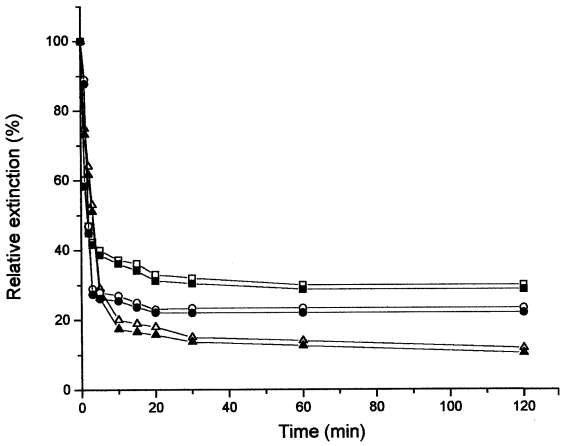

Fig. 3. Enzymatic degradation in enzyme-water solution (0.4 mg/ml) at room temperature of empty nanoparticles by protease (■), (α-chymotrypsin (●), trypsin (▲), and of loaded nanoparticles by protease (□); α-chymotrypsin (○) and trypsin (△).

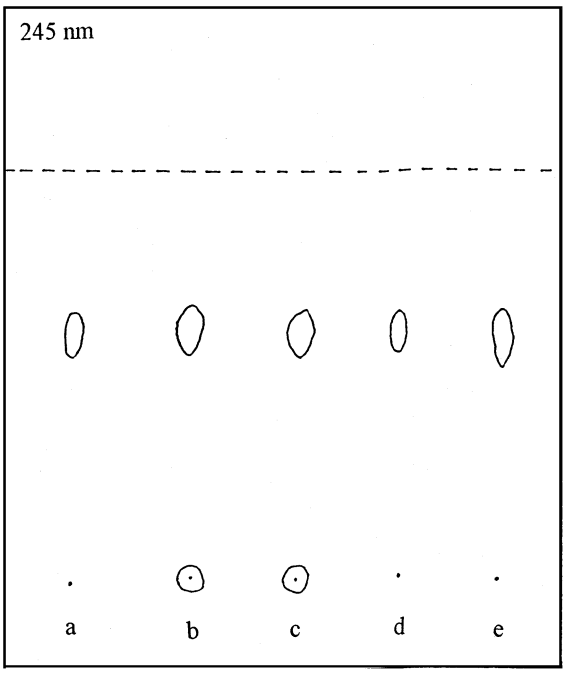

Fig. 4. TLC assays. (a): free DXR; (b): loaded nanoparticles; (c): degraded-loaded nanoparticles; (d): empty nanoparticles/DXR; and (e): degraded empty nanoparticles/DXR.

Fig. 4 shows the spots resulting from TLC studies. Free drug: (a) migrates from the origin in the solvent system used ($R_f = 0.67$). For the loaded nanoparticles (b) two orange spots were recovered: a spot with an $R_f = 0.67$, suggesting that free DXR was present in the loaded nanoparticles, and a spot on the origin attributable to drug associated to the carrier. For degraded-loaded nanoparticles (c) two orange spots were recovered also. A spot with an $R_f = 0.67$ attributable to the free drug and another one on the origin. In this case, according to Chen et al. (1988), the spot on the origin may be attributed to DXR covalently bound to peptide fragments resulting from trypsin digestion. In order to confirm this hypothesis, the physical mixtures (d) and (e) (empty nanoparticles/DXR and degraded-empty-nanoparticles/DXR, respectively) were assayed. As Fig. 4 shows, in both cases no spots were detectable on the origin. Therefore, the orange spot on the origin of degraded-loaded nanoparticles (c) was due to DXR covalently linked to peptide residues.

In order to evaluate the DXR amount available as free drug, the loaded nanoparticles were placed in saline solution. In these conditions, only the free drug can be released allowing this amount to be recognised from that of the linked DXR. On the basis of experimental results, the percentage of free DXR in nanoparticles resulted in about 8% of the whole drug loading. To estimate the amount of active drug available in vivo, in vitro release experiments were carried out in saline solution for 72 h (Fig. 5). The results did not show any increase in the percentage of the free drug with respect to the value reported above (about 8%). The incomplete release of DXR from glutaraldehyde cross-linked microspheres was previously reported by Willmott et al. (1985). According to these authors a complete release would occur by degradation of the protein matrix. Therefore, a proteolytic enzyme was added to clarify how much of the remaining 92% could be available. The degradation by protease induced the release of a further 10% of the drug loaded, whereas α-chymotrypsin and trypsin allowed a higher amount of DXR to be released (15 and 20%, respectively). As the amount of DXR released agrees with the degradation efficiency of the enzymes, it may be attributable to a fraction of the free drug entrapped in the protein matrix. The remaining part of the drug (approx. 70%) should correspond to the fraction linked to the protein fragments produced by the nanoparticle digestion, as DXR bound to the protein matrix should not be cleaved by proteolytic enzymes.

In conclusion, gelatin nanoparticles cross-linked by glutaraldehyde resulted in a biodegradable carrier in which the major part of DXR (about 70%) was bound to the protein matrix via glutaraldehyde, forming a drug-conjugate. DXR-protein conjugates improve the cytotoxic activity of the drug, allowing DXR to escape from the multidrug resistance mechanism (Ohkawa et al., 1993).

Further in vivo evaluations will be carried out to assess toxicity and antitumor activity of DXR in these biodegradable drug-conjugate nanoparticles.

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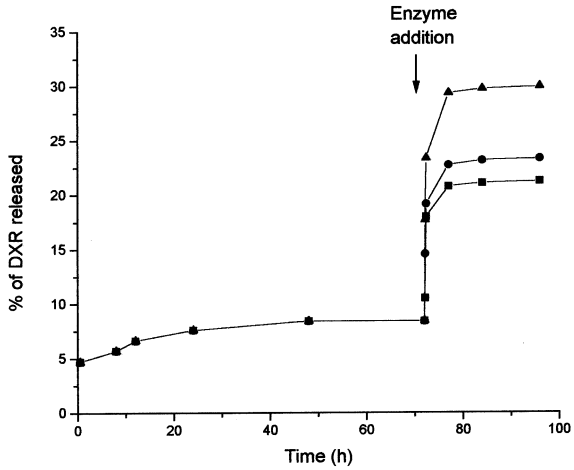

Fig. 5. Doxorubicin (DXR) in vitro release from nanoparticles before and after treatment with different proteolytic enzymes. Key: protease (■); α-chymotrypsin (●); trypsin (▲).

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