Pharmaceutical Aerosol Deposition Device On Cell Cultures (PADDOCC): Development of an *in vitro* test system based on pulmonary epithelial cells

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Meiner Familie

"Das Ende eines Dinges ist der Anfang eines anderen."

Leonardo Da Vinci (1452-1519)

Contents

1 Short summary

Drug application via the lung is a convenient route of administration, because of its easy handling and the special anatomy of the lung. A large surface area, rapid absorption through the thin alveolar epithelium, low enzymatic activity and a direct access to the blood circulation are the advantages of the administration of local as well as systemic drugs to the lung. However, new drugs and formulations have to be tested for their safety and efficacy, especially since new particle types like nanoparticles or liposomes are in the focus of the development of new drugs. Such testing is often done by animal experiments due to a lack of appropriate *in vitro* models. Therefore a new *in vitro* model was developed allowing to test aerosolisation, deposition as well as subsequent absorption of aerosol formulations. The so-called "Pharmaceutical Aerosol Deposition Device On Cell Cultures" (PADDOCC) system relies on sedimentation, which is the main deposition mechanism in the deep lung. The PADDOCC system, comprising of air flow control unit, aerosolisation unit and deposition unit, is able to generate the dry powder aerosol and deposit only the respirable fraction simultaneously onto three air-liquid interface grown cell monolayers. After the deposition several endpoints such as cytotoxicity or absorption are determined. Budesonide and salbutamol sulphate, two important drugs, which are used to treat asthma were tested, and different absorption profiles compared to standardised transport studies could be detected.

2 Kurzzusammenfassung

Die Lunge ist ein erfolgversprechender Ort für eine Arzneistoffgabe, da ihre Anatomie viele Vorteile bietet. Sie besitzt eine große Oberfläche, eine schnelle Absorption des Arzneistoffes durch das dünne Alveolarepithelium, eine geringe enzymatische Aktivität sowie einen direkten Zugang zum Blutkreislauf. Neu entwickelte Arzneistoffformulierungen zur inhalativen Anwendung müssen in Bezug auf ihre Sicherheit und Wirksamkeit getestet werden, in besonderem Maße seitdem neue Partikeltypen wie Nanopartikel oder Liposomen in den Focus der Neuentwicklungen gerückt sind. Diese Tests werden meistens mit Tiermodellen durchgeführt, da es keine oder kaum geeignete *in vitro* Modelle gibt. Deshalb wurde ein neues *in vitro* Modell entwickelt, mit dem die Aerosolisierung, die Deposition sowie die nachfolgende Absorption einer Arzneistoffformulierung untersucht werden kann. Das "Pharmaceutical Aerosol Deposition Device On Cell Cultures" (PADDOCC) System basiert auf der Sedimentation der Aerosolteilchen eines Arzneistoffes auf einem Zellmonolayer, da die Sedimentation der Hauptdepositionsmechanismus in der tiefen Lunge ist. Das PADDOCC System, bestehend aus einer Kontrolleinheit, einer Verneblungseinheit sowie einer Depositionseinheit, generiert ein Trockenaerosol und deponiert nur die lungengängige Fraktion gleichzeitig auf drei, an der Luft-Grenzschicht gewachsenen, Zellmonolayer. Danach können verschiedene Endpunkte wie Zytotoxizität oder Absorption bestimmt werden.

Budesonid und Salbutamolsulfat, die zwei bedeutende Therapeutika in der Asthmatherapie darstellen, wurden getestet und es wurden veränderte Absorptionsprofile im Vergleich zu standardisierten Transportexperimenten mit diesen Arzneistoffen gefunden.

3 Introduction

Parts of this chapter have been published in:

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Particulate carriers for pulmonary drug delivery in: P. Gehr, C. Mühlfeld, B. Rothen-Rutishauser, F. Blank (Eds.) Particle lung interactions 2nd edition, Informa Healthcare, New York (2009), ISBN: 978-1420072563

Drug administration by inhalation is well established since many years, but has mainly been used for locally treating diseases in the lungs such as asthma or chronic obstructive pulmonary disease (COPD). During the last two decades, though, increasing attention has been paid to using the healthy lung as a convenient route to treat diseases such as diabetes mellitus by aerosol delivery of insulin. Pulmonary delivery offers many advantages as a non-invasive method for both local and systemic drug delivery due to the characteristics of the lung. The lung has a large surface area, offers rapid absorption through the thin alveolar epithelium, there is low enzymatic activity, and it affords direct access to the circulation. However, most drugs, approved for inhalation therapy, consist of the pure drug and some excipients like lactose or sodium chloride which stabilise the formulation, but do not offer the possibility of prolonged release. The development of such prolonged drug formulations is difficult due to the defense mechanisms of the lung. These defense mechanisms protect the body from airborne particles of the environment, but they do not distinguish between particles which could possibly harm the body (e.g. viruses, bacteria, particulate matter) or drug particles which are able to heal or ameliorate the disease. New particle approaches like liposomes or nanoparticles, among other things, try to evade the defense mechanisms to offer an efficient therapy without severe side effects with a convenient administration for the patient. Nevertheless, safety issues of the materials used in these new formulations are a big challenge, because nowadays only very few excipients are approved for inhalation therapy, and make the conversion of innovative delivery technologies into marketed drug products a rather slow process.

3.1 Structure of the respiratory tract

3.1.1 Anatomy of the lung

The main function of the lung is gas exchange, meaning the oxygen uptake from the atmosphere into the bloodstream and the carbon dioxide excretion from the bloodstream into the atmosphere. The functional structure can be classified by the conducting airways and the gas exchange part. The conducting airways consist of trachea, bronchi, bronchioles and terminal bronchioles and open out into the gas exchange area, comprising of respiratory bronchioles, alveolar ducts and alveoli. The inhaled air is filtered, warmed up to 37°C and humidified in the conducting airways. The trachea is divided into two main bronchi, a left and right side. Afterwards the bronchi branch repeatedly into two smaller bronchi until the alveolar region. The so formed generations of airways result in an exponentially increased area of tissue. Generation 0 - 16 is formed by the conducting airways and 17 - 23 is located in the gas exchange region (Figure 1).

Due to the different functions of the conducting and respiratory zone, there are differences in the morphology of the cells present. Bronchioles mainly consist of ciliated cells and goblets cells [2], whereas the alveolar region is covered by a flat monolayer of epithelial type I and II cells. Epithelial type I cells cover about 95% of the surface and are specialised in gas exchange [3]. The other 5% are covered by epithelial type II cells which secret surfactant to prevent collapsing of the lung during exhalation [4]. The surfactant is composed of 90% lipids, mostly phospholipids and 10% proteins. The surfactant proteins are divided in the hydrophilic SP-A and SP-D plus the hydrophobic SP-B and SP-C which all have different functions on the alveolar surface in the context of biophysics and immunology [5]. The alveolar region has a very large (100 m^2), but thin (< 0.5 μm) surface to provide a rapid gas exchange, and it is also suited for drug absorption into the bloodstream.

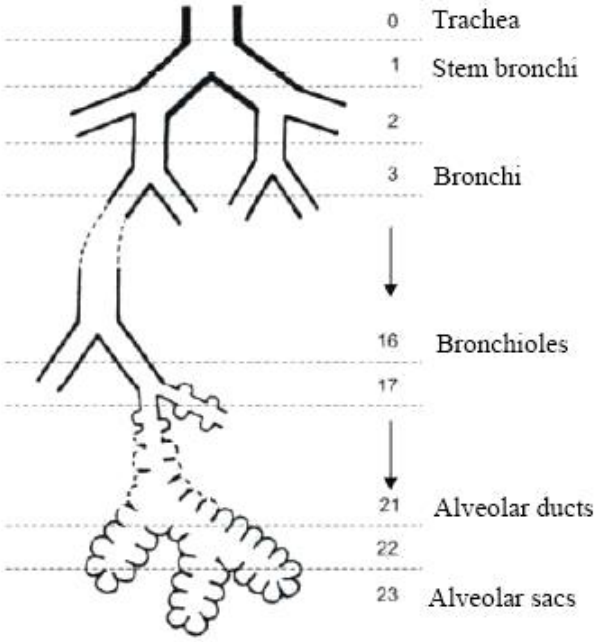


Figure 1: Different generations of the respiratory tract due to the branching of the trachea (modified according to [1])

3.1.2 Clearance mechanisms of the lung

The lung has certain clearance mechanisms to protect the human body from the environment. These mechanisms function as a barrier to withhold bacteria, viruses or other pathogenic particles, but they also prevent drugs to take effects in the lung. Deposited drug particles will also be eliminated because the defense mechanisms are not able to distinguish between pathogenic and drug particles. Indeed, establishing a depot for prolonged release from drug particles is desirable, but still appears to be rather difficult. In order to make further progress, the particles must be able to evade the clearance mechanisms of the lungs such as macrophages and mucociliary clearance without affecting them, otherwise the integrity of the respiratory tract could be compromised.

Mucociliary clearance

Dependent on size, density etc., not all particles are deposited in the deep lung and reach the alveolar region. Most of the particles are deposited in the conducting airways during in- and exhalation. To remove the particles from this region, the respiratory tract is equipped with a so-called mucociliary clearance system. Ciliated cells covered with mucus are lining the parts between the trachea and the terminal bronchioles. These ciliated cells beat in a metachronal coordinated wave pattern, thereby transporting particles, deposited on the mucus, towards the throat, where they can be swallowed [6]. This special mucus, secreted by the goblet cells, consists mostly of water and in minor parts of glycoproteins, proteins, lipids, and inorganic salts [7]. Depending on different experimental techniques, the clearance velocity of healthy non-smokers varies from 4 to 20 mm/min [8, 9], but the interactions between the ciliary cells, the mucus and deposited particles are not yet explained. An *in vitro* model was developed to investigate the mucociliary clearance [10, 11]. An embryonic chicken trachea was used to determine the influence of particle size or material properties on the clearance velocity. Clearance rates of polystyrene particles of different sizes (50-6000 nm) did not differ significantly in this *in vitro* model. Thus, it remains unclear which particle characteristics influence particle clearance.

Alveolar macrophages

Alveolar macrophages are present on the alveolar surface. These macrophages are derived from monocytes in the bone marrow. Afterwards they migrate to the alveoli and represent the first line of defense against inhaled particles because they have a high phagocytotic and microbicidal potential [12]. Particles which are deposited in the alveolar region, will be phagocytosed by those alveolar macrophages. Champion et al. [13] demonstrated that particle shape and the position to the macrophage influences the internalisation by

phagocytosis. Alveolar macrophages are also able to secrete a variety of cytokines or other mediators and to attract other cells of the non specific immune system. Macrophages that are full of phagocytosed particles are eliminited by migrating to the bronchial tissue and then escape through the mucociliary escalator, where they are swallowed at the upper end.

3.2 Pulmonary drug targeting with different particulate carriers

In order to be effectively deposited in the deep lung, it is well known that an aerosol must fulfill some requirements. The particle size of the administered drugs needs to have a median mass aerodynamic diameter between 1 and 5 μm, because smaller particles are exhaled while bigger particles will not reach the alveoli and are confined to the upper airways instead. This is due to different deposition mechanisms, depending on aerodynamic diameter and inhalation manoeuvre. Particles with a diameter > 5 μm are mostly deposited in the upper airways by impaction forces [14, 15], whereas smaller particles are deposited by sedimentation (1-5 μm) or Brownian diffusion (< 1 μm) in the deep lung (Figure 2). Ultra-fine particles of 5-10 nm are also efficiently deposited and not exhaled. They may remain in the deep lungs as well, but this size range is not used for aerosol medicines at the moment. This is probably due to a lack of appropriate formulation technologies that can generate ultra-fine drug particles, along with intrinsic limitations on the dose of an active pharmaceutical ingredient that ultra-fine particles can deliver within a reasonable aerosol volume or time of inhalation.

Regardless of their size, particulate drug carriers for inhalation aerosol must be non-toxic and well tolerated. However, only a few materials are approved by the United States Food and Drug Administration (FDA) for inhalation. Current aerosol drug products essentially nebulise the "naked" drug via different systems such as nebulisers, pressurised metered dose inhalers or dry powder inhalers [17, 18]. Apart from a few propellants and solvents, excipients other than lactose or NaCl are virtually uncommon in pharmaceutical aerosols due to their unproven safety and a lack of regulatory approval for use in inhalation aerosols. Biodegradable pharmaceutical polymers such as poly(lactic-co-glycolic-acid) (PLGA) and chitosan are common in controlled release formulations for oral or even parenteral admin-

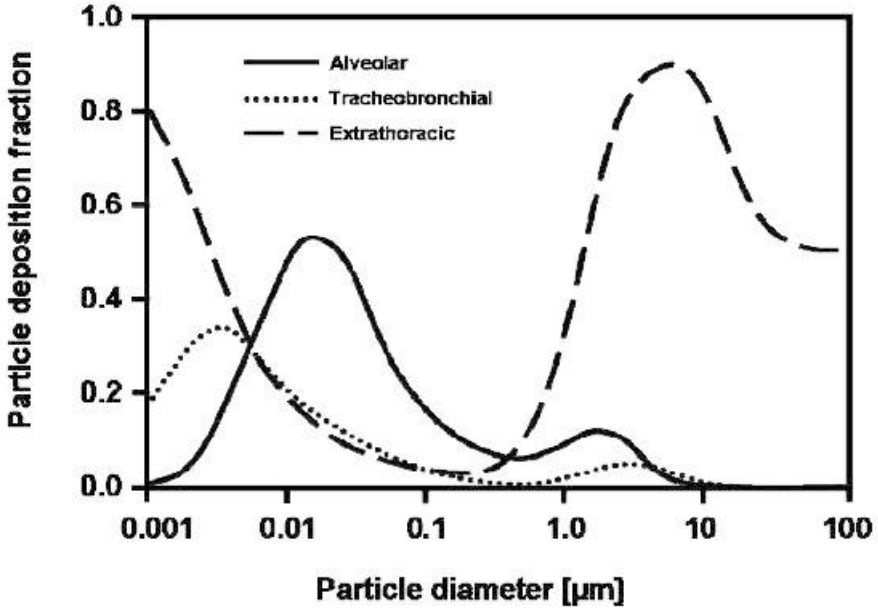


Figure 2: Deposition in the different regions of the lung is dependent on particle size [16]

istration, but they have yet to be approved for use in humans by inhalation.

Limited by a very short list of approved excipients, the development of inhalation medicines has so far focussed on aerosol production to optimise deposition in preferred lung areas. Controlled release of the active ingredient from the carrier which is a common strategy for many oral or parenteral drug formulations has yet to be achieved for inhaled drugs. A controlled-release system for an inhaled drug is rather challenging because once it is deposited in the lungs it will be prone to a variety of very efficient clearance mechanisms, such as mucociliary clearance in the upper airways [19] and macrophage clearance in the lower respiratory tract [20, 21]. These mechanisms are physiologically important to protect the body from inhaled particles a priori, regardless of whether they are toxic or not. Therefore advanced particulate drug carriers must overcome these clearance mechanisms in order to achieve long-term sustained release or enhanced absorption into the bloodstream for systemically acting drugs. At the same time, however, such carriers must be absolutely

biocompatible and well-tolerated by the patient. In order to avoid any long-term accumulation in the lung or other compartments in the body, they must be biodegradable within an appropriate timeframe. Advanced carriers also offer an opportunity to target drugs to a specific site of action, for example by triggering cell-specific uptake mechanisms, reducing side effects while decreasing the dose that is required for treatment.

3.2.1 Systemic drug delivery via the lungs

The aim of aerosol delivery systems employed in systemic formulations is to provide good systemic bioavailability by allowing convenient, pain-free inhalation and rapid uptake into the bloodstream. This approach may be of interest whenever oral delivery of an active pharmaceutical ingredient is not feasible and intravenous injection is not desired. These formulations need to cross the diffusional barrier at the alveolar epithelial barrier and avoid the clearance mechanisms of the lung that will act to diminish or inhibit uptake. Another consideration for the inhaled formulation is whether the drug needs to act with a rapid onset of action or over prolonged period of release.

Large molecules

It is difficult to deliver large molecules like insulin or heparin to the systemic circulation with acceptable bioavailability by any route other than parenteral administration. Nebulising and administering them via the lungs has been shown to be feasible, but the bioavailability is usually rather low. This may, however, be further improved if advanced particulate drug carriers and novel excipients are employed in conjunction with improved aerosol technology. There has been strong interest in developing an inhalable insulin formulation for many years due to an increase in diabetes mellitus patients and because many patients suffer from their daily regime of injections. The first FDA-approved product was

Exubera®, which came on market in 2006. It was a microparticle powder produced by a spray-drying technique containing recombinant human insulin, mannitol, glycine and sodium citrate. Only 10% of rapid acting insulin was absorbed into the systemic circulation compared to subcutaneous administered insulin [22]. Although certainly an important pioneer, one must acknowledge that Exubera® was in principle still a conventional formulation where no attempt was made to enhance absorption or modify release from the carrier. Exubera® failed to gain wide acceptance and in October 2007 Pfizer decided to phase out Exubera® for mainly economical reasons. Other firms were also working on inhalable insulin formulations, but after the withdrawal of Exubera®, Novo Nordisk (AERx® insulin) and Eli Lilly (AIR® insulin) decided to stop their clinical phase III trials because of economical reasons, too.

One example of an enhanced insulin formulation is Technosphere® insulin from the MannKind Corporation. It consists of pH-sensitive carrier particles and monomer insulin, which is the bioactive form of insulin. The insulin is loaded onto pH-sensitive organic molecules that self-assemble into small particles under the neutral pH conditions in the lung [23]. The monomers diffuse into the bloodstream and show rapid uptake with 30-50% bioavailability compared to subcutaneous administration [24, 25]. The FDA accepted submission for Technosphere® insulin which is now called AFRESA® in May 2009. Other formulations have been tested *in vitro* and in animals. Grenha et al. [26] developed insulin loaded lipid/chitosan nanoparticle complexes that were spray-dried with mannitol into microspheres. Testing *in vitro* showed that the lipids provide controlled release of the insulin. Other groups have used large porous particles for delivering insulin into the systemic circulation [27]. These particles were first introduced by Edwards et al. [28] and are characterised by large sizes (> 5 μm) but small mass densities. These particles can be deposited into the deep lung, and since they are too big to be phagocytosed, they are an attractive

delivery system for systemic drug application. To avoid accumulation in the lungs caused by the reduced phagocytosis, these particles need to be biodegradable. Another advantage is that the large porous particles aggregate less than other nonporous particles because smaller particles have stronger cohesive forces [29]. Huang et al. [30] encapsulated insulin into liposomes and administered them to mice and produced a decrease in plasma glucose levels compared to mice administered empty liposomes. Cagnani et al. [31] produced an inhalable insulin powder with spray-drying technique using clear mild acidic solutions of insulin. *In vitro* studies showed that these particles had respirable aerodynamic diameters and a "raisin-like" morphology that showed no agglomeration tendency. While these studies have all paid attention to insulin delivery, others have focussed on improving the stability of the formulations. Amidi et al. [32] produced insulin-loaded microparticles with N-trimethyl chitosan and dextran as carriers using a supercritical fluid-drying technique and showed that the particle characteristics and the insulin structure were maintained for one year. All of these carriers mentioned here could be employed in the lungs offering enhanced systemic delivery, perhaps even sustained release properties, and therefore allow diabetes patients to avoid daily injections.

Another important macromolecule for systemic delivery via the lungs is heparin. To prevent deep vein thrombosis, a low molecular weight heparin (LMWH) was connected to a positively charged dendrimer in order to enhance absorption by reducing the negative surface charge density of the LMWH. The drug-dendrimer complex was administered to rats and it was as efficacious as subcutaneously administered LMWH and it had no toxic effects on the lungs [33]. Yang et al. [34] tested LMWH formulations with tetradecyl-β-maltoside or dimethyl-β-cyclodextrin *in vitro* and *in vivo* and showed that both formulations enhance the pulmonary absorption of LMWH. They also showed that tetradecyl-β-maltoside formulations were more potent than dimethyl-β-cyclodextrin formulations.

Small molecules

Small molecules can also be systemically delivered via the lungs by inhalation. This is an attractive option when the drug molecules are not stable or water soluble enough to be delivered via the gastrointestinal tract, or when an extremely rapid onset of action is desired, such as analgesia. Some nebulised drugs are in clinical trials that are administered to the alveoli in order to achieve a rapid onset of action. MAP0004 is a dihydroergotamine mesylate [35] used to treat migraine, however intravenous administration of this drug causes some serious side effects. When MAP0004 was applied to the lungs by a pressurised metered dose inhaler there was a decreased rate of side effects in healthy volunteers. MAP0004 (now: Levadex) is currently phase 3 studies [36, 37] and the new drug application (NDA) submission is planned for the first half in 2011. Another strategy for inhaled migraine therapy is described by Rabinowitz et al. [38, 39]. It is a single dose thermal aerosol device with a thin (about 5 μm) film of pure drug (e.g. rizatriptan). Breath-activation of the device by patients causes rapid heating of this film and a vapour is formed in less than one second followed by condensation of the vapour phase drug into aerosol particles during inhalation (Figure 3). The emerging solid particles are spherical with an amorphous form and a mass median aerodynamic diameter of 1-3 μm, which is optimal for alveolar deposition. Drugs delivered in this way will act rapidly, because the particles dissolve in the alveolar liquid lining upon deposition and rise immediately into the systemic circulation. Unfortunately, this delivery method is only suitable for drugs with specific properties: they have to be able to sublimate and they need to be thermostabile because the drug is heated to 400°C in the device. About 175 different drugs, such as rizatriptan, fentanyl, zolpidem or loxapine have been used with this technology without thermal decomposition. The device was developed further to the Staccato system which is now in several trials with different drugs. Loxapine finished phase 3 studies and an NDA was submitted in December

2009. It is used in the acute treatment of agitation in emergency medical aid. Studies with Staccato loxapine showed rapid absorption and was well-tolerated [40].

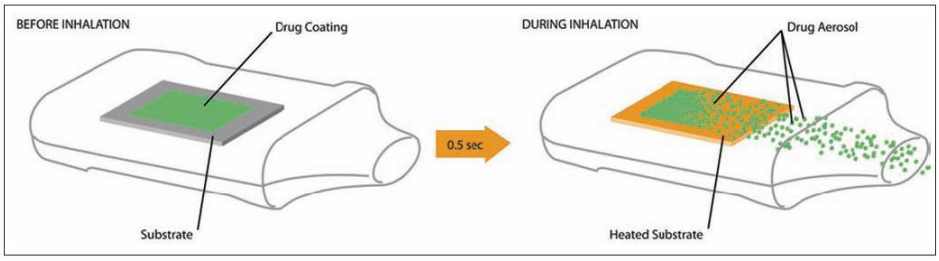


Figure 3: Staccato loxapine before and after inhalation [40]

In pain control, there is not only a need for a rapid onset of action, drug plasma levels need to be maintained above a minimum level for a prolonged period of time while avoiding an initial peak that exceeds the maximum tolerance level and undesired drug effects. From a pharmaco-kinetic point of view, a controlled-release delivery system addresses all of these issues.

There is a strong incentive to develop inhalable controlled-release formulations to treat pain in cancer patients. There have been some attempts to use nebulised fentanyl for pain relief [41] and the early stages of development are summarised by Farr et al. [42]. One product is a composition of free and liposome-encapsulated fentanyl (AeroLEF$^{\text{TM}}$) and it passed the phase II trials in 2007. The free fentanyl provides a rapid onset of analgesia while an extended period of analgesia is achieved with the liposome-entrapped fentanyl. Liposomes show many advantages for pulmonary delivery because they are made of different phospholipids, such as dipalmitoylphosphatidylcholine (DPPC) and dilauroylphosphatidylcholine (DLPC), which naturally occur in the lung and therefore are compatible with lung surfactant. Hydrophilic as well as lipophilic substances can be incorporated into liposomes and they enable sustained drug release. Liposomes are not as stable as microparticles,

though some attempts have been made to prolong their stability by producing a liposomal dry powder by lyophilisation [43, 44] or spray-drying [45].

3.2.2 Controlled loco-regional delivery to the lungs

The field of inhalation therapy was established many years ago in order to develop drug delivery systems to treat loco-regional diseases like asthma and COPD. These early drug formulations often showed no controlled- or sustained-release because the particles were not modified to circumvent the clearance mechanisms of the lungs. Therefore new particulate systems were developed to prolong residence times of the drug particles that are used to treat several lung diseases.

Asthma/COPD

There is a wide range of aerosol drug products on the market for the treatment of asthma and COPD. Most of these formulations are made of drug particles mixed with lactose as a carrier material (Figure 4) and are administered by a dry powder inhaler or by nebulising the drug dissolved/dispersed in a propellant with a pressurised metered dose inhaler. Although these formulations achieve efficient pulmonary deposition, they were not designed to provide sustained- or controlled-release. If they are long acting (e.g. β-agonists), it is due to the pharmacological half-life of the drug and not the delivery system. Indeed, the particle technology of these conventional formulations aims to improve the aerodynamic properties and thus the deposition rate of the aerosol particles. Some of the new approaches in this area try to find formulations that show sustained release properties so as to reduce the dose frequency for patients and improve bioavailability in the lung.

Arya et al. [46] coated budesonide particles with a very thin film of polylactic acid using the pulse laser ablation technique. They administered coated and uncoated budesonide

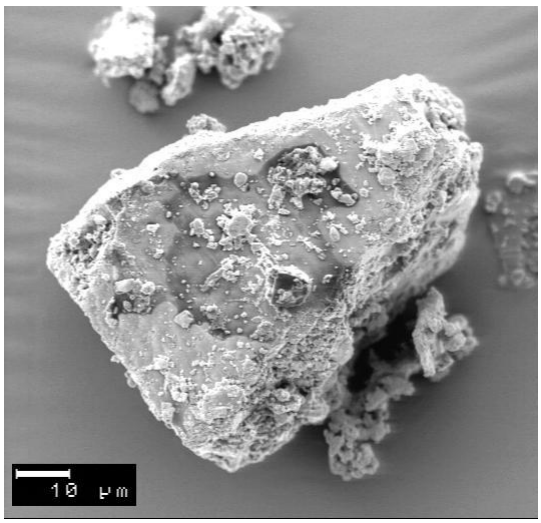


Figure 4: Typical dry powder formulation comprising of large carrier particle covered with small drug crystals

intratracheally to neonatal rats and observed higher AUC levels in the lung with coated budesonide, while the systemic exposure of budesonide was reduced compared to uncoated budesonide. In another study the poly-(ethylene oxide)-block-distearoyl phosphatidyl-ethanolamine polymer (mPEG-DSPE) was used to prepare beclomethasone loaded micelles [47]. The lyophilised beclomethasone loaded polymeric micelles showed high entrapment efficiency and *in vitro* drug release studies showed a sustained release over six days. In another study, salbutamol acetonide was incorporated into solid lipid microparticles (SLMs) after increasing its lipophilicity [48]. SLMs show physicochemical stability and compatibility and no acute toxicity *in vivo* in rats [49] and *in vitro* release studies demonstrated that salbutamol acetonide SLMs had a slower release of the drug than pure salbutamol acetonide. Thus, SLMs promise to provide sustained pulmonary drug delivery, which in turn will reduce the number of doses required by patients.

Liposomes have also been considered as another carrier for lung delivery of anti-inflammatory drugs. Saari et al. [50] investigated the distribution of ^{99m}Tc-labeled beclomethasone

dipropionate DLPC and DPPC liposomes in healthy volunteers. They found that the clearance of DPPC liposomes was slower than DLPC liposomes, perhaps because of the different phase transition temperatures, but in both formulations about 80% of the deposited radioactivity remained in the lungs 24 hours after inhalation. Learoyd et al. [51] produced chitosan-based terbutaline sulphate particles where chitosan acted to modify drug release. Different molecular weights of chitosan were used and high molecular weights of chitosan increased the duration of terbutaline release.

Pulmonary arterial hypertension

Pulmonary arterial hypertension is a severe lung disease with increased pulmonary arterial pressure resulting in right ventricular failure [52]. The vasodilator iloprost is FDA-approved for pulmonary administration, but because of its short-acting properties, multiple inhaled doses (6-9 per day) are necessary [53]. Therefore Kleemann et al. [54] developed iloprost-containing liposomes for sustained release. Liposomes containing DPPC and cholesterol showed good stability and iloprost loading efficiencies, so further investigations are under way *in vivo* to develop a suitable carrier system for prolonged iloprost release in the lungs.

Immunosuppressives

Pulmonary drug delivery is being evaluated for loco-regional application of immunosuppressive drugs to lung transplant patients. Intravenous or oral formulations of tacrolimus are available for therapy of lung transplantation but they are poorly tolerated. Sinswat et al. [55] created nanostructured aggregates containing amorphous (with lactose) or crystalline tacrolimus nanoparticles by an ultra-rapid freezing technique. These aggregates could be delivered by nebulisation and showed high drug absorption in the lungs of mice. Another immunosuppressive drug, cyclosporine A is very hydrophobic so aerosol formulations were

based on ethanol and propylene glycol dissolutions [56, 57], but these excipients were sometimes poorly tolerated in animals as well as humans. Thus, cyclosporine A liposomes were produced and they were efficiently absorbed into lung tissue and the formulations were well tolerated [58, 59]. In another approach, Chiou et al. [60] produced cyclosporine A powders with confined liquid impinging jets (CLIJ) technique and subsequent spray-drying. They optimised this technique to obtain suitable particles for pulmonary delivery of proteins. All of these advanced formulations of cyclosporine A promise to reduce systemic plasma levels and thus toxicities to other organs like kidneys.

From the discussion above it is clear that the majority of approaches either improve bioavailability, control the release properties or reduce the dose frequency for the drug with the aim of improving patient compliance and the quality of therapy. However, one problem remains unsolved. There is no existing technology able to inhibit or circumvent the clearance mechanisms of the respiratory tract. As a consequence, the potential of such carriers to act as a platform for sustained drug delivery for longer periods cannot be entirely exploited, even when it is possible to design aerosol drug carriers that show sustained release profiles for 24 hours or longer. New carrier systems are needed that avoid clearance and achieve a powerful drug depot in the respiratory tract.

3.2.3 Drug targeting within the respiratory tract

Targeted delivery of drugs is particularly important when the therapy causes severe side effects, such as in the treatment of lung cancer. These drugs are often administered to the systemic circulation, but in order to achieve an acceptable drug level at the site of action, high plasma levels may be required and can produce side effects in other tissues. Therefore, targeting drugs to the lungs by inhalation therapy promises to protect other tissues and therefore reduce side effects.

Vaccines

Delivering vaccines by the pulmonary route is easy, fast and non-invasive, and therefore a powerful strategy in the fight against infectious diseases, particularly in the developing world. Furthermore, this immunisation route allows mass vaccination campaigns to be carried out without the need for medical personnel. Many pulmonary vaccines are in development for several infectious diseases such as influenza [61, 62], measles [63–65], diphtheria [66] and hepatitis [67].

Pulmonary vaccine delivery can induce local immune responses in the lungs as well as systemically [68]. Below the pulmonary epithelium, there is an array of immune cells, such as antigen-presenting cells that continuously sample inhaled antigens and subsequently present it to T cells, and the broncho-alveolar lymphoid tissue (BALT) that is induced by local infection [69]. Local activation of the pulmonary immune response has the advantage of targeting pathogens directly at the port of entry and is suitable for diseases like influenza. Several formulations for intranasal administration of influenza vaccine have already been tested and shown to elicit a modest systemic immune response [61, 70]. Smith et al. [71] encapsulated inactivated or subunit split influenza virus vaccines into spray-dried microparticles containing DPPC as well as distearoylphosphatidylcholine (DSPC) and administered them intratracheally to mice and rats. This formulation showed improved local bioavailability to the BALT, and increased antigen-loading of antigen-presenting cells, IgG antibodies, and T cell responses locally as well as systemically. In another study, an influenza subunit vaccine powder stabilised by inulin was prepared by spray freeze-drying and delivered to the lungs of mice [62]. This formulation produced enhanced IgG and IgA levels compared to the conventional intramuscular administered influenza vaccine, proving that the modified vaccine can enhance local and systemic antibody production.

Another infectious disease that is transmitted by the airborne route is measles. Several

research groups administered wet mist aerosols of live attenuated measles vaccine to people and the immune response was greater compared to injected vaccine [72, 73]. However, the stability of the vaccine was a big problem because of cold-chain maintenance caused by the thermolability of the vaccine. Therefore different research groups have developed powder vaccines with increased stability. De Swart et al. [63] administered two different dry powder measles vaccines to macaques, but the vaccination was less efficient than intramuscular vaccination or nebulised vaccination. Thus, more work is required to improve the composition of the dry powders to obtain a formulation that can boost serum antibody levels with acceptable properties for administration by dry powder inhalers.

Antiinfectives

One third of the world population is infected with tuberculosis [74]. The treatment of tuberculosis is a great challenge, because Mycobacterium tuberculosis invades and replicates within macrophages. Drugs against tuberculosis are given orally and over a long period, though side effects and a high dose frequency result in many interruptions to therapy. Targeting macrophages could decrease systemic exposure, reduce the dose that is needed, and decrease side effects though a special targeting strategy is needed to both channel the drugs into infected macrophages and provide prolonged drug release once it is delivered. Therefore different formulations for pulmonary administration are being developed. Pandey et al. [75] produced biodegradable PLG nanoparticles with three anti-tubercular drugs (ATD) (rifampicin, isoniazid and pyrazinamide) and administered the aerosolised nanoparticles to infected guinea pigs. They found that the bioavailability of all three drugs was increased compared to intravenous administration and that the drugs remained above a therapeutic concentration for 11 days after inhalation. Sharma et al. [76] tried to improve the bioavailability of ATDs by producing bioadhesive wheat germ agglutinin-coated PLG nanoparticles

with ATDs. Wheat germ agglutinin was used because it is known to bind to the alveolar epithelium [77] and the results showed that the concentrations of the nebulised ATDs were in a therapeutic range for about 15 days. Other investigations have employed alginate nanoparticles [78].

As already discussed above, liposomes are well suited for administration to the lungs because their similarity to surfactant prevents them from acting as an irritant once deposited in the lungs. Zaru et al. [79] designed different rifampicin-loaded liposomes and showed that rifampicin-liposomes were less toxic to alveolar epithelial cells (A549) compared to the free drug. Stealth liposomes are sterically stabilised liposomes that avoid elimination through the reticuloendothelial system [80] and are used for intravenous cancer therapy (e.g. Caelyx®/Doxil® (stealth liposomal doxorubicin)). Deol et al. developed stealth liposomes for pulmonary delivery by modifying the surface with O-stearylamylopectin to increase the affinity for the lung tissue of mice. The encapsulated drugs isoniazid and rifampicin showed reduced toxicity for peritoneal macrophages in infected mice compared to free drugs [81, 82]. Another targeting strategy exploits the mannose receptors that are expressed on alveolar macrophages through mannosylation of liposomes. Wijagkanalan et al. [83] reported efficient targeting of mannosylated liposomes to alveolar macrophages after intratracheal instillation to rats, as did Chono et al. [84] when they administered ciprofloxacin-loaded mannosylated liposomes for pulmonary intracellular parasitic infections. In another study, ciprofloxacin nanoparticles were encapsulated in large porous particles and showed controlled release over 2-4 weeks [85].

Other groups have developed microspheres with different polymers to act as carrier systems for anti-infective drugs. Takenaga et al. [86] demonstrated that lipid microspheres loaded with rifampicin could be delivered to alveolar macrophages *in vitro* as well as *in vivo* with reduced side effects in the liver. Hirota et al. [87] examined the phagocytic

activities of alveolar macrophages to rifampicin-containing PLGA microspheres of different sizes. They found that 3 μm particles were the most efficient for drug delivery to alveolar macrophages. Inhalable PLGA microspheres have been investigated for the treatment of tuberculosis by several other research groups [88–91]. Capreomycin is used in the treatment of multidrug-resistant tuberculosis, but it shows severe side effects when it is administered intravenously. Garcia-Contreras et al. [92] developed large porous capreomycin sulphate particles and administered them to the respiratory tract of guinea pigs, reporting a decrease in both inflammation and bacterial burden in the lung tissue.

New approaches for delivering anti-infectives to the respiratory tract are not limited to tuberculosis therapy though. Tobramycin is an anti-infective that is used to treat Pseudomonas aeruginosa, which often exists in cystic fibrosis patients. Pilcer et al. [93] formulated lipid-coated tobramycin particles and showed that they were in a respirable range, and that the lipid coating reduced agglomeration, further improving drug deposition. In another approach, moxifloxacin was loaded onto chitosan microspheres that were crosslinked with glutaraldehyde and tested in an *in vitro* model with Calu-3 cells. In these experiments, the microspheres retarded the absorption of moxifloxacin compared to free moxifloxacin [94].

Pulmonary gene therapy

Cystic fibrosis (CF) disease is caused by mutations in the gene that is encoding the cystic fibrosis transmembrane conductance regulator. CF is characterised by abnormal mucus production, inflammation in the respiratory tract and chronic bacterial infection [95]. Treatment of CF by gene therapy is an interesting field because by replacing the defective gene with a gene transfer vector, mucus production can be normalised and infection suppressed. Therefore cystic fibrosis transmembrane conductance regulator gene transfer was

one of the first targets of gene therapy. There are other genetic disorders like α-1 antitrypsine deficiency [96] or haemophilia [97] where gene therapy could beneficially affect the aetiopathology, but one of the biggest gene therapy fields is concerned with the treatment of different types of cancer [98].

Nebulisation of naked plasmid DNA leads to low transfection rates and poor stability of the DNA [99]. Therefore DNA must be delivered to the mucosal surface of the lung by carrier systems that protect the DNA from enzymatic degradation, improve long-term expression of the antigen, and enhance transfection efficiency. There are two different types of DNA carriers, viral and non-viral vectors, and both have their own advantages and disadvantages.

The viral vectors that are used for DNA delivery have a high efficiency in gene transfer even though they have been modified to eliminate their pathogenicity. Since DNA delivery by viral vectors is not based on particle technologies, they will not be discussed in this chapter, except to note that they are immunogenic [100], which is a major disadvantage compared to non-viral vectors, because multiple dosing therapies are not possible. Non-viral vectors can be administered in multiple doses, but gene transfer is less efficient than viral vectors. Most of the carriers are positively charged so that they can interact with the DNA, which is negatively charged, by complexation or adsorption. Non-viral vectors need to be biocompatible, non-toxic and able to carry DNA across various cellular barriers to the nucleus. For these reasons, liposomes and polymers are perfectly suited carriers for DNA delivery, and they are also easy to generate.

Chitosan is a very popular polymer for gene delivery that has been utilised by many research groups [101–105] because of its mucoadhesive properties [106]. Li et al. [105] developed lipid/polycation condensed plasmid DNA chitosan particles and showed that the *in vitro* deposition of chitosan-modified powders was higher than unmodified powders,

and that the level of reporter gene expression was enhanced. Another polymer that is used for pulmonary gene delivery is polyethylenimine (PEI) [107–109]. Kleemann et al. [110] developed TAT-PEG-PEI conjugates to deliver plasmid DNA and reported enhanced DNA protection and higher transfection efficiencies *in vivo* compared to unmodified PEI. Cationic lipids, such as lipofectin, have also been used as carriers for gene delivery. Bhattarai et al. [111] administered lipofectin polymer (poly(p-dioxanone-co-L-lactide)-block-poly(ethylene glycol) micelles with the tumour suppressor gene PTEN to C57BL/6 mice with a melanoma and observed significantly improved gene expression of PTEN in the lungs without any toxicity and longer survival times. It also is worth noting many of these delivery particles have also been employed to deliver antisense DNA/RNA or siRNA to the lungs to treat several diseases by gene therapy. Similar to plasmid DNA, these smaller nucleotide sequences also need to be formulated with a carrier system that can protect them and enhance transfection rates.

Lung cancer therapy

The therapy of cancer, especially lung cancer, is still very toxic for the patient because most cytostatic drugs are not sufficiently specific in their action. Drugs against lung cancer are administered systemically and they act systemically, causing serious side effects in healthy organs such as the liver, heart or kidneys. Thus pulmonary administration offers the opportunity to achieve higher local effects and even sustained release in the lung while reducing systemic exposure to cancer drugs. Several approaches have been adopted to target different cancer drugs to the lungs.

Hitzman et al. [112] administered aerosolised lipid-coated nanoparticles loaded with 5-fluorouracil to hamsters with squamous cell carcinoma of the lung. They chose lipid-coated nanoparticles because earlier studies had shown that these particles have sustained

release properties [113]. 5-fluorouracil levels were much lower in the serum than in the lungs in these experiments, indicating effective local exposure and sustained release can be achieved with this approach. Paclitaxel-loaded albumin nanoparticles were approved for injection by the FDA in 2005 to treat breast cancer [114], and while there are no published studies on their effectiveness in the lungs, the potential of this technology for inhalation therapy deserves to be investigated. Liposomes have been used in rats by Zhang et al. [115] to achieve sustained release of 9-nitrocamptothecin in the lungs while reducing accumulation in other tissues. In another approach, the toxic effects of cisplatin were reduced by sustained release lipid inhalation targeting (SLIT) [116]. SLIT-cisplatin is a dispersion of cisplatin encapsulated in lipid vesicles that releases 50% of the dose immediately while the other 50% remains in liposomes for sustained release [117]. This phase I study showed that the administration of SLIT-cisplatin is feasible and safe unfortunately the deposition efficiency (10-15%) was very low.

Some cell-specific targeting systems have the potential to further improve cancer therapy in the respiratory tract. For example, lectin-functionalised liposomes bind specifically to the tumor-derived cell line A549 [77, 118] and therefore may act as an effective targeting system. Abu-Dahab et al. in our laboratories investigated the effect of nebulisation on the stability of lectin-functionalised liposomes and their binding to A549 cells with promising results. A more specific target may be the transferrin receptor, which is over-expressed in many human tumour cells. Anabousi et al. [119, 120] examined the uptake levels and cytotoxicity of transferrin-conjugated liposomes and showed enhanced uptake with increased cytotoxicity. Additive PEGylation of these liposomes increased their stability for aerosolisation. Finally, an interesting emerging target for cancer therapy is telomerase because it is present in most human cancers [121](see Shay et al. [122] for a recent review). Inhibiting telomerase may represent a novel therapy for lung cancer, except specific

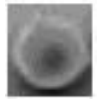
telomerase inhibitors like the antisense oligonucleotide 2'-O-methyl-RNA (2-OMR) need a special carrier system to exert a biological effect in targeted cells. Beisner et al. [123] administered this telomerase inhibitor in different liposomal formulations containing DOTAP (N-[1-(2,3-Dioleoyloxy)]-N,N,N-trimethylammonium propane methyl-sulphate), which is a cationic lipid, or a mixture of DOTAP and cholesterol to A549 cells. These reagents enhanced transfection of A549 cells and efficiently inhibited the telomerase. Nafee et al. [124] used chitosan-coated PLGA nanoparticles, which were developed by Kumar et al. for plasmid DNA delivery [125], as a carrier for the antisense oligonucleotide 2-OMR. Because of a cationic surface modification by chitosan, PLGA is able to form nanoplexes with nucleotide-based drugs, protecting these molecules from premature degradation and facilitating their cellular uptake. Taetz et al. [126] used cationic chitosan/PLGA nanoparticles to deliver 2-OMR to A549 cells, and observed enhanced uptake of 2-OMR nanoplexes into A549 cells, efficient telomerase inhibition, and significant shortening of telomeres compared to 2-OMR alone. Obviously, these kind of nano-technology based carrier systems represent an interesting new platform for the safe and efficient delivery of telomerase inhibitors in the context of lung cancer.

Table 1: Overview of different particle types for pulmonary drug delivery

	Particle type	Requirements on drugs	Advantages	Disadvantages	References
 ∅ 50μm (carrier) ∅ 1 - 3μm (drug)	Microparticles: large lactose carrier particles with adherent pure drug crystals	Solid, crystalline drugs (conventional asthma drugs)	➢ good stability ➢ free of critical excipients	➢ no controlled release	[127, 128]
 ∅ 20nm - 100μm	Liposomes	Sufficient solubility in either aqueous or lipidic phase (fentanyl, cyclosporine A, iloprost, beclomethasone)	➢ similar to lung surfactant ➢ biocompatible ➢ biodegradable ➢ incorporation of hydrophilic or lipophilic drugs ➢ controlled release	➢ stability problems ➢ short shelf-life ➢ high production cost ➢ low encapsulation efficiency ➢ large scale production difficult ➢ effective aerosolisation not trivial	[30, 50, 54, 58] [59, 79, 83, 84] [115, 118–120]
 ∅ 10-1000nm	Nanoparticles: (PLGA, chitosan) Solid NP (matrix controlled)	Stable against production procedure (antituberculosis drugs)	➢ high stability ➢ high carrier capacity	➢ materials not approved for pulmonary delivery	[26, 75, 76, 124] [126, 129]

(continued)

continued

	Particle type	Requirements on drugs	Advantages	Disadvantages	References
 ∅ 10-1000nm	Core-shell NP (membrane controlled)	needs protection from enzymatic breakdown (insulin, tacrolimus)	➢ Incorporation of hydrophilic or lipophilic drugs ➢ protection of sensible drugs ➢ controlled release ➢ large surface ➢ biodegradable ➢ high number of particles leading to a high number of sites of deposition (macrophage targeting in therapie of tuberculosis)	➢ complex behaviour in physiological environment (risk of aggregation) ➢ Effective aerosolisation not trivial	
 ∅ > 5μm	Large porous particles	advantageous for drugs with rapid clearance and/or poor bioavailability (insulin, heparin)	➢ less aggregation ➢ reduced phagocytosis by macrophages	➢ sophisticated production	[27, 28]
 ∅ 1 - 3 μm	amorphous solid particles	thermostabile, sublimable drugs (rizatriptan, fentanyl, zolpidem)	➢ rapid onset ➢ no additives ➢ particle generation in situ	➢ only a few drugs are suitable for this procedure	[38, 39]

3.3 *In vitro* models for testing pulmonary particle deposition

There are many aerosol formulations on the market for the treatment of "classical" lung diseases, like asthma and COPD, and up until now, significant improvements have been achieved mainly by improving the aerosol properties of the formulations. The technology already exists to produce a variety of particles, such as nanoparticles, liposomes and large porous particles that can be efficiently deposited by aerosol inhalation in the lungs - even under patho-physiological conditions. More work is needed though to control what happens to these particles after they are deposited in the respiratory tract. Most of these new particle approaches (Table 1) that are in the pipeline as described in the previous chapter are tested *in vivo* by intratracheal instillation or inhalation as an aerosolised formulation as well as by testing a solution of the new formulation onto several lung cell culture models *in vitro*. To obtain more realistic data, *in vitro* models are needed that not only mimic the absorption of the drug, but also a realistic deposition onto cell cultures. There is a big difference between the deposition of a particle in the lung and the testing of a drug by pipetting a drug solution onto a cell monolayer. This chapter gives an overview both about established pulmonary cell culture models and the current *in vitro* models of particle deposition in environmental toxicology and pharmacology.

3.3.1 Cell culture models of the respiratory tract

There are many cell culture models available to determine absorption or cytotoxic effects of particles *in vitro*. Some are cell lines, others are primary cells. An overview of the current cell culture models of the respiratory tract is given by Steimer et al. [130]. This paragraph will only focus on the Calu-3 cell line, because it was the cell line that was used in this thesis.

Figure 5: Liquid covered cultivation (left side) differs from the air-liquid interface cultivation (right side). LCC cells are covered with medium from both sides, whereas ALI cells are exposed to air at their apical side

Calu-3 cell line

Calu-3 is an adenocarcinoma cell line from a 25-year old caucasian male. Calu-3 cells can be cultivated in liquid covered culture (LCC) as well as air-liquid interface culture (ALI). LCC cells are covered in medium from both sides when cultivated in a Transwell system whereas the ALI cultivation only has medium supply from the basolateral compartment (Figure 5). The air-liquid interface culture represents a more *in vivo* like situation, because the lung surface is only covered by a thin lining fluid.

When cultivated at ALI conditions Calu-3 cells built mucus on their surface which can be detected by Alcian blue staining. In LCC cells no such mucus is found, maybe because it is diluted in the medium and washed away [131]. Calu-3 cells are an appropriate cell line for transport studies due to its presence of tight junctions building a tight monolayer which can be determined by transepithelial electrical resistance (TEER) measurement or by immunostaining for zona occludens-1 (ZO-1) or claudin. However, significant differences in their values are detected when using different cultivation methods. LCC Calu-3 cells show higher TEER values ($> 800\ \Omega{*}cm^2$) compared to ALI grown Calu-3 cells ($> 350\ \Omega{*}cm^2$) due to different tight-junctional protein distribution (Figure 6). Grainger et al. [132] reported a higher amount of ZO-1 in immunostained LCC cells by confocal microscopy. Although the Calu-3 cell line is of bronchial origin, it is often used for transport studies to

simulate the deep lung, because other alveolar cell lines like e.g. A549 do not form tight monolayers. Its usefulness for transport studies has been demonstrated in several studies [133, 134]. Hamilton et al. showed that Calu-3 cells express an energy-dependent Pgp efflux pump and possess MRP1 functional activity [135, 136]. Permeability characteristics of passive and actively transported drugs could also be correlated to *in vivo* data from rats [137].

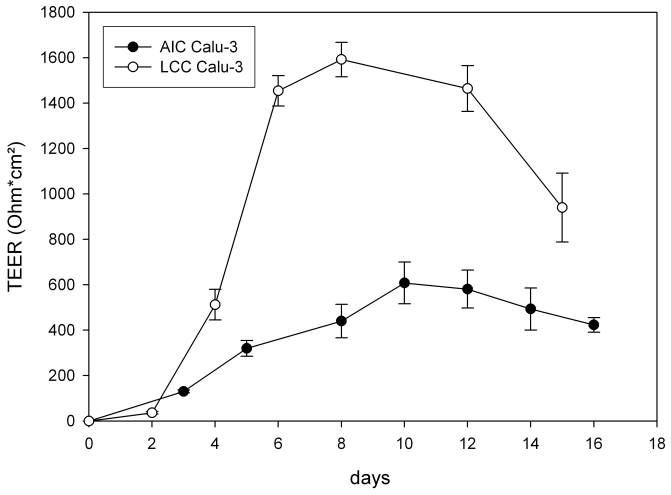


Figure 6: Calu-3 cells show different TEER values dependent on their cultivation due to different tight-junctional protein distribution

3.3.2 *In vitro* models in toxicology

In environmental toxicology the effects of aerosols of natural or artificial origin are investigated to determine their harmful potential for humans or animals. There are several attempts to test these aerosols and determine mostly the long-time effects with the help of *in vivo* as well as *in vitro* models. Due to the emerging nanotechnology industry new particle types have to be tested for their toxic potential to be able to prevent diseases, especially of the lung, caused by (accidently) inhaled particles. First *in vitro* experiments

were performed by pipetting a solution or dispersion of the compound on top of a cell layer and determine the effects [138]. However, this application did not represent the situation *in vivo*. Therefore setups were developed where particles were deposited onto air-liquid interface grown cells. The CULTEX model [139, 140] is designed to provide direct access of complex gas mixtures to lung cell culture models which are grown at air-liquid interface. The apparatus houses three vessels with cell culture inserts, provides access to culture medium at the basolateral side and allows sample-taking during the experiments. The gas mixture is drawn into the system by low-pressure and mainly deposited by impaction or sedimentation (Figure 7). In first experiments the influence of airborne pollutants (e.g. diesel exhaust) to human bronchial epithelial cells was investigated. The CULTEX system was modified [141, 142] to also test cigarette smoke, or other pollutants [143] like fly ash as did Diabate et al. [144]. They collected fly ash in a commercial municipal incinerator, resuspended it in filtered air and deposited the aerosol onto BEAS-2B cells, a human bronchial cell line, which was co-cultured with THP-1 macrophages. Release of IL-8, a proinflammatory mediator, was induced as a function of exposure time, showing that this model is suitable to detect interactions of the aerosol with the lung cells. Further developments of the CULTEX system which is now marketed by Vitrocell (Waldkirch, Germany) were used to study various effects of different gas mixtures mostly to A549 cells [145–150]. Another approach was described by Tippe et al. [151]. They reconstructed a commercially available perfusion cell (Minucell, Bad Abbach, Germany) to allow aerosol exposure by stagnation point flow. Ultrafine carbonaceous particles were uniformly deposited onto air-liquid interface cultured A549 cells with an efficacy of 2% [152] and the low flow velocity did not affect the cell monolayer showed by testing with clean air.

A different setup of collecting particles onto cell monolayer is electrostatic precipitation. By charging particles and applying an electrical field higher deposition efficacies of the

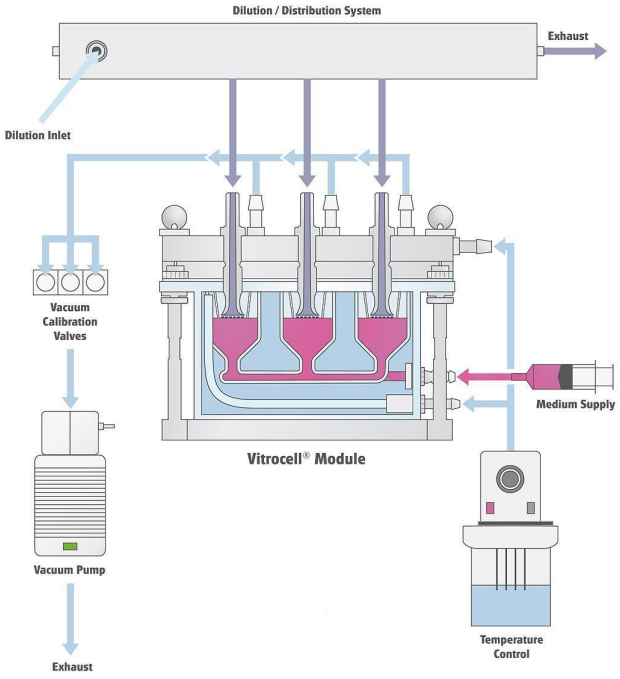


Figure 7: An overview of the CULTEX-Vitrocell system: the gas mixture is drawn to the three vessels which contain air-liquid interface cultured cell monolayer, viability of cells is ensured by medium supply and temperature control (modified according to [153], by courtesy of VITROCELL®)

particles are obtained. Mainelis et al. [154, 155] first deposited airborne microorganisms onto agar plates in an electrostatic precipitator. In this device two ionisers charge the particles if they do not carry a sufficient charge for collection. Savi et al. [156] developed an exposure chamber to deposit polystyrene particles onto cells directly out of a conditioned air flow. Before entering the chamber, particles pass a bipolar Kr-85 charger. Afterwards about 40-70% of those particles are charged and they are deposited by an alternating, square-wave electrical field of 4 kV/cm which is between the particle delivery tube and an electrode placed underneath the filter inserts with the cells. Deposition efficacy of this method is about 15-30% of all particles. Gaschen et al. [157] used the same model to

deposit secondary organic aerosol particles like 1,3,5-trimethylbenzene or α-pinene onto A549 or human alveolar macrophages to determine toxic effects. EAVES, the Electrostatic Aerosol *in vitro* Exposure System, is another setup based on electrostatic precipitation [158]. Volckens et al. [159] modified the EAVES system to increase cell capacity and aerosol flow rate. They compared direct particulate matter deposition to NHBE cells with liquid-interface deposition and detected differences between these two different ways of application. Cellular responses from air-liquid interface deposition occured at concentrations that were one order of magnitude lower than from liquid-interface exposures. This was probably due to the different composition of the particulate matter due to physiological changes caused by extraction, lyophilisation and resuspension of the liquid-interfacce deposited particles. The direct particle deposition represents a more *in vivo* like situation and allows a more accurate assessment of particle toxicity. Stevens et al. [160] used the electrostatic particulate dosage and exposure system (EPDExS) to deposit 1,4-naphthoquinone particles onto murine alveolar type II epithelial cells. The EPDExS consists of six chambers which have cylindrical cage electrodes on the bottom. Culture dishes are placed on top of the electrode separated by an insulating pedestal. The aerosol is generated by a nebuliser and receives a bipolar charge distribution by passage through a Kr-85 charger. Deposition efficacy was 100% for the particle range of 40-530 nm.

3.3.3 *In vitro* models in pharmacology

As mentioned before, in pharmaceutical context the focus is on high dose deposition in short period of time, but in contrast to environmental toxicology only very few models are available. The first group who used an impactor as a deposition system in the context of pharmaceutical technology was Schreier et al. [161]. They used an Andersen Mark II cascade impactor as a "simulated lung setup" and put 2-CFSMEo-cells grown on micro-

scope coverslips onto the steel plates of the impactor. A Pari LL Jet nebuliser was used to aerosolise several liposome DNA complexes onto these 2-CFSMEo-cells on several stages of the impactor in order to investigate the cellular uptake of these plasmid gene products. Although the air flow was set to 28.3 l/min, which is the flow of air during tidal breathing in humans, the cell viability, tested with aerosolised trypan blue, was not affected. Fiegel et al. [131] used an Astra-type liquid cascade impinger to aerosolise and deposit large porous particles onto Calu-3 cells. The Calu-3 cells were grown in Transwell® systems and then placed under the second stage nozzle of the impinger. Afterwards polymeric large porous particles labelled with fluorescein were aerosolised, deposited and the effects on the cell monolayer were determined. A similar model was developed by Cooney et al. [162]. A viable cascade impactor, which was originally developed for the identification of airborne microbes [163], was used to deposit fluorescein isothiocyanate (FITC)-dextran solutions onto small airway epithelia cell (SAEC) monolayers to test for permeability coefficients. Furthermore, Calu-3 cells were used to determine the monolayer integrity after an airflow of 60 l/min in the impactor. In the models of Cooney and Fiegel, the Transwells® were just placed under the nozzles of the impactor stages. Therefore Bur et al. [164] cultivated the cells on the underside of the Transwells® to prevent turbulences in the impactor caused by the bowel-like form of the Transwells®. A multi stage liquid impinger (Figure 8) was used with the inverted cell culture to deposit budesonide and salbutamol sulphate powders.

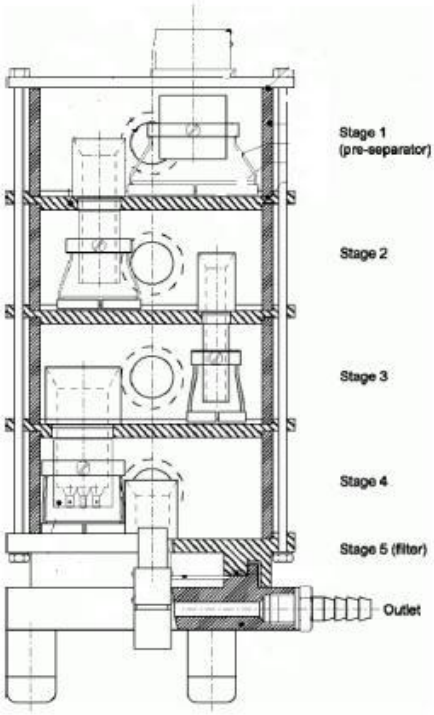


Figure 8: Schematic view of a multi-stage liquid impinger which was used by Bur et al. [164]

These dry powder formulations, approved for the treatment of asthma, consist of large carrier particles and small drug particles which are separated during the aerosolisation process and deposited onto several stages in the impinger by impaction. Therefore only the drug particles deposit onto the cell monolayers and only the influence of the drug particles is investigated. Absorption profiles of budesonide and salbutamol sulphate were significantly different compared to standardised transport experiments (liquid culture) due to the high concentration gradient on the apical side because of the air-liquid interface cultivation in the apical compartment. A twin-stage impinger (Figure 9) was used by Grainger et al. [165] who placed a Transwell® insert at the bottom of this impinger

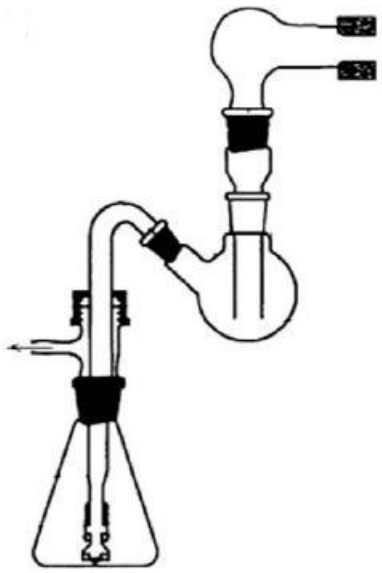


Figure 9: Schematic view of a twin-stage impinger which was used by Grainger et al. [165]

and deposited aerosolised FITC-dextran particles of different molecular weights onto air-liquid interface cultured Calu-3 cells and compared them to transport of dextran solutions through the monolayer. Transport rate of particles was significantly higher due to the high driving concentration generated by the dissolution in the low volume of fluid in the apical compartment.

3.4 Aim of this thesis

All those described pharmaceutical models rely on impaction as the main deposition mechanism. However, the main deposition mechanism in the deep lung is sedimentation. To obtain a more *in vivo* like situation, our new *in vitro* model, designed for dry powder aerosols, should be able to aerosolise a dry powder aerosol, and separate both carrier and drug particles during this aerosolisation process. Furthermore it should only deposit the respirable fraction of particles onto air-liquid interface grown lung cells and provide a gentle deposition (without high airflow) by sedimentation to protect these sensitive cell monolayers. Reproducible and simultaneous deposition onto multiple cell monolayers should be required.

The aims of this thesis were:

1. **to develop an *in vitro* model to investigate the influence of the "naked" particle onto air-liquid interface cultured lung cells**
2. **to optimise and validate this new *in vitro* model**
3. **to deposit pharmaceutical relevant dry powder aerosols and subsequently perform transport studies to determine differences to conventional transport studies**

4 Development of the PADDOCC system

Parts of this chapter have been published in:

S. Hein, M.Bur, T. Kolb, B. Müllinger, U.F. Schäfer, C.-M. Lehr

Pharmaceutical Aerosol Deposition Device On Cell Cultures (PADDOCC) system *in vitro*: Design and experimental protocol, Alternatives to Laboratory Animals, 38(4):285-295, 2010

4.1 Summary

The development of aerosol medicines typically involves numerous tests on animals, due to the lack of adequate *in vitro* models. A new *in vitro* method for testing pharmaceutical aerosol formulations on cell cultures was developed consisting of an aerosolisation unit fitting a commercial dry powder inhaler (HandiHaler®, Boehringer Ingelheim, Germany), an air flow control unit (Akita®, Activaero, Germany) and a custom made sedimentation chamber. This chamber holds three Snapwell® inserts with monolayers of pulmonary epithelial cells. The whole setup, referred to as Pharmaceutical Aerosol Deposition Device On Cell Cultures (PADDOCC system) is aimed to mimic the complete process of aerosol drug delivery, encompassing aerosol generation, aerosol deposition onto pulmonary epithelial cells and subsequent drug transport across this biological barrier to investigate new aerosol formulations in an early stage of development. We here describe the development of the design and the protocol for this device. By testing aerosol formulations of budesonide and salbutamol sulphate, respectively, reproducible deposition of aerosol particles on, and integrity of pulmonary cell monolayer could be demonstrated.

4.2 Introduction

The development of pharmaceutical aerosol medicines typically involves numerous tests on animals prior to a first clinical evaluation in man. These experiments are usually performed by intra-tracheal instillation or forced inhalation, and afterwards blood and tissue samples are analysed. However, these animal experiments often fail to provide useful information as neither the method of aerosol administration and deposition, nor the subsequent absorption and disposition in the animal model used are easily transferable to humans. Apart from ethical reasons and the obligation to follow the 3R principle the development of new aerosol medicines could be made much more time effective and cost efficient if adequate *in vitro* models were available. A number of *in vitro* models of the so called air-blood barrier based on pulmonary epithelial cells have already been described [130] including their culture and drug transport studies at an air-liquid interface (ALI) e.g. with Calu-3 cells to mimic the physiological situation as closely as possible [132]. Most *in vitro* deposition models have been developed in the context of toxicology [139, 143, 151, 152, 156] where the focus is typically on high-dose and/or long-term exposure scenarios. However, in order to evaluate the safety and efficacy of new aerosol medicines, it is important to study the effects of a single aerosol bolus ("puff") after deposition of relevant doses on the epithelial surface within a relatively short time period. The probably most relevant endpoints of such studies are i) the rate and extent of absorption (i.e. permeability) of the active pharmacological ingredient across the pulmonary epithelial barrier, and ii) possible changes of the latter, which might be either a symptom of some undesired toxic effects of the drug or its formulation and excipient, or be elicited on purpose to temporarily enhance drug absorption (e.g. by modulating the tightness of intercellular junctions or the activity of some transporter/efflux pumps).

There have been some attempts to adopt impinger systems which are described in most pharmacopoeia for analytical aerosol fractionation. By integrating epithelial monolayers grown on permeable filter systems (Transwells®) into the impinger, the behaviour of the aerosol to these cell culture models can be tested [131, 162, 164, 165]. Originally designed to characterise the particle size distribution of an aerosol, impinger systems are based on impaction forces to deposit particles on the various stages of the system. This implicates, however, that inserted cell cultures are exposed to high air streams (up to 60 l/min) which is not physiological with respect to the situation in the deep lung and may negatively influence epithelial integrity and cell viability. Other approaches rely on sedimentation as deposition mechanism [166]. This is certainly advantageous, as sedimentation and diffusion are main mechanisms of particle deposition in the deep lung [167], and occur in an atmosphere with low air streams. However, these deposition chambers, designed in the context of environmental or work place toxicology, collect the entire spectrum of particles in a given atmosphere and do not distinguish between respirable and non-respirable particles.

Nonetheless, the low mechanical forces of sedimentation process do have the advantage of not disturbing sensitive cell monolayers, so sedimentation appeared as good starting point for the design of a new system that suffices the particular research and development needs of aerosol medicines. Pharmaceutical dry powder inhalers (DPIs) generate aerosols that are often mixtures of large carrier particles and small drug crystals. These are separated during the inhalation process in such a way that only the respirable particle fraction (i.e. the drug) penetrates into the lung for subsequent deposition on the bronchial or alveolar epithelium, while the larger carrier particles (typically lactose) do not pass beyond the pharynx. As the sedimentation of particles is a predictable process [168, 169] particles can be separated by size differences based on differential sedimentation velocities. A system optimised in this way would therefore consist of an aerosol generation unit where drug

particles are separated from their carrier particles and a deposition unit where the drug particles can deposit onto cell cultures to test the behaviour of aerosolised particles onto pulmonary epithelial cells.

The development and initial evaluation of such a new Pharmaceutical Aerosol Deposition Device On Cell Cultures (PADDOCC) as well as some essential technical details are described in this chapter.

4.3 Materials and Methods

4.3.1 PADDOCC system

The system consists of three parts: the aerosolisation unit, the air flow control unit and the deposition unit, connected by flexible silicone tubes (inner ⌀ 7 mm, outer ⌀ 9 mm, Shore-Hardness A 70°, RCT, Heidelberg, Germany). The apparatus is schematically depicted in Figure 10. For simplification, only one of three ports is shown at the top of the deposition unit.

Aerosolisation unit

The aerosolisation unit (Figure 10 II) consists of an inhaler chamber, connected to a commercially available dry powder inhaler HandiHaler® (Boehringer, Ingelheim, Germany, "HandiHaler® chamber", Figure 10, b) sitting on top of an aerosolisation chamber (Figure 10, c), so that the mouthpiece of the HandiHaler® protrudes in this chamber. The aerosolisation chamber has a volume of 300 ml.

Air flow control unit

The Akita® device (Activaero, Gemünden/Wohra, Germany, Figure 10 I) is normally used to optimise aerosol inhalation during patients administration process and can be programmed by a SmartCard to generate different breathing patterns. In the PADDOCC system the Akita system controls both aerosol generation and subsequent transport to the sedimentation chamber by different aerosol manoeuvre with variable periods of dispersion impulses (60 l/min) and ventilation flows (6 l/min). At one end the Akita® device is connected via tubing (aerosolisation tube, Figure 10, d) to the cap of the cylindrical inhaler chamber. At the other end the Akita® is connected by means of a Y-shaped tube (Figure 10, e) to the base of the aerosolisation chamber. The Y-shaped tube has a syringe on one

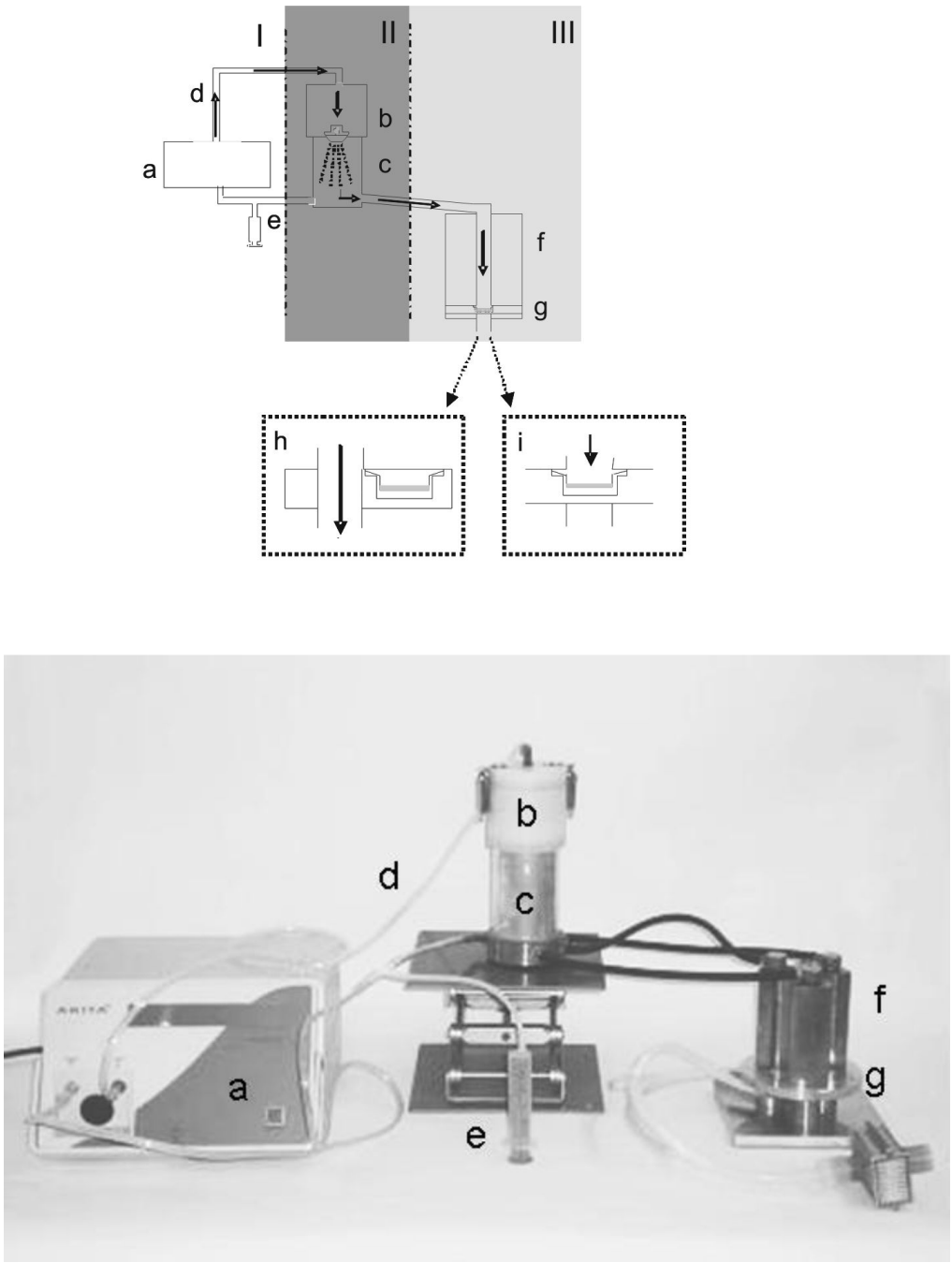


Figure 10: Schematic view of the PADDOCC system I: air flow control unit, II: aerosolisation unit, III: deposition unit, a: Akita® device, b: HandiHaler® chamber with capsule, c: aerosolisation chamber, d: aerosolisation tube, e: y-shaped tube with syringe, f: sedimentation chamber, g: sampling unit, h: sampling unit (with Snapwell®) in ventilation mode, i: sampling unit (with Snapwell®) in deposition mode

Figure 11: Aerosolisation unit of the PADDOCC system

end to trigger aerosolisation by the pulling and pushing the plunger, thus generating a temporary low-pressure impulse.

Deposition unit

The deposition unit (Figure 10 III) consists of a brass block with three sedimentation chambers (Figure 10, f), a sampling unit (Figure 10, g) and a pedestal where discharged air is deflected. The cylindrical sedimentation chambers have a diameter of 1.2 cm and a length of 10 cm. They are arranged on top of a revolving sampling unit. The sampling unit has three ventilation holes alternating with three sampling wells (Figure 13). Therefore two different positions/modes of operation are possible: In ventilation mode (Figure 10, h) (during the dispersion impulse and ventilation flow) the sedimentation chambers are placed directly on top of the ventilation holes of the sampling unit so that the air streams generated by the Akita device can escape through the pedestal, not reaching the sampling

wells. In deposition mode (Figure 10, i) just after the end of the ventilation flow the sampling unit is rotated so that the sampling wells are placed directly on the bottom of the sedimentation chambers to allow the particles to sediment simultaneously onto three sampling wells.

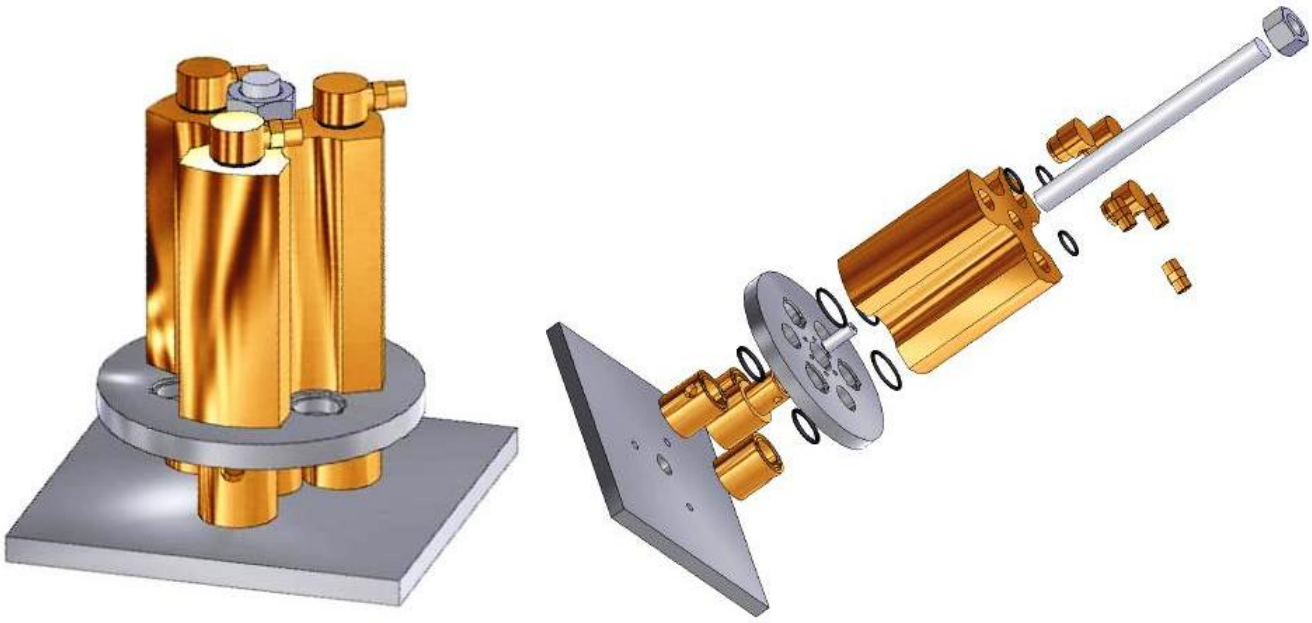


Figure 12: Deposition unit of the PADDOCC system

The sampling wells are filled with 500 μl of buffer in the basolateral compartment covered by a Snapwell® insert (⌀ 1.2 cm), on which a pulmonary epithelial cell monolayer has been previously grown. Holes in the sampling unit permit sample aliquots to be taken from the basolateral compartment with a pipette (Figure 13). The pedestal with integrated ventilation holes underneath the sampling unit constitutes a continuous connection in the ventilation mode between the sedimentation chamber and the sampling unit to tubes, ending with a filter to protect the environment from non-deposited aerosol particles.

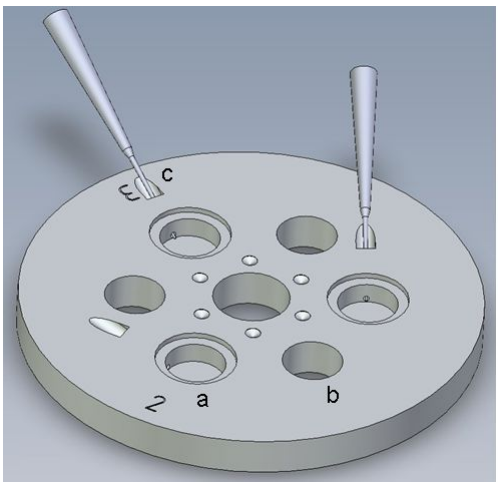


Figure 13: Sampling unit with alternately sampling wells (a) and ventilation holes (b) as well as pipetting ports for sample taking (c)

4.3.2 Experimental protocol

The experimental protocol is summarised in Table 2.

Before starting the experiment a dry powder capsule is placed in the HandiHaler® and the capsule is pierced by pressing and releasing the green piercing button on the side of the HandiHaler®. Two holes are made in the capsule by two needles, thus allowing the powder to be released when the dispersion impulse is started. The deposition chamber is positioned in "ventilation mode". The Akita® device is turned on. By pulling and pushing the plunger of the syringe a temporary low-pressure is generated in the system. This activates the Akita® device to produce a dispersion impulse. The pressure wave of the dispersion impulse is propagated from the Akita® via the tubing to the HandiHaler®. The capsule inside begins to vibrate and releases the dispersed powder into the aerosolisation chamber. The aerosol cloud thus generated, is carried forward by the ventilation flow of the Akita® to the deposition unit. The progress of the different steps can be monitored by the display of the Akita® apparatus. After the ventilation flow stage, the deposition chamber is positioned in "deposition mode" and the Akita® is turned off. During the experiment

Table 2: Experimental protocol for the aerosolisation and deposition process of the PADDOCC system, The period of the aerosolisation manoeuvre depends on the programmed SmartCard used, see Table 3.

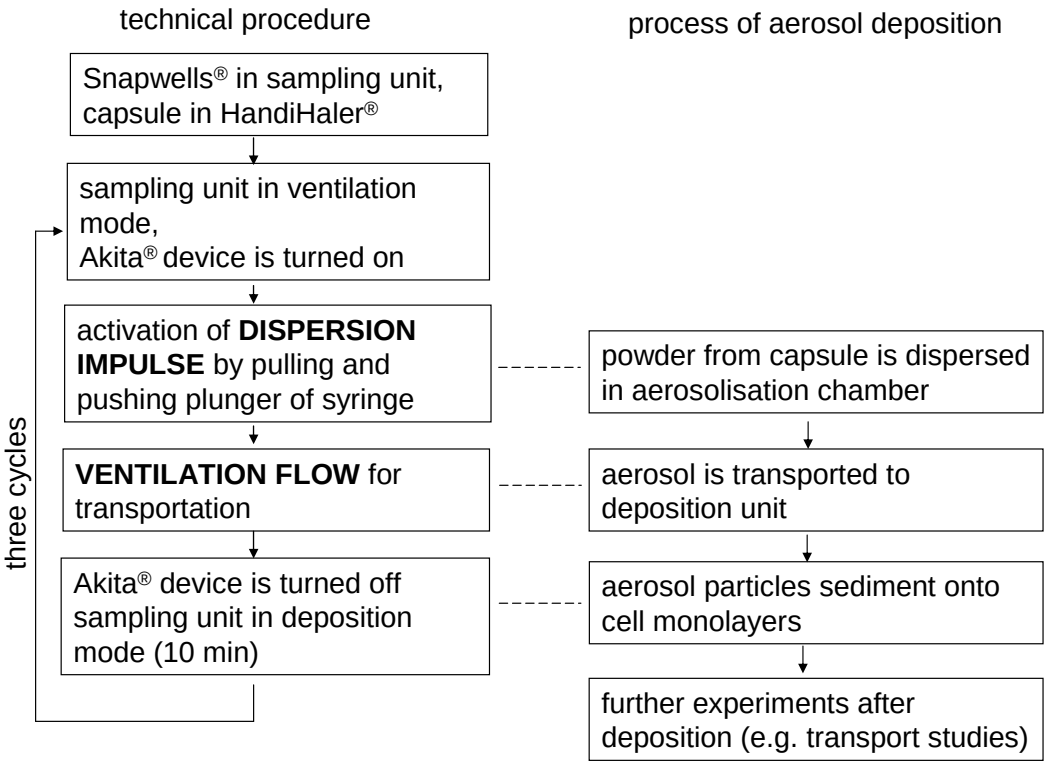


Table 3: Technical data of the different SmartCards tested in the PADDOCC system

SmartCard No.	dispersion impulse in s (60 l/min)	ventilation flow in s (6 l/min)	generated volume during dispersion impulse (ml)	generated volume during ventilation flow (ml)
SC1	0.2	2.0	200	200
SC2	0.3	1.5	300	150
SC3	0.3	2.0	300	200
SC4	0.3	3.0	300	300
SC5	0.4	1.0	400	100

the PADDOCC system (without Akita®) is placed in an incubator at 37°C to maintain physiological conditions while transport of the deposited drug across the cell monolayer is taking place and is studied by taking samples from the basolateral compartment at given time points The dispersion impulses that were tested were in the range of 0.2 s to 0.4 s resulting in a volume of 200 ml to 400 ml. The ventilation flows which were tested were in the range of 1.0 s to 3.0 s resulting in a volume of 100 ml to 300 ml (Table 3). The sedimentation of respirable particles (2-5 μm) takes about 10 min in this sedimentation chamber (calculated according to Dua et al. [125]). After deposition the amount of particles can be analysed by rinsing the well with 1 ml of mobile phase and quantifying the amount by HPLC analysis. To empty the capsule in the HandiHaler® completely three cycles of aerosolisation are required meaning a window of 30 min for the whole aerosolisation and sedimentation process. In initial experiments SmartCard 4 (see Table 3) was used and capsules of budesonide (Cyclocaps® Budesonid 400 μg, PB Pharma, Meerbusch, Germany) or salbutamol sulphate (Cyclocaps® Salbutamol 400 μg PB Pharma, Meerbusch, Germany) were aerosolised.

4.3.3 Optimisation of aerosol deposition

Within the aforementioned protocol, further attempts were made to increase the deposition amount in the sampling wells. Multiple SmartCards with different dispersion impulses and ventilation flows (Table 3) were tested in the Akita® device in combination with different lengths of the connecting tubes to try to increase and optimise the amount deposited in the sampling wells. Long tubes were 265 mm in length and were provided by the manufacturer of the sedimentation chamber. The short tubes were 55 mm in length to obtain the shortest connection between the aerosolisation and deposition unit. Five different SmartCards were tested by aerosolising a capsule of budesonide (three cycles) and determining the deposited amount on the sampling wells.

4.3.4 Deposition reproducibility

After optimisation of the deposition amount, experiments to show reproducibility of deposition were performed by aerosolising a dry powder capsule within the PADDOCC system (three cycles) and after sedimentation each well of the sampling unit (without cells) was washed with 1 ml of HPLC mobile phase and quantified by HPLC analytics. For these experiments SC1 was used in combination with long connecting tubes which had previously identified as being the optimised combination.

4.3.5 Separation of lactose carrier and drug particles

To confirm that the powder mixture containing lactose carrier and drug particles in the capsule could be separated through the aerosolisation and deposition processes, samples of the original mixture from the capsule (Cyclocaps® Budesonid 400 μg) were analysed by HPLC analysis. After aerosolisation and sedimentation in the PADDOCC system,

drug content was determined in samples collected from the pedestals of the sedimentation chamber. The system deposits drug particles in the sampling wells of the deposition unit by sedimentation forces, whereas the lactose carrier particles are collected in the aerosolisation chamber by impaction during the dispersion impulse, or in the pedestal during the ventilation flow due to their sedimentation velocity. The concentration of drug in the mixture was calculated as μg/mg powder mixture.

4.3.6 Scanning electron microscopy

Samples of salbutamol sulphate and budesonide powders from capsules were aerosolised and deposited in the PADDOCC system onto carbon tabs (Plano Leit-Tabs G3347, Plano, Wetzlar, Germany). The same powders were placed onto the tabs with a spatula without aerosolisation. The specimens were then mounted on stubs and sputtered with gold to a layer thickness of 10 nm. Scanning electron micrographs were recorded on a PhilipsXL 30 SEM (FEI Co. Philips Electron Optics, Zürich, Switzerland) at 5 kV.

4.3.7 HPLC analysis

A Dionex RP-HPLC System was used comprising a 690 pump, ASI 100 automated sampler, UVD 340 U UV/VIS detector (Dionex, Idstein, Germany) and a LiChrospher® 100 RP-18 column (125 mm x 4.0 mm, Merck, Darmstadt, Germany). For analysis of salbutamol sulphate a mobile phase of triethylamine-phosphate buffer (0.03 M triethylamine, 0.03 M NaH_2PO_4 dihydrate in 1000ml water, pH adjusted to 6.0 with phosphoric acid 85%) and methanol (90:10, v/v) was used. The detector was set to 276 nm, flow rate 1.0 ml/min, column temperature 40° C and the injection volume was 80 μl. Under such conditions, the lower limit of quantification was 100 ng/ml. Linearity was proven between 100 ng/ml and 250 μg/ml. For analysis of budesonide, the mobile phase was phosphate

buffer pH 3.0 (Ph.Eur.) and acetonitrile (60:40, v/v) and detector was set to 240 nm. The flow rate was 1.7 ml/min, the column temperature at 30° C and the injection volume 80 μl. The lower limit of quantification was 50 ng/ml and linearity was proven between 50 ng/ml and 250 μg/ml. All reagents were obtained from Sigma Aldrich (Taufkirchen, Germany). Chromatograms were analysed by estimating the area under the peak in the curve by employing a computerised data integration program (Chromeleon 6.5, Dionex) and compared to external standards.

4.3.8 Cell culture

Pulmonary epithelial Calu-3 cells, clone HTB-55, were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and used at passages 38-42. Calu-3 cells were grown to 90% confluence in 75 cm^2 T-flasks with RPMI 1640 (PAA, Pasching, Austria) supplemented with 10% FBS Gold (PAA, Pasching, Austria) and 1 mM sodium pyruvate (Lonza, Verviers, Belgium). Culture medium was changed three times a week. The incubator temperature was set to 37°C in an atmosphere of 90% relative humidity and 5% CO_2. After trypsinisation, cells were seeded on Snapwells® (pore size 0.4 μm, 1.13 cm^2, Snapwell® type 3801, Corning Costar, Bodenheim, Germany) at a density of 100.000 cells/cm^2 with 1.5 ml medium in the basolateral compartment and 500 μl in the apical compartment. After two days, the cells were set to air-interface conditions with 1.0 ml medium in the basolateral compartment. After 10-14 days the cells formed a tight monolayer (TEER > 450 Ω*cm^2) and were then deemed ready for the deposition experiments. Buffer for monolayer integrity experiments was KRB. Composition was as follows: 1.41 mM $CaCl_2$, 3.00 mM KCl, 2.56 mM $MgCl_2$, 142.03 mM NaCl, 0.44 mM K_2HPO_4, 4.00 mM D-glucose and 10.0 mM HEPES. All these reagents were obtained from Sigma Aldrich (Taufkirchen, Germany). KRB was adjusted to pH 7.4 by means of NaOH.

All chemicals were of highest available grade.

4.3.9 Integrity of cell monolayer

Tight monolayers can be identified by their transepithelial electrical resistance (TEER). It is an indicator of the integrity of a cell monolayer. As a consequence of the air-liquid interface cultivation the apical compartment has to be filled with 500 μl pre-warmed buffer and the basolateral compartment with 1.5 ml before TEER measurement and equilibrate in an incubator for 30 min. TEER values of these Calu-3 cells on Snapwell® filters were then measured with an EVOM device (WPI, Berlin, Germany) equipped with chopstick electrodes. Afterwards the buffer in the apical compartment was removed and the Snapwells® were put into the sampling unit which is filled with 500 μl pre-warmed KRB buffer in the basolateral compartment. Deposition experiments (three cycles) were performed and the Snapwells® were put back into the well plate. The apical compartment was filled with 500 μl buffer and the basolateral compartment with 1.5 ml buffer and after equilibration for 30 min TEER was measured again in order to detect any damage to the cell monolayer which might have occured during the deposition experiments.

4.3.10 MTT cytotoxicity assay

The MTT assay allows the quantification of the metabolic activity of cells. It is based on the cleavage of the yellow tetrazolium salt MTT to purple formazan salts (Figure 14) by metabolic active cells which can be quantified by spectrophotometry [170]. Snapwell® plates with Calu-3 cells at air-liquid interface conditions were grown and after 10 days the MTT assay was performed. Medium was removed from the basolateral compartment, replaced by KRB buffer, and equilibrated for 30 min in an incubator. The buffer was then removed and the Snapwells® were placed into the PADDOCC system and the deposition

experiments (three cycles) were performed with Cyclocaps© Salbutamol 400 μg. The wells were put back into a 6-well plate and 400 μl of KRB buffer was filled into the apical compartment. Cells were incubated for two hours. Then 40 μl of MTT reagent (Sigma, Taufkirchen, Germany) was added to the apical compartment and the cells were incubated for four hours. The liquid in the apical compartment was removed and 200 μl DMSO (Sigma, Taufkirchen, Germany) was added. The samples of the single wells were pipetted into a 96-well plate and absorption was measured at a wavelength of 550 nm with an UV/VIS reader (infinite M200, Tecan, Crailsheim, Germany). Positive control was KRB buffer with 1% Triton X (Sigma, Taufkirchen, Germany) and negative control was just buffer in the apical compartment without the deposition experiments.

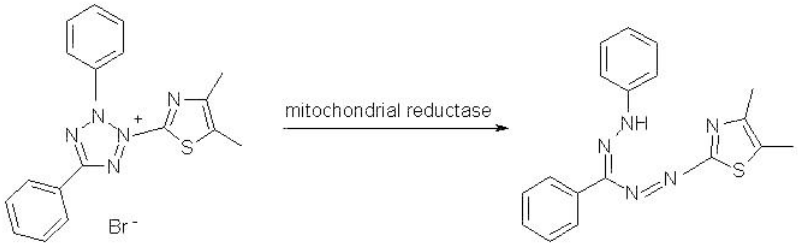


Figure 14: Metabolisation of MTT to a formazan salt by viable cells

4.3.11 Statistical analysis

Data are expressed as mean ± SD. Statistical analysis was performed by SigmaStat 3.0 software (Systat Software GmbH, Erkrath, Germany). Data of different SmartCards were compared by one-way ANOVA followed by the Holm-Sidak method for differences between two or more groups (n=9). Data of the TEER measurements before and after deposition experiments as well as comparison between powder mixtures before and after aerosolisation were compared by Student's t-test. Differences were deemed statistically significant if $p < 0.05$.

4.4 Results

4.4.1 Deposition experiments

After aerosolisation and deposition, drug particles were detected in the sampling wells of the PADDOCC system by HPLC analysis and by SEM photography. SEM images of salbutamol sulphate and budesonide powders showed differences before and after aerosolisation in the PADDOCC system. Before aerosolisation, salbutamol sulphate particles were attached to their lactose carrier particles (Figure 15a). After aerosolisation almost no lactose particles could be seen onto the sampling wells, whereas small salbutamol sulphate particles were now homogenously distributed onto the sampling well (Figure 15b). Same results were obtained with budesonide (Figure 15c + d).

4.4.2 Optimisation of deposited amount

Based on the fact that the initial experiments, performed with SC4 and long connecting tubes resulted in a low deposition (0.92 μg/well), further investigations with various combinations of SmartCards and long or short connecting tubes were performed. These experiments showed no significant change in deposition amount when using the other SmartCards in combination with the short tubes compared to SC4 with the short tubes (Figure 16). Deposition amount was always about 1 μg/well when using the short tubes and much of the powder was found in the pedestal in these cases. Experiments with different SmartCards and long tubes showed differences in the deposition amounts between the various SmartCards and a general significant increase in deposition amount compared to the short tubes (Figure 16). SC1 gave the highest deposition amount with 2.23 μg/well (n=9), which is a 2.5-fold increase compared to SC4.

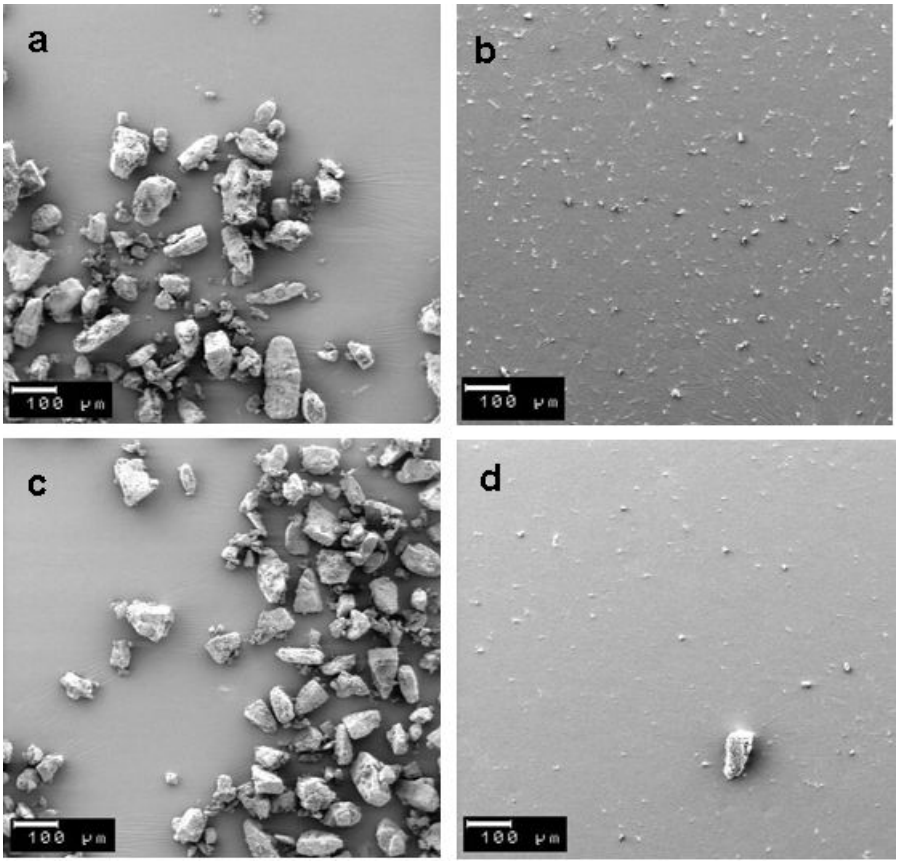


Figure 15: a) Salbutamol sulphate particles attached to lactose carrier particles before aerosolisation; b) homogenously-distributed salbutamol sulphate particles in the sampling well after deposition; c) budesonide particles attached to lactose carrier particles before aerosolisation; and d) homogenously-distributed budesonide particles in the sampling well after deposition.

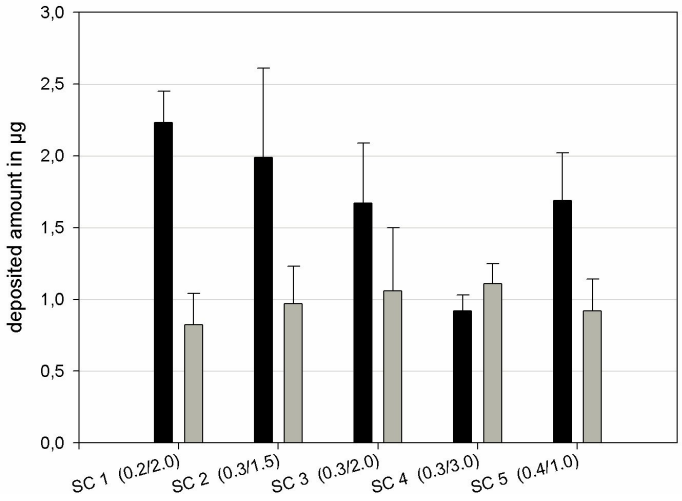


Figure 16: Deposited amounts of budesonide 400 μg when using different SmartCards (dispersion impulse/ventilation flow in s) and connecting tubes (n=9, mean ± SD). SC4 and long tubes show a significant difference to other SmartCards used with long tubes

4.4.3 Reproducibility experiments of deposition

The aerosolised deposition of budesonide 400 μg in a sampling well, by means of SC1 and long connecting tubes was about 1.82 ± 0.44 μg/cm^2 (n=27). Salbutamol sulphate capsules of 400 μg were similarly deposited at 2.23 ± 0.44 μg/cm^2 on each well (n=9). No significant differences in deposition in the three different sampling wells were evident, with either budesonide 400 μg or salbutamol sulphate 400 μg (Figure 17).

4.4.4 Separation of drug and carrier particles during aerosolisation

The drug content of budesonide 400 μg was 13.68 ± 3.7 μg/mg mixture in the capsule (n=10). After aerosolisation and deposition in the pedestal the budesonide amount decreased significantly to 8.83 ± 0.9 μg/mg mixture (n=10) indicating the separation of the drug crystals from the lactose carriers (Figure 18).

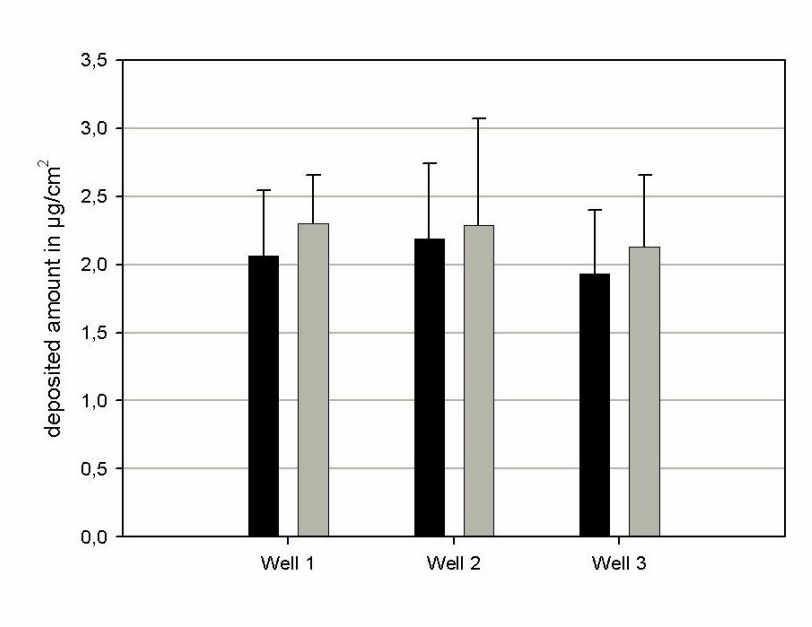


Figure 17: The deposited amounts of budesonide 400 μg and salbutamol sulphate 400 μg onto the three circularly-arranged sampling wells For budesonide 400 μg, n = 9 and for salbutamol sulphate 400 μg, n = 3. Data are all shown as the mean ± SD. Black bars = budesonide; grey bars = salbutamol sulphate.

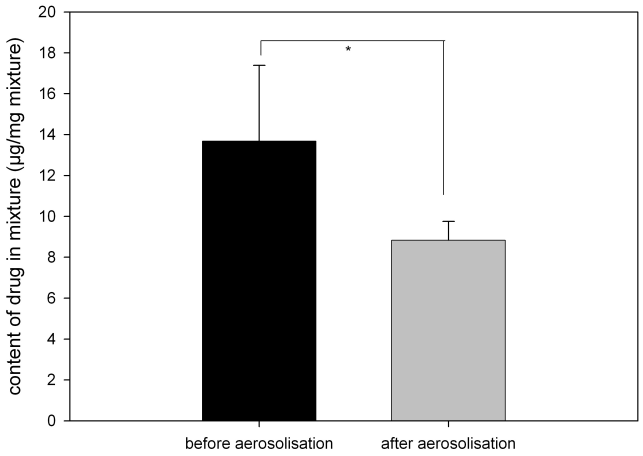


Figure 18: The content of drug in powder mixture, before and after aerosolisation. Aerosolisation sampling point was in the pedestal. A significant separation of drug and carrier particles is shown. Data are all shown as the mean ± SD; n = 10.

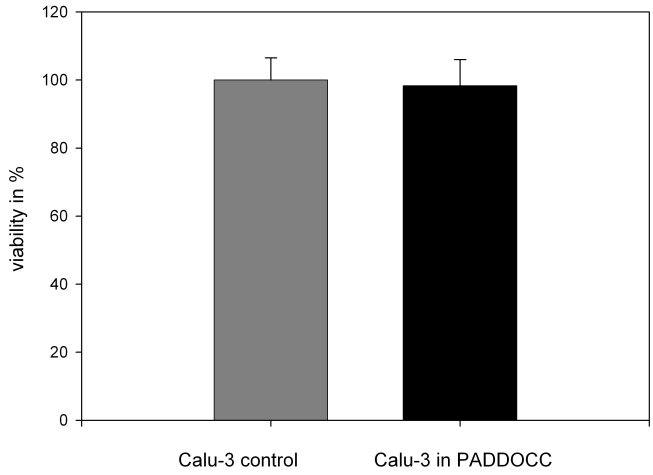


Figure 19: MTT assay showed no significant differences in viability of Calu-3 cells when used in PADDOCC system compared to untreated control

4.4.5 Integrity of cell monolayer

TEER values of the Calu-3 cells, grown air-liquid interface, plateaued at day 8-14 after forming a tight monolayer on a level of 500-600 Ω*cm^2. This is in accordance with reports from Fiegel et al. [131] or Foster et al. [171]. TEER consistency experiments showed a TEER value of 508 $\pm$ 51 Ω*cm^2 before the experiments. 30 min after deposition procedures in the PADDOCC system TEER values were 525 $\pm$ 82 Ω*cm^2 showing no significant differences in monolayer integrity before and after the deposition experiments.

4.4.6 MTT assay

As can be seen in Figure 19 there is no significant difference between the cells in the PADDOCC system and the untreated control. Viability of treated cells was about 98 $\pm$ 7.7% compared to control.

4.5 Discussion

The main objective of this work was to develop an *in vitro* test system in which dry powder aerosols can be generated and deposited onto cell monolayers without influencing cell monolayer integrity and thus the possibility to conduct further studies, e.g. permeability studies. In the *in vitro* system, the dry powder aerosol which consists of large carrier particles and small drug crystals should be properly separated through the aerosolisation process in order to mimic the situation which occurs by inhalation of dry powders by patients. After aerosolisation, deposition of the drug crystals should occur on air-liquid interface cell monolayers which have been previously grown on permeable filters placed in the sampling wells of the PADDOCC system. thus permitting the investigation of the interactions between the dry aerosol particles and the cell monolayers. In particular, the possibility should be raised of conducting transport experiments over the cell monolayer.

Our new *in vitro* model for testing aerosol formulations combines an aerosol generation step with deposition of particles onto sampling wells where permeable filters covered with cell monolayers are integrated. Experiments showed that the PADDOCC system deposits drug particles (2-5 μm) in the sampling wells of the deposition unit by sedimentation forces whereas the lactose carrier particles (> 50 μm) are collected in the aerosolisation chamber by impaction during the dispersion impulse or in the pedestal during the ventilation flow due to their sedimentation velocity. Hence, only very few lactose carrier particles can be found in the sampling unit. After deposition, drug particles which are smaller than 5 μm are homogenously distributed in the sampling unit (Figure 15b + d). A comparison of drug amount between the original powder mixture and the aerosolised powder mixture in the pedestal showed that the drug amount significantly decreases during aerosolisation. This implies that the lactose carrier particles are separated from the drug particles during

the aerosolisation process (Figure 18). This is important because these carrier particles are designed to prevent agglomeration of the small drug crystals in the capsule, but during inhalation the particles have to separate so that the carrier particles deposit in the oropharyngeal area and the small drug particles can reach the lower respiratory tract [172]. The initial deposition experiments were carried out with SC4 and long connecting tubes which resulted in a deposition amount of 0.92 ± 0.11 μg budesonide per well (Figure 16). To increase the deposition amount in the system other programmed SmardCards as well as short tubes were tested. Shorter tubes were tested in the hope of decreasing deposition of the drug within the tubes, but this led to an increased deposition in the pedestal (data not shown) indicating that a large proportion of the aerosol is transported through the sedimentation chamber during the ventilation flow (100-300 ml). This was observed for all of the SmartCards used, resulting in no significant increases of deposition amount (Figure 16) when using the short tubing. Therefore longer tubes are more suitable for experiments although parts of the drug already deposit in the tubes during the ventilation flow. Further experiments with long tubes, in combination with the other SmartCards, showed significant differences in the deposition amount compared to SC4 which was used in the initial experiment. The highest deposition was obtained with SC1 (2.23 μg/well), so that a 2.5-fold increase was realised compared to our first settings with SC4 (0.92 μg/well). Experiments with salbutamol sulphate using long tubes showed similar results, with the highest deposition amount also being achieved with SC1 in this case (data not shown). Deposited amount in the three different sampling wells of the deposition unit showed no significant differences with either budesonide or salbutamol sulphate (Figure 17) Therefore, three experiments can be performed in parallel without influencing the results.

The integrity of the cell monolayer in the PADDOCC system was tested to ensure that drug permeation studies can be performed after deposition. Calu-3 cells were used

which are known to form a tight monolayer under air-liquid interface conditions [171, 173]. As expected, experiments showed that there were no significant differences between TEER values of the Calu-3 cells before and after the aerosolisation and deposition process. MTT assay results of the cells which were put into the PADDOCC system also showed no significant differences compared to the untreated cells (Figure 19). The integrity of the cell monolayer results from protection of the cells in the ventilation mode from any air streams of the aerosolisation process and their viability is maintained by buffer support and an atmosphere of 37°C. This is in contrast to the impinger systems where the cell monolayer are exposed to high air flows [131, 162, 164]. Thus, the PADDOCC system is able to deposit aerosolised particles onto cells in such a way that consecutive transport experiments can be performed with an intact cell monolayer.

This prototype, featuring a combination of three sedimentation chambers is superior to other deposition systems where only one well of cells can be investigated at time [165]. Our system could also be modified by integrating more sedimentation chambers to obtain up to 6 or 8 experiments in parallel and would represent a suitable method to get representative and reproducible data in short time.

4.6 Conclusion

The PADDOCC system is a further step toward the realistic mimicking of the deposition of a dry powder aerosol onto the lung surface *in vitro*. This permits the investigation of the interactions between these aerosolised particles and the different cell types of the lung without changing the aerosol properties. By depositing aerosols delivered from a commercially available dry powder inhaler directly to the air-liquid interface of an epithelial cell monolayer we consider that this model has potential value in the development of new aerosol medicines. Beyond merely studying deposition the PADDOCC system may also provide information on formulations and excipient effects on barrier function, as well as on drug absorption across the pulmonary epithelium. By using human derived lung cell cultures the PADDOCC may help to overcome inter-species differences and to reduce animal experiments.

5 Transport studies with PADDOCC system

Parts of this chapter have been accepted for publication:

S. Hein, M.Bur, U.F. Schäfer, C.-M. Lehr

A new Pharmaceutical Aerosol Deposition Device On Cell Cultures (PADDOCC) to evaluate pulmonary drug absorption of metered dose dry powder formulations, European Journal of Pharmaceutics and Biopharmaceutics, 2010

5.1 Summary

Absorption studies with aerosol formulation delivered by metered dose inhalers across cell and tissue based *in vitro* models of the pulmonary epithelia is not trivial due to the complexity of the processes involved: i) aerosol generation and deposition, ii) drug release from the carrier, and iii) absorption across the epithelial air-blood barrier. In contrast to the intestinal mucosa, pulmonary epithelia are only covered by a thin film of lining fluid. Submersed cell culture systems would not allow to studying the deposition of aerosol particles and their effects on this delicate epithelial tissue. We developed a new Pharmaceutical Aerosol Deposition Device On Cell Cultures (PADDOCC) to mimic the inhalation of a single metered aerosol dose and its subsequent deposition on filter-grown pulmonary epithelial cell monolayers exposed to an air-liquid interface. The reproducibility of deposition of these dry powder aerosols and subsequent drug transport across Calu-3 monolayers with commercially available dry powder inhalers containing salbutamol sulphate or budesonide could be demonstrated. In the context of developing new dry powder aerosol formulations PADDOCC appears as a useful tool, allowing to reducing animal testing and faster translation into clinical trials.

5.2 Introduction

Dry powder inhalers represent an important platform both for local as well as systemic pulmonary drug delivery. As for all new medicines, aerosol powder formulations need to be tested for safety and efficacy. Mostly these experiments are done *in vivo* by animal experiments or on *ex vivo* lung preparations [174, 175]. Based on the 3R principle of animal welfare there is a demand for *in vitro* test systems to determine the efficacy of these aerosols, but they are rarely available. For the characterisation of aerosol properties and pulmonary deposition physical devices like the multistage liquid impinger (MSLI) or Andersen impactor are widely established. However, these methods allow no conclusions about the influence of formulation factors on drug permeability and absorption across the pulmonary epithelial barriers, nor do they provide any information about the biocompatibility with or possible toxic effects on those cells and tissue. Therefore an apparatus allowing to simultaneously studying both deposition and subsequent drug absorption of pharmaceutical aerosols appears to be highly desirable. Aerosol effects on lung cells are often addressed in environmental toxicology [139, 140, 143, 144, 151, 152]. There are different methods available, such as e.g. electrostatic precipitation [156, 158–160] to collect particles from atmospheric aerosols, typically having a rather low particle density, onto cell monolayers. The main focus of those investigations is long-term effects of low-dose exposed materials like diesel dust or particulate matter. In contrast, pharmaceutical aerosols are aimed to deliver a specific dose of a given drug with a single bolus or puff. However, there are very few models available to study the effects of aerosolised drug powders on cell culture systems. One approach is the integration of cell monolayer in a liquid impinger system. Cooney et al. [162] used an Andersen viable cascade impactor as a deposition device and delivered aerosolised FITC-dextran solutions to cell monolayers. Fiegel et al.

[131] deposited large porous particles onto Transwell® filters which were placed on the stages of a liquid cascade impinger. In both setups no particular attention was paid to the turbulences that were caused of the bowel-like form of the Transwell® filters. Therefore Bur et al. [164] refined this approach by integrating upside down Transwell® filters in the bottom of a multi-stage liquid impinger to minimise turbulences in the air streams and subsequently increase deposition efficiency on the Transwell® grown cell monolayers. Still, these models rely on impaction as the main deposition mechanism. Besides impaction and diffusion the prevailing deposition mechanism in the deep lung is sedimentation. Sedimentation is a predictable process [168, 169] and does not cause changes of the aerosol properties like electrostatic precipitation does. Therefore we decided to develop a system where the deposition of aerosol particles mainly occurs by sedimentation at the air-liquid interface of filter-grown pulmonary epithelial cells. To study drug absorption and permeability *in vitro* tight cell monolayers are needed. The respiratory Calu-3 cell line forms tight junctions and produces mucus, making it suitable for modelling the airway epithelial barrier [171, 173]. Another advantage of the Calu-3 cell line is the formation of tight monolayers on permeable filter supports at an air-liquid interface (ALI), yielding closer resemblance to the native epithelium than under liquid covered culture (LCC) [132]. This study describes a further evaluation of the new Pharmaceutical Aerosol Deposition Device On Cell Cultures (PADDOCC) based on filter-grown cell monolayers placed on the bottom of a sedimentation chamber. While the development and the experimental protocol of the apparatus with focus on optimisation of the deposited amount have already been described elsewhere [176], we here report additional validation steps by conducting combined deposition and transport experiments using commercially available dry powder aerosol formulations of salbutamol sulphate and different doses of budesonide. The results were compared with the standard procedure of liquid-interface transport studies.

5.3 Materials and Methods

5.3.1 Materials

Snapwell® permeable filters (pore size 0.4 μm, 1.13 cm^2, Snapwell® type 3801) and Transwell® permeable filters (pore size 0.4 μm, 1.13 cm^2, Transwell® type 3460) were purchased from Corning Costar (Bodenheim, Germany). RPMI 1640 (without phenol red) and fetal bovine serum (FBS Gold) were obtained from PAA (Pasching, Austria) and sodium pyruvate was obtained from Lonza (Verviers, Belgium). Buffer for transport experiments was KRB. Composition was as follows: 1.41 mM $CaCl_2$, 3.00 mM KCl, 2.56 mM $MgCl_2$, 142.03 mM NaCl, 0.44 mM K_2HPO_4, 4.00 mM D-glucose and 10.0 mM HEPES. All these reagents were obtained from Sigma Aldrich (Deisenhofen, Germany). KRB was adjusted to pH 7.4 by means of NaOH. Budesonide and salbutamol sulphate powders were a gift from Boehringer (Ingelheim, Germany). All other chemicals were of highest available grade.

5.3.2 Cell culture

Calu-3 cells, clone HTB-55, were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and used at passages 38-52. Calu-3 cells were grown to 90% confluence in 75 cm^2 T-flasks with RPMI 1640 supplemented with 10% FBS Gold and 1mM sodium pyruvate. Culture medium was changed three times a week. The incubator temperature was set to 37°C in an atmosphere of 90% relative humidity and 5% CO_2. After trypsinisation, cells were seeded on Snapwells® or Transwells® at a density of 100.000 cells/cm^2 with 1.5 ml medium in the basolateral compartment and 500 μl in the apical compartment. After two days, the cells in the Snapwells® were set to air-liquid interface conditions with 1.0 ml medium in the basolateral compartment. After 10-14 days the cells

were ready for the deposition experiments.

5.3.3 Bioelectrical measurements

Transepithelial electrical resistance (TEER) was measured to monitor the tightness of the cell monolayer. As a consequence of the air-liquid interface cultivation the apical compartment has to be filled with 500 μl pre-warmed medium and the basolateral compartment with 1.5 ml before TEER measurement and equilibrate in an incubator for 30 min. TEER was measured with an Electrical Volt-Ohm Meter (EVOM, WPI, Berlin, Germany) equipped with chopstick electrodes. Thereafter the medium in both compartments was removed and the basolateral compartment was filled with 1.0 ml medium again. TEER was measured until 2-3 days before deposition experiments to assure the recovery of the mucus layer on the cells, and after the transport experiments to confirm the integrity of the cell monolayer during the experiments.

5.3.4 Dose dependent deposition

For all the follwing experiments, the PADDOCC system was used, as described previously [176]. Experiments to demonstrate a linear dependence of the deposited amount of drug were performed first by aerosolising a dry powder capsule with different concentrations of budesonide (Cyclocaps® Budesonid 200 μg, 400 μg and 800 μg, PB Pharma, Meerbusch, Germany) and depositing the aerosol in the sampling wells (i.e. without cells). After three aerosolisation-deposition cycles each well of the sampling unit was washed with 1 ml of HPLC mobile phase and quantified by HPLC analytics.

5.3.5 Deposition experiments and subsequent transport studies on ALI Calu-3 monolayers

60 min before the experiments, cell monolayers grown in Snapwells® were transferred to a 6-well culture plate, each containing 750 μl pre-warmed KRB buffer in the basolateral compartment and equilibrated in an incubator. After this pre-equilibration, they were placed into the sampling wells of the PADDOCC, filled with 500 μl pre-warmed KRB buffer, and the apparatus was then assembled to conduct the deposition experiment. To keep the temperature constant, the entire system (without Akita® device) was placed inside an incubator at 37°C. Commercially available capsules of dry powders were aerosolised and deposited onto ALI grown Calu-3 cells via the deposition system. The studies were performed with Cyclocaps® Salbutamol 400 μg (with 480 μg salbutamol sulphate, PB Pharma, Meerbusch, Germany) and Cyclocaps® Budesonid 400 μg (PB Pharma, Meerbusch, Germany) and dose dependent studies were performed with Cyclocaps® Budesonid 200 μg and 800 μg (PB Pharma, Meerbusch, Germany). Sedimentation for respirable particles (i.e. with a MMAD in a range between 2 and 5 μm) takes no longer than 10 min in this chamber (calculated according to Dua et al. [168]) and performing three deposition cycles resulted in a window of 30 min for the aerosolisation and deposition process. After these 30 min the Snapwells® were then transferred back to the 6-well culture plate filled with 750 μl pre-warmed KRB buffer in the basolateral compartment and rotated gently on a shaker (150 rpm). Samples of 100 μl were taken at different time points from the acceptor compartment and replaced by 100 μl fresh buffer. Samples were quantified by HPLC analysis. After the transport experiments TEER values were measured to assure the integrity of the monolayer. To determine the total amount of drug deposited at the end of the experiment, the cells were lysed with 200 μl dimethylsulfoxide in case of budesonide

as model drug, or with 200 μl isopropanol for salbutamol sulphate, unified with the apical and basolateral compartment (total volume 2.2 ml) and centrifuged for 3 min at 14000 min^{-1}. The supernatant was then quantified by HPLC.

5.3.6 Liquid interface transport experiments of budesonide and salbutamol sulphate

Transport experiments were also carried out with Calu-3 cells after dissolving of the drugs in buffer and pipetting the resulting solution to the apical cell compartment. The donor concentration of budesonide was 30 μM and of salbutamol sulphate was 1000 μM. Samples from the acceptor compartment were taken at different time points and quantified by HPLC analysis. P_{app} values were calculated as follows:

$$P_{app} = \frac{J}{A * C}$$

where J is the flux, A is the area (1.13 cm^2) and C is the initial concentration in the donor compartment.

5.3.7 HPLC analysis

HPLC analysis of budesonide and salbutamol sulphate has already been described in detail in chapter 4.3.7

5.3.8 Data analysis and statistics

The area under the curve ($AUC_{absorption}$) as a parameter for absorption rate after 4 hours in the transport experiments was calculated by SigmaPlot 8.0 software (Systat Software

GmbH, Erkrath, Germany). Data are expressed as mean ± SD. Statistical analysis was carried out using SigmaStat 3.0 software (Systat Software GmbH, Erkrath, Germany). Data were compared by one-way ANOVA followed by the Holm-Sidak method for differences between two or more groups (n>6). Differences were deemed statistically significant if $p < 0.05$.

5.4 Results

5.4.1 Deposition experiments

Deposition experiments with salbutamol sulphate (400 μg) and different amounts of budesonide (200, 400 and 800 μg) showed a uniform and reproducible deposition of about 0.5% of the aerosolised dose per well. There was no significant difference in deposition amount between the three wells. As can be seen in Figure 20 the different doses of budesonide resulted in a proportional deposition in the sampling wells.

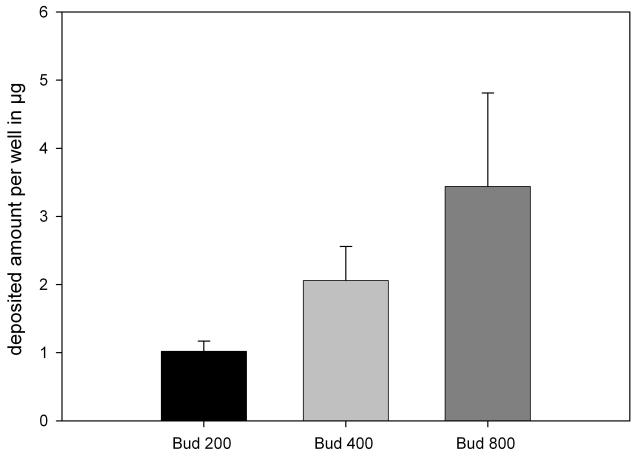


Figure 20: Deposition behaviour of budesonide in dependency of different doses. The deposited amount of budesonide per sampling well (in μg) is proportional to the original dose (200,400 und 800 μg per capsule) which is equal to about 0.5% of the original dose (mean $\pm$ SD).

5.4.2 Deposition experiments and subsequent transport studies

Transport experiments after deposition of budesonide aerosol powder (400 μg) showed a linear increase in the transported amount in the first 60 min, but then the curve flattened, resulting in a total transport of about 71 $\pm$ 11% of the deposited amount during four hours (Figure 21). Transport of salbutamol sulphate showed a similar time course, with a

flattening of the curve after 90 minutes and a total transport of 19 ± 6% after four hours (Figure 22).

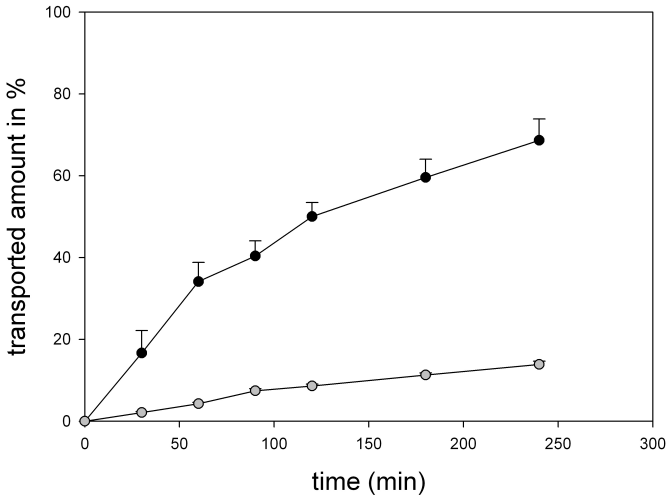


Figure 21: Transport of budesonide across filter-grown Calu-3 monolayers: Deposition and subsequent transport experiments for the aerosol powder formulation using the PADDOCC system (full circles, 400 μg capsule, n=12) versus the dissolved drug using a conventional Transwell® setup (open circles, 30 μM solution, n=5)

5.4.3 Liquid interface transport studies

Transport studies performed with dissolved budesonide in a conventional Transwell® setup showed a linear transport during the first four hours (Figure 21). P_{app} was 8.37 ± 0.36 $*$ 10^{-6} cm/s and total transport was about 13.8 ± 0.8% after four hours. Salbutamol sulphate transport studies also showed a linear transport in the first four hours (Figure 22), but total transport was only 0.11 ± 0.08% and P_{app} was calculated to be 0.126 ± 0.09 $*$ 10^{-6} cm/s.

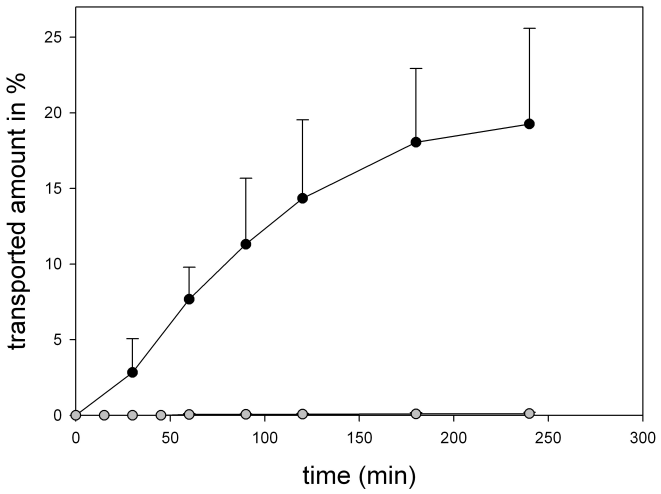


Figure 22: Transport of salbutamol sulphate across filter-grown Calu-3 monolayers: Deposition and subsequent transport experiments for the aerosol powder formulation using the PADDOCC system (full circles, 400 μg capsule, n=9) versus the dissolved drug using a conventional Transwell® setup (open circles, 1000 μM solution, n=5)

5.4.4 Dose dependent transport studies

After the deposition experiments, the budesonide capsules were aerosolised and deposited with the PADDOCC system, but now equipped with Calu-3 monolayers grown on Snapwell® filter inserts. As can be seen in Figure 23 there is a dose dependent transport rate of the budesonide molecules through the Calu-3 monolayer. Higher dosing of the drug resulted in an increased transported amount while the fraction of the dose in % remained constant. The area under the curve ($AUC_{absorption}$) as a parameter for absorption rate of the transport curves is proportional to the original dose in the budesonide capsules (Figure 24).

5.5 Discussion

Ideally, an *in vitro* model for testing new aerosols and formulations should be able to mimic the deposition of aerosol particles on epithelial cell cultures by involving the same processes of impaction, sedimentation and diffusion as they occur in the lung *in vivo*. The new PADDOCC system aims to approximate this by the integration of Snapwells® with pulmonary epithelial cells in a sedimentation chamber. There the aerosol particles from dry powders will deposit by sedimentation which is the prevailing deposition mechanism for particles sizes between 1 and 5 μm in the deep lung [177, 178]. Before this respirable aerosol fraction reaches the sedimentation chamber, the PADDOCC system separates larger particles, such as e.g. lactose carriers, corresponding to the *in vivo* separation of larger particles by the throat and pharynx, allowing only smaller drug particles to reach the deep lung [127]. In the PADDOCC system larger carrier particles are already deposited in the pedestal, due to higher sedimentation velocities, whereas the drug particles sediment onto the sampling wells which has previously been shown by SEM imaging [176]. Therefore, transport studies are not affected by the carrier particles.

In this study budesonide and salbutamol sulphate were chosen as model drugs for aerosolisation because they are widely used to treat pulmonary diseases like asthma. Budesonide, as a biopharmaceutics classification system (BCS) class II model drug, is highly permeable and poorly soluble and therefore its pulmonary bioavailability is likely to be limited by its solubility in the lining fluid of the cell monolayer. In contrast, pulmonary bioavailability of the highly soluble salbutamol sulphate, a BCS class III drug, may be limited by its low permeability. As for systemic bioavailability after oral administration, solubility and permeability are most likely as important as for pulmonary bioavailability after aerosol delivery to the lung. Apart from effective deposition, adequate absorption

across the pulmonary epithelia is pivotal. With the PADDOCC system, the ability of drug to cross the biological absorption barriers of the lung, e.g. by a Calu-3 monolayer, can be determined for dry powder aerosol formulations in a rather early stage of the development process.

The total amount of drug recovered after deposition in the three sampling wells is about 1.5% of the total amount in the capsule. This may appear low compared to electrostatic precipitation methods [156, 158] where deposition efficacies up to nearly 100% occur. However, the total surface area of the epithelial cell cultures ($\sim$ 3 cm^2) in the PADDOCC is even much smaller than the total surface area of the deep lung ($\sim$ 100 m^2). Assuming homogeneous deposition of a single aerosol bolus in the 0.1 - 1 mg range would theoretically lead to a density of deposited particles on the alveolar mucosa in the order of 0.1 - 1.0 ng/cm^2. This is still at least 1000 times lower compared to the actually collected particle concentration on the Snapwell® filter surface in our setup. Further reducing the deposition density would at the one hand demand much more analytical efforts. On the other hand, the $\sim$ 1000 times higher concentration of particles to which the cells are exposed in this *in vitro* setup appear as a useful "safety margin" to detect any possible adverse effects of a given drug or excipient, e.g. by a change of TEER or other cytotoxicity indicators.

Aerosol generation with a dispersion impulse of 60 l/min for 0.2 s is similar but shorter compared to other studies [131, 162] where impactors with flow rates of 28.3 and 60 l/min over 30 s occur. The main advantage is that the sedimentation process of the particles onto the cells happens without any disturbing air streams, so that the cells were not affected. In this study Calu-3 cells were used which were cultivated at air-liquid interface conditions, reflecting the *in vivo* properties more than liquid covered cultured Calu-3 cells [132]. They are more differentiated than LCC, but their TEER values are much lower than LCC due to the cultivation conditions. Several studies for air-liquid interface grown Calu-3 cells

show that TEER of about 450 Ω*cm^2 are needed to obtain a tight monolayer [131, 179], therefore only results of transport studies with TEER after experiments of more than 450 Ω*cm^2 were used. However, aerosol generation and deposition did obviously not affect the barrier properties of the cell monolayers, as indicated by practically the same TEER values before and after the deposition [176].

Epithelial transport experiments of budesonide, which is known to be highly permeable but poorly water soluble, after deposition with the PADDOCC system show a linear increase in the first 60 min in the transport rate (Figure 21), and then flattens, indicating a depletion in the donor compartment. After deposition of the aerosol on the monolayer, the budesonide particles dissolve only partially, yielding a saturated solution around themselves and some concentration gradient between the particle surface and the lining fluid. Another concentration gradient exists between the lining fluid with dissolved budesonide and the basolateral compartment containing KRB buffer. This concentration gradient leads to a rapid transport through the monolayer to the basolateral compartment. After one hour the transport curve flattens because the concentration gradient between the budesonide particles and the lining fluid decreases. It runs out of sink conditions and the permeation through the monolayer is limited by the dissolution process of the budesonide particles. As a result the transport over the monolayer decreases resulting in a slower slope in transport curve. However, the hydrophilic salbutamol sulphate is dissolved in the lining fluid after deposition. In this case permeation is the limiting factor for transport. Due to the high concentration gradient in the beginning salbutamol is transported through the monolayer into the basolateral compartment (Figure 22). After about 90 min the transport rate decreases due to depletion in the donor compartment. As expected, the transport rate of the hydrophilic salbutamol sulphate through the Calu-3 monolayer is much lower than of the lipophilic budesonide. The high local drug concentrations on the Calu-3 monolayer due to

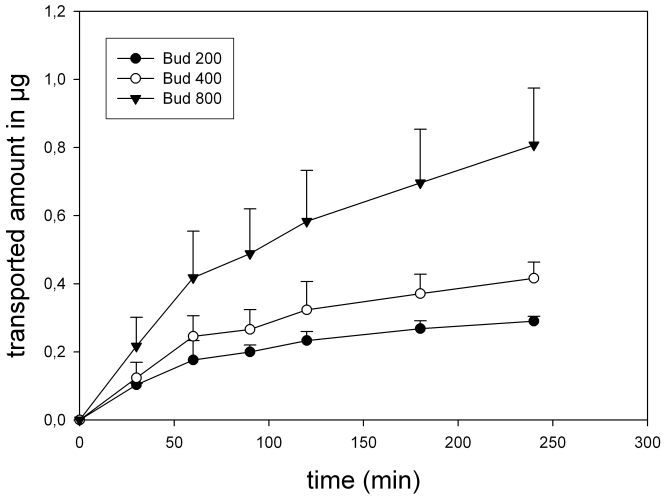


Figure 23: Transported amount of different masses of deposited budesonide (after aerosolisation and deposition in the PADDOCC system from a 200, 400 and 800 μg capsule, n=6) across a Calu-3 monolayer

the landing of dry drug particles onto a cell monolayer with only a thin film of fluid cause the high absorption rates compared to submersed transport studies where budesonide and salbutamol sulphate show much lower absorption rates (Figure 21 and 22). The P_{app} values of the submersed transport studies calculated for budesonide and salbutamol sulphate correlate very well with data from Bur et al. [180]. Similar observations of an increased transport when using air-liquid interface cell monolayers were made by Grainger et al. [165] where FITC-dextran particles which were deposited by a twin-stage impinger onto air-liquid interface Calu-3 monolayers showed a 20 fold-higher transport rate compared to FITC-dextran solutions.

When using budesonide capsules with different drug content, the ratio of the amounts deposited on the sampling units of the PADDOCC were in good agreement with those of the labelled dose (i.e. 1:2:4, Figure 20). Deposition and transport of increasing amounts of budesonide show an increased transport (Figure 23) resulting in proportional increasing of

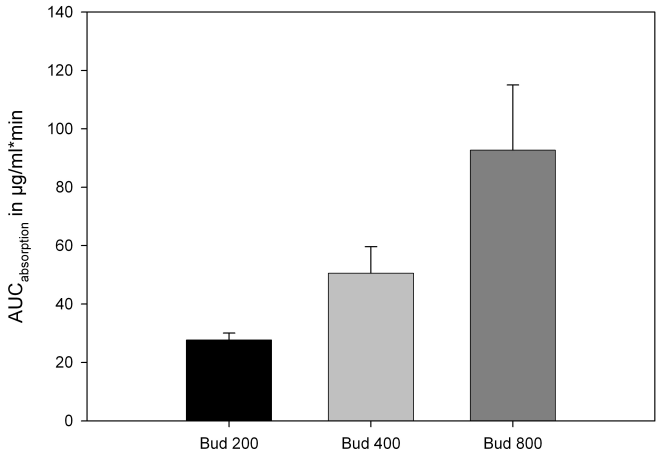


Figure 24: The absorbed dose ($AUC_{absorption}$ in μg/ml$*$min) across the Calu-3 monolayer from capsules of 200, 400 and 800 μg. The transport is proportional to the aerosolised dose (mean $\pm$ SD)

area under the curve ($AUC_{absorption}$) values as a parameter for absorption rate (Figure 24) indicating that the PADDOCC system is able to distinguish between different amounts of the deposited drug. The ratio of the $AUC_{absorption}$ (1:1.8:3.3) is also in good accordance with the ratio of the dosing strength (1:2:4) in the budesonide capsules. While the processes of aerosol deposition and absorption *in vivo* are much more complex, the PADDOCC system aims to reduce this complexity to a level which at the one hand remains practically feasible, but at the other hand may provide some insight into what happens when a dry powder aerosol formulation reaches the epithelial air-blood barrier of the lungs. Such information goes clearly beyond mere deposition studies of aerosol particles in a conventional impactor device and beyond mere permeability testing of dissolved drug in a conventional Transwell® setup. Alternative to the bronchial epithelial cell line Calu-3, an extension of the current setup towards more sophisticated cell culture systems, like human alveolar epithelial cells [181] or co-cultures with endothelial cells and macrophages [182], also featuring a more complex apical lining fluid (e.g. to model the surfactant film), appears easily feasible.

5.6 Conclusion

The PADDOCC system allows to combining the aerosolisation and deposition of pharmaceutical aerosol formulations with subsequent absorption studies across filter-grown cell cultures of pulmonary epithelial cells. Sedimentation as the main deposition mechanism is gentle, thereby not affecting cell monolayer integrity. Moreover, it allows to separate respirable drug particles from non-respirable carrier particles due to different sedimentation velocities. Air-liquid interface deposition of drug particles directly on the apical cell membrane, covered by only a minimal amount of lining fluid, makes an important difference to pipetting the drug dissolved in a buffer solution in a conventional Transwell® setup. For the development of pharmaceutical aerosol powder formulations, we trust that this setup could be very useful, replacing animal experiments and facilitating the translation of new concepts for pulmonary drug delivery into the clinic.

6 Summary and Outlook

Development of new inhalative drug formulations is increasing because pulmonary diseases like tuberculosis or asthma are on the rise. There are also new approaches for local therapies of lung cancer in the pipeline with advanced particle types like liposomes or nanoparticles. All these new approaches have to be tested for their safety and efficacy, which is at the moment mostly done in animal experiments.

There is a need for such an *in vitro* model to reduce animal experiments in the early phase of drug formulation development, because *in vitro* models are, besides ethical reasons, more time-effective and cost-efficient. The work presented in this thesis dealt with the development of a new *in vitro* model to characterise aerosol particles which have been deposited onto air-liquid interface grown cell monolayers. Other models used in pharmacological context rely on impaction as the deposition mechanism, but our model focuses on sedimentation, the main deposition mechanism in the deep lung.

The first part of this thesis is about the development and first evaluation of our Pharmaceutical Aerosol Deposition Device On Cell Cultures (PADDOCC) system, which consists of three components: air flow control unit, aerosolisation unit and deposition unit. The air flow control unit aerosolises the dry powder formulation into the aerosolisation unit and transports the aerosol to the deposition unit where these aerosolised drug particles are deposited onto human lung cell culture monolayer by sedimentation. These cell monolayers are grown at air-liquid interface conditions to represent a more *in vivo* like structure. The dry powder formulation which typically consists of large carrier particles (> 50 μm) and small drug crystals (2-5 μm) is separated during the aerosolisation process similar to *in vivo* situation, so that only the drug crystals are deposited onto the cell monolayer. Experiments showed a reproducible, uniform deposition of drug particles onto these cell

monolayers and due to the gentle deposition mechanism no damage of the cell monolayer was detected.

The second part focuses on the deposition and absorption of drug particles. Aerosolisation of different masses of drug particles resulted in a proportional deposition. Absorption studies performed with air-liquid interface grown Calu-3 cells showed significant differences compared to transport studies with liquid interface grown Calu-3 cells. This was due to the high concentration gradient caused by the small amount of fluid on top of the ALI cell monolayer.

Surely there are further developments needed to obtain a perfect *in vitro* model of the respiratory tract where all the deposition mechanisms occur onto the relevant cell culture models, but our model is a first step to simulate deposition in the deep lung by sedimentation and may help to improve safety and efficacy testing of new aerosol formulations *in vitro*.

This thesis was only focussing on the deposition of dry powder aerosols. However, there are also liquid formulations on the market and in development. Promising approaches like liposomes and nanoparticle suspensions need also to be tested for their efficacy and safety before approval. To reduce animal experiments also in this area, *in vitro* models for liquid formulations are needed. Therefore the next step would be to modify the PADDOCC system to investigate the influences of liquid formulations onto cell monolayers, too.

7 Zusammenfassung und Ausblick

Die Erforschung von neuen inhalativen Arzneistoffformulierungen entwickelt sich stetig weiter, da Krankheiten der Lunge wie Tuberkulose oder Asthma auf dem Vormarsch sind. Es gibt auch neue Ansätze für lokale Therapien zur Behandlung des Lungenkrebses, die auf neuen Partikelarten wie Liposomen oder Nanopartikeln basieren sowie Versuche andere Krebsarten durch systemisch wirkende Aerosole zu behandeln. Diese neuen Formulierungsansätze müssen hinsichtlich ihrer Sicherheit und Wirksamkeit getestet werden. Dieses geschieht zur Zeit zum größten Teil mit Tierversuchen. Deshalb werden *in vitro* Modelle benötigt, die die Tierversuche in der frühen Phase der Arzneistoffformulierung ersetzen können. *In vitro* Modelle haben den Vorteil, dass sie, neben den ethischen Gründen, zeitsparender und kosteneffektiver sind. Diese Arbeit beschäftigte sich mit der Entwicklung eines solchen *in vitro* Modells, das den Einfluß von deponierten Aerosolpartikeln auf an der Luft-Grenzschicht gewachsenen Zellmonolayern untersucht. Andere Modelle, die im pharmazeutischen Bereich genutzt werden, basieren auf der Impaktion als Depositionsmechanismus. Unser Modell beruht auf der Sedimentation, die auch den Hauptdepositionsmechanismus in der tiefen Lunge darstellt.

Der erste Teil dieser Arbeit beschreibt die Entwicklung sowie eine erste Evaluation des Pharmaceutical Aerosol Deposition Device On Cell Cultures (PADDOCC) Systems. Es besteht aus drei Hauptkomponenten: einer Kontrolleinheit, einer Verneblungseinheit und einer Depositionseinheit. Die Kontrolleinheit vernebelt die Pulverformulierung in der Verneblungseinheit und transportiert danach das entstandene Aerosol zur Depositionseinheit, wo die Aerosolpartikel auf Lungenzellkulturen durch Sedimentationsprozesse deponiert werden. Diese Zellmonolayer sind an der Luft-Grenzschicht gewachsen, da sie so eine ähnlichere *in vivo* Struktur aufweisen. Die Pulverformulierung besteht üblicherweise

aus größeren Trägerpartikeln (> 50 μm) und kleinen Arzneistoffpartikeln (2-5 μm), die während des Aerosolisierungsprozesses, ähnlich der *in vivo* Situation, getrennt werden, so dass nur die Arzneistoffpartikel auf dem Zellmonolayer deponiert werden. Versuche in dem PADDOCC System zeigten eine reproduzierbare und gleichmäßige Deposition der Partikel auf den Zellmonolayern und durch den schonenden Depositionsprozess konnten keine Beschädigungen am Zellmonolayer festgestellt werden.

Der zweite Teil der Arbeit beschreibt Depositions- und Absorptionsversuche mit verschiedenen Arzneistoffpartikeln. Die Verneblung von unterschiedlichen Massen der Arzneistoffpartikel resultierte in einer zur Masse proportionalen Deposition. Absorptionsstudien, die mit an der Luft-Grenzschicht kultivierten Calu-3 Zellen durchgeführt wurden, zeigten signifikante Unterschiede im Absorptionsverhalten verglichen mit Transportstudien, die mit submers kultivierten Calu-3 Zellen durchgeführt wurden. Dies ist bedingt durch den großen Konzentrationsgradienten, der aufgrund der geringen Flüssigkeitsschicht auf dem Zellmonolayer, entsteht.

Sicherlich sind noch einige Weiterentwicklungen nötig, um ein perfektes *in vitro* Modell des Respirationstraktes zu erhalten, das alle relevanten Depositionsmechanismen auf die verschiedenen Zelltypen simuliert, aber unser Modell ist ein erster Schritt, um die Sedimentation von Arzneistoffen in der tiefen Lunge darzustellen. Das PADDOCC System könnte in der Lage sein, Sicherheits- und Wirksamkeitsstudien von neuen Formulierungen *in vitro* durchzuführen, um Tierversuche reduzieren oder gar teilweise zu ersetzen.

Diese Arbeit beschäftigte sich mit der Deposition von Pulverformulierungen. Ein anderer großer Part der Inhalanda besteht aber aus Flüssigformulierungen, die schon auf dem Markt sind (Lösungen von Arzneistoffen) oder sich aber in der Entwicklung befinden. Diese, sich in der Entwicklung befindlichen, neuartigen Partikeltypen wie Liposome oder Nanopartikelsuspensionen müssen jedoch auch hinsichtlich ihrer Sicherheit und Wirksamkeit

getestet werden bevor sie zugelassen werden können. Deshalb wird auch in diesem Feld ein *in vitro* Modell benötigt, dass die Auswirkungen dieser Formulierungen auf Zellkulturen untersucht. Der nächste, konsequente Schritt wäre deshalb eine Modifikation des PADDOCC Systems, so dass auch flüssige Fomulierungen getestet werden können.

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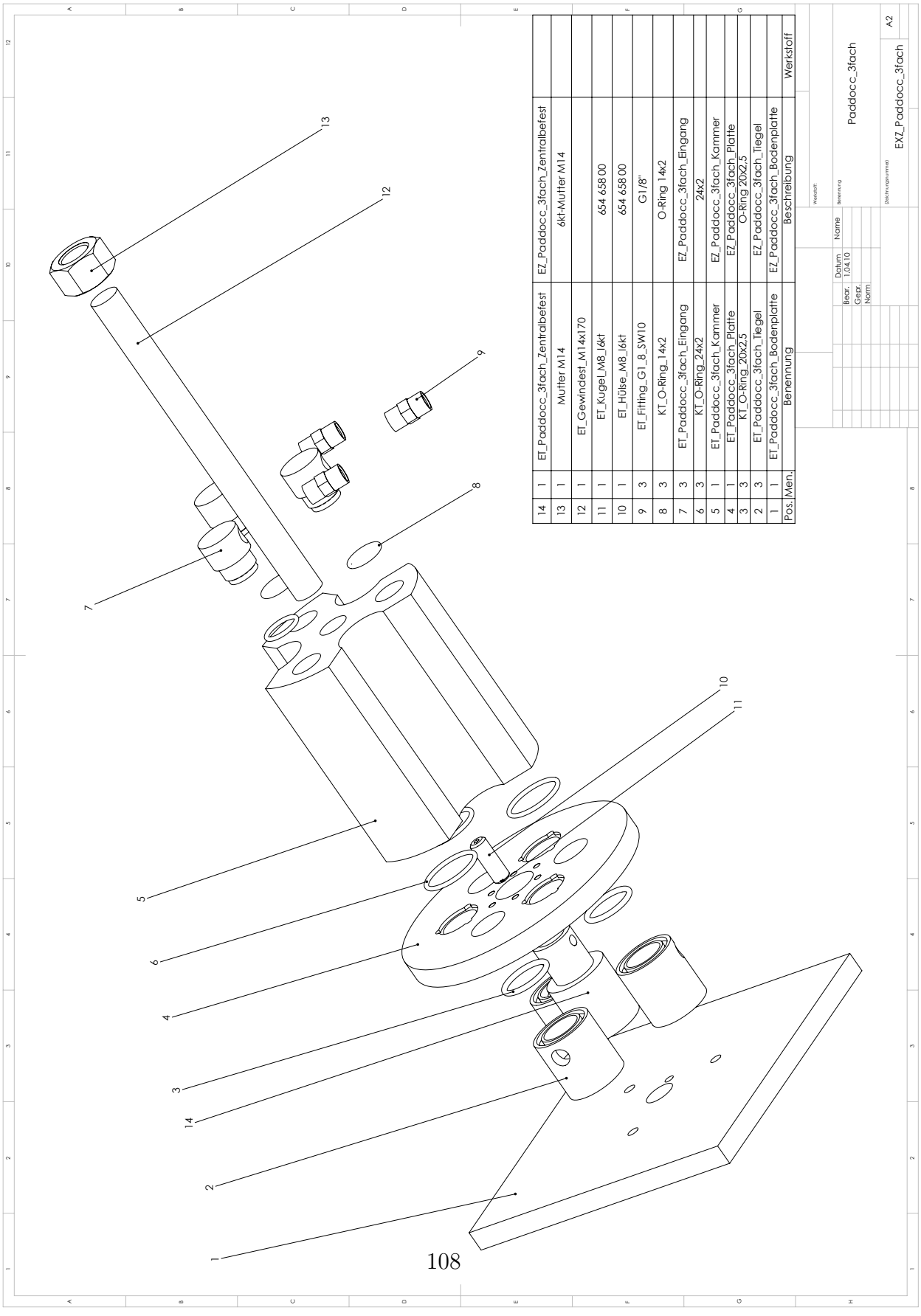
A Abbreviations

3R	Replace, reduce, refine
ALI	Air-liquid interface
ATD	Antitubercular drug
AUC	Area under the curve
BALT	Broncho-alveolar lymphoid tissue
CF	Cystic fibrosis
CLIJ	Confined liquid impinging jet
COPD	Chronic obstructive pulmonary disease
DLPC	Dilauroylphosphatidylcholine
DMSO	Dimethylsulfoxide
DPI	Dry powder inhaler
DPPC	Dipalmitoylphosphatidylcholine
DSPC	Distearoylphosphatidylcholine
EAVES	Electrostatic aerosol in vitro exposure system
EPDExS	Electrostatic particulate dosage and exposure system
EVOM	Electric Voltohmmeter
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FITC	Fluoresceinisothiocyanat
HPLC	High pressure liquid chromatography
KRB	Krebs Ringer buffer
LCC	Liquid covered culture
LMWH	Low molecular weight heparine
MTT	Methyl-thiazolyl-tetrazolium
PADDOCC	Pharmaceutical Aerosol Deposition Device On Cell Cultures
PLG	Poly(lactide)-co-poly(glycolide)
PLGA	Poly(lactic-co-glycolic-acid)
SAEC	Small airway epithelial cells
SLIT	Sustained release lipid inhalation targeting
SLM	Solid lipid microparticles
TEER	Transepithelial electrical resistance

B Blueprints of the PADDOCC system

The blueprints of the PADDOCC system are listed below.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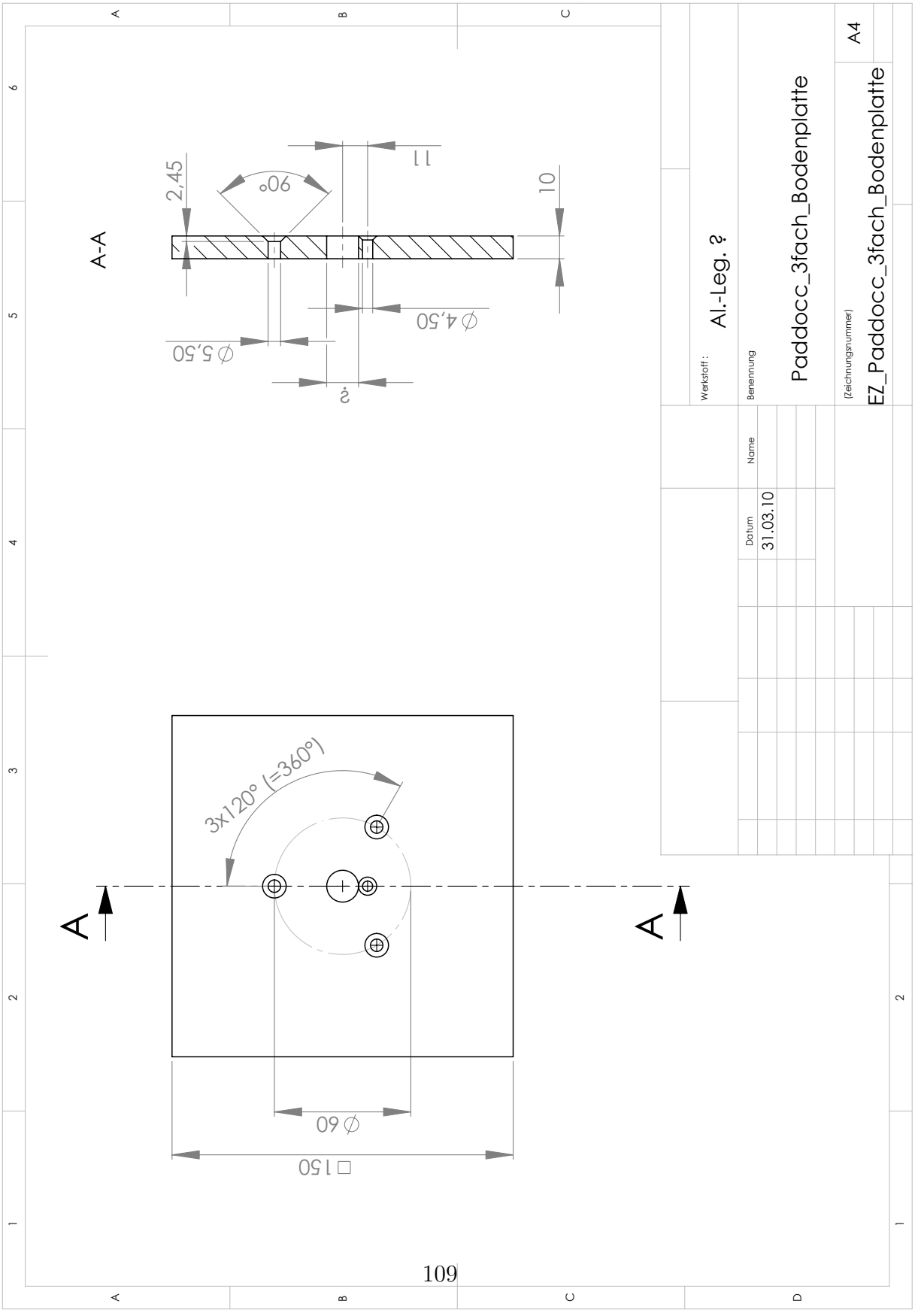
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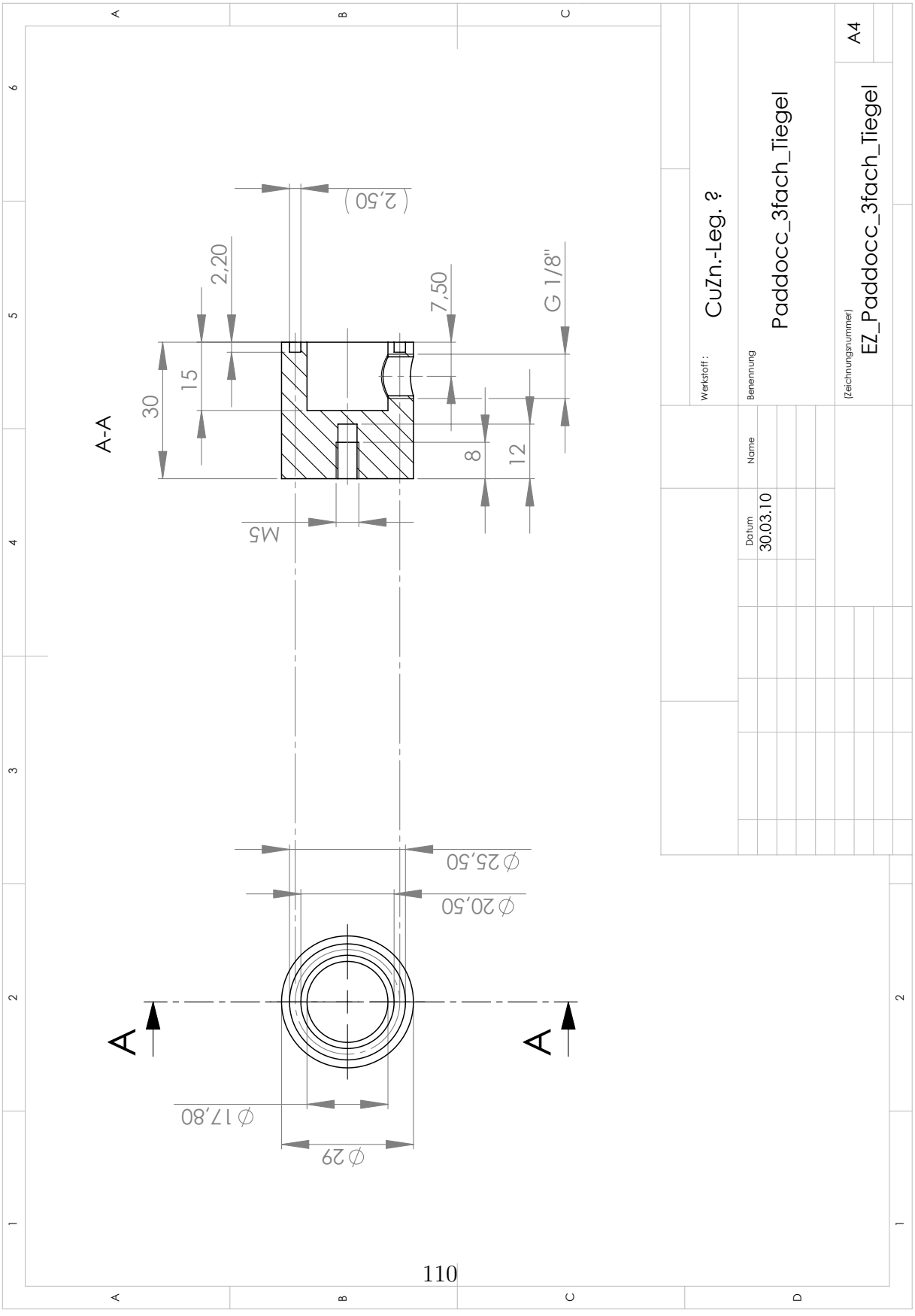
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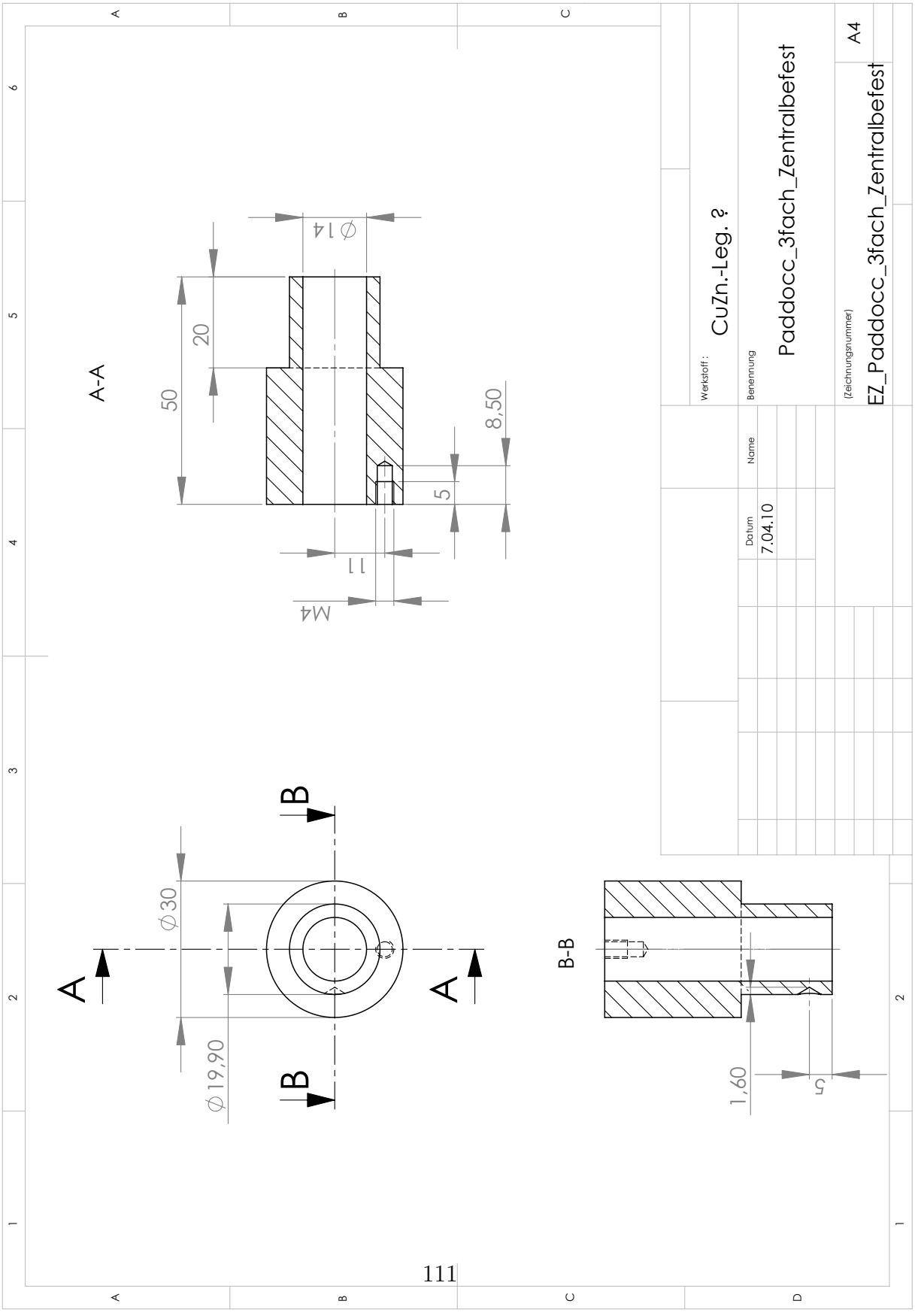
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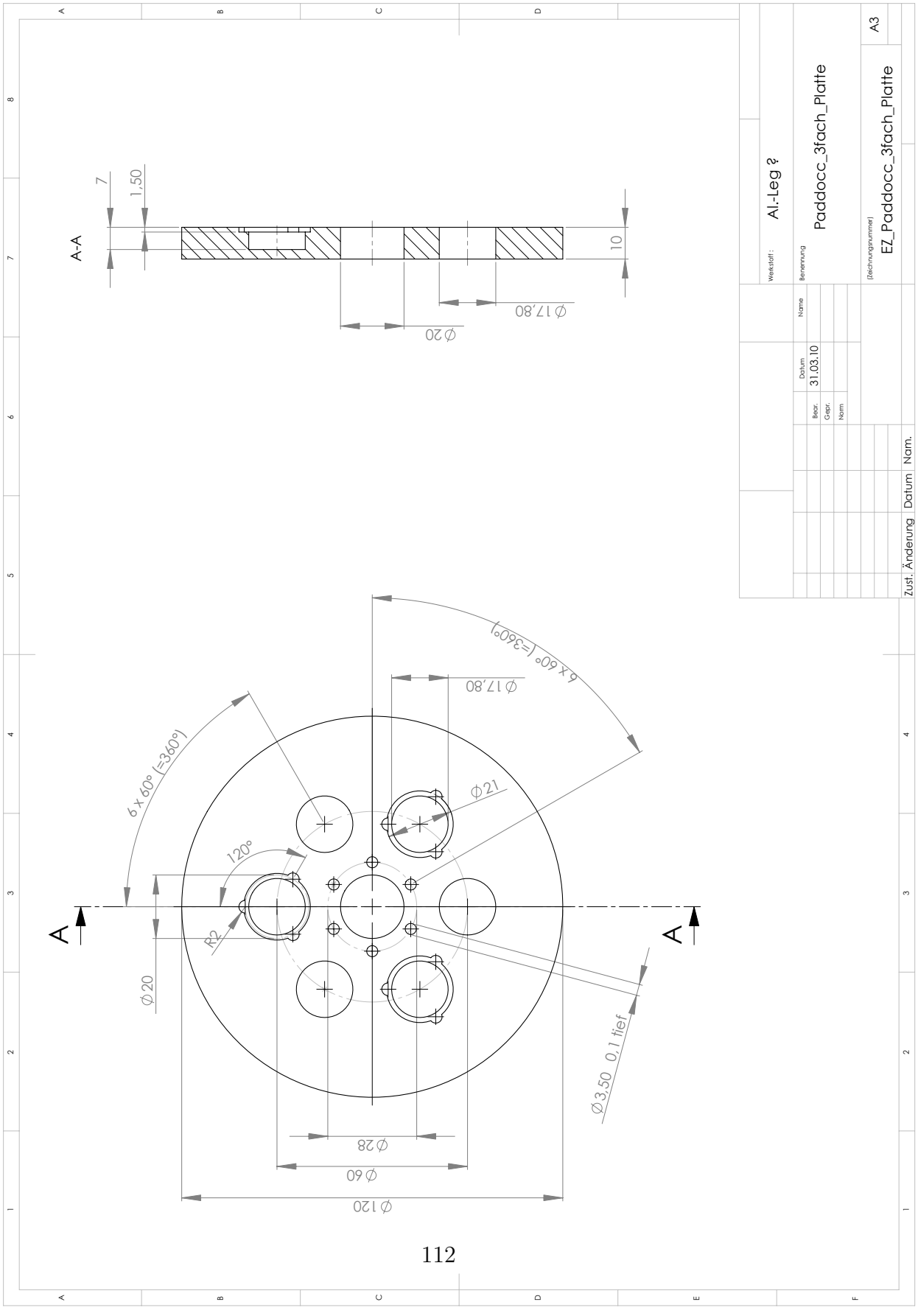
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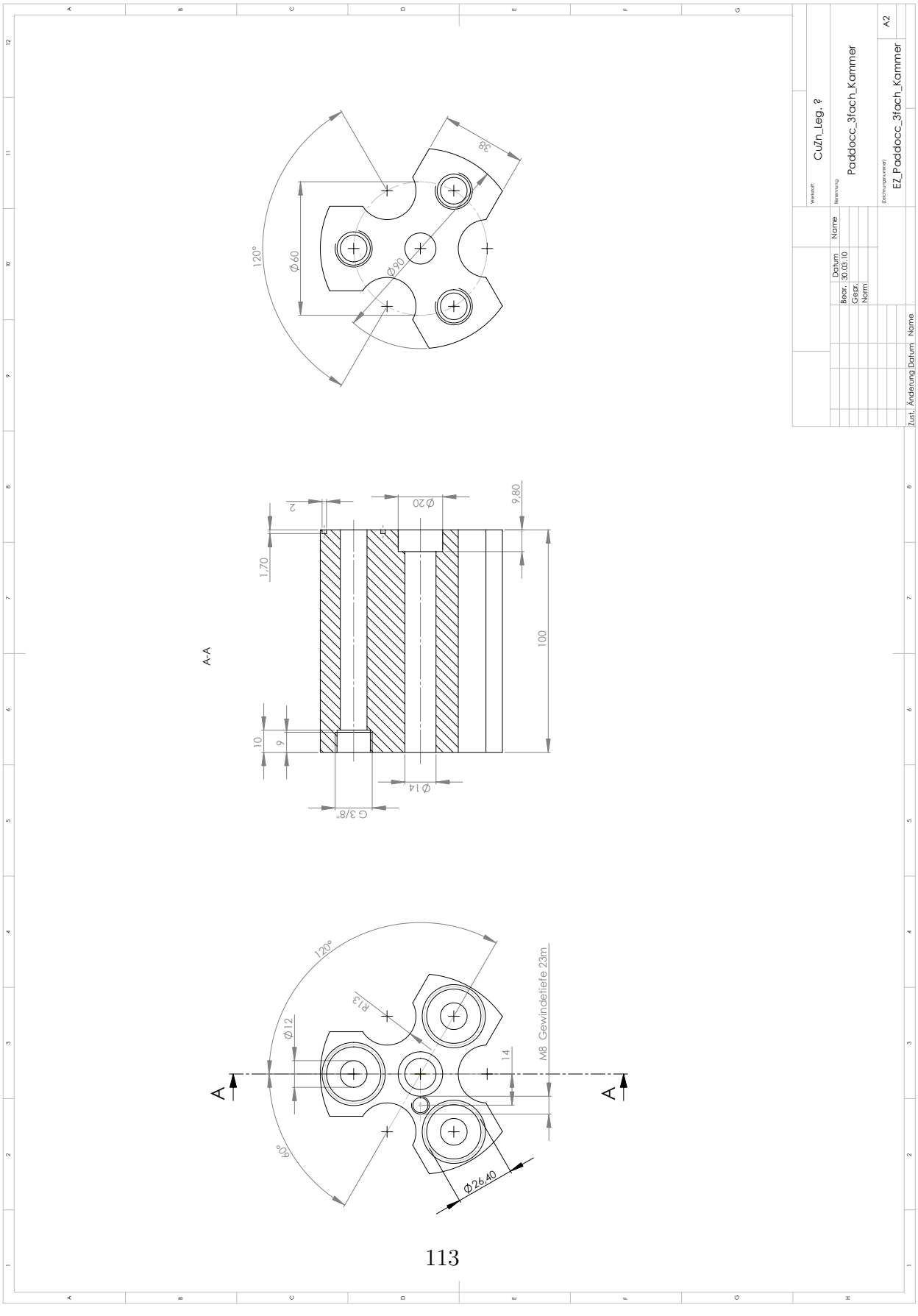
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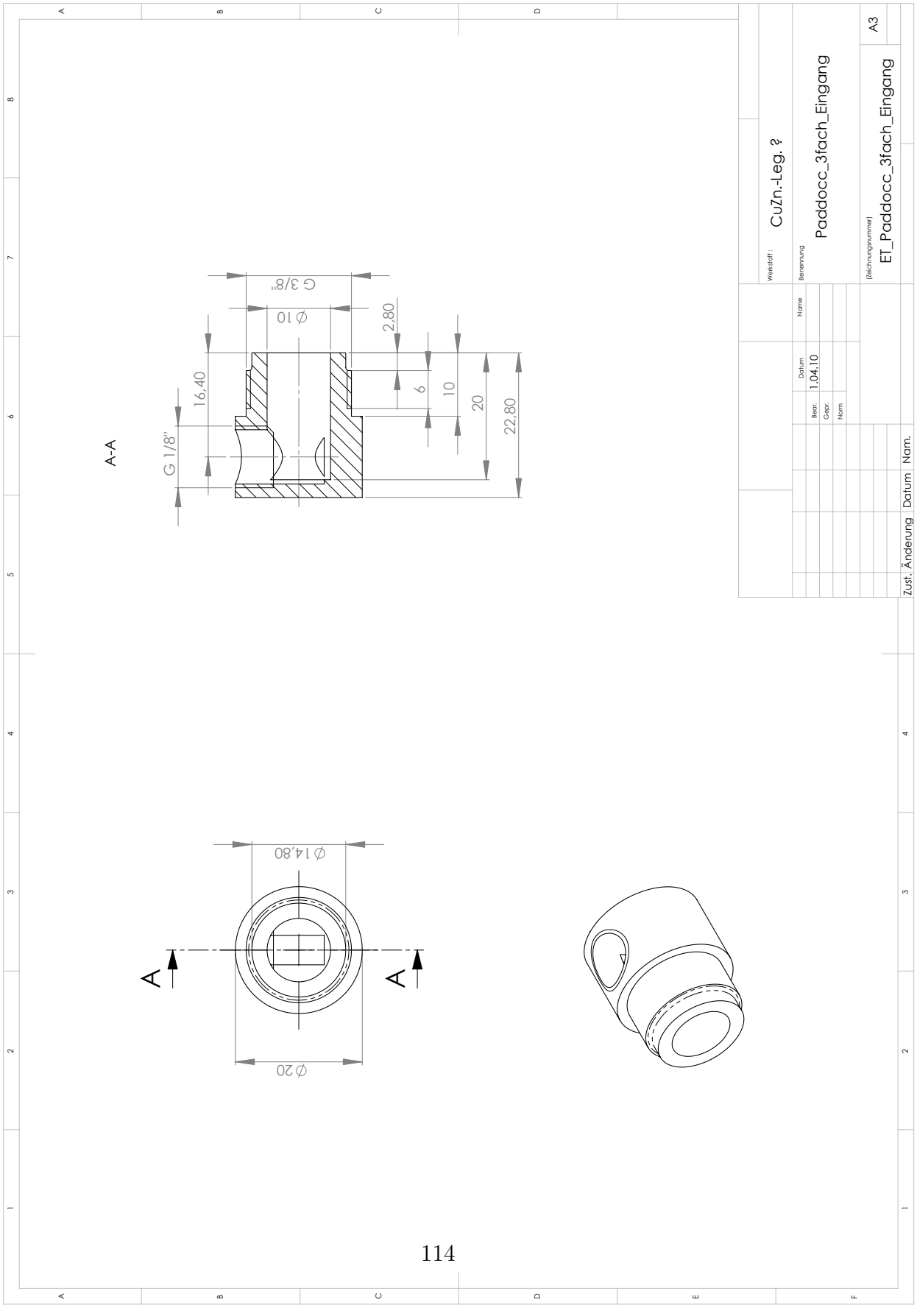
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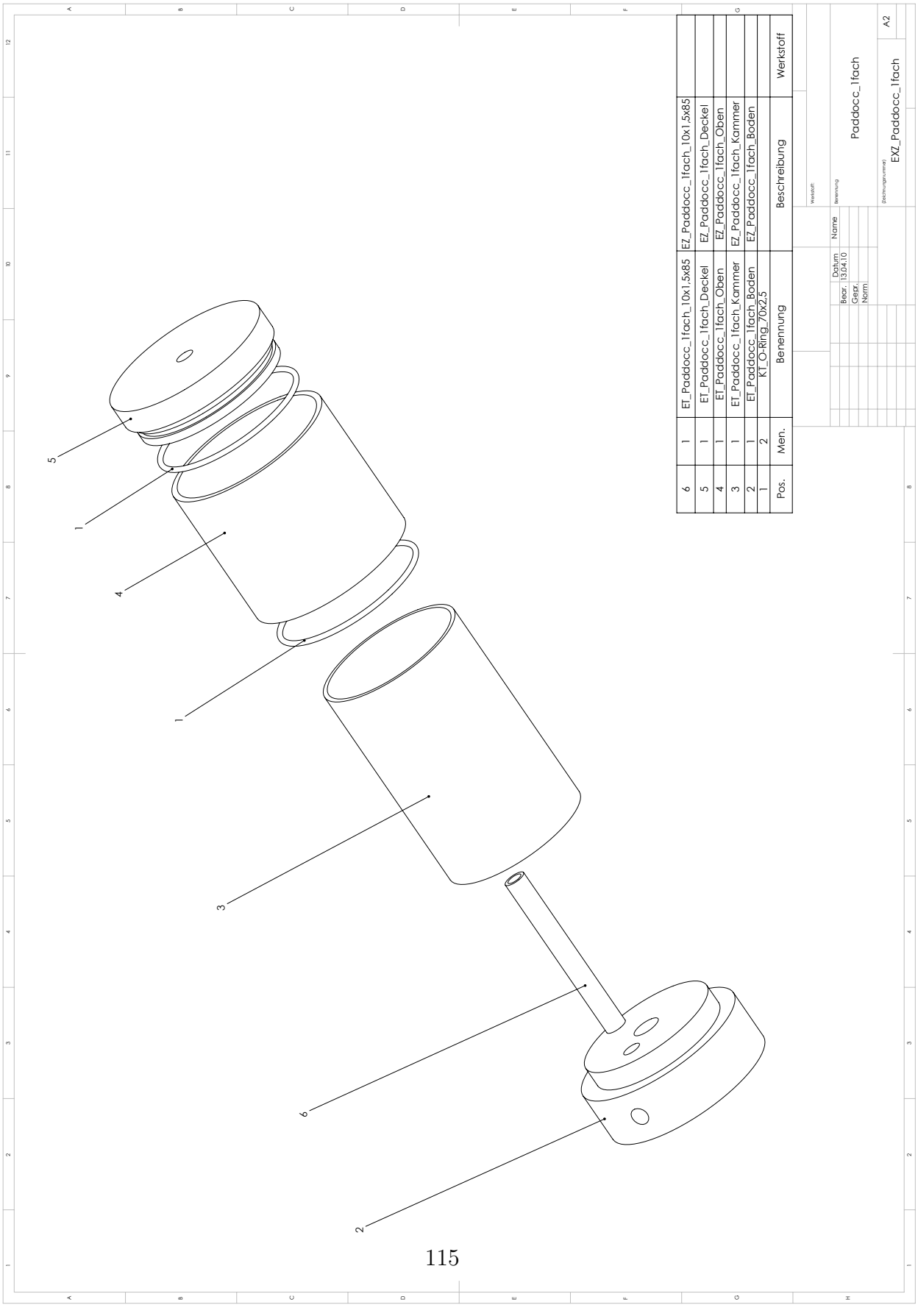
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4 1 ET_Paddocc_1fach_Oben EZ_Paddocc_1fach_Oben
3 1 ET_Paddocc_1fach_Kammer EZ_Paddocc_1fach_Kammer
2 1 ET_Paddocc_1fach_Boden EZ_Paddocc_1fach_Boden
1 2 KT_O-Ring_70x2,5
Pos. Men. Benennung Beschreibung Werkstoff
Datum Name
Bearb. 13.04.10
Gezr.
Norm
Paddocc_1fach
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A2
5
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3
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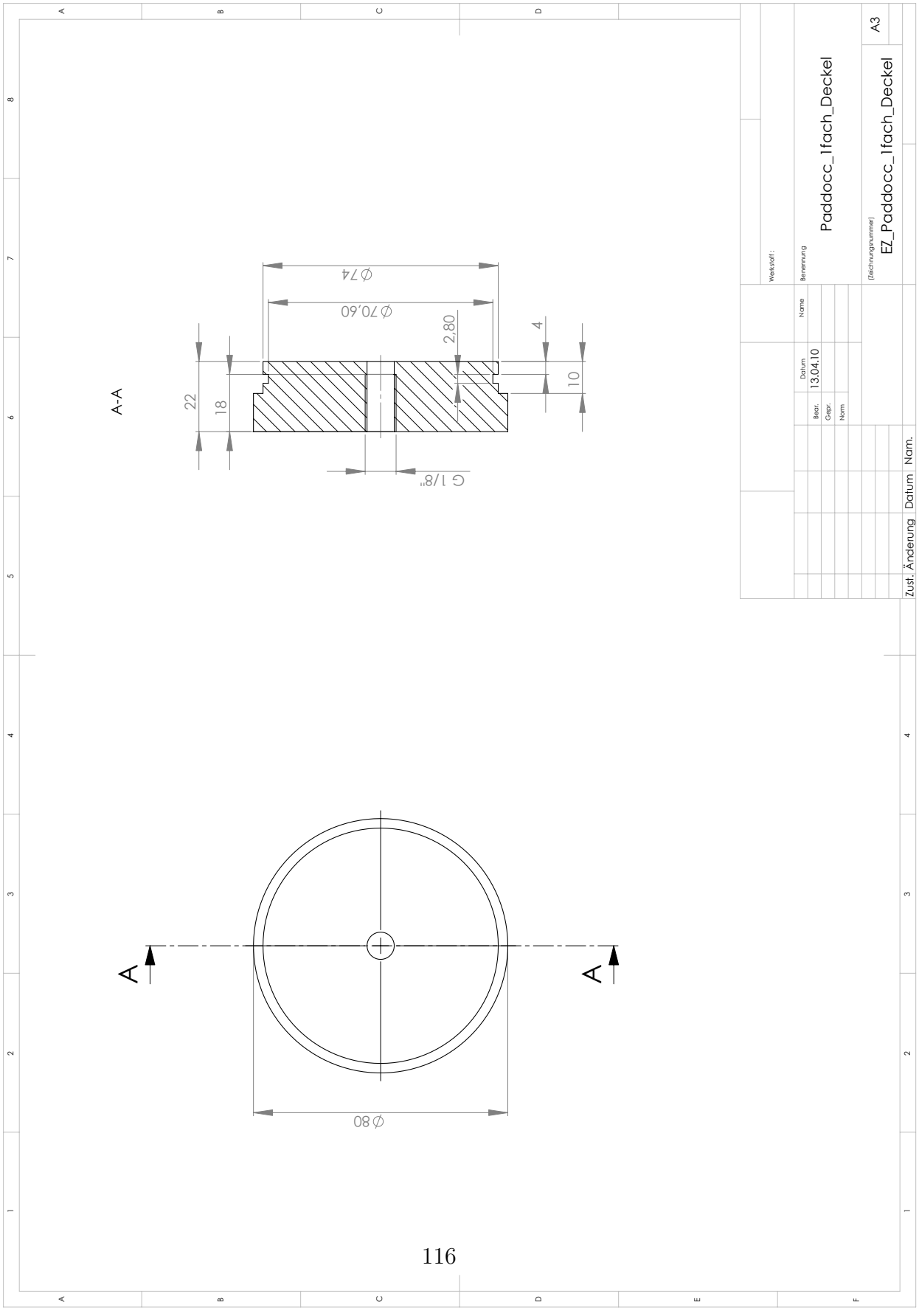
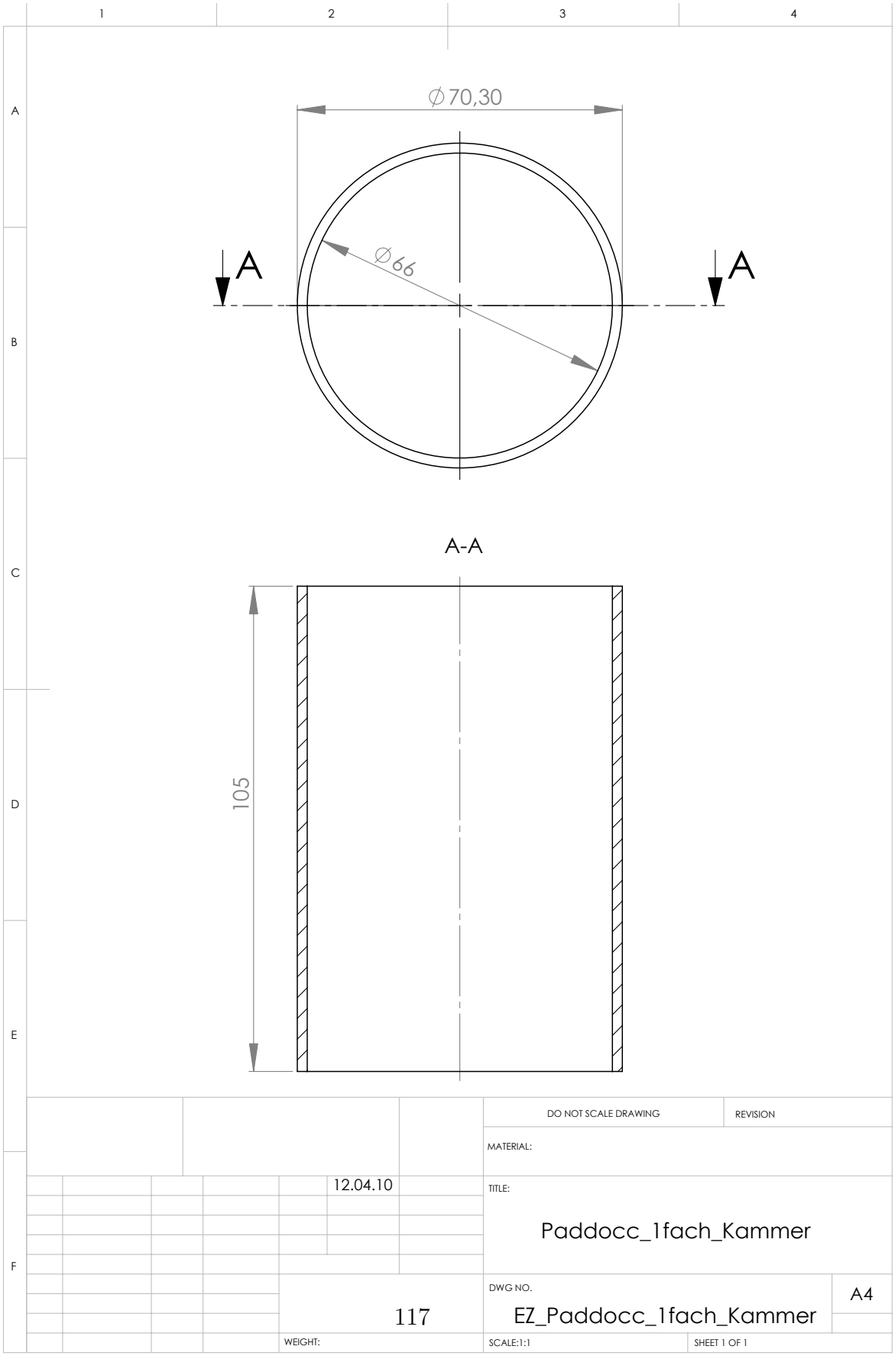
2


A-A
Ø74
Ø70,60
2,80
4
10
22
18
G 1/8"
A
A
Ø80
Werkstoff :
Benennung
Paddocc_1fach_Deckel
(Zeichnungsnummer)
EZ_Paddocc_1fach_Deckel
A3
Name
Datum
13.04.10
Bearb.
Gepr.
Norm
Zust. Änderung
Datum
Nam.


1
2
3
4
A
B
C
D
E
F
∅70,30
∅66
A
A
A-A
105
DO NOT SCALE DRAWING
REVISION
MATERIAL:
12.04.10
TITLE:
Paddocc_1fach_Kammer
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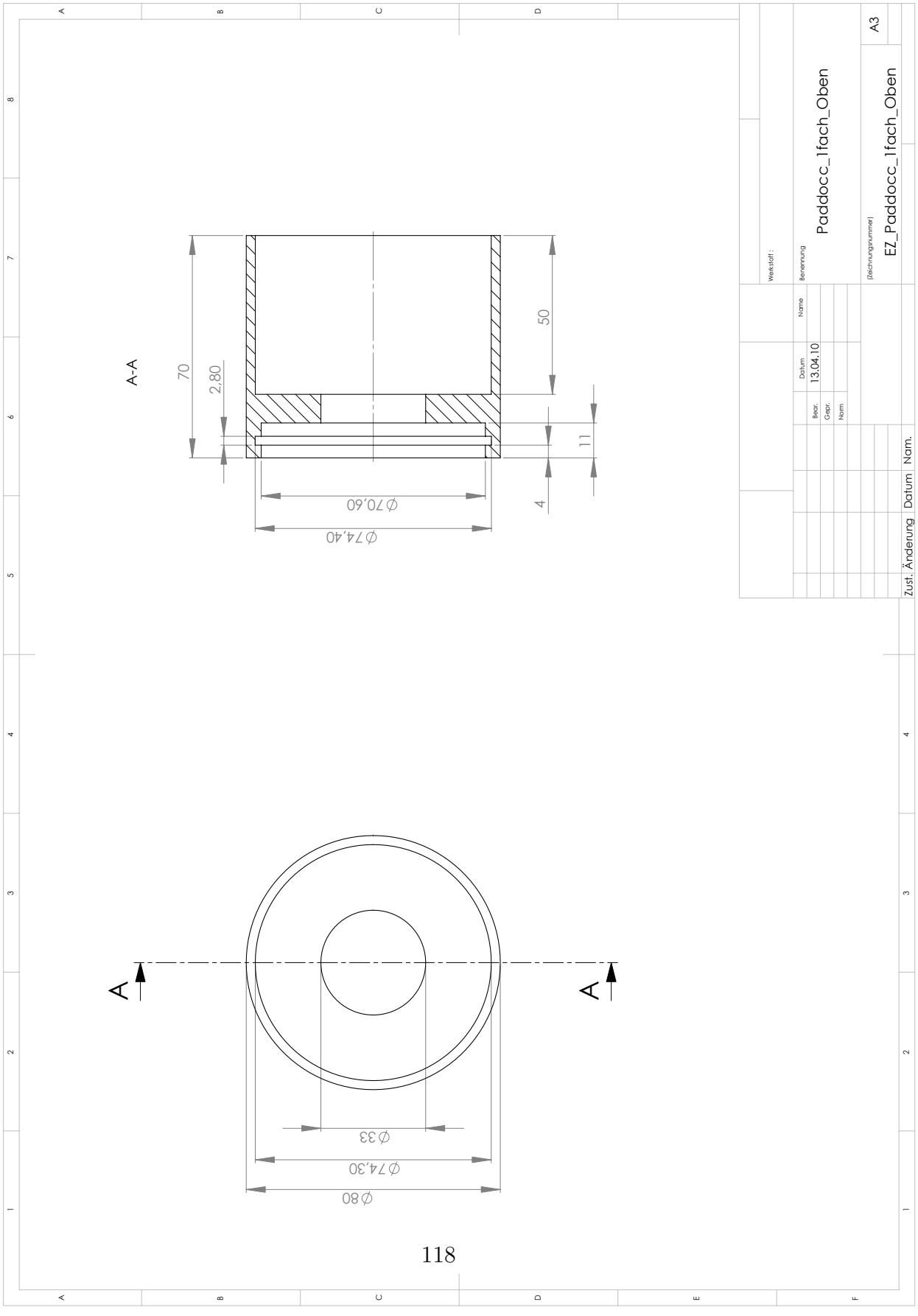
A4
117
WEIGHT:
SCALE:1:1
SHEET 1 OF 1


A-A
70
2,80
50
11
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Werkstoff :
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Paddocc_1fach_Oben
(Zeichnungsnummer)
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Gepr.
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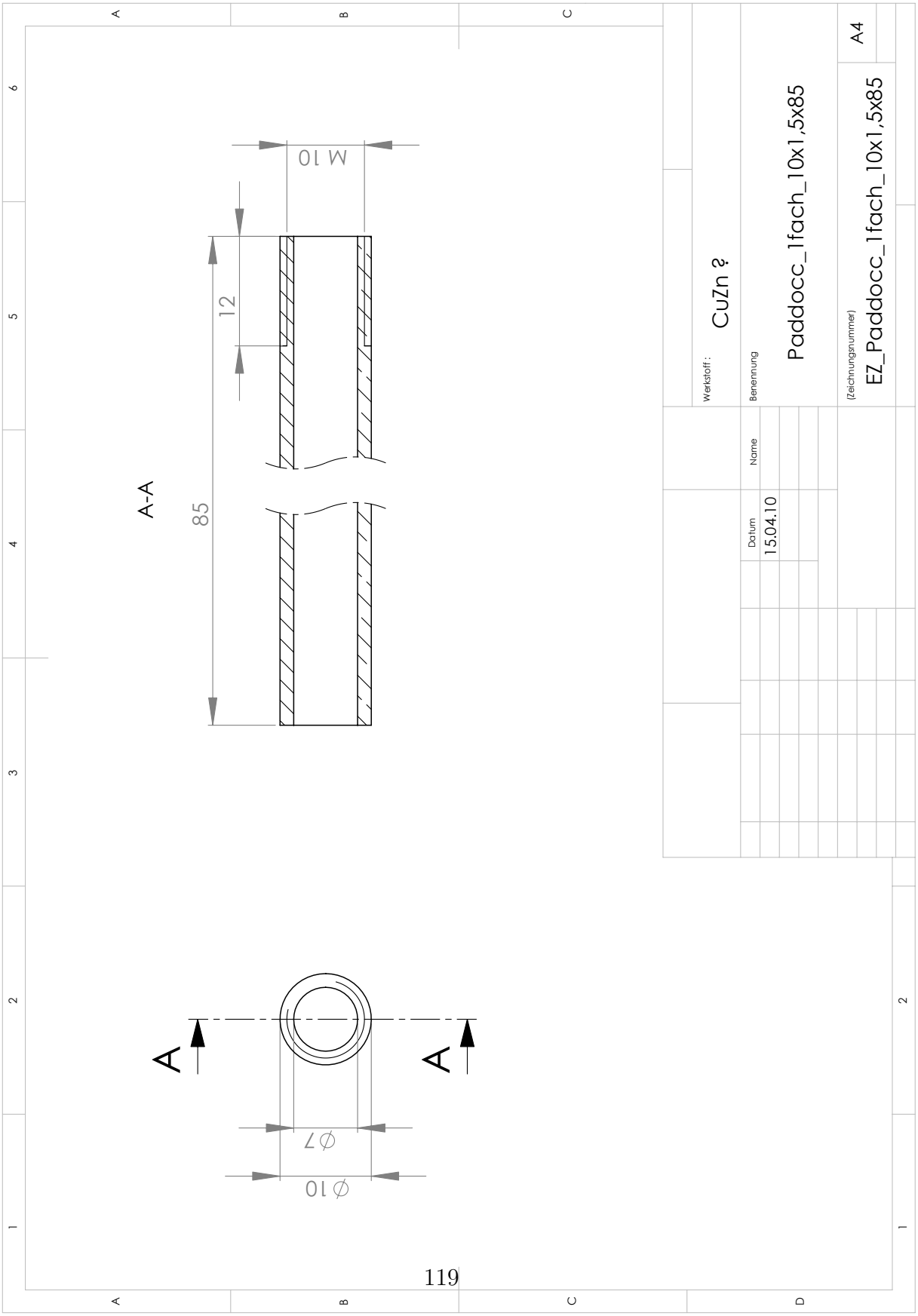
M 10
12
85
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A
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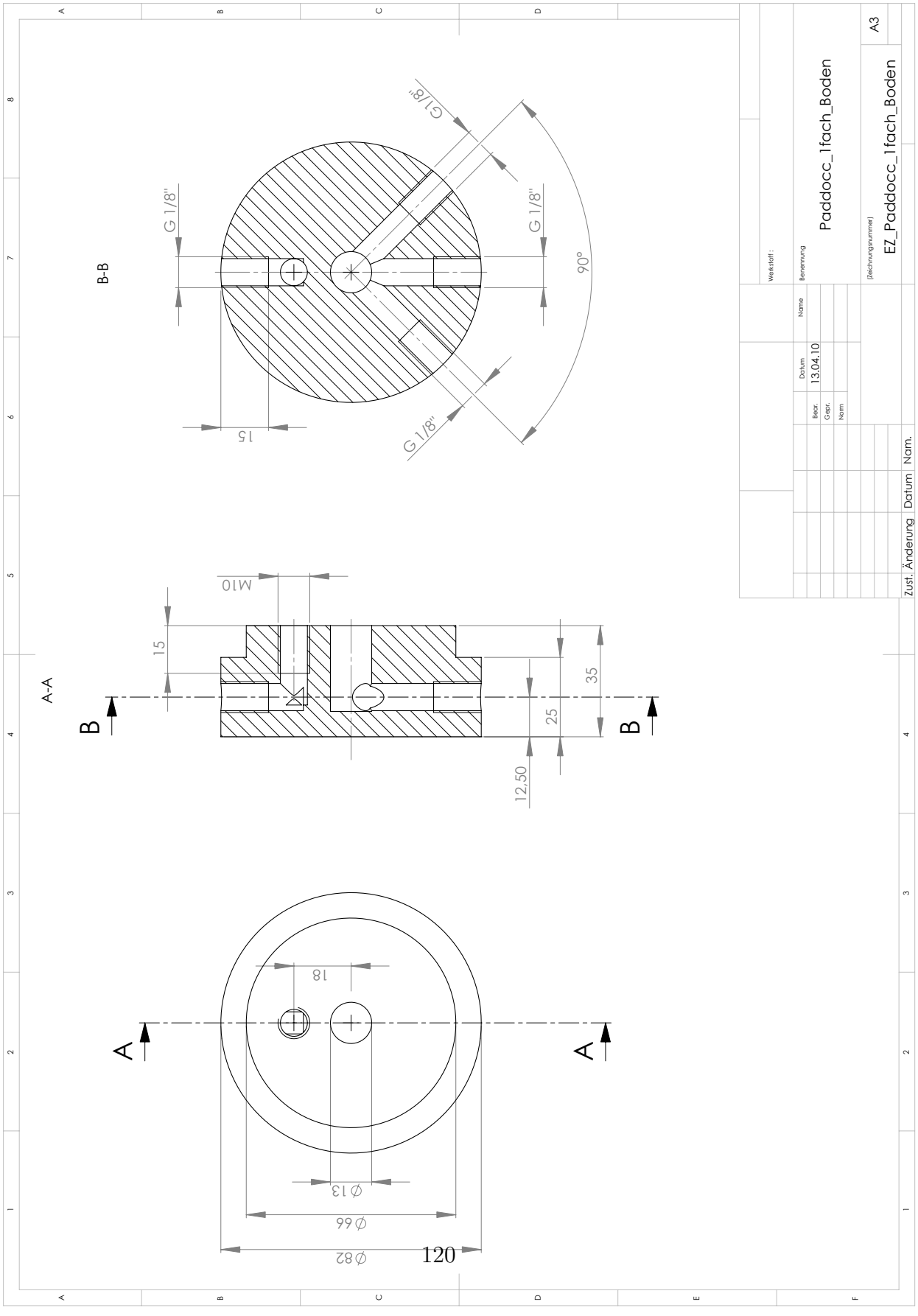
A4


B-B
G 1/8"
G 1/8"
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90°
15
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M10
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35
25
12,50
B
B
A
A
18
Ø 13
Ø 66
Ø 82
Werkstoff :
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Name
Datum
13.04.10
Bearb.
Gepr.
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(Zeichnungsnummer)
EZ_Paddocc_1fach_Boden
A3
Zust. Änderung
Datum
Nam.

C Curriculum vitae

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doctoral thesis

2007-2010	Biopharmaceutics and Pharmaceutical Technology, Saarland University, Saarbrücken, Germany

diploma thesis

2006-2007	Pharmaceutical Technology, Martin-Luther-University Halle-Wittenberg, Halle, Germany "Simulation des Walzenkompaktierprozesses mit nachfolgender Tablettierung und Charakterisierung von pharmazeutischen Hilfsstoffen"

undergraduate studies

2001-2006	Pharmacy, Martin-Luther-University Halle-Wittenberg, Halle, Germany

school

1992-2001	Bischöfliches Cusanus-Gymnasium, Koblenz
1988-1992	Grundschule Weißenthurm

D Publication list

Research papers

Hein S., Picker-Freyer K.M., Langridge J., Simulation of roller compaction with subsequent tableting and characterization of lactose and microcrystalline cellulose, Pharmaceutical Development and Technology, 13(6):523-532, 2008; DOI: 10.1080/10837450802288972

Hein S., Bur M., Kolb T., Muellinger B., Schäfer U.F., Lehr C.-M., Pharmaceutical Aerosol Deposition Device On Cell Cultures (PADDOCC) *in vitro*: design and experimental protocol, Alternatives to Laboratory Animals, 38(4):285-295, 2010

Hein S., Bur M., Schäfer U.F., Lehr C.-M., A new Pharmaceutical Aerosol Deposition Device On Cell Cultures (PADDOCC) to evaluate pulmonary drug absorption of metered dose dry powder formulations, European Journal of Pharmaceutics and Biopharmaceutics, accepted, 2010; DOI: 10.1016/j.ejpb.2010.10.003

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Hein S., Henning A., Bur M., Schneider M., Lehr C.-M., Particulate carriers for pulmonary drug delivery in: P. Gehr, C. Mühlfeld, B. Rothen-Rutishauser, F. Blank (Eds.) Particle lung interactions 2nd edition, Informa Healthcare, New York (2009), ISBN: 978-1420072563

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DOI: 10.1080/08958370902962283

Henning A., **Hein S.**, Schneider M., Bur M., Lehr C.-M., Pulmonary Drug Delivery: Medicines for Inhalation in: M. Schäfer-Korting (Ed.) Handbook of Experimental Pharmacology, Vol. 197: Drug Delivery, Springer, Heidelberg (2010), ISBN: 978-3642004766 DOI 10.1007/978-3-642-00477-36

Daum N., Kühn A., **Hein S.**, Schäfer U.F., Lehr C.-M., Isolation, Cultivation and Application of Human Alveolar Epithelial Cells in: Methods in Molecular Biology - Human

Cell Culture, submitted, (2010)

Poster presentations

Hein S., Bur M., Lehr C.-M., A new Pharmaceutical Aerosol Deposition Device On Cell Cultures (PADDOCC) as alternative method for biocompatibility and ADME screening, 7th World Congress on Alternatives and Animal Use in the Life Sciences, Rome, August 30 - September 3, 2009

Hein S., Bur M., Lehr, C.-M., A new Pharmaceutical Aerosol Deposition Device On Cell Cultures (PADDOCC) as method for combined measurement of aerosolisation, deposition, and absorption, DPhG Jahrestagung, Jena, September 28 - October 1, 2009

Hein S., Bur M., Schäfer U.F., Lehr C.-M., A new *in vitro* model for testing drug permeation of aerosol formulations: the Pharmaceutical Aerosol Deposition Device On Cell Cultures (PADDOCC), ZEBET-Symposium: 20 Jahre ZEBET, Berlin, October 26-27, 2009

Hein S., Bur M., Schäfer U.F., Lehr C.-M., The Pharmaceutical Aerosol Deposition Device On Cell Cultures (PADDOCC) as an *in vitro* model for testing dry powder formulations on pulmonary epithelial cells, 8th International Conference and Workshop on Biological Barriers - *in vitro* Tools, Nanotoxicology, and Nanomedicine, Saarbrücken, March 21 - April 1, 2010

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