Laser-activated drug implant for controlled release to the posterior segment of the eye

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Abstract

Posterior segment eye diseases such as age-related macular degeneration (AMD), diabetic macular edema (DME) and proliferative vitreoretinopathy (PVR) are serious choric diseases that may result in vision loss. The current standard of care for the posterior segment eye diseases involves frequent intravitreal injections or intravitreally injectable sustained-release implants. However, dosage is not controllable once the implant is inserted in the vitreous, resulting in serious local side effects, such as elevated intraocular pressure and cataract formation.

We previously developed a size-exclusive nanoporous biodegradable PLGA capsule and combined with light-activatable drug-encapsulated liposomes, to create a light-activated dose-controllable implant for posterior eye disease treatment. We demonstrated the stability and safety of the implant in rabbit eyes for 6 months. In this study, we focused on the drug release from the dose-controllable implant by laser irradiation both in vitro and in vivo. Drug release kinetics upon laser irradiation were analyzed with two different total dosages. Drug release by laser irradiation in the rabbit eyes was determined by fluorescence intensity. Optical and histology examination confirmed no damage on the retina. The results demonstrated feasibility of using the implant as a on-demand dose-controllable drug delivery system to the posterior segment of the eye.

1. Introduction

Posterior segment eye diseases such as age-related macular degeneration (AMD), diabetic macular edema (DME) and proliferative vitreoretinopathy (PVR) are serious choric diseases that may result in vision loss. More than 100 million people around the world are suffering from the chronic posterior eye diseases. The current standard of care for posterior segment eye disease (e.g. wet age related macular degeneration, diabetic macular edema, edema from retinal vein occlusions) involves long term use of intravitreal medications given monthly, every 8 weeks, or every 12 weeks depending on the medication and the patient's response to treatment. Frequent

injections are unavoidable to maintain therapeutically effective dosage because drug molecules are cleared from the vitreous relatively quickly (within hours – days). [1] However, there is a significant burden to the patient, the patient's family, and the health system because current intravitreal therapies require between every 4 and 12 week administration over many years.

To resolve the issue, intravitreally injectable sustained-release implants have been developed to prolong therapeutic efficacy. Non-biodegradable implants are claimed to be used up to 3 years for diabetic macular edema treatment (Iluvien).[2] Biodegradable implants, such as OZURDEX®, were developed using PLGA (poly lactide-co-glycolic acid) as a drug depot designed for 6 months.[3] However, most of the drug is released within 2 months, resulting in high initial drug concentration in the vitreous or retina. This high initial dose or burst release of drug from the implant are considered to cause local side effects such as elevated intraocular pressure and cataract formation. [4]

We previously developed a size-exclusive nanoporous biodegradable PLGA capsule and combined with light-activatable drug-encapsulated liposomes, to create a light-activated dose-controllable implant for posterior eye disease treatment.[5] We demonstrated the stability and safety of the implant in rabbit eyes for 6 months. In this study, we focus on the drug release from the implant by laser irradiation both in vitro and in vivo to demonstrate feasibility of using the implant as a dose-controllable drug delivery system to the posterior segment of the eye. Drug release kinetics in vitro were also analyzed utilizing implants with two different total dosages. The light-activatable liposomes co-encapsulate methotrexate (MTX) and Cy5 fluorecence dye. MTX is an anti-inflammatory drug, used to treat PVR, and Cy5 was used to quantitively analyze hydrophilic molecules released by laser trigger in vivo.

2. Materials and methods

2.1 Materials

Poly(lactide-co-glycolide) acid (L/G 90/10, M.W. 200,000) was purchased from PolyScitech, Inc (West Lafayette, IN). Dichloromethane (DCM), chloroform, and polyethylene glycol (PEG, Average MW 3350) were purchased from Fisher Chemical (USA). Stearylamine was purchased from Tokyo Chemical Industry CO.,LTD (Tokyo, Japan). Cholesterol was obtained from MP Biomedicals, LLC (Solon, OH). 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-5000 (DSPE-PEG 5000), and 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Gold-nanorods were purchased from NanoPartz, Inc. (Loveland, CO). Methotrexate (MTX) (Thymoorgan Pharmazie Gmbh, Germany), hydrocortisone and neo-poly-bac ointments were purchased from University of Cincinnati Pharmacy. Sulfo-Cy5 carboxyl acid (Cy5) was purchased from Lumiprobe Co. (Hunt Valley, MD).

2.2 Synthesis of MTX/Cy5 encapsulated liposomes

MTX liposomes and MTX/Cy5 co-encapsulated liposomes were synthesized by a modified reverse-phase evaporation (REV) method reported in our previous publication.[5] MTX liposome was prepared for in vitro drug release test. MTX/Cy5 co-encapsulated liposome was used for in vivo drug release test. The MTX or MTX/Cy5 loaded liposome was purified by PD-10 column to remove free drug or dye solution. 50 µL gold nanorod suspension (~4.7×10^{13} /mL) (NanoPartz, Loveland, CO) was added in the purified sample, corresponding to a number ratio of 2:1 for a gold nanorod to a liposome. The mixture was gently shaken for at least 40 min to have the gold nanorods attach on the surfaces of liposomes via the electrostatic interaction. The attached gold nanorod-coated liposome suspension was concentrated by centrifugation at 6000 rpm for 30 min to achieve a higher drug concentration (accuSpin Micro 17, Fisher Scientific). After centrifugation, the suspension of concentrated drug/dye loaded liposome (approximate 350 µL) was first frozen in the -20 °C for 2 hours and then transferred into -70 °C for another 2 hours until solidly frozen. Then the frozen liposome suspension was lyophilized in a freeze-dryer (FreeZone 2.5, LABCONCO, Kansas City, MO) for 4 hr.

After lyophilization, to reach 250 mg/mL MTX concentration for 1000 µg dosage loading, 36.5 µL of PBS buffer was added into the lyophilized MTX and MTX/Cy5 liposome samples separately. The rehydrated sample was incubated in the room temperature overnight. Before sample loading, a 30-gauge syringe needle was used to manually homogenize the sample for 5 mins. Then the gel-like high concentration MTX liposome suspension was centrifuged at 3000 rpm for 3 min to remove any potential bubbles that may occur during the mixing.

The mixed samples were injected immediately to avoid evaporation.

2.3 Synthesis of nanoporous PLGA sheet

Nanoporous PLGA sheets were synthesized by following our previous study.[5] Briefly, it was synthesized by a solvent casting and particulate leaching method using polyethylene glycol (PEG) as porogen at 0.1 PEG to PLGA weight ratio.

2.4 Fabrication of laser-activated drug implant

A dried nanoporous PLGA sheet was cut into $1\ cm \times 3\ cm$ rectangular shape. The PLGA sheet was rolled using a 22-gauge needle as a template, creating a double-layer tube. Bottom end of the tube was sealed by a preheated straightener at 60 °C. 4 µL of the MTX/Cy5 co-encapsulated liposome suspension (around 250 mg/ml MTX concentration) was then injected into the tube by a 30-gauge syringe blunt tip needle. The other end of the tube was sealed by a preheated straightener at 60 °C. After

sealing, the implant was incubated in a 1.5 mL test tube with 1 mL PBS buffer in a 35 °C water bath for a week to remove free MTX and Cy5.

2.5 In vitro NIR laser irradiation of implant

Two different initial dosage (1000 μg and 500 μg MTX) of implants were prepared for 15 mins irradiation test in vitro by NIR laser.

Implants were put in the 1.5 ml test tube with 1 ml 35 °C PBS buffer individually. Each test tube was held horizontally on a petri dish. A pulsed NIR (1064 nm) laser (Italy) box was setup above the test tube. The implant was separated into 4 parts from left to right equally. The pulsed NIR laser was irradiated from left part of the implant to the right part of the implant. Our hypothesis is, by irradiation, the gold nanorod would induce phase transition of the liposome membrane to induce drug release. The released free drug would pass through the nanoporous structure of implant to the surrounding media (Figure 1). For each part, we irradiated 5 seconds then we moved to the next parts until the end of the implant, then we start from the first (left) part and repeated the cycle for another 3 times. By irradiating in this pattern, the effective irradiation time is 1 minute, then cooling down for 3 minutes to reduce the chance of melting gold nanorod. After cooling section, the irradiation was applied repeatedly until the total effective irradiation achieved 15 minutes. The released MTX (drug) outside of the implant was quantitively analyzed by using UV-vis spectrometry at absorption wavelength 370 nm. A calibration curve was prepared for MTX concentration in PBS buffer corresponding to its optical density (O.D). The measurement for the solution outside of implant was first characterized 10 hours after irradiation. We took out the 1 ml solution outside of the implant and mixed well with pipette to get homogeneous solution then gently put it back to the test tube. The concentration in vial was monitored after another 14 hours to reach the first 24-hour time point after irradiation, new PBS buffer was replaced after every 24 hours, O.D was measured before and after each buffer replacement, then the cumulative MTX released dosage was calculated after each measurement.

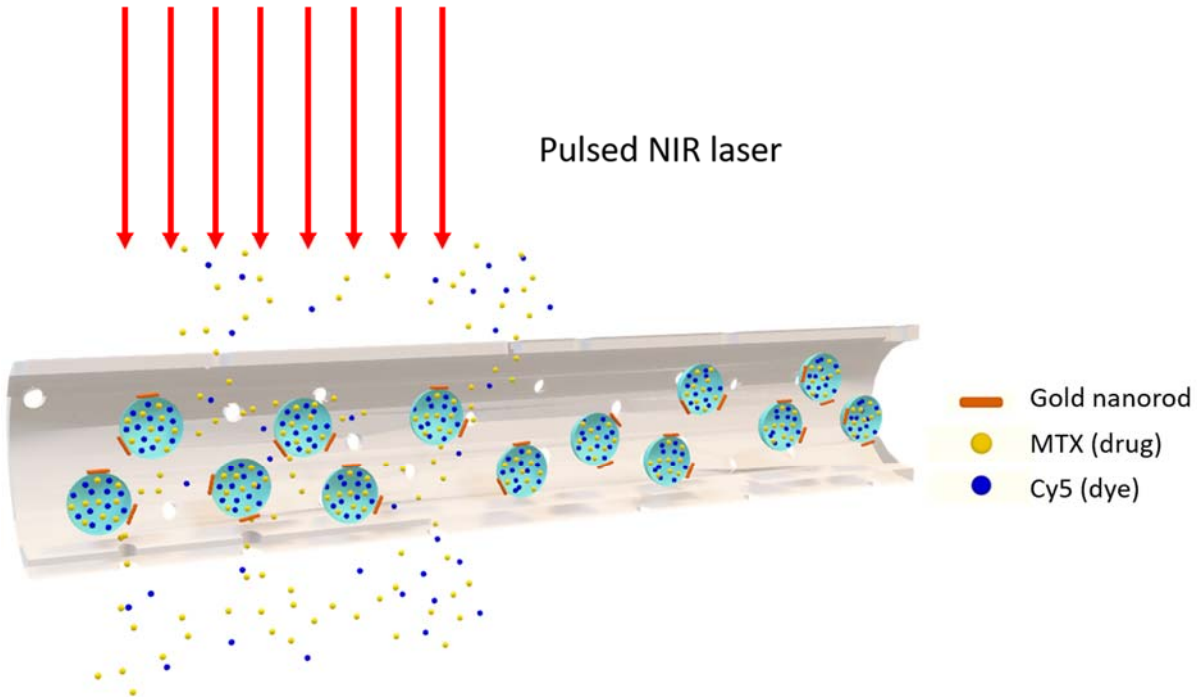


Figure 1. 3D schematic of light-activated drug release implant under pulsed NIR laser irradiation. Drug and dye molecules are released from nano pores of the implant.

2.5 Modelling analysis of drug release

According to the cumulative MTX dosage release data above, we analyzed the drug release kinetics and mechanism by fitting drug release curve into following models. The fitting of each model was evaluated based on correlation coefficient (R^2) values.

2.5.1 Zero order

Zero order which diffusion coefficient is independent of concentration of drug in the system. The equation is as below:

$$M_t = M_0 + k_0 t$$

M_0: total drug released from implant; M_t: amount of drug release in time

t: time in hours

2.5.2 First Order

First order demonstrates a system which diffusion coefficient of drug release is dependent of concentration. The equation could be described as below:

$$ln\frac{M_0 - M_t}{M_0} = -k_1 t$$

M_0: total drug released from implant; M_t: amount of drug release in time

K_1: first order constant; t: time in hours

2.5.3 Static analysis of drug release kinetics

Statistical comparisons between slope (k_1 and k_0) values of release kinetics of different dosage implants were made by Single Factor ANOVA, p-value <0.05 was considered significant. All experiments were at least repeated three times.

2.6 In vitro fluorescence calibration of remaining MTX in implant

Implants were loaded with 700 μg, 800 μg and 900 μg MTX dosage of concentrated MTX/Cy5 co-encapsulated liposome suspension. These implants were characterized by optical imaging and fluorescent imaging. The distance between implants to lens was around 1 cm to 1.2 cm. The fluorescent intensity of in the implant was analyzed by ImageJ with mean gray values. A calibration curve was made based on the mean gray value in the implant corresponding to the dosage loaded.

2.7 In vivo drug release of implant

All animal studies were performed at the Laboratory Animal Medical Services and approved
by the IACUC at the University of Cincinnati. The drug release of our laser-activated implant was determined in the New Zealand White rabbit (~2 kg). Before injection, the implant was sterilized by ultraviolet (UV) for 30 minutes and then inserted into a sterilized 17-gauge needle.
The implant was injected into the vitreous at the pars plana through the sclera around 3−5 mm away from the limbus performed by a surgeon.

2.7.1 Characterization of in vivo drug release test

Rabbit was characterized by optical imaging, ultrasound imaging and fluorescent imaging before and after injection. A guide beam was used to aim the center of injected implant. After alignment, our near infrared pulsed laser (1064 nm, 1.1 W/cm2) replaced the position of the guide beam and tested the alignment again for 10 seconds. Then irradiation for the implant started from the left of the implant, after each 5 seconds irradiation, the beam moved to another spot, until 4 spots were irradiated. We started from the first spot from the left again, this cycle had been done 3 times before 3 mins cooling time. After cooling, the same irradiation pattern was performed as described above until we reached 10 mins effective irradiation in total. After the laser irradiation, optical imaging, ultrasound imaging and fluorescent imaging were used to characterize the implant and surrounding area in the vitreous. Fluorescent imaging was used to capture the fluorescent signal of the Cy5 encapsulated in implant and in the vitreous. During the measuring, the lens of microscopy was contacted with the cornea of the rabbit with eye gel (Genteal Tears, Fort Worth, TX). The distance between the lens to the implant was fixed (around 1 cm). The fluorescent source intensity was adjusted to maximum value and maintained during characterization.

2.7.2 In vivo drug release analysis

Drug release analysis was achieved by analyzing the fluorescent intensity in the implant. The fluorescence intensity was quantitively analyzed by mean gray value using ImageJ. The range of the mean gray value was as follows: min = 0 and max = 255. By using the calibration curve prepared in vitro, we could estimate the remaining drug in the implant after irradiation. The different between the remaining drug after each characterization was the estimated dosage of released drug.

2.8 Tissue processing and histological examination

3 Results

3.1. Characterization of laser-activated drug implant

The thickness of the implant 2-layer wall was around 33 μm. The inner radium of the implant was around 364.25 μm (Figure 2 middle). Rehydrated MTX/Cy5 liposome was diluted 400 times by PBS and characterized by UV-vis spectrometry and TEM. The diameter was around 1 μm. The concentration of MTX (around 250 mg/ml) was calculated based on the calibration. The lumen was filled with rehydrated MTX/Cy5 liposome. The liposome loaded implant then was inserted into a 17-gauge needle (Figure 2 right).

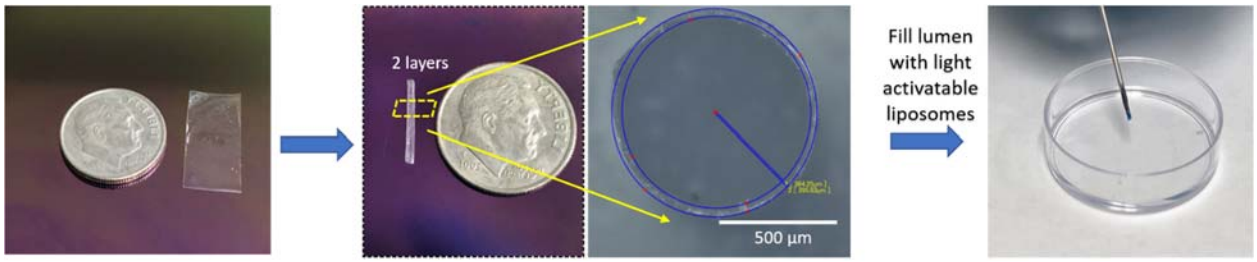


Figure 2. Process of preparation of dye/drug co-encapsulated liposome loaded in a two-layers PLGA implant.

3.1 In vitro drug release of implant after laser irradiations

Cumulative release profiles of MTX from 1000 μg implant and 500 μg implant after 15 mins irradiation were demonstrated in Figure 3. Implant initially loaded with 1000 μg implant showed a rapid release within first 24 hours. From the 24 hours to 168 hours of the 1000 μg implant, the trendline was getting less steep with time. Until the 168 hours, the trendline was close to even. For 500 μg implant, the trendline was relatively linear with steady cumulative dosage increasement.

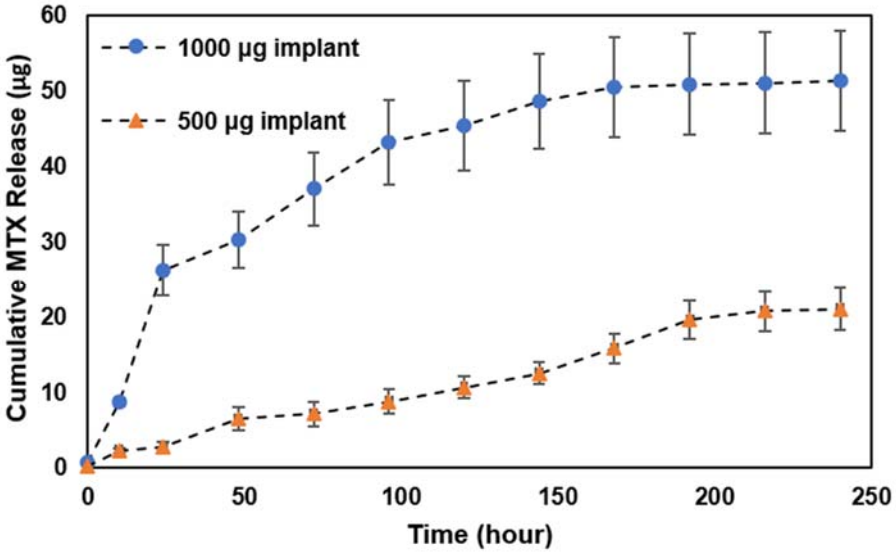


Figure 3 Cumulative dosage of MTX released from implants after 15 mins irradiation with guidelines (Blue circle) 1000 µg initial MTX dosage implant (orange rectangular) 500 µg initial MTX dosage implant.

3.2 Modeling of in vitro drug release kinetics

Accumulative MTX release data was analyzed by fitting into first order release model and zero order release model. For the first order release model, 1000 µg showed a higher correlation coefficient (R^2=0.9759) than 500 µg implant (R^2=0.9201). At the meantime, for the zero-order release model, 500 µg implant showed a relatively linear trendline with R^2=0.9814. However, the 1000 µg implant zero order fitting curve was way off the trendline with R^2=0.3597.

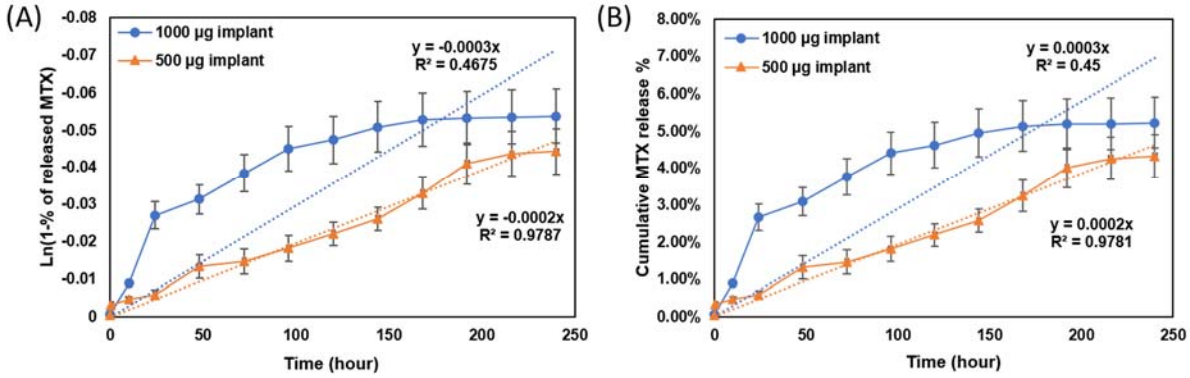


Figure 4 MTX release kinetics fitting (A) first order drug release (B) zero order drug release (Blue: 1 mg implant, Orange: 500 µg implant) after 15 mins pulsed NIR laser irradiation.

The P-value for first order fitting groups was 0.023227.

The P-value for zero order fitting groups was 0.00164.

3.5 Implant injection and in vivo laser irradiation

Characterization of implant before and after injection and the setup for laser irradiation were shown in the Figure 5. Figure 5(A) and 5 (C) were optical images of rabbit eye before implant injection and after implant injection, respectively. Ultrasound images for rabbit eye before and after were revealed in Figure 5 (B) and (D). In Figure 5 (D), cylindrical shape implant was in the center of the eye. Rabbit under anesthesia was ready for irradiation after laser alignment was demonstrated in Figure 5 (E).

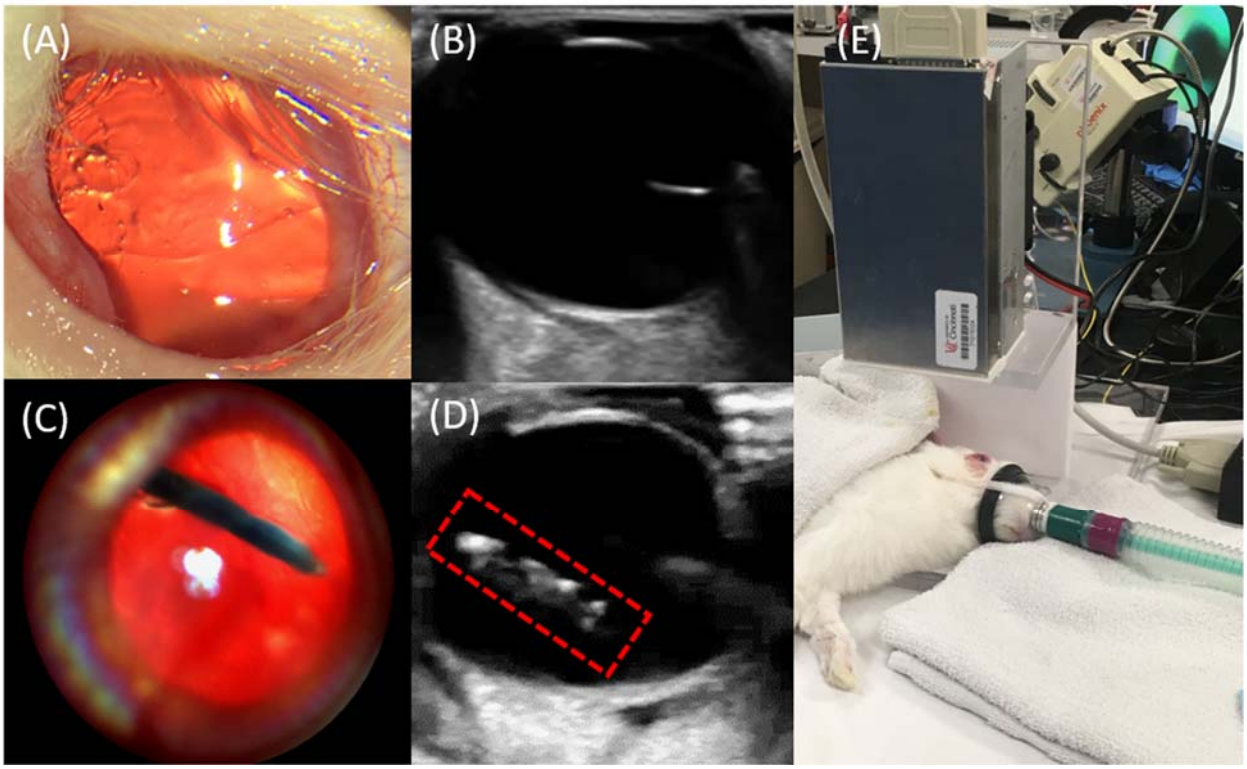
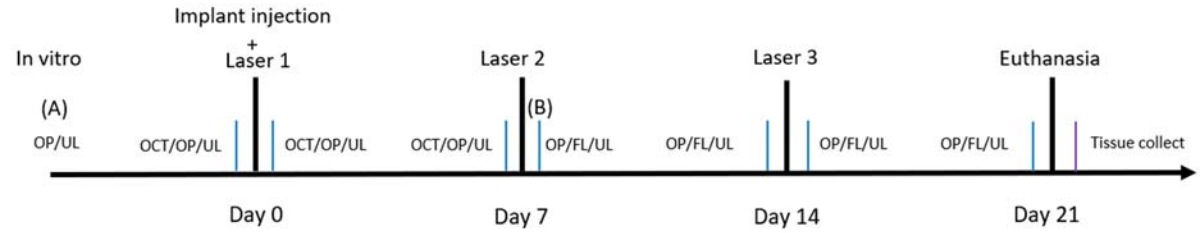


Figure 5 (A) and (C) are optical images of rabbit eye before and after implant injection. (B) and (D) are ultrasound images before and after implant injection. (E) NIR laser irradiation setup after alignment.

3.6 In vivo drug/dye release after laser irradiation

Implant injection, irradiation and detail examination time schedule in this study was shown in Scheme 1. Examination included optical imaging (OP), ultrasound imaging (UL) and fluorescent imaging (FL). Time for examination was around 40 mins. For example, before Laser 1, OP, UL and FL were applied and they took around 40 mins, then Laser 1 with 10 mins effective irradiation was applied. The total time for irradiation process was around 40 minutes which included the cooling time, then the following examination was applied right after last cooling cycle. The time between (C) and (D)

was around 1.5 hours. An individual 1000 μg MTX/Cy5 implant was prepared for fluorescent intensity comparison (A). Examination of (B) was right after the Laser 2.



Scheme 1 Laser and examination schedule in this study. Examination is set before and after 40 minutes of each laser irradiation.

Mean gray of 1000 μg implant without irradiation was shown in Figure 6(A). Mean gray value of in vivo drug release test in the implant and in the vitreous at (B), (C) and (D) were demonstrated in Figure 6. From Figure 6 (B) to Figure 6 (C), the mean gray value next to implant in vitreous decreased from around 33 to 15. Before Laser 3, in Figure 6 (C), the mean gray values in the implant were 70, 40 and 62 from top end to bottom end, respectively. After irradiation in Figure 6 (D), the mean gray values in implant decreased to 62, 38 and 58 which indicated that MTX/Cy5 had been released after irradiation within 1.5 hours. At the meantime, the mean gray values in the vitreous increased with distance trend (small font). The closer area demonstrated higher mean gray values than the far area.

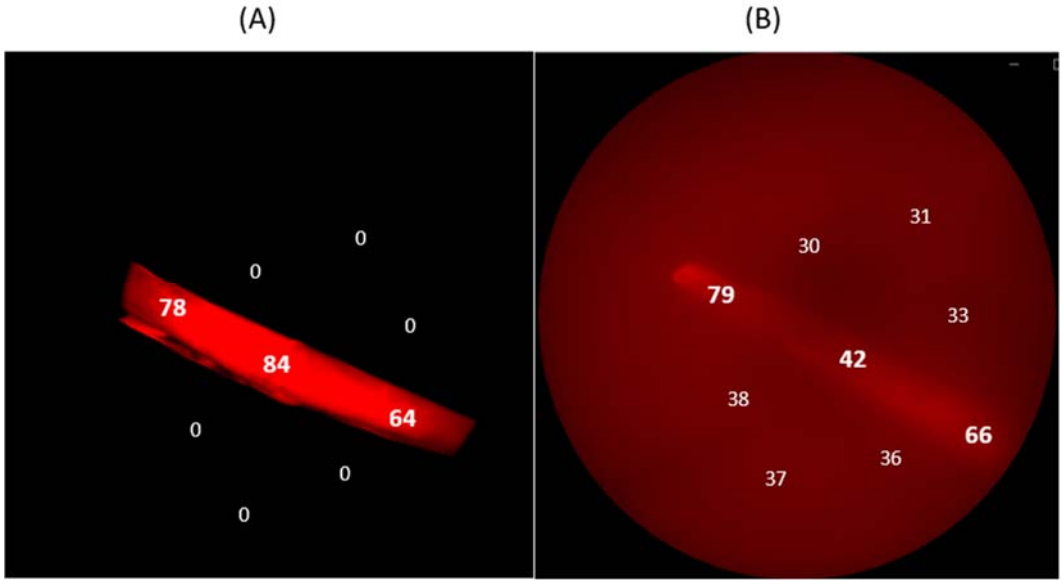


Figure 6 Mean gray values of fluorescent images of different areas (A) in vitro without laser and in rabbit eye at (B) 40 minutes after Laser 2.

Mean gray value of entire implant and whole vitreous were shown in Table 1. MTX dosage remained in implant was estimated from the calibration curve of MTX dosage in implant related to mean gray value. From Figure 6 (A) to Figure 6 (B), the mean gray value in the vitreous decreased from 41.5 to 40.9 corresponding to dosage reduced from 873.2 μg to 868.7 μg.

Image	(A)	(B)
Mean gray value in implant	64.1	44.6
Mean gray value in vitreous	0	33.9
Estimated MTX remained in implant (μg)	1000 μg	898.7

Table 1 Mean gray value of Cy5 fluorescent intensity in the implant and in the vitreous at (B) day 7 after 2nd irradiation (C) day 14 before the 3rd irradiation (D) day 14 after 3rd irradiation with estimated MTX dosage remaining in the implant.

3.7 Safety of laser irradiation ()

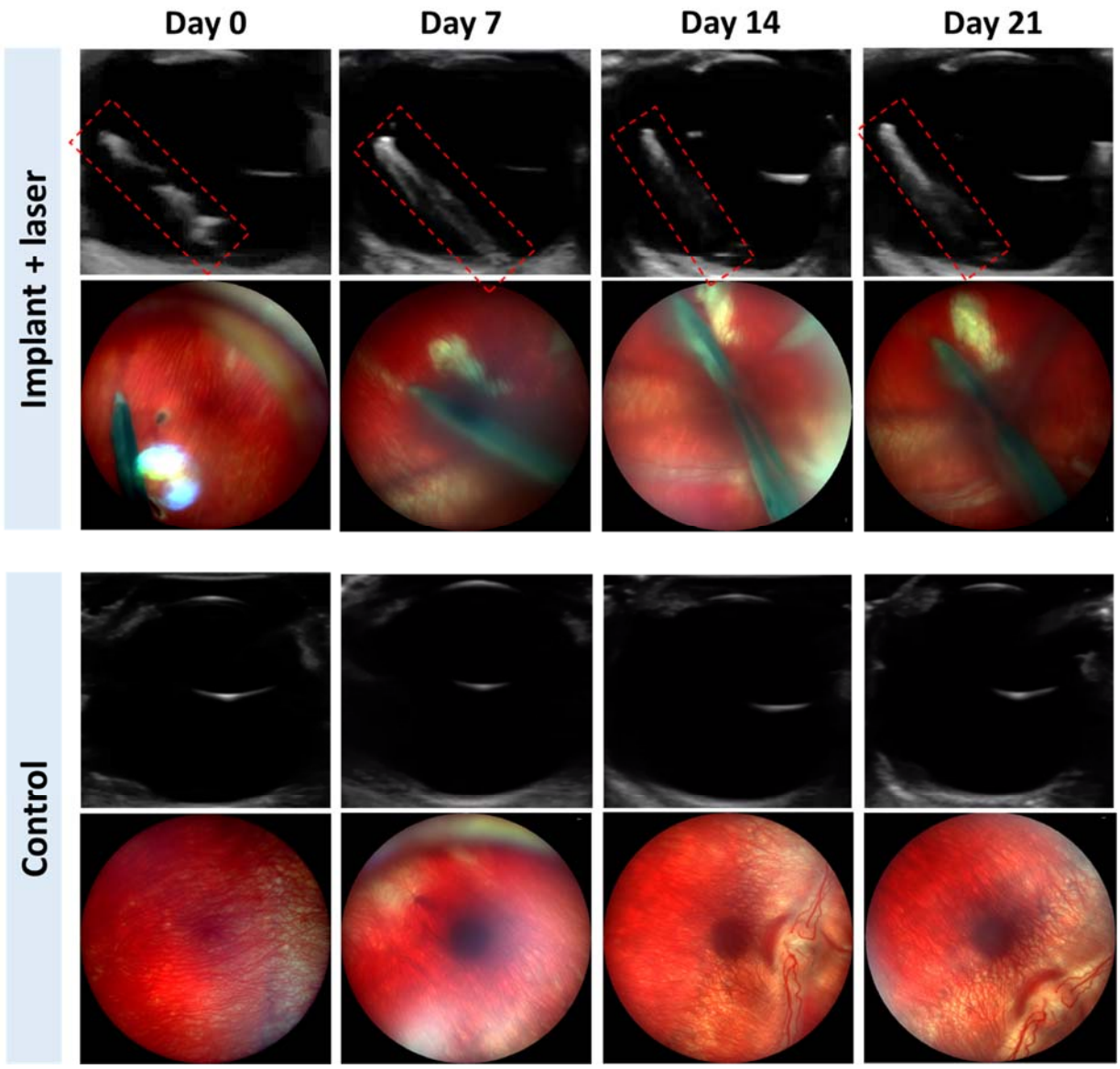


Figure 7. Ultrasound and optical images of the implant-injected eye showing the retina and the implant, and the control eye on Day 0, Day 7, Day14 and Day 21, respectively.

Figure 8. Gross picture of the implant-injected rabbit eye after removing the lens and cornea, showing the intactness of the implant.

4 Discussion

From the in vitro drug release test results, 1000 μg implant showed a relatively rapid release especially at the first 72 hours, however, 500 μg demonstrated a gradual release. The drug release kinetics demonstrated that 1000 μg implant release pattern followed first order kinetics while 500 μg implant followed zero order kinetics. In terms of correlation coefficient R^2,

At the meantime, single-factor ANOVA results proved that p-values were all below 0.05 which showed significant different between these two different dosage groups. The different release pattern of different dosage might be due to the packing density of gold nanorod. 1000 μg implant theoretical contained twice amount of gold which could generate more heat and resulted in a rapid release at the beginning. The heat generated by gold nanorod could contribute to the liposome membrane melting and the diffusion energy providing. (Reference searching)

In vivo drug release was successfully achieved by showing the number changes in the vitreous and in the implant (Figure 6 (B), (C) and (D)).From Figure 6 (B) to Figure (C), these number change in the vitreous revealed the clearance functionality of vitreous. Significant amount of Cy5 was cleaned out by vitreous within 7 days. At the meantime, compared with (C) and (D), which (C) was captured before Laser 3 and (B) was taken after laser. The increasing mean gray value in vitreous demonstrated the quick response of laser-activated drug release from implant which happened within around 1.5 hours. The increasement also proved that we could release the drug from the implant by laser irradiation repeatedly. From the dosage change, after Laser 2, the estimated remaining dosage in the implant was around 898 μg. The average drug release for Lase 1 and Laser 2 was around 50 μg (less than 50 μg due to purification stage). However, compared with (B) and (D), around 30 μg MTX released by Laser 3. It

also showed the evidence that most of our photothermal reagent, the gold nanorod, still maintained the shape which could generate heat to melt the liposome membrane after. (cite Zheng's paper)

The histological …..

5 Conclusion

In this study, we successfully released the drug from our nanoporous PLGA implant by using pulsed NIR laser irradiation both in vitro and in vivo. We analyzed the drug release kinetics in vitro by fitting our data in zero order and first order models. At the meantime, we took advantage of the fluorescent signal in the dye/drug co-encapsulated liposome to estimate the amount of drug remaining in the implant. This could potentially be used for quantitatively predict the drug release kinetics in vivo without scarifying animals frequently. In addition, the safety of in vivo drug release

Acknowledgment

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References

Supplementary materials

1. Calibration curve of MTX concentration vs O.D from UV-vis spectrometry

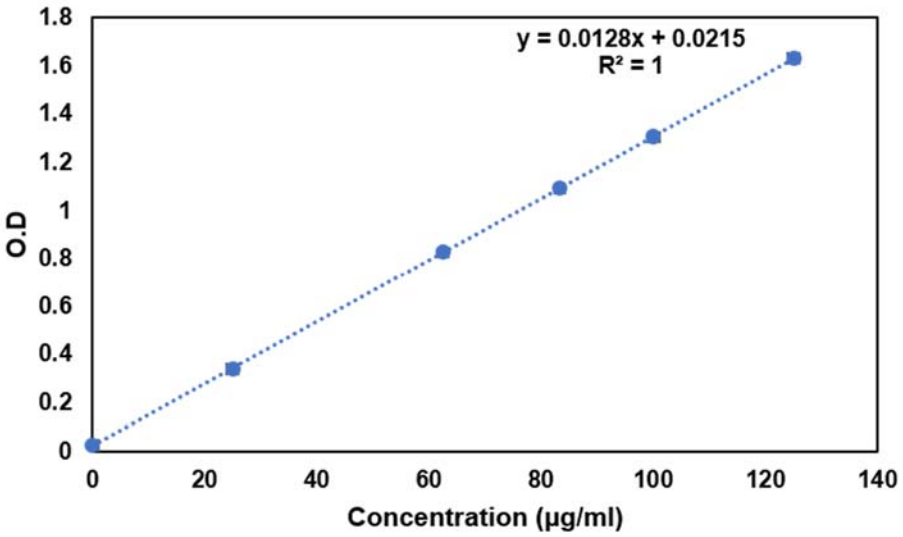

Figure 8 Calibration curve of MTX concentration in PBS with O.D.

2. Calibration curve of MTX dosage with mean gray value

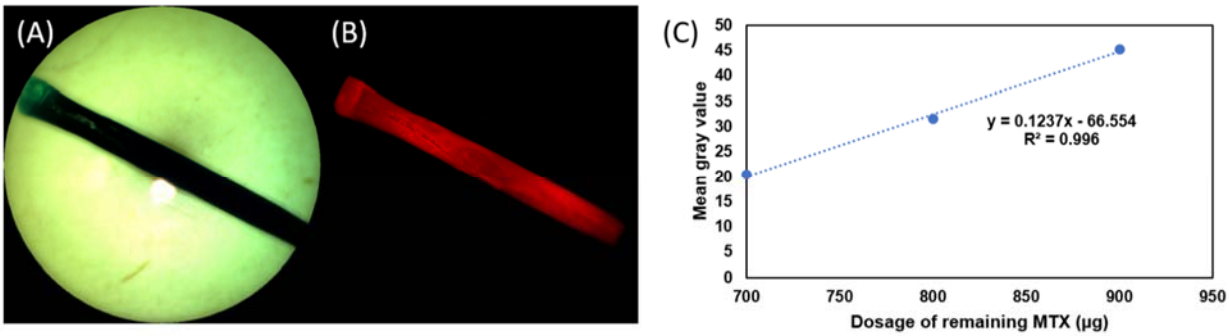

Figure 9 MTX/Cy5 co-encapsulated liposome implant contains MTX 900 µg (A) optical image (B) Fluorescent image (C) calibration of implant contains different dosage.

1. del Amo, E. M.; Rimpelä, A.-K.; Heikkinen, E.; Kari, O. K.; Ramsay, E.; Lajunen, T.; Schmitt, M.; Pelkonen, L.; Bhattacharya, M.; Richardson, D.; Subrizi, A.; Turunen, T.; Reinisalo, M.; Itkonen, J.; Toropainen, E.; Casteleijn, M.; Kidron, H.; Antopolsky, M.; Vellonen, K.-S.; Ruponen, M.; Urtti, A., Pharmacokinetic aspects of retinal drug delivery. *Progress in Retinal and Eye Research* **2017,** *57*, 134-185.

2. Kane, F. E.; Burdan, J.; Cutino, A.; Green, K. E., Iluvien™: a new sustained delivery technology for posterior eye disease. *Expert Opinion on Drug Delivery* **2008,** *5* (9), 1039-1046.

3. Bezatis, A.; Spital, G.; Höhn, F.; Maier, M.; Clemens, C. R.; Wachtlin, J.; Lehmann, F.; Hattenbach, L. O.; Feltgen, N.; Meyer, C. H., Functional and anatomical results after a single intravitreal Ozurdex injection in retinal vein occlusion: a 6-month follow-up – The SOLO study. **2013,** *91* (5), e340-e347.
4. Allergan, Ozurdex ten years **2019**.
5. He, X.; Yuan, Z.; Kao, W.; Miller, D.; Li, S. K.; Park, Y. C., Size-Exclusive Nanoporous Biodegradable PLGA Capsules for Drug Delivery Implants and In Vivo Stability in the Posterior Segment. *ACS Applied Bio Materials* **2020,** *3* (3), 1722-1729.