Section of Clinical Neuropharmacology[1], Department of Neurosurgery; Freiburg Materials Research Centre[2]; University Hospital Pharmacy[3], Albert-Ludwigs University, Freiburg, Germany

Valproate release from polycaprolactone implants prepared by 3D-bioplotting

M. Kammerer[1], M. Fabritius[2], C. Carvalho[2], R. Mülhaupt[2], T. J. Feuerstein[1], R. Trittler[3]

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Professor Dr. T.J. Feuerstein, Neurocentre, University Medical Centre, Breisacher Str. 64, 79106 Freiburg, Germany thomas.feuerstein@uniklinik-freiburg.de

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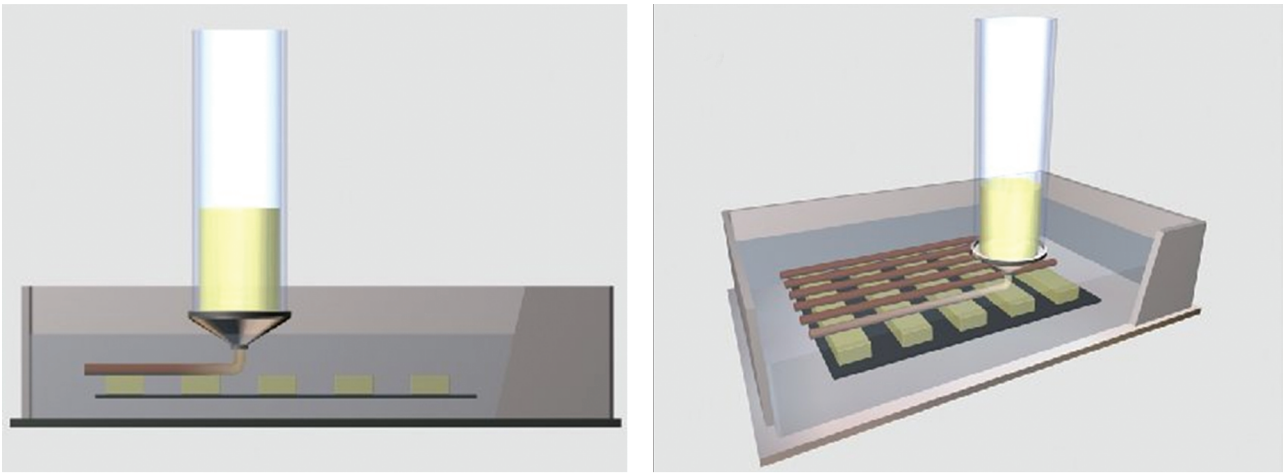
In this study we examined the release kinetics of valproate from polycaprolactone (PCL) implants constructed for local antiepileptic therapy. The PCL implants were produced with a novel 3D-Bioplotting technology. Release kinetics were determined by superfusion of these implants. Valproate was measured in the superfusate fractions with high pressure liquid chromatography (HPLC). The HPLC measurements were linear over a concentration range of 10–500 μg/mL for valproate and the limit of quantification was found to be 9 μg/mL. The HPLC method used is simple, accurate and sensitive. Within the first day, valproate (10%w/w)-PCL implants released already 77% of the maximum possible liberated amount whereas (5%w/w)-PCL implants released only 53%. After four days, 88% of valproate was released from (10%w/w)-PCL implants and 94% valproate from (5%w/w)-PCL implants. When valproate was ground before the 3D-Bioplotting process, only 63% from (10%w/w)-PCL implants was released within the first day. This released amount of ground valproate was significantly lower compared to that which was not ground from the (10%w/w)-PCL implants. After three days of superfusion a total amount of 89% of *ground* valproate within the implants was released, corresponding to 88% of non-ground valproate after four days. The fast releasing PCL implants can be used to study *acute* effects of locally applied valproate on epileptogenesis *in vivo* after initiation of an epileptic focus in an animal model. The corresponding biocompatibility may also be analysed.

1. Introduction

The widely used antiepileptic drug valproate is a simple branched short-chain fatty acid and is applied in the treatment of diverse epileptic syndromes, e.g., absence seizures, generalized tonic-clonic seizures, complex partial seizures, juvenile myoclonic epilepsy and seizures associated with Lennoux-Gastaut syndrome (Michoulas and Farrell 2010). To achieve a sufficient antiepileptic concentration at target sites in the brain, the reasonably high valproate plasma concentration is 300 μM (Neels et al. 2004). However, even with this plasma concentration some seizures cannot be treated sufficiently, because the locally acting valproate tissue concentration is still too low (Serralta et al. 2006). Insufficient local valproate concentrations are caused, for instance, by multidrug transporters, which actively remove drugs out of the neurons (Lazarowski et al. 2007; Löscher 2007). The alternative to systemic drug therapy, i.e., epilepsy surgery, is not always applicable: Many epileptic foci are located in brain areas where they cannot be removed, since the focus area is covering a relevant function, as the primary motor, speech or visual region. Systemic side effects of valproate at (too) high plasma levels include mild to moderate dysfunctions as increase of sleep duration, weight gain (Grosso et al. 2009) and alopecia (Chateauvieux et al. 2010). In addition, there are numerous *severe* side effects, as decreased fertility in 25% of the patients and teratogenity with an approximately threefold increase in major anomalies (Clayton-Smith and Donnai 1995; Genton et al. 2006). Furthermore, the occurrence of parkinsonism as a valproate side effect has been reported (Jamora et al. 2007). Life-threatening side effects are coagulation disorders, which occur with an incidence of 4% in children (Gerstner et al. 2006), hepatotoxicity (Dreifuss et al. 1987, 1989) and finally depression with an increased suicide rate (Olesen et al. 2010). Overall, only two out of three epileptic patients on systemic pharmacotherapy show an adequate seizure control with acceptable, i.e., tolerable, side effects (Kwan and Brodie 2000). To obtain a better therapeutic ratio, direct drug delivery of antiepileptic drugs to the central nervous system as target area seems to be a promising solution. Furthermore, a direct intracerebral drug delivery circumvents the blood-brain-barrier and may allow to use potential antiepileptic drugs which do not pass this barrier, but by direct application can reach a concentration high enough at the target site. In other brain diseases, the idea of a locally-applied polymer, which releases the drug over time, has already been realized. For example, the chemotherapeutic carmustin (BCNU)-containing wafer (trade name: Gliadel®) is nowadays often implanted after resection of high-grade gliomas. For the treatment of epilepsy, this new route of drug delivery still has to be elaborated. Therefore, the present study investigated the release of valproate from polycaprolactone (PCL) implants, which were produced using an innovative Rapid Prototyping technology (see below).

Natural and synthetic polymers are widely applied in the biomedical field (Lendlein 1999). The spectrum reaches from



(a) (b)

Fig. 1: Concept of 3D-Bioplotting. (a) Plotting material is pressed out of the heatable syringe; (b) Strands get oriented to form one layer

bone repair (Jagur-Grodzinski 2006) over hip joint replacement (Mathiesen et al. 1986) to the design of vascular stents (Altman et al. 2003). The controlled release of drugs from a variety of devices is also studied intensively (Wang et al. 2007; Chang et al. 2008). PCL is a thermoplastic aliphatic polyester, which is fully resorbable by the human body. Due to its high biocompatibility and good processability it is often used in tissue engineering and its application for targeted drug delivery is extensively studied. Ozkan et al. (2009) used a new construction process to generate porous tissue engineering scaffolds out of PCL comprising bovine serum albumin.

One of the objectives of the present study was to evaluate the possible appropriateness of the 3D-bioplotting technology in the construction of drug delivery devices. PCL can be readily processed by the 3D-bioplotter. Its low melting point of 60 °C allows a direct mixing of the molten polymer with solid additives without using organic solvents. This is preferable as no further purification is needed.

Rapid Prototyping establishes the direct construction of complex objects without the use of moulds, combining computer-assisted design with computer-assisted manufacturing. First, a 3D model is designed at the computer and then cut into a sequence of layers. In the following building process these layers are created one after another by the chosen Rapid Prototyping technique to form the desired object (Solid Freeform Fabrication SFF; Wohlers 2007). In comparison to many conventional casting and moulding processes this method enables the construction of complex internal structures. By now different Rapid Prototyping methods are used in the biomedical field, yet each method is limited to a relative narrow set of processable substances. In 2000 the first construction of 3D scaffolds by melt processing of thermoplastic biodegradable polymers, as for example PCL, by fused deposition modelling was reported (Hutmacher 2000). Since then a lot of progress has been made. Landers and Mülhaupt constructed an innovative Rapid Prototyping technique called 3D-bioplotting at the materials research centre in Freiburg/Germany (Landers et al. 2002a; Pfister et al. 2004). 3D-bioplotting is based on the method of fused deposition modelling. The plotting material is transferred into a heatable syringe and pressed through a nozzle by air pressure to form uniform strands. These strands form layers which can be orientated to form multi-layer mesh structures. Depending on the processed material the plotting process can take place in air or in solution (Fig. 1).

The bioplotting technology allows to process a wide set of different materials reaching from polymer melts to hydrogels. This enables the construction of either hard or soft constructs with the same method (Al-Ahmad et al. 2008; Haberstroh et al. 2010). Its broad application and outstanding flexibility makes 3D-bioplotting especially suitable for tissue engineering and medical purposes (Al-Ahmad et al. 2008; Haberstroh et al. 2010).

2. Investigations and results

The release of disc-shaped valproate-loaded PCL implants containing 5 or 10 wt% drug, produced using the 3D-Bioplotting technology, was measured. The size of the implants (diameter range 4.9 mm to 5.1 mm, thickness range 0.9 mm to 1.1 mm) was adapted to the size of the planned craniotomy in rats whith a local neocortical epilepsy focus.

2.1. HPLC as a method to quantify valproate concentrations

A typical chromatogram of valproate in buffer is shown in Fig. 2. Valproate was well separated with a retention time of 2.5 min with no endogenous interfering peak.

The calibration curve was linear in the concentration range of 10–500 µg/mL. The equation of the calibration curve of the valproate standards was $y = 0.0436x$, where x represents the concentrations of valproate standards and y represents the corresponding peak area. The CI_{95} of the slope was [0.0434, 0.439]. The limit of quantification (signal/noise ratio 1:10) was found to be 9 µg/mL.

The precision of the assay at a concentration of 25 µg/mL, expressed as the within-run coefficient of variation was 2.1% ($n = 11$) and the inter-day coefficient of variation was 3.3%

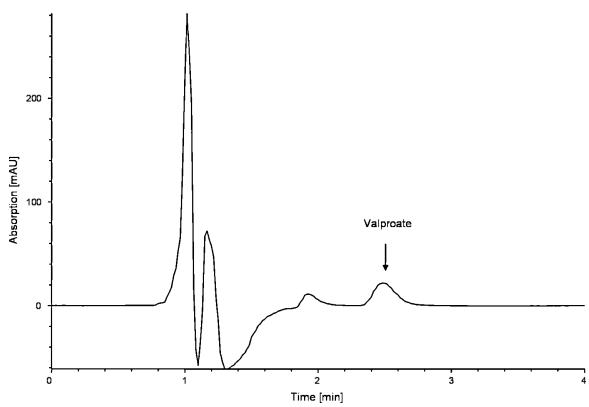


Fig. 2: Typical HPLC chromatogram (absorption-time diagram) of valproate at a concentration of 100 µg/mL. mAU = milli absorption unit

Table 1: Peak area mean values (coefficient of variation < 3.0%) are given for each concentration for freshly prepared standard solutions and the same standard solutions after one freeze-thaw cycle

Valproate-concentration (μg/mL)	Peak area (mAU*min) of freshly prepared valproate standards	Peak area (mAU*min) of valproate standards after one freeze-thaw cycle
1000	20.10	20.59
500	10.41	10.46
200	4.25	4.20
100	2.07	2.12
50	1.01	1.06

(n = 3). After one freeze-thaw cycle the same results as with freshly prepared samples were obtained (see Table 1).

2.2. In vitro release experiments

2.2.1. Valproate release from valproate-loaded (5% w/w) PCL implants

The release of valproate from drug-loaded (5% w/w) PCL implants over a period of four days is shown in Fig. 3. On the first day of perfusion, 52.6% (CI_{95} = [47.9, 57.3]) of the stored valproate was released. On subsequent days, the liberation of valproate decreased so strongly that by day 5 the released amount was underneath the limit of quantification. The corresponding means were 21.7% (CI_{95} = [20.7, 22.7]) for day 2, 12.6% (CI_{95} = [11.1, 14.1]) for day 3, and 7.1% (CI_{95} = [5.3, 8.9]) for day 4. After four days of perfusion, approximately a total of 94% of the stored valproate was released.

2.2.2. Valproate release from valproate-loaded (10% w/w) PCL implants

The release of valproate from (10% w/w) PCL implants produced by the two different fabrication methods is illustrated in Fig. 4.

For valproate-implants of fabrication method I (see Fig. 4, triangles) 77.4% (CI_{95} = [75.3, 79.6]) of valproate were already released during the first 24 h of perfusion. On the following days the release was markedly diminished to only 5.4% (CI_{95} = [4.0, 6.9]) on day 2, to 2.7% (CI_{95} = [2.2, 3.2]) on day 3, and to 2.0% (CI_{95} = [1.7, 2.4]) on day 4. By day 5, an almost negligible amount of valproate was released, which was no longer

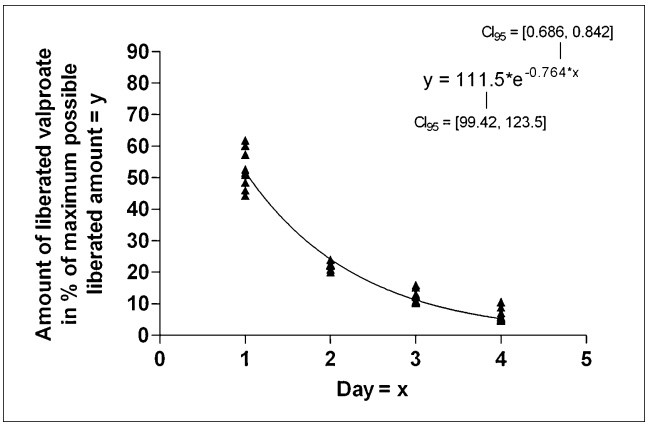


Fig. 3: Valproate release from three valproate-containing (5% w/w) PCL implants, which were produced by fabrication method I. Each single data point represents the liberated amount of valproate per day, in percent of the maximum possible liberated amount of valproate. All measured triplicate data points for each implant are given

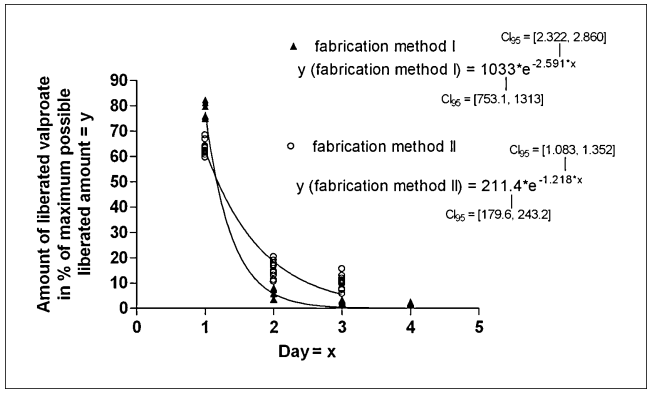


Fig. 4: Valproate release from valproate-containing (10% w/w) PCL implants, which were produced by the two different fabrication methods. For fabrication method I the release from three implants was measured, for fabrication method II that from four. Each single data point represents the liberated amount of valproate per day, in percent of the maximum possible liberated amount of valproate. All measured triplicate data points for each implant are given

detectable with the HPLC method used. After four days of superfusion, a total of 88% valproate was released.

For valproate-implants produced by fabrication method II the corresponding values were for the first day: 62.9% (CI_{95} = [61.2, 64.6]), on day 2: 15.4% (CI_{95} = [13.1, 17.8]), and on day 3: 10.2% (CI_{95} = [8.4, 12.1]) (see Fig. 4, circles). At day 4 an almost negligible, no more detectable amount of valproate was released. After three days of superfusion a total of 89% valproate was released. The release of valproate from valproate-implants differed significantly depending on the fabrication method (see Fig. 4, equations).

2.2.3. Microscopic studies

Microscopic studies were carried out to yield information about the surface of the implants. Figure 5 shows pictures of the implant's surface.

The pictures on the left (I day 0, II day 0) are representative for the used implants before the release study. Although comprising the same material, a significant difference between the two pictures is obvious. Picture I day 0 shows big needle like crystals of sodium valproate on the surface, whereas picture II day 0 does not show any of these forms. The missing needle phenomenon in II day 0 is attributed to the grinding of the valproate used in fabrication method II. After release, picture I day 4 shows a rough and brittle surface, whereas the surface of II day 4 is relatively smooth.

These pictures provide an explanation for the higher release rate of the implants processed by fabrication method I. As the sodium valproate crystals are highly water soluble, they dissolve quickly within the first day of measurement and leave a roughened surface and channels. This allows more water to diffuse into the implant thus causing a faster release of valproate than the smooth surface of implants produced by fabrication method II.

3. Discussion

This study tested the release kinetics of uniform PCL implants loaded with valproate. The PCL implants were produced by 3D-bioplotting applying two different fabrication methods. The thickness (1 mm) of these implants was comparable to the thickness of Gliadel® wafers which are already used as implants after tumour resection in humans. To examine the amount of valproate released, a simple, quick, accurate, sensitive and robust HPLC method has been established, as it was described earlier by Kishore et al. (2003), who established a method for valproate concentration measurements in human serum. Release studies,

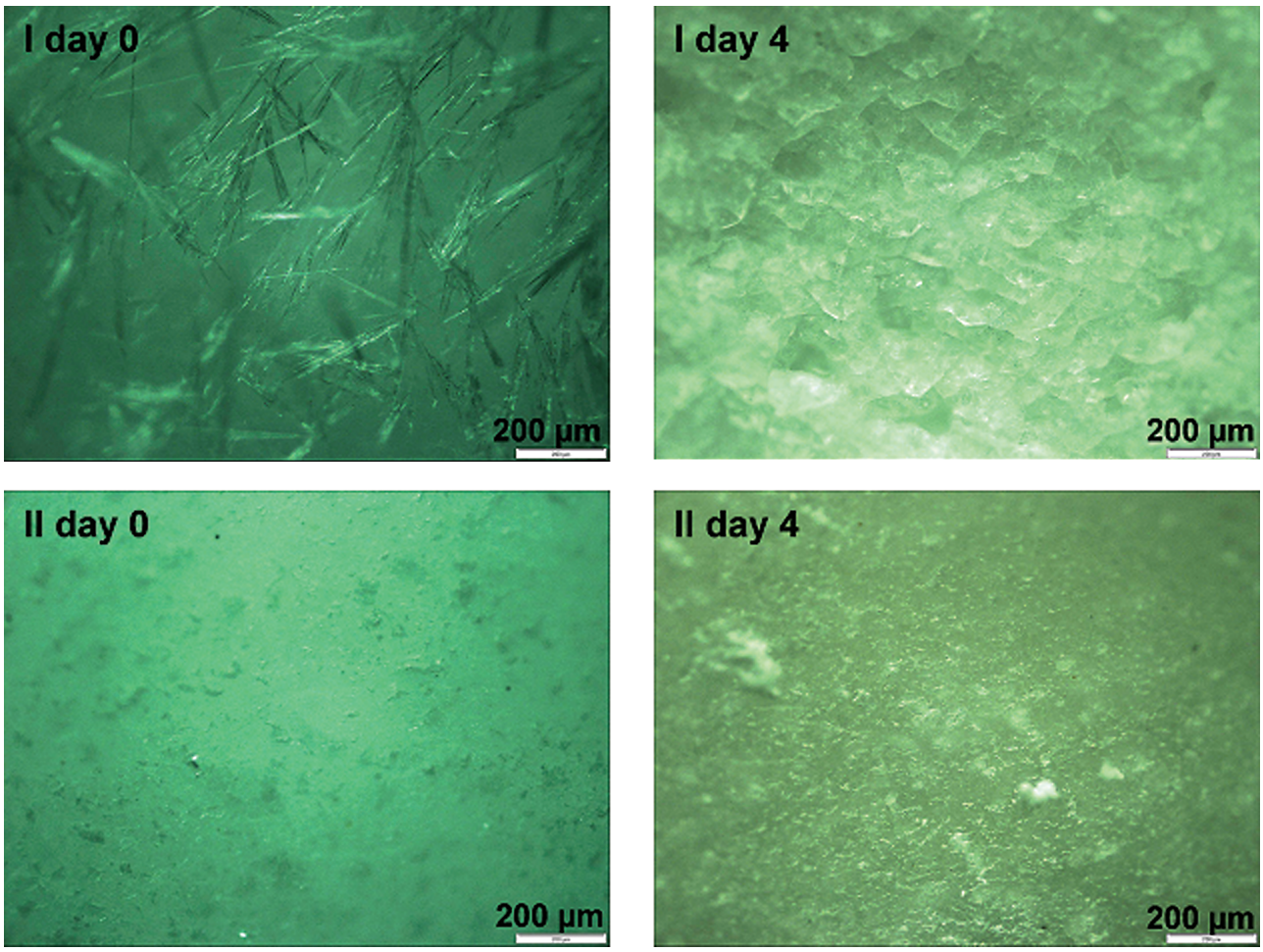


Fig. 5: Magnification of the used implants before (day 0) and after (day 4) the release studies. Fabrication method I (I), fabrication method II (II). Picture I day 0 shows needle like crystals of sodium valproate that are attributed to the lack of grinding. In contrast, picture II day 0 doesn't show any of these crystals as the material was ground before mixing with the polymer. The higher release rates of implants produced by fabrication method I is attributed to the quick dissolving of the shown crystals and the roughened surface

using the validated HPLC method, showed a burst release within the first few days. PCL is known to release drugs over days continuously. Carcaboso et al. (2008), for instance, produced melt-molded PCL implants, loaded with the antiepileptic drug gabapentin. These coated implants released gabapentin, after a moderate burst effect, relatively linear over a period of seven days. Four factors may contribute to the above mentioned burst release of valproate: (1) its hydrophilicity, (2) the relative amount of loaded drug, (3) the distribution of valproate in the disc, and (4) the chain length of PCL. Valproate is known to dissolve easily in water. With the higher concentration of valproate amount in the PCL implants, a faster release of valproate was found, which may be due to the higher concentration gradient of 10% valproate compared to 5% between the inner part of the implant and the buffer. The grinding of the valproate particles before the 3D-bioplotting process led to a more homogenous distribution and to smaller valproate particles in the PCL implant, which resulted in a smaller burst release on the first day. PCL is hydrolysable at the ester linkage (Goldberg 1995). Free carboxylic functions cause the cleavage of the polyester backbone inducing an autocatalytic reaction. The molecular weight of PCL is also crucial for the degradation process. PCL with a higher molecular mass and therefore a longer chain length takes longer to degrade. All the above mentioned factors may contribute to the fast valproate release from loaded PCL implants produced by 3D-bioplotting. These fast releasing PCL implants can be used, for instance, to study early effects of valproate on an early phase of epileptogenesis *in vivo* after initiation of an epileptic focus in an animal model and its corresponding biocompatibility.

In future research, a longer lasting release of valproate from polymer-implants produced by 3D-bioplotting needs to be achieved, so that long-term effects of locally administered valproate can also be investigated. Different possibilities to reach this goal exist. Carcaboso et al. (2008) proposed coating the implants. Another possibility is PCL with a higher molecular mass. A further possibility is the use of non-biodegradable polymers. For instance, ethylene-vinyl acetate provided a controlled-release of the antiepileptic drug phenytoin over 105 days (Tamargo et al. 2002).

In the present study, a simple and reliable HPLC method to determine the concentration of quickly released valproate has been established. This measurement method can also be applied in further studies for liberation measurements of valproate polymers with sustained release characteristics. 3D-bioplotting can be used for the design of tailor-made drug delivery devices. It offers the possibility to easily control the active surface area of the construct and thus the rate of release of a drug. In upcoming studies, we will concentrate on the design of longer term drug delivery devices using non-biodegradable polymers as matrix material.

4. Experimental

4.1. Fabrication of valproate-PCL implants

4.1.1. Fabrication method I

PCL was molten at 150 °C on a Petri dish. Sodium valproate powder was added to the clear melt and stirred firmly for 5 min to create a milky suspension. The melt was cooled slowly while stirring to avoid the aggregation of

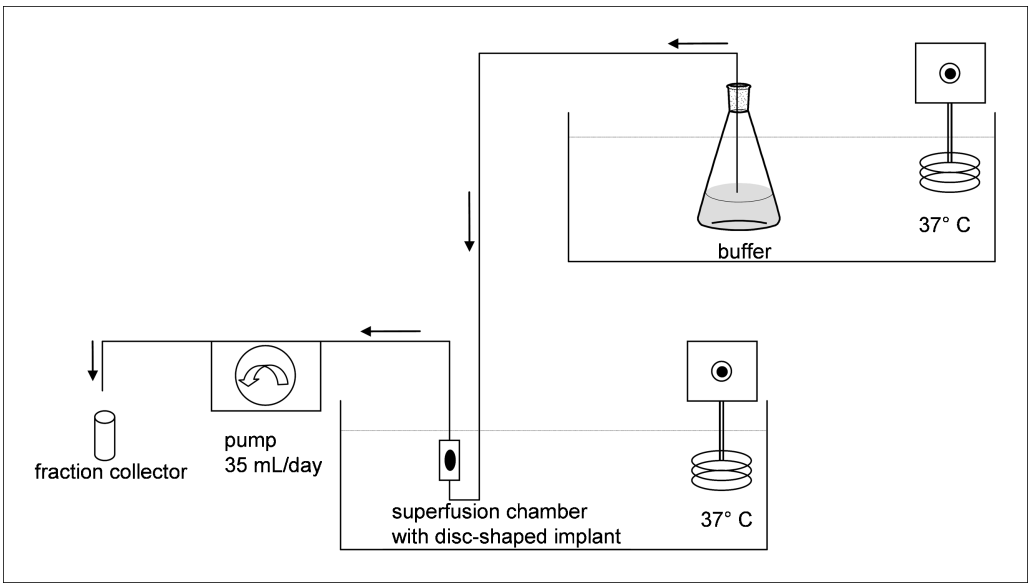


Fig. 6: Scheme of the superfusion apparatus. Only one of twelve superfusion chambers arranged in parallel is shown

the valproate particles. Once cooled to room temperature, the white material was inserted into a glass syringe. This syringe was then fitted into the high temperature head of the 3D-bioplotter. The syringe with the PCL-valproate mixture was heated to 80 °C for 1 h until a homogeneous melt was achieved. The instrumental data of the plotting process are shown in Table 2.

4.1.2. Fabrication method II

PCL was frozen in liquid nitrogen and ground (10.000 RPM, Retsch ZM 200) to form granulated material. Sodium valproate and PCL were dried in vacuum (40 °C) over night. Sodium valproate is a strongly hygroscopic substance and, therefore, it was ground quickly after drying using a preheated pestle and mortar. Together with granulated PCL it was transferred into a glass flask and thoroughly mixed. The mixture was heated (80 °C, 10 min) and stirred constantly to achieve a uniform distribution of the valproate particles in the polymer melt. The mixture was cooled to room temperature to form a solid composite. The polymer mixtures were immersed into the heatable dispensing cartridge of the 3D-bioplotter and plotted onto a Teflon foil to uniform, disc-shaped implants. The instrumental data of the plotting process are shown in Table 2.

4.2. PCL implant characterization

The disc-shaped implants were characterized by optical microscopy before and after the release studies using the Axioskop HB050 (Carl-Zeiss, Jena, Germany), the Softimaging system CC12 and the analySIS software (Olympus, Münster, Germany).

Table 2: Experimental data of the 3D-bioplotting process is indicated according to the two different fabrication methods

	Fabrication method I	Fabrication method II
3D-Bioplotter (Envisiontec)	1. Generation	3. Generation
Plotting medium	Air	Air
Temperature plotting material (°C)	80	70
Temperature plotting medium (°C)	20	20
Inner nozzle diameter (mm)	1.2	0.45
Excess air pressure (x 10^5 Pa)	0.1–0.2	0.49–0.55
Moving speed of plotting head (mm/s)	500	300
Layer thickness (mm)	0.3	0.5
Corner delay/(s)	0.1	0.1

4.3. In vitro release experiments using the superfusion method

Three to four PCL implants containing valproate were transferred to three to four acrylic glass superfusion chambers (void volume 100 µL) and continuously superperfused with fresh buffer (composition: see below) with a rate of 35 mL/day to collect valproate liberated out of it. During superfusion, buffer and chambers were immersed into a water bath at 37 °C to simulate the *in vivo* situation. The superfusate of each implant was collected in eight fractions per day. The buffer used for the release experiments contained (in mM) NaCl 121, KCl 1.8, $CaCl_2$ 1.3, KH_2PO_4 1.2, $MgSO_4$ 1.2 and $NaHCO_3$ 25 to imitate the physiological cerebrospinal fluid. Before use, buffer pH was adjusted to 7.4 and prewarmed to 37 °C. 1000 µL of the daily superfusate fractions were transferred to screw-capped (8 mm) HPLC glass vials (WICOM, Heppenheim, Germany) and stored at −20 °C until HPLC measurements were performed. A schematic drawing of the superfusion equipment is shown in Fig. 6.

4.4. Chromatographic method for valproate analysis

4.4.1. Instrumentation

The HPLC system consisted of a SIL-10 AD VP autoinjector (equipped with a 100 µL sample loop), a CTO-10 AS VP column oven (Shimadzu, Duisburg, Germany) to keep the column temperature at 40 °C, a Wellchrom HPLC pump K-1001, a solvent organizer Wellchrom K-1500, a Wellchrom degasser, and a diode-array detector Wellchrom DAD K-2700 with the Wellchrom lamp K-2701 (Knauer, Berlin, Germany). Chromatography was performed on an analytical column CC 125/4 Nucleodur 100–5 C18 ec, preceded by a guard column CC 8/4 Nucleodur 100–5 (Macherey Nagel, Düren, Germany).
The mobile phase consisted of methanol plus 0.1% trifluoroacetic acid (72: 28 vol/vol). The flow rate of the mobile phase was maintained at 1 mL/min. The column effluent was monitored with the detector wavelength set to 210 nm. EuroChrom® for Windows, basic edition V3.05 (Knauer, Berlin, Germany), was used as computer software. All samples were measured in triplicate.

4.4.2. Quantification

Quantification was done by reference to valproate standards which were obtained at each batch of samples. Every analytic run also incorporated quality control standards at a concentration of 25 µg/mL valproate. The unknown concentration of valproate was quantified using linear regression analysis of the peak area (sample/standard peak area ratio) versus valproate concentration.

4.4.3. Calibration standards

A stock solution of valproate was prepared at a concentration of 1.0 mg/mL in buffer. The stock was diluted with buffer to produce valproate concentrations of 10, 15, 20, 25, 50, 100, 250, 500 µg/mL to study linearity.

4.4.4. Precision

The intraday precision of the method was assessed by calculating the coefficient of variation for replicates (n = 11) of the quality control standards. The intraday precision was determined from the quality control standard's mean values obtained at three different days. Furthermore, the peak mean values after one freeze-thaw cycle of valproate standards were determined and compared to freshly prepared valproate standards.

4.5. Drugs

Valproic acid sodium salt and $CaCl_2$ were purchased from Sigma (Taufkirchen, Germany).
NaCl, KCl, KH_2PO_4, $MgSO_4$ and $NaHCO_3$ were obtained from Merck (Darmstadt, Germany).
Methanol was obtained from J.T. Baker (Devent, Netherlands) and trifluoroacetic acid from Merck (Darmstadt, Germany). Polycaprolactone (M_n = 10.000 g/mol) was purchased from Aldrich, St. Louis, USA.

4.6. Calculations and statistics

Results in the text are given as arithmetic means with 95% confidence intervals (CI_{95}) to indicate statistical probability (Altman 1991). The coefficient of variation (ratio of the standard deviation to the mean) was applied to show a normalized measure of variation.

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