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POLYMERIC IMPLANTS FOR DELIVERY OF GREEN TEA POLYPHENOLS

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Abstract

Polymeric implants (millirods) have been tested for local delivery of chemotherapeutic agents in cancer treatment. Modeling of drug release profiles is critical as it may provide theoretical insights on rational implant design. In this study, a biodegradable poly (ε-caprolactone) (PCL) polymeric implant delivery system was tested to deliver green tea polyphenols (GTPs), both *in vitro* and *in vivo*. Factors including polymer compositions, supplements, drug loads and surface area of implants were investigated. Our data showed that GTPs were released from PCL implants continuously for long durations, and drug load was the main determining factor of GTPs release. Furthermore, the rates of *in vitro* release and *in vivo* release in the rat model followed similar kinetics for up to 16 months. A mathematical model was deduced and discussed. GTPs implants have the potential to be used locally as an alternative strategy. GTP implants have the potential to be used systemically and locally at the tumor site as an alternative strategy.

INTRODUCTION

Chemotherapy is one of the most important strategies against cancer. However, considering the high toxicity caused by standard drug treatment, local delivery of cancer drugs at the cancer sites in addition to other strategies, create the possibility of improving both the efficacy and safety of chemotherapy ¹. In fact, it becomes extremely necessary and potentially helpful in some situations, e.g. for unresectable tumors, prevention of local recurrence, etc. Several studies have demonstrated the feasibility and efficacy of local delivery of chemotherapeutic agents by polymeric implants (millirods) in cancer treatment in nude mice, rats, rabbits, dogs and humans ^{2–9}. However, the use of polymeric implants has not been attempted until recently ¹⁰. Furthermore, the optimization of polymeric implants remains a challenge. In order to optimize the efficacy of these polymeric implants over time, modeling of drug release profiles is critical as it may provide theoretical insights on rational implant design.

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CONFLICT OF INTEREST

The authors declare that there is no personal financial or non-financial conflicts of interests.

Polymeric implants can be prepared using biodegradable or non-degradable materials. Poly (ϵ -caprolactone) (PCL) is one of the most common biodegradable materials used in subdermal grafting and importantly, its medical application has been approved by the FDA. PCL is ideally suitable for long-term delivery because of its slow degradation rate ¹¹. *In vivo*, the hydrolytic degradation of PCL converts the polymer into smaller molecular weight polymers leading further to complete degradation and absorption of the polymeric materials. The drug is released in concert with the physical erosion of the implant, particularly, by diffusion into the blood and tissues as interstitial fluid penetrates into the polymer and solubilizes the drug ¹².

Green tea is the second most popular drinks in the world, only next to water. Green tea consumption is believed to associate with many beneficial health effects ^{13–16}. It has been drawing more attention because of its possible anti-tumor effects ^{17–19}. Green tea polyphenols (GTPs), including epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG) and epigallocatechin gallate (EGCG) are believed to be the bioactive components. *In vitro*, GTPs have been shown to inhibit tumor cell proliferation and induce cell apoptosis through different mechanisms ^{18,20}. *In vivo*, GTPs decreased tumor incidence and tumor multiplicity at different organ sites in animal models for skin, lung, esophagus, stomach, intestine, colon, liver, mammary gland, prostate and other cancers ^{20,21}. Polyphenon E (poly E), a standardized green tea extract, has been approved by the FDA for use to treat genital and perianal warts in ointment form (Veregen®, MediGene AG, Munich, Germany). Poly E has also been investigated in clinical trials for prostate cancer ²². A Phase II study is currently underway to investigate the effects of poly E (Polyphenon Pharma, New York, NY) in patients with chronic lymphocytic leukemia at the Mayo Clinic in Rochester, Minnesota. Our own *in vitro* data showed significant antiproliferative activity against human lung and cervical cancer cells (H. Kauser, R. Munagala, R.C. Gupta, Unpublished data).

We successfully developed PCL implants containing various chemopreventives, including GTPs using a melt-extrusion method, and these implants can be grafted subcutaneously ¹⁰. In this study we report various factors including polymer composition, supplements, drug load and surface area of implant that affect the rate of release of GTPs from the implants. These data were used to deduce a mathematical model. We also correlated the rates of release of GTPs from the implants *in vitro* and *in vivo*.

MATERIALS AND METHODS

Chemicals

PCL, F68, γ -cyclodextrin, PEG8000 and GTP60 (a green tea extract preparation containing 60% catechins) were purchased from Sigma-Aldrich (St. Louis, MO) and GTP60 was used for all *in vitro* studies. Polyphenon E (a standard GTPs preparation containing approximately 90% catechins) was obtained from the National Cancer Institute and Pharma Foods International Co., Ltd, Kyoto, Japan was used for the *in vivo* study.

Preparation of GTP-PCL implants

Polymeric implants were prepared using the methodology described elsewhere¹⁰. Briefly, PCL and GTPs were dissolved in dichloromethane and ethanol, respectively before mixing, followed by removal of the solvents using vacuum dryer. The polymeric material was then filled in a disposable syringe attached to silastic tubing (I.D. 3.2 mm), heated at 70°C and extruded. The implants were removed from the silastic tubing mold and excised to desired lengths (0.5–3 cm). Polymer composition (PCLs with different mol. wts.), supplements in the implant (F68, cyclodextrin, PEG8000) and GTPs load were varied based on the purpose of studies.

Electron microscopy studies

The implants were subjected to surface morphology studies using scanning electron microscopy by preparing a 2 mm cross-section of the implants and imaged in Zeiss Supra 35 VP unit under a low accelerated voltage of 2 kV using secondary electron detectors.

Calorimetric studies

To study the uniform distribution of the GTPs in the PCL implants, differential scanning calorimetry was performed where the melting temperature of the PCL, GTPs and PCL embedded with GTPs were performed by placing them in the standard aluminum pans and heated with a temperature range between 20 – 300 °C at a 5 °C/min rate under nitrogen spurge. The generated melting curve was analyzed in TA Universal analysis software.

In vitro release of GTPs from the implants

Release of GTPs was investigated as described elsewhere²³. Briefly, implants were stirred in 5 ml of phosphate-buffered-saline (PBS) supplemented with 10% bovine serum, pH 7.4 at 37°C to simulate the *in vivo* extracellular fluid environment. Release medium was changed every day. For long-term release study, the measurement was done only on select days, even though the media was replaced every day. The amount of catechins released was measured spectrophotometrically at 540 nm after reaction with a dyeing solution containing 0.1% ferrous sulfate and 0.5% potassium sodium tartrate tetrahydrate²⁴. A calibration curve was established using a series of known concentration of GTP60 solution react with the dyeing solution and repeated at least three times.

Overall degradation rate of GTPs *in vitro*

The overall degradation rate of GTPs in an *in vitro* environment was measured as follows: A known amount of GTPs was dissolved in PBS containing 10% serum and kept in amber colored vials at 37°C. An aliquot of this GTPs solution was sampled and measured spectrophotometrically at different time points.

Effect of polymer composition on GTPs release *in vitro*

Implants of PCL of mol. wt. 65,000 (P65) and 15,000 (P15) with different compositions (0:90, 9:81, 27:63, or 45:45 wt%) and 10% GTPs were prepared. Release of GTPs from 1 cm implants was investigated by stirring the implants in PBS containing 10% bovine calf

serum, pH 7.4 at 37°C. The amount of catechins released was measured spectrophotometrically as described above.

Effects of drug load and implant surface area on GTPs release *in vitro*

In order to determine the effects of the variable amounts of GTPs embedded in the implants as well as variable surface areas of the implant on GTPs release into the medium, we tested GTPs release from 0.5 – 2 cm implants (corresponding to surface areas of 1.075 – 2.3 mm²/mg) with 5 – 20% of drug loads. A total of 9 groups were assigned containing 20% GTPs of 0.5, 1.0 or 2.0 cm in length; and 5 or 10 % GTPs load of 1.0, 1.5 or 2.0 cm in lengths. All test implants contained P65:F68 in 9:1 ratio. Release of GTPs from implants *in vitro* was measured spectrophotometrically as described above.

Effect of supplements in the implants (F68, cyclodextrin, PEG8000) on GTPs release *in vitro*

In order to determine the effect of water-soluble supplements on GTPs release from PCL implants, implants were formulated containing P65:GTPs (90:10), P65:F68:GTPs (81:9:10), P65:cyclodextrin:GTPs (81:9:10) and P65:PEG8000:GTPs (81:9:10). Release of GTPs from 1 cm implants *in vitro* was measured as described above.

GTPs release from implants with blank PCL coating *in vitro*

In order to determine whether coating implants with a thin layer of blank PCL can improve the release of GTPs from implants, GTPs-PCL implants were prepared as described above and then dipped in 8% P65 solution in dichloromethane for 1 sec followed by air dry and repeated sequentially six times. The release profile of these implants was tested *in vitro* comparing with the implants without PCL coating.

GTPs release *in vivo* and potential toxicity

All animal experiments were performed after obtaining approval from the Institutional Animal Care and Use Committee (IACUC). We determined the rate of GTPs release from the implants by grafting them subcutaneously in 5–6 week-old female ACI rats (Harlan Laboratories, Indianapolis, IN). The implants were formulated with P65:F68 (9:1) with 20% poly E. They were sterilized before implantation using 70% ethanol. Groups of animals (n=5) were grafted with one 2-cm implant (200 mg) onto the back of the rat subcutaneously. At 1, 2, 3, 5, 8, 18, 36, 42 and 70 wks, animals were euthanized and the implants were removed for further analysis. Local tissues surrounding the implants were harvested at 3 weeks and fixed in 10% formaldehyde, embedded in paraffin wax and sectioned at 5 µm thickness. Sections were deparaffinized, rehydrated, and stained with hematoxylin and eosin (H&E). Sections were studied using Nikon Eclipse 80i microscope. The residual amounts of poly E in the implants removed from the animals were measured by dissolving the implant in a mixture of dichloromethane and ethanol, extracting the poly E in PBS, followed by reaction with a dyeing solution containing 0.1% ferrous sulfate and 0.5% potassium sodium tartrate tetrahydrate and spectrophotometric detection as described elsewhere²³. A calibration curve was obtained using polyE solution with serial known concentrations react with the dyeing solution and repeated at least three times. The amount of poly E released at

selected time points was calculated by subtracting the residual amounts of poly E from the initial amount loaded in the implants.

RESULTS

Overall degradation rate of GTPs *in vitro*

Degradation of GTPs varied as a function of time, faster initially and slower at later time points. The rate of degradation of GTPs in PBS with 10% serum was found to be approximately 49.4% in 24 h. Hence all subsequent release data were corrected accordingly.

Distribution of GTPs in polymer matrix

The GTPs were evenly distributed in the polymer matrix in amorphous form as is established by lack of crystallization evidenced by scanning electron microscopy (Supplementary Figure S-1). The texture of the polymer had changed due to the presence of GTPs at 20% drug load. The amorphous distribution of the drugs were also confirmed with differential scanning calorimetry where no melting curve of GTPs were observed when the polymer loaded with GTP was used (Supplementary Figure S-2).

Effect of polymer composition on GTPs release *in vitro*

The GTP content of fresh prepared implants was found to be within 5% of the target content by dissolving the implants followed by PBS extraction and measured spectrophotometrically. GTPs from the polymeric implants showed a continuous release as a function of time (Figure 1A). For example, the release of the GTPs from the implants containing 50% P65 fits in a mathematic equation $y = 9.5227x^{-0.856}$ ($R^2 > 0.98$), in which y is percent daily GTPs release and x is the time in days (Figure 1B). The observed release of GTPs was high initially followed by a steady decline. A theoretical cumulative release of GTPs was obtained by summing up the daily release calculated by the equation above, and it can be expressed as $y = 14.846 \ln(x) + 10.307$ ($R^2 > 0.98$), in which ' y ' is the theoretical cumulative release and ' x ' is the time in days (Figure 1C).

The release pattern of implants with different compositions was similar, although implants containing 50% P65 showed significant lower release at day 1 and 7 in comparison with 0% P65 ($p < 0.05$, two-way ANOVA with Bonferroni correction). The PCL implants remained intact during the entire duration of 9 months. The high percentage (50%) of P65 used in one of the implant formulations increased the plasticity of the implants which makes them less fragile.

Effects of drug load and implant surface area on GTPs release *in vitro*

The results showed that drug load is the main determining factor of GTPs release (Figure 2). One-cm implants with 20% GTPs load released more GTPs than implants with 10% and 5% load (Figure 2A). Similarly, 2 cm implants with 20% GTPs load released more GTPs than implants with 10% and 5% drug load (Figure 2B). Thus the release was proportional to the drug load, with a high correlation coefficient (> 0.98).

Comparison of the implant size, 0.5, 1 and 2 cm containing 20% GTPs load resulted in an initial burst release of GTPs by the smaller implant, but a lower release for the next several days (Figure 2C). Similar results were observed with 1, 1.5 and 2 cm implants with lower drug loads of GTPs (Figure 2C). It should be noted that these data have been normalized by implant weight, since the size of the implants and surface area are not directly proportional. We found that the implants with a larger surface area initially resulted in a higher rate of drug release. However, subsequently the rate of GTPs release was dictated by the drug load.

Effect of implant supplements (F68, cyclodextrin, PEG8000) on GTPs release *in vitro*

Our results indicated that there were statistical differences between the 1st day F68, 3rd and 4th day cyclodextrin as well as PEG and control group ($p < 0.01$). However, at all other time points, there was no significant difference. Additionally, no radical differences in the release pattern of GTPs from the implant in the presence of the various water-soluble additives (Figure 3). However, the presence of such an additive facilitated in the extrusion of the polymeric implants, as well as the formation of micro pores for the extracellular fluid after the water-soluble polymer dissipates in a few days after implantation.

GTPs release from implants with blank PCL coatings

The data showed that the release of GTPs from implants with blank PCL coatings reduced the initial burst release as compared with implants without the coatings, dropping from approximately 9.5% to 3.8% on day 1 and 4.9% to 2.4% on day 2. In fact, the GTPs release from these two groups was almost identical after 9 days, with levels of 1.2% and 1.0% respectively, which indicates that the implants coated with blank polymer released GTPs at more constant rate than those implants without the PCL coating (Figure 4). The release equation for implants with a PCL coating was $y = 3.5885 \times^{-0.4884}$ ($R^2 > 0.98$) and that for implants without coating was $y = 8.6788 \times^{-0.7474}$ ($R^2 > 0.99$).

GTPs release from subcutaneous implants *in vivo*

The implants gradually changed their color from reddish to brownish starting from two to three months after implantation, suggesting GTPs released from the implants over time. There was no significant change in terms of implant surface. Implants weights remained roughly the same initially but dropped somewhat after several months. Our results showed that *in vivo* release of GTPs from the implants followed similar release kinetics as found *in vitro*, with an initial burst release followed by a gradual decline but the release occurred continuously (Figure 5). The total amount of GTPs released after 1, 2, 3, 5, 8, 18, 36, 42 and 70 week(s) of the implantation was approximately 18%, 30%, 38%, 42%, 47%, 60%, 80%, 86% and 89%, respectively. The release equation was expressed as $y = 16.752 \ln(x) + 17.616$ ($R^2 > 0.96$), in which 'x' is the time in weeks.

Histopathological study at site of implantation

The histological study of the subcutaneous tissue surrounding the GTPs implants showed the presence of extensive influx of macrophages and large multi-nucleated giant cells consistent with a foreign body granulomatous reaction (Figure 6).

DISCUSSION

The studies were conducted to characterize and model the release of GTPs from polymeric implants both *in vitro* and *in vivo*. *In vitro* studies were conducted using an extracellular fluid environment to mimic the *in vivo* situation in order to establish a baseline for further *in vivo* evaluation. It is known that GTPs are not stable under alkaline environment¹⁷. Our results also indicated that approximately 50% of GTPs released into the media were degraded within 24 h. The degradation of GTPs was also confirmed by HPLC (details of HPLC method as reported earlier²³), with EGCG being relatively more unstable than the other catechins (data not shown). All the data presented have been corrected by applying the degradation factor as reported elsewhere²³.

The amorphous distribution of the GTPs in the polymer by scanning electron microscopy and differential scanning calorimetry substantiated that the GTPs are homogeneously and physically distributed within the matrix.

In vitro release of GTPs from the implants showed a continuous decline with time. Interestingly, the release of GTPs from implants can be expressed as $y = Ax^B$, in which 'y' is the daily GTPs release, 'x' is the time in days and 'A' and 'B' are constants for each specific formulation but vary between different formulations (Figure 1B). This mathematical expression of GTPs release describes firstly that the release of GTPs from the implant is comparatively high during the early time points, while the drug release is much slower subsequently. Secondly, it suggests that the release of GTPs is based on diffusion process, in which the GTPs molecules released from implants are inversely proportional to the square of the distance between the molecules and the implant surface. This is confirmed by cumulative release of GTPs that fit into equation $y = C\ln(x)+D$, in which 'y' is the accumulative release, 'x' is the time expressed in days and 'C' and 'D' are constants for each specific group (Figure 1C). We mathematically modeled the equations for GTPs that are hydrophilic compounds. However, the release of hydrophobic compounds that are also released from the implants by diffusion should also follow a similar pattern. For example, the daily release of curcumin from PCL implants published from this laboratory²⁵ can also be re-plotted and expressed in the form of $y = Ax^B$ with the only difference being the values of A and B. Thirdly, effect of degradation of implant by bulk erosion on drug release is very limited since the GTPs in the implant remain bound to the polymer matrix during the time of the study.

Since the GTPs release from the implants is based on diffusion rather than bulk erosion, we expected a significant increase in the release profile when water-soluble polymer additives were added to the base polymer. But we did not observe any significant increase in the drug release. This could be due to the high water solubility of GTPs. However, as expected, an increase in the drug release proportional to the surface area was observed, supporting our notion that the release is based on diffusion of the drug by the extracellular fluid.

A potential limitation of the GTPs polymeric implants is that the release of the GTPs is relatively high initially due to burst release, while the release was much lower at the later time points. The initial burst release could possibly result in toxicity and the subsequent

lower release may be insufficient *in vivo*. In order to improve the release profile of the implants, a coating with blank PCL was used to limit the initial burst release. This experimental manipulation diminished the burst release phenomenon and the subsequent release was also found more uniform (Figure 4). The presence of the blank polymer coatings presumably blocked the release of the surface-bound drug thus diminishing the burst-release effect.

We have previously demonstrated by scanning electron microscopy that curcumin as a model compound ²⁵ was dispersed homogenously in the matrix. The polymer matrix was also demonstrated to be stable in the release medium for more than 3 months. At the end of that study, analysis of the implants demonstrated a slight crystallization of the amorphous drug in the polymer was observed due to contact with aqueous release media.

The release of GTPs *in vivo* follows the same pattern as *in vitro* (Figure 1C and Figure 5) suggesting the release *in vivo* is also a simple diffusion process. Pearson product-moment correlation coefficient was calculated to see if there is any correlation between these two sets of data and results showed a very high correlation ($r=0.9792$), which further endorse this conclusion. Because the GTPs used for *in vitro* and *in vivo* were different extracts (GTP60 vs Polyphenone E), no direct comparison of the amount of GTPs release could be made in this study. The systemic effects caused by these GTPs implants have been reported elsewhere ²³.

A foreign body reaction was observed at the site of implantation. However, this reaction is no more severe than the reaction to sham implants (data not shown). Previous studies have shown that this FDA approved polymeric material do cause a mild reaction and may not be of major concern ^{26–28}.

CONCLUSION

In conclusion, our studies showed that GTPs are released from these PCL implants by a simple diffusion process both *in vitro* and *in vivo* which can be mathematically modeled. The initial burst release of the drug embedded in implants can be optimized by coating implants with blank polymer and adjusting the drug load. GTP implants have the potential to be used locally at the tumor site as an alternative strategy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

GTPs	green tea polyphenols
poly E	polyphenone E
PCL	Poly (ϵ -caprolactone)

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Figure 1.

In vitro release of green tea polyphenols (GTPs) in PBS with 10% serum. **A.** Daily release of GTPs. Data are expressed as mean $\pm$ SD (n=3). P65: Poly (ϵ -caprolactone) of mol. wt. 65,000; P15: Poly (ϵ -caprolactone) of mol. wt. 15,000. The inset represent the release data at later time points provided in for better clarity. **B.** Daily release of GTPs from implants containing 50% P65 ($y = 9.5227x^{-0.856}$; $R^2 > 0.98$). **C.** Theoretical cumulative GTPs release from implants containing 50% P65 ($y = 14.846\ln(x) + 10.307$; $R^2 > 0.98$). Daily values were calculated from the equation given in Figure 1B. **D.** Picture of the sham and GTP-loaded millirods (3 cm long, 3.2 mm dia).

Figure 2.

Effect of GTPs load on daily release based on 1 cm (A) and 2 cm (B) implants. C. Effect of surface area on daily release (normalized by implant weight). The insert represents the release data at later time points for better clarity. Data are expressed as mean $\pm$ SD (n=3). GTPs: green tea polyphenols.

Figure 3.

Effect of supplements (F68, cyclodextrin, polyethelene glycol) on daily GTPs release. Data are expressed as mean $\pm$ SD (n=3).

Figure 4.

GTPs released from polymeric implants with blank Poly (ϵ -caprolactone) coatings ($y = 3.5885 \times^{-0.4884}$, $R^2 > 0.98$) and without coatings ($y = 8.6788 \times^{-0.7474}$, $R^2 > 0.99$). Data are expressed as mean $\pm$ SD (n=3). GTPs: green tea polyphenols.

Figure 5.

GTPs release from subcutaneous implants in female ACI rats ($y = 16.752\ln(x) + 17.616$; $R^2 > 0.96$). Data are expressed as mean $\pm$ SD (n=3). GTPs: green tea polyphenols.

Figure 6.

Local reaction caused by subcutaneous implants in female ACI rats. A, low magnification (10 $\times$); B, high magnification (40 $\times$).