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Enhancement of niacin availability in the ileocolonic region by microencapsulation and coating

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Abstract

In the recent past, the human gut microbiota has gained increasing interest as a target for the delivery of substances due to the observed interaction between the microbiome, nutrition and health. The water-soluble vitamin niacin showed first effects on the composition of the gut microbiota. In order to enhance the availability of niacin in the ileocolonic region, where most of the microorganism are located, an early systemic absorption of niacin in the stomach and small intestine must be prevented. Therefore, two active forms of niacin (nicotinic acid (NA) and nicotinamide (NAM)) were encapsulated by a protective shellac coating applied via fluidized bed coating. The modulation of the release profile of niacin was assisted by a newly designed *in vitro* dissolution test and verified by a human bioavailability study with 10 healthy volunteers for each group. Different intrinsic effects on the release profile such as derived from the properties of the encapsulated compound were investigated. The active substance core was produced by a one-step spray granulation and a two-step process (extrusion with subsequent spheronisation). The latter method achieved a higher particle yield and required a lower use of shellac in relation to the mass of active substance. As an alternative to shellac coated microcapsules, the incorporation of niacin into pectin-zein hydrogel beads was characterized. A precise targeted release of niacin into the ileocolonic region *in vitro* and *in vivo* was reached by using a shellac based coating with adapted intermediate subcoatings consisting of either citric acid for NAM or sodium bicarbonate of NA. The subcoatings were able to control the effect of the physico-chemical properties of the encapsulated compound and to influence the pH-dependent dissolution of the surrounding shellac coatings. The water-solubility in combination with the aqueous pH value and the surface characteristics of the compound were factors influencing the release profile. On the other hand, premature release from the pectin-zein hydrogel beads could not be prevented due to the high water solubility and low molecular weight of NAM. Consequently, the enhancement of the availability of niacin was reached using a shellac based coating formulation adapted to the compounds properties, which are predominantly determining the encapsulation technique and materials. The designed *in vitro* dissolution test for the targeting provided a precise instrument to predict the release behavior in humans.

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2py	N1-methyl-2-pyridone-5-carboxamide
4py	N1-methyl-4-pyridone-3-carboxamide
β-LG	beta-lactoglobulin
ACMS	2-amino-3-carboxy-muconate semialdehyde
ACMSD	ACMS-decarboxylase
ADP	adenosine diphosphate
ADPR	ADP ribolysation reactions
AOAC	Analysis of the Official Analytic Chemists
Aox	aldehyde oxidase
ATP	adenosine triphosphate
AUC	area under the curve
cAMP	cyclic adenosyl monophosphate
C_{max}	maximum serum concentration
CoA	coenzyme A
DA	degree of amidation
DAD	diode array detection
DE	degree of esterification
DNA	deoxyribonucleic acid
EFSA	European Food Safety Authority
Gln	glutamine
Glu	glutamic acid
HAAO	3-hydroxyanthranilate
HDL	high-density lipoprotein
HM	high methoxylated
HPLC	high performance liquid chromatography
IDDM	insulin-dependent diabetes mellitus
IDO	indoleamine 2,3-dioxygenase

ISO	International Organization for Standardization
KF	kynurenine formylase
KMO	kynurenine 3-monooxygenase
KYNU	kynureninase
LDL	low-density lipoprotein
LM	low methoxylated
LOAEL	lowest observed adverse effect level
MS	mass spectrometry
NA	nicotinic acid
NAD/H	nicotinamide adenine nucleotide
NADG	NAD-glycohydrolase
NADK	NAD-kinase
NADP/H	nicotinamide adenine nucleotide phosphate
NADS	NAD-synthetase
NAM	nicotinamide
NAMDA	NAM-deamidase
NAMNAT	NAM-mononucleotide-adenyltransferase
NAMPRT	NAM-phosphoribosyltransferase
NAPRT	NA-phosphoribosyltransferase
NE	niacin equivalent
NMNAT	NA-mononucleotide-adenyltransferase
NMNT	NAM-N-methyltransferase
NOAEL	no observed adverse effect level
NRK	NAM-riboside-kinase
NVS	Nationale Verzehrsstudie
PP	pellagra preventing
PPi	pyrophosphate

PRPP	phosphoribosylpyrophosphate
QPRT	quinolinate phosphoribosyltransferase
RDA	recommended daily allowance
SAH	S-adenosylhomocystein
SAM	S-adenosylmethionine
SCF	Scientific Committee on Food
SCFA	short-chain fatty acids
TDO2	tryptophan 2,3-dioxygenase
t_{max}	time with the highest serum concentration
UKSH	University Hospital Schleswig-Holstein
UL	upper limit
USP	United States Pharmacopeia
VLDL	very low-density lipoprotein
WPI	whey protein isolate

1 Motivation and objectives

Nowadays, the focus has increasingly shifted from disease cure and therapy to disease prevention. Therefore, nutraceuticals are of growing interest. The term nutraceutical was coined by Stephen DeFelice and is a *portmanteau* of the words nutrition and pharmaceutical. On the one hand, a nutraceutical consists of a food or a part of a food; on the other hand, it provides medical or health benefits, including the prevention and treatment of diseases (DeFelice, 1995; Santini et al., 2017). If a nutraceutical is proven for safety, bioavailability and clinically beneficial health properties, it can be used to prevent the onset of pathological conditions, to conserve the well-being, to avoid or delay the need of pharmaceuticals, to reduce the costs of chronic therapies and to become a proactive medicine approach (Santini et al., 2017). Obesity and its comorbidities are an increasing problem in industrialized and developing countries (Friedman, 2009). Recent studies showed an association between the composition of the gut microbiome and obesity (Bäckhed et al., 2004; Le Chatelier et al., 2013; Ley et al., 2006; Turnbaugh et al., 2006). *Bacteroidetes*, beside *Firmicutes*, one group of the dominant beneficial bacteria in the human gut, is decreased in obese people compared to lean people (Ley et al., 2006), which has an influence on the metabolic potential of the gut microbiota (Turnbaugh et al., 2006). Therefore, a positive effect on the microbiome would be desirable with regard to the prevention of obesity and the co-morbidities such as insulin resistance and diabetes.

Niacin is a water-soluble vitamin, which is also known as vitamin B3. The two active forms are nicotinic acid (NA) and nicotinamide (NAM) (Friedrich, 1987; Kirkland, 2007). In a mouse model, the administration of tryptophan as well as NAM led to an altered composition of the intestinal microbiota, which is concluded to be a result of induced expression of antimicrobial peptides from epithelial cells (Hashimoto et al., 2012; Waetzig and Seegert, 2012). Singh et al. also supported a beneficial effect of niacin, that suppressed colitis and colon cancer by mediating the colon receptor Gpr109a (Singh et al., 2014). Thus, the tryptophan metabolites NA and NAM can act as protective substances by beneficially influencing the gut microbiome (Schreiber et al., 2014).

However, simply increasing the content of NA and NAM in food would lead to a nearly total absorption in the upper intestine. Therefore, niacin need to be protected until it reaches the site of action (Yang et al., 2017), in this case the ileocolonic region, where most of the microorganisms are located (Sekirov et al., 2010). Microencapsulation is one opportunity to create a nutraceutical with a targeted delivery (Kunz et al., 2003). In many cases, a coating is

applied that allows gastric resistance and that dissolves in the colon due to colon specific conditions (Czarnocka and Alhnan, 2015; Maroni et al., 2013). Regarding a legislation as nutraceuticals, the used materials must be approved as a food additive or GRAS-deemed, which are still scarce. Therefore, the encapsulation of niacin is challenging due to a limited number of food-grade materials, which are suitable for an (ileo)colon targeted delivery. Furthermore, previous *in vitro* dissolution tests for colon-targeted delivery systems are not prescribed and are dependent on the desired formulation. Thus, it is sophisticated to develop a precise instrument to predict the release behavior in humans, which is needed for the adaption of the encapsulation formulation. In addition, physico-chemical properties of encapsulated compounds can influence the resulting release profile (Menge, 2016; Ozturk et al., 1988; Ragnarsson et al., 1992; Sousa et al., 2002), which is a technological challenge for the different compounds NA and NAM.

The aim of the present thesis is thus to develop and evaluate a new niacin nutraceutical with a targeted delivery in the ileocolonic region using food-grade materials. The systemic resorption should be minimal, whereby the availability for the gut microbiome is ensured. Therefore, *in vitro* tests of different formulations were developed and considered before the release behaviour of two formulations was characterized *in vivo* in cooperation with the Department of Internal Medicine 1, Nutrition and Metabolic Medicine, UKSH Kiel, Germany.

Hypothesis 1: pH-sensitive food-grade film and gel forming materials are suitable for a colon-targeted delivery of niacin.

a) **Due to the high pk_a, a coating using the ammonium salt of shellac enables gastric and small intestine resistance and a pH-dependent release of niacin in the (ileo)colonic region. (Paper I and Manuscript II)**

Background

The choice of colon-targeted coating materials for food use is rather limited compared to pharmaceutical materials. Shellac is a food-grade material and is legislated as a food additive with the E-number E904. Due to its acidic character and a high pk_a value of 6.9 – 7.5, it is insoluble in the acidic gastric milieu and could lead to a delayed and/or colon targeted release in the human lower intestine with higher pH values (Al-Gousous et al., 2015; Pearnchob et al., 2003a; Roda, 2007).

Experimental approach

Food-grade niacin microcapsules coated with shellac will be developed by fluidized bed coating. *In vitro* dissolution tests will be conducted to predict the release of niacin under simulated gastrointestinal conditions.

b) **Due to its molecular structure and low digestibility, pectin in combination with zein features crucial properties for retarded release formulations. Morphology of the pectin-zein particles determines the coating properties and the resulting release profile. (Manuscript V)**

Background

Low methoxylated pectins can form gels in the presence of calcium ions by cross-linking of free carboxyl groups with the calcium ions. The resulting calcium pectinate is described to act as colon-targeting material due to its insolubility and the enzymatic degradation by gut microbiota (Liu et al., 2003; Sriamornsak, 2011; Willats et al., 2006). Zein is used as water barrier (Liu et al., 2006), which is suitable especially for the encapsulation of water soluble substances like niacin. Furthermore, the production process parameters for hydrogel beads can influence the morphology (Smrdel, 2008), which also can affect the coating properties and the resulting release profile (Guo and Kaletunç, 2016; Harvestine et al., 2014).

Experimental approach

For the encapsulation of niacin, the process of ionotropic gelation will be examined using pectin and zein as core and coating materials. To study the effect of the process parameters on the morphology, two different processes with varying conditions will be conducted: a one-step gelation and coating process and a two-step process, which will separate the gelation and coating procedure. The resulting encapsulation efficiency and the gastrointestinal release profile will be determined by *in vitro* digestion.

Hypothesis 2: The use of a triple shellac coating enables a robust targeted release of niacin despite the influence of extrinsic factors such as the production process, storage conditions and pH values of simulated gastrointestinal fluids. (Manuscript II)

Background

Process parameters during coating can influence the resulting release profile (Buch et al., 2009; Farag and Leopold, 2009; Farag and Leopold, 2011b; Farag and Leopold, 2011c; Thoma and Bechtold, 1999). Shellac undergoes ageing, which influences the release profile and can result in a lower gastric resistance and a lower solubility at higher pH values (Farag and Leopold, 2009; Penning, 1996), whereby the use of the ammonium salt form reduces this ageing effect (Limmatvapirat et al., 2007). Furthermore, residence times and pH values in the gut can vary due to inter- and intraindividual differences (Dressman et al., 1998; Ibekwe et al., 2008; Russell et al., 1993; Shani-Levi et al., 2017), that mainly affects the dissociation of shellac and therefore the release of the encapsulated compound. This could lead to premature or insufficient release.

Experimental approach

The robustness of shellac coated pellets will be evaluated based on the resulting *in vitro* release profile and scanning electron microscope pictures. Therefore, several coating processes will be replicated and compared. Furthermore, the pellets will be exposed to various storage conditions over a period of 24 months. Different possible physiological conditions of residence time or pH values will be simulated *in vitro*.

Hypothesis 3: Adjustment of shellac coating for niacin by *in vitro* assay enables targeted release in the ileocolonic region *in vivo*. (Paper I and Manuscript II)

Background

To achieve a sufficient delivery of niacin into the ileocolonic region, the absorption in the upper gastrointestinal tract must be prevented (Schreiber et al., 2014). Using *in vitro* assays to simulate digestion fluids and anticipating residence times in the gastrointestinal tract allows the development of a target delivery system (Jorgensen and Bhagwat, 1998; Zahirul and Khan, 1996).

Experimental approach

An *in vitro* assay will be designed based on transit times for pellets (Abrahamsson et al., 1996; Clarke et al., 1993; Davis et al., 1987) and pH values in the gut segments (Dressman et al., 1990; Evans et al., 1988; Ibekwe et al., 2008; Russell et al., 1993). The bioavailability will be confirmed in an *in vivo* study with 10 healthy subjects in each group (nicotinic acid and nicotinamide), conducted by the research group of Prof. Dr. Matthias Laudes, University Hospital in Kiel. The study will comprise the free vitamin as well as the microencapsulated form using various dosages. The serum level of nicotinic acid and nicotinamide will be quantified.

Hypothesis 4: A precise targeting can be achieved by modulating simultaneously the influencing intrinsic factors of release.

a) **Physico-chemical properties of the encapsulated compound such as intrinsic pH value, water solubility and surface structure influence the coating properties and the resulting release profile. (Manuscript III)**
b) **Different subcoatings will precisely modulate the release profile of the microencapsulated compounds with varying physico-chemical characteristics. (Manuscript III)**

Background

Due to small amounts of water penetrating the shellac coat during the digestion process, the encapsulated compounds will be partly solubilized and thereby influencing the pH-value in the surrounding shellac layer (Menge, 2016; Ozturk et al., 1988; Ragnarsson et al., 1992;

Sousa et al., 2002). Furthermore, the water solubility can also affect the water penetration and solubilisation of the compounds (Ragnarsson et al., 1992; Sousa et al., 2002). These processes will influence the pH-dependent dissociation of shellac and the diffusion of the encapsulated compound. Subcoatings may counteract the effect of solubilized compounds and affect the release profile (Farag and Leopold, 2011a).

Experimental approach

Different compounds with different physico-chemical characteristics will be optimized for their release in the ileocolonic region by individual coating formulations that control the effect of the encapsulated compounds. Riboflavin 5'-monophosphate sodium salt hydrate will be used as reference substance. Further, the effect of NA and NAM will be compared. *In vitro* dissolution tests will give information about the release profile after the application of different subcoatings.

Hypothesis 5: The wet extrusion and spheronisation process is superior compared to spray granulation in order to obtain high yield of spherical NAM pellets requiring lower addition of shellac. (Manuscript IV)

Background

The formation of spray granulated NAM pellets resulted in spherical and compact particles, which were suitable for a uniform and functional coating. However, due to the high amount of unsuitable particles, only a narrow range could be subjected to coating, which led to a rather low yield. Further, a high amount of shellac was required to achieve an ileocolon-targeted release, which is related to the high specific surface area. Wet extrusion followed by spheronisation is known as a method to prepare pellets of higher and uniform particle size (Knop and Kleinebudde, 2005).

Experimental approach

For the wet extrusion/spheronisation process, a twin-screw extruder will be used. To calculate the effects of the process parameters on the resulting properties of NAM pellets, a Design of Experiment approach will be examined. Finally, the release profile of coated NAM extrudates will be compared with coated NAM spray granulates.

2 Theoretical background

2.1 Niacin

The collective term niacin, also known as vitamin PP (pellagra preventing) or vitamin B_3, refers to all forms with vitamin activity of nicotinic acid (NA) (pyridine-3-carboxylic acid; **Figure 2.1A**), nicotinamide (NAM) (pyridine-3-carboxamide; **Figure 2.1B**) and pyridine nucleotide structures like nicotinamide adenine dinucletides (Friedrich, 1987; Kirkland, 2007). Pyridine nucleotides are soluble coenzymes, which are consisting of adenosine monophosphate and nicotinamide mononucleotide. They are present as oxidized or reduced nicotinamide adenine dinucleotides either in their unphosphorylated (NAD^+ (**Figure 2.1C**) and NADH) or phosphorylated ($NADP^+$ (**Figure 2.1D**) and NADPH) forms (Nakamura et al., 2012). NA as well as NAM can be transformed to the biologically active forms of niacin, NAD^+ and $NADP^+$. NAD^+ and $NADP^+$ are describing the total pool of all four forms as mentioned above (Kirkland, 2007).

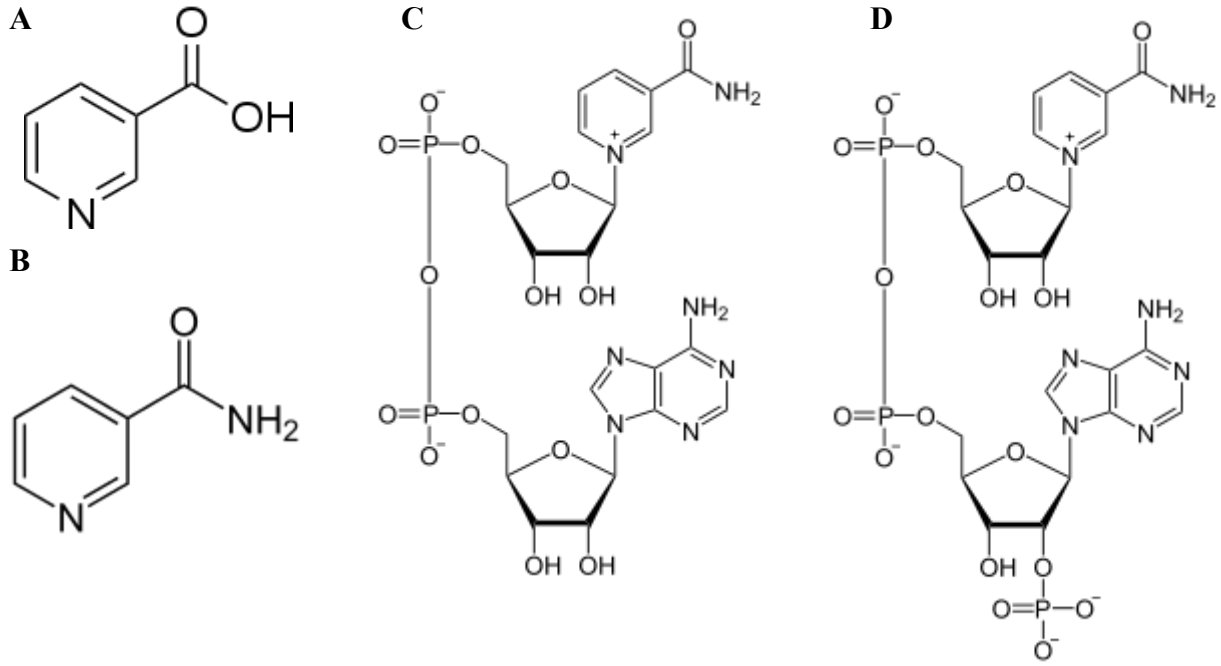

Figure 2.1: *Chemical structures of niacin compounds. (A) nicotinic acid, (B) nicotinamide, (C) nicotinamide adenine dinucleotide, (D) nicotinamide adenine dinucleotide phosphate*

2.1.1 Chemical and physical characteristics

The chemical and physical characteristics of NA and NAM are summarized in **Table 2.1**. Both, NA and NAM, are crystalline and colourless/white substances with a low molecular weight of 123.11 and 122.12 g/mol. They are water-soluble, whereby NAM is highly (1000 g/l) and NA moderately soluble in water (16 g/l). NA has a melting point of 236 – 237°C with sublimation. NAM is melting at a temperature of 129 – 132°C. The pH value of a saturated aqueous NA solution is 3 and the pk_a is 4.8 (25°C). NAM leads to an aqueous solution with a pH value of 6. The pk_a of NAM is 3.3 (20°C). The maximum UV absorbance of NA or NAM in water is at 260 – 263 nm, the absorption intensity is pH dependent. The free vitamin does not fluoresce. The reduced forms of the coenzymes (NADH and NADPH) have a strong UV absorption at 340 nm. The oxidized forms have a weaker absorption at 260 nm (Ball, 2006; Eitenmiller et al., 2008; Friedrich, 1987; Kirkland, 2007).

Table 2.1: *Chemical and physical characteristics of nicotinic acid and nicotinamide (Ball, 2006; Eitenmiller et al., 2008; Friedrich, 1987; Kirkland, 2007)*

	Nicotinic acid (NA)	**Nicotinamide (NAM)**
Appearance	Crystalline powder	Crystalline powder
Colour	White	White
Molecular weight	123.11 g/mol	122.12 g/mol
Water solubility	16 g/l	620 – 1000 g/l
Melting point	236 – 237°C	129 – 132°C
pH value	≈ 3 (in H_2O)	≈ 6 (in H_2O)
pk_a value	4.8 (25°C)	3.3 (20°C)
Max. UV absorbance	260 – 263 nm	260 – 263 nm

Generally, niacin is known as a very stable vitamin: it is resistant to air, heat, light, acids and alkalis. NAM can be partially hydrolysed to NA by acid and alkali, but it does not lose its biological activity (Ball, 2006; EFSA, 2012; Harris, 1988). It is also stable during food processing, storage and cooking of foods, except of leaching losses (Ball, 2006). Furthermore, it was found that nicotinic acid powder was stable in 0.1 N HCl, HCl medium with a pH value of 2.2 and in a phosphate buffer with a pH value of 6.8 for a period of up to 76 hours (Chuong et al., 2010). The EFSA concluded from technical dossiers that the shelf life of NA and NAM is 36 months at 25°C. Under accelerated conditions (40°C) the shelf life is reduced to 12 and 24 months, respectively (EFSA, 2012).

Niacin powders are almost white and odourless. NAM, however, generates a bitter taste (Ball, 2006; EFSA, 2012; Friedrich, 1987).

NA shows poor flowability properties (Chuong et al., 2010), whereby NAM powder has a high electrostatic charge, which is disadvantageous for handling (Hojgaard and Wittendorff, 2013).

2.1.2 Food content, food supplementation

Niacin is available in plant, as well as in animal products, whereas NA is mainly present in plant products. However, much of NA exists in poorly understood bound forms of heterogeneous mixtures of polysaccharides and glycopeptides esterification. The consumption of products with high levels of bound NA results in low bioavailability, which could finally lead to niacin deficiency and the development of pellagra. Especially the consumption of corn products was associated with pellagra. For an effective absorption of NA from corn, an alkaline treatment is necessary to release NA and to achieve a higher bioavailability (Friedrich, 1987; Kirkland, 2007).

Animal products mainly offer niacin in form of the coenzymes NAD/H and NADP/H. During aging and cooking of meat, as well as during intestinal digestion, NAM will be released from the coenzyme form. Beside the niacin content of human foods, the content of the amino acid tryptophan plays an important role in the supply of niacin. 60 mg tryptophan can be converted to 1 mg NA. Therefore, the niacin content in food is expressed as niacin equivalents (NE) and is defined as niacin content (mg) + 1/60 tryptophan content (mg) (Friedrich, 1987; Kirkland, 2007). Recommended daily allowances (RDA) of niacin are varying depending on the target group: the RDA is ranging from 2 mg preformed niacin per day (infants) to 16 - 18 NE/day (during pregnancy) (Kirkland, 2007; Strohm et al., 2016). Niacin is not assigned to vitamins with a critical intake. German data show that the median intake of niacin is within or even higher than the range of reference (NVSII, 2008; Strohm et al., 2016). To ensure a diet rich in niacin equivalents, a consumption of protein containing foods such as fish, meat, beans, peanuts and mushrooms is recommended. A supplemental intake of niacin is not necessary for healthy people with a healthy lifestyle (Strohm et al., 2016). However, niacin is supplemented beside other vitamins (NVSII, 2008). Both, NA and NAM are authorised for the use in food and food supplements and for addition to foods for specific nutritional purposes and particular nutritional uses, to processed cereal-based foods and baby foods for infants and young

children and to infant formulae and follow-on formulae. Furthermore, niacin is listed as pharmacologically active substance (EFSA, 2012).

2.1.3 Synthesis, absorption, distribution and metabolism of niacin in humans

Synthesis

Beside the nutritional intake of NA, NAM and pyridine nucleotides, the amino acid tryptophan can act as precursor for the synthesis of niacin in mammals (**Figure 2.2**). However, the majority of tryptophan is catabolized through kynurenine and 2-amino-3-carboxymuconic-6-semialdehyde (ACMS) to Acetyl-CoA. If ACMS accumulates, some of it reacts spontaneously to quinolinic acid, which can be enzymatically transformed to NAMN, predominantly in the kidney and liver. Via Preiss-Handler pathway, NAD^+ can be synthesized from NAMN. Both, NA and NAM can also be transformed to NAD^+: NA via Preiss-Handler and NAM via Dietrich pathway (**Figure 2.2**) (Kirkland, 2007).

The utilization of tryptophan for niacin synthesis is affected by individual variations because of genetics, disease state and dietary components. Additionally, hormones and the energy, protein, pyridoxine and riboflavin status can influence the tryptophan-niacin-conversion. The niacin status is less incorporated into the regulation. First, dietary tryptophan is used to restore the nitrogen balance, afterwards, to restore blood pyridine nucleotides and last, to be excreted as niacin metabolite (Kirkland, 2007; McDowell, 2000).

Absorption

NAM and NA can be absorbed in stomach and small intestine, whereby the absorption in the small intestine is more rapid. Intact nucleotides (NADP/H) can be prior converted into NAM by pyrophosphatase in the upper small intestine (Kirkland, 2007). It is described that both vitamin forms are absorbed via a carrier-dependent (Nabokina et al., 2005) and/or sodium dependent mechanism (Evered et al., 1980). At higher concentrations, the absorption is triggered by passive diffusion (Kirkland, 2007). After entering the enterocytes, either NAM or NA are converted via Dietrich or Preiss-Handler pathway into NAD^+ to be stored or they can be released in their native forms into the portal circulation, whereby only minor amounts of native NA are circulating. Most of NA is converted through NAD^+ to NAM. The principal circulating form of niacin is NAM (Kirkland, 2007; McDowell, 2000).

Additionally, some microorganisms of the gut microbiota are capable to synthesize niacin, which is absorbed to colonocytes. The transport is a carrier-mediated mechanism. However, no data is available regarding the influence on the systemic niacin status (Biesalski, 2016; Kumar et al., 2013).

Distribution and metabolism

First, the circulating niacin compounds are either internalized by erythrocytes or transported to the liver, whereby the latter is the central processing organ for niacin. Both organs receive NA and NAM and can convert them into the nucleotides for storage (Kirkland, 2007). The storage of NAD^+ is used to supply other tissues with niacin, although most tissues can utilize NAM and sometimes NA to synthesize their own NAD^+. The most circulating form is NAM, which is released of NAD^+ via NAD glycohydrolases (Dietrich pathway) (Henderson, 1983; McDowell, 2000). Compounds for urinary excretion are metabolized in the liver, depending on the niacin status of the organism (Kirkland, 2007). NAM is primarily methylated to N1-methylnicotinamide (Friedrich, 1987; Kirkland, 2007), which can be further oxidized to N1-methyl-2-pyridone-5-carboxamide and N1-methyl-4-pyridone-3-carboxamide (Felsted and Chaykin, 1967). A second enzymatic system is suggested to oxidize NAM to NAM-N-oxide (Real et al., 2013). NA is conjugated with glycine to nicotinuric acid. High intake of dietary niacin can increase the levels of untransformed niacin in the urine (Kirkland, 2007). N1-methylnicotinamide is an often used metabolite of the urine to assess the niacin status (Friedrich, 1987).

2.1.4 Physiological, pharmacological and toxicological effects

The main function of niacin is that of the coenzyme form NAD/H and NADP/H. Enzymes containing NAD/H and NADP/H are important for biological oxidation-reduction systems because of their capacity to act as hydrogen-transfer agent. NAD^+ is reduced to NADH in several reactions of glycolysis, citric acid cycle, β-oxidation of fatty acids and other cellular oxidation reactions. Afterwards, the formed NADH functions as hydrogen donor to the respiratory chain for ATP production. Therefore, NAD(H) plays a central role in catabolic reactions, whereas NADP(H) is present in biosynthetic (anabolic) reactions. $NADP^+$ can be synthetized from NAD^+ via NAD kinase. The reduction of $NADP^+$ during the pentose phosphate pathway or during the malate-pyruvate shuttle across the mitochondrial membrane

results in NADPH, which serves as reducing agent for fatty acid production, cholesterol synthesis, manufacture of desoxyribonucleotides and several other cellular macromolecules (Kirkland, 2007; McDowell, 2000).

Furthermore, both NAD/H and NADP/H are integrated in the synthesis and degradation of amino acids. NAD^+ plays a role in ADP-ribosylation of proteins and poly(ADP)ribose. These poly ADP-ribolysated proteins are important for mechanisms like DNA-repair, DNA-replication and cell differentiation (McDowell, 2000).

Beside the essential physiological effects of niacin, several pharmacological functions of niacin are described. NA is associated with the treatment of hyperlipidaemia. The effect is related to a decrease of lipolysis in adipose tissue due to the binding of NA to a G-protein receptor (GPR109A) and a resulting inhibition of the activity of the enzyme adenylate cyclase. This leads to decreased cAMP (cyclic adenosine monophosphate) levels, which results in decreased mobilization of fatty acids. Therefore, VLDL (very low-density lipoprotein) formation in the liver is supressed and LDL (low-density lipoprotein) levels are decreasing. Furthermore, the positive HDL (high density lipoprotein) can be increased due to NA treatments (Gille et al., 2008; Kirkland, 2007). As a result, NA is an effective agent to reduce risk factors for atherosclerosis. However, high doses of orally administered NA causes unwanted side effects such as flushing, nausea and diarrhoea. A dose of 50 – 100 mg NA leads to a flush of the face and the upper body. Increasing the dose to 500 – 1000 mg the rest of the body is also affected by strong flushes. Flushing is explained by induced vasodilation. Typical symptoms are burning or stinging sensations, similar to sunburn but for shorter lasting (30 – 90 minutes). Although the flushing decreases after several weeks of continuous treatment, intolerable flushing is one reason of discontinuing the NA treatment. Doses up to 2000 mg NA could result in gastrointestinal side effects, whereby doses up to 3000 mg can lead to effects with liver toxicity and damage, a reduction of glucose tolerance and an increase in plasma uric acid (BfR, 2012; Domke et al., 2004; Gille et al., 2008; Kirkland, 2007).

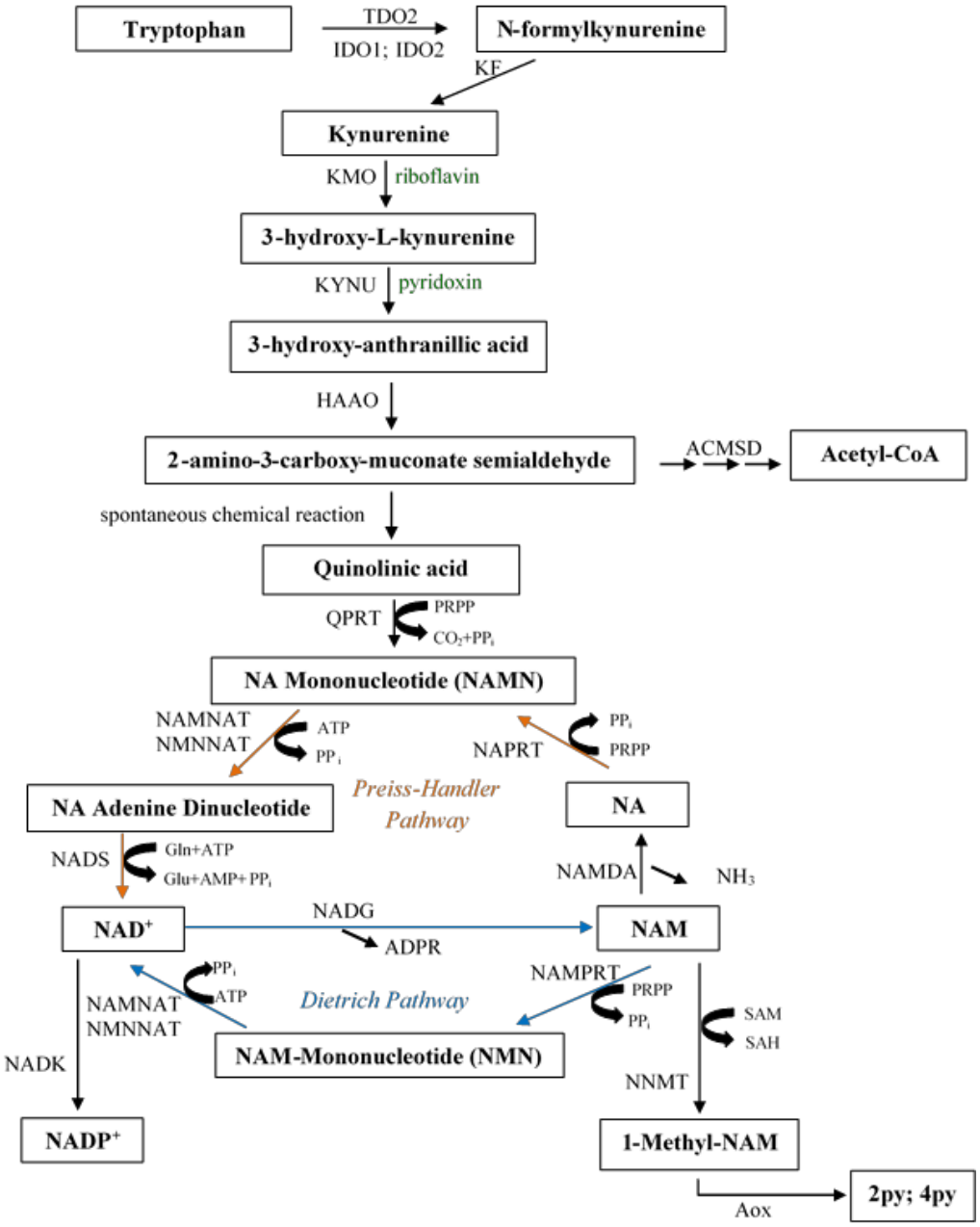

Figure 2.2: *De novo biosynthesis and metabolism of NAD(P)$^+$. (Friedrich 1987; Favennec et al. 2015; Chen und Guillemin 2009; Pissios 2017; Kirkland 2007; Berdanier und Adkins 1998).* *Abbreviations: 2py: N1-methyl-2-pyridone-5-carboxamide; 4py: N1-methyl-4-pyridone-3-carboxamide; ACMS: 2-amino-3-carboxy-muconate semialdehyde; ACMSD: ACMS-decarboxylase; ADP: adenosindiphosphate; ADPR: ADP ribolysation reactions; ATP: adenosintriphosphate; Aox: aldehyde oxidase; Gln: glutamine; Glu: glutamic acid; HAAO: 3-hydroxyanthranilate; IDO1: indoleamine 2,3-dioxygenase I; IDO2: indoleamine 2,3-dioxygenase II; KF: kynurenine formylase; KMO: kynurenine 3-monooxygenase; KYNU: kynureninase; NA: nicotinic acid; NAM: nicotinamide; NAD: nicotinamide adenine dinucleotide; NNMT: NAM-N-methyltransferase; NMNAT: NA-mononucleotide-adenylyltransferase; NAMNAT: NAM-mononucleotide-adenylyltransferase; NADG: NAD-glycohydrolases; NADK: NAD-kinase; NADS: NAD-synthetase; NAPRT: NA-phosphoribosyltransferase; NAMPRT: NAM-phosphoribosyltransferase; NAMDA: NAM-deamidase; NRK: NAM-riboside-kinase; PRPP: phosphoribosylpyrophosphate; PPi: pyrophosphate; SAM: S-adenosylmethionine; SAH: S-adenosylhomocysteine; TDO2: tryptophan 2,3-dioxygenase; QPRT: quinolinate phosphoribosyltransferase*

Therefore, the SCF (Scientific Committee on Food) defined a LOAEL (Lowest Observed Adverse Effect Level) of 30 mg NA per day. The UL (Tolerable Upper Intake Level) was set to 10 mg NA per day for adults (except for pregnant and nursing women) (Domke et al., 2004). Slow-release formulations (e.g. Niaspan®) were developed to suppress peak plasma levels and to achieve a continuous NA absorption over time (Gille et al., 2008; Knopp et al., 1998). However, some slow-release formulations could lead to constitutional or gastrointestinal symptoms (Knopp et al., 1985). Liver damages were already detected after doses of 1000 – 2000 mg NA per day (BfR, 2012). The colon targeted encapsulation of niacin of the present thesis, which aimed a minimal systemic exposure, is innovative and new.

In contrast to NA, NAM is under discussion regarding the prevention or delay of the onset of IDDM (Insulin-Dependent Diabetes Mellitus) (Kirkland, 2007). Furthermore, a recent study showed in a mouse model, that the topical administration of NAM influenced the gut microbiota and showed anti-inflammatory effects in the colon. It is suspected that NAM or tryptophan are transported on the luminal surface of epithelial cells, where mTOR is activated. Antimicrobial peptides are expressed and can affect the composition of the gut microbiota (Hashimoto et al., 2012). Despite the fact, that some gut microorganisms are also capable to synthesize niacin coenzymes (NAD/H) *de novo* or from the vitamin precursors (NA, NAM or nicotinamide riboside), further effects on systemic processes are unknown (Biesalski, 2016; Gazzaniga et al., 2009). Additionally, it was hypothesized that microbial produced niacin could bind to the colon receptor Gpr109a, which leads to a beneficial effect on colitis and colon cancer (Singh et al., 2014). The present thesis gives new approaches for a topical application of niacin in the colon.

With regard to toxicological effects of NAM, no adverse side effects on an acute basis are known (Domke et al., 2004; Kirkland, 2007). Larger doses (up to 10 g/day) can lead to gastrointestinal disorders and liver injuries. No flush symptoms are related to NAM. The SCF did not define a LOAEL for NAM, whereby the NOAEL (No Observed Adverse Effect Level) was set to 25 mg/kg/day and the UL to 900 mg/day for adults. Due to lower adverse side effect of NAM, compared to NA, it is often used for fortification and supplementation formulations (Domke et al., 2004).

2.1.5 Quantification

A wide range of chemical or microbiological assay techniques are available to detect niacin in food. Analytical methods can be based on official methods of USP/BP, AOAC or ISO/CEN/BS. Regarding AOAC methods, NA and NAM can be determined using colorimetric or microbiological assays. The colorimetric method is based on the Koenig reaction with cyanogen bromide. The coupling of the derivates (pyridinium compound, the result of the reaction of niacin and cyanogen bromide) to aromatic amines forms coloured compound, which is proportional to the niacin content, and can be measured spectophotometrically at an absorption maximum of 463 nm (Eitenmiller et al., 2008; Lawrence, 2015). This assay has been criticized because of its lack in specificity and sensitivity and the high toxicity of the reagent cyanogen bromide (Kim et al., 2011; Rose-Sallin et al., 2001). For microbiological assays, *Lactobacillus plantarum* is used. Niacin enables the proportional growth of *Lactobacillus plantarum* in a supporting medium. However, this method requires considerable laboratory set-up and maintenance time. Additionally, it can be subject to contamination. The most common methods are based on the UV absorbance of NA and NAM. Therefore, a UV/VIS-spectrophotometer (McLaren et al., 1973; Sheen et al., 1992) or a high performance liquid chromatography (HPLC) combined with a diode array detector (DAD) was used (Kirchmeier and Upton, 1978; Sood et al., 1977; Walker et al., 1981). The absorption maximum is measured at 261 or 262 nm. If niacin is determined simultaneously with other vitamins like pyridoxine, riboflavin and thiamine, a wavelength of 254 nm is chosen (Walker et al., 1981). However, due to the low specificity in the UV region of interest, interference with other food ingredients could lead to a low specificity and sensitivity (Kim et al., 2011; Lawrence, 2015). For determination of niacin in pure substance, injectable solutions and vitamin tablets and capsules, spectrophotometric and HPLC methods are suitable, as recommended by USP/BP (Lawrence, 2015). Further HPLC methods with fluorimetric detection after a post-column derivatisation with UV irradiation were developed to quantify niacin in food, although the pure niacin does not fluoresce (Lahély et al., 1999; Lawrence, 2015; Rose-Sallin et al., 2001). The combination of HPLC and mass spectrometry (MS) is also used for the determination of niacin. However, to overcome matrix effects, stable isotope-labelled compounds were used as internal standard e.g. deuterated niacin compounds (Goldschmidt and Wolf, 2007; Lawrence, 2015).

Depending on the food matrix and the type of niacin to be determined, different extraction processes are needed to determine the niacin content. Determining the total niacin content,

usually an alkaline hydrolysis is used. During the alkaline hydrolysis, bound niacin (NAD/H and NADP/H) is released from food matrix, whereby NAM is converted to NA. As a result, the total niacin content, independent from bioavailability for humans, can be determined. If only the free or added niacin needs to be quantified, an aqueous or acid hydrolysis is required, which can lead to a conversion from NA to NAM (Lawrence, 2015).

2.2 Microencapsulation

Microencapsulation is defined as a process, to encapsulate solid, liquid or gaseous substances, which can be released under specific conditions in controlled rates (Kunz et al., 2003). The size of the resulting microcapsules is ranging from 0.1 to 999 µm (Jackson and Lee, 1991). Encapsulation processes are commonly used by the pharmaceutical and food industry. The compound is protected towards external environmental influences like moisture, temperature, light, oxygen and interaction with incompatible substances. The reduced interaction leads to an improved product stability and shelf life (Bule et al., 2010; Champagne and Fustier, 2007; Gibbs et al., 2009; Heinzelmann and Franke, 1999; Kunz et al., 2003; Nedovic et al., 2011). Furthermore, microencapsulated products are mostly common as solids (powders or pellets), which are more stable and easier to handle than fluids, especially for slightly soluble substances (Gibbs et al., 2009). Microencapsulation techniques can also be used to mask unpleasant odours and tastes (Favaro-Trindade et al., 2010; Nedovic et al., 2011; Sohi et al., 2004) and to achieve a targeted release in the human gut to enhance the bioavailability or to influence the microbiota (Anal and Singh, 2007; Champagne and Fustier, 2007; Desai and Jin Park, 2005; Takahashi et al., 2009). The microencapsulation procedures are various (Arshady, 1993; Jackson and Lee, 1991; Kunz et al., 2003) e.g. physicochemical processes such as coacervation and liposomic entrapment (Frenzel et al., 2015), chemical processes such as interfacial polymerisation and physical processes such as extrusion, spray drying (Serfert et al., 2013) and fluidized bed coating (Berg et al., 2012; Schell and Beermann, 2014). Latter techniques are more common in food industry (Kunz et al., 2003). The following techniques are used for the experiments of the present thesis.

2.2.1 Fluidized bed coating

The application of a coating layer is one opportunity to cover and protect a compound and to influence the surface properties of the covered particles with regard to aesthetic or protective purposes. The coating should uniformly cover the surface of every single particle. Agglomerates should be avoided. Fluidized bed coating is used for smaller particles (100 µm to a few millimetres) (Gouin, 2004; Kumpugdee-Vollrath and Krause, 2011; Sandadi et al., 2004; Teunou and Poncelet, 2002). The coating process is based on suspended solid particles and the drying of the atomized liquid coating material around them (Kumpugdee-Vollrath and Krause, 2011; Nedovic et al., 2011).

The detailed process during the fluidized bed coating is shown in **Figure 2.3**. Small solid particles are suspended and maintained in a fluidising bed by a temperature (and humidity) controlled air stream. The fluidising air is flowing bottom-up. Depending on air velocity and particle flow properties, the state of the fluid bed is varying (Teunou and Poncelet, 2002). The coating material, which is dissolved in an acceptable solvent for food (e.g. water or ethanol), is transferred by a peristaltic pump to the nozzle, where it is atomized using compressed air. The atomized coating droplets are sprayed onto the particles. After reaching the top of the chamber, the wetted particles move downwards, while the solvent of the coating is evaporating. The coating material solidifies and distributes on the particles. Due to the fluidized bed, this wetting and drying process will be repeated several times, until a uniform film with the desired thickness is reached (Arshady, 1993; Desai and Jin Park, 2005; Kumpugdee-Vollrath and Krause, 2011).

Depending on the nozzle position, three spray modes are differentiated: bottom spray, top spray and tangential spray. The bottom spray mode is demonstrated in **Figure 2.3**. The nozzle is placed in the middle of the gas distributer plate and the coating solution is sprayed from the bottom to the top in parallel flow with the fluidization air. Processing chambers with a narrow diameter, like laboratory size equipment, lead to a well-organized particle flow, which results in reproducible coating successes. For upscaling processes with a larger diameter, a cylindrical Wurster insert is employed, which is a special form of the bottom spray mode. The Wurster process facilitates efficient separation between spraying and drying zone. The Wurster cylinder is inserted around the nozzle in a small distance to the distributor plate. The distributor plate for a Wurster process is characterised by larger holes in the cylinder and nozzle zone compared to the outer parts. This constellation leads to a more organized flow of the particles. The fluidized particles are horizontally transported through the gap between the

Wurster insert and the distributor plate into the spraying zone. Afterwards, they are moved with high velocity into the expansion zone. The particles decelerate and fall into the area around the Wurster cylinder, where the drying process is continued. After that, the process starts from the beginning (Kumpugdee-Vollrath and Krause, 2011; Teunou and Poncelet, 2002; Werner et al., 2007; Yang et al., 1992). Problems could occur if the fluidized particles tend to adhere to the walls due to electrostatic charge or remaining solvent. Especially, in case of a Wurster insert, the fluidized bed could finally break down, which makes an optimal adjustment of temperature and spraying rate indispensable (Fukumori et al., 1991).

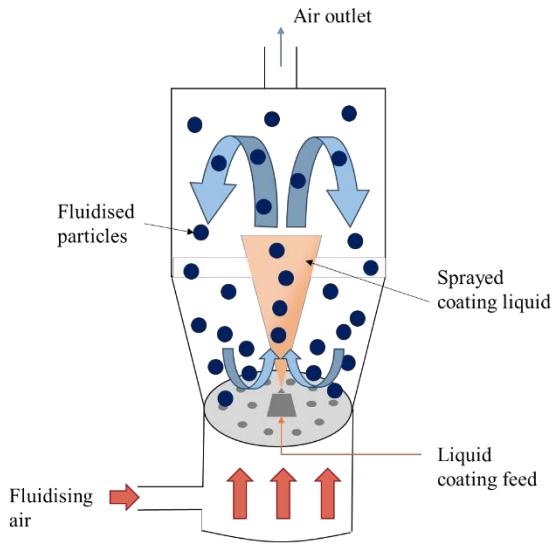

Figure 2.3: *Schematic process of fluidized bed coating with bottom spray, modified according (Kumpugdee-Vollrath and Krause, 2011; Teunou and Poncelet, 2002)*

In the top spray mode, the nozzle is placed in the expansion chamber. The fluidization air and the sprayed coating liquid are arranged in a counterflow (Yang et al., 1992). During the top spray configuration, coating imperfection is possibly caused by premature droplet evaporation before contacting the particles (Desai and Jin Park, 2005). The third spraying mode is the tangential-spray mode. The nozzle is located at the side of the product chamber. In contrast to top and bottom spray, for tangential spray an additional rotating disk is added to generate a centrifugal, gravitational and vertical particle flow in order to maximize the coating efficiency (Yang et al., 1992). The coating quality is comparable to a Wurster bottom spray apparatus, whereby the tangential spray is not suitable for friable materials, because of the high agitation (Teunou and Poncelet, 2002). Therefore, the bottom spray process, especially the Wurster configuration, is commonly used for coating processes.

Fluidized bed coating can be conducted as a batch or continuous process. The focus in the present thesis is on the batch process as shown and explained in **Figure 2.3**. Until now, the batch process is more common for coating processes, although a continuous process is desirable because of a time saving. During a single bed continuous process, particles with a pre-defined size are automatically discharged by a pipe at the bottom of the chamber that is determined by a controlled airflow. Due to gravity, coated particles are classified from uncoated particles. Dust and small particles are transferred back to the product chamber as well as crushed oversized particles. Additionally to the single bed continuous process, horizontal and multicell continuous fluid beds are existing with product chamber separated in smaller chambers. All continuous processes are prepared with the top spray configuration. As mentioned, the advantage of continuous processes are a higher flow rate and therefore lower production costs, which is important for the food industry. However, the coating quality can be imperfect, if the coating material is not covering the total surface. This would lead to a loss of protection. This could be a problem, especially, if the difference between the size of coated and uncoated particles is too small (Kumpugdee-Vollrath and Krause, 2011; Teunou and Poncelet, 2002).

In order to achieve a uniform coating result and a high coating efficiency the adjustment of process parameters is important. Variables like inlet air temperature, inlet air velocity, spray rate, solution temperature and dry matter content, atomization pressure, ambient air temperature and relative humidity as well as outlet air temperature and relative humidity are influencing the fluidized bed operation. Especially the degree of agglomeration and premature drying are detrimental for the coating result (Arshady, 1993; Desai and Jin Park, 2005). Furthermore, agglomeration can result in a defluidisation that could lead to a process shutdown and product loss. To avoid agglomeration, the droplet size of the atomized coating material should be smaller than the particle size (Gouin, 2004; Werner et al., 2007). The droplet size is depending on process parameters such as the air and fluid velocity and material characteristics like viscosity, density and surface tension. A very small droplet size would lead to premature droplet evaporation, which results in a lower coating efficiency (Dewettinck and Huyghebaert, 1998).

The application of a uniform coating is particularly important, when a targeted release profile is desired. The more spherical and regular the particle, the more uniform is the coating (Chopra et al., 2002). Additionally, different sizes and weights of particles lead to different coating thicknesses (Wesdyk et al., 1990). Therefore, a narrow size distribution of the

fluidized particles is important (Gibbs et al., 2009; Wesdyk et al., 1990). Methods such as pelletization disc, high shear granulator, fluid bed granulator and extrusion/spheronisation are commonly used for pelletization. In the present thesis, spray granulation and wet extrusion/spheronisation were used for producing spherical pellets and will be described in the following sections.

2.2.2 Spray granulation

Spray granulation can be defined as a particle-forming process by which a solid containing liquid is converted into solid granules by simultaneous drying (Link and Schlünder, 1997). Hot air is used to fluidize the produced particles. The temperature-controlled air also leads to an evaporation of the solvent, which is sprayed through an atomizing nozzle. The dissolved material will adhere on the surface of the seeding particle, which results in an increase of the granule diameter. External material or undersized particles of the previous batch, can be used as seeds or starting material. During the process, spray dried particles (drying prior to contact with fluidized particles), dust, abrasion and fragments are used for further granulation. In contrast to agglomeration, the particle growth proceeds by surface layering, comparable to the fluidized bed coating. For spray granulation the top spray mode is often used. The process is easy to handle in continuous process (as described in **Chapter 2.2.1**). The final granules (pellets) are consisting of the dry matter of the sprayed solution formed by spray granulation. Granules characteristically have a well-rounded and uniform “onion skin” layered structure, which is optimal for a further protecting coating step and a high particle strength (Link and Schlünder, 1997). The process parameters need to be adjusted to avoid agglomeration and to achieve spherical granules with a narrow particle size distribution. The particle size range can be controlled by regulating the air stream for classification (see **Chapter 2.2.1**) (Kumpugdee-Vollrath and Krause, 2011; Teunou and Poncelet, 2002).

2.2.3 Extrusion-spheronisation

Formation of round and small pellets is achieved by extrusion and subsequently spheronisation. During extrusion, a wet mass is shaped into rods by passing an extruder screen. Four main extruder classes are existing: screw, sieve and basket, roll and ram extruder. In the present thesis, a twin-screw extruder was used. Two screws are mixing the

added powder (consisting of the drug and some pelletizations aids) and granulating liquid (e.g. water), while simultaneous transferring the wetted mass to the die at the end of the barrel, where cylindrical extrudates are formed (**Figure 2.4A**). The barrel can be tempered if necessary. After the extrusion process, the wet extrudates are transferred into a spheronizer. A spheronizer is equipped with a rotating cross-hatched friction plate, where the extrudates are broken up into smaller cylinders (**Figure 2.4B**). However, not every wetted mass can be successfully extruded and spheronized. Therefore, spheronization aids, in most cases microcrystalline cellulose (MCC), are used to form spherical pellets. Due to its high water absorption and retention property, a cohesive plastic mass is formed, which is brittle enough to be broken down but not to disintegrate completely and which is plastic enough to be rolled into spheres but not too wet to agglomerate (Dukić-Ott et al., 2009; Vervaet et al., 1995).

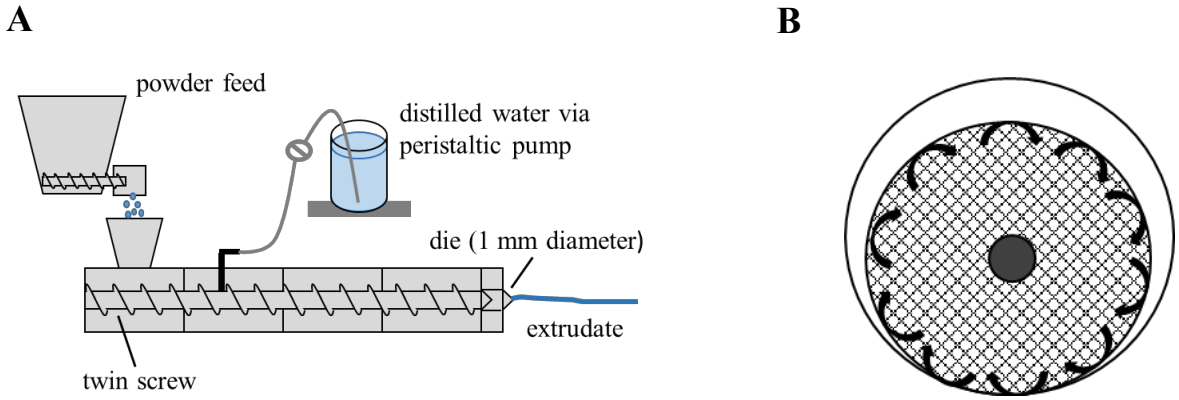

Figure 2.4*: Schematic demonstration of a twin-screw extruder (A) and a spheronizer (B)*

2.2.4 Hydrogel bead production – ionotropic gelation

Hydrogels are defined as three dimensional, hydrophilic networks. The incorporation of compounds is possible, whereby the release rate in the human gastrointestinal tract can be influenced. Hydrogel beads are often prepared by ionotropic gelation. This technique is based on the ability of some polymers to cross link in the presence of counter ions. Examples for natural polymers used for drug encapsulation via ionotropic gelation are alginates, gellan gum and pectin as anionic materials and chitosan as cationic material. To achieve a hydrogel bead, an aqueous solution of the polymer (and the desired compound to encapsulate) are added dropwise into a solution of the counter ions. The anions/cations of the polymer react with the cations/anions of the cross linking solution. For anionic polymers, calcium chloride is often

used as cross-linking solution. Through electrostatic and ionic bonding a proper gel is formed. The polymers can also act as release rate retardant (Patil et al., 2010; Patil et al., 2012; Smrdel, 2008; Sriamornsak, 2011).

2.2.5 Materials for colon targeted delivery

Depending on the desired functionality of the formulation, different materials are suitable. Independently of the application as a coating or matrix material, the material is required to ensure that the formulation is delivered to the target site, where the encapsulated compound is released in adequate amounts. Regarding a colon targeted delivery, the active ingredients need to be protected during the entire gastric and small intestine transit. The site-specific release should be indicated by differences between the small and large bowel. These are more difficult to seize compared to the passage from stomach to the small intestine. However, differences in the composition of the microbiota, which is more abundant in the large intestine than in the small intestine, the intraluminal pressure, the total transit time, both increases from proximal to distal, and the differences in pH values of the fluids can trigger the release of the encapsulated substance (Maroni et al., 2013).

For influencing the microbiota, a release in the distal ileum and proximal colon region, where most of the living microorganisms are located, is desirable (Sekirov et al., 2010). In the human gastrointestinal tract, the pH values are varying from stomach to proximal small intestine, distal small intestine and large intestine. Detailed information are listed in **Chapter 2.3**. The pH value is increasing in the ileum and in the ascending colon the pH value is decreasing slightly (Evans et al., 1988; Ibekwe et al., 2008; Lesmes and McClements, 2009), due to microbial fermentation products (short-chain fatty acids (SCFA)) (MacFarlane et al., 1992). Therefore, the application of pH-sensitive coatings an approach to achieve an (ileo-)colon targeted release e.g. shellac.

The gastrointestinal tract (GIT) is the most heavily colonized organ in comparison to the human skin, genitourinary and pulmonary tract, which are inhabited by a number of microorganisms. The number as well as the variety of microorganisms in the GIT is increasing from proximal to distal. In the stomach a number of 10^1 cells per gram are documented, which is increasing to 10^3, 10^4, 10^7 and 10^{12} cells per gram from duodenum, jejunum, ileum to colon. The dominating phyla of the human gut microbiota are *Bacteroidetes* and *Firmicutes*. *Proteobacteria*, *Verrucomicrobia*, *Actinobacteria*, *Fusobacteria* and

Cyanobacteria representing minor microorganisms (Sekirov et al., 2010). The inhabiting microbiota can catalyze many enzymatic reactions, which are not proceeded by enzymes of human digestion fluids. Therefore, materials, which are not susceptible to the digestion in the small intestine and which will be degraded by colonic bacteria, may serve as colon delivery aids e.g. pectin (Maroni et al., 2013; Rubinstein, 1990).

2.2.5.1 Shellac

Shellac is the refined form of the natural insect resin lac. The lac is secreted by insects *Kerria lacca* in India, Thailand or Southeast Asia, which are parasitic on certain trees. After the insects permeate the bark of the trees, they convert the sap internally to a polyester resin. Afterwards, they secrete the resin through their body surface. Thick encrustations of the resin are formed on the twigs. The resin should protect the brood from extreme temperatures and predators. After hatching, the encrustations are harvested (seedlac) and further processed to achieve the final refined product called shellac. Depending on the insect species, host trees and refining process, the chemical composition, color and the properties such as release behaviour of shellac is varying. Three different refining processes are used: melting process, bleaching process or solvent extraction process, whereby latter process does not change the chemical structure of shellac. The resulting shellac film is crushed to flakes or milled into a fine powder (Buch et al., 2009; Farag and Leopold, 2011b; Penning, 1996).

Shellac consists of polyesters of mainly aleuritic acid, shellolic acid, jalaric acid, butolic acid and small amounts of free aliphatic acids (Christie et al., 1963; Wang et al., 1999). The soft fraction of shellac consist of single esters of a sesquiterpenoid acid and a hydroxyl fatty acid, whereby the hard fraction are single esters polymerized by ester linkages (Wang et al., 1999).

Shellac is a food grade material and legislated as food additive under the E-Number E904. It is used in the food, food supplement, nutraceutical and pharmaceutical industry for different applications. Shellac is suitable as moisture barrier and gloss coat (Pearnchob et al., 2003b; Trezza and Krochta, 2001), therefore, it is often used as glazing agent for citrus fruits and confectionaries. In the nutraceutical or pharmaceutical industry, shellac is frequently used as enteric coating material for a delayed and/or colon targeted release in the human gut, due to its acidic character and the presence of carboxylic groups (Al-Gousous et al., 2015; Pearnchob et al., 2003a; Roda, 2007). The pk_a value of shellac is between 6.9 and 7.5, therefore shellac

dissolves at a pH value of about 7.0. In the acidic milieu of the stomach, shellac is still insoluble, because of protonated carboxylic groups (Limmatvapirat et al., 2007). These properties make shellac suitable for formulations with desired gastric resistance and delayed release in the ileocolonic region.

Indeed, natural products are a potential source of batch-to-batch variations and alterations (Krause and Müller, 2001; Penning, 1996). Shellac, which is soluble in solvents like ethanol and methanol, is often described as an instable and hardening material over time. Aging of shellac leads to changes in the release characteristics like loss of gastric resistance and a decrease in intestinal fluid solubility, which is disadvantageous compared to synthetic materials (Farag and Leopold, 2009; Penning, 1996). During ageing, polymerization via esterification of the carboxylic and alcohol groups occurs, as shown in **Figure 2.5**. The aqueous shellac solution produced by addition of alkali salts, e.g. the volatile ammonium carbonate can hinder the polymerization reaction of the carboxylic and the alcoholic groups within the film (**Figure 2.5**) (Limmatvapirat et al., 2007; Penning, 1996). However, during coating, curing and storage a part of the present ammonium can evaporate as ammoniac, which results in protonated carboxyl groups and could be polymerized in further processes (Al-Gousous et al., 2015; Limmatvapirat et al., 2004; Schmidt and Teuber, 1991). Regarding the water vapour permeability and the water uptake, the aqueous ammonium shellac is more permeable than the native acid form of shellac (Hagenmaier and Shaw, 1991; Pearnchob et al., 2004). However, gastric resistance is also given. After exposition to gastric fluids, the carboxyl groups are protonated, which results in an insoluble and protecting outer layer (Henning et al., 2012; Penning, 1996). The water-soluble shellac is also used for the present studies.

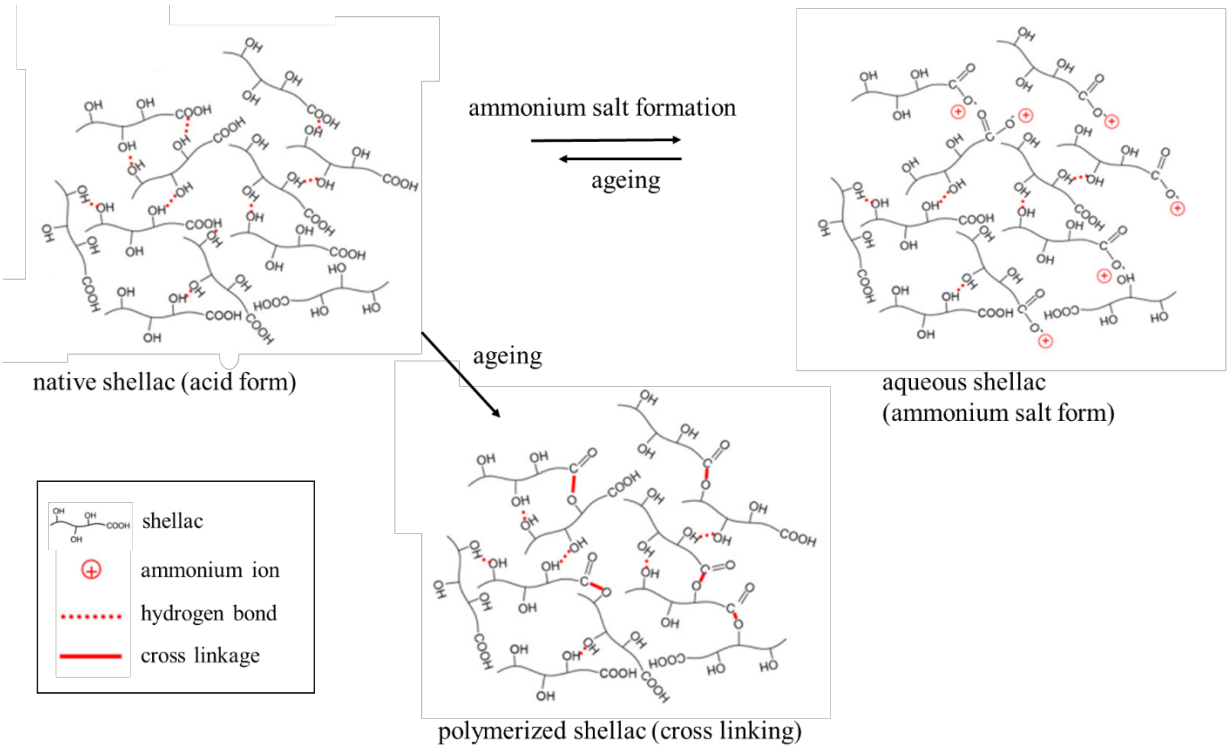

Figure 2.5*: Chemical structure of native shellac and its ammonium salt form and the effect of ageing (Limmatvapirat et al., 2007)*

The film formation of either the dissolved native shellac in organic solvents or the water-soluble ammonium salt shellac solution occurred by evaporation of the solvent. The sprayed droplets coalesce on the surface, the polymer concentration and interaction is increasing, whereby an intermediate gel-like stage is reached. After further evaporation, a continuous solid polymeric film is obtained (Banker, 1966; Schmidt and Teuber, 1991).

2.2.5.2 Pectin

Pectin is a natural polysaccharide, which occurs in higher plants as cell wall structural carbohydrate. It is a heterogeneous molecule, which contains α-1,4-glycosidic bonded poly-D-galacturonic acid. Neutral sugars, like L-rhamnose, can be inserted or attached to the main chain of pectin. The carboxyl groups of the pectin molecule are partially esterified with methanol. Depending on the degree of esterification (DE), low and high methoxylated (LM: DE < 50 %, HM: DE > 50 %) pectins are differentiated. Amidated pectins possess carboxamide groups instead of carboxylic groups, when ammonia was used for the de-esterification process (Liu et al., 2003; Willats et al., 2006). Pectin is broadly used in the food

and pharmaceutical industry because of its gelling ability and known as E440. Depending on temperature, pectin type, DE, degree of amidation (DA), pH value, sugar, calcium and other solutes, gels with different physico-chemical properties can be formed. HM pectin is building a three-dimensional network by cross-linking of galacturonic acid via hydrogen bridges and hydrophobic forces to methoxyl groups. This reaction occurs in the presence of high sugar concentrations and low pH values. LM pectins form gels by cross-linking of free carboxyl groups with calcium ions, as described in **Chapter 2.2.4**. The formed complexes are also known as "egg box" complexes (**Figure 2.6**). Calcium pectinate shows a reduced solubility, which could lead to an inhibited release of the encapsulated compound. Furthermore, calcium pectinate is still suitable for enzymatic degradation in the colon. However, calcium pectinate matrices seem to be limited for lipophilic or extremely low water-soluble substances. For water-soluble compounds, the application of an additional coating layer or a combination with other polymers is proposed. Some combinations have already been studied (Liu et al., 2003; Willats et al., 2006). One option is the combination with an additional zein coating, which should enhance the water resistance (Chotiko and Sathivel, 2017; Liu et al., 2006).

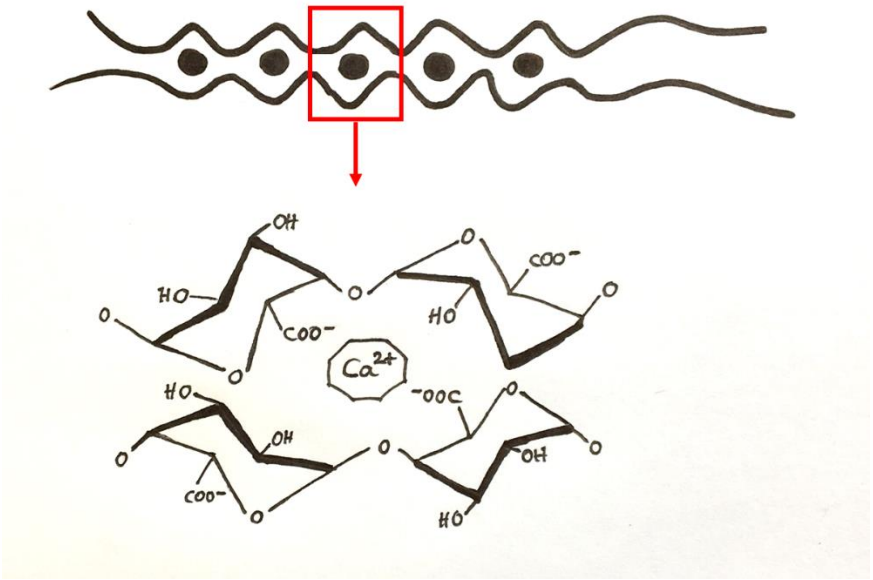

Figure 2.6: *Egg box model for the gelation mechanism of low methoxylated pectin in the presence of calcium ions (Sriamornsak, 2011)*

2.2.5.3 Zein

Zein is the major storage protein of corn. About 45 – 50 % of corn protein is zein. It is distributed as "zein-bodies" in the cytoplasm of the endosperm cells. The molecular weight of

zein is about 40 kDa and it belongs to the class of prolamins. Four classes of zein are known: α-, β-, γ- and δ-zein with different protein fractions. Zein is a natural biopolymer and approved by the FDA (Food and Drug Administration) (Patel and Velikov, 2014). Regarding its amino acid composition, zein has a deficiency in acid and basic amino acids, except of glutamic acid, and a high proportion of nonpolar amino acids (leucine, proline, alanine). This amino acid composition leads to a low solubility in water, alcohol and alkalis (pH value > 11). Due to its property to form tough, glossy, flexible and hydrophobic coatings, it is applicable for controlled release formulations and as moisture and water barrier (Paliwal and Palakurthi, 2014; Shukla and Cheryan, 2001; Zhang et al., 2015). Liu et al., combined pectin and zein for the encapsulation of indomethacin and BSA into the hydrogel and achieved a colon-targeting (Liu et al., 2006).

2.3 *In vitro* dissolution testing

In vitro tests are conducted to evaluate the dissolution and release behaviour of dosage forms simulating the human gastrointestinal tract. Nevertheless, *in vivo* studies are still the "golden standard" for bioaccessibility, absorption, bioavailability, metabolism and excretion. However, *in vitro* dissolution methods are less labour and cost intensive, more rapid and have lower bioethical restrictions (Shani-Levi et al., 2017). Especially for early stages of formulation design and optimization, *in vitro* dissolution tests are feasible (Jorgensen and Bhagwat, 1998; Zahirul and Khan, 1996).

Regarding European Pharmacopeia, the dissolution specification for a delayed release formulation has to be decided from case to case according to the formulation design. Due to prolonged gastrointestinal residence of the intact dosage form and variabilities in physiological conditions, the establishment of a proper dissolution test for controlled release oral dosage forms is very difficult, compared to immediate release formulations (Zahirul and Khan, 1996). To simulate the *in vivo* release for an (ileo-)colon targeted delivery, the *in vitro* dissolution tests need to be adjusted to the gastrointestinal conditions from stomach to colon and to the individual drug delivery system (pH-, time-, pressure- or microflora-activated system) (Yang et al., 2002). An overview of the human gastrointestinal conditions regarding pH value and transit time is given, which are the most important factors influencing the release from shellac coated particles. For pectin beads, microbial enzymes in the large bowel are considered. Nutraceuticals, which are supposed to be swallowed, are only validated simulating gastric and intestinal conditions.

Stomach

The pH value of gastric fluid is low compared to the rest of the gastrointestinal tract. It was found that the pH value varies between fasted and fed state. In fasted state pH values of about 1 – 3 were found (Dressman et al., 1990; Gruber et al., 1987; Ibekwe et al., 2008; Russell et al., 1993), whereby in fed state the pH value can increase to pH 4 – 6 (Dressman et al., 1990; Russell et al., 1993). However, pH values higher than 6 are also documented (Dressman et al., 1990; Lindahl et al., 1997; Russell et al., 1993). Within two or three hours, the pH value returns to fasted state values, depending on meal size (Dressman et al., 1998). The requirements of the Pharmacopeia for gastric resistance are fulfilled, if less than 10 % of the encapsulated drug is dissolved after two hours at pH value 1.2 (Tarcha, 1991). To simulate fed stomach conditions, higher pH values (pH 2 – 4) (Czarnocka and Alhnan, 2015) and more complex media compared to buffer solutions are used (Galia et al., 1998; Jantratid et al., 2008; Klein et al., 2004).

Regarding residence time in the stomach, a high variability from 15 – 420 min is described due to food composition, food structure and size, calorie content and biological factors (Clarke et al., 1993; Clarkston et al., 1997; Davis et al., 1987, 1987; Dressman et al., 1998; Guerra et al., 2012; Ibekwe et al., 2008). Pellets, which are smaller than 2 mm, are able to pass the pylorus independently of gastric emptying, which reduces the residence time (Davis et al., 1987).

Small intestine

The small intestine is compartmented in duodenum, jejunum and ileum from proximal to distal. Depending on fasted or fed state, the pH value in the small intestine can vary. Values for the pH are documented between 5.4 – 7.5 (Dressman et al., 1990; Guerra et al., 2012; Russell et al., 1993; Shani-Levi et al., 2017). An increasing pH value from proximal (6.2 – 6.6) to mid (6.7 – 7.4) and distal (7.4 – 7.5) small bowel was found (Evans et al., 1988; Ibekwe et al., 2008). For *in vitro* dissolution tests a pH value between 6.5 and 6.8 is often used, which is representative for the complete small intestine (Czarnocka and Alhnan, 2015; Dressman et al., 1998; Farag and Leopold, 2011a; Galia et al., 1998; Klein, 2010; Marques, 2004; U.S. Pharmacopeial Convention, 2005), whereby simulated fed state intestinal fluids can have a lower pH value of about 5.0 (Klein, 2010; Marques, 2004; Marques et al., 2011).

The transit time in the small intestine was found to vary between 2 and 5 hours (Guerra et al., 2012), depending on the combined food size, whereby the effect of the gastric emptying time is mostly responsible for this variability (Clarke et al., 1993; Davis et al., 1987; Dressman et al., 1998; Ibekwe et al., 2008; Malagelada et al., 1984). For pellets, a median small intestine transit time of 2.7 – 3.1 h (Clarke et al., 1993) and 1.5 – 5.7 h (Abrahamsson et al., 1996) is described.

Large intestine

From proximal to distal large intestine, the pH value increases from 6.37 to 6.61 and 7.04 (Evans et al., 1988), whereby the pH value in the ascending colon is lower compared to the ileum (Gruber et al., 1987; Guerra et al., 2012; Lesmes and McClements, 2009; Shani-Levi et al., 2017). For *in vitro* simulations of the colon, phosphate buffers were used (Abraham and Srinath, 2007), sometimes with added enzymes like galactomannase (Singh et al., 2004) or pectinase (Liu et al., 2006). Added enzymes should simulate the enzymes produced by gut microbiota neglecting complexity as well as the anaerobic conditions. Therefore, adapted *in vitro* simulations were developed with added faeces inoculum, to mimic the fermentation in the lower gut (Barry et al., 1995; Glei et al., 2006; Stein et al., 2011).

The transit time of the large intestine is characterized by a huge variability ranging from hours to days (Shani-Levi et al., 2017). Depending on the dosage form, different colon transit times were detected. Smaller pellets resulted in a longer transit time of 28.4 h, compared to tablets (15.2 h) (Abrahamsson et al., 1996). The longer residence time of smaller particles was explained due to a slower transport (Abrahamsson et al., 1996) and to the extensive mixing in the proximal colon (Dressman et al., 1998). The caecal arrival time (C_{50}) of pellets is about 3.6 – 7.9 h after oral intake, whereby the variability is affected due to the average gastric emptying and the small intestine residence (Clarke et al., 1993; Davis et al., 1987; Ibekwe et al., 2008). The ascending and transversal colon is of interest for further drug absorption. The distal parts of the colon only show small amounts of fluids to dissolve the encapsulated compound, because of its leading function to form faeces (Dressman et al., 1998; Schiller et al., 2005).

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3 Paper I: Targeted microbiome intervention by microencapsulated delayed-release niacin

Targeted microbiome intervention by microencapsulated delayed-release niacin beneficially affects insulin sensitivity in humans

Short running title: Microbiome as a therapeutical target

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3.1 Abstract

OBJECTIVE: Gut microbiota represent a potential novel target for future prediabetes and type 2 diabetes therapies. In that respect, niacin has been shown to beneficially affect the host-microbiome interaction in rodent models.

RESEARCH DESIGN AND METHODS: We characterized more than 500 human subjects with different metabolic phenotypes regarding their niacin (nicotinic acid (NA) and nicotinamide (NAM)) status and their gut microbiome. In addition, NA and NAM delayed-release microcapsules were engineered and examined *in vitro* as well as *in vivo* in two human intervention studies (bioavailability study and proof-of-concept/safety study).

RESULTS: We found a reduced α-diversity and *Bacteroidetes* abundance in the microbiome of obese human subjects associated with a low dietary niacin intake. Therefore, we developed delayed-release microcapsules targeting the ileo-colonic region to deliver increasing amounts of NA and NAM to the microbiome while preventing systemic resorption to avoid negative side effects (e.g. facial flushing). *In vitro* studies on these delayed-release microcapsules revealed stable conditions at pH 1.4, 4.5 and 6.8 followed by release of the compounds at pH 7.4, simulating the ileo-colonic region. In humans *in vivo*, gut-targeted delayed-release NA but not NAM produced a significant increase of the *Bacteroidetes* abundance. In the absence of systemic side effects, these favorable microbiome changes induced by microencapsulated delayed-release NA were associated with an improvement of biomarkers for systemic insulin sensitivity and metabolic inflammation.

CONCLUSION: Targeted microbiome intervention by delayed-release NA might represent a future therapeutic option for prediabetes and type 2 diabetes.

Keywords: obesity, type 2 diabetes, microbiome, niacin, tryptophan, coating, delayed-release, microencapsulation

3.2 Introduction

Rather than being viewed as simple commensals, the gut microbiome is now seen as playing an active role in the control of energy homeostasis and in the mediation of the adverse consequences of obesity (Turnbaugh et al., 2006). In the recent past several studies in humans and rodents revealed that obesity is associated with a reduction in *Bacteroidetes* (Ley et al., 2005; Ley et al., 2006; Turnbaugh et al., 2009) as well as a lower diversity compared to healthy and lean subjects (Cotillard et al., 2013; Turnbaugh et al., 2009). Of physiological relevance, the composition of the gut microbiota can be altered by diet, as weight loss interventions have been reported to influence the abundance of *Bacteroidetes* (Ley et al., 2006) and the overall microbial diversity (Cotillard et al., 2013; Heinsen et al., 2016).

Administration of niacin (nicotinic acid (NA) and nicotinamide (NAM)) has been shown to beneficially effect the host-microbiome interaction in a mouse model (Hashimoto et al., 2012). Nicotinamide adenine dinucleotide (NAD+) is the central co-factor of metabolism, mediating fuel oxidation, ATP generation, reactive oxygen species (ROS) detoxification, biosynthetic processes, DNA repair and nutritionally sensitive gene regulation (Belenky et al., 2007). In vertebrates, NAD+ is synthesized *de novo* from tryptophan (Trp) and from three vitamin precursors. NAM and NA are the classical NAD+ precursor vitamins. Nicotinamide riboside (NR) was discovered to be a NAD+ precursor vitamin much more recently (Bogan and Brenner, 2008). The NAD+ metabolome has been shown to be dysregulated in obesity and type 2 diabetes (T2D) in mice (Trammell et al., 2016). Moreover, NR repletion has been shown to blunt weight gain on high fat diet (Cantó et al., 2012) and to oppose fatty liver on high fat high sucrose diet (Gariani et al., 2016), largely by increasing the activity of SIRT1, an NAD+-dependent protein lysine deacetylase.

These data on the beneficial effects of niacin on both, the gut microbiome and the systemic glucose metabolism suggest this micronutrient to be an interesting candidate for future targeted microbiome interventions, e.g. to prevent manifestation of type 2 diabetes from prediabetes. However, because the upper gastrointestinal tract efficiently resorbs soluble micronutrients, simply increasing the NA and/or NAM nutritional load would not be expected to deliver these molecules into the ileo-colon region, where most of the microbiome is located (Sekirov et al., 2010). The aim of the present study was therefore (I) to examine NA and NAM in humans in relation to obesity and the gut phylogenome in a large human cohort of >500 well characterized individuals and (II) to use a microencapsulation procedure to develop a novel delayed-release system to deliver significant amounts of NA and NAM into the

human colon in order to beneficially affect the gut microbiome and the systemic metabolism while preventing systemic side effects.

3.3 Research Design and Methods

3.3.1 Study cohorts and study designs

511 subjects of the Food Chain Plus (FoCus) cohort, which has been reported earlier (Müller et al., 2015), were included in the present investigation. Fasted serum samples and stool samples were collected and anthropometric measurements were performed within subjects of the FoCus cohort. Further, a 12-months retrospective food frequency questionnaire (EPIC) was completed by n=481 subjects (Kroke et al., 1999) in order to determine niacin (NA+NAM) nutritional intake. Baseline characteristics of the FoCus-subset are shown in **Table 3.1**. Two human intervention studies were performed to evaluate NA/NAM microcapsules *in vivo*: (I) a bioavailability study including 20 healthy subjects (mean age 26.85±4.86a, 50% female, median BMI 22.78 kg/m^2 (21.25; 25.57)) (**Supplementary Table 3.2**) and (II) a proof-of-concept and safety study including 10 metabolically healthy subjects without manifest metabolic diseases and normal glucose and triglycerides levels (mean age 44.80±11.06a, 80% female, BMI 26.67±3.68 kg/m^2) (**Supplementary Table 3.3**). In those human interventions, NA- and NAM-effects observed under different dosages of microencapsulated NA (=”NA group”) or NAM (=”NAM group”) were compared with effects after ingestion of a reference dose of free NA (30 mg) or NAM (900 mg). During bioavailability study one subject of NA-group dropped out after two study days due to persistent difficulties in blood sample collection. Within the proof-of-concept and safety study one subject of NA-group had to be excluded after week 5 due to elevated aspartate transaminase (AST) levels, and one subject of NAM group dropped out in week 2 due to an accident not associated with the study.

Subjects were recruited at the University Hospital Schleswig-Holstein (Kiel, Germany). Niacin interventions (D439/15) and FoCus study (A108/08) were approved by local ethic committee and written confirmed consent was obtained from each subject.

3.3.2 Biochemical analysis

Blood samples were subjected to routine laboratory analyses at the central laboratory of the University Hospital Schleswig-Holstein (Kiel, Germany). Homeostasis Model Assessment

(HOMA) index was calculated as glucose (mg/dl)*insulin (µU/ ml)/405. Serum was stored immediately at -80°C. NA and NAM serum levels were measured by liquid chromatography and mass spectrometry (LC-MS/MS; Agilent 1100 HPLC/CTC-PAL Autosampler/Sciex API 4000 Triple Quadrupole) in an external specialized laboratory (Medizinisches Labor Bremen). Systemic metabolic parameters in the serum samples were measured by Enzyme-linked Immunosorbent Assay (ELISA) using myostatin test kit (SEB653Hu, Cloud-Clone Corp.), fetuin-A test kit (SEA178Hu, Cloud-Clone Corp.) and osteopontin test kit (SEA899Hu, Cloud-Clone Corp.), following manufacturer's instructions. Gut microbiome analysis was performed by 16S rDNA amplicon sequencing as described by Heinsen et al. (Heinsen et al., 2016).

3.3.3 Production of NA and NAM microcapsules and *in vitro* evaluation

Microcapsule cores, including NA or NAM (SternVitamin, Ahrensburg, Germany), were prepared. In order to achieve higher daily doses of NAM, NAM cores were prepared by a ProCell fluidized bed granulator with Vario 3 from an extern company (Glatt Ingenieurtechnik, Weimar, Germany). In contrast, NA was applied to Cellets350 in a Mini Glatt fluidized bed coater with bottom spray. In the next step, NA/NAM cores were both coated with an inner shellac (SSB Aquagold, Bremen, Germany) coating (2 % weight gain (w.g.)/50 % w.g.), an intermediate layer of a pH-modulating substance, sodium bicarbonate for NA (1 % w.g.) and citric acid for NAM (1 % w.g.) and at last an outer shellac coating (20 % w.g./10 % w.g.). Afterwards, microcapsules were dried at 50 °C in an oven for 1 h. For *in vivo*-studies NA and NAM microcapsules and NA- and NAM-powder (free niacin) were filled into standard size 0 gelatin capsules using a manual capsule filler. Differential dosing was achieved by administration of different amounts of full or partially filled capsules.

Dissolution tests were performed in triplicate with 0.5 g of NA or NAM microcapsules in 250 ml simulated gastric fluid (pH 1.4), citrate buffer (pH 4.5) and phosphate buffers (pH 6.8&7.4) using a standard dissolution paddle apparatus at 100 rpm and 37 °C (DT 70, Pharmatest Group, Hainburg, Germany). The exposition to the release media was set to 1 h at pH 1.4, 0.5 h at pH 4.5, 2h at pH 6.8 and 1.5 h at pH 7.4. Niacin release was recorded every 30 min by UV/vis spectrophotometer at 262 nm (Helios gamma. Thermo Fisher Scientific, Dreieich, Germany).

3.3.4 Scanning electron microscopy of microcapsules

Microcapsules from stool samples were isolated, washed in demineralized water and dried overnight. Afterwards the isolated digested microcapsules, as well as undigested control microcapsules, were prepared on a holder with Leit-C conductive carbon cement. Before examination in a Hitachi S-4800 (Hitachi High Tech., Tokyo, Japan) scanning electron microscope (SEM) at an accelerating voltage of 3 kV, microcapsules were sputter-coated with a layer of 8-10 nm gold-palladium using a Leica EM SCD 500 (Leica Microsystems GmbH, Wetzlar, Germany) high-vacuum sputter coater. SEM pictures of the capsules isolated from stool samples were compared with SEM pictures of undigested microcapsules.

3.3.5 Statistical Analyses

Statistical analyses were carried out employing SPSS version 22.0 for windows (SPSS, Chicago, IL; USA) and graphic data analysis was performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA). Data were checked for normality by using Shapiro-Wilk-tests and presented as means ± SDs (normal distribution) or median and interquartile range (not normally distributed). Independent samples t-tests and Mann-Whitney-U-tests were used to determine between group differences for continuous variables, and x^2-test was used for categorical variables. To compare more than two groups Kruskal-Wallis-test was used for non-parametric data. Welch-ANOVA with Games-Howell-Post-Hoc-test was used for parametric data with heterogeneity of variances. According to distribution Spearman correlation analysis was performed. Area under the curves (AUCs) were calculated with GraphPad Prism. In case of serum levels after 12 h not matching baseline serum levels, the NAM-serum level curves were extrapolated. Extrapolation was performed by means of trend lines in Excel version 2010 (Excel, Microsoft Cooperation, Redmond, WA, USA). According to distribution Wilcoxon test or paired sample t-test were used to determine differences between AUCs of unformulated niacin and AUCs calculated under the application of microencapsulated niacin. Changes in systemic metabolic parameters during the study weeks were determined by Friedman-test and Wilcoxon test or paired t-test according to distribution. To determine significant changes in the microbial composition under niacin intervention GraphPad was used to perform over time repeated ANOVA and paired t-test. Statistical significance was set at $p<0.05$.

3.4 Results

3.4.1 Niacin status in humans in relation to obesity, type 2 diabetes and the gut microbiome

First, we examined the association of NA and NAM with the microbiome in relation to human obesity and type 2 diabetes in n=511 subjects from our FoCus cohort (**Table 3.1**). In agreement to earlier reports (Ley et al., 2005; Ley et al., 2006; Turnbaugh et al., 2009), the gut microbial composition of obese individuals showed significantly lower α-diversity measures of genera ($p=0.036$) and OTU ($p<0.001$) as well as significantly lower *Bacteroidetes* abundance on phylum level ($p=0.027$) compared to lean subjects. In obese subjects a low niacin intake was associated with both, a reduced α-diversity (rho=0.286, $p=0.001$) and a lower *Bacteroidetes* abundance (rs=0.191, $p=0.026$). This was further supported by a significant correlation of low NAM serum levels with a reduced α-diversity in obesity (rho=0.176, $p=0.032$). Of interest, these findings were only significant in insulin resistant obese subjects without clinical manifest type 2 diabetes (**Figure 3.1**), suggesting that a niacin related microbiome intervention might be most promising in the prevention rather the treatment of type 2 diabetes. In this respect, it has to be taken into account, that in the group of type 2 diabetic subjects several patients were treated with metformin which is known to influence the composition of the gut microbiome (Wu et al., 2017) and therefore might beneficially interfere with the niacin-microbiome interaction.

3.4.2 Development of novel delayed-release niacin microcapsules and *in vitro* evaluation

Having found an association of a low niacin intake with adverse microbiome changes we developed a microencapsulation procedure to deliver increasing amounts of NA or NAM into the ileo-colonic region where most of the gut bacteria are localized. In order to detect differential effects, two separate microcapsules were engineered, containing either NA or NAM (see material and methods). Subsequently, these microcapsules were compared to free NA and NAM in two human intervention studies: (I) a bioavailability study and (II) a proof-of-concept and safety study (see below). After the coating process of the cores, both microcapsules, NA and NAM, were analysed by SEM for the verification of a homogenously distributed coating material. As shown in **Figure 3.2A+B**, the outer shellac coating formed a homogenous layer covering the complete capsule surface indicating a sufficient protection of the encapsulated compounds. To evaluate the *in vitro* dissolution profile, NA and NAM

microcapsules were subjected to simulated gastrointestinal fluids according to modified pharmaceutical standards (USP, 2011). The dissolution profiles in **Figure 3.2C+D** show gastric resistance of NA and NAM microcapsules for 1h at pH 1.4 and 0.5 h at pH 4.5 (release < 3%). Afterwards, the whole amount of encapsulated NA or NAM was released at pH 7.4. In contrast to NA microcapsules, NAM microcapsules already released about 35 % after 2 h at pH 6.8.

Table 3.1: *Characteristics of the FoCus subset study cohort*

	BMI < 20 kg/m² n= 66	**BMI 20-25 kg/m²** n=149	**BMI > 30 kg/m²** without T2D n=148	**BMI > 30 kg/m²** with T2D n=148	p_{total}	p_1	p_2	p_3	p_4	p_5	p_6
age (years)	45.53 ± 15.58[a]	52.90 ± 10.82	52.82 ± 10.86	52.93 ± 10.83	<0.001	<0.001	<0.001	<0.001	n.s	n.s	n.s.
gender (% female)	86.4 %	67.1 %	66.9 %	66.2 %	<0.05	<0.01	<0.01	<0.01	n.s.	n.s	n.s
height (m)	1.70[b] (1.64; 1.75)	1.72 (1.68; 1.79)	1.70 (1.64; 1.78)	1.70 (1.64; 1.80)	n.s.						
weight (kg)	55.45 (50.38; 58.43)	66.80 (61.35; 74.40)	111.30 (95.73; 132.20)	123.25 (102.50; 148.08)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
BMI (kg/m²)	19.09 (18.18; 19.73)	22.81 (21.45; 23.98)	37.08 (32.40; 45.13)	42.80 (36.75; 47.94)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
mmHg (sys)	120.00 (110.00; 130.00)	120.00 (115.00; 130.00)	130.00 (130.00; 140.00)	140.00 (130.00; 140.00)	<0.001	0.004	<0.001	<0.001	<0.001	<0.001	n.s.
mmHg (dia)	80.00 (70.00; 80.00)	80.00 (70.00; 80.00)	80.00 (80.00; 90.00)	80.00 (80.00; 90.00)	<0.001	0.005	<0.001	<0.001	<0.001	<0.001	<0.001
glucose (mg/dl)	88.00 (85.00; 95.25)	93.00 (87.00; 99.00)	100.00 (91.00; 108.00)	123.00 (104.25; 162.00)	<0.001	n.s.	<0.001	<0.001	<0.001	<0.001	<0.001
insulin (mIU/l)	5.25 (3.90; 8.30)	6.50 (5.10; 9.28)	17.20 (11.00; 24.23)	25.50 (15.53; 43.88)	<0.001	n.s.	<0.001	<0.001	<0.001	<0.001	<0.001
HOMA-IR index	1.14 (0.83; 1.89)	1.49 (1.08; 2.26)	4.15 (2.57; 5.71)	7.87 (4.18; 16.94)	<0.001	0.005	<0.001	<0.001	<0.001	<0.001	<0.001
trigylcerides (mg/dl)	68.00 (54.75; 92.50)	87.00 (64.00; 112.50)	121.50 (91.25; 179.25)	166.00 (122.00; 237.75)	<0.001	0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Lp(a) (mg/l)	117.00 (95.00; 361.00)	121.00 (95.00; 320.00)	148.00 (95.00; 378.50)	108.00 (95.00; 324.25)	n.s.						
IL-6 (pg/ml)	2.15 (1.50; 3.73)	2.50 (1.50; 4.20)	4.00 (2.90; 5.35)	5.20 (3.33; 7.08)	<0.001		<0.001	<0.001	<0.001	<0.001	0.001
CRP (mg/l)	0.90 (0.90; 1.13)	0.90 (0.90; 1.90)	4.35 (1.75; 8.65)	5.85 (2.90; 10.88)	<0.001	n.s.	<0.001	<0.001	<0.001	<0.001	n.s.

[a]mean ± sd (all such values); [b]median; 25th, 75th percentiles in parentheses (all such values); p_{total} = p-value for overall comparison, $p_{1\text{-}6}$=pairwise comparison: p_1=*BMI < 20 kg/m²* vs. *BMI 20-25 kg/m²*; p_2=*BMI < 20 kg/m²* vs. *BMI > 30 kg/m² without T2D*; p_3= *BMI < 20 kg/m²* vs. *BMI > 30 kg/m² with T2D*; p_4= *BMI 20-25 kg/m²* vs. *BMI > 30 kg/m² without T2D*; p_5= *BMI 20-25 kg/m²* vs. *BMI > 30 kg/m² with T2D*; p_6=*BMI > 30 kg/m² without T2D* vs. *BMI > 30 kg/m² with T2D*. Abbreviations: BMI= body mass index; mmHg=mm of mercury column; HOMA-IR= Homeostasis Model Assessment insulin resistance index; Lp(a)= lipoprotein (a); IL-6= interleukin-6; CRP=C-reactive protein, T2D= type 2 diabetes; n.s.= not significant.

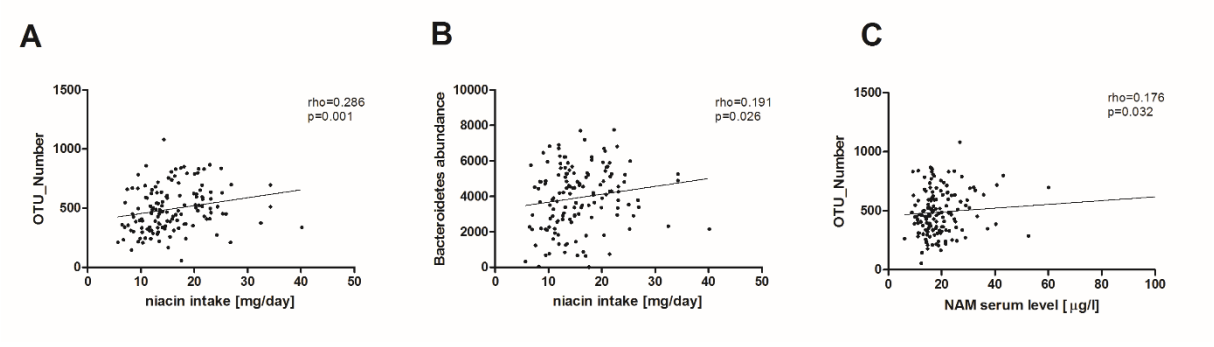

Figure 3.1: *Niacin intake and serum levels in relation to the composition of the gut microbiome Scatterplots and spearman correlation analyses of (A) niacin nutritional intake and alpha-diversity, (B) niacin nutritional intake and* Bacteroidetes *abundance and (C) nicotinamide (NAM) serum levels and alpha-diversity of obese (without type 2 diabetes).*

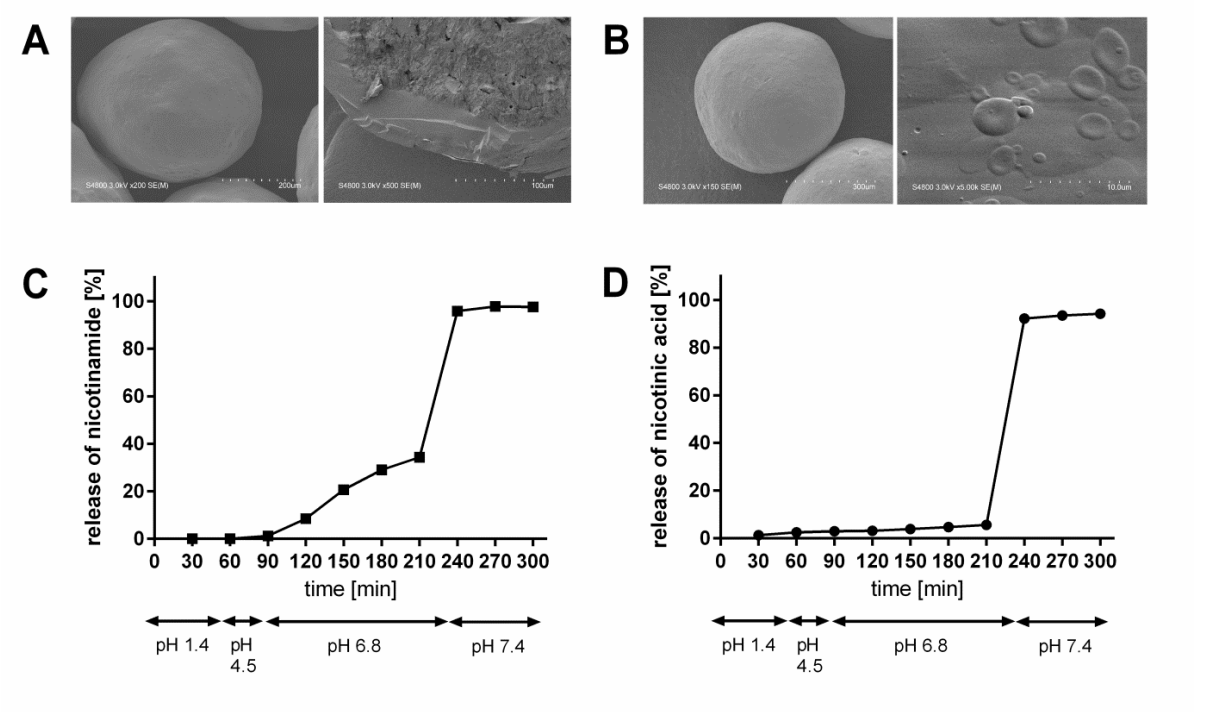

Figure 3.2: *In vitro evaluation of novel delayed-release niacin microcapsules*
Scanning electron microscope pictures of (A) undigested whole coated nicotinamide (NAM) microcapsule (left), cross section of an undigested nicotinamide microcapsules showing tight coating with nicotinamide core inside (right), (B) undigested whole coated nicotinic acid (NA) microcapsule (left) and higher magnified smooth coating surface of undigested microcapsules (right). (C) In vitro release of nicotinamide from nicotinamide microcapsules using pH change test. (D) In vitro release of nicotinic acid from nicotinic acid microcapsules using pH change test. In vitro results shown as mean ± SD (n=3).

3.4.3 Bioavailability study in healthy human subjects

In the bioavailability study, 20 human volunteers received single doses of NA or NAM microcapsules followed by blood sampling in defined time intervals for 12 h (60 min intervals for 8 h after ingestion and two additional blood samples after 10 and 12 h). The procedure was repeated with increasing doses (NA=30, 150, 300 mg, NAM=900, 1500, 3000 mg) with washout phases of 6 days in between. The microcapsules showed open and empty coating shells after gastrointestinal passage, indicating release of the ingredients (**Figure 3.3A**). The NAM serum levels in the bioavailability study showed a delayed release 4h after oral ingestion suggesting opening of the capsules in the ileo-colonic region **(Figure 3.3B)**. Due to the rapid metabolism of NA into NAM (Kirkland, 2007), in the NA-group only minimal fluctuations of NA serum levels could be observed, but no consistent and dose-dependent increase (data not shown). Of importance, for both NA and NAM, even under the highest dose of the microencapsulated compounds (NA= 300 mg, NAM=3000 mg) the serum concentrations were not significantly increased compared to the reference dose of 30 mg free NA or 900 mg free NAM. This finding clearly indicates that the delayed-release microcapsules deliver high amounts of NA or NAM to the microbiome without significant alterations in serum concentrations. This is of importance, since for NA high systemic levels are known to be associated with negative side effects like facial flushing and liver dysfunction (SCF, 2002).

3.4.4 Proof-of-concept and safety study in healthy human subjects

In the proof-of-concept and safety study we analyzed the microbiome and determined the systemic insulin sensitivity in n=10 metabolically healthy human volunteers receiving either NA or NAM microcapsules on a daily basis for a total of 6 weeks with a weekly increase in the dosage (NA=30 up to 300mg, NAM=900 up to 3000 mg). In the NA-group a significant increase in *Bacteroidetes* abundance over the time period of 6 weeks was observed (p=0.0025) (**Figure 3.3C**). In contrast, no significant change in the *Bacteroidetes* abundance could be observed in the NAM-group. This specificity of NA versus NAM is explained by the finding that *Bacteroidetes* are deficient in the enzymes *nicotinamidase* and *nicotinamide phosphoribosyltransferase* resulting in the inability to metabolize NAM (Gazzaniga et al., 2009). Since obesity is associated with a reduction in *Bacteroidetes* abundance, these data suggest that NA is a stronger candidate for a targeted microbiome intervention compared to NAM. This is further supported by the findings on systemic insulin sensitivity: biomarkers for

insulin resistance were only significantly improved by microencapsulated NA, but not by microencapsulated NAM. For examples, microencapsulated NA in contrast to free NA or any NAM formulation induced a significant decrease in myostatin (1.78±0.67 to 1.55±0.48 ng/ml, $p<0.05$) and Fetuin-A levels (3.29±1.10 to 2.66±0.95 ng/ml, $p<0.05$), which serve as markers for skeletal muscle and liver insulin resistance. In addition, microencapsulated NA resulted in a significant reduction of circulating osteopontin levels (2.77±1.39 to 2.09±0.78 ng/ml, $p<0.05$), suggesting an improvement of metabolic inflammation which is often found in prediabetic and diabetic individuals. In terms of safety profile, one subject of the NA-group experienced a mild elevation of aspartate aminotransferase (AST)-levels at week 5, which returned to normal within 4 days. Apart from that, no safety signals or facial flushing occurred.

3.5 Conclusions

The impact of the nutritional load (Cotillard et al., 2013) and dietary patterns (Kong et al., 2014) on the composition of the gut microbiome has been reported by several studies, however, to the best of our knowledge, our observation connecting niacin micro-nutrition and the human gut microbiome is novel. The positive correlations of niacin intake and niacin serum concentrations with alpha-diversity as well as the *Bacteroidetes* abundance suggest a favorable effect of niacin on the human gut microbial composition. To test this hypothesis, we developed delayed-release NA and NAM microcapsules to deliver increasing amounts of the micronutrients into the ileo-colonic region. *In vitro* analyses showed that NA and NAM microcapsule cores were protected by the coating layer (**Figure 3.2**). Because of the ability of microcapsules (< 2 mm) to pass the human pylorus independent of gastric emptying (Davis et al., 1986), the simulated gastric phase was reduced to 1 h. Both formulations released the whole amount of encapsulated niacin after reaching a pH of 7.4 (**Figure 3.2**) because of the exceeded dissolution pH of shellac (7.3) (Limmatvapirat et al., 2007). This dissolution pH is in agreement with the postulated pH of the human ileum (Evans et al., 1988). However, the pH in the desired ileo-colonic region can vary inter- and intra-individually and can be lower than the dissolution pH of shellac (Gruber et al., 1987). Furthermore, a decreasing pH in the colon is documented (Lesmes and McClements, 2009). Therefore, a partial release at pH 6.8 is desirable as shown in **Figure 3.2C**.

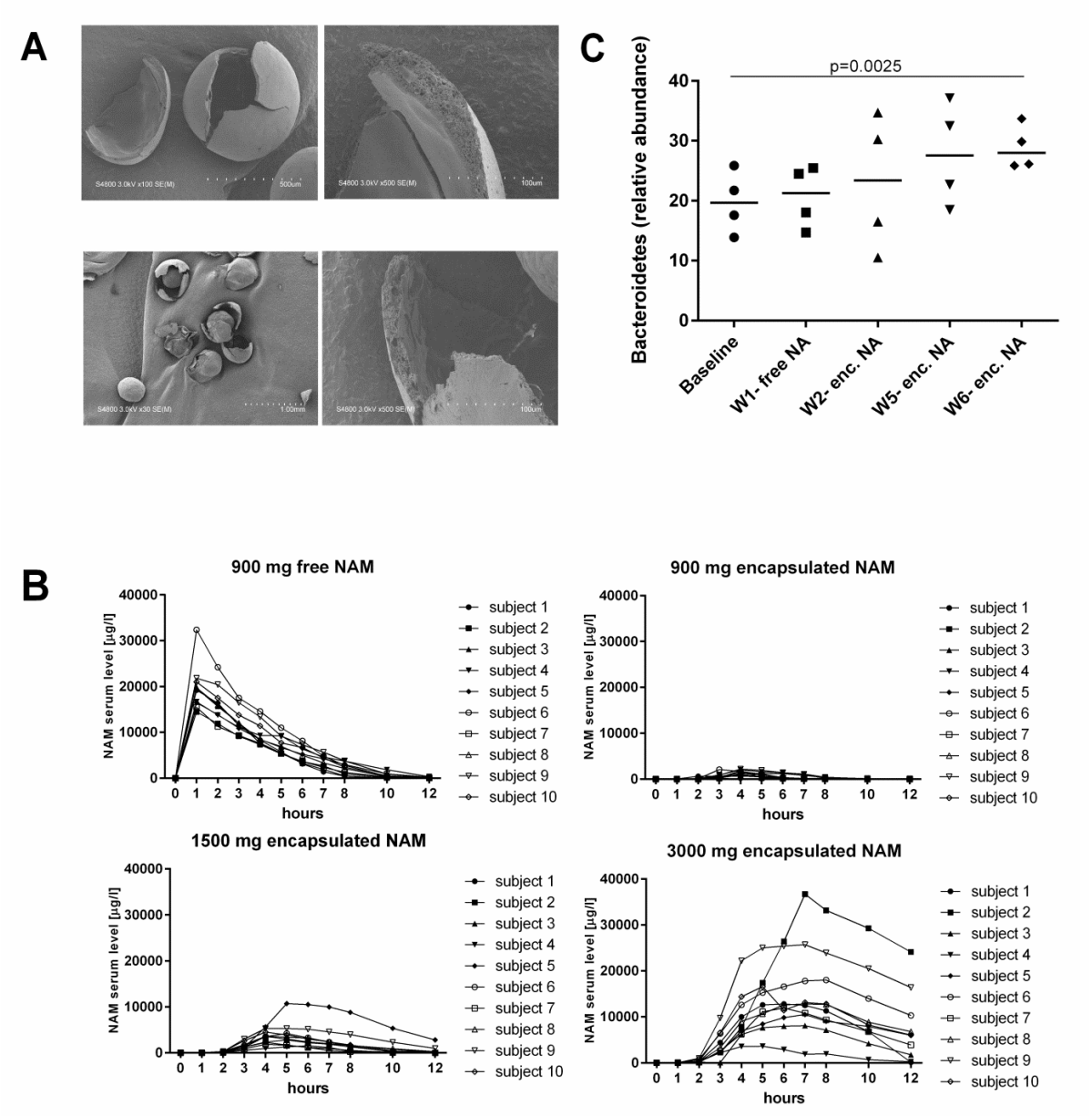

Figure 3.3: *In vivo evaluation of novel delayed-release niacin microcapsules*
Scanning electron microscope pictures of (A) whole opened nicotinamide (NAM) microcapsules (above, left), porous and spongy surface and profile of nicotinamide coating shell (above, right), whole opened nicotinic acid (NA) microcapsules with indigestible cellulose-core inside (bottom, left) and porous and spongy surface and profile of opened nicotinic acid microcapsules (bottom, right) after passage in the human volunteer. (B) Pharmacokinetic curves of free nicotinamide (NAM) and different doses of microencapsulated NAM. (C) Relative abundance of Bacteroidetes of NA-group in the course of proof-of-concept and safety study.

Taking these information into account our results showed a sufficient release of NA and NAM from our microcapsules and indicate a targeted release in the ileo-colonic region, where the tested pH-values were found. As reviewed by Pišlar et al. (2015), median time of gastric

emptying after ingestion of non-disintegrating tablets accounts to approx. 35 min, the small intestinal transit time to 215 min (min/max: 60/544) and the colon arrival time to 254 min (min/max: 117,604) (Pišlar et al., 2015). Thus, our bioavailability curves with serum peak levels after 4−8 h indeed suggest a pH-dependent release of NA and NAM in the ileo-colonic region. We note, that the gastro-intestinal transit times reported by Pišlar et al. were examined in a group of subjects, receiving the first meal at 4 h post-dose whereas meals given earlier can increase the time of gastric emptying or shorten small intestine transit time (Pišlar et al., 2015). During our bioavailability study, the first meal was given 3 h post dose. Nevertheless, the transit times still seem comparable to the ones reported by Pišlar et al. (2015), because pellets smaller than 2 mm are emptied from the stomach very rapidly and are unaffected from the digestive state of the subject (Davis et al., 1986). Comparison of AUCs under dosage with microencapsulated and free NAM proofed, that there is no significant increase in the total systemic niacin resorption. This finding clearly shows that our microencapsulation is able to deliver high amounts of NA/NAM into the colon without a significant increase in total systemic NA/NAM uptake in comparison to systemic uptake under application of the reference dose of free NA/NAM.

Under dosage with our novel niacin formulations, no severe safety signals occurred according to laboratory parameters. Besides that no clinical symptoms or flush phenomenon was observed. In contrast, in the past, different niacin formulations to treat lipid disorders have been reported to induce several adverse side effects. For instance it is commonly known, that NA doses higher than 30−50 mg can induce facial flush, and doses of 300−2000 mg NA can cause gastrointestinal symptoms (BfR, 2012; SCF, 2002). Further, administration of high NA doses can lead to liver-disorders or even hepatitis and liver failure (BfR, 2012; SCF, 2002). Compared to NA, side effects of NAM are fewer and only appear under doses ≥3000 mg (BfR, 2012; Knip et al., 2000). Of importance, side effects like flush or liver-disorders also occurred under dosage with sustained- or extended-release formulations (Knopp et al., 1985; McKenney et al., 1994). However, these pharmacological niacin formulations contained much higher doses (>1000 mg NA) and aimed a maximal systemic exposure to specifically treat lipid-disorders. In complete contrast, in our approach we aimed a topical exposure of NA/NAM in the colon with minimal increase in systemic resorption to improve the gut microbial composition in obesity and type 2 diabetes. In summary, our data indicate that the developed NA and NAM microcapsules show a preferable safety profile with no severe side effects and no systemic accumulation in healthy human volunteers.

In the present study, we found that the effect on both, the *Bacteroidetes* abundance and the systemic metabolism is specific to microencapsulated NA, while no effect was seen for any form of the NAM formulations. In this respect it is important to mention that bacteria have been classified by virtue of their ability to synthesize NAD *de novo* from aspartic acid and/or the vitamin precursors NAM, NA and NR (Gazzaniga et al., 2009). Thereby, the ability to use NAM was predicted on the basis of possession of homologs of *nicotinamidase* (PncA) and nicotinamide *phosphoribosyltransferase* (NadV). Remarkably, *Bacteroidetes* were reported to be deficient in both genes resulting in the inability to metabolize NAM (Gazzaniga et al., 2009). This finding explains the specificity of the increase in *Bacteriodetes* abundance to the delayed-release NA intervention found in our study.

It has to be mentioned that the proof-of-concept and safety study reported here represents a "phase 1 clinical trial" equivalent and was therefore performed in healthy human volunteers showing no major insulin resistance (**Supplementary Table 3.3**). The HOMA index was normal in those subjects at the beginning and did not significantly change within the normal range during the intervention. However, we would like to point out that this finding does not argue against an effect of microencapsulated NA on systemic insulin sensitivity, since even a metformin therapy over a time period of 6 months does not further improve a normal HOMA in non-obese subjects as shown by independent researchers (Trolle et al., 2007). In addition, the HOMA index is known not to detect early stages of insulin resistance, therefore the emphasis has recently shifted towards myokines and hepatokines as alternative clinical biomarkers for liver and skeletal muscle insulin resistance (Park et al., 2015). Myostatin is expressed in skeletal muscle (Hittel et al., 2010) and is causally involved in muscle insulin resistance, since myostatin null mice are protected from insulin resistance induced by diet-induced obesity (Park et al., 2015). In humans, myostatin plasma levels have been shown to be strongly correlated to insulin resistance (Hittel et al., 2010). On the hepatic site, fetuin-A was shown to be a natural inhibitor of insulin receptor tyrosine-kinase (Srinivas et al., 1993) and to be associated with insulin resistance in human subjects (Stefan et al., 2006). Of interest, strong associations of fetuin-A with the degree of insulin resistance were reported especially in non-diabetic subjects (Ishibashi et al., 2010), making this hepatokine an interesting candidate for our proof-of-concept and safety study. Indeed, both myostatin and fetuin-A levels were significantly reduced by microencapsulated NA, suggesting beneficial effects on skeletal muscle and liver insulin resistance. Importantly, both factors did not respond to free NA, indicating that the effect is most likely of an indirect nature, presumably via the beneficial changes of the microbiome. Beside biomarkers for insulin sensitivity of

skeletal muscle and liver we measured osteopontin serum concentrations as a marker for adipose tissue inflammation (Lund et al., 2009). Osteopontin levels also decreased under microencapsulated NA, suggesting anti-inflammatory properties in addition to the beneficial metabolic effects (Kiefer et al., 2010).

In summary, in the present study we present evidence (I) that the reduced α-diversity and *Bacteroidetes* abundance found in obese subjects is associated with a lower dietary niacin intake and (II) that a gut-targeted NA supplementation by delayed-release microcapsules is able to beneficially affect both, the microbiome and the systemic insulin sensitivity.

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Duality of Interests

Georg H. Waetzig and Dirk Seegert are employees of CONARIS Research Institute AG, and Stefan Schreiber is the chair of the supervising board. Karin Schwarz, Julia K. Keppler, Eva-Maria Theismann, Jörg Knipp, Daniela Fangmann, Matthias Laudes, Stefan Schreiber, and Georg H. Waetzig are inventors on a patent describing controlled-release formulations for nicotinic acid and nicotinamide, which is owned by CONARIS Research Institute AG (Kiel, Germany). Charles Brenner is a member of the scientific advisory board of ChromaDex, Inc. . No other potential conflicts of interest relevant to this article were reported.

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3.7 Supplemental Information

Supplementary Table 3.2: *Baseline characteristics of the niacin bioavailability cohort*

	NAM group	NA group	all subjects
age (years)	27.50 ± 4.81[a]	26.20 ± 5.07	26.85 ± 4.86
gender	5 male, 5 female	5 male, 5 female	10 male, 10 female
height (m)	1.75 ± 0.08	1.78 ± 0.79	1.76 ± 0.08
weight (m)	73.70 ± 13.66	75.16 ± 19.22	74.43 ± 16.25
BMI (kg/m^2)	22.78 (21.66; 26.80)[b]	22.91 (20.40; 24.90)	22.78 (21.25; 25.57)
NAM (µg/l)[c]	12.67 ± 2.76*	18.53 ± 7.52*	15.60 ± 6.28
uric acid (µmol/l)	284.80 ± 81.79	296.20 ± 63.60	290.50 ± 71.55
creatinine (µmol/l)	80.80 ± 13.11	86.70 ± 9.56	83.75 ± 11.57
eGFR (ml/min/1.73)	102.80 ± 12.18	94.20 ± 13.26	98.50 ± 13.16
AST (U/l)	20.15 (17.85; 26.70)	20.65 (19.82; 29.88)	20.65 (19.03; 26.35)
ALT (U/l)	18.85 (9.15; 31.55)	20.55 (11.80; 26.33)	20.55 (11.43; 30.75)
γ-GT (U/l)[d]	17.00 (9.00; 28.28)	16.50 (12.00; 26.75)	17.00 (12.00; 26.50)

[a]mean ± sd (all such values); [b]median; 25th, 75th percentiles in parentheses (all such values); [c]baseline NA serum levels of all subjects were below the detection limit of 12 µg/l and are therefore not included in this table; [d]for γ-GT one value was slightly below the detection limit of 7 U/l and set tot 7.0 for the calculations; *significant differences between NA- and NAM-group; unpaired t-test ($p<0.05$). Abbreviations: BMI= body mass index; NAM=nicotinamide; GFR= glomerular filtration rate; AST= aspartate aminotransferase; ALT= alanine aminotransferase; γ-GT= Gamma-glutamyl transpeptidase

Supplementary Table 3.3*: Characteristics of niacin proof-of-concept and safety study cohort at baseline and after niacin intervention*

	NAM group		**NA group**	
	Baseline	**End of treatment**	**Baseline**	**End of treatment[a]**
age (years)	39.40 ± 12.10[b]	-	50.20 ± 7.50	-
gender (female %)	100%	-	60%	-
height (m)	1.70 ± 0.08	-	1.75 ± 0.06	-
weight	73.52 ± 10.23	71.03 ± 10.46	85.64 ± 15.50	84.86 ± 15.70
BMI	25.55 ± 2.86	25.34 ± 3.45	27.97± 4.33	27.71 ± 4.35
mmHg (sys)	121.00 ± 7.42	108.70 ± 2.5	119.00 ± 11.40	116.00± 11.40
mmHg (dia)	72.00 ± 4.47	72.50 ± 2.89	75.00 ± 11.18	78.00 ± 4.47
glucose (mg/dl)	91.40 ± 5.50	89.50 ± 5.26	96.80 ± 7.63	93.40 ± 5.77
insulin (mIU/l)	7.10 ± 5.77	5.70 ± 0.51	8.56 ± 3.24	8.98 ± 3.75
HOMA-IR index	1.56 ± 1.19	1.26 ± 0.10	2.06 ± 0.81	2.05 ± 0.83
uric acid (µmol/l)	274.60 ± 45.23	285.75 ± 55.17	304.20 ± 37.38	298.60 ± 32.73
LDL (mmol/l)	2.88 ± 1.02	3.01 ± 0.49	3.50 ± 1.08	3.67 ± 1.08
HDL (mmol/l)	1.75 ± 0.32	1.95 ± 0.35	2.07 ± 0.52	2.11 ± 0.48
Lp(a) (nmol/l)	17.10 (7.60, 193.95)[c]	108.10 (9.18, 219.48)	24.50 (7.35, 127.70)[d]	23.30 (9.60; 137.70)[e]
triglycerides (mmol/l)	1.04 ± 0.52	0.80 ± 0.14	1.06 ± 0.17	0.98 ± 0.19
CRP (mg/l)	2.07 (0.99, 4.13)	2.27 (0.77, 3.28)	1.52 (0.89, 7.71)	1.30 (0.84; 6.14)
AST (U/l)	21.90 (19.10, 22.90)	22.95 (21.93, 24.73)	24.80 (15.10, 27.50)	22.60 (15.90; 73.15)
ALT (U/l)	20.30 (10.25, 24.50)	18.15 (11.65, 20.30)	19.10 (15.35, 24.65)	18.70 (16.90; 36.80)
γ-GT (U/l)	28.00 (11.00, 28.50)	22.50 (11.75, 33.25)	23.00 (19.00, 30.00)	20.00 (16.00 ± 25.50)
bilirubin (µmol/l)	5.60 ± 0.79	8.88 ± 2.04	6.70 ± 2.83	7.50 ± 2.22
creatinine (µmol/l)	65.20 ± 7.09	70.50 ± 7.77	85.00 ± 8.06	85.40 ± 8.79

[a]since one subjects of NA-group was withdrawn from the study after week 5 (due to an elevation in AST-levels), values measured at the end of week 5 were included instead of values measured at the end of week 6; [b]mean ± sd (all such values); [c]median; 25th, 75th percentiles in parentheses (all such values); [d]for Lp(a), values for one subject was slightly below the detection limit of 7 nmol/l and set to 7.0 for the calculations. Abbreviations: BMI= body mass index; HOMA= Homeostasis Model Assessment index; mmHg=mm of mercury column; LDL= low density lipoprotein; HDL= high density lipoprotein; Lp(a)= lipoprotein; CRP= C-reactive protein; AST= aspartate aminotransferase; ALT= alanine aminotransferase; γ-GT= gamma-glutamyl-transferase

4 Manuscript II: Precision of *in vitro* and *in vivo* shellac coated niacin release

Precision of in vitro and in vivo shellac coated niacin release

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4.1 Abstract

For ileocolon-targeted delivery systems of niacin, a precise release profile is desired. Therefore, shellac coated niacin microcapsules were evaluated regarding the robustness of the release profile in the ileocolonic region *in vitro* and *in vivo*. The effects of storage and various simulated gastrointestinal conditions were analyzed by using a dissolution tester. Furthermore, the *in vivo* pharmacokinetic curves of nicotinamide (NAM) were characterized from 10 healthy human volunteers regarding the release mechanism and interindividual variation.

The results showed a high robustness of the release profile *in vitro* for varying gastrointestinal conditions. Gastric resistance was given in simulated fasted (pH 1.4) and fed state (pH 4.5) for at least 2 h. A reduced simulated pH value in the ileocolonic region also indicated a sufficient release *in vitro* (80 – 100 % after 26 h). Despite higher variations for encapsulated than for the free NAM (reference dose) *in vivo*, a delayed absorption time (t_{max}: 1 vs. 4 h) was obtained for all individuals indicating a precise release in the target region. Furthermore, the ileocolon-targeted NAM formulation resulted in decreased C_{max} (1,322.7 vs. 19,690.0 µg/l) and AUC levels (4,207.15 vs. 17,334.20), when compared to the same reference dose. The profile indicated a slow diffusion driven release of NAM from the microcapsules over a longer period, resulting in a low systemic availability.

4.2 Introduction

In recent years, there has been a growing interest in the prevention of diseases and the maintenance of a well-being status using alternative and more natural approaches with regard to pharmacologic therapies (Santini et al., 2017). Obesity and its comorbidities are a major problem in industrialized countries, with growing incidence also in the developing countries (Friedman, 2009). In the recent past, several studies showed an association between the composition of the gut microbiome and obesity (Bäckhed et al., 2004; Le Chatelier et al., 2013; Ley et al., 2006). Therefore, a nutraceutical with a targeted impact on the gut microbiome could be beneficial for preventing obesity and comorbidities. In a previous study, the bioavailability of a colon-targeted nutraceutical formulation comprising the water-soluble vitamin niacin with its two active forms nicotinic acid (NA) and nicotinamide (NAM) was evaluated. We confirmed the targeted release of shellac coated NA and NAM in the ileocolonic region *in vitro* and *in vivo*, which is necessary to influence the gut microbiota. The intake of the microencapsulated NA led to favourable changes of the gut microbiome and an associated improvement of biomarkers for systemic insulin sensitivity and metabolic inflammation in healthy subjects. NAM, however, did not induced these changes (Fangmann et al., 2018). In a mouse model it was shown that the intake of NAM is capable to positively regulate the gut microbiota, the production of antimicrobial peptides and the propensity of inflammation (Hashimoto et al., 2012).

However, when developing controlled delivery systems, it is important that they meet several requirements: targeted release in desired region of the gastrointestinal tract, reproducibility of the release profile and batch-to-batch reproducibility as well as stability of the release profile over a longer period of time (Dressman et al., 1998; Pearnchob et al., 2003). *In vivo* genetic variations, ethnic background and states of diseases mainly influence interindividual variabilities, whereby intraindividual variability is a result of circadian rhythm, stress and physical activity level as well as food ingestion. These individual differences affect for example the composition of gastrointestinal fluids regarding the buffer capacity, enzyme and surfactant concentration, volume, residence time and pH value (Dressman et al., 1998; Ibekwe et al., 2008; Nugent et al., 2001; Russell et al., 1993; Shani-Levi et al., 2017). The fasted and fed state in humans is strongly influencing the pH value of the gastric fluid, resulting in pH values from 1.3 to 5.0 (Dressman et al., 1990; Russell et al., 1993). However, Feldman and Barnett also reported that the basal gastric pH value (fasted state) can increase up to 6.8 in healthy subjects (Feldman and Barnett, 1991). In addition, drugs, such as proton pump

inhibitors, were found to increase the gastric pH value (Peghini et al., 1998). Furthermore, depending on meal size the gastric residence time of small pellets can increase to 125 – 141 min (Clarke et al., 1993; Davis et al., 1984). Physiological measured pH values in the target area of the distal small bowel are ranging from 6.5 to 8.0 (Dressman et al., 1998; Evans et al., 1988; Gruber et al., 1987; Ibekwe et al., 2008). Furthermore, when reaching the ascending colon, always a decreasing pH value of approximately 6.4/6.5 is observed (Evans et al., 1988; Ibekwe et al., 2008), due to short-chain fatty acids, which are produced by microbial fermentation (Macfarlane et al., 1992). In addition, diseases such as inflammatory bowel disease (IBD) can lead to a reduced be reduced intraluminal pH value in the colon (Nugent et al., 2001). All these pH value and residence time variations can influence the release from shellac coated niacin pellets.

The aim of the present study was to characterize the robustness of the release profile of shellac coated NA and NAM pellets with a colon-targeted release. Firstly, the reproducibility and stability of the formulation was examined. Further physiological conditions were simulated by *in vitro* dissolution tests with altered pH values and residence time in fluids. Finally, intra- and interindividual variation were evaluated.

4.3 Materials and Methods

4.3.1 Materials

Nicotinic acid (NA) and nicotinamide (NAM) were purchased from SternVitamin (Ahrensburg, Germany). Shellac solution (SSB Aquagold) was obtained from Stroever Schellack Bremen (Bremen, Germany). Microcrystalline pelletized cellulose (Cellets®350) was obtained from iPc Process Center (Dresden, Germany). Citric acid monohydrate was obtained from Jungbunzlauer (Basel, Switzerland) and sodium bicarbonate was purchased from Dr. August Oetker (Bielefeld, Germany). As a binder hydroxypropylmethylcellulose (HPMC) (AnyAddy®) was obtained from Harke Group (Mülheim an der Ruhr, Germany) and pea starch (Lycoat™ RS 780) was from Roquette (Lestrem, France). Other used chemicals were purchased from Carl Roth (Karlsruhe, Germany).

4.3.2 Methods

4.3.2.1 Preparation of the coating solutions

All coating solutions were dissolved and stirred at room temperature. The coating compositions are listed in **Table 4.1**. The same batch of every substance was used for the production of NA and NAM pellets.

Table 4.1: *Composition of the coating solutions of the investigated pellet formulations [in % w/w]*

Coating	Ingredient 1	[%]	Ingredient 2	[%]	Ingredient 3	[%]	Water [%]
Nicotinic acid	Nicotinic acid	9.3	HPMC	0.7	-	-	90
Citric acid subcoat	Citric acid	1	Maltodextrin	9	-	-	90
Sodium bicarbonate subcoat	Sodium bicarbonate	2.33	Lycoat RS 780	4.65	Glycerol	0.023	93
Shellac	SSB Aquagold	60	-	-	-	-	40

4.3.2.2 Production of NA pellets

The production of the microcapsules was the same as described by Fangmann et al. (Fangmann et al., 2018). However, a more detailed description is the following: First, the nicotinic acid was applied to 175 g Cellets in a Mini Glatt fluid bed coater (Glatt, Binzen, Germany) with bottom spray using a 0.5 mm two-way nozzle, an atomizing air pressure of 0.6 bar, an inlet air pressure of 0.4 bar and a spraying rate of 1.2 g/min. Inlet air temperature was set to 39 °C, which resulted in a product temperature of about 32.4 °C. The final weight gain (w.g.) was about 16 %. During the application of the NA-coating, the coating solution was continuously stirred. Afterwards, an inner shellac coating (2 % w.g.), a sodium bicarbonate subcoating (1 % w.g. referred to sodium bicarbonate) and an outer shellac coating (20 % w.g.) was applied to 200 g vitamin coated Cellets. The atomizing air pressure was set to 0.6 – 0.7 bar, the inlet air pressure to 0.35 bar, a product temperature of about 33.7 °C and the spraying rate was increased from 0.43 – 0.87 g/min, except for the subcoating with 0.57 g/min. After the last coating step, the pellets were dried at 50 °C in a drying oven for 1 h.

4.3.2.3 Production of NAM pellets

As mentioned above, the production of NAM pellets is also described by Fangmann et al. (Fangmann et al., 2018). First, vitamin cores, consisting of 96 % NAM and 4 % HPMC as a binder, were produced from an extern company using a ProCell fluidized bed granulator with Vario 3 (Glatt Ingenieurtechnik, Weimar, Germany). Afterwards an inner shellac coating (50 % w.g), a citric acid subcoating (1 % w.g. referred to citric acid) and an outer shellac coating (10 % w.g.) were applied to 150 g NAM-cores (315 – 400 µm). The conditions for the two shellac coatings were similar to the shellac coatings of NA pellets. For the application of the citric acid, a higher product temperature of about 36 °C and a lower spray rate of 0.58 g/min were chosen. After the inner and the outer shellac coating, the pellets were dried at 50 °C in a drying oven for 1 h.

4.3.2.4 In vitro dissolution test

All *in vitro* drug release studies were carried out using a standard dissolution paddle apparatus at 100 rpm and 37 °C (DT 70, Pharmatest Group, Hainburg, Germany). Each experiment was conducted in triplicate with 0.5 g of pellets in 250 ml of the dissolution medium. During the experiments, the amount of released NA or NAM was recorded by UV/vis spectrophotometer (Helios γ, Thermo Fisher Scientific, Waltham, USA) at the wavelength of 262 nm.

As dissolution media were used the following: Simulated gastric fluid (pH 1.4) was adjusted to USP gastric fluid without pepsin (U.S. Pharmacopeial Convention, 2005), citrate buffer (pH 4.5) was modified according to (Gomori, 1955) and phosphate buffer (pH 6.8 and 7.4) was prepared according Soerensen's buffer. A detailed composition of the buffers is shown in **Manuscript III** (in submission).

To calculate the percentage of released vitamin, the maximum NA or NAM amount was determined by stirring (100 rpm) 0.5 g of NA or NAM pellets in 250 ml phosphate buffer at pH 7.4 for 1.5 h at 37 °C in the above-mentioned dissolution tester. The niacin concentration of the solution corresponded to 100 % niacin release.

4.3.2.5 Reproducibility and stability of the in vitro dissolution profile of NA and NAM pellets

The general *in vitro* profile to check the reproducibility and stability of NA and NAM pellets was conducted as described in previous studies (Fangmann et al., 2018; Manuscript III, in submission). Briefly, 1h at pH value 1.4; 0.5 h at pH value 4.5; 2h at pH value 6.8 and 1.5 h at pH value 7.4.

For stability tests, NA and NAM pellets were stored in a drying oven at 20 °C with a relative humidity (r. h.) of 31 % for a period of 24 months. The first year, every three months and afterwards every 6 months, an *in vitro* dissolution test was examined. After 18 months, a part of the stored NA and NAM pellets was transferred to a yoghurt cup and stored in a fridge (4 °C; r. h.: 47 %) for six or eight weeks. The *in vitro* dissolution profile was checked after 1, 2, 4, 6 (and 8) weeks of storage.

4.3.2.6 Robustness of the in vitro dissolution of NA and NAM pellets in varied gastric conditions

Gastric resistance of NA and NAM pellets under varied conditions was conducted for 2 h in pH value 1.4, 4.5 and 6.8 under the same conditions as described above.

4.3.2.7 Robustness of the in vitro dissolution of NA and NAM pellets in varied conditions of the target region (ileum/colon)

The elevated dissolution profiles started with 1 h pH value 1.4 and 0.5 h pH value 4.5. After 2 h in phosphate buffer at pH value 6.8 either the exposition was elevated for 22.5 h or the pellets were transferred to another vessel filled with phosphate buffer at pH value 7.0 for 22.5 h. These experiments were conducted in triplicate instead of the elevated profile to pH value 7.0, which was a single measurement.

4.3.2.8 Scanning electron microscope pictures

After the elevated dissolution profile at pH 6.8, NA and NAM pellets were separated from the dissolution medium and dried in a desiccator over night. Afterwards microcapsules were prepared on a holder with carbon Leit-tabs (Plano GmbH, Wetzlar, Germany). Some of them

were cut using a scalpel. Before examination in a Hitachi S-4800 (Hitachi High Tech., Tokyo, Japan) scanning electron microscope (SEM) at an accelerating voltage of 3 kV, microcapsules were sputter-coated with a layer of 8-10 nm gold-palladium using a Leica EM SCD 500 (Leica Microsystems GmbH, Wetzlar, Germany) high-vacuum sputter coater.

4.3.2.9 In vivo bioavailability study

The bioavailability study was examined according to Fangmann et al. (2018). Ten subjects received either NA or NAM in its free form (30 or 900 mg) and in its encapsulated form with increasing doses (NA: 30, 150 and 300 mg; NAM: 900, 1500 and 3000 mg). For 8 hours every hour and after 10 and 12 h blood samples were taken to be determined by the concentration of NA and NAM in serum. A detailed description is given by Fangmann et al. (Fangmann et al., 2018).

In order to calculate the half-life ($t_{1/2}$) the following equations were used. k_e is the elimination constant. Whereby C_{max} is defined as the highest measured NAM concentration and C_{12} as the reached NAM concentration after 12 h. Δt is the difference of the time between C_{max} and C_{12} in h. It was assumed that the elimination follows a first order kinetic. Half-life was calculated for every subjects. Afterwards the results were presented as mean.

$$t_{\frac{1}{2}} = \frac{\ln(2)}{k_e} \qquad k_e = \frac{\ln C_{max} - \ln C_{12}}{\Delta t}$$

4.3.2.10 Statistical analysis

Statistical analyses were performed using SPSS version 24.0 for windows (SPPS, Chicago, IL; USA). GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA) was used for graphic data analyses and calculation of area under the curves (AUCs), in order to evaluate total uptake of niacin. If serum levels after 12 hours were not matching baseline serum levels (for 3000 mg dose), NAM serum level curves were extrapolated by means of trend lines in Excel version 2010 (Excel, Microsoft cooperation, Redmond, WA, USA). Data are presented as means ± SD (standard deviation). Saphiro-Wilk-test was used to determine deviations from normal distribution and according to distribution Spearman correlation analysis was performed. The half-life was analysed by ordinary one-way ANOVA with Tukey multiple comparison test and a significance level of 0.05 using GraphPad Prism Software.

4.4 Results and Discussion

To evaluate the precision of the site-specific release of the encapsulated niacin, 1.) the reproducibility, 2.) the robustness and stability of the release profile, 3.) the release mechanism and 4.) the interindividual variation were determined in *in vitro* and *in vivo* studies.

4.4.1 Reproducibility and stability of in vitro dissolution profile

The *in vivo* validity of the *in vitro* release profile was confirmed in a previous study (Fangmann et al., 2018). NA pellets were produced in duplicate and caused similar release profiles (**Figure 4.1A**). Four NAM batches showed differences of about 9.4 percentage points after 120 minutes and 6.62 percentage points after 210 minutes (**Figure 4.1B**). These minor variations are in accordance with the results of Pearnchob et al., who described a good conformity between different trials of a shellac coating (Pearnchob et al., 2003).

The stability of the mixture of all trials of NA and NAM pellets at 20 °C and 4 °C is shown in **Figure 4.2.** At a storage temperature of 20 °C, the stability was examined for a period of 24 months (**Figure 4.2A+B**). With increasing storage time the vitamin release was reduced especially after 18 and 24 months to 15.9 % and 18.3 %. Directly after the coating process, 34.3 % NAM were released at pH value 6.8 after 210 min and after 3, 6 and 9 months the release of NAM was 31.4 %, 29.6 % and 33.0 %. Similar results were obtained regarding NA pellets. After 210 min at pH value 6.8, the release rate decreased from 5.6 % (after coating) to 2.6 %, 3.5 %, 3.2 %, 3.2 %, 0.9 % and 1.2 % after 3, 6, 9, 12, 18 and 24 months of storage, respectively. The reduced release of NA and NAM is related to changes of the shellac coating. Stability tests of the vitamins themselves, showed no differences in the absorbance profile and intensity. Furthermore, the total vitamin content was similar compared to the beginning of the production process (data not shown).

Figure 4.2C+D show the stability of the release profile for NA and NAM at a lower temperature (4 °C). NAM pellets were stable for up to eight weeks with no changing release rates. NA pellets were only stable for two weeks. After four weeks, the release rate at pH value 1.4, 4.5 and 6.8 was increased. At the end of week six, about 90 % of NA was released immediately at pH value 1.4 after 30 min.

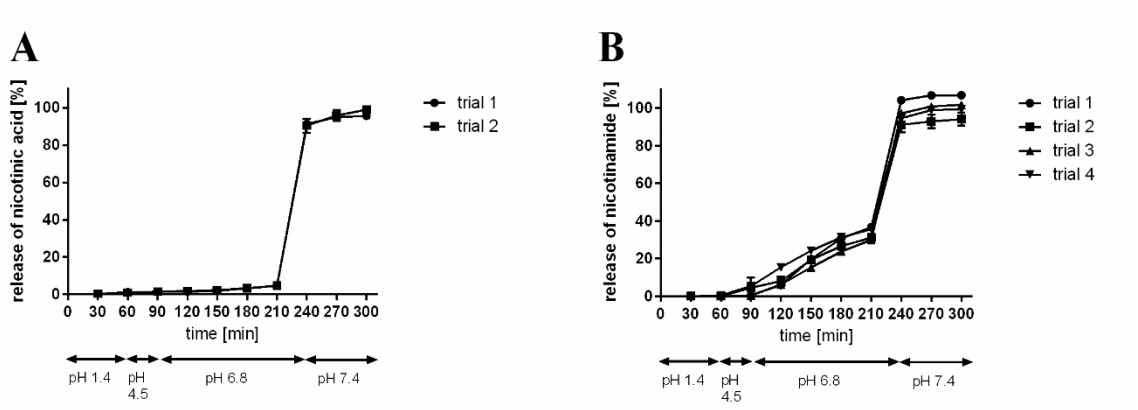

Figure 4.1: *Reproducibility of (A) NA and (B) NAM release from triple coated pellets with the same batch but different trials. All results are given as mean ± SD (n=2). NA = nicotinic acid; NAM = nicotinamide*

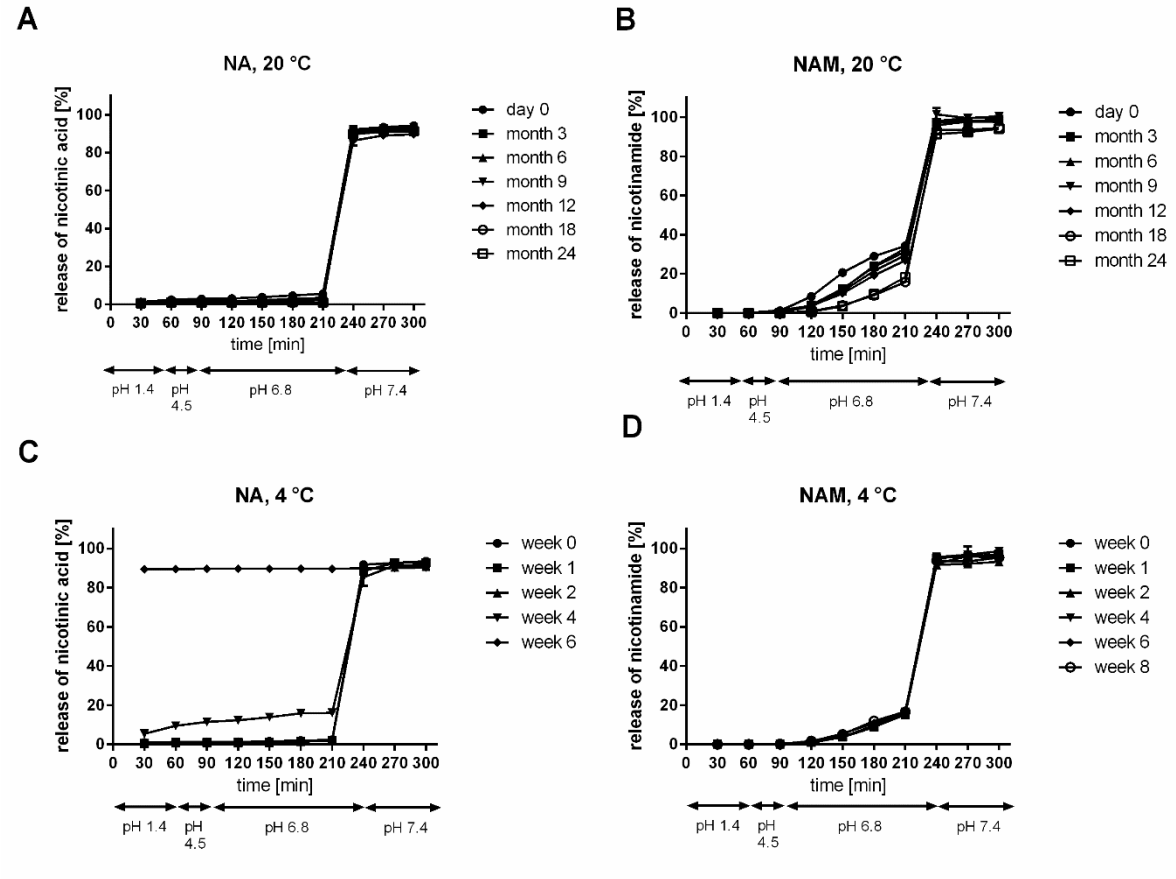

Figure 4.2: *Stability of (A+C) NA pellets and (B+D) NAM pellets after storage (A+B) at 20 °C for up to 24 months and after storage (C+D) at 4 °C for up to 8 weeks. All results are given as mean ± SD (A+B: n=3; C+D: n=2).*

The observed instability of shellac coatings during storage is explained by polymerization via esterification of the large amount of hydroxyl and carboxyl groups of the shellac components

(Farag and Leopold, 2009; Limmatvapirat et al., 2005; Limmatvapirat et al., 2007). Ammonium salt shellac is described to be more stable compared to the ethanolic shellac (Limmatvapirat et al., 2005), however, Limmatvapirat et al. also detected an increase of insoluble solids (about 25 %) of the ammonium salt form of shellac at pH value 7.0 after 3 months of storage at 40 °C and 75 % RH. After 6 months, the amount of insoluble solids increased to about 50 % (Limmatvapirat et al., 2007). Pearnchob et al. also documented the release stability of shellac coated pellets after 6 months of storage. They demonstrated that the release kinetics only showed slight changes (Pearnchob et al., 2003). However, they used triethyl citrate as plasticizer, which also influences the stability and the amount of insoluble solids. Whereas, depending on the used plasticizer, hydrogen bonding between the active sites of shellac reduce the polymerization of shellac (Luangtana-Anan et al., 2010). Additionally to the reduced solubility of shellac at higher pH values with increasing storage time, it is also described, that shellac can lose its gastric resistance during storage (Farag and Leopold, 2009). This observation was not confirmed in the present study, where the stability was investigated at a storage temperature of 20 °C for 24 months. However, at a storage temperature of 4 °C, NA pellets lost their gastric resistance. This may be attributed to the influence of the sodium bicarbonate subcoating in case of NA pellets. As described in detail in **manuscript III** (in submission), sodium bicarbonate led to an increase of the released compound due to an enhancement of the intrinsic pH value and the production of CO_2 in combination with moisture. The relative humidity was higher at +4 °C (47 %) compared to 31 % at +20 °C. The higher relative humidity of 47 % could lead to a partial dissolution of the sodium bicarbonate, which enhances the solubility of shellac and results in a premature release.

4.4.2 Robustness of the in vitro dissolution profile of NA and NAM pellets under varied gastrointestinal conditions

To study the robustness of the *in vitro* release profile, NA- and NAM pellets were subjected to simulated fluids of the gastric and ileocolonic region representing different gastrointestinal conditions.

Stomach conditions

The release profile was evaluated in simulated gastric fluids representing fasted and fed state at pH values of 1.4 and 4.5 for 2 h (**Figure 4.3**). Both formulations were stable at a pH value of 1.4 and 4.5 (**Figure 4.3A+B**). The release was below 10 % after an exposition for 2 h. At pH value 4.5, the release of NA or NAM (3.6 % and 2.3 %) was only minimal higher compared to pH value 1.4 (2.5 % and 0.5 %). These results are in accordance with Czarnocka and Alhnan, who showed no significant release of a shellac-alginate formulation during pH value 2, 3 and 4 for 2 h (Czarnocka and Alhnan, 2015). Thus, a high robustness of NA and NAM pellets is given under varying gastric conditions. Furthermore, robustness for a longer gastric residence time than previously tested by Fangmann et al., was observed. They simulated the gastric phase with only one hour (Fangmann et al., 2018), due to the ability of pellets smaller than 2 mm to pass the pylorus independently of gastric emptying (Davis et al., 1987).

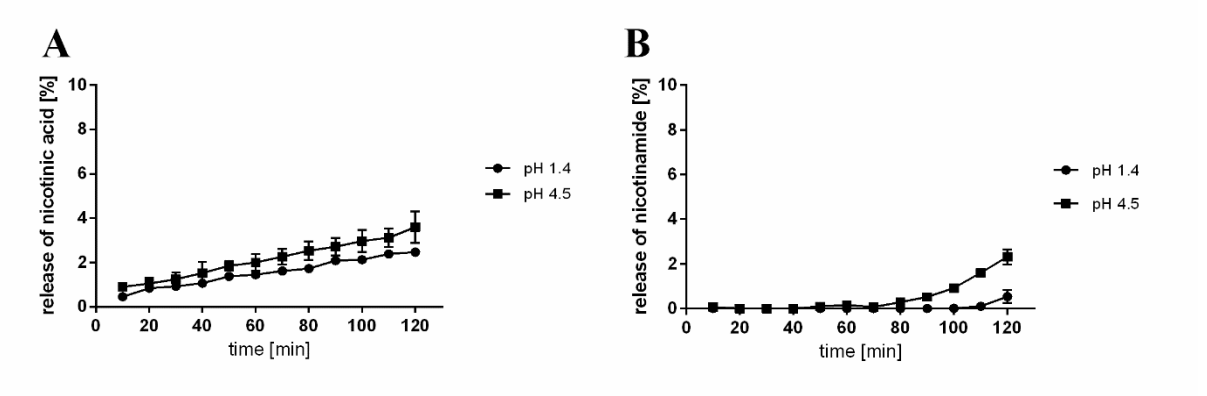

Figure 4.3: *In vitro release of (A) NA and (B) NAM in pH 1.4 and 4.5 for 2h. All results are given as mean ± SD (n=3).*

As shown in **Figure 4.4**, the dissolution profile at pH value 6.8 differed completely compared to a pH value of 1.4 and 4.5. At pH value 6.8, about 83 % NA and 53 % NAM were released after 60 minutes; an increase to 97 % and 94 % was documented after 120 minutes, respectively. These results indicated no delayed release, if a pH value of 6.8 occurs in gastric lumen. This would lead to a total absorption of NA and NAM in the stomach and the upper small intestine, which would not affect the gut microbiota.

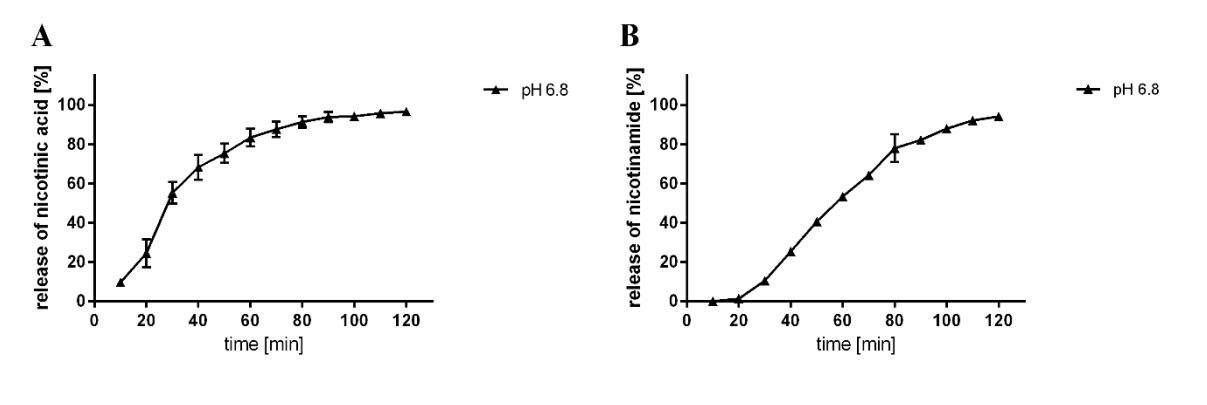

Figure 4.4: *In vitro release of (A) NA and (B) NAM in pH 6.8 for 2 h. All results are given as mean ± SD (n=3).*

Target area of the ileum/colon

The conditions in the target area, ileum and colon, in the gastrointestinal tract were varied as it is important, that a high amount of NA and/or NAM is available for gut microbiota. Therefore, an adjusted *in vitro* release profile was chosen in order to predict the release of NA and NAM under lower pH values of 6.8 and 7.0 (**Figure 4.5**). Both formulations released only minor amounts of NA (1.4 %) and NAM (0.5 %) during the first 90 min. Whereby, NA was released very slowly at a pH value of 6.8 (3.1 % after 210 min), 33.2 % NAM was liberated after 210 min. Afterwards, the pellets were exposed either to a pH value of 7.0 or were left at pH value 6.8 for 22.5 h. At pH value 6.8, the release increased slowly to 11 % NA after 420 min in total. Finally, an entire release was achieved. In contrast to NA pellets, a complete release was not achieved during the whole exposure time of 26 h. The maximum release was 82.8 % NAM. A higher final pH value of 7.0 led to a faster release. The complete NA and NAM concentration was liberated after 260 – 420 min (**Figure 4.5**). Differences between NA and NAM pellets can be explained due to differences in their physicochemical properties, their different production method and the use of different subcoatings. All of the mentioned parameters are influencing the dissolution profile. The citric acid subcoating in case of NAM pellets will be the factor, which is responsible for the incomplete release of NAM at pH value 6.8 (**Manuscript III**, in submission).

In general, it can be noticed that the lower the pH value the slower the release rate of NA and NAM due to a decrease of the coating swelling, which leads to a reduced diffusion capacity. After a total residence time of 300 min, 5.8 % and 45.4 % NA were released at a pH value of 6.8 and 7.0, respectively. NAM pellets released 43.2 % and 75.4 % NAM at pH value 6.8 and 7.0 after 300 min. The results of Fangmann et al. showed a release of about 95 % NA or

NAM after 300 min with an end pH value of 7.4. However, although the release of NA and NAM was slowed down with decreasing pH values, the long residence time in the colon should lead to a sufficient release in the targeted region. The human colon transit time can vary between 8 – 72 h (Kararli, 1995) with an average of about 36 h (Lesmes and McClements, 2009). Small pellets resulted in a longer transit time of 28 h compared to single unit dosages (15 h) (Abrahamsson et al., 1996). Therefore, a dissolution method with an elongation for 26 h in total was chosen, which is comparable to other *in vitro* colon simulations (Krishnamachari et al., 2007; Stein et al., 2011). It is documented that pellets are slowly transferred during the ascending colon, because of intensive mixing (Davis et al., 1984). Abrahamsson et al. observed a storage of administered pellets for up to 14 h after oral intake in the ascending colon and up to 48 h in the transversum. Regarding the *in vitro* profile of the present study (**Figure 4.5**), during this time a release of about 50 % NA and 60 % NAM at pH value 6.8 is expected. At a pH value of 7.0, a total release of NA occurred already after 6.5 h, whereby a total release of NAM after 18 h is expected to occur in the region of the ascending/transversal colon.

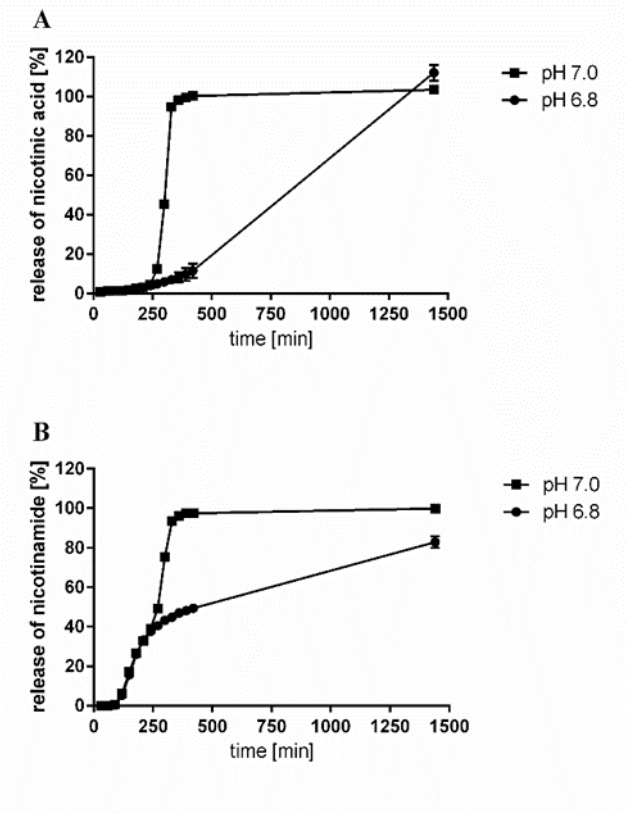

Figure 4.5: *In vitro release of (A) NA and (B) NAM with elevated exposure at pH 6.8 or 7.0 after 1h at pH 1.4, 0.5 h at pH 4.5 (and 2 h at pH 6.8). All results are given as mean ± SD (pH 6.8: n=3; pH 7.0: n=1).*

Structural properties of the pellets

As demonstrated in microscope (**Figure 4.6A+G)** and SEM pictures **(Figure 4.6B+H)**, most of the capsules were intact after the *in vitro* digestion for 26 h at an end pH value of 6.8. Open NAM pellets were empty and the intact Cellet was found in the NA capsule (**Figure 4.6C+I**). The structure of the cross section of the remaining shellac coating showed a spongy and porous structure in both microcapsules (**Figure 4.6D+J**). In contrast to NA pellets, the outer areas of the shellac coating of NAM pellets were less perforated than the middle area. This could be explained due to the thicker coating layer of NAM pellets and also due to the sustaining property of the citric acid subcoating (**Manuscript III**, in submission). In addition, intact NAM pellets showed only a few pores on the surface and some of them were still including a small NAM core (**Figure 4.6E+F**). These cores may be attributed to the lower *in vitro* release after 26 h. NA was totally released from NA pellets and additionally the surface of closed microcapsules was rough and porous (**Figure 4.6K+L**). It is suspected that the swelling and perforation of the shellac coating led to a penetration of the surrounding fluid, dissolution of the encapsulated compound and finally to a diffusion driven release of the vitamin, which is also confirmed by the concentration dependent slope of the *in vitro* curve after reaching a pH value of 6.8. In addition, both, NA and NAM have a low molecular weight of 123.11 and 122.13 g/mol and are moderately or highly water-soluble (15 g/l and 691 g/l), which enables an easy diffusion (Huang and Brazel, 2001).

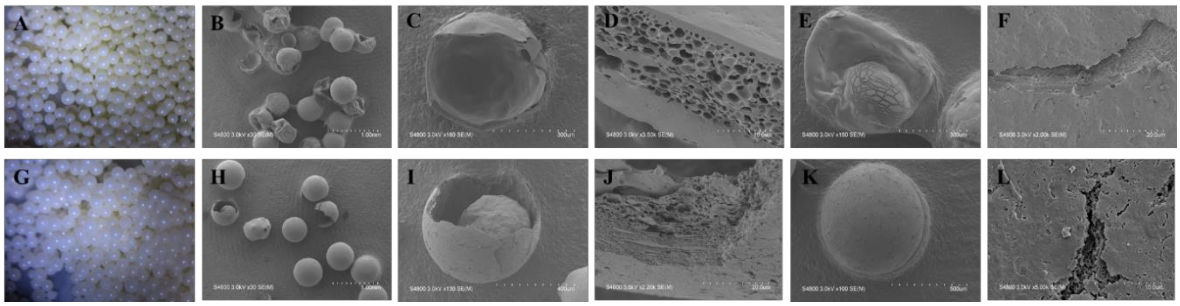

Figure 4.6: *Light microscope pictures (A+G) and SEM pictures (B-F and H-L) of dried NAM pellets (top) and NA pellets (bottom) after 26 h in vitro dissolution as shown in figure 4.4 with a final pH of 6.8. A,B,G,H: overview of pellets; C+I: open pellet; D+J: cross-section of open pellet; E+K: intact pellet; F+L: surface of intact pellet*

Furthermore, the results of the elongated dissolution profile show differences compared to the results as illustrated in **Figure 4.4**. A significant difference was observed in the release rate at pH value 6.8 with or without previous contact to a lower pH value. Whereby the pellets,

which were exposed directly to a pH value of 6.8 showed an almost complete release after 2 h, pellets, which were first added to pH value 1.4 and 4.5 resulted in a (very) slow release rate at pH value 6.8 after 2 h (up to 6 % NA and 34 % NAM). This effect was also documented for a tablet coating with Eudragit L30 D-55, which is also a pH dependent coating material that dissolves above a pH value of 4 – 6 It was suggested, that the penetration of acid delays the dissolution of the polymer and therefore the release. With increasing exposure time in gastric fluids, the subsequent release rate at higher pH values was decreasing (Menge, 2016). Thus, it is suggested, that only minor amounts of NA or NAM are released at pH 6.8, when the pellets were previously exposed to gastric fluids with a lower pH value.

4.4.3 Dissolution mechanism and the bioavailability of NAM

A diffusion driven *in vivo* release of NAM is also hypothesized with regard to the curve characteristics measured in the human bioavailability study (**Figure 4.7**). The same dose of microencapsulated NAM compared to the free administered NAM resulted in a significantly delayed absorption (t_{max}: 4 vs. 1 h) without sharp peak and lower AUC levels (4,207.15 vs. 17,334.20), which could be related to low absorption rates due to a slowed release from the microcapsules and therefore to lower NAM levels in the gut lumen. Furthermore, as shown in **Figure 4.8**, the half-life ($t_{1/2}$) is slightly enhanced from 1.4 h for free NAM to 1.8 h for encapsulated NAM (900 and 1500 mg dose), although this effect was not statistically significant (p=0.4531). In comparison to NA, the half-life of NAM is much longer, both in the liver and the blood of rats (1.0 h vs. 5.3 h) (Petrack et al., 1966). In humans, 1 g of administered NAM resulted in a comparable half-life of about 1.7 h, in a dose-dependent manner. The higher the administered amount of NAM the higher the half-life (Stratford et al., 1992). In the present study, the half-life tended to increase for the encapsulated form despite the lower systemic NAM levels. This finding strengthen the hypothesis that NAM is released from shellac coated microcapsules over a longer period in the distal intestine, which results in absorption over a longer period and thus enhances the half-life. Furthermore, t_{max} increased from 900 to 3000 mg encapsulated NAM (**Table 4.2**). This result confirms a retarded release of NAM within the first 3 – 4 h i.e. low uptake in the small intestine. Afterwards, NAM is liberated slowly, whereby the release occurred over a longer period of time resulting in an accumulation of NAM and a shift in t_{max}. In comparison, colon-targeted pharmaceutical formulations such as guar gum-based 5-fluorouracil or flurbiprofen tablets for the treatment of colon cancer or colon inflammation, showed a higher t_{max} and increased AUC levels for their

encapsulated drug compared to the free form. This indicated a high systemic availability despite the delayed release. Higher AUC levels were attributed to a slowly absorption of the drugs in the colon due to less surface area. Furthermore, they detected a sharp absorption peak which was related to an erosion based release after guar gum degradation by microbial enzymes (Krishnaiah et al., 2003; Vemula and Bontha, 2013). These results support the hypothesized mechanism for a diffusion driven release of NAM from the present microcapsules *in vivo*. In addition, the *in vitro* release of NA and NAM from shellac coated microcapsules is mainly driven by diffusion, when the pH value did not exceed a pH value of 7.3. After reaching a pH value of 7.4, a combination of diffusion and erosion processes is suggested, due to a fast dissolution of the shellac itself. The reduced affinity to NAM carriers in the colon (K_m= 2.5 ± 0.8 µM) (Kumar et al., 2013) compared to the small intestine (K_m= 0.53 ± 0.08 µM) (Nabokina et al., 2005), will also affect the systemic availability and results in lower t_{max} concentrations and AUC levels.

The presented SEM pictures of shellac shells after *in vitro* digestion showed a spongy structure similar to the structure of the digested capsules by Fangmann et al. This indicate a similar pH value for *in vitro* and *in vivo*, e.g. between 6.8 and 7.3 (Fangmann et al., 2018). Some capsules from the stool samples were completely open, which can be related to shear stress under *in vivo* digestion compared to *in vitro*. Intense muscle contractions, a higher viscosity and pressure in the lumen of the human lower intestine can affect the integrity of the capsules (Takaya et al., 1998), whereas *in vitro* the low viscosity of the aqueous solution causes lower shear stress during mixing.

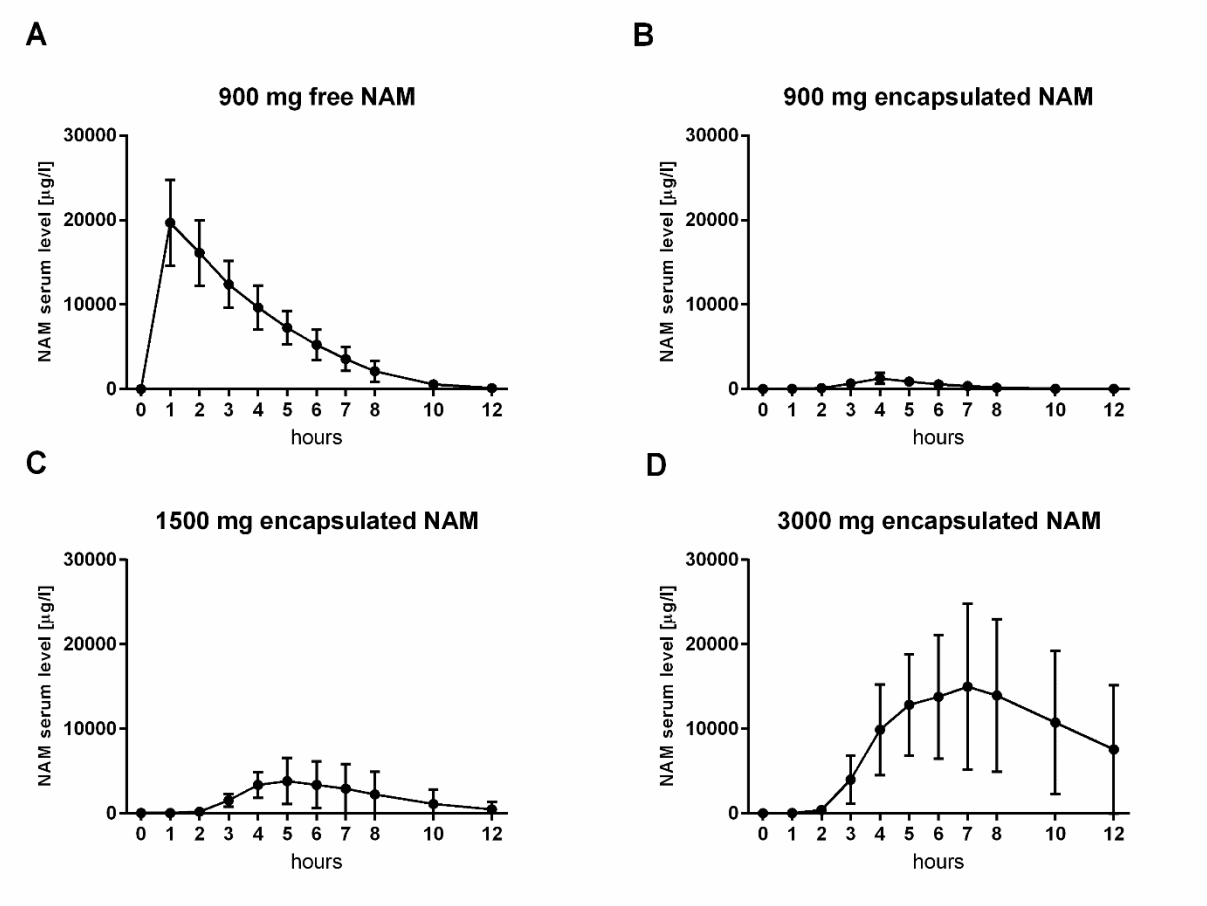

Figure 4.7: *Pharmacokinetic curves after the administration of (A) 900 mg free NAM as reference dose, (B) 900 mg encapsulated NAM, (C) 1500 mg encapsulated NAM and (D) 3000 mg encapsulated NAM (mean ± SD, n=10).*

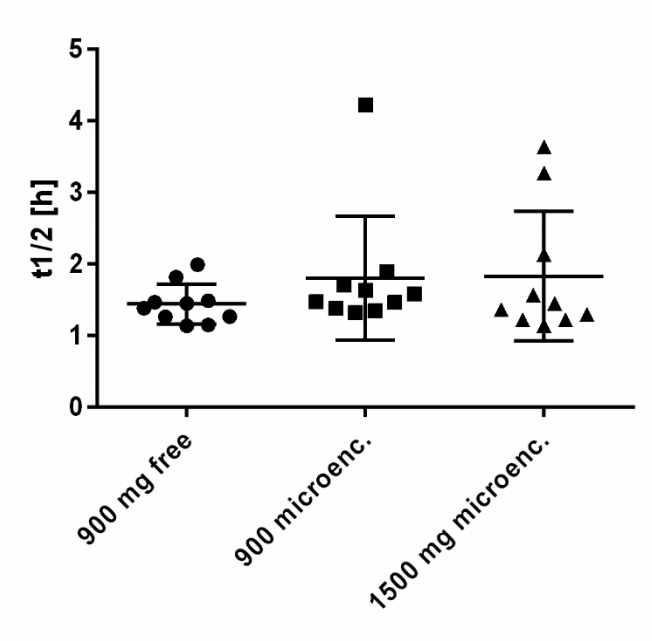

Figure 4.8: *Half-life ($t_{1/2}$) of NAM after administration of 900 mg free, 900 mg and 1500 mg encapsulated NAM*

Table 4.2: *t_{max} after oral administration of free or microencapsulated (enc.) nicotinamide (NAM)*

subjects	t_{max} 900 mg free NAM [h]	t_{max} 900 mg enc. NAM [h]	t_{max} 1500 mg enc. NAM [h]	t_{max} 3000 mg enc. NAM [h]
S1	1	4	4	6
S2	1	4	5	7
S3	1	4	4	7
S4	1	4	6	5
S5	1	4	5	7
S6	1	3	5	8
S7	1	4	4	6
S8	1	4	4	7
S9	1	4	4	7
S10	1	3	4	5
Mean	1	3.8	4.5	6.5

4.4.4 Robustness of NAM microcapsules in vivo

As summarized in **Table 4.3**, the human bioavailability study indicated higher variabilities of serum NAM level after oral administration of encapsulated niacin compared to the reference free dose. The coefficient of variation increased with increasing dose. This result indicated that the release of NAM is strongly influenced by individual variations, which is enhanced for higher doses. To determine the robustness of the NAM release from microcapsules *in vivo*, the reproducibility of the main release area (referred to t_{max}) and the dose-dependent increase of C_{max} and AUC within the subjects were evaluated. No significant correlations of the AUC levels ($p=0.098$) or C_{max} ($p=0.206$) within the subjects was found between the 1500 mg and the 3000 mg NAM dose (**Figure 4.9A+B**). Only, 30.4 % of the variation in AUC and 19.2 % of the variation in C_{max} is determined by the dose increase from 1500 to 3000 mg NAM. The high variation is mostly dependent on intra- and interindividual fluctuations. Thus, the subject with the highest AUC or C_{max} at 1500 mg NAM has not the highest levels after 3000 mg NAM. These effects are additionally illustrated in **Figure 4.9C+D**. Despite individual variabilities in C_{max} and AUC, t_{max} showed a maximal variability of 3 h between the subjects (**Table 4.2**). Thus, a robust targeted release in the desired region of the ileocolon is proposed, which is also related to the use of a multiparticular dosage form. The risk of loss of the functionality of the coating is significantly reduced compared to a single unit dosage form like tablets (Srivastava and Mishra, 2010; Wesdyk et al., 1990). Furthermore, small pellets are not dependent from gastric emptying, which is the most influencing factor of the gastrointestinal transit time, and therefore are less influenced by fasted or fed state (Clarke et al., 1993; Davis et al., 1987; Dressman et al., 1998; Ibekwe et al., 2008; Malagelada et al., 1984).

Table 4.3: *Overview of the variations in AUC levels of NAM group*

AUC	mean ± SD	Coefficient of variation [%]
900 free	78,334.20 ± 19,538.11	24.94
900 enc.	4,287.15 ± 2,571.91	59.99
1500 enc.	21,011.90 ± 17,616.94	83.84
3000 enc.	135,748.20 ± 105,826.64	77.96

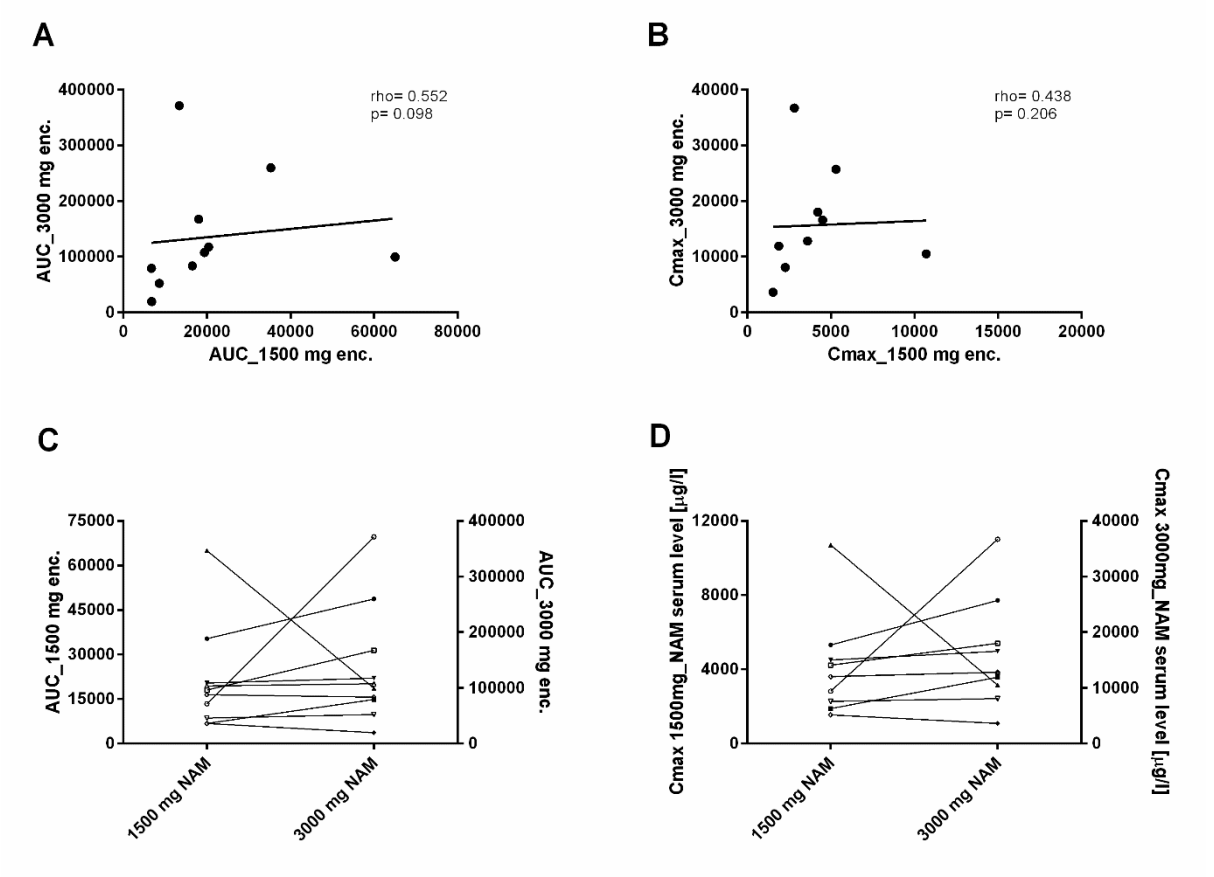

Figure 4.9*: Interindividual variations within the NAM group (A) correlation of AUC levels between 1500 mg and 3000 mg encapsulated NAM; (B) correlation of C_{max} between 1500 mg and 3000 mg encapsulated NAM; (C) AUC levels of single probands after administration of 1500 mg and 3000 mg NAM; (D) C_{max} of single probands after administration of 1500 mg and 3000 mg NAM*

4.5 Conclusion

A high robustness of the coating process as well as the release profile of the colon-targeted developed NA and NAM pellets (Fangmann et al., 2018) was given under different varied gastrointestinal conditions *in vivo* and *in vitro*.

The coating process of NA and NAM pellets with ileocolon-targeted delivery resulted in a good reproducibility. Gastric resistance is maintained for up to 24 months at 20 °C storage conditions, whereby the intestinal release was slightly reduced with increasing storage time. Targeted release characteristics of shellac coated NA and NAM pellets are provided for a storage at 20 °C for up to 12 months. Storage of 4 °C with a relative humidity of 47 % resulted in a loss of gastric resistance for NA after 4 weeks. NAM pellets were intact after eight weeks of storage at 4 °C.

In vitro, both formulations showed gastric resistance at elevated pH values up to 4.5, corresponding to a fed status of the stomach in vitro. Even unexpected lower pH values than described for the targeted region still resulted in a sufficient release of NA and NAM, especially in combination with a prolonged transit time.

The *in vivo* serum levels of NAM resulted in high variations in C_{max} and AUC levels after the administration of microencapsulated NAM due to intra- and interindividual variations. The time of release and absorption, however, was relatively robust and occurred in the human ileocolonic region.

Both, the *in vitro* and the *in vivo* results proposed a diffusion driven release of NAM from the capsules.

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5 Paper III: Controlling the interactions of coating and bioactive compound

Adjustment of triple shellac coating for precise release of bioactive substances with different physico-chemical properties in the ileocolonic region

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5.1 Abstract

Formulations for the controlled release of substances in the human terminal ileum and colon are essential to target the gut microbiome and its interactions with the intestinal mucosa. In contrast to pharmaceutical enteric coatings, reliable food-grade alternatives are still scarce. Shellac coatings have been used for various active ingredients, but their stability is affected by the physicochemical properties of the encapsulated substances. It is well known, that shellac release can be modulated by an acidic subcoating. Here, we hypothesized that a triple shellac coating with an adjusted intermediate coating (acidic or alkaline) can be effectively used to counteract the differences in pH value of various encapsulated substances, allowing a precise targeting of the desired release pH value. First, the system was tested with riboflavin 5'-monophosphate sodium salt dihydrate (RMSD) as a characteristic model substance. Secondly, it was transferred to nicotinic acid (NA) and nicotinamide (NAM) as bioactive compounds with different physicochemical properties: NAM, an alkaline crystalline and highly water-soluble substance, led to a premature release from conventional shellac microcapsules, whereas RMSD and NA with their medium solubility and neutral to acidic pH properties delayed the shellac dissolution. A precise modulation of the release profile of each substance was possible by the addition of different intermediate subcoatings: an acidic layer with citric acid counteracted the premature release of the alkaline and highly soluble NAM. In contrast, an alkaline sodium bicarbonate intermediate subcoating enhanced shellac swelling and delayed the release of NA and RMSD. In conclusion, the novel triple-layer shellac coating provides a much higher adaptability and reliability for nutritional formulations aiming at a targeted release in the ileocolonic region.

Keywords

enteric coating; fluidized-bed; shellac; subcoat; targeted release; colon

5.2 Introduction

The development of formulations targeting the (ileo)colonic region is gaining increasing interest because recent studies showed many correlations between the gut microbiome, nutrition and health. Nutritional compounds were found to achieve a health-promoting effect due to their direct influence on the microorganism composition in the lower intestine (Albenberg and Wu, 2014; Kau et al., 2011; Vos et al., 2010; Vos and Vos, 2012). We have recently shown that NA and NAM have beneficial effects on the host-microbiome interaction, particularly when they are administered with a targeted release in the ileocolonic region of mice and humans. NA results in an increase in *Bacteroidetes* in the microbiota profile and improves the markers for insulin sensitivity. NAM, on the other hand, is proposed to be a key regulator of the gut microbiota and of the propensity to inflammation (Fangmann et al., 2018; Hashimoto et al., 2012; Waetzig and Seegert, 2013). Simply increasing the content of NA or NAM in food would lead to a nearly total absorption in the upper gastrointestinal tract. Therefore, a nutritional encapsulation with an (ileo-)colon-targeted delivery mechanism is necessary to avoid a systemic uptake of the bioactive compound before reaching the ileocolonic region to ensure a high bioavailability of NA or NAM for the microbiome and the intestinal mucosa. The leading technology to protect bioactive compounds from the gastrointestinal environment is the application of an enteric coating as a protective layer (Czarnocka and Alhnan, 2015). The coating material needs to resist the acidic environment during the residence time in the stomach and small intestine. Suitable coating materials for oral colonic delivery are subject to various mechanisms that differ between the small intestine and the colon to ensure dissolution or erosion of the coating layer in the targeted region. Common examples are enzymatic-degradable, pH-sensitive, pressure-sensitive or time-dependent film coatings (Maroni et al., 2013). Commonly used materials are pH-dependent methacrylic acid copolymers such as Eudragit® or Eudraguard biotic®. The advantage of these synthetic polymers is a high batch-to-batch consistency. However, they can only be administered for pharmaceutical or solid nutraceutical products. For an application in foods, shellac can be used due to its legislation as food additive. Shellac is a purified resin from the lac insect Laccifer Lacca, which lives on trees mainly in India and Thailand. Shellac has a relatively high dissolution pH value of about 7.3, which allows a targeted release in the lower intestine (Buch et al., 2009; Farag and Leopold, 2011a; Limmatvapirat et al., 2007; Roda et al., 2007). The major disadvantages of a natural polymer such as shellac are the variability of the raw material and the loss of gastric resistance combined with a decreased solubility in intestinal fluids with increased storage time due to polymerization (Farag and Leopold, 2009).

However, shellac films from aqueous ammoniacal solutions show a good long-term stability (Limmatvapirat et al., 2005; Pearnchob et al., 2003), which supports its application as food coating material with ileocolon targeting properties. The high dissolution pH value of shellac implements that the human ileum can reach pH values of >7.3, which was verified in studies of (Evans et al., 1988; Ibekwe et al., 2008), despite lower pH values were also identified similar to the upper intestine (>pH 6.5) (Dressmann et al., 1998; Gruber et al., 1987). Further, a lack of free water in the lower intestine can result in an incomplete dissolution and drug release in the desired region (Schiller et al., 2005). However, our previous work showed a precise release of NAM in the ileocolonic region *in vivo* in 10 human study subjects by using the triple shellac coating and the *in vitro* profiles as demonstrated in the present manuscript (Fangmann et al., 2018).

Targeted release formulations based on pH-dependent coatings triggering a local release are commonly used (Khan et al., 1999; Liu et al., 2009; Liu et al., 2010; Liu and Basit, 2010; Roda et al., 2007; Varum et al., 2013). Partially neutralised Eudragit® S as inner coat and standard Eudragit® S result in an accelerated and precise release in the ileocolonic region due to simultaneous dissolution from its inner and outer surface (Liu et al., 2010; Varum et al., 2013). Regarding shellac, Farag and Leopold (Farag and Leopold, 2011a) developed sustained release pellets by using citric acid as subcoating to decrease the intrinsic pH value, when water penetrates the outer shellac coating (Farag and Leopold, 2011a). However, it is known that the properties of the encapsulated compound may also affect its rate of release (Menge, 2016; Ozturk et al., 1988; Ragnarsson et al., 1992; Sousa et al., 2002a). Following this, the subcoating has to be adjusted to the physicochemical characteristics of the microencapsulated compounds in order to precisely modulate the release profile.

The aim of the present study was therefore to provide an ileocolon targeted shellac formulation optimized for the release of three representative compounds by controlling the effect of the encapsulated compound on shellac by using individual adapted coating formulations. The influence of the encapsulated substance and a double shellac coating with a pH-modulating intermediate subcoating (*i.e.,* triple layer coating) on the release profile was investigated and the interplay between the three applied coating layers was examined with regard to a targeted release. First, RMSD was analysed as a commonly used model substance to simulate the in vitro-dissolution of coating formulations (Bajpai and Sharma, 2005; Guo et al., 2002; Li et al., 2013; Pajander et al., 2008). Second, the effect of the encapsulated compound on the transferability from a model system was evaluated regarding the

development of NA and NAM shellac coated microcapsules with a desired ileocolonic release profile. All three compounds (RMSD, NA and NAM) are hydrophilic, but differ in their properties such as acidity, water solubility and physical form.

5.3 Materials and methods

5.3.1 Materials

NA and NAM were purchased from SternVitamin (Ahrensburg, Germany), and RMSD was from Sigma-Aldrich (Taufkirchen, Germany). Shellac solution (SSB Aquagold) was obtained from Stroever Schellack Bremen (Bremen, Germany). Microcrystalline pelletized cellulose (Cellets®350) was obtained from iPc Process Center (Dresden, Germany). Citric acid monohydrate was purchased from Jungbunzlauer (Basel, Switzerland), and sodium bicarbonate was from Dr. August Oetker (Bielefeld, Germany). The binders hydroxypropylmethylcellulose (HPMC) (AnyAddy®) and pea starch (Lycoat™ RS 780) were obtained from Harke Group (Mülheim an der Ruhr, Germany) and from Roquette (Lestrem, France). All other chemicals were purchased from Carl Roth (Karlsruhe, Germany).

5.3.2 Methods

5.3.2.1 Preparation of coating solutions

For the preparation of the coating solutions, the excipients were blended with a binder: HPMC for coating NA or NAM microcapsules, and Lycoat for all intermediate subcoatings and the coating of RMSD microcapsules. After blending, the mixtures were dissolved at room temperature under moderate stirring in water using a magnetic stirrer. The composition of each formulation is listed in **Table 5.1**.

Table 5.1: *Composition of the coating solutions for the investigated microcapsule formulations [in % w/w]*

coating layer	active ingredient	[%]	binder	[%]	plasticizer	[%]	water [%]
NA	NA	9.3	HPMC	0.7	-	-	90
NAM	NAM	9.3	HPMC	0.7	-	-	90
RMSD	RMSD	2.33	Lycoat RS 780	4.65	Glycerol	0.023	93
Citric acid subcoat	Citric acid	2.33	Lycoat RS 780	4.65	Glycerol	0.023	93
Sodium bicarbonate subcoat	Sodium bicarbonate	2.33	Lycoat RS 780	4.65	Glycerol	0.023	93
Shellac	SSB Aquagold	60	-	-	-	-	40

5.3.2.2 Production of microcapsules

A schematic view of all coated microcapsules is shown in **Figure 5.1** and will be described in the following section. All formulations investigated in this study are summarized in **Table 5.2**.

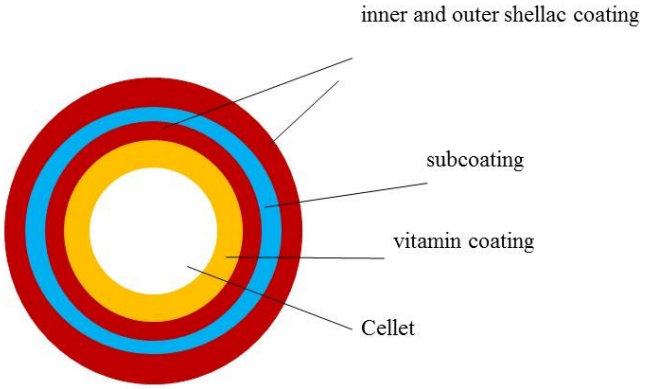

Figure 5.1: *Schematic overview of the coated microcapsules*

5.3.2.2.1 Production of RMSD microcapsules

The RMSD solution was applied to Cellets in a Mini Glatt fluidized bed coater (Glatt GmbH, Binzen, Germany) with bottom spray using a 0.5-mm two-way nozzle. An atomizing air pressure of 0.5–0.6 bar, an inlet air pressure of 0.3–0.4 bar and a spraying rate of about 0.6 g/min were used. Inlet air temperature was set to 40–45°C, which resulted in a product temperature of 33.1–35.0°C. The weight gain (w.g.) of RMSD was calculated to be 2%, whereby the total w.g. including pea starch as binder was 6% (see **Table 5.2**). Afterwards, the inner shellac coating, the intermediate coating sodium bicarbonate, and the outer shellac coating were applied to RMSD coated Cellets under the same conditions as described above with a product temperature of about 33°C (**Table 5.2**). For the administration of the intermediate subcoating, pea starch was also used as binder **(Table 5.1**). In the following sections, only the w.g. of the modulating substance is stated. Finally, the microcapsules were dried in an oven at 50°C for 1 h.

Table 5.2: *Composition of the investigated microcapsule formulations (w.g. = weight gain)*

Formulation	Vitamin coating	[%w.g.]	Inner shellac coating [%w.g.]	Subcoating	[%w.g.]	Outer shellac coating [%w.g.]
1	RMSD	2	0	Sodium bicarbonate/Lycoat	0/0	20
2	RMSD	2	2	Sodium bicarbonate/Lycoat	0.5/1.0	20
3	RMSD	2	2	Sodium bicarbonate/Lycoat	1.0/2.0	20
4	RMSD	2	2	Sodium bicarbonate/Lycoat	1.5/3.0	20
5	RMSD	2	2	Sodium bicarbonate/Lycoat	1.7/3.4	20
6	RMSD	2	2	Sodium bicarbonate/Lycoat	2.0/4.0	20
7	NA	10	0	Sodium bicarbonate/Lycoat	0/0	20
8	NA	10	2	Sodium bicarbonate/Lycoat	0/1.5	20
9	NA	10	2	Sodium bicarbonate/Lycoat	0.5/1.0	20
10	NA	10	2	Sodium bicarbonate/Lycoat	1.0/2.0	20
11	NA	10	2	Sodium bicarbonate/Lycoat	1.2/2.4	20
12	NA	10	2	Sodium bicarbonate/Lycoat	1.5/3.0	20
13	NA	10	2	Sodium bicarbonate/Lycoat	2.0/4.0	20
14	NAM	10	0	Citric acid/Lycoat	0/0	20
15	NAM	10	2	Citric acid/Lycoat	0/6.0	20
16	NAM	10	2	Citric acid/Lycoat	1.0/2.0	20
17	NAM	10	2	Citric acid/Lycoat	2.0/4.0	20
18	NAM	10	2	Citric acid/Lycoat	4.0/8.0	20
19	NAM	10	2	Citric acid/Lycoat	2.0/4.0	25
20	NAM	10	10	Citric acid/Lycoat	2.0/4.0	25

5.3.2.2.2 Production of NA and NAM microcapsules

First, the NA or NAM layer was applied to 195 g of Cellets in a Mini Glatt fluidized bed coater with bottom spray using a 0.5 mm two-way nozzle. An atomizing air pressure of 0.6 bar, an inlet air pressure of 0.35–0.4 bar and a spraying rate of 0.7–1.2 g/min were used. The inlet air temperature was set to 39–42°C, which resulted in a product temperature of 32.4–33.4°C. During the application of the NA coating, the coating solution was continuously stirred. The final w.g. was about 10%. Afterwards, the inner shellac coating was applied to 100 g of NA- or NAM-coated Cellets under the same conditions. In the next step, the intermediate subcoating, including the pH-modulating substances sodium bicarbonate (NA microcapsules) or citric acid (NAM microcapsules), was applied to 90 g of Cellets coated with NA or NAM, respectively. In the following text, only the calculated w.g. of the modulating substance is stated. The intermediate subcoating with 0% sodium bicarbonate or citric acid acts as a blank subcoating without modulating substance and is comparable to the formulation with 0.5% sodium bicarbonate for NA microcapsules and with 2% citric acid for NAM microcapsules. Finally, the outer shellac coating was applied to 80 g of Cellets coated with NA or NAM, inner shellac coating and pH-modulating intermediate subcoating under the

same conditions as used for the inner shellac coating. All formulations are summarized in **Table 5.2**. After the last coating step, the microcapsules were dried at 50°C in a drying oven for 1 h.

5.3.2.3 Characterization of the encapsulated compounds and shellac-coated microcapsules

5.3.2.3.1 pH values of the encapsulated compounds

For the characterization of the encapsulated compounds, their pH value was measured in solution (demineralized water) at room temperature using a pH meter (inoLab_IDS, Multi9420, WTW GmbH, Weilheim, Germany). The concentration of the measured solutions corresponds to the concentration in the coating solution (**Table 5.1**).

5.3.2.3.2 Physical form of the encapsulated compound

For measuring the physical form of the encapsulated compounds, differential scanning calorimetry (DSC) was used. First, dissolved RMSD, NA and NAM were separately spray-dried in a Mini Glatt fluidized bed coater to get the same physical form as during coating on the cellulose core. The spray-drying conditions were as followed: inlet air pressure: 0.3 bar; atomizing air pressure: 0.6 bar; product temperature: about 34°C; spraying rate: 0.75–1.2 g/min. Afterwards, the spray-dried substances were removed from the vessel walls and filters. About 4 mg of the powder were weighed and sealed in hermetic aluminum pans. Three samples of each substance were measured under nitrogen atmosphere by DSC (Q2000, TA Instruments, New Castle, DE, USA). The initial temperature was set to 20°C, afterwards a ramp up to 200°C (or 300°C in the case of NA) was programmed with an increase rate of 20 K/min. Glass transition temperatures, melting and crystallization peaks were determined using the TA Instruments software Universal Analysis 2000. The equipment was calibrated with indium. The baseline was generated without a pan.

5.3.2.3.3 Optical characteristics of coated microcapsules

To get a further insight into the structure of RMSD, NA and NAM coated microcapsules, scanning electron microscope (SEM) images were taken. For this purpose, microcapsules

were prepared on a holder with carbon Leit-Tabs (Plano GmbH, Wetzlar, Germany). Before examination in a Hitachi S-4800 SEM (Hitachi High Tech, Tokyo, Japan) at an accelerating voltage of 3 kV, microcapsules were sputter-coated with a layer of 8–10 nm gold-palladium using a Leica EM SCD 500 high-vacuum sputter coater (Leica Microsystems GmbH, Wetzlar, Germany).

5.3.2.3.4 Calculation of coating thickness

For calculation of the coating thickness, the particle size of coated and non-coated Cellets was determined by laser diffraction (Horiba LA-950V2, Retsch Technology GmbH, Haan, Germany). To get comparable results, RMSD, NA and NAM microcapsules with the following coating weight gains were measured: 2% w.g. from the inner shellac coating; 1% w.g. from the sodium bicarbonate or citric acid in the intermediate layer; 20% w.g. from outer shellac coating. The measurements were performed using the dry feeder with vacuum-driven forced ejection, a vibration power alteration of 120 and a compressed air pressure of 0.3 MPa. Refractive indices were the following: cellulose: 1.63, shellac: 1.52, NA: 1.49 and NAM: 1.47. All samples were measured in triplicate. Afterwards the increase in particle diameter and radius were calculated to obtain the total coating thickness.

5.3.2.4 Dissolution of shellac-coated microcapsules

In vitro dissolution tests were carried out in duplicate at 37°C with 0.5 g of NA or NAM microcapsules or 1 g of RMSD microcapsules in 250 ml simulated gastrointestinal fluids using a paddle apparatus at 100 rpm (Model DT 70, Pharmatest Group, Hainburg, Germany). Dissolution tests were run for 1 h at pH 1.4 to simulate fasted gastric conditions, 0.5 h at pH 4.5 (citrate or acetate buffer), 2 h at pH 6.8 and 1.5 h at pH 7.4 (Soerensen phosphate buffers) (Fangmann et al., 2018; Theismann et al., 2019). The pH of 4.5 was used to simulate the transfer from a fed gastric to the duodenal passage, whereby the phosphate buffers simulated the distal parts of the small intestine. Previous results showed that citrate and acetate buffer only differed in handling and not in influencing the release rate (data not shown). For buffer exchanges, microcapsules were retrieved by filtration using paper filters, and microcapsules were transferred to the next buffer. The release was recorded at 30 min intervals using a UV/vis spectrophotometer (Helios gamma, Thermo Fisher Scientific, Waltham, MA, USA) at a wavelength of 262 nm for NA or NAM and at 445.5 nm for RMSD using 1 x 1 cm quartz cuvettes. In order to calculate the percentage of released compound, the total amount of

RMSD, NA and NAM or in coated microcapsules was determined at a pH of 7.4 after 30 min (full release) and set to 100%.

According to the definition of the USP, gastric resistance is achieved if less than 10% is released during 2 h in 0.1 N HCl (pH 1.2) (Tarcha, 1991). In the present study, gastric resistance was defined as less than 10% release after passing a pH value of 1.4 for 60 min and a pH value of 4.5 for 30 min. The reduction of exposition time and the increased pH value was chosen because of the ability of microcapsules (< 2 mm) to pass the pylorus independent of gastric emptying (Davis et al., 1987).

5.3.2.5 Characterization of the swelling properties of shellac

In order to evaluate the swelling of the shellac coating, the microcapsules (n=3) were analyzed in the above-mentioned buffers using a transmitted light microscope (Meade Instruments Europe GmbH, Kempten, Germany) in combination with an electronic eyepiece (MikroOkular-II, Bresser GmbH, Rhede, Germany). Every 5 min, an image was taken at a magnification of 100x.

5.4 Results

5.4.1 RMSD microcapsules

5.4.1.1 Physico-chemical properties of RMSD microcapsules

RMSD was chosen as a widely used reference compound to modulate its in vitro dissolution profile. Regarding coating and dissolution processes, RMSD showed a moderate water solubility and a pH value of 6.2 in aqueous solution (2.3 % (w/w)) (**Table 5.3**). **Figure 5.2A** shows that RMSD was present in an amorphous form with a glass transition temperature of 81.0 ± 0.86°C. An exothermic DSC peak occurred at 128.6 ± 1.36 °C, followed by an endothermic irregular signal. In combination with pea starch as binder, RMSD coated on Cellets resulted in a smooth surface, which was optimal for a subsequent uniform enteric coating (**Figure 5.2B**).

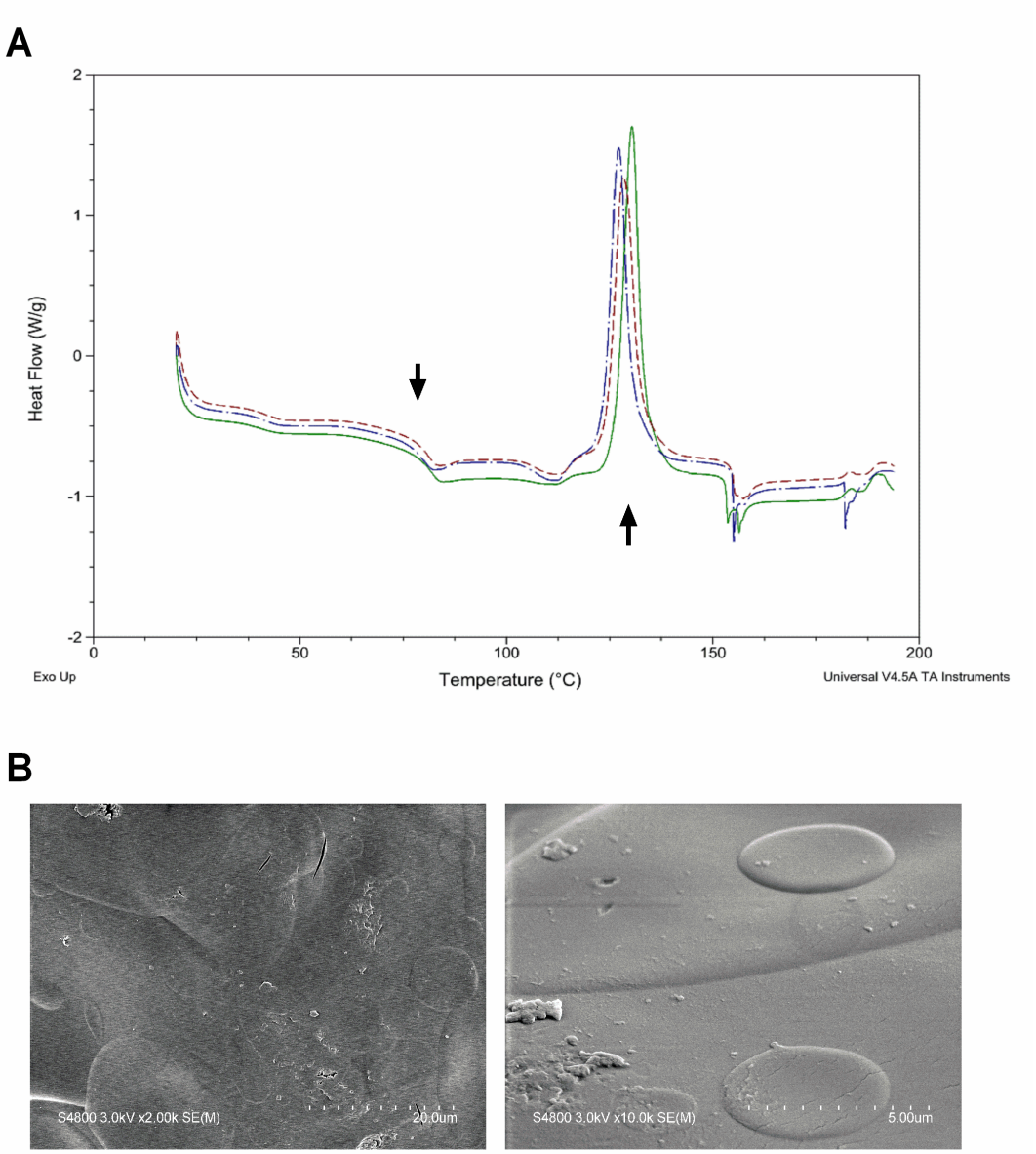

Figure 5.2: *Characterization of RMSD. (A) DSC thermogram of spray dried RMSD. Arrows indicate the glass transition and the recrystallization peak. (B) SEM images of the surface of RMSD-coated Cellets.*

5.4.1.2 In vitro release profile modulation of RMSD microcapsules

For modulation of the release profile of RMSD, single shellac-coated (20% w.g.) RMSD microcapsules were dissolved in vitro (**Figure 5.3A**). Gastric resistance was given. At pH value 6.8, 10% were released after 210 min. Reaching a pH value of 7.4 resulted in a RMSD release of 78.6%. To enhance the RMSD release, especially at pH 6.8, which is related to an ileocolonic release as shown by (Fangmann et al., 2018), a triple coating formulation, consisting of an inner and outer shellac coating and an intermediate sodium bicarbonate

coating, was applied. As shown in **Figure 5.3B**, the addition of sodium bicarbonate led to an earlier and increased release at pH 6.8 compared to the release without intermediate subcoating. The application of 0.5 or 1% sodium bicarbonate resulted in a release of 24% or 20% RMSD after 210 min, respectively, despite gastric resistance. Applications of 1.5–2.0% sodium bicarbonate led to a loss of the gastric resistance.

5.4.1.3 Cross section and coating thickness of RMSD microcapsules

As shown in **Figure 5.3C**, the coating layers (RMSD, inner shellac, sodium bicarbonate, and outer shellac layer) were clearly distinguishable by SEM. The coating thickness of every layer was calculated based on the cross section: approximately 5.0, 1.5, 1.0, and 18.0 µm for RMSD, inner, intermediate and outer coating layers, respectively.

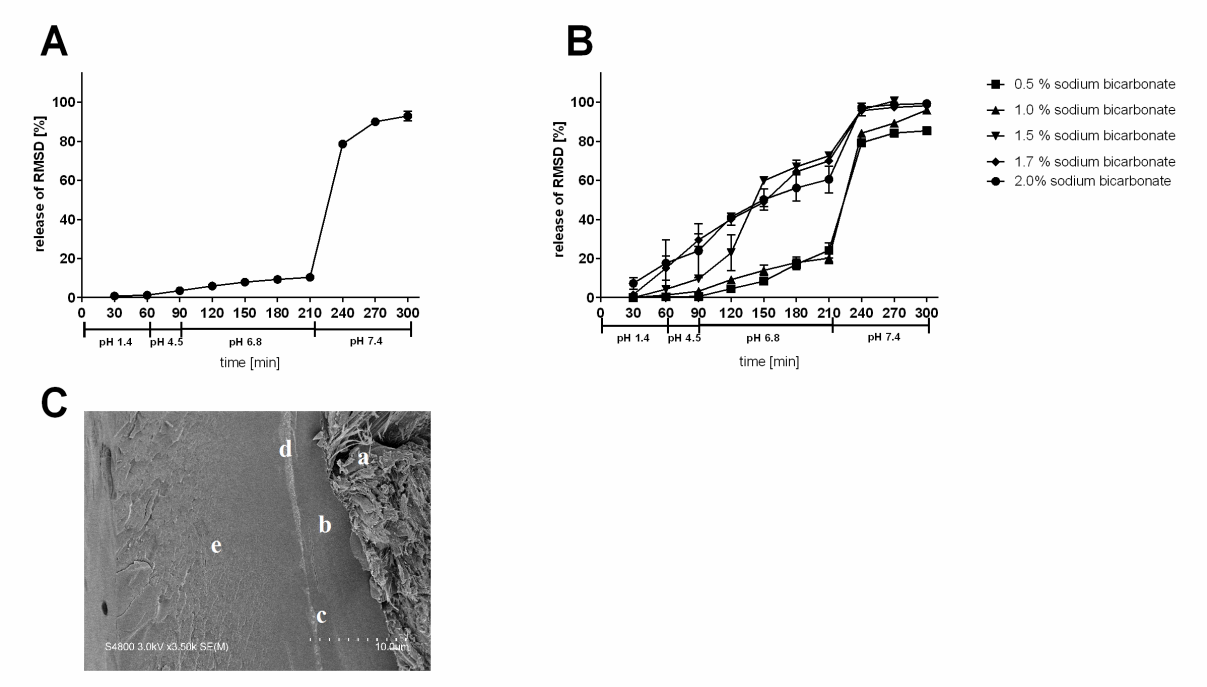

Figure 5.3: *In vitro release profile and cross sections of shellac-coated RMSD microcapsules. (A) RMSD microcapsule coated with a single shellac coating (20% weight gain (w.g.)). (B) RMSD microcapsules coated with an inner shellac coating (2% w.g.), a sodium bicarbonate intermediate subcoating (0.5–2.0% w.g.) and an outer shellac coating (20% w.g.). All results are shown as mean ± standard deviation (n=2). (C) Representative SEM image of a cross section of an RMSD microcapsule consisting of (a) Cellet core, (b) RMSD (2% w.g.), (c) inner shellac (2% w.g.), (d) sodium bicarbonate (0.5% w.g.), and (e) outer shellac layer (20% w.g.)*

5.4.2 NA and NAM microcapsules

5.4.2.1 Physico-chemical properties of NA and NAM microcapsules

In contrast to RMSD, NA and NAM were chosen because of their beneficial effect on the intestinal microbiome (Fangmann et al., 2018; Hashimoto et al., 2012; Waetzig and Seegert, 2013). Although NA and NAM have some biological functions in common, they differ in their physico-chemical properties (**Table 5.3**). DSC results (**Figure 5.4A**) indicated that most of the NA existed in its crystalline form. Two endothermic peaks were observed at 182.7 ± 0.00°C and at 236.4 ± 0.08°C. The weak endothermic peak was a solid-to-solid transformation, whereas the second peak represented the melting point of NA. However, we also measured a glass transition temperature at 275.6 ± 0.42°C. Due to the peak height, the main part of NA appears to be present in its crystalline form. NAM only existed in its crystalline form with a melting point of 129.8 ± 0.31°C (**Figure 5.4A**). The surface of NA after coating on Cellets, as determined by SEM, showed some crystalline irregularities, but appeared predominantly smooth (**Figure 5.4B**). In contrast, the NAM-coated surface showed many cavities and irregularities (**Figure 5.4C**). The physico-chemical properties of RMSD, NA and NAM are summarized in **Table 5.3**.

Table 5.3: *Comparison of the physico-chemical properties of the compounds RMSD, NA and NAM*

	RMSD	NA	NAM
pH	6.2 (2.3% w/w)	3.4 (9.3% w/w)	6.2 (9.3% w/w)
pKa[1]	-	4.85	3.3
pKa (strongest acid)[2]	-	2.79	13.39
Water solubility (g/l)[1]	50	10-15	1000
Glass transition temperature (DSC) (°C)	81.0 ± 0.86	275.6 ± 0.42	-
Melting temperature (DSC) (°C)	> 130	182.7 ± 0.00 236.4 ± 0.08	129.8 ± 0.31
Recrystallization temperature (DSC) (°C)	128.6 ± 1.36	-	-
Surface (SEM)	Smooth	Moderate irregularities	Cavities/irregularities

1 (ChemBook) 2 (HMDB)

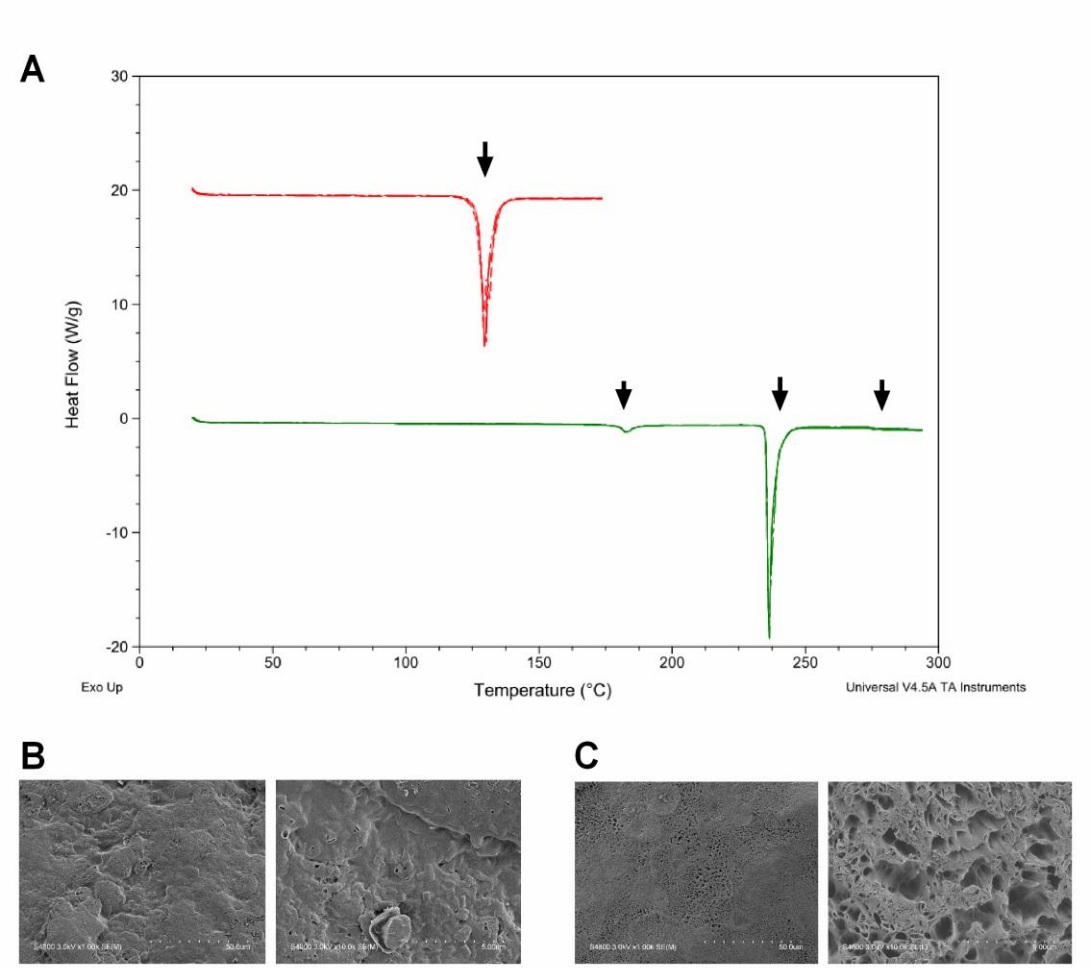

Figure 5.4: *Characterization of NA and NAM. (A) DSC thermogram of spray dried NA (green) and NAM (red) .The arrows indicate the melting peaks and the glass transition. (B, C) SEM images of the surface of a NA-coated (B) or NAM-coated Cellet (C).*

5.4.2.2 In vitro release profile modulation of NA and NAM microcapsules

Figure 5.5A+B shows the in vitro release profiles of NA and NAM microcapsules coated with a single shellac layer. NA microcapsules with a single shellac coating showed a very slow release: until a pH of 7.4 was reached (**Figure 5.5A**). In contrast, NAM microcapsules with a single shellac layer did not show gastric resistance (**Figure 5.5B**). In order to modulate the release profile of NA and NAM, different intermediate subcoatings based on sodium bicarbonate or citric acid were tested: for NA, sodium bicarbonate was chosen as a pH-modulating substance in the intermediate subcoating because of its effects on the release profile of RMSD (**Figure 5.3B**). As expected, the incorporation of a sodium bicarbonate subcoating dose-dependently increased NA release (**Figure 5.5C**). The objective of 20–40 % release after 210 min was achieved using 1.2% sodium bicarbonate. In contrast to RMSD, 1.5

and 2.0% sodium bicarbonate already led to a loss of gastric resistance (**Figure 5.5C**). In addition, the results show that the earlier and enhanced release was due to sodium bicarbonate itself and not to the intermediate subcoating and the resulting diffusion barrier in general (control: 0% sodium bicarbonate). In the case of NAM, citric acid was used to aim for a reduced and delayed release (**Figure 5.5D**). After 210 min, the control intermediate subcoating without citric acid (0%) led to a NAM release of 58%, the addition of 1.0%, 2.0%, or 4.0% citric acid resulted in a reduced release. The control intermediate subcoating with 0% citric acid demonstrated that the effect was mainly triggered by citric acid, but indicated that the subcoating and the added diffusion barrier itself may also play a role. Gastric resistance was only obtained with 4.0% citric acid in the intermediate subcoating, although it was only marginally acceptable with 10% NAM release at pH 4.5 after 90 min.

In addition to the variation of the intermediate pH-modulating subcoating, the thickness of the inner shellac coating can also be modified to adapt the release profile to the desired site and time course (**Figure 5.5E**). Increasing the inner shellac subcoating layer from 2% w.g. to 10% w.g. in combination with 2% citric acid in the pH-modulating subcoating and an outer shellac coating with 25 % w.g. reduced the NAM release from 31% to 21% after 210 min (**Figure 5.5E**). Furthermore, gastric resistance was achieved. Previous results showed that a pH-independent inner layer, such as maltodextrin, did not provide sufficient separation and resulted in a higher release compared to shellac (data not shown).

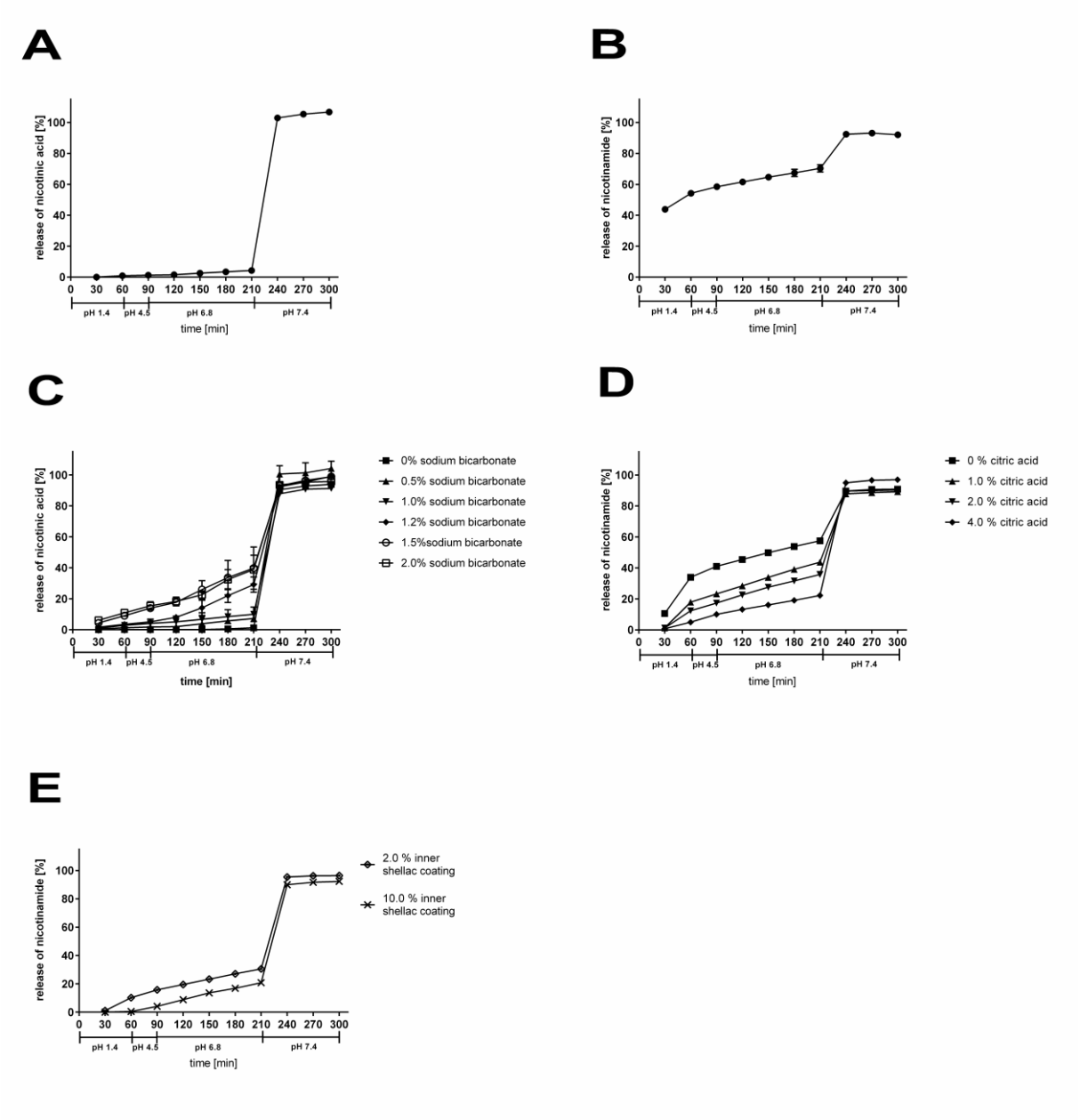

Figure 5.5: *In vitro release profiles of shellac-coated NA and NAM microcapsules. (A) NA microcapsules coated with a single shellac layer (20% w.g.). (B) NAM microcapsules coated with a single shellac layer (20% w.g.). (C) NA microcapsules coated with an inner shellac coating (2% w.g.), a sodium bicarbonate intermediate subcoating (0.0–2.0% w.g.) and an outer shellac coating (20% w.g.). (D) NAM microcapsules coated with an inner shellac coating (2% w.g.), a citric acid intermediate subcoating (0.0–4.0% w.g.) and an outer shellac coating (20% w.g.). (E) NAM microcapsules coated with an inner shellac coating (2 or 10% w.g.), a citric acid intermediate subcoating (2.0% w.g.) and an outer shellac coating (25% w.g.). All results are shown as mean ± standard deviation (n=2).*

5.4.2.3 Cross section and coating thickness of NA and NAM coated microcapsules

An SEM cross section of a NA-coated microcapsule is shown in **Figure 5.6A**, showing four clearly distinguishable layers and the Cellet core. The sodium bicarbonate layer is seen as a small layer punctured by holes. The NA layer also shows some cracks. The indicated layer thicknesses based on the cross section were about 10, 2, 1.5 and 16.5 µm for the NA, inner, intermediate and outer coating, respectively. A similar total coating thickness of NA microcapsules was measured by laser diffraction: 25.59 ± 1.5 µm (1 % w.g. of sodium bicarbonate in the intermediate subcoating). The SEM cross section of NAM (**Figure 5.6B**) differed strongly from cross sections of RMSD (**Figure 5.3C**) and NA microcapsules (**Figure 5.6A**), as only two different types of layers were detectable. The outer layer had a thickness of about 19 µm. In total, a coating thickness of about 31 µm was indicated from SEM cross sections (including 4% w.g. of citric acid in the intermediate subcoating). The laser diffraction method calculated a total thickness of 23.26 ± 0.11 µm (1 % w.g. of citric acid in the intermediate subcoating).

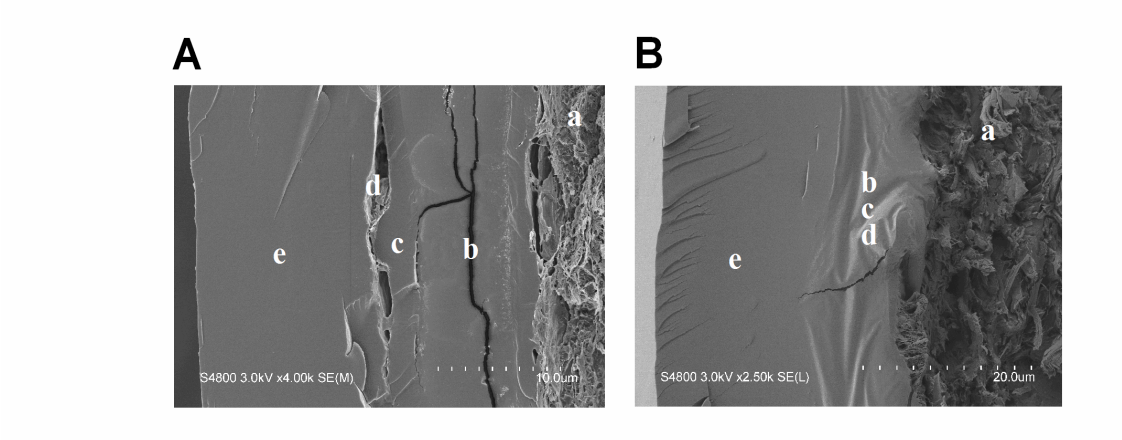

Figure 5.6: *(A) SEM image of cross section of a NA microcapsule consisting of (a) inner Cellet, (b) NA (10% w.g.), (c) inner shellac (2% w.g.), (d) sodium bicarbonate (1.2% w.g.), (e) outer shellac coating (20% w.g.). (B) SEM image of cross section of a NAM microcapsule consisting of (a) inner Cellet, (b) NAM (10% w.g.), (c) inner shellac (2% w.g.), (d) citric acid (4% w.g.), (e) outer shellac coating (20% w.g.).*

5.4.3 Effects of the encapsulated compounds and subcoatings on the swelling properties of shellac

In order to investigate the effects of the encapsulated substances and the chosen pH-modulating intermediate subcoatings on the swelling properties of shellac, light microscopy images were taken using the different buffers with pH values of 1.4, 4.5, 6.8 and 7.4 also employed for the release profiles.

Due to the known effects of the encapsulated compounds on the release profiles (Ozturk et al., 1988; Ragnarsson et al., 1992), the swelling behaviour of the single shellac coating of RMSD, NA and NAM microcapsules was analyzed. **Figure 5.7** shows microscope images of shellac-coated RMSD, NA and NAM microcapsules after 0, 15 and 30 min at pH 7.4. Cellets coated with NA and shellac (**Figure 5.7A**) exhibited only a slight swelling after 15 and 30 min. In contrast, RMSD and NAM microcapsules (**Figure 5.7B+C**) showed a clearly enhanced swelling after 15 min and an indication of the dissolution of the shellac coating after 30 min. According to these results, an influence of the encapsulated compound on the release profile was assumed, as NA appeared to inhibit the swelling of the shellac coating, whereas RMSD and NAM seemed to enhance it.

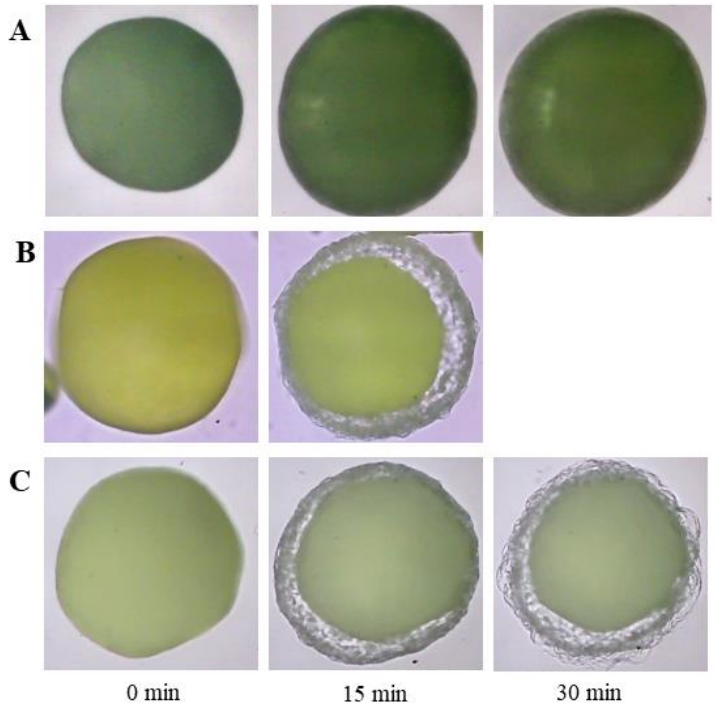

Figure 5.7: *Light microscope images showing the effect of (A) encapsulated RMSD, (B) encapsulated NA, (C) encapsulated NAM on the swelling of a single shellac coating (20% w.g.) at pH 7.4 after 0, 15 and 30 min (100x magnification).*

In **Figure 5.8**, the effect of the intermediate subcoating with sodium bicarbonate is shown. In **Figure 5.8** A-C, CO_2 bubbles arising from NA microcapsules with 1.2%, 1.5% or 2.0% w.g. of sodium bicarbonate at pH 6.8 are evident after 2–5 min. In the case of encapsulated RMSD with 2.0% w.g. of sodium bicarbonate in the intermediate subcoating (**Figure 5.8D+E**),

bubbles were not observed, although local swellings appeared, followed by swelling of the whole shellac coating after 2 and 3 min.

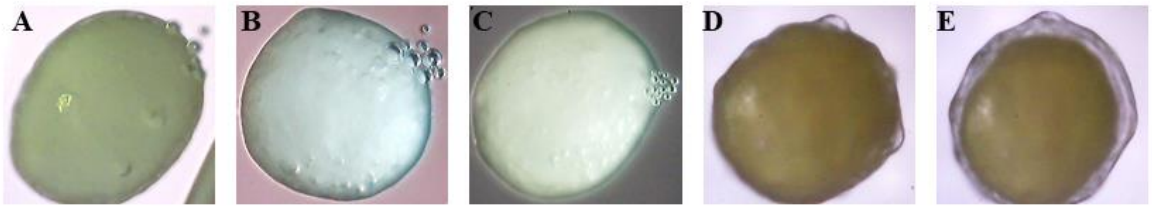

Figure 5.8*: Light microscope images showing the effect of sodium bicarbonate intermediate subcoatings at pH 6.8. (A-C) NA microcapsules with (A) 1.2% w.g. sodium bicarbonate, (B) 1.5% w.g. sodium bicarbonate, or (C) 2.0% w.g. sodium bicarbonate. (D, E) RMSD microcapsules with 2.0% w.g. sodium bicarbonate after 2 and 3 min, respectively (100x magnification).*

The contrary effect was observed, when citric acid was used in the intermediate subcoating to reduce the fast NAM release (**Figure 5.9**). No or only minimal swelling of citric acid-coated NAM microcapsules was observed at pH 7.0 or 7.4, whereas microcapsules without an intermediate subcoating or microcapsules with a subcoating without citric acid increased significantly in their diameter due to swelling of shellac (**Figure 5.9**). Moreover the swelling of the shellac at pH 7.4 was stronger than at pH 7.0.

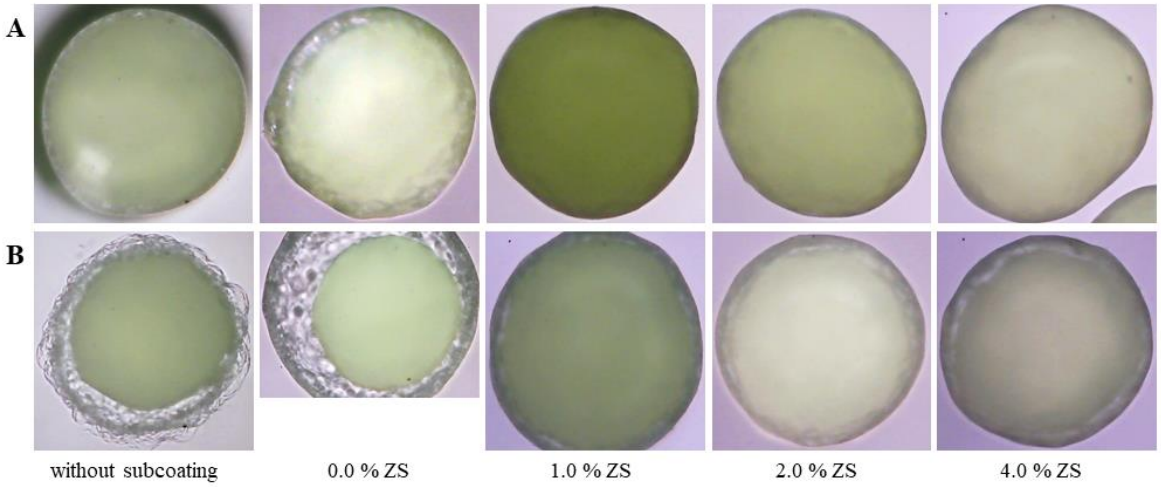

Figure 5.9: *Light microscope images showing the effect of citric acid as a pH-modulating substance in the intermediate subcoating on the swelling properties of NAM double shellac-coated microcapsules at (A) pH 7.0 and (B) pH 7.4 after 30 min (100x magnification).*

5.5 Discussion

To improve the precision of shellac coatings for targeted release in the ileocolonic region, the interactions between the coating and various encapsulated compounds were investigated. For this purpose, a triple coating with two shellac layers and intermediate pH-modulating subcoating was developed. The intermediate layer should control the effects of the encapsulated substance. To the best of our knowledge, this formulation concept is novel (PCT/EP2017/058733 and PCT/EP2017/058741) and adds important new options to the toolbox of fully nutritional controlled-release coatings.

5.5.1 Effect of physico-chemical properties of the encapsulated compounds on the release profiles

The attempt to transfer the coating processes tested for RMSD microcapsules to NA and NAM microcapsules showed the dependence of the release profile on the compound to be encapsulated. A completely different release profile was observed for NAM microcapsules (**Figure 5.5A**) than for NA (**Figure 5.5B**). NA and NAM have different solubility, acidity and coating quality (**Table 5.3**). While it is known that several properties of the encapsulated compound can influence the solution profile, including particle size, solubility and pK_a (Menge, 2016; Ozturk et al., 1988; Ragnarsson et al., 1992), we found that the pH value of the encapsulated substance has a significant modulating influence on the pH-sensitive shellac coating. At pH values below the pK_a of shellac, its carboxyl groups are protonated, which results in an insoluble coating and hinders the release of the encapsulated compound. As the pH of the surrounding medium increases, carboxyl groups dissociate, promoting water penetration and swelling of the coating material (Farag and Leopold, 2011a; Henning et al., 2012; Penning, 1996). The swelling of shellac at pH 6.8 is known. This behaviour promotes the dissolution of an applied subcoating or the encapsulated substance itself, which in turn influences the dissociation of shellac and the release of the substance (Farag and Leopold, 2011a). The pH value in aqueous solutions of NA is 3.44, which is lower than the pH values of NAM (pH 6.23) or RMSD solutions (pH 6.19). The lower pH value of NA could therefore delay the dissociation and dissolution of shellac. In contrast, the higher pH value of NAM and RMSD could lead to an increased intrinsic pH value, an earlier dissolution and a stronger swelling of shellac above pH 6.8 compared to NA. This was confirmed by the microscopic analyses shown in **Figure 5.7**. The faster and stronger swelling of the outer shellac coating could lead to a faster dissolution of the encapsulated substance and thus to a diffusion-related release at pH 6.8. At pH 7.4, shellac generally dissolves and a burst release occurs (Farag and

Leopold, 2011a; Qussi and Suess, 2005). In contrast to NAM, NA and RMSD microcapsules showed gastric resistance. Normally, shellac is insoluble at gastric conditions due to protonated carboxyl groups. However, small amounts of acid water can penetrate shellac, especially if it is present in its ammonium salt form, as in the present study (Hagenmaier and Shaw, 1991; Pearnchob et al., 2004). In consequence, an interaction between NAM and shellac during the coating and dissolution process is plausible, which could be related to the higher water solubility of NAM (1000 g/l) compared to RMSD (50 g/l) or NA (15 g/l). The higher the water solubility of the encapsulated compound, the lower the mean dissolution time or lag time (with only a few exceptions) (Ozturk et al., 1988; Ragnarsson et al., 1992; Sousa et al., 2002a). Aqueous coating solutions can dissolve the drug during the process, resulting in the formation of channels in the outer coating (Guo et al., 2002; Heinämäki et al., 1994). The highly soluble NAM is probably more susceptible to this than NA and RMSD, although obvious external channels in the outer shellac coating could not be observed in the SEM (data not shown). A high amount of dissolved drug could also induce an increased osmotic influx rate of water and subsequently lead to a higher expansion of the coating and a premature release of encapsulated substance (Ragnarsson et al., 1992). Little irregularities or small pores in the coating can accelerate the release process (Bruce et al., 2003; Förmer et al., 2006; Henning et al., 2012; Phan The et al., 2008). These coating irregularities can be caused by the microcapsule shape or surface characteristics (Chopra et al., 2002). The shape of the microcapsules used in the present study was equally spherical due to the inert spherical Cellet core. Interestingly, the NAM layer (but not the NA or RMSD layers) exhibited a porous and uneven structure. The porosity of microcapsules affects the substance release profile, which is inversely related: The higher the porosity, the shorter the average dissolution time. Possible explanations could be the altered ability of an additional coating to adhere to the underlying surface, the incorporation of abraded particles from the surface during the coating process due to increased brittleness, or the unevenly distributed final coating due to increased surface roughness (Chopra et al., 2002; Sousa et al., 2002b; Tunón et al., 2003).

DSC measurements reflect the thermal behaviour of spray-dried compounds and provide information on their physical form, which in the present study could be related to the surface properties of the RMSD, NA and NAM layers. As shown in **Table 5.3**, RMSD and NA were partially present in their amorphous or semicrystalline form, while NAM was only present in crystalline state. This could be responsible for the rough surface of NAM-coated Cellets. NA also showed a solid-to-solid transformation and a melting point of the crystalline form, which is in accordance with other reports (Wang et al., 2004). RMSD was recrystallized after

exceeding the glass transition temperature, which is typically seen for amorphous or semicrystalline substances (Liu et al., 1997; Mano et al., 2004). The resulting melting signal illustrated irregularities that may occur as a result of overlapping melt and decomposition peaks. Different physical characteristics have different effects on coating quality and release rate. The crystallinity of NAM can lead to crystal defects, which can also affect coating properties such as surface roughness (Murr and Inal, 1979): Penetration of the applied coating material into pores of the substance layer is possible if the penetration time exceeds the spreading time. This is the case when porosity and pore size in the substance layer increase, resulting in loss of coating material and reduced coating yields (Kennedy and Niebergall, 1996; Werner et al., 2007). The pores of the NAM microcapsule surface can thus lead to incorporation of shellac during the coating process, which could influence the gastric protection properties of shellac (Chopra et al., 2002; Guo et al., 2002; Heinämäki et al., 1994; Tunón et al., 2003). Indeed, the cross section of NAM microcapsules showed no clear separation of the four administrated layers (NAM, inner shellac, citric acid, outer shellac coating) compared to the RMSD and NA microcapsules, which suggests an interaction of NAM and the shellac coating. One possibility to reduce the porous structure of NAM layers is the heightening of the binder level (Sinchaipanid et al., 2004).

5.5.2 Effect of sodium bicarbonate on the release profile of RMSD and NA

The combination of sodium bicarbonate as an intermediate subcoating and shellac was used in the present study in order to counteract the slow dissolution of shellac at pH 6.8. A beginning release at pH 6.8 showed a low systemic availability and a high availability in the (ileo-)colonic region (Fangmann et al., 2018). Furthermore, pH values of lower than 7.3 can exist in the human gut (Dressmann et al., 1998; Gruber et al., 1987), which makes a controlled release at a pH value of about 6.8 advantageous. The results confirmed that the application of such a subcoating adapts the release profile of the encapsulated compounds to the different requirements. In all experiments, the pH-modulating subcoating with sodium bicarbonate led to an earlier release of RMSD and NA. The observed effects of the selected intermediate subcoating substances on the release profile could be explained by the altered intrinsic pH value and the dissolution behaviour of shellac (Farag and Leopold, 2011a). Above pH 6 and below the dissolution pH of 7.3, shellac swells and water penetrates into the microcapsule where it can dissolve the subcoating material (Limmatvapirat et al., 2004; Limmatvapirat et al., 2007). Thus, the strongest effect of an intermediate subcoating is assumed to take place at

pH 6.8. Sodium bicarbonate and water are generating hydroxide ions ($HCO_3^- + H_2O \leftrightarrow H_2O + CO_2 + OH^-$), which result in a pH of about 7.9 (2.5 g/50 ml) (pk_a=10.329). As a result, sodium bicarbonate leads to a local increase in pH and thus to accelerated shellac dissociation. Furthermore, the production of CO_2 could also lead to an earlier release of the encapsulated compound, as the gastric juice resistance was suddenly lost above a critical amount of sodium bicarbonate used (i.e., 1.5% w.g. in NA and RMSD microcapsules). In addition, the presence of NA seemed to increase the CO_2-producing effect of sodium bicarbonate and the penetrating water. Ichikawa et al. (Ichikawa et al., 1991) used sodium bicarbonate in combination with tartaric acid as an effervescent layer below an outer shellac layer with polyvinyl acetate. The CO_2 formation resulted in a pill swollen like a balloon (Ichikawa et al., 1991). This floating drug led to a prolonged gastric residence, the drug was released continuously and could be absorbed in the absorption window closely after the pylorus (Surana and Kotecha, 2010). This, however, would be completely undesirable for formulations targeting the ileocolonic region. Floated microcapsules were not observed during the dissolution experiments, but the addition of an acid, in this case NA, could plausibly lead to a higher CO_2 production (Binnewies et al., 2016; Choi et al., 2002; Lin et al., 2017). The resulting bubbles can rupture the outer shellac coating and result in the release of the encapsulated compound. Due to the fact that the shellac coating was applied without using any plasticizer, the elasticity and flexibility of the coating was low (Pearnchob et al., 2004; Qussi and Suess, 2006). This mechanism may also explain higher variabilities in release rate with increasing amount of sodium bicarbonate. The higher the production of CO_2 bubbles, the more uncontrolled and irregular the release. The use of a plasticizer to increase the elasticity of the coating could reduce the release variability. However, this also may lead to a premature release in the upper intestine because of an increased water vapour permeability resulting from the use of hydrophilic plasticizers (Luangtana-Anan et al., 2010; Pearnchob et al., 2003). This depends on the plasticizer (Pearnchob et al., 2003; Rao and Diwan, 1997)and sometimes even no effect is visible (Krogars et al., 2003).

5.5.3 Effect of citric acid on the release profile of NAM

In contrast to sodium bicarbonate, citric acid with its pH value of 1.87 (pK_{a1}=3.3; pK_{a2}=4.76; pK_{a3}=6.4) reduces the intrinsic pH-value and delays the dissolution of shellac, leading to a slower release rate (Farag and Leopold, 2011a). In addition, the present results showed that citric acid reduced the swelling properties of shellac immensely (**Figure 5.9**). The only

disadvantage of the citric acid subcoating was the challenging application during the coating process, because of a high risk of agglomeration. Citric acid is hygroscopic and can lead to increased stickiness (Dewettinck et al., 1998). Furthermore, citric acid could induce hydrolysis of the pea starch molecules used as binder, leading to a reduction in amylose and an increase in glucose (Wang et al., 2003), which further increases the stickiness. Hence, both effects could result in agglomeration. Another alternative to pea starch is polyvinylpyrrolidone (PVP), which was previously used by Farag and Leopold (Farag and Leopold, 2011a). However, the permission to declare pea starch as food additive in food stuff is advantageous over PVP. In EU, the administration of PVP as a food additive is only allowed in solid nutraceuticals or as a sweetener (EG Nr. 1333/2008). Due to the demanding handling of the citric acid subcoating, it was desirable to shorten the process time and to obtain a comparable release profile by adapting of the inner shellac coating. The inner shellac coating was administered to separate NAM from the pH-modulating intermediate coating. As expected, the release of the active ingredient was reduced by increasing of the inner shellac coating due to a thicker diffusion barrier (Chopra et al., 2002; Czarnocka and Alhnan, 2015; Farag and Leopold, 2011a, 2011b; Qussi and Suess, 2005; Tunón et al., 2003). Since it was shown that the chosen substance strongly influences the release rate, the inner shellac coating ensures the separation of the layer with the bioactive compound from the pH-modulating intermediate subcoating layer and could therefore lead to a separation of these two modulating effects, which is more efficient than a pH-independent inner layer. This inner shellac is an invention resulting from the present study (PCT/EP2017/058733 and PCT/EP2017/058741) and may considerably expand the applicability of shellac coating systems on cores with pH-influencing active ingredients.

5.6 Conclusions

In summary, the properties of the encapsulated compound have a great impact on the release profiles of shellac-coated microcapsules. As demonstrated in the present study, the combination of the aqueous pH value, the water solubility, and the crystallinity of the encapsulated substance are factors influencing the release profile from shellac microcapsules. Therefore, a simple formulation transfer from one substance to other compounds with different properties is not possible. The novel triple-layer formulation concept described here shows that a precise modulation of the release profile is possible by using different acidic or basic pH-modulating intermediate subcoatings, e.g., sodium bicarbonate or citric acid,

counteracting the properties of the encapsulated compounds. We also demonstrate that sodium bicarbonate as a subcoating leads to an earlier and dose-dependent increased release rate by affecting the dissolution of shellac at pH 6.8. Therefore, sodium bicarbonate as a subcoating is suitable for the enhanced release of compounds with a relatively low water solubility, such as NA and RMSD, and can counteract the slow dissolution of shellac at lower pH values. In contrast, citric acid reduces the swelling of shellac, delays the release profile, and can counteract a premature release, for example for alkaline and highly water-soluble compounds, such as NAM. The present results describe valuable tools for the production of shellac microcapsules for different substances with the aim of a targeted release in the (ileo-)colon, e.g. to influence the gut microbiome. However, due to the adaptability of the triple coating layer, also other applications are conceivable to target more proximal or distal parts in the human intestine.

Acknowledgements

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6 Paper IV: Nicotinamide pellets produced by wet extrusion/spheronisation process

Modelling the effect of process parameters on the wet extrusion and spheronisation of high-loaded nicotinamide pellets using a quality by design approach

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6.1 Abstract

The aim of the present study was to develop an alternative process to spray granulation in order to prepare high loaded spherical nicotinamide (NAM) pellets by wet extrusion and spheronisation. Therefore, a Quality by Design approach was implemented to model the effect of the process parameters of the extrusion and spheronisation process on the roundness, roughness and useable yield of the obtained pellets. The obtained results were compared to spray granulated NAM particles regarding their characteristics and their release profile in vitro after the application of an ileocolon targeted shellac coating. The wet extrusion and spheronisation process was able to form highly loaded NAM pellets (80 %) with a spherical shape and a high useable yield of about 90 %. However, the water content range was rather narrow between 24.7 and 21.3 %. The Design of Experiments (DoE), showed that the spheronisation conditions speed, time and load had a greater impact on the quality attributes of the pellets than the extrusion conditions screw design, screw speed and solid feed rate (hopper speed). The best results were obtained using a low load (15 g) combined with a high rotation speed (900 m/min) and a low time (3 – 3.5 min). In comparison to spray granulated NAM pellets, the extruded NAM pellets resulted in a higher roughness and a higher useable yield (63 % vs. 92%). Finally, the coating and dissolution test showed that the extruded and spheronized pellets are also suitable for a protective coating with an ileocolonic release profile. Due to its lower specific surface area, the required shellac concentration could be reduced while maintaining the release profile.

Keywords: wet extrusion/spheronisation; Quality by Design; Design of Experiment (DoE); Niacin; controlled release

6.2 Introduction

Advancements in drug delivery systems were and are highly present in the pharmaceutical and food research area. Novel approaches include macro, micro and nano particulate systems as well as uniparticulate or multiparticulate systems (Zaman et al., 2016). Nano particulate systems are often used for enhancing the bioavailability or solubility of poorly soluble compounds e.g. by coaxial electrospraying (Yu et al., 2019) or liposomes (Frenzel and Steffen-Heins, 2015). However, for high daily doses, nano systems could lead to high weight of the final formulation. Therefore, the improvement of micro and macro systems with high drug loads is of recent interest. Macro or micro particulate systems can also provide an enhanced bioavailability (Mueller et al., 2018; Egert et al., 2012; Petersen et al., 2016) and/or a side-specific delivery in the human intestine. Pelletization techniques are playing a leading role in the field of drug delivery systems. Pellets are small free flowing, spherical particles with a diameter from 0.5 – 2 mm. Pellets, as drug delivery systems, provide technological as well as therapeutical advantages in comparison to uniparticulate systems such as simplicity of coating, less bowel irritation, uniform distribution in the gastrointestinal tract and decreased peak plasma fluctuations (Zaman et al., 2016; Knop and Kleinbudde, 2005; Lustig-Gustafsson et al., 1999). Fluid-bed processes and wet extrusion-spheronisation provide high drug load capacity and uniformity of pellets (Zaman et al., 2016; Verstraete et al., 2017; Politis and Rekkas, 2011) including moderate to highly water-soluble compounds such as nicotinamide (NAM). NAM is one active form of the vitamin niacin. A colon-targeted delivery of NAM can lead to beneficial effects on the host microbiome (Hashimoto et al., 2012). Due to the high bioavailability of NAM and its high absorption rate in the upper intestine, a protective coating is suitable for a targeted delivery in the ileocolonic region. In a previous study, the topical delivery to the colon area of NAM was achieved by application of an adapted shellac coating. Shellac is a natural resin from the lac insect *Laccifer Lacca*. It is often used as pH-sensitive enteric coating due to its acid character and its high dissolution pH value of about 7.3 (Buch et al., 2009; Limmatvapirat et al., 2007; Farag and Leopold, 2011a). However, an adapted subcoating to the characteristics of the encapsulated compound can be advantageous for a targeted release (Farag and Leopold, 2011a; Fangmann et al., 2018). In recent publications, shellac was used as a coating for a nanocomposite with a colon targeted release using a modified electrospraying process (Yang et al., 2018; Wang et al., 2018). Additionally, shellac can be used for food as edible coatings (Kumar et al., 2018; Chauhan et al., 2015; Chitravathi et al., 2014) and/or nutraceuticals (e.g. niacin) because of its GRAS status and its legal approval as a food additive (Czarnocka and Alhnan, 2015). The NAM cores were

produced by continuous spray granulation, which fulfilled the requirements for the application of a functional coating, such as sphericity and compactness. However, the useable size range was rather narrow (315- 400 µm), which led to a cost and time intensive production process. Furthermore, a rather thick coating layer was required to achieve the desired release profile (Fangmann et al., 2018). Therefore, NAM cores produced by wet extrusion/spheronization process will be compared to the spray granulated cores in this study as an alternative process. The requirements for the NAM cores correspond to the definition of pellets, which are favored due to their small particle size (< 2mm) and their ability to pass the pylorus independently from gastric emptying, which lead to smaller individual variations. Additionally, the round shape is ideal for coating and the risk of dose dumping is lower compared to a coated single unit/tablet (Knop and Kleinbudde, 2005). The aim of the present study was therefore to modulate the extrusion/spheronisation process in order to produce highly loaded NAM pellets and to compare their properties to spray granulated NAM pellets. A Quality by Design (QbD) approach, which is often applied in the pharmaceutical industry to guarantee the product quality (Sovány et al., 2016; Zhang and Mao, 2016) was used to define the Quality Target Product Profile (QTPP) with its Critical Quality Attributes (CQAs). The effect of the Critical Material Attributes (CMAs) and the Critical Process Parameters (CPPs) on the CQAs was determined by performing Design of Experiments (DoEs) for the extrusion and spheronisation processes, respectively. For the wet extrusion/spheronization process, a high drug load of 80 % NAM combined with 20 % MCC (Avicel PH101) was used. MCC is known as the "golden standard" for extrusion-spheronisation processes. It is characterized by its high water absorption and holding capacity, which improves the wetted mass plasticity and the spheronisation (Dukić-Ott et al., 2009; Jain et al., 2010). Finally, the release profile of the coated extrudates will be compared with coated spray granulates.

6.3 Materials and Methods

6.3.1 Materials

For wet extrusion and spheronisation processes, nicotinamide (NAM) were purchased from SternVitamin (Ahrensburg, Germany) and microcrystalline cellulose (MCC) Avicel Type PH 101 was ordered from FMC Health and Nutrition (Philadelphia, USA). For the following coating process, shellac solution (SSB Aquagold) was obtained from Stroever Schellack Bremen (Bremen, Germany). Citric acid monohydrate was obtained from Jungbunzlauer (Basel, Switzerland) and C* Dry maltodextrin 01915 from Cargill (Minneapolis, MN, USA).

Other chemicals used for dissolution tests were purchased from Carl Roth (Karlsruhe, Germany).

6.3.2 Methods

6.3.2.1 Pellet preparation

Micronisation

In order to avoid lumps, the batch of NAM was micronized using a milling machine U5-0024 from Quadro Engineering Inc. (Waterloo, Canada). The square impeller and the screen 7L094R (=2.39 mm) were inserted.

Extrusion and spheronization

The wet extrusion was applied using the twin-screw extruder nano 16 from Leistritz (Somerville, USA) with a 1mm diameter die. Prior to the extrusion, 1000 g of micronized NAM and MCC, in a ratio of 80:20 (% w/w), were dry blended in a V-shaped blender (Pharmatech, Birmingham, England) for 20 minutes and filled in a single screw hopper (Brabender FlexWall, Duisburg, Germany) which fed the powder mixture into the extruder. Distilled water was used as binder liquid and was fed using a peristaltic pump (Watson-Marlow Pumps, Type 120S, Falmouth, Cornwall, UK) at 4 rpm (feed rate of 1.74 g/min). The potentially critical factors identified for the extrusion process were the screw configuration, screw speed and powder feed rate. In the present study, the hopper speed was varied to achieve an altered solid-to-liquid ratio. Therefore, a calibration curve of the powder feed rate corresponding to the hopper speed was done (powder feed rate (g/min) = 0.0382*hopper speed – 3.6991). The used solid feed range varied between 5.3 – 7.9 g/min, which resulted in a liquid/solid ratio of 0.33 – 0.22. The screw configurations used are shown in **Figure 6.1**. After the extrusion process, the extrudates were spheronized using a spheroniser (made in house, De Montfort University, UK) with cross-hatched rotation plate with a diameter of 12 cm. The potentially critical factors identified for the spheronisation process were rotation speed, time and mass load. Two spheronisation processes were carry out for every extrusion run. The spheronized pellets were dried at 40 °C in a drying oven over night.

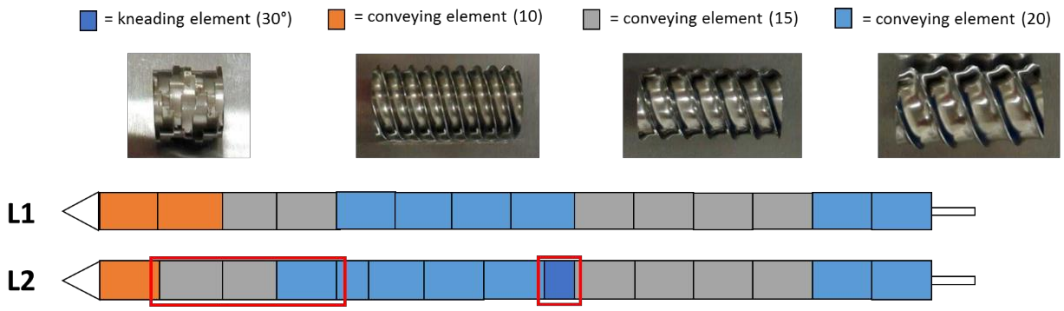

Figure 6.1: *Configuration of the two different screw designs (L1 and L2) used in the present extrusion DoE*

Spray granulation of NAM

For spray granulation of NAM, a ProCell fluidized bed granulator with Vario 3 was used (Glatt Ingenieurtechnik, Weimar, Germany). For a high NAM load and a continuous process with a high useable yield, the following conditions were chosen after the conduction of preliminary experiments (not shown). First, 30 % (w/w) nicotinamide including 4 % (w/w) of hydroxypropyl methylcellulose (HPMC) were dissolved in tap water. The solution was sprayed through a bottom-spray nozzle with an increasing spraying rate of 5 – 24 g/min. The conditions of the process were the following: inlet air temperature: 80 – 90 °C, product temperature: 50 °C, air rate: 80 – 90 m3/h and an atomizing air pressure of 2 – 2.8 bar.

Fluidized bed coating

For application of a coating, a fluidized bed coater (MiniGlatt, Glatt Ingenieurtechnik GmbH, Binzen, Germany) was used. The applied coating consisted of an inner shellac coating, an intermediate citric acid coating and an outer shellac coating in accordance to previous experiments (Fangmann et al., 2018) to achieve an ileocolon targeted delivery of NAM. Therefore, an inner shellac layer was applied with a weight gain (w.g.) of 20 %, followed by a citric acid coating, which generated a citric acid w.g. of 0.65 % and a w.g. of 5.85 % of maltodextrin, which was used as binder. Afterwards, an outer shellac coating (7.5 % w.g.) was applied. The calculated coating w.g. were based on the coating levels for coated NAM cores produced by spray granulation (see below) including a roughness factor of 54 %, which doubled the calculated coating mass application. The triple coating was sprayed onto 80 g extruded and spheronized NAM: MCC pellets under the following conditions: inlet air pressure of 0.4 – 0.55 bar, atomizing air pressure of 0.55 – 0.70 bar, inlet air temperature of 37 – 42 °C and a spraying rate of 0.5 – 0.95 g/min. In the end, the coated pellets were dried in a drying oven at 50 °C for 1 h.

Calculation of the coating level and coating process

The coating level (CL) [mg/cm^2] is determined as the mass of shellac ($m_{shellac}$ in mg) applied to the pellet surface (A in cm): $CL = \frac{m_{shellac}}{A}$ as described by (Farag and Leopold, 2011b). $M_{shellac}$ is the difference between the mass of the sample (m_{sample} in mg) and the mass of the cores (m_{cores} in mg) in the sample. The mass of the cores in the sample was calculated to the following equation: $m_{cores} = 100\frac{m_{NAM\ sample}}{c_{uncoated\ pellets}}$, whereby $m_{NAM\ sample}$ is the NAM mass in the sample (mg) and $c_{uncoated\ pellets}$ the NAM content of uncoated pellets (%). The surface area of the sample was calculated from the average radius and the number of pellets (n): $A = 4\pi r^2 n$. The number of the pellets is the relation of m_{cores} to $m_{uncoated\ pellet}$. The average mass of pellets was calculated from 500 pellets. The average radius was measured by particle size measurements by laser diffraction using a using the dry feeder of the particle analyzer (Horiba LA-950V2, Retsch Technology GmbH, Haan, Germany) with vacuum-driven forced ejection, a vibration power alteration of 120 and a compressed air pressure of 0.3 MPa. A refractive index of 1.47 was used for NAM. The NAM content, after the application of the coating layer, was determined spectrophotometrically at 262 nm after dissolution of about 50 mg pellets in 100 ml Soerensen phosphate buffer at a pH value of 7.4 (Helios gamma, Thermo Fischer Scientific, Waltham, USA).

6.3.2.2 Extrudate characterization

Process yield

During every run, the process yield, which was defined as the extruded mass per minute (g/min), was examined by weighing. The standard deviation of three measurements was calculated to control the process variability.

Moisture content

To measure the moisture content during process, freshly produced extrudates were analyzed before the spheronization process. About 2.5 g sample were weighed before and after drying at 105 °C in drying oven for 24 h. The experiments were done in triplicate. The water content is expressed as the percentage of the total weight of the wet mass.

6.3.2.3 *Pellet characterization*

Pellet size distribution - sieve analysis

The sieve analysis gives information about the particle size distribution after the extrusion-spheronisation process. The dried pellets of two spheronisation processes were sieved in a sieve shaker AS 200 control (Retsch GmbH, Haan, Germany) using sieve sizes of 2, 1.4, 1, 0.5 and 0.25 mm to separate the pellets according their size. The amplitude was set to 0.5 mm/"g" and the total sieve time to 3 minutes. Afterwards, the percent weight of the fractioned pellets was calculated.

The particle size analysis of spray granulated NAM pellets was done by Glatt Ingenieurtechnik GmbH (Weimar, Germany) using a Camsizer XT (Retsch Technology, Haan, Germany).

Pellet shape – photo image and scanning electron microscopy (SEM)

The shape of the spheronized pellets (n= ≥6) was evaluated by optical inspection and by SEM. For SEM, pellets with a diameter between 1 and 1.4 mm were prepared on a holder with carbon Leit-tabs (Agar Scientific, Stansted, UK). Before examination in a Zeiss EVO 15 (Carl Zeiss Ltd, Cambridge, UK) at an accelerating voltage of 10 kV, microcapsules were sputter-coated with a layer of 15 nm gold using a Quorum Q150RS (Quorum Technologies Ltd, Laughton, UK) rotary pumped sputter coater. The pellets were categorical evaluated regarding their roundness and their roughness of the surface: roundness (category 1 – 5): 1 = irregular (like agglomerates); 3 = spherical; 5 = dumbbell-shaped or strains; roughness (category 1 – 5): increasing category for increasing roughness.

For SEM pictures of the spray granulated NAM pellets were prepared and analyzed as described in [15].

Dissolution testing after application of coating

The in vitro drug release study was carried out using a standard dissolution paddle apparatus at 100 rpm and 37 °C (DT 70, Pharmatest Group, Hainburg, Germany) (Berg et al., 2012). The experiment was conducted in duplicate with 0.5 g of coated extrudated NAM: MCC pellets in 250 ml of the dissolution medium. During the experiments, the amount of released NAM was recorded by UV/vis spectrophotometer (Helios γ, Thermo Fisher Scientific, Waltham, USA) at the wavelength of 262 nm. The following dissolution media were used to simulate the human gastrointestinal tract from stomach to ileum to evaluate the release profile: Simulated gastric fluid (pH 1.4) was adjusted to USP gastric fluid without pepsin

(U.S. Pharmacopeial Convention), citrate buffer (pH 4.5) was modified to (Gomori, 1955) and phosphate buffer (pH 6.8 and 7.4) was prepared according Sorensen's buffer. To calculate the percentage of released vitamin, the maximum NAM amount was determined by stirring (100 rpm) of 0.5 g coated NAM extrudates in 250 ml phosphate buffer at pH 7.4 for 1.5 h at 37 °C in the above mentioned dissolution tester. The NAM concentration of the solution corresponded to 100 % NAM release.

6.3.2.4 Quality by Design

Quality target product profile (QTPP) and Critical Quality Attributes (CQAs)

The target dosage form is a pellet for oral administration. It should have a uniform and spherical shape, a smooth surface and a narrow size distribution for an appropriate application of an additional coating in a further step. The size should be smaller than 2 mm to be able to pass the pylorus independently from gastric emptying. Furthermore, the vitamin load should be high to prevent a high number of capsules, which have to be swallowed to reach the daily dose.

The following CQAs were determined based on previous experiments: vitamin load: ≥ 80 %; a roundness of category 2.0 – 4.0; a roughness of category ≤ 2 and a maximal useable yield (pellets with a diameter between 1 and 2 mm and an acceptable roundness).

Critical Material Attributes (CMAs) and Critical Process Parameters (CPPs)

The most critical material attributes are the high water solubility of nicotinamide (1000 g/l) and the corresponding vitamin to binder ratio. For these studies, the NAM:MCC ratio was kept constant, 80:20 % w/w. The influence of potentially CPPs such as powder mixture feed rate, screw speed and screw configuration (for the extrusion process) and rotation speed, rotation time and mass load (for the spheronisation process) on the CQAs was investigated. The identification of CMAs and CPPs were based on preliminary risk assessment and prior knowledge. Note: CMA is not an ICH (International Conference for Harmonisation) term but it is used here to describe the quality attributes of the materials.

Design of experiments (DoE)

The effects of the CPP's were determined using a DoE approach using the statistical software JMP Pro (SAS Institute, Cary, North Carolina, USA). For the extrusion process, two 22 full factorial designs were determined for two different screw designs, which were already shown in Figure 1. Therefore, 12 experiments were run in total, including 2 replicates of the central

point for every investigated DoE. For the spheronisation process, a 2^3 full factorial design with 10 experiments, including 2 replicates for the central point, were performed. An overview of every single experiment is listed in **Tables 6.1** and **6.2**. Variations in the moisture content of the extruded mass were set by changing the hopper speed from 250 to 270, which resulted in a powder feed rate from 5.8 to 6.6 g/min according to the calibration curve (powder feed rate (g/min) = 0.0382*hopper speed – 3.6991).

Table 6.1: *Design of Experiment of the extrusion process and the resulted responses (for all runs the spheronisation process was constant with 9 min time, 75 % speed and 15 g load)*

run	pattern	screw design	screw speed [rpm]	hopper speed[1] [rpm]	mc [%]	roundness	roughness	py SD	uy [%]
1	100	L1	200	260	*23.87*	*3*	*2*	*0.08*	*91.62*
2	1+−	L1	300	250	*23.86*	*3*	*4*	*1.19*	*91.18*
3	1−−	L1	100	250	*23.79*	*2*	*4*	*0.41*	*86.62*
4	1++	L1	300	270	*21.70*	*3*	*2*	*0.23*	*95.79*
5	100	L1	200	260	*23.55*	*3*	*3*	*0,12*	*98.12*
6	1−+	L1	100	270	*21.39*	*3*	*2*	*0.23*	*97.53*
7	200	L2	200	260	*23.15*	*2*	*2*	*0.36*	*98.04*
8	2−−	L2	100	250	*24.09*	*5*	*4*	*1.3*	*0*
9	2+−	L2	300	250	*22.78*	*4*	*4*	*0.56*	*42.42*
10	2++	L2	300	270	*21.85*	*3*	*3*	*0.26*	*95.96*
11	200	L2	200	260	*22.12*	*3*	*2*	*0.65*	*97.49*
12	2−+	L2	100	270	*22.78*	*3*	*2*	*0.22*	*96.43*

[1]hopper speed set to 250, 260 and 270 resulted in a feed rate of 5.8, 6.2 and 6.6 g/min. mc = moisture content; py = process yield; uy = useable yield

Table 6.2: *Design of Experiment of the spheronisation process and the resulted responses (for all runs the extrusion process was constant with screw design L1, 130 rpm screw speed and 270 rpm hopper speed)*

run	pattern	time [min]	speed[1] [%]	load [g]	uy [%]	roundness	roughness	sy [%]
1	+−+	9	50	65	0	4	2.5	97.62
2	−++	3	100	65	27.05	3.5	3	97.00
3	+−−	9	50	15	71.43	3	2	94.27
4	−−−	3	50	15	0	5	3.5	96.93
5	++−	9	100	15	90.99	3	2	91.93
6	000	6	75	40	65.29	2.5	2.5	96.28
7	−−+	3	50	65	0	5	4	98.94
8	−+−	3	100	15	86.44	3	2	94.60
9	000	6	75	40	81.38	3	2.5	96.83
10	+++	9	100	65	29.29	2	3	97.86

[1]rotation speed of 50, 75 and 100 % are in accordance with 430, 680 and 900 m/min. uy = useable yield; sy = spheronisation yield

6.4 Results and Discussion

6.4.1 Preliminary tests: establishing the range of solid-to-liquid ratio

For a high vitamin load, a ratio of 80 % w/w NAM and 20 % w/w MCC was chosen. In order to get a paste that can be spheronised without blocking the die of the extruder, the range of the solid-to-liquid ratio had to be evaluated before a systematic investigation. Distilled water was added to the extruder with a constant rate of 1.74 g/min. The solid feed rate was increased from 5.3 to 7.9 g/min to evaluate the particle shape changes (**Figure 6.2**). 15 g of the formed extrudates were spheronised for 3 min, dried and photographed. A low solid feed rate resulted in big and irregular agglomerates (A-C), whereby with increasing solid feed rates the amount of fines and dumbbell-shaped particles or strands increased (G-I). The optimal solid feed rate was between 6.2 and 6.6 g/min, which led to spherical particles (D-F). The differences regarding the particle size distribution of different solid feed rates were confirmed by sieve analysis (**Figure 6.3**). The lower the solid feed rate, the higher the amount of large particles (> 14 mm and > 2mm). The higher the solid feed rate, the higher the amount of fine particles (< 1 mm). The highest amount of the useable particles (1 – 2 mm) was obtained for a solid feed rate of 6.2 – 6.7 g/min.

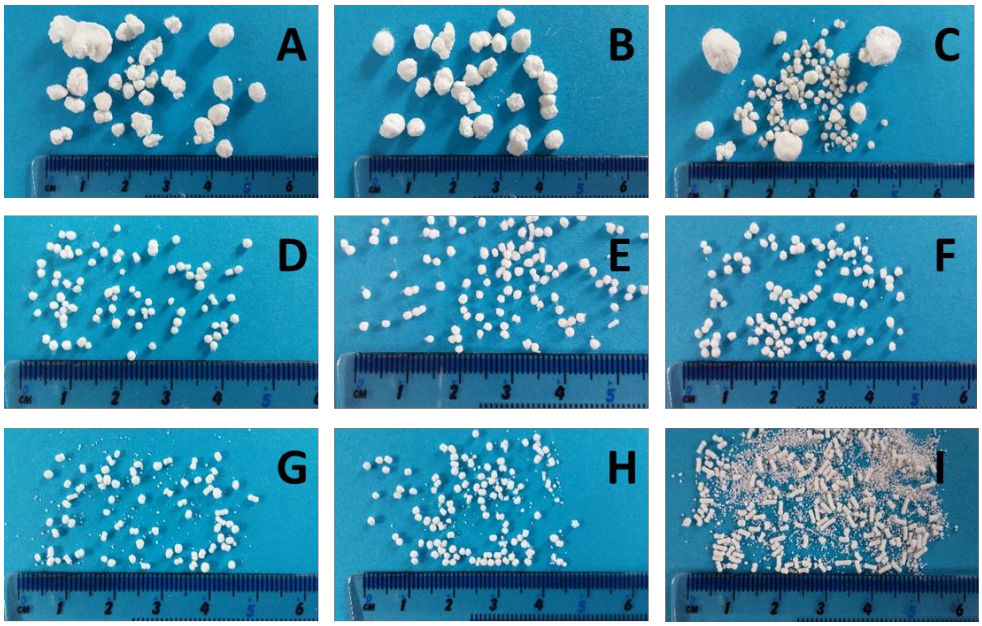

Figure 6.2: *NAM-MCC-Pellets (80:20) after extrusion and spheronisation (3 min, 15 g) with different solid feed rates (g/min): 5.3 (A), 5.5 (B), 5.6 (C), 6.2 (D), 6.4 (E), 6.6 (F), 6.7 (G), 7.4 (H), 7.9 (I). The granulating liquid was distilled water with a constant feed of 1.74 g/min.*

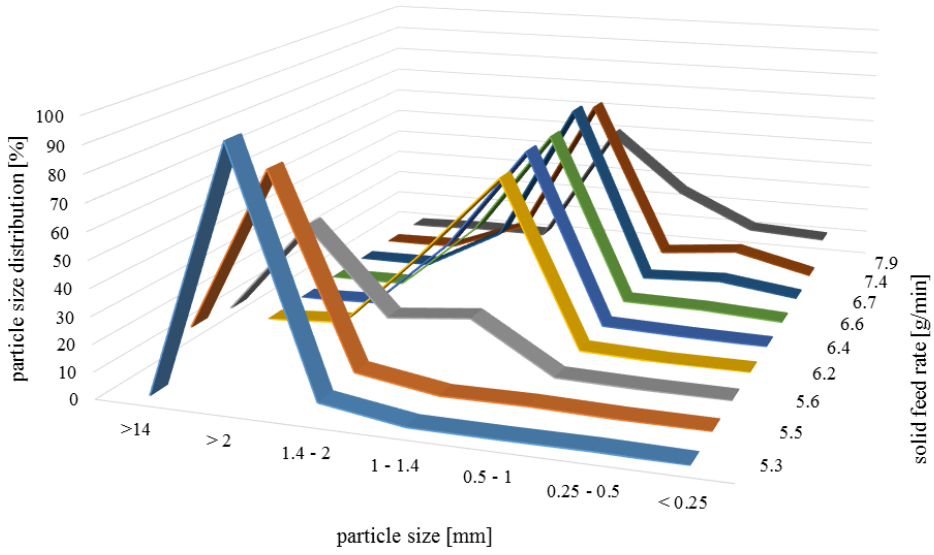

Figure 6.3: *Particle size distribution (%) after sieve analysis of NAM-MCC-Pellets (80:20) after extrusion and spheronisation (3 min, 15 g) with different solid feed rates (g/min). The granulating liquid was distilled water with a constant feed of 1.74 g/min.*

The effect of the solid feed rate on the moisture content of the extrudates is illustrated in **Figure 6.4**. Furthermore, the resulting particles formed after spheronisation are shown in **Figure 6.4**. A low solid feed rate resulted in big and irregular particles due to a high moisture content of 25.5 %. The wet extrudates tended to stick together during spheronisation and formed irregular and large particles, like a “rolling snowball”. A low moisture content (19.2 %) produced brittle extrudates that formed dumbbell-shaped particles. Only in a narrow water content range between 24.7 and 21.3 % (corresponding to a solid feed rate of 5.5 and 6.6 g/min respectively), spherical particles were generated. It is known that the moisture content of extrudates influences the spheronisation success. The extrudates must be brittle enough to be broken down into short strands during the spheronisation, but not so friable, that they disintegrate completely. Furthermore, the material has to be sufficiently plastic, that the broken strands can be rolled into spheres by the action of the friction with the plate. However, if the material is too wet, lumps will be formed during spheronization (Dukić-Ott et al., 2009; Jain et al., 2010; Vervaet et al., 1995). The range of water level over which spherical pellets were formed is influenced by the incorporated drug characteristics. It is documented that the ideal water level decreased with increasing water solubility of the drug, due to loss of solid matter by dissolution of the drug (Lustig-Gustafsson et al., 1999, Jover et al., 1996). The optimal water content for glucose, which has the same water solubility as NAM (1000 g/l), was 30.0 % vs. 23.6 % for NAM. The difference is explained by the higher amount of drug

used in the present study (80 %) compared to others, who used 50 % drug and 50 % MCC (Lustig-Gustafsson et al., 1999). Decreasing the MCC fraction to a lower concentration as 50 % (w/w) can lead to negative effect on the process yield and on the pellet properties (Verstraete et al., 2017). As shown in the present study, Podczeck et al. also showed pellets with good properties using 20 % MCC and 80 % drug (lactose, ≈195 g/l water solubility). The optimal water content was 23.66 % for a formulation with 80 % lactose. A lower water content (21.66 %) was required for the more water-soluble ascorbic acid (≈400 g/l) (Podczeck et al., 2008).

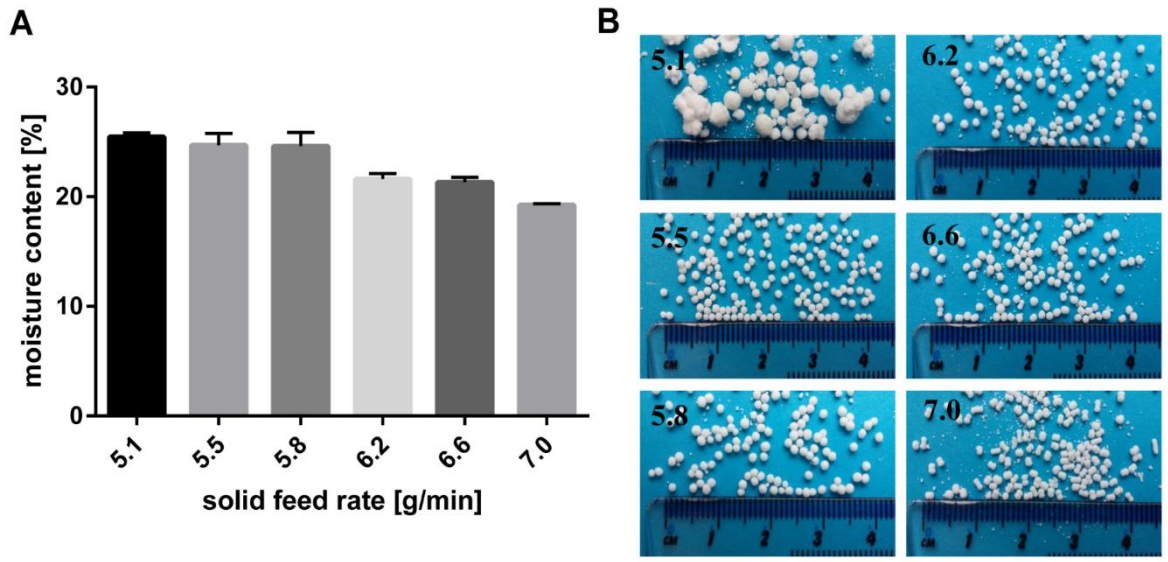

Figure 6.4: *(A) Effect of solid feed rate (g/min) on moisture content of wet extrudates (NAM: MCC; 80:20) and (B) the resulting shape of spheronised particles. The granulating liquid was distilled water with a constant feed of 1.74 g/min.*

6.4.2 Design of Experiment – Extrusion process

To evaluate the effect of the extrusion process parameters on the particle characteristics, the parameters for the spheronisation process (load: 15 g, rotation speed: 75 %, time: 9 min) as well as the formulation composition were held constant. During the extrusion process the factors screw design, screw speed and hopper speed (= solid feed rate; powder feed rate (g/min) = 0.0382*hopper speed – 3.6991) were varied. The effect of the above factors on the responses such as moisture content and process yield variations (SD of process yield) of the extrudates and roundness, roughness and useable yield of the spheronised particles was examined using a DoE approach. The coefficient of determination (r2) varied in a broad range depending on the responses. The r2 of the

roughness (0.845) and the useable yield (0.817) indicated a good fitting between the independent variables and responses in the model, in contrast to the moisture content (0.577), the process yield SD (0.544) and the roundness (0.470). However, only the model for the prediction of the roundness gave no significant results (p=0.1471). The outcomes of all tested combinations are shown in **Table 6.1**. The changes on the screw speed values showed no significant effect on all responses measured. Therefore, the screw speed was excluded from the model for a better predictability. The hopper speed had a significant effect on the roughness (p=0.0002) and the useable yield (p=0.0087) of the particles as well as on the process yield variations (p=0.0154) and the moisture content (p=0.0041). Furthermore, the combination of hopper speed and screw design influenced the useable yield significantly (p=0.0221). The interaction profiles between screw design and hopper speed on the responses are shown in **Figure 6.5**. As can be seen, there was no interaction between screw design and hopper speed for the responses roughness of the particles, moisture content and process yield of the extrusion (B, D, E). A lower hopper speed, however, led to an increased roughness, a higher moisture content and a higher variability of the process yield (B, D, E). There was a clear interaction between screw design and hopper speed for the responses roundness and useable yield (A, C). The useable yield of the particles was relatively constant and independent from the screw design when using a hopper speed set to 270 (6.6 g/min). The lower hopper speed set to 250 (5.8 g/min) in combination with screw design L2 resulted in a drastic decrease of the yield (C). This result was in accordance with the roundness of the particles: the higher hopper speed (270) led to more spherical particles in combination with screw design L1 (A). SEM pictures of all runs are shown in **Figure 6.6**. They were used to assess the roundness and roughness of the particles. In general, it is seen that the screw design L2 always resulted in more irregular shaped particles. Modelling a maximization of the desirability (0.95) of all responses, a combination of the screw design L1 and a hopper speed set to 263 was proposed (**Figure 6.7**).

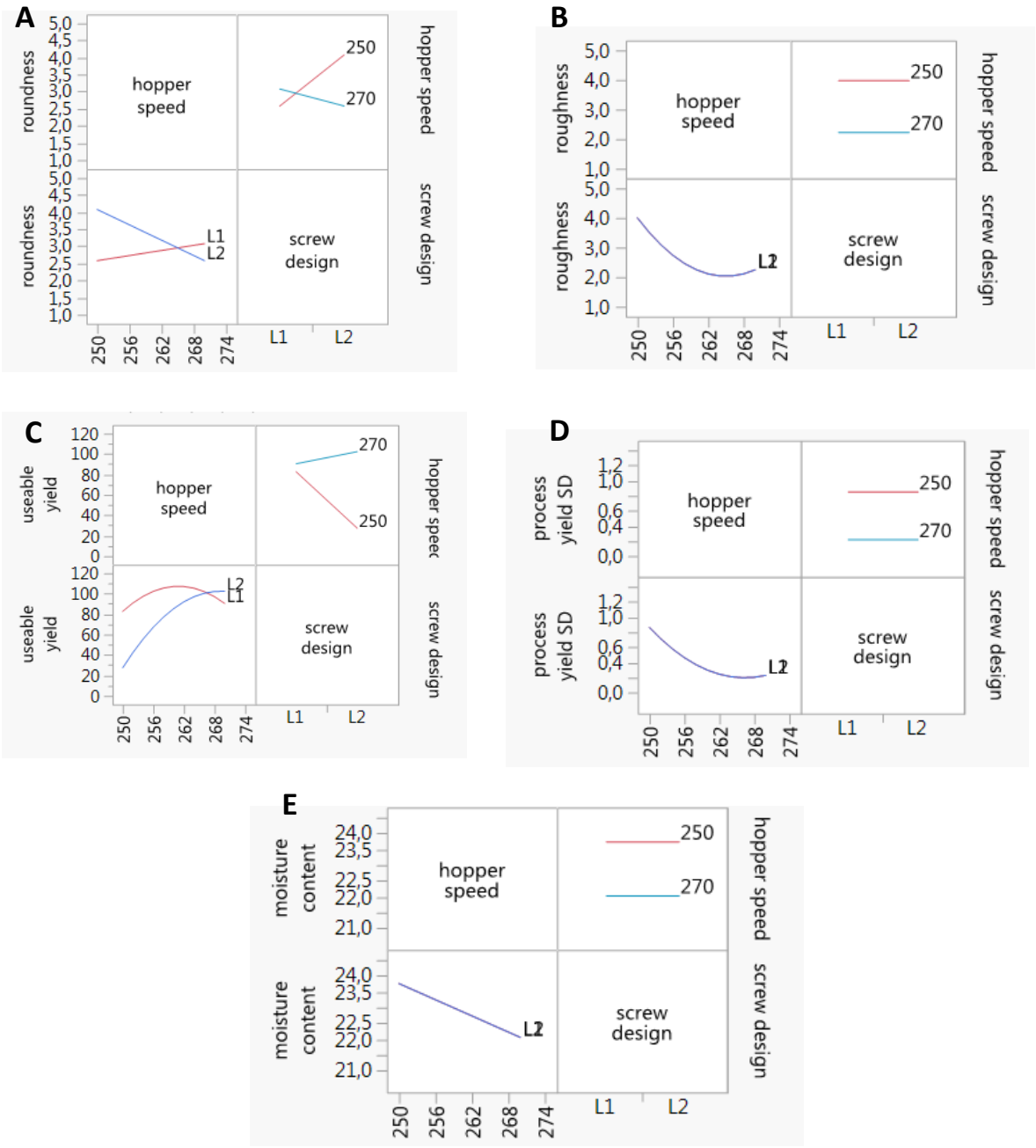

Figure 6.5: *Interaction profile of the extrusion process variables hopper speed and screw design on the responses roundness (A), roughness (B), useable yield (C), process yield SD (D) and moisture content (E).*

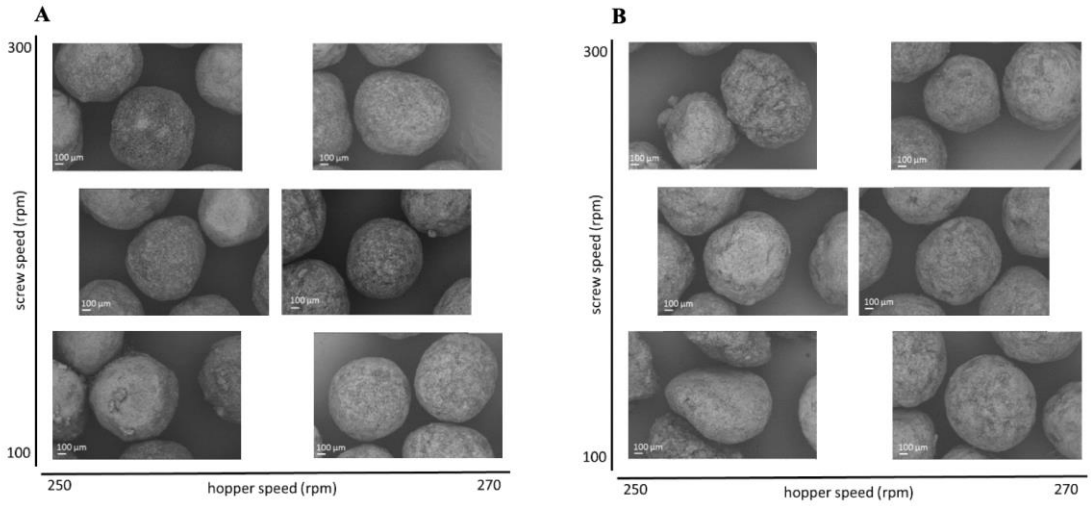

Figure 6.6: *SEM pictures of spheronised NAM: MCC-particles after DoE for extrusion process with screw design L1 (A) and L2 (B) with varying screw speed (y-axis) and hopper speed (x-axis).*

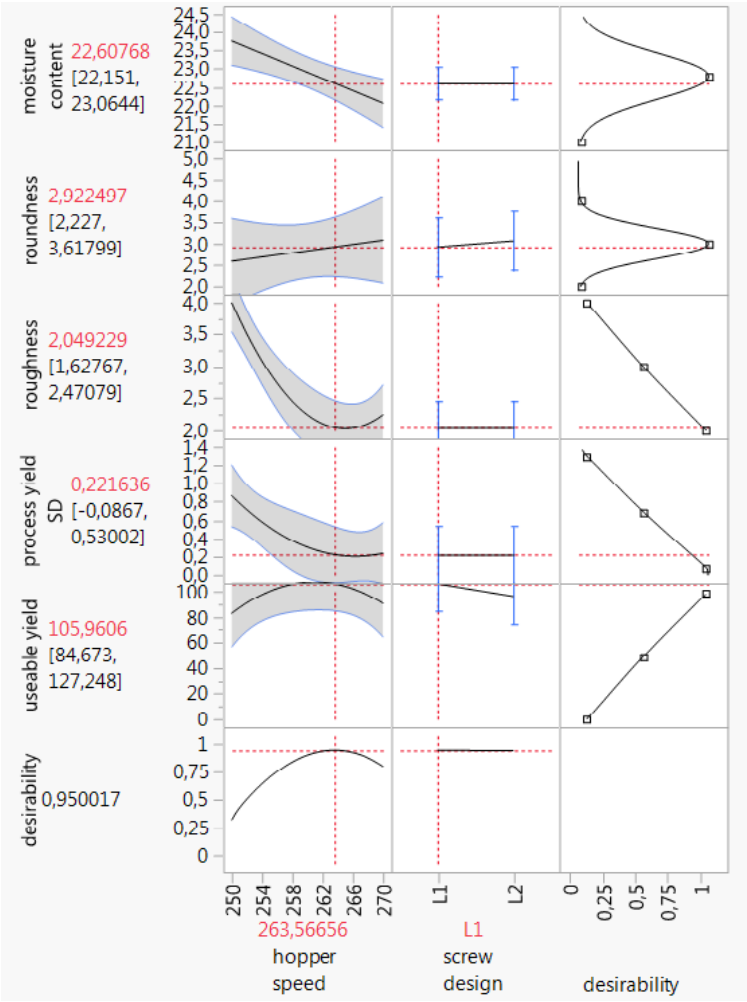

Figure 6.7: *Proposed extrusion configurations for a maximized desirability of all responses (CQAs) measured (useable yield, process yield SD, roughness, roundness, moisture content).*

In the present study, a lower hopper speed (= higher water content) resulted in a higher roughness, although a higher density was documented for a higher water content after spheronisation (Baert and Remon, 1993). However, it is reported that for twin-screw granulation a higher powder feed leads to an increased channel fill and torque, which increases the strength and decreases the porosity of the resulting granules (Dhenge et al., 2011). This could be an explanation for the observed lower roughness of the spheronised particles of the present study using a higher powder feed. Furthermore, the implementation of kneading elements can result in denser granules after extrusion (Seem et al., 2015), which was not shown in the present results. The screw speed showed no significant effects on the particle characteristics, although the screw speed can also influence the barrel fill and the density of the extrudates. However, if the screw speed was too low, blockages were observed, which is in accordance with a high fill level and an increased material compaction (Seem et al., 2015; Dhenge et al., 2010). The roundness of the particles was not significantly affected by variations of the extrusion process, which could be related to a higher impact of the spheronisation process and the moisture content (Di Pretoro et al., 2012; Hasznos et al., 2008) and will be discussed later. Whereby, the moisture content is significantly influenced by the hopper speed, due to an independent feed of the powder and the granulating liquid.

6.4.3 Design of Experiment – Spheronisation process

The results of the DoE for the spheronisation process are shown in Table **6.2**. The extrusion process was done at the following constant conditions: screw design L1, screw speed of 130 rpm and a hopper speed set to 270 (6.6 g/min). During spheronisation, the effects of the factors load, speed and time on the roundness, roughness, useable yield and spheronisation yield were investigated. Compared to the extrusion process, the spheronisation process showed a better fitting between the independent factors and the responses, which is demonstrated by higher r2 of the roughness (0.976), spheronisation yield (0.958), roundness (0.741) and useable yield (0.614). The spheronisation load had a significant effect on the useable yield ($p=0.0380$), the roughness ($p=0.0026$) and the spheronisation yield ($p=0.0016$), whereby the speed affected significantly the roundness ($p=0.0202$), roughness ($p=0.0111$) and the spheronisation yield ($p=0.0238$). The rotation time showed significant influences on the roundness ($p=0.0426$) and roughness ($p=0.0026$) of the particles and the spheronisation yield ($p=0.0321$). Furthermore, the combination of speed and time significantly affected the particle roughness ($p=0.0026$). The interaction profiles of the factors load, speed and time are exhibited in **Figure 6.8**. Regarding roundness, a higher speed always resulted in rounder

particles, independently of the load. A low spheronisation time leads to more dumbbell-shaped particles and strands, especially in combination with a low speed. However, this effect was reduced in combination with a high speed. If the rotation time was too long, while rotating with a speed of 100 %, the resulting particles were more irregular (A). The roughness of the particles was increased by increasing load and due to a low speed in combination with a low rotation time (B). The useable yield is only affected by the rotation speed and load: the yield was always higher with the highest speed and the lowest load (C). The spheronisation yield was dependent on all process parameters, whereby a high load, a low speed and a low time resulted in the lowest abrasion of the particles and therefore in the highest spheronisation yield (D).

The corresponding SEM pictures of the ten runs (**Figure 6.9**), show irregular particles due to a high load and dumbbell-shaped particles after spheronisation with a low speed. In comparison to the extrusion process, it is demonstrated that the spheronisation process has a higher impact on the desired responses (CQAs) of the particles. For a maximization of the desirability (0.77) of all responses, a combination of a load of 15 g, a rotation time of 3 min and a rotation speed of about 91 % is proposed after modelling (**Figure 6.10**). The effect of the spheronisation conditions on the pellet characteristics has often been evaluated and always showed a great impact, which is in line with the findings of the present study. As expected, the spheronisation yield (remained quantity after abrasion during spheronisation) was affected by all three tested parameters time, speed and load. The lower the speed, the higher the load and the lower the time, the lower would be the particle-plate interactions, which lead to abrasions and loss of material (Baert et al., 1993; Dukić et al., 2007; Newton et al., 1995). The particle-plate and particle-particle interactions are relevant for the roundness, roughness and useable yield of the spheronised particles. The spheronisation process depends on friction between particles-particles and particles-plate. Low speeds resulted in low interactions and therefore in failure of rounding the particles (Newton et al., 1995) which is in accordance with the present results, where the particles were still strands or dumbbell-shaped. Other authors also documented a high impact of the spheronisation speed on the roundness of the particles, whereby an increase of spheronisation speed resulted in a higher pellet sphericity (Sovány et al., 2016; Baert et al., 1993; Dukić et al., 2007; Krueger et al., 2013). A recent article showed that an initial high-speed spheronisation was required to reduce the extrudates. Followed by a medium speed to minimize the fines but to ensure enough round off of the extrudates (Shah et al., 2017). Furthermore, for a low water content, the spheroniser speed became more important than the time (Baert et al., 1993). Whereby, the time also played a major role for the

roundness of the particles also in dependence of the speed (Baert et al., 1993; Pandey et al., 2018). In the present study, a longer rotation time led to more spherical particles at a low speed, compared to a shorter time, but this relationship was statistically not significant. The load had no significant effect on the roundness with is in accordance with Krueger et al. (2013). However, for a higher load a longer spheronisation time was needed to form round particles (Newton et al., 1995), which makes an adjustment of the spheroniser speed, time and load indispensable. Additionally, the roughness of the particles was significantly influenced by spheroniser speed and time. Especially, a high rotation speed for a short time resulted in the lowest roughness of the particles. It is documented, that the spheronisation speed has to be optimized to obtain the desired densification of the particles, which can be insufficient for low speeds (Verveat et al., 1995; Newton et al., 1995) and would explain the higher roughness at low speeds in the present study. However, contrary results were shown regarding the rotation time. Krueger et al. reported a lower porosity and denser particles with increasing times, whereby the reduction of the porosity was lower, when the pellets consisted of the drug chloramphenicol instead of lactose-monohydrate (Krueger et al., 2013). Therefore, the higher surface roughness after longer spheronisation times could also be related to the encapsulated NAM.

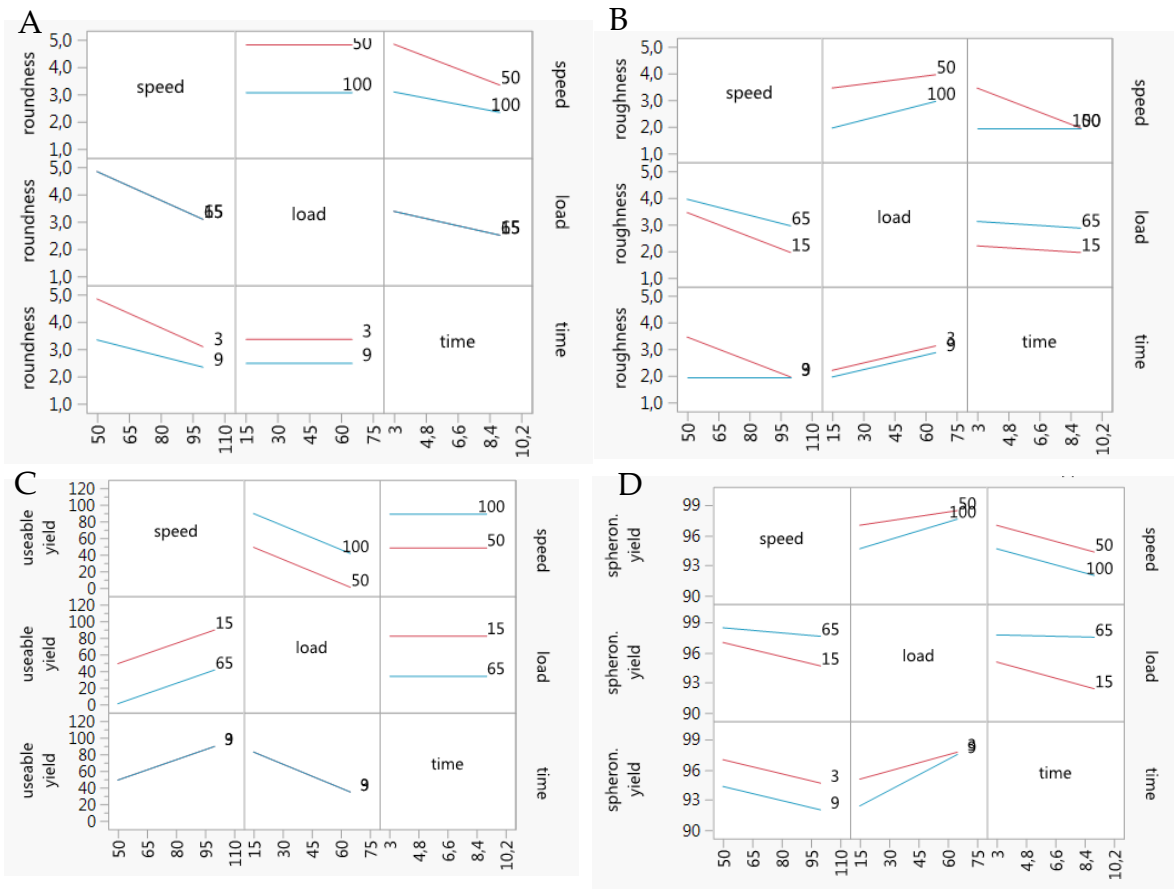

Figure 6.8: *Interactions of the spheronisation process variables rotation time, speed and load on the responses roundness (A), roughness (B), useable yield (C) and spheronisation yield (D).*

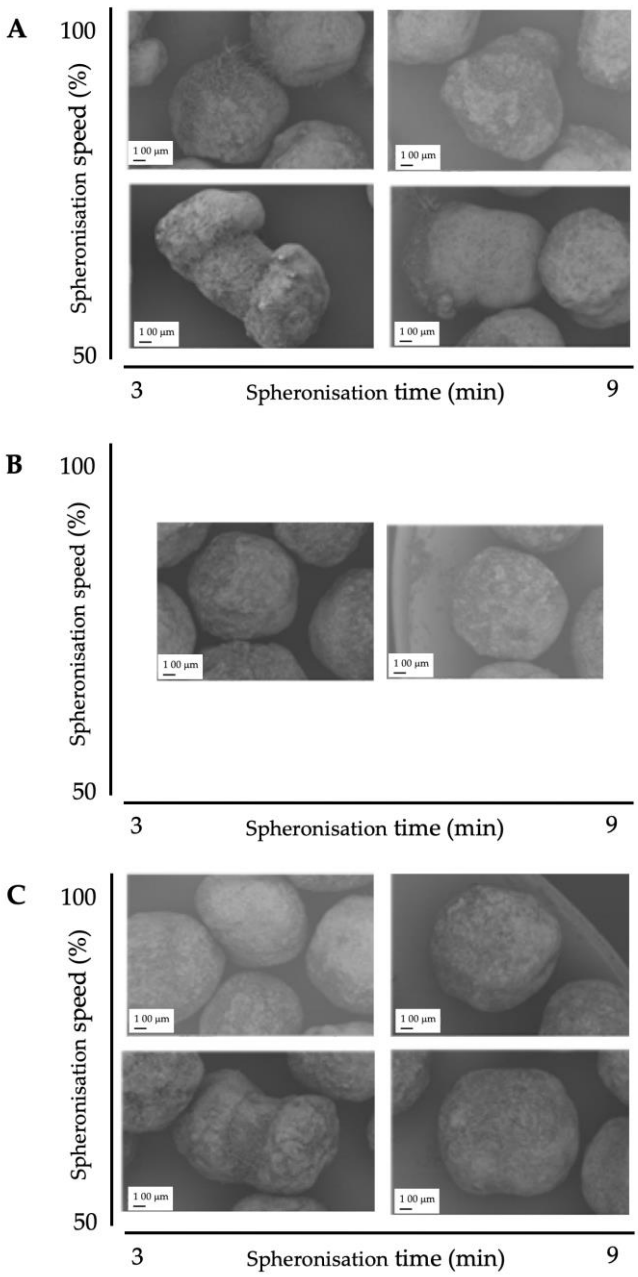

Figure 6.9: *SEM pictures of spheronised NAM: MCC-particles after DoE for spheronization process with the highest spheroniser load (65 g) (A), midpoint of the spheroniser load (40 g) (B) and the lowest spheroniser load (15 g) with varying spheronisation speed (y-axis) and time (x-axis)*

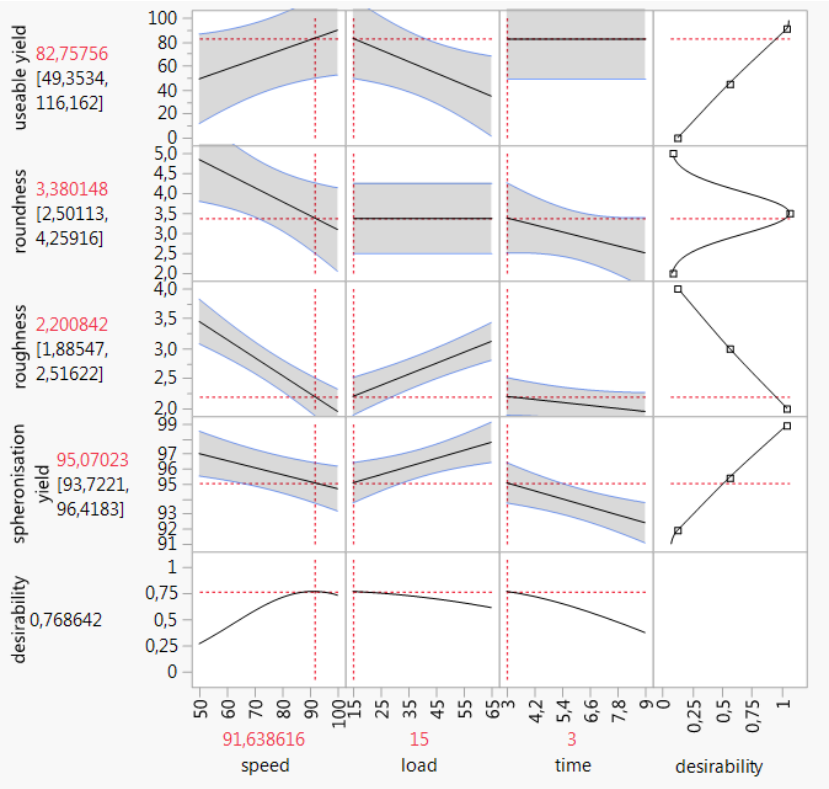

Figure 6.10: *Proposed spheronisation configurations for a maximized desirability of all responses*

6.4.4 Verification of the spheronisation model

As already mentioned, the coefficient of determination (r^2) varied according to the responses useable yield (r =0.614), spheronisation yield (r =0.958), roughness (r =0.976) and roundness (r =0.741). The obtained equations for these responses in term of the used factors (rotation speed, time and load) are as follows:

$$spheronisation\ yield = 96.226(intercept) - 0.7237(time) - 0.7962(speed) + 1.711(load) + 0.6087(time * load) + 0.37125(speed * load)$$

$$useable\ yield = 45.187(intercept) + 20.2925(speed) - 24.065(load)$$

$$roughness = 2.7(intercept) - 0.375(time) - 0.25(speed) + 0.375(load) + 0.375(time * speed) + 0.125(speed * load)$$

$$roundness = 3.4(intercept) - 0.5625(time) - 0.6875(speed) + 0.1875(time * speed)$$

For the practical verification of the calculated spheronisation model, the following extrusion and spheronisation conditions were used to achieve a high desirability (0.70) of the measured responses: screw design L1, screw speed 200 rpm, hopper speed set 270 (6.6 g/min), rotation speed of 100 %, rotation time of 3.5 minutes and a load of 15 g. Therefore, only the

spheronization model was verified due to similar extrusion conditions as used for the DoE. The run was conducted for about 3 hours. Every 15 minutes, the moisture content of the extrudates was measured. The mean was 23.6 ± 0.58 % for 13 measurements in total. The process yield of 44 measurements during the process was 7.95 ± 0.65 g/min, which confirmed a constant process. Furthermore, all runs resulted in well-formed particles with a spherical shape (roundness: 2.5) and a low roughness of 2 (**Figure 6.11**). In comparison, the model proposed a roundness of 3 and a roughness of 1.95. The mean useable yield of three different runs was 91.61 ± 1.83 % compared to the predicted 89.54 %. Thus, the model prediction of the spheronisation process is in good accordance with the measured values.

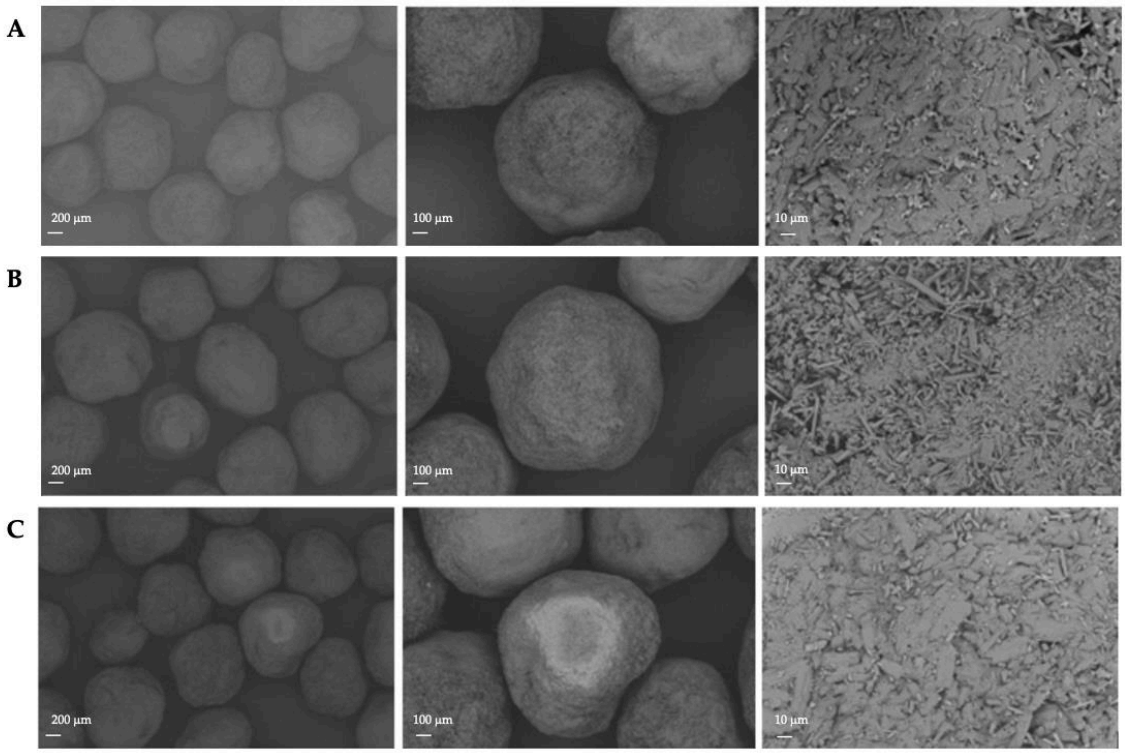

Figure 6.11: *SEM pictures of spheronised particles (NAM: MCC) of three different runs (A-C) with the same process parameter constellation. Extrusion: screw design L1, screw speed 200 rpm, hopper speed set to 270 (6.6 g/min); Spheronization: speed 100 %, load 15 g, time 3.5 min. Magnification: 50-times, 100-times and 1000-times from left to right.*

6.4.5 Comparison of NAM pellets produced by wet extrusion/spheronization and spray granulation

As indicated in **Figure 6.3** and **6.12**, the resulting pellet diameter is bigger for NAM extrudates than for NAM spray granulates. This is due to the die diameter of 1 mm for the extruder. A smaller die diameter would reduce the particle diameter. However, the useable size of the NAM pellets produced by spray granulation was in a narrow range between 315

and 400 µm (Fangmann et al., 2018); bigger particles were irregular and therefore not applicable for the coating process. The useable yield of the spray granulated particles was about 63 % (**Figure 6.12**) and lower compared to the spheronised NAM extrudates (92 %). Using a high drug load of 96 % in the spray granulated pellets, the conditions were optimized in pre experiments (data not shown) to achieve a high yield, spherical particles and a continuous and stable granulation process. Possibly, the coating of a NAM-HPMC-mixture on spherical cores (like Cellets®) could lead to a higher yield but would also reduce the relative load of NAM. Furthermore, as shown by SEM pictures in **Figure 6.13**, the spray granulated NAM pellets showed some irregularities in their shapes. The crystalline structure of NAM is clearly demonstrated, whereby the roughness is less pronounced compared to the extrudates, although a higher concentration of NAM is present in the granules (94 % vs 80 %). This suggests a higher compactness of the pellets, which were produced by spray granulation.

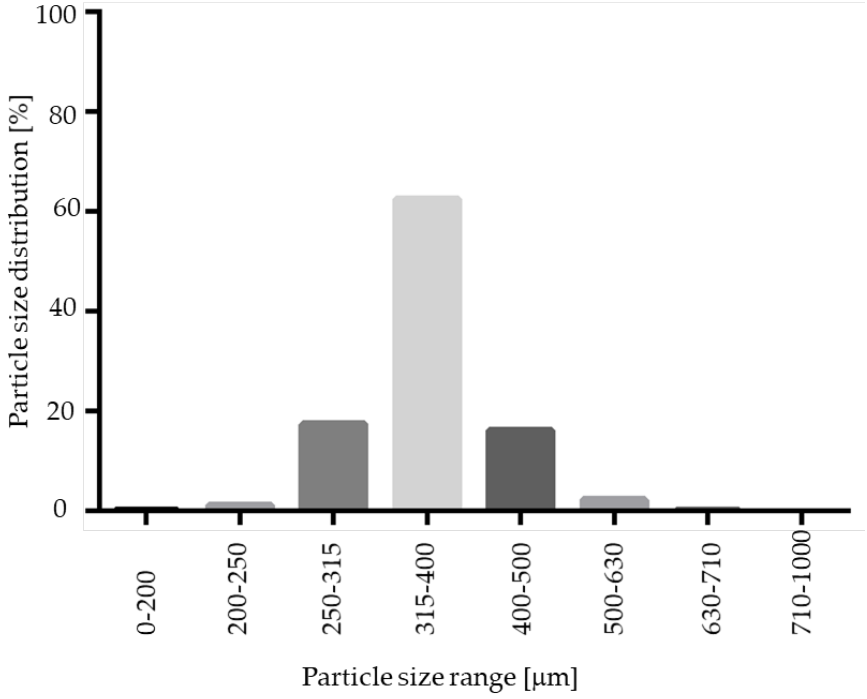

Figure 6.12: *Particle size distribution of spray granulated NAM pellets*

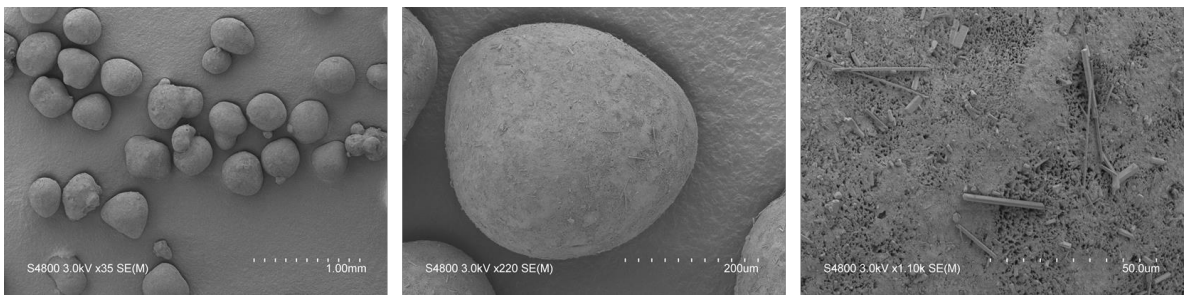

Figure 6.13: *SEM pictures of NAM pellets produced by spray granulation*

After the extrusion/spheronisation process, the resulted pellets were evaluated regarding their dissolution profile without and with an enteric coating, according to Fangmann et al. [15] (**Figure 6.14A+B).** In a simulating fluid with a pH value of 6.8, both, the spray granulated as well as the extruded uncoated NAM pellets liberated NAM immediately within 10 minutes. Thus, no delay of NAM is attributed to the usage of MCC or HPMC in the formulation as it is described for poorly water-soluble compounds (Dukić-Ott et al., 2009; Manda et al., 2019). This is mainly explained due to the very high water solubility of NAM (1000 g/l). After the application of the shellac coating with an intermediate citric acid sub-coating on the NAM extrudates, the release of NAM was clearly delayed in simulated gastrointestinal fluids (pH 1.4 – 7.4) (**Figure 6.14B**). The modified shellac coating prevented a premature release at gastric conditions (2.5 % after 60 min at pH 1.4), followed by a continuous release at pH value 6.8 (from 22.5 % after 30 min to 82.9 % after 120 min) and a complete release is reached at a pH value of 7.4. The release profile of the spray granulated NAM pellets with a comparable coating level showed a slower release profile at pH 6.8 (up to 34.4 % after 210 min in total) (**Figure 6.14B**) (Fangmann et al., 2018). This could be related to the higher roughness of the extruded pellets, which increases the porosity of the coating layer and therefore increases the release rate of NAM. A 1.6-fold higher coating thickness (5.8; 7.6 and 10.0 mg/cm^2) of the extruded NAM pellets (**Figure 6.14C**), resulted in a slower release at pH 6.8 (up to 12.2 % after 210 min in total). The exact accordance of the release profile of coated extruded and spray granulated particles would be existing with a coating layer in between. However, both processes are suitable for the production of NAM pellets and a further uniform coating. Furthermore, the spray granulation process as well as the twin-screw extrusion can be easily scaled up and performed as continuous processes. On the one hand, the narrow water level range (< 10 %) may cause problems for further scale-up processes for wet extrusion and spheronisation (Lustig-Gustafsson et al., 1999). On the other hand, the release profile indicates that lower shellac amount is required for a targeted release. Only 3.6 % (w/w) coating weight gain was needed for the application of a coating level of 1 mg/cm2, compared to 12 % (w/w) for the spray granulates. Related to 100 g NAM, the required addition of shellac was reduced from 60 g to min. 27.5 g for spray granulated compared to wet extruded NAM pellets, respectively. Due to the resulting thicker coating level for extruded pellets, it is proposed that the required amount could be further decreased (max. to 16.5 g of shellac per 100 g NAM).

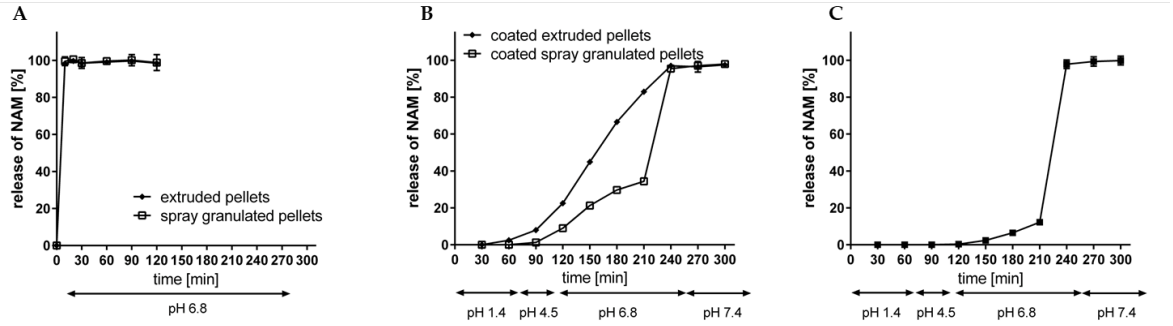

Figure 6.14: *Release rate of NAM from (A) uncoated spray granulated and extruded NAM and (B) from triple shellac coated spray granulated and extruded NAM pellets with a coating thickness of 3.7/4.9/6.2 mg/cm² and 3.6/4.6/6.0 mg/cm² and (C) from triple shellac coated extruded NAM pellets with a coating thickness of 5.8/7.6/10.0 mg/cm² at simulated gastrointestinal conditions (mean ± SD; n=2).*

6.5 Conclusion

All in all, the extrusion/spheronisation process demonstrates an opportunity to produce highly NAM loaded pellets with a useable yield of about 90 %. The pellets were spherical and had a rough surface. In comparison to spray granulated NAM pellets, the extruded NAM pellets resulted in a higher roughness and the useable yield was increased. Finally, the coating and dissolution test showed that the extruded and spheronised pellets are also suitable for a protective coating with an ileocolonic release profile *in vitro*. The Quality by Design (QbD) proved to be a suitable method to better understand the effects of the Critical Process Parameters (CPPs) on the Critical Quality Attributes (CQAs) of the desired product in an efficient way. It was shown, that the prediction model was in good agreement with the measured outputs. The Design of Experiments (DoE), showed the spheronisation conditions speed, time and load had a greater impact on the CQAs of the pellets than the extrusion conditions screw design, screw speed and solid feed rate (hopper speed). The roundness was significantly affected by the rotation speed and time, whereby the roughness varied depending on the spheroniser load, time, speed*time and the solid feed rate. Furthermore, the spheroniser load, solid feed rate and the solid feed rate*screw design significantly impacted the useable yield. The screw speed showed no significant effect. The moisture content is one of the most critical factors for an optimal spheronisation result. Therefore, the moisture content need to be adjusted to the formulation characteristics to ensure an optimal product. The spheronisation conditions must be adjusted using a multivariate approach.

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Author Contributions:

Conceptualization, W.S., E.M.T, J.K.K. and K.S.; Methodology, W.S. and E.M.T.; Software, M.O.; Validation, E.M.T and W.S.; Formal Analysis, E.M.T and M.O.; Investigation, E.M.T and W.S.; Resources, K.S. and W.S.; Writing – Original Draft Preparation, E.M.T.; Writing – Review & Editing, W.S., K.S., J.K.K. and M.O.; Visualization, E.M.T. and W.S.; Supervision, W.S. and K.S.; Project Administration, K.S.; Funding Acquisition, K.S. and J.K.K.

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7 Manuscript V: Niacin loaded pectin-zein-hydrogel beads

Encapsulation of water-soluble niacin in pectin-zein-hydrogel beads: Effect of production process and materials on the morphology, encapsulation efficiency and gastric resistance

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7.1 Abstract

The aim of the present study was to encapsulate niacin in pectin-zein hydrogel beads by ionotropic gelation as a vegan colon-targeted delivery system of niacin. The effect of niacin, and process parameters for two different production process (one-step vs. two-step) were evaluated regarding the resulting morphology, encapsulation efficiency (EE) and release profile of niacin *in vitro*. The results showed, that a direct dripping into a $CaCl_2$-zein solution (one-step) always resulted in red blood cell shaped particles, whereby a separate cross-linking and coating step (two-step) produced flat beads. The EE of varied in a broad range (< 1 - > 50 %) depending on the water solubility of the encapsulated compound, production process and particle formulation. The relatively low EE is explained due to the high water solubility of niacin (NA: 18 g/l; NAM: 1000 g/l). Furthermore, none formulation was able to prevent a premature release of niacin during the simulated gastric phase independently of the morphology and the production process. Due to a successful encapsulation and release of whey protein isolate (WPI) into the pectin-hydrogel beads, further developments are needed to protect a water-soluble molecular such as niacin.

7.2 Introduction

Niacin is a water-soluble vitamin and includes the two active forms nicotinic acid (NA) and nicotinamide (NAM). Their coenzymes NAD/H and NADP/H are essential for the energy metabolism in humans (Kirkland, 2012). Furthermore, a recent study showed a beneficial effect of a gut targeted release of nicotinic acid on the abundance of *Bacteroidetes*, which is associated with an improvement of biomarkers for systemic insulin sensitivity and metabolic inflammation. The application of NAM did not result in such beneficial effects (Fangmann et al., 2018). However, Hashimoto et al. and Singh et al. postulated NAM related anti-inflammatory effects in the colon (Hashimoto et al., 2012; Singh et al., 2014). The colon targeted release of NA and NAM was achieved using an adapted shellac coating applied via fluidized bed coating (Fangmann et al., 2018). Shellac is the refined form of the natural insect resin lac. Due to its acidic character, it is often used as coating material for a delayed or colon targeted release (Al-Gousous et al., 2015; Pearnchob et al., 2003; Roda, 2007). However, fluidized bed coating in combination with a high amount of shellac to get the desired release profile is cost intensive and is therefore only of restrictive use (Desai and Jin Park, 2005; Teunou and Poncelet, 2002). Thus, an alternative encapsulation material and process for niacin is desirable.

Hydrogel beads formed of a combination of pectin and zein, already showed promising results regarding a colon targeted release profile for encapsulated indomethacin and bovine serum albumin (BSA) (Liu et al., 2006). In general, hydrogels beads can be formed using the ionic gelation technique. An aqueous polymer solution is added dropwise into a crosslinking solution with counter ions, which results in a formation of spherical particles (Patil et al., 2010; Patil et al., 2012). Pectin is able to form beads in the presence of calcium ions and can further act as colon-targeted material due enzymatic degradation by the gut microbiota (Sriamornsak, 2011; Willats et al., 2006). Zein, a water insoluble hydrophobic storage protein from corn, is used as additional coating material to achieve low water penetration (Chotiko and Sathivel, 2017; Liu et al., 2006). Furthermore, pectin is a natural polysaccharide and still postulated as safe food additive without ADI (acceptable daily intake) after the re-evaluation if the EFSA in the year 2017 (Mortensen et al., 2017). The re-evaluation of shellac is still in progress (EFSA, 2018). Furthermore, the substitution of shellac against pectin and zein would result in a product, which is also applicable for a vegan lifestyle.

Before evaluation of the pectin-zein-system as an alternative drug delivery system with a delayed release of niacin, the bead formation was studied due to a documented influence of the bead shape on the release (Guo and Kaletunç, 2016; Harvestine et al., 2014). Therefore, the aim of the present study is to evaluate the pectin-bead formation while encapsulation of niacin. The effects of the process conditions and different encapsulation materials were analysed regarding the morphology of the hydrogel beads and the resulting encapsulation efficiency. Furthermore, first experiments were done to evaluate the gastric resistance characteristics.

7.3 Materials and Methods

7.3.1 Materials

Nicotinic acid (NA) and nicotinamide (NAM) were purchased from SternVitamin (Ahrensburg, Germany). Three different types of pectin were used from Herbstreith & Fox (Neuenbürg, Germany). Their characteristics are shown in **Table 7.1**. Zein (was purchased from Sigma-Aldrich GmbH (München, Germany). Shellac solution (SSB Aquagold) and shellac flakes (SSB 57 Pharma FL) were obtained from Stroever Schellack Bremen (Bremen, Germany). Whey protein isolate (WPI) BiPro® was from Davisco Foods (Minnesota, USA). Pectinase Fruktozym® was obtained from Erbslöh Geisenheim AG (Geisenheim, Germany). The activity was declared with ≥ 75 ASV-U/ml. Other used chemicals were purchased from Carl Roth (Karlsruhe, Germany).

Table 7.1: *Characteristics of the three pectin types used in the present study*

	Pektin Classic CU 902 (P5.9)	**Pektin Classic AF 707 (P36)**	**Pektin Classic CU 201 (P69)**
Appearance/colour	yellowish	yellowish	yellowish
Degree of esterification	5.9 %	36 %	69 %
Degree of amidation	-	-	-
Content of galacturonic acid	76 %	79 %	88 %
pH value (2.5 % in dist. H_2O, 20 °C)	4.0	3.0	3.1

7.3.2 Methods

7.3.2.1 One-step procedure of pectin-zein hydrogel bead production

The one-step production was conducted according to Liu et al. (2006). First, pectin (P69, P36 or P5.9) were dissolved in milliQ water, P36 was heated up to be dissolved. The pectin concentration was either 2 % or 4 % (w/w). NA or NAM was added to the polymer solution (0.5, 5 or 20 % (w/w)). The pectin(-vitamin) solution was directly dropped into a cross-linking solution consisting of calcium chloride (0.5 % (w/w)) and zein (1, 3 or 5 % (w/w)) dissolved in 75 % ethanol under constant stirring (100, 150 or 200 rpm) from a dropping distance of 9.5, 1.5 or 0.5 cm above the water surface. The used technique in the present study is illustrated in **Figure 7.1** and a scheme of the procedure is shown in **Figure 7.2A**. For bead formation, a tube with a diameter of 0.9 mm was used, which was connected to a syringe pump. The beads cured for 15 minutes in the cross-linking solution (non-isotonic process). In some experiments, the cross linking solution contained the same vitamin concentration as in the polymer solution (isotonic process). The formed beads were collected in a sieve. Ethyl acetate and distilled water were used for washing. Beads were air dried on a filter paper over night.

7.3.2.2 Two-step procedure of pectin-zein hydrogel bead production

In the first step, the pectin solution (P5.9, 4 % (w/w) or P36, 8 % (w/w)) combined with different amounts of NAM (0, 5 or 20 % (w/w)) was dropped into the cross-linking solution (**Figure 7.1**) only consisting of calcium chloride dissolved in distilled water (0.5, 2 or 4 % (w/w)) under constant stirring (150 rpm). The formed beads were cured for 30 minutes in the cross linking solution (non-isotonic or isotonic conditions). In the second step, the beads were stirred in a zein solution (1, 5 or 15 % (w/w)), which was dissolved in 75 % ethanol, for 60 minutes at 250 rpm (**Figure 7.2B**). The pectin-zein beads were washed and dried as described in the one-step-procedure. In some cases, multiple zein coating were conducted with an intermediate drying step.

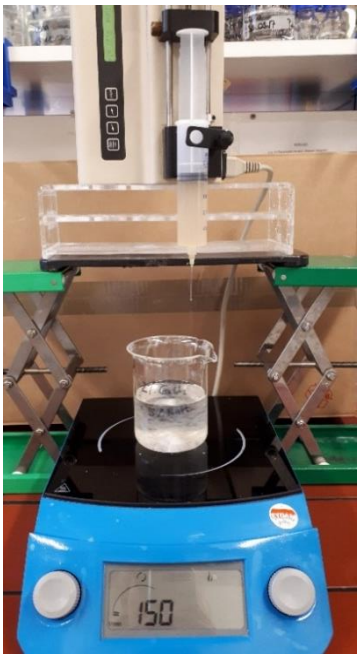


Figure 7.1*: Picture of the pectin bead preparation technique used in the present study*

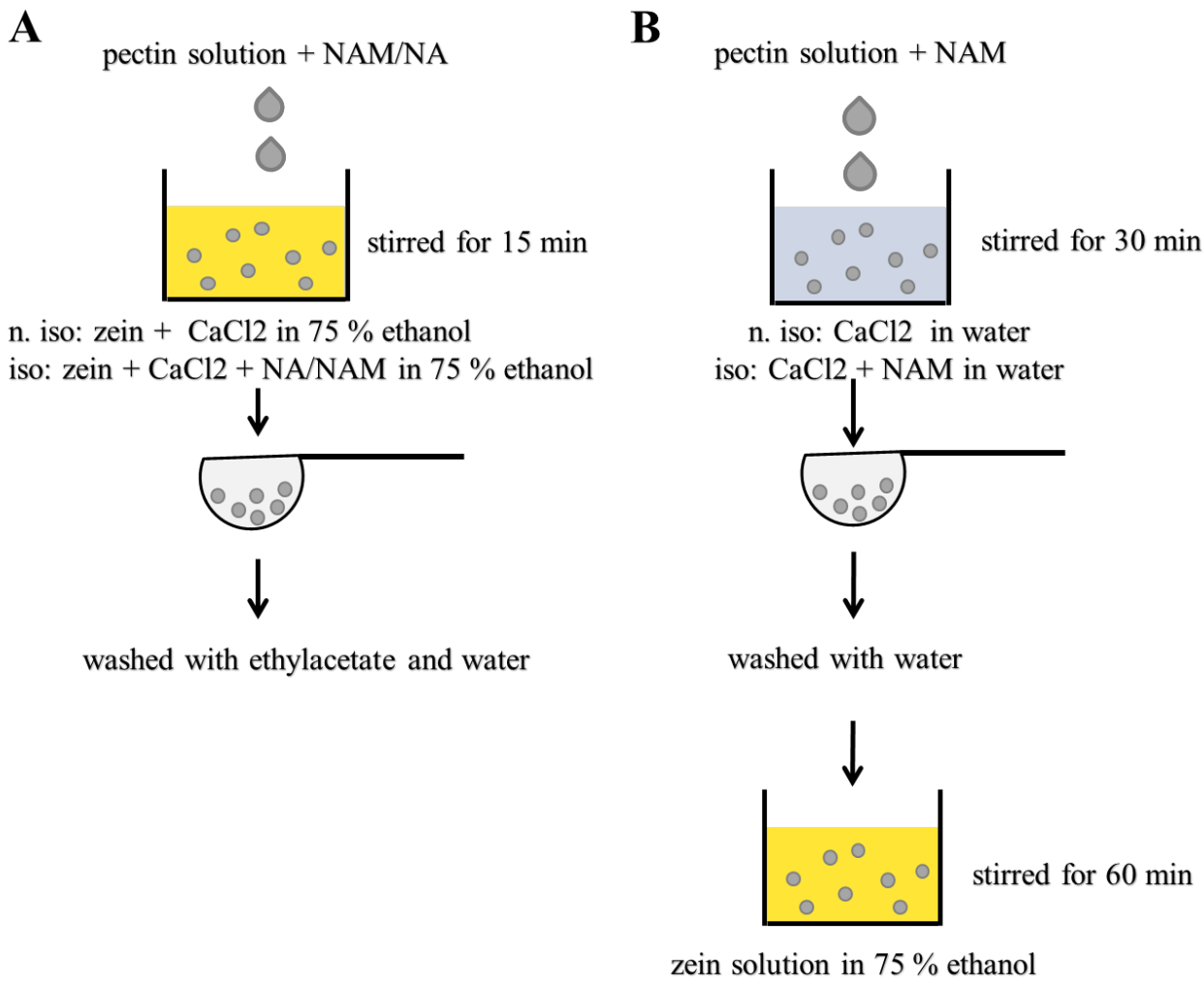


Figure 7.2: *Overview of the one-step production process (A) and the two-step production process (B) for pectin bead formation*

7.3.2.3 Feasibility study of the encapsulation processes using whey protein isolate (WPI)

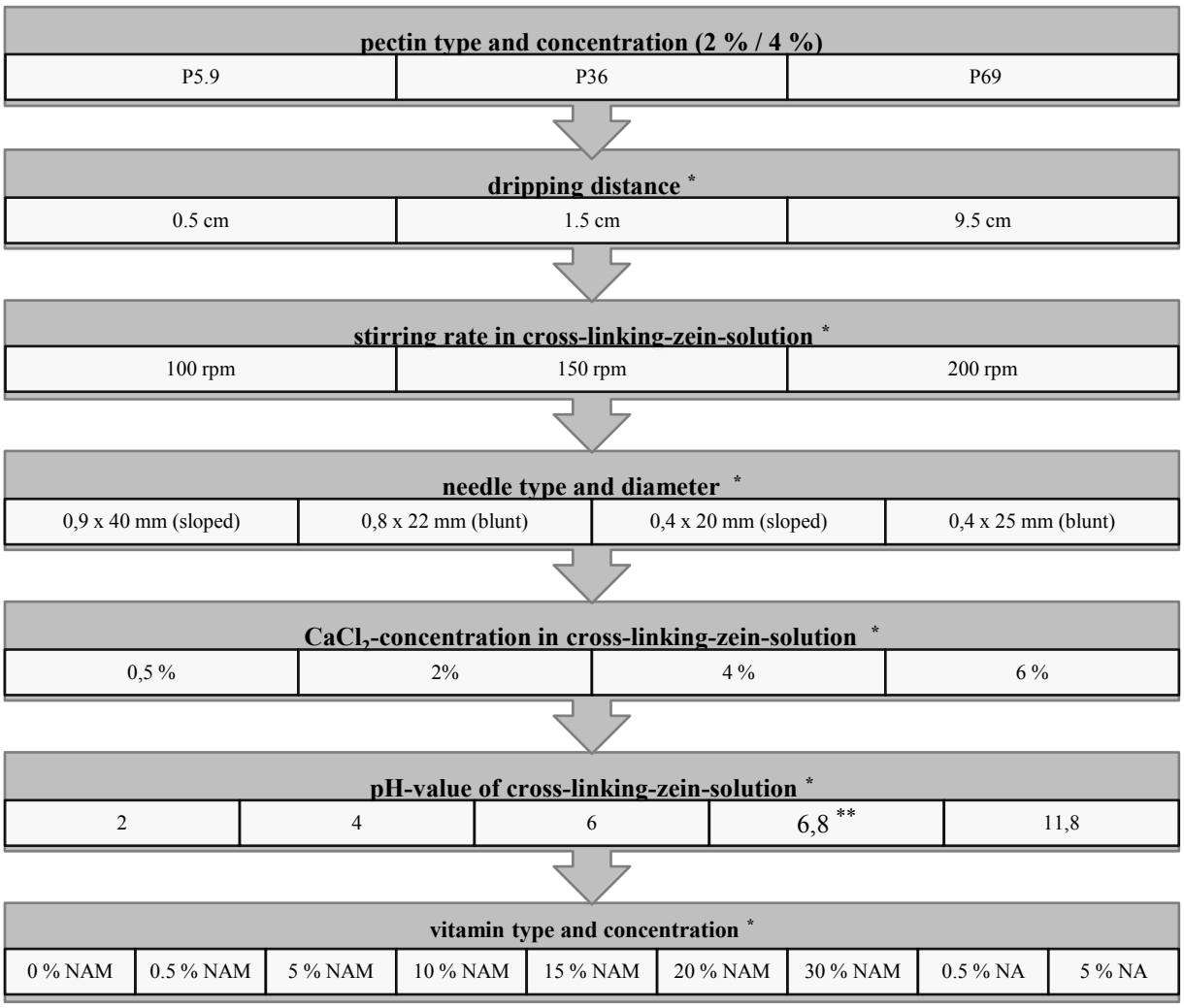
Instead of niacin, WPI was encapsulated to study the feasibility of the two production processes. The influence on the morphology, the encapsulation efficiency and the release profile was examined. Therefore, 2.5 % (w/w) of WPI was added to the P5.9 pectin solution (4 % w/w). This solution was dropped into a non-isotonic cross-linker solution by the one-step and the two-step procedure. Afterwards the beads were dried over night.

7.3.2.4 Two-step procedure of pectin-zein-fibrils hydrogel bead production

Whey protein isolate (WPI) fibrils were added to the pectin-vitamin solution and produced as described in the two-step procedure. Only pectin P5.9 was used. The production of the WPI-fibrils was according to (Serfert et al., 2014). WPI was added to milliQ-water (2.5 % (w/w)). Afterwards the pH value was adjusted to pH 2 (±0.05) using 6N HCl. The solution was stirred at 250 rpm in a water bath at 90 °C for 5 hours, cooled down and stored at +4 °C. Before adding the final solution to the pectin, NAM (3.3 % (w/w)) was added to the fibril solution (1.67 % (w/w)) and the pH value was adjusted to pH 4 (±0.05) using 6N HCl. The resulting fibril concentration is related to the concentration of WPI in the stock solution. Subsequently, pectin (4 % (w/w)) was added. The cross-linking bath was prepared isotonic with a calcium chloride concentration of 6 % (w/w). The following steps were equivalent to the two-step procedure of pectin-zein hydrogels with a zein concentration of 5 % (w/w).

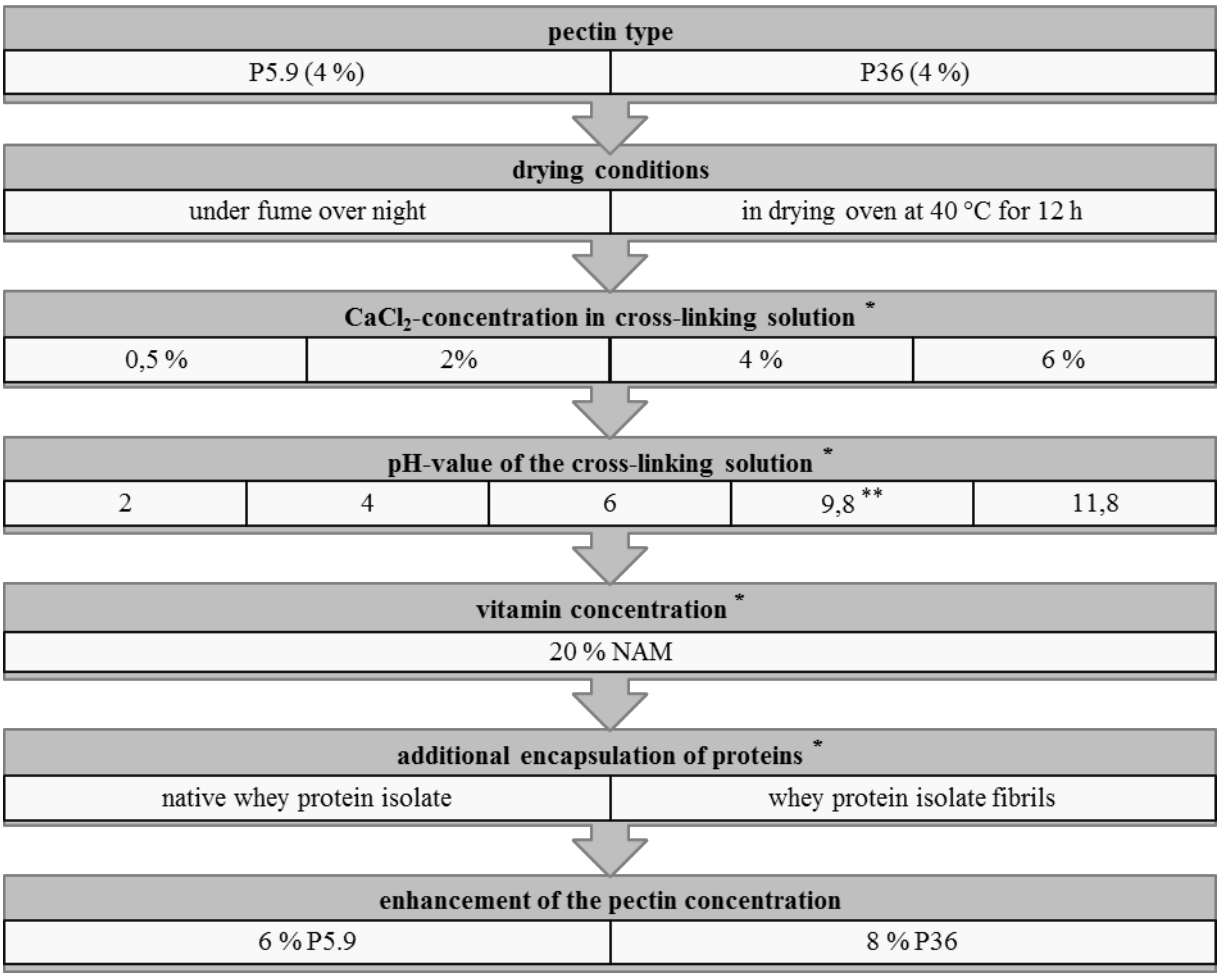
7.3.2.5 Two-step procedure of pectin-shellac(-fibrils) hydrogel bead production

To get a direct comparison of the pectin-zein-hydrogels, also pectin-shellac hydrogels were developed and characterized. Therefore, shellac from ethanolic solution and shellac from an aqueous solution of alkali salts (water dispersable shellac; wd-shellac) was used as outer coating material instead of zein. The used pectin type was P5.9 (4 % (w/w)) in combination with or without fibrils (1.67 % (w/w)) as described above. The NAM concentration was 5 % (w/w) for pure pectin beads and 3.3 % (w/w) for pectin-fibril beads. After dropping the pectin solution into the calcium chloride bath (6 (with fibrils) or 2 % (w/w)), the formed beads were stirred at 250 rpm for 1 h in an ethanolic shellac solution or in wd-shellac (5 % w/w) for 1, 5 or 15 minutes. Afterwards, the beads were air dried overnight.



* experiments were only done using P5.9
** pH-value of the cross-linking-zein solution without adjustment

Figure 7.3: *Overview of the one-step procedure process parameter variations to study their effects on the bead morphology*



* experiments were only done using P5.9
** pH-value of the cross-linking-zein solution without adjustment

Figure 7.4: *Overview of the two-step procedure process parameter variations to study their effects on the bead morphology*

7.3.2.6 Viscosity of polymer solutions

The viscosity of the polymer solution before and after the addition of NAM was examined using a viscometer (Haake Viscotester iQ, Thermo Fisher Scientific, Waltham, USA). About 15 ml of the solution was added. Viscosity tests were run at 20, 50, 100 and 200 rpm.

7.3.2.7 Morphology of hydrogel beads

The gross morphology of the hydrogel beads was documented by using a light microscope (Motic Microscoped, Wetzlar, Germany), which was connected to a camera (Canon Legria HF20, Tokyo, Japan). The images were recorded with a 20-times and 50-times magnification.

Samples were prepared either from dried hydrogel beads or from fresh beads after different production steps.

For scanning electron microscope (SEM) pictures beads were prepared on a holder with carbon Leit-tabs (Plano GmbH, Wetzlar, Germany). Some of them were cut using a scalpel. Before examination in a Hitachi S-4800 SEM (Hitachi High Tech., Tokyo, Japan) at an accelerating voltage of 3 kV, microcapsules were sputter-coated with a layer of 8-10 nm gold-palladium using a Leica EM SCD 500 (Leica Microsystems GmbH, Wetzlar, Germany) high-vacuum sputter coater.

7.3.2.8 Encapsulation efficiency

The encapsulation efficiency (EE) of NA or NAM was defined as the ratio of the actual content of the encapsulated substance and the theoretical amount in dried hydrogel beads, which was calculated according to the following equation:

$$EE\ [\%] = \left(\frac{actual\ concentration\ \left[\frac{mg}{ml}\right]}{sample\ weight\ [mg] * \left(\frac{added\ concentration\ [\frac{mg}{ml}]}{dry\ mass\ [\frac{mg}{ml}]} \right)} \right) * 100$$

Before measuring the EE, dried pectin-zein(-protein) hydrogel beads were dissolved in McIlvaine citrate-phosphate buffer with a pH value of 5.0. Furthermore, pectinase was added to macerate the beads and to achieve a total release of the encapsulated vitamin. Samples were taken after 5 hours under stirring. Afterwards, the samples were filtered through 0.2 µm syringe filters (regenerated cellulose membrane, Macherey-Nagel GmbH & Co. KG, Düren Germany) and frozen at -18 °C until they were subjected to HPLC analysis.

For pectin-shellac hydrogel beads and for WPI loaded beads, the same sample preparation was done with the exception of used Soerensen phosphate buffer with a pH value of 7.4 instead of the citrate-phosphate buffer with a pH value of 5.0, to dissolve the shellac coating. In case of WPI loaded beads, a higher pH value was chosen due to the IEP of WPI at pH value 5.35 – 5.41, which would result in a low solubility of WPI and therefore in an

incomplete determination of the encapsulated amount. Furthermore, the sample collection was taken after stirring overnight.

7.3.2.9 In vitro gastric resistance

In vitro dissolution tests were carried out in triplicate at 37 °C at 100 rpm using a basket apparatus (DT 70, Pharmatest Group, Hainburg, Germany). Depending on the EE, amounts of 150 – 5000 mg beads were weight into 250 – 400 ml simulated gastric fluid. As simulated gastric fluid either a McIlvaine buffer of pH 3.5 or a buffer with a pH of 1.4 was used as described in Manuscript III (in submission). After 30 and 60 minutes, a sample was taken to determine the released amount of vitamin. The samples were filtered through 0.2 µm syringe filters and frozen at –18 °C.

For the shellac coated beads, the whole gastrointestinal tract was simulated. Therefore, after the gastric residence time, the beads were transferred to Soerensen buffers with a pH value of 6.8 for 2 h, followed by a pH value of 7.4 for 1.5 h. In addition, the release of β-LG from WPI loaded beads was documented during a simulated gastric phase (pH 3.5) for 1 h, followed by the small intestine at a pH value of 7.4 for 4 h and the colon at a pH value of 5.0 with added pectinase.

7.3.2.10 Determination of NA and NAM

Frozen samples were defrosted and mixed thoroughly prior to the NA and NAM content analysis by RP-HPLC (reversed phase high performance liquid chromatography) using the Agilent 1100 Series with diode array detector (Agilent Technologies, Santa Clara, USA) and C18-Sphinx column (Nucleodur Sphinx RP, 5 µm, Macherey-Nagel, Düren, Germany). The injection volume was 10 µl at a flow rate of 1 ml/min using eluent A (0.1 % (v/v) TFA in milliQ water) and eluent B (0.1 % (v/v) TFA in acetonitrile). The elution used gradient steps of 95 % A (0 – 1 min), 95 – 5 % A (1 – 10 min), 5 % A (10 – 10:50 min) and 5 – 95 % A (10:50 – 12 min). The column temperature was set to 20 °C and the detection wavelength was 262 nm.

7.3.2.11 Determination of beta-lactoglobulin (β-LG) from WPI

Bevor quantification of β-LG, samples were filtered through 0.2 µm syringe filters (PET-30/13 membrane, Macherey-Nagel GmbH & Co. KG, Düren Germany). Afterwards the measurements were performed using a Thermo Scientific Dionex UltiMate 3000 HPLC (Waltham, USA) with a diode-array detector and PLRP-S column (300 Å, 8 µm, 150 x 4.6 mm, Agilent Technologies, Santa Clara, USA). For the analytical separation the injection volume was 40 µl at a flow rate of 1 ml/min and a column temperature of 40°C using Eluents A (0.1 % (v/v) TFA in water) and B (0.1 % (v/v) in ACN). The elution used gradient steps of 35-38 % B (1-8 min), 38-42 % B (8-16 min), 42-46 % B (16-22 min), 46-100 % B (22-22.5 min) and 100-35 % B (23-23.5 min). The detection wavelength was 205 nm.

7.3.2.12 Statistical analysis

All results are expressed as arithmetic means and standard deviation of the replicated analyses. The EE was analyzed by ordinary one-way ANOVA with Tukey multiple comparison test and a significance level of 0.05. The statistics and the figures were done with GraphPad Prism (version 6, GraphPad Software, San Diego, CA, USA).

7.4 Results

The viscosity of the pectin solutions used for hydrogel bead formation was determined before and after addition of NAM (**Figure 7.5A+B**). In general, the P36 indicated a higher viscosity at room temperature compared to P5.9 and the viscosity decreased if temperature was increased to 45 °C. Furthermore, the higher the pectin concentration, the higher the viscosity. The addition of fibrils (1.67 % (w/w)) led to a higher viscosity with an increase of about 100 mPa·s, whereas the addition of WPI resulted in no change of the viscosity. Moreover, as illustrated in **Figure 7.5B**, the addition of NAM resulted in a significant reduction of the viscosity. Different viscosities showed a different behavior in dripping. The pectin solution of 8 % P36 (w/w) at room temperature was too viscous to drop (about 1800 mPa·s).

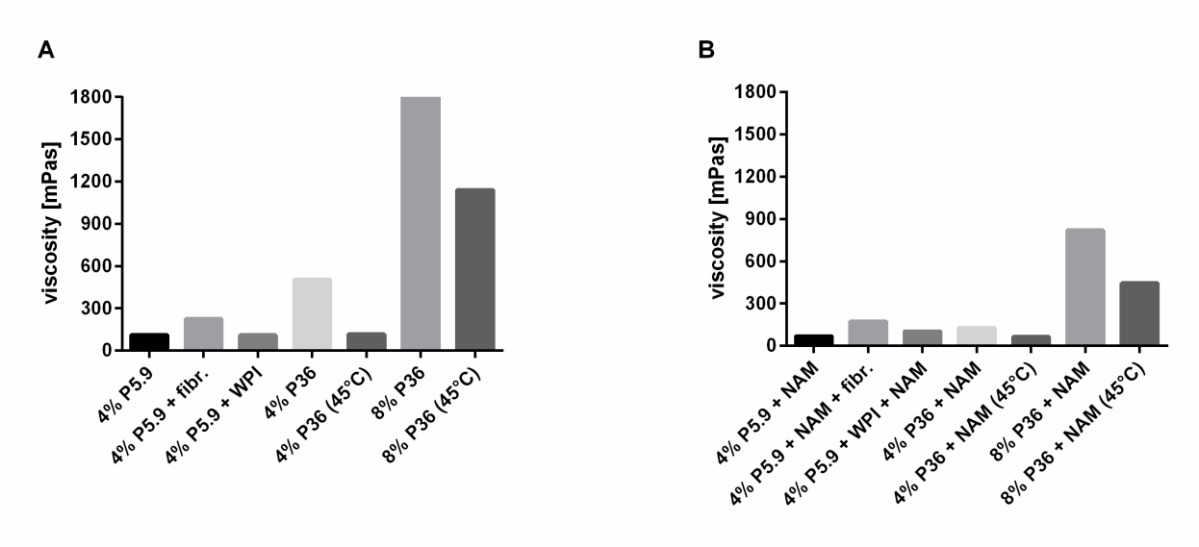


Figure 7.5: *Viscosity (mPas) of polymer solution without (A) and with (B) added nicotinamide (NAM)*

7.4.1 Feasibility study of the encapsulation of WPI

According to the results shown by Liu et al. (2006), the encapsulation of bovine serum albumin (BSA) into pectin-zein hydrogel beads led to a colon-targeted release. In the present study, WPI was encapsulated to prove the production process and the process parameters regarding their feasibility to encapsulate and to achieve a colon-targeted release.

As shown in **Figure 7.6**, the morphology of WPI loaded pectin beads depends on the production process. Whereas the two-step procedure led to spherical particles, the one-step beads resulted in red blood cell shaped beads. The encapsulation efficiency varied from 26.9 % (one-step) to 19.6 % (two-step). The release of β-LG from WPI loaded pectin beads was recorded (**Figure 7.7**). During the first 90 minutes, no release occurred, afterwards the release increased to 90 % after 300 minutes at a pH value of 7.4. The release of β-LG was slower, when the beads were produced by the two-step method. These results indicated appropriate parameters of the pectin-zein hydrogel bead preparation and a low release during the gastric phase (pH 3.5). WPI is mainly consisting of β-lactoglobulin (75 %) and α-lactalbumin with a molecular weight (MW) of about 18 and 14 kDa, which is lower compared to BSA (66.5 kDa) and could explain the faster drug release. Despite the fact that WPI tends to build some oligomers (<10 nm) at a pH value of 3.5 (Keppler et al., 2017), which could delay the release of WPI at this pH value, the same study also showed that the zeta-potential is approximately +20 mV, thus the protein is able to dissolve in the dissolution medium. In addition, Liu et al.

gave evidence that BSA encapsulated in similar beads was also able to be liberated at these conditions (Liu et al., 2006), although BSA has a higher molecular weight and its isoelectric point (pH 4.5 – 5.0) (Phan et al., 2015) is even lower than WPI (pH 5.0) (Keppler et al., 2017).

In conclusion, the feasibility of the process is given despite less favorable properties of the protein source WPI compared to BSA.

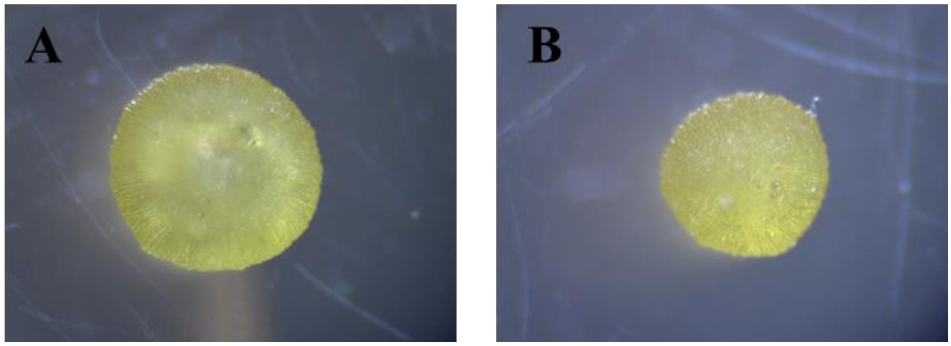


Figure 7.6: *Morphology of WPI loaded pectin hydrogel beads prepared by the (A) one-step procedure and (B) two-step procedure. Light microscope, 50-times magnification.*

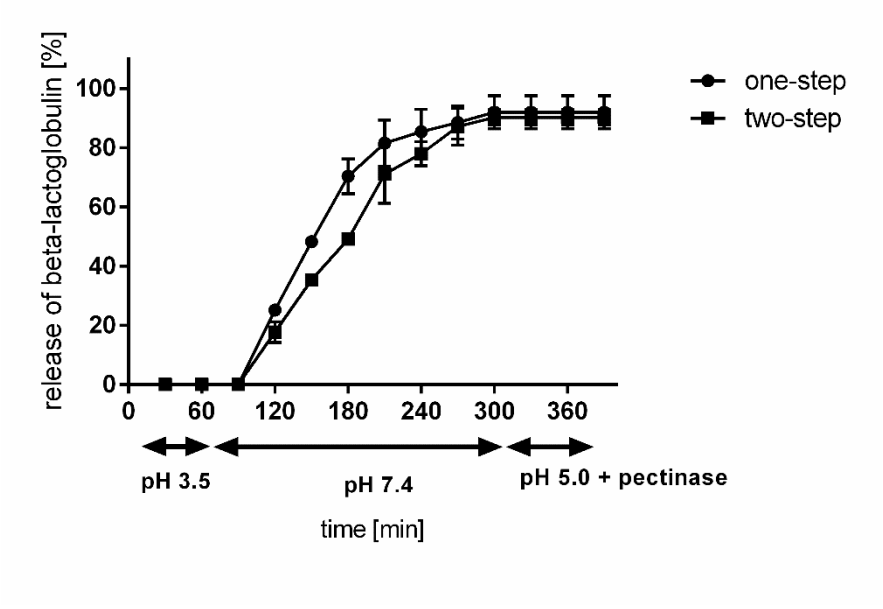


Figure 7.7: *Release of beta-lactoglobulin from WPI loaded pectin hydrogel beads produced by the one-step and the two-step procedure (mean ± SD, n=3).*

7.4.2 Morphology of pectin hydrogel beads prepared by one-step procedure

Three different pectin types (DE 69 %; 36 % and 5.9 %) with a concentration of 2 and 4 % (w/w) were compared in their ability to form hydrogel beads with encapsulated NAM (**Figure 7.8**). As expected, the hydrogel bead formation using a pectin with the highest DE (69 %) resulted in very soft hydrogel beads after dripping, whereby the beads lost their cohesion after drying. In contrast, LM pectin with a DE of 36 and 5.9 % led to a bead formation at a concentration of 2 and 4 % (w/w). Non-spherical particles were formed with a shape of red blood cells (biconcave disk with a flattened center). Higher pectin concentration of 4 % (w/w) always resulted in the formation of more regular and voluminous beads.

Further experiments were conducted with the pectin type P5.9 exhibiting the lowest DE and a processing at room temperature. Regarding the effect of the encapsulation compound on the morphology, pectin-zein hydrogel beads were prepared without NAM (**Figure 7.9A**). No effect on the sphericity has been seen. Both, before and after drying the beads showed a hollow on their surface. The exclusion of ethanol in the cross-linking solution (**Figure 7.9B**) resulted in beads that are more spherical after dripping, which formed a disky shape after drying.

Figure 7.8*: Comparison of one-step-procedure hydrogel beads made from different pectins P69 (orange box), P5.9 (green box) and P36 (purple box) with different concentration of 2 % (w/w) (always left) and 4 % (w/w) (always right) before (top) and after (bottom) drying. Light microscope pictures; 20 times magnification.*

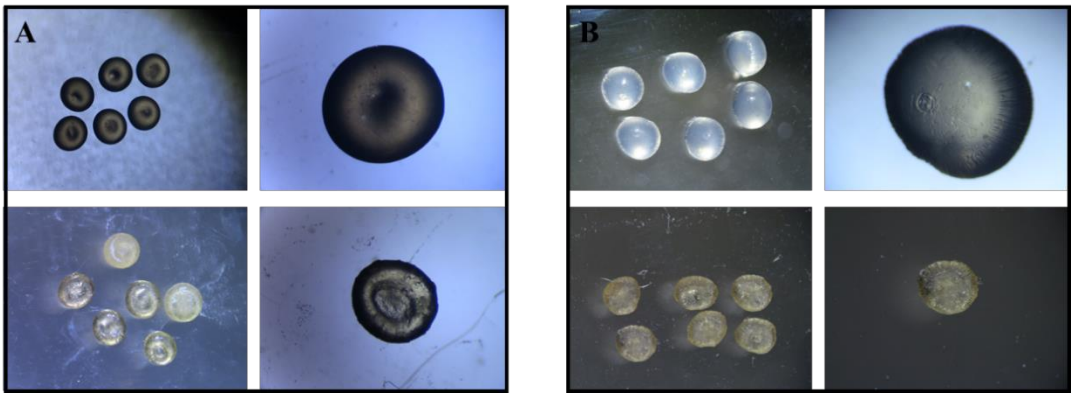


Figure 7.9: *Effect of encapsulated NAM and zein coating on the morphology of P5.9 hydrogel beads. (A) Hydrogel beads of 4 % (w/w) P5.9 without encapsulated NAM produced in a one-step-procedure with zein-$CaCl_2$-precipitation (75 % EtOH) bath before (top) and after (bottom) drying. (B) Hydrogel beads of 4 % (w/w) P5.9 with 0.5 % (w/w) NAM dripped in $CaCl_2$-precipitation bath dissolved in water before (top) and after (bottom) drying. Light microscope pictures; 20 times magnification (left); 50 times magnification (right).*

7.4.2.1 Dripping height

However, the influence of further process parameters was investigated to analyze the resulting bead shapes during the one-step procedure. As shown in **Figure 7.10**, the dripping height (distance between needle and solution surface) was reduced from 9.5 cm to 1.5 and 0.5 cm. The highest distance resulted in irregular and flattened particles, whereby a distance of 1.5 cm produced red blood cell shaped particles as already observed. A minimal distance of about 0.5 cm resulted in drop-shaped beads. For further experiments, a dripping height of 1.5 cm was chosen.

Parameters such as stirring rate (100, 150 and 200 rpm), $CaCl_2$ concentration (0.5, 2, 4 and 6 % (w/w)) and pH value of the precipitation bath (2, 4, 6, 6.8 and 11.8 by addition of HCl or NaOH) had no effect on the morphology of formed beads. The use of a smaller needle (0.4 mm inner diameter) resulted in smaller beads with the same shape compared to the bigger needle (0.9 mm inner diameter) (data not shown).

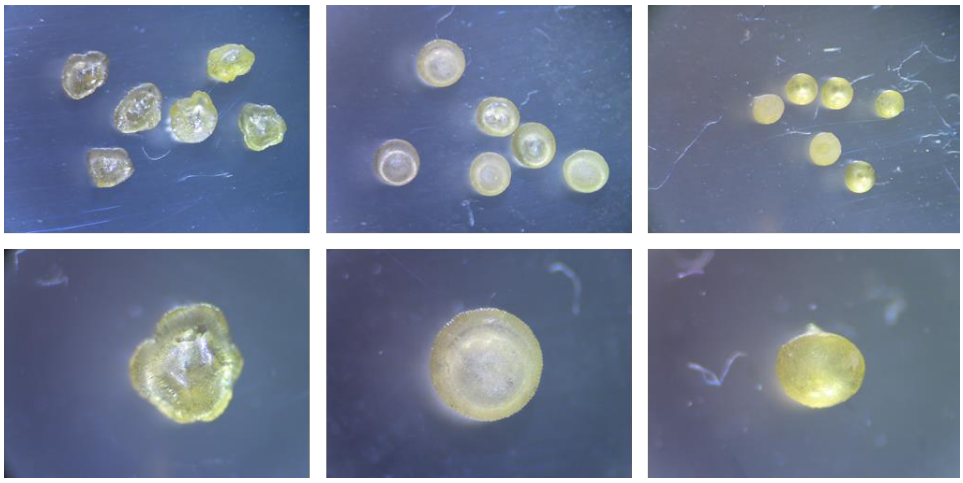


Figure 7.10: *Effect of dripping height on P5.9 hydrogel beads formation in a one-step-procedure. Left: 9.5 cm; middle: 1.5 cm; right: 0.5 cm distance between needle and solution surface. Light microscope pictures; 20 times magnification (top); 50 times magnification (bottom).*

7.4.2.2 NAM and NA concentration

The addition of NAM to the pectin solution was increased from 0.5 to 5, 10, 15, 20 and 30 % (w/w). With increasing NAM concentration the total encapsulation of NAM was reduced, whereby a notable crystallization of NAM on the bead surface occurred (**Figure 7.11A**). The concentration of 0.5 and 5 % NA (w/w) showed the same shape (hollow surface) as demonstrated with NAM. In case of 5 % NA a clearly separation of the NA in the inner core was found (**Figure 7.11B**).

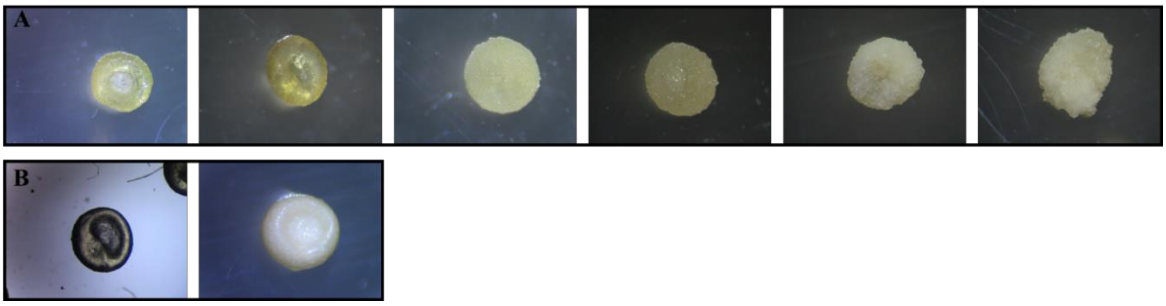


Figure 7.11: *Effect of the encapsulated compound at its concentration on dried P5.9 hydrogel beads of one-step-procedure. (A) 0.5, 5, 10, 15, 20 and 30 % (w/w) NAM. (B) 0.5 and 5 % (w/w) NA. Light microscope pictures; 50 times magnification.*

7.4.3 Morphology of pectin hydrogel beads prepared by two-step procedure

The results for the pectin P5.9 and P36 beads regarding morphology in the two-step procedure are illustrated in **Figure 7.12**. It is shown, that a pectin concentration of 4 % (w/w) resulted in almost spherical particles with a small hollow at the surface. Furthermore, the surface seemed to be more wrinkled compared to the one-step produced particles. The enhancement of the pectin concentration to 6 % (P5.9) or 8 % (P36) led to bigger and more regular and spherical particles.

Furthermore, the effect of the $CaCl_2$ concentration in the cross-linking solution was examined using 4 % P5.9 pectin (**Figure 7.13**). The results showed no clear trend regarding the sphericity of the hydrogel beads. However, the lowest $CaCl_2$ concentration of 0.5 % resulted in beads with the smallest diameter. The enhancement to 4 % $CaCl_2$ led to very irregular beads, whereby a concentration of 6 % showed more or less regular and round beads.

Process parameter such as drying in a drying oven and pH value of the $CaCl_2$ bath (2, 4, 6, 9.8 and 11.8 adjusted with HCl or NaOH) had no influence on the morphology of the beads.

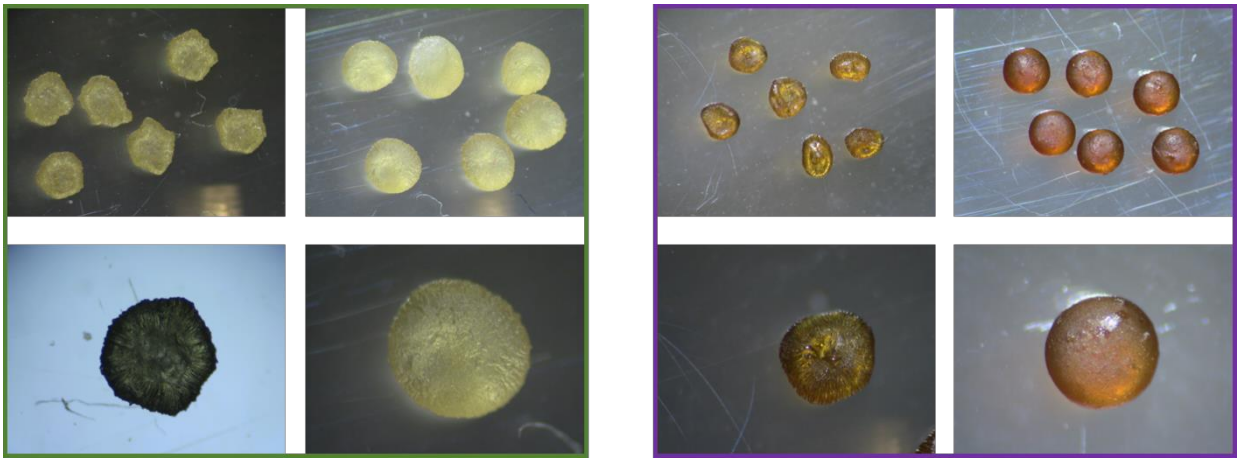


Figure 7.12: *Comparison of two-step-procedure hydrogel beads made from different pectins P5.9 (green box) and P36 (purple box) with different concentration of 4 % (w/w) (always left hand side) and 6 % (w/w) (right hand side for P5.9) or 8 % (w/w) (right hand side for P36) after drying. Light microscope pictures; 20 times magnification (top); 50 times magnification (bottom).*

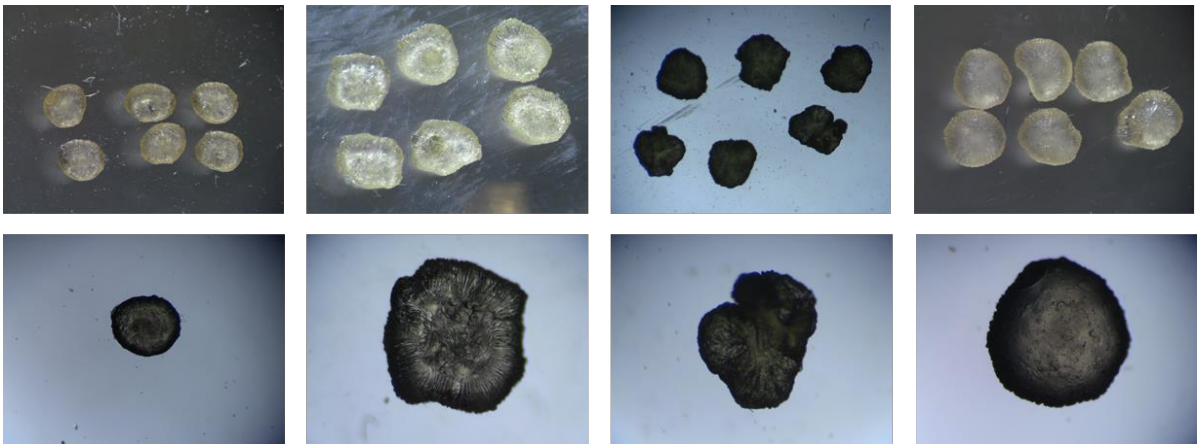


Figure 7.13*: Effect of different $CaCl_2$ concentrations on P5.9 hydrogel bead formation in a two-step-procedure after drying from the precipitation bath. From left to right: 0.5, 2, 4 and 6 % $CaCl_2$ (w/w). Light microscope pictures; 20 times magnification (top); 50 times magnification (bottom).*

The enhancement of the NAM concentration from 5 to 20 % (w/w) in the dripping solution resulted in drop shaped particles (**Figure 7.14**). After drying, NAM crystallized at the surface.

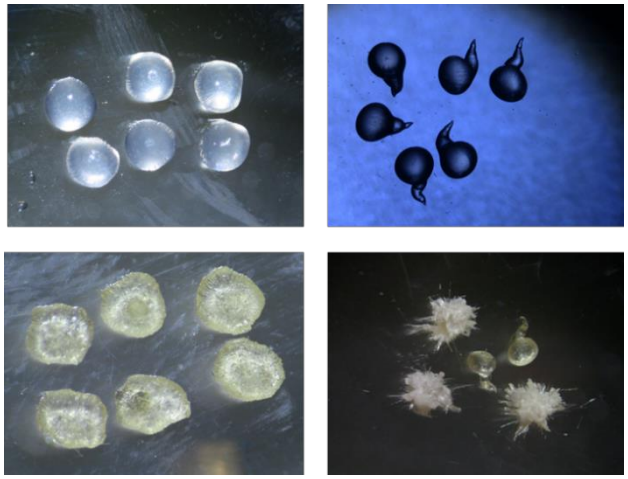


Figure 7.14*: Effect of NAM concentration on P5.9 hydrogel bead formation after dripping in $CaCl_2$ bath before (top) and after (bottom) drying. Left: 5 % (w/w) NAM, right: 20 % (w/w) NAM. Light microscope pictures; 20 times magnification*.

Further, WPI fibrils were added to enhance the sphericity. As demonstrated in **Figure 7.15**, the inclusion of fibrils caused a structuring effect leading to spherical beads.

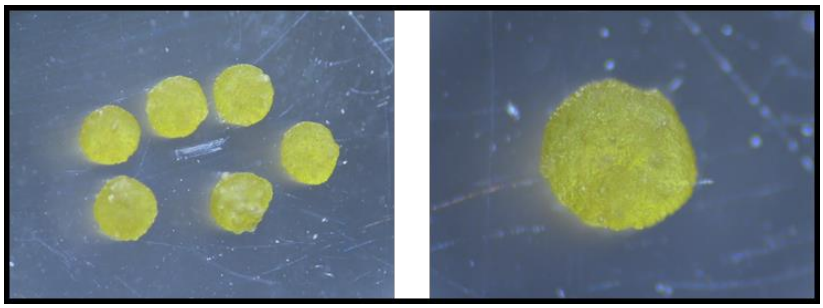


Figure 7.15: *Effect of fibril inclusion on morphology of P5.9 hydrogel beads (two-step-procedure) after drying. Light microscope pictures; 20 times magnification (left); 50 times magnification (right).*

Additionally, P5.9 beads were also evaluated after a coating step in eth. shellac or wd-shellac (instead of zein). As demonstrated in **Figure 7.16**, the shape was not changed and was still flat with hollow surface for eth. shellac (A) and wd-shellac for 1 min (B), whereby the wd-shellac began to gel after approximately 5 minutes, which resulted in irregular coated beads (C).

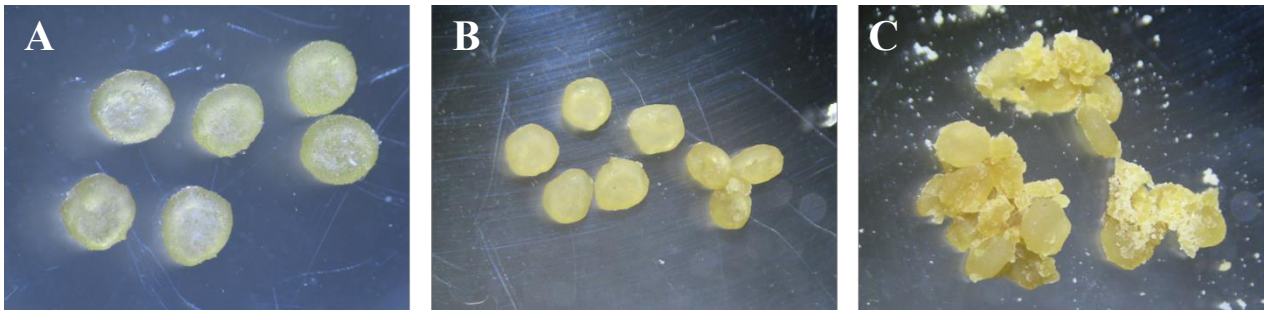


Figure 7.16*: Effect of ethanolic shellac coating for 60 min (A), wd-shellac coating for 1 min (B) and wd-shellac coating for 5 min (C) on morphology of P5.9 hydogel beads (two-step-procedure) after drying. Light microscope pictures; 20 times magnification (A); 10 times magnification (B, C).*

7.4.4 Encapsulation efficiency (EE) of NA and NAM

All hydrogel bead combination, which were evaluated for the EE are shown in **Table 7.2**. In general, the two different procedures significantly influenced the EE. A higher EE was generated using the one-step procedure. Furthermore, beads showed always a significant higher EE, when the conditions were isotonic compared to non-isotonic: 52.63 vs. 18.72 % EE of NAM for one-step produced pectin-zein beads and 18.10 vs. 3.75 % EE of NAM for two step produced pectin-zein beads. Furthermore, it was evident, that the EE of NA (43.64

%) was significantly higher than of NAM (18.72 %). The enhancement of the zein concentration showed no significant effect on the EE, regardless of the pectin type P5.9 or P36 and the pectin concentration. The use of 8 % P36 resulted in a significant higher EE (24.64 %) compared to 4 % P5.9 (18.72 %). In case of the two-step procedure, it was evident that the use of 4 or 8 % of P36 and 1 % zein had no significant effect on the EE. The EE for different coating materials with isotonic procedure showed the following order: ethanolic shellac (28.41 %) > zein (18.10 %) > wd-shellac (11.84 %). The lower incubation time (1 min) in wd-shellac resulted in a higher EE compared to an incubation time of 5 or 15 min. Moreover, the addition of fibrils led to a significant reduction in the EE for all coating formulations with zein (11.81 %), ethanolic shellac (17.52 %) and wd-shellac (5.96 %). The lowest EE was observed for beads, which were subsequently coated three times in a zein coating solution (0.68 %). If the beads were dried between the coating steps, the EE was 8.73 % after four coating cycles.

Table 7.2: *Encapsulation efficiency (EE) of NA or NAM of different formulations after one-step- or two-step-procedure (n=3). Abbreviations: NA=nicotinic acid; NAM: nicotinamide; iso.=isotonic; n. iso.= non isotonic. Different letters indicate a statistically significance (p=0.05) in one group (one-step or two-step-procedure).*

procedure	vitamin	pectin conc.	pectin type	iso. vs. n. iso.	coating material conc.	coating material	additives	EE ± SD (sign.)
one-step	NA	4 %	P5.9	n. iso.	1 %	zein (15 min)	-	43.64 ± 1.95 % (a)
one-step	NAM	4 %	P5.9	n. iso.	1 %	zein (15 min)	-	18.72 ± 0.35 % (b)
one-step	NAM	4 %	P5.9	iso.	1 %	zein (15 min)	-	52.63 ± 0.89 % (c)
one-step	NAM	4 %	P5.9	n. iso.	3 %	zein (15 min)	-	18.10 ± 0.35 % (b)
one-step	NAM	4 %	P5.9	n. iso.	5 %	zein (15 min)	-	17.89 ± 0.19 % (b)
one-step	NAM	8 %	P36	n. iso.	1 %	zein (15 min)	-	24.64 ± 0.34 % (d)
one-step	NAM	8 %	P36	n. iso.	5 %	zein (15 min)	-	23.48 ± 0.25 % (d)
two-step	NAM	4 %	P5.9	n. iso.	1 %	zein (60 min)	-	3.75 ± 0.18 % (ac)
two-step	NAM	4 %	P5.9	n. iso.	15 %	zein (60 min)	-	2.71± 0.01 % (ab)
two-step	NAM	4 %	P5.9	iso.	1 %	zein (60 min)	-	18.10 ± 1.00 % (d)
two-step	NAM	4 %	P5.9	iso.	5 %	zein (3x wet; 60 min)	-	0.68 ± 0.06 % (b)
two-step	NAM	4 %	P5.9	iso.	5 %	zein (4x dried; 60 min)	-	8.73 ± 0.11 % (e)
two-step	NAM	4 %	P5.9	iso.	5 %	eth. shellac	-	28.41 ± 0.13 % (f)
two-step	NAM	4 %	P5.9	iso.	5 %	wd-shellac (1min)	-	26.98 ± 2.73 % (f)
two-step	NAM	4 %	P5.9	iso.	5 %	wd-shellac (5min)	-	10.80 ± 0.23 % (eg)
two-step	NAM	4 %	P5.9	iso.	5 %	wd-shellac (15min)	-	11.84 ± 1.11 % (g)
two-step	NAM	4 %	P36	n. iso.	1 %	zein (60 min)	-	2.94 ± 0.06 % (ab)
two-step	NAM	4 %	P36	iso.	1 %	zein (60 min)	-	17.99 ± 0.12 % (d)
two-step	NAM	8 %	P36	n. iso.	1 %	zein (60 min)	-	3.88 ± 0.08 % (ah)
two-step	NAM	8 %	P36	n. iso.	5 %	zein (60 min)	-	5.51 ± 0.28 % (ch)
two-step	NAM	8 %	P36	iso.	1 %	zein (60 min)	-	16.82 ± 0.11 % (d)
two-step	NAM	4 %	P5.9	iso.	5 %	zein (60 min)	+ fibrils	11.81 ± 0.03 % (g)
two-step	NAM	4 %	P5.9	iso.	5 %	eth. shellac (60 min)	+ fibrils	17.52 ± 0.77 % (d)
two-step	NAM	4 %	P5.9	iso.	5 %	wd-shellac (15min)	+ fibrils	5.96 ± 0.52 % (ah)

7.4.5 Gastric resistance of pectin-zein hydrogel beads

Several bead formulations were evaluated for their release profile in an *in vitro* dissolution tester simulating gastric conditions (pH 3.5 for 1 h) (**Figure 7.17**). It is clearly shown that none of the formulation covered with zein by one-step or two-step procedure (**Figure 7.17A+B**) showed gastric resistance. After 30 min of incubation 80 – 100 % NA or NAM were released. The release was independent of the vitamin, the pectin type and concentration, the production process, the EE (high vs. low vitamin content), the zein concentration and the sphericity.

In comparison, the release after coating with ethanolic shellac was comparable to zein coated beads and resulted in no delayed release. In contrast, the incubation of NAM-pectin beads for 15 min in wd-shellac led to a gelation of the whole shellac solution. However, it also resulted in a reduction of the release: below 40 % released NAM were detected after 60 min at pH value 3.5 (**Figure 7.17C**).

The addition of fibrils caused no effect on the release: The zein and ethanolic shellac coated beads indicated a complete release of NAM after 30 min, whereby the release of NAM from pectin-wd-shellac beads was slightly higher compared to the formulation without fibrils. Interesting is, that the release of NAM was only slightly enhanced to about 60 % after crushing the wd-shellac coated beads (**Figure 7.17D**).

Based on these results, the release profile of NAM from pectin-wd-shellac beads after an incubation for 1 and 5 min in the shellac solution was evaluated under simulated gastrointestinal conditions (stomach to ileum) (**Figure 7.18**). After 60 min at gastric conditions (pH 3.5), the beads were transferred to small intestine conditions (pH 6.8) and conditions of the ileum (pH 7.4). The release increased from 36 or 53 % after 30 min at pH 3.5 to 73 or 88 % after 180 min at pH 6.8 for 5 and 1 min shellac incubation, respectively. After 240 min at pH 7.4, the whole amount of encapsulated NAM was released. The release was slightly higher for beads coated only 1 min vs. 5 min.

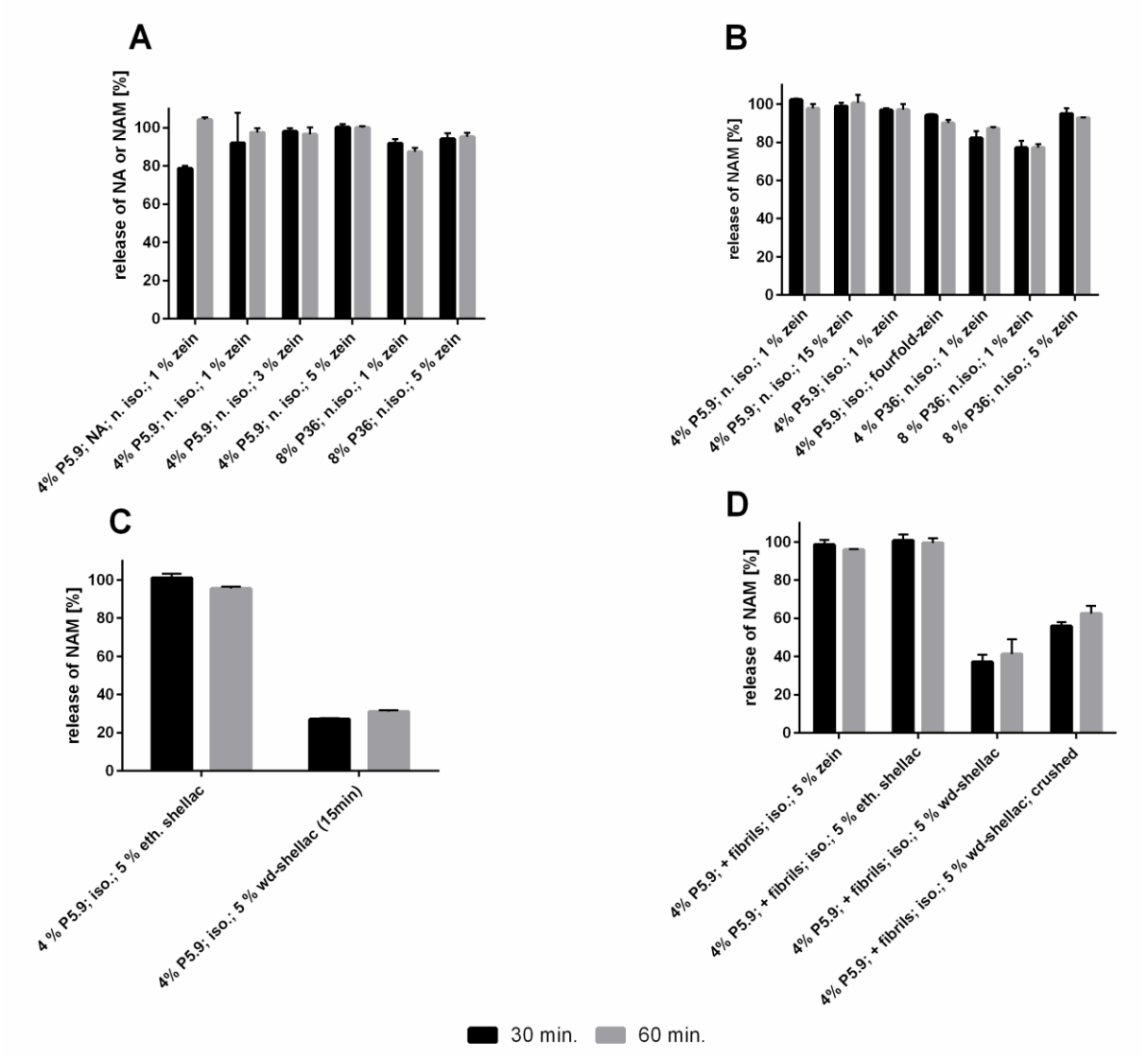


Figure 7.17: *Release of nicotinic acid (NA) or nicotinamide (NAM) from hydrogels at pH 3.5 after 30 and 60 minutes (mean±SD, n=3). (A) Hydrogels prepared by one-step-procedure with zein coating; (B) Hydrogels prepared by two-step-procedure with zein coating; (C) Hydrogels prepared by two-step procedure with shellac coating; (D) Hydrogels prepared by two-step-procedure with added fibrils and different coatings. Unless otherwise noted, NAM was encapsulated.*

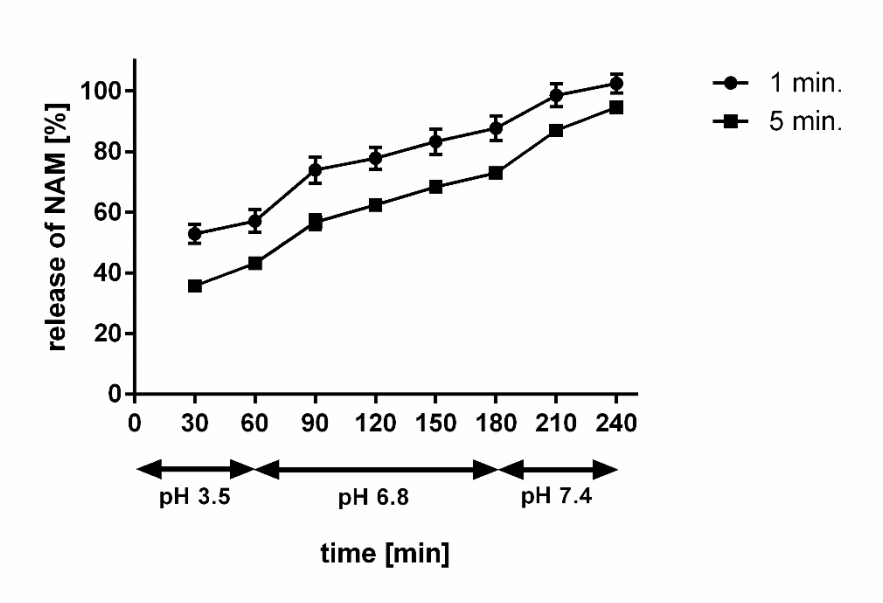


Figure 7.18: *Release profile of two-step-hydrogels with NAM encapsulated in 4 % P5.9 and coated with wd-shellac for 1 or 5 minutes (mean±SD, n=3).*

7.4.6 Surface characteristics of coated pectin hydrogel beads

The surface of pectin hydrogel beads was characterized by SEM (**Figure 7.19)**. All bead formulations showed large cracks and pores (highlighted by red arrows and circles). Furthermore, the two-step produced beads led to a wrinkled surface (**C-F**). The enhancement of the zein concentration to 15 % led to a clear separation of pectin bead and zein covering (**E**). In general, the surface of one-step produced beads showed a smoother surface (**A+B**) compared to the two-step process. Bead containing fibrils had a rough surface with some holes in the inner core (**G**).

The wd-shellac coated NAM-pectin bead is shown in **Figure 7.20**. A part of the shellac coating was already lost and showed a small gap between the pectin bead and the outer shellac coating. Furthermore, the cross section of the shellac coating reflected small crystalline areas. The surface was rough and small microstructures were detected.

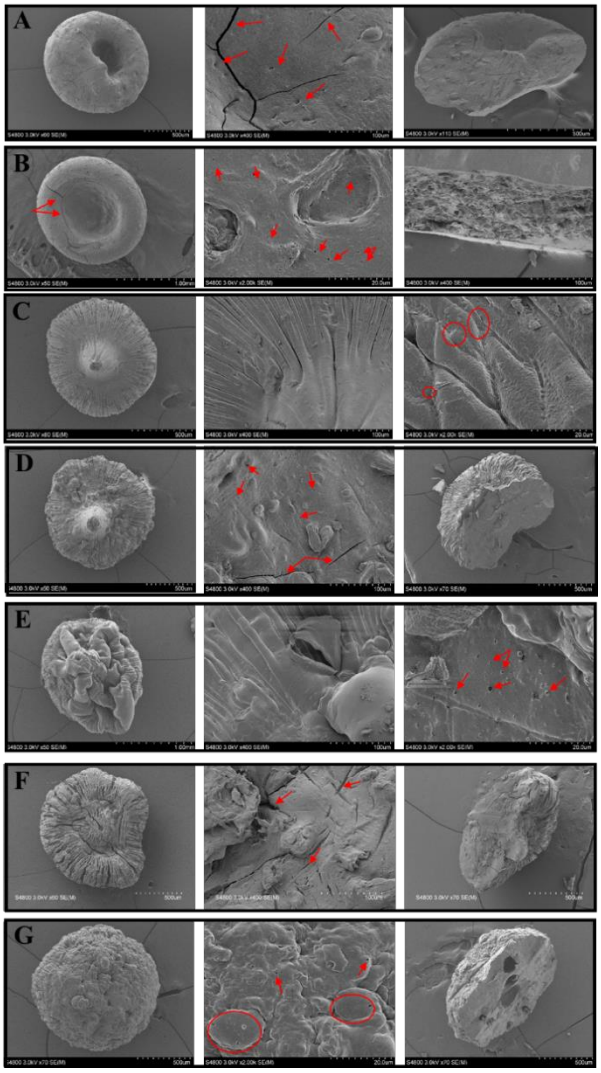


Figure 7.19: *SEM pictures of surface characteristics of different hydrogel beads.(A) One-step P5.9 bead; 5 % NAM; n. iso.; 1 % zein. (B) One-step)P5.9 bead; 5 % NA; n. iso.; 1 % zein. (C) Two-step P36 bead; 5 % NAM, n. iso; 1% zein. (D) Two-step P5.9 bead; 5 % NAM, n. iso; 1% zein. (E) Two-step P5.9 bead; 5 % NAM, n. iso; 15% zein. (F) Two-step P5.9 bead; 5 % NAM, n. iso; 4x zein. (G) Two-step P5.9 bead with added fibrils; 3.3 % NAM; n. iso.; 1 % zein*

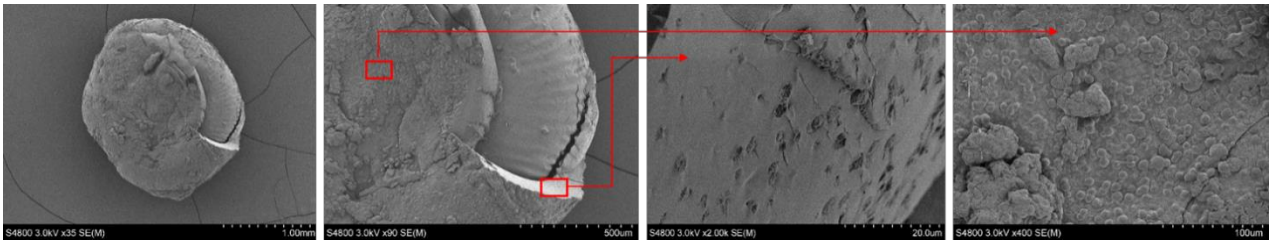


Figure 7.20: *SEM pictures of two-step P5.9 hydrogel bead coated with wd-shellac for 1 min.*

7.5 Discussion

In general, the shape of particles can influence the release rate of the encapsulated compound. The low specific surface area of spherical particles is associated with a lower dissolution rate than for disc or red blood cell shaped particles (Guo and Kaletunç, 2016; Harvestine et al., 2014). The shape of such particles can be influenced by dripping distance, the polymer viscosity and surface tension, the drying conditions, hardening addition and hardening time, crosslinker type, concentration and temperature, addition of fillers and the surface tension of the gelling bath (Bourgeois et al., 2005; Chan et al., 2009; Chan et al., 2011; Davarcı et al., 2017; Guo and Kaletunç, 2016; Henning et al., 2012; Nithitanakool et al., 2013; Smrdel, 2008; Sriamornsak and Nunthanid, 1999). Therefore, in the present study varying process conditions were studied for effect on the shape of niacin loaded pectin hydrogel beads. Furthermore, the influence of different forms and formulations of pectin hydrogel beads were evaluated regarding their encapsulation efficiency and gastric resistance.

7.5.1 Morphology of pectin hydrogel beads

As expected, pectin with the highest DE (P69) was not able to form stable hydrogel beads, whereas P36 and P5.9 produced stable beads. This can be explained by the higher amount of free carboxylic groups that react with the calcium ions to form a gel. Zones with a low DE in the molecule are necessary to obtain crosslinking by Ca^{2+} (Sande, 2005). However, the DE did not influence the bead morphology, both DE resulted in bowl-like indentations of the bead surface. This is contrary to the results of Liu et al., who used the same one-step production procedure. They generated roughly spherical pectin and pectin-zein beads, while using an alkaline de-esterified citrus pectin (2 % w/v) with a corresponding DE of 10 – 16 % (Liu et al., 2004; Rubinstein et al., 1993)). This was comparable to the DE of P5.9 in the present study, which was desterified using an alkaline process. They encapsulated indomethacin and BSA (Liu et al., 2006), which could also influence the morphology of hydrogel beads due to the lower water solubility of indomethacin compared to niacin and the higher molecular weight of BSA. However, the encapsulation of WPI in the feasibility study also resulted in flattened particles with a hollow, when produced by the one-step method, despite WPI has a lower solubility and a higher molecular weight (MW) compared to NAM. In fact, other publication also showed calcium pectinate beads (DE: 27 – 32 %) with a hollow at the surface (Bourgeois et al., 2005; Nithitanakool et al., 2013; Sriamornsak and Nunthanid, 1999), which

is comparable to the present results. They explained the deformation as a result of insufficiently strong beads and a collapse during the drying process. In the present study, the hollow surface was formed already prior to the drying step of the one-step produced particles. Therefore, the influence of ethanol as solvent in the zein bath and $CaCl_2$ cross-linking solution was considered. It is mentioned, that ethanol could lead to a precipitation of alginate and a gel collapse, which results in a decreased volume (Moe et al., 1993). This could also apply to the present observation with pectin, due to loss of elasticity (Chotiko and Sathivel, 2017). Furthermore, the surface tension influences the bead formation due to changed impacts between the liquid pectin drop and the cross-linking bath. Davarci et al. documented a higher penetration depth of alginate droplets when the surface tension of the $CaCl_2$ solution decreased, which is in accordance to the present results. The reduced surface tension also led to more spherical beads, which was explained due to a higher penetration depth (> 15 mm) (Davarcı et al., 2017). Despite 70 % ethanol solution used in the present study exhibits a lower surface tension of 25.48 mN/m (Vazquez et al., 1995) compared to the lowest surface tension of the $CaCl_2$ solution measured by Davarci et al. (37.05 mN/m) were deformed. For Ca-alginate beads it was documented, that the formation of spherical beads was strongly dependent on the liquid properties such as surface tension and viscosity of the alginate solution. When an alginate bead was dropped into the cross-linking solution, two forces were competing, which affected the drop shape: the viscous-surface tension forces of the drop and the impact-drag forces of the surrounding fluid. If the viscosity of the drop was too low, the viscous-surface tension forces were lower than the minimum forces required to counteract the impact-drag forces, which led to a deformation of the drop. For alginate a minimal viscosity of 50 – 60 mPa*s was documented (Chan et al., 2009). The P5.9 solution of the present study had a viscosity of 112 and 69 mPa*s without and with 5 % (w/w) NAM, which is above the critical viscosity to counteract the impact-drug forces. Nevertheless, a deformation was observed with both solutions.

In the present study, the dripping distance showed a significant influence on the bead shape. This was also described for alginate beads produced by ionotropic gelation. A dripping distance of more than 6 cm illustrated flattened particles (Fundueanu et al., 1999; Smrdel, 2008), whereby smaller distances led to beads with a short tail (Fundueanu et al., 1999). Hydrogel beads consisting of a combination of pectin and alginate resulted in disk shaped particles with increasing dripping distance (Guo and Kaletunç, 2016), which is in accordance with the present results. However, the red-blood cell shape could not be prevented by changing the dripping distance. The stirring rate showed no effect on the shape of pectin

beads, which could be explained by the low sheer stress at a stirring rate of 100 to 200 rpm. During the formation of flattened particles were formed above 600 rpm (Smrdel, 2008).

The variation of the $CaCl_2$ concentration only affected the shape of pectin beads in the two-step procedure. In comparison with others, the effect of $CaCl_2$ concentration was often studied with regard to its effect on the EE and the release of the encapsulated compound. Sriamornsak showed a slower release of BSA with increasing $CaCl_2$ concentrations, which was explained due to a stronger gel (Sriamornsak, 1999). A stronger gel at higher $CaCl_2$ concentrations could be an explanation for a stronger cohesion and therefore for a more spherical bead. Sande et al. postulated a high gel strength for pectin calcium gels with 12 – 14 mg Ca/g pectin. In the present study, significantly higher ratios were used amounting to 680 – 2770 mg Ca/g pectin.

The increase of the NAM concentration above 5 % (w/w) in the pectin solution resulted in overloaded beads with visible NAM crystals on the surface. The crystallization could occur due to the high NAM content in the zein coating solution, which diffused from the pectin core during dripping process. NAM will be adsorbed at the surface and crystallizes during the drying process. Furthermore, the encapsulated NAM could migrate to the periphery of the beads along with the water flux during drying, which lead to an accumulation in the surface (Shariff et al., 2007; Smrdel, 2008). These overloaded beads would lead to a premature release of NAM due to an insufficient incorporation and coating. Therefore, only NAM concentration below 10 % will be suitable for encapsulation by pectin hydrogels. While dropping the pectin solution with 20 % NAM into a $CaCl_2$ bath, drops did not immerse into the cross-linking solution, but floated on the water surface, which resulted in coalescence of the single beads. Therefore, the dripping height was adjusted, which led to the formation of drop-shaped beads. This could be related to the significantly decreased viscosity after the addition of NAM. A decreased viscosity led to a decreased velocity of the drop, which results in a lower immersion into the cross-linking solution and a sticking to the liquid surface (Davarcı et al., 2017; Guo and Kaletunç, 2016). Furthermore, the low ratio of pectin to NAM could result in an inefficient encapsulation of NAM and a less compact gel (Nithitanakool et al., 2013).

The addition of WPI and WPI-fibrils resulted in voluminous and spherical particles, this was related to the structuring and/or filling effect of the proteins on the loose pectin network (Mohammadian and Madadlou, 2018). Although the viscosity of the pectin-fibril solution was higher than the pectin-WPI solution, no effects on the shape were observed.

7.5.2 Encapsulation efficiency

The EE of niacin in the present study was low compared to others studies encapsulating indomethacin, BSA, FITC-dextran, sulphamethoxazole, anthocyanins and beta-lactamase (Bourgeois et al., 2005; Chotiko and Sathivel, 2017; Henning et al., 2012; Liu et al., 2006; Munjeri et al., 1997; Sriamornsak and Nunthanid, 1998). The differences could be related to many different process parameters such as different pectin types (Jung et al., 2013; Sriamornsak and Nunthanid, 1998), pectin concentrations (Lee et al., 2009; Nithitanakool et al., 2013), different production processes (Lee et al., 2009; Sriamornsak et al., 2010), different drug loads (Lee et al., 2009; Sriamornsak and Nunthanid, 1998) and different characteristics of the encapsulated compound (Munjeri et al., 1997; Sriamornsak and Nunthanid, 1998). The present study showed no clear effect of pectin type/DE and concentration on the EE, which supports the hypothesis, that the water-solubility of niacin is the main influencing factor regarding the low EE. Niacin, especially NAM, is highly water-soluble (NA: 15 g/l; NAM: 1000 g/l), which could lead to a high diffusional loss of NA and NAM from the beads into the cross-linking solution (Sriamornsak and Kennedy, 2007). This loss will be mainly driven by a concentration gradient, which also explains the higher EE of isotonic conditions. Munjeri et al. also documented a lower EE for soluble substances like sulphamethoxazole (67 – 82 %) than for the insoluble indomethacin (82 – 98 %) (Munjeri et al., 1997). This is also confirmed by the encapsulation of NA, which have a higher EE compared to NAM with the highest water-solubility. Furthermore, the lower EE in case of two-step beads could be explained due to a longer incubation time (Lee et al., 2009), a second bath (Henning et al., 2012) and no protecting coating in the cross-linking bath (Chotiko and Sathivel, 2017), which enables the leaching of NAM. As a result, the pectin-zein beads, which were coated three times by zein showed the lowest EE. The leaching of NAM was reduced, when ethanolic shellac was used as coating material, due to a lower solubility of NAM in ethanol compared to water (about 666 g/l). Therefore, the ionic gelation is widely used for encapsulation of hydrophobic compounds (Kurozawa and Hubinger, 2017). For hydrophilic compounds changes in the formulation e.g. an additional coating or polymers are necessary (Liu et al., 2003; Sande, 2005). Chotiko and Sathivel also observed, that the addition of WPI led to an enhanced EE due to a blockage of the diffusion. However, this effect was not significant and was not confirmed in the present study when fibrils were added (Chotiko and Sathivel, 2017). The lower NAM content in the dripping solution with fibrils could also be responsible for the lower EE compared to the beads without fibrils (Nithitanakool et al., 2013).

7.5.3 Gastric resistance

The present study showed no influence of the shape or sphericity of the pectin beads on the release of NAM. All formulations, except of the wd-shellac coated beads, resulted in an approximately total release already after 30 min. In comparison to Liu et al., who also used the one-step procedure for pectin-zein hydrogel beads, a successful encapsulated indomethacin and BSA with a colon-targeted delivery was achieved (Liu et al., 2006). However, Chotiko and Sathivel also prepared pectin-zein(-WPI) hydrogel beads to incorporate anthocyanin extract and documented a release of 50 – 80 % anthocyanin extract after 30 min at simulated gastric fluid. They observed, that zein only randomly covered the pectin bead. Furthermore, wide cracks were seen on the SEM pictures similar to the present studies. This could lead to a premature release of niacin. Additionally, as already mentioned, niacin is highly water-soluble and has a low molecular weight of about 121 Da, which supports a high diffusion rate and therefore a fast release (Chotiko and Sathivel, 2017; Sriamornsak and Kennedy, 2007). This is an explanation for the lacking effect of the shape on the release: NAM diffused very fast, leading to no detectable difference of the release after 30 minutes.

The additional experiments of encapsulating WPI confirmed this theory. Both, the one-step and two-step produced WPI pectin hydrogel beads covered with zein protected the β-LG for 90 minutes followed by a continuous release to 90 % at a pH value of 7.4 after 5 h in total. β-LG has a MW of about 18 kDa and therefore diffuses slowly compared to niacin. Further, it was found that the α-lactalbumin (14 kDa) was released slightly faster than β-LG (data not shown). Furthermore, the different bead shapes, resulting from the different production methods, led to a faster release from the red blood cell shaped particles compared to the spherical beads.

The addition of fibrils did not delay the release of NAM, although fibrils lead to a protection of drugs and a reinforcement of the structure of microcapsules, when applied by a number of layers (Mohammadian and Madadlou, 2018; Rossier-Miranda et al., 2010).

The only significant effect on NAM release was found when using wd-shellac as coating solution. Therefore, a total replacement of shellac while generating gastric resistant beads was not possible. However, depending on the EE, the use of shellac could be reduced to 0.37 g shellac per g NAM compared to the applied amount of shellac in our previous study (0.63 g shellac/g NAM), when a coating time of 1 min was chosen. The delayed release when using

wd-shellac is explained by the formation of hydrogen bonds between carboxylate groups of shellac via calcium ions (Ben Messaoud et al., 2016). This forms irreversible water unsoluble salts, which decreases the film swelling and could delay the release of niacin (Farag and Leopold, 2011). However, an improvement of the gastric resistance need further investigations. The adjustment of the pH value during the application of the shellac coating (Henning et al., 2012) or an additional heating of the capsules above the T_g of shellac (about 60 °C) (Ben Messaoud et al., 2016) could be interesting with regard to an influence on the release.

7.5.4 Surface characteristics of pectin-zein hydrogel beads

In general, the surface of the one-step produced beads seemed to be smoother than the two-step produced beads, which could be attributed to a stronger interaction between pectin and zein during the one-step procedure (Liu et al., 2006). The observed cracks and pores on the surface of the pectin-zein hydrogel beads are in accordance with the results shown by Chotiko and Sathivel (Chotiko and Sathivel, 2017). Liu et al. did not describe this phenomenon and showed gastric resistance, which indicates no or only minimal cracks in the coating, that would not induce a premature release of the large BSA molecule or the low soluble indomethacin (Liu et al., 2006). As shown in the present study as well as by others, when zein was used without further additives, brittle coatings are formed (Parris and Coffin, 1997), which could induce crack formation during drying. One opportunity to soften the zein coating and to prevent cracks could be the addition of plasticizer. Glycerol or polyethylene glycol increased the flexibility of zein film and could prevent the formation of cracks (Kadam et al., 2017; Paramawati et al., 2001; Parris and Coffin, 1997), whereby the water vapor permeability also rose (Paramawati et al., 2001; Parris and Coffin, 1997), which could be contraindicative for the present aim. The integration of catechin led to highly flexible zein films while maintaining their integrity and hydration barrier. Other polyphenolic acids could result in films with a higher porosity or the loss of their integrity (Arcan and Yemenicioğlu, 2011). Furthermore, the addition of titanium dioxide to zein film could enhance the hardness of zein films, which lead to a reduction in brittleness and an increase in hydrophobicity (Kadam et al., 2017).

The addition of fibrils resulted in more spherical particles with an irregular surface. Furthmore, the holes inside the bead, as shown by SEM pictures, were formed due to the foaming properties of fibrils (Mohammadian and Madadlou, 2018).

With respect to the surface characteristics of shellac coated pectin beads, a rough surface was observed. It is suggested, that the visible microspheres on the surface were generated under the reaction between shellac and calcium (Xue and Zhang, 2009). Furthermore, an incorporation of NAM into the shellac layer is suggested because of crystalline areas in the SEM pictures. This would explained the continuous release of NAM after crushing. Moreover, SEM pictures showed no direct contact between pectin core and shellac layer. This interspace was also observed in a combination of alginate beads and shellac. The authors suggested an induced shrinking by depletion of cross-linked calcium ions, which resulted in an interspace (Ben Messaoud et al., 2016).

7.6 Conclusion

Encapsulation degree of niacin (NA and NAM) into pectin-zein and pectin-shellac beads varied in a broad range < 1 % to > 50 %. In most cases, flattened, non-spherical particles were formed with a red blood cell shape, when produced by the one-step procedure. This was independent from the encapsulated compound (NAM, NA or WPI) or the process parameters. Only the incorporation of whey protein isolate (WPI), or WPI fibrils and an enhancement of the pectin concentration resulted in almost spherical particles by using the two-step procedure. Only the release profile of β-LG was influenced by the shape of the beads. The more spherical bead resulted in a slower release of β-LG. No effect on the niacin release profile was observed due to the fast release of NAM during the first 30 min. The small niacin molecule facilitated an easy diffusion through the small cracks observed in the zein coating, so none of the tested pectin-zein formulations protected niacin against gastric conditions. Only a coating of wd-shellac (ammonium salt of shellac) resulted in a reduced release due to the formation of unsoluble calcium shellac. The low encapsulation efficiency (EE) of niacin was due to the high water-solubility of niacin, which resulted in a high leaching especially in case of the two-step procedure.

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8 General discussion

The objective of the present thesis was to develop a nutraceutical formulation with an enhanced availability of the vitamin niacin (NA and NAM) in the ileocolon region of the human gastrointestinal tract to induce beneficial effects on the gut microbiome. Therefore, a shellac based triple coating on niacin pellets and niacin loaded pectin-zein hydrogel beads were evaluated regarding its suitability as colon delivery system. The evaluation was based on *in vitro* dissolution profiles and a human bioavailability study in case of shellac coated niacin microcapsules. The pharmacokinetics *in vivo* were examined by the research group of Prof. Dr. Matthias Laudes (UKSH Kiel) and are also part of the dissertation of Daniela Fangmann (Fangmann, 2017). Further insights were gained into the interactions of the encapsulated compounds and the protecting materials, which influenced the release profile.

8.1 Suitability of food-grade film and gel forming materials for a colon-targeted delivery of niacin

In order to beneficially influence the gut microbiota, a targeted release into the (ileo)colonic region has to be ensured (Schreiber et al., 2014). Thus, in the pharmaceutical industry, pH-dependent coating materials (e.g. Eudragit® S100, S 12,5 or FS 30 D) are commonly applied. These polymers are anionic with a high amount of functional carboxylic groups, which are protonated and therefore unsoluble at low pH values. At higher pH values higher than 7, the carboxylic groups will be deprotonated and the polymer gets soluble, which leads to a release of the encapsulated compound (Evonik; Yoshida et al., 2013). However, these polymers are only permitted for the use in pharmaceuticals. Due to the high demand for nutraceuticals (Czarnocka and Alhnan, 2015; Das et al., 2012; Santini et al., 2017), recent developments of Evonik Industries combined the same polymers with a permission for the use in solid nutraceuticals or supplements (Eudraguard® biotic) with an upper limit of 100.000 mg/kg.

Food-grade alternatives for colon-targeted delivery systems are limited and still scarce. Referring to **hypothesis 1**, it was proved that pH-sensitive film and gel-forming materials such as shellac and pectin are suitable for a colon-targeted delivery of niacin (**Paper I, Manuscript II and Manuscript V**). This hypothesis was based on the properties of the materials as described in the sections below. Both, shellac and pectin are legislated as food

additives without restrictions concerning the concentration. An acceptable daily intake-level (ADI) does not exist.

8.1.1 Shellac as coating material for niacin pellets

In **Paper I and Manuscript II** it was hypothesized (**Hypothesis 1a**) that shellac is a suitable food-grade coating material for niacin to prevent a previous liberation under gastric and small intestine conditions and to lead to a sufficient release in the ileocolonic region. This was confirmed by *in vitro* dissolution tests simulating gastrointestinal conditions from stomach to ileum and a human bioavailability study (**section 8.3**). However, only the adjustment of the shellac coating formulation to the properties of NA and NAM using a novel triple shellac coating with a pH-influencing intermediate subcoating led to a precise release (**section 8.4**). Independently from the encapsulated compound, shellac behaves similar as the synthetic coating polymers (Eudragit®). The carboxylic groups within the shellac molecule are protonated and insoluble at low pH value. Above a pH value of 7.3, shellac dissolves after deprotonation of the carboxylic groups (Al-Gousous et al., 2015; Limmatvapirat et al., 2007; Pearnchob et al., 2003; Penning, 1996) and can liberate the encapsulated compounds. The pk_a of shellac is between 6.9 and 7.5, thus the deprotonation begins at a pH value of 6.8. This leads to swelling of the shellac coating into a gel-like stage (Farag and Leopold, 2011a), which promotes the water influx and the solubilization and release of the protected material such as niacin. The ammonium salt form of shellac is soluble in water, which is advantageous because organic solvents are dispensable. The administration of the water soluble ammonium salt of shellac on NAM pellets or NA coated Cellets (diameter of 350 – 500 µm) by fluidized bed coating was successful without further complications. Good coating results with a smooth surface and a uniform coating layer were achieved as demonstrated by SEM pictures (**Figure 3.2A+B**), which also confirmed a sufficient protection of the encapsulated compound.

8.1.2 Pectin as material for niacin loaded hydrogel beads

Due to its lower production cost as well as being derived from plant material, pectin is an interesting alternative to shellac. Therefore, the mechanism of ionotropic gelation using pectin to encapsulate niacin was evaluated in **Chapter 7**. It was hypothesized (**Hypothesis 1b**) that pectin in combination with a zein coating exhibits similar features as the shellac

encapsulation. The feasibility was demonstrated for β-lactoglobulin. The free carboxylic groups of low methoxylated pectin are able to form a gel by cross-linking with calcium ions, which results in a reduced solubility while maintaining the degradation by enzymes of the gut microbiota (Liu et al., 2003; Maroni et al., 2013; Willats et al., 2006). Additionally, the water insoluble zein coating should led to a reduced water absorption, which could reduce the release during stomach and small intestine (Liu et al., 2003; Liu et al., 2006; Rubinstein, 2000). However, none of the pectin-zein hydrogel beads were sufficient to protect a premature release of niacin under simulated gastric conditions. The rapid and complete release of niacin is explained due to the physico-chemical properties of niacin in contrast to β-LG and will be discussed below (**Chapter 8.4**). The zein coating was not able to inhibit the release of niacin loaded pectin beads, which was confirmed by large cracks in SEM pictures (**Figure 7.19**). Thus, in contrast to the hypothesis, no effect of the morphology of the pectin-zein beads on the release profile have been seen. In comparison to the zein coating, wet pectin-niacin hydrogel beads were also coated with water-soluble shellac under stirring. *In vitro* dissolution tests resulted in a reduced release at gastric conditions of about 50 % after 60 min compared to the zein coating, whereby a gastric resistance was not achieved (**Figure 7.17**). This effect is explained by the formation of irreversible insoluble calcium shellac, which can delay the release (Farag and Leopold, 2011a).

8.2 Robustness of the release profile *in vitro* of triple shellac coated niacin pellets despite influencing extrinsic factors

In contrast to synthetic materials, natural materials are determined by natural fluctuations, which can influence their properties and the resulting release of the encapsulated compound during storage (Buch et al., 2009; Farag and Leopold, 2011b). Further, variations in intestinal fluid compositions can influence the dissolution profile. In **chapter 4**, it was hypothesized (**Hypothesis 2**) and confirmed that the novel triple shellac coating enables a robust release profile despite the changing extrinsic factors.

8.2.1 Influence of the storage conditions on the release profile

Coated NA and NAM pellets were stored for a period of 24 months at 20 °C. With increasing storage time, a slight reduction of the released vitamin was observed, especially at a pH value of 6.8 (**Figure 4.2A+B**). However, the gastric resistance was maintained. The reduced release

is caused by aging of shellac. This leads to polymerization processes via esterification of the large amount of hydroxyl and carboxyl groups of the shellac components (Farag and Leopold, 2009; Limmatvapirat et al., 2005; Limmatvapirat et al., 2007). Due to the use of the ammonium salt form of shellac, only slight effects on the release profile were detected. The ammonium acting as counter ion for the carboxyl groups, which hinders the esterification. This process is reversible due to evaporation of ammonia (Limmatvapirat et al., 2007).

The storage of the microencapsulated NA and NAM at 4 °C for 8 weeks showed no differences in the release of NAM, whereby the NA release was increased after 4 weeks. After 6 weeks, 90 % of the encapsulated NA was already liberated during the first 30 min (**Figure 4.2C+D**). This effect could be attributed to the sodium bicarbonate containing intermediate subcoating in NA pellets. The higher relative humidity of 47 % could lead to a partial dissolution of the sodium bicarbonate, which enhances the solubility of shellac and results in a premature release. Thus, the robustness of the release profile is dependent on the storage conditions especially in case of NA pellets using a sodium bicarbonate intermediate subcoating.

8.2.2 Influence of simulated gastrointestinal conditions on the release profile

Shellacs dissolution is dependent on the pH value of the surrounding solution. The pH value of the stomach can vary from 1.3 to 5.0 in fasted or fed state, with pH level peaks of 6.7 (Dressman et al., 1990; Russell et al., 1993). The present NA and NAM pellets showed gastric resistance at a pH value of 1.4 and 4.5 for 2 h due to protonated carboxyl groups in the shellac coating (**Figure 4.3**). If the stomach pH value starts with a pH value of 6.8, as it can be present in a few healthy people (Feldman and Barnett, 1991) or in patients with a proton-pump-inhibitor (Peghini et al., 1998), NA and NAM are completely liberated within 2 h (**Figure 4.4**). This is mainly explained by the application of the water-soluble ammonium salt shellac. In contrast to ethanolic shellac, the carboxylic groups in the ammonium salt shellac film are affiliated to the ammonium ion (Limmatvapirat et al., 2005), which enables a higher solubility and water vapor permeability. After contact to acid solutions, the carboxyl groups are getting protonated and the ammonium ion is replaced, which results in an insoluble and protecting outer layer (Henning et al., 2012; Penning, 1996). Without further exposition to an acidic milieu, the water uptake of water-soluble shellac is increased, which resulted in a fast release at a pH value of 6.8. An exposition to the gastric fluid (pH 1.4) for 1 h, therefore,

resulted in a reduced release in the subsequent digestion fluids. This was also documented for a tablet coating with Eudragit L30 D-55, which is also a pH dependent coating material that dissolves above a pH value of 4 – 6. It was suggested, that the penetration of acid delays the dissolution of the polymer and therefore the release. The longer the exposition the more delayed was the following release (Menge, 2016). Except for people with an enhanced basal gastric pH value up to 6.8, the present shellac coated pellets indicated a high robustness during the simulated gastric residence time.

In contrast to the stomach and the upper intestine, in the targeted ileocolonic region, a maximal amount of released vitamin is desired. However, especially regarding the high dissolution pH value of shellac (7.3), it is often criticized (Farag and Leopold, 2009; Limmatvapirat et al., 2007), that the pH value of the human digestion fluids does not reach such high values (Dressman et al., 1998; Gruber et al., 1987), which could led to an insufficient release. Therefore, the amount of released NA and NAM from shellac coated particles were recorded in a simulated prolonged phase in the ileocolonic and colonic region at lower pH values of 6.8 and 7.0. The results showed a retarded release at lower pH values , however, after 26 h 80 % of NAM and 100 % of NA was released at a pH value of 6.8 (**Figure 4.5**), which suggest a suitable amount for the gut microbiota and a robustness for varying pH values in the colonic region. The lower release of NAM is attributed to the citric acid subcoating as described below. SEM pictures of *in vitro* digested pellets after 24 h at a pH value of 6.8, showed remaining shellac shells with a porous structure, which indicated a sufficient release of NA and NAM through pores (**Figure 4.6**). These pictures were in accordance to the SEM pictures of the digested pellets from stool samples of human subjects (**Figure 3.3A**). Therefore, a lower pH value than the dissolution pH value of shellac is obvious for humans, but due to the porous shellac, coating a sufficient release of the water-soluble vitamin niacin is assumed. This could be more problematic for low water-soluble substances.

8.3 *In vitro* dissolution test and *in vivo* bioavailability

In **Paper I** and **Manuscript II** the hypothesis (**Hypothesis 3**) was confirmed that the adjustment of the shellac coating for niacin by *in vitro* assay enables a targeted release in the ileocolonic region in humans. The *in vitro* assay simulated the pH conditions and residence times in the human stomach, small intestine and ileum. The gastric residence time was

reduced to 60 – 90 min due to the fact that microcapsules smaller than 2 mm, as in the present study, are able to pass the pylorus independently from gastric emptying. After 60 minutes, the pH value was enhanced to 4.5 for 30 min to simulate the transition from fasted to fed status. During this phase no or only minor amounts (maximum 10 %) of niacin should be liberated. The small intestine was represented at a pH value of 6.8 for 2 h. During this pH value a slowly release of niacin was desired (up to 40 %) due to a documented comparable pH value in the target region of the ileum. Finally, the ileum was simulated with a pH value of 7.4 for 1.5 h, which should lead to a total release of the encapsulated niacin. The transit times and pH values simulating the gut from stomach to ileum are based on human studies with comparable pellet sizes in the literature (Abrahamsson et al., 1996; Clarke et al., 1993; Davis et al., 1987; Dressman et al., 1990; Evans et al., 1988; Ibekwe et al., 2008; Russell et al., 1993). According to this profile, which was hypothesized to give a precise prediction of the release behavior *in vivo*, the coating formulation to the encapsulation of NA and NAM was adapted. The applied shellac amount, drying time and coating composition to achieve a sufficient barrier with gastric and small intestine resistance was based on previous experiments with the reference model substance RMSD. These experiments showed that a shellac w.g. of 15 % could not lead to a gastric resistance. A w.g. of 20 % shellac resulted in a sufficient release protection under simulated gastric condition, whereby the release at pH 6.8 was low. Therefore, a triple coating layer with an intermediate pH-modulating subcoating was developed, which should lead to a precise release in the ileocolonic region (**Chapter 8.4.4**). Further transfer on the bioactive substances NA and NAM required an adjustment of the coating formulation to the properties of NA and NAM, which also influenced the release profile (**Chapter 8.4**). However, the adaption of the coating formulation resulted in an *in vitro* profile with no release of NA and NAM at the simulating gastric conditions (pH 1.4 and pH 4.5); followed by a beginning release at pH value 6.8 (NA: 6 % and NAM: 35 % after 2 h) and a burst release at pH value 7.4 (**Figure 3.2C+D**), which suggested a targeted release in the human ileocolonic region.

The human study, conducted by the research group of Prof. Dr. Laudes (**Chapter 3**), confirmed the designed *in vitro* model and the ileocolon-targeted release of niacin. Further, it suggested a comparable diffusion driven release from the microcapsules as hypothesized after the *in vitro* dissolution test. These observations based on the pharmacokinetic curves of free and microencapsulated NA and NAM, which were administered to 10 healthy subjects each. NAM serum levels were measured hourly for 8 h and after 10 and 12 h. No flush symptoms occurred, although microencapsulated vitamin doses up to 300 mg NA were administered.

High blood concentrations of niacin are associated with facial flushes and liver dysfunctions (SCF, 2002). The microencapsulation of NA resulted in no significant increase of the serum concentration compared to the reference dose (free NA) due to the triple shellac coating. This would explain the absence of side effects. Furthermore, the pharmacokinetic curve of NAM showed four-times lower niacin serum levels (AUC: 4,207.15 vs. 17,334.20) and ten-times lower C_{max} (1,322.7 vs. 19,690.0 µg/l) for the dose of microencapsulated NAM (900 mg) compared to the same reference dose (900 mg free NAM). In addition to the reduced niacin absorption, also delayed serum peak levels were observed for microencapsulated NAM after 4 – 8 hours in contrast to 1 h for free NAM (**Figure 3.3B and 4.7**). The delayed peak levels and the low AUC levels indicated a negligible release in the first 3 h and a slow diffusion driven release process of NAM after 3 – 4 h over a longer period, which corresponds to the ileocolonic region regarding the average human gastrointestinal transit time (Clarke et al., 1993; Pišlar et al., 2015). This observation is in accordance with the *in vitro* release profile and confirms the ability of the developed assay to predict an ileocolon targeted release *in vivo*. However, a reduced affinity to NAM carriers in the colon compared to the small intestine could also influence the lower systemic availability (Kumar et al., 2013; Nabokina et al., 2005). Although the NAM serum level were affected by inter- and intraindividual variations (**Table 4.3**), the AUCs under application of 3000 mg NAM was not significantly increased compared to the reference dose. Furthermore, t_{max} was only slightly affected (**Table 4.2**), which could be explained by the use of the multiparticular dosage form, which is less influenced by fasted or fed state regarding its transit time during gastric and small intestine (Clarke et al., 1993; Davis et al., 1987; Dressman et al., 1998; Ibekwe et al., 2008; Malagelada et al., 1984). Therefore, a high availability of niacin for the gut microbiome was proposed for the novel shellac encapsulation of niacin, which was confirmed by Fangmann (Fangmann, 2017). Microencapsulated NA led to a significant increase in *Bacteroidetes* abundance in stool samples (**Figure 3.3C**). Fangmann also demonstrated (**Chapter 3)**, that the microencapsulated NA beneficially affected biomarkers such as myostatin, fetuin-A and osteopontin, which are associated with insulin resistance of skeletal muscle and liver as well as adipose tissue inflammation (Hittel et al., 2010; Ishibashi et al., 2010; Lund et al., 2009; Park et al., 2015; Stefan et al., 2006). This effect is attributed to an indirect effect via beneficial changes of the microbiome (Fangmann, 2017) and thus a targeted release in the ileocolonic region.

8.4 Modeling and controlling intrinsic factors influencing the release profile of the encapsulated compound

In **Paper III**, it was hypothesized (**Hypothesis 4**) and confirmed that a precise targeting can be achieved by modulating simultaneously the influencing intrinsic factors of release. Further, it was confirmed that the main intrinsic factors are determined by the properties of the encapsulated compounds such as the intrinsic pH value, the water solubility and the surface structure and influences the shellac coating properties and the resulting release profile. Furthermore, the effect of the encapsulated compound such as water solubility and molecular weight was also evaluated producing pectin-zein hydrogel beads in **Manuscript V**. In case of shellac coated NA and NAM microcapsules, it was confirmed, that different subcoatings of the triple shellac coating can precisely modulate the release profile by counteracting the influence of the encapsulated compound. This was achieved by controlling the pH-dependent swelling and dissolution of the surrounding shellac layer (**Paper III**).

8.4.1 Influence of the water solubility and the molecular weight

In case of the produced pectin hydrogel beads by ionotropic gelation, the high water solubility of NAM and the low molecular weight (MW) of niacin from about 122 g/mol were the leading factors for the low encapsulation efficiency (EE) and the fast release (**Table 7.2 and Figure 7.17**). NAM, the encapsulated compound with the highest water solubility of 1000 g/l, resulted in the lowest EE of 19 or 4 %, depending on the one-step or two-step production process; followed by β-lactoglobulin (β-LG) in form of whey protein isolate (WPI) with a lower solubility (about 30 g/l) and an EE of 21 and 15 %. NA showed the highest EE of 43 % for the one-step procedure due to the lowest water solubility of 18 g/l. Substances with a high solubility in the cross-linking solution lead to high diffusion loss during bead formation (Jyothi et al., 2010; Munjeri et al., 1997). Furthermore, niacin loaded pectin hydrogel beads always resulted in an immediate release after 30 min under gastric conditions, independently of the production process, the coating concentration, the pectin type and concentration or the addition of WPI-fibrils as fillers. Thus, the hypothesized effect of the morphology on the release profile was not confirmed for niacin. In contrast, the encapsulation of WPI/β-LG were resistant at a pH value of 3.5 for 1 h with an increasing release up to 92 % at a pH value of 7.4 over a period of 4 h (**Figure 7.7**). Furthermore, the more spherical particles resulted in a slower release compared to the red blood cell shaped particles. This indicated an effect of the morphology on the release profile, whereby the effect is attributed to the properties of the

encapsulated compound. Substances with a high solubility and a low MW facilitates an easy diffusion through small pores, cracks and a swollen pectin gel, which was also documented for anthocyanin molecules with a MW of 449 Da (Chotiko and Sathivel, 2017; Munjeri et al., 1997). β-LG has a MW of about 18 kDa, which delayed the release. This also explains, why the encapsulation of the low water soluble indomethacin and of bovine serum albumin (BSA) with a high MW of about 66.5 kDa resulted in a colon-targeted release while using the same one-step encapsulation method as in the present thesis (Liu et al., 2006). A targeted delivery using pectin hydrogel beads is depending on the water solubility and the MW of the encapsulated compound and is more efficient for compounds with low solubility and higher molecular weight.

The water solubility of the compound also influenced the release profile using shellac as coating material administered by fluidized bed coating. Therefore, RMSD, NA and NAM were coated on Cellets to directly compare their influence on the applied shellac coating. In contrast to NA and RMSD pellets, single shellac coated NAM Cellets resulted in a premature release under gastric conditions (**Figure 5.3 and 5.5**). NAM is the vitamin with the highest water solubility of 1000 g/l compared to RMSD (50 g/l) or NA (18 g/l). The higher the water solubility the higher the interaction between an aqueous coating material and the compound itself. In the present thesis, the water-soluble ammonium salt of shellac was used, which could induce channel formations in the coating layer. This can lead to an increased water influx, dissolution and release of the compound (Guo et al., 2002; Heinämäki et al., 1994). Furthermore, in case of highly water soluble substances the amount of dissolved drug in the core increases, which is associated with a higher osmotic driven influx rate of water through the coating and finally resulting in a faster release (Ragnarsson et al., 1992).

8.4.2 Influence of the surface characteristics of the encapsulated compound

The formation of channels or infiltration of coating material is enhanced for porous subsurfaces and can lead to a reduced coating thickness (Chopra et al., 2002; Guo et al., 2002; Heinämäki et al., 1994; Tunón et al., 2003; Werner et al., 2007). SEM pictures of the surface from RMSD, NA and NAM coated Cellets showed a porous and irregular surface for NAM (**Figure 5.2B and 5.4B+C**). Further, the cross-section of triple coated NAM pellets showed no distinguishable coating layers in comparison to RMSD and NA coated pellets, which indicated an incorporation of the coating material into the existing NAM pores during the

coating process (**Figure 5.6B**). DSC measurements indicated that NAM was present in its crystalline form and not in an amorphous form (**Figure 5.2A and 5.4A**), which could induce a higher surface roughness (Murr and Inal, 1979). Therefore, the physical form and the resulting surface characteristic is of immense importance.

8.4.3 Influence of the aqueous pH value of the encapsulated compound

Due to the pH-dependent dissolution of shellac, the pH value of the encapsulated compound can also influence the dissolution of shellac and therefore its release (Menge, 2016; Ozturk et al., 1988). This was demonstrated by microscope pictures in the present study (**Chapter 5**), the swelling of the single shellac layer at pH value 7.4 was reduced if the acidic NA was encapsulated compared to the neutral to slightly alkaline RMSD and NAM (**Figure 5.7**). A reduced swelling and dissolution of the coating layer results in a lower water uptake and therefore in a lower dissolution and release of the encapsulated compound (Farag and Leopold, 2011a).

8.4.4 Adaption of a pH-modulating subcoating to control the effect of the encapsulated compound

A triple coating, consisting of an inner shellac coating, an intermediate subcoating and an outer shellac coating, was able to counteract the influence of RMSD, NA or NAM to achieve an ileocolon-targeted release *in vitro*. In case of NAM Cellets, the intermediate subcoating contained citric acid. Citric acid was already described to prolong the release of shellac encapsulated theophylline when applied as a subcoating due to a decreased intrinsic pH value which delayed the dissolution of shellac (Farag and Leopold, 2011a). In the present study, the citric acid coating reduced the swelling of shellac at pH value 7.0 and 7.4 (**Figure 5.9**) and therefore was able to prevent the premature release of the high water soluble and crystalline NAM. Depending on the citric acid concentration, the *in vitro* dissolution test showed gastric resistance with a slow increasing release at a pH value of 6.8 and a total release after reaching a pH value of 7.4 (**Figure 5.5D**).

In contrast, RMSD and NA with a lower water solubility showed only minor amounts of released vitamin at pH value 6.8, when a single shellac layer was applied. Therefore, a novel alkaline intermediate subcoating including sodium bicarbonate was applied. The sodium

bicarbonate led to an increased intrinsic pH value, which accelerated the swelling and dissolution of shellac at a pH value of 6.8 and finally increased the release of NA or RMSD (**Figure 5.8**). With the optimal sodium bicarbonate concentration in the intermediate subcoating, the release at pH value 6.8 was enhanced from 4 to 29 % for NA and from 10 to 24 % for RMSD after 2 h compared to the single shellac coating (**Figure 5.5C and 5.3B**).

The interplay between the encapsulated substance, the coating and the intermediate subcoating is crucial for the development of targeted release systems by influencing the pH-dependent swelling and dissolution of shellac. In the present thesis, it was shown that the adapted intermediate subcoating to the encapsulated compound, embedded from an inner and outer shellac coating, resulted in a controlled and precise release of NA and NAM in the ileocolon region *in vitro* and *in vivo*. The inner shellac coating also led to a more separated effect of the encapsulated compound and the effect of the intermediate subcoating (**Figure 5.5E**). This shellac microcapsule formulation and composition is patented for the topical intestinal delivery of niacin (PCT/EP2017/058733, **Supplemental 11.2**).

8.5 NAM pellets produced by a two-step wet extrusion-spheronisation process

In **Paper IV**, it was hypothesized (**Hypothesis 5**) and confirmed that the wet extrusion and spheronisation process is superior compared to the spray granulation in order to obtain high yield of spherical NAM pellets requiring lower addition of shellac. A mixture of NAM and MCC was extruded and afterwards spheronized in order to obtain spherical and smooth particles. The influence of the process parameter on the roundness, roughness and useable yield were evaluated in an experimental design (DoE) (**Chapter 6**). The main effect was reported for the moisture content of the extrudates and the spheronisation speed, time and load. In order to get spherical particles, the water content of the extrudates needed to be adjusted in a narrow range to generate an optimal brittleness and plasticity to form spherical particles (**Figure 6.4**) (Dukić-Ott et al., 2009). Only spherical particles provide a uniform distribution of the coating material. NAM required a low water content (23.6 % ref. to total mass), due to loss of solid by dissolution of NAM during the extrusion process (Jover et al., 1996; Lustig-Gustafsson et al., 1999). This is attributed to the high water-solubility of 1000 g/l of NAM. Regarding the spheronisation process, a combination of a high speed, a low load and a short rotation time resulted in the highest yield with the most spherical and smooth particles. The effect of the spheronization process on the morphology of the particles is

mainly attributed to the resulting plate-particle and particle-particle-interactions, which varied with the spheroniser speed, load and time. Low rotation speed results in low interactions and therefore in failure of rounding the particles (Baert et al., 1993; Dukić et al., 2007; Krueger et al., 2013; Newton et al., 1995; Sovány et al., 2016). In addition, for a higher load a longer spheronisation time is needed to form round particles (Newton et al., 1995), which makes an adjustment of the spheroniser speed, time and load indispensable. The yield of the extrusion-spheronisation process was markedly higher (92 %) than for the spray granulation process. The larger diameter of about 1.2 mm, measured by laser diffraction method, resulted in a lower specific surface area. The coating level was calculated as used by Farag and Leopold (2011b) with an additional roughness factor (+ 54 % additional coating material), due to an observed higher roughness of the extruded particles compared to the spray granulated particles (**Figure 6.11 and 6.13**). The additional coating material was not required and resulted in higher coating levels for the extruded particles, which led to a reduced NAM release (**Figure 6.15C**). However, an ileocolonic release was achieved and required a lower use of shellac as coating material based on the NAM content. Related to 100 g NAM, the required addition of shellac was reduced from 64 g to 34 g for spray granulated and wet extruded NAM pellets, respectively. Due to the resulting thicker coating level for extruded pellets, it is proposed that the required amount could further be decreased to about 22 g of shellac per 100 g NAM.

8.6 Closing remarks and outlook

The present thesis clearly indicated that the design of a specific *in vitro* digestion for the ileocolon target region enables to precisely modulate the coating of various vitamins. The encapsulation of niacin by a shellac based coating inhibited *in vivo* an early systemic uptake and a precise release in the ileocolonic region as it was proposed by the *in vitro* release profile. The targeted release of nicotinic acid led to beneficial effects on the gut microbiota, confirming again the successful targeting of the ileocolonic region. The application of different subcoatings enabled to precisely modulate the release profile by affecting the swelling and dissolution of the shellac coating and to counteract interfering effects of the encapsulating substance. *In vitro* dissolution tests promised a robust release in the ileocolonic region for a broad range of gastrointestinal variations regarding pH value and residence time. The presented system is of high practical relevance and resulted in the following patent (PCT/EP2017/058733, **Supplemental 11.2**). The high potential of the proposed targeting

system is demonstrated by the following patent for dye-based chromoendoscopy (PCT/EP2017/058741, **Supplemental 11.3**). In the future, NAM cores produced by wet extrusion/spheronisation instead of spray granulation could lead to a less cost-intensive process due to higher pellet yield and lower amounts of shellac required for coating. Until now, pectin-zein hydrogel beads were not able to substitute shellac as colon-delivery system, which is related to the high water solubility and low molecular weight of niacin and not to the system itself.

Nonetheless, some further questions arose during this work. The release of the encapsulated compound through the shellac coating was strongly depending on the compound itself and on the surrounding conditions. It is of interest to get further insights in direct effects on the shellac film properties such as the water vapor, water and acid permeability. Therefore, free films could be prepared and analyzed. Additionally, the investigation in different methods e.g. Electron Paramagnetic Resonance (EPR) may clarify the release mechanism from shellac coated particles with respect to different formulations and treatments.

Further developments of pectin hydrogel beads for the encapsulation and colon delivery of niacin is interesting. Therefore, the influence of drying conditions, coating materials and addition of fillers on the bead formation and release profile could be investigated.

As indicated in the present thesis, the upscaling of the wet extrusion/spheronisation process for preparing NAM cores could cause problems due to the narrow water level range. Therefore, it is of interest to develop an inline measurement to control the moisture content and moisture distribution e.g. by using near-infrared spectroscopy (NIRS) as a process analytical technology (PAT).

8.7 References

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9 Summary

The aim of the present thesis was to enhance the bioavailability of the water-soluble vitamin niacin in the ileocolonic region by microencapsulation with food-grade materials. The targeted release should beneficially influence the gut microbiota. Therefore, processes were developed to form spherical pellets (spray granulation and wet extrusion-spheronisation), coated pellets (fluidized bed coating) and hydrogel beads (ionotropic gelation) as carrier for niacin. To precisely predict the release profile and to modulate the processes an *in vitro* digestion test was designed. For the ileocolon target area, the test considered relevant pH values in the digestion fluids and anticipated the transit times in the gastrointestinal tract. The precision of the release profile of niacin from these shellac coated microcapsules was confirmed in a human bioavailability study with ten healthy subjects for each group examined by the research group of Prof. Dr. Matthias Laudes (**Chapter 3 and 4**). The effects of the encapsulated compound in interaction with an adapted intermediate subcoating was characterized for a targeted release in the ileocolonic region, which gave further insights in the pH-dependent dissolution mechanism of shellac (**Chapter 5**). Two production processes (spray granulation and wet extrusion-spheronisation) for high NAM loaded cores were compared regarding their properties for the application of the functional shellac coating (**Chapter 6**). An alternative system, based on pectin-zein hydrogel beads, was evaluated regarding a colon-targeted delivery (**Chapter 7**).

The *in vitro* release profile of shellac coated niacin microcapsules was precise and showed an efficient protection during the simulated fasted and fed gastric phase (pH 1.4 and 4.5) for at least 2 h. The release of NA or NAM began at a pH value of 6.8 due to an increased swelling of the shellac coating, which led to liberation of niacin by diffusion driven processes. Reaching the pH value of 7.4 of the human ileum, a complete release of encapsulated niacin was observed, which is related to the exceeded dissolution pH value of shellac. The designed *in vitro* dissolution test provided a targeted release of niacin in the ileocolonic region, which was confirmed by the pharmacokinetic curves of the human bioavailability study. In comparison to the free NAM, the same dose of microencapsulated NAM showed a delayed t_{max} (1 vs. 4 h), decreased C_{max} and decreased AUC levels. The increase of the dose for microencapsulated NA and NAM resulted in no significant higher AUC levels compared to the reference dose. These results indicated a diffusion driven release from the microcapsules in the ileocolonic region with a low systemic absorption of niacin, which makes it available

for local action in the colon. Microencapsulated NA led to an increase in *Bacteroidetes* and a beneficial effect on systemic insulin resistance.

The precise targeted release in the ileocolonic region was reached by adaption of the coating formulation on the varying properties of NA and NAM. It was shown that the combination of the aqueous pH value, the water solubility, and the crystallinity of the encapsulated substance are factors influencing the release profile. The application of an intermediate subcoating surrounded by an inner and an outer shellac layer was able to control these effects. The sodium bicarbonate subcoating enhanced the release of compounds with a relatively low water solubility such as NA, due to an increased intrinsic pH value and therefore a faster dissolution of the outer shellac coating. In contrast, citric acid reduced the swelling of shellac, delayed the release profile and counteracted a premature release of the alkaline and highly soluble NAM. Independently of the NAM core production process, both spray granulation and wet extrusion/spheronisation resulted in spherical NAM pellets, which were suitable for the coating process and an ileocolon targeted release profile. However, the resulting larger particles of the wet extrusion/spheronisation process reduced the requirement of shellac concentration, which could reduce the production process costs.

In contrast to the modified shellac coating, pectin-zein hydrogel beads were not able to protect niacin during the simulated gastric residence time. The vitamins were completely liberated within 30 minutes. This was explained by the high solubility combined with the low molecular weight (≈123 Da) of niacin, which supported a high diffusion through the pores and cracks in the coating. The larger molecule β-lactoglobulin (≈ 18 kDa) was prevented from a premature release using pectin-zein hydrogel beads. Therefore, an effect of the hydrogel bead morphology on the release profile was only observed for β-lactoglobulin, whereby the more spherical beads delayed the release in the simulated small intestine.

In conclusion, the microencapsulation of niacin using a shellac based coating lead to an enhanced availability of niacin in the human ileocolonic region. The designed in vitro dissolution test provided a precise instrument to predict the release behavior in humans. The usage of food-grade materials provides the potential use as nutraceutical especially with regard to beneficially effect on the gut microbiome and the prevention of prediabetes.

10 Zusammenfassung

Ziel der vorliegenden Arbeit war es, die Bioverfügbarkeit des wasserlöslichen Vitamins Niacin im Bereich des Ileocolons durch den Prozess der Mikroverkaselung zu erhöhen. Die gezielte Freisetzung sollte die Darmmikrobiota günstig beeinflussen. Daher wurden verschiedene Verfahren entwickelt, um Niacin zu verkapseln. Das Vitamin wurde entweder als kugelförmige Pellets verkapselt, die mittels Sprühgranulation und Nassextrusion-Sphäronisierung hergestellt und mittels Wirbelbettcoating beschichtet wurden oder es wurde in Hydrogelkügelchen mit Hilfe der ionotropen Gelierung eingelagert. Um das Freisetzungsprofil genau vorherzusagen und zu modulieren, wurde ein *in vitro* Verdauungstest entwickelt. Für den Zielbereich des Ileocolons berücksichtigte der Test relevante pH-Werte in den Verdauungsflüssigkeiten sowie die Transitzeiten im Magen-Darm-Trakt. Die Präzision des Freisetzungsprofils von Niacin aus Schellack-beschichteten Mikrokapseln wurde in einer humanen Bioverfügbarkeitsstudie mit zehn gesunden Probanden pro Gruppe durch die Arbeitsgruppe von Prof. Dr. Matthias Laudes (**Kapitel 3 und 4**) bestätigt. Die Effekte der verkapselten Substanz in Wechselwirkung mit einer angepassten Zwischenschicht wurden für eine gezielte Freisetzung in der Region des Ileocolons charakterisiert, was weitere Einblicke in den pH-abhängigen Auflösungsmechanismus von Schellack lieferte (**Kapitel 5**). Zwei Produktionsverfahren (Sprühgranulation und Nassextrusion-Spheronisierung) mit einer hohen Beladung von NAM im Kern wurden hinsichtlich ihrer Eigenschaften für die Anwendung der funktionellen Schellackbeschichtung verglichen (**Kapitel 6**). Ein alternatives System, basierend auf Pektin-Zein-Hydrogel-Kügelchen, wurde hinsichtlich einer zielgerichteten Freisetzung im Colon untersucht (**Kapitel 7**).

Das *in vitro* Freisetzungsprofil von Schellack-beschichteten Niacin-Mikrokapseln war präzise und zeigte einen wirksamen Schutz während der simulierten Magenpassage für mindestens 2 Stunden sowohl unter nüchternen als unter nicht-nüchternen Bedingungen (pH 1,4 und 4,5). Die Freisetzung von NA oder NAM begann bei einem pH-Wert von 6,8, was durch erhöhtes Quellen der Schellackbeschichtung zu erklären ist. Die Freisetzung des Niacin scheint dadurch diffusionsgetrieben zu sein. Bei Erreichen eines pH-Wertes von 7,4, wie er im menschlichen Ileum vorliegt, wurde die gesamte Menge des verkapselten Niacin freigesetzt, was mit dem Erreichen des Auflösungs-pH-Wertes von Schellack zusammenhängt. Das entwickelte *in vitro* Freisetzungprofil versprach eine gezielte Freisetzung von Niacin in der Region des Ileocolons, was durch die pharmakokinetischen Kurven der humanen

Bioverfügbarkeitsstudie bestätigt wurde. Im Vergleich zum freien NAM zeigte die gleiche Dosis von mikroverkapseltem NAM einen verzögerte Absorptionszeitpunkt (t_{max}) (1 vs. 4 h), verringerte maximale Serumkonzentrationen (C_{max}) und verringerte AUC-Spiegel. Die Erhöhung der Dosis für mikroverkapselte NA und NAM führte zu keinen signifikant höheren AUC-Werten im Vergleich zur Referenzdosis. Diese Ergebnisse verwiesen auf eine diffusionsgetriebene Freisetzung aus den Mikrokapseln im Bereich des Ileocolons mit einer geringen systemischen Absorption von Niacin, wodurch das Niacin für lokale Wirkungen im Dickdarm zur Verfügung steht. Mikroverkapseltes NA führte *in vivo* zu einem Anstieg der *Bacteroidetes* und einer positiven Wirkung auf die systemische Insulinresistenz.

Die präzise und zielgerichtete Freisetzung im Bereich des Ileocolons wurde durch Anpassung der Coatingformulierung an die variierenden Eigenschaften von NA und NAM erreicht. Es wurde gezeigt, dass die Kombination des pH-Wertes, der Wasserlöslichkeit und der Kristallinität der eingekapselten Substanz Faktoren sind, die das Freisetzungsprofil beeinflussen. Die Anwendung einer Zwischenschicht, die von einer inneren und einer äußeren Schellackschicht umgeben ist, konnte diese Effekte steuern. Die Natriumbicarbonatschicht erhöhte die Freisetzung von Substanzen mit einer relativ geringen Wasserlöslichkeit, wie NA, aufgrund eines erhöhten intrinsischen pH-Wertes und daher einer schnelleren Auflösung der äußeren Schellackbeschichtung. Im Gegensatz dazu reduzierte Zitronensäure die Quellung von Schellack, verzögerte das Freisetzungsprofil und wirkte einer vorzeitigen Freisetzung der alkalischen und gut löslichen NAM entgegen. Unabhängig vom NAM-Kernherstellungsverfahren ergaben sowohl die Sprühgranulation als auch die Nassextrusion/Sphäronisation sphärische NAM-Pellets, die für den Beschichtungsprozess geeignet waren, und eine Freisetzung im Ileocolon ermöglichten. Die resultierenden größeren Partikel des Nassextrusions-/Spheronisierungsverfahrens verringerten jedoch die benötigte Schellackmenge, was die Produktionsprozesskosten verringern könnte.

Im Gegensatz zu der modifizierten Schellackbeschichtung waren Pektin-Zein-Hydrogelpartikel nicht in der Lage Niacin während der simulierten Verweilzeit des Magens zu schützen. Die Vitamine wurden innerhalb von 30 Minuten vollständig freigesetzt. Dies wurde durch die hohe Löslichkeit in Kombination mit dem niedrigen Molekulargewicht (≈123 Da) von Niacin erklärt, das eine hohe Diffusion durch die Poren und Risse in der Beschichtung induzierte. Eine frühzeitige Freisetzung des größeren Moleküls β-Lactoglobulin (~18 kDa) konnte hingegen mit Pektin-Zein-Hydrogelpartikeln entgegengewirkt werden. Daher wurde auch ein Effekt der Morphologie der Hydrogelpartikel auf das Freisetzungsprofil

nur für β-Lactoglobulin beobachtet, wobei die runderen Kügelchen die Freisetzung im simulierten Dünndarm verzögerten.

Zusammenfassend kann gesagt werden, dass die Mikroverkapselung von Niacin unter Verwendung einer Schellack-basierten Beschichtung zu einer verbesserten Verfügbarkeit von Niacin in der menschlichen Ileocolon-Region führt. Das konzipierte in vitro Freisetzungsprofil erwies sich dabei als gutes Vorhersageinstrument für das Freisetzungsverhalten im Menschen. Die Verwendung von *food-grade* Materialien bieten eine potentielle Verwendung als Nutraceutical, insbesondere im Hinblick auf die vorteilhafte Wirkung auf das Darmmikrobiom und die Prävention von Prädiabetes.

11 Supplemental

11.1 Experimental and analytical approach for unpublished manuscripts

11.1.1 Fluidized bed coating for production of shellac coated niacin microcapsules

To influence the surface properties of particles with regard to aesthetic or protective purposes, a coating covering the surface of every single particle uniformly, can be applied. To coat small particles (100 µm to a few millimetres), usually the fluidized bed coater is used (Gouin, 2004; Kumpugdee-Vollrath and Krause, 2011; Sandadi et al., 2004; Teunou and Poncelet, 2002).

During fluidized bed coating, the small solid particles are suspended and maintained in a fluidized bed through a temperature-controlled airflow (bottom-up). The dissolved coating material is transferred to the nozzle with a peristaltic pump and atomized with compressed air. The atomized coating droplets are sprayed onto the flowing particles. The solvent of the coating material evaporates, while a polymer film is distributed on the particles surface (Arshady, 1993; Desai and Jin Park, 2005; Kumpugdee-Vollrath and Krause, 2011). In the present study, the nozzle was placed on the bottom of the chamber and had a diameter of 0.5 mm. Depending on the process parameters such as inlet air temperature, inlet air velocity, spray rate, product temperature, atomization pressure of the coating solution and outlet air temperature, the fluidized bed operation and the coating efficiency is influenced. Therefore, similar process parameters were chosen to compare different processes and formulations. However, the process parameters need to be adjusted to the coating material used in order to avoid agglomeration (Arshady, 1993; Desai and Jin Park, 2005; Dewettinck and Huyghebaert, 1998). The amount of the applied coating material was calculated and reported as additional weight gain in percent. In the present study, a Mini Glatt fluidized bed coater (Glatt Ingenieurtechnik GmbH, Binzen, Germany) was used to microencapsulate nicotinic acid, nicotinamide or riboflavin with a shellac coating to achieve a targeted release. The inlet air temperature, the spraying rate, the inlet and atomizing air pressure were adaptable. The ranges, used in the present thesis, were adjusted to the process and are shown in **Table 11.1**. After the coating process, shellac coated pellets were dried at 50 °C for 1 h in order to remove some of the ammonium and to strengthen the coating layer.

Table 11.1: *Process parameter for fluidized bed coating used in present thesis*

Process parameter	Applied range
Inlet air temperature	39 – 45 °C
Spraying rate	0.6 – 1.2 g/min
Inlet air pressure	0.3 – 0.4 bar
Atomizing air pressure	0.5 – 0.7 bar

11.1.2 Spray granulation for production of nicotinamide cores

Spray granulation is a drying process of liquids with simultaneous formation of granules. Granulation is achieved by spray drying of the dissolved or suspended substance through a nozzle into a temperature-controlled chamber. In the further process, the particle size increases by surface layering, comparable to the fluidized bed coating process. In the end, the formed granules consist of the dry matter of the sprayed solution. Characteristically, granules show a well-rounded and uniform “onion skin” layered structure, which is ideal for a further protecting coating step and a high particle strength (Link and Schlünder, 1997). In the present thesis, the spray granulation of nicotinamide was done by Glatt Ingenieurtechnik GmbH (Weimar, Germany). They harmonised the process parameters to achieve spherical granules with a narrow particle distribution and a high load of nicotinamide. This was necessary to meet the requirements of a higher dose in the bioavailability study and a reduced number of capsules the subjects would have to swallow.

For spray granulation, a ProCell fluidized bed granulator with Vario 3 was used (Glatt Ingenieurtechnik, Weimar, Germany). 30 % (w/w) nicotinamide including 4 % (w/w) of hydroxypropyl methylcellulose (HPMC) was dissolved in tap water. The solution was sprayed through a nozzle. The process parameters of the spray granulation of nicotinamide are listed in **Table 11.2**. Afterwards the granules were coated in a fluidized bed coater as described above.

Table 11.2: *Process parameter for spray granulation of nicotinamide used in present thesis*

process parameter	applied range
inlet air temperature	80 – 90 °C
product temperature	50 °C
spraying rate	5 – 24 g/min
air rate	80 – 90 m^3/h
atomizing air pressure	2 – 2.8 bar

11.1.3 Extrusion-Spheronisation process for production of nicotinamide cores

During extrusion, a wet mass is shaped into long rods by passing an extruder screen. In the present thesis, a twin-screw extruder was used. Two screws mix the added powder (consisting of the drug and some pelletizing aids) and the granulating liquid (e.g. water), with simultaneous transfer of the wetted mass to the nozzle at the end of the barrel, where cylindrical extrudates are formed (**Figure 11.1**). The barrel can be tempered if necessary. After the extrusion process, the wet extrudates are transferred into a spheronizer. A spheronizer is a machine with a rotating cross-hatched friction plate, where the extrudates are broken up into smaller cylinders. With increasing spheronisation time, the small cylinders become spherical pellets, which is beneficial for further coating processes (Dukić-Ott et al., 2009; Vervaet et al., 1995). In the present thesis, the conditions of the extrusion and spheronization process were varied to examine their influence on the desired product profile of NAM cores for coating applications (e.g. roundness and roughness). Therefore, a Quality by Design approach with integrated statistical Design of Experiments was used (see below).

In general, the twin-screw extruder nano 16 from Leistritz (Nuremberg, Germany) with a 1mm diameter die in the end was used for all experiments. The spheronizer was custom-made with a cross-hatched plate and a diameter of 12 cm (**Figure 11.1**).

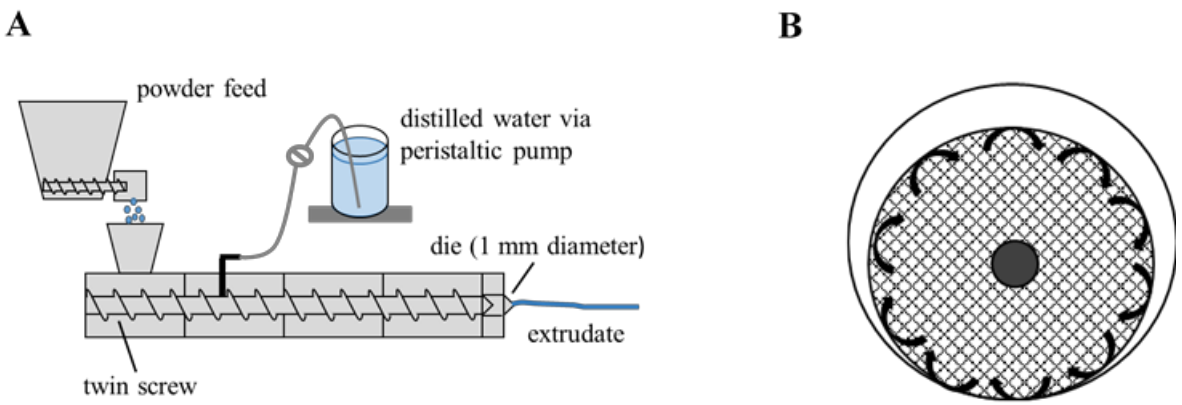

Figure 11.1: *Schematic demonstration of the (A) twin-screw extruder and (B) spheronizer used in the present study*

11.1.4 Ionotropic gelation for production of pectin-zein-niacin hydrogel beads

Ionotropic gelation is a commonly used procedure to prepare hydrogel beads, that contain bioactive substances. This technique is based on the ability of some polymers to spherical cross-link in the presence of counter ions. Examples for natural anionic polymers are alginates, gellan gum and pectin. In order to form beads, an aqueous solution of the polymer (and the desired compound to encapsulate) is added dropwise to a solution of counter ions. In case of an anionic polymer a cationic cross-linking solution is used (Patil et al., 2010; Patil et al., 2012; Smrdel, 2008; Sriamornsak, 2011). In the present thesis, pectin was used as anionic polymer for the encapsulation of the vitamins nicotinic acid or nicotinamide. The mixture of pectin and vitamin was dropped into a cationic cross-linking solution consisting of calcium chloride. In some cases, zein (corn protein) was added to the calcium chloride solution in order to cover the formed pectin beads (Liu et al., 2006). Pectin with a low degree of methoxylation forms gel beads by cross-linking free carboxyl groups with calcium ions. The formed calcium pectinate shows a reduced solubility. For incorporation of water-soluble substances such as nicotinic acid and nicotinamide, however, an additional coating is suggested. In the present thesis, the formed pectin-niacin-hydrogel beads were added to a solution, that contained zein to apply a coating layer. Afterwards, the pectin-niacin-zein-beads were dried at room temperature for further analyses.

11.1.5 Dissolution tests for characterization of vitamin release rate

In vitro dissolution tests are conducted to simulate and evaluate the dissolution and release behaviour of dosage forms throughout the human gastrointestinal tract. These studies are less labour, time and cost intensive compared to *in vivo* studies. Especially in early stages of formulation design and optimization, *in vitro* dissolution tests are extremely important (Jorgensen and Bhagwat, 1998; Shani-Levi et al., 2017; Zahirul and Khan, 1996). *In vitro* dissolution tests are performed in a dissolution test apparatus. Adapted to the formulation design, different dissolution specifications and the composition of the simulated gastrointestinal fluids are suggested by European Pharmacopeia. However, some specifications have to be decided case by case (Zahirul and Khan, 1996). In general, different stages of the gastrointestinal tract are simulated – stomach, small and large intestine. Depending on the simulated region, the test conditions vary regarding e.g. pH value, residence time, enzyme and salt concentration (Yang et al., 2002). For the coating material shellac, which dissolves depending on the pH value, the pH value and the residence time in the simulated gastrointestinal fluids were changed in the present thesis. With regard to the dissolution of pectin beads, microbial enzymes in the large bowel were considered (Guerra et al., 2012). Therefore, in the case of dissolution of pectin hydrogel beads, the enzyme pectinase was added to the simulated colon fluid.

In the present thesis the dissolution tester DT 70 (Pharmatest Group GmbH, Hainburg, Germany) was used with a paddle apparatus for shellac-coated pellets and with a basket apparatus for pectin hydrogel beads. Various simulated gastric, small intestine and large intestine fluids were tested to evaluate the release of the encapsulated vitamin over time. To quantify the released vitamin in the surrounding fluids, UV-VIS spectroscopy or HPLC analyses were used (see below). The amount of released vitamin was related to the total amount of encapsulated vitamin to obtain a percentage result.

To quantify the total amount of the encapsulated vitamin, shellac coated pellets were incubated for 1.5 h at pH value 7.4 (Soerensen phosphate buffer), which is above the dissolution pH value of shellac. Pectin-zein hydrogel beads were incubated for 5 h under agitation in a McIlvaine citrate-phosphate buffer (pH value 5.0) with additional pectinase to macerate the beads.

11.1.6 Light microscope/Optical microscope for characterization of particle shape

A light microscope or optical microscope is used to magnify images of small samples by visible light and a system of lenses. An image can be captured by normal cameras adapted to the microscope system. For the present thesis, light microscope pictures of shellac coated particles were taken to observe their swelling properties during contact with different solvents and to analyze the shape of pectin-niacin-zein hydrogel beads. Depending on the size of the particles, the following microscopes with different magnifications were used: Motic Microscope (Wetzlar, Germany), which was connected to a camera (Canon Legria HF20, Tokyo, Japan) and from Meade Instruments Europe GmbH (Kempten, Germany) in combination with an electronic eyepiece (MikroOkular-II, Bresser GmbH, Rhede, Germany).

11.1.7 Scanning electron microscope (SEM) for characterization of particle surface

A SEM is an electron microscope that scans the surface of a sample using a focused beam of electrons. Due to the interaction of the electrons with atoms in the sample, secondary electrons from the sample are generated and detected by a sensor that gives information about the surface of the sample such as their topography and composition. The detected signals are converted into an image of the sample. The resolution of a SEM is more than one nanometer (Bozzola and Russell, 2006). In the present study, SEM images are taken to compare surface characteristics of different shellac coated and vitamin coated pellets, as well as of the pectin-niacin-zein hydrogels. For SEM analyses, the samples were dried and prepared on a holder with carbon Leit-tabs (Plano GmbH, Wetzlar, Germany). To observe the cross-section, some of the beads were cut using a scalpel. Before examination in a Hitachi S-4800 SEM (Hitachi High Tech., Tokyo, Japan) at an accelerating voltage of 3 kV, microcapsules were sputter-coated with a layer of 8-10 nm gold-palladium using a Leica EM SCD 500 (Leica Microsystems GmbH, Wetzlar, Germany) high-vacuum sputter coater.

11.1.8 Differential scanning calorimetry (DSC) for characterization of physical form of vitamins

DSC is a thermoanalytical technique that measures thermal transitions in a material. More specifically, the difference in heat flow rate between a sample and a reference as a function of

temperature and time is analyzed. During the analysis, both the sample and the reference are exposed to a similar temperature profile: the temperature increases or decreases linearly as a function of time. Therefore, temperature-dependent properties of materials can be analyzed by DSC. This includes e.g. glass transition, crystallization, melting and decomposition (Waters GmbH). In the present thesis, the physical form (amorphous or crystalline) of the applied vitamin via fluidized bed coating was investigated using DSC. Therefore, about 4 mg of spray-dried vitamin, either riboflavin, nicotinic acid or nicotinamide, was weighed and sealed in hermetic aluminum pans. The samples were measured in triplicate under nitrogen atmosphere with DSC (Q2000, Waters GmbH, TA Instruments, New Castle, USA). The initial temperature was set to 20 °C, afterwards a ramp to 200 °C (or 300 °C in case of NA) was programmed with an increase of 20 K/min. Glass transition temperatures, melting and crystallization peaks were determined by TA Instruments software Universal Analysis 2000. The equipment was calibrated with indium. The baseline was generated without pan.

11.1.9 Viscometer for characterization of fluid characteristics (viscosity)

A viscometer is an apparatus to measure the viscosity of a fluid. The measured fluid remains stationary, while an object rotates in the fluid. The required force to turn the object at a known speed is a function of the fluids viscosity (Barnes et al., 1989). In the present thesis, the viscosity of the pectin solutions for production of hydrogel beads was measured before and after the addition of the vitamin nicotinamide, because it was hypothesized that the viscosity influences the bead formation. About 15 ml of the pectin(-niacin)-solution was added to the viscometer (Haake Viscotester iQ, Thermo Fisher Scientific, Waltham, USA) and measured at 20, 50, 100 and 200 rpm.

11.1.10 Laser diffraction analysis for characterization of particle size

Laser diffraction analysis gives information about the geometric dimension or size of the measured particles. Diffraction patterns of a laser beam pass through a sample, which consists of particles ranging from nanometers to millimeters. When the laser beam hits a particle, the light is diffracted, reflected, refracted and absorbed, which is detected by detectors. The particle size is inversely proportional to the angle of the scattered light: large particles scatter light at smaller angles while small particles scatter light through larger angles (Retsch

Technology GmbH, 2018). In the present thesis, uncoated and coated particles were measured with regard to their increase in diameter to calculate the coating thickness. Due to the use of dry particles, the measurements were performed using the dry feeder of the particle analyzer (Horiba LA-950V2, Retsch Technology GmbH, Haan, Germany) with vacuum-driven forced ejection, a vibration power alteration of 120 and a compressed air pressure of 0.3 MPa. The following refractive indices were used: cellulose: 1.63, shellac: 1.52, NA: 1.49 and NAM: 1.47. All samples were measured in triplicate. Afterwards the increase in particle diameter and radius were calculated to get the total coating thickness.

11.1.11 Sieve analysis for characterization of particle size distribution

Sieve analysis is a common practice to analyze the particle size distribution of granular material. The added material is separated into its fractions depending on the size. Therefore, sieves with different meshes are required. A representative weight of the sample is poured into the upper sieve with the largest mesh size. Each sieve below has smaller mesh openings. At the bottom, a receiver pan is placed. The sieve column is shaken to separate the sample according to size. Afterwards, the weight of the sample retained by each sieve is taken and divided by the total weight to obtain the percentage size fraction.

In the present thesis, dried pellets were analysed in a sieve shaker AS 200 control (Retsch GmbH, Haan, Germany) using sieve meshes of 2, 1.4, 1, 0.5 and 0.25 mm. The amplitude was set to 0.5 mm/”g” and the total sieve time was 3 minutes.

11.1.12 Moisture content quantification

The moisture content of solid samples can be examined in a drying oven by determining the weight loss. The sample is weighed into a tray and dried at 105 °C for 24 h. The sample is then cooled in a desiccator and weighed again. The weight loss is equated to the moisture content and expressed as a percentage of the total wet mass.

11.1.13 Ultraviolet-visible spectrophotometer (UV-VIS spectrophotometer) for vitamin quantification

UV-VIS spectrophotometry is routinely used for analytical chemistry for qualitative and quantitative determination of different substances (mostly dissolved in a solvent). This method refers to the absorption spectroscopy in the UV-VIS spectral region. Some molecules can absorb the energy in the form of ultraviolet or visible light of a suitable frequency. Then the molecule is raised from its ground state to an electronically excited state. Organic compounds can absorb light in the UV-VIS range (Hesse et al., 2005). Niacin, as used in the present thesis, has a maximum UV absorbance of 260 – 263 nm in water. One of the most common methods for niacin determination is based on the UV absorbance of NA and NAM. Therefore, a UV-VIS-spectrophotometer (McLaren et al., 1973; Sheen et al., 1992) or a high performance liquid chromatography (HPLC) combined with a diode array detector (DAD) is used (Kirchmeier and Upton, 1978; Sood et al., 1977; Walker et al., 1981).

In the present study, the UV/VIS-spectroscopy (Helios gamma, Thermo Fischer Scientific, Waltham, USA) was used to determine released niacin during the dissolution tests (see above). The wavelengths used were the absorption maxima for the measured substance/vitamin (niacin: 262 nm and riboflavin: 445.5 nm). Calibration curves for each of the fluids used were prepared for quantification of the vitamins, because the absorption is dependent on the pH value of the solvent, which was varied to simulate the gastrointestinal release (see above). If necessary, the solution was diluted to reach a measurable concentration.

11.1.14 High-performance liquid chromatography (HPLC) for vitamin and protein quantification

HPLC is an analytical technique to separate, identify and quantify components in a mixture. In principle, during HPLC analysis, a pressurized liquid (mobile phase) and a sample mixture is passed through a column, which is filled with a solid adsorbent material (stationary phase). Depending on the component properties, such as hydrophobicity, each compound in the sample interacts differently with the adsorbent material. This results in different elution times for the components and therefore leads to the separation of the sample mixture. The detector generates a signal proportional to the concentration of the eluded component, which allows quantification after previous calibration. For quantification, different detectors can be used.

The most common detector is a UV-VIS absorbance detector, also known as diode array detector (DAD) (Waters GmbH). The mechanism is the same as described for UV-VIS spectrophotometer. In the present thesis, the vitamin niacin was quantified at a wavelength of 262 nm. The analysis of niacin by HPLC was performed for quantification of the encapsulation efficiency and the vitamin release during dissolution test of pectin-niacin-hydrogel beads. The UV-VIS spectrophotometer was not applicable due to interferences of pectin in the absorption area.

The niacin samples were analyzed by RP-HPLC (reversed phase high performance liquid chromatography; mobile phase: polar; stationary phase: non-polar) using the Agilent 1100 Series with diode array detector (Agilent Technologies, Santa Clara, USA) and C18-Sphinx column (Nucleodur Sphinx RP, 5 µm, Macherey-Nagel, Düren, Germany). The injection volume was 10 µl at a flow rate of 1 ml/min using eluent A (0.1 % (v/v) TFA in milliQ water) and eluent B (0.1 % (v/v) TFA in acetonitrile) as mobile phase. The elution used gradient steps of 95 % A (0 – 1 min), 95 – 5 % A (1 – 10 min), 5 % A (10 – 10:50 min) and 5 – 95 % A (10:50 – 12 min). The column temperature was set to 20 °C and the detection wavelength was 262 nm.

For the determination of β-lactoglobulin, the samples were filtered through 0.2 µm syringe filters (PET-30/13 membrane, Macherey-Nagel GmbH & Co. KG, Düren Germany). Afterwards the measurements were performed using a Thermo Scientific Dionex UltiMate 3000 HPLC (Waltham, USA) with a diode-array detector and PLRP-S column (300 Å, 8 µm, 150 x 4.6 mm, Agilent Technologies, Santa Clara, USA). For the analytical separation the injection volume was 40 µl at a flow rate of 1 ml/min and a column temperature of 40°C using Eluents A (0.1 % (v/v) TFA in water) and B (0.1 % (v/v) in ACN). The elution used gradient steps of 35-38 % B (1-8 min), 38-42 % B (8-16 min), 42-46 % B (16-22 min), 46-100 % B (22-22.5 min) and 100-35 % B (23-23.5 min). The detection wavelength was 205 nm.

11.1.15 Encapsulation efficiency (EE) of the encapsulated compound in pectin-zein hydrogel beads

As mentioned above, the HPLC analysis was conducted to calculate the encapsulation efficiency of nicotinic acid, nicotinamide and β-lactoglobulin in the pectin-zein hydrogel beads. Therefore, the relation of actual active compound content in dried beads to the

theoretical maximal content of the compound was calculated according to the following equation:

$$EE\ [\%] = \left(\frac{actual\ concentration\ \left[\frac{mg}{ml}\right]}{sample\ weight\ [mg] * \left(\frac{added\ concentration\ [\frac{mg}{ml}]}{dry\ mass\ [\frac{mg}{ml}]} \right)} \right) * 100$$

To quantify the encapsulated vitamin, the dried hydrogel beads were incubated for 5 h under stirring in a McIlvaine citrate-phosphate buffer (pH value 5.0) with additional pectinase to macerate the beads and to get a total release of the encapsulated vitamin.

11.1.16 Quality by Design approach with Design of Experiment for analysis of interactions of process parameters

The Quality by Design approach is a quality assurance system and present in the pharmaceutical industry. It is used for pharmaceutical developments at a risk-based approach, where the effects of Critical Process Parameters (CPPs) and Critical Material Attributes (CMAs) and their interactions on the Critical Quality Attributes (CQAs) of the drug product are evaluated. It aims for a more flexible production process and the reduction of failure costs due to a design-space based production. The design space is the accepted region of the studied CQAs. Modifications of CPPs and CMAs within this space are not regarded as change and thus do not result in a different product (ICH Q8, 2009).

In order to study the interactions of CPPs and CMAs on the CQAs a Design of Experiments (DoE) can be done. A DoE is an essential tool for the QbD approach. A DoE is defined as a systemic way to vary process parameters (input or independent variables) and to analyze their effect on the output or response variables (dependent variables). Furthermore, the relationships between all variables and the random variability of the process will be determined while using the minimum number of experiments, compared to the One-factor-at-a-time method (Kleppmann, 2013).

In the present study, a full factorial design was used for the NAM core production by extrusion and spheronisation (see above), where all possible factor combinations are

examined. For the extrusion process two 2^2 full factorial design with one center point in duplicate were evaluated by varying the screw design, screw speed and feed rate of the twin-screw extruder. For evaluation of the spheronisation process, a 2^3 full factorial design by variations of the rotation speed, time and load was done. Therefore, the DoE software JMP Pro from SAS Institute (Cary, North Carolina, USA) was used.

11.2 Patent PCT/EP2017/058733: Shellac microcapsule formulations and compositions for topical intestinal delivery of vitamin B3

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

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(54) Title: SHELLAC MICROCAPSULE FORMULATIONS AND COMPOSITIONS FOR TOPICAL INTESTINAL DELIVERY OF VITAMIN B3

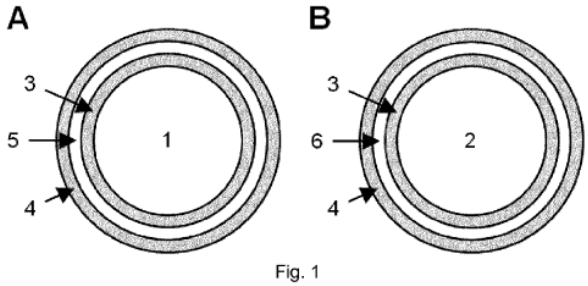

Fig. 1

(57) Abstract: The present invention relates to microcapsules comprising a core containing vitamin B3, which are characterised by a coating layer system comprising two layers of shellac and a pH-modulating substance provided between the two layers of shellac.

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(54) Title: SHELLAC MICROCAPSULE FORMULATIONS AND COMPOSITIONS

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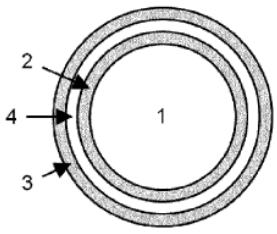

Fig. 1

11.4 References

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