Pharmaceutical Research, Vol. 26, No. 9, September 2009 (© 2009)
DOI: 10.1007/s11095-009-9922-2

Research Paper

Hydrogel Matrix Entrapping PLGA-Paclitaxel Microspheres: Drug Delivery with Near Zero-Order Release and Implantability Advantages for Malignant Brain Tumour Chemotherapy

Sudhir Hulikal Ranganath,[1] Irene Kee,[2] William B. Krantz,[1] Pierce Kah-Hoe Chow,[3,4] and Chi-Hwa Wang[1,5]

Received February 10, 2009; accepted June 1, 2009; published online June 20, 2009

Purpose. To develop paclitaxel-delivering PLGA microspheres entrapped in a gel matrix with sustained drug release properties and implantability advantages for local glioma chemotherapy.
Methods. Paclitaxel-loaded PLGA microspheres were fabricated using electrohydrodynamic atomization and entrapped by electrospray and gelation. The physicochemical characterizations were performed using scanning electron microscopy and differential scanning calorimetry. The influence of various parameters on the disintegration time was investigated. *In vitro* release of paclitaxel was quantified using high performance liquid chromatography. Cytotoxicity of the formulations was assessed by the quantification of IC_{50} and caspase-3 activity against C6 glioma cells *in vitro*. The formulations were tested against a subcutaneous C6 glioma tumour in mice.
Results. Highly monodisperse gel beads containing a uniform microsphere distribution were obtained. Gelation using Ca^{2+} ions ensured entrapment of microspheres with high loading efficiency. With an increase in the gelation time, gelling bath concentration and decrease in microsphere loading, it was more difficult to disintegrate the beads and release the microspheres. The formulations demonstrated sustained drug release for more than 60 days at a near-constant rate and a low initial burst. Cell culture studies proved the cytotoxicity against C6 glioma and improved performance in comparison to Taxol®. The formulations could reduce subcutaneous tumour volume to a greater extent compared to Taxol® and the control.
Conclusions. Paclitaxel-loaded PLGA microspheres entrapped in an alginate gel matrix could be potential local chemotherapy implants to treat malignant glioma with critical advantages of implantability and sustained drug release with low initial burst.

KEY WORDS: chemotherapy; electrohydrodynamic atomization; glioma; hydrogel; microspheres; PLGA-paclitaxel.

INTRODUCTION

The critical challenge faced by modern clinical pharmacology in treating brain neoplasms, such as malignant glioma, is to deliver the therapeutic agents to the target site at the necessary therapeutic concentration level(s) while reducing systemic levels of the drug to minimise systemic toxicities. The limited ability of most chemotherapeutic agents to penetrate the blood-brain barrier (BBB) (1-4) has been addressed by the development of polymeric devices for local delivery of chemotherapeutic agents (5-7); these devices bypass the BBB while reducing the risk of systemic toxicity effects.

Ever since Gliadel® was used in clinical trials and showed promise (8), a myriad of drug-polymer combinatorial delivery devices has been developed and evaluated with promising preclinical results (9-15). However, the need to develop polymeric drug delivery implants that possess features such as easy intracranial application, near-constant drug release for extended time periods facilitating high drug penetration into the tumour bed, slow sustained drug release resulting in low systemic and local toxicity, and bio-compatibility still remains to be addressed. The critical factors that decide the efficacy of such implants are properties of the encapsulated drug, type of polymer and geometry of the implant. Properties of the encapsulated drug, such as its molecular weight, diffusivity in the brain tissue and drug elimination constant, are keys to determining the penetration distance of the drug in the brain tissue, whereas drug cytotoxicity is crucial to treatment efficacy. Selection of the polymer as the implant material is critical because of the desired properties, such as biocompatibility and controllable degradation. The geometry of the implant and surface-area-volume ratio play key roles in determining polymer degradation and drug-release kinetics.

Paclitaxel is a very widely used chemotherapeutic agent for treating various cancers, but has proven to be ineffective

[1] Department of Chemical and Biomolecular Engineering, National University of Singapore, 4 Engineering Drive 4, Singapore, 117576, Singapore.
[2] SingHealth Experimental Medicine Center, Singapore General Hospital, Block 9, Level 3, Outram Road, Singapore, 169608, Singapore.
[3] Duke-NUS Graduate Medical School, 2 Jalan Bukit Merah, Singapore, 169547, Singapore.
[4] Department of General Surgery, Singapore General Hospital, Block 6, Level 7, Outram Road, Singapore, 169608, Singapore.
[5] To whom correspondence should be addressed. (e-mail: chewch@nus.edu.sg)

against malignant gliomas when administered systemically (16-18) at the maximum tolerated doses due to the resulting very low levels in the brain (19). In contrast, paclitaxel delivered locally by polymeric devices (4,20) is known to reach therapeutic concentrations at least 3 mm from the implant site in the brain. However, implants with more favourable release kinetics, sustainability and penetration in the brain need to be developed. Paclitaxel has a higher molecular weight, lower diffusivity in the brain, higher elimination constant and higher cytotoxicity against glioma compared to other drugs such as carmustine (Gliadel®) and 4-HC (21). Thus, poly(D,L-lactic-co-glycolic)acid (PLGA), an FDA approved biodegradable polymer that undergoes hydrolytic degradation (that can be controlled by varying the co-polymer ratio), could be ideal to encapsulate highly hydrophobic paclitaxel in comparison to matrices such as PCPP-SA (15) and polilactofate (20).

Several forms of paclitaxel-delivering implants have been developed, such as compressed discs (21-23), cubic phases (4), micro/nano-fiber discs or sheets (24), microparticles (15,25, 26) and nanoparticles (27). Paclitaxel encapsulated in discs, wafers and sheets provides efficient intracranial application; however, due to the low surface area available for polymer degradation and drug diffusion, the release rate is generally low, which could subsequently lead to low therapeutic efficacy. On the other hand, paclitaxel-loaded biodegradable micro/nanoparticles provide a higher release rate due to a larger surface area (15,25-27), but they have been shown to exhibit a large initial burst that could result in significant neurotoxicity effects. Nevertheless, microsphere-based local drug delivery to treat glioma is promising due to the fact that the gliomas tend to recur within 2 cm of the resected tumour site post surgery (28); microspheres could reach remote tumour regions in the cavity by cerebrospinal fluid flow and edema. However, a challenge remains to contain the microspheres (suspension) in the cavity during the injection and treatment period and also to minimize or even prevent the initial drug burst. Consequently, we hypothesized that to provide containment, a solid matrix could be utilized to entrap the microspheres. In addition, the matrix could act as a barrier to drug diffusion from the microspheres and thus could inhibit the initial burst. One such matrix could be alginate, a natural polysaccharide derived from seaweed and algae that has realized numerous applications in the microencapsulation of cells (29) and biomolecules, tissue engineering (30) and drug delivery (31,32) due to its biocompatibility and hydrogel-forming abilities. Alginates undergo instantaneous gelation under mild reaction conditions in the presence of divalent cations such as Ca^{2+} and form cross-linked three-dimensional hydrogel matrices.

Thus, in this study, the main objectives were to design, develop and evaluate a novel composite implant that couples the sustained release properties of PLGA-paclitaxel microspheres with the intracranial implantability advantages of the alginate matrix for post-surgical chemotherapy to treat malignant glioma. PLGA-paclitaxel microspheres were fabricated using Electrohydrodynamic Atomization (EHDA), since this process has been shown to be very efficient in controlling the microsphere size, morphology and monodispersity (25). An electrospray in dripping mode was utilized to entrap the microspheres in alginate hydrogel beads owing to its ease of operation and demonstrated monodispersity (33). Furthermore, the effects of various parameters, such as gelation time, gelation bath concentration and microsphere loading on *in vitro* drug release, were studied, followed by cell culture studies (evaluation of cytotoxicity and paclitaxel-induced apoptosis in C6 glioma cell *in vitro*) and an *in vivo* anti-tumour efficacy study in a subcutaneous tumour model.

MATERIALS AND METHODS

Materials

All materials and reagents used were of analytical grade unless otherwise indicated. The co-polymer used in microsphere fabrication, PLGA 50:50 (Av. Mol Wt, 40,000–75,000 Da), was purchased from Sigma Aldrich. Paclitaxel was supplied courtesy of Bristol-Meyers Squibb (New Brunswick, NJ). The low viscosity sodium salt of alginic acid (sodium alginate) from macrocyctis pyrifera (Mol Wt, 12,000–80,000 Da) and polyoxethylene-sorbitan monoleate (Tween 80) were purchased from Sigma Aldrich; calcium chloride dehydrate from Nacalai Tesque (Kyoto, Japan); Taxol® from Bristol-Meyers Squibb (Princeton, NJ); HPLC-grade dichloromethane (DCM) and acetonitrile (ACN) from TEDIA (Fairfield, OH, USA); phosphate buffer saline (PBS) containing 0.1 M sodium phosphate, 0.15 M sodium chloride (pH 7.4) from Sigma Aldrich;. Dulbecco's modified eagle medium (DMEM); penicillin-streptomycin and trypsin-EDTA from Sigma Aldrich; and fetal bovine serum (FBS) from Gibco (Life Technologies, AG, Switzerland). A Takara Bio Inc. (Japan) Premix WST-1 cell proliferation assay system and a Biovision (CA, USA) Caspase-3/CPP32 fluorometric assay kit were used.

Fabrication of PLGA-Paclitaxel Microspheres by the EHDA Process

Fabrication of PLGA-paclitaxel microspheres was done using EHDA as reported earlier (25). Briefly, PLGA (50:50) was dissolved in DCM in 8% (*w/v*) proportion (i.e., 1 ml DCM to 0.08 g polymer) along with 16.7% (*w/w*) paclitaxel. A potential difference was applied between the nozzle (10–12 kV) and ring (9 kV) using high voltage generators (Glassman High Voltage Inc., NJ, USA) in order to obtain highly monodisperse particles with an average diameter of approximately 12 μm and to ensure that a single spray cone was maintained during the fabrication process. The nitrogen flow rate was set at 40 l/min to act as a sink for solvent evaporation from the dispersed droplets. The polymer-drug solution was pumped at a rate of 5 ml/h through the nozzle forming a liquid cone with a thin emerging jet. Jet break-up then occurred causing the formation of mono-dispersed droplets. The subsequent evaporation of DCM caused the accumulation of solid microspheres onto a petri-dish placed at the sample collection point at the side opening of the chamber. The fabricated microspheres were frozen in liquid nitrogen (roughly at a temperature of −196°C that is much lower than the melting point of DCM (−96°C)) and later freeze-dried for 5 days to remove any residual DCM.

Fabrication of Alginate Beads Entrapping PLGA-Paclitaxel Microspheres by Electrospray

The electrospray process as shown in Fig. 1 was employed to entrap the PLGA-paclitaxel microspheres in the alginate beads. The apparatus consisted of a high-voltage generator, syringe pump with a metal tip (tapered conical section made of copper sheet) and a grounded stage. The metal tip was connected to the syringe and used instead of a needle to avoid clogging by the microspheres. A dish filled with $CaCl_2$ solution was placed on the grounded stage. PLGA-paclitaxel microspheres were suspended in deionized water (with 0.1% *w/v* of Tween 80 surfactant) to form a uniform dispersion. Sodium alginate was added to the dispersion at 1% *w/v* and then completely dissolved to create a viscous suspension. The suspension was then pumped at 1 ml/min using the syringe pump with the metal tip connected to the high-voltage generator at 6 kV. Due to the high potential difference at the metal tip, the suspension broke into monodisperse droplets that were dripped into the $CaCl_2$ solution. The alginate in the droplets instantly underwent gelation in the $CaCl_2$ solution, thereby resulting in calcium-cross-linked hydrogel beads that entrapped the microspheres within their matrix. Additionally, paclitaxel-loaded alginate beads were fabricated to compare the *in vitro* release profiles. An equivalent amount of paclitaxel (free-form) was dispersed into a 1% *w/w* sodium alginate solution and dripped into a $CaCl_2$ solution using the electrospray technique as described earlier.

The following preparative variables were evaluated in an effort to understand their effect on the drug release profiles: Gelation time (1, 5, 15 min), $CaCl_2$ concentration (0.5, 1, 2% *w/v*) and microsphere loading (50, 80, 90% *w/w*). Following gelation, the beads were washed with deionized water (to remove any non-cross-linked Ca^{2+} ions) and freeze-dried for at least 7 days to remove water. The beads were sterilized by exposure to UV light for 12 h before using them for the cell culture and animal studies. Table I summarizes the different formulations that were prepared and studied.

Physicochemical Characterization of Hydrogel Beads

Morphology, Size Distribution and Uniformity of Microsphere Entrapment

The surface morphology of the microspheres and uniformity of microsphere entrapment in the hydrogel beads were examined using Scanning Electron Microscopy (SEM) (JEOL JSM 5600LV, Tokyo, Japan). The free microspheres were evenly spread onto a copper stub using double-sided adhesive tape to expose the surface. Similarly, the hydrogel beads were cut in half and mounted on a copper stub to expose their cross-section. The samples were then sputtered with platinum for 60 s using a JFC-1300 Platinum Coater (JEOL, Tokyo, Japan) prior to the analysis. Scanning of the samples was performed using a high-energy electron beam with an accelerating voltage of 15 kV. Analyses of the hydrogel bead size were done using SmileView software (version 2.05, JEOL) at $n>50$. A Coulter LS230 particle size analyzer (Beckman Coulter, CA, USA) was used to analyze the size distribution of the microspheres. The samples were prepared by resuspending the microspheres in distilled water with 0.1% *w/v* Tween-80. The volume size distribution was analyzed by a computerized analysis for spheres.

Encapsulation and Loading Efficiencies

The amount of paclitaxel present in each hydrogel bead depends on two factors: the drug encapsulation efficiency (EE) and the microsphere loading efficiency (LE), defined as follows:

$$\text{Encapsulation Efficiency (\%)} = \frac{\text{Actual drug loading in microspheres}}{\text{Theoretical drug loading in microspheres}} \times 100 \quad (1)$$

$$\text{Loading Efficiency (\%)} = \frac{\text{Measured amount of microspheres in a bead } (\mu g)}{\text{Total amount of microspheres } (\mu g) \,/\, \text{number of beads fabricated}} \times 100 \quad (2)$$

The EE for the paclitaxel-loaded PLGA microspheres was determined as previously reported (25). To quantify the loading efficiency of the hydrogel beads, each bead was accurately weighed in triplicate and completely dissolved in 5 ml of 0.1 M sodium citrate. Then the suspension was centrifuged at 12,000 rpm for 40 min after which the supernatant was separated from the collected microspheres. Two milliliter of DCM was then added to the supernatant solution, vortexed and left to stand for 2 days to allow mass transfer. The aqueous supernatant was discarded and the DCM was allowed to evaporate. The centrifuged microspheres were dissolved in 1 ml of DCM that

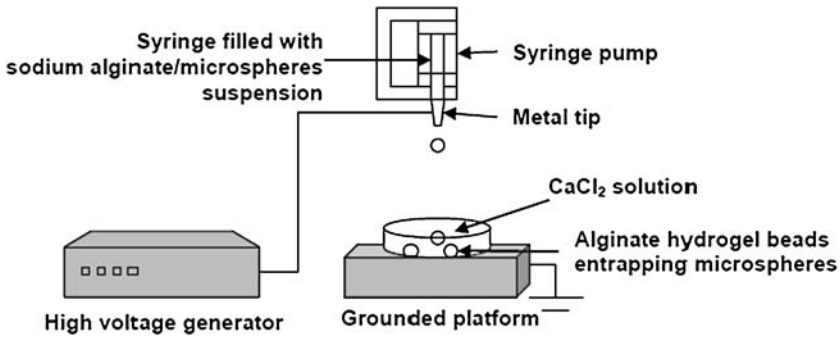


Fig. 1. Schematic for the fabrication of alginate beads entrapping PLGA-paclitaxel microspheres by electrospray dripping followed by $CaCl_2$ gelation.

Table I. Preparative Variables for Hydrogel Bead Fabrication and Respective Formulations

Preparative variables	Formulations								
	H50	M50	L50	H80	M80	L80	H90	M90	L90
Microsphere Loading (% w/w)	50	50	50	80	80	80	90	90	90
Gelation Time (min)	15	5	1	15	5	1	15	5	1
$CaCl_2$ Concentration (% w/v)	2	1	0.5	2	1	0.5	2	1	0.5

was then allowed to evaporate completely. Subsequently all the samples were re-dissolved in 5 ml of an acetonitrile-water solution (50:50 *v*/*v*) followed by filtering the solution through a 0.22 μm syringe filter (Millipore, MA, USA) into 2 ml sample vials. The amount of paclitaxel in each sample was then analyzed using high-performance liquid chromatography (HPLC). Paclitaxel was detected at a wavelength of 227 nm with an ultraviolet variable wavelength detector after the sample solution was pumped through a C-18 column using acetonitrile-water (50:50 *v*/*v*) as the mobile phase. A calibration graph was obtained by plotting the reported peak areas from the HPLC analysis and corresponding known concentrations of paclitaxel. The concentration of paclitaxel in the samples was then obtained using the calibration plot. The amount of microspheres in each bead was estimated from the encapsulation efficiency and amount of paclitaxel determined from HPLC. The loading efficiency was then calculated using Eq. (2).

Thermal Analysis of Hydrogel Beads

Thermal analysis was performed using Differential Scanning Calorimetry (DSC) (Mettler Toledo, 822e, Greifensee, Switzerland) to evaluate the physical state of the drug after encapsulation and entrapment. Beads weighing between 3 and 4 mg were hermetically sealed in a standard 40 μl aluminum crucible; an empty crucible was used as the reference. Thermal analysis was conducted over a temperature range of 25 to 300°C using a heating rate of 10°C/min. The equipment was purged with N_2 at a rate of 30 ml/min and liquid nitrogen was used to re-establish the starting temperature. Calibration of the instrument's temperature and enthalpy were done using pure indium. Characteristic peaks were obtained by analyzing alginate hydrogel beads with PLGA microspheres without paclitaxel (control) and with paclitaxel. The results were assessed using the accompanying STARe software. Normalization was performed on the obtained results with respect to the respective sample masses to facilitate comparison.

***In Vitro* Characterization of Hydrogel Beads**

Hydrogel Bead Disintegration Study

This study was conducted to evaluate the effects of gelation time, $CaCl_2$ concentration and microsphere loading on the disintegration time of the beads. All the formulations reported in Table I were fabricated under the specified conditions and were used in this study. Each bead was suspended in 5 ml of PBS at 37°C and agitated at 120 rpm in a water bath to shorten the experiment duration. The time required for total disintegration of the bead was determined by direct observation. The density of cross-linking in the alginate matrices was quantified by estimating the total Ca^{2+} ion content in the release media after total disintegration using inductively coupled plasma-optical emission spectroscopy (ICP-OES) with the Ca^{2+} absorbance at 317.33 nm (ICP Optima 3,000 DV, Perkin Elmer, USA).

In Vitro Paclitaxel Release from Hydrogel Beads

An *in vitro* paclitaxel release study was conducted by immersing each bead (PLGA-paclitaxel-loaded alginate bead or paclitaxel-loaded alginate bead) into a 1 ml PBS buffer (pH 7.4) containing 0.05% (*w*/*v*) Tween 80 (to enhance the paclitaxel solubility) in 12- or 6-well plates and incubated at 37°C in a water bath to simulate physiological conditions of the body. Ten data points (days) were taken, each of which involved three beads. At each specified time interval, the buffer was withdrawn for analysis and the beads discarded. In order to maintain a constant sink condition, fresh PBS (1 to 3 ml depending on the incubation duration) was added to the remaining beads (for subsequent data points). Subsequently 2 ml of DCM was added to the withdrawn buffer solution to extract the paclitaxel from the aqueous phase by allowing mass transfer for 2 days. A mixture of acetonitrile/water (50:50, *v*/*v*) was added to the extracted paclitaxel after the DCM had evaporated. HPLC analysis was performed as explained previously for the loading efficiency study.

***In Vitro* Cell Culture Study**

Cell Culture and Maintenance

Rat C6 glioma (ATCC® Number: CCL-107™) was employed as the tumour cell line in this study. The cells were grown in DMEM (Sigma Aldrich) supplemented with 10% FBS and 1% penicillin/streptomycin in a humidified incubator under the conditions of 37°C, 5% CO_2 and 90% relative humidity. The cells were washed in PBS and detached from the T-flask using trypsin-EDTA after they had reached 90% confluence. The cells were centrifuged at a speed of 1,500 rpm for 6 min and re-suspended to obtain a concentration of 3×10^5 cells/2.5 μl before inoculation into a 75 cc tissue culture flask containing 15 ml of fresh media.

In Vitro Cytotoxicity Against C6 Glioma Cells

The cytotoxicity of selected formulations (H80 and M80 beads) was evaluated via a cell viability study using a premix WST-1 cell proliferation assay. 5×10^3 C6 glioma cells were meticulously seeded into 48-well plates. Two hundred microliter of fresh medium was added per well before the plates were left

to incubate for 24 h. Thereafter, the spent medium was removed and replenished with fresh medium. Hydrogel beads were added to the respective wells to give the specified paclitaxel concentrations (predicted using the *in vitro* release profiles). Each 48-well plate was prepared to consist of at least one row of control (i.e., no drug administered to the wells) for the accurate monitoring of growth conditions that could vary from plate to plate. At the end of the 72-h exposure to the drug, the used medium and beads were removed. Each well was then filled with 400 μL of fresh medium coupled with 40 μL of the assay (volume ratio of 1:10). Addition of premix WST-1 was done in darkness due to the light sensitivity of the assay. To ensure accuracy, the plates were wrapped in aluminium foil after assay addition and left to incubate for an additional 4 h. Thereafter, the plates were observed by a microplate reader (Safire2, TECAN, Switzerland) at 430 nm with a reference wavelength of 620 nm.

Cell viability was calculated by normalizing the absorbance values of cells treated with hydrogel beads against the absorbance value of the control wells defined as follows:

$$\text{Cell Viability}\,(\%) = \frac{\text{Absorbance of test cells}}{\text{Absorbance of control cells}} \times 100 \quad (3)$$

In Vitro Cellular Apoptosis

Paclitaxel-induced apoptosis in the C6 glioma cells was quantified after the treatment with H80 and M80 beads and subsequently compared with three groups: blank control (no drug and implant), placebo control (implant with no drug) and Taxol® control. Each group consisted of triplicates. First, all the groups were inoculated with 1×10^6 C6 glioma cells each in 75 ml tissue culture flasks with 30 ml of DMEM. Before the treatment was initiated, the cells were allowed to attach and grow in the flask for 2 days (day 0). Studies of cell density and apoptosis were performed on days 2, 4 and 6. C6 cells in the Taxol® control group received commercial Taxol® on day 2 at doses equivalent to the paclitaxel released from H80 beads after 6 days (from *in vitro* release profiles), and, subsequently, the Taxol®-laden media were removed after 24 h and the cells were washed twice by PBS before replenishing with fresh medium. The Taxol® control was employed in this study to simulate the systemic chemotherapy (acute paclitaxel exposure and subsequent clearance). Cellular recovery was anticipated after drug clearance in the Taxol® group. For the H80 and M80 groups, the respective beads were introduced on day 2. Cell counting was performed using a haemocytometer after days 2, 4, and 6 to obtain the cell density.

On days 2, 4 and 6, 1×10^6 cells were collected for caspase-3 activity-level measurement (indicative of apoptosis extent) of the samples using a caspase-3/CPP32 fluorometric assay kit. Apoptotic (caspase-3) activity level was quantified by re-suspending the cells in 50 μl chilled lysis buffer and incubating them on ice for 10 min before adding 50 μl of 2× reaction buffer (with 1% 10 mM DTT) to each sample. Subsequently, 5 μl of 1 mM DEVD-AFC substrate was added before the samples were transferred to 96-well plates. The plates were then incubated at 37°C for 2 h in a humidified incubator before reading the samples in a microplate reader (fluorometer) from Tecan Genios, Switzerland, equipped with a 405 nm excitation filter and 485 nm emission filter. Upon cleavage of the substrate by CPP32 or related caspases, free AFC emits a yellow-green fluorescence (max=505 nm). Absorbance for the experimental groups was then compared with the controls for caspase-3 activity (extent of apoptosis) and paclitaxel-induced apoptosis.

In Vivo Evaluation

Animal Care and Maintenance

All animal experiments were performed at the Sing-Health Experimental Medicine Center (SEMC), Singapore General Hospital (SGH) with approval from the SGH Institutional Animal Care and Use Committee (IACUC). Experimental practices were conducted in accordance with the National Advisory Committee for Laboratory Animal Research (NACLAR) guidelines (guidelines on the care and use of animals for scientific purposes) in facilities licensed by the Agri-food and Veterinary Authority of Singapore (AVA).

BALB/c nude male mice of about 5 weeks were purchased from the Animal Resource Centre, Murdoch, Western Australia under an import license from the AVA. They were housed in cages of fives and left to acclimatize for a week at the SEMC prior to an experiment. Free access to food and water was given. The anesthesia administered before surgical procedures contained 50 mg/kg Ketamine and 5 mg/kg Valium. The painkiller contained 5 mg/kg Toradol and antibiotics contained 15–20 mg/kg Baytril.

In Vivo Subcutaneous Tumour Volume Study

Animals were randomized into different groups with five animals in each group: three control groups, namely control group (no beads and no drug), placebo control (beads without drug) and Taxol®; and two experimental groups, namely H80 and M80. Animals in the H80 and M80 groups were implanted with three beads each, weighing in total about 2 mg with an average paclitaxel amount of 162 μg per animal. On day 0, all the animals were inoculated with 5×10^5 C6 glioma cells subcutaneously by injecting using a 27G needle to the right flank. The tumour was allowed to grow for 7 days after which treatment was commenced. For animals under placebo and the M80 groups, the beads were implanted by aseptic surgery as follows: a small incision was made into the skin and the tumour was reached after which another incision was made into the tumour in order to implant the beads. The wound was closed using subcutaneous suturing. However, for the animals in the H80 group, the beads were implanted around the tumour. Animals in the Taxol® control group were administered with a dose equivalent to the total amount of paclitaxel administered using the beads (0.03 ml of commercial Taxol® injection (concentration of 6 mg paclitaxel/ml)) directly into the tumour mass. Equal amounts of paclitaxel were administered into animals from the Taxol® group and the experimental groups for an equitable comparison of the treatments. Every seven days, the lengths of the major (longest diameter) and minor axes (perpendicular to the major axis) of the tumour were measured to determine the ellipsoidal tumour volume using the formula ($Volume = [\Pi/6] \times A \times B^2$), where A and B represent the lengths of the major and minor axes of the tumour, respectively. Weight monitoring of the animals was carried out once a week in order to detect any experimental

abnormalities. For easy comparison, the percent reduction in tumour volume (RTV) was calculated for the H80 and M80 groups against the blank control and Taxol® groups using the formula % $RTV = 100(V_C - V_E)/V_C$, where V_C and V_E represent the tumour volume of animals from the control (blank or Taxol®) and experimental groups, respectively as shown in Table III.

Statistical Analysis

An ANOVA single-factor test at a significance level of 0.05 was used to determine if the apoptotic levels between the M80 and H80 groups were significantly different from that of the blank and Taxol® groups. In the subcutaneous tumour study, statistically significant differences ($p<0.05$) in the tumour volume among groups were analyzed by one-way analysis of variance (ANOVA).

RESULTS

Physicochemical Characterization of Hydrogel Beads

Morphology, Size Distribution and Uniformity of Microsphere Entrapment

PLGA-paclitaxel microspheres fabricated using the EHDA process yielded highly monodisperse microspheres with a spherical shape and smooth surface morphology as shown in Fig. 2a, and the size distribution analysis revealed that the microspheres had a mean diameter of 11.76±2.79 μm with 86% of the total volume comprising microspheres between 9 to 18 μm. The electrospray process followed by gelation at the specified operating parameters listed in Table I yielded monodisperse beads with the average bead diameters shown in Table II. Importantly, the microspheres were uniformly distributed throughout the hydrogel matrix and maintained their original morphology, which is confirmed in the representative images in Fig. 2b, c and d. All the formulations had a uniform distribution of microspheres inside the gel matrix.

Encapsulation Efficiency and Loading Efficiency

Paclitaxel was well-encapsulated in the microspheres (82± 0.1%) after the EHDA process. Also, the electrospray process and instantaneous gelation facilitated achieving good microsphere loading efficiency (~98%) in the gel matrix.

Thermal Analysis of Hydrogel Beads

Figure 3 shows the characteristic DSC thermographs obtained for representative samples. The endothermic peak centered close to 85°C represents the thermogram for pure calcium alginate described in the literature (34). Another endothermic

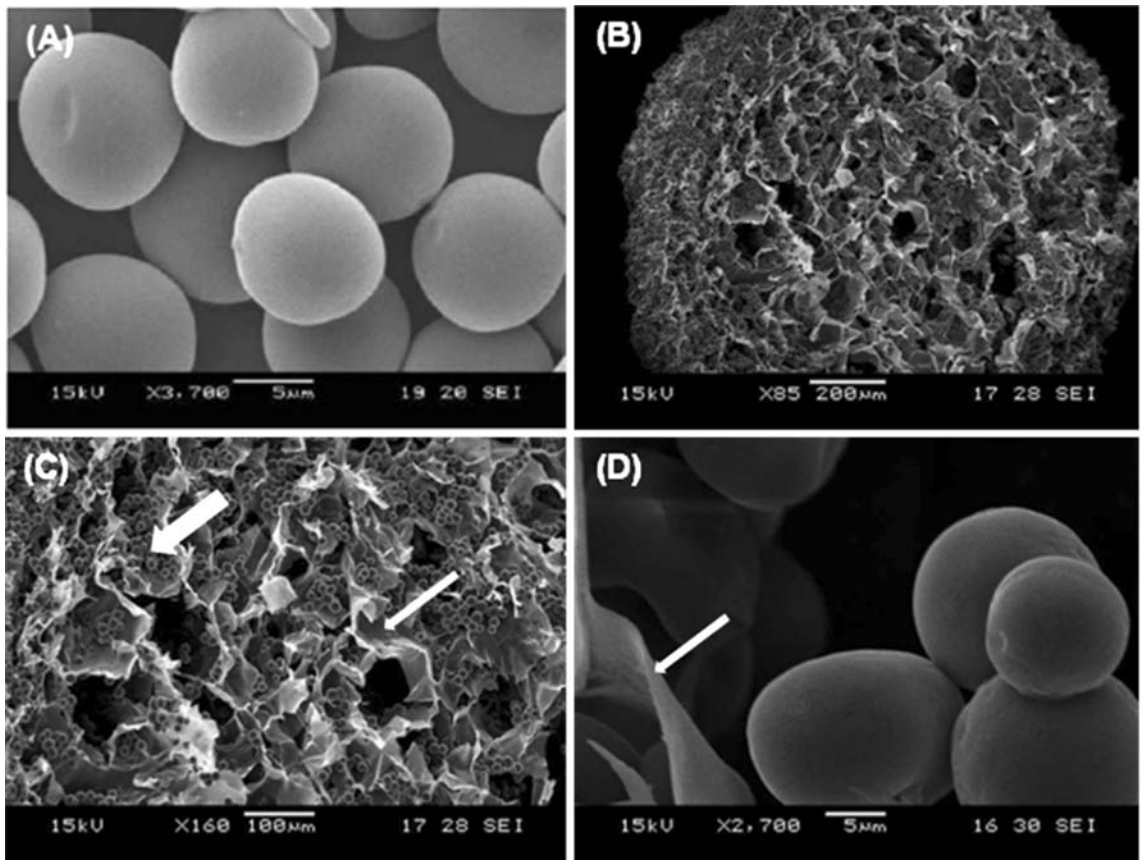


Fig. 2. Representative SEM images of **a** PLGA-paclitaxel microspheres fabricated by the EHDA process; **b** cross-sectional view of an alginate gel bead entrapping PLGA-paclitaxel microspheres; **c** high magnification image of **b** with the large arrow showing the microspheres and the small arrow pointing to the gel matrix; **d** higher magnification of the alginate matrix with the arrow showing the hydrogel.

Table II. Physicochemical Characterization of Hydrogel Beads Fabricated by Electrospray

Formulations	Average bead diameter (mm)	Loading efficiency (%)
50% microsphere loaded beads	1.61±0.04	98.02±1.07
80% microsphere loaded beads	1.65±0.06	98.50±2.02
90% microsphere loaded beads	1.68±0.05	97.10±3.77

peak was observed at around 45 to 50°C corresponding to PLGA 50:50 that has been reported to have a distinctive glass transition temperature (T_g) at about 45°C (25). Interestingly, with an increase in alginate content in the beads, the T_g of PLGA increased slightly as seen for the H90 thermogram in Fig. 3. Pure crystalline paclitaxel has been reported to show a sharp endothermic melting peak at 223°C (25), but none of the formulations exhibited an endothermic melting peak at 223°C. However, an exothermic peak at around 246°C was observed in the samples containing paclitaxel.

In Vitro Characterization of Hydrogel Beads

Hydrogel Bead Disintegration Study

The nine formulations whose preparative variables are summarized in Table I constituted the entire range for this study. The prefixes H, M and L refer to the extent of cross-linking in the beads, (high, medium and low) brought about by the corresponding conditions of gelation time and gelation bath concentration. The other variable studied was the microsphere loading that was fixed at 50, 80 and 90% (*w*/*w*). For example, H80 would mean that the bead was fabricated using 2% (*w*/*w*) $CaCl_2$ solution, gelled for 15 min and contained 80% (*w*/*w*) microspheres. Results from Fig. 4a reveal that the disintegration time of the beads decreased with an increase in microsphere loading. Furthermore, at the same microsphere loading, an increase in the extent of cross-linking resulted in slower disintegration of the beads.

To understand the effect of gelation time, gelling bath concentration and microsphere loading on the cross-link density of the beads, the total Ca^{2+} ion content was measured in the beads. Data from Fig. 4b suggest that at any microsphere loading, increase in gelling bath concentration and gelation time would increase the cross-link density. However, with an increase in microsphere loading there is an optimum cross-link density observed.

In Vitro Paclitaxel Release from Hydrogel Beads

This study was performed to evaluate the effect of various preparative variables on the paclitaxel release from the alginate matrix entrapping microspheres in an *in vitro* environment. Figure 5a, b and c show the paclitaxel release profiles for the various formulations. Generally it can be seen that in comparison to the free PLGA-paclitaxel microspheres (25), the initial burst from all the formulations was smaller and decreased as the microsphere loading increased. Importantly, near-zero-order release kinetics was exhibited by most of the formulations. In case of the 50 and 90% microsphere-loaded beads, an increase in the extent of cross-linking usually resulted in lower release rates indicated by the percent cumulative release after 60 days. Interestingly, for the 80% microsphere-loaded beads, an increase in the extent of cross-linking brought about an increase in the release rate as seen from Fig. 5b. Moreover, the percent drug released after 60 days decreased as the microsphere loading was increased. For the paclitaxel-loaded alginate beads, we noticed that the alginate matrix disintegrated within 1 or 2 days depending on the extent of cross-linking and resulted in 100% drug release during this period.

In Vitro Cell Culture Study

In Vitro Cytotoxicity Against C6 Glioma Cells

Based on the release study, two formulations (H80 and M80) were screened to be used in the evaluation of *in vitro* cytotoxicity against C6 glioma cells. The cytotoxic potency was assessed based on the quantification of IC_{50} of the formulations in comparison to Taxol® (25). IC_{50} refers to the concentration of the formulation required to inhibit 50% of cell growth *in vitro*. Figure 6 shows a gradual decrease in cell viability after 3 days of treatment with varying concentrations of the beads (or paclitaxel concentrations). IC_{50} values of M80 and H80 were approximately 1,500 μg/ml and 4,800 μg/ml, respectively (marked by the two arrows in Fig. 6 and with good regression coefficients), which corresponds to about 25 μg/ml of paclitaxel based on the *in vitro* release profiles.

In Vitro Cellular Apoptosis

Figure 7 presents the extent of apoptosis or caspase-3 activity measured by fluorescence intensity in the C6 glioma cells being treated with various groups. Clearly, the blank and placebo groups showed negligible signs of apoptosis even after 6 days. On day 2, the Taxol® group exhibited significant apoptosis in comparison to the blank ($p<0.05$) and H80 groups ($p<0.05$), while the difference was not significant when compared to the M80 group. Interestingly, the level of apoptosis started to decrease gradually on days 4 and 6 for Taxol®. On all the treatment days for the H80 and M80 groups, the extent of apoptosis was significantly higher than that of the blank ($p<0.05$). In contrast to Taxol® the H80 and M80 groups demonstrated a gradual increase in the apoptosis level. On day 6, both the H80 and M80 groups had a significantly higher apoptosis level compared to Taxol®.

Table III. Reduction in Tumour Volume for H80 and M80 Groups in Comparison to Control and Taxol® Groups After 21 Days of Treatment

Formulations	RTV (%) against control	RTV (%) against Taxol®
H80 beads	52	32
M80 beads	85	78

RTV reduction in tumour volume

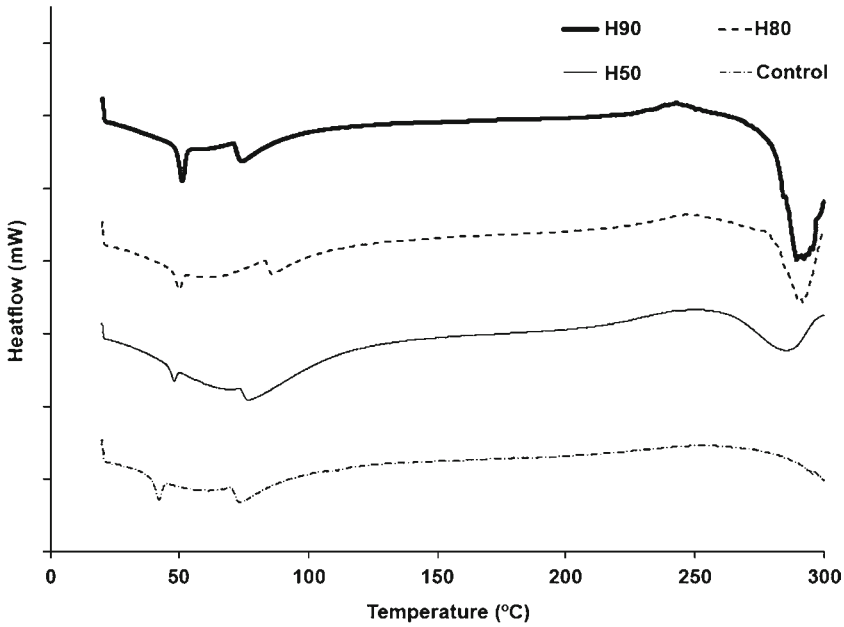


Fig. 3. Representative DSC thermographs of alginate beads entrapping PLGA-paclitaxel microspheres under the gelation conditions of 2% (*w/v*) $CaCl_2$ and a 15-min gelation time. The *Control* represents the alginate beads entrapping blank microspheres without paclitaxel.

In Vivo Subcutaneous Tumour Volume Study

As proof of concept, this study was intended to evaluate the efficacy of the H80 and M80 formulations in treating the subcutaneous C6 glioma tumour in mice in comparison to the blank control and Taxol® groups. Figure 8 shows the change in tumour volume after 7, 14 and 21 days of treatment for the various groups. The blank control and placebo control showed similar tumour growth for the entire period of study. In comparison to the blank control, animals in the Taxol® group had smaller tumours, but the difference was not significant ($p>0.05$). For both the H80 and M80 treated animals, the tumours were significantly smaller in comparison to those treated with the blank control ($p<0.05$) for the entire treatment period. However, in comparison to the Taxol® group, the tumours were not significantly smaller for the H80 treated mice ($p>0.05$), but the M80 treated animals had significantly smaller tumours ($p<0.05$) on days 7 and day 21. The H80 group exhibited about 52 and 32% smaller tumours in comparison to the blank control and Taxol® groups, whereas the M80 group showed about 85 and 78% smaller tumours compared to the blank control and Taxol® group, respectively.

DISCUSSION

Since EHDA is a proven simple and efficient fabrication technique, it was utilized to produce the microspheres in this study. The monodispersity of the microspheres was achieved by having a ring voltage to stabilize the electric field resulting in a single spray cone and a uniform particle size distribution (35). Spherical shape and smooth morphology were obtained by controlling the flow rate, polymer concentration and nitrogen flow rate across the jet that aids in the evaporation of the organic solvent. The electrospray process to drip the suspension of microspheres into the gelation bath proved to be efficient in obtaining high monodispersity of the beads with high throughput. Furthermore, the uniformity of the microsphere distribution inside the gel matrix was facilitated by the presence of the surfactant Tween 80 in the sodium alginate solution, which helped the highly hydrophobic microspheres to stay uniformly suspended during the dripping. Also, the cross-linked alginate gel layer could be seen both on the surface and within the bead, which confirms that Ca^{2+} ions were able to diffuse into the bead and form the gel, thus entrapping the microspheres within them. The paclitaxel encapsulation efficiency in the microspheres was high, suggesting that the EHDA aided in uniformly dispersing the amorphous paclitaxel within them. The high microsphere loading efficiency proved that the electrospray process provided uniformly sized beads. Also, the instantaneous gelation of the bead with Ca^{2+} ions prevented the escape of microspheres from the matrix, thereby resulting in a high loading efficiency.

The absence of an endothermic melting peak at 223°C in the thermal analysis (Fig. 3) revealed that paclitaxel was in a molecular-dispersion or amorphous state in the microspheres even after encapsulation and subsequent entrapment in the gel matrix. The slight increase in T_g for PLGA with an increase in microsphere loading was probably due to additional endothermic overlap owing to the energy required to overcome the microstructure of the polymer (36). Broad peaks observed for alginate were in correspondence with the high poly-dispersity of alginate chains (37). Polymer-drug crystallinity has been a key concern related to drug delivery efficiency. It has been established that molecular dispersions give better release profiles relative to particulate dispersions of the same drug (38). Hence, in view of the goal of the current formulations to provide constant drug release at a relatively higher rate, molecular dispersion of paclitaxel seems to be well-suited for the intended drug delivery applications.

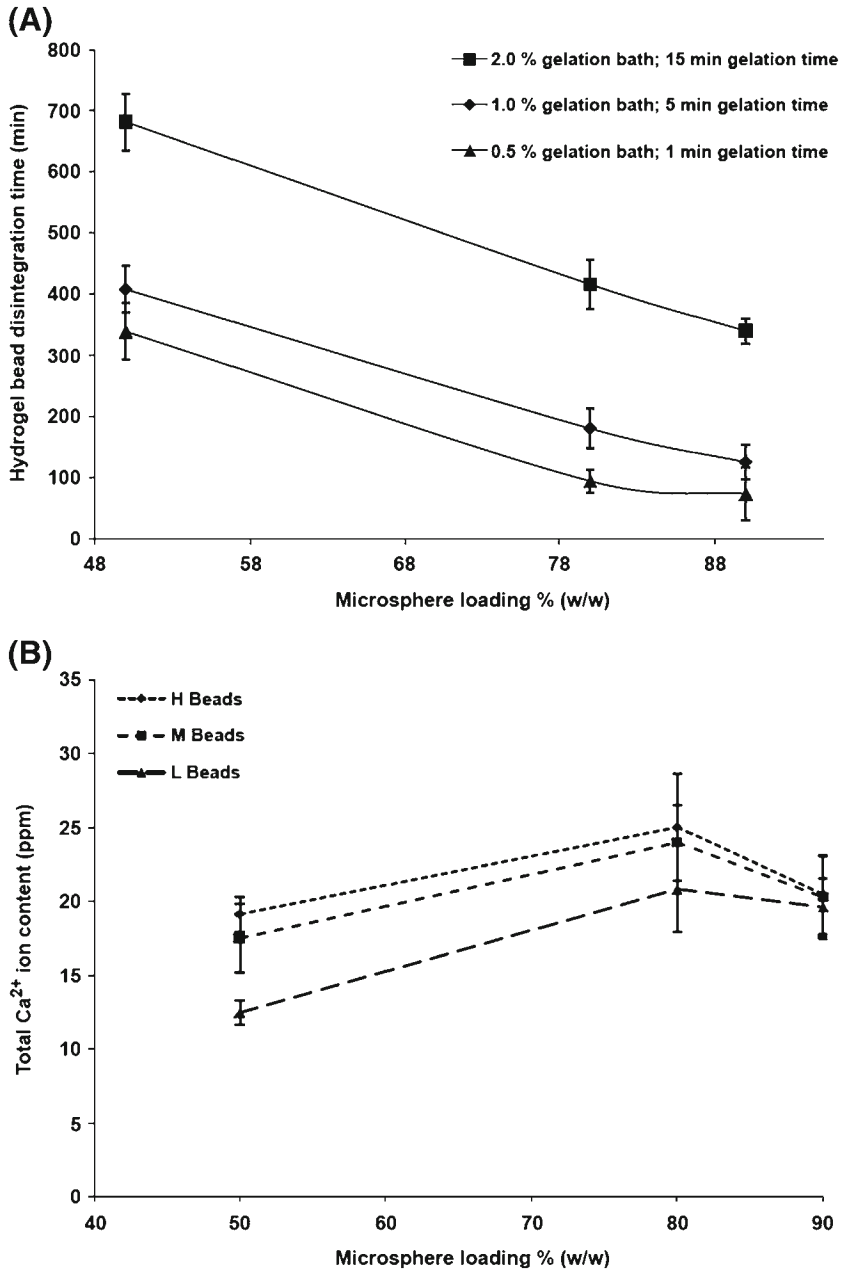
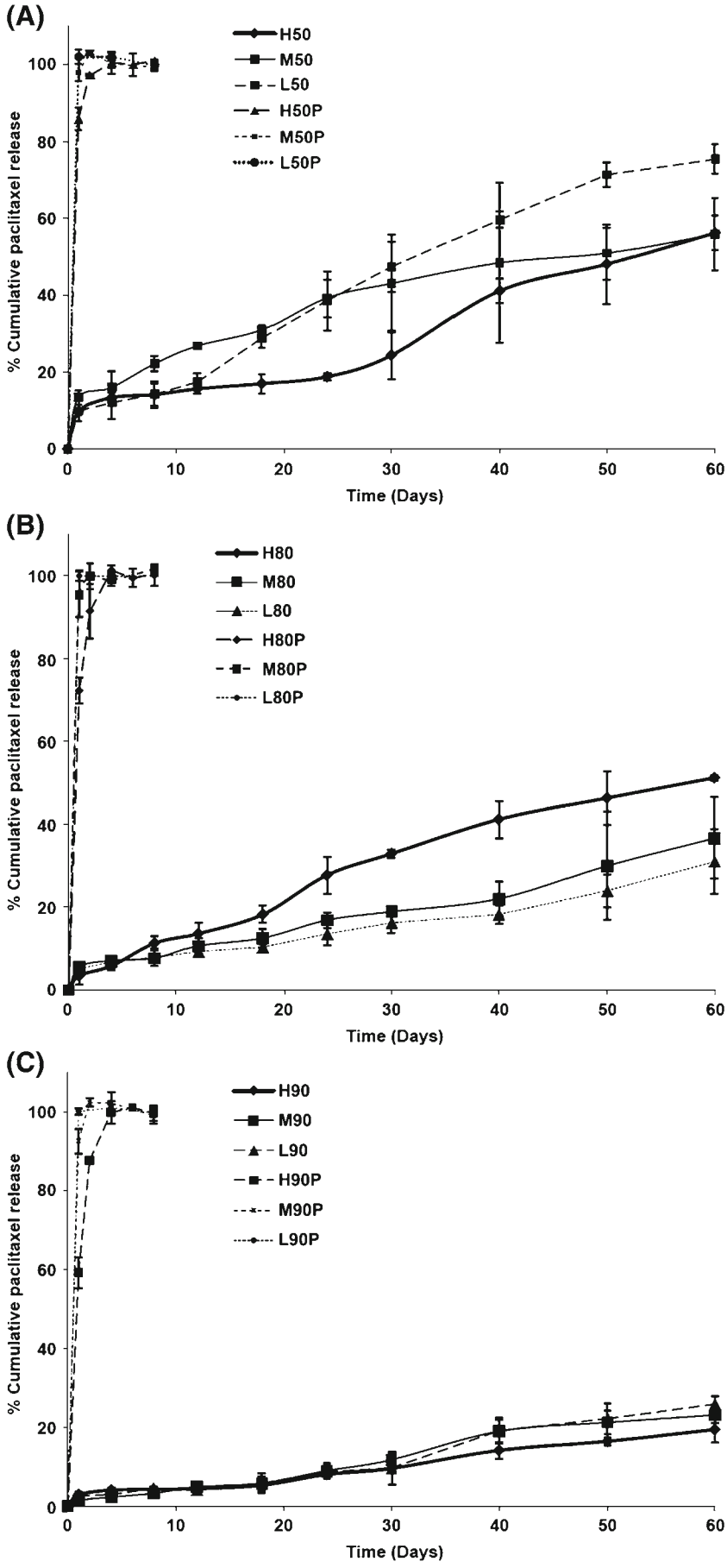


Fig. 4. **a** Effect of microsphere loading on the disintegration of the alginate beads in PBS solution at 37°C and 120 rpm at different gelation conditions. Each *data point* represents the average of triplicate samples and the *error bars* represent the standard deviation. **b** Effect of microsphere loading on the total Ca^{2+} ion content of the alginate beads after complete disintegration in PBS solution at 37°C and 120 rpm at different gelation conditions. Each *data point* represents the average of triplicate samples and the *error bars* represent the standard deviation.

Disintegration of the gel matrix due to dissociation of the Ca^{2+} cross-links resulting in the release of the microspheres was the controlling process in minimizing the initial burst release. Degradation of alginate has been reported to be very slow (39) and does not contribute to the release of the microspheres and the drug. The disintegration time of the bead is controlled by three major factors, namely gelation time, gelation bath concentration and microsphere loading. An increase in the first two factors increases the extent of cross-linking and thus the time needed to disintegrate the beads would be higher. But with an increase in microsphere loading, the diffusion of Ca^{2+} ions into the bead for cross-linking would be hindered due to a higher density of microspheres and lower porosity leading to a decrease in disintegration time. The disintegration study was performed with agitation (120 rpm) to shorten the duration of the experiment and thus does not adequately represent the actual scenario in a static environment.

For any given volume of the alginate-microsphere suspension dripped into the gelling bath, the cross-link density depends


(A)
H50
M50
L50
H50P
M50P
L50P
% Cumulative paclitaxel release
Time (Days)
(B)
H80
M80
L80
H80P
M80P
L80P
% Cumulative paclitaxel release
Time (Days)
(C)
H90
M90
L90
H90P
M90P
L90P
% Cumulative paclitaxel release
Time (Days)

◀ **Fig. 5.** *In vitro* release of paclitaxel from different formulations of the alginate beads. **a** 50% (*w*/*w*) microsphere-loaded beads and paclitaxel-loaded beads; **b** 80% (*w*/*w*) microsphere-loaded beads and paclitaxel-loaded beads; **c** 90% (*w*/*w*) microsphere-loaded beads and paclitaxel-loaded beads. Each *data point* represents the average of triplicate samples and the *error bars* represent the standard deviation. H90P, M90P, etc. refers to paclitaxel-loaded beads with equivalent amount of paclitaxel as compared to its microsphere loaded beads.

on volume of alginate available for cross-linking and the porosity of the bead. In case of 50% loaded beads, due to larger volume of alginate solution, the cross-link density could be relatively lower. With the 90% loaded beads, since the available alginate volume is lower, it could result in higher cross-linking density. Interestingly, the 80% loaded beads seem to possess the densest cross-linking probably due to the availability of an optimum volume of alginate and penetration of Ca^{2+} ions. These results reveal that the microsphere loading could be more dominant in controlling the disintegration; thus, in Fig. 4a, with an increase in microsphere loading, the disintegration time decreases.

The *in vitro* release of paclitaxel from the entrapped PLGA microspheres is controlled by three important factors. During the initial stages, the degree of cross-linking of the gel matrix and the density of microsphere packing in the matrix are important since they control the disintegration of the bead releasing the microspheres. Much later, the degradation of PLGA would dictate the release.

From the *in vitro* release results, it can be seen that all the formulations were able to release the drug sustainably for more than 60 days with minimum initial burst. It is a well-known fact that the initial burst is due to the surface bound drug that readily dislodges itself from the microsphere surface upon contact with the release medium. Polymer degradation could be safely ruled out because of the time scale of the initial burst. Diffusion of the drug from the core of the microsphere could also be slow since PLGA is hydrophobic and does not readily allow penetration of the aqueous release medium. To better understand the parameters affecting the initial burst, we compared our implants with free PLGA-paclitaxel microspheres reported by Xie *et al.* (25). Clearly, the data suggest that the free microspheres exhibit a high initial burst (around 30%) within 2 days followed by sustained release; the reason has been reported to be due to the surface-bound paclitaxel on the microspheres. In our case, due to the fact that not all the microspheres were exposed to the release medium and due to the presence of the alginate matrix barrier, the initial burst was avoided. Additionally, the data from Fig. 5a, b and c suggest that as the microsphere loading increased (which increased the density of microsphere packing), the initial burst decreased. Thus, the decrease in the initial burst with an increase in microsphere loading could be due to the denser packing of microspheres and reduced porosity that hindered the penetration of buffer into the bead. This seems to contradict the disintegration study results, where the increase in microsphere loading showed a decrease in disintegration time. However, it should be noted that the disintegration studies were performed with agitation at 120 rpm that aided in the dispersion of the microspheres, while the release studies were done statically (without agitation to avoid disturbing the microspheres).

The *in vitro* release was also affected by the extent of cross-linking in the gel. For 50 and 90% microsphere-loaded beads, the decrease in drug release rate with an increase in the extent of cross-linking as seen in Fig. 5a and c was evidently due to two important factors. First, the increase in cross-linking would result in stronger beads, thus prolonging the disintegration time. Second, with higher gel content the beads would swell, thus increasing the drug diffusion pathway resulting in reduced release rates. However, the 80% microsphere-loaded beads behaved contrastingly, suggesting that 80% loading could be

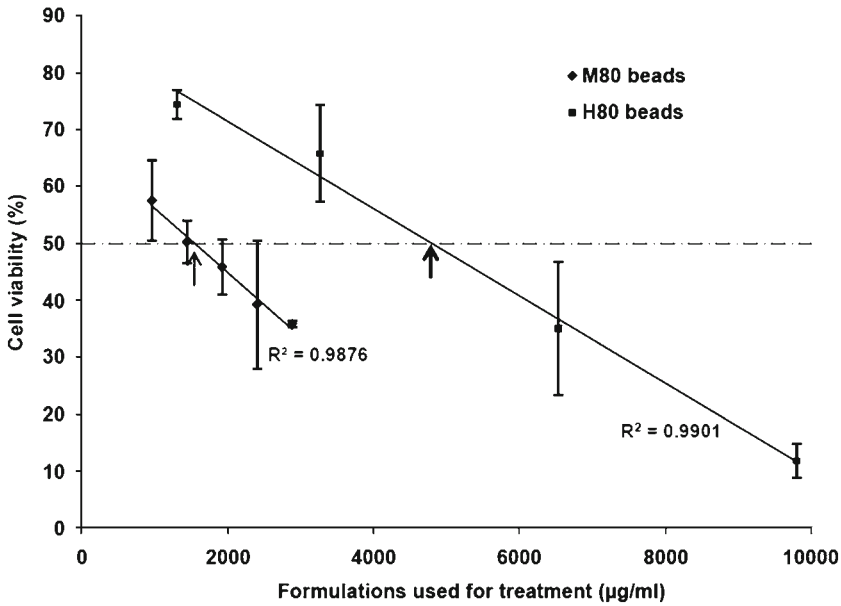


Fig. 6. Cell viability of C6 glioma cells treated with different formulations of the alginate beads for 3 days *in vitro*. The *dotted line* represents 50% cell viability and the arrows point to the approximate IC_{50} value of the respective formulations. Each *data point* represents the average of triplicate samples and the *error bars* represent the standard deviation.

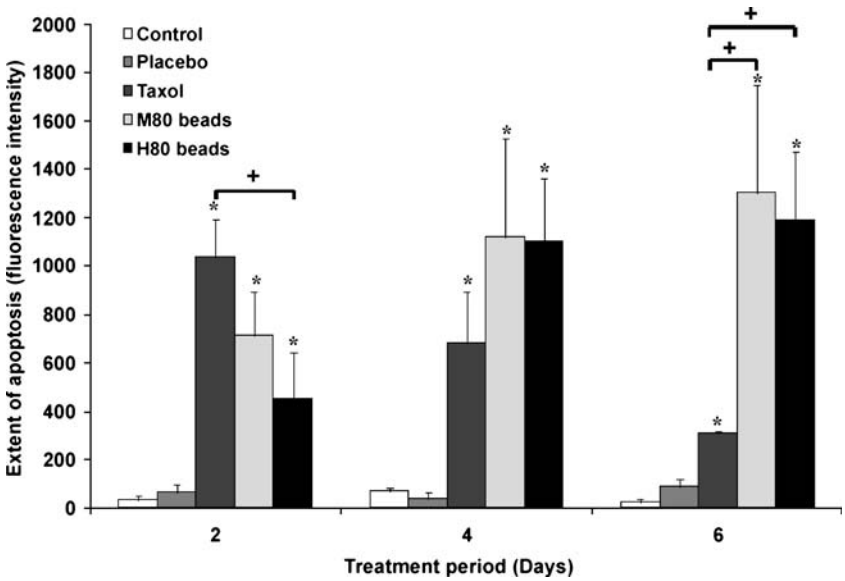


Fig. 7. Extent of apoptosis (measured by absorbance or fluorescence intensity) in C6 glioma cells treated with different groups after 2, 4 and 6 days *in vitro*. The Taxol group represents the equivalent amount of commercial Taxol® corresponding to paclitaxel release from M80 after 6 days (from *in vitro* release profiles). Each *data point* represents the average of three samples and the *error bars* represent the standard deviation. The symbols * represents a significant difference ($p<0.05$) in comparison to the control by ANOVA and + represents a significant difference ($p<0.05$) between the two samples by ANOVA.

optimum (in terms of swelling and density of packing); hence, with an increase in the degree of cross-linking, the release rate increased. Additionally, in the case of paclitaxel-loaded alginate beads, data from Fig. 5a, b and c reveal that due to rapid disintegration of the alginate matrix, paclitaxel was released rapidly and proved that the paclitaxel itself did not provide controlled release.

Furthermore, zero-order kinetics is dictated by the geometry of the carrier and is more or less independent of the drug properties or composition. Hence the near-zero order release profile was exhibited by the formulations that maintained the geometry for a longer period before complete disintegration. For the 50% microsphere loaded beads, the release deviates slightly from zero-order kinetics as compared

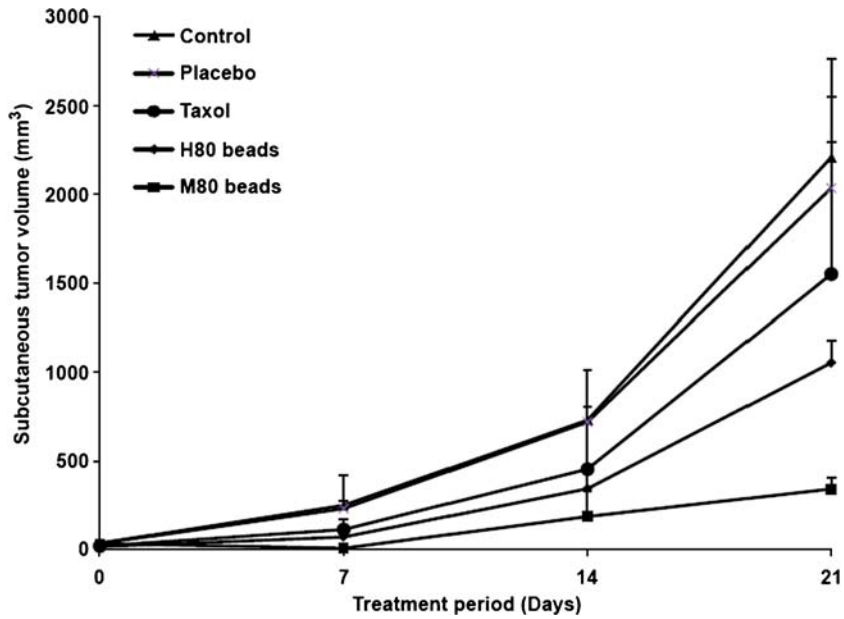


Fig. 8. *In vivo* subcutaneous C6 tumour volume profiles of mice treated with different groups for 21 days. (▲) Control; (X) Placebo control; (●) Taxol® control; (◆) H80 beads; (■) M80 beads. Each *data point* represents five samples ($n=5$ animals). Animals were sacrificed after 21 days. *Error bars* represent the standard deviation.

to the other two. Since the alginate hydrogel de-cross-links and dissolves in the release medium, the drug release then depends on PLGA degradation and drug diffusion into the release medium. But for 80 and 90% microsphere-loaded beads, due to denser packing, the disintegration is slower, thereby leading to a relatively near-zero order release. Owing to the release properties of the H80 and M80 formulations in terms of minimum initial burst, near-zero-order release kinetics and optimum microsphere loading, we chose to use them for the subsequent cell culture and animal studies.

Recently, a trans-lymphatic drug delivery system was reported that constituted a gelation sponge impregnated with PLGA-paclitaxel microspheres (40). However, these implants had very low microsphere loading (1% *w*/*w*), which means gelatine was used just to impregnate the drug-loaded microspheres and has no anti-cancer properties. Also, the spray drying process to fabricate the microspheres had very low yield (12.3%). But most importantly, the release profiles exhibited by PLGA-taxol, Spon-PLGA(taxol) and cross-linked Spon-PLGA (taxol) were not significantly different, thereby pointing to the fact that this previous publication did not demonstrate what parameters would affect the drug release from these implants. In comparison, the implants in the current study achieved very high microsphere loading (90% *w*/*w*) with high EHDA yield (40%) and possessed tuneable controlled drug release by varying different process parameters. Other paclitaxel-delivering microspheres such as PLGA microspheres (25), polilactofate microspheres (20) and PCPP-SA microsphere discs (15) display a significant initial burst relative to the microspheres entrapped in the gel matrix developed in this study. The initial burst caused either by loosely bound drug or fast degrading polymer has been reported to result in sporadic toxicity in experimental animals (15). In comparison, the microspheres entrapped in gel have a minimum initial burst, near constant drug release rate and prolonged sustainability and thus strong clinical implications. Moreover, this composite drug delivery system synergizes the advantages of microsphere containment (implantability) and sustained drug release.

When paclitaxel was administered as Taxol® for three days to inhibit C6 glioma cells *in vitro*, the IC_{50} value was shown to be approximately 30 μg/ml (25). Results from the cytotoxicity tests shown in Fig. 6 reveal that about 1,500 μg/ml of H80 and 4,800 μg/ml of M80 would be necessary to bring about the same cytotoxic effect or reach an IC_{50} paclitaxel concentration. These results highlight the fact that the formulations pose similar cytotoxicity as systemically administered Taxol®. The advantage is that H80 and M80 need to be implanted only once to see the effect, whereas Taxol® needs repeated systemic administration at high concentration. From the apoptosis results shown in Fig. 7, for H80 and M80 the sustainability of paclitaxel-induced apoptosis in C6 glioma cells and low cellular recovery were evident due to near-constant drug release. In contrast, Taxol®, which was cleared off after one day (simulating the drug clearance from the body), resulted in cellular recovery and reduced apoptosis after 6 days. This demonstrates that the beads have the potential to sustain therapeutic levels of paclitaxel and would prevent cellular recovery ensuring apoptosis.

From the subcutaneous tumour volume study, it was evident that alginate or PLGA in the implant did not cause any cytotoxicity to the tumour. Taxol® injection was initially effective in reducing the tumour volume, but later due to drug clearance, a fast increase in the tumour size was seen, which underscores the limitations of systemic drug delivery. However, the two formulations (H80 and M80) showed significantly smaller tumours in comparison to the blank control due to sustained paclitaxel release during the entire treatment period. This accentuates the fact that these implants release therapeutic concentrations of paclitaxel even after 21 days. Since the tumours treated in this study were solid tumours and due to the space constraint, the beads implanted were limited, we could not observe a higher reduction in tumour volume. Nevertheless, these gel-matrix entrapped microspheres could be potential local chemotherapy implants to treat malignant glioma and could be engineered specific to patient requirements.

CONCLUSIONS

A novel drug delivery implant comprising an alginate gel matrix entrapping paclitaxel-loaded PLGA microspheres was developed using the electrospray process in dripping mode that yielded highly monodisperse spherical gel beads with a uniform microsphere distribution and high loading efficiency. Thermal analysis on the implants confirmed that paclitaxel was in an amorphous state after the fabrication process that facilitates its diffusion from the matrix. The hydrogel bead disintegration study revealed that with increase in gelation bath concentration and gelation time, the beads disintegrate slowly, whereas with an increase in microsphere loading the disintegration time decreased. *In vitro* release of paclitaxel revealed that the implants could deliver the drug sustainably for more than 60 days at a near-constant rate and minimum initial burst. The *in vitro* IC_{50} study against C6 glioma cells showed that the beads could induce a similar cytotoxicity effect as Taxol® that overcomes the need for repeated drug administering. *In vitro* cellular apoptosis of C6 glioma cells suggested that the formulations could provide sustainable paclitaxel-induced apoptosis in comparison to Taxol®. Furthermore, subcutaneously implanted gel beads in mice could reduce the tumour size in nude mice more effectively than Taxol® and the control, thereby demonstrating their potential in local chemotherapy for glioma treatment. However, further *in vivo* intracranial studies focusing on paclitaxel biodistribution in the brain, survivability analysis and evaluation of tumour volume regression through bio-imaging are warranted.

ACKNOWLEDGEMENTS

The authors acknowledge the Biomedical Research Council (BMRC), A*STAR and the National University of Singapore for providing support under the grant numbers BMRC/07/1/21/19/508 and R279-000-257-731, respectively. The authors thank Alvin Yang, Ying Ying Chan, Jinghan Huang and Hui Yian Tan for technical assistance in the preparation of this manuscript and Dr. Yilong Fu for his assistance in the animal experiments.

REFERENCES

1. Petri B, Bootz A, Khalansky A, Hekmatara T, Muller R, Uhl R, *et al.* Chemotherapy of brain tumor using doxorubicin bound to surfactant-coated poly(butyl cyanoacrylate) nanoparticles: revisiting the role of surfactants. J Control Release. 2007;117:51–58.

2. Pradilla G, Wang PP, Gabikian P, Li K, Magee CA, Walter KA. Local intracerebral administration of paclitaxel with the Paclimer® delivery system: toxicity study in a canine model. J Neuro-Oncol. 2006;76:131–138.
3. Limentani SA, Asher A, Heafner M, Kim JW, Fraser R. A phase I trial of surgery, Gliadel wafer implantation, and immediate postoperative carboplatin in combination with radiation therapy for primary anaplastic astrocytoma or glioblastoma multiforme. J Neuro-Oncol. 2005;72:241–244.
4. von Eckardstein KL, Patt S, Kratzel C, Kiwit JCW, Reszka R. Local chemotherapy of F98 rat glioblastoma with paclitaxel and carboplatin embedded in liquid crystalline cubic phases. J Neuro-Oncol. 2005;72:209–215.
5. Brem H. Polymers to treat brain tumors. Biomaterials. 1990; 11:699–701.
6. Tamargo RJ, Myseros JS, Epstein JI, Yang MB, Chasin M, Brem H. Interstitial chemotherapy of the 9 L gliosarcoma: controlled release polymers for drug delivery in the brain. Cancer Res. 1993;53:329–333.
7. Brem H, Mahaley MS Jr, Vick NA, Black KL, Schold SC Jr, Burger PC, *et al.* Interstitial chemotherapy with drug polymer implants for the treatment of recurrent gliomas. J Neurosurg. 1991;74(3):441–446.
8. Brem H, Piantadosi S, Burger PC, Walker M, Selker R, Vick NA, *et al.* Placebo-controlled trial of safety and efficacy of intraoperative controlled delivery by biodegradable polymers of chemotherapy for recurrent gliomas. The Polymer-Brain Tumor Treatment Group. Lancet. 1995;345:1008–1012.
9. Menei P, Boisdron-Celle M, Croue A, Guy G, Benoit JP. Effect of stereotactic implantation of biodegradable 5-flurouracil-loaded microspheres in healthy and C6 glioma-bearing rats. Neurosurg. 1996;39:117–124.
10. Bellinzona M, Roser F, Matthies C, Samii M, Saini M. Biopolymer mediated suramin chemotherapy in the treatment of experimental brain tumors. Acta Oncol. 2004;43(3):259–263.
11. Hsu W, Lesniak MS, Tyler B, Brem H. Local delivery of interleukin-2 and adriamycin is synergistic in the treatment of experimental malignant glioma. J Neurooncol. 2005;74:135–140.
12. Chen W, He J, Olson JJ, Lu DR. Carboplatin-loaded PLGA microspheres for intracerebral implantation: *in vivo* characterization. Drug Deliv. 1997;4(4):301–3111.
13. Lesniak MS, Upadhyay U, Goodwin R, Tyler B, Brem H. Local delivery of doxorubicin for the treatment of malignant brain tumors in rats. Anticancer Res. 2005;28(6B):3825–3831.
14. Lu W, Wan J, Zhang Q, She Z, Jiang X. Aclarubicin-loaded cationic-albumin pegylated nanoparticle for glioma chemotherapy in rats. Int J Cancer. 2007;120:420–431.
15. Walter KA, Cahen MA, Gur A, Tyler B, Hilton J, Colvin OM, *et al.* Interstitial taxol delivered from a biodegradable polymer implant against experimental malignant glioma. Cancer Res. 1994;54:2207–2212.
16. Prados MD, Schold SC, Spence AM, Berger MS, Mcallister LD, Mehta MP, *et al.* Phase II study of paclitaxel in patients with recurrent malignant glioma. J Clin Oncol. 1996;14:2316–2321.
17. Chamberlain MC, Kormanik P. Salvage chemotherapy with taxol for recurrent anaplastic astrocytoma. J Neurooncol. 1999;43: 269–276.
18. Chang SM, Kuhn JG, Robins HI, Schol SC Jr, Spence AM, Berger MS, *et al.* A phase II study of paclitaxel in patients with recurrent malignant glioma using different doses depending upon the concomitant use of anticonvulsants: a North American Brain Tumor Consortium report. Cancer. 2001;91:417–422.
19. Glantz MJ, Choy H, Kearns CM, Mills PC, Wahlberg LU, Zuhowski EG, *et al.* Paclitaxel disposition in plasma and central nervous systems of humans and rats with brain tumors. J Natl Cancer Inst (Bethesda). 1995;87:1077–1081.
20. Li KW, Dang W, Tyler BM, Troiano G, Tihan T, Brem H, *et al.* Polilactofate microspheres for paclitaxel delivery to central nervous system malignancies. Clin Cancer Res. 2003;9: 3441–3447.
21. Fung LK, Ewend MG, Sills A, Sipos EP, Thompson R, Watts M, *et al.* Pharmacokinetics of interstitial delivery of carmustine, 4-hydroperoxycyclophosphamide, and paclitaxel from a biodegradable polymer implant in the monkey brain. Cancer Res. 1998;58:672–684.
22. Vogelhuber W, Sprub T, Bernhardt G, Buschauer A, Gopferich A. Efficacy of BCNU and paclitaxel loaded subcutaneous implants in the interstitial chemotherapy of U-87 MG human glioblastoma xenografts. Int J Pharm. 2002;238:111–121.
23. Wang J, Ng CW, Win KY, Shoemakers P, Lee TKY, Feng SS, *et al.* Release of paclitaxel from polylactide-co-glycolide (PLGA) microparticles and discs under irradiation. J Microencapsul. 2003;20(3):317–327.
24. Ranganath SH, Wang CH. Biodegradable microfiber implants delivering paclitaxel for post-surgical chemotherapy against malignant glioma. Biomaterials. 2008;29:2996–3003.
25. Xie J, Marijnissen JCM, Wang CH. Microparticles developed by electrohydrodynamic atomization for the local delivery of anticancer drug to treat C6 glioma *in vitro*. Biomaterials. 2006;27:3321–3332.
26. Elkharraz K, Faisant N, Guse C, Siepmann F, Arica-Yegin B, Oger JM, *et al.* Paclitaxel-loaded microparticles and implants for the treatment of brain cancer: Preparation and physicochemical characterization. Int J Pharm. 2006;314:127–136.
27. Xie J, Wang CH. Self-assembled biodegradable nanoparticles developed by direct dialysis for the delivery of paclitaxel. Pharma Res. 2005;22(12):2079–2090.
28. Geurin C, Olivi A, Weingart JD, Lawson HC, Brem H. Recent advances in brain tumor therapy: local intracerebral drug delivery by polymers. Invest New Drugs. 2004;22:27–73.
29. Joki T, Machluf M, Atala A, Zhu J, Seyfried NT, Dunn IF, *et al.* Continuous release of endostatin from microencapsulated engineered cells for tumor therapy. Nat Biotechnol. 2001;19:35–39.
30. Fantazzini P, Garavaglia C, Gomez S, Toffanin R, Vittur F. Chondrocyte-alginate bioconstructs: an nuclear magnetic resonance relaxation study. J Biomed Mater Res A. 2007;83A (2):345–353.
31. Rokstad AM, Strand B, Rian K, Steinkjer B, Kulseng B, Skjak-Braek G, *et al.* Evaluation of different types of alginate microcapsules as bioreactors for producing endostatin. Cell Transplant. 2003;12:351–364.
32. Chen H, Ouyang W, Jones M, Metz T, Martoni C, Haque T, *et al.* Preparation and characterization of novel polymeric microcapsules for live cell encapsulation and therapy. Cell Biochem Biophys. 2007;47:159–167.
33. Xie J, Wang CH. Electrospray in the dripping mode for cell microencapsulation. J Colloid Interf Sci. 2007;312:247–255.
34. Soares JP, Santos JE, Chierice GO, Cavalheiro ETG. Thermal behavior of alginic acid and its sodium salt. Ecletica Quimica. 2004;29(2):53–56.
35. Xie J, Lim LK, Phua Y, Hua J, Wang CH. Electrohydrodynamic atomization for biodegradable polymeric particle production. J Colloid Interf Sci. 2006;302:103–113.
36. Dubernet C. Thermoanalysis of microspheres. Thermochimica Acta. 1995;248:259–269.
37. Donth EJ. Relaxation and thermodynamics in polymers. Glass transition. Berlin: Akademie Verlag; 1992.
38. Freiberg S, Zhu XX. Polymer microspheres for controlled drug release. Int J Pharm. 2004;282:1–18.
39. Boontheekul T, Kong HJ, Mooney DJ. Controlling alginate gel degradation utilizing partial oxidation and bimodal molecular weight distribution. Biomaterials. 2005;26:2455–2465.
40. Liu J, Meisner D, Kwong E, Wu XY, Johnston MR. A novel trans-lymphatic drug delivery system: implantable gelatin sponge impregnated with PLGA-paclitaxel microspheres. Biomaterials. 2007;28:3236–3244.