Preparation and Characterization of Poly(Lactic Acid) Nanoparticles for Pharmaceutical Use

Samuli Hirsjärvi

ACADEMIC DISSERTATION

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Helsinki 2008
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Abstract


Nanoparticles have versatile potential for efficient exploitation of different drug delivery formulations and routes because of the properties provided by their small size. These possible benefits include controlled release, protection of the active pharmaceutical ingredient and drug targeting. Nanoparticles are expected to offer new solutions e.g. for gene therapy and delivery of peptide drugs. Generally, nanoparticles are applied as an injectable or oral solution, but their use as dried material in formulations such as tablets or inhalable powders is equally conceivable. Although the research on pharmaceutical nanoparticles has been extensive during recent years, breakthrough of products to the market has not yet occurred. Problems like poor drug encapsulation efficiency and difficulties in controlling and scaling up of the preparation process have inhibited progress.

In this study, nanoparticles were prepared from a biodegradable poly(lactic acid) (PLA) polymer. Knowledge and understanding of properties of the materials used in the particle preparation as well as behavior of the nanoparticles in different environments are essential in order to create successful nanoparticle formulations and to predict their performance in the body. Therefore, the effect nanoprecipitation, a nanoparticle preparation method, on the physicochemical properties of the polymer and model drugs encapsulated in the nanoparticles as well as the effect of the drugs on the polymer were studied by thermoanalytical and spectroscopic methods. The suitability of capillary electrophoresis for drug quantitation in the nanoparticle formulations was estimated. Surface pressure measurements were applied for the assessment of stability and aggregation of the nanoparticles. The protective ability of carbohydrates to improve the freeze-drying process of the nanoparticles was evaluated. Finally, a layer-by-layer polyelectrolyte coating process, which modifies the particle surface properties, was introduced. Analytical methods such as electron microscopy, size measurements and zeta potential determinations were included in the nanoparticle characterization.

Both the preparation process as well as the drugs affected the physicochemical characteristics of the nanoparticles such as particle size and crystallinity of the polymer, and, further, the stability of the PLA nanoparticles. With a combination of surface pressure measurements and electron microscopy observations, it was possible to gain information about conditions and progression of nanoparticle aggregation and the role of the stabilization mechanisms. Stability of nanoparticle dispersion was a prerequisite for completion of the layer-by-layer coating. Utilization of protective excipients, glucose and lactose in this study, was found to be indispensable for the freeze-drying. Capillary electrophoresis appeared to be a promising tool for the quantitation of drug encapsulation and subsequent drug release. The results of this study provide information about the materials used and the methods applied for further studies that aim at development of biocompatible and biodegradable nanoparticles for human use.
Acknowledgements

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Helsinki, January 2008

Samuel
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This thesis is based on the following publications of Samuli Hirsjärvi (né Hyvönen):


The publications are referred to in the text by their roman numerals I-V. Reprinted with the permission of the publishers.
Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
</tr>
<tr>
<td>BDP</td>
<td>Beclomethasone dipropionate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CE</td>
<td>Capillary electrophoresis</td>
</tr>
<tr>
<td>CHL</td>
<td>Chloroform</td>
</tr>
<tr>
<td>D,L-PLA</td>
<td>Poly(D,L-lactic acid)</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DLCA</td>
<td>Diffusion-limited cluster aggregation</td>
</tr>
<tr>
<td>DSC</td>
<td>Differential scanning calorimetry</td>
</tr>
<tr>
<td>ESCA</td>
<td>Electron spectroscopy for chemical analysis</td>
</tr>
<tr>
<td>ESEM</td>
<td>Environmental scanning electron microscope</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier transform infrared spectroscopy</td>
</tr>
<tr>
<td>GI</td>
<td>Gastro-intestinal</td>
</tr>
<tr>
<td>GPC</td>
<td>Gel permeation chromatography</td>
</tr>
<tr>
<td>HIC</td>
<td>Hydrophobic interaction chromatography</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>LbL</td>
<td>Layer-by-layer</td>
</tr>
<tr>
<td>L-PLA</td>
<td>Poly(L-lactic acid)</td>
</tr>
<tr>
<td>MEEKC</td>
<td>Microemulsion electrokinetic chromatography</td>
</tr>
<tr>
<td>MPS</td>
<td>Mononuclear phagocyte system</td>
</tr>
<tr>
<td>$M_w$</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>PAH</td>
<td>Poly(allylamine hydrochloride)</td>
</tr>
<tr>
<td>PCS</td>
<td>Photon correlation spectroscopy</td>
</tr>
<tr>
<td>PE</td>
<td>Polyelectrolyte</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly(ethylene glycol)</td>
</tr>
<tr>
<td>PLA</td>
<td>Poly(lactic acid)</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly(lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>PSS</td>
<td>Poly(styrene sulfonate)</td>
</tr>
<tr>
<td>RLCA</td>
<td>Reaction-limited cluster aggregation</td>
</tr>
<tr>
<td>SCG</td>
<td>Sodium cromoglycate</td>
</tr>
<tr>
<td>SEC</td>
<td>Size exclusion chromatography</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SS</td>
<td>Salbutamol sulphate</td>
</tr>
<tr>
<td>$T_c$</td>
<td>Cold crystallization temperature</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>$T_g$</td>
<td>Glass transition temperature</td>
</tr>
<tr>
<td>$T_m$</td>
<td>Melting temperature</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>XRPD</td>
<td>X-ray powder diffraction</td>
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</tbody>
</table>
1 Introduction

Pharmaceutical nanoparticles are submicron-sized, colloidal vehicles that carry drugs to the target or release drugs in a controlled way in the body. After preparation, nanoparticles are usually dispersed in liquid. Such a system can be administered to humans for example by injection, by the oral route, or used in ointments and ocular products. Alternatively, nanoparticles can be dried to a powder, which allows pulmonary delivery or further processing to tablets or capsules.

In drug delivery, nanoparticles should readily be biocompatible (not harmful for humans) and biodegradable (deteriorate and expulse in the body conditions). These properties, as well as targeting and controlled release, can be affected by nanoparticle material selection and by surface modification. Materials such as synthetic polymers, proteins or other natural macromolecules are used in the preparation of nanoparticles. To process these materials into nanoparticles, a variety of preparation techniques exist ranging from polymerization of monomers to different polymer deposition methods [1, 2].

Nanoparticles in pharmaceutical applications have gained plenty of research attention during recent decades [3]. Although the research concerning formulation of nanoparticles into drug delivery devices has been extensive, only a few polymeric nanoparticulate products have reached the market. One known product, Abraxane™, consist of intravenously administered 130-nm nanoparticles prepared from the protein albumin bound with paclitaxel, a drug used in cancer therapy [4]. Another cancer drug, Doxorubicin Transdrug®, consisting of doxorubicin-loaded poly(isohexylcyanoacrylate) nanoparticles is currently at the Phase II/III clinical trials [5]. Among the drugs used in nanoparticle formulations, particularly cancer therapeutics are widely studied because the formulation might reduce toxicity of the drug while improving efficacy of the treatment [6]. In addition to drug molecules, other candidates to be encapsulated in or coupled with nanoparticles include macromolecules like proteins, peptides and genes (nucleic acids) [7, 8]. These kinds of molecules tend to be inactivated in the body by enzymatic degradation. In terms of controlled release, nanoparticles provide protection against the body conditions resulting in sustained release and maintenance of bioactivity before the drug reaches the target.

After intravenous administration, nanosized particles are mainly taken up by the macrophages of the mononuclear phagocyte system (MPS) [9, 10] and, thus, can be localized in the liver, spleen and lungs [11-13]. By modifying particle surface, e.g., by coating, defence mechanisms of the body can be avoided to some extent leading to longer circulation times of nanoparticles in the blood [14]. Tailored coating also enables another promising application of nanoparticles: drug delivery across the blood-brain barrier (BBB) [15]. From the intestine, after oral administration, intracellular uptake may occur and prior to that, the nanoparticles can adhere to the mucosa (bioadhesion) and thus improve pharmacokinetics of the drug [16].

As a summary of the above mentioned facts, the benefits of nanoparticles include protection of the encapsulated pharmaceutical substance, improved efficacy, fewer adverse effects, controlled release and drug targeting. Correspondingly, potential active substances in nanoparticle formulations could be expensive molecules applicable in small amounts.

At the present time, several successful laboratory-scale nanoparticulate drug targeting systems are available, (reviewed e.g. in [3, 17]), and some processes have been scaled up [18].
As the time approaches when an increasing number of nanoparticulate drug delivery systems reach the market and the systems are transferred from animal tests to human use, concerns about the safety of the products are emerging. More attention will be paid to stability and toxicology of nanoparticles and their constituents. According to recent opinion, proper physicochemical characterization of nanoparticles should be included in risk assessment and toxicology considerations of nanoparticulate systems, in addition to pharmaceutical \textit{in vitro} and \textit{in vivo} testing [19].

In this thesis, nanoparticles were prepared from poly(lactic acid) polymers using the nanoprecipitation technique and model drug substances were encapsulated in the nanoparticles. Properties of the nanoparticle surface were modified by layer-by-layer coating whereas freeze-drying was applied to further expand the processing possibilities of nanoparticles. The focus in this study was on physicochemical characterization and evaluation of nanoparticle-behavior during different stages of preparation, surface modification and stabilization in different environments. Increasing knowledge about these kinds of systems extends the understanding and facilitates the prediction of the behavior of similar systems in man.
2 Review of the literature

2.1 Preparation and processing of polymeric nanoparticles

Biodegradable nanoparticles for pharmaceutical use are prepared from a variety of synthetic and natural polymers. Synthetic polymers such as polyacrylates, polycaprolactones, polylactides and its copolymers with polyglycolides are widely used and discussed [20, 21]. Natural polymers include e.g. albumin [22, 23], alginate [24], gelatin [25, 26], and probably the most studied natural polymer for pharmaceutical nanoparticle applications: chitosan (reviewed in [27]).

Several methods exist for the preparation of nanoparticles. When synthetic polymers are used, they are typically dissolved in a convenient solvent followed by precipitation in a liquid environment leading to nanoparticle formation. The drug intended to be encapsulated in the particles is usually incorporated in the process during the polymer solvation and precipitation. Emulsification/solvent diffusion, emulsification/solvent evaporation, nanoprecipitation and salting-out methods are widely applied techniques and they have been discussed in several reviews [2, 20, 21, 28]. In emulsification/solvent evaporation, the dissolved polymer is emulsified with aqueous phase with the help of a high-energy source such as ultrasound or homogenization followed by solvent evaporation (vacuum, heat). In the salting-out method, polymer precipitation is obtained via phase separation (organic solvent - aqueous phase) by the addition of a salting-out agent, e.g. electrolytes in the case of acetone as a solvent for the polymer. Similarly, in emulsification/solvent diffusion, nanoparticles are formed when the saturation limit of a partially water-miscible solvent (e.g. benzyl alcohol) is exceeded by addition of water. In both techniques, the phase separation is accompanied by vigorous stirring. The separated solvent is removed e.g. by cross-flow filtration. The nanoprecipitation method is presented in chapter 2.1.2. Techniques such as supercritical fluid technologies [29, 30] are different approaches to nanosized particle preparation. In addition to polymers, nanoparticles can be created directly from monomers using different polymerization techniques as presented in comprehensive reviews [1, 2, 21].

The properties and fate in vivo of a nanoparticulate system depend to a great extent on the surface properties of the particles. Thus, stability, drug release and targeting can be tailored by surface modification. Two strategies exist. First, polymers with grafted molecules, such as poly(ethylene glycol) (PEG), can be used at the surface (covalent attachment) [31]. Alternatively, nanoparticles can be coated by adsorbing molecules on the surface by techniques like layer-by-layer adsorption (non-covalent attachment) [32].

Nanoparticles are usually in (aqueous) liquid dispersion after preparation, which may cause degradation of the polymer and drug leakage into the medium. In addition, handling (storage, transportation) of a liquid particle system is laborious. Stability and facilitated handling as well as readiness for further processing (formulation of tablets, capsules, powders) and conservation of the particle structure of the nanoparticulate system are achieved by drying. Freeze-drying in appropriate conditions is a method that conserves the structure of particles in an acceptable and efficient manner.
2.1.1 Poly(lactic acid)

Poly(lactic acid) (PLA) is a linear aliphatic polyester derived from lactic acid monomers (Fig. 1). PLA among its copolymers with glycolic acids (PLGA) are widely used in micro- and nanoparticulate drug delivery systems because of their biocompatibility and biodegradation properties; they possess the Generally Regarded as Safe (GRAS) status of the Food and Drug Administration (FDA). In addition to drug delivery devices, these polymers are used in several medical devices such as sutures, tissue screws and tacks, tissue regeneration membranes, bone fixation devices and systems for meniscus and cartilage repair [33-35].

![Chemical structure of poly(lactic acid).](image)

PLA exists in two optical forms. Polymers of L(+)-lactic acid and D(-)-lactic acid are partially crystalline, while the racemic poly(D,L-lactic acid) is amorphous. Of the two enantiomers, the polymeric L-lactic acid is commonly employed because it is the one degrading into the endogenous lactic acid in the body. Low molecular weight (MW) PLAs (< 3000 g/mol) are produced by direct condensation of lactic acid, whereas higher MW PLA polymers are obtained usually from ring opening polymerization of lactide [36]. PLAs are soluble in organic solvents such as chloroform and dichloromethane, but insoluble in common alcohols like ethanol [36]. They are also insoluble in water, although low MW PLAs are more hydrophilic and contain oligomers with some aqueous solubility [37, 38]. Unlike racemic D,L-PLA, enantiomeric PLAs are insoluble in some water miscible organic solvents like acetone and acetonitrile, which makes the use of co-solvents indispensable e.g. in the nanoprecipitation process.

The presence of ester linkages in the polymer backbone (Fig. 1) allows hydrolytic degradation of the polymer in an aqueous environment [39, 40]. The degradation products are biocompatible and metabolizable; they are removed from the body by the citric acid cycle. The degradation rate is dependent on several parameters such as crystallinity (crystalline PLA degrades slower), $M_n$ (low $M_n$ PLAs degrade faster) environment (pH, ionic strength, temperature) and particle morphology (porous particles degrade faster) [38, 41, 42]. At neutral pH (in vitro liquid environment without enzymes), PLA nanoparticles [41] and microparticles [38] remain relatively stable over tens of days. On the contrary, polyester microparticles are reported to degrade substantially faster when injected subcutaneously or intravenously to rats compared to in vitro tests in buffer solutions [43, 44]. Also in gastric and intestinal fluids, degradation of D,L-PLA nanoparticles in vitro [45] and in vivo [46], and PLGA nanoparticles in vivo [47] was fast due to enzymatically catalyzed hydrolysis. Drugs encapsulated in the particles are known to affect the degradation. Both acidic and basic drugs may catalyze the degradation. For example, basic drugs can catalyze ester linkage
scission [48, 49] but, on the other hand, they may also neutralize the carboxylic acid chain end groups of the polymer and slow down the hydrolysis [50].

2.1.2 Nanoprecipitation

In nanoprecipitation, introduced by Fessi and co-workers [51], the particle formation is based on precipitation and subsequent solidification of the polymer at the interface of a solvent and a non-solvent. Thus, the process is often called solvent displacement or interfacial deposition. The polymer is dissolved in a water miscible organic solvent (or solvent mixture) and added to an aqueous solution, in which the organic solvent diffuses (Fig. 2). Particle formation is spontaneous, because the polymer precipitates in the aqueous environment. According to the current opinion, the Marangoni effect is considered to explain the process [28]: solvent flow, diffusion and surface tensions at the interface of the organic solvent and the aqueous phase cause turbulences, which form small droplets containing the polymer. Subsequently, as the solvent diffuses out from the droplets, the polymer precipitates. Finally, the organic solvent is typically evaporated with the help of a vacuum. No emulsification step (which is usually part of a nanoparticle preparation process), laborious processing conditions or special laboratory ware is needed. The size of the nanoparticles prepared by nanoprecipitation varies typically from 100 to 500 nm.

![Figure 2. Schematic illustration of the nanoprecipitation process.](image-url)

Usually, surfactants or stabilizers are included in the process to modify the size and the surface properties, or to ensure the stability of the nanoparticle dispersion (especially during the early stages of the precipitation). However, presence of surfactants/stabilizers is not indispensable for the formation of the particles. The drug substance to be encapsulated is, depending on its solubility, dispersed as an aqueous solution or dissolved in the organic
Review of the literature

solvent before the fusion of the phases. The nanoprecipitation technique suffers from poor encapsulation efficacy of hydrophilic drugs, because the drug can diffuse to the aqueous outer phase during polymer precipitation [52]. By modifying the solubility of the drug by changes in pH, the encapsulation has been increased [53-55]. For example, decrease of solubility of procaine hydrochloride (pKa 8.8) by increasing the aqueous phase pH from 5.8 to 9.3, increased the drug entrapment from 11% to 58% [54]. Correspondingly, increased encapsulation was achieved by decreasing the aqueous solubility of sodium cromoglycate (pKa 2.0) by lowering the pH [55]. An accelerated precipitation rate of the polymer, modified solvent composition and increase in the $M_w$ of the polymer are among the other means to improve the encapsulation efficiency. Examples of different nanoprecipitated formulations are listed in Table 1.

**Table 1. Examples of polymeric nanoparticles prepared by the nanoprecipitation method.**

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Drug/study and reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eudragit L100-55</td>
<td>A study on the preparation variables [56]</td>
</tr>
<tr>
<td>Hydroxypropyl methylcellulose phthalate (HP55)</td>
<td>A study on the preparation variables [57]</td>
</tr>
<tr>
<td>Poly(D,L-lactic acid)</td>
<td>Isradipine [58], tyrphostin [59], cloriciromene [53], indomethacin [51, 60], diclofenac [61], atovaquone [62], acyclovir [63], clofibrate [64], primaquine [65], docetaxel [66], a model poorly water-soluble crystalline drug [67], a study on the preparation variables [68, 69], a stability study [70]</td>
</tr>
<tr>
<td>Poly(L-lactic acid)</td>
<td>Sodium cromoglycate [55, 71, 72]</td>
</tr>
<tr>
<td>Poly(D,L-lactic acid) - poly(ethylene glycol)</td>
<td>Procaine hydrochloride [73], a study on the preparation variables [74],</td>
</tr>
<tr>
<td>Poly(D,L-lactic-co-glycolic acid)</td>
<td>Isradipine [58], xanthone/3-methoxyxanthone [75], atovaquone [62], benzathine penicillin G [76], nifedipine [77], procaine hydrochloride [54], docetaxel [66], flurbiprofen [78], rose bengal [79], DNA plasmids [80], a study on the preparation variables [68, 69], a stability study [70]</td>
</tr>
<tr>
<td>Poly(D,L-lactic-co-glycolic acid) - poly(ethylene glycol)</td>
<td>Docetaxel [81], paclitaxel [82]</td>
</tr>
<tr>
<td>Poly(D,L-lactic-co-glycolic acid) - poly(vinyl alcohol)</td>
<td>DNA [83]</td>
</tr>
<tr>
<td>Poly(sebaic acid) terminated with lithocholic acid and poly(sebaic acid-co-lithocholic acid)</td>
<td>A model poorly water-soluble crystalline drug [67]</td>
</tr>
<tr>
<td>Poly(ε-caprolactone)</td>
<td>Cyclosporin A [12, 84], isradipine [58], atovaquone [62], primidone [85], Parsol MCX (a sunscreen agent) [86], griseofulvin [87], rhodamine (a fluorescent dye) [88], a stability study [70]</td>
</tr>
<tr>
<td>Poly(ε-caprolactone) - poly(ethylene glycol)</td>
<td>Rhodamine (a fluorescent dye) [88]</td>
</tr>
</tbody>
</table>
Overall, the challenge in nanoprecipitation is to find a drug/polymer/solvent/non-solvent combination, which allows successful nanoparticle production and drug encapsulation. However, scale-up of nanoprecipitation from laboratory-scale to pilot-scale has been recently reported [18].

2.1.3 Surface modification

Surface modification or coating by biocompatible (hydrophilic) polymers protects nanoparticles against uptake of the MPS and enhances stability. Polymers for this purpose include e.g. PEG, and the ethylene oxide / propylene oxide block copolymers poloxamers and poloxamines [31, 79, 88-93]. Mucoadhesive coatings by polymers like chitosan, poly(acrylic acid), sodium alginate and poloxamers improve bioavailability of the encapsulated drug by prolonging the residence time of nanoparticles at the absorption site (mucus layer) [88, 94-96]. Polysorbates and PEG on the particle surface are known to enable the transport across the blood-brain barrier [15]. In addition, the drug release profile may be modulated by surface modification.

Surface modifiers can be attached on the nanoparticles by adsorption (e.g. hydrophobic insertion) [90-93, 97, 98], grafting via covalent bonds [31, 99, 100], or by electrostatic interactions [32, 101, 102].

In layer-by-layer (LbL) coating, based on electrostatic interactions, polyelectrolytes (PEs) are adsorbed sequentially on charged substrates (Fig. 3) [32, 103]. For example, PLA nanoparticles are good substrates for LbL coating because of the carboxylic acid groups on the particle surface (generally negatively charged in dispersion). In LbL coating, the physicochemical properties of the resulting, coated nanoparticles can be controlled by the conditions of assembly such as pH, ionic strength, number of layers and type of PEs. Examples of polycations (positively charged PEs) applied to LbL coating are poly(allylamine hydrochloride) (PAH), poly(ethylenimine) and chitosan, while poly(styrene sulfonate) (PSS), dextran sulphate, poly(acrylic acid), chitosan sulphate, alginic acid and DNA molecules represent polyanions (negatively charged PEs) [32, 101, 102, 104-109].

![Figure 3. Sequential adsorption of polyelectrolytes on a charged particle.](image-url)
The most central challenge in an LbL process is to remove the unadsorbed PEs among the particles prior to the next layer. To overcome that, two methods have been suggested: (1) utilization of exact amounts of PEs with regards to the particle surface charge [32]; (2) intermediate removal of excess PEs by ultracentrifugation [32, 105, 110], or ultrafiltration [101] before each new layer. Determination of exact amounts might be difficult, and in some cases nanoparticle structure does not tolerate the forces of ultracentrifugation. However, once the processing conditions are optimized, the coating process can possibly be continued to tens of layers. Several LbL coating studies have been carried out using non-biodegradable polymers such as PAH and PSS [32, 101, 107, 109]. In the future, biocompatible and functional PEs are needed to develop surface-modified nanoparticles with MPS-avoidance and controlled release properties.

2.1.4 Freeze-drying

Briefly, in freeze-drying (lyophilization), solvent (e.g. water) in the system is frozen followed by its sublimation in vacuum. At present, freeze-drying is the method of choice to remove water from the system in nanoparticle formulations [12, 111-120]. However, freeze-drying of nanoparticles is a complicated process because the stability of the colloidal nanoparticle dispersion is, in most cases, disturbed, leading to aggregation before the particulate population is dry. Several variables affect the final result: freezing rate and temperature, drying temperatures, pressure, use of excipients like bulking agents or cryo- and lyoprotectants, and changes in pH or ionic strength. After successful drying, one should be able to re-disperse particles in their initial dispersing medium without causing significant changes in their size and drug content.

Freezing (temperature and rate) is the most crucial part of the process for particle survival. Freezing temperature affects the rate, e.g. if the product is frozen with the help of liquid nitrogen (fast freezing), but usually the freezing rate can be controlled. In general terms, slow freezing rates lead to a smaller number of large ice crystals and high freezing rates lead to a larger number of small ice crystals. It is possible that large ice crystals destroy the particles mechanically, which promotes the use of faster freezing [112]. On the contrary, in another study, slow freezing enabled survival and efficient drying of poly(caprolactone) nanoparticles [114], which could be explained on the basis of the more porous and permeable structure (for the water vapor) of the slowly frozen material [121]. In addition to the freezing protocol, changes in the dispersant phase may cause particle instability. During freezing, the nanoparticle concentration of the non-frozen phase is increased (more interactions between the particles) accompanied by possible changes in ionic strength and pH, which may induce aggregation.

Appropriate protective excipients are indispensable for a successful freeze-drying process, and the choice of the excipients has to be evaluated for each different nanoparticulate composition [12, 111, 115, 116, 119, 120]. In the case of poly(caprolactone) nanoparticles, when optimal amounts of protectants were applied, even the freezing rate did not significantly affect the result [122]. Cryoprotectants protect the product during freezing, whereas lyoprotectants provide stability during the drying step. Glycine, sucrose, glucose and poly(vinyl pyrrolidone) are cryoprotectants, while disaccharides such as trehalose, sucrose and...
lactose act usually as lyoprotectants [123-125]. The protective mechanism of disaccharides is understood to originate from an amorphous matrix, which is formed when the sugar is in contact with water [126]. Protection arises, when the amorphous matrix forms hydrogen bonds with the nanoparticulate product and act as a water substitute inhibiting the destructive effect of ice (water) crystals. Therefore, it is essential to maintain the amorphous material in the glassy state (below the glass transition temperature, $T_g$), to avoid crystallization of the sugar. For example, the $T_g$ of water-plasticized trehalose is approximately -30°C [126], which is higher than $T_g$s of most of the other sugars (disaccharides and monosaccharides). Thus, the use of trehalose enables a wider temperature scale for drying.

Examples of successfully freeze-dried nanoparticles are presented in a comprehensive review article [127].

### 2.2 Nanoparticle characterization

Colloidal systems like nanoparticles differ from macroscopic objects because of sub-micron properties such as high surface area and energy, and movement of the particles by diffusion (Brownian motion). The different behavior of nanoparticles leads, to some extent, to the use of a different pattern of characterization methods. Extensive characterization of a nanoparticle system is essential for understanding and prediction of the performance of the system in the body. As the field of pharmaceutical nanoparticles is evolving constantly, the need for more thorough characterization and comprehensive understanding of the systems increases.

Size, morphology and physical state of the encapsulated drug as well as $M_w$ and crystallinity of the polymer influence drug release and degradation of the nanoparticles. Meanwhile, size, surface charge and hydrophobicity/hydrophilicity are parameters that affect the body distribution and interactions with the biological environment. Stability of nanoparticles is also a general issue governing the above mentioned properties. In the following chapters, some common characterization methods of pharmaceutical nanoparticles are presented.

#### 2.2.1 Size, morphology, surface properties and stability

Conventional light microscopy is not suitable for nanoparticle characterization because its resolution is limited to about 1 μm. Instead, techniques for the characterization of nanoparticle size and morphology are scanning electron microscopy (SEM) [53, 56, 58, 68, 80] (Fig. 4) and transmission electron microscopy (TEM) [51, 54, 59, 64, 69, 74, 75, 99]. Nowadays, nanoparticles are usually studied with 5 000 – 30 000-fold magnifications. In a SEM setup, the nanoparticulate sample, coated to be conductive (e.g. platinum), is scanned in a high vacuum chamber with a focused electron beam [128]. Secondary electrons, emitted from the sample, are detected and the image formed. The environmental scanning microscope (ESEM) allows working even with moist samples, without conductive coatings and high vacuum. In TEM,
electrons scattered by the sample are detected; the sample is between the electron gun and the detector [129]. Another technique in pharmaceutical nanoparticle characterizations is atomic force microscopy (AFM) (scanning probe microscopy) [101, 130-133].

Photon correlation spectroscopy (PCS), a technique based on dynamic (laser) light scattering, is widely used in size determinations of nanoparticles [54, 56, 58, 59, 64, 67, 70, 73, 77, 79, 80]. PCS measures the intensity variation (because of the Brownian motion of nanoparticles) of scattered light, and relates it to the particle size with the help of an autocorrelation function [134]. As a result, a hydrodynamic diameter is obtained (PCS presumes all the particles being spherical). If the nanoparticle population is not monodisperse, the data can be further processed with the help of algorithms to give size distributions. PCS is a fast technique, sensitive to nanoscale particles, and provides information about the whole particulate population. On the other hand, the dispersion has to be diluted, filtered and the results are based on mathematical calculations (dispersant viscosity, temperature and refractive index should be known). Therefore, one should be vigilant when interpreting size information from PCS experiments. Instead, SEM/TEM provides visual and descriptive information, a real overview about the nanoparticle population. However, this information is not usually quantitative (size distributions). As a conclusion, micrographic images (electron microscopy) and a computational technique (light scattering) should be used together in the initial size determinations.

**Figure 4.** An SEM image of poly(styrene) nanoparticles with the size of 460 nm, 5 000-fold magnification.

Surface charge of nanoparticles, for its part, determines the performance of the nanoparticle system in the body, e.g. interactions with cell membranes. Zeta (ζ) potential measurements provide information about the particle surface charge [135]. ζ-Potential is the charge at the electrical double layer, created by ions of the liquid, which exists around each particle. The mobility of charged particles is determined with the help of electric potential (electrophoretic mobility) and transferred to ζ-potential, e.g. with the help of Smoluchowski’s equation. ζ-Potential is a function of conditions of the dispersing medium such as pH and electrolyte concentration [136]. E.g. polystyrene nanoparticles are found to exhibit the most extreme ζ-potential values at very low electrolyte concentrations [137]. Correspondingly, carboxylic acid groups at the PLA nanoparticle surface are well ionized at basic pH which decreases the ζ-potential value. ζ-Potential can also be altered by surface
modification [32] or stabilizer concentration [138]. ζ-Potential measurements are useful e.g. in the Lbl coating to verify the success of the process [32, 101].

Nanoparticles dispersed in aqueous solutions can be stabilized either by electrostatic stabilization (surface charge) or by steric stabilization (surfactants or other molecules at the particle surface), or by a combination of both [139, 140]. ζ-Potential represents a measure of an electrostatically stabilized colloidal dispersion: adequately high ζ-potential absolute values provide stability for nanoparticle dispersion. Values beyond +/-30 mV are considered characteristics for a stable colloidal dispersion [141]. According to the DLVO theory, aggregation occurs when attractive van der Waals forces between the particles become dominant. This loss of stability leads in an increase in polydispersity (detected e.g. by PCS) and, finally, in visual cloudiness. Aggregation is usually determined by turbidity measurements (ultraviolet (UV) spectroscopy); increasing turbidity indicates decreased stability [73, 105, 142].

The amount of the functional, charged groups on the surface of nanoparticles can be determined by e.g. acid number titration [143] or by conductometric titration [144]. Hydrophobicity of nanoparticles is characterized by hydrophobic interaction chromatography (HIC): hydrophilic particles pass the column faster while elution of hydrophobic particles is retarded [92, 98, 99, 145]. As an example of another technique, coating of polystyrene and PLA nanoparticles by a Vitamin E derivative [131, 146], and PLA nanoparticles by PEG [147] were verified by x-ray photoelectron spectroscopy (XPS). This technique, also known as ESCA (electron spectroscopy for chemical analysis), reveals the elemental and chemical composition at the surface. Other surface chemistry analysis methods include Fourier-transform infrared spectroscopy (FTIR) [148] and nuclear magnetic resonance spectroscopy (NMR) [149].

### 2.2.2 Drug-polymer interactions

Drug loading can be performed during the preparation of nanoparticles or by adsorbing/absorbing in preformed particles. Within the particle-forming polymer, drug can be present as a solid solution (individual drug molecules) or as a solid dispersion (amorphous/crystalline drug). It can be adsorbed on the particle surface [150] or bound chemically within the nanoparticles [151]. The preparation process can also modify the crystal structure of the drug. The polymer is usually amorphous or semi-crystalline. Differential scanning calorimetry (DSC), (powder) x-ray diffractometry (XRPD) and FTIR are commonly used techniques to reveal the physicochemical state and possible interactions of the drug and the polymer in pharmaceutical micro- and nanoparticles. Polymer $M_W$ is determined e.g. by size exclusion chromatography (SEC) [58, 70] (the term gel permeation chromatography (GPC) is interchangeably used [61, 152]).

DSC detects phase transitions such as glass transition, (exothermic) crystallization and (endothermic) melting: the nanoparticle sample is heated and changes in heat flow, compared to reference, are registered [153]. Crystallinity/amorphicity properties are obtained from XRPD analysis when diffraction pattern of the x-ray from the sample is determined as a function of scattering angle [154]. In FTIR, a vibrational spectrum, characteristics for a given crystal structure, is obtained [155].
Absence of the drug melting peak and diffraction peaks of the crystal structure of the drug in DSC thermogram and XRPD pattern, respectively, are usually signs of amorphous or molecularly dispersed drug within the polymer [156-164]. It can also indicate that the amount of drug is lower than the detection limit of the instrument [50, 160, 161, 165, 166]. Drug-polymer interactions (e.g. plasticizing effect of drug on polymer) or polymorph change of the drug can be detected as peak shifts in DSC thermogram, band shifts in FTIR spectra or as new reflections in XRPD pattern [162, 166-169]. Correspondingly, smoothened XRPD pattern, increased cold crystallization exotherms (DSC) or some band shifts to higher wavenumbers (FTIR) indicate increased amorphicity of the polymer [167].

2.2.3 Drug loading and release

Drug entrapment or encapsulation efficiency is a percentage value that describes the quantity of the drug material in the nanoparticles out of the total amount used in the process. The drug content (or drug loading) percentage is the drug amount compared to the nanoparticle mass. Because of the colloidal nature of nanoparticles, determination of drug encapsulation, drug loading and in vitro release may be problematic. However, the encapsulation is determined e.g. by separating nanoparticles from the dispersion medium by ultracentrifugation or ultrafiltration. Drug content is then quantified from the supernatant or after solvation of the nanoparticle pellet [12, 64, 80, 84, 88]. In the case of drug quantitation after freeze-drying, the polymer and drug are first dissolved [54, 63, 73, 94, 170]. For example, PLGA nanoparticles containing procaine hydrochloride were dissolved in acetonitrile and the drug was quantified from the solution by UV spectroscopy, because the polymer was found not to exhibit absorbance interference at the wavelengths used [54]. In another case, D,L-PLA nanoparticles containing acyclovir were dissolved in dichloromethane followed by extraction of the drug by an aqueous buffer [63]. The drug quantitation was performed by high performance liquid chromatography (HPLC).

Drug release from nanoparticles may occur by diffusion through the particle, by desorption from the surface or after degradation. Although the drug release environment in vivo is complex and may be difficult to simulate, important information about the release in vitro can be collected by several techniques. Ultracentrifugation is often exploited [63, 79, 85, 91, 94, 99, 170]. E.g. D,L-PLA nanoparticles containing savoxepine were stirred in a buffer solution [91]. At predetermined intervals, a sample was withdrawn and ultracentrifugated. Subsequently the drug content was analyzed from the supernatant by UV spectroscopy. Similarly, the withdrawn sample can be treated by ultrafiltration [54, 171]. In a dialysis setup, nanoparticles are placed either in small dialysis bags in a stirred receptor (aqueous) medium [53, 58, 86] or in the medium containing drug-free dialysis bags (reverse dialysis) [64, 75, 88]. Because the nanoparticles do not permeate the dialysis bags, the released drug is quantitated from the compartment not containing the particles. In addition to dialysis bags, diffusion cells separated with a membrane provides a possibility to monitor the drug diffusion (excluding particle diffusion) from one compartment to another [172, 173].
2.3 Nanoparticles in drug delivery applications

While the research in the field on pharmaceutical nanoparticles is extensive (reviewed e.g. in [20, 21, 152, 174-177]), introduction of commercialized products has not yet occurred. At the same time, several microparticulate drug delivery systems prepared from the same biodegradable materials are already on the market. Using as an example the polymer used in this study, PLA, and its copolymers with glycolic acid (PLGA), marketed microparticulate systems are listed in Table 2. These products offer mostly extended duration of therapeutic effect when injected into tissue (intramuscularly, subcutaneously). Instead, research in the field of biodegradable nanoparticles has concentrated on the formulation of systems that take advantage of their smaller size. The following paragraphs present examples of recent, in vivo tested nanoparticulate drug delivery applications based on PLA and PLGA polymers.

Table 2. Examples of marketed PLA and PLGA microparticulate drug delivery systems.

<table>
<thead>
<tr>
<th>Product</th>
<th>Drug / polymer</th>
<th>Indication</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arestin®</td>
<td>minocycline / PLGA</td>
<td>periodontitis, powder administered into periodontal pocket</td>
<td>OraPharma</td>
</tr>
<tr>
<td>Decapeptyl® Depot</td>
<td>triptorelin / PLGA</td>
<td>prostate cancer, edometriosis, i.m.</td>
<td>Ferring</td>
</tr>
<tr>
<td>Decapeptyl® SR</td>
<td>triptorelin / PLA, PLGA</td>
<td>prostate cancer, endometriosis, i.m.</td>
<td>Ipsen</td>
</tr>
<tr>
<td>Lupron Depot®</td>
<td>leuprolide / PLGA</td>
<td>prostate cancer, endometriosis, i.m.</td>
<td>TAP</td>
</tr>
<tr>
<td>Nutropin®</td>
<td>human growth hormone / PLGA</td>
<td>treatment of growth failure, s.c.</td>
<td>Genetech</td>
</tr>
<tr>
<td>Risperdal® Consta™</td>
<td>risperidone / PLGA</td>
<td>schizophrenia, i.m.</td>
<td>Janssen-Cilag</td>
</tr>
<tr>
<td>Sandostatin® LAR</td>
<td>octreotide / PLGA-glucose</td>
<td>acromegaly, i.m.</td>
<td>Novartis</td>
</tr>
<tr>
<td>Somatuline® PR</td>
<td>lanreotide / PLGA</td>
<td>acromegaly, i.m.</td>
<td>Ipsen</td>
</tr>
<tr>
<td>Suprecur® MP</td>
<td>buserelin / PLGA</td>
<td>endometriosis, i.m.</td>
<td>Sanofi-Aventis</td>
</tr>
<tr>
<td>Trelstal™ LA / Depot</td>
<td>triptorelin / PLA</td>
<td>prostate cancer, i.m.</td>
<td>Pfizer</td>
</tr>
<tr>
<td>Vivitrol®</td>
<td>naltrexone / PLGA</td>
<td>alcohol depence treatment, i.m.</td>
<td>Alkernes</td>
</tr>
</tbody>
</table>

i.m. = intramuscularly injected; s.c. = subcutaneously injected

After oral administration, nanoparticles (usually in suspension) are uptaken and transported across the mucosal epithelium by enterocytes or the M cells of Peyer’s patches because of their small size [16]. Before reaching these locations, nanoparticles protect the active ingredient in the gastro-intestinal (GI) tract and/or prolong the residence time of its contents on the mucous membrane. For example, oral absorption of peptides such as salmon calcitonin (osteoporosis treatment) using PLGA nanoparticles [178] and elcatonin using chitosan-coated PLGA nanoparticles [94] have been reported. Similarly, improved oral bioavailability (sustained release) of e.g. mifepristone [179] and estradiol [47] loaded in PLGA
nanoparticles has been shown due to the enhanced mucoadhesion. The animals used in these studies were rats. Delivery of antigens, such as tetanus toxoid with PLA-PEG nanoparticles [106], ovalbumin with PLGA-PEG nanoparticles [180] and bovine serum albumin (BSA) with PLGA nanoparticles [181] has led to better immunization responses because of the targeted uptake in the M cells of the test animals (mice). These kinds of systems are promising for oral vaccination.

When nanoparticles have reached the blood circulation, e.g. after intravenous injection or absorption from the GI tract, they are rapidly recognized and uptaken as foreign objects by macrophages. Therefore, strategies to escape the clearance mechanism and thus to improve the in vivo biodistribution are developed. Most of these approaches are based on nanoparticle surface engineering. Several examples can be found in the field of tumor/cancer treatment. Due to the prolonged time in blood circulation, e.g. camptothecin-loaded PEG/poly(propylene glycol)-coated PLA nanoparticles prolonged the mean residence time of the drug in mice to 25 hours (compared to 0.6 h of the free drug) [182]. Paclitaxel-loaded methoxy-PEG-PLA nanoparticles provided approximately 3-fold longer half life and effective concentration of the drug in rats, without concentration peaks above toxic level compared to a conventional formulation [82]. On the other hand, sustained drug release by nanoparticles may not always be a key factor determining the drug efficacy: PEG-PLA nanoparticle-delivered doxorubicin has performed similar anti-cancer effects in mice compared to the free doxorubicin [183]. However, reduced toxicity of the drug was emphasized also in this case.

Targeting of drugs by the natural body distribution of nanoparticles is thought to be an alternative to treat tumors in fenestrated and phagocytically active tissues such as liver, lung and spleen. For example, oridonin-loaded PLA nanoparticles have maintained the drug concentration high in these tissues of mice (36 h) compared to the free drug (0.5 h) [13]. Recently, tumor targeting, independent of body distribution, has been reported: paclitaxel-loaded poly(vinyl alcohol)-PLGA nanoparticles have been successfully targeted to prostate tumors of mice with the help of an RNA aptamer (binds to the tumors) on the particle surface [81].

Another studied application of nanoparticles is drug delivery across the blood-brain barrier (BBB). For that purpose, the particles should be coated by suitable surfactants (e.g. Polysorbate 80, PEG) [15]. Thus far, most of these studies have been carried out with poly(alkylcyanoacrylate) nanoparticles [184]. Brain localization has also been achieved using the probably safer poly(lactic acids): intranasally administered (in rats) PLA-PEG nanoparticles functionalized by lectin [185], intravenously injected (in mice) PLA-PEG nanoparticles bearing cationic BSA coating [186], PLA-polysorbate 80 nanoparticles (in mice) [187], and PLGA-peptide nanoparticles (in rats) [188]. Recently, loperamide has been delivered to the central nervous system of rats with sub-200 nm PLGA-peptide nanoparticles and sustained effect of the drug was observed [189]. However, the mechanisms of particle transport across the BBB are not entirely clarified; several different processes are probably involved depending on the nanoparticle characteristics [15].

In gene delivery studies, DNA is coupled to nanoparticles to create non-viral vectors protecting the DNA in body conditions. A size of around 100 nm and the positive charge of the particles facilitate the delivery and interactions with the cell membrane, respectively. For example, orally administered PLA-PEG nanoparticles have protected the encapsulated DNA and resulted in high efficiency transfection in mice [190]. With chitosan coating on the
Review of the literature

particles, the gene expression was even more pronounced. Intramuscularly injected PLGA nanoparticles in mice have been reported to release DNA in a prolonged manner which promoted the antigen presentation [191]. Increasing the amount of DNA encapsulated in the particles, while maintaining the small nanoparticle size, is one of the key challenges in nanoparticulate gene delivery applications.

Nanoparticles with mucoadhesive properties can improve ocular drug delivery by prolonging the residence time of the drug in the tear film, controlling release and reducing irritation after topical administration. Because of these benefits, e.g. flurbiprofen (an anti-inflammatory drug) encapsulated in PLGA nanoparticles showed higher anti-inflammatory effect than the corresponding commercial eye-drops [78]. Similarly, acyclovir-loaded PEG-PLA nanoparticles provided enhanced ocular bioavailability compared to the free drug: a 12.6-fold increase in the total drug concentration was detected and the concentration remained at an effective level three times longer [63]. These experiments were performed using rabbits.

Despite the successful \textit{in vivo} tests with animals, the relevance of these findings to human body conditions as well as long-term effects of the formulations is uncertain. However, biodegradable colloidal drug delivery systems seem to provide promising treatment for various diseases using different administration routes.
Aims of the study

The purpose of this study was to prepare nanoparticles from poly(lactic acid) polymer and to characterize physicochemical properties of the starting materials and the nanoparticles by different methods. The aim was to evaluate and understand the effect of different process conditions and environments on the nanoparticle characteristics.

Specifically, the following themes were examined:

- Effect of the nanoparticle preparation method, nanoprecipitation, on the physicochemical properties of the polymer and the encapsulated drugs
- Application of capillary electrophoresis, a novel method in the field of biodegradable pharmaceutical nanoparticles, in the quantitation of the encapsulated drugs
- Evaluation of stability and aggregation behavior of the prepared nanoparticles with the help of surface pressure measurements – an alternative method to characterize pharmaceutical nanoparticles
- Improvement of the freeze-drying process of the nanoparticles by the use of cryo- and lyoprotectants
- Introduction and development of a layer-by-layer coating process for poly(lactic acid) nanoparticles
4 Experimental

4.1 Materials

Materials used in this work and references to the corresponding publications are summarized in Table 3. All other used materials were of analytical grade.

Table 3. Materials used in this work.

<table>
<thead>
<tr>
<th>Material</th>
<th>Publication</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>II, III</td>
<td>Riedel-de-Haën, Seelze, Germany</td>
</tr>
<tr>
<td>Beclomethasone dipropionate (BDP)</td>
<td>I, II</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
</tr>
<tr>
<td>Chloroform (CHL)</td>
<td>I, IV, V</td>
<td>Riedel-de-Haën, Seelze, Germany</td>
</tr>
<tr>
<td>D-glucose</td>
<td>IV</td>
<td>Fischer Scientific UK Ltd., Leicestershire, UK</td>
</tr>
<tr>
<td>Dichloromethane (DCM)</td>
<td>I, V</td>
<td>Riedel-de-Haën, Seelze, Germany</td>
</tr>
<tr>
<td>Ethanol (96% v/v)</td>
<td>I, IV, V</td>
<td>Primalco, Rajamäki, Finland</td>
</tr>
<tr>
<td>Hydrochloric acid (HCl)</td>
<td>III, V</td>
<td>Shannon Co., Clare, Ireland</td>
</tr>
<tr>
<td>Lactose</td>
<td>IV</td>
<td>DMV International, Veghel, The Netherlands</td>
</tr>
<tr>
<td>Paper filters (11 µm)</td>
<td>II-V</td>
<td>Whatman, Brentford, UK</td>
</tr>
<tr>
<td>Poloxamer 188 (Lutrol® F 68)</td>
<td>III</td>
<td>BASF, Ludwigshafen, Germany</td>
</tr>
<tr>
<td>Poly(allylamine hydrochloride), $M_w$ 70 000 g/mol (PAH)</td>
<td>V</td>
<td>Sigma-Aldrich, Steinheim, Germany</td>
</tr>
<tr>
<td>Poly(L-lactic acid) (L-PLA), $M_w$ 2 000 g/mol</td>
<td>I, IV, V</td>
<td>ICN Biomedicals Inc., Aurora, USA</td>
</tr>
<tr>
<td>Poly(D,L-lactic acid), IV 0.20 dl/g (D,L-PLA)</td>
<td>II, III</td>
<td>PURAC Biomaterials, Gorinchem, The Netherlands</td>
</tr>
<tr>
<td>Poly(sodium 4-styrenesulfonate), $M_w$ 70 000 g/mol (PSS)</td>
<td>V</td>
<td>Sigma-Aldrich, Steinheim, Germany</td>
</tr>
<tr>
<td>Polycarbonate membrane filters (0.2 µm)</td>
<td>III, V</td>
<td>Millipore, Molsheim, France</td>
</tr>
<tr>
<td>Polysorbate (Tween) 80</td>
<td>IV</td>
<td>Fluka Chemie, Buchs, Switzerland</td>
</tr>
<tr>
<td>Propylene glycol (PG)</td>
<td>I, II, IV, V</td>
<td>YA Kemia, Helsinki, Finland</td>
</tr>
<tr>
<td>Salbutamol sulphate (SS)</td>
<td>I, II</td>
<td>A donation from Orion Pharma, Espoo, Finland</td>
</tr>
<tr>
<td>Sodium chloride (NaCl)</td>
<td>III, V</td>
<td>Riedel-de-Haën, Seelze, Germany</td>
</tr>
<tr>
<td>Sodium cromoglycate (SCG)</td>
<td>II, IV</td>
<td>ICN Biomedicals Inc., Aurora, USA</td>
</tr>
<tr>
<td>Sodium hydroxide (NaOH)</td>
<td>III, V</td>
<td>Shannon Co., Clare, Ireland</td>
</tr>
<tr>
<td>Water (ultrapurified)</td>
<td>I-V</td>
<td>Millipore, Molsheim, France</td>
</tr>
</tbody>
</table>
4.2 Methods

Experimental equipment and methodology used in this work together with references to the corresponding publications are summarized in Table 4. Detailed descriptions of the methods are given in the original publications or references therein.

Table 4. Methods and equipment used in this work.

<table>
<thead>
<tr>
<th>Method / equipment</th>
<th>Publication</th>
<th>Equipment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capillary electrophoresis (CE)</td>
<td>II</td>
<td>HP CE equipped with a photodiode array detector (Hewlett-Packard, Waldbronn, Germany)</td>
</tr>
<tr>
<td>Differential scanning calorimetry (DSC)</td>
<td>I, V</td>
<td>DSC822e with STARe software (Mettler Toledo, Columbus, USA)</td>
</tr>
<tr>
<td>Fourier transform infrared spectroscopy (FTIR)</td>
<td>I</td>
<td>Spectrum One with Universal ATR Sampling Accessory (Perkin-Elmer, Boston, USA)</td>
</tr>
<tr>
<td>Freeze-drying</td>
<td>IV</td>
<td>HETO LyoPro 3000 (Heto-Holten A/S, Allerød, Denmark)</td>
</tr>
<tr>
<td>Layer-by-layer coating (LbL)</td>
<td>V</td>
<td>Vacuum filtration equipment (Millipore, Molsheim, France)</td>
</tr>
<tr>
<td>Photon correlation spectroscopy (PCS)</td>
<td>II-V</td>
<td>Zetasizer 3000HS (Malvern Instruments, Worcestershire, UK)</td>
</tr>
<tr>
<td>Scanning electron microscope (SEM)</td>
<td>I, III-V</td>
<td>Agar Sputter Coater (Agar Scientific Ltd., Essex, UK) and DSM 962 (Zeiss, Jena, Germany)</td>
</tr>
<tr>
<td>Surface pressure measurements</td>
<td>III</td>
<td>KSV Minitrough (KSV Instruments, Helsinki, Finland)</td>
</tr>
<tr>
<td>X-ray powder diffractometry (XRPD)</td>
<td>I</td>
<td>Bruker axs D8 (Bruker, Karlsruhe, Germany)</td>
</tr>
<tr>
<td>ζ-Potential measurements</td>
<td>III-V</td>
<td>Zetasizer 3000HS equipped with MPT-1 titrator (Malvern Instruments, Worcestershire, UK)</td>
</tr>
</tbody>
</table>
4.2.1 Preparation, coating and freeze-drying of nanoparticles

Nanoparticles were prepared either by the nanoprecipitation method ([II, III]) [51] or by a modified nanoprecipitation method ([I, IV, V]) [72]. In the nanoprecipitation method, 25 mg of D,L-PLA was dissolved in acetone (2 ml). If beclomethasone dipropionate (BDP) was included in the process, it was dissolved with the polymer whereas salbutamol sulphate (SS) and sodium cromoglycate (SCG) were first dissolved in water (0.15 ml) before mixing the solutions together. This inner phase was then added into the outer phase, 4 ml of water, with a syringe and a gauge. If Poloxamer 188 was included in the formulation, it was dissolved (20 mg) in the outer phase. In the modified method, 25 mg of L-PLA together with 175 mg of propylene glycol (stabilizer) were dissolved in 2 ml of chloroform (CHL) or dichloromethane (DCM). In the formulations containing BDP, the drug was dissolved with the polymer and the stabilizer. In the case of SS and SCG, the drug was dissolved in 0.15 ml of water and 0.7 ml of 96% ethanol was added (a co-solvent). In all the formulations prepared by the modified method (drug or no drug), the polymeric solution was mixed to the water-ethanol solution. The formed dispersion was added dropwise to 5 ml of 70% ethanol solution under mild stirring. In the both methods ([I-V]), the organic solvents were evaporated in a fume cupboard at room temperature. Prior to filtration through a paper filter, the dispersions were diluted with water or NaCl solution (Zetasizer analysis and LbL coating), or with isopropanol (surface pressure experiments).

LbL coating (V) was performed on a membrane using a vacuum filtration setup (Fig. 1 in V). 10 ml of nanoparticle dispersion in 0.02 M NaCl, stirred gently throughout the process, was used in the coating experiments. PEs were dissolved in 0.02 M NaCl (1 mg/ml). Particles were exposed to each PE solution, 200 µl, for 5 min followed by two washing cycles: vacuum filtration until the volume of the dispersion was about 2 ml followed by expansion of the volume to 10 ml by 0.02 M NaCl. A weak overpressure under the membrane together with gentle brushing was applied to detach the particles from the membrane after filtration. The membrane was replaced by a new one after each coating layer.

In freeze-drying (IV), the protective excipients glucose (50-400 mg) and lactose (100-300 mg), as aqueous solutions, were added to the nanoparticle dispersions after evaporation of the organic solvents. The nanoparticle dispersions were frozen at -32 °C for a minimum of 12 h and freeze-dried at -55 °C and 0.5 kPa for 24 h. Additional freeze-thawing (freezing and subsequent melting at room temperature) experiments were conducted to evaluate the performance of the protectants during the freezing step.

4.2.2 Characterization of nanoparticles

ζ-Potentials and size distributions were determined with Zetasizer ([II-V]). Electrophoretic mobilities were converted to ζ-potentials using Smoluchowski’s equation. In PCS, the results were analyzed by the CONTIN algorithm and the sizes were presented as intensity distributions.
Materials and methods

Surface pressure versus surface area (π vs. A) isotherms on different aqueous solutions (subphases) were recorded by the Wilhelmy plate technique using a Langmuir trough (III). 40-75 µl of the particle dispersion was spread dropwise on the subphase surface with a Hamilton microsyringe. The surface was compressed at a speed of 10 mm/min. For the SEM analysis, particle populations were deposited on trichloro octadecyl silane -functionalized silicon plates by touching the surface with a horizontal plate (Langmuir-Schaefer deposition).

Other SEM samples of nanoparticles were prepared by depositing a drop of particle dispersion (I, IV) or by attaching (with two-sided tape) a piece of a filter membrane containing the particles (V) on a metal plate. The metal and silicon plates were sputtered for 20 s with platinum prior to the SEM analysis.

Drug encapsulation in the nanoparticles was determined by capillary electrophoresis (CE) (II). The samples were injected and analyzed in fused-silica capillaries. In the case of SS and SCG, the nanoparticles (in water), filtrates or water solutions obtained after particle decomposition with chloroform were detected at a wavelength of 200 nm (UV detection). For BDP, the nanoparticles as such (in water), and the filtrate (particles separated by filtration through 100 nm membrane filter) or dispersing medium of the particles (methanol, microemulsion or aqueous sodium dodecyl sulphate solution) were detected at a wavelength of 254 nm (UV detection).

Before DSC experiments (I, III), dispersions were allowed to dry and weighed (2-4 mg) in aluminium pans. Powder samples were used as such. The pans were sealed with aluminium caps. The samples were heated at the rate of 10 °C/min. Prior to 2nd scan (I), the samples were cooled down after the 1st scan to -10 °C at a rate of 20 °C/min. The locations of the thermal transitions and surface areas of the peaks (V) were obtained from the thermograms. Similarly, dry powder samples were also used in FTIR experiments (I).

Samples for XRPD analysis (I) were prepared by depositing and drying the nanoparticle dispersions on silicon plates. Powder samples were analyzed as such. The experiments were performed in symmetrical reflection mode. The scattering was recorded from 5° to 30° with steps of 0.1° (20 s per step). Crystallinity of the samples was estimated by comparing the intensities of crystalline and amorphous samples. The single crystal data of the Cambridge Structural Database (CSD) was used in the identification of crystal structures.

The success of the freeze-drying (IV) was also evaluated by visual inspection of the dried formulations (volume and porosity of the cakes), as well as by redispersibility of the nanoparticles. The cake was redispersed in 10 ml of water or 2.5 ml of aqueous Tween 80 solution (1.2 mg/ml). Aggregate formation and the Tyndall effect in the redispersions were observed.
5 Results and discussion

5.1 Characteristics of the nanoparticles

5.1.1 Preparation of the nanoparticles

Generally, the PLA nanoparticles prepared were smooth and spherical (Fig. 5). The polydispersity indices obtained from the PCS measurements were around 0.1 or lower indicating narrow deviations in sizes.

Figure 5. Examples of PLA nanoparticles. SEM images of empty nanoparticles prepared with DCM (left) or CHL (middle) as solvents for L-PLA, and SS containing L-PLA nanoparticles prepared with CHL as a solvent (right).

Low $M_w$ polymers were selected for this study because of their faster biodegradation compared to higher $M_w$ polymers ($M_w$s well above 10 000 g/mol) [38, 41]. Fast degradation is desirable if the retention of the polymer in the body is unfavourable, e.g. after pulmonary delivery. However, production of particles from low $M_w$ PLA sets up certain challenges compared to higher $M_w$ polymers. Low $M_w$ PLA has higher solubility in some commonly used organic solvents and, therefore, precipitation of the polymer occurs more slowly and more solvent diffusion is needed to precipitate the polymer during preparation [143, 165]. The surface of the low $M_w$ PLA nanoparticles is reported to be more adhesive than that of the particles from polymers with higher $M_w$, which causes an aggregation tendency [164]. The particle yield has also been reported to decrease as the water soluble fraction was increased by the addition of low $M_w$ PLA in the composition [143].

The L-form of PLA was selected for this study (I, IV, V) in order to evaluate the properties of this semi-crystalline polymer with drugs and in different processes. Moreover, the L-PLA polymer, compared to the D,L-PLA polymer, is seldom used in nanoparticulate applications. Preparation of the nanoparticles from L-PLA was performed by a method optimized for this particular polymer [71, 72] because of the solubility limitations of the semi-crystalline L-form: it is soluble only e.g. in CHL or DCM. A co-solvent, ethanol in this case, was needed to increase the mutual miscibility between the solvent of the polymer and the outer aqueous phase. The nanoparticles were prepared using either CHL (IV, V) or DCM (I, V) as solvents for
Results and discussion

The (empty) particles using DCM were slightly smaller, approximately 350 nm compared to 400 nm in the case of CHL. Solubility of PLA polymers is higher in DCM than in CHL and DCM has slightly higher miscibility in water. This obviously led to faster diffusion of DCM into the outer phase (70% ethanol), which created smaller polymer-containing droplets ready to precipitate as smaller nanoparticles compared to the formulations with CHL as a solvent. Correspondingly, the lower the interfacial tension is or, similarly, the interaction parameter between the organic solvent and the outer phase, the smaller are the nanoparticles formed.

The nanoparticles from D,L-PLA were prepared by the original nanoprecipitation method with or without a surfactant, Poloxamer 188. Nanoparticles with the surfactant were larger than the surfactant-free nanoparticles (approximately 290 nm compared to 240 nm), probably due to surfactant adsorption on the particle surfaces. The D,L-PLA nanoparticles were smaller than those prepared from the L-PLA polymer. Acetone, the solvent used in the preparation in this case, is miscible in water in all proportions, which affected the droplet size in the outer phase, polymer precipitation and, thus, led to smaller particle size. The D,L-PLA nanoparticles were prepared by injecting the inner phase into the outer phase using a fine gauge needle. In contrast to the dropwise addition, this approach was expected to spread the organic solvent better into the outer phase as the density of acetone is smaller than that of water.

When SS or SCG were encapsulated in the nanoparticles, the particle size was bigger (~50 nm difference) compared to the BDP-loaded or empty nanoparticles (Fig. 5). When the phases were fused during the nanoprecipitation, water soluble drugs (such as SS and SCG) in the inner phase created a solvent flow of the outer aqueous phase towards the inner phase because of a concentration gradient (no drug initially in the outer phase). The osmotic pressure thus formed increased the polymeric droplet size before polymer precipitation leading to bigger particles.

Although the nanoprecipitation process is relatively simple to perform, the spontaneous particle formation process involves complex interfacial phenomena that can be described as outer phase/solvent, outer phase/polymer and solvent/polymer interactions. In more detail, the organic (polymer) phase viscosity, stabilizer, drug, solvent properties and polymer properties (e.g. $M_n$ and ability to precipitate), among others, give their contribution to the final particle properties. This variety enables speculative discussion concerning the role of each parameter e.g. on the particle size, as was the case of the PLA nanoparticles in this study.

5.1.2 Physical characterization of the polymer and the encapsulated drugs

The physical state of the PLA polymer and the encapsulated drugs was studied by DSC, XRPD and FTIR. These analytical methods provide extensive information about the interactions between the polymer and the drugs, as well as about the influence of the nanoparticle preparation process on the materials. A selection of DSC thermograms describing the behavior of the PLA polymers is presented in Figure 6 (also Fig. 4 in and Fig. 6 in V). In the thermograms of L-PLA, an endothermic event at 152 °C is indicative for the melting of the crystalline polymer. Correspondingly, the only detectable thermal event of the
amorphous D,L-PLA is glass transition, which is located at 45 °C. Generally, the nanoprecipitation process decreased the crystallinity of L-PLA because the polymer network did not have enough time to be organized prior to the precipitation during the solvent diffusion into the outer phase [167]. This could be observed as reappearance of the glass transition and cold-crystallization (at 100 °C, crystallization of amorphous material) thermal events (Fig. 6). The same observation could also be noticed from the XRPD data: the calculated crystallinity proportion of L-PLA decreased from 59% to 39% as a consequence of nanoprecipitation (Table 2 in I). The L-PLA nanoparticles prepared with CHL were more crystalline than the particles prepared with DCM (V). Again, the solvent diffusion probably explains the difference: slower diffusion of CHL to the outer phase during nanoprecipitation left more time for the polymer to obtain favourable conformation for the crystallization.

Figure 6. Examples of the PLA thermograms.

Salbutamol sulphate and beclomethasone dipropionate were used as model drugs in the evaluation of the nanoprecipitation process on the properties of L-PLA and the drugs (I). No interactions between the drugs and PLA were detected. However, the physical state of the components was affected. Effect of the nanoprecipitation was studied by increasing the amount of the drug used in the process.

In the case of SS, although the amount of the drug (the drug being more crystalline than L-PLA according to the XRPD studies) was increased with respect to the polymer, the relative amount of the crystalline PLA remained high (Table 2 in I). As this was opposite to the finding that the nanoprecipitation decreased the crystallinity of L-PLA, it seemed obvious that the presence of SS enhanced the crystallinity of L-PLA. During the DSC experiments, when these samples were cooled down after the thermogram registration, the polymer exhibited a tendency to crystallize even though the cooling rate was 20 °C/min. In addition, cold-crystallization exotherm of L-PLA was decreased during re-heating as the amount of SS was increased, which indicated increased crystallinity. SS acted as plasticizer for L-PLA by enhancing the polymer chain mobility and thus promoting crystallization tendency [195, 196]. Instead, although amine drugs such as SS are known to interact electrostatically with carboxylic acids (chain ends of PLA) [197], no such interactions were found in this study.
When BDP was used as a model drug, it did not seem to modify the crystallinity of L-PLA compared to the empty nanoparticles. BDP is reported to form solvates with a variety of solvents such as alcohols [198] and halogenated hydrocarbons [199]. In this study, the anhydrous BDP used was changed to monohydrate as a result of nanoprecipitation (Figs. 3 & 9 and Table 2 in I). BDP-containing samples were also treated by cooling and re-heating. Contrary to the SS-PLA samples, neither the polymer nor the drug crystallized during the cooling. The \( T_g \) and \( T_c \) of an amorphous material (a portion of L-PLA in this study) can be increased by the addition of another amorphous compound having a higher \( T_g \) (BDP) [200]. Indeed, during the re-heating, the amorphous BDP increased the \( T_g \) and \( T_c \) of the polymer.

It should be noted that the crystallinity values calculated from the XRPD results were estimations based on a mathematical model and the error marginal in the values was approximately 10%. However, comparison of the values e.g. as a result of increased drug amount provided useful information about the changes in the crystalline/amorphous nature.

FTIR results supported the findings of the DSC and the XRPD studies: no detected interactions between SS and PLA and transformation of the anhydrous BDP to the monohydrate form.

Overall, these findings indicated that the nanoprecipitation process influences the physical properties of the polymer and the drugs. Even though the drug was molecularly dispersed within PLA in the nanoparticles, the drug may modify the polymer and nanoparticle properties. Also phenomena such as drug crystallization, affecting the drug properties, may occur during storage or further processing.

### 5.1.3 Determination of drug content

In paper II, applicability of CE in quantitation of drugs encapsulated in the D,L-PLA nanoparticles was estimated. CE is a versatile and sensitive technique for the separation and determination of various substances [201].

A method for the determination of water soluble SS and SCG could be developed. The results demonstrated that, with the loading protocol used, no drugs (SS or SCG) could be found inside the nanoparticles at the detection moment; all the drugs used for the preparation could be found outside the nanoparticles in the water phase. These results are consistent with the observation that the entrapment of hydrophilic drugs inside nanoparticles by the nanoprecipitation method is problematic [52]. No increase in the encapsulation could be achieved when the solubility of SS (pKa 9.3) in aqueous outer phase was decreased by increasing the pH of the phase from neutral to 8 (Fig. 4 in II). To successfully entrap hydrophilic drugs inside the PLA nanoparticles, the preparation method should be further optimized by changing the compositions of the outer and inner phases (of nanoprecipitation). However, significant changes e.g. in pH and ionic strength expose the particles to aggregation as reported in the chapter 5.1.5 and in paper III.

A suitable method for the determination of hydrophobic BDP was developed using microemulsion electrophoretic chromatography (MEEKC) [202]. In MEEKC, hydrophobic substances are solubilized and detected from the microemulsion phase. In more detail, the optimized microemulsion of this study consisted of octane, 1-butanol, sodium dodecyl sulphate, and sodium tetraborate buffer. Approximately 30% of the drug used in the
nanoparticle preparation was found inside the particles. The results with the BDP-loaded nanoparticles suggest that the quantitation technique could even detect the drug inside the nanoparticles: BDP was detected from the nanoparticulate dispersion (in water) but not from the filtrate (Fig. 5 in II). Instead, when the nanoparticles were mixed with the microemulsion, almost 90% of the successfully encapsulated BDP was found in the filtrate. This finding may provide a novel approach to drug encapsulation determination as the existing conventional techniques require separation of the particles from the dispersing medium.

Overall, electromigration techniques based on CE can be successfully used in the determination of different drugs used in nanoparticle preparation processes.

5.1.4 Surface charge of the nanoparticles

Stability of the PLA nanoparticles was evaluated by ζ-potential measurements (III, V). ζ-Potential, determined by the state of the functional groups (carboxylic acid in the case of PLA) at the surface of nanoparticles, is a function of environment, i.e. ionic strength and pH.

ζ-Potentials of the PLA nanoparticles as a function of the added electrolyte concentration and pH are presented in Figure 7. Generally, the nanoparticles were most charged at low ionic strengths (0.02 mol/l), when NaCl concentration of the dispersing medium was increased gradually from zero (Fig. 3 in V). The lowest ζ-potential values of the L-PLA and D,L-PLA nanoparticles were approximately -56 mV (V) and -76 mV (III), respectively. The difference between the ζ-potential values of the two PLA nanoparticles probably originated from the difference of the polymer chain: the D,L-PLA in question was tailored to carry uncapped chain ends (free carboxylic acids).

Figure 7. ζ-Potential of the L-PLA (●) and the D,L-PLA (○) nanoparticles as a function of NaCl addition (left) and pH (right).

At some point during the electrolyte addition, PLA nanoparticles are known to start to aggregate [203]. When the electrolyte concentration was increased towards 0.1 mol/l and beyond, ζ-potential approached zero and the particles started to aggregate. At low electrolyte concentrations, the range of the electrical double layer around the nanoparticles was high (ζ-potential maximum) and according to the DLVO theory, repulsive forces extended to a large distance [140]. At high electrolyte concentrations, free ions in the
medium decreased the double layer magnitude and the particles could approach each other giving rise to attractive van der Waals forces leading to aggregation. Decreasing pH to acidic, ζ-potential approached zero as the carboxylic acid groups on the surface were deionized. When pH is titrated to acidic values e.g. by HCl addition, the dissociated H⁺ and Cl⁻ ions may act first as electrolytes, which surpass the surface charge-decreasing effect of the pH decrease. This was observed in the case of D,L-PLA nanoparticles (Fig. 1 in III).

Variation in the ζ-potential values was also observed if the processing conditions were modified although the polymer type remained the same. Nanoparticles prepared from L-PLA using CHL as the solvent were more charged (ζ-potential ~ -50 mV) than the particles prepared with DCM (ζ-potential ~ -30 mV) (V). An explanation could be based on the slower diffusion rate of CHL to the outer phase (compared to DCM) and subsequent slower precipitation of PLA, as the hydrophilic chain ends of the polymer (ionized carboxylic acid groups) had more time to be organized at the solvent - outer phase interface towards the aqueous outer phase making the formed surface more charged. In the case of D,L-PLA nanoparticles prepared with the surfactant, Poloxamer 188 (III), adsorption of the surfactant molecules on the nanoparticle surfaces was detected as minor ζ-potential (absolute) values compared to the surfactant-free nanoparticles.

5.1.5 Stability and aggregation studies by surface pressure measurements

Behavior of colloidal particles (stability, aggregation, film forming ability etc.) at interfaces such as water-air or water-oil has been a topic of several studies during recent years. Surface pressure can be studied by the Wilhelmy plate technique when the particle population is compressed as a monolayer at an interface (π vs. A isotherms). Surface pressure determinations are a widely used tool in the characterization of inorganic particles, but its application in the studying of organic nanoparticles has been less frequent. Thus far, mainly polystyrene nanoparticles have gained such research attention [131, 204-206]. In paper III, stability and aggregation behavior of D,L-PLA nanoparticles in different environments were characterized by surface pressure measurements and SEM visualizations.

The π vs. A isotherms and corresponding SEM images of the surfactant free PLA nanoparticles clearly revealed that the particle aggregation had started at pH 4.0 during the pH decrease (Fig. 8 and also Figs. 2 & 6 in III). The isotherms registered on subphases at pH 4.0 or below exhibited increase in the surface pressure at larger surface area and higher surface pressure in the end of the compression. The formed particle aggregates obviously resisted (mechanically) the compression more than the nanoparticle population consisting of the more charged individual particles (pH above 4.0), which could be rearranged in a more flexible way.

When the nanoparticles were prepared with Poloxamer 188, the surfactant molecules located on the nanoparticle surface created a steric barrier that resisted the compression at longer distances compared to the surfactant-free nanoparticles. This could be seen as an earlier increase in the surface pressure during the compression (Fig. 8 and also Fig. 3 in III). Repulsion (pressure) was similar in proximity of the most compressed state in the cases of the both particles. Therefore, contribution of the surface charge was probably dominant at these identical sections of the isotherms. The surfactant, i.e. steric stabilization, protected the
nanoparticles against pH-induced aggregation, which could be seen as a non-aggregated particle population even at pH 4.0 (Fig. 7 in III). These findings promote the role of both the surface charge and the surfactant in the nanoparticle stabilization [203].

**Figure 8.** $\pi$ vs. $\Lambda$ isotherms (left) and SEM images (right, 1 000-fold magnification) of the surfactant-free nanoparticles (except * indicating the isotherm of the nanoparticle prepared with Poloxamer 188) on different subphases.

Increased surface charge ($\zeta$-potential) and, thus, increased repulsion between the surfactant-free nanoparticles, induced by electrolyte addition (0.02 mol/l NaCl), caused a rise in the surface pressure values (Fig. 4 in III). Cluster formation as a sign of aggregation was observed in SEM images, when the electrolyte concentration was further increased to 0.15 mol/l. Also this time, Poloxamer 188 prevented particle aggregation at the higher NaCl concentration.

Aggregate formation of the nanoparticles was treated by the classification system of Robinson and Earnshaw [207]. If the aggregation occurs spontaneously by particle diffusion and collisions, the process is referred to diffusion-limited cluster aggregation (DLCA). Correspondingly, if the probability for the aggregation is lower, e.g. due to repulsion or steric barriers between the particles, the process occurs by reaction-limited cluster aggregation (RLCA). The aggregation due to the pH decrease occurred by DLCA and resulted in percolated particle networks (Fig. 8 and also Fig. 6 in III), if the surfactant was not present. With surfactant, or if the surface charge was high enough as a result of electrolyte addition, the aggregation followed the RLCA regime. Under such conditions, the formation of networks was prevented and the aggregates remained as clusters (Figs. 7 & 8 in III).

### 5.2 Freeze-drying

Protective excipients, such as carbohydrates, are widely used in freeze-drying to ensure redispersibility and to avoid aggregation or size changes of nanoparticles [12, 111, 113, 115, 116]. In paper IV, glucose and lactose were evaluated as cryo- and lyoprotectants for the L-
PLA nanoparticles because these nanoparticles could not survive during the drying process without protectants.

Even the smallest tested amount of glucose (weight ratio glucose:nanoparticles 1:4) was found to protect the nanoparticles (Fig. 3 and Table 2 in IV), although the appearance of the dried material was translucent and sticky, and its redispersibility was poor. When lactose was used as a protectant, it enhanced the appearance of the cake (the dried material) as a white powder, eligible for a freeze-dried formulation. Redispersion of the nanoparticle was possible, but as a form of visible aggregates. Further freeze-thawing experiments revealed that already the freezing step (with lactose) destroyed the particles (Fig. 4 in IV). Next, the two carbohydrates were used together to combine the cryoprotective functionality of glucose and the lyoprotective functionality of lactose. The best result, prolonged Tyndall effect (opalescence in the dispersion) after redispersion of the dried formulation and good-quality nanoparticles (Fig. 6 in IV) were obtained, when the amount of lactose was double the amount of glucose. The weight ratios of glucose and lactose to the nanoparticles were 1:2 and 1:1, respectively. Additionally, when an extra stabilizer, Tween 80, was used during the nanoparticle preparation or during the redispersion, the freeze-dried cake could be redispersed more easily with increased stability (prolonged Tyndall effect).

The good cryoprotective results with glucose probably arise from its ability to bind water molecules to the amorphous phase which it forms during the freezing step [12]. Part of the water in the frozen glucose remained non-frozen (even 32% w/w). That water acted as a plasticizer and as a spacing matrix reducing the pressure of ice crystals against the nanoparticles and preventing harmful aggregation caused by freeze concentration, respectively. At the same time, insufficient cryoprotective function of lactose derived most likely from its lower water binding activity. However, as a combination with glucose, lactose reduced the amount of water to a level where the interaction of glucose with water was reduced [208] and, thus, the formation of ice crystal was slightly promoted. This enabled sufficient evaporation of water during the drying and formation of a proper cake. Tween 80 improved the freeze-drying result as it acted as a steric stabilizer and increased the hydrophilicity of the nanoparticles. An hydrophilic surface enhances the redispersion properties of the freeze-dried nanoparticles [115, 122].

5.3 Layer-by-layer coating

To successfully perform the LbL coating process, the core particles should be stable enough and carry sufficient surface charge. These properties are needed to enable adsorption of PEs and to avoid particle aggregation and destruction during different stages of the coating process. Therefore, in paper V, influence of process parameters including polymer solvent, coating medium, PE concentration and filtration membrane type, were evaluated to obtain suitable conditions in relation to nanoparticle surface charge and PE adsorption.

PAH (+) and PSS (-) were selected as model coating PEs because of their known usability in LbL processes [32, 101, 209]. Knowing their nature as non-biodegradable plastics, they should be replaced by biocompatible PEs in further studies. Suitable macromolecules for that could be e.g. chitosan and alginic acid.
Although the nanoparticle preparation process with either of the solvents (CHL or DCM) was successful producing spherical particles (Fig. 2 in \textit{V}), coating of the particles prepared with DCM failed (instant aggregate formation). The reason for the failure was the less charged (surface) nature of the nanoparticles in question, which led to unwanted electrical interactions between the particles and the PEs.

0.02 mol/l NaCl solution at pH \( \sim 7 \) was selected as the medium for coating because the nanoparticles possessed the highest surface charge in these circumstances (Fig. 7 and also Figs. 3 & 5 in \textit{V}). Low ionic strength of the medium was also favourable in relation to the flexibility of the PEs [210, 211]. 1 mg/ml concentration of PEs in the coating solutions applied as a volume of 200 µl provided excess amounts of PEs [103]. It was possible to perform the LbL process using several types of membranes. However, 0.2 µm polycarbonate membranes were preferred due to their durability and finely tailored structure (uniform, black dots in the background of the nanoparticles in Fig. 9 (particles after coating, below) are membrane pores). The particles blocked the membrane during subsequent filtrations, if they were not properly detached with the help of pressure and gentle brushing with a hair pencil. Otherwise, the vacuum below the membrane was increased because of the poor flow of the coating/washing medium. This led to stress on the particles, which promoted aggregation.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure9.png}
\caption{Left: ζ-Potential (●) and size (○) as a function of LbL coating layers. Right: Nanoparticles before coating (above) and after coating (below).}
\end{figure}

The success of the coating process was studied by monitoring the alternation of the ζ-potential and increase in size (PCS, SEM) as a function of coating layers (Fig. 9 and also Figs. 7 & 8 in \textit{V}). After six coating layers, the size of the nanoparticles was increased to above 500 nm. The size distribution was slightly broadened during the coating; the polydispersity index increased from \( \sim 0.1 \) to \( \sim 0.3 \). In addition, adsorption of PEs (PLA-CHL nanoparticles) and the failure of the coating (PLA-DCM nanoparticles) was verified by heats of thermal transitions detected by DSC. In the case of the CHL particles, heats of \( T_c \) and \( T_m \) of PLA decreased from 3.07 J/g and 15.6 J/g to 1.19 J/g and 5.64 J/g, respectively, when two layers were adsorbed (PAH+PSS) (Table 1 in \textit{V}). Such a decreasing tendency was not observed with the PLA-DCM particles. The decrease of heats indicated adsorption of PEs, because the relative amount of PLA (of the sample mass) was decreased due to the presence of the PEs. In the case of PLA-DCM particles, the PEs were not adsorbed and were most probably filtered away during the
washing step. No new thermal events were found in the thermograms indicating that the structure of PLA was not affected by any interactions between the polymer and the PEs.
6 Conclusions

Nanoparticles could be successfully prepared by the nanoprecipitation method using either semi-crystalline poly(L-lactic acid) or amorphous poly(D,L-lactic acid). Applying different characterization methods, notably scanning electron microscopy, X-ray powder diffraction, differential scanning calorimetry and infrared spectroscopy, changes could be detected in the physicochemical characteristics of the nanoparticles induced by the preparation process and by the model drugs used in the preparation process. These characteristics included size, crystallinity of the polymer and state of the drugs. Capillary electrophoresis could be used in the quantitative concentration determination of the model drugs.

Stability of the nanoparticle dispersion was affected by the dispersion medium conditions such as pH and ionic strength. Surface pressure measurements together with scanning electron microscopy imaging proved to be a suitable tool in the evaluation of the stability and the aggregation properties of these biodegradable nanoparticles. During the freeze-drying process, stability of the nanoparticles could be enhanced by the addition of carbohydrates, which acted as cryo- and lyoprotectants inhibiting aggregation of the particles and facilitating the redispersion properties.

Surface properties of the nanoparticles could be modified by the layer-by-layer polyelectrolyte coating technique. A filtration approach in the coating together with the optimized process conditions enabled successful attachment of the polyelectrolytes on the nanoparticle surface.

Overall, the main focus in this work was in the physicochemical characterization of nanoparticles during preparation, in different processes and in changing environments. Some of the applied techniques – capillary electrophoresis, surface pressure measurements and layer-by-layer coating – bring novel aspects to the characterization of poly(lactic acid) nanoparticles and other biodegradable pharmaceutical nanoparticles. All the results of this thesis provide useful information for the future studies aiming at development of drug delivery formulations consisting of nanoparticles.
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