Abstract. Background: The purpose of our study was to evaluate the application of thermoreversible gelation polymer (TGP) as a local drug delivery system for malignant glioma. Materials and Methods: Polymeric microspheres or liposomes loaded with doxorubicin (sphere-dox or lipo-dox) were combined with TGP to provide continuous drug delivery of doxorubicin (dox) for kinetic release studies and cell viability assays on glioma cell lines in vitro. For in vivo studies, TGP loaded with dox alone (TGP-dox) was combined with sphere-dox or lipo-dox. Their antitumor effects on subcutaneous human glioma xenografts were evaluated in nude mice. Results: In vitro, TGP combined with sphere-dox or lipo-dox released dox for up to 30 days. In vivo, TGP-dox combined with sphere-dox or lipo-dox inhibited subcutaneous glioma tumor growth until day 32 and day 38, respectively. Conclusion: TGP in combination with microspheres or liposomes successfully prolonged the release of dox and its antitumor effects.

Malignant gliomas are the most common primary brain tumor and remain a major therapeutic challenge. Despite advances in neurosurgical techniques, radiation and drug therapies, the prognosis of patients with malignant glioma remains dismal. More than 80% of patients with this disease experience local recurrence of tumor within centimeters of the original lesion, which contributes to their poor prognosis (1). Chemotherapy for these patients is not effective, in part because there is poor penetration across the blood-brain barrier (BBB) into the central nervous system (CNS) after systemic administration. In addition, even with drugs that penetrate the BBB, it is difficult to reach sufficient therapeutic concentrations in brain tumor tissue without causing considerable systemic toxicity (2). Therefore, development of new local management strategies may provide a therapeutic advantage in the adjuvant medical management of glioma. Local delivery of chemotherapeutic agents directly to tumor sites using a sustained-release device overcomes many obstacles associated with systemic delivery by bypassing the BBB and minimizing toxicity. Thus recent efforts have been focused on designing new devices that deliver chemotherapeutic agents more effectively (3-8).

The use of thermoreversible gelation polymer (TGP) as a novel drug delivery system (DDS) for local chemotherapy of malignant glioma is based on its unique temperature kinetics (9). TGP is a water-soluble aqueous solution at low temperature, whereas it becomes a solid at body temperature. TGP is a copolymer composed of the thermo-responsive polymer poly(N-isopropylamido-co-n-butyl methacrylate) (poly(NIPAAm-co-BMA)) and the hydrophilic polymer polyethylene glycol (PEG). Temperature-responsive polymers are hydrophilic below the liquid-gel transition temperature (LgTT), whereas above this temperature they become hydrophobic, and make crosslinks between intermolecular poly(NIPAAm-co-BMA) blocks due to hydrophobic interactions. The liquid-gel transition occurs quickly, and it is reversible. The LgTT can be set at any temperature from 5 to 70˚C. Furthermore, TGP is biocompatible and completely pathogen-free. These unique features have made TGP particularly useful in medicine (10-17). The benefit of TGP as a local DDS is that it can provide a practical means of delivering drugs by local administration based on its unique features. In other words, TGP can be injected into the tumor cavity via a catheter at low temperature, where it will remain at the site of disease because it will solidify at body temperature. In our previous study, we used doxorubicin (dox), a well-known anticancer drug and an anthracycline ring antibiotic, with a
broad spectrum of antitumor activity on a variety of human and animal solid tumors. The kinetics of dox release from TGP and its antitumor activity on glioma cells in vitro and in vivo have been demonstrated (18), but a significant drawback of this study was the rapid release of dox from TGP.

In the present study, we investigated release of dox by combining TGP with poly(lactic-co-glycolic acid) (PLGA) microspheres and liposomes. These delivery systems were then tested for inhibition of growth of subcutaneous (s.c.) glioma xenografts in nude mice.

Materials and Methods

Cell cultures. Human glioblastoma cell lines U87MG and LN229 were obtained from the American Type Culture Collection (Manassas, VA, USA), and G55 was provided by Dr. Manfred Westphal. These cells were cultured in Modified Eagle’s Medium (MEM) alpha supplemented with 10% fetal bovine serum, 2 mM/l L-glutamine, 2 mM/l nonessential amino acids, 2 mM/l sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml fungizone (Invitrogen, Grand Island, NY, USA). Cells were maintained in 5% CO2 incubator and were routinely passaged at confluency.

Microsphere and TGP preparation. Dox was obtained from Sigma Chemical Co., Inc (St. Louis, MO, USA). Liposomal dox (dip-dox) is commercially available as Doxil from Alza Pharmaceuticals, Inc. (Foster City, CA, USA). Dox-loaded microspheres (sphere-dox) were prepared using the double-emulsion solvent extraction method with slight modifications as reported previously (19). Dox was loaded in particles made of 200 mg PLGAS50:50 (RG502; Boehringer Ingelheim, Germany) lactic to glycolic acids ratio (20,000 kDa). Briefly, polymers were dissolved in 0.5 ml of dichloromethane (Frutorom, Israel). A predetermined solution of dox was added to the dissolved polymer and the solution was homogenized using an ultra-turax (type DI-18; IKA, Germany) for 1 min leading to the formation of the first emulsion polyvinyl alcohol of 85-89 kDa (Sigma-Aldrich Chemical, St Louis, MO, USA) saturated with dichloromethane, which was rapidly added to the first W/O emulsion, and the solution was homogenized again for 20 seconds. The resulting solution was mixed for 5 minutes and a volume of 50 ml of 0.1% w/v aqueous polyvinyl alcohol containing 5% (v/v) 2-propanol solution (J. T. Baker, Holland) was added.

After 30 minutes of extensive stirring the microspheres were centrifuged, washed three times and lyophilized. A total loading of 4 mg dox in 115 mg of microsphere was obtained. TGP was obtained from Mebiol Inc. (Tokyo, Japan).

TGP is a co-polymer composed of thermoresponsive poly NIPAAm-co-BMA and hydrophilic PEG (9). Upon heating the aqueous solution, the co-polymer solidifies, forming what is referred to as a hydrogel; when cooled the hydrogel liquefies. It has been demonstrated that polyNIPAAm aggregates and precipitates in aqueous solution upon heating above the LgTT due to hydrophobic interactions. The LgTT can be adjusted by randomly co-polymerizing NIPAAm with other monomers. The LgTT increases or decreases when NIPAAm is co-polymerized with a more hydrophilic monomer, such as acrylamide, or a hydrophobic monomer, such as n-butyl methacrylate. LgTT was set at 20˚C in our experiment, and it was observed to solidify when heated above 20˚C. Lyophilized TGP was dissolved in phosphate buffered saline (PBS) or dox solution at 4˚C overnight for preparing TGP alone or TGP loaded with dox alone (TGP-dox) respectively. For the preparation of TGP combined with microspheres or liposomes, these materials were dissolved in TGP alone or TGP-dox and stored at 4˚C until use. TGP content was 9.1% w/v.

There were five different treatment regimens with TGP (Table I). For the in vitro experiments, three groups were designed: TGP-dox, TGP combined with sphere-dox (TGP+sphere-dox) and TGP combined with lipo-dox (TGP+lipo-dox). The dox concentration in the TGP was 1.0 mg/ml as we previously reported (18). For the in vivo experiments, two other groups were used: TGP-dox combined with sphere-dox (TGP-dox+sphere-dox) and TGP-dox combined with lipo-dox (TGP-dox+lipo-dox). These groups were designed to extend the tumor inhibition observed with TGP-dox alone. The dox concentration for the in vivo studies in TGP-dox was 0.1 mg/ml, which was the 90% inhibitory concentration of dox on U87MG cells. The final concentration of dox loaded in the microspheres or liposomes was 2.0 mg/ml, based on a local chemotherapy protocol presently being used for bladder cancer (20).

In vitro drug release kinetics assay. For the in vitro drug release kinetic studies, 0.6 g of TGP alone, TGP-dox, TGP+sphere-dox and TGP+lipo-dox were placed in Transwell inserts (Corning, Corning, NY), and put into the lower chamber containing 3.0 ml of PBS. The chambers were maintained in a 5% CO2 incubator at 37˚C, and the PBS was replaced every day for 60 days. The absorbance of dox in PBS was measured by multiwell scanning spectrophotometer (Fisher Scientific International Inc., Hampton, NH, USA) at 492 nm.

<table>
<thead>
<tr>
<th>Group</th>
<th>TGP-dox (mg/ml)</th>
<th>Sphere-dox (mg/ml)</th>
<th>lipo-dox (mg/ml)</th>
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<tbody>
<tr>
<td>TGP alone</td>
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<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>TGP-dox</td>
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<tr>
<td>TGP+sphere-dox</td>
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<td>N/A</td>
<td>1.0</td>
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<tr>
<td>TGP+lipo-dox</td>
<td>N/A</td>
<td>N/A</td>
<td>1.0</td>
</tr>
<tr>
<td>TGP-dox+sphere-dox</td>
<td>0.1</td>
<td>2.0</td>
<td>N/A</td>
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<tr>
<td>TGP-dox+lipo-dox</td>
<td>0.1</td>
<td>N/A</td>
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N/A, Not applicable.
In vitro drug release kinetics assay. In this assay, the release profile of dox from TGP in the TGP-dox, TGP+sphere-dox, and TGP+liposome-dox groups was determined by measuring the cumulative percentage of dox released over time (Figure 1). TGP-dox released 78%, 94% and 100% of dox after 2, 4 and 12 days respectively. TGP+sphere-dox gradually released dox, with 48% and 94% released after 14 and 30 days, respectively. TGP+liposome-dox also released dox slowly, with only 9% released in the first 8 days; for the subsequent time period, the release rate increased to 24%, 64% and 94% release of dox after 14, 30 and 54 days, respectively. These results confirmed that TGP-dox released dox very quickly. In contrast, TGP combined with microspheres or liposomes loaded with dox prolonged the release of dox.

In vitro cytotoxicity assay. Glioma cell viability assays were carried out to confirm the retention of the biological activity of dox released from TGP. In this assay, cell viability was measured as the mean±SD as a percentage of the control viability (=100%).

In vivo growth inhibition on mouse subcutaneous human glioma xenograft model. Male Swiss nude mice (5 to 6 weeks old; Charles River, Wilmington, MA, USA) were inoculated s.c. with 5x10⁶ U87MG cells. Ten days later, when tumors reached ~200 mm³, animals were randomly divided into four groups of five animals each. Each group received a single s.c. injection of 100 μl of TGP alone, TGP-dox, TGP-dox+sphere-dox, and TGP-dox+liposome-dox, respectively, adjacent to the observed tumor. The tumor growth was measured transcutaneously with a caliper every other day for 24 to 38 days. The tumor volume was calculated and expressed as the mean±SD as a percentage of the control viability (=100%).

In vivo cytotoxicity assay. The cytotoxic effect of TGP alone, TGP-dox, TGP+sphere-dox and TGP+liposome-dox was evaluated in vitro. TGP at 0.6 g for each treatment group was placed in Transwell inserts, and put into the lower chambers containing 3.0 ml of medium. The chambers were maintained in a 5% CO₂ incubator at 37°C, and the medium was replaced with fresh daily for 30 days and stored at -20°C until used. U87MG, LN229, or G55 cells (5x10³ cells/well) were plated in 96-well plates (Corning, Inc., Acton, MA, USA). After 6 hours, the medium was replaced with the media collected from the TGP incubations. The cells were then cultured for an additional 48 hours. Quantification of cell viability was calculated with a colorimetric assay using Cell Counting Kit-8 (Dojindo Molecular Technologies, Gaithersburg, MD, USA). All experiments were conducted in triplicate. Cell viability was expressed as the mean±SD as a percentage of the control viability (=100%).

In vivo growth inhibition on mouse subcutaneous human glioma xenograft model. Mice were inoculated by a right flank s.c. injection of 5x10⁶ U87MG cells. The tumors reached a size of 206.2±58.6 mm³ 10 days post-injection, at which time the TGP was injected adjacent to the tumor. TGP-dox inhibited the growth of U87MG glioma xenografts for 14 days, which was followed by aggressive tumor growth until day 24, the termination of the studies. A much longer duration of tumor inhibition was observed in the TGP-dox combined with microspheres and liposomes groups (p<0.01, TGP alone or TGP-dox versus TGP-dox+sphere-dox; p<0.01, TGP alone or TGP-dox versus TGP-dox+liposome-dox; Figure 3a). The growth of gliomas was significantly suppressed with TGP-dox+sphere-dox and TGP-dox+liposome-dox when compared to TGP alone and TGP-dox. Moreover, TGP-dox+sphere-dox inhibited the tumor growth until day 32, when the tumors again started to increase.
in volume. In contrast, TGP-dox+lipo-dox inhibited tumor growth up to day 38, the time of experiment termination ($p<0.01$). These results confirmed that TGP-dox combined with microspheres or liposomes loaded with dox inhibited the growth of s.c. glioma tumors and showed long-term antitumor activity. Injected TGP-dox, TGP-dox+sphere-dox and TGP-dox+lipo-dox were removed from the animals at the termination of the experiments for inspection. TGP-dox was colorless, suggesting that the dox had been completely released from the TGP (Figure 3b). On the other hand, TGP-dox+sphere-dox material was light orange in color, suggesting that a small amount of dox was retained (Figure 3c). In contrast, TGP-dox+lipo-dox was bright orange in color, suggesting the retention of large amounts of dox (Figure 3d).

**Immunohistochemistry.** Histological analysis of the subcutaneous tumors is shown in Figure 4a. The mean proliferative indices of TGP alone, TGP-dox+sphere-dox and TGP-dox+lipo-dox were 55.5%, 30.6% and 22.3% respectively. The mean apoptotic indices of each group were 0.4%, 6.9% and 4.3% respectively. These results revealed a significant decrease in the proliferative index in tumors treated with TGP-dox+sphere-dox or TGP-dox+lipo-dox, compared with the control group ($p<0.01$, Figure 4b). The apoptotic index was significantly increased in all treatment groups compared with the controls ($p<0.01$).

**Discussion**

Malignant gliomas are the most common primary brain tumor and remain a major therapeutic challenge. The median patient survival is 14.6 months with radiation therapy plus temozolomide (22). Despite intensive multimodal treatment such as surgical resection, radiation and drug therapies, the prognosis of patients with this tumor remains dismal due to local recurrence. Therefore, glioma patients with persistent or recurrent disease are ideal candidates for local therapy. The development of new local management strategies may provide a therapeutic advantage in the adjuvant treatment of gliomas. Direct delivery of controlled-release anticancer agents maintains optimal concentrations at the tumor site, provides sufficient time for drug diffusion, and acts directly on the tumor cells. Moreover, chemotherapeutic agents whose therapeutic potential is limited by the BBB or dose-dependent systemic toxicity should be used for local drug delivery. A local DDS overcomes many obstacles associated with systemic delivery because it can bypass the BBB and minimizes drug toxicity. Therefore, there has been much interest in a variety of approaches to local tumor treatment. Several local DDSs, including biodegradable polymers for interstitial chemotherapy, intra- and peritumorally injected micro-/nanoparticles, and targeted toxins administered by osmotic minipumps or convection-enhanced delivery (CED) have been used successfully for the treatment of experimental brain tumors and in clinical trials (3, 4, 6-8, 19, 23-27). Each of these DDSs has its advantages and disadvantages. For example, wafers are stable in the tumor bed, but surgery is required for administration. Micro-/nanoparticles can be directly injected into the brain parenchyma, but there is an increased risk of bleeding, stroke or brain contusion. CED offers a greater volume of distribution than simple diffusion and is designed to direct a drug to a specific target site. It is associated with some problems, such as spillover or reflux in the vicinity of sulci and ventricles, or toxicity to the surrounding normal brain tissue.

TGP has some unique features. It is liquid at low temperatures and becomes a solid at body temperature. The transition occurs quickly, and it is reversible and controllable. TGP is biocompatible and completely pathogen-free. It has been evaluated for use in a variety of medical fields, such as cell culture, regenerative medicine, tissue engineering and blood vessel embolization (10-17). We hypothesized that TGP may be a novel DDS for the treatment of malignant glioma. This means that TGP can be injected repeatedly and remains at the site of disease. In
other words, TGP as a liquid can be injected into the tumor resection cavity and it will solidify at body temperature. The solid TGP in the tumor cavity will release the drug continuously. Conversely, if the chemotherapeutic solution is injected directly into the tumor resection cavity, it will be easily washed away from there with the cerebrospinal fluid. The purpose of our study was to evaluate the application of TGP for DDS, and this report represents an interesting first step in the development of a novel DDS. We previously demonstrated that TGP provided a practical means of local delivery of dox to human glioma xenografts (18). Dox was used for three reasons in our study. Firstly, dox does not penetrate the BBB when administered systemically. Secondly, the therapeutic potential with systemic delivery is limited by its dose-dependent cardiotoxicity and myelosuppression. Direct delivery would focus high concentrations of dox at the tumor site without causing systemic toxicity. Finally, dox has been used successfully as a local chemotherapeutic agent for the treatment of experimental brain tumors and glioma patients (4, 7, 25, 26 28-30). Moreover, there were no clinically significant

Figure 2. In vitro cytotoxicity assay of TGP alone, TGP-dox, TGP+sphere-dox and TGP+lipo-dox on malignant glioma cells. a: TGP alone did not reduce tumor cell viability for 30 days. b: TGP-dox only reduced tumor cell viability until day 10. c: TGP+sphere-dox reduced tumor cell viability starting at day 2 until day 30. d: TGP+lipo-dox reduced tumor cell viability from day 10 to day 30. Values are the mean cell viability as a percentage of that of the control; bars, SD; *p<0.01.
adverse reactions either in the brain or systemically (27, 28). Our previous report demonstrated dox-release kinetics from TGP and its antitumor efficacy in vitro and in vivo (18). In addition, we showed that TGP remained in the liquid state at room temperature allowing injection adjacent to the s.c. tumor, and became a solid at body temperature remaining for up to two weeks. The rapid release of dox from TGP limited the efficacy of this system. Therefore, this study focused on using TGP in combination with microspheres or liposomes for local chemotherapy as previously demonstrated (4, 19, 21, 25, 31).

PLGA microspheres have been used as a controlled delivery system for many proteins, drugs, and others substances, such as cytokines, hormones, enzymes, vaccines, and chemotherapeutic agents (4, 32-34). The protein is
released from the PLGA microspheres by the degradation of the polymeric matrix. The composition of the PLGA allows control of the degradation rate and therefore the control on the release kinetics. Most importantly, PLGA microspheres are biocompatible and can be used in humans. On the other hand, liposomes are nano- or microscale carriers typically consisting of a phospholipid membrane shell surrounding a hollow core that can be used to encapsulate small molecules such as drugs and genes. One potential advantage of the liposome-encapsulated cytotoxic drug over corresponding unencapsulated agents is the sustained release of the drug, prolongation of its half-life, and an increase in its therapeutic index (35-39). Recently, efforts have been made to increase the vehicular efficiency of liposomes and to direct therapeutic agents to specific target sites, not only by systemic administration but also by local administration (25, 26, 30). Lastly, dox-loaded microspheres and liposomal-dox have been used for the treatment of tumors in experimental models and show no toxicity in normal tissue (4, 30, 40).

We hypothesized that a combined drug delivery approach featuring TGP combined with microspheres or liposomes loaded with dox could overcome the disadvantages faced when using TGP alone, and evaluated the drug release kinetics of these systems and their antitumor effects on malignant glioma cells in vitro and in vivo. Our results confirm that these systems successfully prolonged drug release and its antitumor effects both in vitro and in vivo. Only in combination with microspheres and liposomes is the antitumor activity enhanced by TGP, and we cannot exclude the possibility that enhanced efficacy is the result of the spheres or liposomes themselves. While these preliminary results are promising, we would highlight the advantage of diffusion-based DDS for the treatment of malignant glioma. Sufficient effective concentrations of the drug do not reach infiltrating tumor cells. Therefore, one might want to investigate the use of an alternative DDS, such as injectable micro-/nanoparticles or CED, to address infiltrating malignant cells.

In conclusion, TGP in combination with microspheres or liposomes successfully prolonged the release of dox while maintaining its biological activity. Furthermore, local administration of these materials significantly inhibited s.c. human malignant glioma growth in nude mice. This significant inhibition was achieved with only a single drug administration. The unique thermoreversible kinetics of TGP may allow repeated local administrations and stable drug release at the site of disease. Moreover, the use of these delivery systems together may offer a way to combine different inhibitors as well as chemotherapy with one single administration. Our data indicate that local drug delivery by TGP in combination with polymeric microspheres or liposomes is an effective modality for the treatment of malignant gliomas. Future studies should evaluate the therapeutic efficacy of these local DDS in an orthotopic glioma model.

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1063


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