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KINETIC STUDY OF RELEASE OF IMMUNOSUPPRESSANT CYCLOSPORIN A FROM NANOFIBERS FOR MEDICINAL PURPOSES

Studium kinetiky uvolňování imunosupresiva cyklosporinu A z nanovláken pro medicinální účely

Diploma thesis

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ABSTRACT

In this thesis, the cyclosporin A-loaded poly(L-lactic acid) nanofibers fabricated by needle-less electrospinning technology were studied. Optimized LC-MS/MS method has been used for quantification of cyclosporin in nanofibers extracts following the extraction in water/phosphate buffered saline at 37.5 °C and 140 RPM. Five different types of cyclosporin A-loaded nanofibers which differed in content of cyclosporin and presence of various molecular weight hydrophilic additive poly(ethylene glycol) were extracted for one week in order to assess release behavior of cyclosporin A. The results showed that none of the studied nanofibers provide appropriate cyclosporin A release profile. The best results were obtained with nanofibers containing cyclosporin 10% (m/m) and poly(ethylene glycol) 6000 15% (m/m). High and steady release rate was observed for the first ten hours and then the release rate was gradually falling but maintained for the whole studied period. It has been concluded that although the nanofibers were not suitable for the intended use in transplantation patients the release profile of cyclosporin would be appropriate if the period of initial release was prolonged. This thesis provides basic information on the cyclosporin A release behavior and it may serve as basis for further research with relevant adjustments.

Key words

Cyclosporin A
Immunosuppression
Transplantation
Nanofibers
Poly(L-lactic acid)
Release kinetics
Local administration
LC-MS/MS

ABSTRAKT

V této diplomové práci byla studována nanovlákna z kyseliny polymléčné s inkorporovaným cyklosporinem A, která byla připravena tzv. needle-less elektrospinningem. Cyklosporin byl z nanovláken extrahován do vody/fosfátového pufru o fyziologické koncentraci při teplotě 37 °C a rychlosti třepání 140 RPM. Pro stanovení cyklosporinu v extraktech byla optimalizována LC-MS/MS metoda. Bylo provedeno týdenní uvolňování cyklosporinu A z pěti typů nanovláken, která se lišila obsahem cyklosporinu a přítomností a molekulovou hmotností přidaného polyethylenglykolu. Výsledná data ukázala, že žádná z použitých nanovláken nevykázala vhodný profil uvolňování cyklosporinu A. Nejlepších výsledků bylo dosaženo s nanovlákny obsahujícími 10 % cyklosporinu a 15 % polyethylenglykolu o molekulové hmotnosti 6000. Uvolňování cyklosporinu z těchto nanovláken bylo stabilní po dobu prvních 10 hodin experimentu, poté rychlost uvolňování postupně klesala, cyklosporin však byl uvolňován po celou dobu sledování (tj. jeden týden). Ačkoli ani tato nanovlákna pro zamýšlené použití nejsou vhodná, prodloužení doby počátečního stabilního uvolňování alespoň o jeden týden by bylo dostačující pro použití těchto nanovláken u pacientů po transplantaci. Tato práce poskytuje základní informace o uvolňování cyklosporinu A z nanovláken z kyseliny polymléčné a může posloužit jako podklad pro další rozšířené sledování kinetiky uvolňování.

Klíčová slova

Cyklosporin A
Imunosuprese
Transplantace
Nanovlákna
Kyselina polymléčná
Kinetika uvolňování
Lokální podání
LC-MS/MS

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

A..... peak area

ACN..... acetonitrile

c..... concentration

C18..... octadecyl

CI..... confidence interval

CsA..... cyclosporinA

h..... peak height

LC..... liquid chromatography

LOQ..... limit of quantification

M_r..... relative molecular weight

m/m..... mass percent

m/z..... mass-to-charge ratio

MeOH..... methanol

MRM..... multiple reaction monitoring

MS/MS..... tandem mass spectrometric detection

PBS..... phosphate buffered saline

PCL..... poly(ε-caprolactone)

PEG..... poly(ethylene glycol)

PLA..... poly(lactic acid)

R^2..... coefficient of determination

RPM..... rounds per minute

RSD..... relative standard deviation

s_r..... standard deviation

t..... time

t_r..... retention time

v/v..... volume percent

w..... peak width at base

$\tilde{x}$..... median

INTRODUCTION

Cyclosporin A is a widely used immunosuppressive agent indicated particularly in patients undergoing organ transplantation. Like any medicine also cyclosporin can cause side effects, e.g. renal dysfunction, hypertension, malignancies or liver impairment. The side effects are dose-dependent so the transplantation patients are very prone to get the side effects as their treatment requires higher doses administered for longer period of time. [1]

Local administration has a potential to reduce the incidence of adverse reactions observed after systemic administration of cyclosporin. Topical cyclosporin has been studied in patients with dry eye syndrome (liposomal formulation) [2], ocular inflammation or autoimmune disorder [3] or cornea transplantation patients [4] (cyclosporin-loaded poly(lactic acid) nanoparticles).

Nanofibers studied in this thesis were produced by a technique called needle-less electrospinning (its modification Nanospider^{TM} patented in 2005) which uses electrostatic force to produce nanoscale fibers.[5]

Nanofibers formed from natural or synthetic polymers are used in several fields, e.g. in medicine, biochemistry, environmental engineering or defense and security.

Use of nanofibers as drug carriers for topical administration have been already studied with e.g. antibiotics [6] [7] or analgetics [8] [9]. It has been shown that properties of drug-loaded nanofibers and drug release profile can be modified by modifying parameters of electrospinning and content of a polymer solution. Especially addition of hydrophilic polymer poly(ethylene glycol) has been extensively studied as it has been shown to have significant effect on drug release profile [10] [11].

It has been proven that cyclosporin A pharmacological activity is maintained after its incorporation into poly(L-lactic acid) nanofibers [12] and therefore cyclosporin-loaded nanofibers might serve as a route of local cyclosporin A administration.

Aims of thesis

The aim of this thesis is to assess release profile of the immunosuppressive agent cyclosporin A from electrospun poly(L-lactic acid) nanofibers.

Firstly a method suitable for quantification of cyclosporin A will be found by optimizing parameters of analysis of cyclosporin by high performance liquid chromatography connected with tandem mass spectrometric detection.

Secondly extraction of cyclosporin A from poly(L-lactic acid) nanofibers will be performed. The experiments will be conducted with nanofibers that differ in content of cyclosporin and presence and molecular weight of added poly(ethylene glycol).

Finally, the aspects of cyclosporin A release will be compared and evaluated with regard to intended use of the nanofibers, i.e. use for local delivery of cyclosporin in patients undergoing organ transplantation. The impact of cyclosporin content and addition of poly(ethylene glycol) will be assessed in order to find optimal nanofibers for the planned use.

THEORETICAL PART

1 Cyclosporin A

Cyclosporins are a group of cyclic undecapeptides produced by two strains of deuteromycetes, *Cylindrocarpum lucidum* and *Tolypocladium inflaturn* [13], as secondary metabolites, with over 25 members that vary in primary structure or in stereoisomerism (e.g. in position 7: cyclosporin A: L-Abu, cyclosporin B: L-Ala, cyclosporin C: L-Thr; in position 5: cyclosporin A: L-MeVal, cyclosporin E: L-Val, cyclosporin H: D-MeVal) [14]. Among the cyclosporin family cyclosporin A (CsA) exhibits the strongest immunosuppressive activity and since 1978 it has been used in clinical practice. The mechanism of cyclosporin A action is based on selective binding to cyclophilin (intracellular receptor of cyclosporin). The formed complex blocks activity of calcineurin which is a protein phosphatase necessary for expression of proteins needed for B-cell function (interleukin 4) or for activation of T-cells (interleukin 2) [15].

1.1 Structure and properties

Basic properties [14]

Formula	$C_{62}H_{111}N_{11}O_{12}$
M_r	1 203
Definition	Cyclo[[(2S,3R,4R,6E)-3-hydroxy-4-methyl-2-(methylamino)-oct-6-enoyl]-L-2-aminobutanoyl-*N*-methylglycyl-*N*-methyl-L-leucyl-L-valyl-*N*-methyl-L-leucyl-L-alanyl-D-alanyl-*N*-methyl-L-leucyl-*N*-methyl-L-leucyl-*N*-methyl-L-valyl]
Appearance	White to almost white powder
Solubility	Poorly soluble in water, soluble in apolar solvents (e. g. anhydrous ethanol)

Structure

Cyclosporin A is a hydrophobic cyclic undecapeptide (see Figure 1). Seven of the aminoacids are *N*-methylated, which might prevent cyclosporine from inactivation in gastrointestinal tract [16], the four unmethylated amino acids may form intramolecular H-bonds that stabilize the molecule by forming a β-sheet structure. The structure then contains a *cis* amide bond between Mle9 and Mle10.

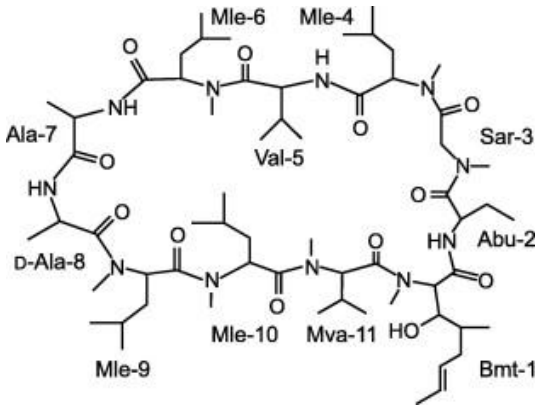

Fig. 1: Primary structure of cyclosporin A [17]

For activity of CsA the formation of a complex with cyclophilin 18 is essential and it requires major conformational change - the H-bonds in CsA have to be broken and the *cis* amide bond has to be transformed to *trans* conformation [18]. It has been postulated that this change is inducted by binding of CsA to metal ions, in particular magnesium ions. [19]

Solubility

Cyclosporin A shows unusual solubility behavior when assessing the solubility as a function of temperature. For most drugs the heat of solution is endothermic [20] which results in increase of solubility with rising temperature. In contrast cyclosporin A shows decrease of solubility with rising temperature, the heat of solution is exothermic. This has been described by dissolution of cyclosporin A in aqueous media at different temperatures and subsequently by calculating the enthalpy of the dissolution process. The solubility of cyclosporin A in water was 7.3 µg/mL at 37 °C and in contrast 101.5 µg/mL at 5 °C. [21]

1.2 Marketing status in the Czech Republic

Medicinal products containing cyclosporin A as the active substance are marketed by four marketing authorisation holders. There are fifteen marketed products (out of 49 registered products), these are summarized in the Table 1. All the marketed products are reimbursed from health insurance. [22]

Table 1: Summary of the products marketed in the Czech Republic

Product name	Marketing authorisation holder	Pharmaceutical form
CICLOSPORIN MYLAN 25 MG	Generics UK Ltd., Station Close, Potters Bar, Hertfordshire, Great Britain	Capsule, soft
CICLOSPORIN MYLAN 50 MG	Generics UK Ltd., Station Close, Potters Bar, Hertfordshire, Great Britain	Capsule, soft
CICLOSPORIN MYLAN 100 MG	Generics UK Ltd., Station Close, Potters Bar, Hertfordshire, Great Britain	Capsule, soft
CYCLAID 25 MG	Medis International a.s., Karlovo náměstí, Praha, Česká republika	Capsule, soft
CYCLAID 50 MG	Medis International a.s., Karlovo náměstí, Praha, Česká republika	Capsule, soft
CYCLAID 100 MG	Medis International a.s., Karlovo náměstí, Praha, Česká republika	Capsule, soft
EQUORAL	TEVA Czech Industries s.r.o., Opava-Komárov, Česká republika	Oral solution
EQUORAL 25 MG	TEVA Czech Industries s.r.o., Opava-Komárov, Česká republika	Capsule, soft
EQUORAL 50 MG	TEVA Czech Industries s.r.o., Opava-Komárov, Česká republika	Capsule, soft
EQUORAL 100 MG	TEVA Czech Industries s.r.o., Opava-Komárov, Česká republika	Capsule, soft
SANDIMMUN	Novartis s.r.o., Praha, Česká republika	Concentrate for solution for infusion
SANDIMMUN NEORAL 25 MG	Novartis s.r.o., Praha, Česká republika	capsule, soft
SANDIMMUN NEORAL 50 MG	Novartis s.r.o., Praha, Česká republika	Capsule, soft
SANDIMMUN NEORAL 100 MG	Novartis s.r.o., Praha, Česká republika	Capsule, soft
SANDIMMUN NEORAL 100 MG/ML	Novartis s.r.o., Praha, Česká republika	Oral solution

1.3 Therapeutic area

Cyclosporin A is widely recognized as a transplantation-associated agent. It is used to prevent graft rejection following lung, heart, liver, kidney or bone marrow transplantation and to prevent graft-versus-host disease.

Apart from transplantation-associated indications cyclosporin A has several non-transplantation indications – for example psoriasis, atopic dermatitis, rheumatoid arthritis, nephrotic syndrome. For psoriasis, atopic dermatitis and rheumatoid arthritis cyclosporin A is not considered medication of first choice - it is used in particular in patients in whom the conventional agents were ineffective or inappropriate. [1]

1.4 Posology in transplantation patients [1]

Daily dose of cyclosporin A should be devided in two doses distributed equally throughout the day.

Treatment should be initiated 12 hours (solid organ transplantation) or one day (bone marrow transplantation) before the surgery. The dose should be maintained for 1 to 2 weeks after the transplantation then the dose is gradually decreased to reach maintenance dose.

The daily doses may be changed in accordance with blood levels, concomitant treatment or gastrointestinal disturbances.

Children generally require higher doses than the adults (given in mg/kg).

1.5 Undesirable effects

Use of cyclosporin A is complicated by the wide range of side effects associated with systemic administration. Selected reported adverse effects are:

- renal dysfunction, tremor, diarrhoea, anorexia, nausea, vomiting, hirsutism, hypertension, increased risk of infections, lymphoma, malignancies (particularly of the skin) (observed during clinical trials)
- thrombotic microangiopathy, migraine, hepatotoxicity (cholestasis, hepatitis, jaundice, liver failure) (reported during post-marketing experience)

The adverse events are dose-dependent so usually the adverse events are more frequent and more severe in patients who undergo the transplantation as the initial doses of cyclosporin are higher and maintenance treatment is longer than in patients with other indications. [1]

1.6 Methods of administration

Almost all the marketed products are administered orally - soft capsules and peroral solutions are available on the Czech market. The only exception is Sandimmun® that may be administered also intravenously. Intravenous administration is recommended in patients with gastrointestinal disturbances which could affect absorption of the drug or in patients shortly after surgery (transplantation) who are not able to take cyclosporin orally. The formulation of the concentrate contains polyethoxylated castor oil which has been reported to cause anaphylactoid reaction consisting of e.g. respiratory distress, flushing of the face, blood pressure changes or tachycardia. The patients on intravenous therapy should be closely monitored and should be transferred to oral therapy as soon as possible. [1] Ocular desease called dry eye syndrome may be treated with locally administered medication Restasis®. It is an emulsion of cyclosporin in glycerin and castor oil at concentration of 0.5 mg/mL [23]. This product is not registered in the Czech Republic.

All the above mentioned methods of administration except the cyclosporin emulsion are systemic, which leads to wide spectrum of side effects. Bioavailability after oral administration is individual and may be affected by food. The daily dose of cyclosporin should be devided in two parts and the treatment is often complicated by the patient compliance with the medication. These factors raised the need for targeted immunosuppression.

Local administration of cyclosporin was studied with regard to a treatment of ocular inflammatory or autoimmune disorders [3] or to prevent graft rejection following the cornea transplantation [4]. The studies evaluated cellular toxicity, uptake, release kinetics and cornea penetration, ocular distribution, efficacy in rats when using CsA-loaded nanoparticles for treatment of inflammation/autoimmune disorder and corneal graft rejection prevention. Carriers of the drug were micelles consisting of poly(lactic acid) (PLA) and different supplemental substances. Both studies concluded that CsA-loaded nanoparticles showed great potential for effective local administration of cyclosporin A.

Cyclosporin A-encapsulated liposomes consisting of phosphaditylcholine from soybeans and egg lecithins were studied with regard to treatment of dry eye syndrome. The liposomes were prepared by supercritical fluid method. Efficacy of liposomes was assessed and compared to efficacy of cyclosporin emulsion already registered for use in eye treatment (Restasis®). It has been concluded that liposomal formulation of cyclosporin is more effective and less irritating in rabbit eyes than the emulsion. [2]

Cyclosporin A was also studied in PLA nanofibers. It has been proven that cyclosporin A does not lose its pharmacological activity after incorporation into nanofibers. The activity was assessed by addition of the nanofibers into cultures of mouse spleen cells stimulated with concanavalin A (stimulator of T cell activity) - the proliferation of T-cells was inhibited and production of T-cell cytokines was suppressed. The release kinetics was investigated by assessing the level of local production of proinflammatory cytokins in skin allografts covered with the CsA-loaded nanofibers. It has been concluded that the CsA nanofibers can be used as drug carriers for local suppression of inflammatory reactions or as scaffolds for cell-based therapy. [12]

1.7 Further proceedings

There are a number of open clinical trials which aim to widen the use of cyclosporin A. Regarding new methods of topical administration new ophtalmic formulations are tested (Phase II studies and one Phase IV study). [24] There is also Phase 1 pilot clinical trial studying aerosol liposomal cyclosporin and its efficacy against chronic rejection following lung transplantation in patients with bronchiolitis obliterans syndrome [25].

Use of cyclosporin is tested as adjuvant medicine in treatment of refractory immune thrombocytopenia[26], advanced solid tumors or colorectal cancer[27], idiopathic membranous nephropathy[28] or acquired aplastic anemia[29].

Other open trials study drug-drug interactions in patients with concurrent disease and/or concomitant treatment.[30][31]

Finally there are open trials designed to collect safety data from the post-marketing cyclosporin use mainly in treatment of dry eye disease, psoriasis, organ (kidney) rejection and graft-versus-host disease. [24]

2 Nanofibers

2.1 Electrospinning

Elestrospinning is a technique for preparation of nanofibers which uses electrostatic forces to form fibers with diameter from nanometer to micrometer from polymer solutions. The thinner diameter enables production of nanofibers with larger specific surface area which makes the nanofibers effective carriers of various active ingredients as the surface is easily accessible to liquid or gaseous media.

High voltage source is necessary for injection of a certain polarity charged polymer in solution. Due to opposite charge polarity of the polymer solution and the collector (counter electrode) the repulsive force overcomes the polymer surface tension force at certain value of applied charge [32]. A charged jet of polymer solution then forms a Taylor cone and the jet is accelerated towards the collector. Rapid and unstable whipping of the jet between the solution and collector leads to the solvent evaporation [33] and subsequently a fiber with thin diameter is deposited on the collector.

In a standard arrangement the polymer solution is in a tube with a capillary at the end (which is connected to a high voltage source) and when the repulsive forces overcome the surface tension force the jet of polymer solution is ejected through the capillary. The drawback of this arrangement is limited process efficiency as only one nanofiber at a time can be formed.

Alternate approach is called needle-less electrospinning and this modification overcames the main disadvantage of the standard process which is a low production rate.

In 2004 a method called upward needleless electrospinning has been presented. There is a two-layer system consisting of ferromagnetic suspension (lower layer) and a polymer solution (upper layer). After applying normal magnetic field vertical spikes of the lower suspension perturbe the interlayer and the free surface. Then normal electric field is applied in addition and multiple jets of the polymer solution are directed upward from the pertrubation sites to the upper counter-electrode. [34]

Another modification called Nanospider^{TM} has been also presented in 2004; this procedure was patented by European Patent Office in 2005.

Nanospider™ [5]

The polymer solution is in a container, continuous intake of polymer solution is ensured by an inlet, amount of the solution is regulated by the position (height) of outlet. One of the electrodes is a rotating cylinder (connected to positive charge). The rotating cylinder is partially immersed in the polymer solution and it draws the solution out of the container. Electric field between the cylinder electrode and the counter electrode covered by a backing fabric leads to formation of Taylor cones on the surface of the solution on the cylinder electrode. The newly formed nanofibers are collected on the backing fabric which is rotating in the opposite direction to the cylinder electrode (see Figure 2). The thickness of the nanofibers layer can be easily regulated by the speed of the running backing fabric. Nanofibers in diameters from 50 nm to 800 nm are formed. Also another three alternate arrangements of this technique have been presented in the patent.

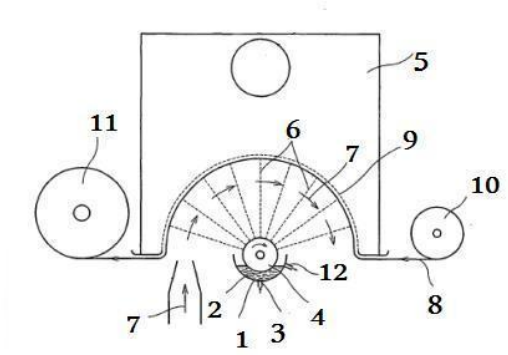

Fig. 2: Nanospider technique arrangement [5]. 1 container; 2 polymer solution; 3 inlet; 4 rotating cylinder electrode; 5 low pressure chamber; 6 newly formed nanofibers; 7 drying air; 8 backing fabric; 9 counter electrode; 10 unreeling device; 11 reeling device; 12 outlet.

2.2 Materials for production of nanofibers

2.2.1 Polymers

Nanofibers can be formed from many types of polymers both natural and synthetic, e.g. nanofibers from proteins [35] [36], polysaccharides [37] or nucleic acids [38] were produced.

Nanofibers formed from natural polymers are generally more biocompatible and less immunogenic, compared to the nanofibers formed from synthetic polymers. Natural polymers carry specific sequences which enable binding of cells to the formed nanofibers [39], they are, however, more prone to degradation/change of properties when put to inappropriate environment (special attention is needed when selecting a solvent for dissolution of polymer) [40].

Natural polymers include e.g. silk fibroin, fibrinogen, collagen, cellulose, gelatin, chitin or chitosan.

Nanofibers formed from synthetic materials offer many advantages compared to natural polymer nanofibers - their mechanical properties (strength, viscoelasticity) and degradation rate are more flexible and enable wider range of produced types of nanofibers of various structure, properties and behavior.

Synthetic polymers used for formation of nanofibers are e.g. poly(ε-caprolactone), poly(lactic acid), polyglycolide, polyacrylonitrile, polyamide or polyurethane.

2.2.2 Solvents

Solvent used in electrospinning has a significant impact on the produced nanofibers. It is thus essential to use a solvent of properties suitable for intended use of the formed nanofibers. Solvent used for dissolution of polymer should have certain properties - good volatility, suitable boiling point and vapor pressure and the integrity of the polymer solution should be maintained. Depending on the type of solvent interaction in the system polymer-solvent may be attractive of repulsive [41].

The solvents are often organic and toxic which requires certain safety measures during the production and subsequent effective wash-out procedure of residual leachables (i.e. residual monomers and solvents). The safest system regarding solvent toxicity is water - polymer, however, in that case subsequent crosslinking would be inevitable for long-term release as the nanofibers would be unstable in humid environment. Nevertheless, the nanofibers prepared from water solution instable in humid environment have been used with advantage for preparation of orally dissolving web. Donepezil HCl (Alzheimer's disease medication)-loaded poly(vinyl alcohol) nanofibrous web formulation was studied in order to obtain a formulation with ultrafast release that would provide suitable way of oral administration (alternative to orally disintegrating tablets) for people with swalowing difficulties (elderly, pediatric patients etc.).[42]

The examples of solvents used for electrospinning are chloroform, ethanol, or dimethylformamide, influence of different solvents on the properties of formed nanofibers was studied by various research groups [43][44].

2.3 Key parameters of electrospinning [45][46][47]

Viscosity

Viscosity is affected by molecular weight of polymer and concentration of solution, composition of used solvents and temperature of the mixture. In a solution of low viscosity surface tension is dominant and therefore only beads or beaded fibers can be produced. Generally, with increasing viscosity the nanofibers of larger and more uniform diameter are formed.

Surface tension

High surface tension leads to instability of the jet which results in formation of droplets, beads and beaded fibers so generally low surface tension enables production of continuous uniform nanofibers.

Conductivity

Conductivity of the solution depends mainly on polymer type and solvent used. With increasing conductivity the fiber diameter decreases, low conductivity may lead to formation of beads. It has been demonstrated that addition of ionic salt (NaCl, NaH_2PO_4) increases conductivity of polymer solution [48].

Distance between electrodes

Certain minimum distance should be set to allow the solvent to fully evaporate. It has been concluded that the distance between the electrodes also affects the formed nanofibers. Flatter fibers were formed from silk-like polymer with closer electrode distance.[49]

Applied voltage

It is necessary to find optimal value of voltage for each polymer type and concentration. Generally application of higher voltage results in thinner diameter but it also leads to higher probability of formation of beads.

2.4 Applications

Electrospun fibers provide high specific surface area, high porosity, desired physico-mechanical properties, which can be influenced during the electrospinning process, and that enable use in various applications. Nanofibers are used in medicine (tissue engineering scaffolds, drug delivery systems, wound healing, as biosensors, vascular graft implants; selected medicinal applications will be described later), as filters (air filters, purification of biomolecules), affinity membranes, for immobilization of enzymes, environmental engineering, for energy storage and energy generation, in defense and security.

2.4.1 Scaffolds for tissue engineering

In tissue engineering basic requirements are biocompatibility and biodegradability of the nanofibrous scaffold. Main advantage of nanofibers is that their structure is very similar to the extracellular matrix. Chemical biocompatibility is well provided by natural polymers such as collagen, silk fibroin, fibrinogen and others. Also blending of natural polymer into synthetic polymers improves biocompatibility of the synthetic scaffold [50]. Biodegradability is required as the scaffolds are used temporarily to support cell seeding, proliferation and differentiation.

Nanofibrous scaffolds have been studied for use as scaffolds in engineering cartilages [51][52], dermal substitutes [53], bones [54], heart [55], or blood vessels [56].

2.4.2 Wound healing

Ideal dressing for wound healing should be efficient as bacterial barrier and absorb excess exudates. Also the adaptability to the wound area and suitable adherence to healthy but not to wound tissue are important characteristics. The dressing must enable appropriate gaseous exchange and have sufficient moisture vapor permeability. It should then be painless to patient, easy to remove and have low cost [57]. These requirements are well met when using nanofibers as wound healing dressings. Among others collagen [51], silk [58], gelatin and poly(ε-caprolacton) [36], polyurethane [59], poly(L-lactide) [60] or chitosan whose hemostatic activity has been known since 1997[61] and therefore chitosan dressings are used in trauma patients (both civilian and military) [62].

2.4.3 Drug delivery systems

Biocompatible polymer matrices can be designed for programmed dissemination so they are mostly used as carriers of therapeutic agents.

Poly(D,L-lactide) and poly(ethylene glycol)-co-poly(D,L-lactide) nanofibers were loaded with paracetamol (analgesic and antipyretic drug). The nanofibers characteristics, degradation of nanofibers and *in vitro* release of paracetamol were studied. The authors concluded that addition of hydrophobic drug, such as paracetamol, leads to better morphology of nanofibers. Degradation of nanofibers was influenced mainly by fiber diameter and porosity, the effect of molecular weight and content of PEG was minor. In the first hours burst paracetamol release was observed followed by a phase of sustained release dependant on degradation of the nanofibers. [8]

Poly(D,L-lactide) (PLA) and poly(ε-caprolactone) (PCL) nanofibers and bicomponent nanofibers PLA-PCL were loaded with antibiotics tetracycline, chlorotetracycline and amphotericine B. The characteristics of polymer solution, release properties and antimicrobial activity were assessed. PCL nanofibers released around 90% of the drug, on the other hand around 10% was released from PLA nanofibers. Release profile of bicomponent nanofibers depended on the amount of each polymer - bicomponent nanofibers with 75% of PCL had similar release behavior as the pure PCL nanofibers. Tetracycline was released at the highest rate, amphotericin B at the slowest rate. The antimicrobial activity tested on *Escherichia coli* and *Staphylococcus aureus* was maintained. [6]

Sandwich-structured poly(D,L-lactide-co-glycolide)/collagen nanofibers with content of vancomycin, gentamicin and lidocaine (antibiotics) were prepared. Drug release (*in vitro* and *in vivo*) and effectiveness in wound healing in rats were assessed. The release profile *in vitro* was triphasic: initial burst release on day 1, then stabilization on lower release rate with a second release peak in week 2. *In vivo* experiments showed only biphasic release with a peak release on day 14. The tests of wound healing showed that nanofibers loaded with antibiotics supported complete healing of *S. aureus* and *E. coli* infected wounds in rats [7].

Poly(vinyl alcohol) nanofibers coated with polyurethane nanofibrous layers were loaded with gentamicin (antibiotic). The structure of the formed nanofibers was observed, the drug release was studied *in vitro* and the antimicrobial efficacy was investigated on *S. aureus* and *P. aeruginosa*. The drug release experiments were led with sandwich-structured nanofibers with non-crosslinked and crosslinked nanofibers. The release from the nanofibers to water was fast as almost 90% of gentamicin was released within one hour with observable influence of thickness of covering layers only during the first part of experiments (the first half hour). The release from the nanofibers to wet agar plates was also fast. Difference was observed in the experiments with thicker covering polyurethane layers which slowed the release to maintain up to 48 hours. The release profile was then assessed by inhibitory tests on *S. aureus*. The area of inhibition in various time periods was evaluated and the results confirmed that only the thicker covering layer can influence (slow) the gentamicin release. [63]

Poly(L-lactic acid) nanofibers were loaded with cyclosporin A. The pharmacological activity, release profile in culture medium and influence of cyclosporin on ability of the nanofibers to serve as scaffolds for cell-based therapy were studied. Maintenance of immunosuppressive activity was proven on spleen cells stimulated with concanavalin A - production of inflammatory interleukins was prevented. The drug release study showed that significant amount of cyclosporin was released only during the first 12 hours, but even after this time cyclosporin was released for at least 96 hours. This experiment was performed in complete RPMI 1640 media and on skin allografts which better correspond to *in vivo* release behavior. It has also been concluded that examined cells (LSCs, MSCs and 3T3 fibroblasts) grew comparably on cyclosporin A-loaded nanofibers and on nanofibers without cyclosporin A and therefore cyclosporin-loaded nanofibers can be used as scaffolds for cell growth. [12]

Apart from the above mentioned there was also ibuprofen incorporated into poly(lactide-co-glycolide) nanofibers [9], cefazolin incorporated into poly(lactide-co-glycolide) nanofibers [64], rifampin incorporated into poly(L-lactic acid) nanofibers [65], and itraconazole incorporated into hydroxypropylmethylcellulose nanofibers [66].

Addition of poly(ethylene glycol)

Poly(ethylene glycol) (PEG) is a hydrophilic polymer. PEG itself is used to manage chronic constipation [67].

It has been added to nanofibers to increase porosity and thus to improve drug release; PEG is more hydrophilic than the incorporated drug and after its release in aqueous matrix the specific area to volume ratio of the nanofibres increases which faciliates release of the drug. [11]

Apart from the drug release rate also the drug release mechanism is altered by the addition of PEG which has been shown for protein release from lipidic implants when alteration of release curves shape has been observed. The release rate remained stable over longer period of time and the total amount of released interferon was higher. [10]

When PEG was added to paclitaxel-loaded poly(lactide-co-glycolide) nanofibers alteration of release profiles was observed as well. Addition of low molecular (8 kD) PEG resulted in high release rate (an average of 12 μg/day/cm^2) for up to 12 days while addition of high molecular (35 kD) PEG resulted in slow release rate (an average of 3.8 μg/day/cm^2) for about a month. [11]

3 Analyses

3.1 Analyses of cyclosporin A

3.1.1 Immunoassays

Immunoassays are used in cyclosporin monitoring in biological samples. In last ten years researchers were trying to examine reliability of this method. Cyclosporin A has a narrow therapeutic index and continuous monitoring of drug levels in blood is necessary to maintain the positive effect of cyclosporin treatment with minimal adverse reactions. It has been proven that performance of immunoassays is often not satisfactory. The monoclonal antibodies cross-react with cyclosporin metabolites which leads to positive biases when assessing the cyclosporin A levels in blood. Such overestimation presents risk for patient as due to apparently high level of drug lower and insufficient doses of cyclosporin are administered and the risk of graft rejection and organ loss increases. It has been concluded that HPLC analyses provide better specificity and reliability and should be therefore preferred for cyclosporin monitoring.[68][69][70]

3.1.2 HPLC - UV/ HPLC - MS(/MS)

High performance liquid chromatography connected with UV spectrophotometer or (tandem) mass spectrometer is nowadays the preferred method for quantification of cyclosporin A. Consequently many systems for analysis of cyclosporin A have been developed and described. Some of these systems are presented in Table 2.

Table 2: Summary of high performance liquid chromatographic methods for analysis of cyclosporin A

Stationary phase	Mobile phase	Temperature	Detection	Reference
Purpose				
Octadecyl (Hypersil)	isopropanol/ACN/water 80/10/10 (v/v/v)	25 °C	UV 227 nm	[71]
Determination of cyclosporin in whole blood				
Octadecyl (Lichrospher)	tetrahydrofuran/phosphoric acid 44/56 (v/v)	75 °C	UV 220 nm	[72]
Determination of cyclosporin and impurities in capsules Neoral and its generic versions				
Phenyl (Spherisorb)	ACN/MeOH/water 47/50/3 (v/v/v)	room temperature	UV 215 nm	[73]
Simultaneous determination of cyclosporin and its major metabolite in human serum				

Stationary phase	Mobile phase	Temperature	Detection	Reference
Purpose				
Octadecyl (Shim-pack)	ACN/water 70/30; 80/20 (v/v)	70 °C	UV 215 nm	[74]
Determination of cyclosporin and lovastatin *in vitro*				
Cyano (Spherisorb)	hexane/isopropanol 90/10 (v/v)	50 °C	UV 212 nm	[75]
Determination of cyclosporin and its metabolites M1, M17 and M21				
Octyl (Ultrasphere)	ACN/water 56/44 (v/v)	60 °C	UV 278 nm (214 nm from min 14)	[76]
Simultaneous determination of cyclosporin and everolimus				
Phenyl (Nova-Pak)	ACN/MeOH/water 20/52/28 (v/v/v)	72 °C	UV 210 nm	[77]
Determination of cyclosporin in human and mouse plasma				
Phenyl (Nova-Pak)	ACN/0.04M monobasic potassium phosphate (pH 2.5) 65/35 (v/v)	75 °C	UV 205 nm	[78]
Determination of cyclosporin in rat blood and plasma				
Octadecyl (Zorbax)	5 mM ammonium acetate/THF gradient	30 °C	MS/MS (1220→1203)	[79]
Determination of cyclosporin in monkey and rat plasma				
Octyl (Altima)	MeOH/50 mM ammonium acetate (pH 5.1) 72/28 (v/v)	70 °C	MS/MS 1203.0→425.4	[80]
Determination of cyclosporin in blood				
Octadecyl (Nucleodur)	ammonium acetate/MeOH formic acid gradient	80 °C	MS/MS 1220→1203	[81]
Determination of cyclosporin A in cat blood				
Octadecyl (Atlantis) cyano (YMC)	ACN/water 52/48 (v/v)	50 °C	MS/MS 1225→1114	[82]
Determination of tacrolimus, sirolimus any cyclosporin in whole blood				
Octadecyl (Ultrasphere)	water/ACN/MeOH 30/37/33 (v/v/v)	80 °C	MS 1225	[83]
Determination of cyclosporin A in whole blood				
Octyl (Symmentry)	10% glacial acetic acid/ACN 25/75 (v/v)	room temperature	MS 601.3	[84]
Determination of cyclosporin in human plasma				
Octyl (Hypersil MOS)	ACN/water gradient	75 °C	MS 1224.9	[85]
Determination of cyclosporin and its metabolites in blood				
Octadecyl	ACN/water 90/10 (v/v)	75 °C	MS 1224.9	[86]
Use of LC/MS in the routine clinical laboratory for monitoring sirolimis, tacrolimus and cyclosporin				

3.2 Drug release

Different methods of extraction of drugs into different media and from different nanofibers have been described. More detailed description of some of those systems is provided below.

Release of paracetamol from paracetamol-loaded poly(D,L-lactide) and poly(ethylene glycol)-co-poly(D,L-lactide) nanofibers was studied. Sections of nanofibers (2 x 2 cm) were placed to tubes containing 20 mL of 0.1 M phosphate-buffered saline (PBS), pH 7.4. The tubes were placed in shaking air bath (37 °C, 120 RPM). Samples (1 mL) were collected at specified time periods for 504 h, 1 mL of fresh medium was added to the incubation solution after each sample taking. The amount of released paracetamol was quantified with UV-Vis spectrophotometer. [8]

Release of tetracycline, chlorotetracycline and amphotericine B from poly(D,L-lactide) (PLA), poly(ε-caprolactone) (PCL) nanofibers and bicomponent nanofibers PLA-PCL was studied. Weighed nanofibers were placed in 50 mL of 0.05 M Tris buffer pH 7.35 and shaked at 37 °C at 100 RPM. Samples were collected for 3 hours and the amount of released drug was quantified with UV-Vis spectrophotometer. [6]

Release of vancomycin, gentamicin and lidocaine from sandwich-structured poly(D,L-lactide-co-glycolide)/collagen nanofibers was observed. Samples of the nanofibres (2 x 3 cm) were inserted into glass test tubes with 1 mL of phosphate-buffered solution (0.15 M, pH 7.4). After 24 h incubation at 37 °C the eluent was analyzed and 1 mL of fresh phosphate-buffered solution was added to the test tube. This procedure was performed for 30 days. The amount of drug was quantified with UV-Vis spectrophotometer (280 nm). [7]

Release of gentamicin from poly(vinyl alcohol) nanofibers coated with polyurethane nanofibrous layers was studied. Discs of diameter 8 mm were cut from the nanofibers and placed in 10 mL of water or on wet agar plate. Discs removed from agar plate were shaken in distilled water for two days to analyze the amount of gentamicin remaining in the nanofibers. The aqueous solutions were analyzed by HPLC-MS with tandem mass spectrometer. The microbial properties of the produced nanofibers were evaluated by placing the nanofibrous mats on the agar previously spread with a suspension containing *S. aureus*. The area of inhibition was measured at certain time points. The discs with residual gentamicin were then placed on a new agar for further evaluation following 24 h incubation at 37 °C. [63]

Release of cyclosporin A from poly(L-lactic acid) nanofibers was observed *in vitro* and *in vivo*. Sections of nanofibers (5 cm x 5 cm) were placed in wells with RPMI 1640 medium containing 10% FCS (fetal calf serum). Nanofibers were transferred into new wells with fresh medium after certain time periods. Supernatants from the wells were tested for inhibition of T-cell proliferation and IL-2 production induced by concanavalin A. In *in vivo* experiments in mice the sections of cyclosporin-loaded nanofibers (10 x 10 cm) were placed on skin grafts and removed after certain time periods. The presence of cyclosporin A was assessed by the ability of inhibition of interleukin production and T-cell proliferation. The amount was quantified by comparing the original nanofibers (before starting the experimens) or with standard of cyclosporin - remaining percentage of cyclosporin was calculated from this comparison. [12]

EXPERIMENTAL PART

4 Experimental conditions

4.1 Materials and reagents

- cyclosporin A, standard (TEVA Czech Industries, Opava, Czech Republic)
- acetonitrile, LC-MS CHROMASOLV® grade, ≥ 99.9% (Sigma-Aldrich, Germany)
- methanol, LC-MS CHROMASOLV® grade, ≥ 99.9% (Sigma-Aldrich, Germany)
- formic acid, 98 - 100% (Merck KGaA, Germany)
- ammonium acetate (Sigma-Aldrich, Germany)
- acetic acid, 99.8% G.R. (Lach-Ner s.r.o., Czech Republic)
- ammonium hydroxide, 25% G.R. (Lach-Ner s.r.o., Czech Republic)
- deionised water
- phosphate buffered saline (PBS), tablet (Sigma-Aldrich, Germany)
- CsA-loaded nanofibers (ElMarco, Litvínov, Czech Republic)
    - 10% (m/m) of CsA; 10.7 g/m^2
    - 5% (m/m) of CsA; 9.6 g/m^2
    - 2.5% (m/m) of CsA; 8.9 g/m^2
    - 10% (m/m) CsA with addition of PEG (poly(ethylene glycol)) 6000 (15%); 6.1 g/m^2
    - 10% (m/m) CsA with addition of PEG 20000 (15%); 6.1 g/m^2

- Agilent 6400 Series Triple Quadrupole LC/MS System (Agilent Technologies, Waldbronn, Germany)
- Ascentis Express C18, 5 μm HPLC Column (15 cm x 3.0 mm) (Sigma-Aldrich, Germany)
- XSelect CSH Phenyl-Hexyl XP Column, 130 Å, 2.5 μm, 4.6 mm x 100 mm (Waters, USA)
- XSelect CSH Phenyl-Hexyl Guard Column, 130Å, 3.5 μm, 4.6 mm X 20 mm (Waters, USA)

- APX-100 Analytical Balance (Denver Instruments, USA)
- Jenway 3510 benchtop pH meter (Jenway, Great Britain)
- ES-20 Shaker-incubator (Grant Instruments Ltd, Cambridge, United Kingdom)
- software: Data Acquisition, Mass Hunter, (Agilent Technologies, Waldbronn, Germany)

  Qualitative analysis, Mass Hunter, (Agilent Technologies, Waldbronn, Germany)

  Origin 6.0 (OriginLab Corporation, USA)

4.2 Stock solution

Stock solution of CsA standard was prepared in methanol by dissolving 10 mg of CsA in 10 mL of methanol.

Stock solution was diluted with a mixture of methanol and water (6/4, v/v) in order to obtain standard in required concentration (200 μg/mL) for optimization of liquid chromatography separation and mass spectrometric detection.

It was necessary to prepare calibration curves for determination of cyclosporin in water and PBS. Due to poor solubility of cyclosporin in water it was necessary to first prepare a solution in concentration of 20 μg/mL from the stock solution by diluting it with mixture of methanol and water (6/4, v/v) and only after that it was possible to dilute it solely with water or PBS to obtain calibration standards and solutions for assessment of validation parameters.

4.3 Chromatographic conditions

To optimize liquid chromatography conditions a standard of cyclosporin A in concentration of 200 μg/mL was prepared.

Mixtures of acetonitrile and water with addition of formic acid (0.1%), acetonitrile and 1 mM ammonium acetate (pH = 4.5), methanol and ammonium acetate were used as mobile phases to find optimal conditions for analysis of cyclosporin on octadecyl stationary phase. The mass spectrometric detection was performed in positive ionization mode.

Mixtures of methanol and ammonium acetate (pH = 4.5), methanol and water with addition of NH_4OH (0.1%) were used as mobile phases to examine the parameters of analysis of CsA on phenyl-hexyl stationary phase. For detection of CsA under using basic mobile phase the negative ionization mode of mass spectrometric detection was used.

In the analyses with mixture of acetonitrile and water with addition of formic acid and with ammonium acetate as a part of mobile phase the ion of m/z 1224.8 was detected, in the analyses with ammonium hydroxide the ion of m/z 1200.8 was chosen for detection.

All the analyses were performed at a column temperature 45 °C and flow rate of the mobile phase 0.6 mL/min.

4.4 Mass spectrometric conditions

The optimized parameters of the mass spectrometer were: fragmentor voltage, collision energy, drying gas flow rate and temperature and nebulizer gas flow rate. The optimal values were determined by comparing the chromatograms of the analyses performed under different settings of the optimized variables.

Fragmentor voltage was tested in the range from 250 to 350 V. Response of the detector was examined in SIM mode for m/z 1224.8. Comparison of the respective outcomes is shown in Figure 4. The most suitable voltage was 300 V.

For examination of the optimal collision energy mode Product Ion was set and the response of the detector for the product ion of m/z 1112.8 (precursor ion m/z was 1224.8) was determined and compared for different values of collision energy (15 - 70 V).

Finally the analyses in MRM mode (1224.8 → 1112.8) with different settings of drying gas flow rate (range 6 - 12 L/min) and temperature (275 - 350 °C) and nebulizer gas flow rate (40 - 60 psi) were performed to find the optimal values of those variables.

4.5 Method validation

Linearity

The linearity of the method for determination of cyclosporin A was assessed from the calibration curve (peak areas) of CsA diluted in PBS buffer. The calibration curve was measured in the concentration range 10 ng/mL - 5 μg/mL.

Precision

Precision of the method was determined by assessing the relative standard deviations of the calibration curve.

Limit of detection

The limit of detection determination was based on visual evaluation. Solutions in concentration of 5 ng/mL and 2.5 ng/mL were prepared and the reliability of the detection was assessed.

Limit of quantification

The limit of quantification determination was based on visual evaluation. The acceptable precision for LOQ was decided to be 10% RSD. The solutions in concentration of 5 ng/mL and 10 ng/mL were analyzed (ten analyses) and the RSD was compared to the limit 10 %.

4.6 Stability of cyclosporin

The stability of cyclosporin A in conditions of extraction was studied. A 50 mL solution of CsA in PBS buffer in concentration of 1.5 μg/mL was inserted to a tempered shaker (set to 37.5 °C and 140 RPM).The samples for analyses (volume 200 μL) were collected at 0, 24, 72 and 144 h after initiation of the experiment.

4.7 Total amount of released CsA

To establish the total amount of CsA releasable from the nanofibers 5 targets (diameter 12.5 mm) of the CsA-loaded nanofibers (the experiments were conducted for all the extracted nanofibers) were put to 50 mL of ethanol. The amount of cyclosporin A was assessed 144 h after initiation of the extraction. The samples were diluted 10 times to fit in the range of calibration curve. Calibration curve in PBS was used for quantification of the CsA amount.

4.8 Extraction of CsA from nanofibers

Extraction of cyclosporin A was conducted in a tempered shaker at the temperature 37.5 °C and at 140 RPM. Five round targets (diameter 12.5 mm) of the CsA-loaded nanofibers were used for each experiment. The experiments were conducted in triplicates. The kinetics of cyclosporin release was studied in a cumulative mode and in a mode with continuous exchange of part of the media. The kinetic studies of the cyclosporin release were performed for five different types of CsA-loaded nanofibers varying in content of cyclosporin and/or content and molecular weight of poly(ethylene glycol).

The cumulative mode experiments were conducted as follows: five targets were put to 50 mL of medium (water or PBS) and samples for analysis (volume 200 μL) were collected at 0.5, 1, 2, 3.5, 6.5, 10, 24, 72 and 144 h after initiation of the extraction.

The continuous exchange of media experiments were conducted as follows: five targets were put to 10 mL of medium (water or PBS). The samples were collected at the same time points as in the cumulative mode experiments. 1 mL of solution was used for the analysis, 3 mL were discarded and then 4 mL of new medium were added into the extraction bottles.

The kinetics of release of cyclosporin from the nanofibers with 10% (m/m) of cyclosporin and without poly(ethylene glycol) in water was compared with release kinetics in PBS buffer. Kinetics of the other types of nanofibers was studied only in PBS.

5 Results and discussion

5.1 Optimization of the liquid chromatography conditions

The data obtained during optimization on C18 stationary phase are summarized in Table 3.

Table 3: Characteristics of the chromatographic analyses of cyclosporin A (c = 200 μg/mL) on the octadecyl stationary phase column, flow rate 0.6 mL/min, 45 °C. t_r...retention time, *A*...peak area, *h*...peak height, *w*...peak width at base

Mobile phase		t_r **[min]**	***A***	***h***	***w*** **[min]**
ACN/H_2O, 0,1% HCOOH	70/30	6.83	103 597 000	992 000	4.62
	80/20	3.15	130 567 000	2 618 000	2.14
	90/10	1.95	67 221 000	2 086 000	1.18
ACN/CH_3COONH_4	70/30	6.54	81 652 000	942 000	3.88
	80/20	3.20	91 916 000	1 763 000	1.98
MeOH/CH_3COONH_4	80/20	8.05	115 260 000	1 556 000	3.18
	90/10	***2.17***	***91 757 000***	***4 181 000***	***0.94***
	95/5	1.44	69 525 000	5 460 000	0.80

The most suitable mobile phase for the analysis was the mixture of methanol and ammonium acetate (90/10, v/v) (data in bold italics in Table 3). Using of the mobile phase with acetonitrile resulted in poor symmetry and unfavorable width of the cyclosporin peak. After changing the composition of mobile phase from ACN/CH_3COONH_4 to MeOH/CH_3COONH_4 the symmetry was significantly better, the ratio of methanol and ammonium acetate 90/10 (v/v) was chosen as the most suitable for further analyses. The ratio 95/5 (v/v) showed better response and narrower peak but with regard to aqueous matrix of real samples the mobile phase with higher content of ammonium acetate was selected.

The data obtained during optimization on phenyl-hexyl stationary phase are summarized in Table 4.

Table 4: Characteristics of the chromatographic analysis of cyclosporin A (c = 200 μg/mL) on phenyl-hexyl stationary phase column, flow rate 0.6 mL/min, 45 °C. t_r...retention time, *A*...peak area, *h*...peak height, *w*...peak width at base

Mobile phase		t_r **[min]**	***A***	***h***	***w*** **[min]**
MeOH/CH_3COONH_4	80/20	12.17	107 573 000	1 670 000	3.02
	90/10	***3.94***	***123 412 000***	***6 798 000***	***0.83***
MeOH/H_2O, 0.1%NH_4OH	80/20	12.24	13 148 000	205 000	2.92
	90/10	3.92	117 763 000	6 634 000	0.77

Mobile phase containing methanol and ammonium acetate (90/10, v/v) (data in bold italics in Table 4) was the most suitable for the analysis on phenyl-hexyl stationary phase as well as for analysis on the C18 stationary phase. Chromatogram of the analysis with the mobile phase containing methanol and water with addition of NH_4OH (0.1 %) showed comparable symmetry, peak width and the response of the detector. For further analyses the mobile phase containing methanol and ammonium acetate (90/10, v/v) was chosen.

Comparison of the analyses on C18 stationary phase and phenyl-hexyl stationary phase with the optimal mobile phase (MeOH/CH_3COONH_4, pH=4.5 (90/10, v/v)) is presented in the Table 5.

Table 5: Comparison of analysis of cyclosporin A (c = 200 μg/mL) on C18 and phenyl-hexyl stationary phase. Mobile phase: MeOH/CH_3COONH_4, pH = 4.5 (90/10, v/v), flow rate 0.6 mL/min, 45 °C. t_r...retention time, *A*...peak area, *h*...peak height, *w*...peak width at base

Stationary phase	t_r **[min]**	***A***	***h***	***w*** **[min]**
C18	2.17	91 757 000	4 181 000	0.94
Phenyl-hexyl	3.94	123 412 000	6 798 000	0.83

Phenyl-hexyl stationary phase showed better response and symmetry and therefore it was chosen for further analyses.

5.2 Optimization of mass spectrometric detection

5.2.1 Fragmentor voltage tested in the range from 250 to 350 V

Comparison of the respective outcomes is shown in Figure 3. The most suitable voltage was 300 V as response of detector was highest.

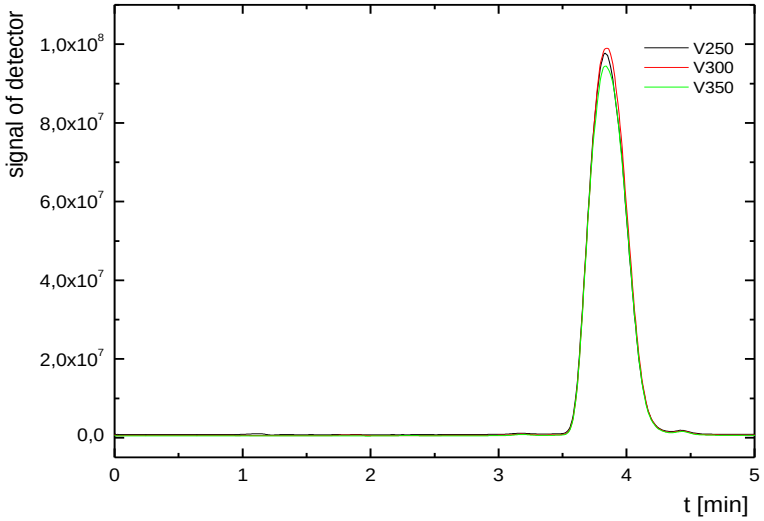

Fig. 3: Comparison of responses of the detector with different values (250 to 350 V) of fragmentor voltage set (values given in volts)

5.2.2 Collision energy tested in the range from 15 to 70 V

The optimal values in MRM mode were assessed in the same way as the optimal fragmentor voltage.

The most convenient collision energy for detection of CsA was 60 V.

5.2.3 Drying gas flow rate and temperature and nebulizer gas flow rate

The optimal values in MRM mode were assessed in the same way as in the assessment of the previous parameters of the mass spectrometer.

Optimal settings found were drying gas flow rate 10 L/min, drying gas temperature 350 °C and nebulizer gas flow rate 50 psi.

5.2.4 Optimal conditions for detection of CsA

The parameters of mass detector chosen for further analyses of cyclosporin A were:

- ionization mode: positive
- fragmentation: 1224.8 → 1112.8
- fragmentor voltage: 300 V
- collision energy: 60 V
- drying gas flow rate: 10 L/min
- drying gas temperature: 350 °C
- nebulizer gas flow rate: 50 psi

5.3 Calibration curves in water

Standard solutions of CsA in concentration range from 10 ng/mL to 5 μg/mL were prepared from stock solution (see Section 4.2). The optimized LC-MS/MS method was used to obtain the necessary data (peak areas and peak heights) for construction of least square calibration curves (Figures 4 and 5). Tables 6a and 6b summarize the statistical data.

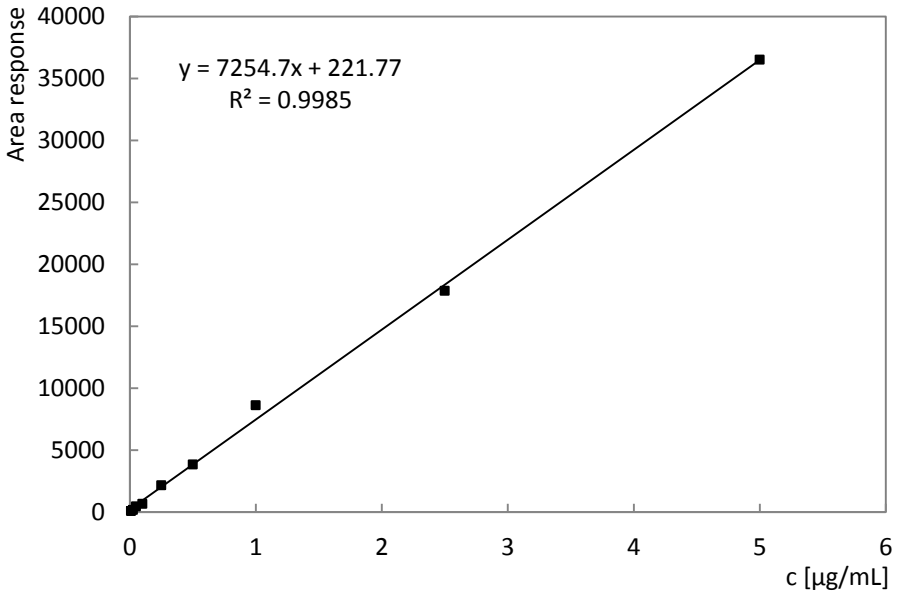

Fig. 4: Calibration curve based on peak areas of CsA in water. Mobile phase: MeOH/CH_3COONH_4, pH = 4.5 (90/10, v/v), flow rate 0.6 mL/min, 45 °C

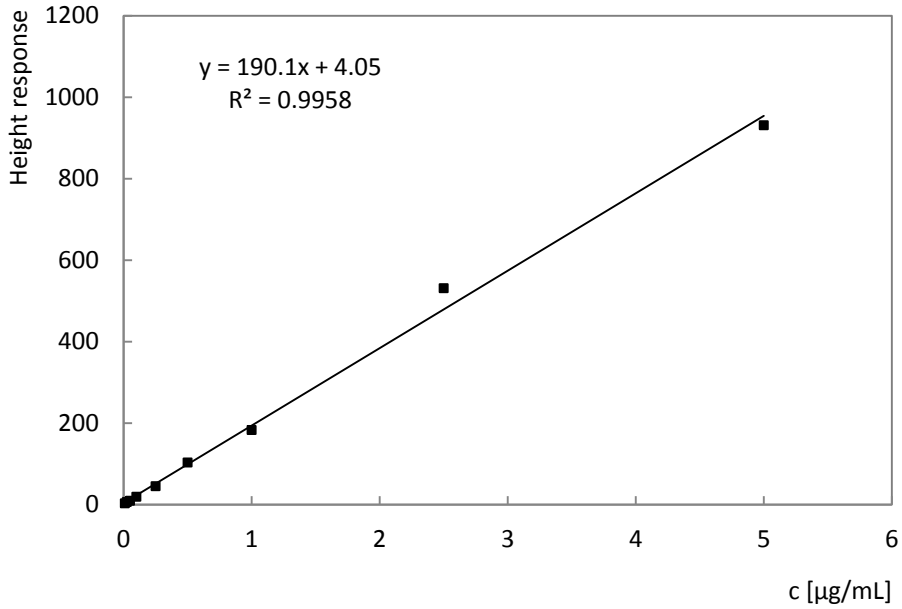

Fig.5: Calibration curve based on peak heights of CsA in water. Mobile phase: MeOH/CH_3COONH_4, pH = 4.5 (90/10, v/v), flow rate 0.6 mL/min, 45 °C

Table 6a: Statistical results for peak areas of CsA in water. *c*...concentration of cyclosporin, $\tilde{A}$...peak area median (n = 5), s_r...standard deviation, RSD...relative standard deviation, 95% CI...95% confidence interval

c (CsA) [µg/mL]	$\tilde{A}$	s_r	RSD [%]	95% CI
5	36511	135.57	0.37	36174 - 36848
2.5	17870	469.65	2.63	16703 - 19037
1	8627	314.24	3.64	7846 - 9408
0.5	3845	73.46	1.91	3663 - 4027
0.25	2175	57.01	2.62	2033 - 2317
0.1	674	19.35	2.87	626 - 722
0.05	467	7.51	1.61	448 - 486
0.025	180	12.86	7.14	148 - 212
0.01	95	4.04	4.25	85 - 105

Table 6b: Statistical results for peak heights of CsA in water. *c*...concentration of cyclosporin, $\tilde{A}$...peak area median(n = 5), s_r...standard deviation, RSD...relative standard deviation, 95% CI...95% confidence interval

c (CsA) [µg/mL]	$\tilde{h}$	s_r	RSD [%]	95% CI
5	931	29.26	3.14	858 - 1004
2.5	531	16.74	3.15	489 - 573
1	183	4.04	2.21	173 - 193
0.5	103	1.00	0.97	101 - 105
0.25	45	2.08	4.63	40 - 50
0.1	19	1.73	9.12	15 - 23
0.05	9	1.53	16.97	5 - 13
0.025	6	1.00	16.67	4 - 8
0.01	3	0.58	19.25	2 - 4

5.4 Calibration curves in PBS buffer

Standard samples of CsA in concentration range from 10 ng/mL to 5 µg/mL were prepared from stock solution (see Section 4.2). The optimized LC-MS/MS method was used to obtain the necessary data (peak areas and peak heights) for construction of least square calibration curves (Figures 6 and 7). Tables 7a and 7b summarize the statistical data.

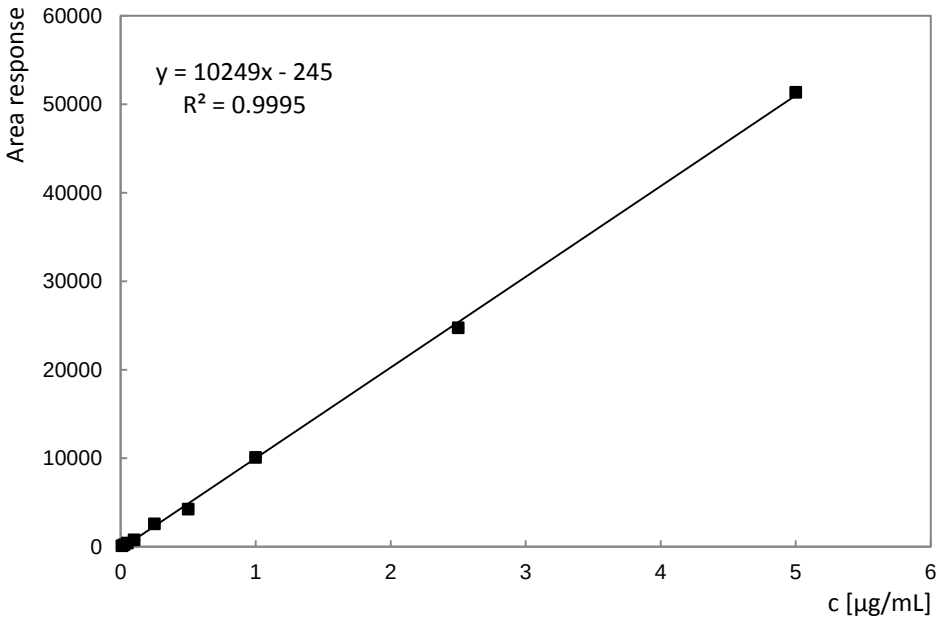

Fig. 6: Calibration curve based on peak areas of CsA in PBS buffer. Mobile phase: MeOH/CH_3COONH_4, pH = 4.5 (90/10, v/v), flow rate 0.6 mL/min, 45 °C

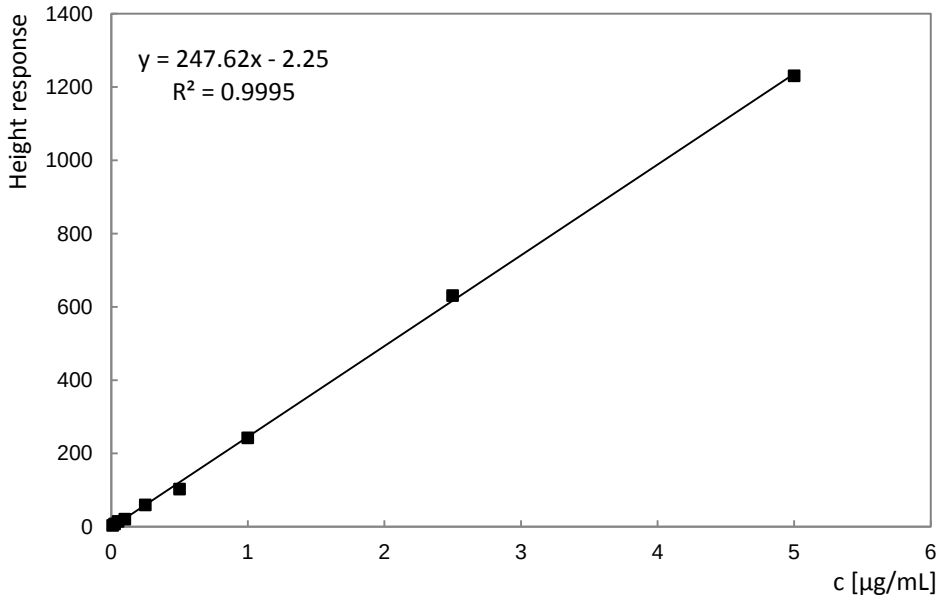

Fig.7: Calibration curve based on peak heights of CsA in PBS buffer. Mobile phase: MeOH/CH_3COONH_4, pH = 4.5 (90/10, v/v), flow rate 0.6 mL/min, 45 °C

Table 7a: Statistical results for peak areas of CsA in PBS buffer. *c*...concentration of cyclosporin, $\tilde{A}$...peak area median (n = 5), s_r...standard deviation, RSD...relative standard deviation, 95% CI...95% confidence interval

c (CsA) [µg/mL]	$\tilde{A}$	s_r	RSD [%]	95% CI
5	51347	576.24	1.12	49916 - 52778
2.5	24739	692.75	2.80	23018 - 26460
1	10089	431.37	4.28	9017 - 11161
0.5	4245	73.46	1.73	4063 - 4427
0.25	2575	57.01	2.21	2433 - 2717
0.1	774	19.35	2.50	726 - 822
0.05	417	5.69	1.36	403 - 431
0.025	206	5.03	2.44	193 - 219
0.01	98	4.16	4.25	88 - 108

Table 7b: Statistical results for peak heights of CsA in PBS buffer. *c*...concentration of cyclosporin, $\tilde{A}$...peak area median (n = 5), s_r...standard deviation, RSD...relative standard deviation, 95% CI...95% confidence interval

c (CsA) [µg/mL]	$\tilde{h}$	s_r	RSD [%]	95% CI
5	1231	29.26	2.38	1158 - 1304
2.5	631	16.74	2.65	589 - 673
1	243	4.04	1.66	233 - 253
0.5	103	1.00	0.97	101 - 105
0.25	60	2.00	3.33	55 - 65
0.1	21	1.15	5.50	18 - 24
0.05	15	0.58	3.85	14 - 16
0.025	8	1.53	19.09	4 - 12
0.01	4	0.58	14.43	3 - 5

5.5 Method validation

5.5.1 Linearity

The data show linearity in whole calibration range, i.e. 10 ng/mL to 5 μg/mL.

5.5.2 Precision

Relative standard deviation in the calibration range varied from 1.12 to 4.28 %. This deviation is low enough and the method is considered precise.

5.5.3 Limit of detection; limit of quantification

a) limit of detection

The limit of detection was determined based on visual evaluation. The limit of detection of CsA is 5 ng/mL, this concentration may be reliably detected.

b) limit of quantification

The limit of quantification was determined based on visual evaluation. The limit of quantification is 10 ng/mL, the relative standard deviation 4.25% did not exceed the limit value (10%). The relative standard deviation of the solution in concentration of 5 ng/mL was 11.18%.

5.6 Stability of cyclosporin

The data obtained in the stability study are summarized in Table 8. For further information about the procedure see Section 4.6.

Table 8: Stability study of cyclosporin A (1.5 μg/mL) in conditions of extraction from the CsA-loaded nanofibers. $\tilde{A}$...peak area median, $\tilde{h}$...peak height median, s_r...standard deviation, RSD...relative standard deviation

t [h]	$\tilde{A}$	$\tilde{h}$
0	13652	358
24	14032	362
72	13821	352
144	14113	372
median	13926.5	360
s_r	208.53	8.41
RSD [%]	**1.50**	**2.34**

The stability data show no relevant deviation in the amount of cyclosporin A. It has been concluded that in the conditions of extraction of cyclosporin A from nanofibers the analyte is stable.

5.7 Total amount of released CsA

The amounts releasable from the nanofibers of different content of cyclosporin A and PEG are provided in the Table 9. For further information about the procedure see Section 4.7.

Table 9: The amount of cyclosporin A released from nanofibers with different content of CsA and PEG. $\tilde{A}$...peak area median (n = 3), theoretical total amount of CsA/target...content of cyclosporin in one target calculated from the amount of cyclosporin in the manufacturing solution and from area density of nanofibers, percent of calculated total amount of CsA...actual values of CsA content observed in the studies given as percents of theoretical amount of cyclosporin in nanofibers

Type of nanofibers	$\tilde{A}$	c_{CsA} **n the analyzed sample [µg/mL]**	**Theoretical total amount of CsA/target [µg]**	**Percent of calculated total amount of CsA**
10% (m/m) of CsA; no PEG	28477	2.80	131.3	213.1
5% (m/m) of CsA; no PEG	8835	0.87	59	147.1
2.5% (m/m) of CsA; no PEG	3053	0.30	27.3	109.9
10% (m/m) of CsA; 15% (m/m) of PEG 6000	8502	0.84	74.9	111.5
10% (m/m) of CsA; 15% (m/m) of PEG 20000	7167	0.70	74.9	94.0

The released amount of cyclosporin A does not correspond with the expected value calculated from the area density of the nanofibers and content of CsA in the solution used for preparation of the nanofibers. The measured content of cyclosporin in the nanofibers is in most cases (except the nanofibers containing PEG 20000) higher than the content in the manufacturing solution. This could be assigned to preferential distribution of each component of the solution used for the electrospinning process. The total amounts of cyclosporin A obtained in the experiments were used for further calculations of percentage of the released cyclosporin A.

5.8 Comparison of release kinetics in water and PBS buffer

The data from continuous exchange of media experiments in water and in PBS buffer are presented in Table 10 and Table 11 (section 5.8.1) and in Figures 8 - 9, respectively, the data from cumulative mode experiments in water and in PBS buffer are presented in Table 12 and Table 13 (Section 5.8.2) and in Figures 10 - 11 (the values of released cyclosporin are given as sum of CsA released in the relevant time interval and the CsA released from the beginning of extraction to the relevant time interval). The comparison of the kinetics in the different media (water and PBS buffer) and modes of extraction is shown in Table 14 in Section 5.8.3. For further information about the procedure see Section 4.8.

5.8.1 Continuous exchange of media

Table 10 The release kinetics of CsA from CsA-loaded nanofibers (10%, m/m) with continuous exchange of media (water); $\tilde{A}$... peak area median calculated from the relevant triplicates; RSD...relative standard deviation; *c*...concentration of CsA in the analyzed sample, *m*...amount of the CsA released in the time intervals t_n-t_{n-1}, released...percentage of the CsA released from the nanofibers in the time intervals t_n-t_{n-1}, release rate...mean rate of the release of CsA in the time interval t_n-t_{n-1}

t **[h]**	$\widetilde{A}$	**RSD($\widetilde{A}$)** **[%]**	***c*** **[ng/ml]**	***m*** **[ng]**	**released** **[%]**	**release rate** **[ng/h]**
0.5	1673	26.94	228.63	2286.32	0.163	4572.64
1	3025	40.73	413.31	2764.08	0.198	5528.15
2	302	39.42	41.22	0.00	0.000	0.00
3.5	270	26.83	36.89	177.17	0.013	88.58
6.5	672	43.78	91.77	696.37	0.050	278.55
10	608	1.04	83.12	282.83	0.020	80.81
24	357	17.71	48.73	0.00	0.000	0.00
72	178	27.03	24.37	0.00	0.000	0.00
144	115	9.65	15.71	10.93	0.001	0.15

Table 11 The release kinetics of CsA from CsA-loaded nanofibers (10%, m/m) with continuous exchange of media (PBS buffer); $\tilde{A}$... peak area median calculated from the relevant triplicates; RSD...relative standard deviation; *c*...concentration of CsA in the analyzed sample, *m*...amount of the CsA released in the time intervals t_n-t_{n-1}, released...percentage of the CsA released from the nanofibers in the time intervals t_n-t_{n-1}, release rate...mean rate of the release of CsA in the time interval t_n-t_{n-1}

t **[h]**	$\tilde{A}$	**RSD($\tilde{A}$)** **[%]**	**c** **[ng/ml]**	**m** **[ng]**	**released** **[%]**	**release rate** **[ng/h]**
0.5	962	15.07	94.48	944.85	0.068	1889.70
1	1047	22.29	102.84	538.09	0.038	1076.18
2	1160	21.23	113.97	417.57	0.030	417.57
3.5	1532	7.58	150.49	821.05	0.059	410.53
6.5	1607	12.36	157.86	768.65	0.055	307.46
10	1310	15.78	128.71	184.38	0.013	52.68
24	1213	20.33	119.21	419.86	0.030	29.99
72	827	18.41	81.22	0.00	0.000	0.00
144	613	20.89	60.26	101.53	0.007	1.41

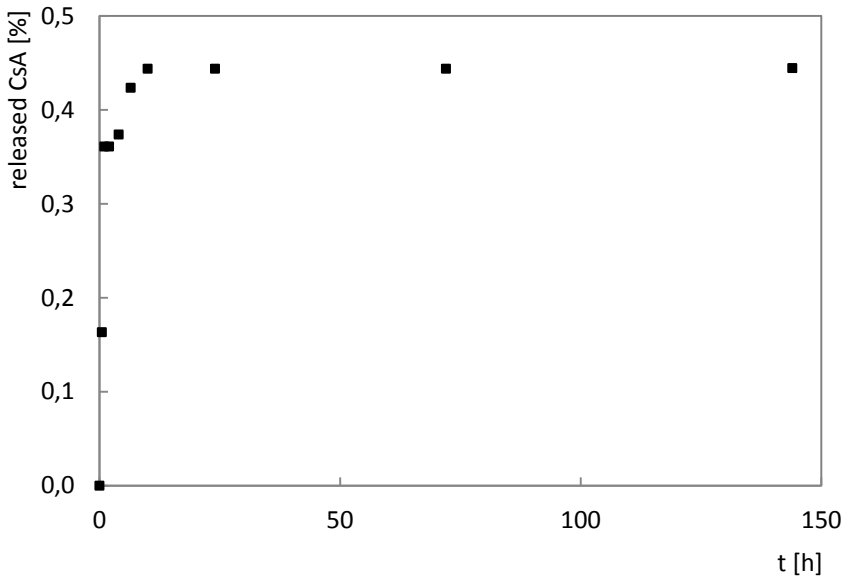

Fig. 8: The release kinetics of CsA from CsA-loaded nanofibers (10%, m/m) with continuous exchange of media (water)

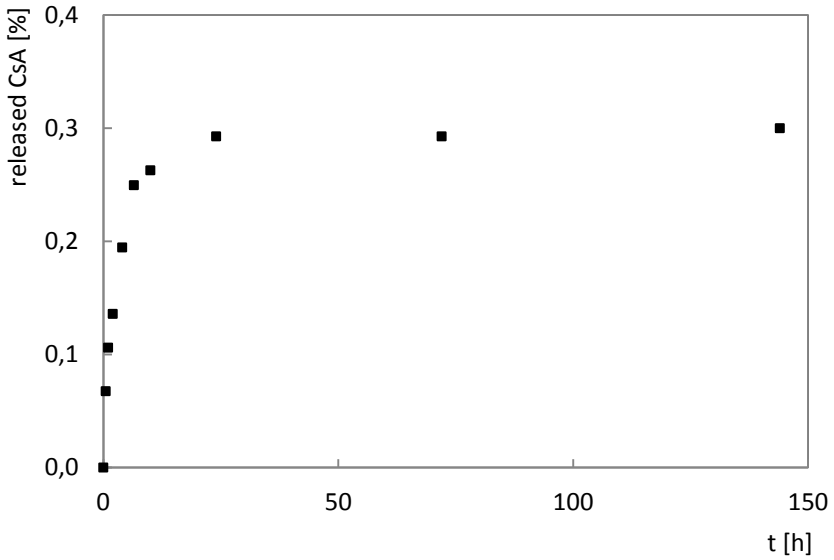

Fig. 9: The release kinetics of CsA from CsA-loaded nanofibers (10%, m/m) with continuous exchange of media (PBS buffer)

5.8.2 Cumulative mode experiments

Table 12 The release kinetics of CsA from CsA-loaded nanofibers (10% m/m) in cumulative mode experiments (water); $\tilde{A}$... peak area median calculated from the relevant triplicates; RSD...relative standard deviation; *c*...concentration of CsA in the analyzed sample, *m*...amount of the CsA released in the time intervals t_n-t_{n-1}, released...percentage of the CsA released from the nanofibers in the time intervals t_n-t_{n-1}, release rate...mean rate of the release of CsA in the time interval t_n-t_{n-1}

t **[h]**	$\widetilde{A}$	**RSD($\widetilde{A}$)** **[%]**	***c*** **[ng/ml]**	***m*** **[ng]**	**released** **[%]**	**release rate** **[ng/h]**
0.5	700	5.49	95.64	4782.14	0.342	9564.28
1	878	12.66	120.01	851.86	0.061	1703.72
2	938	13.40	128.21	1226.64	0.088	1226.64
3.5	1332	12.08	181.95	1846.32	0.132	923.16
6.5	1630	7.50	222.71	2074.49	0.148	829.80
10	1750	13.43	239.11	1556.11	0.111	444.60
24	2375	14.40	324.50	4317.59	0.309	308.40
72	1670	12.63	228.18	0.00	0.000	0.00
144	710	30.47	97.01	0.00	0.000	0.00

Table 13 The release kinetics of CsA from CsA-loaded nanofibers (10%, m/m) in cumulative mode experiments (PBS buffer); $\tilde{A}$... peak area median calculated from the relevant triplicates; RSD...relative standard deviation; *c*...concentration of CsA in the analyzed sample, *m*...amount of the CsA released in the time intervals t_n-t_{n-1}, released...percentage of the CsA released from the nanofibers in the time intervals t_n-t_{n-1}, release rate...mean rate of the release of CsA in the time interval t_n-t_{n-1}

t **[h]**	$\tilde{A}$	**RSD($\tilde{A}$) [%]**	***c*** **[ng/ml]**	***m*** **[ng]**	**released [%]**	**release rate [ng/h]**
0.5	722	38.31	70.90	3545.23	0.253	7090.46
1	1030	37.07	101.20	3494.20	0.250	6988.41
2	1365	36.72	134.11	0.00	0.000	0.00
3.5	1413	9.48	138.86	698.21	0.050	349.10
6.5	1755	16.08	172.43	1274.02	0.091	509.61
10	1815	19.18	178.33	329.24	0.024	94.07
24	1677	10.39	164.73	0.00	0.000	0.00
72	1622	3.88	159.33	0.00	0.000	0.00
144	1358	7.87	133.46	0.00	0.000	0.00

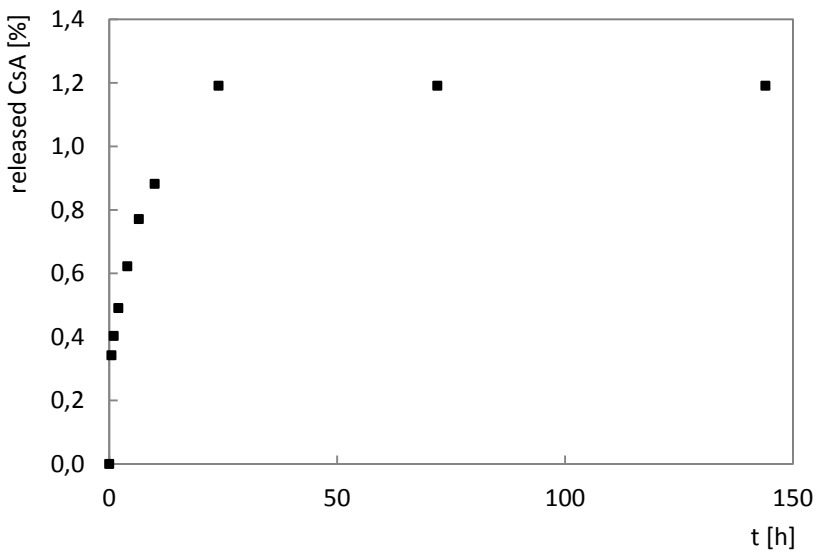

Fig. 10: The release kinetics of CsA from CsA-loaded nanofibers (10%, m/m) in cumulative mode experiments (water)

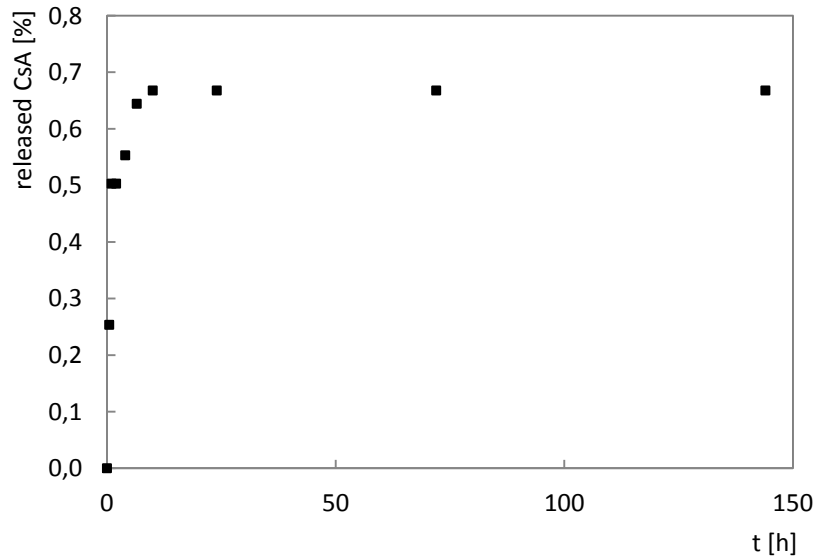

Fig. 11: The release kinetics of CsA from CsA-loaded nanofibers (10%, m/m) in cumulative mode experiments (PBS buffer)

5.8.3 Data comparison of the cyclosporin A release

The comparison of cyclosporin release in different media is provided in Table 14.

Table 14: Comparison of the amount of CsA released 144 h after initiation of the extraction (values given as percents of the total amount of CsA releasable from the nanofibers)

Mode	Medium	% of released CsA
Continuous exchange of media	water	0.444
	PBS buffer	0.300
Cumulative mode	water	1.191
	PBS buffer	0.668

The total amount of cyclosporin A released from the nanofibers was higher in water as the extraction medium than in the PBS buffer.

The media differed in the kinetic profiles in the studied modes of extraction. In continuous exchange of media experiments the kinetic profile was more appropriate in the PBS buffer regarding the planned use - cyclosporin A was being released for a longer period of time, on the other side cumulative mode experiments showed opposite phenomenon - cyclosporin A was being released for a longer period of time in water, in the PBS buffer no cyclosporin was released after 10 h of extraction.

5.9 Release kinetics of cyclosporin from different nanofibers in PBS buffer

5.9.1 CsA-loaded nanofibers with 10% (m/m) cyclosporin, without poly(ethylene glycol)

The data from kinetic studies in the nanofibers with 10% (m/m) of cyclosporin A are presented in section 5.8 Comparison of release kinetics in water and PBS buffer.
The outcome from "continuous exchange of media" experiments show release of cyclosporine only during the first 24 hours, in addition the speed of release gradually falls, which is not consistent with the intended use.

The cumulative mode data only confirm that this type of nanofibers is not appropriate for planned use. Cyclosporin was being released only during the first ten hours and the speed of release was falling sharply.

The cumulative mode is less similar to the physiological model so the emphasis is mainly on the data from continuous exchange of media experiments.

5.9.2 CsA-loaded nanofibers with 5% (m/m) cyclosporin, without poly(ethylene glycol)

The data obtained in kinetic studies of cyclosporin A in the mode with continuous exchange of media and in the cumulative mode are summarized in Tables 15 and 16 and in Figures 12 and 13 (the values of released cyclosporin are given as sum of CsA released in the relevant time interval and the CsA released from the beginning of extraction to the relevant time interval).

Table 15: The release kinetics of CsA from CsA-loaded nanofibers (5%, m/m) with continuous exchange of media; $\tilde{A}$... peak area median calculated from the relevant triplicates; RSD...relative standard deviation; *c*...concentration of CsA in the analyzed sample, *m*...amount of the CsA released in the time intervals t_n-t_{n-1}, released...percentage of the CsA released from the nanofibers in the time intervals t_n-t_{n-1}, release rate...mean rate of the release of CsA in the time interval t_n-t_{n-1}

t **[h]**	$\tilde{A}$	**RSD($\tilde{A}$) [%]**	***c*** **[ng/ml]**	***m*** **[ng]**	**released [%]**	**release rate [ng/h]**
0.5	706	11.33	69.37	693.65	0.160	1387.31
1	949	29.51	93.24	562.78	0.130	1125.56
2	2056	21.28	202.00	1176.46	0.271	1176.46
3.5	1183	19.82	116.23	192.97	0.044	96.48
6.5	1362	38.09	133.82	735.70	0.170	294.28
10	565	32.95	55.51	113.77	0.026	32.51
24	553	10.46	54.33	198.07	0.046	14.15
72	701	17.09	68.87	386.32	0.089	8.05
144	1409	39.22	138.44	1050.11	0.242	14.58

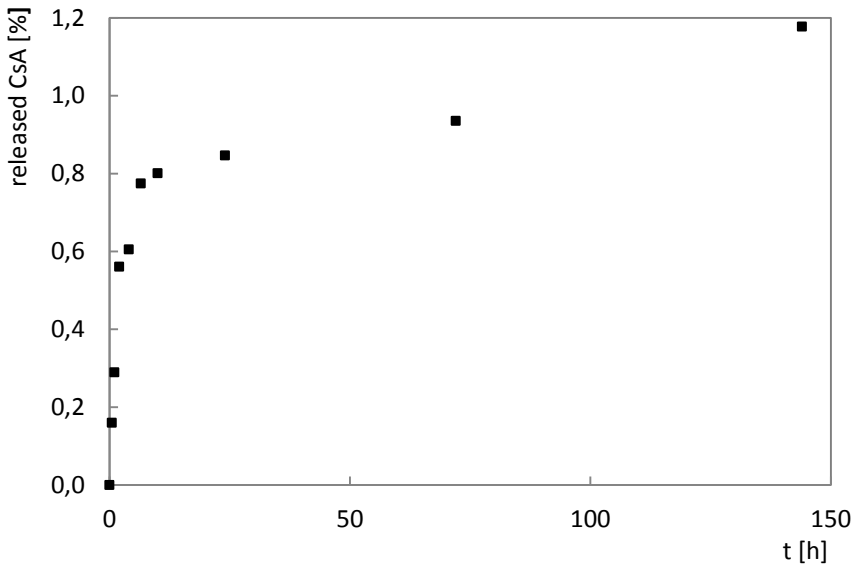

Fig. 12: The release kinetics of CsA from CsA-loaded nanofibers (5%, m/m) with continuous exchange of media

Table 16: The release kinetics of CsA from CsA-loaded nanofibers (5%, m/m) in cumulative mode experiments; $\tilde{A}$... peak area median calculated from the relevant triplicates; RSD...relative standard deviation; *c*...concentration of CsA in the analyzed sample, *m*...amount of the CsA released in the time intervals t_n-t_{n-1}, released...percentage of the CsA released from the nanofibers in the time intervals t_n-t_{n-1}, release rate...mean rate of the release of CsA in the time interval t_n-t_{n-1}

t **[h]**	$\widetilde{A}$	**RSD($\widetilde{A}$)** **[%]**	***c*** **[ng/ml]**	***m*** **[ng]**	**released** **[%]**	**release rate** **[ng/h]**
0.5	235	29.99	23.09	1154.45	0.266	2308.90
1	509	32.68	50.01	1333.29	0.307	2666.57
2	699	29.89	68.68	446.12	0.103	446.12
3.5	714	35.40	70.15	2135.96	0.492	1067.98
6.5	1387	40.81	136.27	1279.84	0.295	511.94
10	699	10.69	68.68	0.00	0.000	0.00
24	794	35.41	78.01	480.43	0.111	34.32
72	371	25.47	36.45	0.00	0.000	0.00
144	299	32.45	29.38	250.52	0.058	3.48

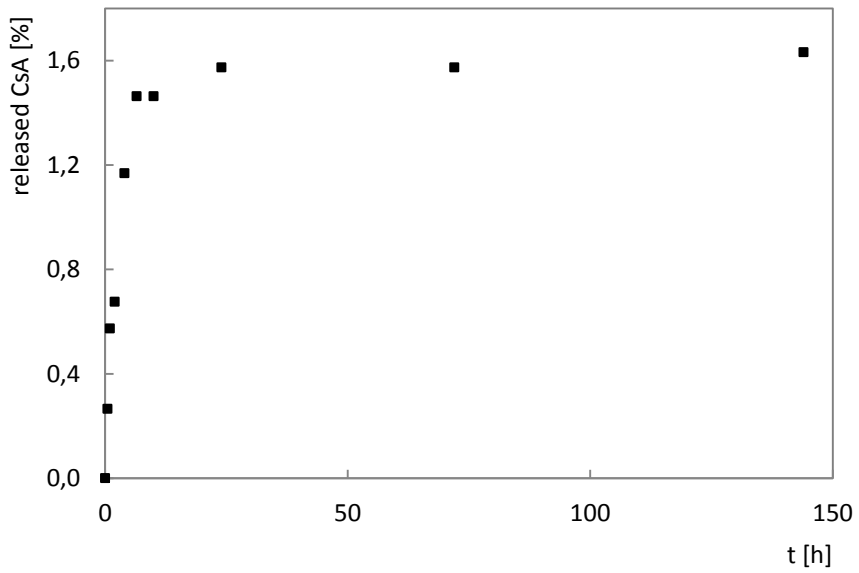

Fig. 13: The release kinetics of CsA from CsA-loaded nanofibers (5%, m/m) in cumulative mode experiments

The nanofibers showed better release kinetics for the intended use than the 10% CsA nanofibers.

In the continuous exchange of media experiments the release rate was highest in the first two hours of extraction, then it lowered and after 24 hours the release speed was stabilized with a potential to further release after the experiment was ended (after 144 hours). Stabilized speed would enable stable delivery of cyclosporin to the transplanted organ. These nanofibers would be suitable for the intended use, but the release in the first 24 hours would have to be slowed down by addition of appropriate agent in the electrospinning solution and become steady.

Cumulative mode showed worse outcomes as well as it was in the experiments with 10% CsA nanofibers, the changes of release rate were variable.

5.9.3 CsA-loaded nanofibers with 2.5% (m/m) cyclosporin, without poly(ethylene glycol)

The data obtained in kinetic studies of cyclosporin A in the mode with continuous exchange of media and in the cumulative mode are summarized in Tables 17 and 18 and in Figures 14 and 15 (the values of released cyclosporin are given as sum of CsA released in the relevant time interval and the CsA released from the beginning of extraction to the relevant time interval).

Table 17: The release kinetics of CsA from CsA-loaded nanofibers (2.5%, m/m) with continuous exchange of media; $\tilde{A}$... peak area median calculated from the relevant triplicates; RSD...relative standard deviation; *c*...concentration of CsA in the analyzed sample, *m*...amount of the CsA released in the time intervals t_n-t_{n-1}, released...percentage of the CsA released from the nanofibers in the time intervals t_n-t_{n-1}, release rate...mean rate of the release of CsA in the time interval t_n-t_{n-1}

t **[h]**	$\tilde{A}$	**RSD($\tilde{A}$)** **[%]**	***c*** **[ng/ml]**	***m*** **[ng]**	**released** **[%]**	**release rate** **[ng/h]**
0.5	264	26.63	25.94	259.38	0.173	518.77
1	293	19.98	28.79	184.12	0.123	368.25
2	211	7.89	20.73	34.58	0.023	34.58
3.5	197	9.72	19.31	63.96	0.043	31.98
6.5	195	15.63	19.11	75.26	0.050	30.10
10	132	0.54	12.92	14.54	0.010	4.15
24	154	5.99	15.08	73.30	0.049	5.24
72	130	5.44	12.77	37.24	0.025	0.78
144	127	2.79	12.43	47.65	0.032	0.66

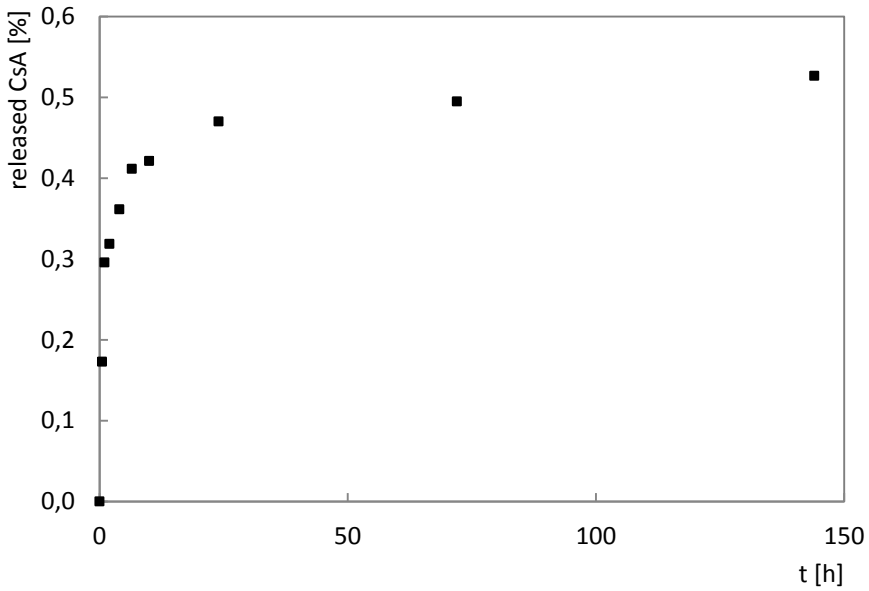

Fig. 14: The release kinetics of CsA from CsA-loaded nanofibers (2.5%, m/m) with continuous exchange of media

Table 18: The release kinetics of CsA from CsA-loaded nanofibers (2.5%, m/m) in cumulative mode experiments; $\tilde{A}$... peak area median calculated from the relevant triplicates; RSD...relative standard deviation; *c*...concentration of CsA in the analyzed sample, *m*...amount of the CsA released in the time intervals t_n-t_{n-1}, released...percentage of the CsA released from the nanofibers in the time intervals t_n-t_{n-1}, release rate...mean rate of the release of CsA in the time interval t_n-t_{n-1}

t **[h]**	$\widetilde{A}$	**RSD($\widetilde{A}$)** **[%]**	***c*** **[ng/ml]**	***m*** **[ng]**	**released** **[%]**	**release rate** **[ng/h]**
0.5	243	26.37	23.88	1193.75	0.796	2387.50
1	207	16.76	20.34	0.00	0.000	0.00
2	235	6.93	23.09	13.89	0.009	13.89
3.5	240	17.65	23.58	29.18	0.019	14.59
6.5	249	6.27	24.46	58.75	0.039	23.50
10	186	18.87	18.27	0.00	0.000	0.00
24	257	5.91	25.25	298.41	0.199	21.31
72	175	7.76	17.19	0.00	0.000	0.00
144	138	2.74	13.56	0.00	0.000	0.00

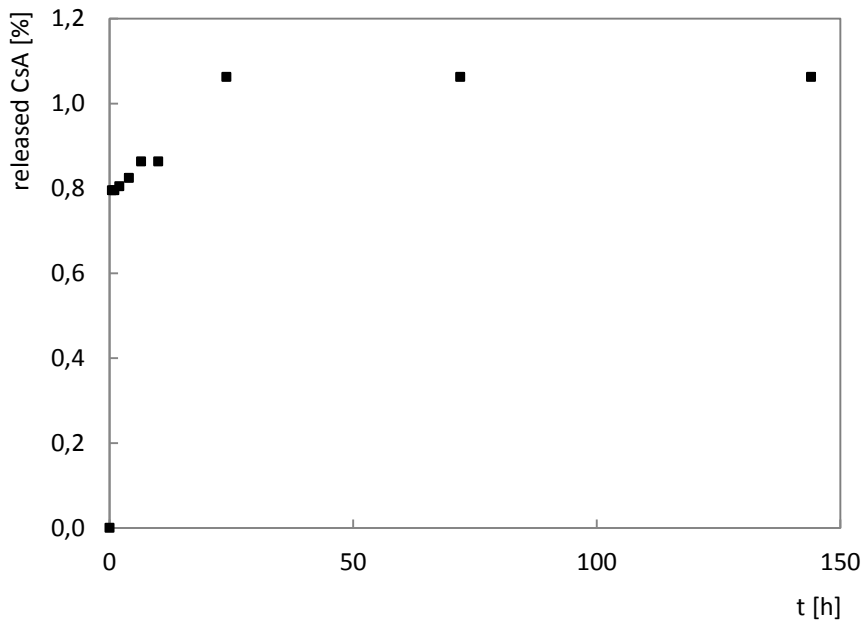

Fig. 15: The release kinetics of CsA from CsA-loaded nanofibers (2.5%, m/m) in cumulative mode experiments

The release speed in the continuous exchange of media was gradually falling, the kinetics reminds the first order kinetics, which is not suitable for intended use. It might be useful for dermatologic indications where gradual decrease of the active substance is desirable.

The outcome from cumulative mode experiments clearly show that most of the cyclosporin was released in the first 0.5 h after initiation of extraction, then the speed decreased immediately and after 24 h no cyclosporin was released.

5.9.4 CsA-loaded nanofibers with 10% (m/m) cyclosporin, 15% poly(ethylene glycol) 6000

The data obtained in kinetic studies of cyclosporin A in the mode with continuous exchange of media and in the cumulative mode are summarized in Tables 19 and 20 and in Figures 16 and 17 (the values of released cyclosporin are given as sum of CsA released in the relevant time interval and the CsA released from the beginning of extraction to the relevant time interval).

Table 19: The release kinetics of CsA from CsA-loaded nanofibers (10%, m/m; 15% PEG 6000) with continuous exchange of media; $\tilde{A}$... peak area median calculated from the relevant triplicates; RSD...relative standard deviation; *c*...concentration of CsA in the analyzed sample, *m*...amount of the CsA released in the time intervals t_n-t_{n-1}, released...percentage of the CsA released from the nanofibers in the time intervals t_n-t_{n-1}, release rate...mean rate of the release of CsA in the time interval t_n-t_{n-1}

t **[h]**	$\tilde{A}$	**RSD($\tilde{A}$)** **[%]**	***c*** **[ng/ml]**	***m*** **[ng]**	**released** **[%]**	**release rate** **[ng/h]**
0.5	1485	15.96	145.90	1459.03	0.349	2918.06
1	2809	12.99	275.99	1884.46	0.451	3768.91
2	5775	17.19	567.40	3837.69	0.919	3837.69
3.5	8163	13.48	802.02	4685.01	1.122	2342.50
6.5	13680	5.96	1344.08	8064.45	1.931	3225.78
10	15019	2.16	1475.63	7222.44	1.730	2063.55
24	18285	7.11	1796.52	9135.59	2.188	652.54
72	20985	4.37	2061.80	10435.45	2.499	217.41
144	19485	16.24	1914.42	6773.43	1.622	94.08

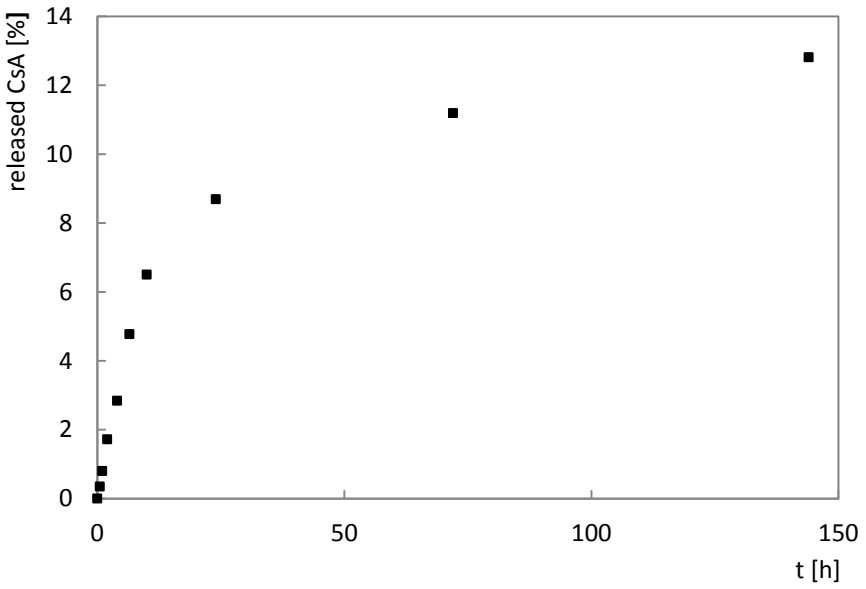

Fig. 16: The release kinetics of CsA from CsA-loaded nanofibers (10%, m/m; 15% PEG 6000) with continuous exchange of media

Table 20: The release kinetics of CsA from CsA-loaded nanofibers (10%, m/m; 15% PEG 6000) in cumulative mode experiments; $\tilde{A}$... peak area median calculated from the relevant triplicates; RSD...relative standard deviation; *c*...concentration of CsA in the analyzed sample, *m*...amount of the CsA released in the time intervals t_n-t_{n-1}, released...percentage of the CsA released from the nanofibers in the time intervals t_n-t_{n-1}, release rate...mean rate of the release of CsA in the time interval t_n-t_{n-1}

t **[h]**	$\tilde{A}$	**RSD($\tilde{A}$)** **[%]**	***c*** **[ng/ml]**	***m*** **[ng]**	**released** **[%]**	**release rate** **[ng/h]**
0.5	1127	26.55	110.73	5536.45	1.326	11072.90
1	1578	14.68	155.04	3323.36	0.796	6646.73
2	4495	34.20	441.64	14502.83	3.473	14502.83
3.5	8269	21.98	812.44	17306.80	4.145	8653.40
6.5	14161	22.45	1391.33	29107.27	6.971	11642.91
10	15475	17.93	1520.44	4941.62	1.183	1411.89
24	19714	13.10	1936.92	30078.46	7.203	2148.46
72	25310	11.19	2486.74	15057.38	3.606	313.70
144	21612	7.37	2123.40	0.00	0.000	0.00

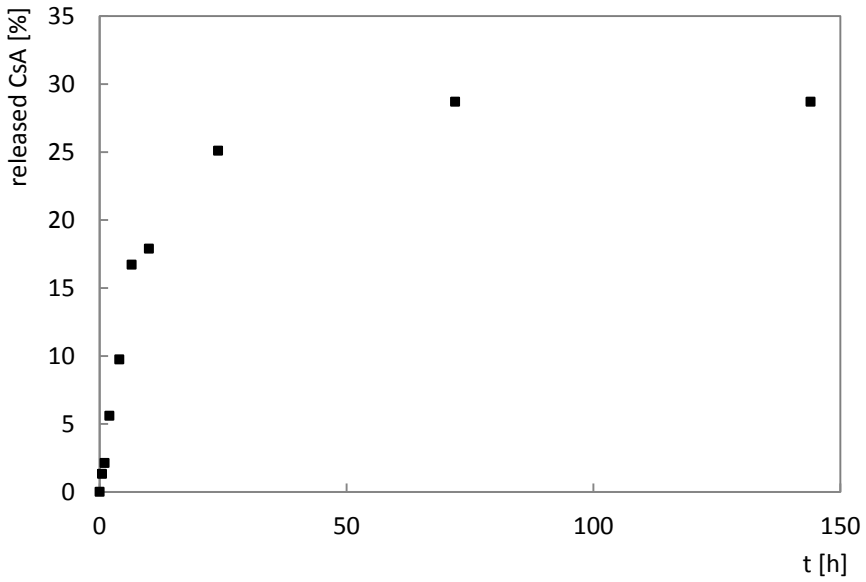

Fig. 17: The release kinetics of CsA from CsA-loaded nanofibers (10%, m/m; 15% PEG 6000) in cumulative mode experiments

The continuous exchange of media experiments showed promising results. The release rate was stable for the first ten hours and was reasonably high. The addition of poly(ethylene glycol) had great impact to the release kinetics compared to the nanofibers containing 10% of cyclosporin (m/m) and no PEG. The release time was extended - cyclosporin was released even after 144 h of the experiment and the release profile was well-balanced.

In cumulative mode cyclosporin was being released for shorter period of time and the release rate fluctuated during whole studied period.

5.9.5 CsA-loaded nanofibers with 10% (m/m) cyclosporin, 15% poly(ethylene glycol) 20000

The data obtained in kinetic studies of cyclosporin A in the mode with continuous exchange of media and in the cumulative mode are summarized in Tables 21 and 22 and in Figures 18 and 19 (the values of released cyclosporin are given as sum of CsA released in the relevant time interval and the CsA released from the beginning of extraction to the relevant time interval).

Table 21: The release kinetics of CsA from CsA-loaded nanofibers (10%, m/m; 15% PEG 20000) with continuous exchange of media; $\tilde{A}$... peak area median calculated from the relevant triplicates; RSD...relative standard deviation; *c*...concentration of CsA in the analyzed sample, *m*...amount of the CsA released in the time intervals t_n-t_{n-1}, released...percentage of the CsA released from the nanofibers in the time intervals t_n-t_{n-1}, release rate...mean rate of the release of CsA in the time interval t_n-t_{n-1}

t **[h]**	$\tilde{A}$	**RSD($\tilde{A}$) [%]**	***c*** **[ng/ml]**	***m*** **[ng]**	**released [%]**	**release rate [ng/h]**
0.5	4269	10.75	419.43	4194.34	1.191	8388.68
1	6310	7.52	619.96	3537.24	1.005	7074.47
2	8896	10.53	874.04	5020.63	1.426	5020.63
3.5	10261	6.26	1008.15	4896.25	1.391	2448.12
6.5	12932	8.57	1270.58	6621.54	1.881	2648.61
10	15550	9.03	1527.81	7918.65	2.249	2262.47
24	20993	35.68	2062.59	11459.03	3.255	818.50
72	21753	3.20	2137.26	8626.65	2.451	179.72
144	22154	2.78	2176.66	8673.61	2.464	120.47

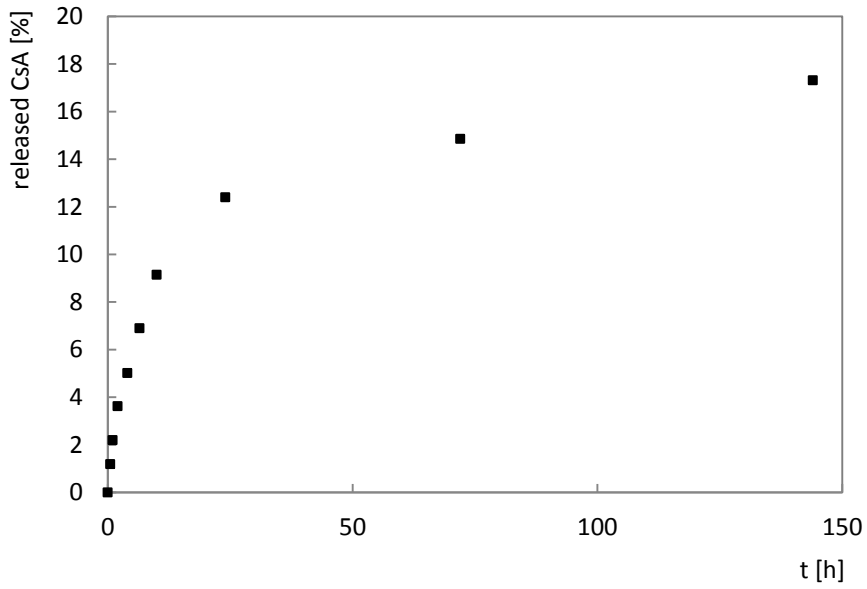

Fig. 18: The release kinetics of CsA from CsA-loaded nanofibers (10%, m/m; 15% PEG 20000) with continuous exchange of media

Table 22: The release kinetics of CsA from CsA-loaded nanofibers (10%, m/m; 15% PEG 20000) in cumulative mode experiments; $\tilde{A}$... peak area median calculated from the relevant triplicates; RSD...relative standard deviation; *c*...concentration of CsA in the analyzed sample, *m*...amount of the CsA released in the time intervals t_n-t_{n-1}, released...percentage of the CsA released from the nanofibers in the time intervals t_n-t_{n-1}, release rate...mean rate of the release of CsA in the time interval t_n-t_{n-1}

t **[h]**	$\tilde{A}$	**RSD($\tilde{A}$)** **[%]**	***c*** **[ng/ml]**	***m*** **[ng]**	**released** **[%]**	**speed** **[ng/h]**
0.5	795	26.38	78.11	3905.48	1.109	7810.96
1	2547	13.97	250.25	7636.73	2.169	15273.45
2	4764	10.93	468.07	12589.57	3.576	12589.57
3.5	6352	23.16	624.09	10678.82	3.033	5339.41
6.5	9930	11.91	975.63	16382.12	4.654	6552.85
10	11585	5.07	1138.24	7672.04	2.179	2192.01
24	15263	7.52	1499.61	13677.13	3.885	976.94
72	19973	3.24	1962.37	25100.02	7.130	522.92
144	21124	32.66	2075.46	6046.83	1.718	83.98

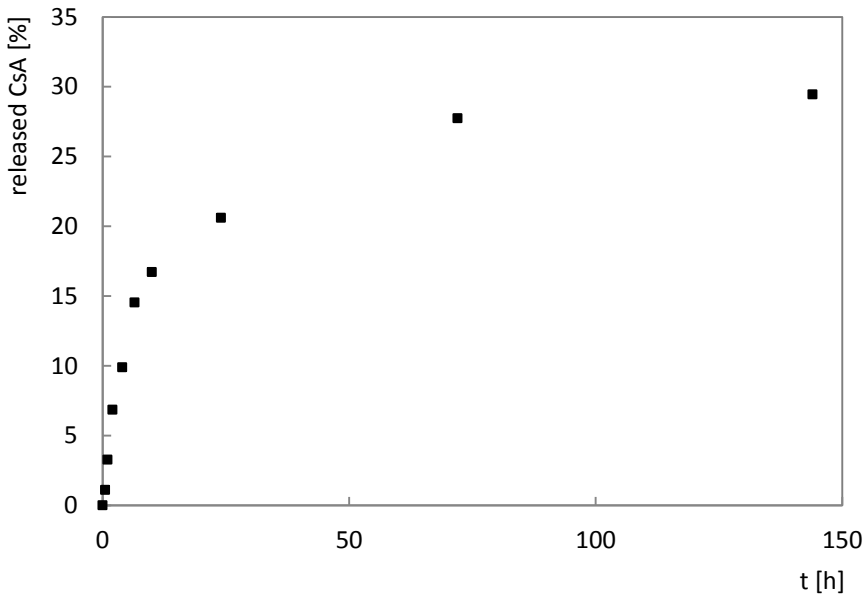

Fig. 19: The release kinetics of CsA from CsA-loaded nanofibers (10%, m/m; 15% PEG 20000) in cumulative mode experiments

The addition of poly(ethylene glycol) with M_r 20000 had similar positive impact to the release kinetics. The release rate decreased in time and the decrease was balanced Kinetic profile in both modes reminds the outcome of experiments with nanofibers containing PEG 6000, only the released amount of cyclosporin is different.

5.9.5 Comparison of the release kinetics in PBS buffer

The comparison of drug release from all the tested nanofibers is provided in Table 23.

Table 23: Comparison of release of cyclosporin A from the tested nanofibers

Type	Mode	Released [%]	Short description
10% (m/m) CsA no PEG	continuous exchange	0.300	Release for 24 hours, release rate gradually falls from 1889.70 ng/h to 29.99 ng/h
	cumulative	0.668	Release for ten hours, release rate falls sharply (~7000→~400→~100→0 ng/h)
5% (m/m) CsA no PEG	continuous exchange	1.178	Release for 144 h (possibly more), release rate gradually falls from 1387.31 ng/h to 14.58 ng/h
	cumulative	1.632	Release for 144 h (possibly more), release rate falls inconsistently (sinusoid decrease) from 2308.90 ng/h to 3.48 ng/h
2.5% (m/m) CsA no PEG	continuous exchange	0.527	Release for 144 h (possibly more), release rate gradually falls from 518.77 ng/h to 0.66 ng/h
	cumulative	1.063	Release for 24 h, random changes in release rate, most of CsA released within the first 0.5 h (speed 2387.50 ng/h)
10% (m/m) CsA 15% PEG 6000	continuous exchange	12.812	Release for 144 h (possibly more), release rate stable for the first ten hours (~2500 ng/h), then gradually falls
	cumulative	28.703	Release for 72 h, sinusoidal decrease with high amplitude (~11072.90 ng/h to 0; maximum value ~14502.83 ng/h)
10% (m/m) CsA 15% PEG 20000	continuous exchange	17.313	Release for 144 h (possibly more), release rate gradually falls from 8388.68 ng/h to 120.47 ng/h
	cumulative	29.455	Release for 144 h (possibly more), release rate after 0.5 h gradually falls (7810.96 ng/h→15273.45 ng/h→83.98 ng/h)

The summary in Table 23 clearly shows that among the nanofibers without addition of poly(ethylene glycol) the most suitable was the content of cyclosporin 5% (m/m). The total amount of released cyclosporin A was also highest for the 5% nanofibers. The main disadvantage of these nanofibers was the kinetic profile - the release rate was gradually decreasing during the 1 week long extraction. This kinetic profile is not desirable for the intended use in organ transplantations where the administration of cyclosporin has to be steady for at least one week after transplantation.

Addition of poly(ethylene glycol) of M_r 6000 had positive effect on the kinetic profile. The release was steady for the first 10 hours of extraction and then continued until at least 144 h after initiation of extraction. This period is still not long enough for the intended use, on the other hand addition of PEG shows possible functional way of adjusting the release profile of cyclosporin. The analyzed nanofibers with PEG 6000 contained 10% (m/m) of cyclosporin in which the previously studied release (without PEG) lasted for 24 hours only. There is a possibility that the nanofibers with 5% (m/m) of cyclosporin in which the release did not end after 144 hours of the experiments would show longer period of steady release rate after addition of PEG 6000. The nanofibers containing PEG 6000 also showed high total amount of released cyclosporin - for the nanofibers without PEG it was around 0.5 - 1.5% of total amount in the nanofibers; in the nanofibers which contained PEG 6000 the total amount was 12.8% and 28.7% in the continuous exchange of medium experiments and in the cumulative mode, respectively.

The addition of poly(ethylene glycol) of M_r 20000 had similar effect to the kinetic profile. Cyclosporin was being released for whole 144 hours, however, the rate of the cyclosporin release was not as stable as in the nanofibers containing PEG 6000. The total amount of released cyclosporin was comparable with the nanofibers containing PEG 6000: 17.3% and 29.5% of cyclosporin was released in the continuous exchange of medium experiments and in the cumulative mode, respectively.
The emphasis was on the data from the continuous exchange of media experiments as this mode is closer to the physiological process. No nanofibers showed desirable kinetic profile of cyclosporin release when the extraction was performed in the cumulative mode.

CONCLUSION

Cyclosporin A release kinetics was studied with five different types of cyclosporin A-loaded poly(L-lactic acid) nanofibers. A newly optimized LC-MS/MS method was developed and used for detection of cyclosporin A.

None of the studied nanofibers was suitable for use in transplantation medicine. Suitable nanofibers should release cyclosporin at high level for 1 - 2 weeks followed by approximately one year of slower release of residual cyclosporin. It would be therefore necessary to extend the longest release period observed in nanofibers with 10% cyclosporin and 15% PEG 6000 approximately 20 times.

Acquired data suggest that different content of cyclosporin or different molecular weight of PEG will not lead to required release profile. Nanofibers with 5% of cyclosporin and 15% of PEG would have longer period of steady release but it would not extend the highest observed period 20 times. In this thesis two types of nanofibers containing PEG were examined and in both of them the content of PEG was 15%. As PEG prolonged release of cyclosporin addition of higher amount of PEG should be considered. In that case special attention should be paid to the concurrently increased amount of released cyclosporin. The total amount of incorporated cyclosporin must correlate with increased release rate as cyclosporin should be released for at least one year in transplantation patients.

Required release profile could be achieved by producing sandwich-structured nanofibers. It has been shown[63] that addition of covering layers to the layer containing a drug can slow release of the drug. Using suitable material of appropriate thickness could prolong the drug release to required time. As this study also showed that thickness of the covering fibers had influence on drug release it might be helpful to examine also PLA nanofibers with different area density.

It has been shown that bicomponent nanofibers express combined characteristics of both components[6] and that provide another possible way of altering the release of cyclosporin from PLA nanofibers. On the other hand it is difficult to predict the results in case of choosing this way because cyclosporin A has not been incorporated to different material yet. Results of studies with different drugs whose properties are similar to those of cyclosporin would be helpful in this case.

If PLA nanofibers did not show suitable release of cyclosporin, it would be necessary to choose completely different material for production of the CsA-loaded nanofibers. This would of course open the whole range of possible modifications - addition of PEG, forming sandwich-structured nanofibers or bicomponent nanofibers etc.

It is necessary to highlight that in this thesis the experiments were performed in solutions which do not correspond well with physiological conditions. The suitability of drug release profile of each promising material will have to be confirmed in more appropriate release conditions, e.g. on tissue cultures.

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