Scalable processes to manufacture nanoparticulate dosage forms for oral vaccination

Dissertation

zur Erlangung des Grades

des Doktors der Naturwissenschaften

der Naturwissenschaftlich-Technischen Fakultät

der Universität des Saarlandes

von

Stefan Schiller

Saarbrücken

2022

Tag des Kolloquiums:	10. Februar 2023
Dekan:	Prof. Dr. Ludger Santen
Berichterstatter:	Prof. Dr. Claus-Michael Lehr
	Prof. Dr. Marc Schneider
Vorsitz:	Prof. Dr. Alexandra K. Kiemer
Akad. Beisitzer:	PD Dr. Martin Frotscher

Die vorliegende Arbeit entstand im Zeitraum von Februar 2011 bis Juli 2022 unter Anregung und Anleitung von Herrn Prof. Dr. Claus-Michael Lehr am Lehrstuhl für Biopharmazie und Pharmazeutische Technologie an der Universität des Saarlandes in Kooperation mit der Merck KGaA, Abteilung Drug Delivery & Innovation.

Table of contents

I Summary

Polymeric nanoparticles are promising drug delivery systems and antigen-carriers for vaccination. They may enhance the severity or the type of the antigen-specific immune response and may facilitate needle-free vaccination *via* the oral or respiratory route. However, the translation of nanoparticulate systems from bench to bedside remains a major challenge. Among the reasons are the limited knowledge and control of critical process parameters during early research, and the poor scalability to and reproducibility in clinical research and the commercial stage. This thesis presents a novel, easily scalable and potentially continuous method for manufacturing antigen-loaded polymeric nanoparticles. The method allows for effective tuning of the nanoparticle size with economically interesting yield, relevant antigen-loading capacity and retained antigen integrity across a batch size range of four orders of magnitude, but with limited loading efficiency. Nanoparticle properties were comparable between scales, but the process parameters were not found to be independent of or proportional to scale. Two continuous methods were developed to simultaneously prepare and dry such nanoparticles for improved process efficiency, and to manufacture enteric-matrix multiparticulates for oral dosing. Further optimization is required to achieve full scalability, improve the cost-effectiveness of the processes, and to demonstrate the functionality for an oral vaccine.

II Zusammenfassung

Polymerbasierte Nanopartikel sind vielversprechende Trägersysteme für therapeutische Anwendungen und Impfungen. Solche Systeme können Umfang und Art der antigen-spezifischen Immunreaktion maßgeblich beeinflussen sowie die orale oder inhalative Gabe ermöglichen. Allerdings stellt die Translation von Forschungsergebnissen in die Klinik eine große Herausforderung dar, die von der eingeschränkten Kenntnis und Kontrolle kritischer Prozessparameter sowie der ungenügenden Skalierbarkeit zu klinischer Entwicklung und kommerzieller Herstellung erschwert wird. Hier wurde eine neue, skalierbare und potentiell kontinuierliche Herstellungsmethode für antigen-beladene polymerbasierte Nanopartikel entwickelt. Sie ermöglicht das Anpassen der Partikelgröße bei wirtschaftlich interessanter Ausbeute, relevanter Antigenbeladung und erhaltener Antigenintegrität über vier Größenordnungen von Losgrößen, allerdings bei limitierter Antigenausbeute. Ähnliche Partikeleigenschaften wurden über verschiedene Losgrößen erzielt, jedoch waren die Prozessparameter nicht unabhängig von oder proportional zum Prozessvolumen. Eine kontinuierliche Methode zur effizienten weil simultanen Generierung und Trocknung von Nanopartikeln wurde entwickelt, sowie eine weitere zur Herstellung magensaftresistenter multipartikulärer Pulver zur oralen Anwendung. Weitere Prozessoptimierung ist notwendig für vollständige Skalierbarkeit und verbesserte Wirtschaftlichkeit, sowie der Beweis der Anwendbarkeit für die orale Impfung.

III Index of abbreviations

API	active pharmaceutical ingredient
CoV	corona virus
COVID-19	coronavirus disease 2019
EC	European Commission
EMA	European Medicines Agency
EuL	Eudragit® L
FDA	United States Food and Drug Administration
FNP	flash nanoprecipitation
GIT	gastrointestinal tract
GM-CSF	granulocyte-macrophage colony-stimulating factor
iFNP	inversed flash nanoprecipitation
ISO	International Organisation of Standardization
IUPAC	International Union of Pure and Applied Chemistry
LNP	lipid nanoparticles
mRNA	messenger ribonucleic acid
P-407	poloxamer 407
PCL	polycaprolactone
PEG	polyethylene glycol
PLA	poly(lactic acid)
PLGA	poly(lactic-co-glycolic acid)
PVA	polyvinyl alcohol
RNA	ribonucleic acid
SARS	severe acute respiratory syndrome
siRNA	small interfering ribonucleic acid
T_g	glass transition temperature
T-VEC	talimogene laherparepvec

1. Introduction

1.1. A brief history of nanotechnology

Nanoparticles have been present in the life of humans long before scientists sought to understand the special nature of nanomaterials. More than 100,000 years ago, archaic humans were using fire as a source of heat and light, to cook, to craft tools, to hunt; and unwittingly created nanoparticles of carbon and ash in the process. Modern humans were consuming casein micelles when they started to consume dairy products several thousand years ago in the Neolithic period, and they were using clay colloids for pottery. The anti-microbial effect of colloidal silver was exploited by using silver vessels to preserve water and food, and by using silver plates or silver preparations for wound treatment since the bronze age (1). Colloidal gold was used as red pigment in glassware and colloidal ferric tannate as pigment in inks since the late antiquity, and the Renaissance saw colloidal gold used as medicine.

However, it was not until the 19th century that humankind made its first conscious efforts to interact with matter on the nanoscale by what became known as colloidal chemistry or colloidal sciences. The International Union of Pure and Applied Chemistry (IUPAC) defines a colloidal system as a dispersion of molecules or particles with a dimension between 1 nm and 1 µm in at least one direction (2). This size range can be traced back to at least 1919, when le Chatelier proposed that the “diameter of the true colloids approaches a millionth of a millimetre, and is always much less than a thousandth of 1 mm" (3). The term colloid (from Ancient Greek κόλλα, “glue”) was introduced by Thomas Graham and first published in 1861. Graham observed in his fundamental experiments on dialysis that solutions of inorganic salts, sugar etc. were membrane-permeable and yielded crystallized particles upon evaporation, while colloidal dispersions were not permeable through a membrane and yielded amorphous gelatinous materials upon evaporation. (3) Graham considered that colloids are made of molecules of very high mass and, thus, did not draw upon the works of Francesco Selmi and Michael Faraday on colloidal silver and gold. The definition of colloids was broadened by Herbert Freundlich, Wolfgang Ostwald, and Peter von Weimarn to include very fine crystalline dispersions (4).

A major breakthrough for the visualization of colloidal systems was the development of the electron microscope in the 1930s by Ernst Ruska (Nobel Prize in 1986), Max Knoll, Bodo von Borries and Helmut Ruska (5, 6). The following years saw many developments of nanoparticles made of metals and oxides of metals and metalloids. The synthesis of gold nanoparticles from tetrachloroauric acid by reduction in aqueous solution was first published by Hauser and Lynn in 1940 (7), further refined by Turkevich et al. (8) and Frens (9), and is still the most commonly used method today (10). The Stöber synthesis, of comparable importance for the synthesis of homogeneous spherical silica nanoparticles, was developed by Stöber, Fink and Bohn in 1968 (11).

The manipulation of matter at the nanoscale is not limited to colloidal systems. In his visionary lecture "There's plenty of room at the bottom" (12) in December 1959, Richard P. Feynman laid out several ideas in the fields of miniaturization of manufacturing devices ("infinitesimal machines"), electronics and data storage; electron microscopy; manipulation of single atoms, and atom-by-atom chemical (or rather physical) synthesis of molecules. While he did not provide probable solutions himself, he wanted to raise awareness for what is possible in principle, for what is in agreement with the laws of physics, as a challenge to scientists to overcome the hurdles. Many of his ideas would later be realized (and some would not):

In 1981, the scanning tunneling microscope was invented at IBM by Gerd K. Binnig and Heinrich Rohrer (who shared a Nobel Prize with Ernst Ruska in 1986) and its successor, the atomic force microscope, was described by Binnig, Quate and Gerber in 1986 (13). These microscopes not only enabled the visualization of single atoms, but for the first time also their manipulation. This ability was impressively demonstrated when IBM scientists spelled out their company's logo with 35 xenon atoms on a nickel substrate in letters measuring only 5 nm in height (14). In 1985, the first page of a novel by Charles Dickens was etched with an electron beam on a 36 μm^2 surface (15), and the goal of a storage density of one bit per 125 atoms was demonstrated in 2002 (16).

Despite its undoubtedly visionary nature and many correct predictions, Feynman's talk was only cited 7 times in the 20 years following its publication (17) in the Caltech magazine Engineering and Science. The number of citations began to rise in the 1990s, and by 2009, references to Feynman's talk were so common that it became an "unwritten rule on *Nature Nanotechnology* that [it] should not be referred to at the start of articles unless absolutely necessary" (18). The actual relevance of Feynman's talk to the early pioneers of nanotechnologies has been put into question, and it may have served rather as a kind of "founding myth" that linked a then young field of science to the genius of a Nobel laureate (17, 19, 20).

Later important discoveries in the field of nanomaterials included the Buckminsterfullerene (21), carbon nanotubes (22) and stable 2D monolayers of carbon (graphene) (23), semiconductor nanocrystals (quantum dots) (24-26), and mechanically interlocked molecules (catenanes) (27) that enabled molecular machines such as "molecular shuttles" (rotaxanes) (28) and molecular motors (29).

Along with the scientific progress of colloidal systems came many commercial applications of nanoparticles, although the nanoscopic aspect of the constituents was rarely advertised. Notable applications of colloidal systems include cements, ceramics, rubber, catalysts, pigments, latex paints, surface coatings (e.g., non-wetting and "self-cleaning" surfaces), photographic films, sunscreen (titanium dioxide or zinc oxide nanoparticles), microelectronics (this thesis is written on a computer

with a processor built with a technology node of 14 nm), volume expanders, synthetic lung surfactant, and eventually also drug delivery systems. By 2019, the Project on Emerging Nanotechnologies lists more than 1800 products that are publicly advertised as employing nanomaterials or nanotechnology (30).

However, not all of these applications would be considered nanotechnology in a narrower sense, one reason being that there exist different understandings and definitions of what constitutes a nanomaterial and what makes them special.

1.2. Definitions of nanoscience, nanotechnology, nanomaterials, and nanoparticles

The Royal Society and the Royal Academy of Engineering define nanoscience as "the study of phenomena and manipulation of materials at atomic, molecular and macromolecular scales, where properties differ significantly from those at a larger scale" (31 p. vii), and attribute the differences mainly to a much higher surface-to-mass ratio and increasingly dominant quantum effects in nanomaterials. They further define nanotechnology as "the design, characterisation, production and application of structures, devices and systems by controlling shape and size at the nanometre scale" (31 p. vii), where nanoscale is understood from 0.2 to 100 nm. This definition is broader than how Norio Taniguchi originally introduced the term "nanotechnology" in 1974 (31 p. 5): "Nanotechnology mainly consists of the processing of separation, consolidation, and deformation of materials by one atom or one molecule" (32).

Many different organizations, scientific and regulatory, have published and adopted more or less differing definitions of what constitutes a nanoparticle or a nanomaterial (Table 1) with size being the most specific feature of all definitions.

The International Organization of Standardization (ISO) adopted the definition of the nanoscale as "length range approximately from 1 nm to 100 nm", a nanomaterial as "material with any external dimension in the nanoscale or having internal structure or surface structure in the nanoscale" (33), and a nanoparticle as a discrete piece of material "with all external dimensions in the nanoscale where the lengths of the longest and the shortest axes of the nano-object do not differ significantly" (34). The aspect ratio is used to differentiate the nanoparticle from a nanofiber or a nanoplate. The ISO definitions do not consider whether the objects in question exhibit special properties due to their size. However, the ISO further specifies engineered nanomaterials ("designed for specific purpose or function) and manufactured nanomaterials ("intentionally produced to have selected properties or composition") to differentiate from incidental nanomaterials.

Table 1. *A selection of scientific, advisory, and regulatory definitions of "nanomaterial".*

Body	Definition of "nanomaterial"	Type	Ref.
ISO	Any dimension or internal or surface structure in the range of 1-100 nm	scientific	(33, 34)
IUPAC	*Nanoparticle:* at least 2 dimensions in the range of 1-100 nm. Up to 500 nm with reference to other phenomenon (e.g., turbidity, stable dispersion)	scientific	(35, 36)
FDA	Any dimension or surface structure in the range of 1-100 nm. Up to 1000 nm if engineered to exhibit properties attributable to dimensions.	advisory	(37)
European Commission recommendation of nanomaterial	≥ 50 % of the particles (number size distribution) with at least one dimension in the range of 1-100 nm; including fullerenes, graphene flakes and single wall carbon nanotubes < 1 nm.	advisory	(38)
European Commission regulation of food information	intentionally produced; any dimension or internal or surface structure in the range of 100 nm or less, including larger structures that retain "properties that are characteristic of the nanoscale"	regulatory	(39)
European Commission regulation of cosmetics	insoluble or biopersistant, intentionally produced; any dimension or internal structure in the range of 1-100 nm	regulatory	(40)

Note: The European Commission has issued several more regulations and approval procedures for nanomaterials, including the REACH framework (Regulation (EC) No 1907/2006), Novel food Regulation (EC) No 2015/2283, and the Medical devices regulation proposal COM(2012)542; summarized by Rauscher et al. (41). Bremer-Hoffmann et al. (42) summarized the regulatory landscape of nanomedicines in further countries. Other national and international institutes for standardization have issued definitions not listed here, including ICH, CEN, ASTM, DIN and more.

The IUPAC does not yet define a nanomaterial, but defines a nanocomposite as a "composite in which at least one of the phase domains has at least one dimension of the order of nanometres" (35). The given size range seems to be similar to the IUPAC definition of a colloid as dispersed particles with "at least in one direction a dimension roughly between 1 nm and 1 μm" (2). However, a nanoparticle is more narrowly defined as a "particle of any shape with dimensions in the 1×10^{-9} and 1×10^{-7} m range" (36), i.e. the same range as the ISO definition, although the aspect ratio is not considered. By note, tubes and fibers with only two dimensions below 100 nm are included in the definition, but plates with only one dimension below 100 nm are not mentioned.

Interestingly, it is specifically mentioned that "because other phenomena (transparency or turbidity, ultrafiltration, stable dispersion, etc.) are occasionally considered that extend the upper limit, the use of the prefix "nano" is accepted for dimensions smaller than 500 nm" (36). This is of special significance for the determination whether a drug delivery system is considered nanoparticulate, because many systems in the range of 100-1000 nm are described in the literature (including systems presented in this thesis) that still interact quite differently with biological membranes and biological processes than larger particles. For instance, such systems may have the ability to cross membranes and to enrich in certain tissues against the classical concept that only dissolved drugs may be absorbed and distributed. They may be taken up by cells in different endocytotic pathways. Crystalline material in that size range does hardly benefit from increased saturation solubility but may still have a considerably increased dissolution rate (43). Such systems may still have an increased surface adhesiveness (e.g. to the skin

or gut wall) that may be exploited for topical formulations, to increase the retention time in the gastrointestinal tract, or for local drug delivery to the gut mucosa (44).

While at first this poses mainly a challenge in interdisciplinary scientific communication, also manufacturers and regulators in healthcare and beyond need to deal with the specific benefits and hazards of nanomaterials and nanotechnology. For example, when nanomaterials pose specific environmental or occupational hazards that require specific risk mitigation strategies, a manufacturer or employer needs to know in which case such risk mitigation strategies are legally binding. Also, when specific properties of nanomaterials or their nature as such may influence the decision of a consumer to buy a product, a clear definition is needed when a nanomaterial needs to be declared. But with an ever-increasing number of vastly different nanomaterials and their applications, it is very challenging for regulators to define a legally binding framework that may keep up to date with the concurrent developments.

The guidelines of health authorities are of special interest to the pharmaceutical scientist. Currently, no dedicated frameworks for nanomedicinal products have been issued in the major markets. Such products are still regulated within the existing frameworks of medical devices and medicinal products., and may occasionally border on regulations for cosmetics or novel foods (45 p. 55).

The European Medicines Agency (EMA) issued a reflection paper on medicinal products employing nanotechnology in 2006 (46). The paper used the definitions of the Royal Society and the Royal Academy of Engineering for nanotechnology, nanomedicine, and the nanometer scale. Further publications by the EMA focused on special considerations that emerged from the review of specific products and are summarized in Table 2.

The United States Food and Drug Administration (FDA) finalized a guidance for industry in 2014, containing non-binding recommendations whether an FDA-regulated product involves the application of nanotechnology (37). This means that manufacturers may prepare for the approval process, but the FDA keeps product assessments a case-by-case decision also with regards to the products potential “nano-aspect”. The definition of the FDA largely reflects the definition of the IUPAC, with a difference in that the FDA assumes an upper limit of 1000 nm if the material is “engineered to exhibit properties or phenomena, including physical or chemical properties or biological effects, that are attributable to its dimension(s)”. Further guidance documents issued by the FDA include liposome drug products, a draft guidance on drug products in general that contain nanomaterials, and guidance on the use of nanomaterials in cosmetic products or food for animals (Table 2).

Table 2. *Guidance Documents issued by EMA and FDA for medicinal products containing nanomaterials*

Body	Title	Issued	Status	Ref.
EMA	Nanotechnology-based medicinal products for human use	2006	adopted	(46)
	Data requirements for intravenous liposomal products developed with reference to an innovator liposomal product	2013	adopted	(47)
	Surface coatings: general issues for consideration regarding parenteral administration of coated nanomedicine products	2013	adopted	(48)
	Development of block copolymer micelle medicinal products	2014	adopted	(49)
	Data requirements for intravenous iron-based nano-colloidal products developed with reference to an innovator medicinal product	2015	adopted	(50)
FDA	Considering Whether an FDA-Regulated Product Involves the Application of Nanotechnology	2014	final	(37)
	Safety of Nanomaterials in Cosmetic Products	2014	final	(51)
	Use of Nanomaterials in Food for Animals	2015	final	(52)
	Drug Products, Including Biological Products that Contain Nanomaterials	2017	draft	(53)
	Liposome Drug Products: Chemistry, Manufacturing, and Controls; Human Pharmacokinetics and Bioavailability; and Labeling Documentation	2018	final	(54)

1.3. Nanomedicine

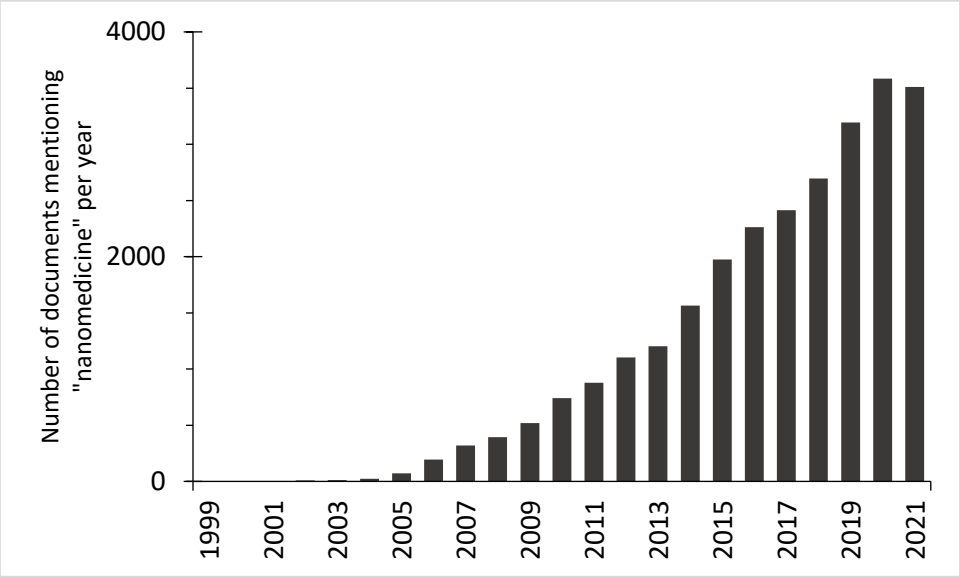

Figure 1. *Number of documents per year using the term "nanomedicine" in the title, abstract, or as keyword, beginning with the first mention in 1999. Search performed on Scopus and SciFinder^n on 06. December 2021. The number of documents for 2021 is expected to rise considerably as more documents published in that year will get properly indexed.*

Nanomedicine is the application of nanotechnology to diagnose, treat or prevent diseases, and may make use of medicinal products or devices. Based on searches on Scopus and SciFinder^n, the term "nanomedicine" first appeared in the title, abstract or keywords of an article in an indexed journal in

1999 (55), and over 3000 documents were published in 2021 (Figure 1). Of course, nanoparticles for biomedical applications were in the focus well before the turn of the millennium. Maybe similar to how nanotechnology is traced back to Feynman's Plenty of Room, the concept of nanomedicine is often traced back to Paul Ehrlich's concept of the "magic bullet" as laid out in his talk before the 17th International Congress of Medicine (56). Ehrlich, the father of modern chemotherapy, observed that dyes injected in living organisms accumulate in different tissues – some in adipose tissue, others in muscle tissue, the peripheral or the central nervous system. He postulated a specific binding between the dye and a structure of the cell, a phenomenon he would investigate in greater detail in his studies of immunology. Drawing upon this experience and on the lock and key principle proposed by Emil Fischer, he would later embark on the search for novel small-molecule anti-infectives that could selectively interact with the pathogen without hurting the host organism („Wir wollen also den Parasiten an erster Stelle möglichst isoliert treffen, daß heißt, wir müssen zielen lernen, chemisch zielen lernen!" (57)). In his development of Salvarsan and Neosalvarsan, Ehrlich foreshadowed several key principles of modern drug discovery, such as lead generation and lead optimization in a series of systematic variations of the structure of an active molecule to establish a structure-activity relationship, and ultimately to increase the therapeutic window.

Although Ehrlich most probably did not have nanoparticular carrier systems in mind, his analogy of the magic bullet was often used to describe the mechanism by which nanomedicine sought to increase the therapeutic window of therapies: Drug carriers that are small enough for safe and efficient distribution through the body were designed to find and to enrich in the target tissue, and to release the drug specifically at the site of action. The first such carrier system goes back to the visualization and description of the self-assembly of phospholipids in sheets, leaflets, cylinders or vesicles by Bangham and Horne in the 1960s (58-61). The phospholipid structures were originally used as model membrane systems to study the properties and different behaviors of blood cells, or particulate matter that is brought in contact with blood: The composition of the hydrodynamic shear plane, the surface charge, opsonization, and clotting behavior. The term "liposome" was first coined by Weissmann (61, 62), and the first liposomal drug carrier as therapeutic agent was described by Gregoriadis et al. in 1971 (63). The authors describe the preparation of liposomes by the film-hydration and probe sonication method, followed by purification by size-exclusion chromatography; lab scale methods that are still in use today. The authors demonstrated a relatively low but successful loading and the retainment of enzymatic activity of amyloglycosidase intended for enzyme replacement therapy, though the liposomes were still relatively large and multilamellar.

Such systems were still commonly referred to as colloidal systems. The prefix nano to refer to drug delivery systems started in the mid-1970s, when "Nanokapseln" (nano-capsules) were first mentioned

in the doctoral thesis of Gerd Birrenbach (64) in 1973, and the first paper (65) on such "nanoparts" was published by Birrenbach and Speiser (66) in 1976. Their work constitutes the first artificially synthesized nanoparticulate drug delivery system and the beginning of research on polymerized micelles for drug delivery. It is also noteworthy because it described for the first time a very promising application of nanoparticles, the modulation of the immune response by altering the presentation and the release profile of an antigen.

Liposomes saw their first commercial use in the cosmetic product Capture by Dior in 1986 and became the first approved nanoparticulate drug delivery system in products like the synthetic lung surfactant Alveofact® (Boehringer Ingelheim), the topical liposomal econazole formulation Epi-Pevaryl® C1 Lipogel (Cilag), the intravenous formulations AmBisome® (Gilead Sciences, amphotericin B) and Doxil® (Alza, doxorubicin).

Vaccination has been another early application of nanotechnology. Virus-derived proteins have been used for the manufacturing of nanoparticles, either as virosomes in combination with lipids, or in self-assembled virus-like particles. Several associated vaccines have been marketed since the 1980's, e.g., Epaxal®, Inflexal®, Recombivax HB®, Cervarix®, Gardasil® and Gardasil® 9.

Nanomedicine has been successfully employed to increase the oral bioavailability of New Chemical Entities coming from high throughput screening with increasingly poor pharmacokinetic properties. In 1996, the FDA approved Taxotere® (then Rhône-Poulenc Rorer), a concentrate for infusion of docetaxel. The original presentation consisted of a solution of the drug at 40 mg/ml and citric acid in polysorbate 80 that yields a clear dispersion of polysorbate micelles upon dilution before infusion, although the nanoparticulate nature of the dosage form was not highlighted at that time. Another successfully translated technology was the so called "carrier-free" nanocrystalline suspensions. The first commercialized technology was the "NanoCrystal" platform by NanoSystems (acquired by Elan in 1998, and by Alkermes in 2011): A concentrated slurry containing particles of an active pharmaceutical ingredient (API), stabilizers, and milling beads is wet milled to a particle size of about 100-400 nm. Nanocrystalline material typically exhibits an increased dissolution rate and, in that size range to a lesser extent, an increased saturation solubility (43). The first approved drug to employ the NanoCrystal platform technology was Rapamune® (2000; Wyeth; sirolimus), followed by Emend® (2003; Merck Sharpe & Dohme; aprepitant) and Tricor® (2004; Abbot; fenofibrate). The technology has been further developed to sustained release parenteral formulations, with Invega Sustenna® (Janssen; paliperidone palmitate) achieving the first FDA-approval in 2009.

Also in 2009, the first (and so far, only) inorganic nanoparticulate drug product was approved with Feraheme™ (AMAG Pharmaceuticals; ferumoxytol) designed for better tolerability of high doses and

rapid infusion rates in the treatment of iron deficiency. The formulation consists of superparamagnetic iron(III) oxide nanoparticles (SPION) that are coated with a stabilizing shell of polyglucose sorbitol carboxymethyl ether.

Many further nanoparticulate systems have been developed, for example polymeric micelles (67-69), polymer-drug conjugates (70), solid or mesoporous silicon oxide nanoparticles (71, 72), polymersomes (73), dendrimers (74), and quantum dot bioconjugates (75, 76). Recently, assemblies of nucleic acids and charged lipids have gained great attention for vaccination.

1.3.1. Nanomedicine for vaccination

Nanomedicines that are intended to alter the biodistribution of a drug often suffer from elimination by phagocytic cells and accumulation in certain organs and tissues. For instance, cytotoxic payloads may not only reach a solid tumor, but may also specifically enrich in the liver, the spleen, the lungs, the kidneys, in draining lymph nodes; or may be cleared before reaching any site of interest (77). The circulation time of nanomedicines can be considerably increased by introducing hydrophilic and steric barriers on the surface of nanoparticles to prevent opsonization and phagocytosis (78), most prominently by using derivatives of polyethylene glycol (79). The success of a specific delivery of drugs to solid tumors *in vivo* has been critically discussed recently (80-82). In contrast, nanomedicines have been very successfully applied when their inherent “weaknesses” were exploited, for example the silencing of genes mainly transcribed in hepatocytes (83), the treatment of intra-cellular parasites (84, 85), or the modulation of the immune system by targeting antigen-presenting or immune-modulating cells in circulation, in lymph nodes, and in the tumor microenvironment (86, 87).

Especially nanomedicines for prophylactic and therapeutic vaccination have gained considerable attention. A nanoparticle can act in one or more roles in a vaccine formulation: as adjuvant, as a carrier encapsulating the antigen, or as a platform presenting the antigen on its surface (88).

Adjuvants are important components of many vaccines to induce an effective and long-lasting immune response in absence of a real infection; they can enable effective responses in immunocompromised populations; they can enable the use of lower antigen doses to support large-scale vaccination in case of a pandemic; they may allow for immunization with fewer doses or increase the speed of the initial response; and they may guide the type of immune response (89).

Aluminum-based adjuvants have been used for more than ninety years and are widely accepted by health authorities as safe and very effective. They are potent to enhance the humoral immune response to an extracellular antigen by an increased activation of T_h2 and T_{fh} $CD4^+$ T cells via the MHC class II pathway (90). A humoral immune response is effective to prevent or fight extracellular bacterial infections, or to inactivate extracellular viral particles to prevent or limit viral infections preferably at

the site of entry. A humoral response alone, however, is not effective at fighting intracellular bacterial or viral infections, or at killing cancer cells. The necessary cellular immune response is mainly driven by $CD8^+$ T cells, but aluminum-based adjuvants are poor to induce such a response (90). Together with ongoing public debates over the safety of aluminum in vaccines (91, 92), this has led to the search for novel adjuvant systems: MF59 and AS03 are adjuvants used in influenza vaccines and are both based on nanoemulsions of squalene in aqueous media; AS01 is a liposomal adjuvant currently used in a vaccine for prevention of herpes zoster and contains monophosphoryl lipid A and the quillaja saponin extract QS-21; several others are in clinical development.

The possibility to induce a potent cellular immune response to one or multiple neoantigens of choice opened a new therapeutic option for cancer treatment. Autologous cell-therapies were among the first to be investigated (93): the patient's own immune cells are primed *ex vivo* either with neoantigens derived from biopsies or with recombinant proteins that are characteristic for certain types of cancer. Sipuleucel-T (Provenge, Dendreon), a therapy of autologous dendritic cells against castration-resistant metastatic prostate cancer, was approved by the FDA in 2010, but commercial success was hampered by the prohibitive cost of the treatment (93). Oncolytic viruses are another therapeutic modality that are designed to infect and kill cancer cells while sparing healthy cells. Talimogene laherparepvec (T-VEC; Imlygic, Amgen) was approved by the FDA and EMA in 2015 for the treatment of melanoma. T-VEC is an engineered herpes simplex virus that replicates in melanoma cells and causes them to burst. It also causes the expression and release of granulocyte-macrophage colony-stimulating factor (GM-SCF) that may promote an immune response against antigens shed from the destroyed cancer cells, leading also to the reduction of lesions that were not injected with the virus (94).

In addition to nanoparticulate adjuvants, nanoparticulate antigen carriers have been shown to be especially effective at cross-presenting endocytosed antigens via the MHC class I pathway (95-97) and to induce an effective cytotoxic $CD8^+$ T cell response against model antigens (98) and relevant tumor antigens like HER2/neu (96, 97) that may be useful in principle for cancer treatment.

Nanoparticles were also used to enable needle-free vaccinations to improve patient acceptance, to increase ease of application, and to reduce costs and the requirements for healthcare infrastructure and cold-chain. Lademann *et al.* (99) showed that nanoparticles can bypass the stratum corneum by penetrating into hair follicles, and subsequent research demonstrated that topical application of nanoparticles can increase the antigen delivery across the skin and elicit an immune response (100-103). This presents an interesting alternative to disrupting the stratum corneum barrier by measures such as abrasion, electroporation, jet injectors and chemical permeation enhancers. Recently, nanoparticles have been combined also with 3D-printed microneedles for self-administration (104).

The stratum corneum barrier can be altogether avoided by mucosal administration. In addition to needle-free administration, mucosal vaccination has the potential to elicit strong humoral and cellular immune responses both systemically and across the so-called “common mucosal immune system” (105). This makes the mucosal route very interesting for prophylactic vaccination, as the mucosal tissues are the main entry point for pathogens, and an effective mucosal immune response can disable pathogens before entry into the body. Nasal administration of nanoparticulate antigen formulations demonstrated an increased immune response over subcutaneous administration in (106), and an *in vivo* proof of concept has been shown for pulmonary vaccination with chitosan nanoparticles that resulted in an effective antigen-specific cytotoxic response (107). Moreover, Chitosan- and poly(lactic-co-glycolic acid)-based nanoparticles delivered *in vitro* to human-derived dendritic cells could increase humoral and cellular immune responses over soluble antigen, and were shown to pass over an intestinal epithelium model (96).

1.3.2. Fighting the COVID-19 pandemic with nanomedicine

Lipid nanoparticles (LNP) are structures where, in contrast to liposomes, also the core consists of a lipid matrix. Solid lipid nanoparticles are a type of LNP that mainly consist of lipids that are solid at room temperature and were proposed as a more economic and/or safer alternative to liposomes and polymeric nanoparticles (108). Several potentially high throughput manufacturing methods have been published in the early 1990’s (109-111). Later developments of LNP included nanostructured lipid carriers, lipid drug conjugates, and polymer-lipid hybrid nanoparticles (112). LNP were further developed as non-viral vector for nucleic acid delivery and became one of the most important assets of nanomedicine by 2021.

In the beginning of the year 2020, more and more case reports of severe acute respiratory syndrome (SARS) caused by a novel corona virus (CoV) were reported. Different to the outbreak of SARS-CoV-1 in 2002, SARS-CoV-2 proved highly infectious even in asymptomatic or presymptomatic individuals (113). Research efforts were focused on finding a vaccine as virologists worldwide began to warn of a potential pandemic. The strategies that helped to curb the outbreak of SARS-CoV-1 (about 8100 confirmed cases and 774 deaths (114)) were largely unsuccessful: by July 2021, the cumulative number of confirmed cases of coronavirus disease 2019 (COVID-19) surpassed 188 million (115, 116), with over 4 million associated deaths (116, 117). With so many cases, it was often no longer possible to trace the chain of infection; the identification and isolation of often asymptomatically infected individuals was only partly successful in the beginning, and repeated lockdowns of the social and economic life of billions of people presented an urgent need for a safe and efficient vaccine.

Several biotech companies saw an opportunity to take the lead in the race for a vaccine with their novel mRNA vaccination platforms. Such platforms have the potential to overcome a major hurdle in

the development of vaccines against rapidly mutating viruses – the speed of vaccine development and high-volume production (118). Delivering on the promise of development speed, the first two COVID-19 vaccines approved by the EMA and FDA were the mRNA vaccines developed by BioNTech/Pfizer and Moderna.

The unprecedented speed at which COVID-19 vaccines were developed represents a breakthrough in vaccine development (119). The genetic sequence of the virus that became known as SARS-CoV-2 was published on 11 January. Already in March, the first human was dosed with the mRNA LNP COVID-19 vaccine candidate of Moderna (120), and the first participants in the Ph I/II study for BioNTech's vaccine candidate were dosed on 23 April (121). At this time, safety data for mRNA vaccines was limited with a total of about 1,400 participants of clinical studies sponsored by Moderna that received mRNA vaccines against different diseases (120). The first approval of a fully tested vaccine followed less than eight months later, or eleven months after the identification of the virus. Previously, the fastest developed vaccine was the 1960's mumps vaccine with 4 years between the isolation of the virus and the approval of the vaccine (122). By the end of April 2021, one year after the first dosing of a COVID-19 vaccine candidate and eleven years after the first direct injection of *in vitro* transcribed mRNA in a human subject (123), more than 300 million doses (124) of Comirnaty® and COVID-19 Vaccine Moderna were administered around the globe.

What makes this feat even more striking is that both mRNA vaccines rely on a novel, nanoparticulate formulation. The lipid nanoparticle carrier systems were carefully designed to stabilize and protect the mRNA cargo, to improve cellular uptake, and to efficiently release the cargo into the cytosol where the mRNA can be translated into the antigens of interest. At the start of the pandemic, the only commercially available LNP product was Onpattro® (Alnylam Pharmaceuticals, patisiran), a small interfering RNA (siRNA) drug with orphan disease designation and with about 950 patients on commercial treatment (125). Nanomedicines were generally developed and approved for the treatment of life-threatening diseases without an available curative treatment so that the potential benefit would outweigh the risks and uncertainties associated with the use of novel nanomaterials. The patient populations were typically small, and the encapsulated active pharmaceutical ingredients (API) were often already approved in conventional formulations. In a stark contrast, the global vaccination programs marked an unprecedented mass administration of nanomedicines relying on a novel modality with a novel type of formulation including several novel excipients that were to be administered to healthy subjects. This is particularly noteworthy in an otherwise very conservative industry that has seen similar (and sometimes even longer) timelines for the adoption of novel excipients as for novel APIs (126, 127). This paradigm shift, together with the expected safety data

from treating hundreds of millions of subjects with a specific type of nanomedicine, may pave the way for other types of nanomedicines as well.

1.4. Production of polymeric drug delivery systems

Numerous methods have been described for the synthesis of polymeric nanoparticles. The preferable method depends on the type of polymer and payload, the intended type of incorporation (encapsulation vs. decoration), the particle size and charge, the route of administration, and the scale of the synthesis. This section will focus on the first methods for polymeric nanoparticle preparation for historic context, and on methods to produce drug carriers from preformed hydrophobic or amphiphilic polymers for protein delivery.

1.4.1. Micelle polymerization

The first polymeric nanoparticles for pharmaceutical applications were described by Birrenbach and Speiser (64, 66) as adjuvant systems. The particles were synthesized by dissolving micelles-forming surfactants (bis-(2-ethyl-hexyl)-sodium succinate and polyoxyethylene(4)-laurylether) in n-hexane, adding water dropwise under stirring to form micelles, dissolving monomers and crosslinking agent (acrylamide and N,N'-methylene-bis-acrylamide) in the dispersion, followed by the antigen (tetanus toxoid or human IgG), inducing polymerization by gamma irradiation, and precipitating the polymeric construct by the addition of an antisolvent (66). The so "congealed" micelles had a diameter of less than 80 nm. The antigen was passively entrapped in the polymeric scaffold, thus forming a stable antigen depot: *In vitro* release studies showed that less than 25% of the antigen was released. (64)

Although the proteins likely retain their enzymatic or immunologic activity (64), attempts to encapsulate "classical" cytotoxic APIs for cancer therapy failed due to degradation of the API by gamma irradiation (128). It could still be demonstrated that the nanocapsules were able to selectively transport fluorescein to lysosomes of rat fibroblasts (129).

The micelle polymerization process was modified by Couvreur et al. by employing biodegradable polymers (polyalkylcyanoacrylates) and radiation-free anionic polymerization in the presence of polysorbate 20 in water (130). This method now allowed the encapsulation of various APIs such as doxorubicin, vincristine, methotrexate, penicillin, ciprofloxacin or acyclovir, as well as several peptides, antisense oligonucleotides or siRNA (128). Vauthier et al. further optimized the method to yield poly(ethylene glycol)-poly(isobutyl 2-cyanoacrylate) nanoparticles (131) intended to avoid rapid clearance by the reticuloendothelial system.

The main drawback of methods involving polymerization during particle formation is the presence of usually toxic monomers and polymerization initiators which are difficult to remove quantitatively and

make such methods less suitable for the preparation of a drug delivery system for human use. Therefore, the remaining chapter will focus on the formation of nanoparticles from pre-formed polymers.

1.4.2. Nanoprecipitation *or* solvent displacement

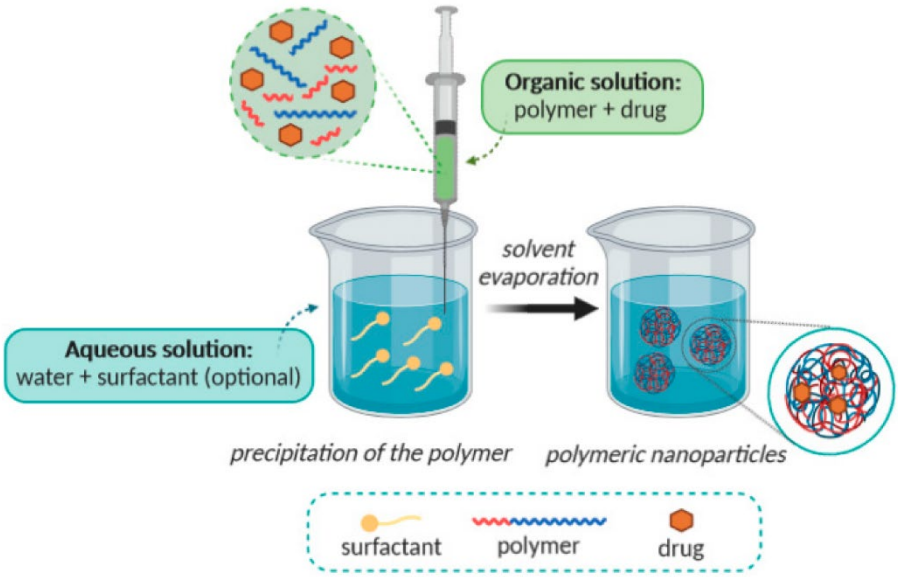

Figure 2. *Preparation of nanoparticles by the nanoprecipitation method. Adapted from (132) under the Creative Commons Attribution (CC BY 4.0) license (http://creativecommons.org/licenses/by/4.0/).*

Nanoprecipitation of polymers was used well before the name was coined or even the concept of nanotechnology was defined (133). It is not surprising that it was also among the first described methods to synthesize a nanoscalic drug delivery system from preformed hydrophobic polymers. Fessi et al. (134, 135) described the manufacturing of nanocapsules by dissolving indomethacin, poly(lactic acid) (PLA), benzyl benzoate and fractionated soy lecithin in heated acetone, and adding the organic solution to an aqueous solution of poloxamer 188 under magnetic stirring (Figure 2). According to the authors, the interfacial turbulence due to the rapid diffusion of acetone into the aqueous phase causes the emergence of nanoscale regions with the polymer enriched at the interface between the “oily core” (indomethacin in benzyl benzoate) and the continuous phase. The PLA subsequently precipitates in a thin shell, encapsulating the indomethacin. The method was thus also called “interfacial polymer deposition following solvent displacement”. Later works used very similar methods to manufacture solid core nanoparticles by using a single solvent instead of a solvent mix.

The working mechanism highlights the main prerequisite for using this method: drug and polymer need a common, miscible pair of solvent and anti-solvent. This is commonly met for hydrophobic small molecular drugs and amphiphilic or hydrophobic polymers. Examples of polymers used for nanoprecipitation are PLA (134), PLGA (136), polycaprolactone (PCL) (137), polymethylmethacrylate

(138), poly(ethyl acrylate-co-methyl methacrylate-co-trimethyl ammonioethyl methacrylate chloride) (Eudragit® RL and RS) (139), polyethylene glycol (PEG) modified poly(alkylcyanoacrylate) (140), hydrophobized polysaccharides like pullulan acetate (141), dextran propionate (142), dextran propionate pyroglutamate (143), various hydrophobic cellulose esters (144), and starch acetate (145). Examples of amphiphilic polymers are PEG-PLA (146), PEG-PLGA (147) and PEG-poly(aspartic acid) (148).

Water-soluble drugs are difficult to incorporate into hydrophobic polymers with acceptable efficiency due to poor interactions between drug and polymer in aqueous solvents (149, 150). This is especially true for proteins. It is possible to inverse the nanoprecipitation method by dissolving a hydrophilic drug and a hydrophilic polymer in a common aqueous solvent, and to precipitate them in an organic non-solvent. Inverse nanoprecipitation has been successfully applied to biopolymers like gelatin (151-153), dextran (154), and maltodextrin (155). Challenges of the method include the interfacial stress that may cause protein payloads to unfold. Furthermore, such particles typically need to be further stabilized to withstand reconstitution in aqueous media and injection into the blood stream, and ultimately, achieve sufficient circulation times. Stabilization strategies may include chemical cross-linking (e.g. using paraformaldehyde, glutaraldehyde, or carbodiimide) and enzymatic cross-linking (156). Baseer *et al.* (157) developed a surface cross-linking strategy to prevent cross-linking and inactivation of the encapsulated protein using a hydrophobic zero-length cross-linker that does not diffuse into the particle core. Gelatin nanoparticles can also be stabilized by enclosing them in a shell of a hydrophobic polymer instead of cross-linking (158).

Nanoprecipitation is very easy to implement in lab scale using a magnetic stirrer, a beaker, and a pipette or syringe. Because the mixing dynamics, solvent interdiffusion and mass transfer are hard to control in this setup, the resulting particle size distribution may be broader and inconsistent from batch to batch (159). This makes the simple setup hardly suitable for scale up and manufacture, where a reliable control of critical process parameters is crucial. The process has been optimized by controlling the feed (e.g. using syringe or peristaltic pumps) and by controlling the mixing in continuous fluidic systems (160, 161). However, the particle formation is still mainly governed by interfacial turbulence and, thus, the interaction of solvent and non-solvent. Microfluidic systems and flash nanoprecipitation were developed to further increase the control of the mixing step.

1.4.2.1. Microfluidics

Karnik et al. (162) were the first to report a nanoprecipitation method based on hydrodynamic flow focusing in a microfluidic chip, allowing to tune the nanoparticle size, size distribution, and drug release of polymeric drug delivery systems. In hydrodynamic flow focusing, the solvent stream is squeezed into a narrow stream by two sheathing non-solvent streams (Figure 3). The resulting width of the

solvent stream may be as small as 0.1 µm (163). This setup achieves a very rapid mixing process with estimated mixing times in the two- to three-digit microseconds range (162), and, ultimately, the control of the interdiffusion of solvent and non-solvent by process parameters, rather than by the chemical composition and miscibility of solvent and non-solvent alone (164). Several other microfluidic setups have been described besides hydrodynamic flow focusing. Ding et al. (164) provide an excellent overview of microfluidic devices to produce polymeric drug delivery systems, including interdigital multilamination micromixers, Y-type micromixers, K-M impact jet mixers, multi-inlet vortex mixers and confined impinging jet mixers (165). Similar to nanoprecipitation, microfluidics were employed for hydrophobic polymers like PLGA (165-168) and for hydrophilic biopolymers like gelatin (169).

The continuous operation in microfluidic reactors is in principle beneficial for scale up, but the intrinsic low flow rates in individual channels limit the overall productivity. The usual approach for increasing productivity is numbering up, that is parallelization of many microfluidic devices (170). This approach is technically very challenging, as the flow dynamics need to be maintained tightly across all parallel devices (171). The channel geometry is also prone to clogging by particulates (164, 172) or if the product stream is too viscous (173), thorough cleaning of the microchannels is very challenging (173), and the necessary effort of an in-process control of the flow in several hundreds or thousands of individual streams is still prohibitive.

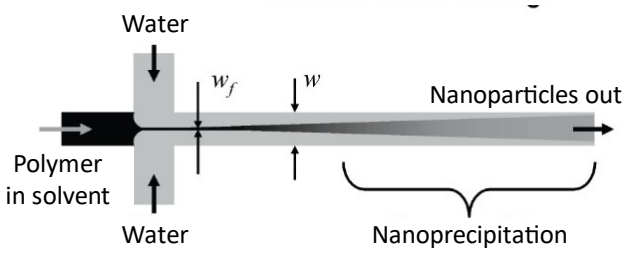

***Figure 3**. Schematic of the production of nanoparticles using a microfluidic device on the principle of hydrodynamic flow focusing. The solvent is focused to a stream of the width w_f between to water streams in a channel of the width w. Adapted from (162) with permission from the American Chemical Society, Copyright 2008.*

1.4.2.2. Flash Nanoprecipitation

Turbulent flow micromixers were introduced by Johnson and Prud'homme (174) design of confined impinging jet micromixers, and applied as "Flash Nanoprecipitation" (FNP) (175) to increase the throughput over the laminar flow designs. Further examples include the multi-inlet vortex mixer (176), and combinations of turbulence with hydrodynamic flow focusing such as a microvortex mixer (177) and a coaxial turbulent jet mixer (178). The production rate of nanoparticles was reported up to 3.15 kg/day (164).

Similar to conventional nanoprecipitation, FNP suffers from poor encapsulation efficiency of hydrophilic drugs. A technique called inversed FNP (iFNP) was developed to load hydrophilic drugs into

hydrophilic or amphiphilic polymers (179) by precipitating the hydrophilic components from a polar solvent (e.g. DMSO) by mixing with a non-polar anti-solvent (e.g. acetone or chloroform). However, the formed nanoparticles need to be stabilized for application in aqueous environments as needed in biomedical applications. This may be achieved by cross-linking the polyacid constituting the nanoparticle's core with a multivalent cation in a process called ionic gelation.

In another variation of FNP, nanoparticle formation and cross-linking are achieved in a single step by mixing of two aqueous streams, containing the polyelectrolyte and the crosslinking agent. The method has been applied as "Ionic Flash NanoPrecipitation" (sometimes also abbreviated as iFNP in the literature) for inorganic-organic hybrid nanomaterials (180) and as Flash Nanocomplexation (FNC) for nucleic acid payloads (181). The method was recently adapted for diffusely charged globular proteins (182).

1.4.2.3. Self-assembly

The preparation of polymeric micelles can be seen as a special case of the nanoprecipitation method using amphiphilic polymers. The polymer and API are dissolved in a common solvent, and the solution is mixed with a common "anti-solvent" (i.e., one in that the polymer does not disperse molecularly). The mixing can be done e.g. by pouring or injection, sometimes followed by co-solvent evaporation (183); or by dialysis (184). Examples of employed polymers include PEG-PLA, PEG-PLGA, PEG-PCL, PEGylated poly-4-(vinylpyridine) (184), chitosan derivatives (185), PEG-distearylphosphoethanolamine (186), PEG-poly(phenylalanine) PEG-poly(2-(4-vinylbenzyloxy)-N,N-diethylnicotinamide) (187), folate-conjugated mPEG-PCL (188), and PEGylated squalenyl derivatives (189).

Some amphiphilic polymers can self-assemble to spherical bilayer vesicles that are called polymersomes for their likeness to liposomes. The methods for the manufacturing of polymersomes are very similar to that of liposomes, including for example film hydration followed by sizing, solvent injection, and microfluidics (190).

The self-assembly method has also been applied with non-polymeric molecules. Couvreur et al. reported that squalenoylation of nucleoside analogues leads to an amphiphilic prodrug that can self-assemble into nanoparticles (191). Cationic squalenyl diethanolamine and anionic squalenyl hydrogen sulfate have been reported to self-assemble with efficient non-covalent API loading (189).

1.4.3. Emulsion methods

1.4.3.1. Emulsification and solvent evaporation

The emulsification solvent evaporation method was the first method to produce polymeric nanoparticles from preformed polymers (192). The method was originally developed to produce artificial latexes (193, 194) and later adapted for pharmaceutical applications by Gurny *et al.* (195-197).

Typically, the polymer and the payload are dissolved in a common solvent (typically methylene chloride, chloroform, or ethyl acetate) and added to an immiscible non-solvent (typically water) containing a stabilizer (Figure 4). The mixture is emulsified e.g. by high-shear or high-pressure homogenization, sonication, spontaneous emulsion (198, 199) or phase inversion (200, 201). Afterwards, the solvent is evaporated by open stirring, elevated temperature, reduced pressure, nitrogen evaporation or similar method. The process of solvent removal and particle formation typically takes several hours, whereas the rate-limiting step of solvent removal may be the mass transport in the liquid phase or in the gas phase (202). The polymer and payload precipitate as nanoparticles at the interface of the emulsion upon solvent removal. The nanoparticles may be washed to remove free drug, excess stabilizer, potential low-molecular-weight polymer, or aggregates.

The method is primarily suitable to encapsulate hydrophobic drugs into hydrophobic polymers like PLGA, PLA and PCL (203-205) or their PEGylated variants as amphiphilic polymers (203, 206, 207). The use of additional stabilizers may be omitted when using amphiphilic polymers (206). The method has also been inversed to incorporate hydrophobic APIs in hydrophilic by using oil-in-water microemulsions (208, 209). To encapsulate hydrophilic drugs into PLGA, Ruiz *et al.* (210) suspended a lyophilized powder of triptorelin in a solution of PLGA in methylene chloride, caused phase separation by the addition of silicon oil and precipitated the PLGA by solvent evaporation. However, the resulting particle size of the drug delivery system is ultimately limited by the size of the primary API particles. Niwa *et al.* (211) modified the method by dissolving nafarelin acetate in an aqueous phase that is emulsified in the organic solution of PLGA. Following coacervation and PLGA precipitation, nanospheres were yielded with a mean diameter of 400-800 nm.

Nevertheless, the method works best for hydrophobic drugs and polymers. Other drawbacks include the use of toxic solvents (192) and the relatively slow process of solvent evaporation and particle formation that can lead to droplet coalescence, particle aggregation and payload degradation (212).

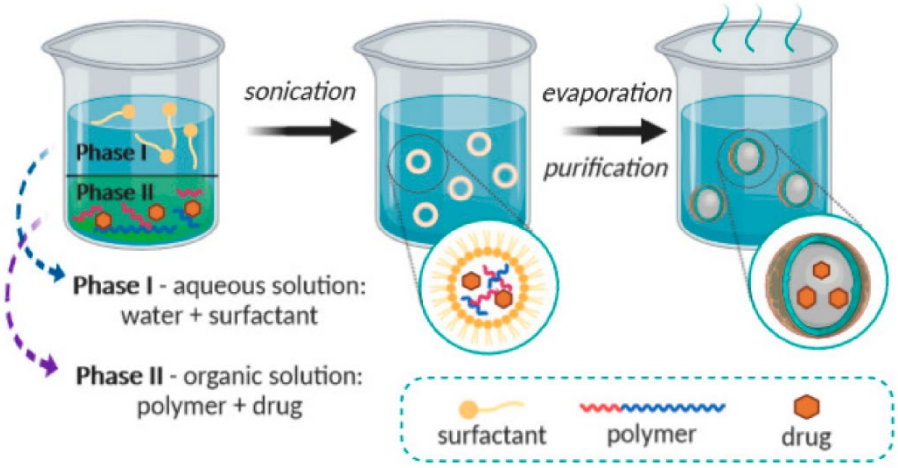

***Figure 4**. Preparation of nanoparticles by the emulsification and solvent evaporation method. Adapted from (132) under the Creative Commons Attribution (CC BY 4.0) license (http://creativecommons.org/licenses/by/4.0/).*

1.4.3.2. Salting-out

The salting-out method (213, 214) was the third method to produce polymeric nanoparticles from preformed polymers after the emulsification and solvent evaporation method and the nanoprecipitation method (192). Like the first method, it relies on an emulsion of a solvent in a non-solvent (water). The used solvent, however, is fully miscible with water, and the initial phase-separation is enabled by a high concentration of a salting-out agent that prevents the diffusion of the solvent into the aqueous phase. Examples of used agents are magnesium chloride (215), sodium chloride (216), magnesium acetate (217) or sucrose (192). Stabilizers are preferred that reduce the surface tension and increase the viscosity, e.g. poly(vinyl alcohol), polysorbate 80, poloxamer 188, and poloxamer 407 have been reported in the literature (192). The mixture is emulsified and subsequently diluted with water below the critical concentration of the salting-out agent. The rapid diffusion of the solvent into the aqueous phase creates an interfacial turbulence (similar to the nanoprecipitation method) and leads to the formation of nanoparticles.

The advantages of the salting-out method over the emulsification and solvent evaporation method are the possibility to use less hazardous or toxic solvents like ethanol, methanol, acetone, or acetonitrile (192). It also allows for a better size control and higher polymer concentrations compared to the nanoprecipitation method (192). The major drawback of the method is the high salt concentration that requires extensive washing after particle formation and bears the risk of forming coacervates of the stabilizer or drug, or to form insoluble salts.

1.4.3.3. Emulsification-diffusion

The emulsification-diffusion method was proposed by Leroux *et al.* (218) and patented by Quintanar *et al.* (219) as an alternative to the salting-out method with reduced purification effort (220). The method relies on the use of a partially water-miscible solvent like ethyl acetate, benzyl alcohol or

methyl ethyl ketone to dissolve the polymer and payload, and an aqueous solution of a stabilizer that has been saturated with the solvent. Both phases are emulsified, and the emulsion is subsequently diluted with water until the polymer precipitates at the interface of the emulsion droplets (Figure 5). Interestingly, the resulting size of the nanoparticles may be much smaller than the size of the emulsion droplets, as multiple particles can form from a single droplet in a phenomenon explained with the "diffusion and stranding" mechanism (221).

The emulsification-diffusion method is more flexible than the salting-out method and can be used with a wide variety of hydrophobic and amphiphilic polymers (205, 220). The process of particle formation is much faster compared to solvent evaporation methods and poses less of a challenge on emulsion or API stability. Still, the use of solvents may negatively affect the structural or chemical integrity of peptides, proteins, or nucleic acids (220). The dilution step also requires bigger reactors and increased efforts for purification, concentration or drying, and wastewater treatment which is especially relevant for scaling up and production.

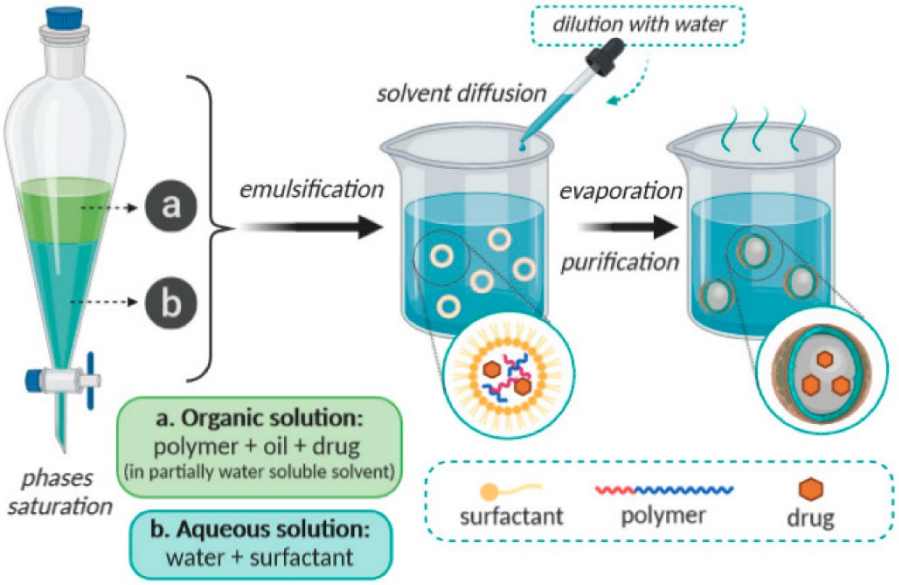

***Figure 5**. Preparation of nanoparticles by the emulsification-diffusion method. Different from the solvent evaporation method, the nanoparticles are formed before the removal of the solvent. Adapted from (132) under the Creative Commons Attribution (CC BY 4.0) license (http://creativecommons.org/licenses/by/4.0/).*

1.4.3.4. Double emulsion methods

Most precipitation-based methods require a common solvent and a common anti-solvent for the payload and the carrier material to form "co-precipitates" with high encapsulation efficiencies. Additionally, solvent and anti-solvent need to be sufficiently miscible. As such, the encapsulation of hydrophilic payloads like proteins into hydrophobic polymers with high efficiency is generally not feasible by precipitation alone. The double emulsion method was developed for this purpose.

Multiple emulsions of the (water-in-oil)-in water type (Figure 6) were discovered in the first half of the 20th century. They were first described in the pharmaceutical field as delivery system to improve the oral absorption of insulin (222) and for the preparation of microcapsules with a wall of polystyrene encapsulating an aqueous solution of gelatin and thiourea (223), and later for the production of microparticulate drug delivery systems (224-226). Blanco & Alonso (227) adapted the method for the preparation of nanoparticles and reported a loading efficiency of bovine serum albumin in PLGA of up to 90% without observed fragmentation or aggregation. The method became widely adopted with many different hydrophilic drugs like small molecules, peptides, proteins and nucleic acids (212, 228), and hydrophobic and amphiphilic polymers, although the method has been reported not to work with desmopressin and PLGA (229). The emulsion step is typically done by high energy homogenization

The double emulsion method has been combined with the different particle formation steps like solvent diffusion (230) or spray-drying (231, 232). The method typically relies on high energy homogenization methods like tip sonication, although more sophisticated methods have been proposed like membrane emulsification (233), microfluidics (233, 234), or coaxial electrospray (233).

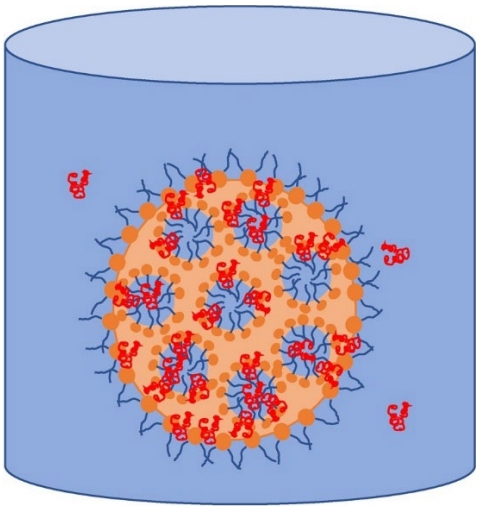

***Figure 6**. Schematic representation of a double emulsion consisting of a continuous outer aqueous phase and a dispersed inner aqueous phase (both in blue), an organic dispersed phase (orange), a stabilizer at the interface, and a protein (red). Reprinted from (235) with permission from Elsevier, Copyright 2017.*

1.4.4. Other

Direct spray-drying of water-in-oil emulsions has been reported to produce microparticles (236-238). Here, the particle size is governed mainly by the droplet size of the spray instead of the droplet size in the emulsion because the polymer is dissolved in the outer phase. Specialized equipment for the generation of nano-sized droplets and the separation of dried nanoparticles from the drying gas exist (239) to produce milligrams to grams of substance in the lab scale, but the throughput is severely limited compared to conventional equipment that has been scaled to the range of kilograms to tons per day. Similarly, rapid expansion of supercritical solutions has been proposed as an alternative to

reduce or replace the use of organic solvents by using supercritical CO_2 or other fluids, but the necessary equipment is comparably complex, while polymers commonly used for drug delivery are poorly soluble and often require still the use of organic co-solvents (212). Among the many more methods that have been described in the literature are ionic gelation (240), membrane reactors (241), aerosol flow reactors, electrohydrodynamic atomization, premix membrane emulsification (242).

2. Aims of the thesis

1. To develop a method to produce antigen-loaded polymeric nanoparticles suitable for small scale screening and supply, to characterize and systematically optimize

    a. Tunability of nanoparticle size distribution
    b. Nanoparticle yield
    c. Antigen loading efficiency
    d. Antigen integrity
    e. Washing protocol
    f. Batch size (scale up to support downstream development)

2. To identify and develop a manufacturing process for dosage forms suitable for animal or human use. Such a process and product should achieve:

    a. Solid dosage form for superior shelf life
    b. Preservation of primary nanoparticles during processing
    c. Protection of nanoparticles from gastric pH
    d. Release of primary nanoparticles at intestinal pH
    e. Ease of dosing for animal use

3. Main Findings

This section presents the main findings from two peer-reviewed publications and an international patent application (granted by the European Patent Office and the patent offices of the United States, Japan, China, Australia, and Israel, at the time of writing), while the full manuscripts are printed in chapter 4.

3.1. Focused ultrasound as a scalable and contact-free method to manufacture protein-loaded PLGA nanoparticles

Scalability of manufacturing processes is a major concern for the translation of nanomedicines from bench to bed (243-246). The first research article describes the development of production and purification methods of protein-loaded PLGA nanoparticles that are sufficiently scalable to be suitable for small scale screening and larger scale production.

The method and equipment were suitable to produce nanoparticles in batch sizes of 1 mg to 2500 mg in the size range of 100-200 nm with a yield of up to 74% and a protein loading of up to 3.6%. The nanoparticle size in screening runs in glass vials using 1 mg PLGA in a reaction volume of 0.3 mL was predictive for experiments performed in glass vials with 30 mg PLGA in 8 mL by normalizing the total incident energy on the reaction volume. Particle size reduction was more efficient when using a flow cell with a polyimide sheet for larger batch sizes (tested up to 2500 mg PLGA). A similar particle size range was achievable with much less incident energy per unit volume. The particle yield was lower, however, especially when working on the lower end of the flow cell working volume. Overall, the particle size reduction and resulting yield could be described with simple mathematical equations that differed only in the parameters used for glass vial or flow cell processing. Larger batch sizes than 2500 mg PLGA may be possible with the described method but were not investigated due to material constraints. A more efficient flow cell design and a more efficient cooling may enable a unidirectional process flow to push the scalability even beyond three orders of magnitude.

The washing protocol was also adapted from batchwise centrifugation to flow processing by using hollow fiber crossflow filtration modules. The developed diafiltration protocol proved to be more efficient, especially in retaining smaller nanoparticles, and helped to further improve the processing yield.

3.2. Towards a continuous manufacturing process of protein-loaded polymeric nanoparticle dry powder formulations

The second research article describes a further streamlined method where the double emulsion is directly fed into a spray dryer without prior precipitation of the nanoparticles. The drying of the double emulsion droplets causes the simultaneous precipitation of the nanoparticles and embedding in a

matrix that allows for better stabilization, size retention and redispersibility upon contact with water. The method is especially interesting because it has the potential for continuous manufacturing of solid formulations containing nanoparticles. A disadvantage of the method is that excess stabilizer used for emulsification and unencapsulated drug will remain in the formulation, although a workup of the emulsion before drying might be possible by column chromatography based on affinity or ion exchange principles.

This method was previously mentioned in the literature in conjunction with batch mode high shear emulsification (231, 232), but no process characterization and only very limited product characterization was described. Chapter 4.2 details the process investigation of the double emulsion spray drying method using a flow-through reaction chamber for focused ultrasound emulsification. Different combinations of emulsion stabilizers and matrix excipients were screened in lab scale spray drying experiments for the resulting nanoparticle size distribution and nanoparticle yield. The investigation showed that this method is suitable to produce PLGA/polyvinyl alcohol nanoparticles with a size distribution comparable to conventional methods. An economically interesting nanoparticle yield of 79% could be achieved when using trehalose and leucine as matrix excipients. This is considerably higher than found previously when using focused ultrasound followed by solvent displacement (chapter 4.1). In contrast to solvent displacement, PLGA/poloxamer 407 nanoparticles could not be manufactured with acceptable yield using the double emulsion spray drying method regardless of the matrix excipient used, even when adding polyvinyl alcohol to the emulsion.

3.3. Preparation of nanoparticles-releasing enteric microparticles

The patent describes continuous and scalable processes to dry and embed PLGA nanoparticles in gastro-resistant microparticles. The microparticles easily disperse in gastric acid or slightly acidic media while still protecting the embedded nanoparticles. If administered orally as a solid dosage form, e.g., in the form of capsules or tablets, the microparticles are expected to disperse in the stomach without agglomeration or gelling. The microparticles-containing powder can also be dispersed for oral dosing as a suspension.

Orally delivered peptide and protein compounds require adequate delivery systems to protect from the harsh environment in the gastrointestinal tract and to foster intestinal absorption (247). PLGA nanoparticles have been investigated as a promising tool for oral peptide and protein delivery and gastro-resistant capsules filled with nanoparticles have been proposed to prevent drug leakage, diffusion and degradation in the gastric environment (248). Gastrointestinal transit times of monolithic controlled delivery dosage forms may vary greatly between doses and between individuals. The variations may lead to differences in bioavailability, and therefore safety and efficacy, of the dosage form. Enteric microparticulate formulations may pass the pylorus independent from fasted or fed state

and the activity of the gastrointestinal tract of the dosed individual. Such formulations may also be designed more easily to release their cargo at a specific region of the intestine, e.g., for colonic delivery of antigens.

The major formulation challenge is to achieve a homogeneous and functional coating while retaining the size and shape of the individual nanoparticles. Enteric coatings based on Eudragit® are usually prepared from organic solutions or aqueous dispersions. The Initial approach of using Eudragit® L (EuL) dissolved in organic solvents proved unsuccessful, as organic solvents either dissolved or softened both EuL and PLGA, or led to visible flocculation. Further approaches relied on aqueous dispersions.

Aqueous spray dispersions of EuL are usually stabilized by adding a certain amount of base. This causes a deprotonation or neutralization of a part of the carboxylic acid groups and results in a negative surface charge of the polymeric particles and electrostatic stabilization. Continuous polymer films can be formed from such latices by the coalescence of individual particles upon the evaporation of the continuous phase. This principle is commonly used to coat tablet cores with Eudragit® films in fluid bed processes. A plasticizer needs to be added to the spray feed to assure a homogeneous distribution and functionality of the films. However, the very nature of plasticizers allows them to move in between polymer chains and, eventually, to leech out of the film. If polymeric nanoparticles were incorporated in such a matrix, it would be reasonable to assume that plasticizer molecules can relocate from the Eudragit® film into the incorporated nanoparticles. This process would be exacerbated by the high surface-to-volume ratio of nanomaterials and the large interface to the matrix. The association of plasticizer at the nanoparticles' surface or diffusion into the particle would soften the particles and likely increase their tendency to deform and agglomerate. This effect would be especially pronounced for polymers with low glass transition temperatures like PLGA: The incorporation of triethyl citrate, a plasticizer commonly used for Eudragit film coating, into PLGA films decreases the glass transition temperature (T_g) of the films to almost room temperature (249). This has major implications not only for the stability during manufacture and storage, but also in vivo performance, where a T_g below body temperature may lead to surface softening and particle agglomeration after administration. Indeed, the resulting sensitivity to temperature and hydration has been used to create stimuli-responsive PLGA/plasticizer systems for both particulates (250-252) and films (249, 253-255).

The present invention describes the facilitation of film formation by using increasing amounts of base instead of a plasticizer. The complete dissolution of EuL in water by adding NaOH leads to a very viscous fluid with good film forming properties. PLGA nanoparticles embedded in such a matrix by spray drying retained their size upon dissolution in phosphate buffered saline pH 6.8. The viscosity of the EuL solution makes it problematic to atomize and dry in a spray dryer. Moreover, neutralized EuL is freely soluble in water, and in gastric buffer, such particles immediately begin to swell and to form sticky gel-

like lumps before the re-protonation of the methacrylate stops the solvation. On the other hand, preparing a standard spray suspension of EuL but without plasticizer did not lead to the formation of an enteric coating and did not protect the PLGA nanoparticles from agglomeration. The patent discloses that it is possible to find a balance between the two effects. Formulations prepared from PLGA nanoparticles and EuL with a degree of neutralization between 15-30% released the nanoparticles at intestinal pH with excellent preservation of particle size while retaining good dispersibility in acidic media. The yielded dosage form is a powder for reconstitution with enteric properties that allows easy and reproducible oral dosing in pre-clinical studies even with small animals like mice. The dosage form is suitable for veterinary medications that need to be dose-adjustable over a wide range of body weights. A powder for reconstitution is less common for human medications except for extemporaneous preparations or in pediatrics.

4. Results: Original publications

4.1. Focused Ultrasound as a Scalable and Contact-Free Method to Manufacture Protein-Loaded PLGA Nanoparticles

Stefan Schiller,[1,2] Andrea Hanefeld,[2] Marc Schneider,[1] Claus-Michael Lehr[1,3]

[1] Department of Pharmacy, Biopharmaceutics & Pharmaceutical Technology, Saarland University, Campus, Building A4.1, 66123 Saarbrücken, Germany

[2] Research Pharmaceutics & Drug Product Development, Merck Serono, HPC D039/002, Frankfurter Str. 250, 64293 Darmstadt, Germany

[3] Department of Drug Delivery (DDEL), Helmholtz Institute for Pharmaceutical Research Saarland (HIPS), Helmholtz Center for Infection Research (HZI), Saarland University Campus, Building A4.1, 66123 Saarbrücken, Germany

Pharm Res. 2015;32(9):2995-3006.

DOI: 10.1007/s11095-015-1681-7

Specification of the contributions:

Stefan Schiller conceived, planned, and carried out the experiments, and analyzed the data. Stefan Schiller, Andrea Hanefeld, Marc Schneider, and Claus-Michael Lehr interpreted the results. Stefan Schiller took the lead in writing the manuscript. Andrea Hanefeld, Marc Schneider, and Claus-Michael Lehr provided critical feedback to the concept of the research and contributed to the manuscript.

Pharm Res
DOI 10.1007/s11095-015-1681-7

RESEARCH PAPER

Focused Ultrasound as a Scalable and Contact-Free Method to Manufacture Protein-Loaded PLGA Nanoparticles

Stefan Schiller[1,2] • Andrea Hanefeld[2] • Marc Schneider[1] • Claus-Michael Lehr[1,3]

Received: 19 December 2014 / Accepted: 18 March 2015

ABSTRACT

Purpose Although nanomaterials are under investigation for a very broad range of medical applications, only a small fraction of these are already commercialized or in clinical development. A major challenge for the translation of nanomedicines into the clinic is the missing scalability of the available lab scale preparation methods and, ultimately, non-identical samples during early and late research.

Methods Protein-loaded PLGA nanoparticles using focused ultrasound in an emulsion solvent diffusion method were prepared in different batch sizes to evaluate achievable mean size, protein loading, and yield.

Results Using the same equipment, nanoparticles could be prepared in batch sizes from 1 mg to 2.5 g. Size and yield were directly controllable by the amount of incident energy with good reproducibility. The nanoparticles displayed similar mean size, protein loading, and nanoparticle yield in batch sizes over three orders of magnitude. A scalable purification method based on diafiltration was established.

Conclusions The proposed method enables for feasibility studies during early research using just a small amount of polymer and protein, while at the same time it allows for larger scale production at later stages. As the proposed method further relies on contact-free energy transmission, it is especially suited for the preparation of clinical research samples.

KEY WORDS drug delivery · focused ultrasound · nanoparticles · nanotechnology · protein

ABBREVIATIONS

AIP	Average incident power
CR	Concentration reduction
DLS	Dynamic light scattering
DV	Diafiltration volumes
E	Total incident energy
EE	Encapsulation efficiency
EtOAc	Ethyl acetate
P-407 / P-188	Poloxamer 407 / 188
PIP	Peak incident power
PLGA	Poly(lactic-co-glycolic acid)
POE	Poly(oxyethylene)
POP	Poly(oxypropylene)
Y	Yield
T	Membrane transmission coefficient

INTRODUCTION

Nanomaterials are used in medicine in a variety of applications such as drug delivery, medical imaging, *in vitro* and *in vivo* diagnostics as well as tissue engineering (1). By encapsulating one or more drugs in nanoscale carrier systems with

Electronic supplementary material The online version of this article (doi:10.1007/s11095-015-1681-7) contains supplementary material, which is available to authorized users.

✉ Stefan Schiller
stefan.schiller@external.merckgroup.com

✉ Claus-Michael Lehr
claus-michael.lehr@helmholtz-hzi.de

[1] Department of Pharmacy, Biopharmaceutics & Pharmaceutical Technology, Saarland University, Campus, Building A4.1, 66123 Saarbrücken, Germany

[2] Research Pharmaceutics & Drug Product Development, Merck Serono, HPC D039/002, Frankfurter Str. 250, 64293 Darmstadt, Germany

[3] Department of Drug Delivery (DDEL), Helmholtz Institute for Pharmaceutical Research Saarland (HIPS), Helmholtz Center for Infection Research (HZI), Saarland University Campus, Building A4.1 66123 Saarbrücken, Germany

Published online: 31 March 2015

specifically designed physicochemical properties and surface modifications, the pharmacokinetics and pharmacodynamics can be distinctly improved (2). Typical applications of nanoparticles include drug solubilization, crossing of biological barriers, controlled release, passive and active targeting, vaccination or immune modulation, and gene therapy (3). Such nanocarriers are often made of synthetic biodegradable polymers with a favorable toxicological profile. One of the best established polymers for biomedical applications is poly(lactic-co-glycolic acid) (PLGA) (3,4). Due to the biodegradability and biocompatibility of PLGA and its hydrolysis products, several PLGA-containing drug products are approved by the FDA.

While the encapsulation of hydrophobic molecules into polymeric nanoparticles usually can be achieved with good efficiency by nanoprecipitation—a method initially developed by Fessi *et al.* (5)—more complex methods are needed to formulate hydrophilic entities like proteins. One of the most widely used approaches is the double emulsion method (6): an aqueous solution of the hydrophilic drug is emulsified into a non- or partially miscible solvent containing the polymer. To this emulsion, a second aqueous phase containing a stabilizer is added and the mixture further homogenized. After solvent removal the polymer precipitates, entrapping the drug within the newly formed particles. Currently employed devices for the critical homogenization step include high shear mixers, probe sonicators, high pressure homogenizers and microfluidic systems, although numerous issues can render them unsuitable for a range of applications. Direct sample contact may lead to cross-contamination or a reduced sample throughput at best, due to the necessity of thorough equipment cleaning. A serious temperature gradient throughout the sample may affect protein stability, while product contamination due to open setups and metal abrasion interferes with parenteral dosing and immunological readouts. Maybe the most important issue of established homogenization techniques is the missing scalability to adapt the produced amount from the bench to the clinic. While there was already some work done on the scale up of lab scale processes (7–10), these mostly include the use of bigger reactors, different geometries, or even different equipment. Furthermore, information on the scale up of actual pharmaceutical nanoparticle production processes for clinical products is very scarce (11). A single piece of equipment that covers early stage formulation screening all the way to proof-of-concept might considerably advance the somewhat obstructed translation of nanomedicines to patients (12).

For this reason, we investigated focused ultrasound as a scalable and contact-free homogenization technique to produce polymeric nanoparticles by the double emulsion method. In contrast to classical probe sonicators which are directly submerged in the sample, focused ultrasound uses a concave transducer which bundles the acoustic waves in a focal point within a closed vessel. The induced cavitation leads, similar to probe sonication, to an energy input and thus to a size reduction of emulsion droplets and subsequently nanoparticles. However, acoustic focusing avoids the formation of complex interference patterns and associated pressure hot spots throughout the sample and cooling bath, as is common with unfocused probe or bath sonication. A slight but constant variation of the emitted acoustic wavelength shifts the focal point for better sample mixing.

This leads to a better energy distribution and process reproducibility. Furthermore, the heat generated by the vibration of the transducer is not absorbed by the sample but rather by a surrounding water bath. In consequence, all these factors help to avoid thermal input into the system and degradation of the drugs. Due to disposable vials and flow cells, the same equipment may be used to process sample volumes from 100 µL to 20 mL in batch mode and from 50 mL up to several liters in continuous mode.

In the present study we show the feasibility to use focused ultrasound to encapsulate the widely used model antigen ovalbumin in PLGA nanoparticles in a production scale from 1 mg to 2.5 g polymer mass. The influence of process parameters on nanoparticle size and yield, drug load, and process scalability was investigated, and a nanoparticle purification protocol suitable for large suspension volumes was successfully established.

MATERIALS AND METHODS

Materials

Poly(lactic-co-glycolic acid) (PLGA, Resomer® RG 503 H; LA:GA = 50:50, 24–38 kD, free carboxylic end group) was purchased from Evonik Industries (Essen, Germany). Ovalbumin grade V was obtained from Sigma Aldrich (St. Louis, USA). Poloxamer 407 (P-407) was kindly provided by BASF (Ludwigshafen, Germany; Lutrol® F127). All other chemicals were obtained from Merck KGaA (Darmstadt, Germany) in analytical or HPLC grade.

Nanoparticle Preparation

All solutions were freshly prepared and filtered through 0.2 µm PTFE (polytetrafluoroethylene; for organic solvents) or PES (polyethersulfone; for aqueous solutions) membrane filters before use. A modified double emulsion solvent evaporation method was used to prepare Ovalbumin-loaded PLGA nanoparticles (13). In brief, 12 mg/mL PLGA was dissolved in ethyl acetate (EtOAc), added to an aqueous solution of 3.75 mg/mL ovalbumin, and the mixture was homogenized using focused ultrasound (Covaris S220x, LGC-KBioscience, Teddington, UK) as described in the section below. To this

primary W/O emulsion, an aqueous solution of 5 mg/mL P-407 was added and the mixture emulsified again by focused ultrasound. EtOAc was removed from this secondary emulsion overnight under magnetic stirring in a fume hood resulting in formation of PLGA nanoparticles. Water lost during evaporation was replaced, yielding an aqueous suspension with nominal concentrations of 0.3 mg/mL ovalbumin, 6 mg/mL PLGA, and 5 mg/mL P-407. The resulting suspension was centrifuged for 15 min at 1,000×*g* to remove larger aggregates. 1 mL aliquots of the supernatant were centrifuged for 10 min at 21,000×*g*, and the nanoparticle pellet was washed and redispersed in particle-free, deionized water (Milli-Q, Merck Millipore, Billerica, USA).

Table I shows the volumes of PLGA, ovalbumin, and P-407 solutions used to prepare PLGA nanoparticles in different batch sizes.

Focused Ultrasound Treatment

The Covaris instrument consists of a concave transducer submerged in a water bath. Acoustic waves are conveyed through the water (degassed and conditioned at 7°C±2°C) to the focal point in the submerged sample vessel. Either closed glass tubes or a sonication flow cell were used. The flow cell is a 22 mL steel cylinder with two tubing connectors and a thin sheet of polyimide (Kapton®) facing the transducer. In our setup, the sample was continuously circulated from a bulk vessel to the flow cell and back by a peristaltic pump (Fig. 1). Samples were taken from the bulk vessel at predetermined time points during the second emulsification step. Device settings and derived parameters are summarized in Table II.

Nanoparticle Characterization

Particle sizes and size distributions were determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) equipped with a helium neon laser (λ=633 nm). Samples were diluted 1:100 with Milli-Q water, measured three times at 25.0±0.1°C using backscatter mode (173°), and data was analyzed using cumulants fit.

The presence of multiple particle populations and the particle size distribution before any washing steps were determined by static light scattering using a Horiba LA-950 V2 (Retsch Technology, Haan, Germany). Particle suspensions

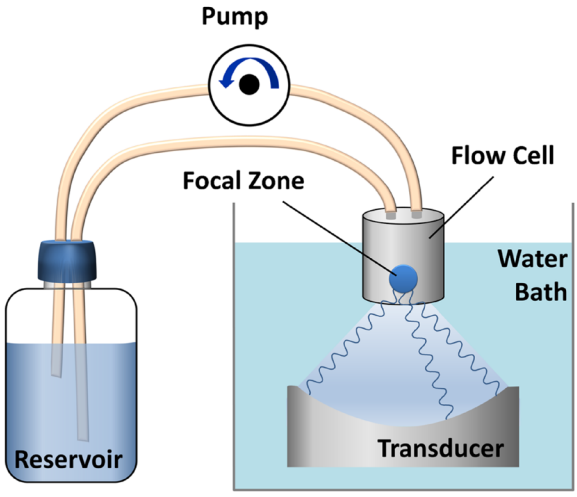

Fig. 1 Schematic of focused ultrasound with flow cell. In the case of batch processing the flow cell would be simply substituted with a closed glass vial.

were added to a MiniFlow circulating system to achieve a transmission between 80% and 90% at λ=405 nm. Analysis was performed according to Mie scattering theory with a refractive index of PLGA of 1.44 – 0.01i (14).

To determine the process yield and ovalbumin loading, 1 mL particle suspension was dried in a rotational vacuum concentrator (RVC 2–33 IR, Martin Christ, Osterode, Germany). The dry nanoparticles were weighed, dissolved in 1 M NaOH, neutralized with 1 M HCl and the protein content measured with a BCA protein assay kit according to the manufacturer's instructions (Merck Millipore, Billerica, USA). The total protein recovery of this method was previously validated by CHN elemental analysis (data not shown). Process yield and ovalbumin loading were calculated using the following equations:

$$\textit{Process Yield} = \frac{\textit{Nanoparticle mass}}{\textit{initial mass of PLGA and Ovalbumin}} \times \textit{100\%} \quad (1)$$

$$\textit{Ovalbumin loading} = \frac{\textit{Ovalbumin mass}}{\textit{Nanoparticle mass}} \times \textit{100\%} \quad (2)$$

Influence of Focused Ultrasound on Ovalbumin Stability

An ovalbumin solution similar in concentration and volume to a typical experiment (0.38 mg/mL in 100 mL) was circulated

Table I Composition of Emulsions Used to Prepare PLGA Nanoparticles in Different Batch Volumes. The Used Sonication Vessels were 300 μL and 16 mL Glass Vials, and a 22 mL Stainless Steel Flow Cell

Identifier	Vial 0.3 mL (μL)	Vial 8 mL (mL)	Flow Cell 95 mL (mL)	Flow Cell 665 mL (mL)
Ovalbumin in water	15.2	0.4	4.8	33.6
PLGA in EtOAc	95	2.5	30	210
P-407 in water	190	5.0	60	420
Total filling volume	300.2	7.9	94.8	663.6

Table II Settings and Derived Parameters for the Covaris Focused Ultrasound Device

Abb.	Parameter	Description
DF	Duty Factor	The percentage of time the transducer emits acoustic energy.
CpB	Cycles per Burst	The number of acoustic oscillations (cycles) during an "on" period of the transducer (burst).
PIP	Peak Incident Power	Sonic power (in W) applied to the sample during an "on" period.
AIP	Average Incident Power	Can be approximated as *AIP*≈*DF x PIP*. The calculated *AIP* may differ from measured AIP due to constructive or destructive interference of generated and reflected sonic waves.
E	Total Incident Energy	The product of *AIP* and treatment time *t*.
E/V	Incident energy per unit volume	The quotient of *E* and batch volume *V*.

through a sonication flow cell and treated with maximum intensity (250 W average incident power) up to 40 min. Samples were taken at predetermined time points and ovalbumin integrity determined by size exclusion high performance liquid chromatography. A TSKgel® SuperSW2000 column (Tosoh Bioscience, Stuttgart, Germany) was used at 25°C with an eluent containing 0.05 M sodium phosphate buffer pH 7.0 and 0.15 M sodium perchlorate. The flow rate was set to 0.45 mL/min and the UV absorption was measured at 214 nm. To calculate the ratio of ovalbumin monomer to aggregates and fragments, all relevant peaks were integrated and divided by the total integrated area before treatment.

Crossflow Filtration Method Development

A purification protocol was developed for MicroKros® hollow fiber crossflow filtration modules (modified polyethersulfone (mPES) membrane, 500 kD MWCO, 20 cm^2 surface area; Spectrum Laboratories, Rancho Dominguez, USA). A diafiltration setup was used for purification, *i.e.*, the volume of the retentate was kept constant by continuously replacing the filtrated volume with fresh medium. The replaced medium is measured in diafiltration volumes (DV), where one DV is defined as the volume of the process solution at the start of the diafiltration. If the membrane transmission of a compound is known, the concentration reduction of the compound in the retentate *CR* can be predicted as follows:

$$CR = 1 - e^{-DV \times \tau} \tag{3}$$

where τ is the transmission coefficient of the solute—with $\tau = 1$ meaning free transmission, and $\tau = 0$ meaning no transmission (15). Equation (3) is only valid if τ is independent from solute concentration and the transmembrane pressure is kept constant. Consequently, the transmission coefficient can be calculated by measuring *CR* and solving Eq. (3) for τ:

$$\tau = -\frac{ln\,(1-CR)}{DV} \tag{4}$$

To determine the transmission coefficient of P-407, 50 mL of a 5 mg/mL P-407 solution were circulated from a bulk vessel to the diafiltration module and back. The filtrated volume was continuously replaced by Milli-Q water. Samples were taken from the permeate and the concentration of P-407 was determined by gel permeation chromatography (GPC) using a Tosoh TSKgel® G3000H_{HR} column at 60°C, dimethylformamide as eluent at 1.2 mL/min, and a refractive index detector. The concentration reduction of P-407 in the retentate *CR* was calculated using the equation:

$$CR = \frac{c_P \times V_P}{m_0} \times 100\% \tag{5}$$

where m_0 is the total mass of P-407 at the start of the diafiltration, c_P is the concentration of P-407 in the permeate and V_P is the volume of the permeate. The transmission coefficient subsequently can be calculated for the respective number of diafiltration volumes by Eq. (4). At the end of each experiment, the residual concentration of P-407 was directly determined from the retentate.

Nanoparticle Suspension Purification

Ten milliliters of a freshly prepared PLGA nanoparticle suspension was diluted to 20 mL with Milli-Q water and circulated at 40 mL/min through a pre-washed MicroKros® module. The permeate was replaced with Milli-Q water at the same rate to perform a diafiltration. At the end of the diafiltration the suspension was concentrated to 7 mL, the filtration module completely emptied, rinsed with 2 mL of Milli-Q water and the combined retentate diluted to 10 mL.

RESULTS AND DISCUSSION

To our knowledge, this is the first study on the preparation of polymeric nanoparticles using focused ultrasound and the double emulsion method. Therefore, optimal treatment

parameters were established experimentally by investigating the influence of the sonication intensity and time on particle size, ovalbumin loading capacity and nanoparticle yield. The study was started with a batch size of 8 mL in disposable glass vials to obtain sufficient material for analysis while keeping the material need at minimum. We could quickly discard settings with low duty factor (DF) or peak incident power (PIP) as a certain minimum sonication intensity is necessary to induce cavitation and therefore to reduce droplet size. There was no pronounced difference in particle size when lowering DF and raising PIP accordingly to keep the average incident power (AIP; equals roughly the product of DF and PIP) constant. Because of the good reproducibility the DF was kept constant at DF 50% for the following experiments. A preliminary stabilizer screening was conducted, including polyvinyl alcohol, poloxamer 188, poloxamer 407, dimethyldidodecylammonium bromide, sodium deoxycholate, and Tween 80 at concentrations between 0.1 and 2% (supplementary material). Poloxamer 407 was chosen due to its ability to form nanoparticles with small size and narrow size distribution. Furthermore, P-407 could adequately stabilize both the emulsion as well as the subsequently formed nanoparticles at a relatively low concentration (Figures S1 and S2).

Nanoparticle Size

Figure 2 shows the correlation between ultrasound treatment time and the resulting mean particle size after collection of the nanoparticle population. At all investigated time points, the nanoparticle size distribution was narrow (polydispersity index <0.2). A mean particle size of 200 nm can be achieved with very short processing times. The size decrease is faster at the beginning of the ultrasound treatment, and the mean nanoparticle size eventually approaches a minimum between 105 and 110 nm. A similar trend was observed previously (16,17), although Feczkó *et al.* reported a minimum size of 140 nm using a similar PLGA concentration, but different solvent and stabilizer. As would be expected, particle size is generally decreased with ongoing treatment, and the size reduction is faster when increasing the average incident power (AIP) from 50 W to 100 W (Fig. 2a). As the particle size is mainly determined by the size of the emulsion droplets, which in turn depends on the energy used for homogenization, it is not surprising that the particle size curves of both treatments become nearly identical when normalizing on total incident energy as the product of AIP and treatment time (Fig. 2b). This interdependence between ultrasound intensity and treatment time makes the process very predictable and therefore provides an advantage during method development.

In this setup, the particle diameter D can be calculated from the incident energy E as follows:

$$\boldsymbol{D(E)} = \frac{\boldsymbol{a}}{\boldsymbol{b}+\boldsymbol{E}} + \boldsymbol{c} \qquad (6)$$

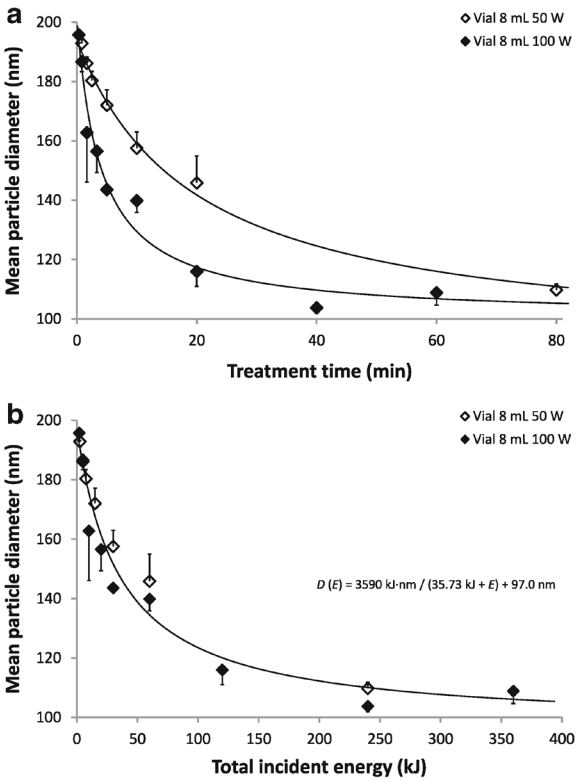

Fig. 2 Mean particle size of formulations manufactured with different Average Incident Power as a function of (**a**) sonication time (starting from several seconds) and (**b**) total incident energy. The size of three independent batches per time point was determined by DLS and the standard deviation depicted as error bars.

where a defines the initial steepness of the curve and as such probably includes factors like temperature, PLGA concentration, emulsion viscosity, sample volume, and the ratio water/organic phase.

The parameter b defines the pole of the function at $E=-b$. The fact that $b>0$ means that a very low amount of nanoparticles may be found even with no second emulsion at all. Due to the removal of microparticles and larger aggregates during the first centrifugation step at $1.000\times g$, this very low amount of nanoparticles is detectable by dynamic light scattering, and a mean diameter for this nanoparticle population can be measured. This phenomenon has its origin most probably in the physical forces upon contact of the first emulsion with the P-407 solution, as EtOAc is partially miscible with water and both phases will saturate each other.

The parameter c defines the asymptote of the function and corresponds to the minimum emulsion droplet size after droplet breakup and recoalescence during ultrasound-induced cavitation. Factors influencing c most probably include surface tension, type and concentration of stabilizer, PLGA concentration, and viscosity.

Nanoparticle Yield

While the nanoparticle size is continuously decreased with progressing treatment time, the nanoparticle yield rises for both the 50 W and 100 W treatments to optimal values of 47–63% at 10–30 kJ total incident energy, but declines when further prolonging treatment (Fig. 3). As expected, a higher intensity leads to a faster increase in yield, and when normalizing again on total incident energy the nanoparticle yield from both treatments follow the same trend, again indicating a predictable process. Interestingly, this biphasic relationship of nanoparticle yield Y and total incident energy E can be described by a simple function (Fig. 3, solid line):

$$\boldsymbol{Y(E)} = \boldsymbol{aE^{-1}} + \boldsymbol{bE} + \boldsymbol{c} \quad (\boldsymbol{a}, \boldsymbol{b} < 0) \tag{7}$$

As a centrifuge step during the manufacturing process separates the nanoparticles from larger particles, it is safe to assume that the major reason for increasing yield in the beginning of the process is the size reduction of bigger emulsion droplets to submicron size. To elucidate this, static light scattering measurements of the particle suspensions were performed before any centrifuge separation during the positive slope of the curve ($E<$ 27 kJ). After 20 s at 100 W AIP (or 2 kJ), two distinct particle populations exist in the submicron and micron size range, respectively (Fig. 4, dotted line). When increasing treatment time, the microparticle population diminishes in favor of the submicron population, and eventually after 20 kJ (Fig. 4, solid line) only the submicron population remains. The transition from one population to the other is not continuous, as the mode of the particle size of the microparticle population does not change and both nanoparticle and microparticle size peaks remain clearly separated. This is in accordance with the mechanism of emulsion droplet size reduction by cavitation which is deformation and sudden break-up of droplets (18).

Consequently, the total conversion of micron to submicron droplets (and therefore the maximum yield gain due to this process) coincides with the observed peak values for the nanoparticle yield between 10 and 30 kJ (Fig. 3). We therefore conclude that due to the steep rise and eventual asymptotic behavior, the term aE^{-1} ($a<0$) from Eq. (7) is reasonable for a mathematical description of the yield gain resulting from droplet size reduction.

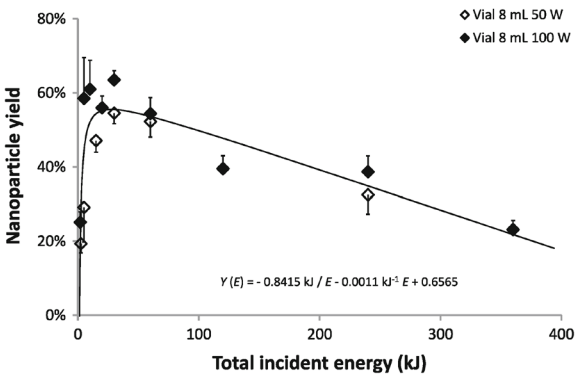

Fig. 3 Nanoparticle production yield as a function of total incident energy. Three independent batches per time point were washed, dried, weighed, and the yield calculated by Eq. (1). *Error bars* represent the standard deviation.

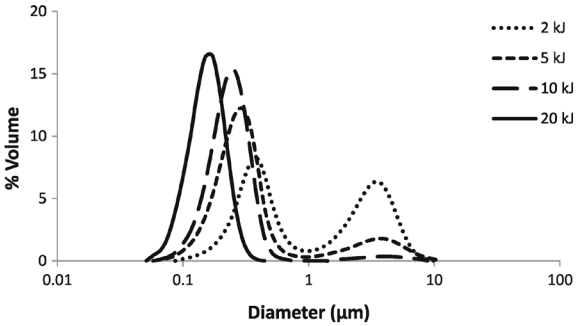

Fig. 4 Particle size distribution of formulations prepared with increasing treatment times and 100 W AIP. Measurements were made by static light scattering before any washing steps.

Yield diminishing influences are accounted for by the second term from Eq. (7) bE ($b<0$). Especially at incident energies below 100–200 kJ the yield reduction coincides with a particle size reduction (Fig. 5). There are several explanations for this correlation. First, if the stabilizer concentration is just enough to stabilize an emulsion, the increased total surface area as a result of emulsion droplet size reduction may lead to a decrease of local stabilizer concentration at the interface and consequently to droplet re-coalescence and emulsion instability after treatment. Second, if the surface of subsequently formed particles is not saturated with stabilizer, aggregation might occur during centrifuge purification. Third, smaller particles are more likely lost during centrifugation as the RCF was kept constant. Consequently, when substituting

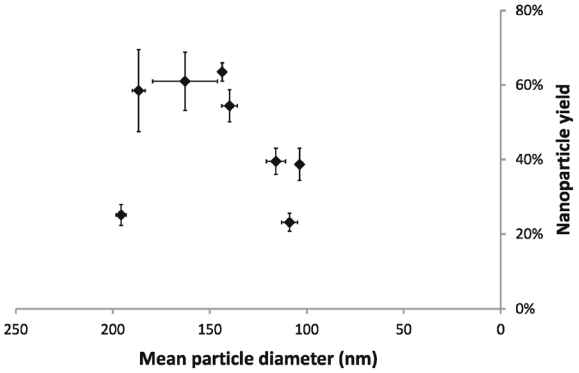

Fig. 5 Correlation between mean nanoparticle size and nanoparticle yield. Eight milliliters emulsion was treated in a glass vessel at 100 W AIP. The horizontal axis was mirrored to clarify the chronological progression from larger to smaller particles during the course of the process. Centrifuge purification is most likely the reason for reduced yield when producing smaller nanoparticles.

centrifuge purification for crossflow filtration, thus eliminating two of the possible reasons, we could significantly increase the yield (see section "Crossflow Filtration").

While the particle size decreases only slightly further at incident energies above 100 kJ, there is still a noticeable drop in nanoparticle yield (Figs. 2b and 3). Considering the small batch volume of 8 mL and the very long treatment time—which is well beyond reasonable process times for production—degradation might occur due to mechanical stress and the generation of free hydroxyl radicals in the process of cavitation. A cleavage of PLGA to water-soluble oligomers and monomers would reduce the mass of precipitable polymer, while a loss of stabilizer could lead to coalescence and aggregation as described above, both decreasing nanoparticle yield. The solvodynamic shear generated by the formation, oscillation and collapse of cavitation bubbles leads to a tension along the elongated polymer backbone and subsequent chain scission (19). Considering the γ-irradiation-induced radical decomposition of PLGA and polyethers like P-407 (20,21), cavitation induced free radicals might lead to a similar autoxidative decomposition pathway: C-H bond cleavage and peroxide formation in the presence of hydroxyl radicals and oxygen, random polymer chain cleavage, and eventually short chain acid formation (21,22).

Indeed, PLGA in dichloromethane was found to experience molecular weight loss when sonicated even at relatively low intensities with relatively short processing times (23). Reich found a significant molecular weight reduction when treating a 6 mL sample at 40 W for 30 s (=0.2 kJ/mL) with a submerged probe sonicator. While we did not systematically monitor the stability of PLGA and P-407 following sonication in this study, no degradation of either polymer could be detected in samples manufactured with an AIP of 50 W and 0.6 kJ/mL energy per unit volume (gel permeation chromatography data not shown). The superior polymer stability in our study may be explained by the controlled and contact-free nature of a focused ultrasound treatment as opposed to probe sonication; the latter suffering from unpredictable interference patterns and energy hotspots across the sample due to the unfocused distribution and reflection of sonic waves. Another factor might be the stabilizer P-407 used in our study, a tri-block copolymer of poly(oxyethylene) (POE) and poly(oxypropylene) (POP) in the form of POE_a-POP_b-POE_a with a = 101 and b = 56. POE was found by Rokita and Ulański to be an effective radical scavenger by investigating competition kinetics in a sonochemical reactor (24). Furthermore, they found that less hydrophobic polymers like POE are enriched at the gas/water interface of the cavitation bubbles up to a factor of 100 compared with the rest of the solution, while more hydrophobic polymers like poly(methyl methacrylate) are evenly distributed (24). Thus, by saturating the interface, the stabilizer might displace other molecules of interest from the zone of greatest stress and therefore additionally protects them from mechanical forces and free radicals.

Only at considerably higher incident energy ($E > 100$ kJ, equal to 12 kJ/mL for 8 mL sample), a decline of nanoparticle yield occurs which is probably related to polymer degradation. The needed energy is 60 times higher than the degradation threshold of PLGA reported by Reich (23). Based on these findings, a systematic assessment of the polymers' integrity depending on the used energy source, incident power and energy per unit volume is necessary for a rational manufacturing process design.

The linear constituent bE from Eq. (7) likely is too simplified to describe all underlying mechanisms of yield decline. However, degradation is only expected when overprocessing a sample, and the manipulation of both particle size and nanoparticle yield is effectively possible before reaching critical levels of incident energy. Therefore we conclude that the process preserves the structure of PLGA and P-407, and that the proposed equation is both mathematically reasonable and adequate for yield optimization.

Ovalbumin Loading

Ovalbumin was used as model protein due to its common usage in early vaccination studies with mice. The ovalbumin loading is at acceptable levels even after short processing times and seems to be higher at lower average incident power. During 50 W treatment the loading reaches a maximum of 3.6 ± 0.1% protein per particle weight at 150 s (equaling 7.5 kJ), while a 100 W treatment peaks at 3.2 ± 0.2% at 200 s (20 kJ) (Table III). This conforms to published work on the encapsulation of ovalbumin in PLGA using probe sonication with reported values from 1.2 to 5.4% (25–27). As we did not optimize the used concentrations of ovalbumin and PLGA

Table III Total Incident Energy (TIE) Necessary to Achieve Either Maximum Ovalbumin Loading or Ovalbumin Encapsulation Efficiency (EE) for Different Average Incident Power (AIP). EE is Calculated from Loading and Nanoparticle (NP) Yield

	TIE (kJ)	Energy per unit volume (kJ/mL)	Ova Loading (%)	NP Yield (%)	Ova EE (%)
AIP 50 W, max. load	7.5	0.9	3.6 ± 0.1	29.5 ± 3.1	22.4 ± 3.3
AIP 50 W, max. EE	30	3.8	3.3 ± 0.2	54.5 ± 4.2	38.2 ± 5.7
AIP 100 W, max. load and EE	20	2.5	3.2 ± 0.2	56.0 ± 3.2	38.1 ± 4.9
Literature (25–28)[a]	*n.a.*	0.1 – 1	1.2 – 5.4	*not reported*	15 – 34

[a] Comparison with probe sonication. Energy per unit volume was estimated as all suitable references lack information regarding one or more of the following: total volume, power source, applied power, pulsing, and ultrasound horn

during emulsification, the ovalbumin loading achievable by focused ultrasound may be higher than reported in this study. Especially when using costly proteins, the encapsulation efficiency (EE; the fraction of ovalbumin that is actually encapsulated in the nanoparticles) is another important factor. It can be calculated from the ovalbumin loading, the nanoparticle yield and the total amount of employed ovalbumin. For the above mentioned loading maxima the EE is 22 and 38% for the 50 W and 100 W treatments, respectively (Table III). This again conforms to published work with reported EE from 15 to 34% (26–28).

Similar to yield, when drastically increasing the incident energy to values above 100 kJ the loading seems to decrease again for the 50 W treatment (2.0% at 240 kJ). A similar trend was observed by Feczkó *et al.* who proposed a greater chance of drug escape from the inner aqueous droplets (which would get encapsulated) to the outer water phase for longer and higher intensity emulsification treatments (29). Interestingly, the loading increases to 6.6% when applying excessive incident energy (360 kJ at a power of 100 W AIP). As the protein visibly aggregates under these harsh conditions, this finding might have been caused by a changed affinity of ovalbumin oligomers to the polymer matrix, the formation of protein aggregates similar in size to the PLGA nanoparticles, or the formation of protein-polymer conjugates. The exact cause was not further investigated as relevant processing energies to achieve acceptable particle size, yield and loading are equal or below 30 kJ and thus more than ten times smaller.

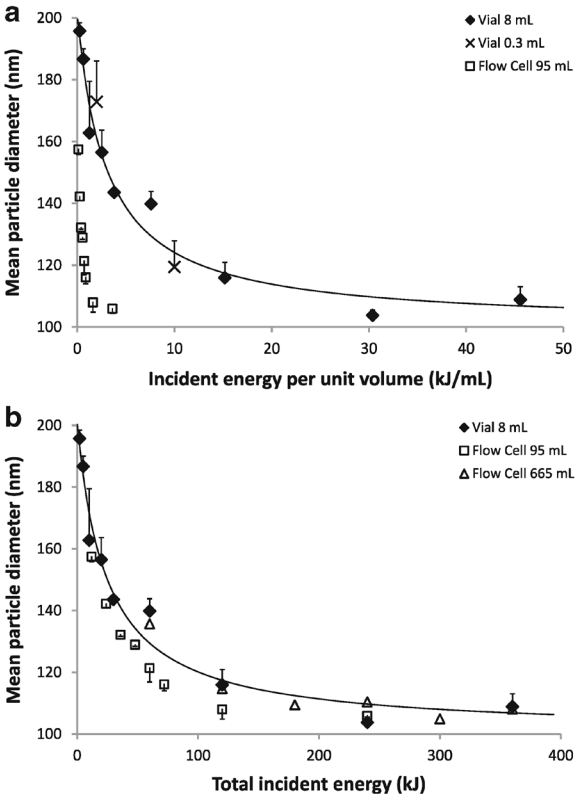

Fig. 6 Mean particle size of formulations with different batch volumes manufactured in different sonication vessels as a function of (**a**) energy density and (**b**) total incident energy. The average incident power was 100 W for the 8 mL and 95 mL batches, and 10 W for the 0.3 mL batch. *Symbols* represent mean ± standard deviation, $n = 3$ for 0.3 mL, 8 mL and 95 mL, $n = 1$ for 665 mL. The *solid line* is a fit for the 8 mL batch according to Eq. (6).

Influence of Batch Volume

To assess the potential for discovery formulation screening with very limited amounts of drug substance, the method was adjusted from 8 mL to fit 300 µL glass sonication vials. This reduced the amount of PLGA necessary for one sample run from 30 mg to 1.1 mg. Although the maximum sonication intensity is limited for the smaller vessels, comparable energy per unit volume can be achieved. When normalizing the total energy intake on batch volume, the particle sizes for the 300 µL batches fit nicely into the curve extrapolated from the 8 mL batches (Fig. 6a). This indicates a good scalability between the two batch sizes and that particle sizes can be predicted by applying simple mathematics.

By using a stainless steel sonication flow cell, batch volumes of up to several liters can be processed with a single unit. As this would equal tens of grams of PLGA nanoparticles, the proposed method would also be suitable for the supply of larger pre-clinical studies. As the process is continuous, contact-free, and all parts with product contact are either sterile consumables or autoclavable, it may possibly be used for the supply of clinical trials. We therefore investigated the upscale potential by producing PLGA nanoparticles in batch sizes of 95 mL and 665 mL (360 mg and 2520 mg PLGA, respectively).

Particle sizes are reduced much more efficiently at same energy per volume levels in the flow cell than in the glass vessels (Fig. 6a). This may be due to a better acoustic transmissibility of the plain polyimide sheet at the bottom of the flow cell, while the curved bottom of the glass vials result in greater sonic wave reflection. In fact, when processing a batch volume of 665 mL the same particle size can be achieved as for the 8 mL batch in a glass vessel by using just the same amount of sonic energy (Fig. 6b).

While the flow cell is more effective regarding particle size reduction at a given energy level, the nanoparticle yield is inferior to the glass vessel application (maximum of 23.9 ± 5.7% at 2.5 kJ/mL *vs.* 63.5 ± 2.5% at 3.8 kJ/mL) (Fig. 7). Possible explanations are surface adsorption in the pump tubing, and incomplete mixing in the bulk vessel. In the resulting "dead volumes" droplets are less likely to be conveyed to the flow cell and may retain above-micron sizes (meaning lower nanoparticle yield), while the rest of the fluid is

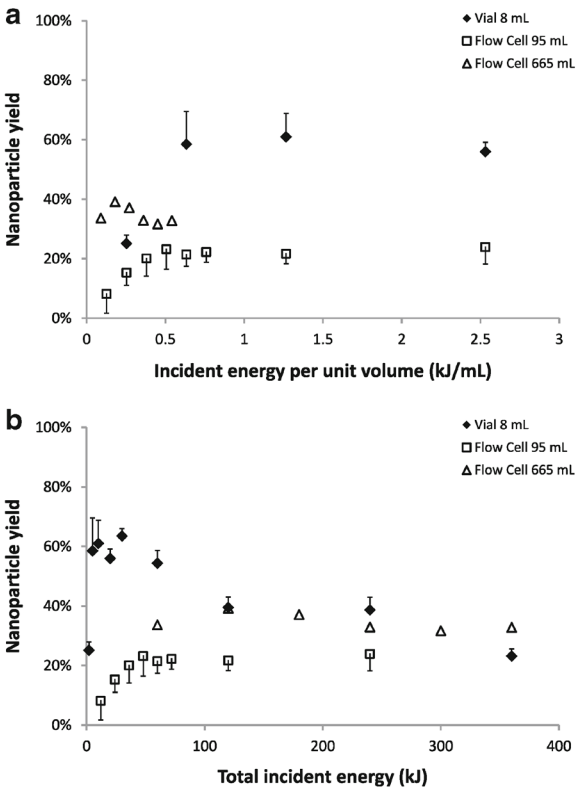

Fig. 7 Nanoparticle yield of formulations using either a glass vial or a flow cell as a function of (**a**) incident energy per batch volume and (**b**) total incident energy. The average incident power was 100 W for all batches. *Symbols* represent mean ± standard deviation, $n = 3$ for 0.3 mL, 8 mL and 95 mL, $n = 1$ for 665 mL.

disproportionately more sonicated (meaning smaller nanoparticles). Consequently, as fluid dynamics were easier to control in a larger scale, the 665 mL batch with a yield of 39.2% was superior to the 95 mL batch, but still not as efficient as the 8 mL process. This difference can be explained by the smaller particle size of flow cell *versus* glass vial preparations at lower energy levels, as smaller particles are lost to a greater extent during centrifuge washing (see section "Crossflow Filtration").

Similar to the nanoparticle yield, the ovalbumin loading achieved during the 95 mL process (0.5–1.0% for 0.1–2.5 kJ/mL) was inferior to the 8 mL process (2.0–3.6% for 0.3–7.6 kJ/mL). When further increasing the batch volume to 665 mL, a comparable loading is achievable by the flow cell process with lower incident energy per unit volume (1.6–2.5% for 0.1–0.6 kJ/mL). This indicates that a certain minimum volume is necessary for a representative flow cell treatment.

Ovalbumin Stability

Figure 8 shows the influence of focused ultrasound on the structure and stability of ovalbumin processed in a flow cell

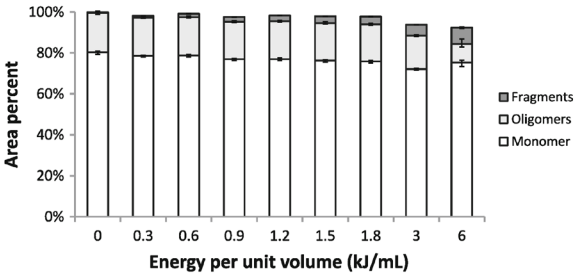

Fig. 8 Stability of ovalbumin during focused ultrasound processing of a solution in a flow cell (AIP 250 W). Protein structure was determined by size exclusion chromatography and quantified by integration of the acquired curve. All integrated peak areas for the respective species are reported as fraction of the total integrated area of an untreated sample (0 kJ/mL). *Bars* represent mean ± standard deviation of three independent treatments.

as obtained by size exclusion chromatography. Before the treatment, ovalbumin is present as 80.3% monomer, 19.2% soluble oligomers and very few to no fragments. At 0.6 kJ/mL (which is enough to reach an acceptable yield for flow cell processing) the monomer content is only slightly reduced from 80.3 to 78.8% of initial total integrated area, while the content of fragments rises from 0.4 to 1.7%. When drastically increasing the energy per unit volume to 6 kJ/mL, the monomer content is still relatively high at 75.3%, but a noticeable increase of fragments to 8.0%. The total integrated area of the size exclusion chromatogram decreases slightly above 0.9 kJ/mL. This is indicative of the formation of some insoluble degradation products. Overall the data shows that focused ultrasound itself has only a very slight effect on the structure of ovalbumin.

Optimization of the Washing Protocol

The nanoparticle mass loss can be partially contributed to the washing step. Higher energy treatments result in smaller particles, which in turn tend to form less dense pellets during

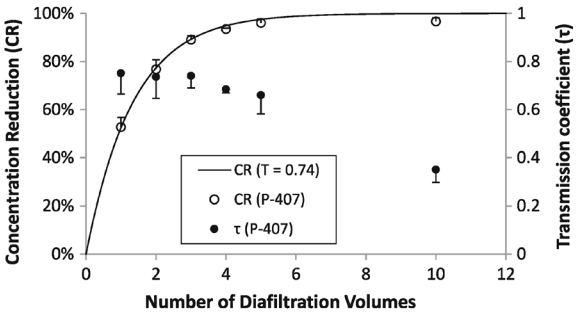

Fig. 9 Concentration reduction (*CR*) of poloxamer 407 in the retentate during diafiltration. The *open spheres* denote the *CR* of P-407 (determined from the permeate). *Solid spheres* denote the apparent membrane transmission of P-407 for the respective time point as calculated from *CR* using Eq. (4). The *line* shows the predicted *CR* for a compound with a concentration independent transmission of 0.74.

Table IV Crossflow Filtration of a Suspension of PLGA Nanoparticles Containing Ovalbumin. Mean and Standard Deviation are Calculated from Three Independent Filtration Experiments Using Different Filtration Modules

	Z-average diameter (nm)	Polydispersity index	Residual P-407	τ (P-407)	Residual ovalbumin
Before washing	122.1	0.098	100%	NA	100%
Crossflow filtration (DV = 5)	122.9 ± 1.6	0.106 ± 0.022	1.7 ± 0.2%	0.82	n.d.
Crossflow filtration (DV = 10)	122.4 ± 1.1	0.104 ± 0.015	<0.03% *	>0.80	<0.3% *

* Below LOQ

centrifugation and exhibit higher loss on washing. A simple solution would be to increase the relative centrifugal force (RCF). While this would reduce losses for smaller particles, preliminary trials suggested that bigger particles from lower energy treatments are more likely to deform or agglomerate at higher RCF, and may even form pellets that cannot be redispersed anymore. To optimize the nanoparticle yield while retaining the key formulation characteristics it would be necessary to establish individual washing protocols depending on the mean particle size of each preparation. Additionally, the feasible processing volume and scalability of centrifugation is limited.

To address these issues, crossflow filtration was established and compared to conventional centrifuge washing regarding purification efficiency, particle size distribution, ovalbumin loading capacity and overall production yield.

Crossflow Filtration

Membrane filtration is widely used for separation and purification purposes in biotechnology (30). In crossflow filtration, a solution or suspension is continuously pumped parallel to a membrane. The created transmembrane pressure forces the solvent and solutes smaller than the membrane's pores across the membrane, while the direction of the stream prevents membrane fouling. The used membranes are usually characterized by their molecular weight cut off (MWCO), indicating a 90% retention for globular macromolecules of that size.

In this study, the sub-micron particles are to be separated from excess ovalbumin (44.3 kDa) and P-407 (12.6 kDa on average). For optimal flux and upscale potential we chose a diafiltration module with a hydrophilic membrane formed to bundled hollow fibers. The fibers are made of modified polyethersulfone (mPES) with a MWCO of 500 kDa, equaling 20 nm according to the manufacturer. The nanoparticle suspension is circulated from a bulk vessel through the interior of the membrane fibers and back. Small molecules like solvents and salts readily cross the membrane to the exterior encasing where they are collected and flushed out. After 7 diafiltration volumes, the concentration of such molecules in the retentate is usually reduced below 0.1% of the initial value. More complex molecules like proteins and polymers may be filtrated if they are smaller than the membrane's pores, but exhibit a reduced transmission.

As this directly influences the efficiency of the purification, the transmission coefficient of P-407 in solution was determined from Eqs. (4) and (5) by repeatedly measuring the concentration of P-407 using GPC in the combined permeate during a diafiltration experiment (Fig. 9). During the first three diafiltration volumes, the transmission coefficient remains relatively unchanged at $\tau = 0.74 \pm 0.05$. With increasing diafiltration volumes (DV) the transmission seems to decrease. At the end of the experiment (after 10 DV), the amount of P-407 found in the permeate was 96.7%, and the transmission was calculated as $\tau = 0.35 \pm 0.05$. However, direct sampling from the retentate revealed that less than 0.1% P-407 (below limit of quantification) actually remained in the retentate, resulting in a transmission of at least 0.70 after 10 DV. Accordingly, the initially observed decline of membrane transmission is not an effect of concentration dependency but rather due to adsorption of P-407 to the tubing and the membrane.

To validate the findings in the presence of nanoparticles, a second diafiltration study was conducted with a freshly prepared nanoparticle suspension (6 mg/mL PLGA) containing ovalbumin. Particle sizes were measured by DLS before and after purification. The concentration of P-407 and ovalbumin was determined directly from the retentate by GPC and BCA assay, respectively. It was found that the particles remained stable during purification with no change in mean particle

Table V Influence of Washing Protocols on Formulation Parameters. Although the Variance Could not be Calculated Due to Sample Pooling ($n = 3$), the Polydispersity Index Is Sufficiently Low to Discern Safely Between the Two Washing Protocols

	Z-average diameter (nm)	Polydispersity index	Ovalbumin loading capacity (%)	Nanoparticle yield (%)
Before washing	172.7	0.084	n.a.	n.a.
Centrifuge washing	190.0	0.134	2.4	60.8
Crossflow filtration	168.9	0.124	2.2	73.8

diameter and size distribution (Table IV). This indicates that poloxamer 407 is effective as stabilizer during particle formation and purification, even after almost quantitative removal from the suspending liquid. This is in accordance with the hypothesis that the tri-block copolymer P-407 irreversibly adsorbs onto the surface of hydrophobic particles with its hydrophobic poly(oxypropylene) (POP) middle block, while the two poly(oxythylene) (POE) chains protrude into the surrounding medium (31,32). In contrast to our results, Quintanar-Guerrero *et al.* found that poly(lactic acid) nanoparticles stabilized with poloxamer 188 (P-188) agglomerate during diafiltration (33). Interestingly, the nanoparticles remained stable when they kept the concentration of P-188 constant during diafiltration, indicating a loose interaction between stabilizer and particle surface. The reason for the superior performance of P-407 is most likely the increased weight of the hydrophobic POP middle block (56 monomers as opposed to 27 in P-188). Indeed, field flow fractionation experiments showed that only the length of the POP chain influences the concentration of different poloxamers adsorbed to the surface of polystyrene nanoparticles of a given size, while the length of POE mainly influences the thickness of the adsorbed layer and the mobility of the protruding POE chains (34).

While a concentrated suspension of particles larger than the filter's pores would immediately block during conventional dead end filtration, no detrimental effect of PLGA nanoparticles on the removal of P-407 could be observed during crossflow filtration. The calculated transmission coefficient of P-407 ($\tau > 0.80$) was coherent with prior observations. It was concluded that a washing cycle of 9–10 DV is sufficient to reduce the amount of free stabilizer and protein to negligible levels (below 1 mg of free stabilizer per gram of nanoparticles).

In contrast to crossflow filtration, the purification of PLGA nanoparticles by centrifugation leads to a significant increase in mean particle size (Table V). While one would expect a somewhat lower ovalbumin load after 2 h of crossflow filtration and corresponding drug release, no difference could be observed to centrifuge purification. As expected, the overall nanoparticle yield could be further improved by the use of crossflow centrifugation. The described method is suitable for the described manufacturing process starting with batch volumes from 10 mL regardless of nanoparticle size. Only minor adjustments would be necessary to process different volumes. As hollow fiber modules are available with the same dimensions but with an increased number of fibers, a scale up to several liters seems to be reasonably simple.

CONCLUSION

A scalable and contact-free method to produce protein-loaded nanoparticles was successfully established based on a commercially available focused ultrasound transducer. Nanoparticles could be produced in batch sizes from 1 mg to 2500 mg using the same equipment. Similar nanoparticle characteristics could be achieved over the range of investigated batch sizes. The mean particle diameter could be controlled between 100 and 200 nm with a maximum yield of 74% and protein loading up to 3.6%. Lower yields for smaller particles and larger batch sizes could be mitigated by the use of diafiltration instead of centrifugation. The influence of device parameters and batch size on nanoparticle size and yield and could be described by simple mathematic relationships. This underlines the robustness and predictability of the process and therefor provides an advantage in method development and scale up. In contrast to already established methods, the proposed nanoparticle manufacturing process is a valuable tool for both screening purposes and manufacturing, and as such could advance the translation of nanomedicine to the clinic.

ACKNOWLEDGEMENTS AND DISCLOSURES

Parts of this work were funded by BMBF (German Federal Ministry of Education and Research) project PeTrA (ref. no. 13N11455). The authors are grateful for the technical assistance of Letícia Pires Rodrigues and Sebastian Franck.

REFERENCES

1. Etheridge ML, Campbell SA, Erdman AG, Haynes CL, Wolf SM, McCullough J. The big picture on nanomedicine: the state of investigational and approved nanomedicine products. Nanomed Nanotechnol Biol Med. 2013;9(1):1–14.
2. Davis ME, Chen Z, Shin DM. Nanoparticle therapeutics: an emerging treatment modality for cancer. Nat Rev Drug Discov. 2008;7(9):771–82.
3. Danhier F, Ansorena E, Silva JM, Coco R, Le Breton A, Préat V. PLGA-based nanoparticles: an overview of biomedical applications. J Control Release. 2012;161(2):505–22.
4. Jain AK, Das M, Swarnakar NK, Jain S. Engineered PLGA nanoparticles: an emerging delivery tool in cancer therapeutics. Crit Rev Ther Drug Carrier Syst. 2011;28(1):1–45.
5. Fessi H, Puisieux F, Devissaguet JP, Ammoury N, Benita S. Nanocapsule formation by interfacial polymer deposition following solvent displacement. Int J Pharm. 1989;55(1):R1–4.
6. Rao JP, Geckeler KE. Polymer nanoparticles: preparation techniques and size-control parameters. Prog Polym Sci. 2011;36(7):887–913.
7. Colombo AP, Briançon S, Lieto J, Fessi H. Project, design, and use of a pilot plant for nanocapsule production. Drug Dev Ind Pharm. 2001;27(10):1063–72.
8. Galindo-Rodríguez SA, Puel F, Briançon S, Allémann E, Doelker E, Fessi H. Comparative scale-up of three methods for producing ibuprofen-loaded nanoparticles. Eur J Pharm Sci. 2005;25(4–5):357–67.
9. Tewa-Tagne P, Briançon S, Fessi H. Preparation of redispersible dry nanocapsules by means of spray-drying: development and characterisation. Eur J Pharm Sci. 2007;30(2):124–35.
10. Rietscher R, Thum C, Lehr CM, Schneider M. Semi-automated nanoprecipitation-system-an option for operator independent,

scalable and size adjustable nanoparticle synthesis. Pharm Res [Internet]. 2014. doi:10.1007/s11095-014-1612-z.
11. Vauthier C, Bouchemal K. Methods for the preparation and manufacture of polymeric nanoparticles. Pharm Res. 2009;26(5):1025–58.
12. Lavik E, von Recum H. The role of nanomaterials in translational medicine. ACS Nano. 2011;5(5):3419–24.
13. Blanco MD, Alonso MJ. Development and characterization of protein-loaded poly(lactide-co-glycolide) nanospheres. Eur J Pharm Biopharm. 1997;43(3):287–94.
14. Schalper K. Herstellung hochdisperser Systeme mit kontinuierlichen Mikromischern [PhD thesis]. Berlin: Freie Universität Berlin; 2002.
15. Kovács Z, Fikar M, Czermak P. Mathematical modeling of diafiltration. Hung J Ind Chem. 2009;37(2):159–64.
16. Feczkó T, Tóth J, Dosa G, Gyenis J. Influence of process conditions on the mean size of PLGA nanoparticles. Chem Eng Process Process Intensif. 2011;50(8):846–53.
17. Bilati U, Allémann E, Doelker E. Sonication parameters for the preparation of biodegradable nanocapsulesof controlled size by the double emulsion method. Pharm Dev Technol. 2003;8(1):1–9.
18. Tadros T, Izquierdo P, Esquena J, Solans C. Formation and stability of nano-emulsions. Adv Colloid Interface Sci. 2004;108–109:303–18.
19. Caruso MM, Davis DA, Shen Q, Odom SA, Sottos NR, White SR, *et al.* Mechanically-induced chemical changes in polymeric materials. Chem Rev. 2009;109(11):5755–98.
20. Conti B, Dorati R, Colonna C, Genta I. Effects of ionizing radiation sterilization on microparticulate drug delivery systems based on poly-α-hydroxyacids: an overview. J Drug Deliv Sci Technol. 2009;19(2):99–112.
21. Kerwin BA. Polysorbates 20 and 80 used in the formulation of protein biotherapeutics: Structure and degradation pathways. J Pharm Sci. 2008;97(8):2924–35.
22. Donbrow M, Hamburger R, Azaz E, Pillersdorf A. Development of acidity in non-ionic surfactants: formic and acetic acid. Analyst. 1978;103(1225):400–2.
23. Reich G. Ultrasound-induced degradation of PLA and PLGA during microsphere processing: influence of formulation variables. Eur J Pharm Biopharm. 1998;45(2):165–71.
24. Rokita B, Ulański P. Studies on the spatial distribution of polymeric reagents in sonochemical reactions: application of competitive kinetics. Polimery. 2005;50(1):29–36.
25. Demento SL, Eisenbarth SC, Foellmer HG, Platt C, Caplan MJ, Mark Saltzman W, *et al.* Inflammasome-activating nanoparticles as modular systems for optimizing vaccine efficacy. Vaccine. 2009;27(23):3013–21.
26. Fievez V, Plapied L, des Rieux A, Pourcelle V, Freichels H, Wascotte V, *et al.* Targeting nanoparticles to M cells with non-peptidic ligands for oral vaccination. Eur J Pharm Biopharm. 2009;73(1):16–24.
27. Mittal A, Raber AS, Schaefer UF, Weissmann S, Ebensen T, Schulze K, *et al.* Non-invasive delivery of nanoparticles to hair follicles: a perspective for transcutaneous immunization. Vaccine. 2013;31(34):3442–51.
28. Slütter B, Bal S, Keijzer C, Mallants R, Hagenaars N, Que I, et al. Nasal vaccination with N-trimethyl chitosan and PLGA based nanoparticles: nanoparticle characteristics determine quality and strength of the antibody response in mice against the encapsulated antigen. Vaccine. 2010;28(38):6282–91.
29. Feczkó T, Tóth J, Dosa G, Gyenis J. Optimization of protein encapsulation in PLGA nanoparticles. Chem Eng Process Process Intensif. 2011;50(8):757–65.
30. van Reis R, Zydney A. Bioprocess membrane technology. J Membr Sci. 2007;297(1–2):16–50.
31. Scholes PD, Coombes AGA, Illum L, Davis SS, Watts JF, Ustariz C, *et al.* Detection and determination of surface levels of poloxamer and PVA surfactant on biodegradable nanospheres using SSIMS and XPS. J Control Release. 1999;59(3):261–78.
32. Blunk T, Hochstrasser DF, Sanchez JC, Müller BW, Müller RH. Colloidal carriers for intravenous drug targeting: Plasma protein adsorption patterns on surface-modified latex particles evaluated by two-dimensional polyacrylamide gel electrophoresis. Electrophoresis. 1993;14(1):1382–7.
33. Quintanar-Guerrero D, Ganem-Quintanar A, Allemann E, Fessi H, Doelker E. Influence of the stabilizer coating layer on the purification and freeze-drying of poly(D, L-lactic acid) nanoparticles prepared by an emulsion-diffusion technique. J Microencapsul. 1998;15(1):107–19.
34. Li J-T, Caldwell KD, Rapoport N. Surface properties of pluronic-coated polymeric colloids. Langmuir. 1994;10(12):4475–82.

Electronic Supplementary Material to

„Focused Ultrasound as a Scalable and Contact-Free Method to Manufacture Protein-Loaded PLGA Nanoparticles"

By S. Schiller[1,2], A. Hanefeld[2], M. Schneider[1], and C.-M. Lehr[1,3]

[1]Department of Pharmacy, Biopharmaceutics & Pharmaceutical Technology, Saarland University, 66123 Saarbrücken, Germany

[2]Research Pharmaceutics & Drug Product Development, Merck Serono, 64293 Darmstadt, Germany

[3] Department of Drug Delivery (DDEL), Helmholtz Institute for Pharmaceutical Research Saarland (HIPS), Helmholtz Center for Infection Research (HZI), Saarland University, 66123 Saarbrücken, Germany

Stabilizer screening for the preparation of PLGA nanoparticles

The following stabilizers were evaluated: polyvinyl alcohol (PVA; Mowiol® 4-88, M_w ~31,000) and dimethyldidodecylammonium bromide (DMAB) were obtained from Sigma Aldrich (St. Louis, USA). Sodium deoxycholate (SDC) and polysorbate 80 (Tween® 80) were obtained from Merck KGaA (Darmstadt, Germany). Poloxamer 188 (P-188) and poloxamer 407 (P-407) were kindly provided by BASF (Ludwigshafen, Germany).

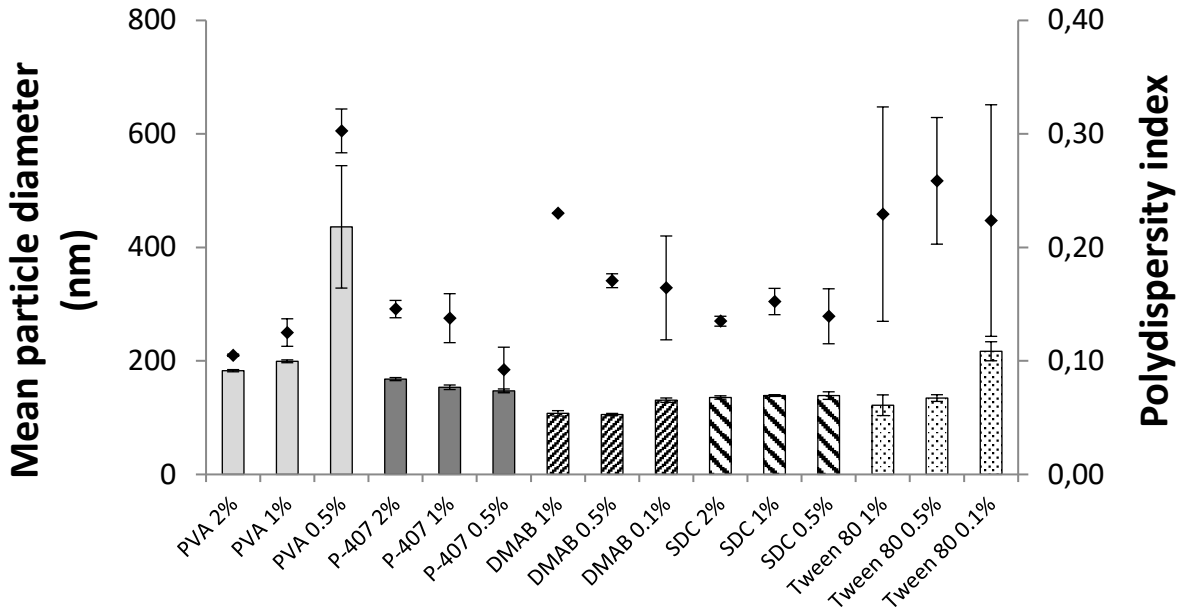

Fig. S1 Mean particle size (bars) and polydispersity index (♦) of formulations manufactured with different stabilizers. The size of three independent batches was determined by DLS, and the standard deviation depicted as error bars. P-188 was omitted due to emulsion instability and subsequent film formation.

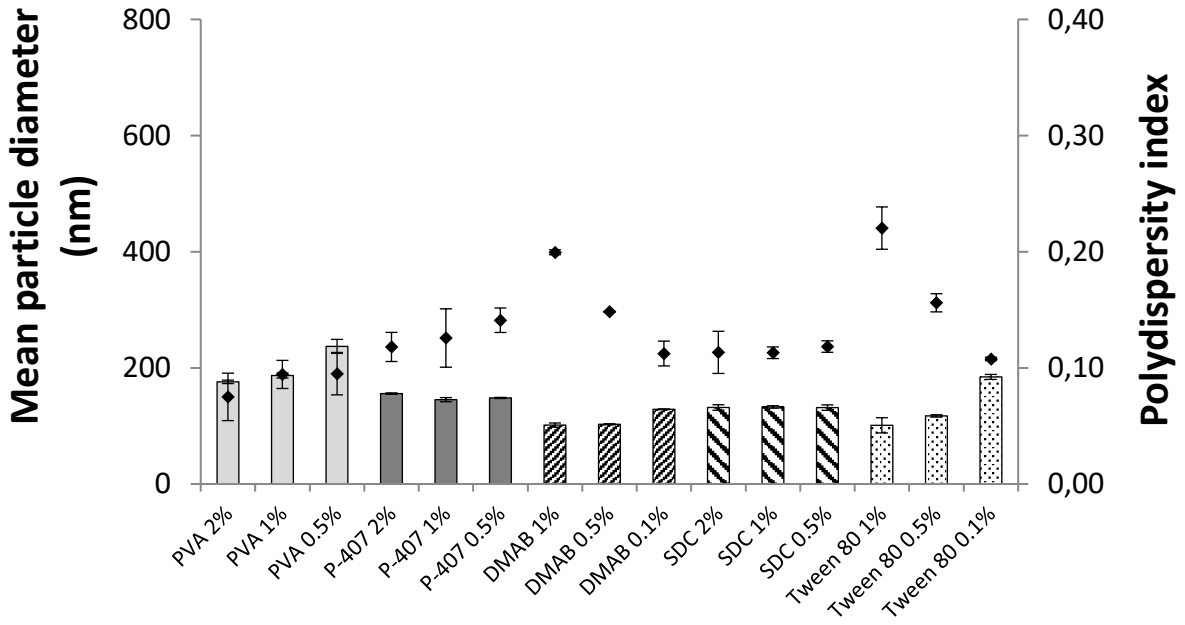

Fig. S2 Mean particle size (bars) and polydispersity index (♦) of formulations manufactured with different stabilizers after removal of aggregates by centrifugation (15 min at 1,000 x g). The size of three independent batches was determined by DLS, and the standard deviation depicted as error bars. P-188 was omitted due to emulsion instability and subsequent film formation.

Nanoparticles were prepared by double emulsion solvent evaporation as described in the methods section. 0.4 mL of an aqueous solution of 3.75 mg/mL ovalbumin, 2.5 mL of 12 mg/mL PLGA in ethyl acetate, and 5 mL of the respective aqueous stabilizer solution were subsequently homogenized by focused ultrasound in a glass tube. The first homogenization was done with 50% duty factor, 1000 cycles per burst, and 200 W peak incident power for 60 s. The second homogenization was done using the same settings for 5 min. After solvent evaporation, the mean particle size was determined by dynamic light scattering before (Figure S1) and after (Figure S2) removal of aggregates by centrifugation (15 min at 1,000 x g) as described in the methods section. The ζ-potential was additionally determined by laser Doppler anemometry (Zetasizer Nano ZS, Malvern Instruments, Worcestershire, UK) when using charged stabilizers: 1 mL of freshly prepared nanoparticle suspension was pelletized by centrifugation (5 min at 21,000 x g), the supernatant removed, the nanoparticles redispersed in 1 mL of 1 mM phosphate buffer pH 7.4, and transferred to a folded capillary cell

(DTS1061). Each sample was measured three times with automatic measurement duration and voltage selection, data recorded using Zetasizer Software v7.01 and analyzed using "General Purpose mode".

The different stabilizers were evaluated for their ability to prevent droplet coalescence and nanoparticle agglomeration during solvent evaporation and after particle formation, respectively. The absence of large aggregates is indicated by minimal differences of mean particle size and polydispersity index between the three independent runs for each stabilizer concentration before removal of aggregates (Figure S1), as well as minimal differences before and after centrifugation (Figures S1 and S2). A good stabilization for both the emulsion and the particle suspension could be observed for PVA at 1 and 2%, P-407 at 0.5, 1 and 2%, DMAB at 0.1%, and SDC at 0.5, 1 and 2%. Visible agglomeration occurred when using PVA at 0.5%, DMAB at 0.5 and 1%, and Tween® 80 at 0.1, 0.5 and 1%. Interestingly, while a minimum concentration of 1% for the nonionic, steric stabilizer PVA was required, formulations using the cationic, non-steric stabilizer DMAB only remained stable at DMAB concentrations of not more than 0.1%. The heavy precipitation observed for DMAB concentrations of 0.5 and 1% may be due to higher charge interactions and crosslinking between positively charged particles, free stabilizer and the negatively charged free ovalbumin. At 0.1% DMAB seems to be feasible for the preparation of small, positively charged PLGA nanoparticles (ζ-potential of 35.0 ± 1.0 mV). Nanoparticles prepared using 0.5% P-407 were slightly negatively charged (-30.4 ± 2.1 mV), while the anionic stabilizer SDC yielded a stronger negative surface charge (-51.7 ± 4.1 mV).

For formulations containing P-188 at 0.5, 1 and 2%, particle size measurement was omitted because of the formation of a continuous film at the surface of the emulsion. The poor emulsification efficiency of P-188 in contrast to P-407 may be explained by the very high hydrophilicity of P-188 (HLB > 24), while P-407 (HLB between 18-23) is more lipophilic due to its more than doubled poly(oxyproylene) middle block.

4.2. Towards a continuous manufacturing process of protein-loaded polymeric nanoparticle powders

Stefan Schiller,[1,2,5] Andrea Hanefeld,[3] Marc Schneider,[1] and Claus-Michael Lehr[1,4]

[1] Department of Pharmacy, Biopharmaceutics & Pharmaceutical Technology, Saarland University, Saarbrücken, Germany.

[2] Department of Pharmaceutical Technologies, Merck Healthcare KGaA, HPC D039/002, Frankfurter Str. 250, 64293, Darmstadt, Germany.

[3] Global Healthcare Operations Innovation Network, Merck Healthcare KGaA, Darmstadt, Germany.

[4] Helmholtz Institute for Pharmaceutical Research Saarland (HIPS), Helmholtz Center for Infection Research (HZI), Saarland University, Saarbrücken, Germany.

[5] To whom correspondence should be addressed. (e-mail: stefan.schiller@merckgroup.com)

AAPS PharmSciTech. 2020;21(7):269.

DOI: 10.1208/s12249-020-01814-w

Specification of the contributions:

Stefan Schiller conceived, planned, and carried out the experiments, and analyzed the data. Stefan Schiller, Andrea Hanefeld, Marc Schneider, and Claus-Michael Lehr interpreted the results. Stefan Schiller took the lead in writing the manuscript. Andrea Hanefeld, Marc Schneider, and Claus-Michael Lehr provided critical feedback to the research and contributed to the manuscript.

AAPS PharmSciTech (2020) 21: 269
DOI: 10.1208/s12249-020-01814-w

Brief/Technical Note

Towards a Continuous Manufacturing Process of Protein-Loaded Polymeric Nanoparticle Powders

Stefan Schiller,[1,2,5] Andrea Hanefeld,[3] Marc Schneider,[1] and Claus-Michael Lehr[1,4]

Received 20 April 2020; accepted 9 September 2020; published online 6 October 2020

Abstract. To develop a scalable and efficient process suitable for the continuous manufacturing of poly(lactic-co-glycolic acid) (PLGA) nanoparticles containing ovalbumin as the model protein. PLGA nanoparticles were prepared using a double emulsification spray-drying method. Emulsions were prepared using a focused ultrasound transducer equipped with a flow cell. Either poly(vinyl alcohol) (PVA) or poloxamer 407 (P-407) was used as a stabilizer. Aliquots of the emulsions were blended with different matrix excipients and spray dried, and the yield and size of the resuspended nanoparticles was determined and compared against solvent displacement. Nanoparticle sizes of spray-dried PLGA/PVA emulsions were independent of the matrix excipient and comparable with sizes from the solvent displacement method. The yield of the resuspended nanoparticles was highest for emulsions containing trehalose and leucine (79%). Spray drying of PLGA/P-407 emulsions led to agglomerated nanoparticles independent of the matrix excipient. PLGA/P-407 nanoparticles pre-formed by solvent displacement could be spray dried with limited agglomeration when PVA was added as an additional stabilizer. A comparably high and economically interesting nanoparticle yield could be achieved with a process suitable for continuous manufacturing. Further studies are needed to understand the robustness of a continuous process at commercial scale.

KEY WORDS: continuous manufacturing; PLGA nanoparticles; focused ultrasound; spray drying; protein delivery.

INTRODUCTION

A major challenge in the translation and commercialization of nanomaterials in medicine is the development of adequate pharmaceutical production processes that work equally well at large scale as at lab scale as (1,2). While several marketed pharmaceutical products employ nanotechnology, very little is publicly known about production processes and the translation from research to commercial scale (3). Continuous processes are often investigated (2,4,5), as they are considered easy to scale and more efficient, allow for simple process monitoring, and typically lead to less batch-to-batch variation. Although continuous nanoparticle precipitation methods—sometimes called flash nanoprecipitation—exist (5–7), in general, they are not suitable to produce nanoparticles of hydrophobic polymers and hydrophilic cargos. Such systems are often produced using the double emulsion method (8): the hydrophilic drug is dissolved in water and emulsified into a non- or partially miscible solvent containing the polymer. This two-phase system is further emulsified into an outer aqueous phase containing a stabilizer. The solvent is subsequently removed and the polymer precipitates to form nanoparticles around the hydrophilic drug. The critical steps of emulsification and solvent removal are typically done with batch processing (9). We previously reported the use of a focused ultrasound transducer coupled with a flow-through cell for a contact-free and scalable emulsification capable of continuous processing (9). The logical next step would be to also adapt the solvent evaporation to a continuous process, for example, by spray drying.

Spray drying is a continuous, fast, and efficient process for solvent removal. It may be used to transform preformed nanoparticle suspensions to dry powders commonly known as "Trojan particles," nanoembedded microparticles, or nanoparticles-in-microparticles (10,11). The direct precipitation and drying of nanoparticles has been described as the emulsion spray drying approach, using single water/oil (W/O)

[1] Department of Pharmacy, Biopharmaceutics & Pharmaceutical Technology, Saarland University, Saarbrücken, Germany.
[2] Department of Pharmaceutical Technologies, Merck Healthcare KGaA, HPC D039/002, Frankfurter Str. 250, 64293, Darmstadt, Germany.
[3] Global Healthcare Operations Innovation Network, Merck Healthcare KGaA, Darmstadt, Germany.
[4] Helmholtz Institute for Pharmaceutical Research Saarland (HIPS), Helmholtz Center for Infection Research (HZI), Saarland University, Saarbrücken, Germany.
[5] To whom correspondence should be addressed. (e–mail: stefan.schiller@merckgroup.com)

emulsions (12–15). The size of the precipitated nanoparticles is controlled by the atomization of the feed rather than by the droplet size of the dispersed phase of the emulsion.

However, the generation of submicron droplets and the collection of submicron particles with sufficient yield are very challenging with conventional equipment, and the throughput of available specialized equipment is severely limited compared with conventional nozzles, cyclones, and filter bags (16).

Spray dying of water/oil/water (W/O/W) double emulsions allows for the *in situ* generation of polymeric nanoparticles with hydrophilic cargo while simultaneously embedding them in a stabilizing powder matrix. The concept has been described for batch mode emulsification and conventional spray drying equipment (17,18), but little is known about the factors influencing nanoparticle size and yield. The aim of this study was to investigate whether poly(lactic-co-glycolic acid) (PLGA) nanoparticles can be produced with acceptable yield and particle size distribution by the double emulsion method using scalable methods fit for continuous manufacturing. Different emulsion stabilizers and matrix components were investigated for their influence on nanoparticle size and yield.

MATERIALS AND METHODS

Materials

Poly(lactic-co-glycolic acid) (PLGA, Resomer® RG 503 H) was purchased from Evonik Industries (Essen, Germany). Poloxamer 407 (P-407), Kollidon® 30 (K30), and Kollidon® VA64 (VA64) were kindly provided by BASF (Ludwigshafen, Germany). Poly(vinyl alcohol) (PVA; Mowiol® 4–88) was obtained from Kuraray Europe GmbH (Hattersheim am Main, Germany). Ovalbumin grade V, as well as all other chemicals, was obtained from Merck KGaA (Darmstadt, Germany). Water was purified with a Milli-Q® system (Merck KGaA; Darmstadt, Germany).

Emulsion Preparation

All solutions were freshly prepared and filtered through 0.2-μm membrane filters (polytetrafluoroethylene for organic solutions or polyethersulfone for aqueous solutions). Double emulsions were prepared using focused ultrasound according to a previously described method (9). In brief, PLGA was dissolved to 12 mg/mL in 70 mL ethyl acetate and added to 14 mL of an aqueous solution of 6 mg/mL ovalbumin. The mixture was circulated through the flow cell of a commercial focused ultrasound transducer (Covaris S2x, Covaris Inc.; Woburn, MA, USA) using a peristaltic pump at 50 mL/min. The mixture was homogenized for 5 min at intensity 10, duty factor 50%, 300 cycles per burst. An aqueous solution, 140 mL of 20 mg/mL PVA or P-407, was added, and the mixture was again emulsified by focused ultrasound for 45 min (same settings). An aliquot of this emulsion was precipitated by solvent displacement (10-fold dilution with purified water). The particle size distribution of the obtained nanoparticle suspension was measured by dynamic light scattering as described below. The results served as a control for in-use emulsion stability and as a target for nanoparticles precipitated by spray drying.

Spray Drying

Aliquots of the double emulsion, consisting of dry mass ratios of 1 part PLGA and 3.3 parts PVA or P-407, were either directly spray dried or spiked with 9 parts matrix excipient. The aliquots were fed at 2 mL/min into a ProCepT 4M8-TriX lab scale spray dryer equipped with a straight and a conical drying column and a small cyclone (ProCepT nv; Zelzate, Belgium); atomized with a 1.0 mm bi-fluid nozzle and 10 L/min nitrogen; and dried at 80°C inlet drying air temperature, 0.4 m^3/min drying air flow, 40–42°C cyclone outlet temperature, and a pressure drop of 60–65 mbar over the cyclone. Ambient conditions were between 20 and 23°C and 40–60% rh.

Particle Size and Yield Determination

Spray-dried powders were reconstituted with purified water at approximately 0.5 mg/mL PLGA by shaking for 30–60 min and passed through a 1.0 μm glass microfiber syringe filter to remove agglomerated PLGA particles and incompletely dissolved matrix excipients. The filtered particle suspensions were analyzed by dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments; Worcestershire, UK; $\lambda = 633$ nm, 25.0 ± 0.1°C, backscatter mode, cumulants fit).

Filtered and unfiltered suspensions were centrifuged for 5 min at 21,000x*g* to collect the PLGA particles. The pellet was washed twice with water, dried in a rotary vacuum concentrator (RVC 2–33 IR, Martin Christ**;** Osterode, Germany), and subsequently weighed. The nanoparticle yield was calculated by dividing the mass of the pellet from the filtered suspension by the mass of the pellet from the corresponding unfiltered suspension.

RESULTS AND DISCUSSION

Double emulsions containing PLGA and either PVA or P-407 as the nanoparticle stabilizer were spray dried and analyzed for changes in particle size distribution and the resulting nanoparticle yield. The emulsions were either sprayed "as is" or after the addition of matrix excipients: trehalose and mannitol as desiccoprotectants, leucine to increase the dispersibility of the spray-dried powders (19)); VA64 and K30 due to their ability to sterically stabilize drug nanoparticles (20); or the further addition of the nanoparticle stabilizers PVA and P-407 also as matrix excipients. Nanoparticle precipitation from PLGA/PVA or PLGA/P-407 double emulsions by solvent displacement without any additives or spray drying served as the control.

Spray drying of double emulsions containing PLGA/PVA yielded nanoparticles of similar size as precipitation by direct solvent displacement (Fig. 1). The addition of different matrix excipients to the emulsions before drying did not influence the resulting nanoparticle size. Only P-407 seemed to decrease the resulting nanoparticle size. P-407 is a surface-active block copolymer suitable for the stabilization of PLGA double emulsions and suspensions alone, resulting in particles of a smaller hydrodynamic diameter when using the same manufacturing method and parameters (9). A possible explanation is that P-407 may replace PVA, to a certain

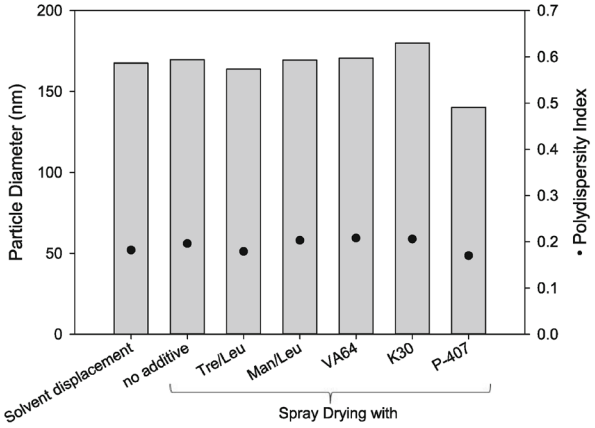

Fig. 1. Resulting nanoparticle sizes after spray drying of PLGA/PVA double emulsions with and without the addition of different matrix excipients. Spray-dried powders were reconstituted in purified water, then aggregates were removed by filtration, and particle size distribution was measured by dynamic light scattering. Nanoparticles precipitated by solvent displacement without spray drying served as the control. Measurements were done in triplicate, RSD < 0.5%

extent, at the liquid/liquid interface before drying or at the particle/liquid interface after drying and reconstitution.

In contrast to the mean particle size, the nanoparticle mass recovery from spray-dried powders is influenced by the choice of matrix (Fig. 2). Spray drying of the emulsion with no further addition of excipients resulted in a nanoparticle yield of 40%. Adding K30 to the emulsion is not beneficial for the nanoparticle yield. Adding mannitol/leucine or VA64 moderately increases the yield to 50–60%. The best result of 79% was obtained when trehalose/leucine were added to the emulsion. A nanoparticle yield of 79% is considerably higher than previously reported for the continuous production of PLGA nanoparticles using focused ultrasound followed by solvent displacement (9). The reported yield may already be economically acceptable when reproduced on a commercial scale.

After the proposed process, excess stabilizer and free drug would remain in the spray-dried powder. The spray-dried powder could be directly used for further downstream processing (*e.g.*, tableting) into a final dosage form for applications where free drug and stabilizer are not an issue. Alternatively, the dry powder intermediate may be purified before further processing. Centrifuge purification works well for PVA and is well established at lab scale, but the scalability is limited and particles may deform or agglomerate under stress (9). Continuous purification methods, such as cross-flow

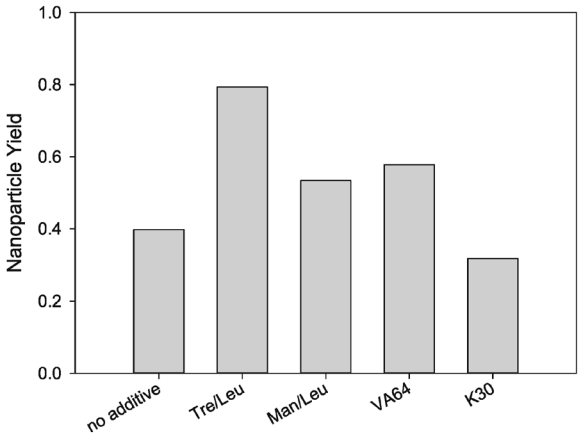

Fig. 2. Resulting nanoparticle yield after spray drying of PLGA/PVA double emulsions with and without the addition of different matrix excipients. Nanoparticles were isolated from the matrix after reconstitution of the complete batch in purified water, then aggregates were removed by filtration, and the mass was determined after drying

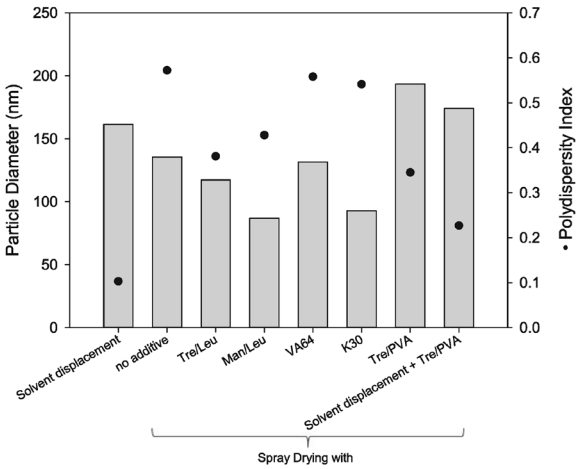

Fig. 3. Resulting nanoparticle sizes after spray drying of PLGA/P-407 double emulsions with and without the addition of different matrix excipients. Spray-dried powders were reconstituted in purified water, then aggregates were removed by filtration, and particle size distribution was measured by dynamic light scattering. Nanoparticles precipitated by solvent displacement without spray drying served as the control. Measurements were done in triplicate, RSD < 0.5%

filtration, are preferred at larger scale, but are challenging for formulations containing PVA: Residual amounts of PVA were found to be several times higher after filtration than after centrifugation (21,22). P-407 has been previously shown to work well with the double emulsion solvent displacement method and is easily removed by crossflow filtration (9). Therefore, we evaluated the potential of using P-407 with the double emulsion spray drying method.

Particles of PLGA/P-407 precipitated from the emulsion by solvent displacement instead of spray drying had a very narrow size distribution (indicated by a polydispersity index [PDI] of 0.10) and served as the control. Spray drying of emulsions stabilized with P-407 yielded agglomerated particles (Fig. 3): Spray drying without further additives resulted in a PDI of 0.57. The addition of VA64 and K30 did not improve the particle size distribution (PDI: 0.54–0.56). Using trehalose and leucine or mannitol and leucine as the matrix excipients resulted in a lower but still unsatisfactory PDI of 0.38–0.43.

As PLGA/PVA emulsions could be successfully spray dried when spiked with P-407, it was tested whether the addition of PVA and trehalose to a PLGA/P-407 emulsion could positively influence nanoparticle stability. The resulting PDI of 0.35 was only slightly better than when using trehalose and leucine. This indicates that the presence of PVA is already needed during the emulsion step to exert its stabilizing effect during spray drying. An acceptable PDI of 0.23 could only be achieved when spray drying a suspension previously precipitated by solvent displacement after the addition of PVA and trehalose. However, the nanoparticle yield was unacceptable for all tested formulations of PLGA/P-407 particles (Fig. 4) and was considerably lower than previously reported for focused ultrasound followed by solvent displacement (9). The superiority of PVA may be attributed to the formation of a thicker and denser surface layer (indicated by the larger resulting hydrodynamic particle sizes) and stronger surface binding and retention (23,24), whereas P-407 adsorbs weaker with its middle poly(oxypropylene) block (25).

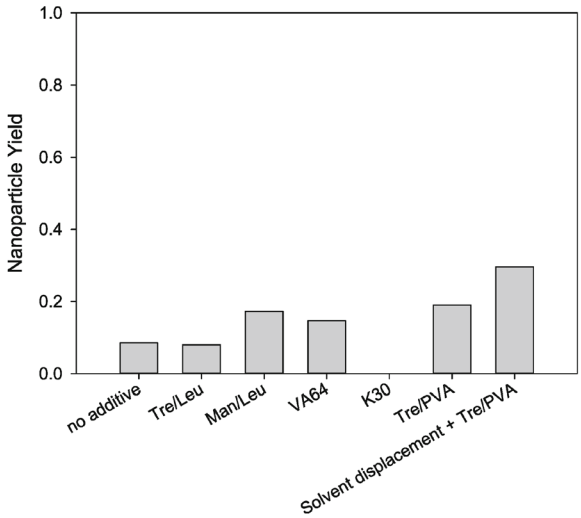

Fig. 4. Resulting nanoparticle yield after spray drying of PLGA/P-407 double emulsions with and without the addition of different matrix excipients. Nanoparticles were isolated from the matrix after reconstitution of the complete batch in purified water, then aggregates were removed by filtration, and the mass was determined after drying. The nanoparticle yield with K30 was too low to determine by weighing and, as such, is reported as 0

CONCLUSION

An economically interesting nanoparticle yield can be achieved using continuous manufacturing and drying methods. PVA stabilizes emulsion droplets and PLGA nanoparticles during spray drying to achieve a high nanoparticle yield and good particle size distribution, especially in the presence of trehalose and leucine. P-407 is not an effective stabilizer for the double emulsion spray drying method, despite being effective for solvent displacement. Further development work is needed to link the individual processes in a continuous line and to investigate process robustness, scalability, and protein loading efficiency. Further optimization of the emulsion step or use of different emerging emulsion techniques, such as microfluidics, would allow for completely continuous manufacturing of polymeric nanoparticles with an acceptable yield for commercial-scale manufacturing.

FUNDING

Open Access funding enabled and organized by Projekt DEAL.

REFERENCES

1. Lavik E, von Recum H. The role of Nanomaterials in translational medicine. ACS Nano. 2011;5(5):3419–24.
2. Valencia PM, Farokhzad OC, Karnik R, Langer R. Microfluidic technologies for accelerating the clinical translation of nanoparticles. Nat Nanotechnol. 2012;7(10):623–9.
3. Vauthier C, Bouchemal K. Methods for the preparation and manufacture of polymeric nanoparticles. Pharm Res. 2009;26(5):1025–58.
4. Makgwane PR, Ray SS. Synthesis of nanomaterials by continuous-flow microfluidics: a review. J Nanosci Nanotechnol. 2014;14(2):1338–63.
5. Rietscher R, Thum C, Lehr CM, Schneider M. Semi-automated nanoprecipitation-system-an option for operator independent, scalable and size adjustable nanoparticle synthesis. Pharm Res. 2015;32(6):1859–63.

6. Sato H, Suzuki H, Yakushiji K, Wong J, Seto Y, Prud'homme RK, et al. Biopharmaceutical evaluation of novel cyclosporine a nano-matrix particles for inhalation. Pharm Res. 2016;33(9):2107–16.
7. Hiemer J, Clausing A, Schwarz T, Stöwe K. MicroJet reactor technology: an automated, continuous approach for nanoparticle syntheses. Chem Eng Technol. 2019;42(10):2018–27.
8. Rao JP, Geckeler KE. Polymer nanoparticles: preparation techniques and size-control parameters. Prog Polym Sci. 2011;36(7):887–913.
9. Schiller S, Hanefeld A, Schneider M, Lehr C-M. Focused ultrasound as a scalable and contact-free method to manufacture protein-loaded PLGA nanoparticles. Pharm Res. 2015;32(9):2995–3006.
10. Ungaro F, D'Angelo I, Miro A, La Rotonda MI, Quaglia F. Engineered PLGA nano- and micro191 carriers for pulmonary delivery: challenges and promises. J Pharm Pharmacol. 2012;64(9):1217–35.
11. Torge A, Pavone G, Jurisic M, Lima-Engelmann K, Schneider M. A comparison of spherical and cylindrical microparticles composed of nanoparticles for pulmonary application. Aerosol Sci Technol. 2019;53(1):53–62.
12. Giunchedi P, Conte U. Spray-drying as a preparation method of microparticulate drug delivery systems: an overview. STP Pharm Pratiques. 1995;5(4):276–90.
13. Gander B, Wehrli E, Alder R, Merkle HP. Quality improvement of spray-dried, protein-loaded D,L-PLA microspheres by appropriate polymer solvent selection. J Microencapsul. 1995;12(1):83–97.
14. Bittner B, Morlock M, Koll H, Winter G, Kissel T. Recombinant human erythropoietin (rhEPO) loaded poly(lactide-co-glycolide) microspheres: influence of the encapsulation technique and polymer purity on microsphere characteristics. Eur J Pharm Biopharm. 1998;45(3):295–305.
15. Gavini E, Chetoni P, Cossu M, Alvarez MG, Saettone MF, Giunchedi P. PLGA microspheres for the ocular delivery of a peptide drug, vancomycin using emulsification/spray-drying as the preparation method: in vitro/in vivo studies. Eur J Pharm Biopharm. 2004;57(2):207–12.
16. Heng D, Lee SH, Ng WK, Tan RBH. The nano spray dryer B-90. Exp Opin Drug Deliv. 2011;8(7):965–72.
17. Booysen LLIJ, Kalombo L, Brooks E, Hansen R, Gilliland J, Gruppo V, et al. In vivo/in vitro pharmacokinetic and pharmacodynamic study of spray-dried poly-(dl-lactic-co-glycolic) acid nanoparticles encapsulating rifampicin and isoniazid. Int J Pharm. 2013;444(1–2):10–7.
18. Semete B, Booysen L, Kalombo L, Ramalapa B, Hayeshi R, Swai HS. Effects of protein binding on the biodistribution of PEGylated PLGA nanoparticles post oral administration. Int J Pharm. 2012;424(1–2):115–20.
19. Vehring R. Pharmaceutical particle engineering via spray drying. Pharm Res. 2008;25(5):999–1022.
20. Ito A, Konnerth C, Schmidt J, Peukert W. Effect of polymer species and concentration on the production of mefenamic acid nanoparticles by media milling. Eur J Pharm Biopharm. 2016;98:98–107.
21. Dalwadi G, Benson HAE, Chen Y. Comparison of diafiltration and tangential flow filtration for purification of nanoparticle suspensions. Pharm Res. 2005;22(12):2152–62.
22. Limayem I, Charcosset C, Fessi H. Purification of nanoparticle suspensions by a concentration/diafiltration process. Sep Purif Technol. 2004;38(1):1–9.
23. Vandervoort J, Ludwig A. Biocompatible stabilizers in the preparation of PLGA nanoparticles: a factorial design study. Int J Pharm. 2002;238(1–2):77–92.
24. Sahoo SK, Panyam J, Prabha S, Labhasetwar V. Residual polyvinyl alcohol associated with poly (D,L-lactide-co-glycolide) nanoparticles affects their physical properties and cellular uptake. J Control Release. 2002;82(1):105–14.
25. Scholes PD, Coombes AGA, Illum L, Davis SS, Watts JF, Ustariz C, et al. Detection and determination of surface levels of poloxamer and PVA surfactant on biodegradable nanospheres using SSIMS and XPS. J Control Release. 1999;59(3):261–78.

4.3. Preparation of nanoparticles-releasing enteric microparticles

Stefan Schiller,[1,2] Andrea Hanefeld,[3] Markus Weigandt,[2] Marc Schneider,[1] and Claus-Michael Lehr[1,4]

[1] Department of Pharmacy, Biopharmaceutics & Pharmaceutical Technology, Saarland University, Saarbrücken, Germany.

[2] Department of Pharmaceutical Technologies, Merck Healthcare KGaA, HPC D039/002, Frankfurter Str. 250, 64293, Darmstadt, Germany.

[3] Global Healthcare Operations Innovation Network, Merck Healthcare KGaA, Darmstadt, Germany.

[4] Helmholtz Institute for Pharmaceutical Research Saarland (HIPS), Helmholtz Center for Infection Research (HZI), Saarland University, Saarbrücken, Germany.

Priority number:	EP14003650
Filed:	28. October 2014
International Patent application no.:	PCT/EP2015/002033
First published:	06. May 2016 as WO2016066249A1

Further publications (as of 08. December 2021):

AU2015341070A1; AU2015341070B2; CA2965858A1; CN107106504A; CN107106504B; EP3212171A1; EP3212171B1; ES2713627T3; IL251946A; JP2017536350A; JP6656244B2; US10561622B2; US2017312229A1; WO2016066249A1

Granted (as of 08. December 2021):

AU2015341070B2; CN107106504B; EP3212171B1; ES2713627T3; IL251946A; JP6656244B2; US10561622B2

Reprinted as granted by the European Patent Office (EP3212171B1), available from the European Patent Register at https://register.epo.org/application?number=EP15783957

Specification of the contributions:

Stefan Schiller, Andrea Hanefeld, Markus Weigandt, Marc Schneider, and Claus-Michael Lehr conceived the experiments. Stefan Schiller planned, and carried out the experiments, and analyzed the data. Stefan Schiller, Andrea Hanefeld, Markus Weigandt, Marc Schneider, and Claus-Michael Lehr interpreted the results. Stefan Schiller took the lead in writing the manuscript. Andrea Hanefeld, Markus Weigandt, Marc Schneider, and Claus-Michael Lehr provided critical feedback to the research and contributed to the manuscript.

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) **EP 3 212 171 B1**

(12) **EUROPEAN PATENT SPECIFICATION**

(45) Date of publication and mention of the grant of the patent:
28.11.2018 Bulletin 2018/48

(21) Application number: **15783957.2**

(22) Date of filing: **15.10.2015**

(51) Int Cl.:
A61K 9/50 *(2006.01)*

(86) International application number:
PCT/EP2015/002033

(87) International publication number:
WO 2016/066249 (06.05.2016 Gazette 2016/18)

(54) PREPARATION OF NANOPARTICLES-RELEASING ENTERIC MICROPARTICLES

HERSTELLUNG VON NANOPARTIKELFREISETZENDEN ENTERISCHEN MIKROPARTIKELN

PRÉPARATION DE MICROPARTICULES ENTÉRIQUES LIBÉRANT DES NANOPARTICULES

(84) Designated Contracting States:
AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO RS SE SI SK SM TR

(30) Priority: **28.10.2014 EP 14003650**

(43) Date of publication of application:
06.09.2017 Bulletin 2017/36

(73) Proprietor: **Merck Patent GmbH**
64293 Darmstadt (DE)

(72) Inventors:
- **HANEFELD, Andrea**
  64297 Darmstadt (DE)
- **WEIGANDT, Markus**
  68259 Mannheim (DE)
- **SCHILLER, Stefan**
  64293 Darmstadt (DE)
- **SCHNEIDER, Marc**
  66125 Saarbruecken (DE)
- **LEHR, Claus Michael**
  66125 Saarbruecken (DE)

(56) References cited:
WO-A1-2012/071013

- **SONIA AL-QADI ET AL: "Microspheres loaded with polysaccharide nanoparticles for pulmonary delivery: Preparation, structure and surface analysis", CARBOHYDRATE POLYMERS, APPLIED SCIENCE PUBLISHERS, LTD. BARKING, GB, vol. 86, no. 1, 11 March 2011 (2011-03-11) , pages 25-34, XP028378188, ISSN: 0144-8617, DOI: 10.1016/J.CARBPOL.2011.03.022 [retrieved on 2011-03-25]**
- **KREUTER J ET AL: "LONG-TERM STUDIES OF MICROENCAPSULATED AND ADSORBED INFLUENZA VACCINE NANOPARTICLES", JOURNAL OF PHARMACEUTICAL SCIENCES, AMERICAN PHARMACEUTICAL ASSOCIATION, WASHINGTON, US, vol. 70, no. 4, 1 April 1981 (1981-04-01), pages 367-371, XP001015815, ISSN: 0022-3549, DOI: 10.1002/JPS.2600700406**
- **AL-QADI S ET AL: "Microencapsulated chitosan nanoparticles for pulmonary protein delivery: In vivo evaluation of insulin-loaded formulations", JOURNAL OF CONTROLLED RELEASE, vol. 157, no. 3, 10 February 2012 (2012-02-10), pages 383-390, XP028897307, ISSN: 0168-3659, DOI: 10.1016/J.JCONREL.2011.08.008**
- **GRENHA A ET AL: "Microencapsulated chitosan nanoparticles for lung protein delivery", EUROPEAN JOURNAL OF PHARMACEUTICAL SCIENCES, ELSEVIER, AMSTERDAM, NL, vol. 25, no. 4-5, 1 July 2005 (2005-07-01) , pages 427-437, XP027803660, ISSN: 0928-0987 [retrieved on 2005-07-01]**

Printed by Jouve, 75001 PARIS (FR)

EP 3 212 171 B1

Description

[0001] The present invention is directed to a process for the preparation of enteric microparticles comprising nanoparticles, wherein the nanoparticles comprise a matrix and an active ingredient. The microparticles obtained by such process are usable for various multiparticulate pharmaceutical formulations such as extemporaneous dosage forms (powder for reconstitution).

[0002] Enteric microparticles retain their enteric properties upon reconstitution in acidic media (pH 3-5) thus protecting the encapsulated nanoparticles from the gastric environment (pH, mucus entrapment). After neutralization in the intestine, nanoparticles are released from the microparticles in the lumen to subsequently cross the intestinal epithelium. Depending on the nanoparticles' design, the active ingredient may be released and elicit a local effect, or enter the blood stream for systemic effect. Nanoparticles for vaccination purposes would be taken up by immunocompetent cells and release the active ingredient (e.g. peptides, proteins, or nucleic acids) to the cytosol, where the active ingredient is processed and the corresponding epitope is presented on the cells' surfaces to elicit an immune response.

[0003] Multiparticulate pharmaceutical formulations when applied as oral suspension have several advantages over oral monolithic dosage forms: They can be easily swallowed and are thus very suitable to be applied to infants or babies as well as to patients suffering from dysphagia (elderly, following chemotherapy etc.); they have a pylorus-independent gastric transit, which lowers the intra- and interindividual variability and avoids food effects; and they are suitable for easy and accurate animal dosing in pre-clinical studies or animal therapeutics.

[0004] Krishnamachari et al. describes the preparation of enteric coated budesonide-loaded PLGA microparticles using an o/o emulsion evaporation method (Krishnamachari, Y., et al. (2007): Development of pH- and time-dependent oral microparticles to optimize budesonide delivery to ileum and colon; International Journal of Pharmaceutics 338(1-2): 238-247). In such method the enteric polymer (Eudragit® S-100) is dissolved in a suitable solvent that does not dissolve the budesonide-loaded PLGA microparticles to be encapsulated and such solution is mixed with the budesonide-loaded PLGA microparticles and emulsified into a viscous oily liquid (liquid paraffin containing 1% (w/v) Span 85 as emulsifier). In subsequent solvent evaporation step the solvent evaporates or disperses into the oil and the enteric polymer precipitates around the nanoparticles. The enteric microparticles obtained are filtered, washed with a further solvent (n-hexane) and dried in vacuum.

[0005] The multistep approach described by Krishnamachari has several disadvantages. Firstly, the filtration step is rather time-consuming due to the non-volatile, very viscous dispersant (liquid paraffin) and the very small pore sizes of the filter needed for retention of the microparticles. Secondly, the washing step involves an excess of a further solvent (n-hexane), which has to be removed thereafter. Thirdly, the overall process is difficult to be up-scaled.

[0006] Nassar et al. describes the preparation of enteric coated docetaxel-loaded PLGA microparticles using spray-drying (Nassar, T., et al. (2011): High plasma levels and effective lymphatic uptake of docetaxel in an orally available nanotransporter formulation; Cancer Research 71(8): 3018-3028). In such method enteric polymer Eudragit® L 100-55 (soluble above pH 5.5) and hydroxypropyl methylcellulose (HPMC; solubility pH independant) are dissolved in phosphate buffer which is adjusted to pH 6.5. Such solution is mixed with an undisclosed amount of Poly(lactide-co-glycolide) nanocapsules (PLGA-NC) and spray dried at 160 °C inlet and 98 °C outlet temperature. The composition of the coating matrix applied to the PLGA-NCs as obtained by the process consists of 40% (w/w) Eudragit® L 100-55, 53% (w/w) HPMC and 7% sodium phosphates.

[0007] The enteric properties of the microparticles obtained by the process described by Nassar et al. remain to be questionable. Firstly, HPMC which contributes 53% (w/w) to the total mass of the coating is a nonionic polymer with a pH independent solubility. Secondly, the spray-drying process is performed with an outlet temperature of 98 °C. As the spray dried product usually reaches a similar temperature this may cause damage to the particulate formulation, especially to the active ingredient but also to NCs as PLGA usually has a glass transition temperature well below 98 °C. Thirdly, the pH of the spraying solution is adjusted to 6.5 with NaOH. As Eudragit® L 100-55 dissolves above pH 5.5, most of such polymer's methacrylic acid groups are deprotonized so that in the spray dried matrix Eudragit® is predominantly present as sodium salt. However, upon reconstitution of the dried microparticles in acidic media, the sodium methacrylate groups lead at first to a partial solvation of the polymer, followed by reprotonation and desolvation, thus leading to swelling and stickiness of the enteric microparticles in suspension. Such effect is even increased by the buffering salts that remain present from the spray-drying solution and which may affect the pH microclimate inside and in the vicinity of the enteric particles. Indeed, as evidenced by scanning electron micrographs the particles obtained by such process are hollow or collapsed (see Fig. 2A of Nassar et al.), which results in an unfavorable surface-to-volume ratio and protrusion of nanocapsules from the enteric matrix. As shown by Fig. 2B the particles are further interconnected after incubation at pH 1.2 for one hour (which pH is comparable to gastric passage), which most likely results from partial solvation and swelling due to excess neutralization as described above. Due to the particles' stickiness in acidic media it is most likely that they cannot be homogeneously dispersed to form a suspension for oral application (enteric microparticles for reconstitution and oral use should be redispersed in slightly acidic solvents having a pH below the solubility threshold of the enteric polymer (e.g. a pH of about 4) to avoid partial salvation/swelling of the microparticles upon their reconsti-

tution). When delivered directly to the stomach in dry form (e.g. as powder in capsule), swelling and sticking of the particles would lead to a partial or complete loss of the described advantages of multiparticulate versus monolithic dosage forms.

[0008] As described the processes known in the art for the production of enteric microparticles comprising nanoparticles have several disadvantages and/or lead to particulate formulations with insufficient properties. It was the object of the present invention to provide a process for the production of enteric microparticles comprising nanoparticles that overcome such disadvantages. The process for production should be easily workable, fast, up-scalable and should lead to a microparticulate formulation that is easily dispersible in aqueous media. Further, the microparticles should maintain their integrity in acidic media (which they have to pass during passage of stomach) and should be able to release the nanoparticles dispersed therein at a pH greater than about 5.5 (as it is present in the intestinal environment) in a reproducible manner without substantial change to the mean particle size and size distribution.

[0009] Surprisingly, it has been found by the present invention that a process meeting such criteria can be made available when the nanoparticles to be contained in the enteric microparticles are suspended in a colloidal dispersion of the enteric coating material and spray-dried or when a suspension of the nanoparticles and a colloidal dispersion of the enteric coating material are co-spray-dried. Accordingly, one object of the present invention is directed to a process for the preparation of enteric microparticles comprising nanoparticles, wherein the nanoparticles comprise a matrix and an active ingredient, such process comprises (i) spray-drying of a suspension of the nanoparticles in a colloidal dispersion of the enteric coating material or (ii) co-spray-drying of a suspension of nanoparticles and a colloidal dispersion of the enteric coating material.

[0010] As used herein, "a" or "an" shall mean one or more. As used herein when used in conjunction with the word "comprising," the words "a" or "an" mean one or more than one. As used herein "another" means at least a second or more. Furthermore, unless otherwise required by context, singular terms include pluralities and plural terms include the singular.

[0011] As used herein, "about" refers to a numeric value, including, for example, whole numbers, fractions, and percentages, whether or not explicitly indicated. The term "about" generally refers to a range of numerical values (e.g., +/- 1-3% of the recited value) that one of ordinary skill in the art would consider equivalent to the recited value (e.g., having the same function or result). In some instances, the term "about" may include numerical values that are rounded to the nearest significant figure.

[0012] The term "microparticles" as used herein refers to particles having a mean size of more than 1 μm. The microparticles can have a regular shape, such as spheres, or an irregular shape. The microparticles are built up of nanoparticles and an enteric polymer that embed the nanoparticles and provides a matrix for them to form microparticles having a sufficient physical stability required for their respective use.

[0013] The term "enteric coating" as used herein generally refers to a barrier applied to oral medication that controls the location in the digestive system where it is absorbed. Enteric refers to the small intestine, therefore enteric coatings prevent release of medication before it reaches the small intestine. The term "enteric" together with "microparticles" as used herein refers to that each of the microparticles is comprised of a matrix that prevents the release of the nanoparticles before the formulation reaches the small intestine. Enteric coatings work by presenting a surface that is stable at the highly acidic pH present in the stomach, but breaks down rapidly at a less acidic (relatively more basic) pH. For example, enteric coatings do not dissolve in the acidic juices of the stomach (pH 1-3) but in the higher pH (above pH 5.5) environment present in the small intestine. The term "enteric coating material" as used herein refers to a material having the properties as described for enteric coating. Such material can be used to embed the nanoparticles and to form the microparticles of the invention and to protect them from degradation during passage of the stomach after oral application.

[0014] The term "nanoparticles" as used herein refers to particles having a mean size of less than 1 μm. The nanoparticles preferably have a regular shape, such as spheres, but may also have an irregular shape.

[0015] The term "matrix" as used herein generally refers to a surrounding substance within which something else is contained. For purposes herein, a matrix refers to the structural properties or architecture of a solid in which other components can be dispersed. In the microparticles of the invention the matrix is provided by the enteric coating material in which the nanoparticles are dispersed.

[0016] The term "active ingredient" means any ingredient that provides a pharmacological or biological effect when applied to a biological system. The active ingredient may be a pharmaceutical drug, biological matter of viral or ling origin. Examples of an active ingredient that may be used in the process of the present inventions are insulin, heparin, calcitonin, hydrocortisone, prednisone, budesonide, methotrexate, mesalazine, sulfasalazine, amphotericin B, nucleic acids, or antigens (peptides, proteins, sugars, or other substances that form surfaces recognized by the immune system, either produced, extracted, or homogenized from tissue, an organism or a virus).

[0017] The term "colloidal" as used herein refers to a state of subdivision, implying that the molecules or polymolecular particles dispersed in a medium have at least, in one direction, a dimension roughly between 1 nm and 1 μm, or that in a given system, discontinuities are found at distances of that order (1972, 31, 605, IUPAC Compendium of Chemical Terminology, 2nd Edition, 1997). The term "colloidal dispersion" as used herein refers to a system in which solid particles

of colloidal size are dispersed in a continuous liquid phase, preferably in an aqueous phase.

[0018] The term "suspension" as used herein refers to a liquid containing one or more components dispersed therein, wherein the components are substantially not dissolved in the liquid. In this context the term substantially means a proportion of at least about 90%, at least 95%, at least about 98%, at least 99% or more. In some embodiments the term substantially includes 100%. In the process of the invention a suspension of nanoparticles in an aqueous solvent is prepared.

[0019] The term "spray-drying", as used herein, refers to a method of producing a dry powder comprising micron-sized particles from a solution or suspension by using a spray-dryer. Spray-drying is, in principle, a solvent extraction process. The constituents of the product to be obtained are dissolved/dispersed in a liquid and then fed, for example by using a peristaltic pump, to an atomiser of a spray-dryer. A suitable atomizer which can be used for atomization of the liquid, include nozzles or rotary discs. With nozzles, atomization occurs due to the action of the compressed gas, while in case of using rotary discs atomization occurs due to the rapid rotation of the disc. In both cases, atomization leads to disruption of the liquid into small droplets into the drying chamber, wherein the solvent is extracted from the aerosol droplets and is discharged out, for example through an exhaust tube to a solvent trap.

[0020] Drop sizes from 1 to 500 μm can be generated by spray-drying. As the solvent (water or organic solvent) dries, the nanoparticles-containing droplets dries into a micron-sized particle, forming powder-like particles.

[0021] A number of commercially available spray drying machines can be used to prepare the microparticles of the invention, for example, suitable machines are manufactured by Buchi and Niro. Examples of suitable spray-driers include lab scale spray-dryers from Buchi, such as the Mini Spray Dryer 290, or a MOBILE MINOR™, or a Pharma Spray Dryer PharmaSD® from Niro, or a 4M8-TriX from Procept NV.

[0022] In a typical spray drying machine the suspension to be dried is pumped from a stirred reservoir to an atomization chamber where it is sprayed from a nozzle as fine droplets (preferably the droplets are in the range of 1 to 20 μm in diameter) into a stream of heated air, for example, inlet temperatures in the range of 50 to 150° C (nitrogen can be used in place of air if there is a risk of undesirable oxidation of the product). The temperature of the heated air must be sufficient to evaporate the liquid and dry the microparticles to a free flowing powder but should not be so high as to degrade the product. The microparticles may be collected in a cyclone or a filter or a combination of cyclones and filters.

[0023] The term "co-spray-drying", as used herein, refers to a method of producing a dry powder comprising micron-sized particles from two or more solutions or suspensions by using a spray-dryer. This method differs from conventional spray drying as described above in that the solutions or suspensions are fed separately to the atomizing device without prior bulk mixing. The separate feeds are brought into contact just in or after the atomizing device. An example of a suitable spray dryer would be a Micro Mist Spray Dryer from Fujisaki Electric.

[0024] Suitable spray-drying techniques, which can be used for preparation of the microparticles, are well known and described, for example, by K. Masters in "Spray-drying Handbook", John Wiley & Sons, New York, 1984. In a preferred embodiment, atomization of the liquid is performed by using a nozzle.

[0025] In the process of the invention spray-drying of the suspension of nanoparticles in a colloidal dispersion of enteric coating material leads to microparticles wherein the nanoparticles are embedded in a matrix of the enteric coating material.

[0026] According to a preferred embodiment of the invention the process comprises the following steps: (a) preparing an aqueous dispersion comprising an enteric coating material; (b) adjusting the pH of the aqueous dispersion prepared by step (a) to a pH slightly below the solubility threshold of the enteric coating material to produce a colloidal dispersion of the enteric coating material; (c) mixing the nanoparticles with the colloidal dispersion prepared by step (b) to produce a suspension of the nanoparticles in such colloidal dispersion; and (d) spray-drying the colloidal dispersion prepared by step (c). Accordingly the invention is also directed to a process comprising the steps

(a) preparing an aqueous dispersion comprising an enteric coating material;
(b) adjusting the pH of the aqueous dispersion prepared by step (a) to a pH slightly below the solubility threshold of the enteric coating material to produce a colloidal dispersion of the enteric coating material;
(c) mixing the nanoparticles with the colloidal dispersion prepared by step (b) to produce a suspension of the nanoparticles in such colloidal dispersion;
(d) spray-drying the colloidal dispersion prepared by step (c).

[0027] For preparation of the aqueous dispersion in accordance to step (a) the enteric coating material is dispersed in an aqueous solvent. The dispersion can be facilitated using suitable techniques known in the art such as stirring or sonification. The term "aqueous solvent" as used herein also refers to water, or a mixture of solvents that contains at least about 50% or 50%, at least about 60% or 60%, at least about 70% or 70%, or about or at 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or higher amounts of water. The aqueous solvent may contain salts, buffers or other solutes that are soluble in water. Preferably the aqueous solvent is water.

[0028] In step (b) the pH is adjusted to a pH slightly below the solubility threshold of the enteric coating material by adding a pH increasing agent. The solubility threshold as used herein refers to the pH, at which the material begins to

dissolve. The solubility threshold is a characteristic of a enteric coating material and is usually given by the manufacturer for a specific material, for example, the enteric coating material Eudragit® L 100-55 is defined to have a solubility threshold of pH 5.5. When increasing the pH in step (b) the enteric coating material dispersed in the aqueous solvent to a pH slightly below the solubility threshold the enteric coating material gets partially deprotonated. The rising surface charge of the dispersed particles and the resulting interparticulate repulsive forces lead to the formation and stabilization of a colloidal dispersion of the enteric coating material. The colloidal dispersion that is prepared by step (b) is characterized by the disappearance of visible particulates and the formation of a homogeneous, milky-white fluid. Preferably, the particle size of the dispersed enteric coating material is below 1 μm. Suitable methods for the determination of the particle size include static light scattering, dynamic light scattering and electron microscopy.

[0029] In one embodiment of the invention the colloidal dispersion obtained in step (b) has a degree of neutralization (DN) of 5 to 40%, preferably 1 to 30%, more preferably 12 to 25% and most preferably about 15% Therefore, the invention is also directed to a process, which is characterized in that the colloidal dispersion obtained in step (b) has a degree of neutralization (DN) of 5 to 40%, preferably 1 to 30%, more preferably 12 to 25% and most preferably about 15% The term "pH increasing agent" as used herein refers to an agent that increases the pH of the aqueous dispersion of enteric coating material when added to such aqueous dispersion. Suitable pH increasing agents are, for example, alkali metal hydroxides such as sodium hydroxide, potassium hydroxide, calcium hydroxide or magnesium hydroxide, carbonates and hydrogencarbonates of alkali metals such as sodium carbonate, potassium carbonate, sodium bicarbonate or potassium bicarbonate, ammonium carbonate, ammonium hydrogencarbonate, diethanolamine, monoethanolamine, triethanolamine, organic amine base, alkaline amino acids such as lysine or arginine, trolamine or NH_3. Preferably the pH increasing agent used for adjustment of pH in step (b) of the process described above are sodium hydroxide, potassium hydroxide, carbonates and hydrogencarbonates of alkali metals, ammonium carbonate, ammonium hydrogencarbonate, or ammonia, more preferably ammonia. Ammonia is especially preferred as evaporates under usual spray-drying conditions leading to that no cation stemming from the pH increasing agent remains in the microparticles after spray-drying.

[0030] It has been found that increasing amounts of alkali cations resulting from the pH increasing agent have a detrimental effect on re-dispersibilty of the spray-dried particles and lead to penetration of sovent and swallowing upon reconstitution in aqueous solutions. Therefore, it is preferred that the pH increasing agent is added in the least possible amount that allows a film formation that is sufficient to build up a flexible matrix for the nanoparticles dispersed therein, to protect them from agglomeration during spray-drying and to form microparticles in which the nanoparticles dispersed therein are protected from gastric environment upon oral administration to a mammal. Depending on the enteric coating material an appropriate pH value slightly below the solubility threshold that allows formation of the colloidal dispersion can be a pH value in the range from ≤ 1 to ≤ 0.01 less than the solubility threshold of the enteric coating material, a pH value in the range from ≤ 0.5 to ≤ 0.01 less than the solubility threshold of the enteric coating material, a pH value in range from ≤ 0.2 to ≤ 0.02 less than the solubility threshold of the enteric coating material or a pH value in the range from ≤ 0.1 to ≤ 0.05 less than the solubility threshold of the enteric coating material.

[0031] According to an alternative preferred embodiment of the invention the process comprises the following steps: (a) preparing an aqueous dispersion comprising an enteric coating material; (b) adjusting the pH of the aqueous dispersion prepared by step (a) to a pH slightly below the solubility threshold of the enteric coating material to produce a colloidal dispersion of the enteric coating material; (c) preparing an aqueous suspension comprising the nanoparticles; and (d) co-spray-drying of the colloidal dispersion prepared by step (b) together with the aqueous suspension prepared by step (c). Accordingly the invention is also directed to a process comprising the steps

(a) preparing an aqueous dispersion comprising an enteric coating material;
(b) adjusting the pH of the aqueous dispersion prepared by step (a) to a pH slightly below the solubility threshold of the enteric coating material to produce a colloidal dispersion of the enteric coating material;
(c) preparing an aqueous suspension comprising the nanoparticles; and
(d) co-spray-drying of the colloidal dispersion prepared by step (b) together with the aqueous suspension prepared by step (c).

[0032] According to a preferred embodiment of the invention the nanoparticles used in the process have a mean size from 20 nm to 1000 nm, preferably from 100 nm to 500 nm, and more preferably from 200 nm to 300 nm. Therefore, the invention is also directed to a process, which is characterized in that the nanoparticles used in the process have a mean size from 20 nm to 1000 nm, preferably from 100 nm to 500 nm, and more preferably from 200 nm to 300 nm.

[0033] The term "mean size" as used herein refers to the hydrodynamic average diameter ("z-average") of the nanoparticle population that moves together in an aqueous medium. The z-average is defined by ISO 22412 as the 'harmonic intensity averaged particle diameter'. To compare z-average sizes measured by different techniques the samples have to be monomodal (i.e. only one peak), spherical or near-spherical in shape and monodisperse (i.e. very narrow width of distribution). The mean size of these systems can be measured by standard processes known by the person skilled in the art, and which are described, for example, in the experimental part (see below).

[0034] The matrix material present in the nanoparticles used in the process of the invention can be any matrix material being suitable for dispersing, dissolving or embedding the active ingredient. In some embodiments of the invention, the nanoparticles comprise a biocompatible anorganic particulate material such as silica, surface-modified silica or a biocompatible organic polymer, preferably a biodegradable polymer. Therefore, the invention is also directed to the process of the invention, which is characterized in that the matrix of the nanoparticles is an anorganic particulate material such as silica, surface-modified silica or a biocompatible polymer, preferably a biodegradable polymer.

[0035] The term "biocompatible" as used herein refers to exhibition of essentially no cytotoxicity or immunogenicity while in contact with body fluids or tissues. The term "biocompatible" together with "anorganic particulate material" or "organic polymer" refers to material which are nontoxic, chemically inert, and substantially non-immunogenic when used internally in a subject and which are substantially insoluble in blood. As used herein, the term "organic polymer" refers to oligomers, co-oligomers, polymers and co-polymers, e.g., statistical, block, multiblock, star, grafted, gradient copolymers and combination thereof. The average molecular weight of the polymer, as determined by gel permeation chromatography, can range from 20,000 to about 500,000. The biocompatible organic polymer can be either non-biodegradable or preferably biodegradable.

[0036] The term "biodegradable" as used herein generally refers to be capable to be decomposed by the action of biological agents. A biodegradable polymer, as used herein, refers to a polymer that degrades or erodes in vivo to form smaller chemical species. Degradation can result, for example, by enzymatic, chemical and/or physical processes. Suitable biodegradable polymers include, for example, poly(lactic acid)s (PLA), poly(glycolic acid)s (PGA), copolymers of lactic acid and glycolic acid (PLGA), polycaprolactones (PLC), polyepsilon caprolactones, copolymers of lactic acid and caprolactone, polyhydroxy butyric acids, chitosans, polyesters, polycarbonates, polyesteramides, polyanhydrides, poly(amino acids), poly(ortho)ester, polyurethanes, polyanhydrides, polyacetyls, polydihydropyrans, polyamides, such as, for example, polyesteramides or polyaminoacids, polysaccharides polycyanoacrylates, polyetheresters, poly(dioxanone)s, poly(alkylene alkylate)s and copolymers of polyethylene glycol, blends and copolymers thereof and derivatives thereof such as pegylated polymers like PEG-PLGA.

[0037] In a preferred embodiment of the invention the matrix of the nanoparticles used in the process is a biodegradable polymer which is poly(lactic acid) (PLA), poly(glycolic acid) (PGA), polycaprolactone (PCL), a copolymer of lactic acid and glycolic acid (PLGA), a copolymer of lactic acid and caprolactone, polyepsilon caprolactone, polyhydroxy butyric acid, chitosan, a polyester, a poly(ortho)ester, a polyurethane, a polyanhydride, a polyacetal, a polydihydropyran, a polyamide, a polysaccharide or a polycyanoacrylate, a blend or copolymer thereof or a derivative thereof such as pegylated polymers like PEG-PLGA. Therefore, the invention is also directed to a process, which is characterized in that the biodegradable polymer is poly(lactic acid) (PLA), poly(glycolic acid) (PGA), polycaprolactone (PCL), a copolymer of lactic acid and glycolic acid (PLGA), a copolymer of lactic acid and caprolactone, polyepsilon caprolactone, polyhydroxy butyric acid, chitosan, a polyester, a poly(ortho)ester, a polyurethane, a polyanhydride, a polyacetal, a polydihydropyran, a polyamide, a polysaccharide or a polycyanoacrylate, a blend or copolymer thereof or a derivative thereof such as pegylated polymers like PEG-PLGA.

[0038] Especially preferred is PLGA as biodegradable polymer. Accordingly, the invention is further directed to a process, which is characterized in that the biodegradable polymer is PLGA.

[0039] The enteric coating material present used to produce the microparticles in the process of the invention can be any enteric coating material that is suitable for dispersing or embedding the nanoparticles used in the process. Preferred enteric coating material used in the process of the invention is cellulose acetate phthalate, hydroxypropyl methylcellulose phthalate, hydroxypropyl methylcellulose acetate succinate, polyvinyl acetate phthalate, carboxymethyl ethylcellulose, cellulose acetate trimellitate, a copolymer of acrylic or methacrylic acid and an acrylic or methacrylic ester, preferably a copolymer of methacrylic acid and a methacrylate or a acrylate ester. Therefore, the invention is further directed to a process, which is characterized in that the enteric coating material is cellulose acetate phthalate, hydroxypropyl methylcellulose phthalate, hydroxypropyl methylcellulose acetate succinate, polyvinyl acetate phthalate, carboxymethyl ethylcellulose, cellulose acetate trimellitate, a copolymer of acrylic or methacrylic acid and an acrylic or methacrylic ester, preferably a copolymers of methacrylic acid and a methacrylate or a acrylate ester. Copolymers of methacrylic acid and a methacrylate or a acrylate ester are commercially available under the trade name Eudragit® (Evonik Industries AG, Essen, Germany).

[0040] Especially preferred copolymers of methacrylic acid and methacrylate or acrylate esters that are usable in the process of the invention are (Poly(methacrylic acid-co-methyl methacrylate) (1:1) (e.g. Eudragit® L 100), (Poly(methacrylic acid-co-methyl methacrylate) (1:2) (e.g. Eudragit® S 100), Poly(methacrylic acid-co-ethyl acrylate) (1:1) (e.g. Eudragit® L 100-55). Accordingly, the invention is further directed to a process, which is characterized in that the copolymer of methacrylic acid and a methacrylate or acrylate ester is (Poly(methacrylic acid-co-methyl methacrylate) (1:1), (Poly(methacrylic acid-co-methyl methacrylate) (1:2), Poly(methacrylic acid-co-ethyl acrylate) (1:1).

[0041] The microparticles produced by the process of the invention have a mean size of 1 μm to 200 μm, preferably of 10 μm to 150 μm and more preferably of 50 μm to 150 μm. Thus the invention is also directed to a process, which is characterized in that the microparticles have a mean size of 1 μm to 200 μm, preferably of 10 μm to 150 μm and

more preferably of 50 μm to 150 μm.

[0042] Advantageously, the parameters in the spray-drying step of the process of the invention are selected and controlled in a way as it is known in the art that the temperature of the dried product is never above the glass transition temperature of the nanoparticles, preferably at least 1 °C below, and more preferably at least 5 °C below the glass transition temperature of the nanoparticles. The product temperature may be calculated by computational fluid dynamics modeling based on device geometry and kinetic studies of the evaporation process in drying droplets (e.g. based on single droplet drying experiments), traced by infrared cameras, or estimated from the temperature at the outlet of the drying chamber. Thus the invention is also directed to a process, which is characterized in that the temperature of the dried product is never above the glass transition temperature of the nanoparticles, preferably at least 1 °C below, and more preferably at least 5 °C below the glass transition temperature of the nanoparticles.

[0043] Parameters that can be selected and varied during the spray-drying process to achieve the desired product temperature and as well as the effect of such parameters on the product temperature are well-known in the art and include, i.a. the kind and/or composition of solvent, the concentrations of starting materials, the flow-rates of the injected materials as well as of the drying gas, the inlet air temperature and inlet air humidity.

[0044] The term "glass transition temperature" generally refers to the temperature at which amorphous polymers undergo a transition from a rubbery, viscous amorphous liquid, to a brittle, glassy amorphous solid. A glass transition temperature as used herein refers to an intermediate point glass transition temperature obtained when the temperature is raised at a heating rate of 10 or 20 °C per minute using a differential scanning calorimeter (DSC).

[0045] The examples explain the invention without being restricted thereto.

Particle Size Analysis of nanoparticles

[0046] Particle size measurements are performed using a Zetasizer Nano ZS (Malvern Instruments) applying dynamic light scattering (DLS). Using cumulants analysis, the z-average (harmonic intensity averaged particle diameter; z-av) and the polydispersity index (estimator of the particle size distribution width; PDI) were calculated according to ISO13321 and ISO22412, using a viscosity of 0.8872 mPas (at 25°C) and a refractive index of 1.330. Each sample is equilibrated to 25°C within 120 seconds and analysis is performed in triplicate.

Nanoparticles used for preparation of Microparticles

[0047] Fluorescent ovalbumin loaded PLGA (Resomer® RG 503 H, Evonik) nanoparticles were used as model nanoparticles (PLGA-NP). They were prepared by a modified double emulsion solvent evaporation method (Blanco, M.D., et al. (1997): Development and characterization of protein-loaded poly(lactide-co-glycolide) nanospheres; Eur J Pharm Biopharm 43(3): 287-294) using polyvinyl alcohol as stabilizer and Coumarin 6 as fluorescent dye. In one embodiment modified PEG-PLGA was used to prepare nanoparticles (mod. PEG-PLGA-NP) according to the method described above. Mean particle sizes of different batches were between 150 - 300 nm.

[0048] Chitsosan nanoparticles are prepared by the ionic gelation method (Grenha, A. (2012): Chitosan nanoparticles: a survey of preparation methods; Journal of drug targeting 20(4): 291-300). Chitosan (Chitoscience, Heppe Medical Chitosan) is dissolved in an acidic acid solution and complexed by e.g. carboxymethylcellulose solution which is prepared by dissolving e.g. Tylose C30 (Hoechst) in purified water and added slowly to the chitosan phase while stirring on a magnetic stirrer.

[0049] Silica nanoparticles are prepared as described in EP 0216278 B1 by hydrolysis of tetraalkoxysilanes in aqueous-alcoholic-ammoniacal medium, where firstly a sol of primary particles is produced, and the SiO2 particles obtained are subsequently brought to the desired particle size by continuous metering-in of tetraalkoxysilane in a controlled manner corresponding to the extent of reaction. The production of 50 g of SiO2 particles having a size of 25 nm requires, for example, 1.2 l of EtOH as solubiliser, 860 ml of deionised water, 167 ml of tetraethyl orthosilicate and 28.5 ml of 25% aqueous ammonia solution.

Enteric coating material

[0050] Enteric polymers such as Methacrylic Acid Copolymers (e.g. Eudragit®) can be sprayed as organic solution (e.g. alcohols, acetone) to achieve a steady film upon drying. While the polymer molecules in solution can freely and ideally rearrange for film formation, the use of solvents in spray drying is less attractive due to environmental restrictions and related cost of equipment. Furthermore, preliminary studies showed that this method is not suitable for the intended purpose. Although alcohols are non-solvents for relevant polymeric nanoparticles (e.g. PLGA), mixing PLGA nanoparticles with a solution of Eudragit® L in ethanol leads to precipitation.

[0051] Although good films can also be produced from aqueous solutions of Eudragit®, the high viscosity is detrimental for nozzle spraying. Moreover, the films are made of polymer with largely neutralized methacrylic acid groups. Contrary

to the free acid, Eudragit® salts are freely soluble in purified, buffer-free water. When dispersing particles made from Eudragit® salts in acidic media they will immediately begin to swell, forming sticky gel-like lumps before the protonation of the methacrylate groups by the medium stops the dissolution process.

[0052] Processing without organic solvents is possible by using aqueous dispersions of Eudragit® which are stabilized electrostatically by partial deprotonation of the methacrylate groups. Upon drying of the coating the Eudragit® particles are eventually held together by capillary forces, but particle coalescence is needed to form a closed film. Therefore, a plasticizer is always added to spray suspensions. However, a plasticizer might also facilitate the coalescence of encapsulated nanoparticles during processing and product storage by decreasing the glass transition temperature of the PLGA-NP (Kranz, H., et al. (2000): Physicomechanical properties of biodegradable poly(D,L-lactide) and poly(D,L-lactide-co-glycolide) films in the dry and wet states; Journal of Pharmaceutical Sciences 89(12): 2899-2605). Hence a plasticizer-free formulation is preferred.

[0053] It has been found that the addition of plasticizer can be avoided when the enteric polymer dispersed in an aqueous solvent is partially neutralized to an extent that leads to that the aqueous dispersion of the enteric polymer is converted to a colloidal dispersion of it as demonstrated in the following.

[0054] Using Eudragit® L as an enteric polymer aqueous spray dispersions having different degrees of neutralization (DN) were tested. The term "degree of neutralization" or "DN" of a polymer as used herein refers to the mole ratio of added NH_3 to the total polymer carboxylic acid groups present in the solution.

[0055] Partially neutralized Eudragit® dispersions with a DN of 6% or 15% and a clear, viscous Eudragit® solution with a DN of 70% were prepared by suspending Eudragit® in purified water and adding the appropriate amount of 1 M ammonia solution dropwise under stirring to yield a concentration of 100 mg/mL Eudragit®.

[0056] To prepare a dispersion of Eudragit® L with a degree of neutralization of 6%, 2.5 g Eudragit® L 100 are dispersed in 20 mL purified water by magnetic stirring. After 5 min stirring, 0.85 mL of 1 N ammonia solution is added dropwise with a syringe pump over 10 min. The dispersion is diluted with purified water to 25.0 g and stirred for 60 min to yield a homogeneous milky white dispersion of 10 % (w/w) Eudragit® L without visible particles or lumps. The pH of the dispersion is 5.56, thus below the solubility threshold of Eudragit® L (pH 6.0).

[0057] To prepare a dispersion of Eudragit® L with a degree of neutralization of 15%, 2.5 g Eudragit® L 100 are dispersed in 20 mL purified water by magnetic stirring. After 5 min stirring, 2.11 mL of 1 N ammonia solution is added dropwise with a syringe pump over 10 min. The dispersion is diluted with purified water to 25.0 g and stirred for 60 min to yield a homogeneous milky white dispersion of 10 % (w/w) Eudragit® L without visible particles or lumps. The pH of the dispersion is 5.88 thus below the solubility threshold of Eudragit® L (pH 6.0).

[0058] To prepare a solution of Eudragit® L with a degree of neutralization of 70%, 2.5 g Eudragit® L 100 are dispersed in 10 mL purified water by magnetic stirring. After 5 min stirring, 9.85 mL of 1 N ammonia solution is added dropwise with a syringe pump over 10 min. The dispersion is diluted with purified water to 25.0 g and stirred for 60 min to yield a clear, viscous solution of 10 % (w/w) Eudragit® L. The pH of the dispersion is 6.09, thus above the solubility threshold of Eudragit® L (pH 6.0). Dispersions of further enteric coating materials are prepared in a similar manner by calculating the amount of base needed for a specific DN from the acid value of the enteric coating material (usually provided as mg KOH per g polymer or similar).

Preparation of Microparticles (general description)

[0059] Spray feeds were prepared by mixing PLGA nanoparticle suspensions with partially neutralized Eudragit® dispersions to yield a total solid content of 55-80 mg/g spray feed. For screening purposes, volume equivalents to 200 mg dry substance were dried with a lab scale spray dryer (4M8-TriX, ProCepT, Zelzate, Belgium) using a feed rate of 6 mL/min, a 0.4 mm bi-fluid nozzle with 20 L/min atomizing air flow, 80±2 °C inlet temperature, 400 L/min drying air flow, 150 L/min cooling air flow, and 32-38 °C outlet temperature. As PLGA has a relatively low glass transition temperature (44 - 48 °C for RG 503 H), a low outlet temperature is preferred to avoid nanoparticle deformation or agglomeration. Experiments were performed at 20-22 °C ambient temperature and 51-60% relative humidity. The microparticles have a final composition as shown in table 1.

Table 1: Composition of enteric microparticles prepared by spray drying

Component	Mass percent (dry mass) of final formulation
Eudragit® L 100	90 %
PLGA-NP	10 %

[0060] Further Microparticles are prepared analogously having the composition as given in table 2:

Table 2: Composition of enteric microparticles prepared by spray drying

Example	Component	Mass percent (dry mass) of final formulation
1	Eudragit® L 100	80 %
	PLGA-NP	20 %
2	Eudragit® S 100	90 %
	PLGA-NP	10 %
3	Eudragit® L 100 D-55	80 %
	PLGA-NP	20 %
4	Eudragit® L 100	90 %
	Mod.PEG--PLGA-NP	10 %
5	Eudragit® L 100 D-55	90 %
	Chitosan-NP	10 %
6	Eudragit® L 100 D-55	90 %
	Silica-NP	10 %

[0061] Alternatively, microparticles can be prepared by co-spray-drying. For this process, a PLGA nanoparticle suspension and a partially neutralized Eudragit® dispersion are fed separately to the atomizing device and spray dried under suitable conditions as described above.

[0062] The formulations were evaluated for the feasibility to produce homogeneous suspensions in acidic media by hand shaking, vortexing and bath sonication. The size of nanoparticles before processing and after release in phosphate buffered saline pH 6.8 was determined by dynamic light scattering to identify possible agglomeration (Table 3).

Table 3: Properties of nanoparticle-releasing enteric microparticle formulations prepared from Eudragit® L 100 with different degrees of neutralization. Meaning of symbols for the dispersibility of the enteric microparticles in HCl: "++": readily dispersible by shaking or vortex;"+": dispersible by bath sonication; "-": not dispersible

Degree of Neutralization	PLGA-NP mass percent	Dispersibility in 0.1 M HCl	Z-av	PDI
	Before spray drying		217 nm	0.26
6%	10%	++	379 nm	0.39
6%	20%	++	655 nm	0.55
15%	10%	+	257 nm	0.26
15%	20%	+	290 nm	0.34
15%	33%	+	1847 nm	0.60
70%	10%	-	259 nm	0.24
70%	20%	-	229 nm	0.23
70%	33%	-	484 nm	0.57

[0063] As shown in Table 3, formulations with DN 6% released only agglomerated nanoparticles, while enteric microparticles prepared with DN 70% underwent gelling and lumping in acidic media. Formulations with DN 15% and a nanoparticle content of 10 % (m/m) release NP at pH 6.8 with a size distribution similar to the untreated NP (Table 3). This indicates that the proposed method does not alter the favorable target product profile of the encapsulated NP. Furthermore, these formulations are homogeneously dispersible in 0.1 M HCl and as such suitable as extemporaneous dosage form for reconstitution in acidic media prior to administration.

[0064] Scanning electron micrographs show that DN 6% does not lead to a closed film as revealed by the black spaces between individual Eudragit® particles (Fig. 1A). Surprisingly, by raising the DN to 15% the particles are now completely bridged, suggesting a closed film and a superior matrix for the protection and spacing of encapsulated PLGA nanoparticles (Fig. 1B). Enteric particles prepared from aqueous Eudragit® solutions (DN 70%) exhibit a smooth surface from film formation (Fig. 1C; the wrinkles are measurement artifacts caused by the shrinkage of the particles under the electron

beam).

[0065] In one example, enteric microparticles were prepared from modified PEG-PLGA-NP and Eudragit® L 100 using DN 30%. The formulation was characterized as described above. The microparticles could be reconstituted homogeneously in 0.1 M HCl, while the PEG-PLGA-NP were released at pH 6.8 with an acceptable increase of the mean particle size and only a minor broadening of the particle size distribution (Table 4).

Table 4: Properties of nanoparticle-releasing enteric microparticle formulations prepared from Eudragit® L 100 and modified PEG-PLGA-NP.

Degree of Neutralization	Dispersibility in 0.1 M HCl	Z-av	PDI
Before spray drying		230 nm	0.13
30%	+	325 nm	0.19

In-vitro release of NP from the enteric microparticles

[0066] To study the enteric properties of the formulation, 20 mg enteric microparticles were homogeneously dispersed in 10 mL 0.1 N HCl. The mean particle size was measured by dynamic light scattering while incrementally raising the pH by addition of NaOH. As expected, particle size drastically decreases above pH 6, indicating the dissolution of the enteric microparticles and the release of the PLGA nanoparticles (see Fig. 2 showing pH titration vs. particle size of nanoparticle-releasing enteric microparticles prepared with DN 15%).

Claims

1. Process for the preparation of enteric microparticles comprising nanoparticles, wherein the nanoparticles comprise a matrix and an active ingredient, such process comprises (i) spray-drying of a suspension of the nanoparticles in a colloidal dispersion of the enteric coating material or (ii) co-spray-drying of a suspension of nanoparticles and a colloidal dispersion of the enteric coating material.

2. Process according to Claim 1, comprising the steps

   (a) preparing an aqueous dispersion comprising an enteric coating material;
   (b) adjusting the pH of the aqueous dispersion prepared by step (a) to a pH slightly below the solubility threshold of the enteric coating material to produce a colloidal dispersion of the enteric coating material;
   (c) mixing the nanoparticles with the colloidal dispersion prepared by step (b) to produce a suspension of the nanoparticles in such colloidal dispersion;
   (d) spray-drying the colloidal dispersion prepared by step (c).

3. Process according to Claim 1, comprising the steps

   (a) preparing an aqueous dispersion comprising an enteric coating material;
   (b) adjusting the pH of the aqueous dispersion prepared by step (a) to a pH slightly below the solubility threshold of the enteric coating material to produce a colloidal dispersion of the enteric coating material;
   (c) preparing an aqueous suspension comprising the nanoparticles;
   (d) co-spray-drying of the colloidal dispersion prepared by step (b) together with the aqueous suspension prepared by step (c).

4. Process according to Claim 2 or 3, **characterized in that** the colloidal dispersion obtained in step (b) has a degree of neutralization (DN) of 5 to 40%, preferably 1 to 30%, more preferably 12 to 25% and most preferably about 15%.

5. Process according to Claim 2 or 3, **characterized in that** the pH is adjusted with a pH increasing agent, preferably with NaOH, KOH, carbonates or hydrogencarbonates of alkali metals, ammonium carbonate, ammonium hydrogencarbonate, or NH_3, more preferably with NH_3.

6. Process according to one or more of Claims 1 to 5, **characterized in that** the nanoparticles used in the process have a mean size from 20 nm to 1000 nm, preferably from 100 nm to 500 nm, and more preferably from 200 nm to 300 nm.

7. Process according to one or more of Claims 1 to 6, **characterized in that** the matrix of the nanoparticles is a biocompatible anorganic particulate material such as silica, surface-modified silica or a biocompatible organic polymer, preferably a biodegradable polymer.

8. Process according to Claim 7, **characterized in that** the biodegradable polymer is poly(lactic acid) (PLA), poly(glycolic acid) (PGA), polycaprolactone (PCL), a copolymer of lactic acid and glycolic acid (PLGA), a copolymer of lactic acid and caprolactone, polyepsilon caprolactone, polyhydroxy butyric acid, chitosan, a polyester, a poly(ortho)ester, a polyurethane, a polyanhydride, a polyacetal, a polydihydropyran, a polyamide, a polysaccharide or a polycyanoacrylate, blends or copolymers thereof or a derivative thereof such as pegylated polymers like PEG-PLGA.

9. Process according to Claim 8 **characterized in that** the biodegradable polymer is PLGA.

10. Process according to one or more of Claim 1 to 9, **characterized in that** the enteric coating material is cellulose acetate phthalate, hydroxypropyl methylcellulose phthalate, hydroxypropyl methylcellulose acetate succinate, polyvinyl acetate phthalate, carboxymethyl ethylcellulose, cellulose acetate trimellitate, a copolymer of acrylic or methacrylic acid and an acrylic or methacrylic ester, especially a copolymer of methacrylic acid and a methacrylate or a acrylate ester.

11. Process according to Claim 10, **characterized in that** the copolymer of methacrylic acid and a methacrylate or acrylate ester is (Poly(methacrylic acid-co-methyl methacrylate) (1:1), (Poly(methacrylic acid-co-methyl methacrylate) (1:2), Poly(methacrylic acid-co-ethyl acrylate) (1:1).

12. Process according to one or more of Claims 1 to 11, **characterized in that** the microparticles have a mean size of 1 μm to 200 μm, preferably of 10 μm to 150 μm and more preferably of 50 μm to 150 μm.

13. Process according to one or more of Claims 1 to 11, **characterized in that** the product temperature during the spray drying process is below the glass transition temperature of the nanoparticles.

Patentansprüche

1. Verfahren zur Herstellung von Nanopartikel enthaltenden magensaftresistenten Mikropartikeln, worin die Nanopartikel eine Matrix und einen Wirkstoff enthalten, wobei das Verfahren umfasst (i) Sprühtrocknen einer Suspension der Nanopartikel in einer kolloidalen Dispersion des magensaftresistenten Überzugsmaterials oder (ii) Co-Sprühtrocknen einer Suspension von Nanopartikeln und einer kolloidalen Dispersion des magensaftresistenten Überzugsmaterials.

2. Verfahren nach Anspruch 1, mit den Schritten

   (a) Herstellen einer ein magensaftresistentes Überzugsmaterial enthaltenden wässrigen Dispersion;
   (b) Einstellen des pH-Wertes der durch Schritt (a) hergestellten wässrigen Dispersion auf einen pH etwas unterhalb der Löslichkeitsschwelle des magensaftresistenten Überzugsmaterials, um eine kolloidale Dispersion des magensaftresistenten Überzugsmaterials zu erzeugen;
   (c) Mischen der Nanopartikel mit der durch Schritt (b) hergestellten kolloidalen Dispersion, um eine Suspension der Nanopartikel in einer solchen kolloidalen Dispersion zu erzeugen;
   (d) Sprühtrocknen der durch Schritt (c) hergestellten kolloidalen Dispersion.

3. Verfahren nach Anspruch 1, mit den Schritten

   (a) Herstellen einer ein magensaftresistentes Überzugsmaterial enthaltenden wässrigen Dispersion;
   (b) Einstellen des pH-Wertes der durch Schritt (a) hergestellten wässrigen Dispersion auf einen pH etwas unterhalb der Löslichkeitsschwelle des magensaftresistenten Überzugsmaterials, um eine kolloidale Dispersion des magensaftresistenten Überzugsmaterials zu erzeugen;
   (c) Herstellen einer die Nanopartikel enthaltenden wässrigen Suspension;
   (d) Co-Sprühtrocknen der durch Schritt (b) hergestellten kolloidalen Dispersion zusammen mit der durch Schritt (c) hergestellten wässrigen Suspension.

4. Verfahren nach Anspruch 2 oder 3, **dadurch gekennzeichnet, dass** die in Schritt (b) erhaltene kolloidale Dispersion

einen Neutralisationsgrad (DN) von 5 bis 40%, vorzugsweise 1 bis 30%, besonders bevorzugt 12 bis 25% und insbesondere bevorzugt etwa 15% aufweist.

5. Verfahren nach Anspruch 2 oder 3, **dadurch gekennzeichnet, dass** der pH-Wert mit einem pH-erhöhenden Mittel, vorzugsweise mit NaOH, KOH, Carbonaten oder Hydrogencarbonaten von Alkalimetallen, Ammoniumcarbonat, Ammoniumhydrogencarbonat oder NH_3, besonders bevorzugt mit NH_3, eingestellt wird.

6. Verfahren nach einem oder mehreren der Ansprüche 1 bis 5, **dadurch gekennzeichnet, dass** die im Verfahren verwendeten Nanopartikel eine mittlere Größe von 20 nm bis 1000 nm, vorzugsweise von 100 nm bis 500 nm und besonders bevorzugt von 200 nm bis 300 nm aufweisen.

7. Verfahren nach einem oder mehreren der Ansprüche 1 bis 6, **dadurch gekennzeichnet, dass** es sich bei der Matrix der Nanopartikel um eine biokompatibles anorganisches teilchenförmiges Material wie Siliciumdioxid, oberflächenmodifiziertes Siliciumdioxid oder ein biokompatibles organisches Polymer, vorzugsweise ein bioabbaubares Polymer, handelt.

8. Verfahren nach Anspruch 7, **dadurch gekennzeichnet, dass** es sich bei dem bioabbaubaren Polymer um Poly(milchsäure) (PLA), Poly(glykolsäure) (PGA), Polycaprolacton (PCL), ein Copolymer aus Milchsäure und Glykolsäure (PLGA), ein Copolymer aus Milchsäure und Caprolacton, polyepsilon-Caprolacton, Polyhydroxybuttersäure, Chitosan, einen Polyester, einen Poly(ortho)ester, ein Polyurethan, ein Polyanhydrid, ein Polyacetal, ein Polydihydropyran, ein Polyamid, ein Polysaccharid oder ein Polycyanoacrylat, Blends oder Copolymere davon oder ein Derivat davon, wie pegylierte Polymere wie PEG-PLGA, handelt.

9. Verfahren nach Anspruch 8, **dadurch gekennzeichnet, dass** es sich bei dem bioabbaubaren Polymer um PLGA handelt.

10. Verfahren nach einem oder mehreren der Ansprüche 1 bis 9, **dadurch gekennzeichnet, dass** es sich bei dem magensaftresistenten Überzugsmaterial um Celluloseacetat-phthalat, Hydroxypropyl-methylcellulosephthalat, Hydroxypropyl-methylcellulose-acetat-succinat, Polyvinylacetatphthalat, Carboxymethyl-ethylcellulose, Celluloseacetat-trimellitat, ein Copolymer aus Acryl- oder Methacrylsäure und einem Acryl- oder Methacrylsäureester, insbesondere ein Copolymer aus Methacrylsäure und einem Methacryl- oder einem Acrylsäureester handelt.

11. Verfahren nach Anspruch 10, **dadurch gekennzeichnet, dass** es sich bei dem Copolymer von Methacrylsäure und einem Methacryl- oder Acrylsäureester um (Poly(methacrylsäure-co-methylmethacrylat) (1:1), (Poly-(methacrylsäure-co-methylmethacrylat) (1:2), Poly(methacrylsäure-co-ethylacrylat) (1:1) handelt.

12. Verfahren nach einem oder mehreren der Ansprüche 1 bis 11, **dadurch gekennzeichnet, dass** die Mikropartikel eine mittlere Größe von 1 μm bis 200 μm, vorzugsweise von 10 μm bis 150 μm und besonders bevorzugt von 50 μm bis 150 μm aufweisen.

13. Verfahren nach einem oder mehreren der Ansprüche 1 bis 11, **dadurch gekennzeichnet, dass** die Produkttemperatur während des Sprühtrocknungsverfahrens unterhalb der Glasübergangstemperatur der Nanopartikel liegt.

Revendications

1. Procédé de préparation de microparticules entériques comprenant des nanoparticules, où les nanoparticules comprennent une matrice et un ingrédient actif, un tel procédé comprend (i) le séchage par pulvérisation d'une suspension des nanoparticules dans une dispersion colloïdale du matériau de revêtement entérique ou (ii) le co-séchage par pulvérisation d'une suspension de nanoparticules et d'une dispersion colloïdale du matériau de revêtement entérique.

2. Procédé selon la revendication 1, comprenant les étapes

   (a) de préparation d'une dispersion aqueuse comprenant un matériau de revêtement entérique ;
   (b) d'ajustement du pH de la dispersion aqueuse préparée dans l'étape (a) jusqu'à un pH légèrement inférieur au seuil de solubilité du matériau de revêtement entérique afin de produire une dispersion colloïdale du matériau de revêtement entérique ;

(c) le mélange des nanoparticules avec la dispersion colloïdale préparée dans l'étape (b) afin de produire une suspension des nanoparticules dans une telle dispersion colloïdale ;
(d) le séchage par pulvérisation de la dispersion colloïdale préparée dans l'étape (c).

3. Procédé selon la revendication 1, comprenant les étapes

(a) de préparation d'une dispersion aqueuse comprenant un matériau de revêtement entérique ;
(b) d'ajustement du pH de la dispersion aqueuse préparée dans l'étape (a) jusqu'à un pH légèrement inférieur au seuil de solubilité du matériau de revêtement entérique afin de produire une dispersion colloïdale du matériau de revêtement entérique ;
(c) de préparation d'une suspension aqueuse comprenant les nanoparticules ;
(d) de co-séchage par pulvérisation de la dispersion colloïdale préparée dans l'étape (b) conjointement avec la suspension aqueuse préparée dans l'étape (c).

4. Procédé selon la revendication 2 ou 3, **caractérisé en ce que** la dispersion colloïdale obtenue dans l'étape (b) possède un degré de neutralisation (DN) allant de 5 à 40%, préférablement de 1 à 30%, plus préférablement de 12 à 25% et tout préférablement d'environ 15%.

5. Procédé selon la revendication 2 ou 3, **caractérisé en ce que** le pH est ajusté à l'aide d'un agent d'augmentation du pH, préférablement à l'aide de NaOH, de KOH, de carbonates ou d'hydrogénocarbonates de métaux alcalins, de carbonate d'ammonium, d'hydrogénocarbonate d'ammonium, ou de NH_3, plus préférablement de NH_3.

6. Procédé selon l'une ou plusieurs parmi les revendications 1 à 5, **caractérisé en ce que** les nanoparticules utilisées dans le procédé possèdent une taille moyenne allant de 20 nm à 1000 nm, préférablement de 100 nm à 500 nm, et plus préférablement de 200 nm à 300 nm.

7. Procédé selon l'une ou plusieurs parmi les revendications 1 à 6, **caractérisé en ce que** la matrice des nanoparticules est constituée d'un matériau particulaire anorganique biocompatible tel que la silice, la silice à surface modifiée ou un polymère organique biocompatible, préférablement un polymère biodégradable.

8. Procédé selon la revendication 7, **caractérisé en ce que** le polymère biodégradable est le poly(acide lactique) (PLA), le poly(acide glycolique) (PGA), la polycaprolactone (PCL), un copolymère d'acide lactique et d'acide glycolique (PLGA), un copolymère d'acide lactique et de caprolactone, la poly-epsilon-caprolactone, l'acide polyhydroxybutyrique, un chitosane, un polyester, un poly(ortho)ester, un polyuréthane, un polyanhydride, un polyacétal, un polydihydropyrane, un polyamide, un polysaccharide ou un polycyanoacrylate, des mélanges ou des copolymères de ceux-ci ou un dérivé de ceux-ci tel que les polymères pégylés comme le PEG-PLGA.

9. Procédé selon la revendication 8, **caractérisé en ce que** le polymère biodégradable est le PLGA.

10. Procédé selon l'une ou plusieurs parmi les revendications 1 à 9, **caractérisé en ce que** le matériau de revêtement entérique est l'acétate-phtalate de cellulose, le phtalate d'hydroxypropyl méthylcellulose, l'acétate-succinate d'hydroxypropyl méthylcellulose, l'acétate-phtalate de polyvinyle, la carboxyméthyl éthylcellulose, l'acétate-trimellitate de cellulose, un copolymère d'acide acrylique ou méthacrylique et d'un ester acrylique ou méthacrylique, notamment un copolymère d'acide méthacrylique et d'un ester de méthacrylate ou d'acrylate.

11. Procédé selon la revendication 10, **caractérisé en ce que** le copolymère d'acide méthacrylique et d'un ester de méthacrylate ou d'acrylate est le (poly(acide méthacrylique-co-méthacrylate de méthyle) (1:1), le (poly-(acide méthacrylique-co-méthacrylate de méthyle) (1:2), le poly(acide méthacrylique-co-acrylate d'éthyle) (1:1).

12. Procédé selon l'une ou plusieurs parmi les revendications 1 à 11, **caractérisé en ce que** les microparticules possèdent une taille moyenne allant de 1 μm à 200 μm, préférablement de 10 μm à 150 μm et plus préférablement de 50 μm à 150 μm.

13. Procédé selon l'une ou plusieurs parmi les revendications 1 à 11, **caractérisé en ce que** la température de produit lors du procédé de séchage par pulvérisation est inférieure à la température de transition vitreuse des nanoparticules.

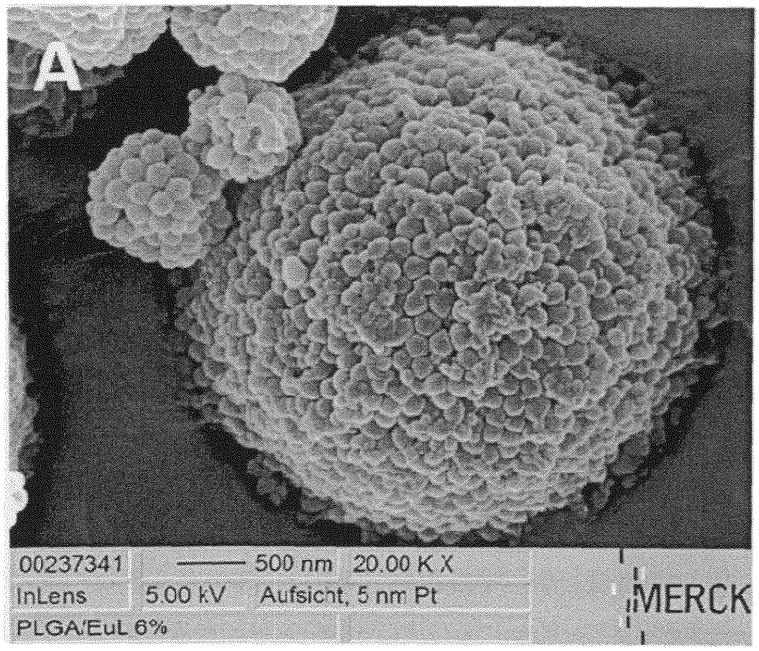

Figure 1A

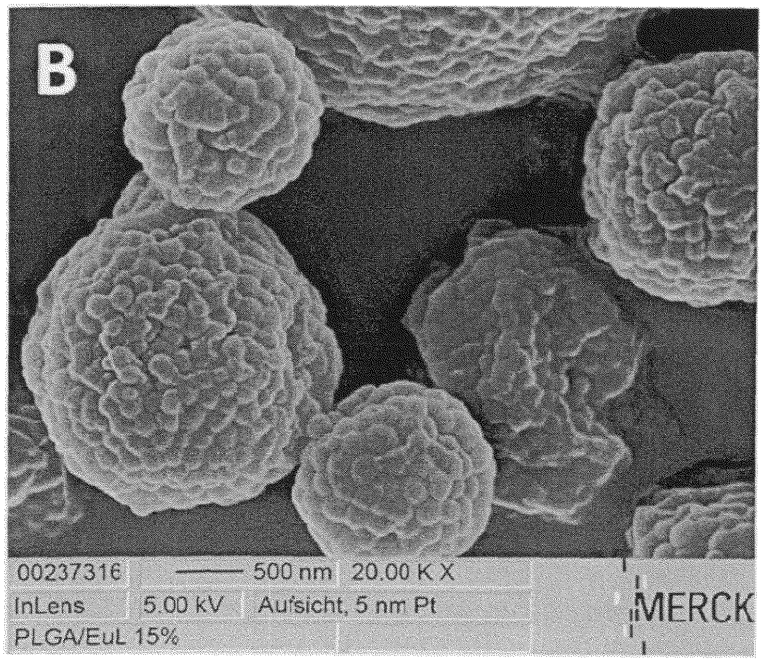

Figure 1B

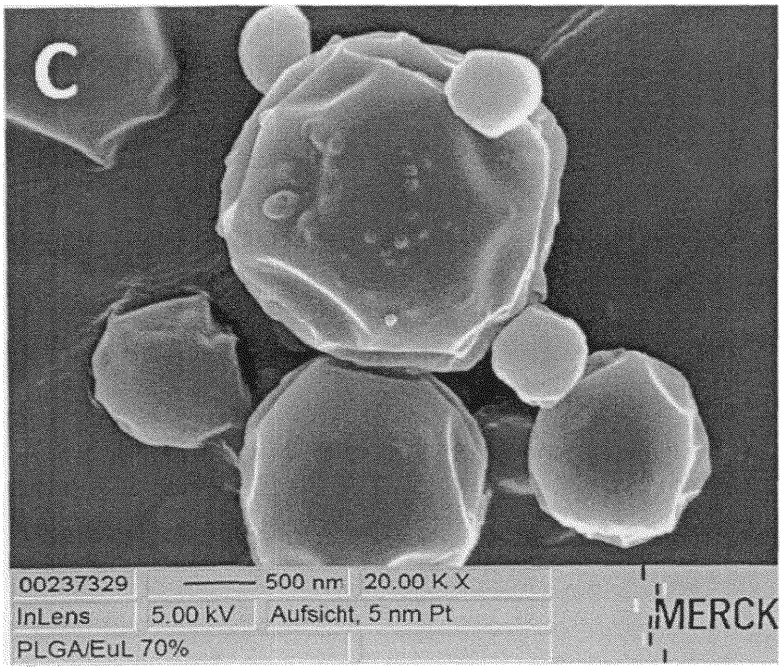
C
00237329
500 nm
20.00 K X
InLens
5.00 kV
Aufsicht, 5 nm Pt
PLGA/EuL 70%
MERCK

Figure 1C

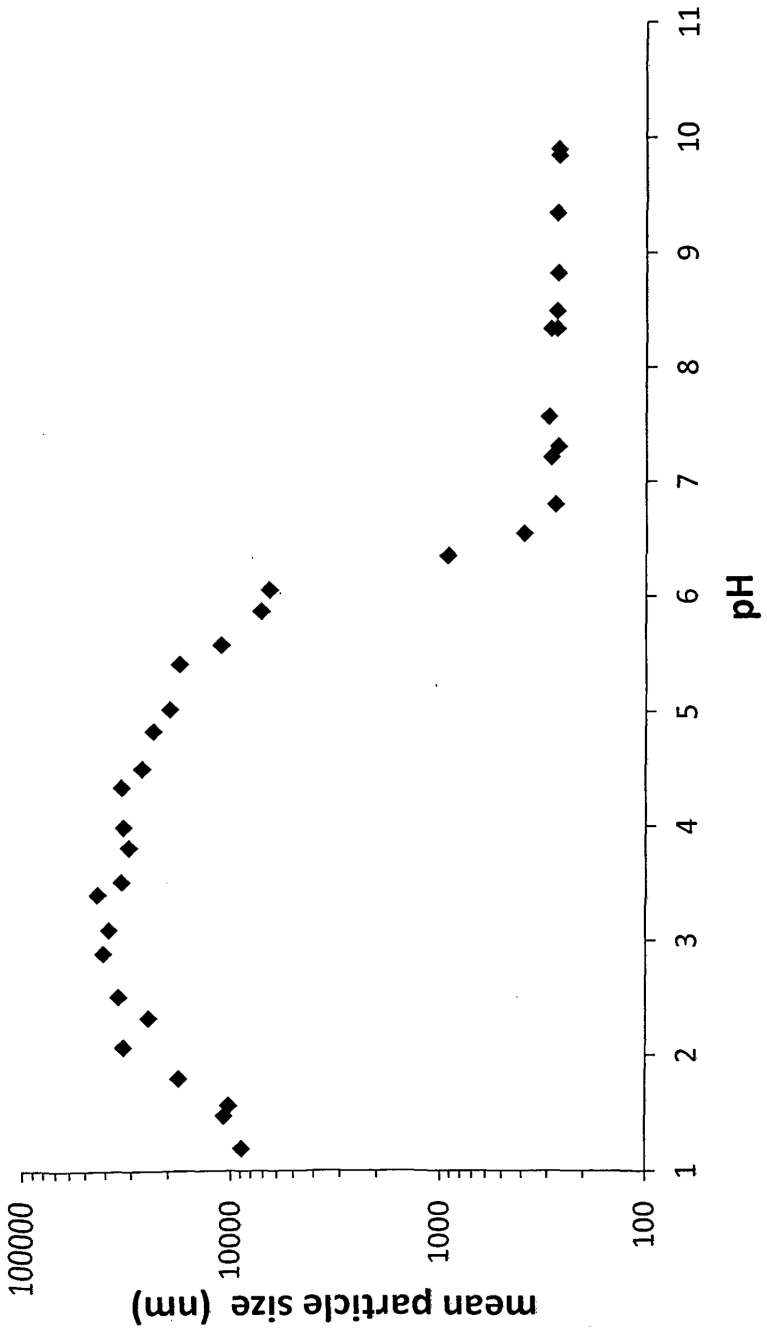
mean particle size (nm)
100000
10000
1000
100
1
2
3
4
5
6
7
8
9
10
11
pH

Figure 2

REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

Patent documents cited in the description

- EP 0216278 B1 **[0049]**

Non-patent literature cited in the description

- **KRISHNAMACHARI, Y. et al.** Development of pH- and time-dependent oral microparticles to optimize budesonide delivery to ileum and colon. *International Journal of Pharmaceutics,* 2007, vol. 338 (1-2), 238-247 **[0004]**
- **NASSAR, T. ; 2011 et al.** High plasma levels and effective lymphatic uptake of docetaxel in an orally available nanotransporter formulation. *Cancer Research,* vol. 71 (8), 3018-3028 **[0006]**
- IUPAC Compendium of Chemical Terminology. 1972, vol. 31, 605 **[0017]**
- **K. MASTERS.** Spray-drying Handbook. John Wiley & Sons, 1984 **[0024]**
- **BLANCO, M.D. et al.** Development and characterization of protein-loaded poly(lactide-co-glycolide) nanospheres. *Eur J Pharm Biopharm,* 1997, vol. 43 (3), 287-294 **[0047]**
- **GRENHA, A.** Chitosan nanoparticles: a survey of preparation methods. *Journal of drug targeting,* 2012, vol. 20 (4), 291-300 **[0048]**
- **KRANZ, H. et al.** Physicomechanical properties of biodegradable poly(D,L-lactide) and poly(D,L-lactide-co-glycolide) films in the dry and wet states. *Journal of Pharmaceutical Sciences,* 2000, vol. 89 (12), 2899-2605 **[0052]**

5. Overall conclusion and perspective

Translating nanomedicines from bench to bedside is a major challenge for their commercialization. Processes that are easily scalable and produce similar results for small scale feasibility trials and commercial manufacturing are of special interest. The aim of this thesis was to develop a scalable process to manufacture antigen-loaded polymeric nanoparticles; to manufacture sufficient quantities of nanoparticle suspensions for the development of downstream processes; and to develop a suitable downstream process for nanoparticle suspensions to a solid dosage form.

The first study of this thesis reported for the first time a modified double emulsion and solvent evaporation method to produce PLGA nanoparticles that were loaded with ovalbumin as model antigen. The emulsion step relies on a contact-less focused ultrasound transducer that is not in contact with the product, thereby eliminating the risk of contamination by abrasion from the tip of an ultrasonic probe sonicator, reducing the cleaning effort, and reducing the risk of cross-contamination between different batches manufactured on the same equipment. Nanoparticle size could be controlled between 100-200 nm with a yield of up to 74% and an antigen loading of up to 3.6% mass. Sonication induced a slight fragmentation of ovalbumin that was found acceptable at relevant incident energy. The loading efficiency of up to 38% is higher than previously published on double emulsion methods using probe sonication. Still, this is comparatively low for commercial processes using costly polymers and antigens, and further optimization would be highly desirable. Nanoparticles were manufactured in batch sizes from 1 mg to 2500 mg in batch mode or quasi-continuous mode, suitable for early screening as well as to supply process development studies of downstream methods. Similar nanoparticle characteristics were found over this range of batch sizes, although the efficiency of the energy transfer was found to be higher, meaning a lower incident energy per unit volume was necessary to produce nanoparticles of a given size in the flow cell compared to the small-scale glass vials. A direct proportionality of the incident energy to the batch volume was found for different volumes using the flow cell. The correlation between incident energy and nanoparticle size and between incident energy and nanoparticle yield could be described mathematically for all batch sizes. A correction factor between scales is generally acceptable, but the predictive power for important parameters like protein integrity may be limited. Finding a setup with direct proportionality of the incident energy to the batch volume across the whole scale would be ideal, and further research into the design of flow cells and vials for screening is necessary to achieve a similar energy efficiency. A readily scalable cross-flow diafiltration method was established for nanoparticle purification. The used hollow-fiber modules are commercially available for batch sizes of 1 mL to 100 L with the same hydrodynamics, allowing for scale-up without further process development. Diafiltration was also

found to considerably improve nanoparticle yield compared to centrifuge purification, especially for smaller particles.

A second study investigated the feasibility of a two-step continuous production of a nanoparticles-containing powder. The first step in this process was performed with the focused ultrasound double emulsion method developed in the first study. The second step is a simultaneous nanoparticle formation and drying step. For this step, different stabilizers and matrix excipients were screened for resulting nanoparticle size and yield. The use of a single batch of double emulsion to investigate the effect of different formulations allowed for superior comparability between individual experiments. A single large batch was also preferrable compared to batch pooling because of a narrower and monomodal particle size distribution. The study showed that the simultaneous nanoparticle formation and drying can result in a comparable size distribution as the drying of preformed nanoparticles. The described method considerably increases processing efficiency by reducing the number of unit operations or by reducing the volume of the spray drying feed. The nanoparticle yield of up to 79% was found to be economically interesting and considerably higher than using focused ultrasound followed by solvent displacement as described in the first study. The described method is limited in applicability because excess stabilizer and unencapsulated protein are not removed from the formulation. A purification of the continuous phase of the double emulsion before drying was not considered but might be feasible continuously or even in-line with column chromatography. Further research would be required to ensure emulsion stability and to prevent protein extraction from the inner aqueous phase. The investigation did not show a completely integrated line of emulsification and spray drying where the throughput of both steps are matched to each other. Also, the continuous addition of the excipients for spray drying while avoiding dilution of the spray feed remains an unsolved challenge.

The third study of this thesis reports the encapsulation of polymeric nanoparticles into plasticizer-free enteric microparticles by spray drying. This study was again made possible by preparing sufficient amounts of nanoparticles using the method described in the first study. In an improvement to the established method, the throughput could be improved by a low-viscosity spray feed that was achieved by suspending the nanoparticles in a colloidal dispersion of Eudragit® L. A functional enteric matrix was achieved without the addition of a plasticizer, but by neutralizing the enteric polymer to a degree that enables film-formation out of individual particles of Eudragit, but that does not compromise the enteric shielding nor the redispersibility in an aqueous administration buffer. The possibility to omit plasticizers excludes a negative impact on glass transition temperature and storage stability by plasticizer migration into polymeric nanoparticles. The particulate nature of the enteric formulation may reduce pharmacokinetic variability, allows dosing of small animals like mice, and allows easy dose

adjustment to the body weight. The dosage form is suitable for veterinary medications, but less common for human medications except for extemporaneous preparations or pediatrics. More research would be necessary to develop a typical oral dosage form like a capsule or a tablet: to optimize powder flowability for dose uniformity in volumetric capsule filling, sachet filling, or tableting processes, or to investigate the tabletability without compromising the enteric property or the primary nanoparticles.

In this thesis, several methods for the preparation of nanoparticles and the downstream processing into solid dosage forms were developed. The methods were easy to combine into quasi-continuous processes, but the technical feasibility of a fully integrated manufacturing line remains to be shown. The studies were limited in the choice of polymers and the model antigen. Additional research is necessary to demonstrate the applicability of the methods for polymers and proteins with different physico-chemical properties; to formulate therapeutically relevant antigens and to ensure retained antigenicity; and, ultimately, to prove the functionality of the developed dosage forms for oral vaccination.

6. References

1. Alexander JW. History of the medical use of silver. Surg Infect (Larchmt). 2009;10(3):289-92.
2. Chalk SJ. IUPAC Compendium of Chemical Terminology, 2nd ed. (the "Gold Book"). 2019 [cited 2021 July 07]. Available from: https://goldbook.iupac.org/terms/view/C01172.
3. le Chatelier H. Crystalloids against colloids in the theory of cements. Transactions of the Faraday Society. 1919;14(0):8-11.
4. Mokrushin SG. Thomas Graham and the Definition of Colloids. Nature. 1962;195(4844):861.
5. Ruska E. The Development of the Electron Microscope and of Electron Microscopy (Nobel Lecture). Angewandte Chemie International Edition in English. 1987;26(7):595-605.
6. Freundlich MM. Origin of the Electron Microscope. Science. 1963;142(3589):185-8.
7. Hauser EA, Lynn JE. Experiments in Colloid Chemistry. New York: McGraw-Hill; 1940.
8. Turkevich J, Stevenson PC, Hillier J. A study of the nucleation and growth processes in the synthesis of colloidal gold. Discuss Faraday Soc. 1951;11(0):55-75.
9. Frens G. Controlled Nucleation for the Regulation of the Particle Size in Monodisperse Gold Suspensions. Nature Physical Science. 1973;241(105):20-2.
10. Wuithschick M, Birnbaum A, Witte S, Sztucki M, Vainio U, Pinna N, Rademann K, Emmerling F, Kraehnert R, Polte J. Turkevich in New Robes: Key Questions Answered for the Most Common Gold Nanoparticle Synthesis. ACS Nano. 2015;9(7):7052-71.
11. Stöber W, Fink A, Bohn E. Controlled growth of monodisperse silica spheres in the micron size range. J Colloid Interface Sci. 1968;26(1):62-9.
12. Feynman R. There's plenty of room at the bottom. Engineering and Science. 1960;23(5):22-36.
13. Binnig G, Quate CF, Gerber C. Atomic Force Microscope. Phys Rev Lett. 1986;56(9):930-3.
14. Eigler DM, Schweizer EK. Positioning single atoms with a scanning tunnelling microscope. Nature. 1990;344(6266):524-6.
15. Newman TH, Williams KE, Pease RFW. High resolution patterning system with a single bore objective lens. Journal of Vacuum Science & Technology B: Microelectronics Processing and Phenomena. 1987;5(1):88-91.
16. Bennewitz R, Crain JN, Kirakosian A, Lin JL, McChesney JL, Petrovykh DY, Himpsel FJ. Atomic scale memory at a silicon surface. Nanotechnology. 2002;13(4):499-502.
17. Toumey C. Plenty of room, plenty of history. Nature Nanotechnology. 2009;4(12):783-4.
18. 'Plenty of room' revisited. Nature Nanotechnology. 2009;4(12):781.
19. Toumey C. Apostolic Succession. Engineering and Science. 2005;68(1):16-23.
20. Toumey C. Reading Feynman into nanotechnology: A text for a new science. Techné: Research in Philosophy and Technology. 2008;12(3):133-68.
21. Kroto HW, Heath JR, O'Brien SC, Curl RF, Smalley RE. C60: Buckminsterfullerene. Nature. 1985;318(6042):162-3.
22. Iijima S. Helical microtubules of graphitic carbon. Nature. 1991;354(6348):56-8.
23. Novoselov KS, Geim AK, Morozov SV, Jiang D, Zhang Y, Dubonos SV, Grigorieva IV, Firsov AA. Electric Field Effect in Atomically Thin Carbon Films. Science. 2004;306(5696):666-9.
24. Ekimov A, Onushchenko A, Tsekhomskii V. Exciton absorption by copper (I) chloride crystals in a glassy matrix. Fizika i Khimiya Stekla. 1980;6(4):511-2.
25. Brus LE. A simple model for the ionization potential, electron affinity, and aqueous redox potentials of small semiconductor crystallites. The Journal of Chemical Physics. 1983;79(11):5566-71.
26. Efros AL, Brus LE. Nanocrystal Quantum Dots: From Discovery to Modern Development. ACS Nano. 2021;15(4):6192-210.
27. Dietrich-Buchecker CO, Sauvage JP, Kintzinger JP. Une nouvelle famille de molecules : les metallo-catenanes. Tetrahedron Lett. 1983;24(46):5095-8.
28. Anelli PL, Spencer N, Stoddart JF. A molecular shuttle. J Am Chem Soc. 1991;113(13):5131-3.
29. Koumura N, Zijlstra RWJ, van Delden RA, Harada N, Feringa BL. Light-driven monodirectional molecular rotor. Nature. 1999;401(6749):152-5.

30. Joubert IA, Geppert M, Ess S, Nestelbacher R, Gadermaier G, Duschl A, Bathke AC, Himly M. Public perception and knowledge on nanotechnology: A study based on a citizen science approach. NanoImpact. 2020;17:100201.
31. Royal Society and Royal Academy of Engineering. Nanoscience and nanotechnologies: opportunities and uncertainties. 30 July 2004. Available from: https://royalsociety.org/-/media/Royal_Society_Content/policy/publications/2004/9693.pdf.
32. Taniguchi N. On the basic concept of nanotechnology. Proceedings of the International Congress on Prod Eng. Tokyo: JSPE; 1974.
33. International Organization for Standardization. Nanotechnologies - Vocabulary - Part 1: Core terms. December 2015. Document No.: ISO/TS 80004-1:2015. Available from: https://www.iso.org/standard/68058.html.
34. International Organization for Standardization. Nanotechnologies - Vocabulary - Part 2: Nano-objects. June 2015. Document No.: ISO/TS 80004-2:2015. Available from: https://www.iso.org/standard/54440.html.
35. Alemán JV, Chadwick AV, He J, Hess M, Horie K, Jones RG, Kratochvíl P, Meisel I, Mita I, Moad G, Penczek S, Stepto RFT. Definitions of terms relating to the structure and processing of sols, gels, networks, and inorganic-organic hybrid materials (IUPAC Recommendations 2007). Pure Appl Chem. 2007;79(10):1801-29.
36. Vert M, Doi Y, Hellwich K-H, Hess M, Hodge P, Kubisa P, Rinaudo M, Schué F. Terminology for biorelated polymers and applications (IUPAC Recommendations 2012). Pure Appl Chem. 2012;84(2):377-410.
37. United States Food and Drug Administration. Guidance for Industry: Considering Whether an FDA-Regulated Product Involves the Application of Nanotechnology. June 2014. Available from: https://www.fda.gov/media/88423/download.
38. European Commission. Commission recommendation of 18 October 2011 on the definition of nanomaterial. Document No.: 2011/696/EU. Available from: https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32011H0696&from=EN.
39. European Commission. Regulation (EC) No 1169/2011 of the European Parliament and of the Council of the European Union on the provision of food information to consumers. Available from: https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32011R1169&from=EN.
40. European Commission. Regulation (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products. Available from: https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:02009R1223-20210617&from=EN.
41. Rauscher H, Rasmussen K, Sokull-Klüttgen B. Regulatory Aspects of Nanomaterials in the EU. Chemie Ingenieur Technik. 2017;89(3):224-31.
42. Bremer-Hoffmann S, Halamoda-Kenzaoui B, Borgos SE. Identification of regulatory needs for nanomedicines. Journal of Interdisciplinary Nanomedicine. 2018;3(1):4-15.
43. Anhalt K. Oral nanocrystal formulations and their biopharmaceutical characterization [dissertation]. Heidelberg: Ruperto-Carola-University; 2012.
44. Müller RH, Shegokar R, Keck CM. 20 Years of Lipid Nanoparticles (SLN & NLC): Present State of Development & Industrial Applications. Current Drug Discovery Technologies. 2011;8(3):207-27.
45. Joint Research Centre of the European Commission. Whitepaper: Anticipation of regulatory needs for nanotechnology-enabled health products. November 27. Document No.: EUR 29919 EN. Available from: https://op.europa.eu/s/plTV.
46. European Medicines Agency, Committee for Medicinal Products for Human Use. Reflection paper on nanotechnology-based medicinal products for human use. June. Document No.: EMEA/CHMP/79769/2006. Available from: https://etp-nanomedicine.eu/wp-content/uploads/2018/10/reflection-paper-nanotechnology-based-medicinal-products-human-use_en-1.pdf.
47. European Medicines Agency, Committee for Medicinal Products for Human Use

Joint MHLW/EMA reflection paper on the data requirements for intravenous liposomal products developed with reference to an innovator liposomal product. February 2013. Document No.: EMA/CHMP/806058/2009/Rev. 02. Available from: https://www.ema.europa.eu/en/documents/scientific-guideline/reflection-paper-data-requirements-intravenous-liposomal-products-developed-reference-innovator_en.pdf.
48. European Medicines Agency. Reflection paper on surface coatings: general issues for consideration regarding parenteral administration of coated nanomedicine products. June 2013. Document No.: EMA/325027/2013. Available from: https://www.ema.europa.eu/en/documents/scientific-guideline/reflection-paper-surface-coatings-general-issues-consideration-regarding-parenteral-administration_en.pdf.
49. European Medicines Agency, Committee for Medicinal Products for Human Use. Joint MHLW/EMA reflection paper on the development of block copolymer micelle medicinal products. January 2014. Document No.: EMA/CHMP/13099/2013. Available from: https://www.ema.europa.eu/en/documents/scientific-guideline/joint-mhlw/ema-reflection-paper-development-block-copolymer-micelle-medicinal-products_en.pdf.
50. European Medicines Agency, Committee for Medicinal Products for Human Use. Reflection paper on the data requirements for intravenous iron-based nano-colloidal products developed with reference to an innovator medicinal product. March 2015. Document No.: EMA/CHMP/SWP/620008/2012. Available from: https://www.ema.europa.eu/en/documents/scientific-guideline/reflection-paper-data-requirements-intravenous-iron-based-nano-colloidal-products-developed_en.pdf.
51. United States Food and Drug Administration. Guidance for Industry: Safety of Nanomaterials in Cosmetic Products. June 2014. Available from: https://www.fda.gov/media/83957/download.
52. United States Food and Drug Administration. Guidance for Industry: Use of Nanomaterials in Food for Animals. August 2015. Available from: https://www.fda.gov/media/88828/download.
53. United States Food and Drug Administration. Draft Guidance: Drug Products, Including Biological Products that Contain Nanomaterials. December 2017. Available from: https://www.fda.gov/media/109910/download.
54. United States Food and Drug Administration. Guidance for Industry: Liposome Drug Products: Chemistry, Manufacturing, and Controls; Human Pharmacokinetics and Bioavailability; and Labeling Documentation. April 2018. Available from: https://www.fda.gov/media/70837/download.
55. Weber DO. Nanomedicine. Health Forum J. 1999;42(4):32.
56. Ehrlich P. Address in Pathology, ON CHEMIOTHERAPY: Delivered before the Seventeenth International Congress of Medicine. Br Med J. 1913;2(2746):353-9.
57. Marquardt M. Unterschied Zwischen Serumtherapie und Chemotherapie. In: Paul Ehrlich. Berlin, Heidelberg: Springer; 1951. p. 102-12.
58. Bangham AD. Physical Structure and Behavior of Lipids and Lipid Enzymes. In: Paoletti R, Kritchevsky D, editors. Adv Lipid Res. 1: Elsevier; 1963. p. 65-104.
59. Bangham AD, Horne RW. Negative staining of phospholipids and their structural modification by surface-active agents as observed in the electron microscope. J Mol Biol. 1964;8(5):660-IN10.
60. Bangham AD, Standish MM, Watkins JC. Diffusion of univalent ions across the lamellae of swollen phospholipids. J Mol Biol. 1965;13(1):238-52.
61. Bangham AD. Surrogate cells or trojan horses. The discovery of liposomes. Bioessays. 1995;17(12):1081-8.
62. Sessa G, Weissmann G. Phospholipid spherules (liposomes) as a model for biological membranes. J Lipid Res. 1968;9(3):310-8.
63. Gregoriadis G, Leathwood PD, Ryman BE. Enzyme entrapment in liposomes. FEBS Lett. 1971;14(2):95-9.
64. Birrenbach G. Ueber Mizellpolymerisate, mögliche Einschlussverbindungen (Nanokapseln) und deren Eignung als Adjuvantien: ETH Zürich; 1973.

65. Couvreur P. Nanoparticles in drug delivery: Past, present and future. Adv Drug Deliv Rev. 2013;65(1):21-3.
66. Birrenbach G, Speiser PP. Polymerized Micelles and Their Use as Adjuvants in Immunology. J Pharm Sci. 1976;65(12):1763-6.
67. Bader H, Ringsdorf H, Schmidt B. Watersoluble polymers in medicine. Die Angewandte Makromolekulare Chemie. 1984;123(1):457-85.
68. Jones M-C, Leroux J-C. Polymeric micelles – a new generation of colloidal drug carriers. Eur J Pharm Biopharm. 1999;48(2):101-11.
69. Gothwal A, Khan I, Gupta U. Polymeric Micelles: Recent Advancements in the Delivery of Anticancer Drugs. Pharm Res. 2016;33(1):18-39.
70. Gros L, Ringsdorf H, Schupp H. Polymeric Antitumor Agents on a Molecular and on a Cellular Level? Angewandte Chemie International Edition in English. 1981;20(4):305-25.
71. Vallet-Regi M, Rámila A, del Real RP, Pérez-Pariente J. A New Property of MCM-41: Drug Delivery System. Chem Mater. 2001;13(2):308-11.
72. Li Z, Mu Y, Peng C, Lavin MF, Shao H, Du Z. Understanding the mechanisms of silica nanoparticles for nanomedicine. WIREs Nanomedicine and Nanobiotechnology. 2021;13(1):e1658.
73. Sharma AK, Prasher P, Aljabali AA, Mishra V, Gandhi H, Kumar S, Mutalik S, Chellappan DK, Tambuwala MM, Dua K, Kapoor DN. Emerging era of “somes”: polymersomes as versatile drug delivery carrier for cancer diagnostics and therapy. Drug Delivery and Translational Research. 2020;10(5):1171-90.
74. Aurelia Chis A, Dobrea C, Morgovan C, Arseniu AM, Rus LL, Butuca A, Juncan AM, Totan M, Vonica-Tincu AL, Cormos G, Muntean AC, Muresan ML, Gligor FG, Frum A. Applications and Limitations of Dendrimers in Biomedicine. Molecules. 2020;25(17).
75. Bruchez M, Moronne M, Gin P, Weiss S, Alivisatos AP. Semiconductor Nanocrystals as Fluorescent Biological Labels. Science. 1998;281(5385):2013-6.
76. Chan WCW, Nie S. Quantum Dot Bioconjugates for Ultrasensitive Nonisotopic Detection. Science. 1998;281(5385):2016-8.
77. Wei Y, Quan L, Zhou C, Zhan Q. Factors relating to the biodistribution & clearance of nanoparticles & their effects on in vivo application. Nanomedicine. 2018;13(12):1495-512.
78. Gulati NM, Stewart PL, Steinmetz NF. Bioinspired Shielding Strategies for Nanoparticle Drug Delivery Applications. Mol Pharm. 2018;15(8):2900-9.
79. Lasic DD, Needham D. The "Stealth" Liposome: A Prototypical Biomaterial. Chem Rev. 1995;95(8):2601-28.
80. Wilhelm S, Tavares AJ, Dai Q, Ohta S, Audet J, Dvorak HF, Chan WCW. Analysis of nanoparticle delivery to tumours. Nature Reviews Materials. 2016;1(5):16014.
81. Lammers T, Kiessling F, Ashford M, Hennink W, Crommelin D, Storm G. Cancer nanomedicine: Is targeting our target? Nature reviews Materials. 2016;1(9).
82. Torrice M. Does Nanomedicine Have a Delivery Problem? ACS Central Science. 2016;2(7):434-7.
83. Akinc A, Maier MA, Manoharan M, Fitzgerald K, Jayaraman M, Barros S, Ansell S, Du X, Hope MJ, Madden TD, Mui BL, Semple SC, Tam YK, Ciufolini M, Witzigmann D, Kulkarni JA, van der Meel R, Cullis PR. The Onpattro story and the clinical translation of nanomedicines containing nucleic acid-based drugs. Nature Nanotechnology. 2019;14(12):1084-7.
84. Owais M, Gupta CM. Targeted Drug Delivery to Macrophages in Parasitic Infections. Current Drug Delivery. 2005;2(4):311-8.
85. Ortega V, Giorgio S, de Paula E. Liposomal formulations in the pharmacological treatment of leishmaniasis: a review. Journal of Liposome Research. 2017;27(3):234-48.
86. Shi Y, Lammers T. Combining Nanomedicine and Immunotherapy. Acc Chem Res. 2019;52(6):1543-54.

87. Sofias AM, Combes F, Koschmieder S, Storm G, Lammers T. A paradigm shift in cancer nanomedicine: from traditional tumor targeting to leveraging the immune system. Drug Discovery Today. 2021;26(6):1482-9.
88. Nguyen B, Tolia NH. Protein-based antigen presentation platforms for nanoparticle vaccines. npj Vaccines. 2021;6(1):70.
89. Coffman RL, Sher A, Seder RA. Vaccine adjuvants: putting innate immunity to work. Immunity. 2010;33(4):492-503.
90. HogenEsch H. Mechanism of Immunopotentiation and Safety of Aluminum Adjuvants. Front Immunol. 2013;3(406).
91. Clements CJ, Griffiths E. The global impact of vaccines containing aluminium adjuvants. Vaccine. 2002;20:S24-S33.
92. Principi N, Esposito S. Aluminum in vaccines: Does it create a safety problem? Vaccine. 2018;36(39):5825-31.
93. Jou J, Harrington KJ, Zocca M-B, Ehrnrooth E, Cohen EEW. The Changing Landscape of Therapeutic Cancer Vaccines—Novel Platforms and Neoantigen Identification. Clin Cancer Res. 2021;27(3):689-703.
94. Conry RM, Westbrook B, McKee S, Norwood TG. Talimogene laherparepvec: First in class oncolytic virotherapy. Hum Vaccin Immunother. 2018;14(4):839-46.
95. Baleeiro RB, Rietscher R, Diedrich A, Czaplewska JA, Lehr C-M, Scherließ R, Hanefeld A, Gottschaldt M, Walden P. Spatial separation of the processing and MHC class I loading compartments for cross-presentation of the tumor-associated antigen HER2/neu by human dendritic cells. OncoImmunology. 2015;4(11):e1047585.
96. Baleeiro RB, Schweinlin M, Rietscher R, Diedrich A, Czaplewska JA, Metzger M, Michael Lehr C, Scherließ R, Hanefeld A, Gottschaldt M, Walden P. Nanoparticle-based mucosal vaccines targeting tumor-associated antigens to human dendritic cells. Journal of Biomedical Nanotechnology. 2016;12(7):1527-43.
97. Rietscher R, Schröder M, Janke J, Czaplewska J, Gottschaldt M, Scherließ R, Hanefeld A, Schubert US, Schneider M, Knolle PA, Lehr CM. Antigen delivery via hydrophilic PEG-b-PAGE-b-PLGA nanoparticles boosts vaccination induced T cell immunity. Eur J Pharm Biopharm. 2016;102:20-31.
98. El-Sayed N, Korotchenko E, Scheiblhofer S, Weiss R, Schneider M. Functionalized multifunctional nanovaccine for targeting dendritic cells and modulation of immune response. Int J Pharm. 2021;593.
99. Lademann J, Richter H, Teichmann A, Otberg N, Blume-Peytavi U, Luengo J, Weiß B, Schaefer UF, Lehr CM, Wepf R, Sterry W. Nanoparticles - An efficient carrier for drug delivery into the hair follicles. Eur J Pharm Biopharm. 2007;66(2):159-64.
100. Mittal A, Raber AS, Schaefer UF, Weissmann S, Ebensen T, Schulze K, Guzm+ín CA, Lehr CM, Hansen S. Non-invasive delivery of nanoparticles to hair follicles: A perspective for transcutaneous immunization. Vaccine. 2013;31(34):3442-51.
101. Hansen S, Lehr CM. Transfollicular delivery takes root: The future for vaccine design? Expert Review of Vaccines. 2014;13(1):5-7.
102. Mittal A, Schulze K, Ebensen T, Weißmann S, Hansen S, Lehr CM, Guzmán CA. Efficient nanoparticle-mediated needle-free transcutaneous vaccination via hair follicles requires adjuvantation. Nanomed Nanotechnol Biol Med. 2015;11(1):147-54.
103. Mittal A, Schulze K, Ebensen T, Weissmann S, Hansen S, Guzmán CA, Lehr CM. Inverse micellar sugar glass (IMSG) nanoparticles for transfollicular vaccination. J Control Release. 2015;206:140-52.
104. El-Sayed N, Vaut L, Schneider M. Customized fast-separable microneedles prepared with the aid of 3D printing for nanoparticle delivery. Eur J Pharm Biopharm. 2020;154:166-74.
105. Hellfritzsch M, Scherließ R. Mucosal vaccination via the respiratory tract. Pharmaceutics. 2019;11(8).

106. Scherließ R, Mönckedieck M, Young K, Trows S, Buske S, Hook S. First in vivo evaluation of particulate nasal dry powder vaccine formulations containing ovalbumin in mice. Int J Pharm. 2015;479(2):408-15.
107. Regina Scherließ AD, Thomas Ebensen, Carlos A. Guzmán, Michael Wolf, Andrea Hanefeld. Chitosan Nanoparticulate Formulation for Pulmonary Vaccination – Formulation and In Vivo Proof of Concept. In: Dalby R, Byron, PR, Peart, J, Suman, JD, Young, PM and Traini, D, editor. RDD Europe 2015. 2. River Grove, IL, USA: Davis Healthcare International Publishing; 2015. p. 269-74.
108. Müller RH, Mäder K, Gohla S. Solid lipid nanoparticles (SLN) for controlled drug delivery – a review of the state of the art. Eur J Pharm Biopharm. 2000;50(1):161-77.
109. Speiser P, inventor; Dr. Rentschler Arzneimittel GmbH & Co, assignee. Lipid nano pellets as drug carriers for oral administration. European Patent EP0167825B1. 1990 August 08.
110. Lucks S, Müller RH, inventors; Medac Gesellschaft für klinische Spezialpräparate mbH, assignee. Arzneistoffträger aus festen Lipidteilchen, Feste Lipidnanosphären (SLN). European Patent EP0605497B1. 1996 March 20.
111. Gasco MR, inventor; Gasco, MR, assignee. Solid lipid microspheres having a narrow size distribution and method for producing them. European Patent EP0526666B1. 1996 April 17.
112. Scioli Montoto S, Muraca G, Ruiz ME. Solid Lipid Nanoparticles for Drug Delivery: Pharmacological and Biopharmaceutical Aspects. Frontiers in Molecular Biosciences. 2020;7(319).
113. Gandhi M, Yokoe DS, Havlir DV. Asymptomatic Transmission, the Achilles' Heel of Current Strategies to Control Covid-19. N Engl J Med. 2020;382(22):2158-60.
114. Centers for Disease Control and Prevention. Severe Acute Respiratory Syndrome (SARS) 2021 [cited 2021 July 16]. Available from: https://www.cdc.gov/sars/about/faq.html.
115. Our World in Data. Cumulative confirmed COVID-19 cases 2021 [cited 2021 July 16]. Available from: https://ourworldindata.org/grapher/cumulative-covid-cases-region.
116. Dong E, Du H, Gardner L. An interactive web-based dashboard to track COVID-19 in real time. The Lancet Infectious Diseases. 2020;20(5):533-4.
117. Our World in Data. Cumulative confirmed COVID-19 deaths 2021 [cited 2021 July 16]. Available from: https://ourworldindata.org/grapher/cumulative-covid-deaths-region.
118. Pardi N, Hogan MJ, Porter FW, Weissman D. mRNA vaccines — a new era in vaccinology. Nature Reviews Drug Discovery. 2018;17(4):261-79.
119. Mathieu E, Ritchie H, Ortiz-Ospina E, Roser M, Hasell J, Appel C, Giattino C, Rodés-Guirao L. A global database of COVID-19 vaccinations. Nature Human Behaviour. 2021;5(7):947-53.
120. Moderna Therapeutics. Moderna Announces First Participant Dosed in NIH-led Phase 1 Study of mRNA Vaccine (mRNA-1273) Against Novel Coronavirus [Press release]. 2020 [cited 2021 May 08]. Available from: https://investors.modernatx.com/news-releases/news-release-details/moderna-announces-first-participant-dosed-nih-led-phase-1-study/.
121. BioNTech SE. BioNTech and Pfizer announce completion of dosing for first cohort of Phase 1/2 trial of COVID-19 vaccine candidates in Germany [Press release]. 2020 [cited 2021 May 15]. Available from: https://investors.biontech.de/news-releases/news-release-details/biontech-and-pfizer-announce-completion-dosing-first-cohort/.
122. Ball P. The lightning-fast quest for COVID vaccines — and what it means for other diseases. Nature. 2021;589(7840):16-8.
123. Sahin U, Karikó K, Türeci Ö. mRNA-based therapeutics — developing a new class of drugs. Nature Reviews Drug Discovery. 2014;13(10):759-80.
124. Our World in Data. COVID-19 vaccine doses administered by manufacturer 2021 [cited 2021 May 13]. Available from: https://ourworldindata.org/grapher/covid-vaccine-doses-by-manufacturer.
125. Alnylam Pharmaceuticals. First Quarter 2020 Financial Results [Press release]. 2020 [cited 2021 May 15]. Available from: https://investors.alnylam.com/press-release?id=24811.

126. Guth F, Schiffter HA, Kolter K. Novel excipients-from concept to launch. Chimica Oggi - Chemistry Today. 2013;31(5):78-81.
127. Kingwell K. Excipient developers call for regulatory facelift. Nat Rev Drug Discov. 2020(19):823-4.
128. Couvreur P. (Poly-cyanoacrylate) nanomedicines for cancer and beyond: Lessons learned. J Control Release. 2021;334:318-26.
129. Couvreur P, Tulkenst P, Roland M, Trouet A, Speiser P. Nanocapsules: A new type of lysosomotropic carrier. FEBS Lett. 1977;84(2):323-6.
130. Couvreur P, Kante B, Roland M, Guiot P, Bauduin P, Speiser P. Polycyanoacrylate nanocapsules as potential lysosomotropic carriers: preparation, morphological and sorptive properties. J Pharm Pharmacol. 1979;31(1):331-2.
131. Peracchia MT, Vauthier C, Puisieux F, Couvreur P. Development of sterically stabilized poly(isobutyl 2-cyanoacrylate) nanoparticles by chemical coupling of poly(ethylene glycol). J Biomed Mater Res. 1997;34(3):317-26.
132. Zielińska A, Carreiró F, Oliveira AM, Neves A, Pires B, Venkatesh DN, Durazzo A, Lucarini M, Eder P, Silva AM, Santini A, Souto EB. Polymeric Nanoparticles: Production, Characterization, Toxicology and Ecotoxicology. Molecules. 2020;25(16):3731.
133. Schubert S, Delaney JJT, Schubert US. Nanoprecipitation and nanoformulation of polymers: from history to powerful possibilities beyond poly(lactic acid). Soft Matter. 2011;7(5):1581-8.
134. Fessi H, Puisieux F, Devissaguet JP, Ammoury N, Benita S. Nanocapsule formation by interfacial polymer deposition following solvent displacement. Int J Pharm. 1989;55(1):R1-R4.
135. Fessi H, Puisieux F, Devissaguet JP, inventors; Centre National de la Recherche Scientifique CNRS, assignee. Procédé de préparation de systèmes colloidaux dispersibles d'une substance, sous forme de nanocapsules. European Patent Application EP0274961A1. 1988 July 20.
136. Chacón M, Berges L, Molpeceres J, Aberturas MR, Guzman M. Optimized preparation of poly d,l (lactic-glycolic) microspheres and nanoparticles for oral administration. Int J Pharm. 1996;141(1):81-91.
137. Molpeceres J, Guzman M, Aberturas MR, Chacon M, Berges L. Application of central composite designs to the preparation of polycaprolactone nanoparticles by solvent displacement. J Pharm Sci. 1996;85(2):206-13.
138. Aubry J, Ganachaud F, Cohen Addad J-P, Cabane B. Nanoprecipitation of Polymethylmethacrylate by Solvent Shifting:1. Boundaries. Langmuir. 2009;25(4):1970-9.
139. Bodmeier R, Chen H, Tyle P, Jarosz P. Spontaneous formation of drug-containing acrylic nanoparticles. J Microencapsul. 1991;8(2):161-70.
140. Peracchia MT, Vauthier C, Desmaële D, Gulik A, Dedieu J-C, Demoy M, d'Angelo J, Couvreur P. Pegylated Nanoparticles from a Novel Methoxypolyethylene Glycol Cyanoacrylate-Hexadecyl Cyanoacrylate Amphiphilic Copolymer. Pharm Res. 1998;15(4):550-6.
141. Zhang H-z, Gao F-p, Liu L-r, Li X-m, Zhou Z-m, Yang X-d, Zhang Q-q. Pullulan acetate nanoparticles prepared by solvent diffusion method for epirubicin chemotherapy. Colloids and Surfaces B: Biointerfaces. 2009;71(1):19-26.
142. Hornig S, Biskup C, Gräfe A, Wotschadlo J, Liebert T, Mohr GJ, Heinze T. Biocompatible fluorescent nanoparticles for pH-sensoring. Soft Matter. 2008;4(6):1169-72.
143. Liebert T, Hornig S, Hesse S, Heinze T. Nanoparticles on the Basis of Highly Functionalized Dextrans. J Am Chem Soc. 2005;127(30):10484-5.
144. Hornig S, Heinze T. Efficient Approach To Design Stable Water-Dispersible Nanoparticles of Hydrophobic Cellulose Esters. Biomacromolecules. 2008;9(5):1487-92.
145. Tan Y, Xu K, Li L, Liu C, Song C, Wang P. Fabrication of Size-Controlled Starch-Based Nanospheres by Nanoprecipitation. ACS Applied Materials & Interfaces. 2009;1(4):956-9.
146. Bazile D, Prud'homme C, Bassoullet M-T, Marlard M, Spenlehauer G, Veillard M. Stealth Me.PEG-PLA nanoparticles avoid uptake by the mononuclear phagocytes system. J Pharm Sci. 1995;84(4):493-8.

147. Almoustafa HA, Alshawsh MA, Chik Z. Technical aspects of preparing PEG-PLGA nanoparticles as carrier for chemotherapeutic agents by nanoprecipitation method. Int J Pharm. 2017;533(1):275-84.
148. Hamaguchi T, Matsumura Y, Suzuki M, Shimizu K, Goda R, Nakamura I, Nakatomi I, Yokoyama M, Kataoka K, Kakizoe T. NK105, a paclitaxel-incorporating micellar nanoparticle formulation, can extend in vivo antitumour activity and reduce the neurotoxicity of paclitaxel. Br J Cancer. 2005;92(7):1240-6.
149. Govender T, Stolnik S, Garnett MC, Illum L, Davis SS. PLGA nanoparticles prepared by nanoprecipitation: Drug loading and release studies of a water soluble drug. J Control Release. 1999;57:171-85.
150. Lassalle V, Ferreira ML. PLA Nano- and Microparticles for Drug Delivery: An Overview of the Methods of Preparation. Macromol Biosci. 2007;7(6):767-83.
151. Lee EJ, Khan SA, Lim KH. Gelatin Nanoparticle Preparation by Nanoprecipitation. J Biomater Sci Polym Ed. 2011;22(4-6):753-71.
152. Khan SA, Schneider M. Improvement of nanoprecipitation technique for preparation of gelatin nanoparticles and potential macromolecular drug loading. Macromol Biosci. 2013;13(4):455-63.
153. Weiss A-V, Fischer T, Iturri J, Benitez R, Toca-Herrera JL, Schneider M. Mechanical properties of gelatin nanoparticles in dependency of crosslinking time and storage. Colloids and Surfaces B: Biointerfaces. 2019;175:713-20.
154. Iakobson OD, Dobrodumov AV, Saprykina NN, Shevchenko NN. Dextran Nanoparticles Cross-Linked in Aqueous and Aqueous/Alcoholic Media. Macromol Chem Phys. 2017;218(10):1600523.
155. Barthold S, Hittinger M, Primavessy D, Zapp A, Groß H, Schneider M. Preparation of maltodextrin nanoparticles and encapsulation of bovine serum albumin – Influence of formulation parameters. Eur J Pharm Biopharm. 2019;142:405-10.
156. Fuchs S, Kutscher M, Hertel T, Winter G, Pietzsch M, Coester C. Transglutaminase: New insights into gelatin nanoparticle cross-linking. J Microencapsul. 2010;27(8):747-54.
157. Baseer A, Koenneke A, Zapp J, Khan SA, Schneider M. Design and Characterization of Surface-Crosslinked Gelatin Nanoparticles for the Delivery of Hydrophilic Macromolecular Drugs. Macromol Chem Phys. 2019;220(18).
158. Khan SA, Schneider M. Stabilization of Gelatin Nanoparticles Without Crosslinking. Macromol Biosci. 2014;14(11):1627-38.
159. Markwalter CE, Pagels RF, Wilson BK, Ristroph KD, Prud'homme RK. Flash nanoprecipitation for the encapsulation of hydrophobic and hydrophilic compounds in polymeric nanoparticles. J Vis Exp. 2019;2019.
160. Xie H, Smith JW. Fabrication of PLGA nanoparticles with a fluidic nanoprecipitation system. Journal of Nanobiotechnology. 2010;8(1):18.
161. Rietscher R, Thum C, Lehr CM, Schneider M. Semi-Automated Nanoprecipitation-System-An Option for Operator Independent, Scalable and Size Adjustable Nanoparticle Synthesis. Pharm Res. 2015;32(6):1859-63.
162. Karnik R, Gu F, Basto P, Cannizzaro C, Dean L, Kyei-Manu W, Langer R, Farokhzad OC. Microfluidic Platform for Controlled Synthesis of Polymeric Nanoparticles. Nano Letters. 2008;8(9):2906-12.
163. Knight JB, Vishwanath A, Brody JP, Austin RH. Hydrodynamic Focusing on a Silicon Chip: Mixing Nanoliters in Microseconds. Phys Rev Lett. 1998;80(17):3863-6.
164. Ding S, Anton N, Vandamme TF, Serra CA. Microfluidic nanoprecipitation systems for preparing pure drug or polymeric drug loaded nanoparticles: an overview. Expert opinion on drug delivery. 2016;13(10):1447-60.
165. Günday Türeli N, Türeli AE, Schneider M. Optimization of ciprofloxacin complex loaded PLGA nanoparticles for pulmonary treatment of cystic fibrosis infections: Design of experiments approach. Int J Pharm. 2016;515(1-2):343-51.

166. Lababidi N, Sigal V, Koenneke A, Schwarzkopf K, Manz A, Schneider M. Microfluidics as tool to prepare size-tunable PLGA nanoparticles with high curcumin encapsulation for efficient mucus penetration. Beilstein Journal of Nanotechnology. 2019;10.
167. Lababidi N, Montefusco-Pereira CV, de Souza Carvalho-Wodarz C, Lehr C-M, Schneider M. Spray-dried multidrug particles for pulmonary co-delivery of antibiotics with N-acetylcysteine and curcumin-loaded PLGA-nanoparticles. Eur J Pharm Biopharm. 2020;157:200-10.
168. Günday Türeli N, Türeli AE, Schneider M. Counter-ion complexes for enhanced drug loading in nanocarriers: Proof-of-concept and beyond. Int J Pharm. 2016;511(2):994-1001.
169. Joseph X, Akhil V, Arathi A, Mohanan PV. Microfluidic synthesis of gelatin nanoparticles conjugated with nitrogen-doped carbon dots and associated cellular response on A549 cells. Chem Biol Interact. 2022;351:109710.
170. Schenk R, Hessel V, Hofmann C, Kiss J, Löwe H, Ziogas A. Numbering-up of micro devices: a first liquid-flow splitting unit. Chemical Engineering Journal. 2004;101(1):421-9.
171. Boskovic D, Loebbecke S. Synthesis of polymer particles and capsules employing microfluidic techniques. Nanotechnology Reviews. 2014;3(1):27-38.
172. Operti MC, Fecher D, van Dinther EAW, Grimm S, Jaber R, Figdor CG, Tagit O. A comparative assessment of continuous production techniques to generate sub-micron size PLGA particles. Int J Pharm. 2018;550(1):140-8.
173. Operti MC, Bernhardt A, Grimm S, Engel A, Figdor CG, Tagit O. PLGA-based nanomedicines manufacturing: Technologies overview and challenges in industrial scale-up. Int J Pharm. 2021;605:120807.
174. Johnson BK, Prud'homme RK. Chemical processing and micromixing in confined impinging jets. AIChE J. 2003;49:2264-82.
175. Johnson BK, Prud'homme RK. Flash NanoPrecipitation of Organic Actives and Block Copolymers using a Confined Impinging Jets Mixer. Aust J Chem. 2003;56(10):1021-4.
176. Liu Y, Cheng C, Liu Y, Prud'homme RK, Fox RO. Mixing in a multi-inlet vortex mixer (MIVM) for flash nano-precipitation. Chem Eng Sci. 2008;63(11):2829-42.
177. Kim Y, Lee Chung B, Ma M, Mulder WJM, Fayad ZA, Farokhzad OC, Langer R. Mass Production and Size Control of Lipid–Polymer Hybrid Nanoparticles through Controlled Microvortices. Nano Letters. 2012;12(7):3587-91.
178. Lim J-M, Swami A, Gilson LM, Chopra S, Choi S, Wu J, Langer R, Karnik R, Farokhzad OC. Ultra-High Throughput Synthesis of Nanoparticles with Homogeneous Size Distribution Using a Coaxial Turbulent Jet Mixer. ACS Nano. 2014;8(6):6056-65.
179. Pagels RF, Prud'homme RK. Inverse Flash NanoPrecipitation for Biologics Encapsulation: Nanoparticle Formation and Ionic Stabilization in Organic Solvents. Control of Amphiphile Self-Assembling at the Molecular Level: Supra-Molecular Assemblies with Tuned Physicochemical Properties for Delivery Applications. ACS Symposium Series. 1271: American Chemical Society; 2017. p. 249-74.
180. Pinkerton NM, Behar L, Hadri K, Amouroux B, Mingotaud C, Talham DR, Chassaing S, Marty JD. Ionic Flash NanoPrecipitation (iFNP) for the facile, one-step synthesis of inorganic–organic hybrid nanoparticles in water. Nanoscale. 2017;9(4):1403-8.
181. Santos JL, Ren Y, Vandermark J, Archang MM, Williford J-M, Liu H-W, Lee J, Wang T-H, Mao H-Q. Continuous Production of Discrete Plasmid DNA-Polycation Nanoparticles Using Flash Nanocomplexation. Small. 2016;12(45):6214-22.
182. Levit SL, Walker RC, Tang C. Rapid, Single-Step Protein Encapsulation via Flash NanoPrecipitation. Polymers. 2019;11(9):1406.
183. Aliabadi HM, Mahmud A, Sharifabadi AD, Lavasanifar A. Micelles of methoxy poly(ethylene oxide)-b-poly(ε-caprolactone) as vehicles for the solubilization and controlled delivery of cyclosporine A. J Control Release. 2005;104(2):301-11.
184. Miller T, van Colen G, Sander B, Golas MM, Uezguen S, Weigandt M, Goepferich A. Drug Loading of Polymeric Micelles. Pharm Res. 2013;30(2):584-95.

185. Qu G, Yao Z, Zhang C, Wu X, Ping Q. PEG conjugated N-octyl-O-sulfate chitosan micelles for delivery of paclitaxel: In vitro characterization and in vivo evaluation. Eur J Pharm Sci. 2009;37(2):98-105.
186. Gao Z, Lukyanov AN, Chakilam AR, Torchilin VP. PEG-PE/Phosphatidylcholine Mixed Immunomicelles Specifically Deliver Encapsulated Taxol to Tumor Cells of Different Origin and Promote their Efficient Killing. J Drug Target. 2003;11(2):87-92.
187. Huh KM, Lee SC, Cho YW, Lee J, Jeong JH, Park K. Hydrotropic polymer micelle system for delivery of paclitaxel. J Control Release. 2005;101(1):59-68.
188. Park EK, Kim SY, Lee SB, Lee YM. Folate-conjugated methoxy poly(ethylene glycol)/poly(ε-caprolactone) amphiphilic block copolymeric micelles for tumor-targeted drug delivery. J Control Release. 2005;109(1):158-68.
189. Ho D-K, Christmann R, Murgia X, De Rossi C, Frisch S, Koch M, Schaefer UF, Loretz B, Desmaele D, Couvreur P, Lehr C-M. Synthesis and Biopharmaceutical Characterization of Amphiphilic Squalenyl Derivative Based Versatile Drug Delivery Platform. Frontiers in Chemistry. 2020;8(937).
190. Rideau E, Dimova R, Schwille P, Wurm FR, Landfester K. Liposomes and polymersomes: a comparative review towards cell mimicking. Chem Soc Rev. 2018;47(23):8572-610.
191. Couvreur P, Stella B, Reddy LH, Hillaireau H, Dubernet C, Desmaële D, Lepêtre-Mouelhi S, Rocco F, Dereuddre-Bosquet N, Clayette P, Rosilio V, Marsaud V, Renoir J-M, Cattel L. Squalenoyl Nanomedicines as Potential Therapeutics. Nano Letters. 2006;6(11):2544-8.
192. Mendoza-Muñoz N, Quintanar-Guerrero D, Allémann E. The impact of the salting-out technique on the preparation of colloidal particulate systems for pharmaceutical applications. Recent Pat Drug Deliv Formul. 2012;6(3):236-49.
193. Burton GW, O'Farrell CP. Preparation of Artificial Latexes. Journal of Elastomers & Plastics. 1977;9(1):94-101.
194. Vanderhoff JW, El-Aasser MS, Ugelstad J, inventors; Lehigh University, assignee. Polymer emulsification process. US Patent US4177177A. 1979 December 04.
195. Gurny R, Peppas NA, Harrington DD, Banker GS. Development of Biodegradable and Injectable Latices for Controlled Release of Potent Drugs. Drug Dev Ind Pharm. 1981;7(1):1-25.
196. Allemann E, Gurny R, Doelker E. Drug-loaded nanoparticles - Preparation methods and drug targeting issues. Eur J Pharm Biopharm. 1993;39(5):173-91.
197. Quintanar-Guerrero D, Allémann E, Fessi H, Doelker E. Preparation techniques and mechanisms of formation of biodegradable nanoparticles from preformed polymers. Drug Dev Ind Pharm. 1998;24(12):1113-28.
198. Niwa T, Takeuchi H, Hino T, Kunou N, Kawashima Y. Preparations of biodegradable nanospheres of water-soluble and insoluble drugs with D,L-lactide/glycolide copolymer by a novel spontaneous emulsification solvent diffusion method, and the drug release behavior. J Control Release. 1993;25(1):89-98.
199. Niwa T, Takeuchi H, Hino T, Kunou N, Kawashima Y. In Vitro Drug Release Behavior of D, L-Lactide/Glycolide Copolymer (PLGA) Nanospheres with Nafarelin Acetate Prepared by a Novel Spontaneous Emulsification Solvent Diffusion Method. J Pharm Sci. 1994;83(5):727-32.
200. Feng J, Esquena J, Rodriguez-Abreu C, Solans C. Key features of nano-emulsion formation by the phase inversion temperature method. Journal of Dispersion Science and Technology. 2021;42(7):1073-81.
201. Fornaguera C, Dols-Perez A, Calderó G, García-Celma MJ, Camarasa J, Solans C. PLGA nanoparticles prepared by nano-emulsion templating using low-energy methods as efficient nanocarriers for drug delivery across the blood–brain barrier. J Control Release. 2015;211:134-43.
202. Wang J, Schwendeman SP. Mechanisms of solvent evaporation encapsulation processes: Prediction of solvent evaporation rate. J Pharm Sci. 1999;88(10):1090-9.

203. Avgoustakis K. Pegylated poly(lactide) and poly(lactide-co-glycolide) nanoparticles: preparation, properties and possible applications in drug delivery. Curr Drug Deliv. 2004;1(4):321-33.
204. Anton N, Benoit J-P, Saulnier P. Design and production of nanoparticles formulated from nano-emulsion templates—A review. J Control Release. 2008;128(3):185-99.
205. Vauthier C, Bouchemal K. Methods for the Preparation and Manufacture of Polymeric Nanoparticles. Pharm Res. 2009;26(5):1025-58.
206. Gref R, Minamitake Y, Peracchia MT, Trubetskoy V, Torchilin V, Langer R. Biodegradable long-circulating polymeric nanospheres. Science. 1994;263(5153):1600-3.
207. Peracchia MT, Gref R, Minamitake Y, Domb A, Lotan N, Langer R. PEG-coated nanospheres from amphiphilic diblock and multiblock copolymers: Investigation of their drug encapsulation and release characteristics. J Control Release. 1997;46(3):223-31.
208. Chin SF, Azman A, Pang SC. Size controlled synthesis of starch nanoparticles by a microemulsion method. J Nanomaterials. 2014;2014:Article 9.
209. Marto J, Gouveia LF, Gonçalves LM, Gaspar DP, Pinto P, Carvalho FA, Oliveira E, Ribeiro HM, Almeida AJ. A Quality by design (QbD) approach on starch-based nanocapsules: A promising platform for topical drug delivery. Colloids and Surfaces B: Biointerfaces. 2016;143:177-85.
210. Ruiz JM, Tissier B, Benoit JP. Microencapsulation of peptide: a study of the phase separation of poly(d,l-lactic acid-co-glycolic acid) copolymers 50/50 by silicone oil. Int J Pharm. 1989;49(1):69-77.
211. Niwa T, Takeuchi H, Hino T, Nohara M, Kawashima Y. Biodegradable submicron carriers for peptide drugs: Preparation of dl-lactide/glycolide copolymer (PLGA) nanospheres with nafarelin acetate by a novel emulsion-phase separation method in an oil system. Int J Pharm. 1995;121(1):45-54.
212. Rao JP, Geckeler KE. Polymer nanoparticles: Preparation techniques and size-control parameters. Prog Polym Sci. 2011;36(7):887-913.
213. Bindschaedler C, Gurny R, Doelker E, inventors; same, assignee. Process for preparing a powder of water-insoluble polymer which can be redispersed in a liquid phase and process for preparing a dispersion of the powdered polymer. . European Patent EP0363549B1. 1992 December 09.
214. Allémann E, Gurny R, Doelker E. Preparation of aqueous polymeric nanodispersions by a reversible salting-out process: influence of process parameters on particle size. Int J Pharm. 1992;87(1):247-53.
215. Konan YN, Gurny R, Allémann E. Preparation and characterization of sterile and freeze-dried sub-200 nm nanoparticles. Int J Pharm. 2002;233(1-2):239-52.
216. Song X, Zhao Y, Wu W, Bi Y, Cai Z, Chen Q, Li Y, Hou S. PLGA nanoparticles simultaneously loaded with vincristine sulfate and verapamil hydrochloride: Systematic study of particle size and drug entrapment efficiency. Int J Pharm. 2008;350(1):320-9.
217. Leroux J-C, Allémann E, De Jaeghere F, Doelker E, Gurny R. Biodegradable nanoparticles — From sustained release formulations to improved site specific drug delivery. J Control Release. 1996;39(2):339-50.
218. Leroux J, Allemann E, Doelker E, Gurny R. New approach for the preparation of nanoparticles by an emulsification-diffusion method. Eur J Pharm Biopharm. 1995;41(1):14-8.
219. Quintanar D, Fessi H, Doelker E, Allemann E, inventors; Université Claude Bernard, assignee. Method for preparing vesicular nanocapsules. European Patent EP1003488B1. 2000 May 31.
220. Quintanar-Guerrero D, de la Luz Zambrano-Zaragoza M, Gutierrez-Cortez E, Mendoza-Munoz N. Impact of the Emulsification-Diffusion Method on the Development of Pharmaceutical Nanoparticles. Recent Patents on Drug Delivery & Formulation. 2012;6(3):184-94.
221. Quintanar-Guerrero D, Allémann E, Doelker E, Fessi H. A mechanistic study of the formation of polymer nanoparticles by the emulsification-diffusion technique. Colloid and Polymer Science. 1997;275(7):640-7.

222. Engel RH, Riggi SJ, Fahrenbach MJ. Insulin: Intestinal Absorption as Water-in-Oil-in-Water Emulsions. Nature. 1968;219(5156):856-7.
223. Vrancken MN, Claeys DA, inventors; Gevaert Photo Producten NV, assignee. Process for encapsulating water and compounds in aqueous phase by evaporation. US Patent US3523906A. 1970 August 11.
224. Ogawa Y, Yamamoto M, Okada H, Yashiki T, Shimamoto T. A new technique to efficiently entrap leuprolide acetate into microcapsules of polylactic acid or copoly (lactic/glycolic) acid. Chem Pharm Bull (Tokyo). 1988;36(3):1095-103.
225. Cohen S, Yoshioka T, Lucarelli M, Hwang LH, Langer R. Controlled delivery systems for proteins based on poly(lactic/glycolic acid) microspheres. Pharm Res. 1991;8(6):713-20.
226. Schwendeman SP, Cardamone M, Klibanov A, Langer R, Brandon MR. Stability of proteins and their delivery from biodegradable polymer microspheres. In: Cohen S, Bernstein H, editors. Microparticulate systems for the delivery of proteins and vaccines. New York: Marcel Dekker; 1996. p. 1-51.
227. Blanco MD, Alonso MJ. Development and characterization of protein-loaded poly(lactide-co-glycolide) nanospheres. Eur J Pharm Biopharm. 1997;43(3):287-94.
228. Pinon-Segundo E, G. Nava-Arzaluz M, Lechuga-Ballesteros D. Pharmaceutical Polymeric Nanoparticles Prepared by the Double Emulsion- Solvent Evaporation Technique. Recent Patents on Drug Delivery & Formulation. 2012;6(3):224-35.
229. Primavessy D, Günday Türeli N, Schneider M. Influence of different stabilizers on the encapsulation of desmopressin acetate into PLGA nanoparticles. Eur J Pharm Biopharm. 2017;118:48-55.
230. Cohen-Sela E, Chorny M, Koroukhov N, Danenberg HD, Golomb G. A new double emulsion solvent diffusion technique for encapsulating hydrophilic molecules in PLGA nanoparticles. J Control Release. 2009;133(2):90-5.
231. Semete B, Booysen L, Kalombo L, Ramalapa B, Hayeshi R, Swai HS. Effects of protein binding on the biodistribution of PEGylated PLGA nanoparticles post oral administration. Int J Pharm. 2012;424(1-2):115-20.
232. Booysen LLIJ, Kalombo L, Brooks E, Hansen R, Gilliland J, Gruppo V, Lungenhofer P, Semete-Makokotlela B, Swai HS, Kotze AF, Lenaerts A, du Plessis LH. In vivo/in vitro pharmacokinetic and pharmacodynamic study of spray-dried poly-(dl-lactic-co-glycolic) acid nanoparticles encapsulating rifampicin and isoniazid. Int J Pharm. 2013;444(1-2):10-7.
233. Chong D, Liu X, Ma H, Huang G, Han YL, Cui X, Yan J, Xu F. Advances in fabricating double-emulsion droplets and their biomedical applications. Microfluidics and Nanofluidics. 2015;19(5):1071-90.
234. Lorenceau E, Utada AS, Link DR, Cristobal G, Joanicot M, Weitz DA. Generation of Polymerosomes from Double-Emulsions. Langmuir. 2005;21(20):9183-6.
235. Martinez NY, Andrade PF, Durán N, Cavalitto S. Development of double emulsion nanoparticles for the encapsulation of bovine serum albumin. Colloids and Surfaces B: Biointerfaces. 2017;158:190-6.
236. Giunchedi P, Conte U. Spray-drying as a preparation method of microparticulate drug delivery systems: An overview. STP Pharma Pratiques. 1995;5(4):276-90.
237. Gander B, Wehrli E, Alder R, Merkle HP. Quality improvement of spray-dried, protein-loaded D,L-PLA microspheres by appropriate polymer solvent selection. J Microencapsul. 1995;12(1):83-97.
238. Bittner B, Morlock M, Koll H, Winter G, Kissel T. Recombinant human erythropoietin (rhEPO) loaded poly(lactide-co-glycolide) microspheres: influence of the encapsulation technique and polymer purity on microsphere characteristics. Eur J Pharm Biopharm. 1998;45(3):295-305.
239. Heng D, Lee SH, Ng WK, Tan RBH. The nano spray dryer B-90. Expert opinion on drug delivery. 2011;8(7):965-72.
240. Calvo P, Remuñán-López C, Vila-Jato JL, Alonso MJ. Novel hydrophilic chitosan-polyethylene oxide nanoparticles as protein carriers. Journal of Applied Polymer Science. 1997;63(1):125-32.

241. Charcosset C, Fessi H. Preparation of nanoparticles with a membrane contactor. J Membr Sci. 2005;266(1):115-20.
242. Ahlin Grabnar P, Kristl J. The manufacturing techniques of drug-loaded polymeric nanoparticles from preformed polymers. J Microencapsul. 2011;28(4):323-35.
243. Colombo S, Beck-Broichsitter M, Bøtker JP, Malmsten M, Rantanen J, Bohr A. Transforming nanomedicine manufacturing toward Quality by Design and microfluidics. Adv Drug Deliv Rev. 2018;128:115-31.
244. Hare JI, Lammers T, Ashford MB, Puri S, Storm G, Barry ST. Challenges and strategies in anti-cancer nanomedicine development: An industry perspective. Adv Drug Deliv Rev. 2017;108:25-38.
245. Ragelle H, Danhier F, Préat V, Langer R, Anderson DG. Nanoparticle-based drug delivery systems: a commercial and regulatory outlook as the field matures. Expert opinion on drug delivery. 2017;14(7):851-64.
246. Eaton MAW, Levy L, Fontaine OMA. Delivering nanomedicines to patients: A practical guide. Nanomed Nanotechnol Biol Med. 2015;11(4):983-92.
247. Smart AL, Gaisford S, Basit AW. Oral peptide and protein delivery: intestinal obstacles and commercial prospects. Expert opinion on drug delivery. 2014;11(8):1323-35.
248. Reix N, Parat A, Seyfritz E, Van Der Werf R, Epure V, Ebel N, Danicher L, Marchioni E, Jeandidier N, Pinget M, Frère Y, Sigrist S. In vitro uptake evaluation in Caco-2 cells and in vivo results in diabetic rats of insulin-loaded PLGA nanoparticles. Int J Pharm. 2012;437(1–2):213-20.
249. Kranz H, Ubrich N, Maincent P, Bodmeier R. Physicomechanical properties of biodegradable poly(D,L-lactide) and poly(D,L-lactide-co-glycolide) films in the dry and wet states. J Pharm Sci. 2000;89(12):1558-66.
250. Dhillon A, Schneider P, Kuhn G, Reinwald Y, White LJ, Levchuk A, Rose FRAJ, Müller R, Shakesheff KM, Rahman CV. Analysis of sintered polymer scaffolds using concomitant synchrotron computed tomography and in situ mechanical testing. J Mater Sci Mater Med. 2011;22(12):2599-605.
251. Desai K-G, Schwendeman SP. Active self-healing encapsulation of vaccine antigens in PLGA microspheres. J Control Release. 2013;165(1):62-74.
252. Jain RA, Rhodes CT, Railkar AM, Malick AW, Shah NH. Controlled release of drugs from injectable in situ formed biodegradable PLGA microspheres: effect of various formulation variables. Eur J Pharm Biopharm. 2000;50(2):257-62.
253. Owen GR, Jackson JK, Chehroudi B, Brunette DM, Burt HM. An in vitro study of plasticized poly (lactic-co-glycolic acid) films as possible guided tissue regeneration membranes: Material properties and drug release kinetics. Journal of Biomedical Materials Research Part A. 2010;95(3):857-69.
254. Wang X, Venkatraman SS, Boey FYC, Loo JSC, Tan LP. Controlled release of sirolimus from a multilayered PLGA stent matrix. Biomaterials. 2006;27(32):5588-95.
255. Schloegl W, Marschall V, Witting MY, Volkmer E, Drosse I, Leicht U, Schieker M, Wiggenhorn M, Schaubhut F, Zahler S, Friess W. Porosity and mechanically optimized PLGA based in situ hardening systems. Eur J Pharm Biopharm. 2012;82(3):554-62.

7. List of scientific publications

Original research articles

Schiller S, Hanefeld A, Schneider M, Lehr C-M. Towards a Continuous Manufacturing Process of Protein-Loaded Polymeric Nanoparticle Powders. AAPS PharmSciTech. **2020**;21(7):269.

Schiller S, Hanefeld A, Schneider M, Lehr C-M. Focused Ultrasound as a Scalable and Contact-Free Method to Manufacture Protein-Loaded PLGA Nanoparticles. Pharm Res. **2015**;32(9):2995-3006.

Patent

Schiller S, Hanefeld A, Weigandt M, Schneider M, Lehr C-M, inventors; Merck Patent GmbH, assignee. Preparation of nanoparticles-releasing enteric microparticles. International Patent Application WO2016066249A1 published 06 May 2016.

Granted (as of 08. December 2021): AU2015341070B2; CN107106504B; EP3212171B1; ES2713627T3; IL251946A; JP6656244B2; US10561622B2

Oral and poster presentations at international conferences

Schiller S, Hanefeld A, Schneider M, Lehr C-M. Focused ultrasound as a scalable and contact-free method to manufacture protein-loaded PLGA nanoparticles. Talk presented at: Braunschweig International Symposium on Pharmaceutical Engineering Research SPhERe; 2015 Oct 19-20; Braunschweig, Germany

Schiller S, Hanefeld A, Schneider M, Weigandt M, Lehr C-M. Focused ultrasound as a contact-free and scalable method to manufacture protein-loaded PLGA nanoparticles. Poster presented at: AAPS Annual Meeting; **2013** Nov 10-14; San Antonio, TX

Schiller S, Hanefeld A, Schneider M, Weigandt M, Lehr C-M. Contact-free encapsulation of proteins in PLGA nanoparticles by focused ultrasound. Poster presented at: Controlled Release Society 40th Annual Meeting & Exposition; **2013** Jul 21-24; Honolulu, HI

8. Acknowledgements

Zuvorderst gilt mein Dank meinem Doktorvater Claus-Michael Lehr, der mir die Promotion auf diesem äußerst spannenden Thema in einem interdisziplinären Umfeld ermöglicht hat und mich auch über die Entfernung wissenschaftlich und strategisch und immer mit Geduld begleitet hat. Vielen Dank für die konstruktive Kritik an den Manuskripten. Ich danke Andrea Hanefeld für die fachliche und organisatorische Betreuung bei Merck, für die vielen wissenschaftlichen Diskussionen, für die Motivation, den stetigen Optimismus und das „can-do"; für die Unterstützung meiner Arbeit und die Vernetzung über die Abteilung und das Unternehmen hinaus. Ich danke Marc Schneider für die wertvolle Unterstützung bei den Veröffentlichungen, den kritischen Blick auf die Manuskripte und die Ermutigung bei manchem Rückschlag und manchem Kommentar eines Reviewers. Ich danke Eva-Maria Collnot für ihre Unterstützung und die universitäre Betreuung in der Anfangsphase meiner Promotionszeit, für ihr kritisches und stets konstruktives Feedback beim Durchleuchten meiner Experimente und Ergebnisse.

Simon Geißler danke ich für die wissenschaftlichen Diskussionen, die organisatorische Unterstützung im Labor, die Vermittlung von Kontakten für Spezialanalytik und vor allem für die Schaffung eines Rahmens neben dem pharmazeutisch-industriellen Tagesgeschäft, in dem die Fremdbezeichnung „akademische Spielwiese" als Kompliment aufgefasst werden konnte. Ich danke Simon, Andrea und Markus Weigandt für die Konzeption und Durchführung des Konsortialprojekts sowie für die Idee für meine Arbeit.

Ich möchte allen Kollegen in Exploratory Development bei Merck danken, vor allem meinen Mitdoktoranden und „Schicksalsgenossinnen und -genossen" Katharina Anhalt, Tobias Miller, Alexandra Hill, Bernd Sterner, Marion Hör, Gudrun Birk, Melanie Hofmann, Mira Oswald, Markus Riehl und Lena Münster für den wissenschaftlichen und technischen Austausch, die Hilfsbereitschaft und das wissenschaftliche Netzwerk, für die vielen guten Gespräche im Labor vor und insbesondere nach Feierabend, für die entstandenen Freundschaften und die gegenseitige Unterstützung auf unseren Wegen. Alex und Bernd für euren Humor und die vielen witzigen Momente. Besonders danke ich meinem ersten „Büro-Mitbewohner" Tobias, von dem ich schon als Praktikant so viel lernen durfte über wissenschaftliches Arbeiten, den Wissenschaftsbetrieb, über Selbst-Kritik und Motivation, über Ausdauer und Beharrlichkeit, über Kreativität und Initiative.

Vielen Dank Gwen für die persönliche Begleitung während der vielen schönen und herausfordernden Momente zwischen Darmstadt, München und Zürich. Dein Charme, Witz, Deine Kreativität und

Leichtigkeit, Deine Liebe haben dafür gesorgt, dass die schönen Momente überwogen und „die Wiese“ mit Alex, Tobi und dem Erlenmeyerkolben wird unvergessen bleiben.

Vielen Dank auch an Letícia Pires Rodrigues und Sebastian Franck für ihre Mitarbeit, ohne Euch wären die Stunden, Tage und Wochen am Zetasizer wirklich endlos geworden. Ich danke allen Doktoranden und Partnern im PeTrA-Projekt, besonders Julia Janke, René Rietscher, Andrea Diedrich, Marius Hittinger, Daniel Primaveßy, Renato Baleeiro, Andreas Kirchner, Rike Wallbrecher, Silko Grimm, Regina Scherließ, Marco Metzger, Peter Walden und Thomas Ebensen, für den wissenschaftlichen Austausch bei den Konsortialtreffen und den Doktorandentreffen und natürlich den „After-Konsortialtreffen“. Afra Torge vom AK Schneider für die Unterstützung mit Fluoreszenz-Konfokalmikroskop-Bildern. Matthias Schröder und den AK Knolle am Institut für Molekulare Medizin und Experimentelle Immunologie für die Ermöglichung zweier Forschungsaufenthalte, für die gute Zusammenarbeit im Labor sowie an der Kletterwand.

Ich danke Jana für die Begleitung, die wertvolle Unterstützung und Motivation zum Abschluss der Dissertation und die vielen Stunden im gemeinsamen „Corona-Home-Office“. Du hast mir den Schwung gegeben, den ich brauchte, um es „endlich hinter mich zu bringen“.

Ein ganz besonderer Dank gilt meinen Eltern und meinem Bruder dafür, dass ihr mich auch „in der Ferne“ immer unterstützt habt und ich mich immer darauf verlassen kann, dass ihr da seid. Ohne euch wäre ich nicht, wo ich jetzt bin.

Danke.