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Aqueous two-phase systems for starch microsphere formulation and encapsulation of live bacteria

A phase behaviour perspective

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Aqueous two-phase systems for starch microsphere formulation and encapsulation of live bacteria

A phase behaviour perspective

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DEPARTMENT OF PROCESS AND LIFE SCIENCE ENGINEERING | LUND UNIVERSITY



Aqueous two-phase systems for starch microsphere formulation and encapsulation
of live bacteria - A phase behaviour perspective

Aqueous two-phase systems for starch microsphere formulation and encapsulation of live bacteria

- A phase behaviour perspective

Zandra Gidlöf



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DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Engineering at Lund University to be publicly defended on Friday 22 November at 9:00 in Lecture Hall KC:C, Kemicentrum, Lund

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Eric Bertoft

Former Associate professor at Åbo Akademi University, Finland

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Foundation for Strategic Research**Title:** Aqueous two-phase systems for starch microsphere formulation and encapsulation of live bacteria
- A phase behaviour perspective

Abstract: The human gastrointestinal tract (GIT) is home to a large community of microorganisms that contribute to human health. Delivering live bacteria to the gut for therapeutic purposes can thus be highly beneficial. However, delivering live biotherapeutic products or probiotic bacteria to the GIT presents challenges because the cells must remain viable during production, storage, and administration. Encapsulating the bacteria in starch microspheres is an interesting approach for this purpose. Starch microspheres can be produced in aqueous two-phase systems (ATPSs). These ATPS can be created by dissolving the starch in water along with polymers of a different chemical nature. Emulsification of the system can generate dispersed starch phase droplets in a continuous polymer phase. Here, the starch can crystallise into solid microspheres, by utilising the natural crystallisation ability of pre-gelatinised starch. The ATPSs can provide a gentle environment for sensitive compounds, such as biologics. Moreover, the digestion of starch in the GIT could potentially be utilised as an oral delivery mechanism for encapsulated cargo.

Starch microsphere encapsulation can thus be regarded as a promising concept in the fields of food and pharmaceutical formulation, and several investigations have been conducted to better understand starch microsphere preparation in ATPSs. However, there is still limited knowledge regarding the starch microsphere formation and how to control the preparation process. One area that has received little focus is phase behaviour in relation to starch microsphere preparation, despite phase behaviour being a fundamental aspect of ATPS science. Moreover, numerous different polymer combinations can make up ATPSs, but polyethylene glycol (PEG) has generally been used as a continuous phase polymer during ATPS starch microsphere preparation.

Therefore, this thesis aims to derive new knowledge about the preparation and formation of starch microspheres in ATPSs and relate this to the phase behaviour of the systems. Our research explored how different parameters (such as the type and size of polymers in the continuous phase) influence ATPS phase behaviour and starch microsphere formation. This thesis also presents a novel method to monitor the starch particle formation process, imaging the crystallisation of starch phase droplets *in-situ*. The findings highlight the potential of utilising different continuous phase polymers, as well as the importance of the phase behaviour (such as water distribution in the ATPS, which is essential for starch crystallisation). Moreover, the thesis demonstrates that it is possible to encapsulate live bacteria in starch-based ATPS, offering a promising concept for further development in food and pharmaceutical sciences.

Keywords: Starch microspheres, Starch microparticles, Microencapsulation, Aqueous two-phase systems, ATPS, Hydrolysed waxy barley starch, PEG, PVP, PEtOx, Phase behaviour, Water distribution, Water activity, Starch crystallisation, Light microscopy, Confocal Raman microscopy, WAXS, Isothermal calorimetry, SEM, DSC, *L. reuteri*, Probiotics, Live biotherapeutic products.

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- A phase behaviour perspective

Zandra Gidlöf



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*“If we knew what it was we were doing,
it would not be called research, would it?”*

– often credited to Albert Einstein

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Abstract

The human gastrointestinal tract (GIT) is home to a large community of microorganisms that contribute to human health. Delivering live bacteria to the gut for therapeutic purposes can thus be highly beneficial. However, delivering live biotherapeutic products or probiotic bacteria to the GIT presents challenges because the cells must remain viable during production, storage, and administration. Encapsulating the bacteria in starch microspheres is an interesting approach for this purpose. Starch microspheres can be produced in aqueous two-phase systems (ATPSs). These ATPS can be created by dissolving the starch in water along with polymers of a different chemical nature. Emulsification of the system can generate dispersed starch phase droplets in a continuous polymer phase. Here, the starch can crystallise into solid microspheres, by utilising the natural crystallisation ability of pre-gelatinised starch. The ATPSs can provide a gentle environment for sensitive compounds, such as biologics. Moreover, the digestion of starch in the GIT could potentially be utilised as an oral delivery mechanism for encapsulated cargo.

Starch microsphere encapsulation can thus be regarded as a promising concept in the fields of food and pharmaceutical formulation, and several investigations have been conducted to better understand starch microsphere preparation in ATPSs. However, there is still limited knowledge regarding the starch microsphere formation and how to control the preparation process. One area that has received little focus is phase behaviour in relation to starch microsphere preparation, despite phase behaviour being a fundamental aspect of ATPS science. Moreover, numerous different polymer combinations can make up ATPSs, but polyethylene glycol (PEG) has generally been used as a continuous phase polymer during ATPS starch microsphere preparation.

Therefore, this thesis aims to derive new knowledge about the preparation and formation of starch microspheres in ATPSs and relate this to the phase behaviour of the systems. Our research explored how different parameters (such as the type and size of polymers in the continuous phase) influence ATPS phase behaviour and starch microsphere formation. This thesis also presents a novel method to monitor the starch particle formation process, imaging the crystallisation of starch phase droplets *in-situ*. The findings highlight the potential of utilising different continuous phase polymers, as well as the importance of the phase behaviour (such as water distribution in the ATPS, which is essential for starch crystallisation). Moreover, the thesis demonstrates that it is possible to encapsulate live bacteria in starch-based ATPS, offering a promising concept for further development in food and pharmaceutical sciences.

Popular scientific summary

This thesis describes the scientific work conducted during my PhD project. As in most projects, this was not a one-person job, but rather the result of many people working together. We explored the formulation of so-called starch microspheres in water-based systems. We also investigated how various parameters affect the formation of starch microspheres and demonstrated a novel application of incorporating live probiotic bacteria into these starch-based particles.

The goal of pharmaceutical formulation is to combine different chemical substances and active drugs to produce a complete medicinal product. Ideally, the formulation should offer good stability, be cost-effective, and be sustainable. More importantly, it must ensure the delivery of the active ingredient to the correct location in the body. Therefore, the medicinal product should be in a dosage form well-suited to its route of administration. Preferably, this route should be patient-friendly, promoting good compliance in pharmaceutical therapy. From a compliance perspective, the oral route is generally preferred. However, recent developments of more complex active pharmaceutical components (such as peptides, proteins, and live cells) have introduced challenges in achieving satisfactory stability and administration options.

Probiotics (and their pharmaceutical counterpart live biotherapeutic products) in the form of bacterial cells present a particular challenge, as the bacteria need to remain viable during production, storage, and administration. The human gastrointestinal tract (GIT) is nonetheless home to a large community of microorganisms, and the gut microbiota has been recognised as having an important and previously underestimated role in human health. This underscores the benefit of delivering live bacteria to the gut for therapeutic purposes.

Starch is an interesting food and pharmaceutical ingredient for oral delivery of live bacteria. Starch molecules make up large chains of repetitive units, known as polymer chains. In starch, the polymers are made up of glucose molecules that are linked together. The starch polymers are naturally digested in the GIT by the body's own enzymes. Digestion of starch can, therefore, be explored as a delivery mechanism in the GIT. Starch can also be considered a safe, abundant, and sustainable material. When treated appropriately, starch polymers can be made to crystallise after being heated in the presence of water, a phenomenon known as retrogradation or recrystallisation. This process involves the gradual rearrangement of starch polymers into more ordered (semi-crystalline) structures. The material hardens as the starch molecules reform, which can be exemplified by a slice of baked bread gradually going from soft and moist to hard and dry. While this phenomenon may be somewhat unwanted in your daily bread, it can be utilised to produce starch microspheres.

Starch microspheres can be produced using a technology called aqueous two-phase systems (ATPSs). An ATPS forms naturally when large starch polymers are

dissolved in water together with polymer chains of a different chemical nature, such as polyethylene glycol (PEG) polymers. If the concentrations of the polymers are high enough, the system separates into two water phases, where one phase contains mostly starch polymers and the other mainly PEG polymers. By applying gentle agitation to the ATPS, the starch phase can be dispersed as emulsion droplets in a continuous PEG phase. Here, the starch phase droplets can form solid microspheres as the starch crystallises, similar to how soft bread hardens. This creates an interesting matrix for microencapsulation in food and pharmaceutical formulation with complex active components, such as biomolecules or live bacteria.

Several investigations have been conducted to better understand starch microsphere preparation in ATPSs. However, there is still limited knowledge regarding the starch microsphere formation process and how different factors influence the preparation. One area that has received little focus is phase behaviour in relation to starch microsphere preparation, despite phase behaviour being a fundamental part of ATPS science.

Therefore, this thesis aims to derive new knowledge about the preparation and formation of starch microspheres in ATPSs, and relate this to the phase behaviour in these systems. We investigated how parameters such as water content and type of polymers in the continuous phase influence ATPS phase behaviour and starch microsphere formation, whereupon the most promising formulations were used to explore microencapsulation of probiotic lactic acid bacteria.

This thesis presents a novel method to follow the starch microsphere formation process, imaging the crystallisation of starch phase droplets *in-situ* using a technique called confocal Raman microscopy. The thesis also highlights the importance of considering ATPS phase behaviour (such as the water distribution between the phases) during starch microsphere formulation. We found that the distribution of water is essential for the starch crystallisation rate, and this distribution can be linked to water activity in the individual polymer materials. The ATPS phase ratios, viscosity, and mixing of the phases during the preparation of microspheres were also found to be important factors to consider, as they affected the size and stabilisation of the starch droplets.

Moreover, the thesis demonstrates how microspheres can be prepared in starch-based ATPSs with different continuous phase polymers by adapting the polymer sizes and concentrations in relation to the ATPS phase behaviour. Lastly, this thesis shows how this formulation approach can be used to encapsulate live bacteria in starch microspheres. More development is needed to obtain a dry dosage form with a prolonged shelf life. Nonetheless, this research demonstrates proof of a promising concept for further formulation development within food and pharmaceutical science.

Populärvetenskaplig sammanfattning

Denna avhandling beskriver det vetenskapliga arbetet som utförts inom mitt doktorandprojekt. Som i de flesta projekt var detta inte ett enmansjobb, utan snarare resultatet av många människors samarbete. Vi utforskade formuleringen av så kallade stärkelsemikrosfärer i vattenbaserade system. Sammantaget har vi undersökt hur olika parametrar påverkar bildningen av stärkelsemikrosfärer, samt visat en ny tillämpning av att inkorporera levande probiotiska bakterier i dessa stärkelsebaserade partiklar.

Målet med farmaceutisk formulering är att kombinera olika kemiska ämnen och aktiva komponenter för att producera en komplett läkemedelsprodukt. Det är önskvärt att skapa formuleringar med god stabilitet, som är kostnadseffektiva och hållbara. Viktigast av allt är att de måste säkerställa leveransen av den aktiva ingrediensen till rätt plats i kroppen. Därför bör läkemedelsprodukten vara i en doseringsform som passar dess administreringsväg. Helst bör denna väg vara patientvänlig, vilket främjar god följsamhet inom farmaceutisk terapi. Ur ett följsamhetsperspektiv föredras i allmänhet oral administrering. Utvecklingen av mer komplexa aktiva farmaceutiska komponenter, såsom peptider, proteiner och levande celler, har emellertid introducerat nya utmaningar för att uppnå tillfredsställande stabilitet och administrationsalternativ.

Probiotika (och den farmaceutiska motsvarigheten levande bioterapeutiska produkter) i form av bakterieceller, är extra utmanande eftersom bakterierna behöver hållas vid liv under produktion, lagring och administrering. Den mänskliga mag-tarmkanalen utgör dock ett hem för ett stort samhälle av mikroorganismer, och tarmfloran har visat sig ha en viktig och tidigare underskattad roll i människors hälsa. Detta betonar den potentiella nyttan med att leverera levande bakterier till tarmen för terapeutiska ändamål.

Stärkelse är ett intressant material för oral leverans av levande bakterier till kroppen. Stärkelsemolekyler består av stora kedjor av repetitiva enheter, kända som polymerkedjor. I stärkelse är polymererna gjorda av glukosmolekyler som är länkade till varandra. Stärkelsepolymererna bryts naturligt ner i mag-tarmkanalen av kroppens egna enzymer. Nedbrytningen av stärkelse har därmed potentialen att nyttjas som leveransmekanism i mag-tarmkanalen. Stärkelse kan också betraktas som ett säkert, rikligt förekommande och hållbart material. När stärkelsepolymererna behandlas på rätt sätt kan de kristallisera efter att ha upphettats med vatten, ett fenomen känt som retrogradering eller rekristallisering. Denna process involverar gradvis omorganisering av stärkelsepolymerer till mer ordnade (semikristallina) strukturer. Materialet hårdnar när stärkelsemolekylerna omordnas, vilket kan exemplifieras av en skiva bakat bröd som gradvis går från mjukt och fuktigt till hårt och torrt. Även om detta fenomen kan vara något oönskat i ditt dagliga bröd kan det utnyttjas för att producera stärkelsemikrosfärer.

Stärkelsemikrosfärer kan produceras i något som kallas vattenbaserade två-fassystem (ATPS). Ett ATPS bildas naturligt när stora stärkelsepolymerer löses i vatten tillsammans med polymerkedjor av en annan kemisk natur, såsom polyetylenglykol (PEG). Om koncentrationerna av polymererna är tillräckligt höga separeras systemet i två vattenfaser, där den ena fasen mestadels innehåller stärkelsepolymerer och den andra mestadels innehåller PEG-polymerer. Genom lättare omrörning kan stärkelsefasen dispergeras som emulsionsdroppar i en kontinuerlig PEG-fas. Här kan stärkelsefasdropparna bli till solida mikrosfärer när stärkelsen kristalliserar, liknande hur mjukt bröd hårdnar. Dessa processer gör stärkelse till en intressant matris för mikroinkapsling inom mat- och farmaceutisk formulering med känsliga komponenter, såsom biomolekyler eller levande bakterier.

Flera vetenskapliga studier har undersökt hur framställning av stärkelsemikrosfärer i ATPS fungerar. Det finns dock fortfarande bara begränsad kunskap om hur stärkelsemikrosfärer bildas och hur olika faktorer påverkar produktionen. Ett område som fått relativt lite fokus är fasbeteende i relation till framställning av stärkelsemikrosfärer, trots att fasbeteende är en grundläggande del av vetenskapen om ATPS. Därför var målet med denna doktorsavhandling att ta fram ny kunskap om framställning och bildning av stärkelsemikrosfärer i ATPS, samt relatera detta till fasbeteendet i dessa system. Vi undersökte hur parametrar såsom vattenhalt och olika polymertyper i den kontinuerliga fasen påverkar fasbeteende och stärkelsemikrosfärernas bildning, varpå de mest lovande formuleringarna tillämpades för mikroinkapsling av probiotiska mjölksyrabakterier.

Denna avhandling presenterar en ny metod för att följa bildningsprocessen av stärkelsemikrosfärer, genom avbildning av kristallisationen i stärkelsegel-droppar *in-situ* med en teknik som kallas konfokal Raman-mikroskopi. Avhandlingen belyser också vikten av att ta fasbeteende (såsom fördelningen av vatten mellan faserna) i beaktning under formulering av stärkelsemikrosfärer. Vi fann att fördelningen av vatten är avgörande för stärkelsens kristalliseringstakt, och denna fördelning kan kopplas till vattenaktiviteten i de enskilda polymermaterialen. Det visade sig att fasförhållandena, viskositeten och blandningen av faserna under mikrosfärtillverkningen också var viktiga faktorer att överväga, eftersom de påverkade storleken och stabiliteten av stärkelsedropparna.

Avhandlingen visar också hur stärkelsemikrosfärer kan framställas i ATPS med olika polymerer i den kontinuerliga fasen, genom att anpassa polymerstorlekarna och koncentrationerna till fasbeteendet. Slutligen belyses hur ATPS kan användas för att kapsla in levande bakterier i stärkelsemikrosfärer. Mer utveckling behövs för att erhålla en torr doseringsform med förlängd hållbarhet. Dock demonstrerar denna forskning ett lovande koncept för vidare utveckling inom mat- och farmaceutisk vetenskap.

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List of Papers

Paper I: Utilising phase diagram to understand barley starch microsphere preparation in an aqueous two-phase system

Zandra Gidlöf, Betty Lomstein Pedersen, Lars Nilsson, Anita Teleman, Marie C. Wahlgren, Anna Millqvist-Fureby. (2023), In: *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 658, 130652. <https://doi.org/10.1016/j.colsurfa.2022.130652>

Paper II: Starch microsphere preparation and phase behaviour in aqueous two-phase systems – effect of continuous phase polymer

Zandra Gidlöf, Josefine Andersson, Lars Nilsson, Randi Nordström, Marie C. Wahlgren, Anna Millqvist-Fureby, *Manuscript*

Paper III: Kinetics of formation of starch microspheres produced in aqueous two-phase systems

Zandra Gidlöf, Illia Dobryden, Lars Nilsson, Jens Sommertune, Wei Zhao, Marie C. Wahlgren, Randi Nordström, Anna Millqvist-Fureby, *Manuscript*

Paper IV: Aqueous two-phase systems for encapsulation of live bacteria in starch microspheres

Zandra Gidlöf, Sebastian Håkansson, Lars Nilsson, Carsten Ravn, Marie C. Wahlgren, Anna Millqvist-Fureby, Randi Nordström, *Manuscript*

Other related publications

The Impact of Formulation and Freeze Drying on the Properties and Performance of Freeze-Dried *Limosilactobacillus reuteri* R2LC.

Nisha Tyagi, Zandra Gidlöf, Daniel Tristan Osanlío, Elizabeth S. Collier, Sandeep Kadekar, Lovisa Ringstad, Anna Millqvist-Fureby, Stefan Roos. (2023), In: *Applied Microbiology*, 3(4), 1370-1387. <https://doi.org/10.3390/applmicrobiol3040092>

Chapter 20 - The use of starch microspheres and nanoparticles in pharmaceutical applications.

Anna Millqvist-Fureby, Zandra Gidlöf, Marie Wahlgren. (2024), In: *Starch in Food (Third Edition)*, 3, 487-501, ISBN 9780323961028. Editor: Lars Nilsson. <https://doi.org/10.1016/B978-0-323-96102-8.00020-6>

Author's contribution to the papers

Paper I

I participated in the study design along with my supervisors. I performed most of the experimental work, and I participated in the asymmetric flow field-flow fractionation measurements and WAXS measurements. I performed the data analysis together with my supervisors. I drafted the manuscript and finalised it together with my supervisors.

Paper II

I designed the study with support from supervisors. I performed most of the experimental work together with my master's student, Josefine Andersson, and I participated in the WAXS measurements. I performed the data analysis together with my supervisors. I wrote the first version of the manuscript and finalised it together with my supervisors.

Paper III

I designed the study with input from my supervisors and co-authors. I performed most of the experimental work, and I participated in the WAXS measurements and the isothermal calorimetry measurements. I performed the data analysis together with my co-authors. I wrote the first version of the manuscript and finalised it together with my supervisors.

Paper IV

I designed the study with input from my supervisors and co-authors. I performed most of the experimental work, and I participated in the WAXS measurements. I performed the data analysis. I wrote the manuscript with input from my supervisors and co-authors.

Abbreviations

API	Active pharmaceutical ingredient
ATPS	Aqueous two-phase system
CFU	Colony-forming unit
DSC	Differential scanning calorimetry
GIT	Gastrointestinal tract
ΔH	Endothermic enthalpy of melting
Mw	Weight-average molecular weight
PEG	Polyethylene glycol
	PEG10: Mw 10 000
	PEG20: Mw 20 000
	PEG200: Mw 200 000
	PEG2mil: Mw 2 000 000
PVP	Polyvinylpyrrolidone
	PVP10: Mw 10 000
	PVP40: Mw 40 000
	PVP360: Mw 360 000
PEtOx	Poly(2-ethyl-2-oxazoline) Mw 50 000
SEM	Scanning electron microscopy
WAXS	Wide-angle X-ray scattering

1 Introduction

This thesis will explore the preparation of starch microspheres, via physical cross-linking in aqueous two-phase systems (ATPSs). The focus will mainly be on examining the starch microsphere formation in relation to ATPS phase behaviour and continuous phase polymers, but a novel application of probiotic encapsulation will also be explored.

1.1 Formulation and delivery of live cells to the gastrointestinal tract

The human body is home to more than 100 trillion microorganisms, with over 1000 species forming a complex community in the human gastrointestinal tract (GIT). This so-called gut microbiota has been recognised as an important and previously underestimated contributor to human health, highlighting the significant role of probiotics in the future prevention and treatment of disease (Afzaal et al., 2022; Wang et al., 2017). Probiotics, in the form of live bacterial cells, have shown promising results in the treatment of various conditions, such as gastrointestinal disorders (Rau et al., 2024) and strengthening the immune system (Abouelela & Helmy, 2024).

The evolving knowledge of microbiota-related health benefits has broadened the therapeutic spectrum, expanding from the general use of probiotics, defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” to further developments such as live biotherapeutic products, defined as “a biological product that contains live organisms; is applicable to the prevention, treatment or cure of a disease or condition of human beings; and is not a vaccine” (Martín & Langella, 2019).

However, delivering therapeutic cells to the gut in a viable state presents several challenges. Firstly, maintaining cell viability during the manufacturing and storage of the finished product is difficult, with viability losses being common (Broeckx et al., 2016; Korona-Głowniak et al., 2019). Secondly, ensuring that the cells reach the intended site, such as the small intestine, in a viable state is another hurdle. Oral administration is the most common and compliance-friendly method for delivering probiotics to the gut. Nonetheless, the passage from the oral cavity to the intestines

presents a hazardous route, including exposure to gastric acid, enzymatic attacks, and bile salts (Wendel, 2021). Lastly, there is also the matter of releasing the cells at the intended site. For this purpose, microencapsulation in polysaccharide matrices can help enhance the survival of bacterial cells and provide controlled release during administration (Lin et al., 2024; Razavi et al., 2021). Starch microspheres is an interesting material for this objective, as will be elaborated upon in the following section.

1.2 Starch: A multifunctional excipient

Starch is a versatile material with significant applications in both pharmaceutical and food formulations. It is considered a safe, abundant, and sustainable resource. Beyond its widespread use as a pharmaceutical excipient, starch is the primary energy source in the human diet (Copeland, 2020; Garcia et al., 2020).

Starch primarily consists of two types of glucose-based polymers: amylose and amylopectin. Amylose is a linear polymer linked by α -1,4-glycosidic bonds, while amylopectin is a branched polymer with both α -1,4- and α -1,6-glycosidic bonds (see **Figure 1**). Native starch is produced by plants in the form of semi-crystalline granules. The size of these granules and the ratio of amylopectin to amylose vary depending on the botanical source of the starch and can also differ within the same plant species. Starches with a high amylopectin content are known as waxy starches (Villa Zabala, 2020).

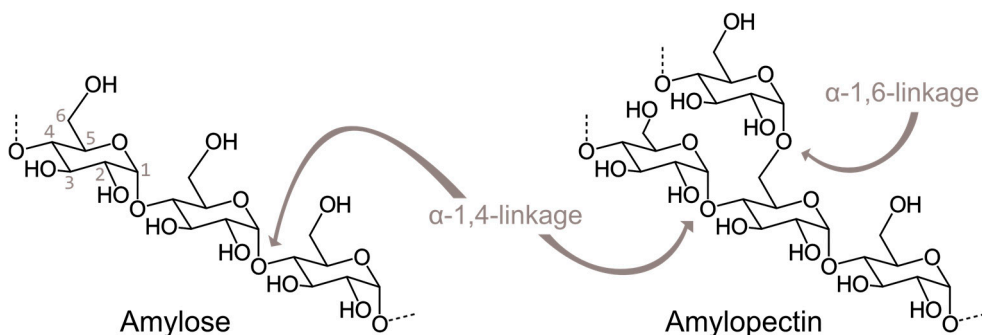


Figure 1. Molecular structure of starch polymers amylose and amylopectin.

One thing that makes starch a compelling material for encapsulation of sensitive active pharmaceutical ingredients (APIs) is its ability to recrystallise after having been gelatinised. Gelatinisation can be broadly described as the disruption of the crystalline order of starch granules when heated in the presence of excess water. The granules swell with water intake, some starch polymers leach out and get

solubilised, and the viscosity increases. Continued heating eventually leads to the bursting of granules, and more starch polymers can flow out into the water to form a disordered hydrogel. The viscosity of the gel will depend on factors such as the amount, size, and structure of the starch, as well as temperature and time (Biliaderis, 2009; Donmez et al., 2021). Subsequently, when the starch gel is cooled, it can gradually crystallise, a process also referred to as recrystallisation or retrogradation. During this process, disordered starch polymers partially rearrange into ordered structures that differ from the original granular arrangements. These polymers exclude some water to physically cross-link with each other, forming double helical arrangements. This process results in a gradual hardening semi-crystalline material. The crystallisation of starch gels can be seen as a continuing process, that generally starts with rapid crystallisation of amylose (within hours), followed by slower crystallisation of amylopectin (over days). Nonetheless, this process is complex and can be influenced by many factors, such as storage conditions, polymer chain length, starch source, water content, and additives (Biliaderis, 2009; Wang, 2020).

Efforts have been made to better understand and hinder starch crystallisation, such as when wanting to prevent bread staling (Cauvain, 2015). However, by dispersing pre-gelatinised starch gel droplets in an emulsion, we can exploit this crystallisation process to produce starch microspheres at mild temperature conditions. Having the starch droplets solidify via physical cross-linkage eliminates the need for additional chemical cross-linkers and harsh reaction conditions. Thus, the production of starch microspheres offers a gentle and promising method for encapsulation of sensitive APIs, such as biologics (Elfstrand et al., 2009b; Yang et al., 2020).

Another factor that makes starch interesting for pharmaceutical formulation is its digestion in the GIT. This generates the opportunity of having encapsulated cargo released in the small intestine as the starch polymers are hydrolysed by the body's digestive enzymes. Using microparticles instead of large tablets can also improve compliance within wider patient groups, and the transit time through the GIT can be more predictable (Aulton & Taylor, 2013; Wilson & Crowley, 2011).

Lastly, these starch microspheres can be produced in emulsified ATPSs, thereby eliminating the need for organic solvents and creating a gentle environment for biological compounds. An ATPS can be formed by mixing sufficient amounts of starch and another chemically incompatible polymer in water, which will be elaborated upon in the next section.

1.3 Aqueous Two-Phase Systems

ATPSs have a broad range of applications (Baghbanbashi et al., 2024; Esquena, 2016). The aqueous nature in both phases, which also generates low interfacial tension, provides a gentle environment for sensitive materials such as enzymes and cells (Albertsson, 1970). The phenomenon was first reported by Martinus Willem Beijerinck in 1896 when he mixed aqueous solutions of starch and gelatine and accidentally found the formation of an ATPS. However, it was several decades later that Per-Åke Albertsson discovered its application in the separation of biomolecules and cells (Albertsson, 1985).

There are different kinds of ATPSs, but the ATPSs used in this thesis are formed by dissolving sufficient amounts of two chemically different hydrophilic polymers in water. This results in a system with two immiscible aqueous phases. The composition of the phases will differ, with one phase being enriched in one polymer and the other phase enriched in the other polymer (as illustrated in **Figure 2**). The separation phenomenon can be broadly explained by the thermodynamic incompatibility of two polymers in a solvent, leading to segregative phase separation. The large size and rigidity of the polymers result in low entropy of mixing, making interactions between the system components a dominant factor in the free energy of mixing (Hatti-Kaul, 2000). The creation of two water phases can also be promoted by the excluded volume effect, here overlapping depletion zones of similar large polymers reject dissimilar polymers (de Kruif & Tuinier, 2001). However, phase separation and partitioning in ATPS are complex and not fully understood to this day, thereby limiting predictive models and necessitating empirical methods to study phase behaviour (Esquena, 2016; Zaslavsky, 1995).

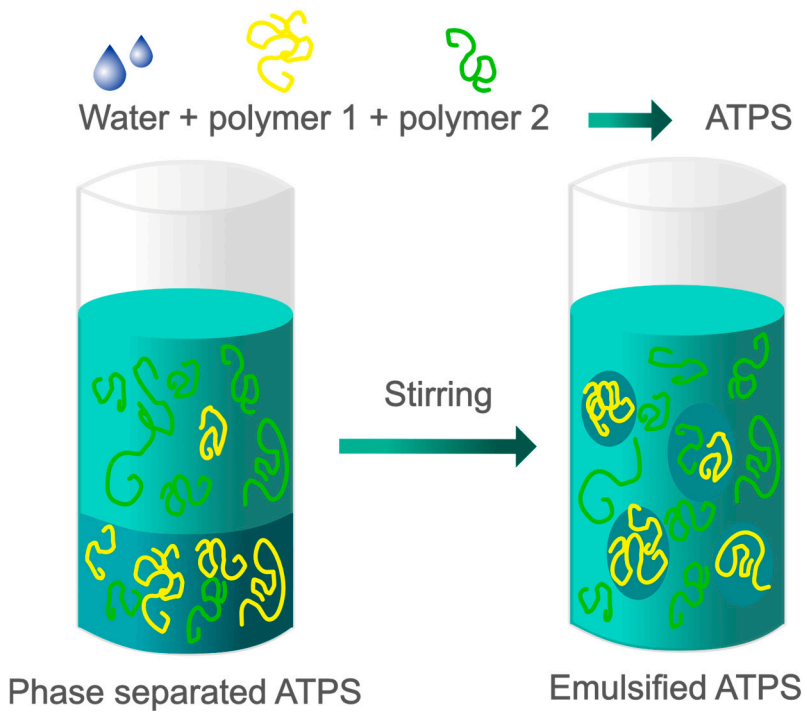


Figure 2. ATPS formed via segregative phase separation of chemically different polymers in water.

Rectangular phase diagrams are typically used to study and describe ATPSs and their phase behaviour under certain conditions. The concentrations of polymers are commonly expressed in weight percent (%w/w) (Albertsson & Tjerneld, 1994; Zaslavsky, 1995). **Figure 3** provides a general representation of an ATPS phase diagram. Generally, the X-axis shows the polymer enriching the bottom phase, while the Y-axis shows the polymer enriching the top phase (Millqvist-Fureby, 2014). The curved line that separates the compositions generating a one-phase system from those generating a two-phase system is known as the binodal curve. Above the binodal curve, two immiscible phases form, while below it, a one-phase system is obtained. At a given total system concentration (point A), the corresponding phase compositions can be found by following the tie line to the nodes on the binodal curve (points B and C). The composition of the phases will stay constant along the tie line, but the phase ratio will change if point A is moved to a different position on that tie line.

The phase ratio at ATPS composition point A can be estimated using the lever rule, which connects nodes B and C on the tie line (Iqbal et al., 2016; Millqvist-Fureby, 2014; Zaslavsky, 1995). The lever rule is expressed as:

$$(V_{\text{top}}\rho_{\text{top}}/ V_{\text{bottom}}\rho_{\text{bottom}}) = AC/BA$$

where V represents the phase volume, ρ represents the phase density and A is the total composition of the system, whereas AC and BA represent the distance along the tie line from point A to C and B to A, respectively (Iqbal et al., 2016; Kronberg & Wall, 2019).

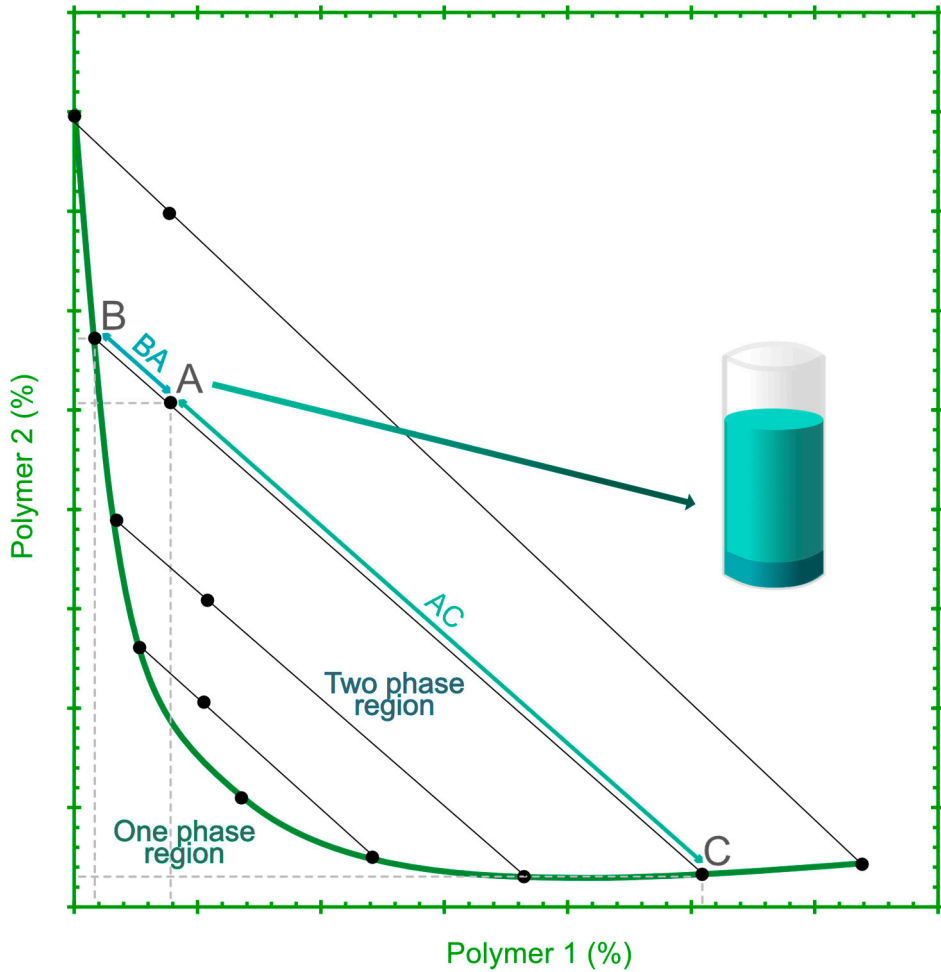


Figure 3. Schematic illustration of a typical ATPS phase diagram composed of polymers 1 and 2. Concentrations above the binodal curve form an ATPS. At a given total system concentration (point A), the corresponding phase compositions can be found by following the tie line to the nodes on the binodal curve (points B and C). The ratio of the phases can be estimated as the ratio of distance BA and AC.

1.4 Aqueous two-phase systems and starch microsphere preparation

Physically cross-linked starch microparticles can be manufactured by mixing pregelatinised starch and another complementary polymer in water. Gentle agitation disperses the starch gel as droplets in a continuous polymer phase, where these droplets can solidify via starch crystallisation. Several studies have explored this approach for preparing physically cross-linked starch microspheres. These investigations have included starches of different botanical origins, such as waxy maize (Elfstrand et al., 2006) and cassava (Xia et al., 2018), with moderate incubation temperatures, such as 4 °C (Xia et al., 2017; Yang et al., 2020), 25 °C (Li et al., 2018), alternating between 4 and 30 °C (Xia et al., 2018), and alternating between 6 and 37 °C (Elfstrand et al., 2009a). The effects of various factors on starch microsphere properties have also been studied, including starch material and molar mass (Elfstrand et al., 2006; Xia et al., 2017), excipients (Elfstrand, Eliasson, Jönsson, Larsson, et al., 2007; Zhang et al., 2024), temperature treatment programs (Elfstrand, Eliasson, Jönsson, Reslow, et al., 2007; Elfstrand et al., 2009a; Xia et al., 2018), and different continuous phase polymer concentrations (Li et al., 2018; Yang et al., 2020).

Despite these efforts, there remains a gap in understanding the starch microsphere formation process, the methods to control the production, and the influence of various factors on the preparation process. Notably, the ATPS phase behaviour, which is a crucial aspect of ATPS science, has been largely overlooked in the context of starch microsphere preparation. Furthermore, polyethylene glycol (PEG) is generally used as a continuous phase polymer in previous investigations, even though ATPSs can be formed from numerous different polymers (Pereira & Coutinho, 2020). Hence, it is essential to expand the variety of polymers utilised in starch microsphere preparation. Lastly, while ATPSs have been applied for the separation of cells (Albertsson, 1970), the potential of utilising ATPSs to encapsulate cells in starch microspheres remains rather unexplored. Thus, delving deeper into this application could uncover valuable insights.

1.5 Aim and objectives

The overall aim of this thesis was to derive new knowledge about the preparation of physically cross-linked starch microspheres in ATPSs, in relation to the phase behaviour in these systems, as well as to investigate the potential of utilising ATPS starch microsphere preparation for encapsulation of probiotics.

The objectives were to first investigate how different parameters, such as continuous phase polymers, influence ATPS phase behaviour, starch microsphere formation, and properties of starch microspheres. Following this, we explored whether the ATPS starch microsphere approach could be applied as a novel method for the microencapsulation of live lactic acid bacteria.

The specific aims of the papers included in this doctoral thesis are:

Paper I: Examine the formation and properties of starch microparticles, while mapping phase behaviour in a starch/PEG ATPS with different water content and phase ratios.

Paper II: Study starch microsphere preparation in starch-based ATPSs with different continuous phase polymer types and weight-average molecular weights (Mw), while controlling the water content in the phases.

Paper III: Investigate the kinetics of starch crystallisation and microsphere formation processes in ATPSs, while utilising different continuous phase polymers.

Paper IV: Explore the potential of ATPS starch microsphere preparation for microencapsulation of live bacteria. Examine how the addition of bacteria and cultivation medium affects starch microsphere preparation. Investigate bacterial encapsulation and viability in starch-based ATPSs, as well as effects of utilising different continuous phase polymers during encapsulation.

2 Materials and methods

This section will present a brief description of the main materials and methods used in the project. For additional methods and detailed instructions, the reader is referred to the methods sections in the individual papers.

2.1 Materials

2.1.1 Starch material

An acid-hydrolysed waxy barley starch was used in this thesis. Native waxy barley starch has a high content of amylopectin and has previously been reported to have an Mw of approximately 106×10^6 g/mol (Rojas et al., 2008). However, our material had been acid-hydrolysed by the supplier, cleaving the polymer chains to reduce the Mw. Reduction of the Mw enables easier handling, due to lower viscosity when boiling highly concentrated dispersion. However, controlling the Mw of the starch material also plays a crucial role during formulation development, influencing factors such as size, shape, yield, crystalline structure, and thermal properties of the starch microspheres. Additionally, other production parameters will interplay with the type of starch material used during the preparation, making the development more complex (Fureby et al., 2024). To focus on the other parameters, the experiments presented in this thesis were conducted with only this acid-hydrolysed waxy barley starch material, which was kindly provided by Lyckeby Starch AB (Product: Lyckeby PU 92–000, Sweden). In Paper I, asymmetric flow field-flow fractionation was used to determine the material Mw as approximately 9×10^5 g/mol.

2.1.2 Continuous phase polymers

The structure of the most promising continuous phase polymers investigated in this thesis can be viewed in **Figure 4**.

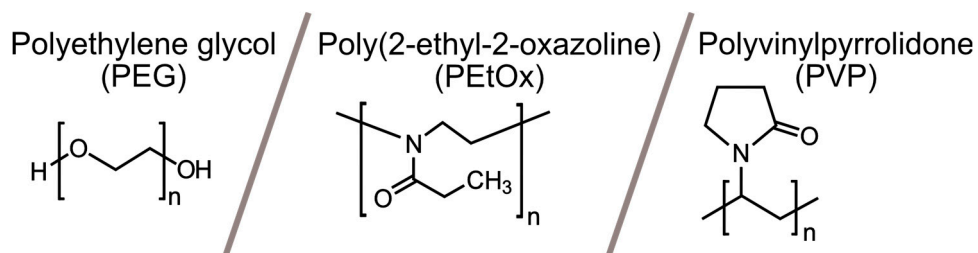


Figure 4. Molecular structure of continuous phase polymers.

PEG is a synthetic polymer that has wide applications within pharma, food, and cosmetics (Gaballa et al., 2024), and this polymer is generally the one used to make up the continuous phase during ATPS starch microsphere preparation (Elfstrand et al., 2009b; Punched-armon et al., 2020; Xia et al., 2018; Zhang et al., 2024). Nonetheless, there are compelling reasons for having other options for PEG in the production of starch microspheres for pharmaceutical use. The primary concerns revolve around PEG immunogenicity and the associated safety risks of antibody development and hypersensitivity reactions (Chen et al., 2021; Ganson et al., 2016; Judge et al., 2006; Savitz & Durning, 2011; Shah et al., 2013). Polyvinylpyrrolidone (PVP) is a synthetic polymer that has broad pharmaceutical usage (Luo et al., 2021). Poly(2-ethyl-2-oxazoline) (PEtOx) is a synthetic polymer that has demonstrated superior chemical stability, compared to PEG (Bauer et al., 2012; de la Rosa, 2014).

In this work, different Mw of these polymers were all acquired from Sigma-Aldrich, Merck KGaA, and were PEG Mw 10 000 (PEG10); PEG Mw 20 000 (PEG20); PEG Mw 200 000 (PEG200); PEG Mw 2 000 000 (PEG2mil); PVP Mw 10 000 (PVP10); PVP Mw 40 000 (PVP40); PVP Mw 360 000 (PVP360); PEtOx Mw 50 000 (PEtOx).

2.1.3 Bacteria

Limosilactobacillus reuteri is Gram-positive facultative anaerobic bacteria. These microbes can be found at various locations of the human body, including the GIT, and have also been linked to several health benefits in the scientific literature, such as strengthening the intestinal barrier and production of vitamins (Mu et al., 2018). *L. reuteri* DSM 17938 was used in this thesis. The bacteria were kindly provided by BioGaia AB (Sweden) as a freeze-dried powder, containing 2.4×10^{11} colony-forming units (CFU)/g. These rod-shaped cells can be viewed in **Figure 5**, displaying light microscopy captures of the bacteria in a wet state.

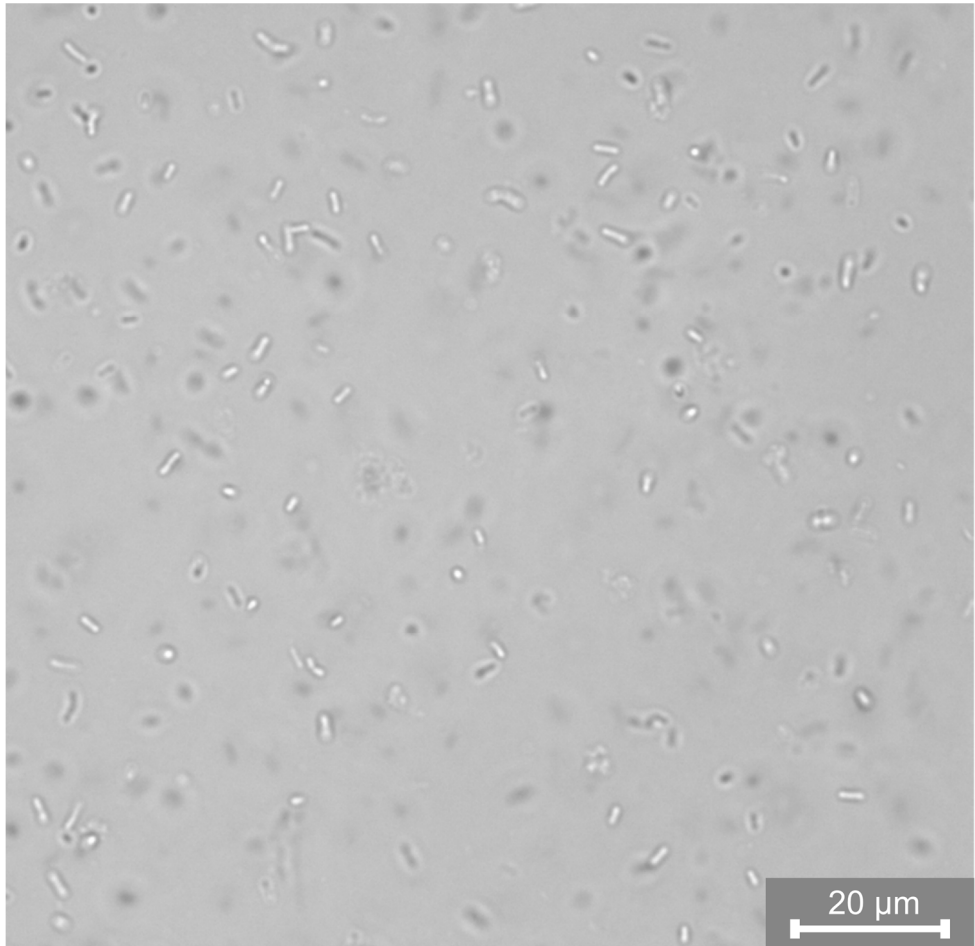


Figure 5. *L. reuteri* DSM 17938. The cells are dispersed in a phosphate buffer and captured with light microscopy.

2.2 Preparation of starch microspheres

Placebo starch microspheres were prepared by essentially the same protocol throughout Papers I-III. In Paper IV, the method was adapted to better accommodate the encapsulation of live bacteria.

2.2.1 Preparation of placebo starch microspheres

The preparation process of starch microspheres is illustrated in **Figure 6**. In essence, ATPSs were prepared in 15 mL centrifuge tubes by adding a pre-boiled starch dispersion to a complementary (continuous phase) polymer solution. The resulting ATPSs were emulsified using a high-shear mixer at 8000 rpm for 1 minute. Subsequently, the tubes were incubated in a vertical test tube rotator at 20 rpm for 24 hours at a constant temperature, being 25 °C in the majority of the experiments.

Following the 24-hour incubation, the solidified material was washed three times with MilliQ water via centrifugation. The newly washed microspheres were then transferred into glass vials using a 7 mL pasture pipette, discarding any material not fine enough to be transferred.

Lastly, the starch microspheres were freeze-dried for approximately 30 hours, including a 2-hour cooling step followed by an additional 2-hour holding period before entering primary drying. After the freeze-drying cycle was finished, the samples were sealed with bromobutyl rubber stoppers and stored over dried silica at ambient temperature between analysis procedures.

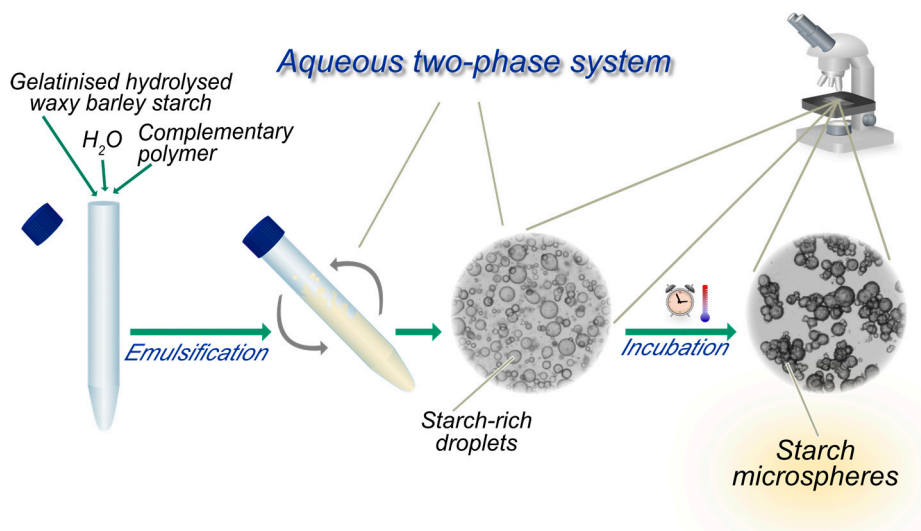


Figure 6. Schematic illustration of the starch microsphere preparation process.

2.2.2 Preparation of microspheres with encapsulated bacteria

The starch and polymer stocks used in Papers I-III were prepared in 0.9-1 (%w/w) NaCl solution. To better accommodate the lactic acid bacteria, the solution was modified by adding 1 part MRS broth (a cultivation medium) to 4 parts of the isotonic 0.9 % NaCl solution when the microbes were included in the formulations.

Alterations were also incorporated into the mechanical process when producing starch microspheres with bacteria. These included upscaling to larger (50 mL centrifuge) tubes, a gentler emulsification step (mixing with a spatula instead of a high-shear mixer), and flushing the formulations with N₂ gas before incubation and when sealing the freeze-dried vials.

Additionally, instead of MilliQ water, the solidified material was washed with a 10.3 (%w/w) sucrose solution. The sucrose solution was also used as the freeze-drying medium around the starch microspheres, as well as in a control formulation. This control formulation contained bacteria in NaCl and MRS solution without starch or polymers but was otherwise handled in the same manner as the ATPS formulations throughout the study.

The cells were included in the ATPSs by mixing them into the pre-boiled and cooled starch dispersion before combining with the polymer solutions. The added bacteria were equivalent to 1.1×10^9 CFU/g ATPS or control formulation (giving 2.4×10^{10} CFU/g starch).

2.3 Phase behaviour characterisation

The phase behaviour was initially characterised by mapping the phase diagram for a starch/PEG ATPS in Paper I. In Paper II, water distribution tie lines, viscosity, and water activity were examined when using different continuous phase polymers. Phase compositions in specific ATPSs with different polymers were also determined in Paper III. Additionally, starch phases were examined with light and confocal Raman microscopy, and these techniques will be elaborated upon in separate method sections.

2.3.1 Mapping of the phase diagram and phase compositions

In Paper I, samples for mapping the starch/PEG phase diagram were prepared by the same method as when manufacturing placebo starch microspheres. However, instead of emulsification with a high-shear mixer, the tubes were placed directly in the vertical test tube rotator for 1 hour at ambient temperature. After 1 hour of rotation, the phases were separated via centrifugation, and the water content was determined by thermogravimetric analysis (TGA) using a TGA 2 STARe System (Mettler-Toledo, Switzerland). A Total Starch Assay Kit AA/AMG (Megazyme Ltd, Ireland) was used to quantify the starch content, and the PEG content was calculated based on the quantified water and starch. The composition of the phases along with the total ATPS composition was used to plot tie lines, and the binodal curve was visualised based on these tie lines (illustrated in **Figure 7**).

Additionally, the phase compositions in selected ATPSs with PVP and PEtOx were determined by quantifying the nitrogen content in dried phase material (by Eurofins Food & Feed Testing Sweden AB (Lidköping, Sweden) according to ISO 16634). Thereafter, the starch content was calculated based on the quantified dry matter and polymer (in Paper III).

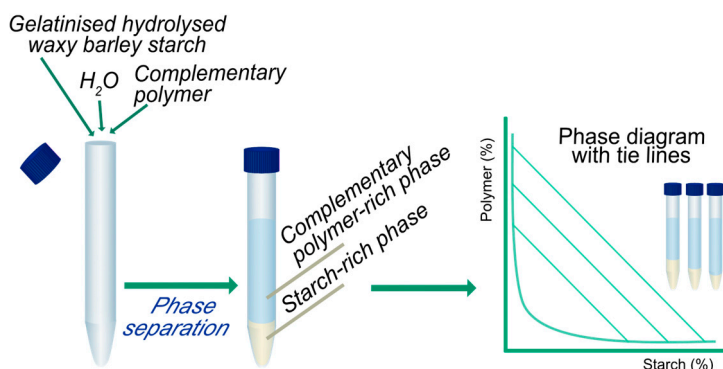


Figure 7. Schematic illustration of mapping phase diagram tie lines in an ATPS.

2.3.2 Water distribution

In Paper II, instead of mapping complete phase diagram tie lines, water distribution was characterised by mapping water distribution tie lines. These tie lines connect the solid contents in the respective phases, assuming that no starch is present in the polymer phase and no polymer is present in the starch phase. Water distribution tie lines provide a reasonable estimate of phase diagram tie lines in highly concentrated ATPSs where the binodal curve is located close to the axes of the diagram, as it is in these studies. Samples for mapping the water distribution were prepared in the same manner as for the phase diagram mapping, with the exception that only the water content was quantified, either by TGA or by drying the separated phases overnight at 105°C.

2.3.3 Viscosity and water activity determination

The viscosity of the continuous phase affects the hydrodynamics during both emulsification and incubation of the ATPSs, and it is influenced by the structure, size, and concentration of the polymers. In this project, the viscosity of separated complementary polymer phases was measured in a Kinexus rheometer (Model KNX2100, Malvern Instruments Ltd, UK). The viscosity of a sample was interpreted as the average shear viscosity in the Newtonian region of the shear rate range (1/s to 250/s) at 25°C (Paper II).

Water activity is the ratio between the partial vapour pressure of water as a solvent and the vapour pressure of pure water (Chang, 2005). It reflects the thermodynamic activity of water as a solvent in an equilibrated system. In this thesis, the water activity was determined for solutions with varying concentrations of polymers and pre-boiled starch, using an AquaLab 4 water activity monitor (METER Group, Inc. USA). MilliQ water with 0.9 % NaCl was used to prepare the solutions (Paper II).

2.4 Investigating the starch microsphere formation, starch crystallisation process, and starch microsphere properties

2.4.1 Light microscopy

Light microscopy observations were conducted using an Axioplan Universal Microscope for transmitted light and incident-light fluorescence (ZEISS, Germany). As the name implies, light microscopes magnify small objects by focusing light through the sample, and this tool was extensively utilised throughout Papers I, II, III, and IV. For instance, it was used to monitor the formation, aggregation, and swelling of starch microspheres, as well as to investigate bacterial partitioning, encapsulation, and release. Additionally, this simple technique provided quick, yet effective input during the screening of both different materials and process parameters, leading up to the results presented in this thesis.

As the author, I would therefore like to highlight the efficiency and elegance of answering research questions using simple methodologies whenever possible. With that said, I would also like to underscore that while light microscopy was an excellent way of providing initial data, the results and conclusions presented in this thesis would not have been possible without combining these observations with data from different techniques.

2.4.2 Wide-angle X-ray scattering

While the natural semi-crystallinity of starch is disrupted during gelatinisation, the recrystallisation of starch generates a new semi-crystalline matrix. Here, the crystal polymorphs are influenced by factors such as water content, chain length, additives, and storage temperature (Wang et al., 2015; Yongfeng & Jay-lin, 2024). This recrystallised starch commonly holds either A-type crystals (made up of compact double helical arrangements with low water content) or B-type crystals (with double helical arrangements that are less compact and have more water localised in the core). The crystals can be identified by characteristic X-ray diffraction patterns (see **Figure 8**) with the A-type structures generating peaks at 15 and 23°(2 θ) and unresolved double signals at 17 and 18°(2 θ). Meanwhile, B-type signals arise at 5.6, 17, 22, and 24°(2 θ). When a mixture of both A-type and B-type crystals is present, the X-ray profile is often referred to as a C-type pattern (Wang, 2020). It should be noted that this technique detects double helices that are organised in repeated arrays but is suggested to be less sensitive to irregularly packed double helices (Cooke & Gidley, 1992; Gidley & Cooke, 1991).

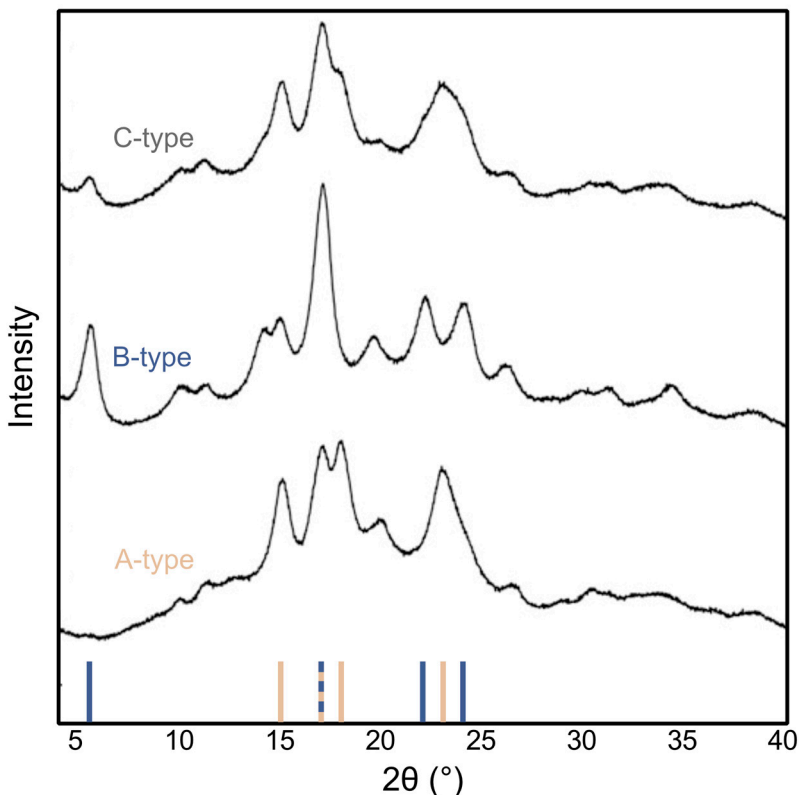


Figure 8. Typical X-ray diffractogram profiles of A-type, B-type, and C-type starch crystalline forms. The C-type emerges from a mix of A-type and B-type crystallites. Characteristic A- and B-type signal positions are illustrated along the x-axis of the graph. (The figure is adapted from (Wang, 2020)).

The X-ray diffraction patterns in this thesis were obtained through Wide-angle X-ray scattering (WAXS) measurements using an Anton Paar SAXSpoint 2.0 (Anton Paar, Austria), with Microsource X-ray beam (Cu K-alpha radiation, wavelength 0.15418 nm) and a $75 \times 75 \mu\text{m}^2$ pixel size detector (Dectris 2D CMOS Eiger R 1M). Diffraction patterns of starch particles were measured in Papers I, II, and IV. In Paper III, diffraction patterns from separated starch phases kept at 25°C were collected every hour for 24 hours, allowing for the growth of crystalline peaks to be followed over time.

2.4.3 Differential scanning calorimetry

Differential scanning calorimetry (DSC) allows us to study the thermal properties of starch. This is done by heating a starch sample and a reference at a controlled rate, while measuring the difference in electric current needed to keep both samples at the same temperature (Chang, 2005). Additional electric current will be needed to heat the starch sample, as it has greater heat capacity compared to the reference. Furthermore, the gelatinisation (or melting of the ordered material) in starch is an endothermic reaction, requiring extra heat during the phase transition. This so-called endothermic enthalpy of melting (ΔH) can be determined by integrating the difference in heat flow during the phase transition (see **Figure 9**). Additionally, the onset, peak, and conclusion temperatures of the gelatinisation can be gained from the DSC thermogram (Wang, 2020).

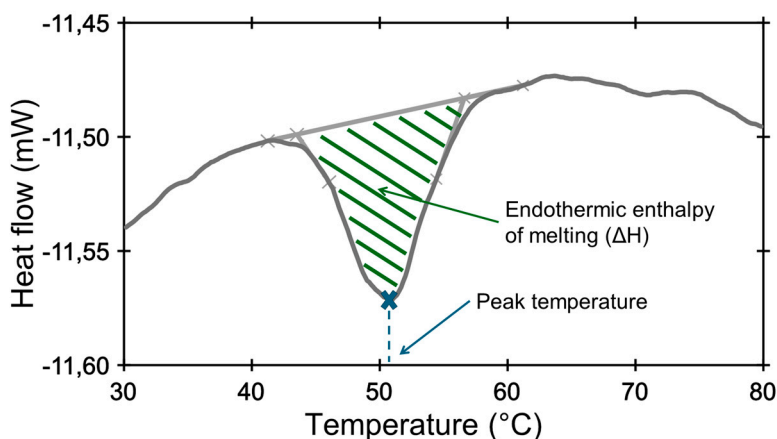


Figure 9. DSC thermogram of waxy barley starch microspheres in excess water. Melting temperatures and the endothermic enthalpy of melting (ΔH) can be determined from the phase transition profile. (Paper I)

DSC thermograms for starch microspheres were recorded in Papers I and II, using a DSC 1 STARe System (Mettler-Toledo, Switzerland) with an empty pan as reference, and a heating rate of $10^{\circ}\text{C}/\text{min}$, going from 20 to 90°C .

DSC and WAXS make for good complementing techniques, as the WAXS diffractogram shows the crystal polymorphs, while the ΔH provided by DSC indicates the amount of ordered structure (including irregularly packed helices).

2.4.4 Scanning electron microscopy and size distribution

Scanning electron microscopy (SEM) is a powerful tool for characterising the shape and structure of particles and has been widely used to analyse the morphology of dry starch microspheres (Elfstrand et al., 2006; Hamdi et al., 1998; Li et al., 2018; Wojtasz et al., 2016; Xia et al., 2017; Yang et al., 2020). Instead of light, a beam of electrons is swept over the sample, with the scattered electrons allowing for high-resolution imaging.

In this thesis, a Quanta 250 FEG ESEM scanning electron microscope (FEI, Czech Republic) was utilised to examine the morphology and interior structure of freeze-dried starch microspheres (in Papers I and II). Additionally, SEM images could be used to compare the size of the dried particles from different formulations (in Papers I, II, and IV). The cumulative volume distribution of the particles was also plotted based on image analysis. Here, the diameters of microspheres from SEM images were determined using ImageJ 1.53k software (Wayne Rasband, USA), whereupon the corresponding spherical volume could be calculated (in Papers I and II).

2.4.5 Isothermal calorimetry

Isothermal calorimetry is a sensitive technique in which a sample is maintained at a constant temperature, while the thermal power (also referred to as heat flow) is continuously measured as a function of time. The heat flow is related to the rate of a process and by integrating the heat flow data, the heat of the reaction can be determined. This heat can then be linked to the extent of the reaction (Wadsö, 2010; Wadsö & Gómez Galindo, 2009).

The recrystallisation of gelatinised starch generates heat that can be measured. However, it should be noted that, as a nonspecific technique, isothermal calorimetry reflects the heat developed in the whole sample, including e.g., the coalescence of droplets. Fortunately, the low interfacial tension in ATPSs (Atefi et al., 2014) makes this heat of coalescence low. Consequently, the crystallisation process in ATPSs was studied using isothermal calorimetry in Paper III. A Thermal Activity Monitor (TAM) IV microcalorimeter system (TA Instruments, NewCastleDE, USA) was used to collect the data.

2.4.6 Confocal Raman microscopy

Confocal Raman Microscopy is a powerful tool in material characterisation. Raman spectroscopy uses inelastic scattering by illuminating a sample with a laser beam, providing molecular fingerprint spectra of the wavenumber shifts. When combined with confocal microscopy, allowing us to focus the laser beam and collect spectra from specific positions within the sample, this technique enables detailed horizontal and vertical spatially resolved mappings within the sample (Hosseinian et al., 2022).

The Raman spectrum of starch is shown in **Figure 10**. As marked in the image, the starch Raman spectrum displays an intense spectral band at around 480 cm^{-1} . The position of this band can shift towards a lower wavenumber as the starch crystallises. Thus, the peak position can be used to monitor starch retrogradation, which has been demonstrated in bulk measurements of starch gels (Fechner et al., 2005).

In this project, we utilised confocal Raman microscopy to map and locate the presence of ATPS continuous phase polymers in the starch phases (in Papers I and III). More importantly, this thesis presents a novel approach to monitor the starch microsphere crystallisation process in ATPSs. Namely, by using confocal Raman microscopy to spatially monitor starch crystallisation within dispersed starch phase droplets *in situ*, as schematically illustrated in **Figure 10** (Paper III). Samples from emulsified ATPSs were incubated in glass cavity slides at 25°C . Starch droplets with a diameter of $20\text{-}30\text{ }\mu\text{m}$ were located, and vertical depth Raman scans were continuously taken for 24 hours. The measurements were conducted using a WITec Alpha 300 RAS system confocal Raman microscope (WITec, Germany) equipped with a $50\times$ Zeiss LD EC Epiplan-Neofluar Dic $50\times /0.55$ objective and a 532 nm excitation laser. The scans were measured with an 1800 g/mm BLZ 500.00 nm grating, collecting $3.5\text{ spots}/\mu\text{m}$. This generates a digital pixel size of 286 nm . However, by multiplying the laser wavelength by 1.22 and dividing this by two times the numerical aperture of the objective, the theoretical spatial resolution could be estimated as around 590 nm (Stewart et al., 2012).

The spectra were analysed using Witec Project 5.1 software (WITec). Fittings of the average 480 cm^{-1} peak were obtained for every pixel of the scan, and heat maps were plotted for the fitted peak positions, providing a visualisation of the crystallisation process throughout the droplets. In addition, histograms of these peak positions were extracted and fitted to gain an average 480 cm^{-1} peak shift within the whole droplet, which could be plotted to show the overall progression of the reaction over time.

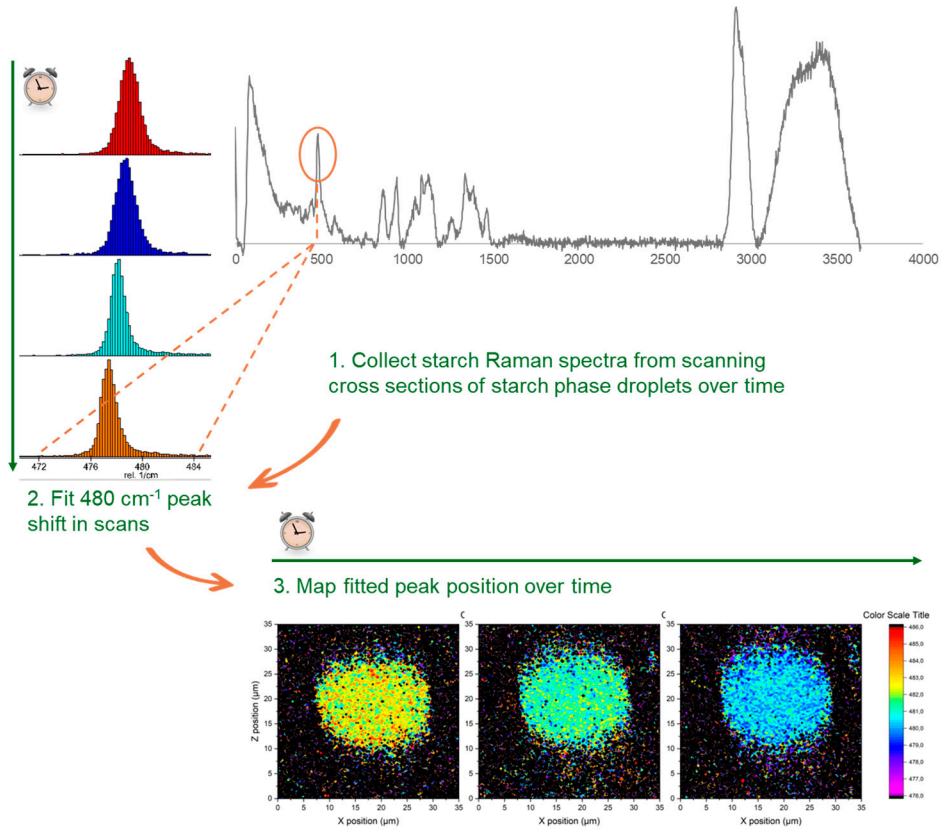


Figure 10. Utilising confocal Raman microscopy to monitor starch crystallisation within a dispersed starch phase droplet. A Raman spectrum of starch displays a characteristic peak around the 480 cm^{-1} band, and this peak can shift towards a lower wavenumber as the starch crystallises. We followed the shift by continuously mapping Raman spectra in a cross section of an ATPS starch phase droplet. The 480 cm^{-1} peak position could then be fitted and plotted as spatially resolved crystallisation heat maps over time.

2.5 Bacterial encapsulation, release, and viability

The bacteria trials were performed in Paper IV, investigating the encapsulation, release, and viability of pre-freeze-dried *L. reuteri* during starch microsphere preparation with different continuous phase polymers.

2.5.1 Starch digestion and release of encapsulated bacteria

The presence and release of encapsulated bacteria were explored using light microscopy and by incubating the starch microspheres with α -amylase. This enzyme is secreted into the GIT via the salivary glands and pancreas. α -Amylase plays a central role in human digestion by hydrolysing the α -1,4-glycosidic bonds of starch into shorter glucose chains (Lentle & Janssen, 2011), making this enzyme particularly interesting for controlled release functions.

2.5.2 Bacterial viability

The bacterial viability in the pre-freeze-dried starting material, as well as during the starch microsphere preparation process and subsequent storage, was assessed by plate counts of serial dilutions. Bacteria that had been encapsulated in starch microspheres were released by hydrolysing the starch with α -amylase before dilutions and plating.

3 Results and discussion

This section will summarise the main results and findings. For more detailed information and comprehensive discussion, the reader is referred to the individual papers I-IV.

3.1 Phase diagram and starch microsphere preparation

3.1.1 Phase diagram

The investigations of the phase behaviour were initiated (in Paper I) by mapping an ATPS phase diagram with starch and the well-established continuous phase polymer, PEG20. This ATPS exhibited very low mixing in the phases. Analysing the phase compositions revealed the presence of some PEG in the starch phase. However, this was primarily in the form of PEG phase droplets within the starch phase, which was confirmed with confocal Raman microscopy, as seen in **Figure 11**.

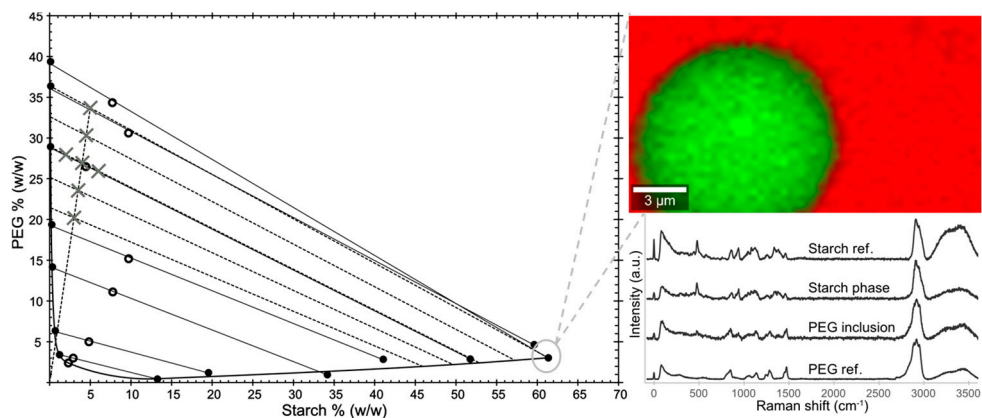


Figure 11. ATPS Phase diagram and confocal Raman microscopy image of a separated starch phase, with hydrolysed waxy barley starch ($M_w 9 \times 10^5$ g/mol) and PEG20 in H_2O with 1 % NaCl, at ambient temperature. For the phase diagram tie lines, the total ATPS compositions are illustrated with open circles, with the fitted tie lines connecting to the top and bottom phase compositions (closed circles). The starch microsphere manufacturing compositions (crosses) are run through by estimated (dashed) tie lines. The binodal curve is visually estimated from the measured phase compositions. The dashed line connected to the origin illustrates a dilution line, associated with experimental conditions for starch microspheres preparation. The confocal Raman microscopy images show a starch phase sample with a spherical PEG inclusion (green) surrounded by the starch phase (red). Reference spectra of starch and PEG are also presented. (Paper I)

3.1.2 Formation of starch microspheres

We examined microsphere preparation at different positions in the phase diagram (compositions shown in **Table 1**), and light microscopy was used to monitor the starch microsphere formation process, as shown in **Figure 12**. As can be seen, the incubation process began with partial coalescence of soft starch phase droplets. During the incubation, these droplets started to aggregate and gradually transitioned to darker solid gel particles. The positioning in the phase diagram influenced the kinetics of starch microsphere formation, with lower water content accelerating the process. Consequently, having a too dilute starch phase hindered the formation of solid starch microspheres within a reasonable time frame. It is therefore noteworthy that the phase diagram revealed a large biphasic region, but only a relatively narrow window to produce starch microspheres with a time frame reasonable for production at $25^\circ C$.

Table 1. ATPS compositions during starch microspheres preparation. The table shows the ATPS compositions, starch phase compositions, relative mass of the starch phases, and the starch phase to PEG20 phase ratio. The phase-related values are estimated from the phase diagram plot. (Paper I)

Abbreviation *	Total starch concentration (%w/w) in the ATPS	Total PEG concentration (%w/w) in the ATPS	Starch concentration in the starch phase (%w/w)	Highest concentration of PEG in starch phase (%w/w)	H ₂ O with 1 % NaCl in starch phase (%w/w)	Relative mass fraction of starch phase (%w/w) in the ATPS	Starch phase to PEG phase ratio in the ATPS
DL-S5P34	5.0	33.7	61	3.0	36	7.9	0.086
DL-S4.5P30	4.5	30.3	57	2.8	40	7.4	0.080
TL-S6P26	6.0	25.9	53	2.5	45	11	0.12
CP-S4P27	4.0	26.9	53	2.5	45	7.3	0.079
TL-S2P28	2.0	27.9	53	2.5	45	3.4	0.035
DL-S3.5P24	3.5	23.6	50	2.3	48	6.6	0.071
DL-S3P20	3.0	20.2	46	2.0	52	5.8	0.062

*The first part of the abbreviation refers to the formulation's position in the experiment set-up, with "CP-" = centre point; "DL-" = dilution line; "TL-" = tie line. The second part of the abbreviation refers to the total system concentration, with an "S" followed by the starch concentration, and a "P" followed by the PEG concentration.

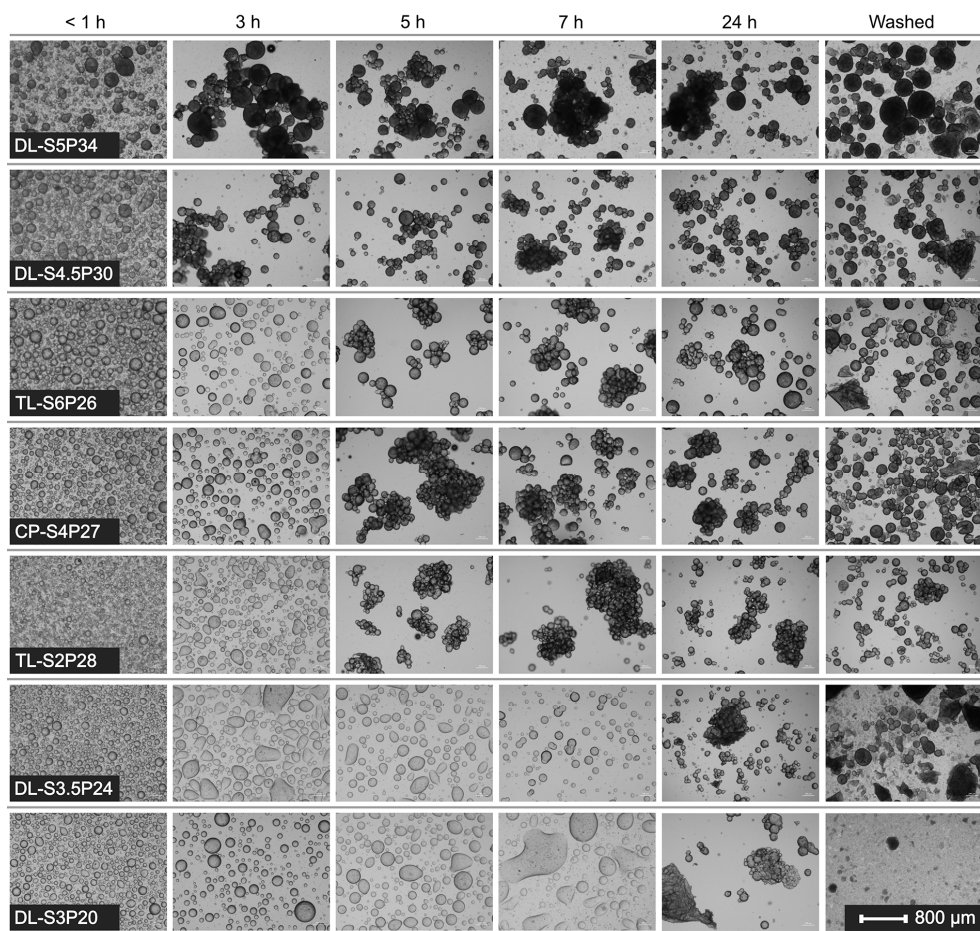


Figure 12. Light microscopy captures of different positions in the phase diagram during the microsphere preparation. The first five columns are samples taken at < 1, 3, 5, 7, and 24 hours of incubation, and the last column is after the microspheres had been washed with water. (Paper I)

3.1.3 Crystals and thermal properties

Before discussing the positions in the phase diagram, it is crucial to recognise the importance of controlling the incubation temperature during the preparation of starch microspheres. This is exemplified in **Figure 13**, which shows crystallite patterns in washed starch microspheres manufactured during initial screening experiments. As demonstrated in the figure, manufacturing at 21°C generated microspheres with predominantly B-type crystals. Increasing the incubation temperature to 27°C significantly enhanced the formation of the A-type polymorph, which became even more dominating at 37°C. Thus, within the investigated

temperature range, moderate changes in incubation temperature can lead to the formation of different crystal types, with lower temperatures favouring the formation of B-type and higher temperatures favouring A-type crystals. It should therefore be noted that the remaining starch microsphere production in this thesis was conducted using a consistent temperature program. Specifically, the phase behaviour experiments and emulsification of ATPSs for starch microsphere preparation were performed at ambient temperature, while the 24-hour incubation period was maintained at 25°C. It is also noteworthy that, while the results in **Figure 13** stress the importance of controlling the operational temperature, they also demonstrate that incubation temperature can be used as a tool to adapt the starch crystallisation process.

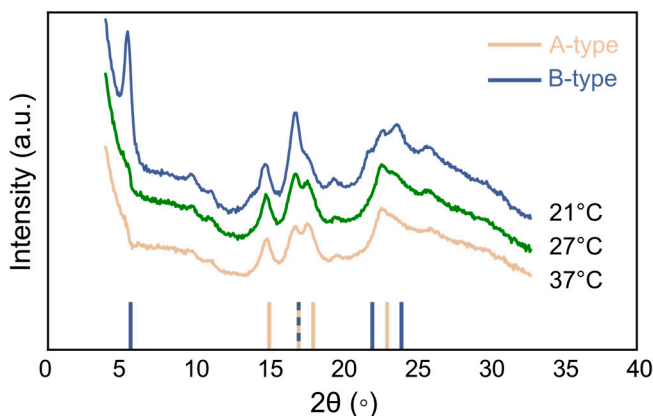


Figure 13. WAXS diffractograms of newly washed starch microspheres manufactured at different incubation temperatures. An ATPS with 2.2 % starch and 27.6 % PEG20 was used to manufacture the microspheres. Characteristic A- and B-type signal positions are illustrated along the x-axis of the graph.

Returning to the positions in the phase diagram, the formulations that had yielded sufficient solid particles for analysis (all formulations except for the most dilute) were washed via centrifugation. Crystalline structures and thermal properties of the wet starch microspheres were examined using WAXS and DSC. As a cereal grain, barley starch generally contains A-type crystals in its native granular state. However, a mixture of A- and B-polymorphs can also occur (Sneh, 2020). We found that both polymorphs were present in recrystallised particles to varying extents, depending on the positions within the phase diagram. This is demonstrated in the left graph in **Figure 14**, showing that gradually increasing the water content in the ATPS transformed the crystallites from A-type to a combination of A- and B-type polymorphs, resulting in a C-type pattern. The presence of more B-type crystals in microspheres that had crystallised in dilute formulations was not unexpected, as the B polymorph contains more water than the A-type (Gidley & Bulpin, 1987; Imberty & Perez, 1988; Osella et al., 2005; Popov et al., 2009). The WAXS results aligned

well with the DSC analysis, which indicated a broader melting temperature range for the positions in the phase diagram with high water content, thereby suggesting less homogeneity in the ordered material. Regarding DSC, it should also be noted that the ΔH did not appear to vary between the formulations, which indicates an even degree of crystallisation between the positions in the phase diagram (Paper I).

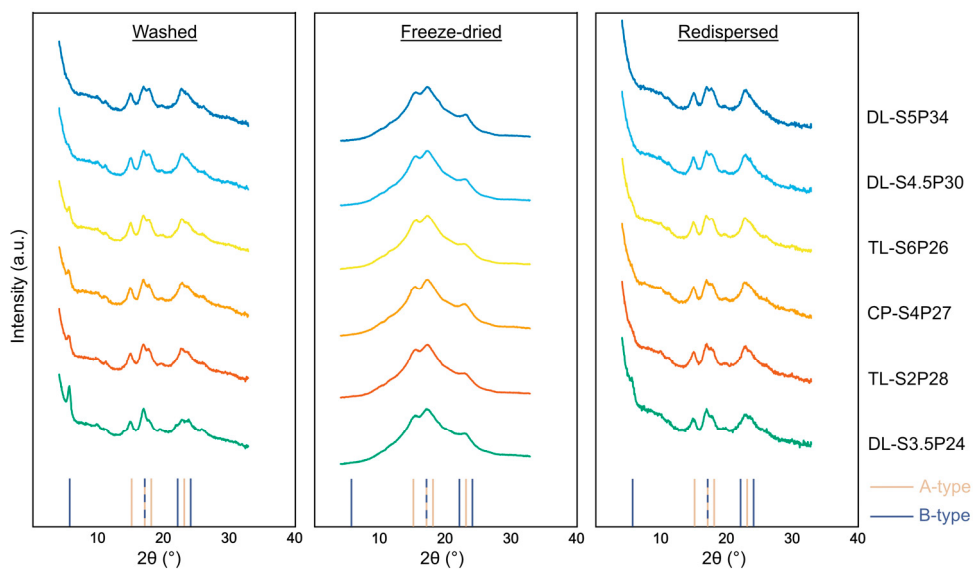


Figure 14. WAXS diffractograms of newly washed, freeze-dried, and redispersed starch microspheres from different positions in the phase diagram. Characteristic A- and B-type signal positions are illustrated along the x-axis of the graphs. (Paper I)

After washing and collecting the starch microspheres, the material was freeze-dried. WAXS analysis of the crystalline structure of newly washed, dried, and redispersed material showed us that some starch crystallites were lost during drying (see the middle graph in **Figure 14**). Upon redispersion in water, some crystalline peaks were regained, with less B-type crystallites being recovered, thus making the difference between positions in the phase diagram less pronounced (see the right graph in **Figure 14**). This outcome seems reasonable when taking into consideration that the A-type crystals contain less water compared to the B-type. Thus, the B-type crystals would be lost at an earlier state during drying, and the A-type crystals could be regained with less water upon rehydration. These WAXS results also aligned well with previous DSC analysis, which showed that freeze-drying reduces the ΔH in starch microspheres (Papers I and II) (Elfstrand et al., 2009a).

3.1.4 Particle size

The particle size distribution was determined for freeze-dried particles, and it was noted that the freeze-drying procedure compacted the starch microspheres and introduced fractures in the larger particles. The implications of these fractures will be elaborated upon in section 3.4.3. Revisiting the phase diagram revealed that both the phase ratio and dilution level of the ATPS influenced the size of the particles (as illustrated in **Figure 15**, showing the size distribution of dried particles). A higher starch phase to PEG phase ratio resulted in larger particles, which can be attributed to the increased starch phase volume in the system. Larger particles in the less dilute systems may be related to a higher viscosity of the phases, making it more challenging to disperse smaller droplets during emulsification.

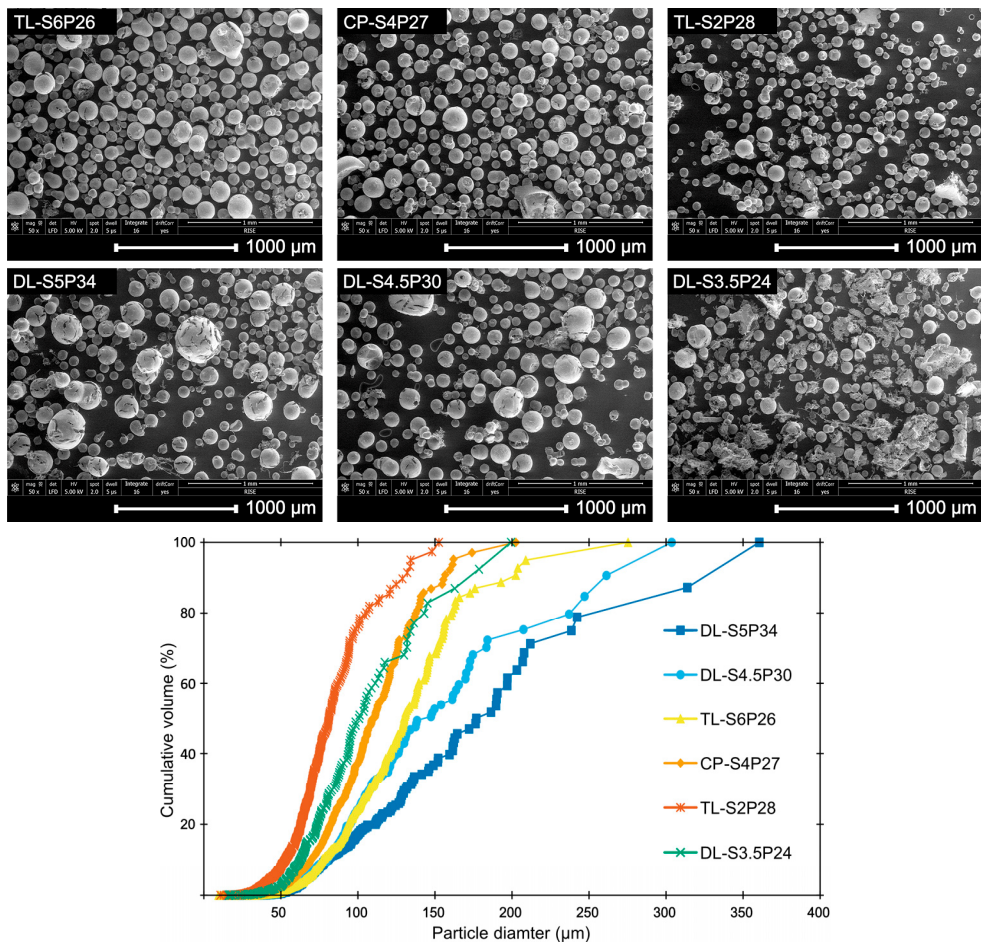


Figure 15. SEM images and cumulative volume distribution of freeze-dried starch microspheres from different positions in the phase diagram. The size distribution is calculated from SEM image analysis. (Paper I)

It is also noteworthy that while the current freeze-drying cycle compacted the particles, upon redispersion in water, the particles rapidly swelled again to assume approximately the same size as before drying (see **Figure 16**). Furthermore, porous dry particles could also be obtained by incorporating a rapid cooling step in liquid nitrogen at the beginning of the freeze-drying cycle, as demonstrated in **Figure 17**. However, the remaining starch microsphere production in this thesis was conducted without liquid nitrogen.

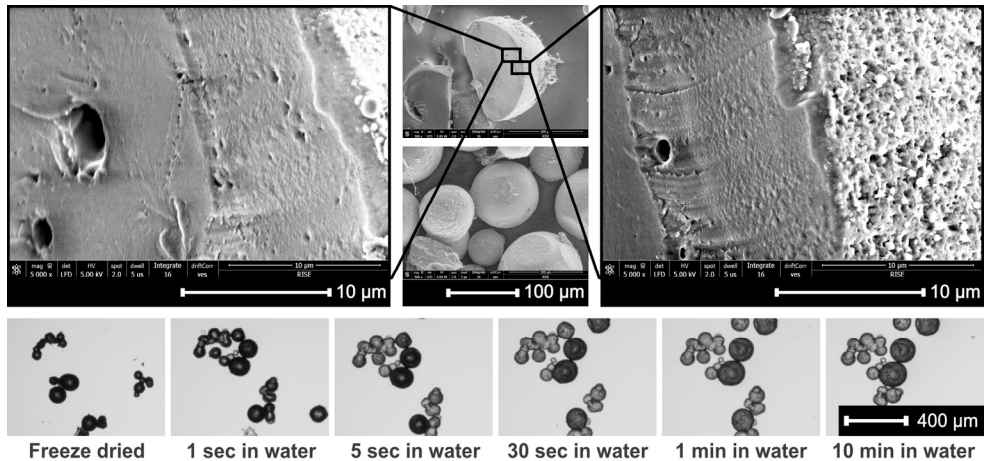


Figure 16. Drying compaction and swelling during rehydration of starch microspheres from the centre point position within the phase diagram. The top row images are of close-ups of the surface and a cross section of freeze-dried particles. The bottom row is light microscopy captures of particles swelling when a droplet of water was dropped onto a dry sample. (Paper I)

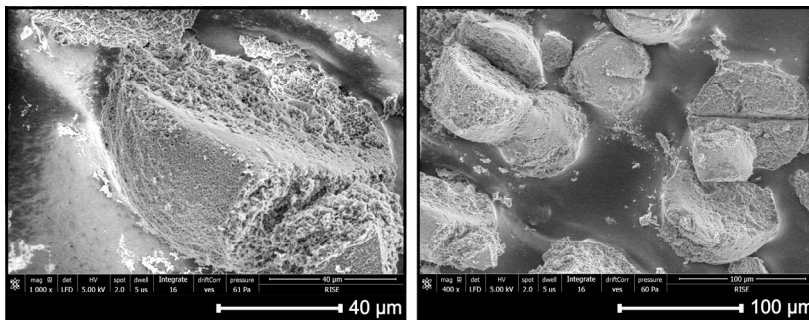


Figure 17. Dry porous starch microspheres of the centre point position within the phase diagram. The SEM images show close-ups of the surface and cross sections of particles that had been quench-frozen in liquid nitrogen before freeze-drying.

Overall, the key finding from the phase diagram studies was that the water content in the starch phase is an important factor to control during formulation development. This is not unexpected, as the water content can influence both the kinetics and the extent of crystallisation (Farhat, 2000; Zeleznak & Hosney, 1986). Our investigations demonstrated that starch phases with 48 % dry matter were too dilute to generate sufficient solid starch particles within a reasonable time frame, as shown in **Table 1** and **Figure 12**. On the other hand, we found that having a starch phase with roughly 55 % dry matter was beneficial for obtaining solid starch microspheres within a reasonable time frame at 25°C. This was used as guidance when we explored the effects of continuous phase polymers, in Paper II.

3.2 Continuous phase polymers

The next step in our investigations was to evaluate the impact of using various continuous phase polymers to make up the ATPSs. This evaluation was conducted by systematically mapping the water distribution within starch-based ATPS with complementary (continuous phase) polymers of different types and Mw. Following this, we explored the potential of preparing starch microspheres in these systems, as outlined in **Figure 18**.

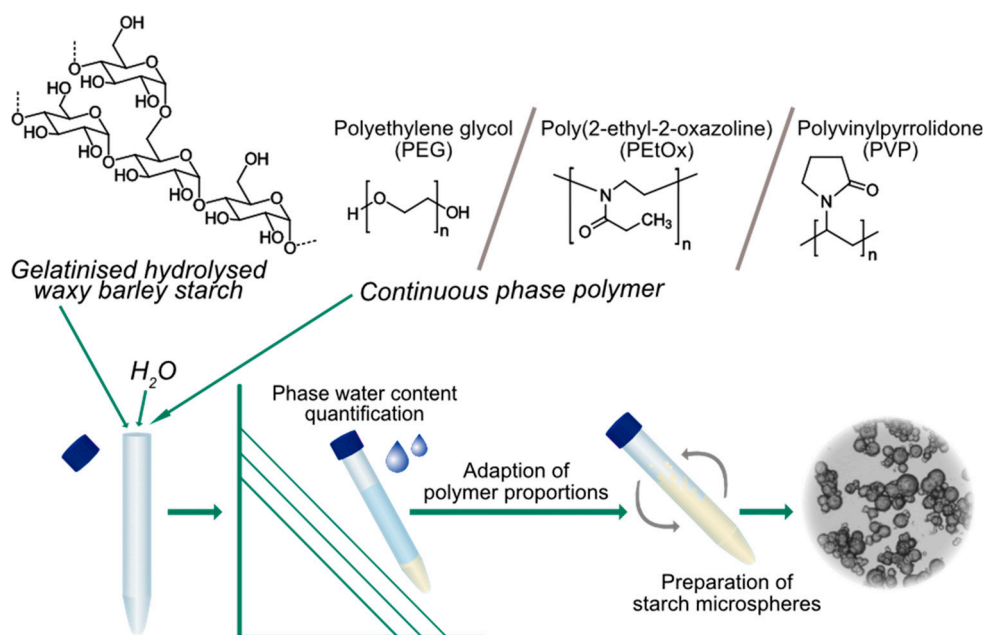


Figure 18. Schematic approach to study phase behaviour and starch microsphere preparation with different continuous phase polymers.

3.2.1 Water distribution

The water distribution was influenced by the type of continuous phase polymer, but not by its Mw within the investigated range. This is illustrated in the left graph of **Figure 19**, showing the slopes of the water distribution tie lines from all the investigated systems. To facilitate the reader's interpretation of the data, the figure also includes a selection of PEG/starch water distribution tie lines. As can be seen, PEGs with different Mw follow the same trend, exhibiting slopes that are less steep compared to the other polymer types. Consequently, higher concentrations of the other polymers are required to achieve the same dry matter content in the starch phase as when using PEGs. Furthermore, there is a clear trend of the water distribution tie line slopes becoming steeper as the ATPS becomes less diluted. These results demonstrate that phase behaviour characterisation is a valuable tool when designing and developing new ATPSs for starch microsphere preparation. However, it should be noted that the water distribution equilibrium may change as the starch crystallises during the incubation period, making it advantageous to collect water distribution data at comparable time points.

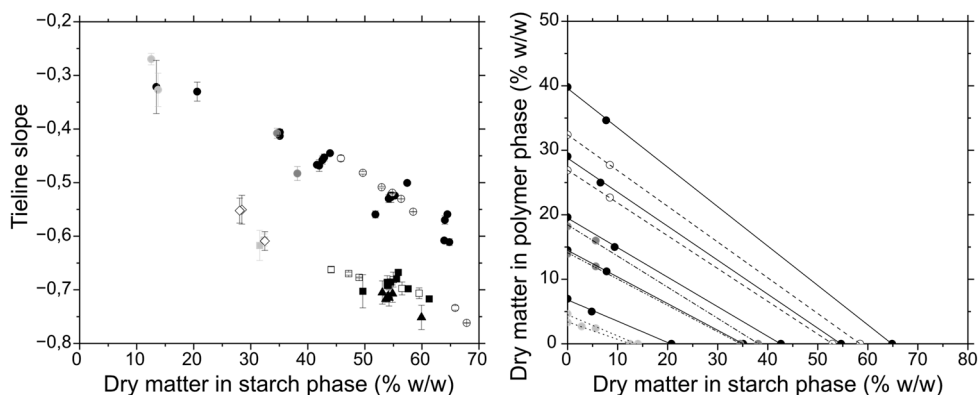


Figure 19. Water distribution in different starch-based ATPSs. The left graph illustrates the ATPS phase behaviour as slopes of water distribution tie lines. The error bars represent the standard error of the linear regression slope coefficient. PEG10 (○), PEG20 (●), PEG200 (●) and PEG2mil (●), PVP10 (□), PVP40 (■), PVP360 (■), PETox (▲) and hydroxypropyl methylcellulose (◇). The right graph shows water distribution tie lines of ATPSs with PEGs of varying Mw. The middle symbols in the tie lines illustrate the total composition of the ATPS, while the nodes show the dry matter of the phases. PEG10 (dashed lines, ○) PEG20 (solid lines, ●), PEG200 (dash-dotted lines, ●) and PEG2mil (dotted lines, ●). (Paper II)

We observed that the dependence of the tie line slope on both polymer type and ATPS dilution level could be correlated with the water activity of the individual polymer solutions in 0.9 % NaCl. **Figure 20** illustrates the water activity as a function of dry matter concentration for the starch and a selection of polymers. Firstly, the starch requires higher concentrations to reduce the water activity to the same extent as for the other polymers, whereas PEG requires the lowest

concentrations. This explains why less PEG polymer is needed to extract enough water from the starch phase under these mixing conditions, since the chemical potential of water, hence also the water activity, should fundamentally be the same across all phases in equilibrium (Chang, 2005). Thus, the greater the relative difference between the starch phase and continuous polymer phase water activity curves, the more the tie line slope will deviate from 1. Secondly, as the concentrations increase, the water activity declines more rapidly while maintaining a roughly similar absolute difference in concentrations of polymer and starch at a given water activity. Thereby, the relative difference between the concentrations at a given water activity is reduced with higher ATPS concentration, making the tie line slopes increase towards 1 with rising ATPS concentration.

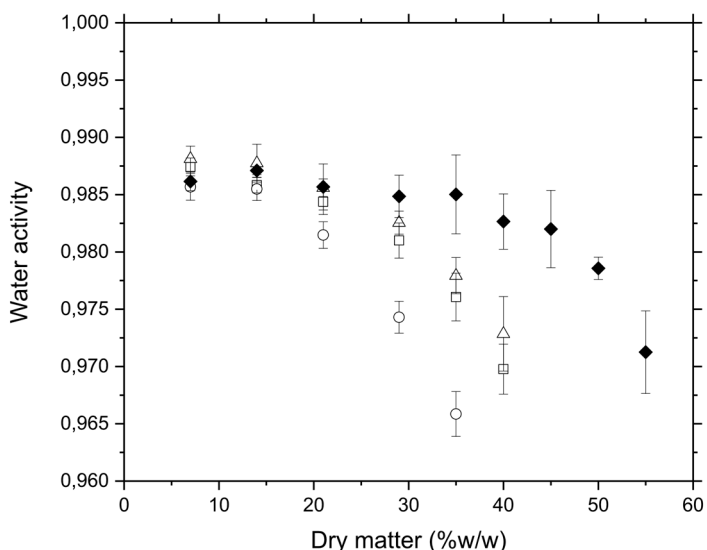


Figure 20. Water activity for different concentrations of pre-boiled starch and polymer solutions. 0.9 % NaCl was used when preparing the solutions. The error bars represent the standard deviation of quadruplicate measurements. PEG20 (○), PVP40 (□), PEtOx (Δ), and starch (◆). (Paper II)

3.2.2 Microsphere preparation and polymer molecular weight

Following the water distribution characterisation, the water distribution tie lines were utilised to adjust the respective polymer concentrations, aiming to generate ATPSs with starch phases containing approximately 55 % dry matter. However, the viscosity generated by the high Mw polymers in solution limited the amount of dry matter that could be mixed into the ATPSs. These limitations prevented the formation of starch phases with sufficiently high dry matter content. Consequently, these starch phases failed to solidify within a reasonable time frame, corroborating

the findings in Paper 1 that the starch phase should not be too dilute during microsphere preparation.

Subsequent investigations focused on using only those polymers with Mw that allowed for sufficient dry matter to be mixed into the ATPSs. These polymers included PEG, PVP, and PEtOx, with molecular weights ranging from 10 to 50 kDa (see **Table 2**).

Table 2. ATPS compositions during starch microsphere preparation with different continuous phase polymers. The table shows the total composition, estimated water content of the phases, and viscosity of the continuous phase. (Paper II)

Continuous phase polymer abbreviation	Starch (%w/w)	Continuous phase polymer (%w/w)	Estimated dry content in continuous phase (%w/w)	Estimated dry content in starch phase (%w/w)	Average shear viscosity of the continuous phase* (Pa s)
PEG10	4.7	26.0	28	55	0.06
PEG20	4.7	26.4	29	55	0.2
PVP10	4.7	34.7	38	55	0.04
PVP40	4.7	34.1	37	55	0.3
PEtOx	4.7	34.9	38	54	1.0

* All phases showed Newtonian flow behaviour between share rate 1-250 s⁻¹

Although high polymer Mw was found to be unsuccessful for starch microsphere preparation, using polymers with too low Mw also proved to be unbeneficial. Specifically, the 10 kDa polymer formulations exhibited material loss due to clumping and surface adhesion, failing to stabilise the dispersed starch phase droplets during the transition from soft to solid gels. A comparison between the bulk of PEG20 and PEG10 after 2 hours of incubation can be seen in **Figure 21**, which also shows that most of the starch phase has migrated from the PEG10 bulk to adhere to the walls of the incubation tube. This phenomenon can be attributed to vigorous hydrodynamics when the viscosity of the continuous phase (stated in **Table 2**) is low. Low viscosity can allow for more droplets to collide and hit the walls of the incubation tube with high velocities. Therefore, a continuous phase polymer with a semi-high Mw is preferred for starch microsphere production. This observation is consistent with the SEM images in **Figure 15** (Paper I), where the most dilute (and thus least viscous) position in the phase diagram displayed more non-spherical formations compared to the other positions.

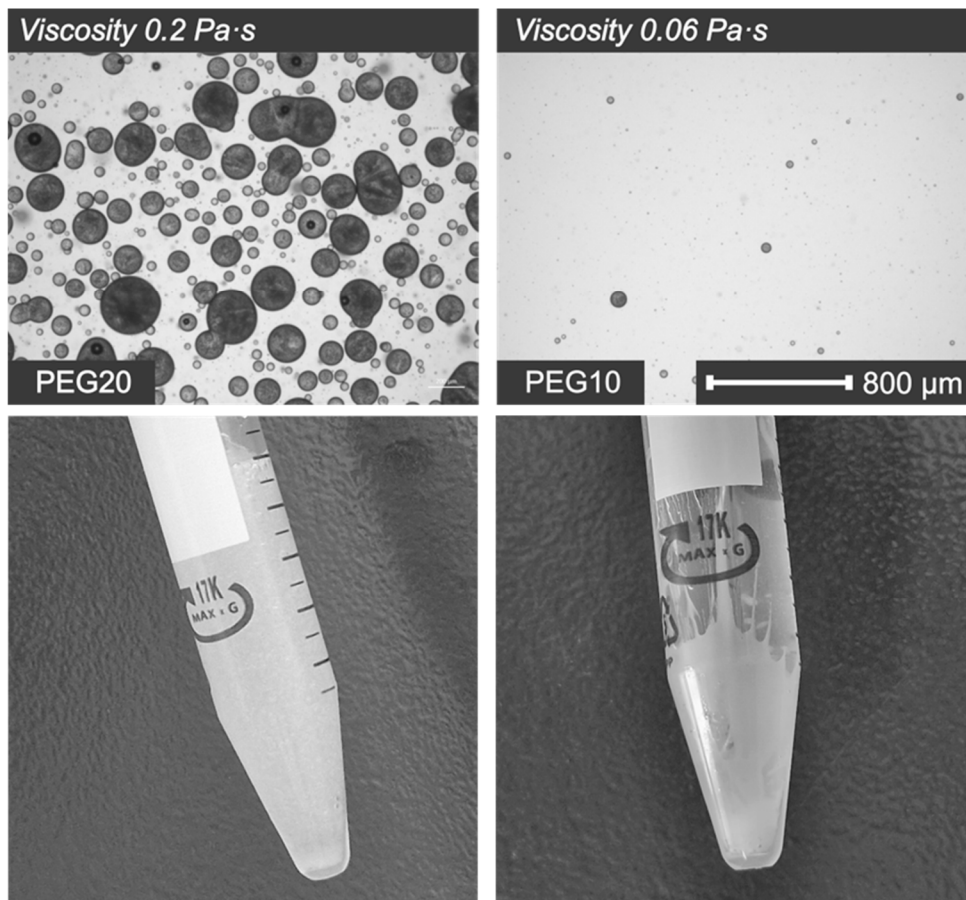


Figure 21. Light microscopy images of ATPS bulk and macroscopic appearance of incubation tubes with different PEG Mw in the continuous phase after 2 hours of incubation. The continuous phase viscosity generated by PEG10 was insufficient to stabilise the dispersed starch phase droplets during incubation. (Paper II)

3.2.3 Microsphere preparation and polymer type

Comparing the semi-high Mw polymers (PEG20, PVP40, and PEtOx), we observed that the polymer type influenced the size and aggregation tendencies of the starch microspheres, as shown in **Figure 22**. The particles dispersed in PEG and PEtOx exhibited aggregation, whereas the particles in PVP displayed very little aggregation. In addition, the starch microspheres that had been produced in PEG were larger, with a median volume distribution particle diameter twice as high as those produced in PVP and PEtOx. Nonetheless, the particle size in PVP and PEtOx could also be increased somewhat by moving along the ATPS water distribution tie

lines to increase the starch phase to polymer phase ratios, thereby increasing production efficiency without producing excessively large particles (see SEM images and tie lines in **Figure 23**).

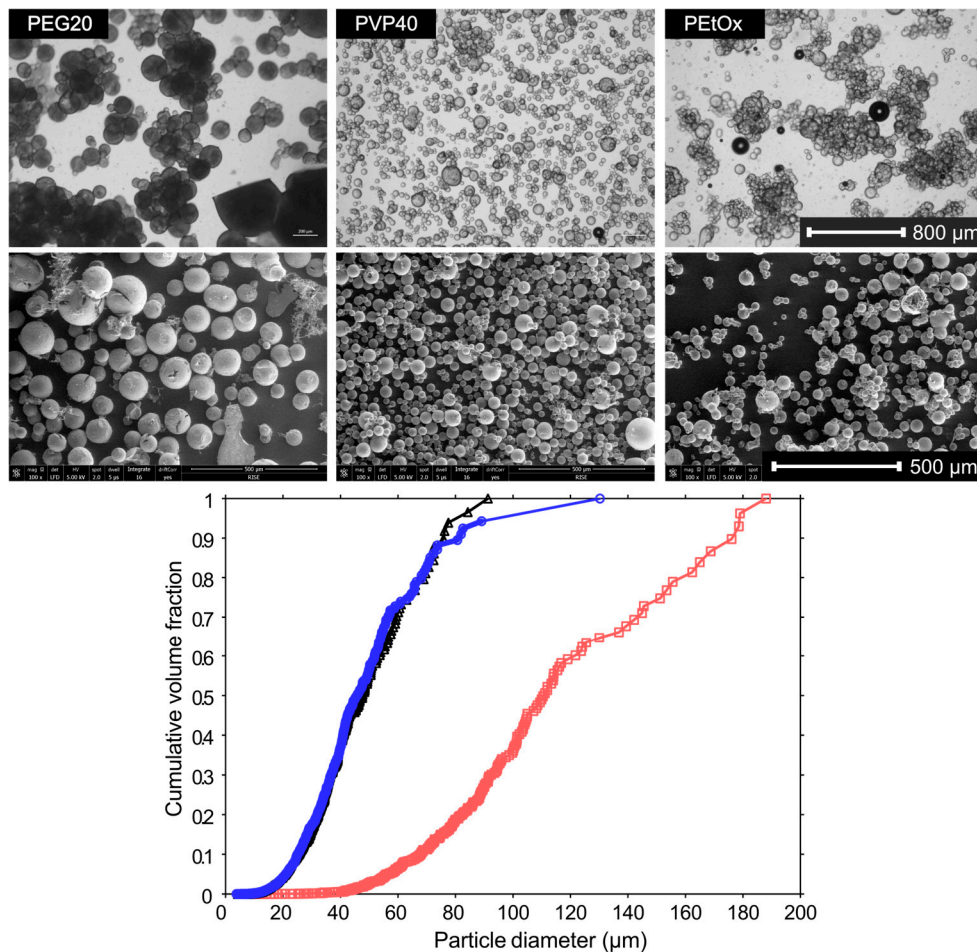


Figure 22. Starch microspheres appearance and size. The light microscopy images (top row) are taken at 24 hours of incubation. The cumulative volume distribution for freeze-dried particles is calculated from SEM image analysis. The continuous phases polymers were: PEG20 (red squares), PEtOx (black triangles), and PVP40 (blue circles). The ATPS compositions can be found in **Table 2**. (Paper II)

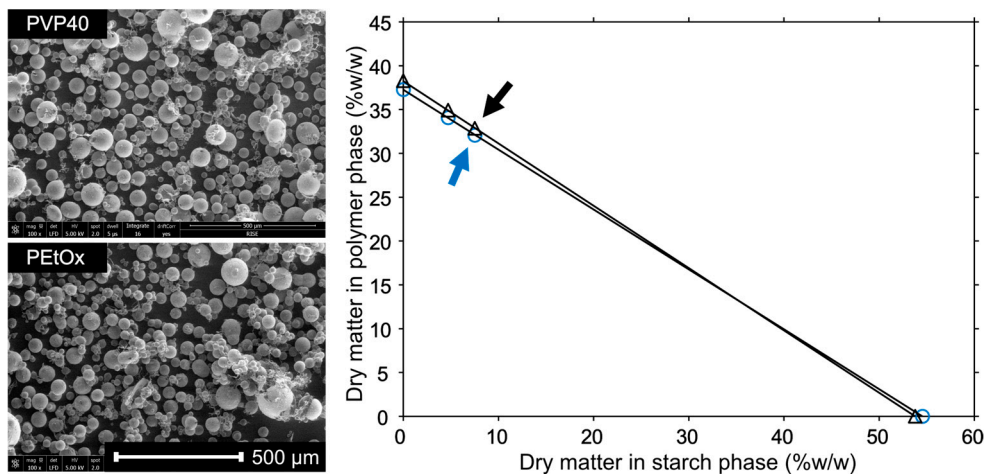


Figure 23. Starch microspheres manufactured in ATPSs with 7.5 % starch and 32.1 % PVP40 or 32.8 % PEtOx. The water distribution tie lines and the manufacturing positions are presented in the graph, PVP (blue circles and arrow), and PEtOx (black triangles and arrow).

Although the type of continuous phase polymer affected the starch particle aggregation and size, the continuous phase polymer did not appear to influence the interior structure, surface morphology, crystal type, or thermal behaviour of the dried starch microspheres (as demonstrated in Paper II). This finding allows for the possibility of altering the continuous phase polymer to suit different requirements, such as influencing the partitioning of different APIs and cells into the starch phase (as will be elaborated upon in section 3.4.1) while still maintaining stable starch matrix properties. Moreover, since the water distribution maintained consistent across different polymer Mw, the continuous phase viscosity with a specific polymer type could be adjusted by adapting the Mw, to acquire satisfactory starch microsphere stabilisation during the incubation process.

Overall, successful starch microsphere preparation was achieved using PEG20, PVP40, and PEtOx in the continuous phase. Therefore, we extended our investigations on these three ATPSs to a more fundamental level (in Paper III), where we examined phase behaviour and explored the starch crystallisation process in greater detail.

3.3 Composition of starch phases, microsphere formation, and starch crystallisation process

The phase behaviour studies were extended by examining the starch phase compositions of the PEG20, PVP40, and PEtOx ATPSs presented in **Table 2**. Thereafter, we conducted more detailed studies of the crystallisation and starch microsphere formation process. This was done using light microscopy, WAXS, isothermal calorimetry, and confocal Raman microscopy.

3.3.1 Starch phase compositions

It was found to be approximately 2-3 % polymer dry matter present in the starch phases. Similar to the phase behaviour characterisations in Paper I (see **Figure 11**), confocal Raman mappings showed that all three polymers were present in the starch phase as inclusions. In addition, light microscopy revealed smaller droplets within some of the dispersed starch phase particles, suggesting that these systems could form double emulsions. Assuming that the polymers are contained within these inclusions and that their water content remains consistent with that in the continuous polymer phase, the weight fraction of the inclusions can be estimated to be 5-9 % of the starch phase (Paper III). This finding should be considered during starch microsphere preparation, as polymer phase residues may be present outside, as well as inside the washed particles.

3.3.2 Starch microsphere formation

The starch microsphere formation was monitored with light microscopy and the solidification was assessed by pushing a coverslip down over the light microscopy samples and observing if the particles could change shape without cracking (see **Figure 24**). This evaluation revealed that the starch particles in PEG solidified at 4-6 hours, while particles in PEtOx needed 5-7 hours, and particles in PVP needed 6-9 hours. Thus, the solidification rate is dependent on the continuous phase polymer, with the fastest rate achieved with PEG and the slowest with PVP. Furthermore, the aggregation of particles in PEG and PEtOx began the hour before the particles started to solidify, whereas the particles in PVP remained well-dispersed. The mechanism behind this is somewhat unclear, but it may be related surface activity of the continuous phase polymers. Unlike PEG, PVP can adsorb to cellulose, a polymer chemically similar to starch (Reid et al., 2017; Voronova et al., 2018). Therefore, it may be possible for PVP polymers to adsorb the surface of the starch phase as it starts to solidify, due to an increased interfacial tension as the starch crystallises. If PVP can adsorb to the surface of solidifying starch droplets, it may help prevent aggregation via steric stabilisation.

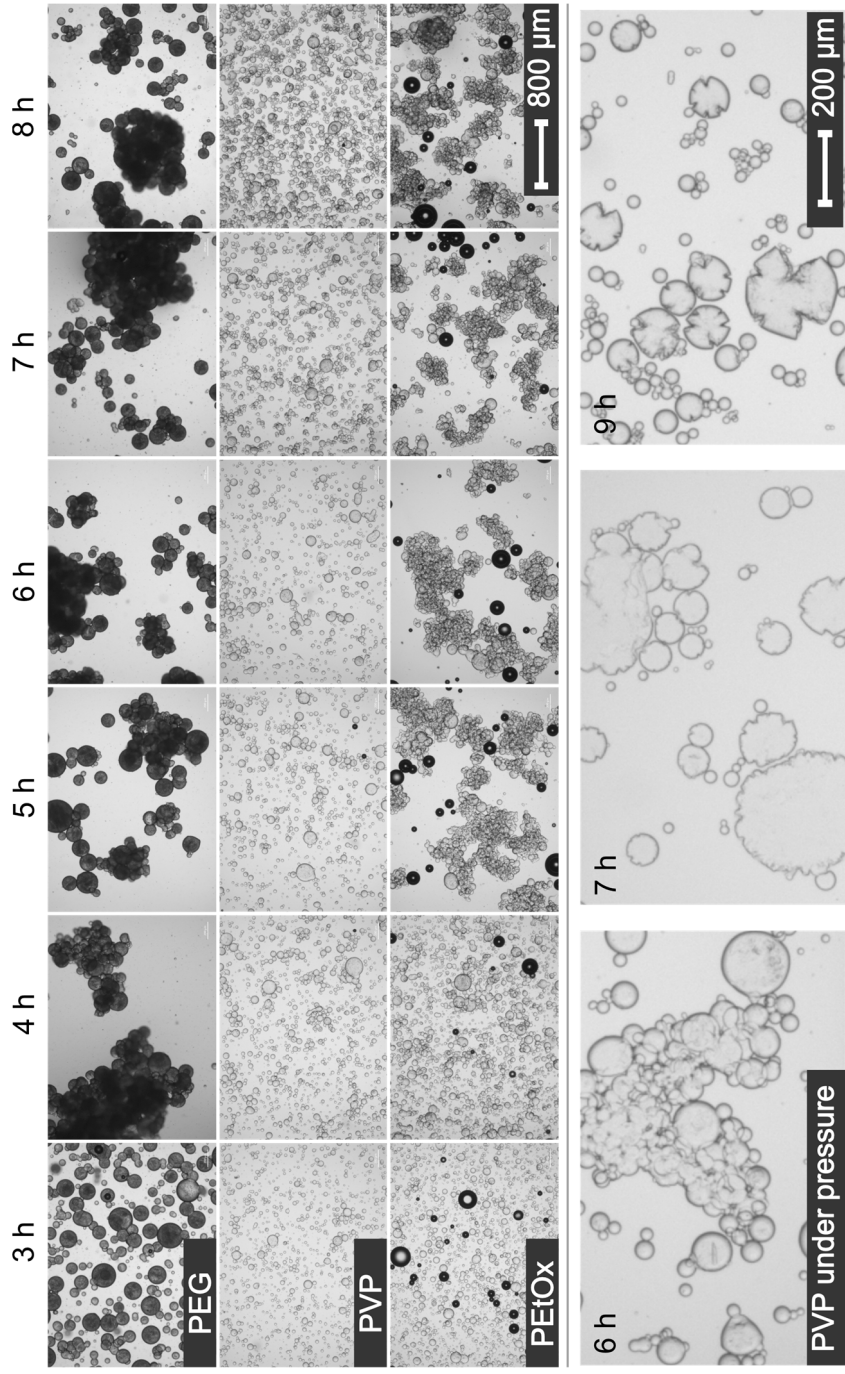


Figure 24. Starch microspheres microsphere formation and solidification. The solidification was assessed once per hour during the first 9 hours of incubation, by pushing a coverslip down over a light microscopy sample. Particles that could change shape without cracking were interpreted as soft particles, while small cracks were interpreted as semi-solid particles, and large cracks as solid particles. The fourth row illustrates how starch microspheres in PVP appear when the coverslip was pressed down on them, with soft starch particles (at 6 hours of incubation), semi-solid particles (at 7 hours of incubation) and solid particles (at 9 hours of incubation). (Paper III)

3.3.3 Starch crystallisation

To better understand the solidification process, the crystallisation in separated starch phases was monitored for 24 hours using WAXS, and the heat development in the ATPSs was followed for 48 hours with isothermal calorimetry. The results are illustrated in **Figure 25**, showing the growth of crystalline peaks and developed heat over time. As shown in the figure, weak WAXS peaks can be detected already at the early hours for the PEG and PEtOx formulations, while the PVP formulation shows a slightly delayed response. The PVP formulation also reaches its peak heat flow later than the other two formulations. Thus, both techniques revealed similar kinetic trends, indicating that starch from the PVP formulation crystallised at a somewhat slower rate compared to when PEG or PEtOx was used. These trends align well with the solidification process trends in **Figure 24**.

The starch crystallisation of individual droplets was also monitored *in situ*, using confocal Raman microscopy. This is visualised in **Figure 26**, which shows the 480 cm^{-1} peak shift inside starch phase droplets. The change in peak position appears to occur rather evenly throughout the droplet. While noting that the spatial resolution may limit the detection of finer details, these results still imply that the process occurs at a similar rate in the whole droplet. Additionally, this method also showed a slower crystallisation kinetic profile in PVP, demonstrated by the lagging change in colours in the heat maps, compared to the other two formulations. The average 480 cm^{-1} peak shift in the droplets over time can be viewed in **Figure 27**, which further confirms a slower crystallisation kinetic profile in PVP.

Furthermore, the results from all three methods used to study crystallisation could be modelled using the Avrami equation:

$$y = y_{max} * (1 - e^{-kt^n})$$

where y is the extent of the crystallisation reaction, k is the rate constant, t is time, and n is the Avrami exponent.

The Avrami fittings indicated random nucleation and diffusion-controlled crystal growth, thus supporting the Raman observations that the crystallisation occurred at a similar rate within the particle, rather than originating from the interface (Paper III).

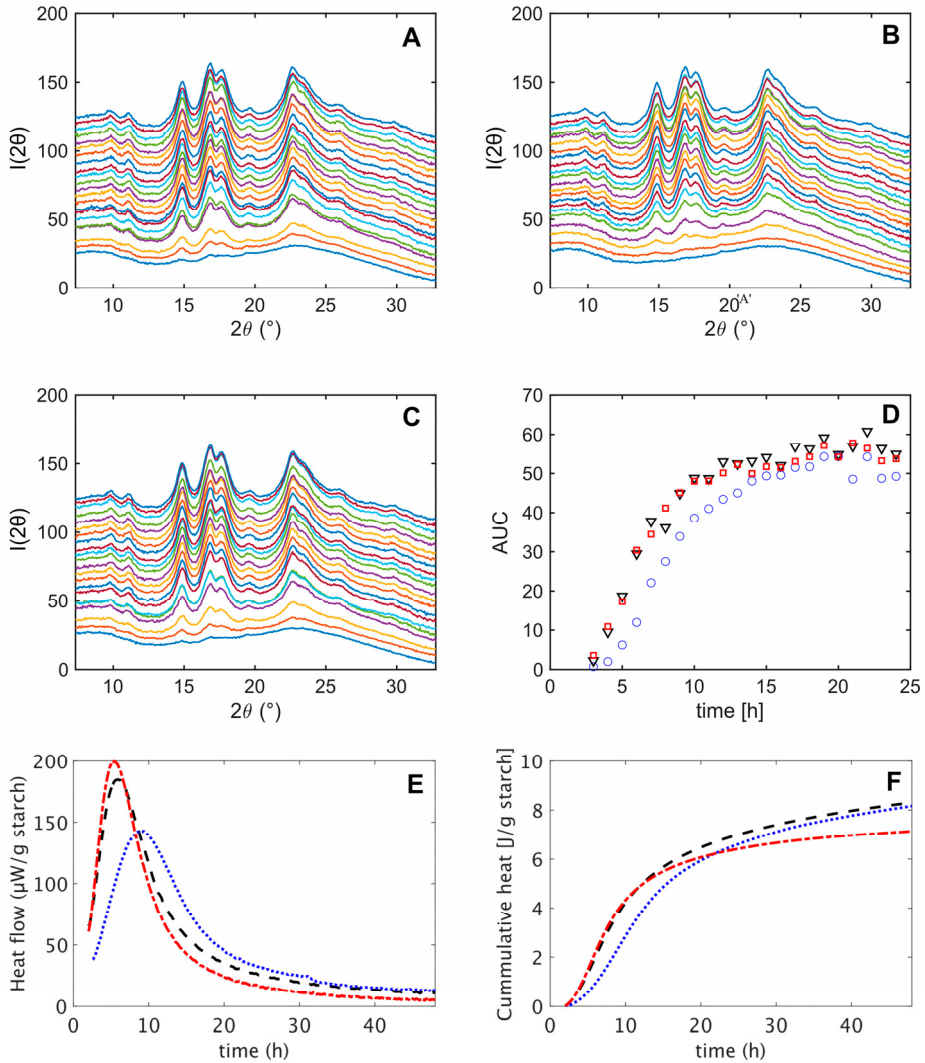


Figure 25. The crystallisation process of starch phases at 25°C, measured by WAXS and isothermal calorimetry. The first and second row (A-D) shows the growth of crystalline peaks in separated starch phases between 3-24 hours, measured by WAXS. The complementary polymers were PEG (A), PVP (B), and PEOx (C). Graph (D) shows the development of crystallinity calculated as the area under the curve between 15.6-18.7°(2θ), with PEG (red squares), PEOx (black triangles), and PVP (blue circles). The third row (E and F) illustrates the heat flow (E) and exothermal heat development (F) in ATPSS incubated between 2-48 hours, measured by isothermal calorimetry. The heat flow curves are the average of duplicate measurements. The continuous phase polymers were PEG (red, dash-dotted lines), PVP (blue, dotted lines), and PEOx (black, dashed lines). (Paper III)

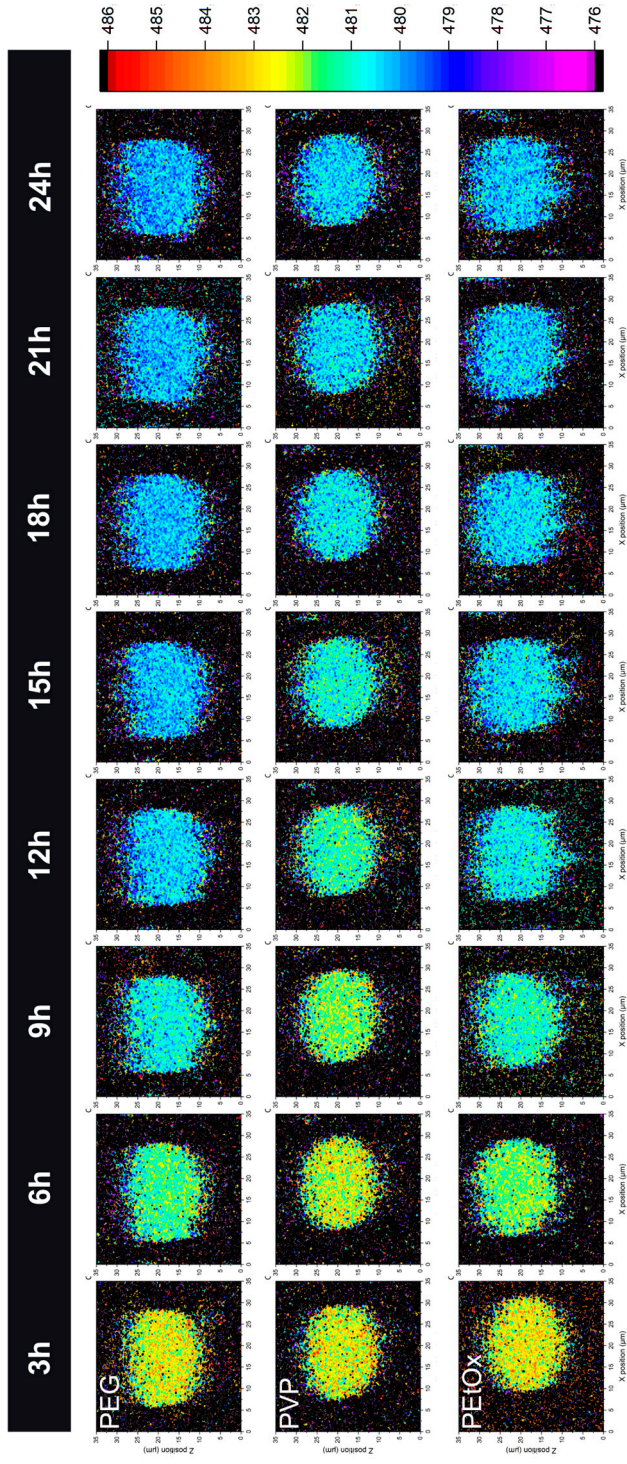


Figure 26. The crystallisation process of starch phase droplets, mapped by confocal Raman microscopy. The heat maps illustrate the 480 cm^{-1} Raman peak shift over time within a cross section of a starch phase droplet that is surrounded by a continuous polymer phase. The continuous phase polymers are PEG in the top row, PVP in the middle row, and PEtOx in the bottom row. (Paper III)

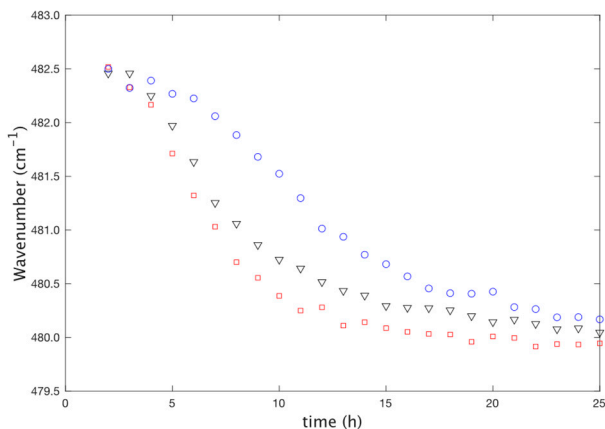


Figure 27. The crystallisation process of starch phase droplets, plotted as the average 480 cm^{-1} peak shift from the confocal Raman microscopy scans. PEG (red squares), PVP (blue circles), and PETox (black triangles). (Paper III)

Keeping in mind that the four techniques utilised measure different aspects of the process, we combined the results from the four methods, in **Figure 28**. Overall, the Raman results appear to be comparable to the WAXS and isothermal calorimetry. Thus, this confocal Raman method can be an effective tool for mapping spatially resolved starch crystallisation, providing a more comprehensive understanding of the starch crystallisation process in ATPSs. Furthermore, from **Figure 28**, it is evident that the solidification occurs already in the early stages of the crystallisation process, which is important to consider with regard to incubation time and crystallinity properties of the starch microspheres.

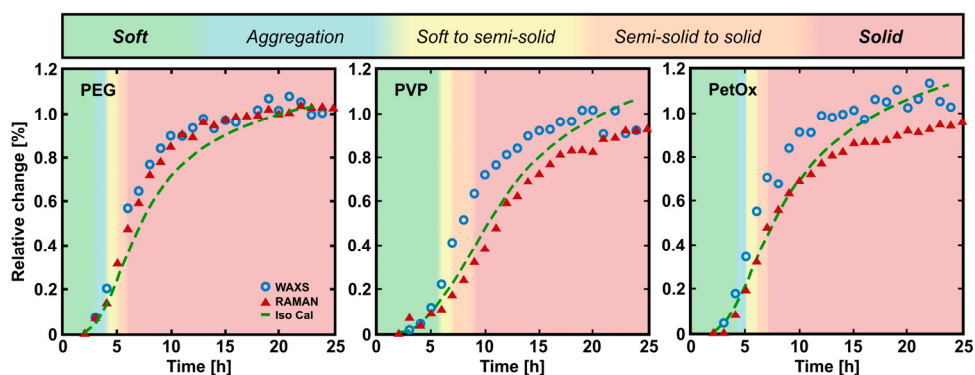


Figure 28. Combined starch microsphere formation and crystallisation observations with different continuous phase polymers. Light microscopy (colour gradient), WAXS (blue circles), isothermal calorimetry (green dotted lined), and Raman microscopy (red triangles). (Paper III)

3.4 Encapsulation of live bacteria and preparation process alterations

Lastly, in Paper IV, we explored the possibility of encapsulating live bacteria in starch microspheres, as conceptually illustrated in **Figure 29**. In these trials, we introduced bacteria and the cultivation medium MRS broth into the PEG20, PVP40, and PEtOx ATPSs previously developed and examined in Papers II and III. We also evaluated the use of another polysaccharide, dextran, as a continuous phase polymer.

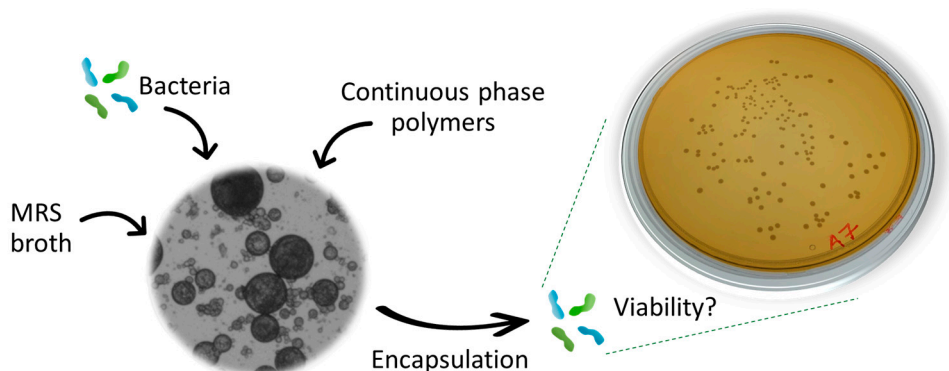


Figure 29. Schematic approach to study encapsulation of live bacteria via starch microsphere preparation in ATPSs with different continuous phase polymers.

3.4.1 Addition and partitioning of bacteria in the aqueous two-phase systems

The partitioning of bacteria was assessed in dilute ATPSs, allowing for the observation of cell distribution via light microscopy. It was found that the selection of continuous phase polymer significantly impacted the partitioning between the phases, as shown in **Figure 30**. Specifically, when PVP, PEG, or PEtOx was used as the continuous phase polymer, the bacteria predominantly partitioned into the starch phase. In contrast, within the starch/dextran ATPS, the bacteria tended to partition into the dextran phase.

The partitioning of APIs and cells in ATPSs is complex and depends on the interaction between the surface properties of the partitioning components (such as surface charge and lipid composition on the cells) with the chemical and physical nature of the phases (Albertsson, 1970; Cabral, 2007). This clearly underscores the necessity of utilising different continuous phase polymers during formulation, as the properties and concentrations of the polymers combined with other additives inherently generate a distinct solvent quality in the phase.

To achieve optimal encapsulation within the starch microspheres, it is naturally desired for the target cells to predominantly partition into the starch phase. Consequently, the starch/dextran system was excluded from further analysis, and the bacteria trials were continued with the PEG, PVP, and PEtOx ATPSs.

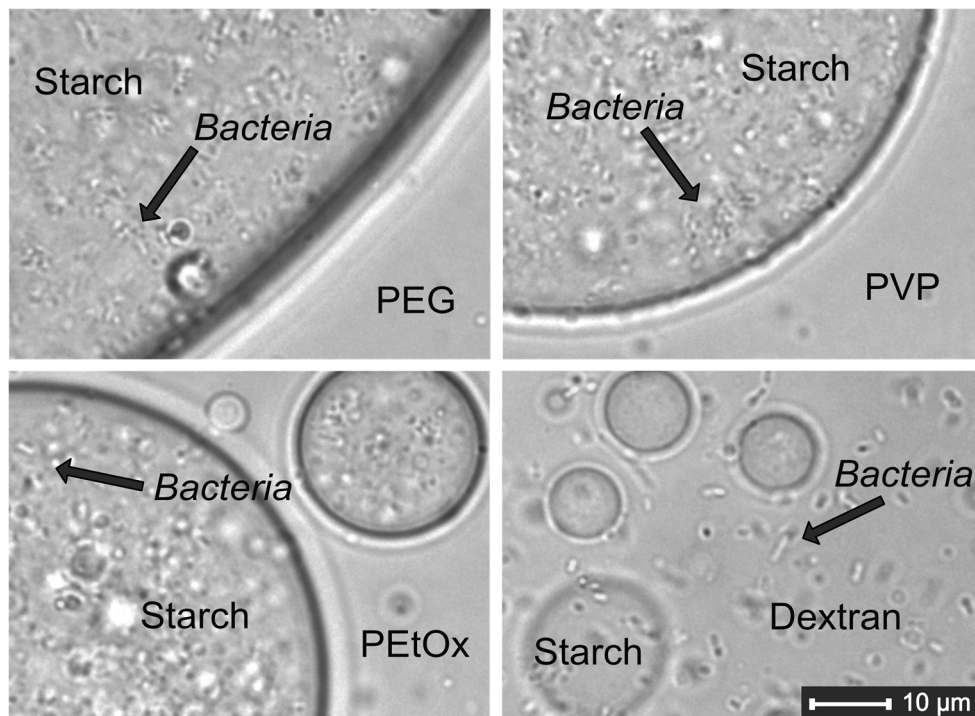


Figure 30. Partitioning of bacteria in starch-based ATPSs with different continuous phase polymers. All systems contain 0.5 % bacteria powder, 5 % starch, and 20 % continuous phase polymer, in NaCl and MRS solution. (Paper IV)

3.4.2 Microsphere formation with bacteria and cultivation media

Upon examining the formation of starch microspheres using light microscopy, we observed that the addition of bacteria and MRS broth influenced the rate of starch phase solidification. More particular, the systems with bacteria and MRS displayed roughly one hour delayed solidification, compared to the placebo formulations (see **Table 3**).

Furthermore, as demonstrated in **Figure 31**, an increase in the clumping and size of the particles formed in the PEG phase was observed. This indicates that the starch/PEG ATPS is more sensitive to alterations than the other two systems.

Table 3. Starch gel transition during ATPS incubation with either MRS and bacteria or without MRS and bacteria (placebo). The transitions were probed every hour by applying pressure onto the light microscopy coverslip atop the samples. Particles that could change shape without cracking were interpreted as soft particles, while small cracks were interpreted as semi-solid particles and large cracks as solid particles. (Papers III and IV)

Continuous phase polymer	Soft (end-point h)	Semi-solid (onset h)	Solid (onset h)	Aggregation (onset h)
PEG	5	6	7	5
PEG Placebo	4	5	6	4
PVP	7	8	9	-
PVP Placebo	6	7	9	-
PEtOx	6	7	8	6
PEtOx Placebo	5	6	7	5

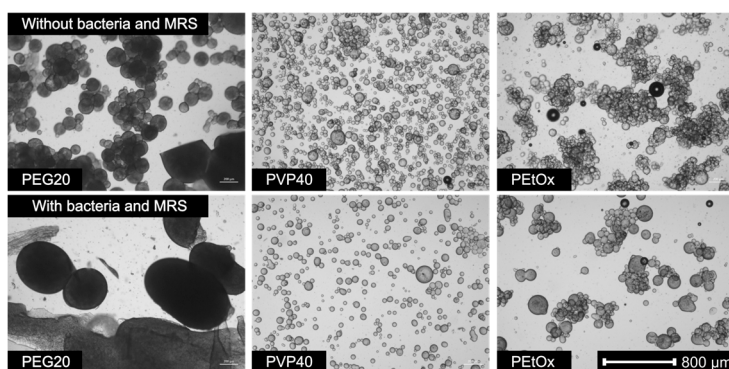


Figure 31. Starch microsphere appearance with and without bacteria and MRS. The light microscopy images are taken at 24 hours of incubation. The top row images are reused from **Figure 22**, to facilitate comparison. (Papers II and IV)

3.4.3 Mechanical process adaptations

To optimise the preparation process to better accommodate the integration of bacteria, alterations were made by flushing the formulations with N_2 gas, as well as testing different emulsification, agitation, and incubation vessels (see **Figure 32** for an overview of evaluated mechanical process parameters). The mechanical process was found to influence the starch particle size, aggregation, and bacterial encapsulation. As shown in the light microscopy images in **Figure 32**, samples that had not been spun in the test tube rotator (but instead left without agitation for 24 hours) had more lumps and broken particles. As can also be seen, these samples exhibited a higher tendency of bacterial leakage into the bulk. The most favourable results (being the most even and non-broken particles, with the least bacteria leaked) were obtained using spatula emulsification, a vertical test tube rotator, and large incubation tubes. Additionally, SEM images of the dried particles revealed that the starch microspheres should not be too large, as the larger particles tended to get

more mechanical stress fractures during drying. These fractures could subsequently lead to bacterial leakage upon redispersion. Therefore, the size of the particles should be controlled, for instance by adapting ATPS phase ratios or polymer type (as described in Papers I and II).

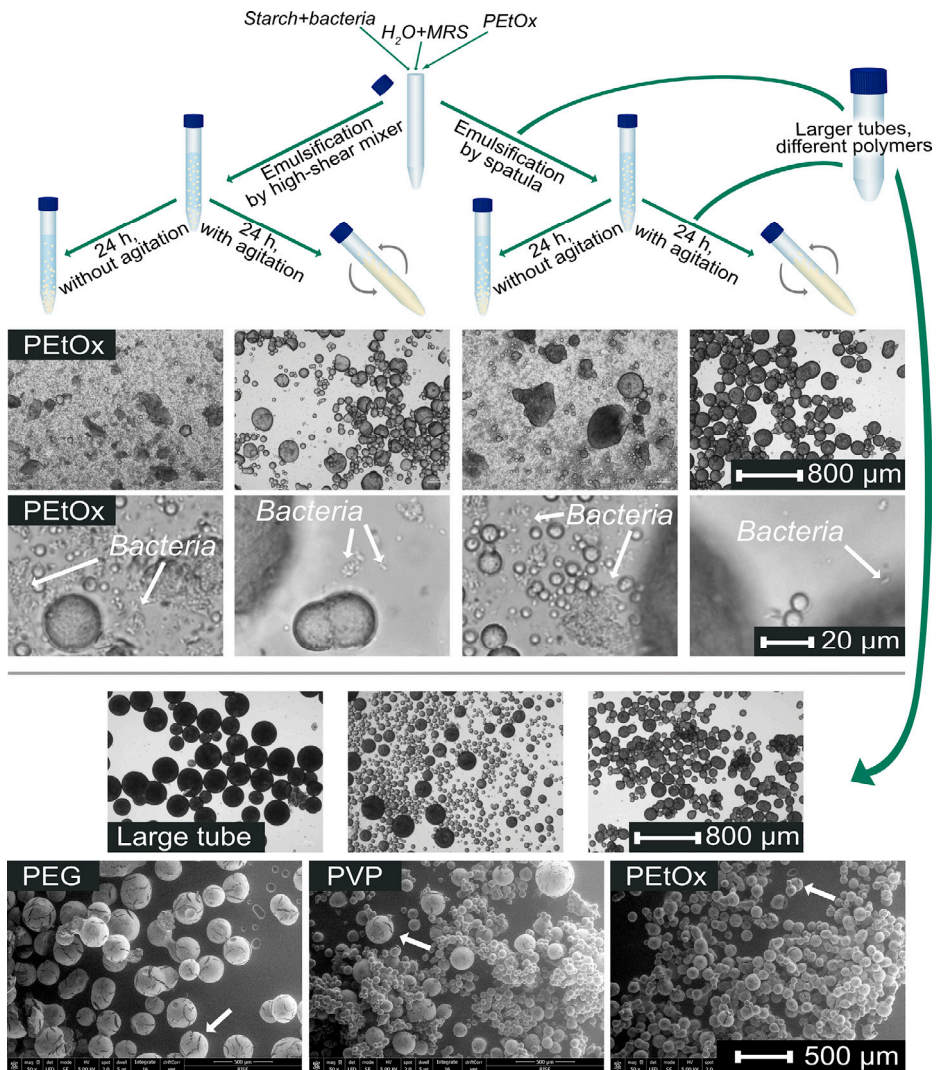


Figure 32. Mechanical process evaluation and appearance of newly washed and dried starch microspheres with bacteria. The light microscopy images (rows 1-3) show material 2-4 hours after having been washed 3 times with 10.3 % sucrose solution. The first and second row displays particles from an ATPS where PEtOx was used as the continuous phase polymer. The third row is of particles from different ATPSs with continuous phase polymers PEG (left), PVP (middle), and PEtOx (right), that were emulsified by a spatula and spun for 24 hours in larger tubes. The fourth row is freeze-dried materials that were prepared by spatula emulsification and spun for 24 hours in larger tubes. The white arrows exemplify leaked bacteria (second row) and fractures in particles (fourth row). (Paper IV)

3.4.4 Bacterial encapsulation and amylase release

To test the release of encapsulated bacteria from solidified microspheres, the collected particles were placed in an amylase buffer. **Figure 33** shows a sample of freeze-dried and redispersed bacteria-loaded particles that have been placed in a cavity glass slide with amylase buffer, at ambient temperature. This demonstrates that the amylase can hydrolyse the starch particles, at the same time as confirming the presence of successfully encapsulated bacteria in the freeze-dried product. These results also suggest that amylase digestion could be used as a release mechanism in the GIT, albeit these studies were conducted with high amylase activity (specified in the caption of **Figure 33**). Therefore, it is noteworthy that the starch microspheres can be effectively digested at amylase activities substantially lower than previously reported recommendations for *in vitro* simulated intestinal digestion, as shown in **Figure 34** (Paper I) (Brodkorb et al., 2019; Minekus et al., 2014; Mulet-Cabero et al., 2020). Consequently, future studies should investigate amylase digestion and bacterial release, as well as how this is influenced by factors such as starch material and pre-treatment, additional excipients, preparation temperature, and time program.

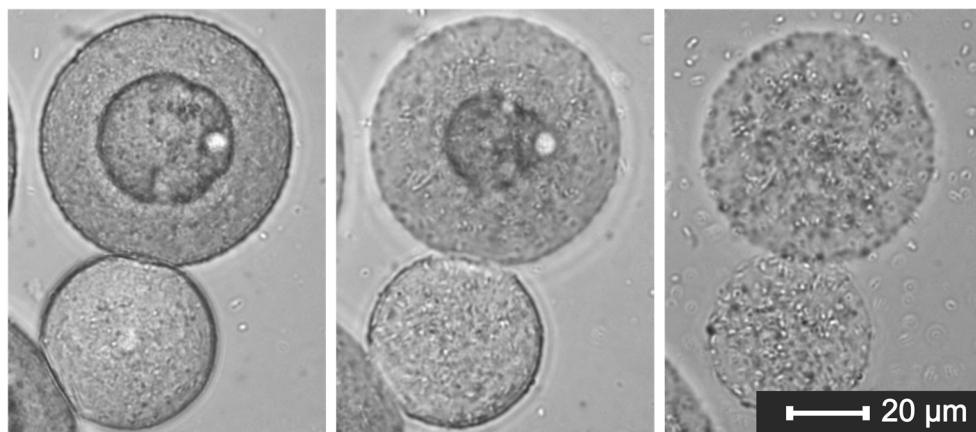


Figure 33. Light microscopy of starch microspheres with encapsulated bacteria incubated with α -amylase at ambient temperature. A droplet of amylase buffer was added to a droplet of redispersed starch particles, and images were taken at 14, 18, and 71 minutes, respectively, from that the amylase buffer was added to the microscopy glass. The amylase buffer contained approximately 940 units per mL, where one unit (as stated by the supplier) would liberate 1.0 mg of maltose from starch in 3 minutes at pH 6.9 at 20°C. (Paper IV)

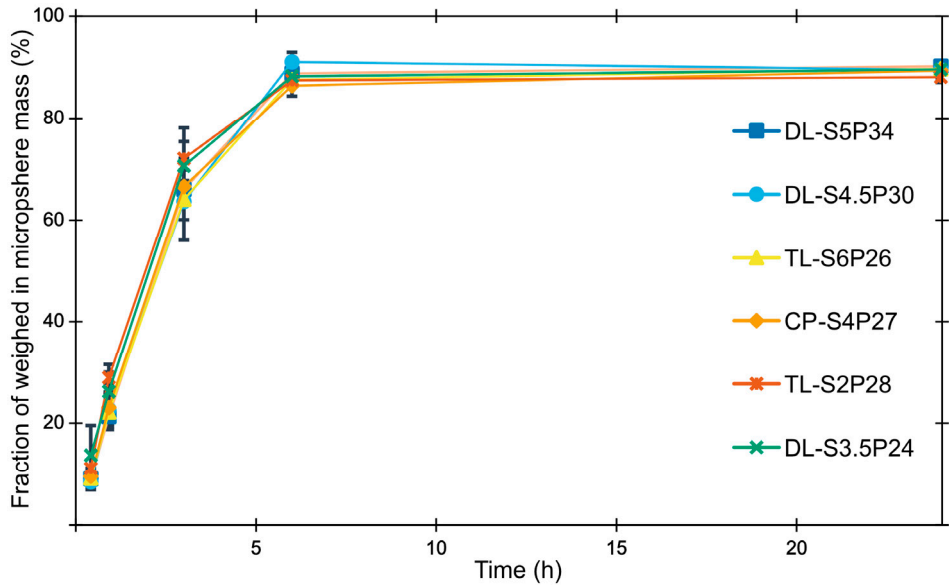


Figure 34. Hydrolysis degree of placebo starch microspheres incubated with 40 units per litre α -amylase for 24 hours, at 37°C. The microspheres were manufactured at different positions within the starch/PEG20 ATPS phase diagram displayed in **Figure 11** and **Table 1**. The error bars represent the standard deviation (n=2). The amylase units were determined with an Amylase Activity Assay Kit MAK009 (Sigma-Aldrich, Germany), where one unit equals the quantity of amylase that cleaves ethylidene-pNP-G7 to produce 1.0 μ mole of p-nitrophenol per minute at 25°C. (Paper I)

3.4.5 Bacterial viability

The final experiments in this thesis tested the viability of the microencapsulated lactic acid bacteria at different stages of the starch microsphere preparation. The number of CFU in the formulations was compared to the starting material CFU (being newly rehydrated pre-freeze-dried bacteria powder). A control formulation was also included in the trials, where the bacteria were incubated in isotonic NaCl and MRS solution and otherwise handled in the same manner as the ATPS formulations.

The results, presented in **Figure 35** and Paper IV, show that the bacteria tolerated the starch solidification well. The CFU count before drying displayed over 80 % survival when the bacteria had been encapsulated in starch microspheres with PEtOx or PEG in the continuous phase, with only 50 % survival in the control samples. However, the bacteria did not fare well having PVP as a continuous phase polymer, as no bacterial growth could be detected in the investigated dilution steps, displayed in **Figure 35**. In addition, the freeze-drying and storage reduced the CFU count by several log units. This highlights the necessity for further development to improve drying survival and stability. However, the bacterial survival after 1 week at 37°C was 58 times better when encapsulated in starch from the PEtOx ATPS, compared to the control formulation that had been freeze-dried in a conventional sucrose matrix. Thus, this method for encapsulation and delivery of live bacteria to the GIT can be regarded as a promising concept, warranting further research to improve and explore the potential of this formulation strategy.

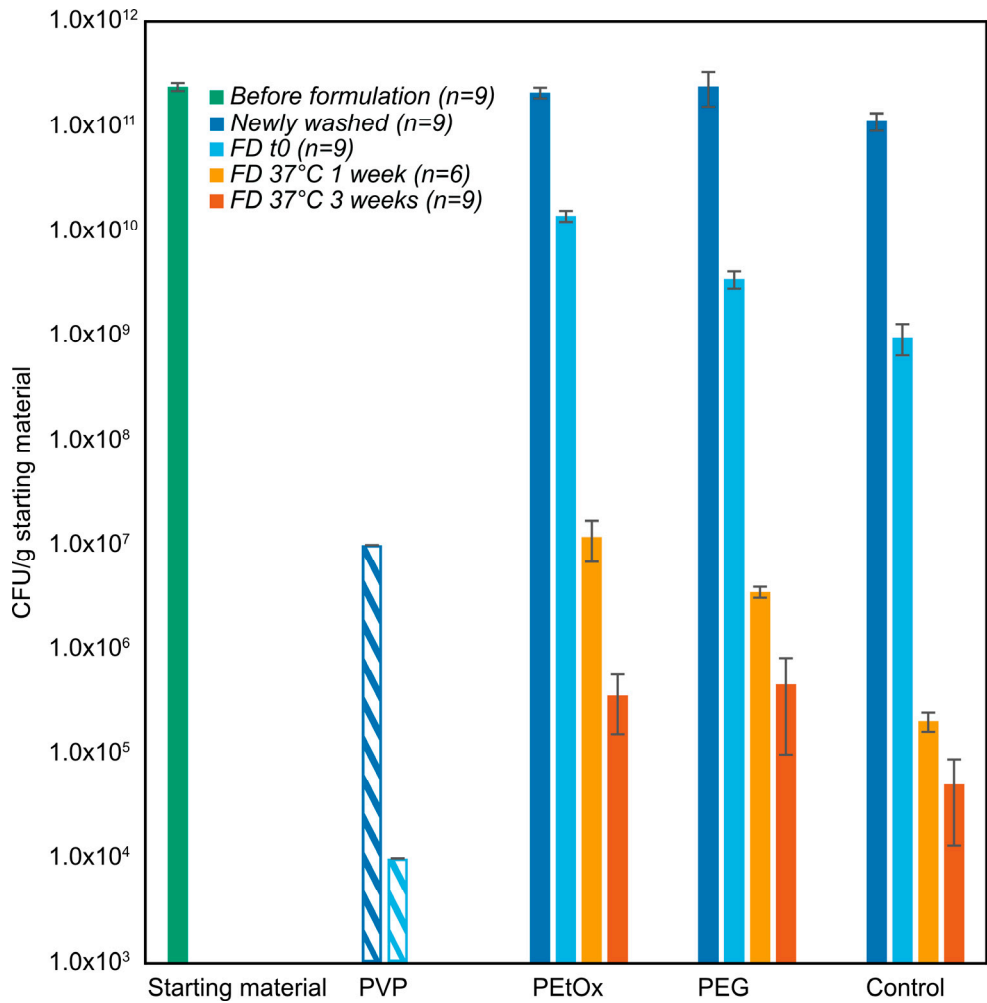


Figure 35. Viable counts for *L. reuteri* in formulations before (newly washed) and directly after freeze-drying (FD t0), as well as after storage at 37°C (FD 37°C...) for 1 and 3 weeks. Error bars represent the standard deviation of n counted plates. Dashed staples are used for samples where no CFU was detected in the lowest dilution step plated, meaning that the presence of CFU under the staple plateau has not been investigated. The starting material CFU comes from newly rehydrated pre-freeze-dried bacteria powder (before formulation).

3.5 Key findings from explored systems and methodologies

Key findings related to the explored systems and methodologies in this thesis were:

- ATPS phase behaviour was an important aspect to consider during the preparation of starch microspheres.
- The ATPS phase diagram revealed a large biphasic region but a relatively narrow window for the production of starch microspheres.
- Mixing of starch in the polymer phase (and vice versa) appeared low but polymer inclusions could be spotted in the starch phase, indicating that these ATPSs can form double emulsions.
- Water content in the starch phase after mixing the ATPSs was key to attaining sufficient starch crystallisation rates for microsphere solidification, with overly diluted systems impairing solidification.
- The ATPS dilution level influenced the starch crystal formation, with lower water content favouring the formation of A-type polymorphs.
- The starch phase to polymer phase ratio and dilution level affected particle size.
- The viscosity of the continuous phase played a considerable role in stabilising the starch droplets. Excessively high viscosity limited the amount of dry matter that could be mixed into the ATPS, while too low viscosity failed to stabilise the dispersed starch phase droplets during incubation.
- Starch microspheres could be prepared with different continuous phase polymers, but different polymer portions were needed to control the water content in the starch phase.
- The water distribution could be correlated to the water activity in individual polymer stock solutions.
- Altering the continuous phase polymer Mw could adjust the ATPS viscosity without significantly affecting the water distribution.
- The continuous phase polymer type could influence starch particle size, aggregation, crystallisation kinetics, and sensitivity to production alterations, but did not have a pronounced effect on interior structure, surface morphology, crystal type, and thermal behaviour of the starch microspheres.

- Solidification of the starch phase droplets started in the early stages of the crystallisation reaction, and the addition of MRS broth and *L. reuteri* decreased the solidification rate.
- Confocal Raman microscopy maps of the crystallisation process were comparable to the WAXS and isothermal calorimetry methods, and indicated an evenly distributed crystallisation inside a dispersed starch phase droplet.
- Moderate changes in ATPS incubation temperature significantly impacted starch crystal formation, with lower temperatures in the investigated range promoting B-type polymorphs.
- During freeze-drying, ordered material was reduced, and crystalline peaks were partly lost. Some crystalline peaks were regained upon redispersion in water, favouring the A-type polymorph.
- Slow cooling before freeze-drying resulted in compacted particles, while rapid cooling promoted porous particles.
- The freeze-dried particles rapidly swelled upon redispersion in water.
- *L. reuteri* could be successfully encapsulated in starch microspheres via ATPSs.
- Mechanical stress fractures occurred in the larger particles during freeze-drying, and leakage of bacteria was observed in samples with broken particles.
- The selection of continuous phase polymer significantly affected the partitioning of *L. reuteri* in the ATPSs.
- *L. reuteri* could maintain good viability during the starch microsphere solidification and could be released from the starch microspheres by amylase digestion.
- Freeze-drying and storage at 37°C reduced the viability of *L. reuteri* by several log units. However, the bacterial survival was up to 58 times better when encapsulated in starch compared to the control formulation freeze-dried in a conventional sucrose matrix.
- The selection of continuous phase polymer significantly affected the viability of *L. reuteri*, highlighting the need to screen appropriate polymers for specific cells and APIs intended for encapsulation.

3.6 Concluding remarks and future perspectives

These studies emphasise the importance of phase behaviour characterisation during formulation development and starch microsphere preparation. They demonstrate that ATPS starch microspheres can be prepared with different continuous phase polymer types and Mw, while keeping in mind that phase behaviour of the ATPS is important for the starch microsphere preparation.

The starch microsphere formation is strongly linked to the water content within the starch phase. Too high water content in the starch phases hinders starch microsphere formation by reducing the solidification rate. Thus, controlling the water distribution within the ATPSs is important for effective starch crystallisation. The water distribution is associated with the water activity dependence in individual polymer solutions, enabling water activity measurements in different polymer solutions to be used as guidance for phase behaviour predictions.

Moreover, this work shows how ATPSs can be utilised to encapsulate pre-freeze-dried live lactic acid bacteria in starch microspheres, and that the bacteria can remain viable as the starch phase crystallises into solid particles. This approach presents a promising formulation strategy, though further development is needed to improve freeze-drying survival and storage stability.

This work also demonstrates that using confocal Raman microscopy to map cross-sectional areas can provide detailed information about specific locations within the sample, thus allowing for a more comprehensive understanding of the starch crystallisation process. Additionally, this method could potentially be used for monitoring *in situ* starch crystallisation simultaneously as mapping encapsulation of APIs, further expanding its applicability in pharmaceutical and material science fields.

Overall, the knowledge and methodologies learnt in this project may be utilised in future food and pharmaceutical development. For instance, a continuous phase polymer type can be chosen to suit desired APIs and applications. Water activity measurements of individual polymer solutions can facilitate the mapping of water distribution, which will allow for tuning of starch crystallisation by controlling the starch phase water content and incubation temperature. Light and confocal Raman microscopy could be utilised to monitor the encapsulation and crystallisation process *in situ*. Additionally, the incubation vessel and Mw of the continuous phase polymer type can then be adjusted to get appropriate hydrodynamics for stabilising the starch particles during the crystallisation process. Furthermore, the size of the starch particles can be modulated by altering the phase ratio along the ATPS manufacturing tie lines.

The potential of these systems should be investigated with newly cultivated bacteria and other excipients, as well as other APIs. The continuous phase polymers warrant additional research, for instance, in relation to functions such as controlled release. Moreover, different starch materials and temperature treatments should be examined to modify digestion and thermal properties, in ways that accommodate suitable release mechanisms for different applications.

To summarise, the preparation of physically cross-linked starch microspheres via ATPSs presents a promising approach for encapsulation and delivery of sensitive cargo. It is nonetheless a procedure that combines the complex phenomena of ATPS phase behaviour and starch crystallisation, making further research a necessity for unlocking the potential of this formulation concept.

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