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Silk polymer based adenosine release: therapeutic potential for epilepsy

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Abstract

Adenosine augmentation therapies (AAT) make rational use of the brain's own adenosine-based seizure control system and hold promise for the therapy of refractory epilepsy. In an effort to develop an AAT compatible with future clinical application, we developed a novel silk protein-based release system for adenosine. Adenosine releasing brain implants with target release doses of 0, 40, 200, and 1,000 ng adenosine/day were prepared by embedding adenosine-containing microspheres into nanofilm-coated silk fibroin scaffolds. *In vitro*, the respective polymers released 0, 33.4, 170.5, and 819.0 ng adenosine/day over 14 days. The therapeutic potential of the implants was validated in a dose-response study in the rat model of kindling epileptogenesis. Four days prior to the onset of kindling, adenosine-releasing polymers were implanted into the infrahippocampal cleft and progressive acquisition of kindled seizures was monitored over a total of 48 stimulations. We document a dose-dependent retardation of seizure acquisition. In recipients of polymers releasing 819 ng adenosine/day, kindling epileptogenesis was delayed by one week corresponding to 18 kindling stimulations. Histological analysis of brain samples confirmed correct location of implants and electrodes. We conclude that silk-based delivery of around 1,000 ng adenosine/day is a safe and efficient strategy to suppress seizures.

Keywords

Silk; Controlled drug release; Drug delivery; Adenosine; Epilepsy; Brain

1. Introduction

Pharmacoresistant epilepsy continues to remain a major health problem. Despite the availability of an armamentarium of antiepileptic drugs, about 30% of all epilepsies cannot be treated with systemic pharmacotherapy for a variety of reasons including pharmacoresistance and intolerable side effects [1]. In contrast to systemic drug delivery, focal drug delivery to the brain is generally devoid of systemic side effects and holds great promise for the therapy of

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epilepsy [2], which frequently is of focal origin [3-5]. Therefore, various cell and gene therapies are currently in development for the treatment of intractable epilepsy [6-10].

The recent identification of focal adenosine deficiency as major cause for seizure generation [11,12] provides a neurochemical rationale for focal adenosine augmentation therapies (AATs). Focal AATs can be accomplished by implanting adenosine releasing devices or cells into the vicinity of an epileptogenic focus [13]. The proof-of-principle for the therapeutic efficacy of AATs has been established in animal models of induced or spontaneous seizures [11,14-16]. These studies demonstrate, that focal AATs can suppress seizures [15,16], but might also be capable of retarding or preventing the progression of epileptogenesis [11,14]. In addition, focal AATs were devoid of systemic and sedative side effects [17], and augmentation of the adenosine response was effective in suppressing experimental seizures that were refractory to standard antiepileptic drugs [18]. Therefore, AATs hold great promise for the therapy of refractory focal epilepsies. However, hitherto used experimental AATs are not suitable for clinical development due to the use of animal cells [13] or the short-term duration of therapeutic effect in some of the approaches [16,19]. Further clinical development of AATs calls for the slow and sustained release of adenosine from safe and biocompatible brain implants. While cell and gene therapies for epilepsy – although promising – might be considered in the future, the most direct and safest route for the development of AATs would be the formation of biocompatible polymer-based brain implants for the sustained delivery of adenosine.

Slow, sustained release of small molecule drugs (e.g. adenosine) remains a major unmet challenge in the field of controlled drug delivery. This challenge emerges from the inability to generate highly crystalline biomaterials with tight control of processing windows, as regulators of diffusion rates. Further, these delivery systems would be advantageous if they were biodegradable, biocompatible and could be prepared and processed under ambient aqueous conditions to avoid loss of bioactivity of the drug to be delivered. While there are a number of biomaterials available for drug delivery devices, purified silk fibroin protein is a unique option that is particularly well suited to the needs listed above. Silk fibroin is biocompatible [20] and biodegrades slowly [21]. Degradation lifetimes of silk can be regulated by the extent of physical crosslinking for beta sheet formation, allowing control of degradation timeframes from weeks to years [21], an important property for sustained release applications. Silk also can be processed under aqueous and ambient conditions in water, thus allowing for the incorporation of labile biomolecules without loss of biological function or activity [22,23]. Further, the frequent use of silk sutures in brain and nerve tissue confirms the feasibility of implantation of silk biomaterials in the brain. For example, the relative safety and efficacy of silk sutures for arteriovenous malformation (AVM) and preoperative endovascular embolization has been demonstrated [24,25].

A diverse range of material formats can be generated from silk fibroin and consequently there exist several options to deliver small molecules using silk protein matrix systems. Silk has been previously explored as a vehicle for drug delivery in the form of drug entrapping films [26], as drug encapsulating microspheres [27] and via silk and drug nanofilm deposition or coatings [27,28]. Peptides and proteins also have previously been chemically coupled to silk fibroin using carbodiimide chemistry [29,30].

This versatility in manipulation of silk protein structure and morphology suggested novel delivery options potentially applicable to adenosine delivery. Since each form of silk has its own release profile that can be modulated via processing conditions, the level of control of small molecule drug release could be enhanced by integration of multiple carrier formats into one device or system, such as combinations of ultra-thin coatings, microsphere and macroscale silk films. The system studied in the present work was based on the integration of several

experimental strategies for a scaled, hierarchically structured protein material delivery approach to orchestrate control of adenosine delivery. The therapeutic potential of these novel AAT devices was tested in the rat kindling-model, in which progressive seizure development, could be studied and quantified as a function to different rates of adenosine release.

2. Materials and Methods

2.1. Implant Design

Implants designed to deliver the target doses (0, 40, 200 and 1,000 ng per day) were designed based on preliminary experiments to determine drug loading. Implants were also designed to split the target drug load roughly evenly between the three systems chosen for integration in the study, microspheres, macroscale films and nanofilms. The three variables used to impact the final adenosine dose were: (1) the concentration of microspheres in the silk solution that were formed into water-based, porous scaffolds, (2) the concentration of adenosine in the adenosine plus silk solution that the porous scaffolds were coated with to form the macroscale silk films, and (3) the number of layers of nanofilms deposited on the system. The composition of the implants and the theoretical amounts of adenosine loaded via each technique are listed in Table 1.

2.2. Implant Fabrication

The implant fabrication process is represented in Figure 1. Silk for the study was prepared from *Bombyx mori* cocoons as we have previously described [29]. Adenosine containing microspheres were prepared according to the MeOH based protocol described previously [27]. Briefly, 200 μ L of 10 mg/mL adenosine stock solution was mixed with 1 mL of 8% (w/v) silk aqueous solution, which was then added to 200 mg of 1,2-Dioleoyl-*sn*-Glycero-3-Phosphocholine (DOPC) phospholipid that had been dissolved in 1 mL of chloroform, then dried under N₂ to a film on the interior of a glass test tube. The solution is diluted, then repeatedly freeze-thawed, then lyophilized. Later, the microspheres were treated with methanol (MeOH) to remove the lipids and induce beta sheet silk physical crosslinks to stabilize the structures.

Water-based porous scaffolds were prepared as previously described [31] using the mixture of microspheres and silk solution to imbue the microspheres in the final porous scaffold. Briefly, 4 g of 500-600 μ m granular NaCl were added to 2 mL of 6% (w/v) silk solution mixed with microspheres in a plastic container and incubated at room temperature for over 24 hours. The scaffolds were then washed for 24 hours to leach out the sodium chloride. To obtain the desired implant geometry (0.6-0.7 mm diameter, 3 mm length), scaffolds were punched out with a 1 mm Miltex biopsy punch and then trimmed with a razor on either end. The porous scaffolds were next coated with macroscale drug-loaded silk films comparable to the films described previously [26]. Implants were soaked in a mixed silk and drug solution for 5 min, then incubated at 60°C for 15-20 minutes, then washed in a 90% MeOH solution.

Finally, nanofilm coatings were applied using our previously described protocol [28] but modified to accommodate coating of a 3D porous scaffold. Scaffolds were first dipped in a 2 mg/mL silk fibroin solution for 2 min, and then washed in 90:10 (v/v) MeOH/water solution for 1 min. Following the methanol wash, the scaffolds were dried at 60°C for 15 minutes. The dried scaffolds were then dipped in a 1 mg/mL adenosine solution for 2 min and dried at 60°C for another 15 minutes. These steps were repeated until the desired number of layers was achieved, ending with a silk layer. After drug loading, all implants were coated with three capping layers (three subsequent silk dips) to delay burst.

2.3. In vitro release studies

For each target dose (40 ng, 200 ng, and 1,000 ng) 3 implants were characterized for release kinetics in vitro (N=3). To evaluate release profiles, implants were immersed in 1 mL of Dulbecco's phosphate buffer, pH 7.2 (PBS) at 37°C. At pre-set time points (1, 2, 3, 4, 6, 10 and 14 days) the PBS was removed and replaced.

Adenosine content in the PBS samples removed from the system was measured using a modified fluorescence assay as previously described [32]. The collected PBS sample was transferred to a 1.5 mL Eppendorf tube and chloroacetaldehyde was added to a final concentration of 220 μ M chloroacetaldehyde. These tubes were capped and boiled for 20 min. Boiling of mixed adenosine and chloroacetaldehyde yields the fluorescent derivative 1,N⁶-ethenoadenosine. The fluorescence of the sample was measured with a plate reader (excitation = 310 nm, emission = 410 nm [33]. For each device at each time point, three fluorescence readings were taken and averaged.

2.4. Animals and surgery

All animal procedures were conducted in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care in accordance with protocols approved by the Institutional Animal Care and Use Committee and the principles outlined in the NIH *Guide for the Care and Use of Laboratory Animals*. Adult male Sprague-Dawley rats were used at a body weight of 280 to 300 g. All rats were acclimatized for one week before being used in the experiments. The rats were housed under a 12 h light / dark cycle (lights on from 8:00 A.M) with food and water provided *ad libitum*.

Anesthesia was induced with 3% isoflurane, 67% N₂O, 30% O₂ and maintained with 1.5% isoflurane, 68.5% N₂O, 30% O₂, while rats (N=22) were placed in a Kopf stereotactic frame. First, polymers with a target release rate of 0, 40, 200, and 1000 ng adenosine per day (N=5-6 per dose) were implanted using a stereotactic implantation device (internal diameter 0.7 mm, external diameter 1 mm) as described [34]. The polymer-loaded device was stereotactically inserted into the brain using a drill hole above the left hemisphere 2 mm rostral to bregma and 1.6 mm lateral to the midline. Using this drill hole the loaded device was inserted into the brain using an angle of 45° from vertical and an angle of 45° from midline. Thus, a diagonal injection tract was created aiming at a coordinate of 5.5 mm caudal to bregma, 5.5 mm to the right of the midline and 7.5 mm below the dura. Upon reaching the target site the 3 mm long polymer was released and deposited within the infrahippocampal cleft by slowly retracting the outer tube of the device. Finally, the device was fully retracted as described previously [34]. Thus, the implanted polymers were deposited within a formed cavity of 3 mm length within the right infrahippocampal cleft and adjacent to the electrode implantation site (Figure 5). The diagonal implantation approach pursued here and previously [11,14] is characterized by a number of important advantages: (i) Coverage of 3 mm of the dorso-ventral extent of the hippocampus by placement of the implants into the infra-hippocampal fissure; (ii) Minimization of damage to the ipsilateral hippocampus; (iii) Compatibility with the electrode-containing head-set of the animals. Next, a bipolar, coated, stainless steel electrode (0.20 mm in diameter, Plastics One, Roanoke, VA) was implanted into the right hippocampus and fixed with a head-set of dental acrylate. Coordinates for the hippocampal electrodes were (tooth bar at 0): 5.5 mm caudal to bregma, 5.5 mm lateral to midline, and 7.5 mm ventral to dura.

2.5. Kindling

Four days after surgery, the animals were stimulated unilaterally 6 times every second or third day with a Grass S-88 stimulator (1-ms square-wave pulses of 5 V at 50-Hz frequency for 10 s, 30-min interval between stimulations). Behavioral seizures were scored according to the scale of Racine [35]. Each animal received a total of 48 stimulations, which was equivalent to

8 test days spread out between day 4 and day 20 following polymer implantation. The electroencephalogram (EEG) was recorded for periods of 1 min before and 5 min after application of each stimulating pulse using a Nervus EEG monitoring system.

2.6. Histology

After completion of the experiments the rats were transcardially perfused with 4% paraformaldehyde in phosphate buffer (0.15M, pH 7.4). Brains were then post fixed in the same fixative for 6 hours and cryoprotected in 10% DMSO in PBS (v/v) before being cut into a total of 72 coronal sections (40 μ m thickness) covering the full extent of the lateral hippocampus (3.5 mm to 6.5 mm caudal to bregma). To characterize gross anatomy of the brain in particular in the implant region, and to verify the precise location of the kindling electrodes, a Cresyl violet stain was performed on every 6th section.

2.7. Statistics

In the *in vitro* studies standard deviations were calculated for the three repetitions per dose. The values for each of the three repetition implants loaded with the same dose were obtained by averaging three fluorescence readings per implant. Standard deviations are listed in Table 2. *In vivo* seizure data are based on N=5 rats for recipients of control polymers (0 ng adenosine) and for recipients of the 40 ng adenosine per day polymers, and N=6 for recipients of the 200 and 1,000 ng adenosine per day polymers. Individual seizure scores and afterdischarge durations were pooled and averaged for each of the 8 test days in each experimental group. Errors are given as $\pm$ SD and data were analyzed using one-way ANOVA with Student-Newman-Keuls Test. * P<0.05, ** P<0.01, *** P<0.001.

3. Results

3.1. Adenosine release kinetics in vitro

The average release of adenosine from the implants at each time point and the cumulative release over 14 days are summarized in Table 2. Over 14 days, the 8 ng target implant released a total of 100.9 ng (average release rate = 7.2 ng/day), the 40 ng target implant released 467.4 ng (average release rate = 33.4 ng/day), the 200 ng target implant released 2387.3 ng (average release rate = 170.5 ng/day) and the 1,000 ng target implant released 11,465.3 ng (average release rate = 819 ng/day). Release rates for each time point are summarized in Table 3.

3.2. Dose dependent suppression of kindling epileptogenesis by infrahippocampal adenosine releasing implants

Infrahippocampal implantation of silk-based polymers with different target release rates of adenosine ranging from 40 to 1000 ng adenosine per day should suppress kindling epileptogenesis in a dose-dependent way. To test this hypothesis, four groups of rats received infrahippocampal implants with target release rates of 0 ng (control, N=5), 40 ng (N=5), 200 ng (N=6), and 1000 ng (N=6) adenosine per day. These target doses were based on previous dose assessments suggesting an effective dose to be in the range of 200 ng adenosine released per day [15,16]. Four days after polymer implantation hippocampal kindling was initiated with each rat receiving a total of 8 kindling sessions every second or third day, with each session comprised of 6 stimulations delivered every 30 min. Thus, during a time span ranging from 4 to 20 days after stimulation, each rat received a total of 48 stimulations (Figure 2A).

Seizure scores were determined after each stimulation according to the scale of Racine [35]. Stages 1 to 3 correspond to partial seizures of progressing intensity, whereas stages 4 and 5 represent generalized seizures with tonic and clonic components. Compared to control (0 ng adenosine) kindling development in recipients of target doses of 40, 200, and 1000 ng was

initially delayed in a dose-dependent relation during the first 24 sessions or until the end of session 4 that corresponds to 11 days after polymer implantation. Most notably, during this time recipients of polymers releasing a target dose of 1000 ng per day were completely protected from any seizure activity (Figure 2A,B). This dose-dependent therapeutic effect was also reflected by the number of stimulations, needed to elicit the first partial (stage 1 or 2) seizure (Figure 3). While in recipients of control implants partial seizures first occurred during session 2 (i.e. around stimulation 10), the first emergence of partial seizures was markedly delayed with increasing doses of released adenosine with recipients of a target dose of 1000 ng adenosine per day needing 2.5 times as many stimulations to reach the same kindling state as the control group (Figure 3).

From stimulation 25 onwards (i.e. session 5 at day 13 after implantation) the therapeutic effects of the implants declined (Figure 2), which is consistent with the reduced rate of adenosine release from the polymers (Table 3). While recipients of control implants reached their kindling criteria (generalized stage 4/5 seizures) from session 5 onwards, all recipients of adenosine releasing polymers showed a delay in the acquisition of generalized seizure responses (Figure 2). Interestingly, the kindling rates (i.e. the progressive increase in seizure stages with time) were similar in recipients of target doses of 0, 200, and 1000 ng adenosine per day, however, with a dose dependent delay in kindling onset, indicating polymer-based adenosine-release suppressed kindling. Most importantly, recipients of a target dose of 1000 ng adenosine per day showed a delay of kindling acquisition of about three sessions or one week, indicating that doses of around 400 ng adenosine per day (Table 3) are sufficient to suppress kindling.

3.3. Infrahippocampal adenosine releasing implants reduce afterdischarge duration in EEG recordings

The therapeutic effect of the adenosine-releasing infrahippocampal implants was further quantified by determination of the duration of electroencephalographic afterdischarges (ADD) elicited by each test stimulus during the kindling acquisition phase (Figure 4).

The ADD was averaged according to session number (N=6 stimulations, each) and treatment target dose (0, 40, 200, and 1000 ng adenosine per day). During the first three sessions a dose-dependent reduction of the ADD became evident. From session 4 onwards ADDs from recipients of target doses of 40 and 200 ng adenosine per day were similar to those from sham controls (0 ng adenosine per day), being consistent with the decline in adenosine released from these polymers with time (Table 3). Remarkably, afterdischarges remained significantly reduced throughout the whole experiment in recipients of polymers releasing a target dose of 1000 ng adenosine per day. At that time the ADD of recipients of target doses of 0, 40, and 200 ng adenosine per day had reached a plateau with average values exceeding 120s, whereas the ADD of recipients of target doses of 1000 ng adenosine per day remained reduced with values of <100s. Thus, the behavioral seizure response as observed in Figure 2 was closely paralleled by the duration of afterdischarges in respective EEGs. These results also indicate that a target dose of 1,000 ng adenosine per day has the potential for prolonged seizure suppression, even if the rates of adenosine release decline with time. After completion of seizure monitoring all animals were sacrificed at day 20 after polymer implantation and subjected to gross histological analysis. In coronal brain sections we detected a cavity resulting from the polymer deposited within the infrahippocampal fissure and traces of the electrode insertion were found in correct locations (Figure 5). No obvious signs for inflammation or bleeding were found.

4. Discussion

In order to provide therapeutic alternatives for patients suffering from pharmacoresistant epilepsy several novel approaches are being developed and tested. These range from electrical

and magnetic stimulation [36-39], focal cooling [40], cell therapies [6,7,13,41,42], and gene therapies [43-46], to the ketogenic diet [47,48]. We recently established a neurochemical rationale for focal adenosine augmentation therapies (AATs). Most importantly, a focal pathological reduction of adenosine – caused by astroglial upregulation of ADK – appears to be a direct cause for seizures [11,12]. Therefore, focal AATs based on transplanting cells engineered to release adenosine into the vicinity of an epileptogenic focus are effective in preventing seizures, and possibly epileptogenesis [11,13,14]; moreover, AATs are effective in the suppression of seizures that are refractory to commonly used antiepileptic drugs [18,49]. It is important to note that some of the alternative therapeutic approaches mentioned above seem to be based on adenosine augmentation: adenosine is liberated as a consequence of deep-brain stimulation [50] and seems to be involved in the therapeutic effects of the ketogenic diet [47]. Thus, focal AATs hold great promise for the treatment of pharmacoresistant partial epilepsy. The therapeutic rationale outlined above prompted us to initiate the development of an AAT compatible with future clinical application. We chose to combine two FDA-approved materials – silk and adenosine – to develop a biocompatible brain implant for the focal delivery of adenosine.

The *in vitro* results presented here suggest that combined, hierarchically designed silk protein devices can be generated to achieve controllable delivery rates of adenosine. All implants continued to release adenosine for at least 14 days, with average release rates close to target release rates. The average release rates were slightly lower than their target rates, likely the result of implants failing to release the entire drug load within 14 days. All implants had high initial release rates, but these rates decreased over 14 days. This may be the result of the initial “burst” release that is characteristic of many delivery systems, but there are several potential strategies to delay burst and prolong release in the future, to achieve near zero order kinetics.

In ongoing studies, coatings of the silk microspheres [27] and chemical coupling to the silk [51] are being explored as strategies to gain further control of the process. Release experiments suggest that increasing the number of silk capping layers, the crystallinity of the silk and increasing the nanofilm thickness can slow the rate of drug release. With more research to correlate the fundamental relationships between material features (e.g., processing conditions, crystallinity, layer thickness) to the release kinetics, predictive models and greater control of the release rates are anticipated.

The *in vivo* results presented here demonstrate that focal adenosine release from silk-based polymers is effective in retarding kindling epileptogenesis in a dose-dependant manner. We used a unique approach combining polymers designed to release specified target doses (0, 40, 200, and 1,000 ng adenosine/day) for a limited 14 day period with a kindling epileptogenesis paradigm extended to 20 days after polymer implantation to document kindling progression after expiration of the polymers. In particular, the reciprocal relation of progressive decline in adenosine release with progressive increase in kindling development allowed us to delineate dose-response relationships. This unique adenosine delivery approach allowed us to draw the following conclusions, which go beyond results obtained from our previous AATs:

(i) Effective dose assessment

It is important to note that first signs of kindling development (e.g. behavioral seizure scores) typically appear with a delay of 1 session comprised of 6 stimulations. Thus, recipients of control implants display first signs of kindling during session 2 (Figure 2); this implies that kindling epileptogenesis is initiated in the session (i.e. at the relevant dose of adenosine released at that time) preceding the first signs of kindling. Consequently, first signs of kindling appear with a dose dependent delay of about 1 session in the control group (stimulation #6), 2 sessions in the 40 ng group (stimulation #10), 3 sessions in the 200 ng group (stimulation #16), and 4 sessions in the 1000 ng group (stimulation #24) (Figure 2A). This close dose-dependency is

also reflected in the average number of stimulations needed to elicit the first stage 1 or 2 seizure (Figure 3) and suggests a linear dose-response relation. Since our experiments were designed to prolong kindling after cessation of adenosine release from the polymers, parallel *in vitro* adenosine release data (Table 3) allowed us to determine the minimal effective dose needed to delay the onset of kindling. This becomes most evident in recipients of a target dose of 1000 ng adenosine per day. These animals continued to be protected from any seizure activity during the first 23 stimulations (i.e. during session 1 to 4) (Figure 2). First seizures occurred during session 5; taking in consideration the one-session-delay of detecting behavioral seizures, we conclude that kindling epileptogenesis was initiated during session 4, corresponding to day 11 after polymer implantation. At that time the polymers released approximately 200 ng adenosine per day (Table 3). Similar calculations performed in recipients of target doses of 40 and 200 ng adenosine per day indicated that kindling epileptogenesis was initiated as soon as daily release rates of adenosine fell below 50 to 100 ng adenosine (Figure 2, Table 3). These calculations allow us to estimate the minimal effective dose to be in the range of 50 to 200 ng adenosine per day. These data are in agreement with previous cell transplantation experiments, in which adenosine release rates of about 20 to 40 ng adenosine released from 10^5 cells was sufficient to suppress kindled seizures [15,17]. It is also important to note that high doses of adenosine (> 2000 ng adenosine per day) appeared to be well tolerated, without any gross behavioral changes of the animals.

(ii) Possible antiepileptogenic effect

An important finding of this study is the observation that kindling acquisition occurs with a dose-dependent delay but at the same kindling rate (i.e. progression in seizure severity). This observation suggests that implant-derived adenosine might prevent epileptogenesis. Our unique experimental design, limiting adenosine release to 14 days (corresponding to session 1 to 5), but extending kindling to 20 days (corresponding to session 8) allowed us to study resuming of kindling after expiration of the polymers. Reduced seizure stages (Figure 2) combined with the presence but reduced duration of ADDs (Figure 4) in recipients of adenosine releasing implants during the early stages of kindling could have two alternative explanations: suppression of seizures, however seizure suppression may mask ongoing epileptogenesis, or prevention of epileptogenesis. In the first case, if seizures were simply suppressed by graft-derived adenosine and epileptogenesis was masked by seizure suppression, cessation of adenosine release from the polymers would imply a sudden, rapid rise in seizure severity. However, this is clearly not the case (Figure 2). Instead, the rate of kindling acquisition was not changed in recipients of adenosine releasing polymers. Thus, kindling development in recipients of a 1,000 ng per day target dose proceeded at the same rate as kindling development in control animals (Figure 2), however, with a delay in kindling onset. These data suggest that focal polymer-based delivery of adenosine might prevent epileptogenesis. It is important to note that afterdischarges that are considered as the “epileptogenic” stimulus are not suppressed by implant-derived adenosine. The interpretation of our data is consistent with previous studies attributing antiepileptogenic effects to increased brain adenosine in adenosine kinase mutant mice [11] or to stem cell derived brain implants that release adenosine [11,14].

5. Conclusion

Our data describe a novel silk-based delivery system for adenosine that fulfills crucial requirements for future clinical application, such as: (i) biocompatibility, (ii) delivery of predetermined doses of adenosine, (iii) safety as opposed to xenotransplantation using animal cells, (iv) sustained function via slow degradation *in vivo*, and (v) therapeutic efficacy in a widely used preclinical model (rat kindling model) that has a high predictive value in drug development. Most importantly, our study for the first time defines minimally effective doses in the range of 50 to 200 ng adenosine per day, and suggests that focal delivery of adenosine

might not only have therapeutic value for the suppression of established seizures, but also for the prevention of epileptogenesis. Although the current polymer design precludes long-term clinical applications due to adenosine depletion over a period of two weeks, the current system would be suited for short-term clinical safety and feasibility trials, in which the implant would be placed into an epileptic focus prior to its scheduled surgical resection. In addition, our data provide a therapeutic rationale for the development of future bioreactors for the sustained long-term delivery of adenosine, a goal that could be achieved by growing human mesenchymal stem cells engineered to release adenosine [52] on silk scaffolds.

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Figure 1.

Schematic of implant fabrication showing individual silk-based drug delivery components: (1) Adenosine-encapsulating microspheres are prepared. (2) Microspheres are mixed with silk solution, then porous scaffolds embedded with microspheres are fabricated. (3) Scaffold is soaked in silk+adenosine solution, filling in pores and coating the implant with a macroscale drug-loaded film. (4) Additional adenosine is loaded with alternating nanofilm deposition

Figure 2.

Retardation of kindling after infrahippocampal implantation of adenosine releasing polymers. Four days after infrahippocampal implantation of silk-based polymers with daily target release rates for adenosine of 0 ng (N=5, red), 40 ng (N=5, dark blue), 200 ng (N=6, light blue), or 1000 ng (N=6, green) kindling stimulations were delivered at a rate of 6 stimulations per day (= session), with session 1 to 8 corresponding to days 4, 6, 8, 11, 13, 15, 18, and 20 following implantation. (A) Seizure stages averaged across animals from each group for each individual stimulation. Kindling curves are shifted to the right with increasing doses of adenosine. Note that recipients of a target dose of 1000 ng adenosine per day are completely protected from any seizures during the first 23 stimulations. (B) Averaged seizure response per session (N=6 stimulations) of the same animals depicted in (A). Note the dose response relationship at session 8. Errors are given as $\pm$ SD. Data were analyzed by ANOVA, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, treatment groups compared to control (0 ng).

Figure 3. Number of kindling stimulations needed to elicit partial stage 1 or 2 seizures. Data were averaged from seizure score tables from the animals of Fig. 4. Errors are given as $\pm$ SD.

Figure 4.

Average duration of afterdischarge in EEG recordings during kindling acquisition. (A) The afterdischarge duration (ADD) was determined after each kindling-stimulation by analyzing the respective EEG recordings. ADDs were averaged for each session ($n = 6$ stimulations) and treatment type: implants releasing target doses of 0 ng (red, $N=5$), 40 ng (dark blue, $N=5$), 200 ng (light blue, $N=6$), or 1000 ng (green, $N=6$) adenosine per day. Errors are given as $\pm$ SD. Data were analyzed by ANOVA: * $P<0.05$, ** $P<0.01$, *** $P<0.001$, adenosine releasing polymer implants versus control (0 ng adenosine). (B) Representative EEG recording at session 5 from an animal with a control implant. (C) Representative EEG recording at session 5 from an animal with a target release dose of 1,000 ng adenosine per day.

Figure 5. Representative Nissl stained coronal brain section 20 days after transplantation. Note that the infrahippocampal location of the polymer and the implantation channel of the stimulating electrode are located in the same anterior-posterior plane.

Table 1
Implant compositions and loading with adenosine. The number of nanofilms includes both a silk nanofilm and the subsequent adenosine nanofilm. Ado = adenosine.

	Nanofilms		Microspheres		Macroscale Film		Total ado loaded for entire implant (in ng)
	Number of nanofilms	Total ado loaded (in ng)	Conc. Of microspheres in silk solution (in mg/mL)	Total ado loaded (in ng)	Conc. of adenosine in silk solution (in mg/mL)	Total ado loaded (in ng)	
40 ng/day	3	250	10	160	0.06	150	560 ng
200 ng/day	12	1,000	50	800	0.4	1,000	2,800 ng
1,000 ng/day	36	3,000	250	4,000	2.8	7,000	14,000 ng

Table 2

Average adenosine release.

Time (in days)	Target: 40 ng		Target: 200 ng		Target: 1,000 ng	
	Ado Release (in ng)	Cumulative Release (in ng)	Ado Release (in ng)	Cumulative Release (in ng)	Ado Release (in ng)	Cumulative Release (in ng)
1	134.4 (±13.8)	134.4	679 (± 25.0)	679	3137 (± 86.5)	3137
2	89.0 (±10.5)	223.4	513.1 (± 21.1)	1192.1	2865.2 (± 55.8)	6002.2
3	33.9 (±9.9)	257.3	202.3 (± 22.3)	1394.4	984.6 (± 79.7)	6986.8
4	66.3 (±4.4)	323.6	330.9 (± 18.4)	1725.3	2042.7 (± 61.2)	9029.5
6	42.7 (±3.4)	366.3	208.4 (± 15.4)	1933.7	853.7 (± 44.0)	9883.2
10	62.4 (±4.5)	428.7	281.3 (± 15.8)	2215.0	914.8 (± 50.6)	10,798
14	38.7 (±10.8)	467.4	172.3 (± 22.3)	2387.3	667.3 (± 45.4)	11,465.3

Table 3

Release rates of adenosine from implants (nanograms per day)

Time (in days)	Target: 40 ng	Target: 200 ng	Target: 1,000 ng
1	134.4	679	3137.0
2	89.0	513.1	2865.2
3	33.9	202.3	984.6
4	66.3	330.9	2042.7
6	21.4	104.2	426.9
10	15.6	70.3	228.7
14	9.7	43.1	166.8
Average	33.4	170.5	819.0