Video Article

Slow-release Drug Delivery through Elvax 40W to the Rat Retina: Implications for the Treatment of Chronic Conditions

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Abstract

Diseases of the retina are difficult to treat as the retina lies deep within the eye. Invasive methods of drug delivery are often needed to treat these diseases. Chronic retinal diseases such as retinal oedema or neovascularization usually require multiple intraocular injections to effectively treat the condition. However, the risks associated with these injections increase with repeated delivery of the drug. Therefore, alternative delivery methods need to be established in order to minimize the risks of reinjection. Several other investigations have developed methods to deliver drugs over extended time, through materials capable of releasing chemicals slowly into the eye. In this investigation, we outline the use of Elvax 40W, a copolymer resin, to act as a vehicle for drug delivery to the adult rat retina. The resin is made and loaded with the drug. The drug-resin complex is then implanted into the vitreous cavity, where it will slowly release the drug over time. This method was tested using 2-amino-4-phosphonobutyrate (APB), a glutamate analogue that blocks the light response of the retina. It was demonstrated that the APB was slowly released from the resin, and was able to block the retinal response by 7 days after implantation. This indicates that slow-release drug delivery using this copolymer resin is effective for treating the retina, and could be used therapeutically with further testing.

Video Link

The video component of this article can be found at http://www.jove.com/video/51563/

Introduction

The treatment of chronic diseases such as diabetes and high blood pressure presents many challenges, as these diseases generally require treatment for extended periods of time, often for life. This has called for the development of slow release drug delivery systems, which reduce the need for frequent dosing. The effectiveness of these slow release methods has been demonstrated through the development of insulin pumps, to reduce the number of insulin injections needed to treat diabetes. Chronic diseases of the eye, particularly those affecting its inner layers, require the frequent administering of drugs through invasive procedures. One such chronic disease affecting the human eye is age related macular degeneration (AMD). It affects the central retina, which is a layer of neural tissue situated at the back of the eye responsible for initiating vision. AMD is the leading cause of blindness in the Western World[1]. A particular challenge with treating retinal diseases is that the drug is required to reach this deep layer in the eye, which often requires invasive methods of delivery. Drugs are usually administered to the vitreous chamber and retina using intravitreal injections. However, with each injection there is a risk of postinjection complications, including endophthalmitis, retinal detachment, cataract and vitreous hemorrhaging[2]. This risk is multiplied with every reinjection of the drug.

Reducing the need for multiple injections would be a major benefit in the treatment of AMD. To treat the wet form of AMD, where new vessel growth is a hallmark, the established therapeutic strategy is to target the endothelial growth factors (VEGF) using VEGF inhibitors[3]. At present, these are delivered through repeated intravitreal injections. Similarly, in the treatment of macular oedema, a common complication of diabetic retinopathy, corticosteroids are delivered through repeated injections[4]. Delivery of these drugs through slow release methods could indeed reduce the risk of postinjection complications.

The notion of a slow release drug delivery system was first described using a silicone rubber vehicle to deliver small molecules into animal tissues[5]. Since then, other slow release methods have been developed to deliver larger molecules, several of which have been tested in the eye. Particle carriers, such as biodegradable microspheres, poly-lactide-co-glycolide (PLGA) nanoparticles and phospholipid vesicles (liposomes) can be useful as delivery vehicles[6,7]. PLGA nanoparticles and liposomes have been compared in an *in vitro* environment for their ability to deliver anti-cancer agents across the sclera over time[7]. Both vehicles were effective in slowly releasing the drugs. However, the study was only conducted in an *in vitro* environment. Bochot *et al.* (2002)[8] tested the efficacy of liposomes to deliver molecules to the retina *in vivo*. They have

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demonstrated that liposomes successfully deliver small oligonucleotides to the rabbit retina. The authors suggested that liposomes could be beneficial in the treatment of retinal diseases[8]. However, the nature of these vesicles to float in the vitreous means that they will likely blur or impair vision[9].

Okabe *et al.* (2003)[10] used non biodegradable polymer discs made up of 33% ethylene-vinyl acetate to apply beta-methasone in rabbits. They implanted discs into a scleral pocket and demonstrated the effective release of the drug into the vitreous and retina for up to one month[10]. However, in this particular protocol, the implant was relatively large and rigid, and required a more complex surgical procedure including a large scleral incision and suturing.

An earlier study investigated the tissue response to various polymer vehicles by implanting them into the cornea of rabbit eyes, and found that ethylene-vinyl acetate copolymers washed in alcohol did not cause inflammation or irritation. These complexes were shown to sustain the delivery of larger compounds into animal tissues for extended periods of time, some exceeding up to 100 days dependent on the drug[11]. One such type of copolymer resin has been developed industrially in the form of Elvax 40W (40% by weight ethylene-vinyl acetate comonomer content with a 'W' amide additive to improve pellet handling). This copolymer resin is an inert substance which is stable at both room and body temperature. It has not been shown to cause allergies or toxicity in biological tissue. This resin has effectively delivered a large variety of drugs in different experimental models that investigate the functions of various systems, such as the mammary ductal system[12], the primary auditory cortex[13,14], and the frog visual system[15]. This resin has also been used in the eye to deliver drugs to developing turtle[16,17], chicken embryo[18,19], and adult ferret retinas[20]. In the rat central nervous system, the resin has only been used in the brain[21-23], but its use in the rat eye has not been documented.

The advantages of using this copolymer resin to deliver drugs slowly to the retina over other methods, is that it is a stable compound that does not cause inflammation or irritation in the eye. Unlike particle carriers, the drug-resin complex would not impair vision after implantation, as it usually remains at the site of delivery instead of floating in the vitreous. It would only require a simple implantation process into the vitreous cavity close to the limbus of the eye, and would not require suturing after implantation. Recently, there has been an emergence in several novel delivery systems, such as encapsulated cell technology (ECT)[24,25], hydrogels[26], and microfilms[27]. However, the method used in the current study for preparing and delivering the drug-resin complex is both easy to follow and inexpensive, thereby being more advantageous for use in a basic research environment. The challenge of using this complex to deliver long-term drug treatments is to determine the optimal concentration of the drug that will maximize the therapeutic benefits of having fewer intravitreal injections.

This paper aims to demonstrate the use of the drug-resin complex for long-term treatment of the adult rat retina. The efficacy of this mode of delivery is tested using the glutamate analogue 2-amino-4-phosphonobutyrate (APB) as the drug. APB blocks the light response of the ON bipolar cells by mimicking glutamate, an endogenous neurotransmitter in the retina[28]. When APB competes with glutamate for its receptor, it blocks the light response. APB has been used in physiological studies to control retinal function and measure its effect using electrophysiological methods such as electroretinography (ERG). In previous studies, APB has been used for both short-term[29] and long-term treatment of the developing retina; the latter involved giving a single dose via intraocular injection daily for 30 days[30]. The same amount of APB (0.092 mg in sterile saline, at a concentration of 50 µM) was used for all injections as suggested in previous work[28,29,31]. We chose APB to test the drug-resin complex as a slow-release vehicle to deliver drugs into the eye. The methods outlined in this study are similar to previously described methods involving the preparation of the drug-resin complex[16,32]; however we also detail its use specifically in the adult rat eye. After surgical implantation of the APB-loaded resin into the eye, ERG was performed to establish whether APB abolishes the retinal light response, and therefore whether APB has been successfully released into the vitreous and retina.

Protocol

All experiments conducted were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and with the approval of the University of L'Aquila Animal Ethics Committee and the Australian National University Animal Experimentation Ethics Committee. Adult rats (P100-200) were used throughout this study.

1. Prepare the Copolymer Resin Pellets

1. Place 20 Elvax 40W pellets into a small glass beaker in the fume cupboard.
2. Fill the beaker with 100% ethanol ensuring to cover all pellets.
3. Cover the beaker with Parafilm, and soak the pellets in 100% ethanol at room temperature for 7-10 days. NOTE: From this step onwards, do not use metal or plastic to handle or store the resin.

2. Prepare the Drug Solution for Loading

1. Dissolve the drug of choice in 0.1% dimethyl sulfoxide (DMSO). Make the drug solution up to 40 µl in volume. Load the resin with three times the concentration of the drug that would be given in a single dose.

3. Prepare and Load the Drug-resin Complex

1. Transfer the 20 washed pellets to another small glass beaker. Dissolve the pellets in 4 ml dichloromethane for approximately 45 min. Cover the beaker with Parafilm while the pellets are dissolving.
2. Prepare a solution of Fast Green FCF by dissolving it in 0.1% DMSO to a concentration of 0.001 mg/ml.
3. Take one pipette, and draw up 40 µl of the drug solution prepared earlier. Load another pipette with 40 µl of Fast Green solution.
4. Add the two solutions simultaneously into the beaker. Mix rapidly using a glass stirring rod until the green dye is uniformly distributed throughout the mixture.
5. Promptly transfer the beaker onto dry ice for 10 min to fast freeze the resin.

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6. Set up an “evaporative chamber” to evaporate the solvent. Fill a larger container up to one-third with calcium sulfate pebbles. Create a well in the pebbles, and carefully place the beaker in the well so that the pebbles reach up to half of the beaker, to stabilize the beaker in the chamber.
7. Cover the larger outside container with Parafilm.
8. Transfer the evaporative chamber to the freezer. Keep at -20 °C for 2-3 weeks.

4. Prepare the Drug-resin Complex for Surgical Implantation

1. Remove the evaporative chamber from the freezer. Transfer the solid drug-resin block to a glass dish kept on ice.
2. With the aid of a microscope or loupe, cut a piece approximately 0.05 mm in diameter and 0.1 mm in length from the block using a trephine or punch tool.
3. Wrap the remainder of the block in freeze-proof material (*e.g.*, aluminum foil) and keep at -20 °C until required.

5. Surgically Implant the Drug-resin Complex into the Rat Eye

1. Prepare the animal for surgery. Anesthetize using an intraperitoneal injection of a mixture of ketamine (100 mg/kg body weight) and xylazine (12 mg/kg body weight). Observe the animal until full anesthesia is achieved. NOTE: The animal should have a loss of movements (except breathing), and a loss of corneal and toe pinch reflexes.
2. Transfer the fully anesthetized animal to the surgery table. Use mydriaticum (*e.g.*, atropine sulphate), and local anesthetic (*e.g.*, tetracaine hydrochloride) drops on the eye(s) undergoing surgery. Apply artificial tears to keep the cornea moist during surgery. Use disposable needles and sterile instruments throughout the surgery.
3. Stabilize the eye using a pair of blunt curved forceps after the pupil is fully dilated. Use an operating microscope to make a full depth puncture wound to reach the vitreous, approximately 2 mm from the limbus using a 25 G needle.
4. Insert the prepared piece of the drug-resin complex into the puncture wound using a pair of fine, pointy forceps (*e.g.*, tweezers) that are able to penetrate the puncture site.
5. As prophylaxis for bacterial infection, apply antibiotic ointment to the eye, to prevent inflammation as well as preventing corneal dryness while the animal is recovering.
6. Observe the animal until full recovery. Do not leave the animal unattended until it has regained consciousness. Do not return the animal to the company of other animals until fully recovered. If the animal shows signs of stress or discomfort, analgesics should be used according to the approved ethics protocol.
7. Follow the usual experimental procedures for collection and analysis of the tissue.
8. At the completion of experiments, euthanize the animal with an intraperitoneal injection of a pentobarbital (60 mg/kg body weight) 2% lidocaine hydrochloride solution.

NOTE: Lignocaine Hydrochloride minimizes local discomfort.

Representative Results

Full-field electroretinography (ERG) was used to detect the effect of APB on the retina. For details on how the ERG was performed, refer to the following studies[33,34]. In brief, after anesthetizing the animal, a gold electrode was placed on the cornea of the eye, and the reference electrode was placed in the anterior scalp between the eyes, to record the electrical activity of the retina. Stimuli were generated using an electronic flash unit and the intensity ranged from 10 to 10^6 photoisomerization, which represents the number of photosensitive molecules activated per rod. In these experiments, ERG recordings were taken at 1 and 7 days post procedure. The right eye of each animal was implanted with the drug-resin complex, whereas the fellow left eye was used as a non treated internal control.

Figure 1 shows the changes in the amplitude of the b-wave with an increase in photoisomerization per rod (φ). In the control eye, the amplitude of the b-wave increases as the flash intensity increases. In **Figure 1A**, retinal response recordings are given 1 day after implantation. The implanted eye showed a similar amplitude pattern to the control eye. The amplitude difference between the two eyes became significantly different at 10^6 φ ($p < 0.0001$). This indicates that at this early stage, the drug-resin complex does not have an effect on retinal response until reaching higher flash intensities. **Figure 1B** demonstrates changes in the ERG response at 7 days after implantation. The b-wave is abolished at all levels of flash intensity in the implanted eye, when compared to the control eye, which has amplitudes comparable to the 1 day recordings. This is significantly different to all amplitudes for the implanted eye at 1 day ($p < 0.0001$). This shows that the retinal response has been abolished by (one week) after the implantation of the drug-resin complex into the eye.

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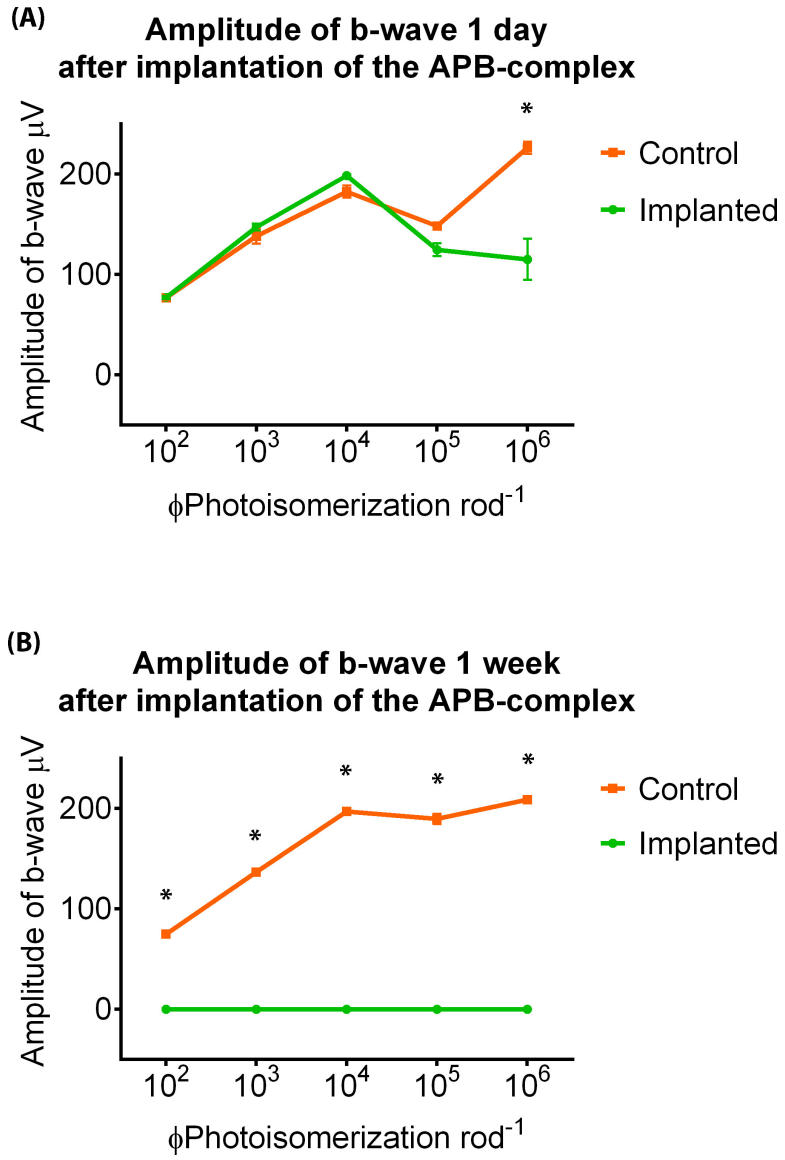

Figure 1. The amplitude of the b-wave at 1 day and 1 week after the implantation of the drug-resin complex. For both panels, the control eye is shown in orange (squares), whereas the implanted eye is shown in green (circles). The symbol * represents significance at this point between the two eyes, where p <0.0001. For all groups, n = 5. (**A**) The amplitude of the b-wave 1 day after the implantation of the drug-resin complex into the eye. The amplitude of the b-wave in relation to the flash intensity (photoisomerization per rod φ) is an indicator of retinal response. There is no significant difference in the b-wave amplitude between the eyes at all flash intensities except at 10^6φ, where the implanted eye has a lower amplitude. (**B**) The amplitude of the b-wave 1 week (7 days) after implantation. The amplitudes recorded from both eyes are significantly different at all flash intensities. In the implanted retina, amplitude of b-wave was not measurable at any stimulus intensities.

Discussion

This paper demonstrated the use of a drug-resin complex for the slow-release delivery of drugs to the retina. We aimed to present a method which is relatively inexpensive and easy to apply in a small animal model.

Given that the function of APB is to act as a glutamate analogue, it will block the retinal response in the eye. The results show that APB caused the blockage of the retinal response by one week postimplantation. This indicates that APB was successfully released into the vitreous and retina, and that its effect was limited to the eye that the drug-resin complex was implanted into. The full effect of the drug became obvious one week after implantation (**Figure 1B**). **Figure 1A** shows that at 1 day after implantation, a significant reduction of the b-wave was only present at the 10^6φ intensity in the implanted eye. This may indicate that at this early time point, only the cone pathway, which responds to higher light intensities, was affected. To determine the length of time that the resin can deliver the APB to the retina, further time points should be investigated. It is proposed that recordings at further time points within the 0-7 day span should be taken, for example, at 3 and 5 days after implantation, to determine exactly when the retinal response is fully abolished. Additionally, recordings should be taken beyond 7 days, to determine how long the APB is released for. This would be evident as the retinal response would continue to be abolished if APB is continually being released slowly into the vitreous.

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In this study, the drug-resin complex was found to be safe for delivery to the retina. APB was dissolved in 0.1% DMSO. At concentrations equal to or higher than 0.6%, DMSO can cause retinal toxicity. However, 0.1% DMSO has been found to be a safe vehicle for the delivery of compounds to the rat eye[35], and this concentration was used throughout all experiments in this study. The implants were inserted transclerally into the vitreous cavity at the ora serrata. The implants were observed to remain at the ora serrata in cryosections of the rat eye, which was collected after the ERG recordings were made. This positioning of the implant guarantees that the drug-resin complex does not cause vision impairment. This method involving the drug-resin complex has been tested with other compounds (publication in preparation), and it was found that no structural or functional damage to the eye was observed up to two months after implantation. A total of 60 animals were implanted, and in 90% of cases the implant position remained unchanged.

Several modifications can be made to the technique if necessary. In these experiments, three times the concentration of a single dose of APB was loaded into the resin (50 µM x 3 = 150 µM), and this should be used as a guideline for preparing the drug solution for loading. However, the effective concentration of the drug in the resin is variable, and should be tested and modified accordingly. The time points where analysis is conducted can also be modified, to determine how long the resin is effective at releasing the drug. In these experiments, it was shown that the stable effective dose of the drug is delivered through the drug-resin complex for at least one week, so it is recommended that analysis of the tissue is performed at least one week after the implantation. During the drug loading step, Fast Green was used to ensure that the mixture had homogenized. Other biologically safe color dyes can be used as an alternative to Fast Green, to visualize the uniformity of the drug within the resin. A limitation of this method is that the Fast Green dye is the only measure of uniform mixing. This technique could also be tested using alternative animal models and drugs, with only minor adjustments to the method. If a higher amount of the drug needs to be delivered, a larger size implant may be necessary. If successful in these models, this copolymer resin could be considered as a therapeutic option for delivering drugs slowly to the retina to have a continued effect over time.

There are several critical steps within the protocol where extra care should be taken. Plastic and metal instruments should not be used throughout the synthesis and handling of the resin. This prevents contamination of the polymer, as dichloromethane can dissolve plastic and corrode metal. Proper dryness of the drug-resin block should be achieved before using it for surgical implantation. Ensure that the beaker containing the drug and resin is fully exposed to the calcium sulfate in order to evaporate the solvent. Alternatively, a lyophilizer can be used to freeze dry the drug-resin. The drug-resin block is ready for use when the solvent has completely evaporated, and only a solid bluish block remains in the beaker. When cutting a piece from the block for implantation, each piece should only be prepared immediately before the surgery as the pieces are very small and are difficult to store. A trephine or punch tool should be used to cut pieces of equal size, ensuring that each piece contains approximately the same amount of the drug. Finally, when inserting the piece into the puncture wound made by the 25 G needle, use fine, pointy tweezers to carefully guide the piece into the wound. An operating microscope or high power loupe should be used as a visual aid.

Only hydrophilic drugs and compounds have been tested using this method. The drug-resin complexes have not shown any undesirable precipitation or byproduct formation, when the drug was mixed with the polymer and the dichloromethane. It is suggested that with each new drug, the concentration, half-life of effect, and any possible reactions with the resin mixture should be tested by the user to determine if the method is suitable.

Disclosures

The authors have nothing to disclosure.

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