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Research paper

A thermosensitive chitosan-based hydrogel for the local delivery of paclitaxel

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Abstract

A novel injectable thermosensitive in situ gelling hydrogel has been developed. The system, which falls under the BST-Gel™ platform technology developed at Biosyntech Inc. (Laval, QC, Canada), consists of a chitosan solution (C) neutralized with β-glycerophosphate (GP) that is liquid at room temperature but gels when heated to body temperature. We propose to use this thermosensitive hydrogel for the sustained release of paclitaxel at tumor resection sites in order to prevent local tumor recurrence. The in vitro release profiles demonstrated controlled delivery over 1 month. The initial drug loading substantially affected the release. Local delivery of paclitaxel from the formulation injected intratumorally was investigated using EMT-6 tumors implanted subcutaneously on Balb/c mice. These experiments showed that one intratumoral injection of the thermosensitive hydrogel containing paclitaxel was as efficacious as four intravenous injections of Taxol® in inhibiting the growth of EMT-6 cancer cells in mice, but in a less toxic manner. Further histological analysis revealed that while the proportion of necrotic areas was similar for the C/GP/paclitaxel and the Taxol®-treated tumors, a disparity between tumor-associated inflammatory cell populations may suggest differing anti-tumor mechanisms.
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Keywords: Chitosan; Thermosensitivity; Hydrogel; Sustained-delivery; Paclitaxel

1. Introduction

Excluding cancers of the skin, breast cancer is the most frequently diagnosed cancer among women. An estimated 211,300 new cases of invasive breast cancer are expected to occur among women in the United States during 2003, and breast cancer will be the second leading cause of cancer death in American women behind lung cancer (American Cancer Society Inc., Surveillance Research, 2003). Almost all women with breast cancer will have some type of surgery in the course of their treatment. The purpose of many of these surgeries is to remove as much of the cancerous tissue as possible, as in a lumpectomy. However, the risk of recurrence stemming from residual malignant cells still exists, and may be averted through administration of local radiotherapy or systemic chemotherapy.

Paclitaxel is one of the best antineoplastic drugs found in nature in the past decades. It interacts with tubulin dimers in the G2 mitotic phase of cell division to promote microtubule polymerisation that results in the formation of highly stable microtubules, thus preventing cell division [1]. Paclitaxel success to date is largely due to its unique mechanism of action against tumors and its ability to work in combination with other anticancer therapeutic agents. It has excellent therapeutic efficacy for a wide spectrum of cancers, especially for ovarian and breast cancers.

However, paclitaxel is a hydrophobic molecule that is poorly soluble in water. Currently, Cremophor® EL, a non-ionic polyethoxylated castor oil solubilizer, is used to enable its clinical administration. Although it has a history of use with other drugs, the amount of Cremophor® EL necessary to deliver the required doses of paclitaxel is significantly higher than that administered with any other marketed drug.

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This causes serious side effects, particularly hypersensitivity reactions, some of which are life-threatening [2–4]. The incidence of these reactions can be substantially reduced by the use of prophylactic antiallergic premedications, but this is undesired as it increases the treatment burden.

In order to eliminate the toxicity of Cremophor® EL, improve efficacy and eliminate premedication, current paclitaxel research is focused on developing new drug delivery systems, which circumvent the Cremophor® EL difficulty associated with its use. A variety of approaches have been investigated including emulsification [5–7], microspheres [8,9], liposomes [10–12], nanoparticles [13,14] and polymeric micelles [15,16].

However, each drug delivery approach has unique inherent difficulties. Stable emulsions are hard to achieve and drug loading is often limited (i.e. high volumes needed to achieve therapeutic concentration) [5]. Likewise, satisfactory entrapment efficiency can sometimes be problematic with nanoparticles, microspheres and liposomes. Harper et al. [8] noted in their paper that high doses of the Paclimer Delivery System (microspheres) were not investigated, in large part because of anticipated difficulties in administering large volumes into tumor nodules. As for micelles, their disassembly upon injection, due to dilution, can result in burst release and toxic effects.

Another therapeutic approach to combating solid tumors and preventing metastasis and tumor re-growth, involves surgical removal of the tumor followed by implantation of a biodegradable device loaded with an antineoplastic agent in the resulting cavity. This method would provide high local drug concentration, effectively destroying surviving malignant cells and would also prevent the systemic side effects of chemotherapy normally associated with its intravenous administration. Since local recurrence of tumors generally occurs near the original excision site, this treatment modality is more desirable than a systemic one. Implantation of drug-loaded devices (including drug-polymer composites) into tumors or tumor resection sites has been investigated by several workers [17–24].

A material, which has large potential for use as an injectable in situ gelling drug delivery device, can be obtained from a chitosan solution (C) neutralized with a polyol counterionic dibase salt such as β-glycerophosphate (GP). This thermosensitive C/GP solution, which falls under the BST-Gel™ platform technology developed at Biosyntech Inc. (Laval, QC, Canada), is liquid at room temperature and solidifies into a hydrogel as temperature is increased to body temperature (Fig. 1) [25–27]. The objective of this work was to evaluate the C/GP thermosensitive formulation as the basis for local chemotherapy. The in vitro release profiles of paclitaxel from within the gel were first investigated, and the anti-tumoral activity of paclitaxel released from the gel was then assessed in vivo using the EMT-6 murine mammary carcinoma model.

2. Materials and methods

2.1. Materials

Medical grade chitosan (Ultrasan™, M_w 228,700, P.I. 1.61) having a deacetylation degree of 95% was obtained from Biosyntech Inc. (Laval, QC, Canada). GP and sodium dodecyl sulfate (SDS) were from Sigma (St. Louis, MO). Paclitaxel was purchased from Bioxel Pharma (Ste-Foy, QC, Canada). Taxol® (Bristol-Myers Squibb) was purchased in a retail pharmacy. All other chemicals were reagent grade. All products were used as received. Deionized distilled water from a Milli-Q water system of Millipore (Fisher Scientific Limited, Nepean, ON, Canada) was used to prepare the aqueous solutions. The EMT-6 murine carcinoma cells were a gift from Professor van Lier of the University Hospital of Sherbrooke (QC, Canada). Cell culture medium (Waymouth) and fetal bovine serum (FBS) were obtained from Invitrogen Canada Inc. (Burlington, ON, Canada).

2.2. Preparation of the chitosan/glycerophosphate solution

First, a chitosan solution was prepared in deionized water and sterilized in an autoclave (121 °C, 10 min) [28]. Second,

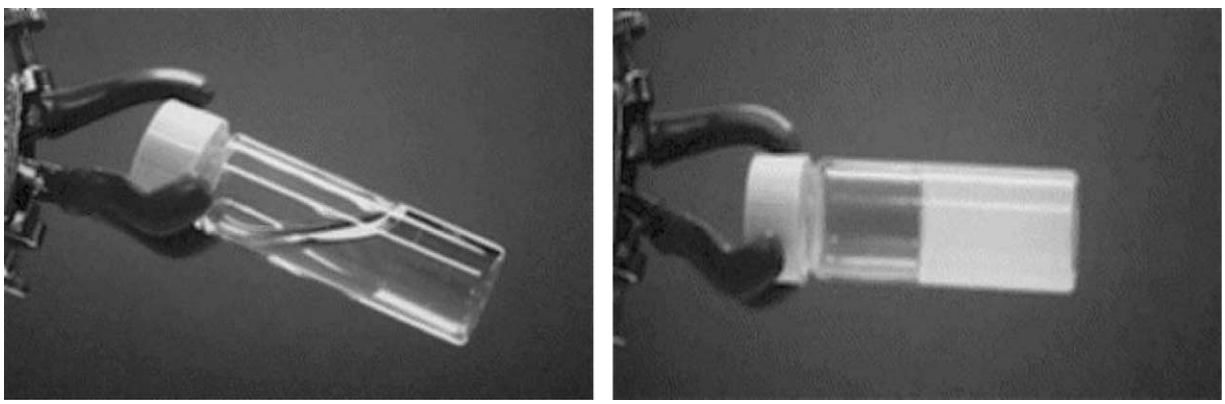

Fig. 1. The C/GP formulation at room temperature (left) and at 37 °C (right).

a GP solution was prepared in deionized water and sterilized by filtration. The two solutions were chilled in an ice bath for 15 min. The GP solution was added dropwise to the chitosan solution under stirring and the resulting mixture was stirred for another 10 min under aseptic conditions. The formulation containing paclitaxel was prepared by pouring the chitosan solution directly on the sterilized drug powder (see Section 2.4) and stirring during 4 h before mixing with the GP solution as described above. The final preparations contained 1.8% (w/w) chitosan, 3.6% (w/w) GP and either 0.64 or 6.4% (w/v) paclitaxel.

2.3. In vitro release studies

Samples of 300 mg of the C/GP solution with or without paclitaxel were placed into circular-shaped molds (diameter 8 mm) and allowed to gel in an incubator at 37 °C for 12 h. The circular-shaped gels were removed, placed into histology cassettes and suspended in 500 mL of isotonic phosphate buffered saline (PBS, pH 7.4) containing 0.3% (w/v) SDS. SDS was included in the release medium to increase the solubility of paclitaxel. The bottles were placed in a shaking incubator at 37 °C and 100 rpm. At the allotted times, 200-μL aliquots of the release medium were collected and stored at −80 °C until analysis. Preliminary studies revealed a slight degradation of paclitaxel after 4 days in the release medium. In order to avoid this phenomenon and to maintain sink conditions, the gels were transferred into fresh medium pre-warmed at 37 °C after each sampling. In the collected fractions, paclitaxel concentration was determined by HPLC using a slightly modified version of a published method [19,21]. The analysis was performed using a mobile phase of acetonitrile/water/methanol (48:41:11 v/v) at a flow rate of 1 mL min^{-1} (Gilson model 302, Middleton, WI), a C_{18} Nova-Pak column (Waters, Milford, MA), and UV detection (Gilson model 116, Middleton, WI) at 242 nm. An internal standard (*N*-heptylbenzamide, NHBZ) was added to each sample in order to correct for inter-injection variation [29].

2.4. Stability of paclitaxel after γ-irradiation

The drug powder was sterilized using 25 kGy γ-irradiation from a ^{60}Co source (MDS Nordion Inc., Laval, QC, Canada). Irradiated paclitaxel samples were stored at 4 °C along with non-irradiated powder. After 1 and 2 months, non-irradiated and irradiated samples were analyzed by HPLC (same conditions as in the in vitro release studies).

2.5. In vivo anti-tumor activity

Balb/c female mice weighing 15–17 g were purchased from Charles River Laboratory (Montreal, QC, Canada) and acclimatized for 7 days after arrival. The mice were provided with food and water ad libitum, and were maintained in an environment with alternating 12 h of light and darkness. Two separate studies were conducted where each mouse was injected subcutaneously in the posteriolateral flank with 2×10^5 EMT-6 cells suspended in 50 μL of Waymouth supplemented with 10% FBS (v/v). The treatment was initiated in the first study when the tumor reached 30 mm^3 ($L \times W \times H \times \pi/6$, between days 6 and 9 after inoculation) and 4 days after inoculation in the subsequent one. For each study, the mice were randomly assigned to one of the four treatment groups. The negative control group received 0.2 mL/day of intravenous (IV) saline via the caudal vein during 4 days (treatment days 0, 1, 2 and 3). The positive control group received 10 mg/kg/day of paclitaxel IV (Taxol® 6 mg/mL diluted in saline to 0.8 mg/mL) via the caudal vein during 4 days (treatment days 0, 1, 2 and 3). One treatment group received 10 μL of C/GP solution intratumorally (IT) and the second treatment group received 10 μL of C/GP solution containing 64 mg/mL of paclitaxel (equivalent to 40 mg/kg) IT, both on treatment day 0. Tumors were measured with calipers and animal weights were obtained every day during the first 9 days and then every other day. The animals were sacrificed after 17 days of observation and the tumors collected for histological analysis. Both studies were terminated at this time point since preliminary studies showed tumor necrosis in non-treated animals after this point. Any tumor effects were evaluated on the basis of the change in tumor volume. Toxicity was assessed by the evolution of the weight of the animals following treatment. All animal care and studies were approved by the Animal Welfare and Ethics Committee of the University of Montreal.

2.6. Histology analysis

Immediately after necropsy, tumors were retrieved whole, along with adjacent tissues and fixed in 10% neutral buffered formalin (Fisher Scientific Limited, Nepean, ON, Canada) for 2 weeks at 4 °C. Each tumor was then bisected manually with a blade, and for C/GP-treated tumors, macroscopic localization of the implant site was attempted. This portion of the tumor was embedded in paraffin, and serially sectioned using 5–6 μm thickness. Staining was accomplished using Saffranin-O/Fast Green, a proprietary process optimized at Biosyntech Inc. (Laval, QC, Canada) for use with BST-Gels™. Histology of stained tumor sections was examined using light microscopy with an Olympus CX40 (Carsen Group Inc., Markham, ON, Canada), and images were captured using a Spot Insight color digital camera (Focus Corp., Seoul, South Korea).

2.7. Statistical analysis

The in vivo data were subjected to a multiple comparison test: analysis of variance (ANOVA) or Kruskal–Wallis if the data did not fulfill the requirements for the ANOVA test. If the null hypothesis was rejected, pairwise comparison was done (Tukey, Scheffé, Nemenyi or Dunn) to determine

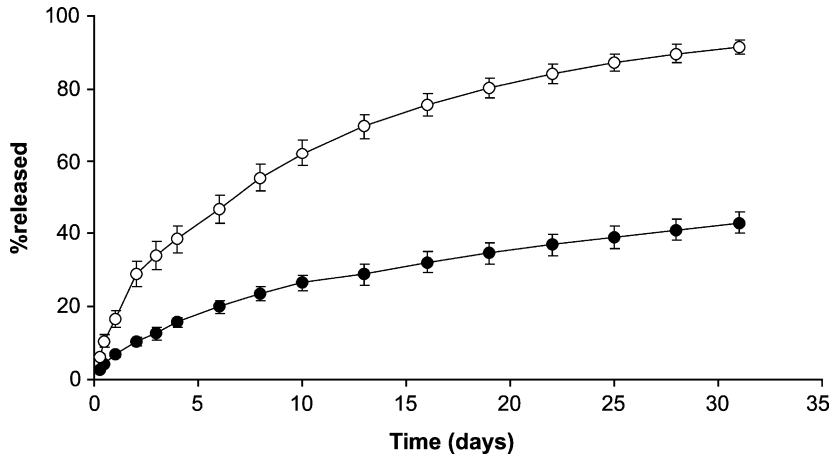

Fig. 2. In vitro cumulative percent release of paclitaxel from the C/GP gel in PBS + 0.3% SDS at 37 °C. Initial paclitaxel loading: 64 mg/mL (closed circles) and 6.4 mg/mL (open circles). Each point represents the mean value ± SEM ($n = 5$).

where the difference lied. $p < 0.05$ was considered significant. Results are expressed as mean ± standard error of the mean (SEM).

3. Results

3.1. In vitro release studies

In order to understand the ability of the C/GP gel to effectively deliver paclitaxel in a sustained fashion, in vitro release studies were performed in PBS/SDS 0.3% at 37 °C under sink conditions. Fig. 2 shows the resulting release profiles of paclitaxel from the C/GP hydrogel. It clearly demonstrates that the initial drug loading substantially affects the release rate, the latter being reduced at higher drug loading. The initial burst effect was lower (7.0 vs 16.6%) and the release rate was slower (2.0%/day from days 2 to 10 vs 4.2%/day) for the 64 mg/mL-loaded gel vs 6.4 mg/mL-loaded gel. This resulted in a 92% cumulative release for the 6.4 mg/mL-loaded gel compared to a 43% cumulative release for the 64 mg/mL-loaded gel after 1 month. Close inspection of the gels at the end of the experiment revealed that the gels loaded with 64 mg/mL of paclitaxel were still white with only a small rim of translucent gel on the outer part, in contrast to the 6.4 mg/mL gels which were almost completely translucent (similar to the unloaded control gel) with only a point of white in the center (data not shown). This suggests a potential for sustained delivery at higher loading rates in this hydrogel.

3.2. Stability of paclitaxel after γ-irradiation

The ability to sterilize an injectable drug is a critical parameter, which we investigated here using γ-irradiation. The HPLC analysis of irradiated paclitaxel gave similar results for all samples (Fig. 3). The samples irradiated at 25 kGy and kept at 4 °C for either 1 or 2 months gave spectra that were identical to those of the non-irradiated samples (control) evaluated in parallel. Irradiation of the paclitaxel powder did not induce degradation since no secondary peaks were detected and the heights of the peaks of the non-irradiated and irradiated samples were similar. This demonstrates that paclitaxel remains stable after irradiation, and can be kept at 4 °C for at least 2 months.

3.3. In vivo anti-tumor activity

Local delivery of paclitaxel from the formulation injected intratumorally was investigated using EMT-6 tumors implanted subcutaneously on Balb/c mice. Two separate studies were conducted, and in the first, the treatment was initiated when the tumors reached a volume of 30 mm^3 (days 6–9 after inoculation). This size was chosen in order to facilitate intratumoral injections. Fig. 4 shows that there was a clear difference in growth between the negative control (saline IV) tumors and all other treatment groups. Over the 17 days of observation, the saline-treated tumors grew to 9.2 ± 1.0 times their original size whereas the other groups showed only a 5.5 ± 0.6 to 5.7 ± 0.4 times increase, which represent a 38–40% growth inhibition. The difference was statistically significant from day 8. In the second study, the treatment was initiated on the fourth day of tumor growth. At this point, the tumors were generally very small and not always visually detectable. This time point was chosen in order to mimic a population of malignant cells remaining after primary tumor removal surgery. Fig. 5 shows that this study also demonstrated a significant difference between tumor growth in the negative control (saline IV) group and both paclitaxel groups (Taxol® IV and C/GP/paclitaxel). However, the same did not hold for the C/GP group, which did not demonstrate a marked growth inhibition. Over the 17 days of observation, the saline-treated tumors grew to 18.5 ± 2.6 times their original size, the paclitaxel groups showed only

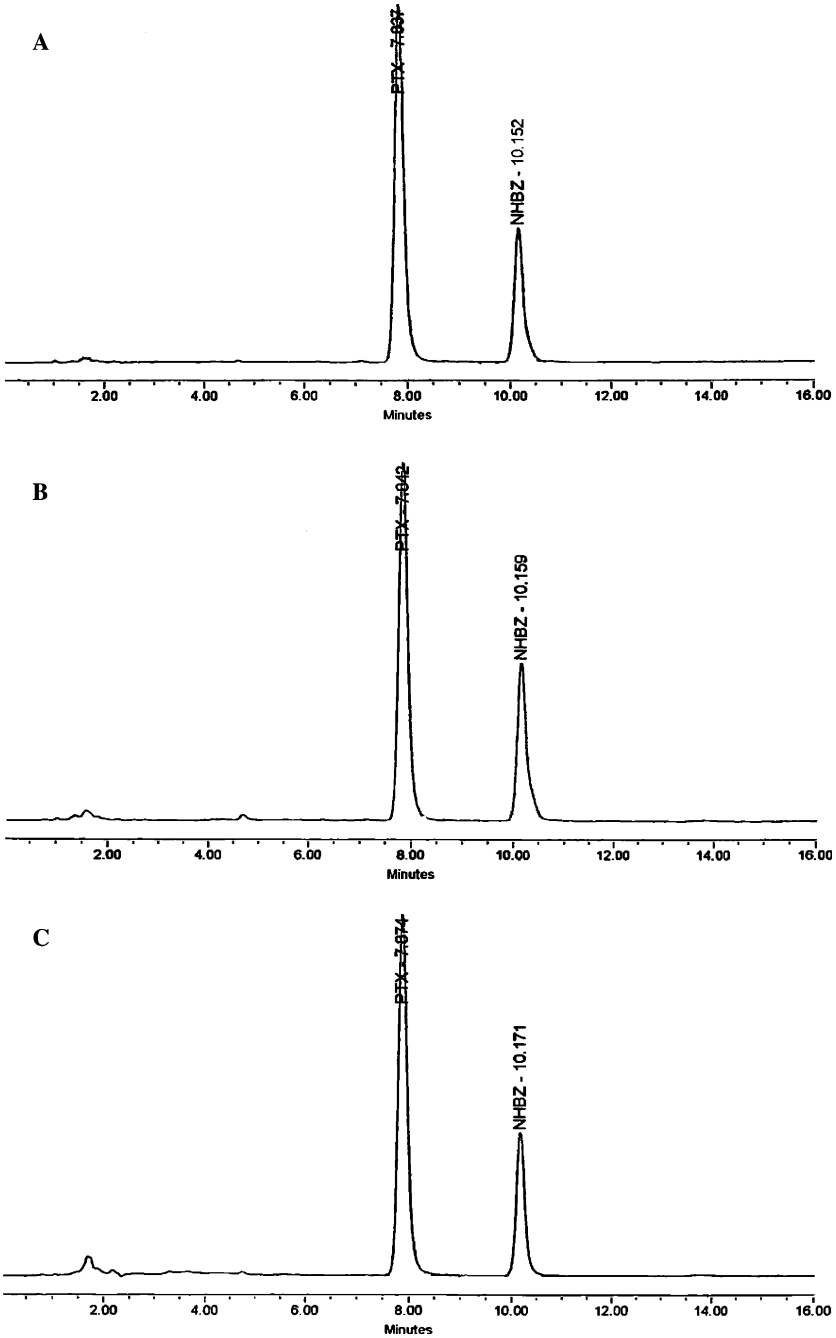

Fig. 3. HPLC spectra of paclitaxel non-irradiated (A), irradiated and stored 1 month at 4 °C (B) and irradiated and stored 2 months at 4 °C (C). Paclitaxel elutes at 7.8 min and the internal standard (*N*-heptylbenzamide) elutes at 10.1 min.

a 5.5 ± 0.8 to 5.6 ± 0.9 times increase and the C/GP-treated tumors grew 12.4 ± 4.4 times. The difference was statistically significant ($p < 0.05$) between the negative control group (saline IV) and the C/GP/paclitaxel group from day 3 of the experiment. The difference was statistically significant ($p < 0.05$) between the negative control group (saline IV) and the positive control group (Taxol® IV) at day 15 of the experiment. These experiments indicate that the C/GP/paclitaxel treatment has a significant ability to delay tumor growth, in a similar manner to Taxol® treatment alone. In addition, it showed that one intratumoral injection of the thermo-sensitive hydrogel containing paclitaxel was as efficacious as four intravenous injections of Taxol® in inhibiting the growth of EMT-6 cancer cells in mice, but in a less toxic manner. The mice receiving Taxol® displayed weight loss throughout the first 6–7 days of the experiments, peaking at days 4–5, whereas the mice receiving C/GP/paclitaxel had weight curves similar to those of the saline-treated mice (Fig. 6). Only one set of data is shown since the results are similar for the two experiments. The difference between the weight of the mice receiving Taxol® and

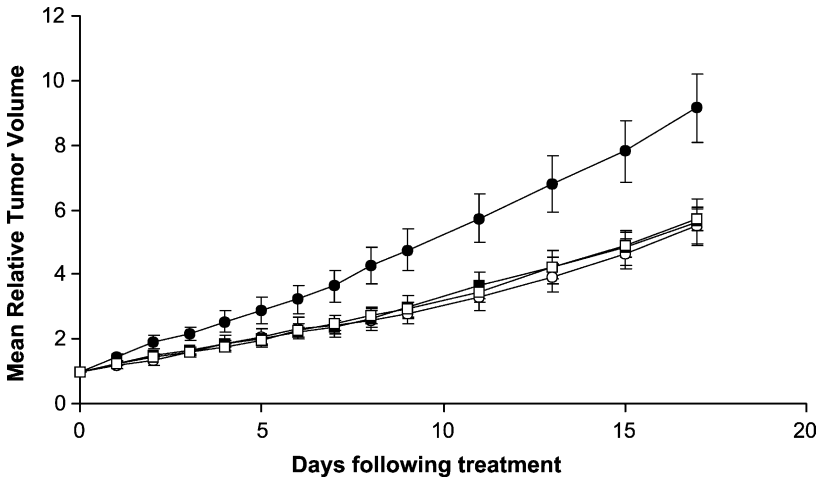

Fig. 4. In vivo anti-tumor effect of the treatments initiated when the tumors reached 30 mm^3. Saline 0.2 mL/day IV × 4 days (closed circles, $n = 10$), Taxol® 10 mg/kg/day IV × 4 days (open circles, $n = 12$), C/GP solution 10 μL intratumoral (closed squares, $n = 15$) and paclitaxel/C/GP solution (64 mg/mL paclitaxel) 10 μL (40 mg/kg paclitaxel) intratumoral (open squares, $n = 15$).

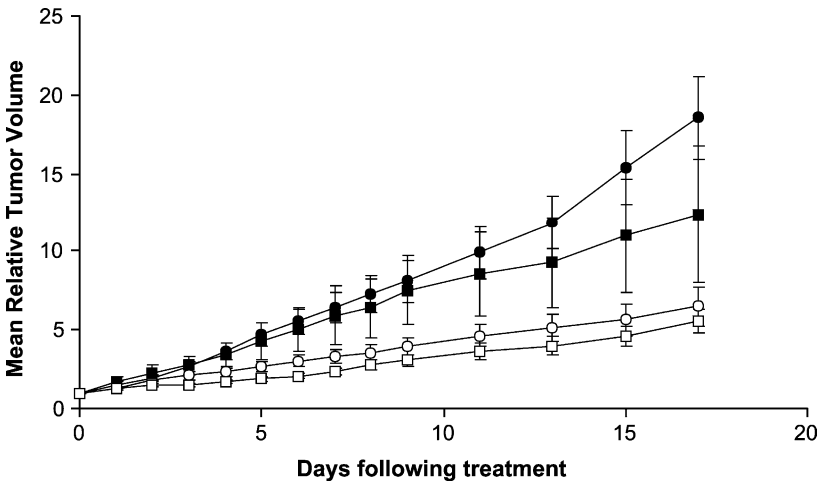

Fig. 5. In vivo anti-tumor effect for treatments initiated on day 4 after tumor inoculation. Saline 0.2 mL/day IV × 4 days (closed circles, $n = 8$), Taxol® 10 mg/kg/day IV × 4 days (open circles, $n = 8$), C/GP solution 10 μL intratumoral (closed squares, $n = 8$) and paclitaxel/C/GP solution (64 mg/mL paclitaxel) 10 μL (40 mg/kg paclitaxel) intratumoral (open squares, $n = 8$).

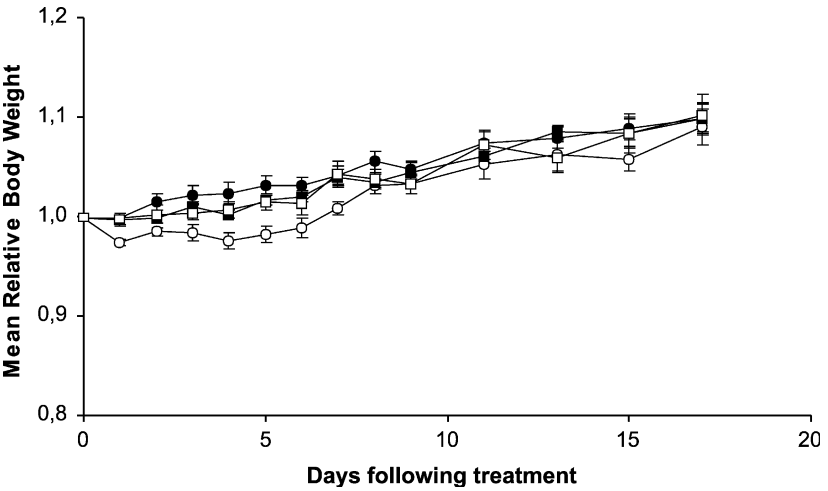

Fig. 6. Body weight of the animals for treatments initiated on day 4 after tumor inoculation. Saline 0.2 mL/day IV × 4 days (closed circles, $n = 8$), Taxol® 10 mg/kg/day IV × 4 days (open circles, $n = 8$), C/GP solution 10 μL intratumoral (closed squares, $n = 8$) and paclitaxel/C/GP solution (64 mg/mL paclitaxel) 10 μL (40 mg/kg paclitaxel) intratumoral (open squares, $n = 8$).

the weight of the others was statistically significant ($p < 0.05$) from day 1 to day 6 or 7, in the second and first studies, respectively.

3.4. Histology studies

All tumors demonstrated some level of necrosis. This was observed as variably sized regions containing necrotic tissue (cell fragments, nuclear debris and/or pyknotic cells) interdispersed between regions of viable tumor cells. The severity of necrosis decreased radially from the center, with all tumors demonstrating necrosis deep in their centers (Fig. 7). Compared with tumors from the positive control group (Taxol® IV) and the two C/GP groups (with or without paclitaxel), tumors from the negative control group (saline IV) demonstrated the lowest necrotic proportions. The intratumoral cell population appeared viable, with a lack of infiltrating leukocytes, and a lack of hematological observations. This is easily observed in Fig. 7A, where darkly stained cell regions are viable tumor zones. This is in contrast with the tumors of the C/GP groups and the positive control group (Taxol® IV), which contained much larger percentages of necrotic regions (Fig. 7B–D). In addition, these groups contained regions composed of tumor cells in various conditions, and in some cases were mixed viable and necrotic populations. This resulted in the appearance of less intense staining. In the C/GP groups, inflammatory cell populations had infiltrated the tumor to a large extent, likely a result of increased permeability of the new vessels visualized within the tumors. Hyperemic areas were also commonly observed in those groups, with mixed erythrocyte and inflammatory cell populations. C/GP material was not identified in all treated tumors, while in others it was found peripheral to the tumor (Fig. 7B, white arrow). This may imply either material degradation or migration following breakup of the gel over time, but the results suggest either mechanism maintains anti-tumoral effects. Tumors with identified C/GP material remaining also contained large macrophage populations. Tumors from the positive control group (Taxol® IV), while also having large necrotic zones, failed to exhibit inflammatory cell infiltration or hyperemia.

Excluding size differences, all tumors from animals receiving treatment either at 4 days or after reaching 30 mm^3 varied in similar manners histologically, according to treatment.

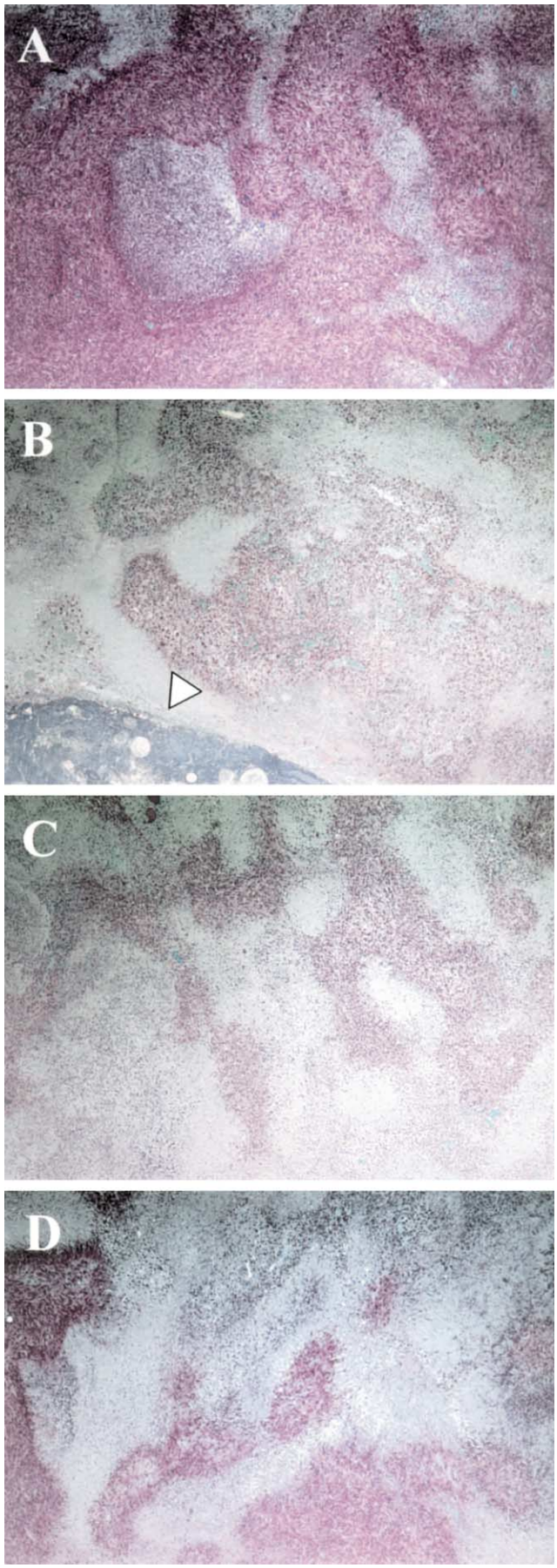

Fig. 7. Tumor cross-sections demonstrating necrotic zones. Differential staining observed represents varying tumor cell conditions. (A) Saline IV: darkly stained zones demonstrate areas with viable and active tumor cells. Spontaneous necrosis (light or no stain) can also be seen. (B) Paclitaxel/C/GP: white arrow identifies remaining C/GP material. Tumor center has a notable lack of darkly stained viable tumor cell zones. (C) C/GP material alone: clearly defined zones of necrosis are visible. (D) Taxol® IV: clearly defined zones of necrosis are visible (magnification 4 ×).

4. Discussion

Local control of tumors is a primary clinical objective and is usually achieved by surgery or radiation. However, local recurrence of tumors generally occurs near the previous surgical excision site of the primary tumor. In order to prevent this phenomenon, locoregional adjuvant chemotherapy has been proposed by several investigators [18,21,23,30]. In the present work, we evaluated a new thermosensitive formulation as the basis for local chemotherapy. The chitosan-based solution we developed remains liquid at room temperature and turns into a gel as temperature increases to body temperature [25–27]. The formulation can be easily injected through a needle at the tumor resection site and, in a uniform manner, cover any residual cancerous cells on the margins of the cavity as it sets into a gel.

The use of paclitaxel in such systems is not new. Zentner et al. [22] reported that a biodegradable thermal poly(lactic acid)-poly(ethylene glycol)-poly(lactic acid) (PLA-PEG-PLA) gel with paclitaxel maintained release in a controlled fashion over 50 days. In their study, the release of paclitaxel over the first 2 weeks was consistent with a diffusion-controlled mechanism, but thereafter, faster release was consistent with a combined diffusion/polymer degradation mechanism. Winternitz et al. [18] used a surgical paste of poly(ε-caprolactone) (PCL) and methoxy-polyethylene glycol (MePEG) for the controlled delivery of paclitaxel which was characterized by an initial burst lasting 1 or 2 days followed by a period of sustained slow drug release. Our lower loading level (6.4 mg/mL) gel which had a similar drug load as the materials used by Zentner et al. [22] (2 mg/mL) resulted in the release of 62% of the drug load after 10 days, and 92% after 1 month compared to approximately 39 and 65% for identical time intervals. In addition, our higher drug-loaded gel (6.4%) which was more similar to the pastes of Winternitz et al. [18] (5%), resulted in the release of 35% after 20 days compared to 20–26% for different pastes. In a previous paper [27], we showed that with low molecular weight hydrophilic compounds (<1000 g/mol), more than 80% of the incorporated drug was released during the first 24 h. For example, calcein, a hydrophilic compound with a molecular weight of 623 g/mol, was completely released from a 0.2% (w/w) loaded gel in 48 h. The C/GP system can thus sustain the delivery of hydrophobic drugs such as paclitaxel for at least 1 month, which is a reasonable time span for an injectable sustained-release delivery system. Moreover, this formulation offers some advantages over other systems. Zentner et al. [22] reported that the onset of gelation of PLA-PEG-PLA was at 14 °C and that the transition to the solid-like gel state was complete around 18 °C for a 23% (w/w) polymer solution. A transition temperature too close to room temperature complicates handling of the preparation. On the other hand, the need to heat the surgical paste of Winternitz et al. [18] for delivery is impractical. Our formulation is liquid at room temperature for several hours and is thus easily injected.

The release of paclitaxel from the BST-Gel™ was concentration-dependent, where higher loading resulted in slower release. Since paclitaxel is dispersed in the formulation, it requires dissolution prior to diffusion from the gel. Higuchi [31,32] developed an equation for the release of a drug from an ointment base and later applied it to diffusion of solid drugs dispersed in homogeneous matrix dosage systems. One form of the equation (Eq. (1)) indicates that the amount of drug released at time t per unit area of exposure (Q) is proportional to the square root of the total concentration, dissolved and undissolved, of drug in the matrix (A), the diffusion coefficient of the drug in the matrix (D), the solubility of the drug in the matrix (C_s), and the time (t).

$$Q = \sqrt{2ADC_s t} \tag{1}$$

In our study, if all the variables except drug loading remained identical, the 10-fold difference between the two drug loadings should result in a 3.2-times difference in the amount of drug released at each time point. In fact, we obtained a 3.0–3.9 ratio between the amount of drug released in the two experiments and excellent linearity was observed ($R^2 > 0.99$) when the cumulative amount released (up to 85%) was plotted against the square root of time.

We needed to confirm that sterilization had no effect on drug stability. γ-Irradiation was chosen for sterilization due to known or anticipated effects with other methods. For example, filtration of the paclitaxel/C mixture was not possible because of high viscosity and large size of drug particles (200–400 μm). On the other hand, autoclaving would have likely resulted in hydrolysis of the paclitaxel. Also, a previous study revealed that γ-irradiation of chitosan solutions has strong negative effects on the gelling properties of C/GP systems [33]. Thus, it was decided to irradiate the paclitaxel alone before incorporation in the sterile chitosan solution. Other authors have investigated the influence of irradiation on paclitaxel release profiles from semi-solid systems and showed minimal effects. The paclitaxel release from a PCL/MePEG paste showed that after 20 days, the release kinetics from both irradiated and non-irradiated samples were not different from each other [18]. In the present study, a comparison of the HPLC spectra obtained from irradiated and non-irradiated samples of paclitaxel revealed the absence of degradation, even after 1 or 2 months of storage (Fig. 3). This implies that the drug can be sterilized and stored at 4 °C until further use. This lack of degradation obviated the need to perform release profiles studies for both irradiated and non-irradiated paclitaxel.

Using the EMT-6 murine mammary carcinoma in Balb/c mice, we have demonstrated that the efficacy of the C/GP formulation containing paclitaxel (one IT injection at 40 mg/kg) was similar to that of the commercial product

Taxol® (4 IV injections at 10 mg/kg) (Figs. 4 and 5). Moreover, the animal treated locally with the gel formulation showed no apparent drug-related adverse effects of treatment, whereas the systemic treatment group showed weight loss during treatment and a few days afterwards (Fig. 6). The high local efficacy and low systemic toxicity can be attributed to the slow, continuous and localized release of paclitaxel from the gel. The efficacy of the treatment was demonstrated in two separate studies representing two stages of tumor growth: (a) 4 days after inoculation when the tumors were generally very small and not always visually detectable and (b) when the tumors reached a macroscopically observable 30 mm^3 (between days 6 and 9). Other investigators have reported similar results regarding the higher efficacy of local treatments compared to systemic treatments in different cancer models. It was demonstrated that a single intratumoral dose of biodegradable PLA-PEG-PLA thermal gel containing paclitaxel was more effective than a maximum tolerated systemic dose of Taxol® against human breast tumor xenografts (MDA231) [22]. The intratumoral treatment groups exhibited a dose response that was equal or superior to that of the systemic treatments. Also, the animals treated with the thermal gel intratumorally showed no drug-related adverse effects of treatment, whereas the systemic treatments groups showed weight loss and two instances of acute toxic death within 2 days of dosing. A reduction of 63 ± 27% in tumor mass in mice with established and palpable MDAY-D2 tumors compared with controls through peri-tumoral injection of paclitaxel–gelatin–PCL paste was also shown [19]. In that study, there was also no significant effect on the weights of the mice following treatment. Unfortunately, the application of such a paste at the tumor site is not trivial. The tumor site has to be opened under anesthesia and the paste has to be heated to around 60 °C to allow extrusion to the tumor site. Positive local drug delivery results were demonstrated using a blend of p(DLLA-*co*-CL)-PEG-p(DLLA-*co*-CL) with MePEG containing paclitaxel [21]. This formulation was a viscous liquid or paste at room temperature that sets into a solid implant within 1 h. A single intratumoral injection used against human prostate LNCaP tumors established in Balb/c mice caused complete regression of the tumors which became non-palpable within 2–4 weeks after treatment. Using a similar base material as our chitosan, Nsereko and Amiji [24] used chitin or chitin–Pluronic F-108 microparticles to deliver paclitaxel in a murine model of Lewis lung carcinoma. Control and paclitaxel-containing chitin microparticles were administered subcutaneously at the base of the tumor. The average tumor volume doubling time increased from 4.70 days in control group to 12.05 days in paclitaxel-containing chitin–Pluronic F-108 microparticles-treated group. However, beyond 8 days, tumor volumes increased in all cases.

Interestingly, our in vivo data revealed that the injection of C/GP gel can impact the growth of tumors in the absence of drug. In fact, when the C/GP formulation was administered intratumorally into 30 mm^3-tumors (6–9 days after inoculation), the growth was decreased to a similar extent as the tumors treated with C/GP/paclitaxel (Fig. 4), and the histology yielded similar observations. But the susceptibility of the tumors to treatment appeared to have a dependence on time of administration, since a pronounced effect of the C/GP without drug was not observed when the tumors were treated at an earlier stage of development (Fig. 5). Insight into this difference comes from others who report anti-tumor characteristics of both chitosan and its derivatives, as well as individual polysaccharides. It appears that the mode by which chitosan imparts this inhibitory activity may be through both direct and indirect manners, although the sequelae which leads to the eventual disruption and inhibition of tumor growth is not known. It is clear that whether through direct or indirect actions, intratumoral injection of chitosan of differing forms has the ability to diminish tumor growth.

Direct exposure to chitosan, which demonstrated growth-inhibitory characteristics, has been reported for different types of tumor cells. Authors have suggested an effect mediated through either the induction of apoptosis, shown on both bladder tumor cells [34] and HL-60 (leukemia) cells [35] or a decrease in glycolysis, for instance in Ehrlich ascites tumor cells [36]. More indirect anti-tumor influences can clearly be attributed to chitosan effects on other immune or inflammatory cell types. It has long been understood that chitosan mediates neutrophil, lymphocyte and macrophage responses in migration, cytokine release and cell signaling [37–40] as well as activation of other cell types like fibroblasts, leading to increased production of cytokines [41]. Furthermore, it has been shown that BST-Gels™ containing chitosans of differing chemistries elicit differential macrophage responses with regards to adhesion, proliferation and cytokine production (Biosyntech, personal communication). In this study, the injection of C/GP or drug containing C/GP likely promoted the migration of inflammatory cells, the activity of which influenced both vascular permeability and/or paracrine signaling.

Intratumoral administration of chitosan compounds alone has previously been shown to promote anti-tumoral effects in metastatic breast cancer models; the tumor responses were augmented when those compounds were used in combinations with other therapies including drugs or laser immunotherapy [42]. Chitosan was also found to activate macrophages into cytotoxic macrophages in vivo, and suppressed Meth-A tumor growth in Balb/c mice [43]. Tokoro et al. [44] showed that two oligosaccharides consisting solely of the two units which compose chitosan, *N*-acetyl-D-glucosamine and D-glucosamine, were growth inhibitory to Meth-A solid tumor transplanted in Balb/c mice when administered systemically. It was proposed that increased sequential production of lymphokines IL-1 and IL-2, led to the manifestation of anti-tumor effect through proliferation of cytolytic T-lymphocytes. Either scenario appears reasonable, and in this study, we observed a significant influx of macrophages and other leukocytes

which support possible roles by both cell types. However, the mechanism by which chitosan is able to infer anti-tumor activities through injection is not clear, and it still remains to be shown whether it is a specific immunological response or simply a result of general inflammation.

Chitosan is not unique as a polysaccharide in its inhibitory effect. Chihara et al. [45] found that certain polysaccharides isolated from an edible mushroom had in vivo anti-tumor activity against sarcoma 180. Likewise, maitake mushroom polysaccharide extracts activated lymphocyte and macrophage responses and demonstrated in vivo anti-tumor effects [46].

Relevant to our study, however, was the Seljelid [47] report that systemic polysaccharide treatment with the water-soluble aminated β1-3D-glucan (AG) caused total regression of Meth A sarcoma grown intradermally in CB6 mice. The key to successful results was the timing of AG administration. In fact, the effect of AG on tumor growth was clearly dependent on the time point at which the substance was administered. A biphasic response was demonstrated when AG was given IV: a dosing at day 3 after tumor inoculation produced incomplete regression, but equal administration at day 7 led to complete regression in all cases, and yet a 14-day administration yielded no significant effect on tumor growth. Further studies [48] demonstrated intratumoral cytokine profiles correlated to tumor susceptibility. This suggests that our observation of differing responses following inoculation at different stages may be related to the susceptibility state of tumors and the cytokine profiles resulting from cellular responses to the C/GP material. Our histological observations demonstrating an increased inflammatory cell population in tumors treated with C/GP material also suggest that the mechanisms through which C/GP drug delivery operate may have underlying differences from the systemic treatment, albeit with similar outcomes. More studies will be pursued to understand time-dependent susceptibility of tumors with regards to both tumor tissue changes and differential responses to our chitosan-based material.

5. Conclusions

In summary, we have provided proof of principle and preclinical efficacy data for a site-directed, injectable, and controlled-release formulation of paclitaxel as an effective treatment for localized solid tumors. In vitro release profiles demonstrated controlled delivery over 1 month. Local delivery of paclitaxel from the formulation injected intratumorally in EMT-6 tumors implanted subcutaneously on Balb/c mice showed that one intratumoral injection of the thermosensitive hydrogel containing paclitaxel was as efficacious as four intravenous injections of Taxol® in inhibiting the growth of cancer cells, but in a less toxic manner. After 17 days of observation, the C/GP hydrogel containing 64 mg/mL of paclitaxel had released 32% of its drug load in vitro and the animals that received this formulation intratumorally displayed a marked tumor growth inhibition. With this information in mind, we can expect a better anti-tumor efficacy if the rates of dissolution and release of paclitaxel were increased. In order to achieve that, a coprecipitate of paclitaxel and a water-soluble polymer could be prepared and incorporated in the C/GP solution. Histological analysis revealed that although the proportion of necrotic areas was similar for the C/GP/paclitaxel and the Taxol®-treated tumors, the difference between tumor-associated inflammatory cell populations may imply differing anti-tumor mechanisms.

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