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Synthesis, controlled release properties and antitumour activity of alginate-*cis*-aconityl-daunomycin conjugates

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Abstract

Covalent conjugates of alginate and the antitumour agent daunomycin (DNM) were synthesized to be stable in the circulation and allow release of the drug in the acidic milieu of the endosomal and lysosomal compartments of tumour cells or the slightly acidic extracellular fluid of some solid tumours. Alginates containing primary amine groups were first prepared by reacting alginate with excess ethylenediamine. DNM was first reacted with *cis*-aconitic anhydride to produce *N-cis*-aconityl-DNM and then subsequently bound to the amino-modified alginate using the water-soluble carbodiimide 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). High ($M_w = 250\,000$) and low ($M_w = 61\,000$) molecular weight alginate-DNM conjugates were prepared. In vitro release studies showed that DMN was released from the conjugates (approx. 22–60%/48 h) under acidic conditions (pH 5 and 6) with minimal release occurring at neutral pH (approx. 2–4%/48 h). Reverse-phase HPLC confirmed that DNM was the only product released from high molecular weight alginate-DNM conjugate (22% released/48 h at pH 5), but the low molecular weight alginate-DNM liberated in addition a DNM derivative (approx. 60% released (total)/48 h at pH 5). In a preliminary experiment to investigate the antitumour activity of alginate-DNM conjugate in vivo, administration of a single intraperitoneal injection of low molecular weight alginate-DNM (equivalent to 5 mg/kg DNM) to mice bearing B16 subcutaneous tumours resulted in a small, but significant delay in the growth of the tumour.

Keywords: Alginate; Daunomycin; Polymeric drug carrier; Acid-sensitive spacer; Macromolecular prodrug; Antitumor activity

1. Introduction

Alginates are biopolymers, composed of (1–4)-linked β-D-mannuronic acid and α-L-guluronic acid (Gacesa, 1988), that stimulate the production of the cytokines tumour necrosis factor-α, interleukin-1 and interleukin-6 from human

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monocytes (Otterlei et al., 1991; Espevik et al., 1993), and display inherent antitumour activity against murine tumour models in vivo (Fujihara et al., 1984; Fujihara and Nagumo, 1992). As it is known that macromolecules accumulate passively within solid tumours (Matsumura and Maeda, 1986), it was considered interesting to explore the possibilities of combining the immunostimulatory properties of alginates with delivery of a covalently bound antitumour agent. It has been shown previously that it is essential during its transport in the bloodstream for an antitumour agent to remain attached to the carrier, but on arrival at the tumour, the drug must be liberated in an active form. Two approaches have been proposed for drug-polymer linkage, both based on the concept of lysosomotropic drug delivery (Trouet et al., 1982). First, the design of peptidyl linkers, which are stable in the bloodstream, but efficiently degraded by lysosomal enzymes (Duncan et al., 1983; Rejmanova et al., 1985). Conjugates of *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers containing the antitumour agents daunomycin (DNM) and doxorubicin (DOX) bound by peptidyl linkers exhibited activity against a wide range of model tumours in vivo (Duncan et al., 1988, 1992; Cassidy et al., 1989). Second, it is possible to exploit the pH difference between the endosome/lysosome and the extracellular environment, and Shen and Ryser (1981) first described an acid-sensitive linker, *cis*-aconityl, which released DNM from model macromolecular carriers only at acidic pH.

Here a method was developed to covalently bind DNM to alginates of either high or low molecular weight via an acid-sensitive linker (*cis*-aconityl). The release of DNM from such conjugates was examined in vitro at pH 5, 6 and 7, and during incubation with mixtures of lysosomal enzymes. Also, their antitumour activity was examined against B16F10 murine melanoma in vitro and in vivo.

2. Materials and methods

2.1. Chemicals

Alginate (sodium salt, from *Macrocyctis pyrifera*) was supplied by Kelco Division of Merck and Co. Inc., San Diego, USA. *cis*-Aconitic anhydride, 1-aminopropan-2-ol, daunomycin (DNM), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) ethylenediamine dihydrochloride, glutathione (reduced form), *o*-phthalaldehyde (OPA) and Triton X-100 were from Sigma Chemical Co. Ltd, Dorset, UK. Doxorubicin (DOX) was from Farmitalia Carlo Erba, Milan, Italy. Phosphate-buffered saline (PBS) was obtained from Flow Laboratories, Hertfordshire, UK. *N*-Hydroxysulphosuccinamide was obtained from Pierce and Warriner, Cheshire, UK. Pullulan molecular weight standards were supplied by Polymer Laboratories, Shropshire, UK.

2.2. Preparation of low molecular weight alginate

Alginate (400 mg) was dissolved in 100 ml acetic acid (1% v/v, pH 3.5) and the solution was heated to 100° C for 5 min. The alginate solution was cooled and the pH adjusted to 7.4 with NaOH (1 M). The resulting solution was dialysed extensively against distilled water and then lyophilised.

2.3. Synthesis of alginate containing primary amine groups (alginate-NH_2)

Alginate (500 mg, 2.5 mmol) was dissolved in water (50 ml) and the pH was adjusted to 4.75. Solid EDC (60 mg, 0.32 mmol) was added to the stirred alginate solution and the pH was kept constant for 1 h by addition of HCl (0.1 M). A 40-fold excess ethylenediamine dihydrochloride solution was prepared by dissolving 13 g (0.1 mol) in 30 ml water and adjusting the pH to 4.75. The alginate solution was added dropwise to the diamine solution while stirring and the pH was maintained at 4.75 by addition of NaOH (0.1 M). After 1 h the pH was increased to 6 (NaOH, 5 M) and the solution left stirring for another 1 h. Enough NaOH (5 M) was added to raise the pH of the solution to 12.0, followed by the addition of NaCl (5 g). The solution was left stirring for 10 min, before it was dialysed extensively against sodium carbonate/bicarbonate buffer, pH 11.3 (0.1 M sodium carbonate, 0.01 M sodium hydrogen carbonate) for 72 h and then against distilled

water for 48 h. Any particulate matter was removed by filtration (0.2 μm filter) and the final solution was lyophilised. The amount of primary amine groups introduced into the alginate was estimated by the OPA method (Roth, 1971) using known standards of 1-aminopropan-2-ol.

2.4. Synthesis of N-cis-aconityl-DNM

N-cis-Aconityl-DNM was synthesised according to the method of Shen and Ryser (1981). DNM (12 mg, 0.02 mmol) was dissolved in 1.9 ml of phosphate buffer, pH 9.0 (0.1 M disodium hydrogen orthophosphate) in a polypropylene tube and cooled on ice. *cis*-Aconitic anhydride (22 mg) was added slowly, the solution was vortexed and the pH was immediately adjusted to 9.0 by careful addition of NaOH (0.5 M). After the pH was stabilised, the tube was wrapped with tin foil and kept on ice for 10 min and then left at room temperature for 15 min. During this period the reaction mixture was subjected to vortexing at regular intervals (every 2 min). The solution was diluted to 3 ml with water and the tube placed back on ice. Hydrochloric acid (1 M) was added slowly while vortexing until a heavy precipitate was formed. After 0.5 h on ice, the precipitate was collected by centifugation ($4000 \times g$, 4° C). The supernatant was removed by aspiration and the pellet redissolved in 4 ml of water with a small amount of NaOH (0.1 M). The solution was acidified again, the final pellet redissolved in 0.5 ml of water with small additions of NaOH (0.1 M) and the pH adjusted to 8.0. The concentration of the product was determined spectrophotometrically at 484 nm ($\varepsilon = 11\,500$ l mol^{-1} cm^{-1}). The final product was analysed by thin-layer chromatography (solvent: dichloromethane/methanol/acetic acid/water, 30:4:1:0.5).

2.5. Synthesis of alginate-cis-aconityl-DNM conjugates

To a solution of *N-cis*-aconityl-DNM, pH 7 (6.2 ml, 0.06 mmol), EDC (16 mg, 0.08 mmol) was added and the reaction mixture was left stirring at room temperature for 20 min. Alginate-NH_2 (320 mg, 0.07 mmol NH_2) dissolved in PBS (25 ml, pH 7) was added to the EDC activated *N-cis*-aconityl-DNM solution and the reaction mixture was left stirring for 18 h at room temperature. Unreacted *N-cis*-aconityl-DNM was separated from polymer bound DNM on a Sephadex G-50 column (3.6×55 cm) eluted with PBS (pH 7.4). The macromolecular fractions containing polymer bound DNM were collected, dialysed against distilled water (pH 7.4) and lyophilised. The amount of DNM bound to the polymer was determined spectrophotometrically at 484 nm ($\varepsilon = 11\,500$ l mol^{-1} cm^{-1}). In an attempt to improve the yield of the water soluble carbodiimide mediated coupling reaction, the following modifications to the method described above were carried out: 2 min following the addition of EDC to *N-cis*-aconityl-DNM, a slight excess of *N*-hydroxysulphosuccinimide (with respect to EDC) was added and the pH readjusted to 7.0 with NaOH (0.1 M). The amount of contaminating free drug in the conjugates was determined by reverse-phase HPLC analysis as described previously (Duncan et al., 1992), using DOX (500 ng) as an internal standard.

2.6. In vitro release of DNM from alginate-cis-aconityl-DNM conjugates

Alginate-*cis*-aconityl-DNM was dissolved in citrate/phosphate buffer at pH 5, 6 or 7 (Dawson et al., 1989) in a total volume of 1 ml to give a final concentration equivalent to 70–100 μg DNM/ml (the exact value determined spectrophotometrically at 484 nm, $\varepsilon = 11\,500$ l mol^{-1} cm^{-1}). In some cases, 0.3 ml of a mixture of rat liver lysosomal enzymes (tritosomes, Trouet, 1974) were added and the buffer (0.7 ml) was modified to include EDTA (1 mM), reduced glutathione (5 mM) and Triton X-100 (0.2% v/v). All incubations were carried out in a water bath at 37° C. Samples (100 μl) were removed at intervals, immediately frozen and the amount of free DNM was determined by reverse-phase HPLC as described before (Duncan et al., 1992).

2.7. Size-exclusion HPLC analysis

Samples (20 μl) were injected onto a TSK G4000 PW analytical column (7.5×300 mm), or

in some instances a TSK G4000 PW column connected in series with the TSK G2000 PW analytical column (7.5 × 300 mm) and eluted with PBS containing 0.4 M NaCl at a flow rate of 1 ml/min. Detection was performed with a refractive index detector (Knauer) maintained at a constant temperature (25° C). The column was calibrated with pullulan molecular weight standards.

2.8. *In vitro cytotoxicity of polymers and polymer-DNM conjugate*

B16F10 mouse melanoma cells were maintained in Minimum Essential Medium (MEM) Eagle supplemented with Earle's salts, sodium pyruvate, non-essential amino acids, vitamins, L-glutamine and foetal calf serum (10% v/v). B16F10 cells were plated in 96-well microtitre plates at a density of 2000 cells/well. Following an incubation of 24 h at 37° C (5% CO_2), the medium was removed by aspiration and in some wells replaced by medium containing polymers/polymer-DNM conjugate at various concentrations (200 μl, six replicates). The remaining wells were replaced with medium only (controls). Due to the poor solubility of alginates in the growth medium-MEM (due to the presence of high concentration of divalent metals), all alginate stock solutions (10 mg/ml) were prepared in Ham's F10 medium, supplemented with foetal calf serum (10% v/v) and L-glutamine (1% v/v). The stock solutions were then sterilised by filtration (0.2 μm filter) and diluted with MEM prior to addition to cells. Any detrimental effects on the cells due to Ham's F10 medium were accounted for by control wells where Ham's F10 medium was diluted in the same ratio with MEM and then added to cells. Following a 72 h incubation (37° C, 5% CO_2), cell viability was assessed by the MTT assay (Sgouras and Duncan, 1990).

2.9. *Antitumour activity of alginate-DNM conjugate against B16F10 melanoma in vivo*

A preliminary experiment was carried out to investigate the activity of alginate-DNM conjugate against B16F10 melanoma in vivo. Male C57BL/6J mice (12–14 weeks, 26–29 g) were inoculated subcutaneously with viable B16F10 cells (10^5 cells/0.1 ml). Tumours were allowed to establish (area approx. 70 mm^2; product of two orthogonal diameters), and a single treatment (free DNM, alginate-DNM conjugate, or alginate/DNM mixture in PBS) at a dose of, or equivalent to 5 mg DNM/kg of body weight administered by intraperitoneal injection. Tumour size and animal weight and survival were monitored. Control animals were not treated.

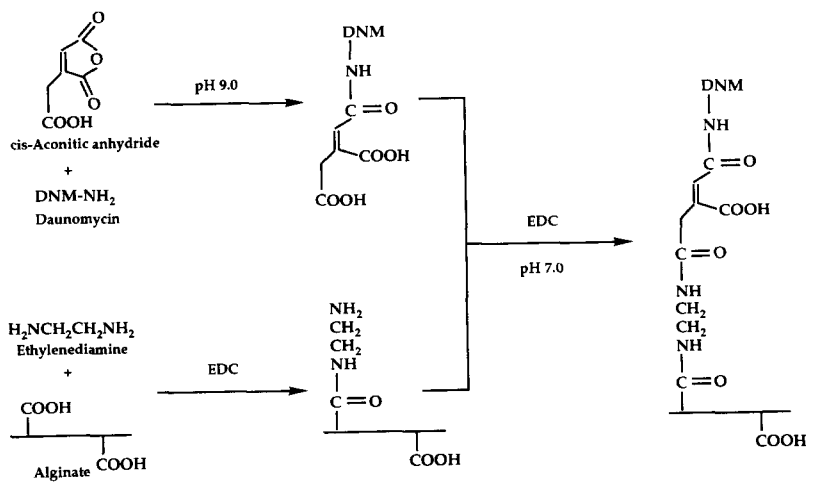


Fig. 1. Synthetic pathway of alginate-*cis*-aconityl-DNM conjugates.

3. Results

3.1. Preparation of low molecular weight alginate

Low molecular weight alginate was prepared by mild acid hydrolysis of alginate (from *M. pyrifera*). Size-exclusion HPLC analysis showed that the low molecular weight alginate had M_w of 131 000 and M_w/M_n of 2.8 compared with M_w of 427 000 and M_w/M_n of 3.1 for the starting material.

3.2. Synthesis of alginate-DNM conjugates

DNM was bound to alginate according to the synthetic scheme shown in Fig. 1. The overall synthetic pathway involved three steps. The first was the preparation of *N-cis*-aconityl-DNM, the second was the preparation of amino-modified alginates and the final step was the binding of the two previous components.

3.2.1. Synthesis of N-cis-aconityl-DNM

This compound was prepared as described previously (Shen and Ryser, 1981). Typically 65–80% of the drug added at the start of the reaction was converted to the *cis*-aconityl derivative, and the remaining unbound drug was removed by centrifugation. The purity of the final product was assessed by thin-layer chromatography which although by visual analysis showed no detectable free DNM, examination under a UV lamp indicated trace contamination with free DNM. A further purification step (precipitation and centrifugation), however, did not result in a purer product. The R_f values for DNM and *N-cis*-aconityl-DNM were 0.09 and 0.32, respectively.

3.2.2. Synthesis of alginate-NH_2

A method was developed to try to incorporate approx. 10 mol% primary amine groups into alginates. Solid EDC was added to alginates in order to activate a theoretical 10% of the carboxyl groups. A 40-fold excess of ethylenediamine was then reacted with the activated alginates. An excess of the diamine had to be used in order to prevent cross-linking and cyclisation of the alginate chains. The amount of primary amine groups introduced was 3.2 and 4.2 mol% for the high and low molecular weight, alginates, respectively. The amount of ionically bound diamine was negligible. An increase in the molecular weight of the amino-modified alginates was not evident on analysis by size-exclusion HPLC (data not shown), hence indicating that cross-linking of the alginate chains did not take place during the reaction with ethylenediamine. Size-exclusion HPLC analysis also showed that high molecular weight alginate-NH_2 had a slightly lower molecular weight than the starting material.

3.2.3. Synthesis of alginate-cis-aconityl-DNM

The binding of *N-cis*-aconityl-DNM to amino-modified alginates was performed using the water-soluble carbodiimide EDC. *N-cis*-Aconityl-DNM was first activated with an equimolar amount of EDC, and then reacted with alginate-NH_2 containing an approximately equivalent amount of primary amine groups. Unreacted *N-cis*-aconityl-DNM was separated from the conjugate by Sephadex G-50 column chromatography. The macromolecular fraction eluting at the void volume was collected, dialysed against distilled water (pH 7.0) and then lyophilised. Conjugations carried out using this procedure resulted in incorporation of 6.1 and 9.2% of the available *N-cis*-aconityl-DNM into high and low molecular weight alginate-NH_2, respectively. In an attempt to improve the coupling yield, *N*-hydroxysulphosuccinimide (Staros et al., 1986) was added to the reaction mixture following activation of *N-cis*-aconityl-DNM with EDC. The activated *N-cis*-aconityl-DNM was then reacted with low molecular weight alginate-NH_2. As a result, the coupling yield was increased from 9.2 to 14.0%. The drug loading of the high molecular weight alginate-DNM (HMW-alginate-DNM) conjugate was 0.8% w/w compared with 1.3% w/w for the low molecular weight (LMW-alginate-DNM) conjugate and 1.6% w/w for the LMW-alginate-DNM conjugate prepared with the addition of *N*-hydroxysulphosuccinimide.

The amount of contaminating free DNM in various alginate-DNM conjugates was determined by reverse-phase HPLC analysis, using DOX as an internal standard. All of the conju-

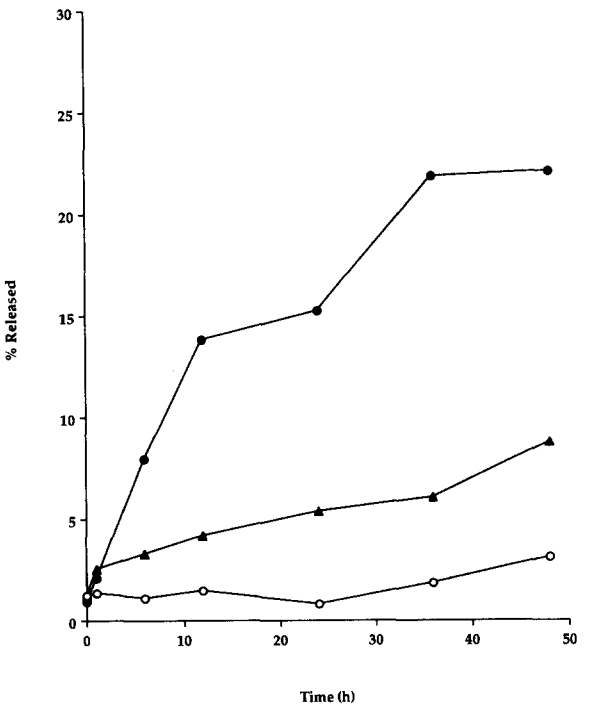


Fig. 2. Effect of pH on the release of DNM from HMW-alginate-DNM conjugate. Release of DNM was quantified by reverse phase-HPLC as described in section 2. Release of DNM at pH 5 (●), pH 6 (▲) and pH 7 (○) is shown. Each point is the mean of two replicates.

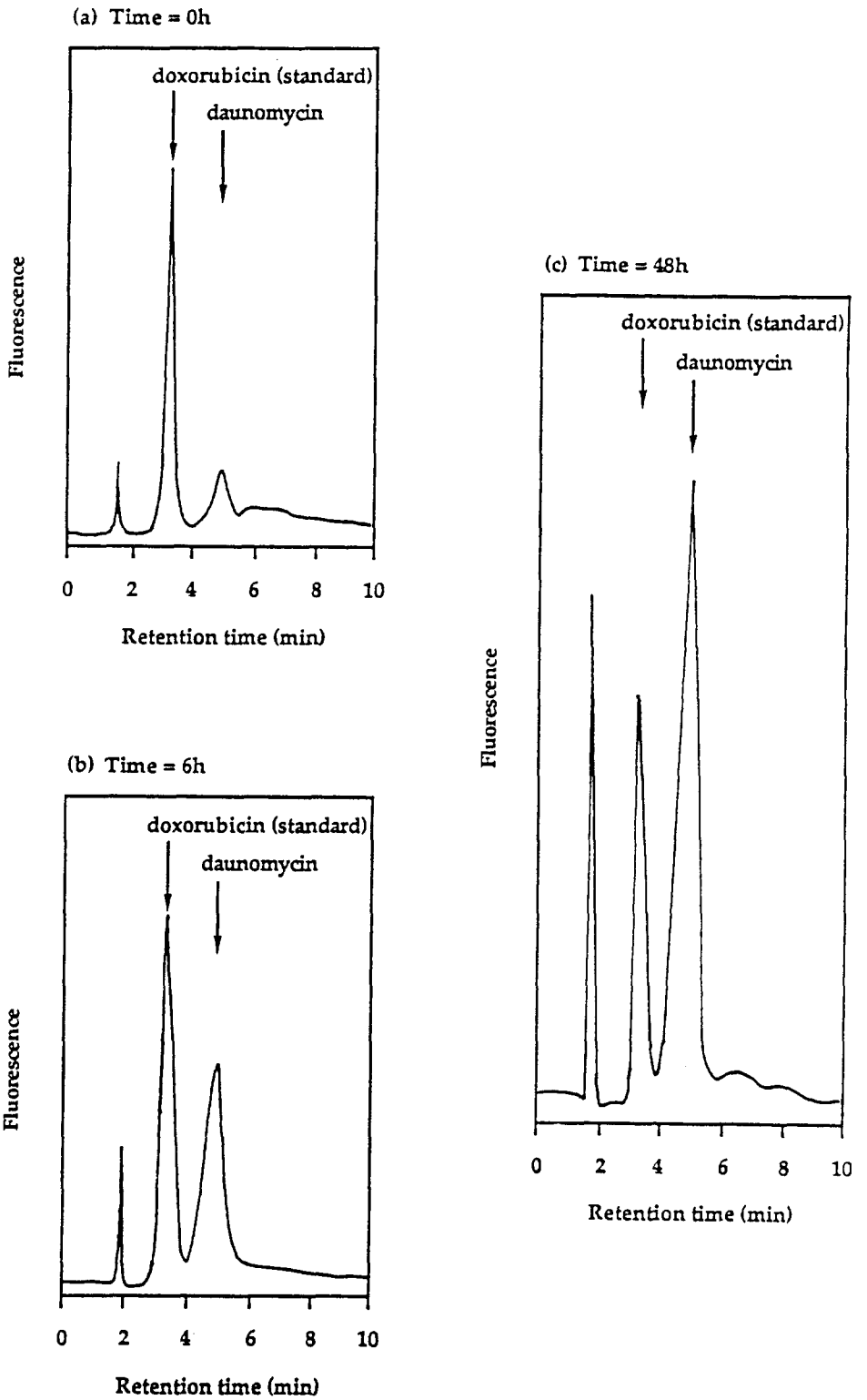
gates analysed contained less than 1% free DNM in relation to the total DNM content. Typically, the extraction efficiency for DNM and DOX was 98 and 92%, respectively.

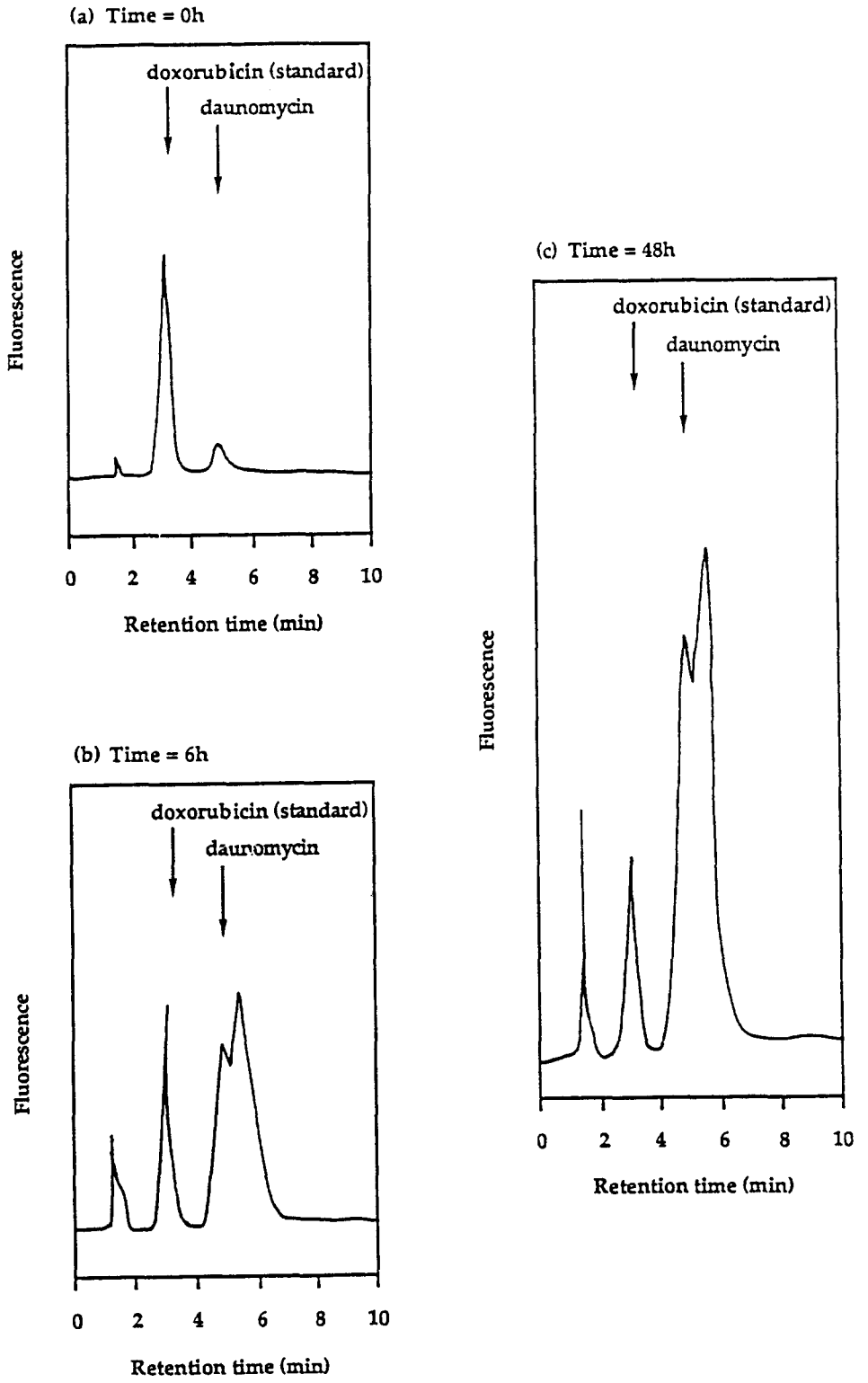
3.3. Release of DNM from alginate-cis-aconityl-DNM conjugates in vitro

The release of DNM from HMW-alginate-DNM conjugate was examined at 37° C in citrate/phosphate buffers at pH values of 5, 6 and 7, and the amount of drug released was quantified by reverse-phase HPLC analysis (Fig. 2). It can be seen that at pH 7, the conjugate was very stable, with only 3.1% DNM released over 48 h. DNM was released significantly faster at pH 5 and 6, and the rate of drug release at pH 5 was approx. 2.5-fold higher than that at pH 6. Although the rate of drug release at pH 6 remained uniform throughout the 48 h incubation period, the rate of daunomycin release at pH 5 appeared to decline after approx. 12 h. The maximum amount of drug released from HMW-alginate-DNM conjugate was 8.7 and 22.0% at pH 6 and 5, respectively.

Drug release from LMW-alginate-DNM conjugate was also examined in vitro. Reverse-phase HPLC analysis showed that unlike HMW-alginate-DNM which released free DNM (represented by a single peak; Fig. 3), LMW-alginate-DNM released a drug derivative in addition to DNM, represented by two unresolved peaks (Fig. 4). Therefore, the area under both peaks was used to determine the amount of drug released, and the results are presented in terms of the percentage of DNM/DNM derivative released (Fig. 5). It can be seen that at pH 5, DNM/DNM derivative was released from LMW-alginate-DNM, whereas at pH 7 the conjugate was very stable with only 1.6% released over 48 h. The maximum amount of DNM/DNM derivative released at 48 h from LMW-alginate-DNM at pH 5 was 61.9%. Similar results were obtained on analysis of the products released from LMW-alginate-DNM prepared using *N*-hydroxysulphosuccinimide, during incubation in citrate/phosphate buffer at pH 5. The release profile of DNM/DNM derivative from this conjugate (Fig. 6) was very similar to that observed with the LMW-alginate-DNM conjugate prepared without the use of *N*-hydroxysulphosuccinimide. Incubation of LMW-alginate-DNM with rat liver lysosomal enzymes (tritosomes) in citrate/phosphate buffer, pH 5 did not enhance the release of DNM/DNM derivative (data not shown).

Fig. 3. Reverse phase-HPLC analysis of release products from HMW-alginate-DNM conjugate following incubation in citrate/phosphate buffer at pH 5 for 0 h (a), 6 h (b) and 48 h (c). Arrows indicate the retention time of the internal standard, doxorubicin and daunomycin.


(a) Time = 0h
doxorubicin (standard)
daunomycin
Fluorescence
0 2 4 6 8 10
Retention time (min)
(b) Time = 6h
doxorubicin (standard)
daunomycin
Fluorescence
0 2 4 6 8 10
Retention time (min)
(c) Time = 48h
doxorubicin (standard)
daunomycin
Fluorescence
0 2 4 6 8 10
Retention time (min)


(a) Time = 0h
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(b) Time = 6h
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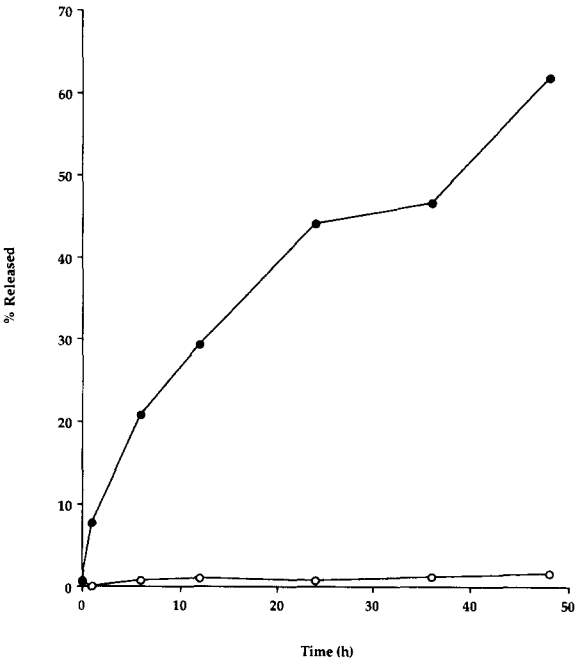

Fig. 5. Effect of pH on the release of DNM from LMW-alginate-DNM conjugate. Release of DNM was quantified by reverse phase-HPLC as described in section 2. Release of DNM at pH 5 (●) and pH 7 (○) is shown. Each point is the mean of two replicates.

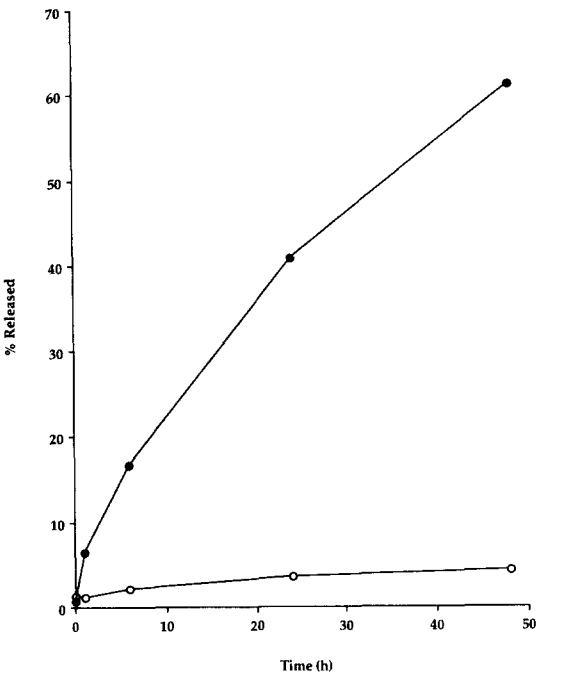

Fig. 6. Effect of pH on the release of DNM from LMW-alginate-DNM conjugate prepared using *N*-hydroxysulphosuccinimide. Release of DNM was quantified by reverse phase-HPLC as described in section 2. The release of DNM at pH 5 (●) and pH 7 (○) is shown. Each point is the mean of two replicates.

3.4. Evaluation of the cytotoxicity of polymers and polymer-DNM conjugate in vitro

HMW-alginate-DNM conjugate displayed dose dependent cytotoxicity against B16F10 cells and gave an IC_{50} value of approx. 0.5 mg/ml (Fig. 7). While alginate was not cytotoxic to cells at the concentration range tested, high molecular weight alginate-NH_2 reduced cell viability to approx. 80% of the controls at the highest concentration of 5 mg/ml. On comparing the cytotoxicity of DNM with that of HMW-alginate-DNM (Fig. 8), it was shown that the conjugate was nearly 700-fold less toxic than the free drug. As can be seen (Fig. 8), the cytotoxicity of the conjugate can be attributed entirely to the small (0.6%) amount of contaminating free DNM, hence making in vitro screening less than ideal for the evaluation of the antitumour activity of alginate-DNM conjugates. The in vitro results described here correlate well with in vitro cytotoxicity results for covalent conjugates of HPMA and DOX (Duncan et al., 1992). Therefore, subsequent testing of the antitumour activity of the conjugates was carried out in vivo.

3.5. Evaluation of the antitumour activity of alginate-DNM conjugate in vivo

Antitumour activity was examined using LMW-alginate-DNM, primarily because this con-

Fig. 4. Reverse phase-HPLC analysis of release products from LMW-alginate-DNM conjugate following incubation in citrate/phosphate buffer at pH 5 for 0 h (a), 6 h (b) and 48 h (c). Arrows indicate the retention time of the internal standard, doxorubicin and daunomycin.

jugate could easily be dissolved in PBS at the concentrations required for in vivo administration (approx. 30 mg/ml), and formed much less viscous solutions than the HMW-alginate-DNM. On analysis by size-exclusion HPLC (data not shown), LMW-alginate-DNM had M_w of 61 000 and M_w/M_n of 5.2. Treatment was administered by a single intraperitoneal injection to mice bearing B16 subcutaneous tumours. Animals treated with LMW-alginate-DNM conjugate at a DNM dose equivalent to 5 mg/kg showed a slower rate of tumour growth, the mean tumour size of these animals on day 7 being significantly ($P < 0.05$) smaller than that of the control group (Fig. 9). Treatment with free DNM (5 mg/kg) or a mixture of DNM (5 mg/kg) and low molecular weight alginate did not significantly delay tumour growth (Fig. 9). Animals treated with the conjugate showed a 14.1% increase in lifespan, compared with 6.3% and 4.2% for DNM and the mixture of DNM and alginate, respectively, although it is noteworthy that the mean survival time of the three treated groups was not significantly different from that of the control. Drug related toxicity was observed in two out of five animals treated with free DNM. In contrast, none of the animals treated with LMW-alginate-DNM or the mixture of DNM and alginate showed signs of toxicity.

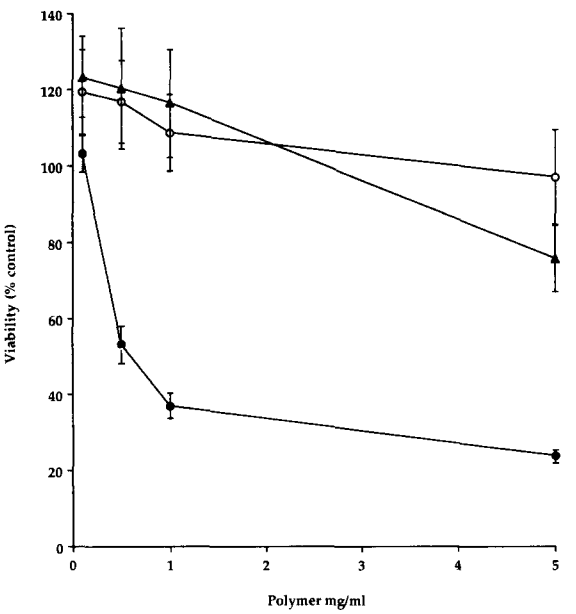

Fig. 7. Cytotoxicity of the parent alginate polymer (○), high molecular weight-amino-modified alginate (▲) and HMW-alginate-DNM conjugate (●) towards B16F10 cells, as assessed by the MTT in vitro cytotoxicity assay. Data (six replicates) are expressed as percentage of untreated control cells.

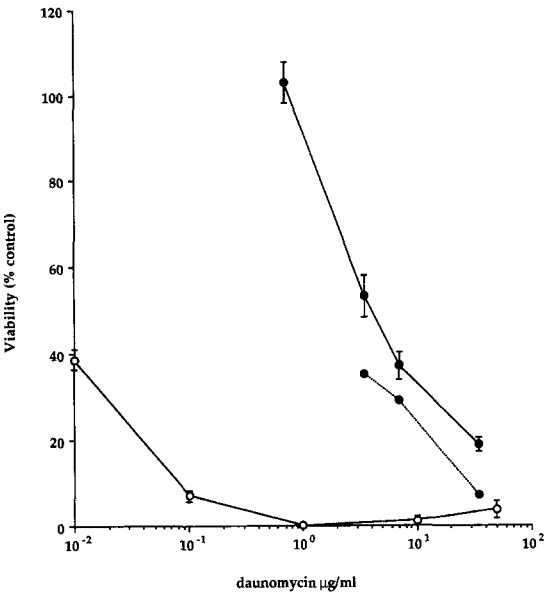

Fig. 8. Cytotoxicity of free DNM (m) and HMW-alginate-DNM conjugate (●, solid line) towards B16F10 cells, as assessed by the MTT in vitro cytotoxicity assay. Data (six replicates) are expressed as percentage of untreated control cells. The estimated cytotoxicity of HMW-alginate-DNM conjugate due to the presense of 0.6% free DNM is shown (●, dotted line).

4. Discussion

Direct conjugation of *N-cis*-aconityl-anthracycline to monoclonal antibodies (Diener et al., 1986; Dillman et al., 1988; Yang and Reisfeld, 1988), poly(D-lysine) (Shen and Ryser, 1981), or branched polypeptides (Hudecz et al., 1992) can be achieved by binding via the amino-terminus and/or the ϵ-amine group of lysine residues. To introduce primary amine groups into alginate structure suitable for coupling to *N-cis*-aconityl-DNM, alginate was reacted with a molar excess of ethylenediamine. This gives a soluble, non-

crosslinked product containing pendant amine groups providing that ethylenediamine complexation with alginate is prevented by increasing the pH to 12.0 at the end of the reaction and also addition of sodium chloride, followed by extensive dialysis. The alginate-DNM conjugates subsequently synthesized had a drug loading of 0.8–1.6% w/w which is relatively low, but sufficient for delivery of highly potent antitumour agents such as the anthracyclines. Here it was demonstrated that DNM remained associated with the conjugate at neutral pH, but was slowly released at acid pH (Fig. 2–6). As we have previously shown that alginate is not preferentially taken up by any particular organ in the body and that it remains in the circulation unless its molecular weight is low enough (≤ 48000) to allow rapid urinary excretion (Al-Shamkhani and Duncan, 1995), it can be safely concluded that DNM would remain associated with the alginate carrier during transport in the circulation. Macromolecules such as polystyrene-co-maleic acid conjugated neocarzinostatin (Matsumura and Maeda, 1986) and HPMA-DOX (Seymour et al., 1994) which persist in the circulation have been shown to accumulate preferentially in tumours due to enhanced permeability of the tumour endothelium and subsequent absence of removal by lymphatic drainage. Alginate-DNM accumulating in a tumour might encounter the slightly acidic extracellular compartment seen in some solid tumours (Tannock and Rotin, 1989) and eventually be directed into intracellular endosomal and lysosomal compartments. Within this acidic environment DNM would be slowly released with time.

It was surprising that the low molecular weight conjugate released a DNM derivative in addition to DNM when incubated at acid pH. This might be explained as *N-cis*-aconityl-DNM can poten-

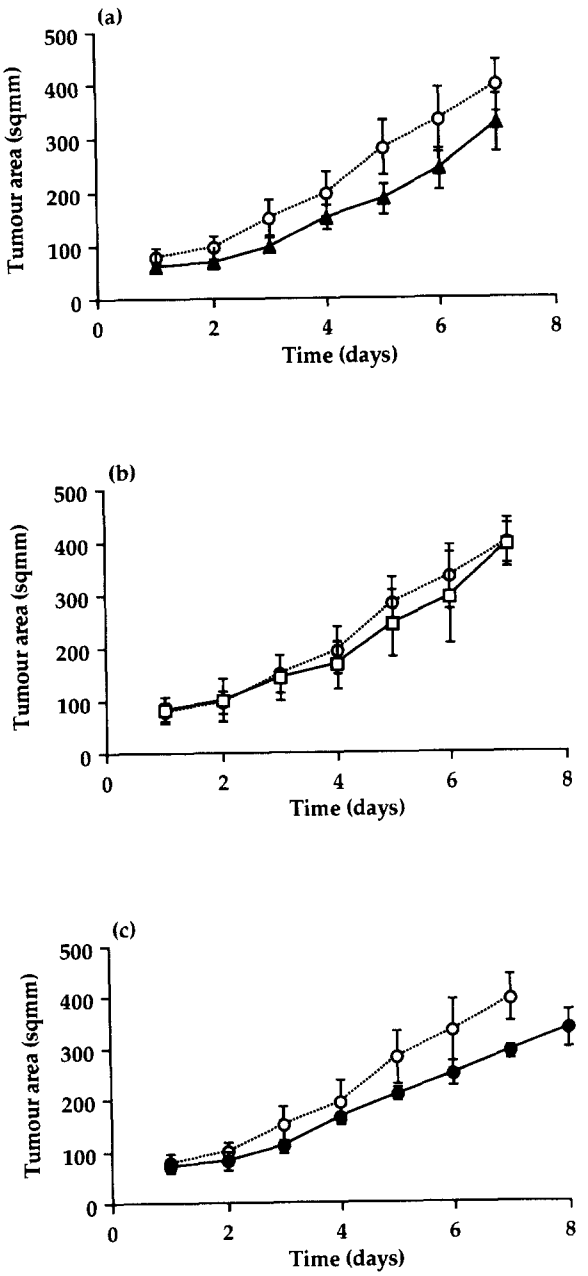

Fig. 9. Antitumour activity of free DNM, a mixture of low molecular weight alginate and DNM and LMW-alginate-DNM conjugate when administered to mice bearing established subcutaneous B16F10 melanoma. Tumour-bearing mice were treated by a single intraperitoneal injection (day 1), and tumour dimensions wre measured daily. (a) Growth of tumours ($n = 5$) following administration of free DNM at 5 mg/kg (▲). (b) Growth of tumours ($n = 3$) following administration of DNM and low molecular weight alginate as a mixture at a dose equivalent to 5 mg/kg DNM (□). (c) Growth of tumours ($n = 5$) following administration of LMW-alginate-DNM conjugate at a dose equivalent to 5 mg/kg DNM (●). Each panel also shows the growth of tumours ($n = 5$) in untreated animals (○, dotted line).

tially bind to low molecular weight alginate via a Schiff base linkage through reaction of the C-13 carbonyl group of DNM with the primary amine groups, whereas similar reaction with the high molecular weight amino-modified alginate is much more unlikely due to steric hindrance. *N-cis*-Aconityl-DNM bound to the alginate by Schiff base linkages, would be released as *N-cis*-aconityl-DNM at acid pH (Bernstein et al., 1978; Ueda et al., 1989).

Administration of LMW-alginate-DNM conjugate resulted in a delay in tumour growth and reduced toxicity when compared to free DNM. Reduction in drug-related toxicity has been reported for a number of other macromolecular formulations of anthracyclines (Trouet et al., 1982; Zunino et al., 1987, 1989; Duncan et al., 1989, 1992; Yeung et al., 1991) and is due to alteration in drug pharmacokinetics (Seymour et al., 1990). Reduced toxicity of the conjugate suggests that the *cis*-aconityl linker is stable in the circulation, however, it is important to note that treatment of mice with a mixture of LMW-alginate and DNM at an equivalent DNM dose (5 mg/kg) also reduced toxicity. DNM could theoretically complex with alginate, and this phenomena might explain the reduced toxicity in this case. Although alginates are known to exhibit inherent antitumour activity in vivo (Fujihara et al., 1984; Fujihara and Nagumo, 1992), co-administration of alginate with DNM in the form of a mixture did not elicit improved antitumour activity. Future studies should investigate further the mechanism of action of the alginate-DNM conjugates; the relative contribution of changed pharmacokinetics, controlled DNM release and immunostimulatory activity of the alginate itself should be more clearly defined. Also, it is interesting to consider that the efficacy of alginate-DNM conjugates may be enhanced by co-administration of agents such as *m*-iodobenzylguanidine (Kuin et al., 1994) which reduce the extracellular pH of solid tumours. As we have previously shown that alginates are non-toxic, and unlike many other natural polymers (e.g., proteins), they elicit only a very low antibody response following repeated administration to mice (Al-Shamkhani et al., 1991), repeated systemic administration of alginate-DNM conjugates could have potential for clinical development.

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