



Resorbable polymer microchips releasing BCNU inhibit tumor growth in the rat 9L flank model

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Abstract

Sustained local delivery of single agents and controlled delivery of multiple chemotherapeutic agents are sought for the treatment of brain cancer. A resorbable, multi-reservoir polymer microchip drug delivery system has been tested against a tumor model. The microchip reservoirs were loaded with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). BCNU was more stable at 37 °C within the microchip compared to a uniformly impregnated polymeric wafer (70% intact drug vs. 38%, at 48h). The half-life of the intact free drug in the microchip was 11 days, which is a marked enhancement compared to its half-life in normal saline and 10% ethanol (7 and 10 min, respectively) [P. Tepe, S.J. Hassenbusch, R. Benoit, J.H. Anderson, BCNU stability as a function of ethanol concentration and temperature, *J. Neurooncol.* 10 (1991) 121–127; P. Kari, W.R. McConnell, J.M. Finkel, D.L. Hill, Distribution of Bratton–Marshall-positive material in mice following intravenous injections of nitrosoureas, *Cancer Chemother. Pharmacol.* 4 (1980) 243–248]. A syngeneic Fischer 344 9L gliosarcoma rat model was used to study the tumoricidal efficacy of BCNU delivery from the microchip or homogeneous polymer wafer. A dose-dependent decrease in tumor size was found for 0.17, 0.67, and 1.24 mg BCNU-microchips. Tumors treated with 1.24 mg BCNU-microchips showed significant tumor reduction ($p=0.001$) compared to empty control microchips at two weeks. The treatment showed similar efficacy to a polymer wafer with the same dosage. The microchip reservoir array may enable delivery of multiple drugs with independent release kinetics and formulations.

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1. Introduction

Brain cancer is still associated with poor prognosis with average survival of less than one year when surgical resection is combined with radiation and chemotherapy [1–6]. Research is ongoing for sustained intracranial delivery of single agents as well as controlled delivery of combination chemotherapeutics via systemic injection, polymers, pumps, and convection enhanced delivery [7–15]. Local controlled treatment of brain tumors with a homogeneous biodegradable polymer, Gliadel[®] wafer, has been particularly successful in improving survival

[16–19]. However, only the non-degradable and degradable microchip platform is able to deliver multiple agents in distinct rounds from a single device [20,21].

The microchip system has a unique array of reservoirs, which enables features not possible with other drug delivery technologies, namely 1) delivery of multiple drugs, 2) independent modulation of drug release profiles, and 3) administration of several drug formulations in a single implantation procedure. The microchip has the potential to deliver subsequent doses of therapeutic agents at a later time to prevent or to treat recurrence of neoplasm. Local delivery by microchips can increase the concentration of chemotherapeutic agent at the tumor site while reducing systemic toxicity. This spatial control is particularly beneficial for brain tumors because they rarely spread outside the brain [22].

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Delivery of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), a commonly used brain cancer chemotherapeutic agent, from a non-degradable microchip was shown to have a dose-dependent inhibiting effect on tumor growth with an efficacy comparable to a subcutaneous injection of an equal dose of the drug [23]. This technology was validated in a similar device for long-term repeat dosing by the triggered release of leuprolide (MW 1205) for up to six months in beagle dogs [24]. These devices are not self-contained, however, and require a transcutaneous or wireless interface. A passively actuated, biodegradable controlled release system can avoid the complications of these components in select applications. Our group has previously reported on the behavior of biodegradable microchips that contain an array of drug reservoirs, each sealed by a thin membrane that degrades according to a predetermined schedule to release drug [20]. The substrate hydrolyzes after all the doses have been released. Herein, we compare the efficacy of this new device to an existing clinical therapy.

Our studies show that the use of a biodegradable polymer-based microchip containing BCNU can significantly limit tumor growth of 9L gliosarcoma challenge in a rat model. The performance of this new drug delivery device is comparable to a polymer wafer uniformly impregnated with BCNU, similar to the FDA-approved Gliadel® therapy. This study provides the foundation for potentially significant improvements in cancer therapies by means of precise local administration of single or multiple drugs in a predetermined manner from a self-contained degradable microchip.

2. Materials and methods

2.1. Microchip fabrication and packaging

The polymer microchips (Fig. 1) were fabricated as previously reported [20]. Briefly, purified poly(L-lactic acid) (PLA, MW 194,000; Medisorb 100L; Alkermes, Cambridge, MA) tablets were compression-molded against a patterned aluminum die and followed by polishing to form the reservoirs. A 12% v/v solution of poly(lactic-co-glycolic) acid (PLGA, MW 11,000; Medisorb 50:50 DL 2A; Alkermes) in 1,1,3,3-hexafluoro-2-propanol was injected to form a membrane inside each reservoir. The microchip was then dried in a vacuum oven

for 2 days at 80 °C. A mixture of BCNU (Bristol-Myers Squibb Co., Evansville, IN) and ¹⁴C-BCNU (Moravak Biochemicals, Brea, CA) was injected into the microchips using a micro-injector (UltraMicroPump II; World Precision Instruments, Sarasota, FL). Each microchip was loaded with a final mass of 0.17, 0.67, or 1.24 mg of BCNU. The final microchip dimensions are approximately 10 mm in diameter and 1 mm in thickness.

Control devices of polymer wafers fabricated from polycarboxyphenoxypropane:sebacic acid (pCPP:SA) were incorporated with 1.24 mg BCNU. Briefly, pCPP:SA in a 20:80 formulation (Guilford Pharmaceuticals, Inc., Baltimore, MD) and BCNU were dissolved together in dichloromethane followed by vacuum desiccation. Polymer disks were then formed by compression molding. Polymers were stored at –80 °C prior to implantation. A detailed protocol is described elsewhere [25].

2.2. BCNU stability and *in vitro* release

The stability of BCNU within the multi-reservoir microchips and the uniformly impregnated wafers was studied. BCNU was microinjected into the polymer microchip and then sealed on both sides with pressure sensitive adhesive. Similarly, BCNU was incorporated into the wafers as described above. The devices were incubated at 37 °C in a capped glass vial for various times. BCNU was extracted from the microchip for 3 min in 3 ml 20:80 ethanol:water. BCNU was extracted from wafers by dissolving with 99.7:0.3 dichloromethane:acetonitrile. The extracted solutions were immediately assayed using the Bratton–Marshall assay.

Intact BCNU was quantified *via* a colorimetric assay using the Bratton–Marshall method [26]. The assay is sensitive to only the whole BCNU molecule. One part of sulfanilamide solution (5 mg/ml in 2 M HCl) was added to two parts of each BCNU solution to be tested, sealed, and heated in a water bath at 50 °C for 45 min. The samples were chilled on ice to room temperature, and 200 µl of the resulting solution and 10 µl of the Bratton–Marshall reagent (3 mg/ml of *N*-(1-naphthyl)ethylene-diamine in deionized water) were transferred to a 96 well plate and the absorbance was read 5 min later at $\lambda=540$ nm. The absorbance followed a linear relationship with BCNU concentration from 0 to 50 µg/ml. Samples were diluted in appropriate solvent before performing the assay to be within the linear range as necessary. The percent of intact BCNU was calculated by dividing the amount of intact BCNU as determined from the Bratton–Marshall assay by the initial amount of BCNU as estimated by radioactive-labeling or mass. All stability measurements were performed in triplicate, and the average value was reported.

Microchips were filled with BCNU, sealed and placed into 3 ml PBS at 37 °C to determine *in vitro* release kinetics. Periodically, the PBS was removed and replaced with fresh PBS. The radioactivity of the release medium was measured using a liquid scintillation counter (Packard TriCarb 2200CA; Perkin Elmer Life Sciences, Wellesley, MA) using ScintiSafe Plus 50% scintillation fluid (Fischer Chemicals, Fairlawn, NJ). The

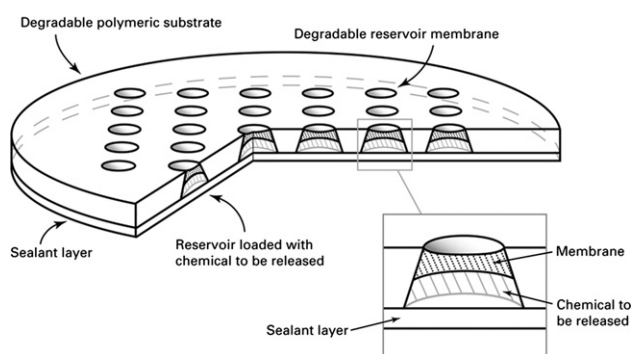


Fig. 1. Schematic showing the polymer microchip. (Reprinted by permission from Macmillan Publishers Ltd: Nature Materials, 2:767–772, 2003).

radioactivity per timepoint was divided by the total radioactivity loaded and integrated over time to describe the cumulative release profile. The results are reported as the median with error bars representing the 25th and 75th percentiles.

2.3. Animal experiments

2.3.1. Orthotopic tumor model

Two separate experiments were performed to evaluate the efficacy of BCNU when delivered *via* the biodegradable microchip. An initial efficacy study examined two dosages of BCNU (0.16 and 0.67 mg) released from the microchip, and a second experiment included a higher dosage of BCNU (1.24 mg).

Forty-one F344 Fischer rats underwent implantation of 9L gliosarcoma in the left flank in the first study. The rats were divided into six groups and received one of the following treatments directly beneath the tumor at ten days: 1) a PLGA microchip containing 0.16 mg BCNU ($n=8$); 2) a PLGA microchip containing 0.67 mg BCNU ($n=8$); 3) an injection of 0.16 mg BCNU ($n=4$); 4) an injection of 0.67 mg BCNU ($n=4$); 5) an empty microchip ($n=8$); and 6) no treatment ($n=9$).

Forty F344 rats underwent 9L gliosarcoma flank implantation in the second study. The rats were randomly divided into five groups and received one of the following treatments: 1) a PLGA microchip containing 1.24 mg BCNU ($n=8$); 2) a pCPP: SA wafer containing 1.24 mg BCNU ($n=8$); 3) an empty PLGA microchip ($n=8$); and 4) empty pCPP:SA wafer ($n=8$); and 5) no treatment ($n=8$).

The microchips and wafers were sterilized by a gamma radiation dose of 480 Gy from a Cesium-137 source (Mark1-68 irradiator; JLS Shepherd, Glendale, CA) prior to implantation. A 2 mm³ piece of solid 9L glioma, an experimental gliosarcoma syngeneic to the Fischer 344 rat (Brain Tumor Research Center; University of California, San Francisco), was implanted into the left flank of experimental animals as described previously [25]. Initial tumor sizes were measured at ten days after tumor implantation, and animals were randomized to experimental groups. The treatment was then placed between the flank muscle and the tumor under aseptic conditions. Microchips were placed with the drug-eluting side facing the tumor. The incision was then closed with sterile autoclips. Tumors were measured three times a week with calipers, and the tumor volume was approximated as an ellipsoid (Eq. (1)) Median tumor volumes *vs.* time were plotted and the delay of tumor growth *vs.* untreated controls was used as the measure of efficacy. The asymmetric, two-tailed Mann–Whitney *U* test was used to compare tumor sizes at various timepoints. *p*-values < 0.05 were considered significant.

$$V_{\text{tumor}} = \text{Length} \times \text{Width} \times \text{Height} \times \pi/6 \quad (1)$$

2.3.2. Kinetics of drug release

Some animals were individually housed in metabolic cages (Nalgene model # 650-0100, Braintree Scientific Inc., Braintree, MA) to assess drug release by radioactive excretion. Radioactivity measurements were conducted on urine after determining that no radioactivity was detected in the fecal samples. One ml urine samples were taken twice daily and

mixed with 5 ml scintillation gel (Ready Gel, Beckman Coulter Inc., Fullerton, CA) in a 7 ml scintillation vial. The radioactivity was measured using a liquid scintillation counter (Beckman Coulter Inc., Fullerton, CA). The amount of radioactivity (nCi) was multiplied by the total volume of urine collected at that timepoint, and integrated over time to produce the drug recovery curves. Selected explanted microchips were sonicated in methanol and then allowed to soak for 1.5 h to extract the remaining radioactive BCNU. The radioactivity was quantified as above.

2.3.3. Animals

Fischer 344 rats (female, 150–200 g) were obtained from Charles River Laboratories (Wilmington, MA). All animals implanted with radioactive BCNU and two animals from the other groups (serving as controls) were individually housed in metabolic cages, which separates feces and urine into tubes outside the cage. All other animals were housed in standard cages. All animals were given standard rat chow and water *ad libitum*. Animals housed in metabolic cages were given sugar water (4 tsp/500 ml), which encouraged the animals to drink more and allowed more frequent collection of urine samples. Animals were checked daily for physical or behavioral evidence of toxicity, such as decreased alertness, impaired grooming, or gait disturbances. Animals were housed and treated in accordance with the policies and principles of laboratory care of the Johns Hopkins University School of Medicine Animal Care and Use Committee and the MIT Committee on Animal Care.

3. Results

3.1. Stability of intact BCNU and *in vitro* release

The half-life of BCNU within the microchip measured by the Bratton–Marshall assay at 37 °C was 11 days (Fig. 2). The stability of BCNU in the microchip and wafer was directly compared in a separate experiment. The microchips contained 74% intact BCNU in the microchip and 62% intact BCNU in the wafer after incubation at 37 °C for 24 h when compared to the initial BCNU content. These values drop to 70% and 38% respectively at 48 h.

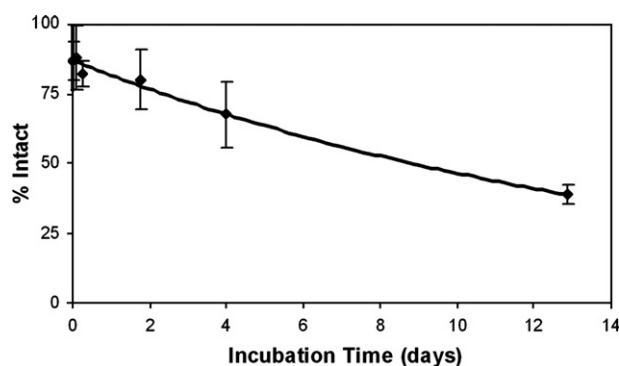


Fig. 2. Intact BCNU in the sealed microchip. The mean ± SD is plotted ($n=3$). BCNU in the polymer microchip has a half-life at 37 °C of 11 days.

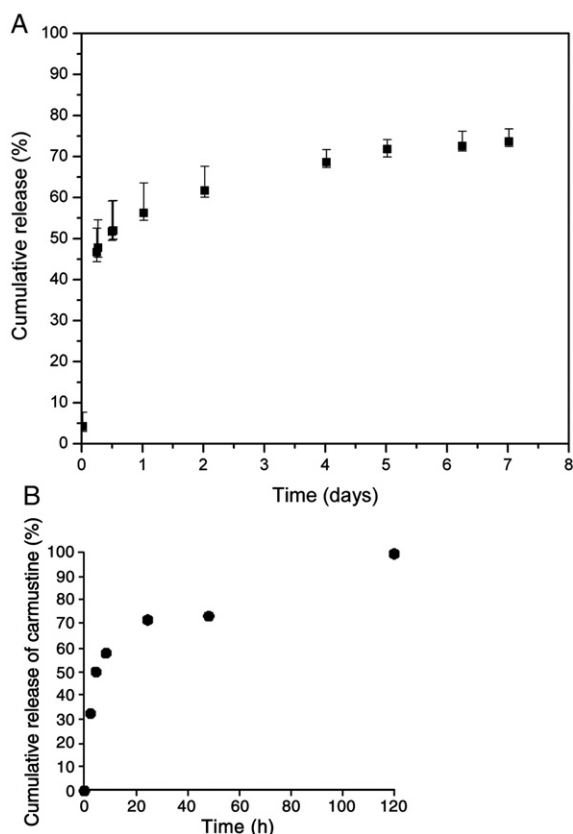


Fig. 3. A) Cumulative release of BCNU from microchip into PBS at 37 °C ($n=6$). The median value is reported with the error bars representing the 25th and 75th percentiles. B) Release of BCNU from pCPP:SA wafers implanted into rat brains. (Reprinted by permission from Elsevier: J Control Release, 42:83–92, 1996).

BCNU was released from the microchips in two distinct phases (Fig. 3A). Approximately 50% of the BCNU from microchips containing 1.24 mg of BCNU was released during

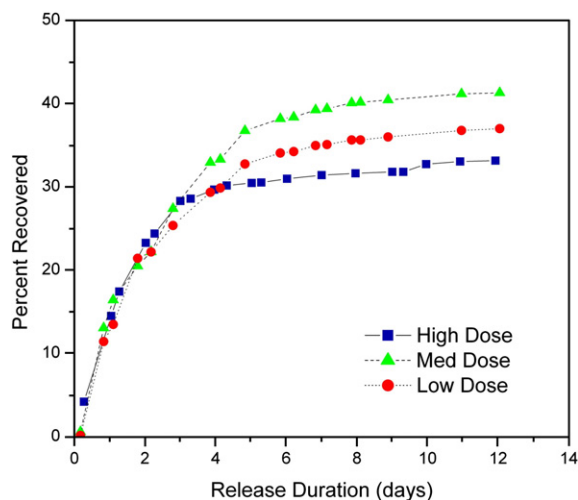


Fig. 4. BCNU release kinetics obtained from quantification of ^{14}C -BCNU excreted in urine after device implantation, normalized by loading amount. There is no statistical difference among three different doses administered (0.17, 0.67, and 1.24 mg). No radioactivity is excreted from the negative controls (not shown).

Table 1

Urinary recovery of ^{14}C from rats with polymer microchips by day 12 ($n=8$ for each group)

BCNU dosage (mg)	Urinary recovery of ^{14}C (% of initial loading)
0.16 ^a	37 ± 10
0.67 ^a	39 ± 13
1.24 ^a	33 ± 8
1.24 ^b	44 ± 4

^a Data from dose escalation study.

^b Data from comparison of microchip vs. wafer delivery.

the initial 12 h. This initial burst was followed by a much slower release of the majority of the remaining contents during the next 5 days.

3.2. *In vivo* release of BCNU

Microchips containing 0.17, 0.67 and 1.24 mg were implanted into the flank of 9L tumor-bearing rats. Animals receiving either no treatment or empty microchips were used as negative controls. The latter group was included to account for possible effects due to the polymer platform itself. The urine from all animals in treatment groups ($n=8$ or 9) and selected animals in negative control groups (both $n=2$) was collected and the BCNU recovered in the urine was integrated over time (Fig. 4 and Table 1). The two control groups showed no radioactivity as expected. The release kinetics from the three BCNU-microchip groups were similar, reaching a plateau around day 6. An average of 37, 39, and 33% of the loaded radioactivity was recovered in the urine at 12 days after microchip implantation from the 0.17, 0.67, and 1.24 mg dose microchips, respectively. The urinary recovery shows that BCNU was released immediately upon implantation as was expected from the *in vitro* data.

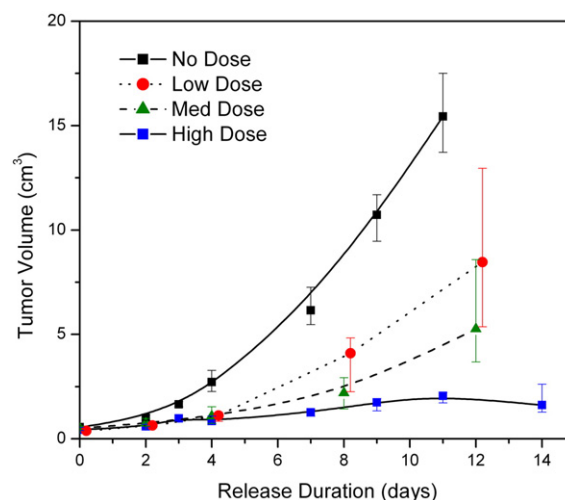


Fig. 5. Dose response to BCNU from the polymer microchip. The high and medium doses showed statistical significance from the negative controls ($p=0.001$ and 0.016 respectively). The tumor reduction from the low dose was not statistically significant ($p=0.074$). The median values are plotted with error bars representing the 25th and 75th percentiles. Values for the medium dose were offset for clarity.

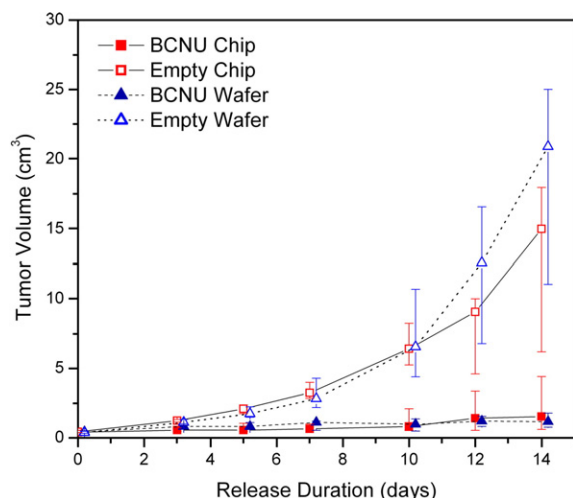


Fig. 6. Tumor response to the same loaded dose of BCNU from the polymer microchip and the polyanhydride wafer. Both microchip and wafer showed tumor reduction compared to their empty control ($p=0.032$ and $p=0.001$ respectively) and were not statistically different from each other ($p=0.156$). The median values are plotted with error bars representing the 25th and 75th percentiles. Values were offset for clarity.

The amount of radioactivity recovered from the microchips after sacrifice was approximately 5% of the initial loading for the 0.17 mg dose microchips, and 10% for the 0.67 mg dose microchips. Data for the residual radioactivity in the 1.24 mg microchips were not available, but is presumed to be of the same order. The unaccounted radioactivity may be distributed in various tissues, feces, or expired as CO_2 [2].

3.3. Microchip delivery of BCNU inhibits 9L tumor growth

A study was performed to examine if the BCNU delivered from the passive microchip retained its tumoricidal activity and to observe the effect of this delivery vehicle on tumor growth. The efficacy of three different doses of BCNU from the microchip was tested against the 9L gliosarcoma. Then, a side-by-side comparison of one dose in the microchip was tested against the wafer formulation with the same amount of BCNU.

The resulting tumor growth data are shown in Fig. 5 for the different treatment groups. No treatment, empty microchips, and empty wafers were used as negative controls to account for possible effects on the tumor growth or growth measurement due to the polymer delivery platform. Overall, the BCNU treatment groups showed a smaller median tumor size than the control groups that received either empty microchips or no treatment. The lowest doses (0.16 mg) showed no significant difference in tumor size from the empty control microchips ($p=0.074$). The difference was significant, however, for the 0.67 mg dose ($p=0.016$), and for the 1.24 mg dose ($p=0.001$), which showed markedly reduced tumor size. Median tumor sizes in treatment groups were compared to empty microchip control at 12 days. Tumors in the low and middle dose groups were 43% and 65% smaller. The tumors receiving high dose microchips were 87% smaller than untreated tumors. The

1.24 mg dose was considered the minimum effective dose and selected for the subsequent study.

The efficacy of BCNU from the microchip was directly compared to that from a uniformly impregnated wafer (Fig. 6). The minimum effective dose of 1.24 mg was microinjected into the polymer microchip or uniformly impregnated throughout the polymer wafers. Negative control groups included blank microchips and blank wafers. The negative control groups showed that the tumor grew by day 14 after microchip implantation to a median of 15.0 and 20.9 cm^3 for the blank microchip and the blank wafer, respectively. In contrast, the tumors in the microchip treatment group grew only to a median of 1.5 cm^3 and the wafer treatment group to 1.2 cm^3 . The microchip and the wafer produced significant tumor reduction compared to their respective empty microchip controls ($p=0.032$ and $p=0.001$ respectively). There was no significance in the difference in tumor sizes between the two treatment groups ($p=0.156$). These results show that the release of BCNU from the microchip is as effective as the release from the wafer.

4. Discussion

This study demonstrates the *in vivo* efficacy of a self-contained degradable microchip that provides precise spatial administration of chemotherapeutics. Specifically, the microchip was used to deliver BCNU against a tumor model and showed a significant reduction in tumor growth (Fig. 5). Tumors treated with the highest loaded microchip showed marked diminished growth compared to empty control microchips ($p=0.001$). The microchip was found comparable to that of the pCPP:SA wafer in release profile (Fig. 3B) and efficacy in reducing tumor size. This demonstrates the first validation of proper microchip operation and efficacy in an *in vivo* model. Previously, only the *in vitro* activity of heparin released from the polymer microchip had been quantified [20], but no *in vivo* results had been demonstrated. A dose-dependent behavior was observed, with the highest dosage of BCNU delivering the strongest inhibiting effect on the tumor growth.

The release of BCNU from the microchip starts on day 1 and continues for nearly 2 weeks (Fig. 3). BCNU is released quickly from the polymer devices after implantation. This is necessary considering the hydrolytic instability of BCNU. The release profile of BCNU from the microchip is comparable to the release from a uniformly impregnated pCPP:SA wafer (Fig. 3B), which when released in rat brains at 37 °C occurred over a period of approximately 5 days [27]. Dang et al., measured the amount of BCNU recovered from pCPP:SA wafer explants and subtracted that amount from the initial loading to calculate the amount released.

High-pressure liquid chromatography (HPLC) and a colorimetric assay using the Bratton–Marshall reagent can both be used to quantify BCNU [2,26–28]. The Bratton–Marshall method was preferred for this study to quantify the *intact* BCNU molecule due to its ability to process larger numbers of samples in parallel. Serial measurement of sample as in HPLC was considered undesirable as BCNU has a short half-life in aqueous solution. BCNU was shown to have a half-life of 11 days when contained

within the microchip at 37 °C (Fig. 2). This represents a substantial extension of the half-life compared to the free drug molecule in normal saline and 10% ethanol ($t_{1/2}$ = 7 and 10 min, respectively) [1,2]. BCNU is also more stable within the microchip compared to the wafer. At 48 h, the microchip contains nearly twice as much intact BCNU as the wafer (70% and 38% respectively). BCNU acts by alkylating DNA, and it is known that its metabolites also have tumoricidal activity. Free BCNU powder dissolved in cell culture medium has been shown to be cytotoxic to XF498 cells after incubation for up to 12 h, at which time the intact BCNU molecule is unmeasurable [28]. The microchip can release BCNU or its metabolically active degradation products, but for long-term, or repeat release, the ability of the microchip to enhance stability of BCNU may also translate to increased effectiveness of other unstable molecules, especially with the use of excipients or stabilizers.

BCNU is reported here to begin release from the 11 kDa PLGA membrane within minutes of incubation in PBS. Previous work from our group has reported release of dextran (MW 70,000), human growth hormone (MW 21,500), or heparin (MW 6000–20,000) from the microchip with the same 11 kDa membrane from between 6 and 17 days in PBS at temperatures from 28 to 33 °C [20]. We believe this earlier release may have occurred for several reasons. All release profiles reported here were performed in a controlled 37 °C incubator. BCNU is small (MW 214.1) and lipophilic compared to the model compounds used previously. It diffuses through the PLGA membrane and may facilitate swelling and opening of the membrane. This is supported by the observation that the membranes showed a visible swelling as early as a few hours after the BCNU-loaded microchips were submersed in PBS at 37 °C, whereas swelling of the membranes in PBS at 28–33 °C was observed later to coincide with the released of dextran [29].

The urinary recovery shows BCNU was released on day 1 of implantation (Fig. 4) as was expected from the *in vitro* data. Approximately one third of the initial loading was recovered by day 12 with a majority being released by day 5 (Table 1). The rate of recovery, however, is slower than the rate of release *in vitro*, attributed to the pharmacokinetics of rodent renal clearance. The total urinary recovery is lower than values reported by other groups (78%, 55%) [30,31], which used different style metabolic cages, animal models, and administration locations. The recovery reported here is consistent with the data from our previous injected control groups and release kinetics measurements [21].

This study shows that the release of BCNU from the microchip is as effective as the release from a clinically approved homogenous polymer matrix. The pCCP:SA wafer with BCNU is widely used to treat brain tumor patients and is commercially available as the Gliadel® wafer [16,17]. Gliadel® is FDA-approved for the treatment of recurrent glioblastoma multiforme and newly diagnosed, high grade malignant gliomas. Six to eight wafers are placed in the cavity that remains after tumor resection, and the BCNU is released as the copolymer degrades. Gliadel® is now used routinely to improve survival in patients with malignant gliomas, but it is capable of delivering only one compound [17–19].

It is expected that delivery of a combination of synergistically acting chemotherapeutic agents can reduce tumor size with fewer side effects beyond that achieved by a monotherapy. Drug resistance and genetic profiling information [32] can be combined with the microchip to customize treatment of tumors. Future studies should exploit combination therapies and the synergistic action of potent combinations using additional anticancer drugs, such as temozolomide [33–35] to achieve greater cytotoxicity with lower drug amounts. The use of solid dosage forms allows the microchip to potentially achieve smaller device dimensions and higher drug:polymer ratios than polymer matrix formulations. An optimized reservoir shape and configuration can deliver the same amount of drug with one tenth less polymer. This represents a solid foundation for improvement in treatments of diseases that can benefit from the precise administration of multiple drug cocktails from a self-contained degradable microchip.

5. Conclusion

BCNU delivered from the polymer microchip inhibited the tumor growth of the 9L glioma in rats. The inhibiting effect on tumor growth was dose-dependent. These results are the first demonstration of *in vivo* efficacy of a polymer microchip brain cancer therapy. The efficacy validation of this platform as well as important dose response information indicates further evaluation of combination chemotherapy using a polymer platform with multiple reservoirs.

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