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Biopolymer-Based Nanoparticles for Drug Delivery

by

Hanna Valo

ACADEMIC DISSERTATION

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Abstract

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Nanotechnology can be used to modify drug delivery by various approaches. Biopolymer-based nanoparticles represent a well-established option to formulate drug delivery systems. New therapeutic compounds are often insoluble or poorly soluble in water, which is a major factor in causing irregular and insufficient absorption, and reduced bioavailability of the drug. Increased dissolution rate can be achieved by decreasing the particle size to nanometer range. Another common reason for the reduced efficacy of the therapeutic compounds is their poor delivery to the desired site of action. Advanced nanoparticle formulations can be used to provide controlled release profiles or they can be combined with ligands for targeted drug delivery. Considering the current needs to produce stable nanoparticle systems for the controlled and actively targeted drug release, the versatile group of biopolymers may offer these functionalities.

One topic of this work was to set-up the electrospray apparatus for the production of poly(lactic acid) (PLA) drug nanoparticles. By utilizing electrospray, it was possible to produce spherical drug-loaded PLA-particles with approximately 200 to 800 nm diameters. The main benefits of the electrospray method were to control the particle size as well as the possibility to entrap both hydrophobic and hydrophilic drugs into the polymeric nanoparticles.

Second topic of this work was to utilize amphiphilic proteins, hydrophobins, to provide a layer around the drug nanoparticles that can be functionalized by protein engineering techniques. Adsorption of the protein onto the particle surface restricted the particle growth after the nanoparticles were formed, and it also produced a layer around the hydrophobic drug that was possible to functionalize further. Hydrophobin-mediated nanoparticle synthesis was a fast and effective process that was also easy to up-scale.

As third topic, nanofibrillar celluloses (NFCs) from various origins were studied as alternative biopolymer carriers for drug nanoparticles. The nanostructured cellulose matrix, an aerogel, prevented the aggregation of the hydrophobin coated nanoparticles during the freeze-drying and storage. Controlled drug release applications could be designed and enabled by utilizing the various modifications of NFC matrices.

As a result of this thesis, knowledge about the versatile biopolymer-based materials was provided as a means to construct stable nanoparticle formulations that can offer versatile applications for pharmaceutical nanotechnology.

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List of original publications

This thesis is based on the following publications. The publications are referred to in the text by their respective roman numerals (**I-IV**).

I Valo, H., Peltonen, L., Vehviläinen, S., Karjalainen, M., Kostiainen, R., Laaksonen, T. and Hirvonen J. Electrospray Encapsulation of Hydrophilic and Hydrophobic Drugs in Poly(l-Lactic Acid)Nanoparticles. Small 5:1791-1798, **2009**.

II Valo, H., Laaksonen, P., Peltonen, L., Linder, M.B., Hirvonen, J. and Laaksonen T. Multifunctional Hydrophobin: Toward Functional Coatings for Drug Nanoparticles. ACS Nano 4:1750-1758, **2010**.

III Valo, H., Kovalainen, M., Laaksonen, P., Häkkinen, M., Auriola, S., Peltonen, L., Linder, M.B., Järvinen, K., Hirvonen J. and Laaksonen T. Immobilization of Protein-Coated Drug Nanoparticles in Nanofibrillar Cellulose Matrices – Enhanced Stability and Release. J. Controlled Release 156:390-397, **2011**.

IV Valo, H., Arola S., Laaksonen P., Torkkeli M., Peltonen, L., Linder, M.B., Serimaa R., Kuga S., Hirvonen, J. and Laaksonen T. Controlled Drug Release from Nanofibrillar Cellulose Aerogels. Submitted manuscript, **2012**.

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Abbreviations and symbols

AFM	Atomic force microscopy
AUC	Area under (*e.g.* the plasma concentration) curve
BC	Bacterial cellulose
BCS	Biopharmaceutics classification system
BDP	Beclomethasone dipropionate
BSA	Bovine serum albumin
C_{max}	Maximum concentration reached by a drug in a physiological fluid
CCD	Charge coupled device
CBD	Cellulose binding domain
DCBD	Double cellulose binding domain
DCM	Dichloromethane
DLVO-theory	Derjaguin, Landau, Verwey and Overbeek-theory
DNA	Deoxyribonucleic acid
DSC	Differential scanning calorimetry
EE	Entrapment efficiency
EtOH	Ethanol
EPR	Enhanced permeability and retention
GFP	Green Fluorescent Protein
MCC	Microcrystalline cellulose
MeOH	Methanol
MT-DSC	Modulated temperature differential scanning calorimetry
HFBI	Hydrophobin I
HFBII	Hydrophobin II
HFBI-DCBD	Hydrophobin I-coupled with two cellulose binding domains
HPLC	High performance liquid chromatography
ITR	Itraconazole
PI	Polydispersity index
PEG	Polyethylene glycol
PKPD	Pharmacokinetics-Pharmacodynamics
PLA	Poly(lactic acid)
PLGA	Poly(lactic-co-glycolic acid)
PCS	Photon correlation spectroscopy
MPS	Mononuclear phagocyte system
MW	Molecular weight
NFC	Nanofibrillar cellulose
RH	Relative humidity
rpm	Rotations per minute
ROP	Ring-opening polymerization
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscopy
SS	Salbutamol sulfate
TEMPO	2,2,6,6,-tetramethylpiperidine-1-oxyl
T_g	Glass transition temperature
T_m	Melting temperature

TEM	Transmission electron microscopy
THF	Tetrahydrofuran
TRE	Trehalose
UV	Ultraviolet
VT-XRPD	Variable temperature x-ray powder diffraction
WAXS	Wide angle x-ray scattering
XRPD	X-ray powder diffraction

1 Introduction

Recent trends have shown that a considerable number of lead optimization compounds suffer from low aqueous solubility or are even completely insoluble in water.[1] These compounds are included in the Biopharmaceutics Classification System (BCS) classes II and IV and they represent about 70-90% of the drugs in the pharmaceutical industry pipelines.[2, 3] If the poorly soluble drug candidate has reasonable membrane permeability (BCS class II), the drug dissolution is the rate-limiting step for absorption. Thus, low solubility causes irregular and delayed absorption, which may finally result in reduced bioavailability of the drug. On this account, the dissolution properties of the drug have far-reaching effects and, therefore, innumerable methods and formulations have been invented to overcome the problem. The improvement of drug solubility can be realized by modifications such as by formation of salt complex [4], pro-drug [5, 6], co-crystals [7], or by hydrates, solvates or amorphous forms [8-10].

Reduction of the particle sizes down to a sub-micron range utilizing different techniques has been seen as a potential answer to the dissolution problems for some time.[2, 3, 11-13] As described by the Noyes-Whitney equation [14], dissolution rate can be increased by increasing the reactive surface area by decreasing the particle size. Smaller drug particles have been shown to further improve the bioavailability.[11] In general, synthetic or mechanic pathways to reach the smaller particle sizes have been divided into top-down and bottom-up approaches. Several comprehensive reviews including both top-down and bottom-up methods concerning particle engineering processes have been written.[15-17]

Besides the particle size reduction methods, many advanced drug delivery platforms have been developed to obtain more precisely controlled and localized drug release that could significantly enhance the therapeutic effect of a drug. The most common ones, all with their own properties, strengths, and weaknesses are cyclodextrins [18,19], emulsions and microemulsions [20, 21], lipid carriers [22, 23], silicon- and silica-based nano-systems [24, 25], dendrimers [26] as well as nanoparticles based on polymer carrier systems [27-30] just to mention some examples.

In this thesis, polymer-based nanoparticles made of very diverse and versatile groups of biodegradable polymers have been taken into consideration as advanced drug delivery systems. As demonstrated, the small size without a doubt improves dissolution rates and *in vivo* bioavailability. However, challenges still exist. One of the main problems in nanoparticle engineering is to maintain the stability of the primary particles since otherwise the advantages of the small particle size are lost.

Physical instabilities are the major challenges with the colloidal nanoparticle systems and typically surfactants and/or polymers are used to stabilize the nanosuspensions. In this thesis, assemblies of biopolymer-based nanoparticles were studied to complete functional pharmaceutical formulations as well as to resolve post-processing and long-term storage challenges for the used methods and excipients. The most commonly used excipients suffer from the fact that they are not able to stabilize the formulation indefinitely, stabilization effect might be lost *e.g.* during the drying process, or during the storage.[31, 32] The current and new stabilizers and surface modifiers are often acting as non-active excipients employed purely to maintain the physical stability of nanoparticles. However, apart from the size, surface properties are key factors controlling the *in vivo* fate of the nanoparticles. Indeed, the surface is a first part that interacts with the components of the surrounding physiological medium and epithelia/tissues. Thus, nanoparticles with a precise drug delivery goal, whether

localization or controlled release, need to show very well defined surface properties.[33] Surface modifiers can bring a more active functional role in targeting, permeation as well as sustained/controlled release of the drug.

As a summary of the above mentioned facts, the main idea of this thesis was to prepare biopolymer-based nanoparticles that increase or control the dissolution rate and also the bioavailability of the drug compounds. Further, nanoparticles with functional coating layer around the hydrophobic drugs provide a way to improved stability and targeting of the system. These complex nanostructured compositions may offer versatile tools for the development of more sophisticated drug delivery systems to exceed obstacles faced by the conventional formulations.

2 Literature review

2.1 Nanoscale drug delivery systems

2.1.1 Particle size

According to the definition of nano, pharmaceutical nanoparticles are considered as submicron-sized drug crystals, particles or drug delivery vehicles, also known as nanocarriers. In general, colloidal dispersions or dry solid particles with dimensions in the range from 10 nm to a few hundred nanometers are preferred depending on the therapeutic target and purpose. In addition to the therapeutic nanocarriers, other micro- and nanostructured materials can be potentially beneficial for the engineering of composite drug delivery applications.

In pharmaceutical technology, the particle size reduction has been used as a powerful tool to enhance the oral administration of poorly-soluble drug compounds. Biopharmaceutics Classification System (BCS) divides the drugs into four classes according to their solubilities and intestinal permeabilities.[34] Low aqueous solubility and high intestinal permeability are the characteristics of BCS Class II poorly-water soluble drugs. Thus, low solubility may strongly restrict the absorption of the drug resulting in incomplete bioavailability. Accordingly, the dissolution rate is the rate-limiting step which determines the bioavailability of the BCS class II drugs. Reduction of the particle size leads to exponential increase of the number of molecules on the surfaces of the particles. Thus, an increased surface area of the system leads to increased surface interactions with particles' surroundings improving e.g. dissolution to gastrointestinal fluids. Therefore by decreasing the particle size the dissolution rate can be increased. The important factors for the kinetics of drug dissolution rate can be expressed by the Noyes-Whitney equation [14] (1),

$$\frac{dm}{dt} = \frac{DA}{h}(C_s - C_b) \tag{1}$$

where the rate of mass transfer of drug (solvate) particles into a solvent (dissolution rate) (dm/dt) is dependent on the surface area available for dissolution (A), the diffusion coefficient of the drug (D), the thickness of the boundary layer of the dissolving drug (h), the saturation solubility of the drug (C_S) and the concentration of the drug at particular time point in the dissolution medium (C_b). After administration, physicochemical factors have a strong influence on the factors shown in Eq. (1). Improved dissolution rate and maximized amount of soluble drug available for absorption are commonly realized due to the particle size reduction. Kawabata et al. (2011) have reviewed that, for example, nanocrystal formulations have been found to show 1.7–60-fold maximum plasma concentrations (C_{max}) and 2–30-fold increases in area under the plasma concentration-time curves (AUC), after oral administration compared to crystalline formulations with micrometer particle size.[11]

Apart from the enhanced dissolution of the poorly-soluble drug compounds by size reduction, another goal in designing pharmaceutical nanoparticles is to control the drug delivery in order to release pharmacologically active agents at the therapeutically optimal rate and site. [2, 31, 35-37] Nanocarriers, like polymer-based nanoparticles composed of advanced materials, can offer possibilities to control and sustain the drug release, leading to

improved pharmacokinetic and pharmacodynamics (PKPD) properties or lowered toxicity. After administration, unmodified free drug compounds are widely distributed throughout the body affecting other organs than the desired tissues or cells. Therefore, the doses of the drugs are often limited due to the adverse side effects at the expense of efficient treatment.[38]

2.1.2 Surface modification

Modification of the nanocarrier surface properties can be utilized to increase the residence time in the blood.[39] If an intravenously administered particle does not dissolve immediately, blood proteins are adsorbed onto its surface resulting in recognition by the immune system (opsonization). The particles are then taken up by the mononuclear phagocyte system (MPS), which leads to fast clearance from blood circulation. Delays in opsonization [40] can be achieved either by the adsorption of polymers or surfactants on the surfaces of the nanocarriers (non-covalent attachment) or by covalent attachments of molecules onto the surfaces. One of the most widely utilized strategies is adsorption or grafting of poly(ethylene glycol) (PEG) molecules on the nanocarrier surface.[41] For example, steric PEG coating increased the half-life of bovine serum albumin (BSA) loaded poly(lactic-co-glycolic acid) (PLGA) nanoparticles from 13.6 min to 4.5 h.[42] Apart from hydrophilic coatings (*e.g.* PEGylation), adjusted surface charges of the nanocarriers may play a significant role in the body.[43] It has been widely shown that nonspecific cellular internalization and protein adsorption during the circulation can be affected by changing the surface charge.[43, 44] Positively charged nanoparticles have higher rates of phagocytosis compared to neutral or negatively charged formulations, resulting in shorter blood circulation half-lifes.

Encapsulation of sensitive drug molecules into particulate nanocarriers can protect them against degradation *in vitro* and *in vivo,* but may also offer more patient friendly administration routes. Polymeric nanoparticles can be highly beneficial for delivering and protecting biologically active entities, such as proteins and peptides, as well as RNA and DNA therapeutics.[45] Enzymatic and hydrolytic degradation can be delayed by encapsulation or coupling of these molecules with nanocarriers, and thus, protecting them against elimination *in vivo*. For example, various nanotechnological carriers based on polymeric materials have been developed to enable the oral administration of insulin.[46]

Nanocarriers, nanosized compositions of drugs and polymer, may enable specifically targeted or passively localized drug delivery.[47] For example, the use of nanocarriers as drug delivery vehicles for anticancer therapeutics are based on the enhanced permeability and retention (EPR) effect at the tumor site, due to the particular physical properties of tumors, such as leaky blood vessels and poor lymphatic drainage.[48-51] With the small size and the hydrophilic surfaces, the nanocarriers tend to increase the residence time in blood, which facilitates the passive accumulation (targeting) to the tumors.[52] Another example of the localized release systems is drug delivery to mucosal surfaces, such as gastrointestinal tract, nose, eyes, lungs or the female reproductive system.[37, 53] A mucoadhesive polymer coating of particles may interact with the mucous surface, leading to an increased residence time in close contact with the mucosa and also to an increased drug stability against digestive enzymes.[54-56]

Active targeting can be reached for example by functionalization of the nanocarriers' surface with specific targeting moieties, such as antibodies [57], receptor binding molecules, peptides [58], proteins [59] and saccharides [60, 61], in order to deliver the therapeutic compound to specific cells, tissues or organs.[62, 63] For example, human serum albumin

nanoparticles coupled to transferrin or transferrin receptor monoclonal antibodies enabled a significant loperamide transport across the blood-brain barrier against the transferrin receptor in the brain.[64] Overall, due to the more advanced and targeted methods, systemic toxicity of the medical treatments can be reduced by using nanoparticles or carriers.

2.2 Preparation methods for drug nanoparticles

Several strategies are available to improve the therapeutic performance of low-soluble drug compounds. To date, no single method or material has emerged as an ideal solution. Examples of these approaches and production methods with their advantages are listed in Table 1. Production methods can be classified into the top-down and bottom-up approaches. Top-down methods involve size-reduction of larger particles to the nanometer level. The most commonly used top-down methods are wet milling and high pressure homogenization, which have resulted in a few commercial products.[12, 65, 66]

In contrast to the top-down techniques, in bottom-up methods the nanocarriers are synthesized starting from the molecular level in a solution based on self-assembly. Conversion of the dissolved molecules into nanodispersed system is accomplished by precipitation or condensation.[67] In spite of the similar principles, the spectrum of the drug nanocarriers made by the bottom-up methods is wide.

Table 1 Examples of nanoscale drug delivery approaches.

Nanocarrier system	Production method	Advantages	References
Lipid based nanocarriers liposomes, niosomes, microemulsions, solid lipid nanoparticles	Formed by phospholipids dispersed in water. The lipid core is stabilized by surfactants.	Biocompatible and biodegradable. Suitable for both hydrophilic and hydrophobic drugs. Avoidance of organic solvents. Size, charge, and surface functionality can be modified. Established liposomal products on the market.	[68-71]
Polymeric /polymer- based nanoparticles	Electrospray, Precipitation, Salting out, Aerosol flow reactor methods, Spray drying, Double-emulsification	High surface modification capacity. Applicable for versatile materials. Possibility to load and stabilize a large range of organic or biological molecules. The drugs can be released in a controlled manner; through surface or bulk erosion, by diffusion through the polymer matrix, swelling followed by diffusion, or in response to the local environment.	[72-74]
Porous silicon- and silica-based nano-systems	Porous particle production by electrochemical etching and milling. Drug loading by immersion of the PSi particles or layers into the loading solution.	Applicable for loading and stabilization of a large range of organic or biological molecules. Solid dosage form. Release profiles can be controlled due to the pore size and functional groups. Carriers can be produced with near monodispersity.	[75, 76]
Nanocrystals (colloidal dispersions of drug crystals, stabilized by surfactants or polymers)	Wet milling. High pressure homogenization.	Cost-effective method. High drug loading. Scalable. Low batch-to-batch variation. Simple to perform. Solution and solid dosage forms. Avoidance for organic solvents. Established products on the market.	[3, 66, 77, 78]
Cyclodextrins	Drug-cyclodextrin complex formation. Kneading, Co-precipitation, Freeze-drying, Spray-drying.	Enhanced stability of therapeutic molecules. Taste masking (reduced bitterness). Established products on the market.	[18, 79]

In spite of the versatile and specific advantages, each delivery system has its limitations *e.g.* instability of the drug and/or carrier, polymorphic changes of the drug, low drug entrapment efficiency, difficulties in controlling size and polydispersity of the nanocarriers, as well as burst and/or uncontrolled drug release. Furthermore, each production method has

drawbacks, such as the need of organic solvents, high batch-to-batch variation and up-scaling, which provide challenges for translating these delivery or production methods for the industrial use.[22, 51, 67, 80, 81] More detailed challenges related to the preparation of biopolymer-based drug nanoparticles are presented in chapter 2.4.

Due to the versatile characters of various polymers and production methods, therapeutic molecules can be entrapped, attached, dissolved or encapsulated into the polymer matrices.[33] Depending on the structural composition of the drug and polymer, they can be classified, *e.g.*, to nanospheres and nanocapsules having different morphologies, properties and drug release characteristics. Nanospheres consist of matrix type structures and nanocapsules are vesicular systems, in which the drug is surrounded by a single polymeric membrane. In general, the term "nanoparticle" has been used to cover these systems. Depending on the preparation method and used biopolymer, the latter can perform a role as a passive colloidal stabilizer or also alternatively as an active substance having a significant role in the nanoparticle synthesis and bioavailability. Some bottom-up methods are based on self-assembly of the polymers, lipids and surfactants, while the others require some processing equipment. Both types of methods are represented in this thesis in detail, the electrospraying method and solvent-antisolvent precipitation method, respectively.

2.2.1 Electrospraying

The phenomenon behind the electrospraying (electrohydrodynamic atomization) has been known since the 19th century, but it was not until 1914 when John Zeleny [82] demonstrated fine droplet formation from a conical shaped meniscus, which was followed by innumerable experimental works, before the method became popular. The interest towards electrospraying has caught on multidisciplinary applications after the Nobel Prize work by Fenn on mass spectroscopy (2002).[83]

In the technique of electrospray, electric field is used to produce very fine droplets of charged liquid out from a thin capillary nozzle. A sufficient electric field between the liquid and counter electrode causes the accumulation of charges on the surface of the liquid. When the potential difference is sufficient, the electrostatic forces overcome the surface tension, the surface becomes unstable and the meniscus develops a conical shape [84]. If the cone is then disturbed by a further charge, it will break into small charged droplets which will detach from the liquid cone and fly towards a counter electrode. Several different spraying modes exist (Figure 1) [85, 86], but for the production of monodisperse nanosized droplets the cone-jet mode is the most desired, known as a Taylor cone [84, 87].

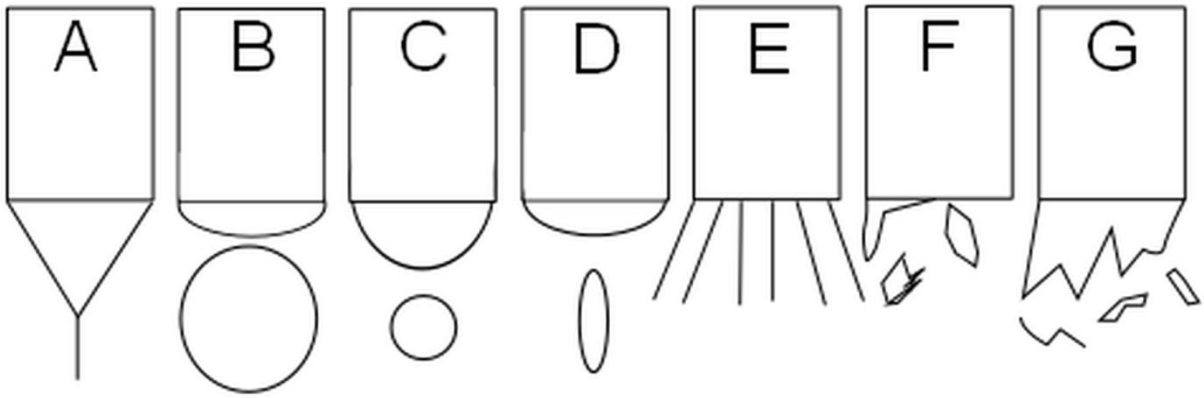


Figure 1 Different geometrical forms of liquid meniscus A) cone-jet (Taylor cone), B) dripping, C) micro-dripping, D) spindle, E) multi-jet, F) multi-spindle, G) ramified-meniscus. Modified from reference [86].

The droplet size and size distribution are controllable to some extent. Several variables, such as liquid properties, conductivity, flow rate, surface tension and viscosity, affect the properties of the end product, but *e.g.* flow rate and conductivity ratio should be fixed in order to obtain a stable spray.[88, 89] Overall, the stable cone-jet mode can be attained within a rather narrow range of flow rates and voltages. During the process, the solvent evaporates from the highly charged droplets and the droplets start to shrink, which also hinders the coalescence of the droplets with the same charge sign.

Electrospray generated by an electrostatic atomization has been applied in numerous studies in many disciplines [90, 91] including the synthesis of polymeric drug micro- and nanoparticles.[90, 92-94] Table 2 lists examples of recent studies of polymeric drug micro- or nanoparticles produced by the electrospray.

Table 2 Recent studies of polymeric drug micro- or nanoparticles prepared by electrospray.

Polymer	Particle size	Therapeutic molecule / References
Poly(lactic-co-glycolic acid) (PLGA)	0.6-1.4 μm 0.2-1.2 μm 0.1 -2.5 μm 5–10 μm 0.2-0.9 μm	Doxorubicin, rhodamine[95] Budesonide[96] Oestradiol[97] BSA and lysozyme[98, 99] Paclitaxel [100]
Poly(lactic acid) (PLA)	3-4.5 μm	BSA[101]
Chitosan	0.3-0.9 μm 0.5 μm	Doxorubicin[102] Ampicillin[103]
Elastin-like polypeptides	0.3–0.4 μm	Doxorubicin[104]
Poly-caprolactone (PCL)	1-15 μm	Taxol[105]

Nanoparticles with controlled size and narrow size distribution can be produced by dissolving the polymer and drug into a sufficiently conductive solvent capable of being electrosprayed. The solvent evaporates during the flight of the droplets towards a ground electrode. At some point, the driving force for crystallization/precipitation of the solute takes over causing particles to form, which can be collected from a liquid, gelatinizing bath or surface. Attaining the desired particle size, size distribution and morphology might be

challenging with this method. The right parameters have to be experimentally found for each case and the correct instrumentation set-up used.

Electrospraying is a suitable method for molecules that do not process well because it does not induce dissociation of the molecules. For example biological materials have been electrosprayed without changing the biological activity.[106] Other benefits of the electrospraying are simplicity and a possibility for continuous one-step production processes. With electrospray ionization, it is possible to process small amounts of materials without extensive losses. The method also enables water-insoluble and soluble drugs to be combined into a single nanoparticle.

2.2.2 Solvent-antisolvent precipitation

Antisolvent precipitation method is scalable bottom-up method to form drug nanoparticles.[107] Two miscible solvents are required. The drug is dissolved in the first solvent (*e.g.* organic solvent) and then added to the anti-solvent solution (*e.g.* water). Precipitation/crystallization is based on the formation of a supersaturated state of the drug due to the mixing of the solvent and the antisolvent, which results in a fast nucleation rate leading to the production of particles. Particle or crystal habits may be affected by lowering the surface energy by the adsorption of different additives, such as surfactants or polymers at growing interfaces of the solids.[108-112] Recently, modifications of the concept have been carried out in order to form variously structured nanoparticles. Examples of nanoprecipitated formulations are listed in Table 3. Overall, in this method and its variants, optimization of the supersaturated state and the growth and morphology of the particles can be affected, by controlling parameters like temperature, agitation, and antisolvent-solvent composition and ratio.[113-115] The nucleation rate is highly dependent on the supersaturation. Therefore, higher supersaturation state due to lower temperature and strong homogenizing mixing result in smaller particles, because a larger number of nuclei are formed.[116]

Table 3 Recent studies of drug micro- or nanoparticles prepared by polymer/surfactant assisted solvent-antisolvent precipitation.

Polymer/ Surfactant	Particle size	Therapeutic molecule / References
Poloxamer/polyvinylpyrrolidone	0.2-0.5 µm	Danazol, naproxen [108]
Hydroxypropyl methylcellulose	3.6 µm	Beclomethasone dipropionate [109]
Hydroxypropylcellulose	5 µm	Siramesine hydrochloride[110]
Hydroxypropyl methylcellulose	0.1-0.3 µm	Triclosan [112]

2.3 Biopolymers as excipients

Numerous synthetic and natural polymers have been extensively investigated as polymeric materials for drug delivery applications. To be considered as a suitable material to deliver drugs *in vivo,* a polymer needs to fulfill several requirements.[117] Firstly, it needs to be biocompatible and the possible degradation products should not be toxic or immunogenic. Secondly, the material should still have acceptable long-term stability and it should allow processing procedures required during the formulation. For the first reason, natural biodegradable polymers have been introduced as platforms and as stabilizing agents of nanoparticles for several drug nanoparticle formulations.

Natural polymers such as cellulose and its derivatives [118, 119], chitosan [120], alginate [122], albumin [123], gelatin [124, 125], and starch [121] and starch derivatives like cyclodextrins and polylactides [126], are widely available in nature and constitute an important class of biopolymers for immobilization of biological and chemical entities.

Biosurfactants are an alternative to the common natural polymers.[127] Biosurfactant is a general term for compounds that are produced by microorganisms, either extracellularly or as a part of the cell membrane, by bacteria, yeasts and fungi, having pronounced surface and emulsifying activities due to the amphiphilicity.[128, 129] Most biosurfactants comprise of hydrophobic moieties consisting of saturated, unsaturated, or hydroxylated fatty acids, or fatty alcohols, together with an anionic/cationic or neutral hydrophilic moiety containing mono-, oligo- or polysaccharides, peptides, or proteins. They are often secondary metabolites of microorganisms having essential functional role for the host biological functions and survival in the environment.[130-132] In addition to the surface activity, properties such as biodegradable nature, low toxicity and degradation to non-toxic end products in an aqueous environment, high tolerance to extreme pH-values, temperature and ionic strength and antimicrobial activity, make them very useful for many applications.[133] Recently, proteins' multifunctionality, biodegradability and uniform characters have been studied in food technology [134], and for drug delivery purposes [59, 135] and therapeutic applications. A typical protein surfactant is *e.g.* fungal hydrophobin, an amphiphilic protein from *Trichoderma reesei*.[136, 137]

Some of the inherent or refined properties of polymeric biomaterials that can affect to their biocompatibility, biodegradation, drug entrapment and release, as well as processing, include crystallinity, chemical structure, molecular weight, solubility, hydrophilicity/hydrophobicity, water absorption, degradation and erosion mechanisms. The interactions, affinity and influence of the drug with the polymeric material have also an impact on nanoparticle formulation and functionality.[138, 139]

2.3.1 Hydrophobins

Hydrophobins are a group of proteins identified first from *Schizophyllum commune* [140] and lately related to filamentous fungi more widely.[141, 142] Hydrophobins are secreted to surroundings or retained in the fungal structures, involving fungal development in many stages dominated by the surface activity and surfactant-like properties.[143, 144] A adsorbed hydrophobin films enable the growth of fungal aerial hyphae, formation of protective structures, and mediate the attachment of fungi to solid surfaces.[140, 145, 146]

Hydrophobins can be considered safe for human use because of their presence in *e.g.* common button mushrooms, *Agaricus bisporus* and fungus-fermented food.[137, 147] Hydrophobins can reduce immune recognition of airborne fungal spores by masking the surface layer and, hence, prevent immune response.[148] On the other hand, they may also act in pathogenic infections by mediating the attachment into the host organism.[149]

Traditionally, hydrophobins have been divided into two classes (referred to as Class I and II) on the basis of differences in amino acid sequences and their hydropathy patterns and also based on the aqueous solubilities of their assembled forms.[150] Both classes have conserved eight cysteine residues in their amino acid sequences, but otherwise the sequences between and within the classes are heterogeneous. Class I hydrophobins (*e.g.* SC3) form aggregates and assemblies which can be only dissociated by using strong acids like trifluoroacetic acid and formic acid.[151] Although, class II hydrophobins (*e.g.* HFBI, HFBII) form monomers, dimers and tetramers depending on their concentration, they are highly

soluble in water, even at concentrations over 100 mg ml^{-1}.[152] The secondary structure of class II hydrophobins, HFBI and HFBII, does not change during the adsorption at the interface. This can also explain the faster self-assembly of class II hydrophobins leading also to a faster reduction in the water surface tension.[153-155] Recently, it has been shown that the strict allocations of many of the identified hydrophobins to either class I or class II is not clear [156] and there are few species that coexpress both hydrophobin classes.[157, 158]

The tertiary structure of hydrophobins deviates from the conventional; the hydrophobic side chains on the surfaces are prevented from turning inside the protein (Figure 2). Thus, instead of the hydrophobic interactions, the protein core is stabilized by the network of the disulfide bonds.[137] Due to this character, the hydrophobin molecule is amphiphilic, owing hydrophilic and hydrophobic parts like ordinary surfactants. Hydrophobins are one of the most surface active biomolecules and they have been widely studied for their unique surface adhesion properties. Although all hydrophobins adhere to surfaces, there is a difference in their binding characteristics. *E.g.* Class II hydrophobin HFBI managed to compete in binding efficiency with class I hydrophobin SC3, but was more easily dissociated than class I hydrophobin, whose adherence was stronger.[155]

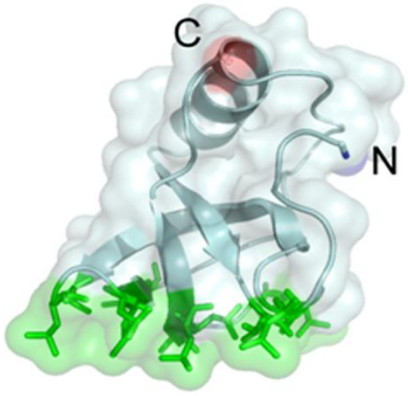


Figure 2 Structure of HFBI. Hydrophobic batch is shown in green. Modified from [154].

The unique natural features of hydrophobins, as well as recombinant technology can be used to modify hydrophobin sequences to have desired or improved properties for a wide range of applications.[137, 159, 160] Hydrophobins can act as tags for purification of recombinant fusion proteins by aqueous two-phase separation.[160] HFBII has an ability to produce exceptionally stable foams.[161, 162] The ability to act as emulsifiers [146] and foaming agents is beneficial to many applications, but on the other hand, having hydrophobins in the products, can cause harm to the beverage industry, such as the gushing of beer.[163] Class I hydrophobin SC3 has been used to formulate suspensions of hydrophobic drug compounds for oral drug administration.[164] The SC3 hydrophobin was used to reduce the particle size to microns and increase the bioavailability of two hydrophobic drugs, nifedipine and cyclosporine A.

Furthermore, hydrophobins can be genetically modified to gain benefits such as adhesion on target surfaces. Binding to cellulose could be mediated via cellulose binding domains (CBDs), which are the non-catalytic part of the cellulose and hemicellulose degrading enzyme.[165, 166] Recently, the cellulose-binding function of hydrophobin (HFBI) was obtained by a fusion with two cellulose-binding domains (CBDs) to bring nanofibrillar cellulose in a controlled manner to different interfaces.[167] CBDs interact with cellulose via

hydrophobic interactions of three aromatic amino acids on one face of the molecule.[168-170].

2.3.2 Poly(lactic acid)

Polyesters, including Poly(lactic acid) (PLA), the linear polymer composed of lactic acid monomers, have attracted much attention as pharmaceutical and medical systems (Figure 3). PLA monomers exist in two optically active isomers; Poly(L-lactic acid) (L-PLA) and poly(D-lactic acid) (D-PLA).[171] The physicochemical properties, such as melting points and partial crystallinity of the optically active isomers, are nearly equal. The polymer composed of racemic mixture of monomers may have very different characteristics than its monomers.

PLA has favorable physicochemical characteristics, like high mechanical strength, compatibility with a wide range of tissues, and a biodegradable nature showing no immunogenicity.[172] Depending on the type of degradation, polymeric biomaterials can be further classified into hydrolytically degradable and enzymatically degradable polymers. Hydrolytically degradable polymers have hydrolytically labile chemical bonds in their backbone. The presence of esters, a functional group susceptible to hydrolysis, allows hydrolytic degradation of PLA.[173] The susceptibility for hydrolytic degradation is a two-sided characteristic due to the undesired effect during processing and storage, but advantageous to the function of the drug formulation in the body. The hydrolytic degradation rate is primarily affected by the exposition to an elevated temperature and humidity, although other parameters like the degree of the crystallinity, morphology (porosity), molecular weight and environment (pH, ionic strength) have an effect on water uptake, which must be considered when the degradability is assessed.[174] Depending on these variables, the degradation time can vary from several hours up to years.[175] For example, the rate of degradation decreases with an increase in crystallinity, because the amorphous structure allows water to penetrate to the structure; resulting in a faster degradation.[44, 176] PLA is commercially available in different compositions and molecular weights, which allow the control of the polymer degradation.[175] In the body, PLA degrades via the citric acid cycle, finally forming innocuous metabolites water and carbon dioxide.[177] Therefore PLA is also a USA Food and Drug Administration (FDA) approved material.

PLA has received much attention as a renewable raw material; l-lactic acid can be produced in large-scale from plant sources by fermentation.[177, 178] Polymeric PLA can be synthesized either by a direct condensation of the lactic acid or by ring-opening polymerization (ROP) of the lactide. From direct condensation, the resultant polymer is a low to intermediate molecular weight material. In the catalytic ROP, molecular weight of the PLA can be controlled and it also enables polymers with higher molecular weights and lower polydispersities. Furthermore, ROP allows the preparation of block copolymers and, therefore, the ROP is often the most preferred.[174, 179]

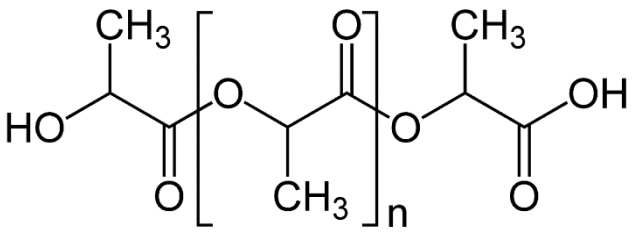


Figure 3 Poly(lactic acid) polymer.

Due to the favorable features like biodegradability and biocompatibility, PLA and its copolymers are one of the most used biodegradable polymers for coatings [74, 180, 181] and matrix materials in drug delivery systems.[182]

2.3.3 Nanofibrillar celluloses

Cellulose is the most abundant and renewable natural polymer on the earth. Inexhaustibility, biocompatibility, high stiffness and strength as well as possibilities for a wide variety of derivative products make cellulose optimal for numerous applications including functional pharmaceutical formulations. The cellulose polymer consists of linear β(1-4) linked anhydro-D-glucose units repeated as dimers, called cellobioses (Figure 4)**.** Each glucose unit has three hydroxyl groups attached to the ring.

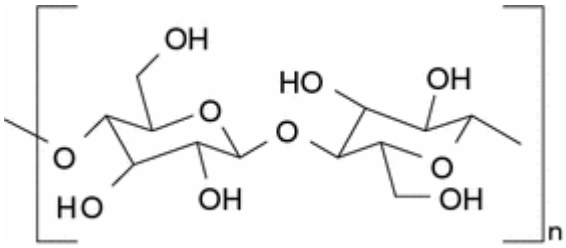


Figure 4 The repeat unit of cellulose, dimer called cellobiose. [183]

The parallel straight cellulose chains are present in lamellae or bundles called microfibers. The formation of microfibers occurs via van der Waals forces and both intramolecularly, by the interaction between OH-groups in the same molecule, or by intermolecularly with the other cellulose chains, bundling the chains together.[184, 185] On plant cell walls, the microfibers are further embedded in combination with hemicelluloses (such as xylan), lignin, and other minor substances (such as pectin), further forming macrofibers and resulting in complex morphologies despite the cellulose's apparent chemical simplicity. Xylan is a polysaccharide made from xylose units and including functional carbonyl groups. Thus, the surface charge of native cellulose develops as a result of the deprotonation of the carboxyl groups found in hemicellulose.[186]

Recently, individual cellulose fibers (referred to *e.g.* as nanofibrillar cellulose, nanocellulose, microfibrillated cellulose, NFC etc.) and crystals (referred to *e.g.* as cellulose nanocrystals, whiskers) with nanometer widths, extracted from wood and plant sources or from bacterial cultures, have gained increasing attention. For comprehensive reviews, please refer to.[183, 187-189] Apart from plants, algae, certain bacteria strains and marine tunicates are also known to synthesize cellulose.[190] Typically nanofibrillar cellulose (NFC) is generated from cellulose microfibers by mechanical treatments consisting of refining and high-pressure homogenization combined with pretreatment processes like alkaline, oxidation treatments or enzymatic hydrolysis (Figure 5).[187, 191, 192]

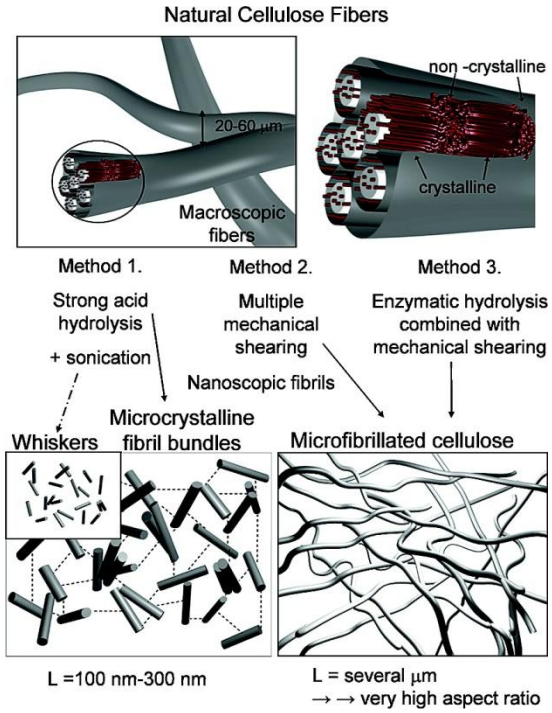


Figure 5 Three methods to disintegrate macroscopic cellulosic fibers into nanoscale fibrils or crystals. Reprinted with permission from [191]. Copyright 2011 American Chemical Society.

The mechanical properties, hydrophilicity, crystallinity, degree of polymerization and the success of the disintegration process of fibrils, strongly depend on the source, extraction and other treatments of the native cellulose.[185] Due to the high aspect ratio (~100-400), fibers are very susceptible to the interactions with surroundings and other molecules containing functional groups, such as water, solvents or nanoparticles that can form hydrogen bonds. The strong bonding of hydroxyl groups between the individual polymer chains makes cellulose water insoluble but provides the capability for chemical modification to obtain specific functionalities.[193]

Differences in the molecular orientations and in the network of hydrogen bonds causes the ordered regions of cellulose to exist in several polymorphs (forms I, II, III, and IV and their allomorphs).[189] In natural fibers, cellulose occurs mainly in the crystalline form I (divided into allomorphs I_α and I_β) mixed with amorphous regions.[194]

Drying of the untreated cellulose can cause a phenomenon called hornification, meaning that cellulose can lose some of its accessibility and reactivity, and the redispersion of fibrous cellulose material is prevented because of irreversible aggregation of the fibrils.[195] Surface modifications, such as 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO)-mediated oxidation of the primary alcohol group at C6 in cellulose molecule, has been used to minimize the adhesion caused by interfibril hydrogen bonds before the mechanical disintegration treatment to gain mostly individualized cellulose nanofibrils and also to prevent aggregation.[196, 197] When the primary hydroxyl groups are oxidized to carboxylic salts by TEMPO-mediated oxidation [197], anionic functionalities are provided to the surface of the microfibrils.[198] Thus, the repulsive effect of the charged surface enhances the separation of individual microfibrils.[186]

In long nanofibrillar cellulose, the aspect ratio is high and, therefore, it can be used in order to form porous structures that can act as templates for nanoparticles in stabilization and drug delivery purposes. Nanofibrillar celluloses have been used to make aerogels either

by itself [199] or in composite formulations, wherein porous NFC templates have formed magnetic [200] or electrically conducting flexible aerogels [201], or aerogels with tunable oleophobicity [202]. Porous structure with a larger surface area of aerogels can interact and prevent the aggregation of the nanoparticles more efficiently compared to the conventional microcrystalline cellulose.[186, 203]

Various bacteria, such as a genus of *Gluconacetobacter* strains, fermentate cellulose in high yields (up to 40%) using glucose as a substrate.[188, 204] Bacterial cellulose (BC) has the same molecular formula as the celluloses derived from plant materials, but have a high water holding capacity, tensile strength and crystallinity (80-90%).[205, 206] Accompanying substances, such as hemicelluloses and lignin, present in plant cellulose are missing from BC and, therefore, no harsh chemical treatments are needed to purify the BC.[207] It has been shown that various cultivation parameters, substrates, additives and bacterial strains have strong influence on the physicochemical properties of the produced cellulose, such as molar mass and homogeneity.[207-209] Bacterial cellulose has been shown to be a remarkably versatile biomaterial for example in food packaging applications [210], paper products [211], electronics [212], and medical devices [213].

2.4 Stability of biopolymer-based nanoparticles

One of the main problems in nanoparticle engineering is to maintain the stability of the primary particles since otherwise the advantages of the small particle size are lost. The surface of a material has an excess energy compared to the bulk. Due to the decreased particle size and increased surface area, the nanoparticles tend towards more thermodynamically stable state driven by an increased free energy. Thus, without sufficient stabilization, the aggregation of nanoparticles in media will occur soon after the particle formation, and at least during long-term storage. Nanoparticles have high particle mobility due to the Brownian motion and, thus, the particles collide with each other. The probability of particle-particle interactions increases with smaller size. If the particles are not sufficiently stabilized, they may aggregate. The irreversible aggregation of the particles following by quality problems such as the sedimentation of the colloidal system are related to these interparticle forces. According to the DLVO-theory [214, 215], solid particles are exposed to attractive van der Waals forces and repulsive forces due to the electrostatic double-layer. Aggregation occurs when the attractive forces exceed the repulsive electrostatic forces. However, the theory can only be used to describe the relevance of electrostatic interactions in colloids, but does not extend or explain other "non-DLVO forces" such as hydrophobic interactions or steric repulsion.[216-218]

In colloids, the stabilization against irreversible aggregation can be realized by using steric (physical barrier) and/or electrostatic stabilization (surface charge) on the surface of the nanoparticles.[31] Ionic or non-ionic surfactants and polymers have been commonly used to provide a barrier against the aggregation of nanoparticles.[65, 112] Steric stabilization can be attained by covering the particles by polymers which prevent the particles' access to the range of attractive forces by forming a physical barrier. Furthermore, a non-ionic amphiphilic polymer/surfactant with a hydrophobic chain around the hydrophobic drug surface allows a hydrophilic part to project into the water and, thus, also increase its hydrophilicity. Steric interactions are more sensitive to temperature fluctuations than electrostatic repulsion.[31] In contrast, ionic stabilizers are very sensitive to changes in pH and ionic strength, and are also incapable of stabilizing nanoparticles in the dry state.[32]

Electrostatically and/or sterically stabilized particles can still move, and thus their agglomeration is just hindered by the prevalence of thermodynamic repulsive forces.[219] The kinetic stabilization can be improved by increasing the viscosity of the liquid, for example by the formation of a polymer network using *e.g.* xanthan gum [219] Overall, when selecting a stabilizer, it has to be recognized as safe for administration, it must wet the surface of poorly water-soluble compounds, and it should withstand the post-processing of the formulation, *e.g.* drying or sterilization.[13, 66]

The long-term storage of nanoparticles in suspensions presents many disadvantages, such as the risk of microbiological contamination, premature polymer degradation by hydrolysis, physicochemical instability due to the particle aggregation, sedimentation and chemical instability of the drug. To avoid such problems, dry formulations are preferred over the suspensions. In general, the transformation of a liquid preparation into a dry product can be achieved using sensitive drying methods such as freeze-drying [203, 220] or spray-drying processes [221].

Polymorphism is common for pharmaceutical solids; they may exist in more than one crystalline solid form. Polymorphic transitions take place easily both during the particle size-reduction processes, post-processing and the storage. The crystalline material has a specific three-dimensional structure, in which molecules are arranged in a regular manner. In contrast, amorphous material has no regular three-dimensional long-range order, but may still have a short-range order, which is present over several molecular dimensions.[222] Production process can cause that crystalline solid to turn completely into an amorphous solid form. Polymers are typically semicrystalline, they contain both amorphous and crystalline regions. In addition, in the nanoparticle formation process, water or organic solvent molecules can be incorporated in the crystal structure. Depending on the solvent type in the crystal lattice, such crystals are known as hydrates or solvates. However, the high energy and molecular mobility also make amorphous solids physically unstable during the long-term storage. Furthermore, during the formulation processing, the polymer and drug may have interactions with each other.

Aggregation of the nanoparticles as well as polymorphism has notable impact on the dissolution rate. Therefore, changes in these may affect the therapeutic efficacy of the pharmaceutical formulation.[223] Amorphous substances are more soluble than their crystalline counterparts, and hydrates show slower dissolution compared to anhydrous forms. Therefore, solid state characterizations are required for both nanosuspensions and dry nanopowders.[222] Aggregation of the particles can be estimated by electron microscopy techniques or by observing phenomena related to light scattering. The appropriate tools for solid state analysis include X-ray techniques (XRPD, WAXS) and thermal analysis (DSC). Thermal events like melting, as well as the changes in diffraction patterns, can be used to detect transformations in the solid state. Variability in the dissolution rates caused by aggregated nanoparticles and polymorphic changes can result in unpredictable variations in drug delivery and bioavailability.[203, 224, 225]

3 Aims of the study

The purpose of this study was to find new nanotechnological ways to produce and stabilize biopolymer-based drug nanoparticles, for controlled drug release or improved solubility and bioavailability of drug compounds with low water-solubility. The nanoparticles were produced with two different techniques, electrospray and nanoprecipitation methods. The performance of the particles was evaluated utilizing 1) different solid state characterization methods, 2) dissolution studies, as well as 3) *in vivo* bioavailability tests. Nanofibrillar celluloses from five different origins and with versatile characteristics were introduced as nanoparticle carriers and matrix formers for controlled drug delivery.

Specifically, the goals were as follows:

- Set-up of the electrospray method for the production of homogenous biopolymer-based drug nanoparticles. The specific target was to control the particle characteristics, such as size and morphology with process variables.
- Preparation of functionalized protein-coated drug nanoparticles by using genetically modified hydrophobin proteins. The specific aim was to obtain particle surfaces that could be further tailored by modifying hydrophilic side of the hydrophobins via protein bioengineering.
- Use of hydrophobin proteins in combination with both native and chemically modified nanofibrillar cellulose (NFC) to facilitate storage and post-processing of nanoparticles. The specific aims were to formulate dry nanoparticle powders and to improve the stability of drug nanoparticles and to control the dissolution rate of drug nanoparticles via optimized characteristics of NFC aerogels.

4 Experimental

Complete experimental details can be found in the original publications (**I-IV**).

4.1 Materials

The model drug compounds were beclomethasone dipropionate (BDP) (Sigma, St, USA), salbutamol sulfate (SS) (Orion Pharma, Finland) and itraconazole (ITR) (Apotechnia, Spain). Itraconazole (Sigma-Aldrich, USA), hydroxyl-itraconazole (OH-ITR) and itraconazole-d5 (Toronto Research Chemicals Canada) were used as references in *in vivo* experiments. Poly(L-lactic acid) (PLA) (MW 2000g mol^{-1}, ICN Biomedicals Inc., USA) was used for the formation of nanoparticles by electrospray. Microcrystalline cellulose (MCC) (Avicel PH101, FMC International, Ireland) was used as a reference to nanofibrillar celluloses (NFCs) (**IV**). The production, purification and conjugation processes of the used cellulose materials and hydrophobins are described in publications listed in Table 4 and Table 5 with corresponding publication references. Green fluorescent Protein (GFP), Double Cellulose Binding Domain (DCBD)

Table 4 Hydrophobins (HFBI, HFBII) and HFBI fusions with Green Fluorescent Protein (GFP) and Double Cellulose Binding Domain (DCBD) used in this work.

Compound	References	Publications
HFBII	[226]	II
GFP-HFBI	[227]	II
HFBI	[226]	III-IV
HFBI-DCBD	[160]	III-IV

Table 5 Nanofibrillar celluloses used in this work.

Compound	References	Publications
Native wood (birch) cellulose	UPM-Kymmene Corporation[228]	III
Bacterial cellulose	VTT[229]	IV
Quince seed cellulose	VTT[230]	IV
TEMPO-oxidized wood cellulose	VTT[197]	IV
Red pepper cellulose	The University of Tokyo	IV

4.2 Methods

The equipment used in the experiments and characterization are summarized with references to the corresponding publications in Table 6.

Table 6 Equipment used in this study.

Method	Publication	Equipment/Manufacturer
Electrospray apparatus	I	Home-constructed system: Power supply (UTK Microfluidic Toolkit, Micralyne, Edmonton, Canada), CCD camera (Watec WA-502A), syringe pump (Harvard Apparatus Pump 5, Playmouth Meeting, PA, USA)
UV-spectrophotometer	I	Ultrospec III, Pharmacia (LKB Biotechnology, Sweden)
Scanning electron microscopy (SEM)	I	DSM 962, (Zeiss, Jena, Germany),
Photon correlation spectroscopy (PCS)	I	Zetasizer 3000HS (Malvern Instruments, Worcestershire, UK)
Modulated temperature Differential scanning calorimetry (MT-DSC)	I-IV	DSC 823e and STAR^e Software (Mettler-Toledo, AG, Switzerland).
Freeze-dryer	II-IV	Kinetics Thermal Systems Lyostar II, (SP Industries Inc., Warminister, USA)
Variable temperature X-ray powder diffractometry (VT-XRPD)	I-IV	Bruker AXS D8 advance, (Bruker AXS GmbH, Germany)
Transmission electron microscopy (TEM)	I-IV	Fei Technai F12, (Philips Electron Optics, The Netherland)
High Pressure Liquid Chromatography (HPLC)	II-IV	Agilent 1100 Series (Agilent technologies, Santa Clara, CA, USA)
Fluorescent Microscope	II	Olympus BX-50. Emission light was filtered with a WG Filter (510-550 nm)
Liquid Chromatography-Tandem Mass spectrometry (LC-MS/MS)	III	Agilent 6410 Triple Quadrupole mass spectrometer Technologies (Palo Alto, CA, USA)
Ultrasound bath	I-IV	GWB Branson 3200, (Danbury, USA)
Dissolution apparatus (small scale)	II, IV	Sotax AT7 (Perkin-Elmer Lambda 2, Germany)
Dissolution apparatus (large scale)	III	Erweka DT-6 (Heusentamm, Germany)
Atomic force microscope (AFM)	IV	A NanoScope IIIa Multimode (E-scanner, Digital Instruments/Veeco), NSCI5/AlBS cantilever (µMASCH)
Fourier transform infrared spectroscopy (FTIR)	IV	Bruker Optics Inc., MA, USA Horizontal Attenuated Total Reflectance (ATR) (MIRacle, Pike Technology, Inc, WI, USA)
Wide angle x-ray scattering (WAXS)	IV	Custom build (SAXS/WAXS) x-ray camera. Cu-anode x-ray tube monocromatized with multilayer optics (Montel). WAXS intensity was measured with MAR345 image plate detector.

4.2.1 Electrospraying

A schematic diagram of the electrospray equipment used in the production of nanoparticles is shown in Figure 6. The sprayed solution was fed by a syringe pump (Harvard Apparatus Pump 5, Playmouth Meeting, PA, USA) and syringe needle (Hamilton, USA) at a

flow rate of 2-10 µl min^{-1} through a silica capillary (outer diameter 0.10 mm, inner diameter 0.05 mm) to a receiving beaker. During the experiments a cone-jet mode was monitored by a CCD camera (Watec WA-502A). The solution was sprayed at an applied voltage of 1–8 kV (UTK Microfluidic ToolKit, Micralyne, Edmonton. Canada). Working electrode was connected to the needle with alligator clips. Grounded counter electrode was a copper wire wrapped around the collection beaker. The distance between the needle tip and the surface of the receiving solution was kept at 3-5 cm. In some experiments a nitrogen flow was used to enhance the evaporation of the organic solvent during spraying.

Conductivity of the spraying liquid was controlled by adding 0.02-0.20% ammonium hydroxide in 96% ethanol. Set-up A: Ammonium hydroxide was included to the sprayed liquid and only nitrogen gas was fed through a nozzle outside of the capillary (Figure 6). Set-up B: The nitrogen gas and the organic salt solution (pumped at flow rate 3.0-8.2 µl min^{-1}) were fed through a T-part connected to the spraying liquid at the end of the silica capillary.

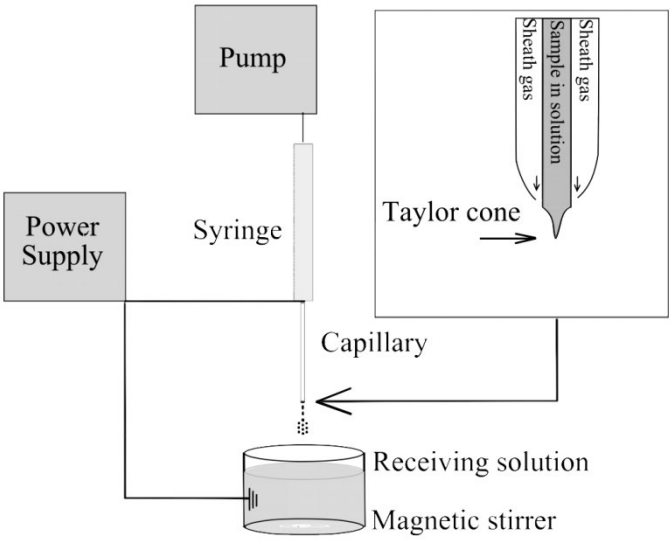


Figure 6 Illustration of the electrospray system (set-up A) used for the production of encapsulated drug nanoparticles. Modified from publication **I**.

4.2.2 Solvent-antisolvent precipitation

Nanoparticles were prepared by using a simple bottom up method, nanoprecipitation **(II-IV)** (Figure 7)**.** In this anti-solvent precipitation method, the hydrophobic drug was dissolved in an organic solvent. Tetrahydrofuran was used as a solvent for itraconazole (ITR) and methanol for beclomethasone dipropionate (BDP). Amphiphilic proteins HFBI, HFBII or HFBI-DCBD were first dissolved in water (0.6 mg/ml) and the solutions were sonicated and placed on an ice bath. Before use, the ITR and BDP solutions (12 mg/ml) were filtered with 0.2 µm PVDF or GHP syringe filters (PALL, Ann Arbor, MI, USA) to remove possible dust particles. Then, the ITR or BDP solutions were rapidly added into the hydrophobin solution. The receiving liquid was stirred vigorously with a magnetic stirrer and temperature of the solution was controlled by keeping the sample on ice. A white precipitate was observed as a turbid solution immediately after the ITR addition, indicating the formation of the nanoparticles.

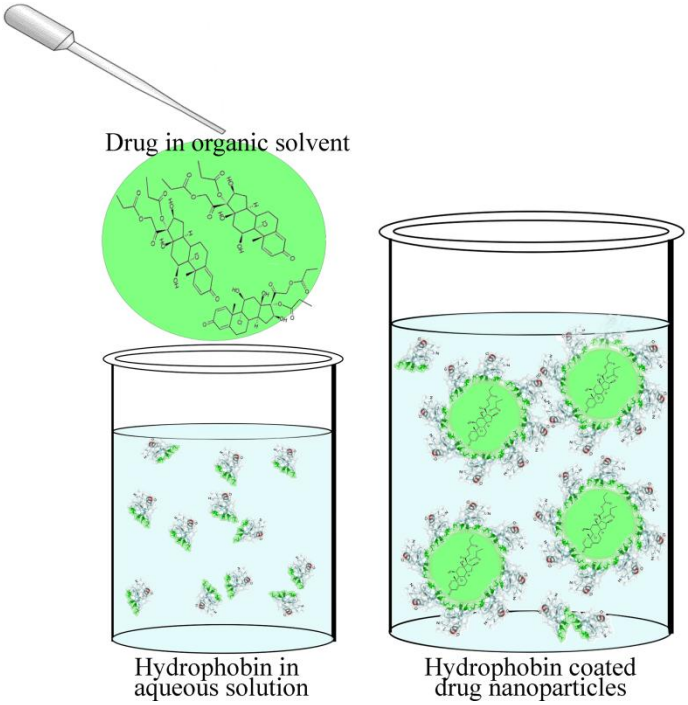


Figure 7 Schematic illustration of the hydrophobin assisted solvent-antisolvent precipitation of hydrophobic drug.

4.2.3 Binding to cellulose nanofibrils

After precipitation, the nanosuspensions were stirred for 20 min to evaporate the residuals of organic solvent. Then the cellulose nanofibrils or microcrystalline cellulose (MCC) in water were added to the nanosuspensions and incubated under mild stirring for 45 min.

In the formulations with different nanofibrillar celluloses (wood cellulose (1.66% hydrogel) bacterial cellulose (0.3% hydrogel), TEMPO-oxidized cellulose (0.8% hydrogel) from quince seeds (0.12% hydrogel) or from red pepper cellulose (dry) and microcrystalline cellulose (dry), the amount of cellulose was expressed in terms of dry weight. The ratio of drug: HFB: cellulose nanofibrils of 1:1:2 was used in all the experiments (**III, IV**).

Regenerated cellulose tubular membrane MWCO 3500 (CelluSep, Spectrum Labs, San Antonio, USA) was used to remove residuals of the organic solvents from the nanoparticle suspensions if the particles were used for *in vivo* animal studies (**III**). The dialysate was ultrapurified water and was changed after 2 and 6 hours during a total dialysis time of 14 hours.

For the removal of water, the suspensions were frozen with liquid nitrogen (**II, IV**) or/and immediately placed into a freeze-drying chamber (Kinetics Thermal Systems Lyostar II, SP Industries Inc., Warminster, USA) (**III**). ITR particle dispersions were dried on the freeze-dryer with or without NFC and D±trehalose (TRE). BDP particles were dried with or without NFC. The ratio of ITR:TRE was 1:1.25. Exact details of the freeze-drying cycles are described in corresponding publications (**II-IV**).

4.2.4 Characterization

Sizes and size distributions of the PLA-nanoparticles were determined with dynamic light scattering (**I**). Particle sizing was based on the photon correlation spectroscopy (PCS); the results were analyzed by CONTIN algorithm and the sizes presented are based on the intensity distributions. The width of the particle size distribution was estimated by the polydispersity index (PI). The PI values range from 0 (perfectly monodisperse) to 1.00 (polydisperse particles, broad distribution), with the limit of monodisperse particles being ca. 0.10.

For the (MT-)DSC experiments the nanosuspensions were allowed to dry at room temperature (**I**) or freeze-dried (**II-IV**). Powders were weighted in aluminum pans. Runs were carried out in pans which were sealed with aluminum caps with pin holes. Thermal behavior of the freeze-dried nanopowders was studied using a differential scanning calorimeter (Mettler-Toledo Inc., Switzerland). The samples were held at 25 °C for 5 min before heating. All the samples were heated from 25 to 250-350 °C depending on the samples (**I-IV**). The heating rate was 2-10 °C min^{-1}. The data was analyzed with STARe software (Mettler-Toledo Inc., Switzerland).

Electron microscopy studies for the nanoparticles were performed by using a scanning electron microscope (SEM) and a transmission electron microscope (TEM). For SEM, the nanopowders were either dried directly (**I**) or freeze-dried and then attached on the metal plates by using two-sided tape (**IV**). The plates were sputtered for 25 seconds with platinum prior to the SEM analysis. For TEM studies, the nanoparticle suspensions were dried or spread (freeze-dried powder) on Formvar film-coated copper grids with the mesh size of 300 (Agar Scientific, Essex, U.K.).

The labeling studies were performed for the fresh BDP-HFBII nanosuspensions. GFP-labeled BDP nanoparticles were studied by using Fluorescence Microscope. The synthesis of the nanoparticles was carried out normally, except that a part of the HFBII was replaced with the GFB-HFBI fusion protein. The GFB labeling of the microparticles was performed after the synthesis by adding GFP-HFBI to the solution containing BDP microparticles. Labeling of the BDP nanoparticles with the mercaptosuccinic acid (MSA) coated Au-nanoparticles was performed by adding MSA-Au particle solution to BDP-HFBII nanoparticles suspension (1:2) and incubating for 1 h before sampling. Particles were imaged by TEM.

Samples for XRPD analysis were either dried on the silicon plates (**I**) or freeze-dried to powder form (**II-IV**). The experiments were performed in a symmetrical reflection mode with Cu Kα radiation (1.54Å) using Göbel Mirror bent gradient multilayer optics. The scattered intensities were measured with a scintillation counter. The angular range was from 5◦ to 40◦. The measurements were made with the steps of 0.02◦, and the measuring time was 0.5-2s/step depending on the samples. In Varied Temperature (VT)-XRPD measurements, the samples were heated on the sample vessels at 24, 55, 90, 106, 115, 145, 180 and 220 ◦C at a speed of 10 ◦C/min and kept at the assigned temperatures during each measurement (**II**). Crystallinities of the samples were estimated by comparing the intensities of crystalline and amorphous samples (**I**). The reference data were retrieved from Cambridge Structural Database (CSD) using Conquest program.

4.2.5 Dissolution studies

Dissolution behaviors of the BDP nanoparticles were studied using either 40 ml of 36% (v/v) ethanol (BDP nanosuspensions, **II**) or 40 ml of 0.3% SDS (freeze-dried BDP powders,

IV) as a dissolution medium. The solubility of BDP in water is so low that the use of ethanol or surfactant in the dissolution mediums was justified for detection aspects. The temperature was maintained at 37 ± 0.5 °C (Sotax AT7, Perkin-Elmer Lambda 2) and the stirring rate was 100 rpm. In the case of suspensions, a 4 ml (0.5 mg/ml) portion of cold (+4 °C) nanoparticle suspension was added to the dissolution media. Powders were loaded inside gelatin capsules (size 0). The released amount of BDP was determined by HPLC (Agilent 1100 series, Agilent technologies, Germany) at a wavelength of 242 nm.

Dissolution studies of the freeze-dried ITR powders were performed immediately after the freeze-drying. Dissolution tests of the ITR samples were performed using a paddle type dissolution apparatus (Erweka DT-6, Germany) with a rotation speed of 150 rpm (**III**). Dissolution medium (400 ml) was 0.2% (w/V) NaCl-HCl pH 1.2 and the temperature was maintained at +37±0.5 °C. Powders were loaded inside gelatin capsules (size 0). A theoretical amount of ITR was 0.5 mg in each sample. The released amount of ITR was determined by a HPLC (Agilent 1100 series, Santa Clara, CA, USA) at a wavelength of 261 nm.

4.2.6 In vivo studies

Investigations of the bioavailability of different itraconazole formulations were performed in University of Eastern Finland, Kuopio. Male Wistar rats were purchased from Laboratory Animal Center (University of Eastern Finland, Kuopio). The animals received intragastrically 1 mg of itraconazole/animal in one of the following formulations: 1) Sporanox® oral solution, 2) HFBI, 3) HFBI + NFC, 4) HFBI-DCBD + NFC or 5) itraconazole powder suspended in water. Blood samples were collected from the saphenous vein into heparinized microcapillaries (Drummond Microcaps, Drummond Scientific Co. Broomall, PA, USA) prior to administration and 0.5, 1, 2, 3, 5, 8, 12 and 24 h after the administration. The analysis of itraconazole and hydroxyl-itraconazole from plasma samples was modified from a previously published method.[231] National Animal Experiment Board of Finland approved the experiments and they were conducted in accordance with the guidelines set by the Finnish Act on Animal Experimentation (62/2006) and European Community Council Directives 86/609/EEC.

5 Results and discussion

5.1 Drug nanoparticles by electrospraying (I)

5.1.1 Preparation

In study **I**, the PLA drug nanoparticles were produced by dissolving the polymer and drug in an organic solvent (or solvent-water in the case of hydrophilic salbutamol sulfate) and spraying them together. Therefore, forming of a strict core-shell structure was hypothetical and a matrix structure could be more descriptive for these nanoparticles. Factors such as the strength of the electric field, the composition, viscosity and electrical conductivity of the spraying liquid, changes in the liquid flow rate and the diameters of the silica capillary, were shown to be essential for the formation of a conical jet shape and, thus, eventually dictating the size of the droplets produced.[89, 234] For instance, the molecular weight of the used polymer has a significant influence on the particle morphology and size due to the viscosity.[235] Previous studies have shown that small and smooth spherical particles could be generated more easily from low- than higher-molecular-weight polymers.[100, 104, 236-238] Furthermore, *in vivo* PLA hydrolysis is dependent on factors such as polymer crystallinity and molecular weight.[239, 240] Low molecular weight PLA (2000 g mol^{-1}) was selected for this study because of faster biodegradation (*e.g.* after pulmonary delivery) compared to higher molecular weight PLA (M_w above 10 000 g mol^{-1}).

For the production of nanometric and monodisperse polymeric particles, stable cone-jet mode has to be formed in the spraying nozzle. In our set-ups, voltage ranges from 2.7–6.2 kV produced a stable jet mode; otherwise the significance of the used voltage was minor compared to the other parameters. Previous studies have shown that electrical conductivity of the spraying liquid has a significant role in maintaining stable spraying conditions.[89] In line with the literature, in this study (**I**) the distinct influence of adequate electrolytic concentration was shown. At an optimal 0.05% ammonium hydroxide concentration, particle size could be controlled by changing the polymer concentration or flow rate, as is shown in Table 7.

Table 7 Electrical conductivity had a significant role in having stable spraying conditions. Particle sizes and standard deviations (nm) of PLA-nanoparticles prepared under different flow rates and polymer concentrations with ammonium hydroxide content of 0.05% and applied voltage 6.2 kV (n = 2-6) are shown. (**I**)

	Particle size (nm) at different flow rates		
PLA content	4 µl min^{-1}	6 µl min^{-1}	8 µl min^{-1}
1%	280 ± 10	310 ± 60	340 ± 50
3%	360 ± 70	450 ± 100	630± 250
6%	550 ± 120	500 ± 60	630 ± 70

When the physical processing parameters were adjusted together with the sprayed solvent properties, a stable cone-jet mode could be attained and particle sizes could be controlled to some extent. The right processing parameters had to be experimentally examined for each case. The correlations between the parameters are presented in Table 8.

Table 8 When the four physical processing parameters, voltage, spraying distance, flow rate and solvent conductivity were adjusted, a stable cone-jet mode could be attained. Production of monodisperse particles was attained only in stable cone-jet mode and particles sizes could be controlled to some extent. The control of the particle size and morphology could be performed by changing the other parameters. The arrow (↑) describes the increasing of the parameter. Table is reproduced with permission from [93]. Copyright 2011 Informa Healthcare.

Parameter	Effects	Product properties affected
Solvent vapour pressure	Evaporation rate of solvent	Particle morphology
Nozzle dimensions (↑)	Droplet size (↑)	Particle size (↑)
Polymer MW (↑)	Viscosity (↑)	Particle size (↑) and morphology
Polymer concentration (↑)	Viscosity (↑)	Particle size (↑) and morphology

Molecular weight of the used polymer and its concentration in the solution has influence on the particle size and morphology due to viscosity. For example the particle size was increased with increasing polymer concentration and flow rate (polymer–drug ratio was kept at a constant level of 10:1). The mean particle sizes could be adjusted between 200 nm to 800 nm by controlling the parameters during the spraying. Polydispersity indices obtained from the photon correlation spectroscopy (PCS) showed moderate size deviations (PI 0.1-0.5) and the nanoparticles were generally spherical with smooth surfaces. Solvent evaporation plays an important role in the mechanism of electrospraying and particle formation. During the process, the solvent evaporates from the droplets and the droplets start to shrink, causing nanometer-sized particles to form. Therefore, it has influence on the structure of the formed particles.[100, 237] Too fast solvent evaporation can cause porous particles and slow solvent evaporation incomplete polymer solidification before the nanoparticles reach the receiving liquid. The evaporation rate could be controlled by adjusting the gas flow rate.

In the case of hydrophilic SS, the spraying solution contained water in addition to the organic solvents. Therefore, a propylene glycol was used to keep the suspensions smooth during the processes. In addition, a surfactant was important in stabilization of the formed nanoparticles. Tween-80 was added to both the spraying solution and also to the receiving liquid. Tween-80 forms a steric layer around the precipitating polymer which prevents the particle aggregation. An amount of the Tween-80 in the receiving liquid influenced the size of the formed nanoparticles. A high concentration (0.1% v/v) of Tween-80 heavily increased the particle size compared to lower amounts of the surfactant (0.01–0.06% v/v). Size distributions were also broader, probably due to aggregated particles. Thus, the surfactant concentrations in the receiving solution gave the contribution to the manufacturing process of monodisperse nanoparticles. Furthermore, 70% ethanol as a receiving liquid was observed to prevent the aggregation of nanoparticles more efficiently than pure water, probably due to the increased miscibility of the PLA and the aqueous phase.

5.1.2 Characterization

The physical state of the solid poorly soluble drug is one of the most important characteristics together with the size affecting the stability, solubility and dissolution, and the bioavailability of the drug.[225] Solid state changes of the drugs and polymers are common both during the manufacturing process and during the storage.[242] In addition, the

materials used in formulations may interact with each other. Therefore, the solid state of the PLA nanoparticles and possible interactions with the drugs and the polymer were examined by using DSC and XRPD.

The electrosprayed PLA nanoparticles were dried at room temperature for solid state characterization (**I**). In DSC thermograms, an endothermic event at around 155 °C indicated the melting of crystalline L-PLA (Figure 8). Generally, the process decreased the crystallinity of PLA due to fast solvent evaporation during the solidification of the polymer in the particle formation process. A crystallization of amorphous material could be seen as a small exothermic peak at 100 °C with the PLA-drug nanoparticle samples when compared the bulk PLA. The melting peak of BDP was not detected in the DSC scan. Its absence was explained by the miscibility of BDP in the PLA, rather than by a change into an amorphous phase during the manufacturing process. Further, the XRPD results confirmed the existence of anhydrate BDP. In contrast, a weak melting peak of SS was detected. The XRPD patterns of the nanoparticles included the reflections of BDP, SS and PLA. Supporting observations could be seen from the XRPD results: the crystallinity of materials was decreased, but no transformations in the crystalline forms were seen. Because no new peaks were seen in the DSC profiles or XRPD, there should not be strong physical or chemical interactions between the drugs and the polymer.

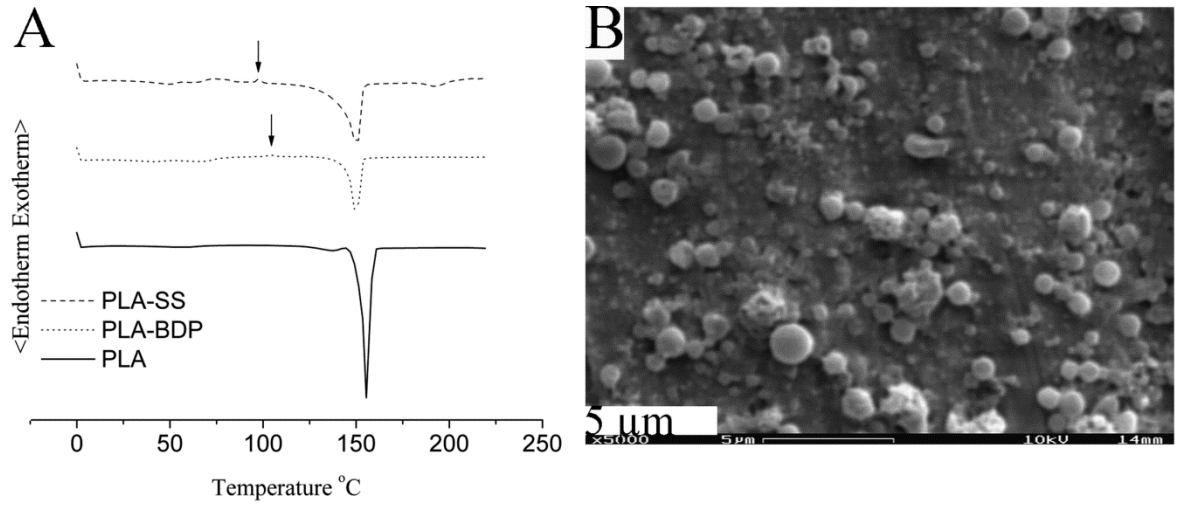


Figure 8 **A)** DSC thermograms of bulk PLA powder, PLA-SS and PLA-BDP nanoparticles. Small exothermic events were observed at around 100 °C (arrows) in both PLA-drug samples. **B)** SEM image of PLA-SS nanoparticles (225 nm). (**I**)

Obviously, the suitability of electrospray for both hydrophilic and hydrophobic drug compounds was the major advantage. In study I, the loss of drug during the electrospraying with salbutamol sulfate was 20%, which was caused mainly by the spreading of the particles to the surroundings during the process. The entrapment efficiency (EE) was used to quantify the amount of the drug entrapped into particles. The EE of hydrophilic salbutamol sulfate (SS) and hydrophobic beclomethasone dipropionate (BDP) into PLA-nanoparticles were more than 50%. The values revealed that the method was able to produce PLA drug nanoparticles with good entrapment efficiencies. For the hydrophilic substance, entrapment into the hydrophobic polymer is advantageous in formulation of drug delivery systems and improving their stability and bioavailability in the body.

Mild processing conditions, low temperature and normal air pressure ensures the suitability of the process also for sensitive therapeutic molecules. Scale-up of the electrospray for industrial applications may require, e.g., several spraying nozzles, which would increase the production costs, but a continuous process mode could be organized without major input.[95]

5.2 Drug nanoparticles by precipitation (II-IV)

5.2.1 Preparation

Precipitation is a commonly used, well-known bottom-up method for producing nanoparticles. It is also easily up-scaled and even a continuous mode can be obtained.[233] However, as mentioned in the context with the electrospray method, the critical parameters such as the solvent-antisolvent ratio, stirring rate, temperature, and selection and amount of the stabilizer, had a major influence on the nanoparticle formation also in the precipitation process. Efficacy of the precipitation was determined on the basis of the amount of free drug in the aqueous outer phase. The BDP and ITR nanoparticles prepared by the precipitation method were almost completely entrapped into the nanoparticles. No or very small amounts of drug could be detected from the outer phase of the nanoparticles immediately after precipitation. Compared to electrospraying, the precipitation method with similar set-ups is not suitable for both hydrophobic and hydrophilic drugs. However, the phenomenon behind the spontaneous crystal formation is complex. Therefore each component and parameter has an implication for particle size, morphology and recovery rate.

In studies **II - IV** spontaneous adsorption of amphiphilic proteins, hydrophobins, was utilized in order to restrict the crystallization and further reduction of the particle sizes. During the precipitation of the drug, hydrophobins self-assembled on the surfaces of the growing drug crystals and, finally, an adequate amount of hydrophobin inhibited the crystal growth completely, leading to the formation of drug nanoparticles. Therefore, the mean sizes of the BDP and ITR nanoparticles could be decreased to a certain level with narrow size distributions.

In study **II**, experiments showed that size and morphology of the BDP nanoparticles were affected strongly by increasing the concentration of the surface active protein HFBII. In the case of BDP, a morphological change of the resulting particles appeared to be abrupt from needles to round shapes (Figure 9) (**II**). As for the ITR, the morphological change was not so dramatic (**III**). No additional changes in the particle sizes or shapes were observed with hydrophobin concentrations exceeding certain, determined levels (drug-protein ratio 1:1). By using the hydrophobin assisted precipitation method, homogenous and round BDP and ITR particles were formed.[241] At the optimum, BDP particles with diameters of 100 ± 30 nm and ITR particles of 100 ± 60 nm were obtained. Apart from the hydrophobin concentration, the optimized precipitation process consisted of a low temperature (+4◦C), high stirring rate and ratio of outer phase/solvent which were responsible for the advanced supersaturation and nucleation during the precipitation.

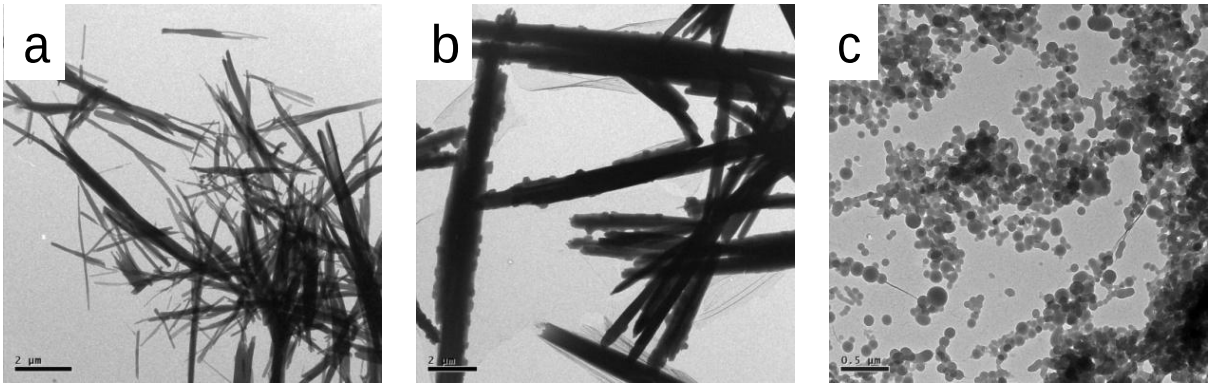


Figure 9 TEM images showing the effect of hydrophobin concentration on crystal habit and size of the BDP particles. BDP particles precipitated with the protein (HFBII) -drug ratio of **a)** 0:1 (pure BDP), **b)** 0.08:1 HFBII **c)** 1:1 (**II**).

5.2.2 Characterization

The solid state of the BDP and ITR nanoparticles was analyzed after lyophilization (**II-IV**). The physical state of the nanoparticles and possible interactions with excipients were examined using DSC, (VT-)XRPD, and WAXS. The drug needs to be dissolved for the precipitation, and thus it is prone to physical changes. Previously, it has been reported that BDP exists in an anhydrate or a monohydrate form or may form solvates with *e.g.* alcohols and halogenated hydrocarbons.[243, 244] In some previous studies BDP has been completely transformed into an amorphous form as a consequence of the processing.[245] Based on the characterization results, BDP was converted partly to a monohydrate or completely to an amorphous form during the precipitation. In the VT-XRPD, changes in BDP crystal structures were observed by heating the samples gradually to set temperatures based on the DSC thermograms (Figure 10).

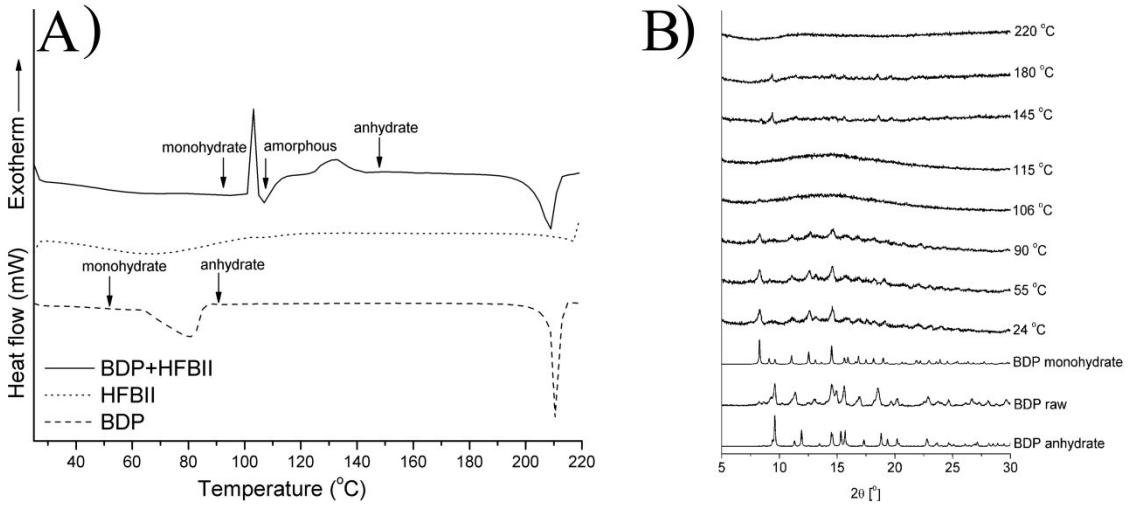


Figure 10 **A)** DSC thermograms of precipitated BDP (dashed line), HFBII (dotted line), and BDP (solid line) precipitated with HFBII. Arrows indicate the crystal structures based on the VT-XRPD results. **B)** VT-XRPD of precipitated BDP nanoparticles with HFBII at various temperatures. Reference patterns are shown at the bottom of the figures (**II**).

The second model drug ITR was partly amorphous and partly crystalline after the precipitation and lyophilization processes. All the precipitated nanoparticle suspensions were freeze-dried before further analyses and, therefore, the crystalline state was undefined immediately after precipitation. Overall, these characterization results indicated that the solid state changes of both ITR and BDP took place during the preparation of the solid nanoparticles. As described, BDP and ITR were at least partly amorphous after the preparation and the original (raw material) crystalline form of BDP was converted from an anhydrate to a monohydrate. Amorphous material dissolves much more easily than its crystalline form, but is very prone to crystallization during storage. In addition, hydrated crystals exhibit slower dissolution rate than their anhydrate counterparts [246] and, therefore, the polymorphic change may decrease the effect of the smaller particle size.

5.3 Properties of nanofibrillar cellulose (III, IV)

In this thesis, nanofibrillar celluloses (NFC) from different sources were harnessed for the colloidal stabilization or controlled drug delivery of drug nanoparticles (**III, IV**). The advantages of the thin cellulose nanofibrils included their modification capacity *via* both chemical modification and specific functionalization. The prior modification was used to provide carboxylic groups on the surface of the fibrils and was obtained by the TEMPO-oxidation process. The latter method was realized using a bi-functional amphiphilic protein, HFBI coupled with two specific cellulose binding domains (DCBD). In this approach, one protein block with aromatic amino acids had affinity towards the cellulose and the other towards the lipophilic nanoparticles. In addition to these two, cellulose fibrils offered inherent sites for functionalization due to the presence of hemicelluloses (native NFC). In wood and plant cell walls, cellulose exist together with hemicelluloses, lignin and other minor components, and even after mild purification and fibrillation processes cellulose can still be considered as a composite structure. Thus, the adsorbed hemicellulose layer may have different properties according to chemical structure and amount of hemicellulose.[247, 248] These functional sites may offer possibilities to affect, *e.g.*, the surface charge of the fibrils and, thus, increase the stability of the individual fibrils and colloidal stability of NFC dispersions.[186]

The studied cellulose nanofibrils were obtained from various sources and extracted using different treatments methods. The studied NFC materials were characterized by AFM, XRPD, DSC and FTIR. The electrostatic stabilization of the fibrils was evaluated based on their ζ-potentials at different pH-values. Moreover, depending on the cellulose origin and extraction method, the fibrils characteristics, such as diameters, pIs and crystallinities, showed differences (Table 9).

Table 9 Characteristics including diameter, isoelectric point (pI), crystallinity of the NFC fibrils and a short description of the cake formation during freeze-drying. (**III, IV**)

NFC	Diameters	pI	Crystallinity*	Cake formation
Bacterial cellulose	5-7 nm, bundles 20 nm	3.2	High	Well-formed
TEMPO-oxidized wood cellulose	3-5 nm	1.5	Low	Well-formed
Quince seed cellulose	3 nm	1.5	Very low	Partially shrunken
Red pepper cellulose	0.9-2.3 nm	1.5	Very low	Collapsed
Wood (birch) cellulose	5-20 nm	1.0	ND	ND

*After freeze-drying, ND = Not determined

XRPD studies of the cellulose nanofibrils were performed in order to investigate the crystalline state of the freeze-dried cellulose fibrils. The XRPD pattern of bacterial cellulose and TEMPO-oxidized cellulose showed crystallinity which was related to the native cellulose crystal structure I. The bacterial cellulose had a high crystallinity compared to the other NFCs. No diffraction was seen in XRPD spectrum of the red pepper or quince seed cellulose and, hence, an amorphous structure was assumed.

Fourier transform infrared spectroscopy (FTIR) was carried out on the freeze-dried cellulose powders in order to identify the functional groups present on the surfaces of the cellulose fibrils. The FTIR spectra confirmed the existence of carboxyl groups, which was related to hemicelluloses, such as xylans in quince seed and red pepper celluloses. Also the specific band present in the cellulose engineered by TEMPO-oxidation was related to the carboxylic acid groups.

The ζ -potential is a measure of the surface charge and is dependent on both the functional groups present on the surface of the fibrils and the environment, *e.g.*, pH and ionic strength. The results showed strong negative charges (-50 mV) for three of the NFCs (quince seed, TEMPO-oxidized and red pepper) at pH 5. These originated from the deprotonated carboxyl groups on the fibrils. Native NFC from wood had a negative charge of -28 mV at pH 4. In contrast, MCC and especially the bacterial cellulose had lower, insufficient charges (-25...-10 mV) considering colloidal stabilization via pure electrostatic interactions. Based on the results, the electrostatic repulsion between the fibrils is sufficient (30 mV, positive or negative) once the charged groups were dissociated due to a sufficiently high pH.

5.4 Drug nanoparticles immobilized in nanofibrillar cellulose (III, IV)

5.4.1 Surface functionalization of drug nanoparticles

Hydrophobin coatings around the drug particles provided a site for surface functionalization. The protein used in the first study (**II**) was a class II hydrophobin HFBII. Class II hydrophobins, HFBII and HFBI have very similar size and behavior, and also mixed layers could be adsorbed on the surfaces of the particles. Therefore, nanoparticles with different hydrophobins could be prepared as described in Chapter 5.2.1. Because of the similarities between the different hydrophobins (HFBI, HFBII), HFBI could be selected for

further studies (**II, III, IV**) on the basis of its specific engineered properties. HFBI could be synthesized as coupled, *e.g.*, with the double cellulose binding domains (DCBD) and with the green fluorescent protein-marker (GFP). After the particle formation, an existence of a hydrophobin layer around the solid drug nanoparticles was confirmed by using the fluorescent character of GFP (**II**). GFP-HFBI coated nanoparticles were successfully detected by using a fluorescence detector of a microscope.

Furthermore, mercaptosuccinic acids (MSA)-coated Au nanoparticles (3 nm) were used to image the drug nanoparticles (**II**). The Au nanoparticles were bound to the drug nanoparticles via electrostatic interactions. This labeling could be used for example for the localization of the particles in electron microscopy or as an alternative route for surface functionalization, *e.g.*, for drug or protein immobilization.[249-251].

5.4.2 Binding to cellulose nanofibrils

In further studies (**III**, **IV**) the cellulose-binding functionality of the nanoparticles was realized through a HFBI coupled with two specific cellulose binding domains (DCBD). CBDs interact with cellulose via aromatic amino acids organized on the surface of the protein (and thus on the surface of the nanoparticles). The more efficient binding capacity of the DCBD compared to ordinary HFBI has been proved recently.[167] Because the long term stability of the nanoparticles can be low, association with the cellulose network was expected to improve the steric stabilization of the particles. Steric stabilization can be considered as an effective mechanism to shelter drug nanoparticles against aggregation and degradation during the post-processing and *in vivo* conditions. It can be more effective than ionic stabilization because of the high ionic strength and variation in pH in the human body, and because electrostatic stabilization might be lost *e.g.* during freeze-drying. However, CBD's specific binding to crystalline cellulose material may have caused also some drawbacks.[252] The crystallinity of the fibrils extracted from plant material was low and the fibrils were partly covered by hemicelluloses, which probably hindered the efficacy of specific binding in the thesis experiments (**IV**).

The nanoparticles were mixed in suspension with the various types of nanofibrillar celluloses (NFC). HFBI coated BDP and ITR nanoparticles were used as controls. On the basis of the TEM images of the suspensions, it was found out that all the hydrophobin coated drug nanoparticles were well-mixed into the NFC networks (Figure 11). No external particles were seen in the suspensions immediately after mixing.

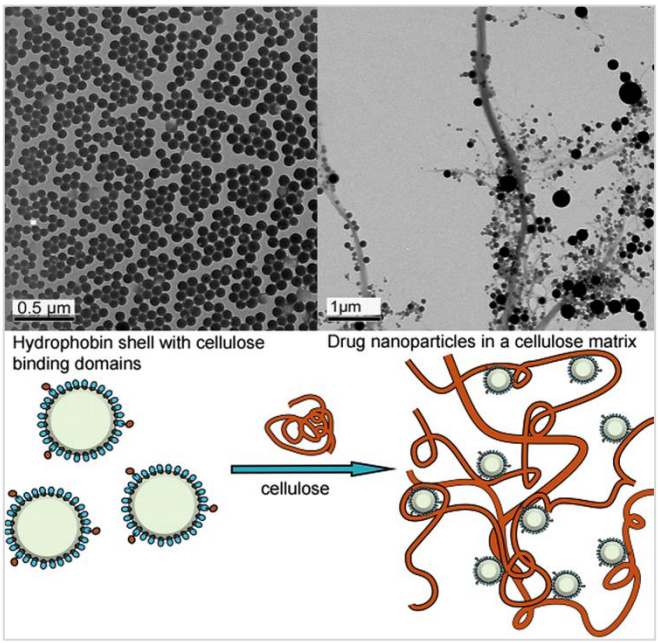


Figure 11 Immobilization of hydrophobin-coated ITR nanoparticles into nanofibrillar cellulose network **(III).**

5.4.3 Stability studies

The effect of pH, temperature and post-processing (dialysis, freeze-drying) on the stability of the hydrophobin coated BDP and ITR particles was studied. (**II**, **III, IV**). Stability of the BDP and ITR nanosuspensions was evaluated by imaging of the suspensions using TEM and SEM.

In suspension, at pH 5, the BDP nanoparticles started to aggregate after 1.5 h, but at pH 8 they could be stored for at least 1 week at +4 ºC (**II, IV**). The results could be explained by the increased electrostatic stabilization since due to the increased pH, the hydrophobins on the surface of the particles were charged. The calculated isoelectric points of the hydrophobins were 6.7 (HFBII), 6.0 (HFBI) and 6.7 (HFBI-DCBD). In the pH-values above pI, the particles were negatively charged. At room temperature, the BDP nanoparticles started to show morphological changes already after 24 h, despite the increased pH (**IV**). An elevated temperature accelerated the physical changes of the particles and, therefore, the particles were more unstable at room temperature compared to +4 ºC. In contrast to BDP particles, ITR nanoparticles were stable at room temperature for weeks, while in cold (+4 ºC) they survived stable for several months (**III**). They were also viable after dialysis in room temperature as the bioavailability studies showed.

Since the BDP nanoparticles showed poor long-term stability in suspension and changes such as particle aggregates were observed, freeze-drying was introduced. According to the previous studies, freeze-drying is a sophisticated way to obtain powders from the nanosuspensions.[203, 220] Usually cryo- and lyoprotectants, such as carbohydrates (*e.g.* trehalose, sucrose or mannitol), have to be used to protect the nanoparticles against morphological changes and aggregation.[253] The BDP nanoparticles remained unchanged through the lyophilization process without additional excipients (**II, IV**). In study **III**, trehalose was used to improve the stability of ITR nanoparticles during the freeze-dying. Long-term (3 months) stability studies for the freeze-dried ITR powders were performed at + 40 °C/75% RH (relative humidity), and the dissolution profiles of the drug-nanoparticle

formulations before and after the storage were compared. The original samples released almost all their contents in the first 5 min, but for the stored BDP-HFBI samples it took some 5 to 20 min more to reach plateau release values, which were also lower level than before storage. Storage had strong influence on BDP-HFBI samples and the drug release was much slower than in the beginning. The original (before storage) release rate could not be reached after storage period. These changes might be due to agglomeration of the stored particles. Trehalose had positive effects on the performance of the stored HFBI-DCBD-coated nanoparticles. The release rates were remained closer to the originals when trehalose was present during the storage.

To improve stability and post-processing of the ITR and BDP nanoparticles, a NFC matrix was introduced to prevent aggregations of both HFBI and HFBI-DCBD coated nanoparticles (**III, IV**). Incorporation of the nanoparticles into the different NFC-matrices did not improve the stability of the BDP nanoparticles in suspension, except for the HFBI coated particles immobilized with bacterial cellulose (**IV**). They retained their size for more than 5 weeks.

However, the effect of CBDs was pronounced during the long-term storage of the ITR nanosuspensions (**III**). After ten month storage at +4 °C in an aqueous environment, morphology of the ITR nanoparticles coated with the ordinary HFBII was changed similarly to the morphology of the microcrystals, whereas the HFBI-DCBD coated particles remained unchanged.

The long-term (3 months) stability studies for the freeze-dried ITR nanoparticles immobilized into the NFC were performed at + 40 °C/75% RH, and the dissolution profiles of the formulations were compared before and after the storage. Trehalose was used also here to improve the performance of freeze-drying. The ITR particles combined with NFC and trehalose were stabilized the most effectively during the freeze-drying and storage, resulting in the most uniform dissolution rates (**III**). Overall, the NFC may have a positive effect on the stabilization of the nanoparticles in suspension or during the freeze-drying.

5.4.4 Drug release studies

In drug delivery, porous matrices can be used as solid scaffolds in different formulations to carry the therapeutic agents. The drug delivery properties are largely influenced by the chemical structure and the textural properties of the matrices; the porosity, wettability, erosion and the surface area all have an effect on the drug release rates. The matrix has an influence on the release profile and, thus, for the bioavailability of the entrapped drug. Aerogels from nanofibrillar celluloses can be formed by removal of the water from the cellulose hydrogels (aqueous suspension) by freeze-drying.[201] In freeze-drying, the NFC network expands upon solvent evaporation. The dry network maintains to some extent its expanded, porous structure when vacuum is released and the network voids are filled with gas. By the definition, these aerogels are very porous solids formed by the replacement of liquid in a gel by gas.

Therefore, nanofibrillar cellulose aerogels were evaluated besides as stabilizers for the nanoparticles during the freeze-drying (**III**), but also as a template for the controlled release (**IV**). Aerogels based on nanofibrillar celluloses with various characteristics (Table 9) were formed from bacterial cellulose, cellulose extracted from red pepper and quince seeds, as well as from TEMPO-oxidized cellulose. The microcrystalline cellulose (Avicel) was used as a reference material.

The specific target of the NFC aerogels was to be able to release the drug from the functionalized nanoparticles in a controlled manner by choosing and modifying the matrix formers. Controlled release of a drug from the matrix is important to ensure a constant drug concentration in vivo. The dissolution behavior of aerogels should be primarily dependent on the penetration of the medium into the matrix and the swelling properties of the porous matrix formed. The collapse of the freeze-dried cake may increase its density, which can lead to a decreased porosity and sublimation rates and, thus, retarded drying. However, the collapse doesn't necessarily have an effect on the moisture content, stability or dissolution rate of the product, as shown previously [254], and here in the study IV. The rapid release of BDP into a medium from the nanoparticles incorporated into MCC or red pepper cellulose aerogels was comparable to the dissolution of BDP nanoparticles (Figure 12). These completely collapsed aerogels released BDP the fastest. The drug release from HFBI-DCBD coated particles was not retarded, although there should be stronger binding to the cellulose fibrils as attested earlier.[167] Freeze-dried aerogels which maintained the shape of the initial suspensions, without shrinkage or collapse, released the BDP slower and, thus, the nanoparticles immobilized into the bacterial cellulose, quince seed cellulose or TEMPO-oxidized wood cellulose, showed sustained release of BDP (Figure 12).

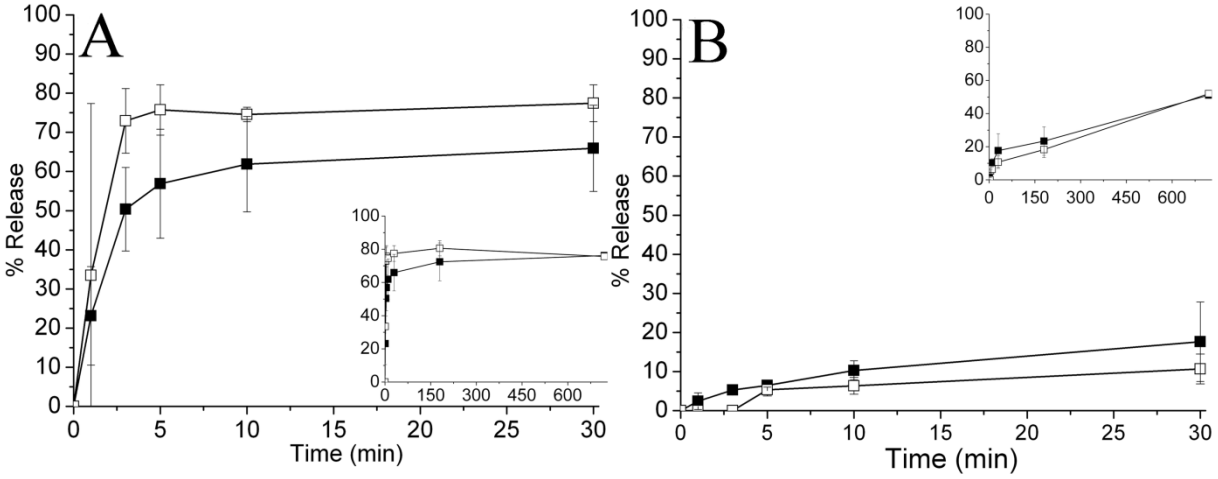


Figure 12 Drug release from two different cellulose aerogels. **A)** BDP was released fast from the nanoparticles immobilized into red pepper cellulose. **B)** Sustained BDP release was obtained from nanoparticles immobilized into quince seed cellulose matrix. Solid and open symbols represent HFBI and HFBI-DCBD coated nanoparticles, respectively. (**IV**)

Based on the correlations between the thermal behavior and release rates of BDP, the interactions between the cellulose materials and the BDP-HFBI(-DCBD) nanoparticles affected the release rates of the drug together with the structured matrices. Therefore, the interactions in the matrix-nanoparticle composition are significant in the design of the NFC aerogels for controlled release drug delivery systems. Overall, the nanoparticles incorporated into the external biodegradable cellulose aerogels could release the drug in a controlled manner by the modulation of the matrix formers. The sustained drug release produced by these kinds of nanocomposite structures can be used to provide the prolonged blood concentrations of the drug compared to single nanoparticles which released their drug contents fast.

5.4.5 *In vivo studies*

In study **III**, the hydrophobin coated drug nanoparticles were shown to improve the bioavailability and to enhance the drug exposure *in vivo*. A pharmacokinetic evaluation of the ITR nanosuspensions was carried out in rats. Plasma concentration versus time profiles of ITR and its active metabolite OH-ITR were studied after oral administration. Itraconazole microsuspension and cyclodextrin-based commercial Sporanox® solution were used as negative and positive control formulations, respectively. The nanosuspensions improved markedly the absorption of ITR. As indicated by the AUC values, the nanosuspensions provided about 20-fold increase in the absorption of ITR, when compared to the ITR microsuspension but did not alter the t_{max} values (Table 10). The improved oral absorption of ITR from the nanosuspensions could be explained by the improved dissolution rate with the decrease in particle size, due to the increased surface area, and the decreased diffusion layer thickness. Compared with the Sporanox® solution, the nanosuspensions had comparable pharmacokinetic values but showed less interindividual variability. The explanation could be that after the nanoparticles were dissolved in stomach, hydrophobins were capable of stabilizing ITR solution until the absorption took a place in small intestine. The AUC ratio of ITR and OH-ITR remained unchanged in all experiments, thus the metabolic activity of ITR was not affected. As shown by these experiments, the hydrophobin and NFC stabilized nanosuspension appears to be a very promising approach to enhance the oral bioavailability for BCS class II drugs.

Table 10 Pharmacokinetic parameters of itraconazole (ITR) and hydroxyl-itraconazole (OH-ITR) after oral delivery of itraconazole formulations. AUC and t_{max} of ITR and OH-ITR are expressed. Total AUC values are compared to Sporanox® (n = 4). Modified from reference **III.**

Formulation	$AUC_{(ITR)}$ [µg/ml h]	$t_{max(ITR)}$ [h]	$AUC_{(OH\text{-}ITR)}$ [µg/ml h]	$t_{max(OH\text{-}ITR)}$ [h]	∑AUC [µg/ml h]	$AUC_{(\sum ITR/\sum SPORANOX)}$ [%]
Sporanox®	0.49	4 (1-5)	2.32	5 (2-5)	2.81	100
HFBI	0.55	4 (2-5)	3.05	10 (3-12)	3.61	128
HFBI+NFC	0.56	3 (3-8)	2.78	3 (3-8)	3.33	119
HFBI-DCBD+NFC	0.54	4 (3-8)	2.99	5 (3-8)	3.53	126
ITR-suspension	0.04	3 (1-5)	0.18	5.5 (3-12)	0.22	8

6 Conclusions

The electrospray apparatus was set-up for the production of poly(lactic acid) - drug nanoparticles. The main benefits of the electrospray method were options to affect the particle size as well as possibilities to entrap both hydrophobic and hydrophilic drugs into the nanoparticles. Therefore, the method is very suitable for the production of the polymeric drug nanoparticles for different purposes in a small scale.

A precipitation method with hydrophobins as stabilizers enabled the successful preparation of small drug nanoparticles. The surface properties of the nanoparticles were modified by coupling two cellulose binding domains to the hydrophilic side of the hydrophobins. The fusion protein facilitated specific binding of drug nanoparticles into nanofibrillar cellulose. Long-term stability of the itraconazole nanoparticles in suspension was improved using specific binding to the nanofibrillar cellulose network and the nanoparticles were stable at least for 10 months.

Nanofibrillar cellulose matrix enabled successful post-processing such as freeze-drying of ITR nanoparticles. Nanofibrillar cellulose aerogels with versatile characteristics could be used to release the drug in a controlled manner. Immediate or sustained release of BDP from the nanoparticles was obtained, depending on the structure and interactions formed by the nanoparticles and the cellulose matrix.

As a summary of the thesis, biopolymer-based nanoparticles can increase and control the dissolution rate of the drugs and, thus, possibly improve the bioavailability of the drug compounds. They offer a competitive alternative to other drug delivery vehicles. In the case of polymer-based nanoparticles, the choice of the polymer is an issue and plays a key role in the formation of the particles, as well as in the physical stability of the system. Nanoparticles accompanied with a very diverse and versatile group of cellulose nanofibrils can be taken into consideration as advanced drug delivery systems.

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