

Sustained and Controlled Release of Daunomycin from Cross-Linked Poly(aldehyde guluronate) Hydrogels

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Received 23 June 1999; revised 16 February 2000; accepted 29 February 2000

ABSTRACT: We have incorporated daunomycin, an antineoplastic agent, into a biodegradable hydrogel through a labile covalent bond. In brief, sodium alginate was chemically broken down to low molecular weight and followed by oxidation to prepare poly(aldehyde guluronate). Adipic dihydrazide was used to incorporate the drug into the polymer backbone and cross-link the polymer to form hydrogels. Daunomycin can be released from the hydrogel after the hydrolysis of the covalent linkage between the drug and the polymer. A wide range of release profiles of daunomycin (e.g., from 2 days to 6 weeks) has been achieved using these materials, and the biological activity of the released daunomycin was maintained. © 2000 Wiley-Liss, Inc. and the American Pharmaceutical Association *J Pharm Sci* 89: 910–919, 2000

Keywords: hydrogel; poly(aldehyde guluronate); daunomycin; covalent cross-linking; controlled delivery

INTRODUCTION

One common approach in the treatment of cancer is the delivery of antineoplastic agents to the tumor. However, to achieve complete eradication of tumors, antineoplastic agents must be administered in high doses intravenously, which unfortunately leads to the killing of healthy cells in addition to the cancer cells. Adverse side effects can include severe immunosuppression, myelosuppression, nephrotoxicity, and cardiotoxicity.^{1,2} In theory, the localized delivery of potent agents at the required concentration should eradicate cancer cells without causing any damage to healthy tissues and organs. This method may then allow one to deliver antineoplastic agents in a controlled manner over a wide range of time frames depending on the type, size, and growth rate of

the tumor. There have been many attempts to deliver antineoplastic agents using various delivery systems to circumvent adverse side effects and to enhance the efficiency of the drugs.^{3–12}

Hydrogels have been widely investigated as delivery vehicles for the localized, sustained release of bioactive agents.^{13,14} Many synthetic and naturally derived materials have been reported to form hydrogels,^{15–18} and one widely used hydrogel is formed from the ionic cross-linking of sodium alginate, a linear polysaccharide isolated from seaweed. Alginate is comprised of (1,4)-linked β -D-mannuronic and α -L-guluronic acid residues arranged in blocks of polymannuronate, polyguluronate, and alternating units of both sugars. Divalent cations, such as calcium, ionically cross-link the carboxylate groups on adjacent alginate strands to form hydrogels. The polyguluronate block of alginate is known to be responsible for this gelling feature.¹⁹ The favorable properties of alginate, including nonimmunogenicity, hydrophilicity, and relatively low cost have prompted

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Journal of Pharmaceutical Sciences, Vol. 89, 910–919 (2000)
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attempts to use this material as wound dressing, dental impression, and immobilization scaffolds for cultured and transplanted cells.^{20–22} Alginate is considered to be a biocompatible polymer,²³ although contaminating factors may induce significant inflammation if the polymer is not suitably purified.²⁴ Alginate hydrogels have been previously proposed for a number of drug delivery applications.^{25–27} One disadvantage of alginate hydrogels is that they are not chemically broken down in mammals. They instead dissolve in an uncontrollable and unpredictable manner following the dissolution of calcium into the surrounding medium. Furthermore, the molecular weight of intact alginate is typically above the renal clearance threshold, thus preventing it from being excreted from the body.²⁸

To overcome these disadvantages, we have recently synthesized new hydrogels composed of low molecular weight polymers derived from alginate.²⁹ In brief, alginate was hydrolyzed under acidic conditions and the polyguluronic acid blocks were isolated, transformed to sodium polyguluronate, and oxidized to yield poly(aldehyde guluronate) (PAG). PAG was then cross-linked with adipic dihydrazide to form biodegradable hydrogels with enhanced and easily controlled mechanical properties. We now report the utilization of these hydrogels to deliver an anti-neoplastic agent, daunomycin, in a controlled manner over a wide range of time frames. Daunomycin was covalently attached to PAG via a dihydrazide spacer, resulting in the formation of hydrazone bonds between the polymer and the daunomycin. The drug was released from the hydrogel by the hydrolysis of the hydrazone bond. The release of daunomycin from the hydrogel was monitored *in vitro* and was controlled by varying the amount of covalent or ionic cross-linking of the hydrogels. The bioavailability of the released drug was tested *in vitro* as well.

EXPERIMENTAL SECTION

Materials

Sodium alginate was purchased from Pronova Biomaterials (Drammen, Norway). Sodium periodate, adipic dihydrazide, ethylene glycol, and calcium chloride were purchased from Aldrich Chemical Company (Milwaukee, WI) and were used as received. Daunomycin hydrochloride was purchased from Fluka Chemical Corporation (Ronkonkoma, NY). Dulbecco's Modified Eagle's

Medium (DMEM) was purchased from Life Technologies (Grand Island, NY). KB cells (an established line of human epidermoid carcinoma cells) were a gift from Dr. John Drach (Department of Biologic & Materials Sciences, University of Michigan).

Instruments

The ultraviolet/visible (UV/vis) spectra were collected on a Perkin Elmer Lambda 12 UV/VIS spectrophotometer. High-performance liquid chromatography (HPLC) analysis was performed with a Hewlett Packard Series II 1090 liquid chromatograph with a photodiode array detector and a Phenomenex Hypersil C18 column. The system was programmed for gradient elution starting with 82/18 ([0.025% aqueous acetic acid]/CH₃CN, v/v) for 2 min then raised to 50/50 during 4 min then back to 82/18. The elution of the drug was monitored at 480 nm wavelength.

Synthesis of Daunomycin–Adipoyl Hydrazide Hydrochloride

A 500-mL round-bottomed flask was wrapped with aluminum foil and charged with daunomycin hydrochloride (25 mg, 44.3 μmol) and adipic dihydrazide (434 mg, 2.49 mmol). Methanol (250 mL) was added, and the reaction was stirred at room temperature in the dark for 5 days. The solvent was concentrated to 50 mL under reduced pressure. The precipitated adipic dihydrazide was removed by filtration, and the filtrates were concentrated to 5 mL under reduced pressure. Chloroform (5 mL) was added in a dropwise manner while shaking, and the mixture was cooled at 4 °C overnight. More adipic dihydrazide precipitated. The solvent was carefully transferred into a 25-mL scintillation vial with a Pasteur pipette, leaving the precipitate behind. The solvent was evaporated under reduced pressure to yield 25.2 mg of product (35 μmol, 80% yield) that was analyzed by thin-layer chromatography (TLC) on Kodak 13181 silica gel in CHCl₃/CH₃OH (80/20) (daunomycin hydrochloride: $R_f = 0.39$; daunomycin–adipoyl hydrazide: $R_f = 0.1$).

Synthesis of the Poly(aldehyde guluronate) (PAG)

PAG was synthesized according to a previously reported procedure.²⁹ Briefly, sodium alginate was hydrolyzed under acidic conditions and the polyguluronic acid oligomers were isolated at pH 2.85. Polyguluronic acid was then dissolved in wa-

ter with the addition of aqueous sodium hydroxide. Sodium polyguluronate was then precipitated with ethanol, filtered, and dried under reduced pressure; MW 6200 Da; M_w/M_n 1.14; FTIR (KBr pellet, cm^{-1}) 3430.5, 2931.2, 1616.4, 1412.5, 1320.5, 1126, 1092, 1028; ^1H NMR (360 MHz, D_2O , δ): 3.90 (br, s), 3.99 (br, s), 4.07 (br, s), 4.45 (br, s), 5.03 (br, s); ^{13}C NMR (360 MHz, D_2O , δ): 67.64, 69.87, 71.73, 82.94, 103.66, 178.29. An aqueous solution of sodium polyguluronate was then oxidized with sodium periodate in the dark at ambient temperature. The product was purified by extensive dialysis (Molecular Weight Cut Off (MWCO 1000)) against double-distilled water for 3 days. The solution was concentrated, frozen, and lyophilized to yield PAG; MW 6000 Da, M_w/M_n 1.4; FTIR (KBr pellet, cm^{-1}) 3336.5, 2942, 1724, 1616.4, 1405.8, 1321.1, 1159.4, 1117.2, 1025.8. The aldehyde content was quantified using trinitrobenzene sulfonic acid.²⁹

Preparation of Covalent Cross-Linked PAG Hydrogels

Daunomycin hydrochloride (10 μL , 25 mg/mL solution in DMSO) was added to aqueous solutions of adipic dihydrazide (25–125 μL , 0.5 M) in 5-mL sterile tubes. After 10 min, aqueous solutions of PAG (150–200 μL , 20% w/w) were added and the total volume was diluted to 500 μL with Dulbecco's Modified Eagle's Medium (DMEM, pH 7.4) containing penicillin and streptomycin (100 units/mL and 100 $\mu\text{g}/\text{mL}$, respectively). The solutions were mixed thoroughly for 30 s and allowed to stand at room temperature for 1 h to form hydrogels. In the Results section of this manuscript, hydrogels are described by their final PAG concentrations (% w/w) and final adipic dihydrazide concentrations (mM).

Preparation of Ionic and Covalent Cross-Linked PAG Hydrogels

Daunomycin hydrochloride (10 μL , 25 mg/mL solution in DMSO) was added to aqueous solutions of adipic dihydrazide (25–125 μL , 0.5 M) in 5-mL sterile tubes. After 10 min, aqueous calcium chloride (20 μL , 1 M) was added followed by aqueous solutions of PAG (150–200 μL , 20% w/w). The total volume was adjusted to 500 μL with DMEM and the gels were formed as already described.

Determination of the Swelling Ratio of the Hydrogels

Hydrogels were formed at various concentrations of PAG, adipic dihydrazide, and calcium chloride

in 24-well plates. The hydrogels were immersed in DMEM (pH 7.4) and incubated at 37 °C for 24 h to reach the equilibrium swelling. The hydrogels were transferred to 2-mL vials and weighed (wet weight). The gels were then frozen and lyophilized, and the dried samples were weighed (dry weight). The swelling ratio was defined as the ratio (wet weight – dry weight)/(dry weight). We have corrected for the contribution of amino acids and salts in DMEM by subtracting this value from the weights of the wet and dry gels.

Determination of the Incorporated Daunomycin

To quantify the amount of daunomycin that is incorporated in the gels, two sets of hydrogels were formed as already described. One set was loaded with daunomycin hydrochloride (0.20 mg, 0.355 μmol) and the other with trypan blue (0.32 mg, 0.333 μmol). After gelation, the hydrogels were frozen and lyophilized. The dried solid in each tube was crushed and 2 mL of double-distilled water was added. The solutions were then sonicated for 30 min, quantitatively transferred to 5-mL volumetric flasks, and diluted with double-distilled water. Each solution was filtered through a 0.22- μm filter, and the concentration of the dissolved drug was quantified spectrophotometrically at 480 nm for daunomycin and 588 nm for trypan blue.

In Vitro Release of Daunomycin in Fresh Medium

Hydrogels were formed in 15-mL sterile tubes as already described. Aqueous solutions of 5 mL of DMEM containing penicillin and streptomycin were added to each gel. The hydrogels were then incubated at 37°C, and the medium was replaced with fresh DMEM (5 mL) periodically. The concentration of the released drug in the medium was quantified spectrophotometrically at 480 nm.

In Vitro Release of Daunomycin in Spent Medium

Spent media was obtained from cultured KB cells. Approximately 2×10^4 cells were seeded in a 162- cm^2 flasks containing 50 mL of media and incubated at 37 °C for 3 to 4 days until cells had approximately reached confluency. The pH of the collected media was not significantly different from fresh media (pH 7.4). Hydrogels containing daunomycin (10 μL , 25 mg/mL in DMSO) were

formed at 6% w/w PAG and 150 mM adipic dihydrazide. The hydrogels were exposed to 2.5 mL of spent Minimal Essential Media with Hanks' salts (MEMH) without phenol red that contained 10% calf serum and penicillin/streptomycin (100 units/mL and 100 μ g/mL, respectively). The medium was replaced weekly, and the concentration of daunomycin that was released from the hydrogels in spent media was determined at 480 nm.

Cytotoxicity Assay with KB Cells

The inhibitory concentrations of the released daunomycin that killed 50% of the cells (IC_{50}) were determined using an established cytotoxicity assay with KB cells as described previously.³⁰ Hydrogels at a final concentration of 6% w/w PAG and 150 mM adipic dihydrazide containing 250 μ g of daunomycin were prepared and incubated in 5 mL of sterile media at 37 °C. The media was tested and replaced with fresh media (5 mL) periodically. As a control, a set of hydrogels were not loaded with the drug and treated in the same fashion as just described. The KB cells were cultured and seeded at a density of 5 ± 10^3 cells/well in 96-well plates with a total volume of tissue culture medium of 200 μ L/well. Cells were allowed to attach for 1 day before adding drug solutions or aliquots from the collected samples. Eight dilutions were used for each sample (each being one-third the concentration of the previous dilution). After 48 h, cells were fixed with 95% ethanol for 5 min, rinsed with tap water, and stained with 2.5 mM crystal violet in 20% aqueous methanol for 5 min. The plates were rinsed again with tap water and 150 μ L/well acidified ethanol (0.01 M HCl) was added to elute the dye from stained cells. The concentration of the dye in each well was measured spectrophotometrically at 570 nm. Dose-response curves were generated by plotting percent inhibition ($[\text{sample absorbance}/\text{control absorbance}] \times 100$) versus the log of the daunomycin concentration and fit to a line. The IC_{50} values were calculated using the equations for the best linear fit. The IC_{50} values of daunomycin released from the hydrogels exposed to both fresh and spent media were compared with those of daunomycin stored in DMSO or daunomycin conjugated with adipic dihydrazide for 15 min before adding to the cells. Solutions containing only PAG, adipic dihydrazide, or material released from the hydrogels without daunomycin were also tested.

RESULTS AND DISCUSSION

Characteristics of Poly(aldehyde guluronate) Hydrogels

Commercially available high molecular weight alginate was hydrolyzed under acidic conditions to breakdown the β -glycosidic linkages between the mannuronate and guluronate residues as previously described.²⁹ Polyguluronic acid was then precipitated at pH 2.85, filtered, and reconstituted in water by adjusting the pH to 7 with sodium hydroxide to yield sodium polyguluronate. Sodium polyguluronate was then oxidized with sodium periodate to yield the PAG. The degree of oxidation was controlled by the mole equivalent of sodium periodate used in each reaction. The degree of oxidation of PAG used in this study was $87 \pm 1\%$.²⁹ The molecular weight and polydispersity of PAG did not significantly differ from the starting sodium polyguluronate. The average number of aldehyde groups per chain was estimated to be 67 groups. PAG was subsequently cross-linked with a bifunctional cross-linker, adipic dihydrazide, to form hydrogels (Figure 1). A high efficiency of this coupling was previously confirmed using Fourier transform-infrared (FT-IR) spectra and quantification of the cross-link density.^{29,31} The degree of swelling of PAG hydrogels was analyzed after the hydrogels had reached the equilibrium swelling in aqueous DMEM. The swelling ratio ranged from 15.5 to 10.5 for hydrogels made with varying extents of cross-linking (Table 1). As expected, increasing the cross-link density resulted in a decrease in the water content in these hydrogels. The swelling was minimally affected by alterations in the concentration of the polymer used in this study. This finding may be attributed to the low molecular weight of the PAG chains utilized to form these hydrogels.

Daunomycin Incorporation

Drugs from the anthracyclin family had been previously incorporated into polymeric carriers for systemic drug delivery via the formation of hydrazone bonds.^{32,33} The ketone group on the C13 position of these drugs reacts with pendant hydrazide groups on the polymer backbone. A similar reaction was utilized in this paper to incorporate daunomycin into hydrogels for localized drug delivery. Aqueous solutions of daunomycin and adipic dihydrazide were mixed for 10 min before the addition of aqueous PAG solutions. During this time, daunomycin reacts with adipic dihydra-

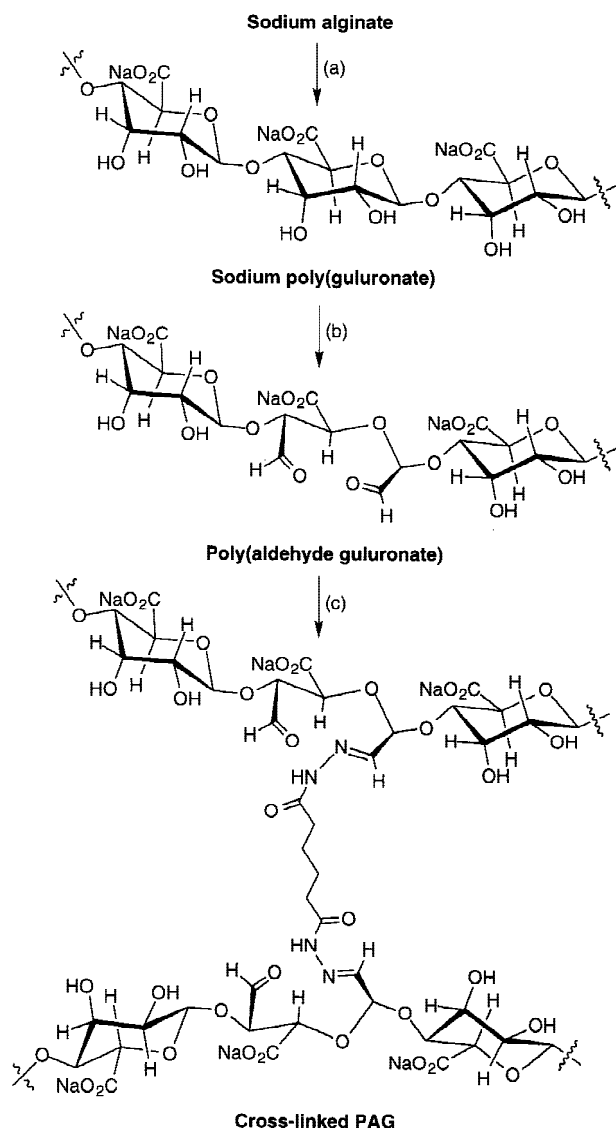


Figure 1. Synthesis of cross-linked poly(aldehyde guluronate) hydrogels: (a) reflux in aqueous hydrochloric acid followed by neutralization; (b) aqueous sodium periodate; and (c) aqueous adipic dihydrazide.

zide to form the daunomycin–adipoyl hydrazide conjugate. In theory, one hydrazide terminal of adipic dihydrazide reacts with the drug to form a hydrazone bond, whereas the other terminal is free because a large excess amount of adipic dihydrazide was used (Figure 2). The free hydrazide group of the daunomycin–adipoyl hydrazide conjugate potentially reacts with an aldehyde group on the backbone of PAG to form a labile hydrazone bond. The rest of the adipic dihydrazides potentially cross-link PAG to form hydrogels.

Daunomycin hydrochloride and adipic dihydra-

Table 1. Degree of Swelling of PAG Hydrogels^a

PAG (% w/w)	Adipic dihydrazide (mM)	CaCl ₂ (mM)	Swelling ratio
6.0	100	40	15.4 ± 0.4
6.0	150	40	11.3 ± 0.1
6.0	200	40	11.0 ± 0.1
6.0	250	40	10.6 ± 0.2
6.0	150	0	14.2 ± 0.2
7.0	150	0	13.3 ± 0.2
8.0	150	0	12.8 ± 0.3

^a Hydrogels were prepared under various conditions; hydrogels were immersed in DMEM (pH 7.4) and incubated at 37°C for 24 h.

zide were mixed in water to form daunomycin–adipoyl hydrazide. The reaction was monitored with TLC. After 15 min incubation at room temperature, unreacted free daunomycin could be detected at all concentrations of adipic dihydrazide (50–250 mM). The reaction was complete after 6 h of incubation at room temperature. HPLC was then used to quantitate the amount of daunomycin that reacted with adipic dihydrazide before the addition of PAG. Daunomycin hydrochloride and adipic dihydrazide were mixed at room temperature and analyzed 15 min later. The mixture

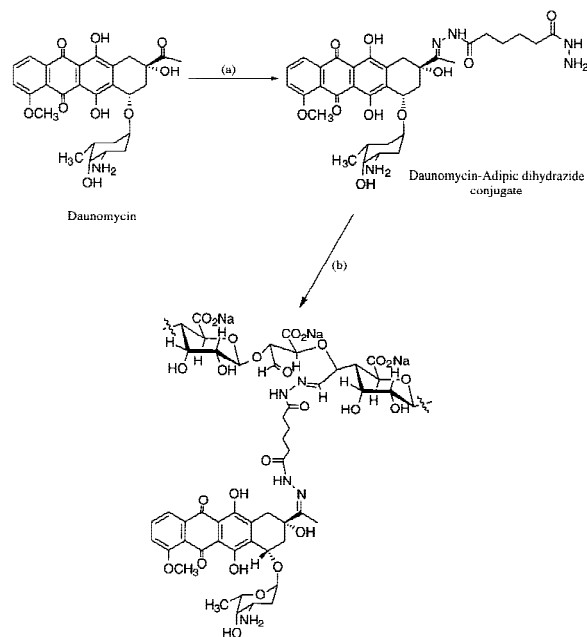


Figure 2. Scheme for the coupling of daunomycin to poly(aldehyde guluronate) hydrogels: (a) aqueous adipic dihydrazide; and (b) aqueous PAG.

contained $88.5\% \pm 0.5$ of the daunomycin–adipoyl hydrazide conjugate and $11.5\% \pm 0.4$ of free drug (Figure 3). The amount of daunomycin that was covalently incorporated into the hydrogel was next determined by quantifying the amount of free drug in the hydrogel. Hydrogels containing daunomycin hydrochloride or trypan blue were synthesized, frozen, lyophilized, and crushed to form powders. The free drug was then dissolved

in water and the resulting mixture was filtered to remove any fine particles. The concentration of the free drug was then determined spectrophotometrically. Trypan blue was used as a control because it does not contain aldehyde or ketone groups for coupling to PAG and has a comparable molecular weight to daunomycin. Only 2% of the trypan blue was retained in the hydrogel. In comparison, $86.9 \pm 0.1\%$ of daunomycin remained in the gelatinous particles. Similar results were obtained when ethanol was used to extract daunomycin and trypan blue. Moreover, hydrogels containing trypan blue that were incubated in DMEM released the entire drug within 24 h (data not shown). To eliminate the possibility that ionic interactions between the carboxylate groups on the polymer backbone and the ammonium group on the sugar ring of daunomycin control drug release, the release of daunomycin and daunomycin–adipoyl hydrazide conjugate from sodium alginate hydrogels was investigated. Alginate hydrogels cross-linked with calcium released the total loaded drug and conjugate within 24 h in DMEM (data not shown). The alginate hydrogels used in this study exhibited the swelling ratios of 33.5 ± 1.9 and 46.9 ± 1.2 before and after drug release, respectively. These findings clearly indicate that daunomycin is covalently coupled to the PAG hydrogel through the adipic dihydrazide molecule. The daunomycin incorporation in the hydrogels correlates well with the percentage of daunomycin–adipic dihydrazide conjugate that was determined by HPLC analysis.

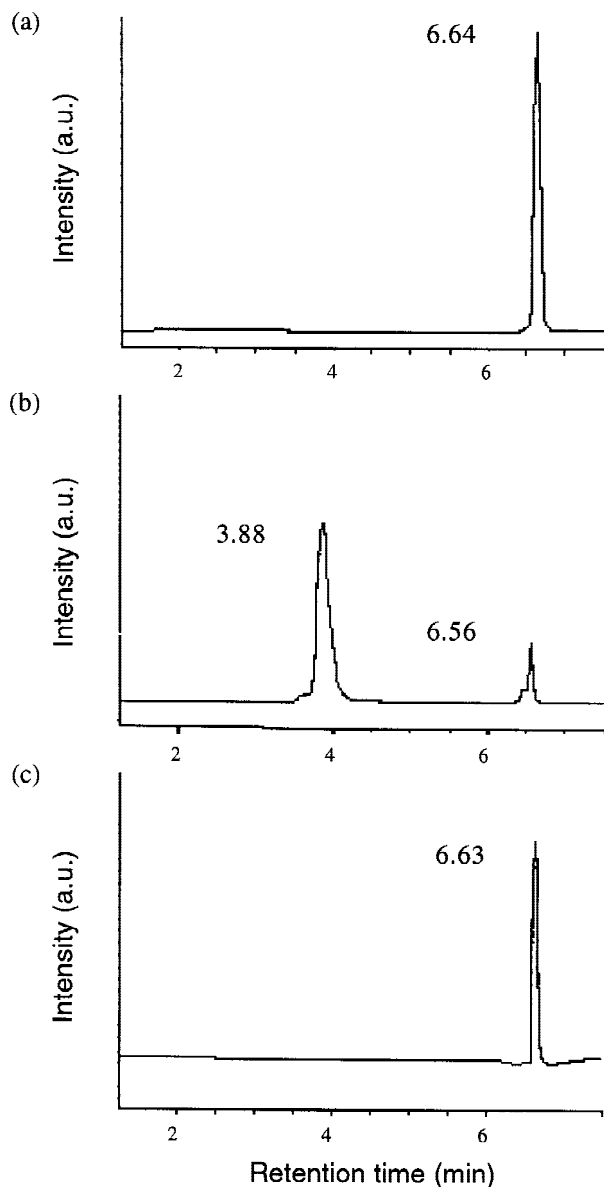


Figure 3. Reversed-phase (Hypersil C18) liquid chromatographic analysis of (a) daunomycin, (b) daunomycin–adipoyl hydrazide, and (c) daunomycin released from PAG hydrogels. The mobile phase consisted of $\text{H}_2\text{O}/\text{CH}_3\text{CN}$ (82/18, v/v) and the aqueous component contained 0.025% v/v acetic acid.

In Vitro Release

The release of the free drug from the hydrogel may be controlled by the chemical hydrolysis of the hydrazone bond between the drug and the adipic dihydrazide spacer, followed by the diffusion of the drug from the hydrogel.³⁴ However, the hydrolysis of the linkage between adipic dihydrazide and PAG could also release the daunomycin–adipoyl hydrazide conjugate. In this case, an inactive form of the drug would be released (pro-drug), which would be later activated by the hydrolysis of the linkage between adipic dihydrazide and daunomycin. To determine the percentage of free drug that is released, samples were periodically analyzed by HPLC. Uncoupled free daunomycin was quantitatively released from cross-linked PAG hydrogels at all times (Figure 3c). These results suggest that hydrolysis of the hy-

drazone linkage between drug and adipic dihydrazide is the dominant reaction in drug release.

In the next series of experiments hydrogels were formed with daunomycin while varying the concentrations of adipic dihydrazide, calcium ions, and PAG. The release profile of the incorporated daunomycin from the hydrogel changed significantly depending on the concentration of adipic dihydrazide, calcium ions in the hydrogel, and PAG. In the first experiment, 6% w/w PAG hydrogels at various concentration of adipic dihydrazide were prepared. Hydrogels cross-linked at 50 mM of adipic dihydrazide released the loaded drug completely in 2 days (Figure 4). As the concentration of the cross-linker was increased, the release rate of daunomycin was decreased. All incorporated drug was released in 2 weeks at 100 mM adipic dihydrazide, and in >6 weeks at elevated concentrations of adipic dihydrazide. It is important to note that at low concentrations of adipic dihydrazide, the complete release of daunomycin was accompanied by the complete dissolution of the hydrogel. Hydrogels with low cross-link density degrade at a faster rate (unpublished results).

Ionic cross-linking of PAG with calcium ions contributes to the physical properties of cross-linked PAG hydrogels in a similar manner as observed with alginate.²⁹ The calcium ions cross-

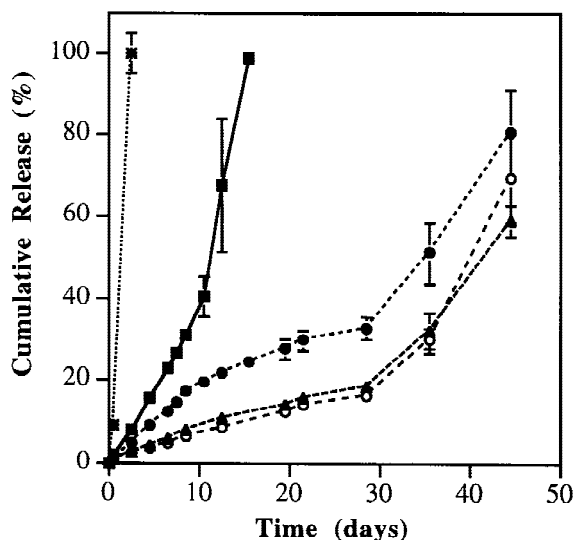


Figure 4. The cumulative release of daunomycin over time from hydrogels cross-linked by 50 mM (*), 100 mM (■), 150 mM (●), 200 mM (▲), and 250 mM (○) of adipic dihydrazide. All hydrogels were formed with 6% w/w PAG and 20 mM CaCl_2 , and release was monitored following incubation in DMEM (pH 7.4) at 37 °C.

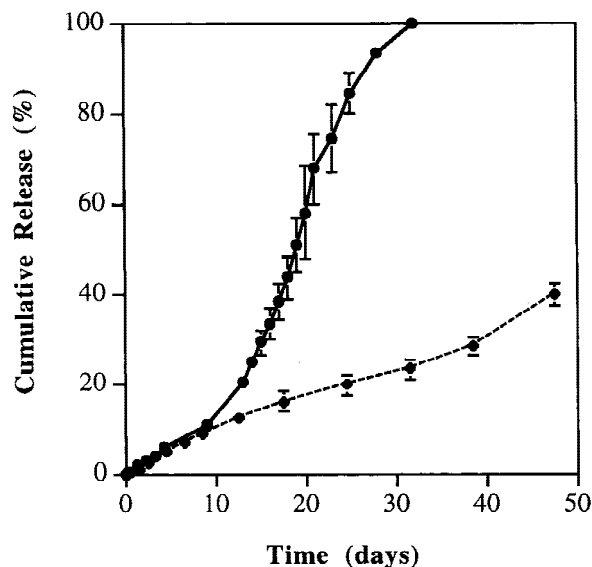


Figure 5. The cumulative release of daunomycin over time from 6% w/w PAG hydrogels cross-linked by 150 mM adipic dihydrazide in the absence of calcium chloride (●) and in the presence of 40 mM calcium chloride (◆). Release was monitored following incubation in DMEM (pH 7.4) at 37 °C.

link the carboxylate groups on adjacent PAG strands. To investigate the effect of calcium cross-linking on the release profile of daunomycin, aqueous solutions of 6% w/w PAG containing 250 μg daunomycin hydrochloride were cross-linked at 150 mM adipic dihydrazide. In the absence of calcium ions in the hydrogels, a linear release of 1% of the incorporated drug per day was observed during the first 10 days followed by ~4% release per day for the following 22 days (Figure 5). The total incorporated drug was released in 32 days. It is important to note that calcium ions are present in the medium at physiological concentrations in these studies. In the presence of 40 mM CaCl_2 in the hydrogel, only 40% of the incorporated drug was released during the first 6 weeks, and it was released at a rate of 0.8% per day. The calcium cross-linking results in hydrogels with a high cross-linking density and a decreased pore size, which likely decreased the release of the drug from the hydrogel.

The extracellular pH of most tumors is slightly lower than that of normal tissues^{35,36} which could affect the release kinetics of drugs from PAG hydrogels. To investigate the possible role of pH on drug release, the release of daunomycin from 6% w/w PAG hydrogels at 150 mM adipic dihydrazide was monitored at different pH conditions (Figure

6). Daunomycin was released at a rate of 1% per day from hydrogels incubated at neutral condition, but daunomycin was released at a significantly higher rate (1.8% per day) from hydrogels incubated in acidic medium (pH 6). This result is likely caused by the higher rate of hydrolysis of hydrazone bonds under acidic conditions.^{37,38} The minimal further change in the release as the pH was further lowered to pH 5 could be related to several factors. The change in hydrolysis from pH 6 to 5 may not be significant. Alternatively, the hydrogel is polyanionic and as the pH decreases, a higher percentage of the carboxyl groups are protonated. This situation may cause changes in the gel structure that alter the drug diffusion (e.g., changing pore size in gel).

Antitumor Activity of Released Daunomycin

The antitumor activity of the daunomycin released from PAG hydrogels was evaluated *in vitro* using a standard cytotoxicity assay with KB cells.²⁹ The media collected from control hydrogels had no effect on cell growth. The IC_{50} of the daunomycin–adipoyl hydrazide conjugate (in the presence of 100-fold excess of adipic dihydrazide) was $0.224 \pm 0.091 \mu\text{M}$ compared with $0.202 \pm 0.048 \mu\text{M}$ for the free daunomycin (Table 2). The activity of the released daunomycin slightly de-

Table 2. Inhibitory Activity of Intact or Released Daunomycin Towards KB Cells over Time

Sample	IC_{50} (μM)
Free daunomycin	0.202 ± 0.048
Daunomycin–adipic dihydrazide	0.224 ± 0.091
Released daunomycin after 1 week	0.560 ± 0.149
Released daunomycin after 2 weeks	0.349 ± 0.176
Released daunomycin after 3 weeks	0.805 ± 0.229

creased during the first 3 weeks, suggesting that the released drug might be partially degraded. The glycosidic linkage between the daunosamine and daunomycinone units of the drug has been previously reported to undergo hydrolytic cleavage under neutral to basic conditions.^{39,40} The activity of daunomycin released in spent media did not differ significantly from that released in fresh media (data not shown). PAG and adipic dihydrazide were also tested to ensure that the noted cytotoxicity was not caused by these components of the hydrogels. Neither aqueous PAG nor adipic dihydrazide had a significant effect on the growth of KB cells over the range of concentrations tested (Table 2).

CONCLUSIONS

We have developed a novel biodegradable hydrogel for the sustained local delivery of daunomycin over time frames ranging from a few days to several weeks. We propose that cross-linked poly(aldehyde guluronate) hydrogels loaded with daunomycin or other antineoplastic agents (containing aldehyde or ketone groups) can deliver these agents *in vivo* in a sustained manner. This system may provide a potent vehicle for the localized delivery of antineoplastic agents in a minimally invasive manner.

ACKNOWLEDGMENTS

The authors thank Regeneration for financial support of this research. The authors also thank Dr. John Drach and Julie Breitenbach for their kind help in the biological evaluations of daunomycin. GMK also gratefully acknowledges the support of a Medical Scientist Training Program Fellowship.

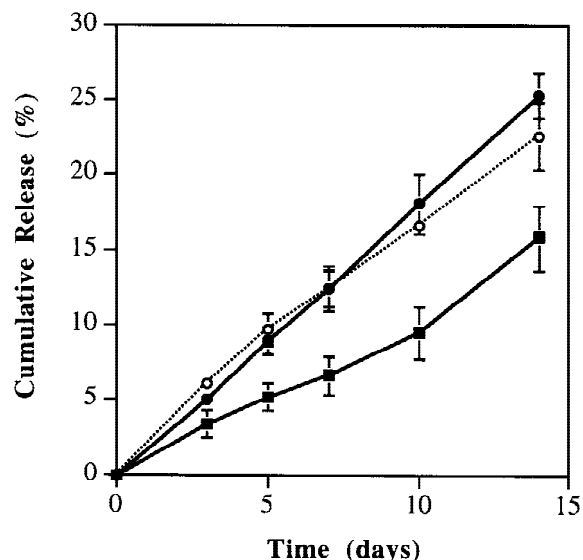


Figure 6. The cumulative release of daunomycin over time at pH 5 (●), pH 6 (○), and pH 7.4 (■). All hydrogels were formed with 6% w/w PAG and 150 mM adipic dihydrazide and release was monitored following incubation in DMEM at 37 °C.

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