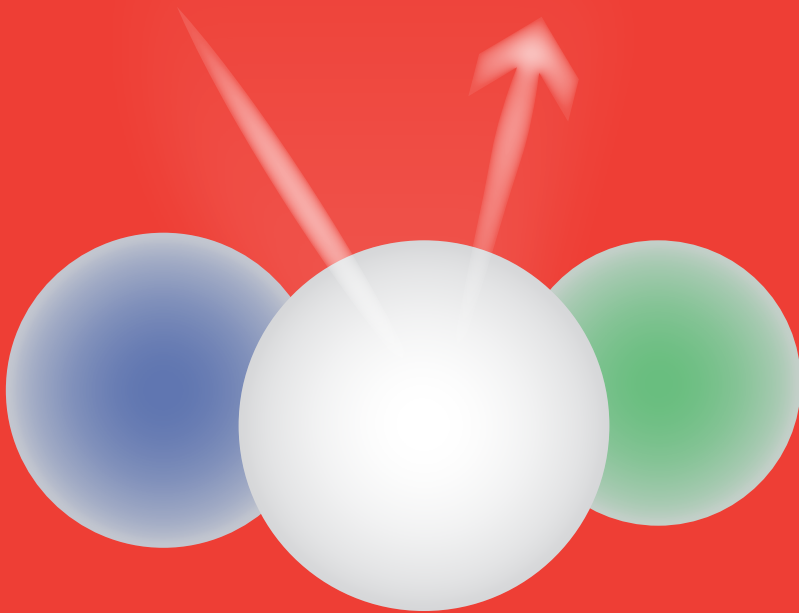


COLLOIDAL COLORANTS

FROM ZEIN PROTEIN
TO COLOR CARRIER



Frankjen Ynske de Boer

Colloidal Colorants

from zein protein to color carrier

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Colloidal Colorants

from zein protein to color carrier

**Colloïdale Kleurstoffen
van zeïne eiwitten naar kleurdrager**
(met een samenvatting in het Nederlands)

Proefschrift

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Chapter 1

Introduction

Abstract

Product appearance is an important factor for consumers to determine the quality of a product, and color is one of the most important factors that contribute to the product appearance. Currently, the safety and consumer acceptance of some colorants used in food products, such as titanium dioxide and some synthetic colorants, are under discussion. Therefore, new ways to use natural colorants as alternatives to these suspect colorants for more applications are investigated. A promising method to increase the applicability for the often-sensitive natural colorants, is the encapsulation of these colorants in colloidal particles by natural polymers such as carbohydrates, lipids and proteins. In recent years, encapsulation has increasingly been used for various purposes regarding several food properties such as color, flavor, and micro-nutrient content. This technique results in improved stability for the often-sensitive natural colorants and opens the possibility to entrap water-insoluble colorants for improved use in an aqueous system. In this Chapter, the encapsulation method used in this Thesis for encapsulation (anti-solvent precipitation) is discussed, including the different types of encapsulants used for encapsulation of colorants for food applications, including the vegetable protein zein which is used for the projects further described in this Thesis.

1.1 Colorants

Color is a fascinating property. People heavily rely on visual information to navigate the world. Evidence suggests that conflicts between visual and other sensory cues tend to be resolved in favor of vision.^{1,2} It has been found that even the color of a plate or cup has a significant effect on how the food or drink is perceived.^{3,4} Not only the color of the product is important, also the color of the surroundings has influence on the perception. Color even affects odor intensity, for example a liquid which is colored (e.g. red) and contains an odorant (e.g. strawberry) will have a stronger perceived smell.⁵ Overall, color is of primary importance in the perceived quality of a product and this initial judgement ultimately influences its acceptance or rejection.⁶⁻⁸

Food colorants can be classified according to several criteria: origin (natural, identical to natural, or synthetic; organic and inorganic), solubility (soluble and insoluble) and covering ability (transparent and opaque). However, these categories often overlap. The classification that is most widely used, is the distinction between soluble and insoluble colorants.⁹

Soluble colorants can be subdivided into natural, semi-synthetic and synthetic ones. Natural dyes are obtained from various fruits and vegetables or other natural materials.¹⁰ Examples include curcuminoids (E100), anthraquinoids¹¹ (E120), chlorophylls (E140), carotenoids (E160 and E161), betalains^{12,13} (E162), and anthocyanins (E163). Another group consist of semi-natural and semi-synthetic dyes. In the case of semi-synthetic dyes, these consist of chemically modified natural dyes to increase their stability, such as copper chlorophyllin¹⁴ (E141). Or, in the case of semi-natural dyes, these are similar molecules to their natural counterparts, however they are obtained through a fully synthetic pathway. Synthetic dyes are produced by chemical synthesis but cannot be found naturally. Examples are azo-dyes and anthraquinoids, which have generally more intensive and permanent color than natural substances. These synthetic dyes often do not impart any flavor to products and are more stable.

Insoluble colorants are also known as pigments. They are very stable colors that exhibit good cover properties and are insoluble in common solvents. Pigments can be inorganic or organic and often have a limited variety of colors available. Examples include: carbon (E153), calcium carbonate (E170), titanium dioxide (E171), and iron oxide (E172). Organic pigments are usually in the form of lacquers which are insoluble complex salts of water insoluble azo-dyes in a wide color palette.

Nowadays, consumers are more and more aware of what they are eating, which is represented in the famous adage: “you are what you eat”. These consumers express a desire for their food products to be natural and should only contain ingredients that, in their opinion, are “natural”. This has caused an increase in the research towards natural food ingredients and colorants.^{10,15} A similar effect is seen in the research towards the safety and possible negative effects of inorganic and synthetic food colorants. The most widely known and used colorant is titanium dioxide (TiO₂) which accounts for 70% of the total production volume of pigments worldwide.¹⁶ TiO₂ is used as a pigment in many industries, primarily in paints and coatings. Additionally, it is also used as a pigment in plastics, paper, inks, textile, photovoltaic cells, biomedical devices, cosmetics and even as a food additive.^{17,18} In the food industry it is used as white colorant and opacifier and it is authorized as a food color in the European Union (EU) as E171. Although TiO₂ is considered food safe, concerns have been raised on the safety of TiO₂ nanoparticles that were found to also be included in food products.^{19,20} The IUPAC definition of a nanoparticle is a particle of any shape in the 1 to 100 nm range, whereas a micro-particle is defined as a particle with dimensions between 0.1 to 100 µm.²¹ Recently, the oral intake of TiO₂ via food and toothpaste was determined based on an average life span of 80 years. It was found that the lifelong daily intake of TiO₂ was 0.19 mg/kg bodyweight, of which 0.62 µg/kg bodyweight were TiO₂ nanoparticles (determined for people living in the Netherlands).²² Using this information, toxicological experiments were performed indicating it is possible there are negative long-term health effects associated with oral intake of TiO₂ nanoparticles. However, additional research on this topic is necessary to provide a good overview of the potential long-term negative effects associated with TiO₂ nanoparticles.^{23,24}

Not only inorganic color additives, such as titanium dioxide, are under discussion, but also some synthetic color additives. Here, questions are raised mainly on reported negative behavioral changes in children, such as ADHD. The colorants in question are Sunset Yellow FCF (E110), Carmoisine (E122), Tartrazine (E102), Ponceau 4R (E124), Quinoline Yellow WS (E104) and Allura Red AC (E129). Parents have reported behavioral changes in their children after consuming these synthetic colorants in the form of hyperactivity.²⁵ In a later study²⁶ by the same group, the same behavioral changes were observed using standardized clinical tests. The researchers concluded: “Artificial colors or a sodium benzoate preservative (or both) in the diet result in increased hyperactivity in 3-year old and 8/9-year-old children in the general population.” However, many aspects still are unclear because of inconsistency in different research investigations on this topic and the possibility that genetic or

environmental factors could also influence these outcomes.²⁷ For example, the same experiment was repeated in Hong Kong²⁸ where the researchers concluded that there are no significant associations between artificial food colorants and a preservative on Chinese children's behavior at the age of 8 to 9. Overall, the results from multiple studies on negative behavioral changes and toxicological effects are not conclusive and research on this topic is ongoing.^{9,29,30}

These inconclusive data on the safety and negative effects of inorganic and synthetic colorants is, at the moment, not very helpful for consumers. Another reason for consumers not to trust non-natural colorants is the fact that several previously approved food additives are no longer currently allowed due to proven side effects, such as toxicity at medium- and long-terms, and a high frequency of health disturbance incidents, whilst these additives were initially considered safe to use.³⁰ This has motivated the search and development of naturally derived and safer alternatives for these questioned synthetic colorants. Recently, Coultate et al. have written an extensive review on this topic,¹⁵ discussing the past, present and future of food colorants. Origins for these natural colorants are plant, animal, microbial and mineral based. Some commonly used and more prevalent natural colorants and additives are: anthocyanins, betalains, carminic acid, carotenoids, chlorophylls, curcuminoids, minerals, phycocyanins, and some vitamins.¹⁵ A disadvantage of using natural colorants is that applications can be limited by their properties. Natural colorants often have a weaker tinctorial strength than their synthetic counterparts, and it is often difficult to match desired hues, especially green and blue. Most are sensitive to heat, light and oxygen; which may result in color loss or shifts. Others may be sensitive to food matrix conditions, such as pH, proteins, metal ions, or some organic compounds.¹⁰

In this Thesis we discuss encapsulation of colorants by the natural polymer zein, which is extracted from corn, for the formation of colloidal particles. Encapsulation offers improved stability for the often-sensitive natural colorants by creating a barrier.³¹ An additional benefit of this is that possible off-flavors of the colorants can be masked by the biopolymer.^{32,33} Also, stability can even be further improved by adding antioxidants and other stabilizers to the particles. Using encapsulation for the delivery of colorants opens the possibility to entrap water-insoluble colorants and use them in an aqueous system,³⁴ which broadens the number of natural colorants that can easily be used in food systems. The encapsulation method has various applications for the purpose of encapsulating colorants, not only in the food industry, but also in the pharmaceutical, cosmetic, textile and other industries.

1.2 Encapsulation

Encapsulation is defined as a technology of packaging solids, liquids, or gaseous materials in matrices (encapsulants) that can sustain and possibly release their contents under specific conditions. Encapsulation can have multiple purposes, the first is to protect sensitive components from light, moisture, or heat. Second, encapsulation can be used for improving the stability of biological pigments as a dispersion in aqueous systems to increase the usability of natural colorants in many industry applications. Another advantage of using encapsulated products is that their protective shell stays intact and therefore the colorants do not easily migrate through the product as conventional colorants do. The brightness of the colorants can also be improved, thus increasing their marketability for commercial use.³⁵ Encapsulation can be achieved using various processes. Examples include emulsification, coacervation, molecular/inclusion complexation, anti-solvent precipitation, emulsification-solvent evaporation technique, supercritical fluid technique, spray-drying, electrospraying, and freeze-drying.^{36,37} In this Thesis, the anti-solvent precipitation technique is used as the method for encapsulation of colorants.

1.2.1 Anti-solvent precipitation

The anti-solvent precipitation technique, also known as nanoprecipitation, liquid-liquid dispersion, or solvent displacement technique, was originally applied to encapsulation and patented by Fessi et al.^{38,39} The technique is straightforward, fast and easy to perform in a single-step procedure. Particle formation using this technique is instantaneous and requires two miscible solvents, in which ideally both the biopolymer and the encapsulate dissolve in the first (the solvent) but not in the second (the anti-solvent). When the biopolymer-containing solvent diffuses into the anti-solvent the biopolymer precipitates (Figure 1.1), which causes immediate entrapment of the encapsulate.^{40–42} Particle sizes obtained following this procedure are often between 20 to 300 nm depending on the specific materials and conditions used for the synthesis.

The term coacervation has also been used to describe this anti-solvent process. However, in the food industry and in colloid science this term is often used to refer to hydrophilic colloids which precipitate by interaction between two oppositely charged colloids upon chemical or physical triggers, such as manipulation of the temperature, salt, pH, and solubility conditions.^{43–46}

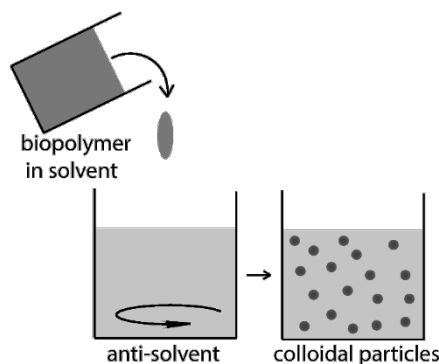


Figure 1.1: Schematic overview of an anti-solvent precipitation process.

An advantage of the anti-solvent precipitation technique is that it does not require extended shearing or stirring rates, sonication, nor very high temperatures, which is beneficial for biopolymers and sensitive encapsulates that could be affected by these conditions. Moreover, surfactants are not always necessary for stability, and toxic organic solvents are generally excluded from this procedure.⁴² A possible disadvantage is that the technique is mostly suitable for compounds having a hydrophobic nature. However, when materials with these properties are used, this method often leads to very high entrapment efficiencies because of close to zero leakage due to the anti-solvent. For the application in dry form, particles synthesized using anti-solvent precipitation then need to be dried. This could be achieved by using freeze-drying or spray-drying as an extra step during particle production to keep particle deformation to a minimum. An example of this is principle was carried out by Patel et al.³⁴ They prepared quercetin loaded biopolymeric colloidal particles by precipitating quercetin (water insoluble) and zein (hydrophobic protein), simultaneously, by adding their hydro-alcoholic solution to the aqueous anti-solvent in the presence of sodium caseinate as a stabilizer.

Anti-solvent precipitation is commonly used to fabricate particles using a simple batch method on lab scale, however this is not directly suitable for industry where continuous production is more desirable. Recently, a continuous technique based on anti-solvent precipitation was developed by Li et al. for production of zein colloidal particles using flash nanoprecipitation with controlled particle sizes on a large scale.⁴⁷ Later also Ebert et al. studied the potential of producing other protein particles by anti-solvent precipitation using a continuous dual-channel micro-fluidization method.⁴⁸

1.3 Types of encapsulants suitable for food applications

Encapsulants suitable for food applications must be food grade, biodegradable, and stable in food systems during processing, storage, and consumption. The most suitable encapsulants for food applications are carbohydrates, lipids, proteins, or mixtures thereof. As discussed previously, encapsulation can be achieved via several processes that lead, depending on the chosen technique and materials, to the formation of particles with various structures (Figure 1.2). Examples of these, including examples of colorants that were successfully encapsulated, will be briefly discussed in this Section and we will conclude this Section with a more elaborate explanation of the encapsulant used throughout this thesis, zein.

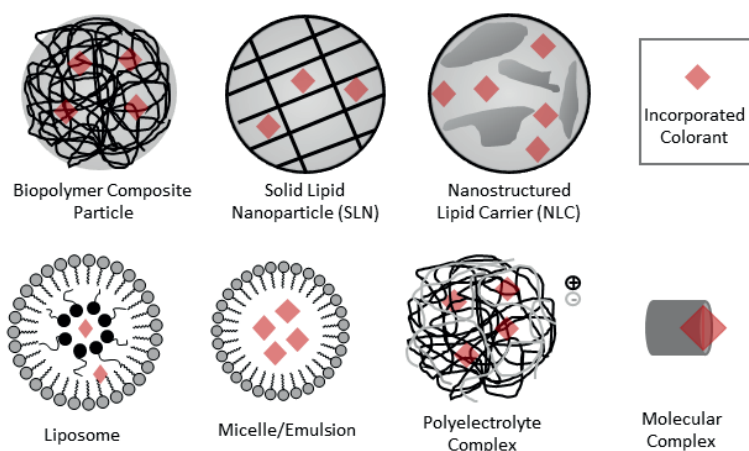


Figure 1.2: Examples of colloidal structures that are formed when a colorant is encapsulated using a biomaterial as encapsulant.

1.3.1 Carbohydrates

Carbohydrates consist of monosaccharides, oligosaccharides, and polysaccharides. They are very suitable for encapsulation of colorants for food applications, since they are already widely used as safe and inexpensive food ingredients. Carbohydrates are considered to be a suitable shell under high temperature processes due to their temperature stability in comparison to lipids or proteins, which might melt or denature when subjected to higher temperatures.⁴⁹ Examples of suitable carbohydrates to encapsulate sensitive natural colorants (such as betalains, anthocyanins, and lycopene) include: starch,^{41,50} guar gum (E412), gum Arabic⁵¹ (E414), xanthan gum⁵² (E415), cyclodextrin,⁵³ maltodextrin⁵⁴ (E1400), and combinations of carbohydrates.^{35,55,56} Carbohydrates are often used for spray drying, which means that these can only be used to encapsulate and stabilize natural colorants in dry form.

1.3.2 Lipids

Among the encapsulation systems, the lipid-based particles are promising systems for encapsulation and delivery of generally poorly-soluble ingredients. For the purpose of encapsulating colorants using lipids not much research has been done so far. Therefore, lipid particles that offer potential use for the application of colorant encapsulation are discussed: solid lipid nanoparticles (SLN), nanostructured lipid carriers (NLC), and liposome vesicles.

Solid lipid nanoparticles

Solid lipid nanoparticles (SLNs) were developed as an alternative carrier system to the existing traditional carriers, such as emulsions, liposomes, and polymeric particles. SLNs are aqueous colloidal dispersions of which the matrix consists of solidified lipids that are highly structured,⁵⁷ see Figure 1.2. Typical particle sizes of these SLNs are in the range of 40-1000 nm.⁵⁸ The particles are prepared either with physiological lipids or lipids with GRAS (generally recognized as safe) status. Primary advantage of SLNs is that they provide protection for sensitive molecules from the external environment (water, light). Disadvantages are their not always controllable particle growth, their unpredictable gelation tendency, their unexpected dynamics of polymorphic transitions, and their inherent low incorporation rate due to the crystalline structure of the solid lipid.^{59,60} Colorant successfully encapsulated in SLNs are curcuminoid extract⁶¹ and β -carotene.⁶²

Nanostructured lipid nanoparticles

A new generation of lipid nanoparticles has been developed as an improvement on SLNs:⁵⁷ nanostructured lipid carriers (NLCs). They are made of biocompatible and biodegradable lipids with well-established safety profiles and toxicological data.^{63,64} NCLs consist of a blend of solid and liquid lipids that creates a less ordered matrix (see Figure 1.2), which allows a higher loading capacity and a lower possibility of drug expulsion during storage compared to SLNs.⁵⁸ The synthesis process can be modified to yield lipid particle dispersions with a 30 to 80% solid content.⁵⁹

Researchers incorporated lutein into colloidal NLCs that could be dispersed in water, which makes it possible to use lutein in water-dispersible food systems.⁶⁵

Liposomes

Liposomes are closed vesicular structures, consisting of bilayers of hydrated phospholipids. The bilayers are separated from one another by aqueous domains and enclose an aqueous core. Liposome vesicles are able to entrap compounds of different solubility, not only hydrophobic but also hydrophilic molecules.⁶⁶ Hydrophobic ingredients will be entrapped in the liposome's bilayer, whereas hydrophilic ingredients will be entrapped in the aqueous core of the liposome (see Figure 1.2). Liposomes can help to overcome the difficulty of the dispersion of hydrophobic substances in food formulations which are predominantly water-based.

It is possible to entrap essential oils, hydrophobic peptides, and carotenoids in liposomes. In a recent study⁶⁷, β -carotene loaded liposomes were prepared and stabilized using xanthan and guar gums as thickeners. The researchers concluded that β -carotene was protected from degradation by the liposomes to a large extent. Particle sizes of liposomes for these applications typically range from 25 nm up to several micrometers and are usually dispersed in an aqueous medium.^{60,67}

1.3.3 Proteins

Proteins are ideal for use as encapsulants for micro- and nanoparticles that have future use in food systems because they are GRAS and contain a high nutritional value.⁶⁸ However, the nutritional value does vary depending on the specific protein source.⁶⁹ Most proteins can be easily digested by the human gastrointestinal tract.⁷⁰ Protein particles can be produced via many different techniques: anti-solvent precipitation, pH induced precipitation, spray-drying, electrospinning, emulsification, etc. Particle sizes that can be obtained using proteins as encapsulants vary from 10 nm to 10 μ m depending on the specific protein and used particle synthesis technique.

Both animal and plant proteins have been used as encapsulants. The most commonly used animal proteins include gelatin, casein, and whey protein. Plant derived proteins commonly used for encapsulation purposes include soy protein, gliadin, and zein.⁷¹

Milk proteins

Milk proteins are biopolymers that are categorized into two main groups: caseins and whey proteins. Caseins^{72,73} are proline-rich, open-structured proteins, which have distinct hydrophobic and hydrophilic domains. 95% of the caseins are naturally self-assembled into casein micelles⁷⁴, spherical colloidal particles with a diameter of 50–500 nm. The major whey

protein in cow milk, β -lactoglobulin, is a small globular protein.⁷⁵ It is folded up into an eight-stranded antiparallel β -barrel with a three-turn α -helix on its outer surface. Milk proteins are widely available, inexpensive, natural, and GRAS raw materials with high nutritional value and good sensory properties. They have many structural properties and functionalities which make them highly suitable as vehicles, or as components for the construction of vehicles, for delivering various bioactives.⁷⁶ Typically, milk proteins form complexes with the ingredients they encapsulate, see Figure 1.2.

Properties such as solubility and photo-stability of several bioactive ingredients were reported to improve after encapsulation and/or binding with whey proteins. Examples include curcumin^{77–79} and anthocyanins.⁸⁰

Gelatin (mixed with various carbohydrates)

Gelatin is a mixture of peptides and proteins⁸¹ produced by partial hydrolysis of collagen extracted from the skin, bones, and connective tissues of animals. Gelatin readily dissolves in hot water and sets to a gel on cooling. When added directly to cold water it does not dissolve well. Gelatin and gum Arabic are often used together because of their opposite charges at low pH, which causes attraction and thereby, by also lowering the temperature, the formation of insoluble particles.⁸² Hydrophilic substances such as gelatin could also be used to encapsulate hydrophobic substances including vegetable oils or oil soluble colorants. Anti-solvent precipitation of gelatin could also be performed in ethanol instead of water to form particles. Encapsulation of colorants in gelatin is often carried out by spray-drying and used in combination with carbohydrates.^{83,84}

Soy proteins

Soy proteins are hydrophilic proteins that supply desirable functional properties when added to a variety of food, for example: emulsification, fat absorption, moisture holding, thickening, and foaming. Soy protein isolate is negatively charged in solutions above its isoelectric point ($\text{pH} \approx 4.5$).^{85–87}

An example including soy proteins is a study performed by Xiao et al. in which capsanthin was successfully encapsulated using soy protein isolate – chitosan coacervation and the encapsulated capsanthin displayed enhanced stability against low and medium moisture, heat, and light.⁸⁸

Gliadin

Gliadin is a plant protein that has widely been used for particle production and encapsulation. It is a proline-rich storage protein from wheat, and therefore sometimes also called wheat gluten.^{89,90} It is insoluble in water but is soluble in alcohol-water mixtures. A potential advantage of utilizing water-insoluble proteins is that no additional hardening step is required to preserve their integrity in aqueous-based products. A potential drawback of using gliadin is that a part of the population has gluten intolerance, and therefore some people have been promoting the elimination of gluten proteins from their diet altogether.⁹¹

An example of encapsulation of a colorant was performed by Yang et al., they encapsulated curcumin using gliadin.⁹² It was possible to reach high encapsulation efficiencies (77-99%) and, more importantly, the stability against UV-light and temperature was improved.

Zein

Zein⁹³ is the major storage protein from corn. It belongs to the class of prolamins (that also includes gliadin, hordein and kafirin).^{94,95} Zein is the encapsulant that we have used in the projects that are described in this Thesis.

Zein is a group of highly hydrophobic proteins and consists of three major types of zein: α -zein (MW 19-24 kDa), β -zein (MW 17-18 kDa) and γ -zein (27 kDa).⁹⁶ The mechanism of formation of zein colloidal particles is well established.⁹⁷ Zein particles are often synthesized via the anti-solvent technique, resulting in particles with a positive surface charge.^{98,99} This positive surface charge often leads to unwanted precipitation of these particles when added to aqueous food products. This can be overcome by using steric stabilizers such as sodium caseinate,^{98,100,101} a combination of Pluronic F68 and lecithin¹⁰² or fish gelatin and sodium alginate.¹⁰³

Zein is insoluble in pure water or in pure ethanol, however it is soluble in an ethanol-water mixture.^{104,105} Zein can easily be converted into spherical particles via a simple anti-solvent technique or into fibers using electrospinning.^{98,106,107} Due to its good barrier properties, biodegradability, and biocompatibility, zein has been extensively researched for encapsulation of various substances (amongst which: drugs, essential oils, colorants, and enzymes).^{108,109} The simplicity of generating zein colloidal particles (often via anti-solvent precipitation), coupled with the ease of loading with functional ingredients, make it an attractive encapsulant. This has resulted in zein having recently received wide spread interest for the generation of functional colloidal structures.¹¹⁰

For example: Patel et al.¹¹¹ showed that it was possible to create colloidal colorants with colors ranging from yellow, to green, to blue. They encapsulated curcumin and indigo-carmin into zein protein particles using anti-solvent precipitation. The different color shades were obtained by loading different ratios of curcumin and indigo-carmin inside of the particles. More importantly, the entrapment of these colorants in colloidal particles resulted in the enhancement of the stability of both curcumin and indigo-carmin to photo-degradation. Another research group¹¹² used anti-solvent precipitation to encapsulate β -carotene with zein, carboxymethyl chitosan and tea polyphenols. They tested the solubility of the freeze-dried powders in water, which is one of the factors to evaluate their practical application for the food industry. It was found that the combination of β -carotene with zein, carboxymethyl chitosan and tea polyphenols was the best combination of encapsulants when compared to β -carotene with the combination of zein and carboxymethyl chitosan without tea polyphenols, or β -carotene with zein alone.

1.4 Conclusion and Thesis Outline

In this Chapter, examples of encapsulation of natural colorants using, carbohydrates, lipids and proteins as encapsulants were discussed. The research results described, reveal that encapsulation of natural colorants using biopolymers provides a significant protection of these colorants against environmental conditions such as increased temperatures, oxidation and photo-degradation. Also, stability can even be further improved by co-encapsulating antioxidants and other stabilizers to the particles. The scalability of the anti-solvent precipitation technique shows the possibility of industrial scale production of fully natural encapsulated particles. They can satisfy the consumers' wish for natural ingredients, i.e. the natural origin of both encapsulants and colorants. Moreover, their improved stability upon encapsulation, facilitates future implementation of these encapsulated colorants in consumer products. Not only in the food industry, but also in the pharmaceutical, cosmetic, textile, and other industries.

In this Thesis, we aim to further study the particle formation and encapsulation properties of the vegetable protein zein without added surfactants. Following this, the optical properties of these colloidal dispersions are studied and compared to theory in order to investigate whether or not it is possible to predict the color of such particle dispersions.

In Chapters 2-4 of this Thesis, we describe experimental studies on how to synthesize particles from the water insoluble protein zein and how to encapsulate colorants. In Chapter

2 the synthesis of white zein colloidal particles is discussed. In this Chapter we discuss the purification of zein and a comparison was made between purified and unpurified zein. Note that this purification method was used for all zein synthesis described in this Thesis. Following the purification, particles were synthesized of which the dispersions have a white appearance and these dispersions are compared to a food grade titanium dioxide benchmark. In Chapter 3, colored particle dispersions were synthesized using anionic dyes, which were found to have affinity with the zein protein. To illustrate this affinity of zein with an anionic dye, a comparison was made between colored particle dispersions that were synthesized using co-precipitation of the dye and dispersions that were synthesized using post-addition. In Chapter 4 we discuss the encapsulation of a natural colorant, lutein, using zein as encapsulant. Following encapsulation, experiments were performed on the photo-chemical stability of the lutein-zein composite particles and the effect of an antioxidant, ascorbic acid, on the particle dispersion was studied.

In Chapters 5 and 6 of this Thesis, we compare experimental studies to light scattering theory. The white zein particles that were synthesized in Chapter 2 are further characterized in Chapter 5 to find the refractive index of the particles, using diluted dispersions. This refractive index was used as an input value to model the optical properties of the concentrated colloidal dispersions which was compared to experiments. The method to measure concentrated dispersions experimentally was improved in Chapter 6 and verified using non-absorbing monodisperse silica particles. After that the setup was used for the slightly absorbing white zein particles (Chapter 2) and absorbing blue zein particles (Chapter 3). In the second part of the Chapter the experimental data were compared to diffusion theory for the case that there is absorption.

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Chapter 2

Synthesis and characterization of white zein colloidal particles.

Abstract

Growing interest in using natural, biodegradable ingredients for food products leads to an increase in research for alternative sources of functional ingredients. One alternative is zein, a water-insoluble protein from corn. In this Chapter, the possibility to use white zein particles as a replacement for titanium dioxide in liquid food systems is investigated.

Zein protein was purified and particles were synthesized via anti-solvent precipitation to obtain stable white colloidal particles in a variety of sizes. Their scattering properties could be tuned by the concentration such that these particles could be used as colorant or clouding agents replacing titanium dioxide, albeit at a higher concentration. Such particles have the potential to be an all-natural edible alternative for TiO_2 as white colorant in wet food products.

2.1 Introduction

Designed colloidal particles from various synthetic materials have found numerous applications in many industries ranging from food¹ to pharmaceuticals² and from ink³ to displays.⁴ This is because colloidal particles offer many possibilities to control product appearance through the manipulation of particle size, composition, and shape.⁵ Product appearance is an important factor that determines the perceived quality of the product by a consumer. Color and other appearance attributes create the first impression encountered by consumers; it has been shown to be of primary importance in the initial judgment of food, ultimately influencing the acceptance or rejection of the food product.⁶⁻⁸

Currently, titanium dioxide (TiO₂) is the best known and most widely used white food colorant. Scattering of the low absorbing and high RI of the TiO₂ particles causes the whiteness that is observed. TiO₂ is authorized for use in the European Union as E171 and is used in foods, such as dairy products and candy. Despite this, concerns have been raised recently regarding the toxicity of nanosized TiO₂ following oral exposure.⁹ However, no conclusive results are available on this topic and research towards the safety of TiO₂ as a food additive is still ongoing.⁹⁻¹¹ According to the current regulations TiO₂ is considered safe as food additive, but all natural fully edible alternatives are highly desirable.

A natural and bio-based class of materials to use and synthesize colloidal particles from is the class of prolamins (e.g. zein, hordein and kafirin). These proteins are extracted as by-product from the seeds of cereal grains. They have many advantageous properties which make these proteins suitable for possible applications in the food industry: they are edible, renewable, and biodegradable. Moreover, they are soluble in a mixture of food safe solvents, such as 85/15 (w/w) ethanol/water; and they have good water barrier properties, both in particulate form and as films.^{1,12-16} Because of these advantageous properties towards food applications, many researchers have investigated the use of zein, a prolamin from corn, for these type of applications.^{17,18}

Recently, zein has been proposed as a fat analogue for fats that are present in mayonnaise.¹⁹ It has also been shown that zein is able to encapsulate colorants for use in certain food applications,^{20,21} or that it can be used for triggered release purposes.²² However, it was not investigated whether zein can be used as a white colorant in food applications to serve as an all-natural and edible replacement for the inorganic TiO₂. Therefore, in this Chapter we will investigate the possible use of zein as an alternative white colorant for wet food products such as mayonnaise, dressings and beverages.

2.2. Experimental

2.2.1 Materials

Ethanol (100% pure) was obtained from Interchema. Hydrochloric acid (HCl) was purchased from Sigma Aldrich. Food grade TiO_2 QS (geometric particle size 376 nm and geometric particle size distribution 1.34) was obtained from ChemoPharma. Zein was purchased from Flo Chemical Corporation (Zein F4000C-FG, lot no.: F40006011C6 (batch 1) this is the one that is used unless otherwise stated, F4000C-FG, lot no.: F40006021C2 (batch 2). The third batch of zein (batch 3) was obtained at Sigma Aldrich and was not food grade, the lot number for this batch was SLBL9380V and water was purified using a Millipore Direct-Q purification system.

2.1.2 Purification of zein powder

Colored impurities present in the zein were removed by washing zein powder in ethanol. Zein (10 g) was stirred in ethanol (1.5 L) overnight, after which the dispersion was left to sediment by gravity. The supernatant was discarded and the sediment was collected. This was repeated two more times. The sediment was used to create a 10 wt% stock solution by dissolving it in 85 wt% aqueous ethanol, this purification process is schematically shown in Figure 2.1. In order to completely dissolve the sediment, the solution was heated to 45°C while stirring, after which the solution was cooled to room temperature on air. Small aggregates were removed by filtration using a 0.45 μm filter (PVDF, Millipore). The resulting solution is further referred to as stock solution.

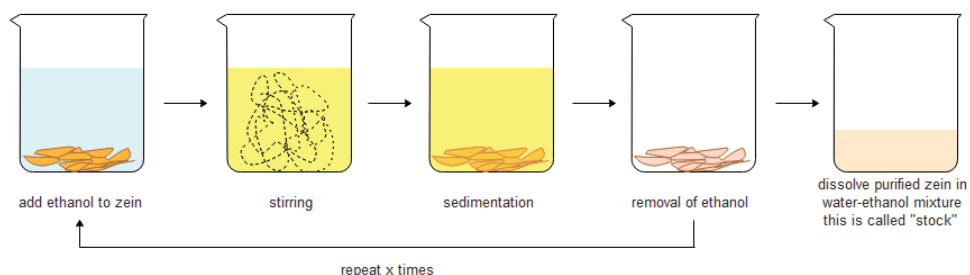


Figure 2.1: Schematic representation of the purification process. Ethanol was added to the zein powder and then stirred for a certain amount of time. After which the zein was left to sediment by gravity and the supernatant (ethanol + impurities) was removed. This process was repeated several times and finally the purified zein was dissolved in aqueous ethanol which is called the stock solution.

2.1.3 Preparation of white zein nanoparticles

The stock solution (10 mL) was quickly added to a beaker with water (120 mL) while stirring at 280 rpm with a stir bar. A two-day dialysis of the resulting dispersion against water adjusted to a pH of 4 with HCl was used to completely remove the ethanol; during this dialysis, the medium was replaced 4 times. Dialysis tubing membranes were purchased at Sigma-Aldrich, and had a molecular weight cut-off of 14,000 Da, as this will retain the 3 major types of zein: α -zein (MW 19-24 kDa), β -zein (MW 17-18 kDa) and γ -zein (27 kDa).²³ The resulting colloidal dispersion was centrifuged for 30 minutes at 222 rcf to remove possible large aggregates, a schematic overview of the synthesis is presented in Figure 2.2. Finally, the samples were stored in the fridge at 5°C. Samples were stored for a maximum of two weeks before measurement. The dry weight of the zein stock solutions and colloidal dispersions were determined by weighing an amount of the solution or dispersion, letting the liquid evaporate at 120°C for 2 hours, and then measuring the dry weight.

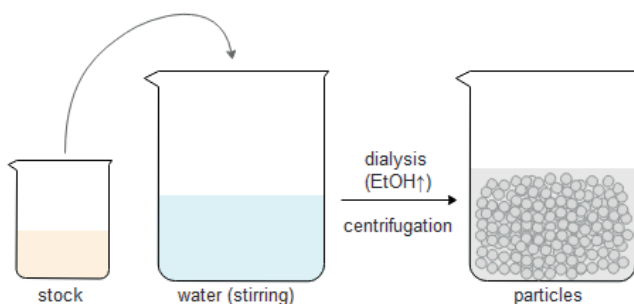


Figure 2.2: Schematic representation of the particle synthesis. Zein stock was precipitated in water while stirring to form particles. Dialysis was performed to remove ethanol and the dispersion was centrifuged to remove possible large aggregates.

2.1.4 Particle shape, size and charge

Particle sizes and charges were measured by dynamic light scattering (DLS) and electrophoresis, using a Zetasizer Nano ZS series, Malvern Instruments. In DLS a CONTIN analysis was used to obtain the size distributions. Prior to the DLS and zeta potential measurements, the samples were diluted, with water adjusted to pH 4, to a suitable concentration, where the samples appear almost completely transparent to the eye, to prevent multiple scattering. Scanning electron microscopy (SEM), Nova Nanolab, FEI, was used for determining the particle shape. To minimize changes in particle size and shape, samples were freeze-dried overnight using a lyophilizer (Virtis). The dry powder, which was loosely

attached to the lyophilizing bottle, was collected and stored in a desiccator until SEM analysis. Prior to SEM analysis a platinum layer of about 4nm was sputtered onto these samples to prevent charging.

2.1.5 Determination of optical properties

UV-vis spectroscopy (HP 8953A spectrophotometer) was used for two purposes: verification of the purification process and for comparative experiments between the opacity of zein particles and TiO₂ particles. Quartz cuvettes of 1 and 10 mm were used.

The purification process was verified by measuring absorption spectra of purified and unpurified solutions of 10 wt% zein and spectra of supernatants of three washing steps. For this purpose, also coatings were made from one unpurified and one purified solution of zein of 10 wt% by applying these solutions, with a brush, onto Leneta cards which were dried in air. These cards were illuminated by the standard daylight illuminant, D65; and images were taken using a DigiEye (VeriVide). For a comparative study between the opacity of zein and TiO₂ particles, zein particles were concentrated using a centrifugal concentrator (Vivaspin 20, pore size 100,000 Da, Sartorius) at a pressure of 3 bar without centrifugation, and then measured at various concentrations. These results were compared to food grade TiO₂ particles in aqueous dispersions of 0.01, 0.1 and 0.2 wt%.

2.1.6 Color determination

The color of strongly scattering samples was determined using total reflectance spectroscopy, which was measured on a home-built set-up. Experiments were carried out with a spectrophotometer (HR4000, Ocean Optics) equipped with a 15 cm diameter integrating sphere (barium sulfate coated, Labsphere) with a sample opening of 12 mm, an opening for the detector port of 3 mm and an opening for the light beam of 6 mm. The total reflectance of concentrated samples was measured using a cylindrical 10 mm quartz cuvette, in the visible wavelength range. As a light source, a Tungsten Halogen light source (HL-2000-FHSA-LL, Ocean Optics) was used and at the detection port a multimode fiber was used to couple the light into a spectrometer (HR4000, Ocean Optics). For a schematic overview of the setup see Figure 2.3.

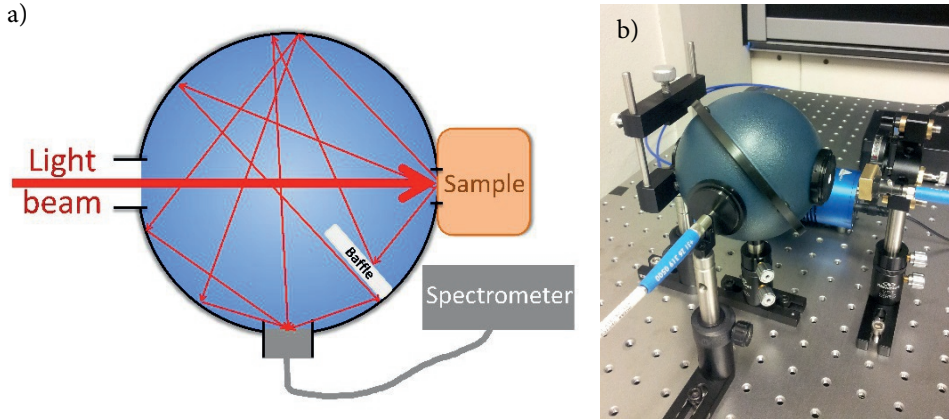


Figure 2.3: Schematic overview of the setup for total reflection measurements (a) and the built-setup (b). An Integrating sphere of 15 cm in diameter was used (Labsphere). The sample opening was 12 mm, the detector port opening was 3 mm and the opening for the light beam was 6 mm.

Total reflectance, $R(\%)$, data is obtained through equation 2.1:

$$R(\%) = \frac{S_\lambda - D_\lambda}{R_\lambda - D_\lambda} \cdot 100\% \quad (2.1)$$

In which S_λ is the measured spectrum of the sample at a certain path length, D_λ the dark spectrum when the shutter of the lamp is closed, and R_λ the reference measurement when there is no sample in front of the port and the lamp is on.

2.3 Results and discussion

2.3.1 Purification of zein

In order to synthesize white particles, zein powder was purified to remove impurities that result in a yellow color, which is attributed to xanthophylls and β -carotene.^{24,25} This purification was carried out by washing zein in 100% ethanol, in which the zein does not dissolve,¹² but the colored impurities do.²⁶ The number of washing steps necessary was determined by collecting the supernatants of three consecutive washing steps and measuring their absorption spectra (Figure 2.4a). These results indicate that one washing step is already sufficient to remove most removable impurities from the zein; this is the number of steps used for particle synthesis. To verify that the impurities due to colorants were indeed removed, the absorption spectra of solutions of unpurified and purified (three washing steps) zein solutions were compared by UV-Vis spectroscopy (Figure 2.4b).

After purification, a lower absorption is observed in the wavelength range 300 to 500 nm than for the unpurified zein, which again indicates that most of the removable impurities were successfully removed. To visualize the effect in a more direct way, coatings were made from one unpurified and one purified solution of zein of 10 wt%, Figure 2.4a and b). These coatings confirm that there were indeed colored impurities in the raw zein as this coating has a yellow appearance. After washing with ethanol, the color is removed to a large extent.

2.3.2 Particle shape, size and charge

The purified zein stock solution was used to prepare colloidal dispersions using anti-solvent precipitation. The particle size of the resulting colloids was tuned by varying the concentration of zein in the stock solution.²⁷⁻³⁰ In SEM it can be observed that the particles are spherical and very polydisperse, see Figure 2.5, which is according to expectations.³¹ Data on the particle size obtained through SEM are presented in Figure 2.5b.

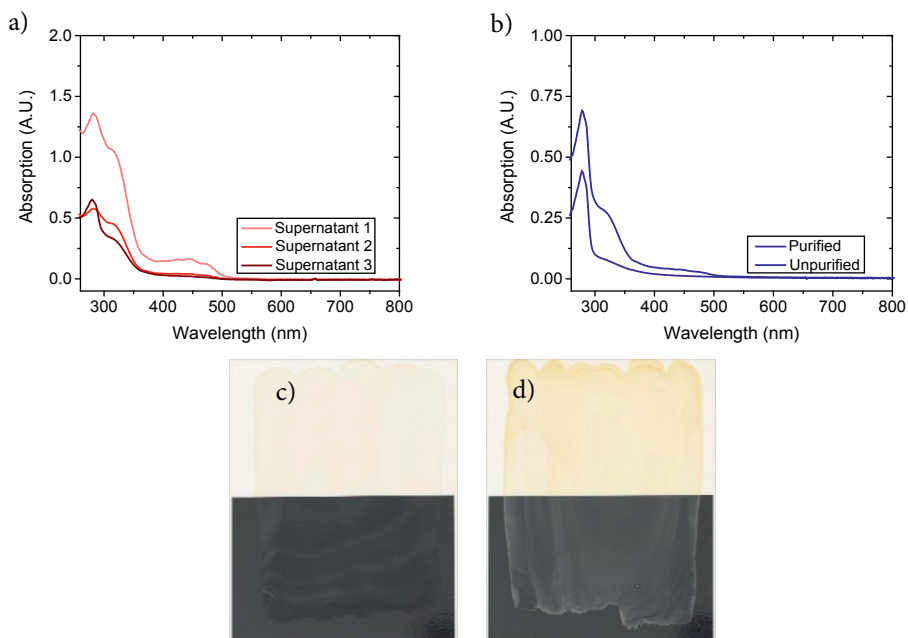


Figure 2.4: UV-Vis absorption spectra showing the effect of purification of zein, by removal of colored impurities by washing zein with ethanol: (a) supernatants after one, two and three washing steps. (b) Purified (after three washing steps) and unpurified solutions of zein. Also, optical photographs of coatings were made from 10 wt% solutions of (c) purified zein and (d) unpurified zein on half-white-half-black Leneta cards. Samples were illuminated by the standard daylight illuminant D65; images were taken using the DigiEye.

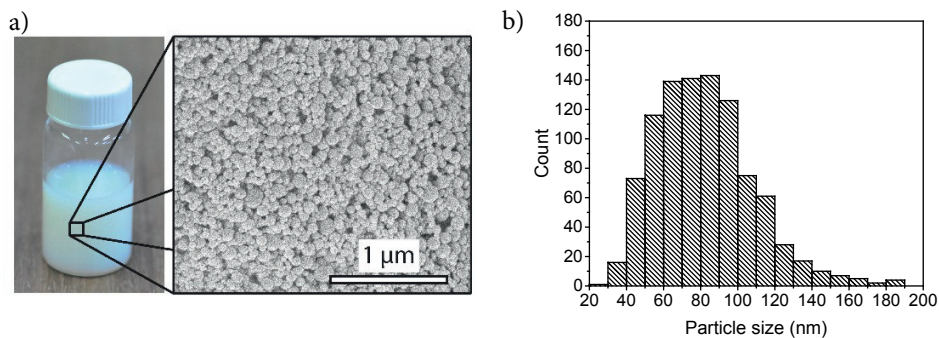


Figure 2.5: (a) A dispersion of white zein particles synthesized from an 8wt% stock solution. And an SEM image of these particles after freeze drying, particles were spherical and had an average diameter of 91 ± 29 nm (counted from SEM images). (b) Manually counted particle sizes (diameters) from SEM images of the sample shown in (a), 960 particles were measured. This sample was synthesized from an 8wt% stock solution.

The samples were prepared with different mean particle sizes, ranging from 35 to 135 nm in diameter (number distribution from DLS), see Figure 2.6a. Particle size distributions are presented in Figure 2.6b. It is observed that the polydispersity, defined as the standard deviation of the distribution, increased with increasing particle size, which is according to expectations.^{32,33} The high polydispersity of the samples is a consequence of the synthesis method. It is possible to synthesize larger particles according to this method using higher zein concentrations in the stock solution. However, then polydispersity will increase, and the particle yield and reproducibility will be low due to severe aggregation. The zeta-potential of the particles varied between 40 and 50 mV for all samples with no specific relation to the particle size. These results are according to expectations.^{34,35} These values are sufficiently high to stabilize the particles without the need for additional stabilizers.

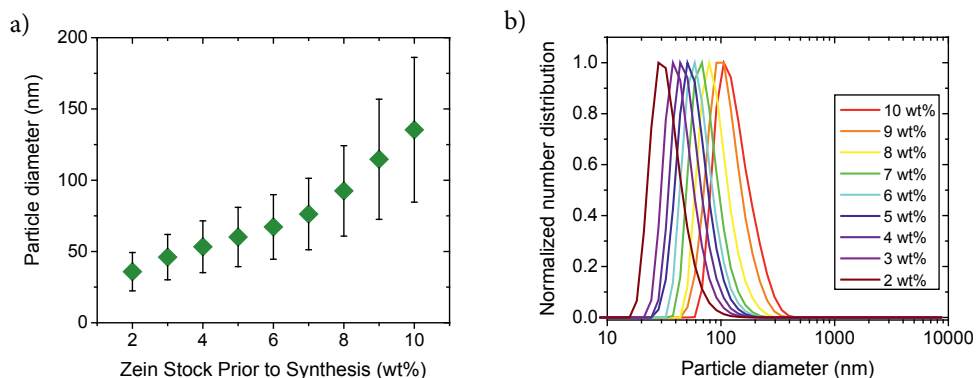


Figure 2.6: A whole range of particle sizes could be synthesized when varying the concentration of zein in the stock solution, mean average sizes (a) were measured in DLS. The vertical bars show the standard deviation of the size distribution from DLS. Normalized size distribution data (number distribution) for particles was also taken from DLS measurements (b).

2.3.3 Reproducibility

During all experiments it was found that the particle size was very reproducible, see Figure 2.7a. This reproducibility is still present when the particles (which were synthesized from purified zein) are compared to particles synthesized from unpurified zein, see Figure 2.7b.

However, when the batch of zein was changed a big difference in the particle sizes is observed, see Figure 2.8. The particle sizes of batch 2 and 3 are both much smaller than the sizes that are obtained from batch 1. Interestingly, when the particle sizes of particles synthesized of purified and unpurified zein from batch 2 and 3 are compared, it seems that then the particle sizes are more comparable to those of batch 1.

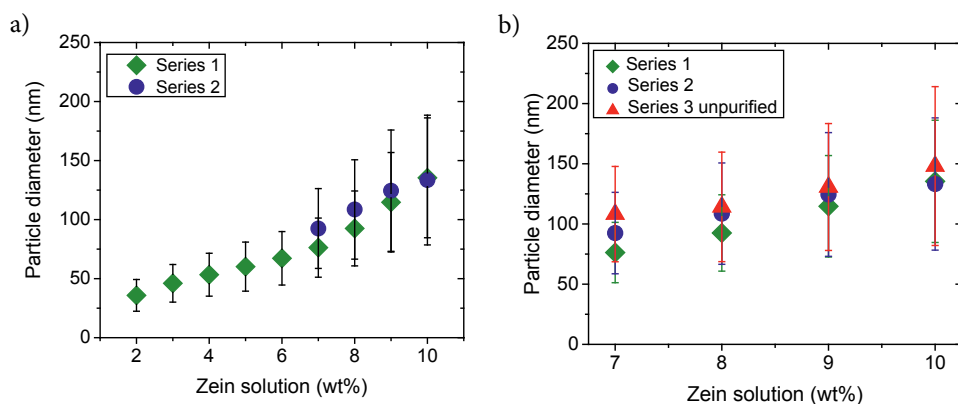


Figure 2.7: Particle sizes measured by DLS of colloidal dispersions for two different series which were prepared at different times (a), and a comparison of these two series to a series of particles which were synthesized from unpurified zein (b).

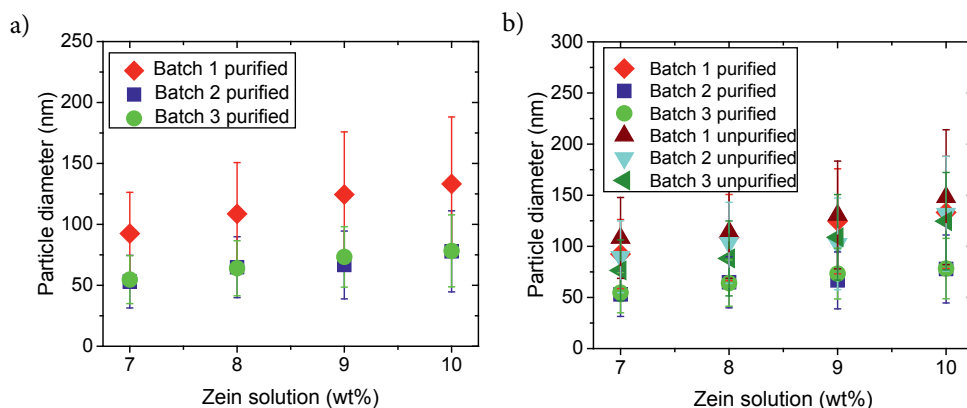


Figure 2.8: Particle sizes measured by DLS of colloidal dispersions created from 7, 8, 9 and 10 wt% zein solutions for the three different batches.

Although the purification steps and particle synthesis were all carried out in the same way, differences in particle sizes were observed. The datasheets from the producers of the batches of the raw zein indicate that every batch is within the specifications. Therefore, it must be concluded that since zein is a natural product, there are inevitable differences between batches. It is recommended when zein is used on a large scale and a batch of zein has to be changed, a thorough quality check must be performed before using the material. It was also observed that apart from the differences between different batches, the reproducibility is good while using the same batch. Which was investigated for batch 1 (Figure 2.7) and for batch 2 (Figure 2.9).

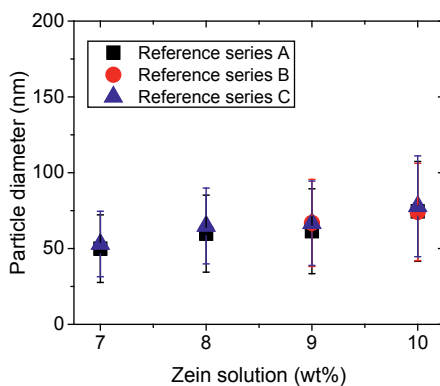


Figure 2.9: Three reproducible particle series (A, B, and C) synthesized from batch 2. Particle sizes were measured by DLS. The vertical bars show the standard deviation of the size distribution.

2.3.4 Determination of color and opacity

The visual appearance of the colloidal dispersions was determined by total reflectance spectroscopy. To determine the color of the sample, the tristimulus values were calculated from the total reflectance data according to Equation 2.2, 2.3 and 2.4:³⁶

$$X = k \sum_{830}^{400} S(\lambda) \bar{x}(\lambda) R(\lambda) \quad (2.2)$$

$$Y = k \sum_{830}^{400} S(\lambda) \bar{y}(\lambda) R(\lambda) \quad (2.3)$$

$$Z = k \sum_{830}^{400} S(\lambda) \bar{z}(\lambda) R(\lambda) \quad (2.4)$$

Here, X , Y and Z are the tristimulus values, λ is the wavelength of the equivalent monochromatic light, $S(\lambda)$ is the spectral distribution of the standard illuminant (D65) (see Figure 2.10a), $\bar{x}$, $\bar{y}$ and $\bar{z}$ are the CIE color matching functions (see Figure 2.10b), $R(\lambda)$ the total reflectance, and $k = 100 / (\sum_{830}^{400} S(\lambda) \bar{y}(\lambda))$. The output can be visualized in a CIE x,y-chromaticity diagram, using the chromaticity coordinates x and y calculated according to Equation 2.5 and 2.6.

$$x = \frac{X}{X+Y+Z} \quad (2.5)$$

$$y = \frac{Y}{X+Y+Z} \quad (2.6)$$

The result from the total reflectance measurement (Figure 2.11a) is presented in a CIE diagram shown in Figure 2.11b where the white point is included as a reference point for optimal whiteness in the CIE color diagram. The zein colloidal particles have a color very close to the white point, which means that the sample is observed as white, making a suitable white colorant.

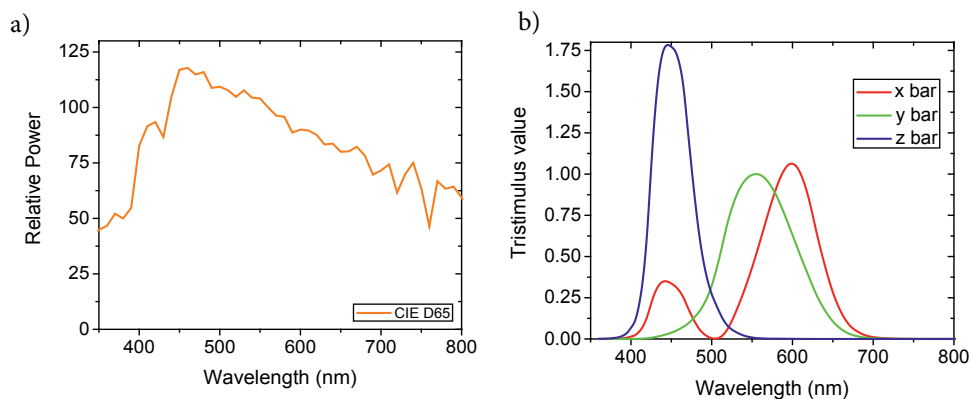


Figure 2.10: Spectral distribution of the standard daylight illuminant D65 (a) and the CIE color matching functions: $\bar{x}$, $\bar{y}$ and $\bar{z}$ (b).

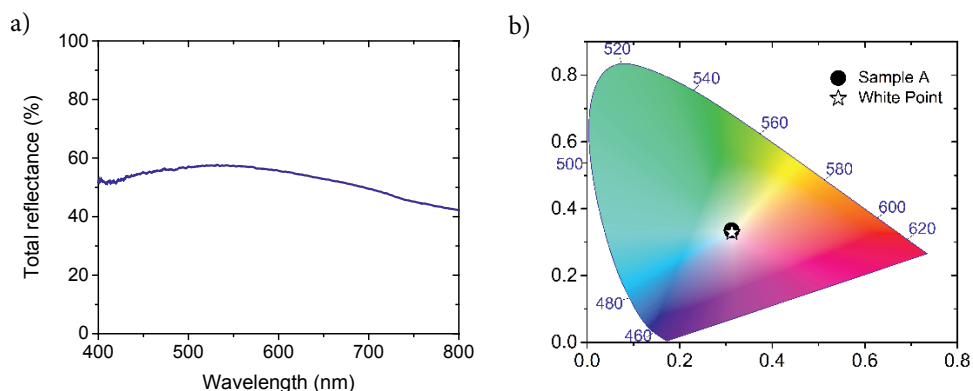


Figure 2.11: Total reflectance measurement (a) and corresponding color coordinates presented in a CIE diagram of synthesized zein particles of sample A (b). Sample A has a particle diameter of 115 ± 41 nm and a concentration of 1.9 wt% and was compared to the theoretical white point.

The hiding power of the white zein particles was determined by their transmission and were compared to food grade TiO_2 particles which functions as a benchmark. The white zein colloids scatter short wavelengths more strongly than long wavelengths, so transmission is higher towards the red wavelength area, as seen in Figure 2.12a. To obtain an opaque dispersion of zein particles, the higher transmission in the red can be reduced by increasing the concentration of the zein particles in the dispersion. Another option would be to increase the particle diameter, since larger particles scatter more strongly. However, processing conditions used here only allow sizes up to about 135nm. TiO_2 is known and used for its good

scattering properties, which are a result of its high refractive index (2.5688 for anatase³⁷). Therefore, food grade TiO₂ particles in aqueous dispersions of 0.01, 0.1 and 0.2 wt% were used as a benchmark. To obtain high opacity dispersions, a relatively low concentration of 0.2 wt% TiO₂ particles is more than sufficient (Figure 2.12b). Since zein particles have a lower refractive index, at similar concentrations, more transparent dispersions are obtained. It is important to note that the zein particles are considerably smaller than the TiO₂ particles, which further limits the achievable opacity of the zein samples. However, when higher concentrations of zein particles are used, a similar opacity can be achieved as for the lower concentrations of TiO₂ particles. An increase in concentration of zein particles of two to seventeen times (2 to 3 wt% zein particles) is needed compared to TiO₂, depending on the wavelength, to achieve a similar transparency.

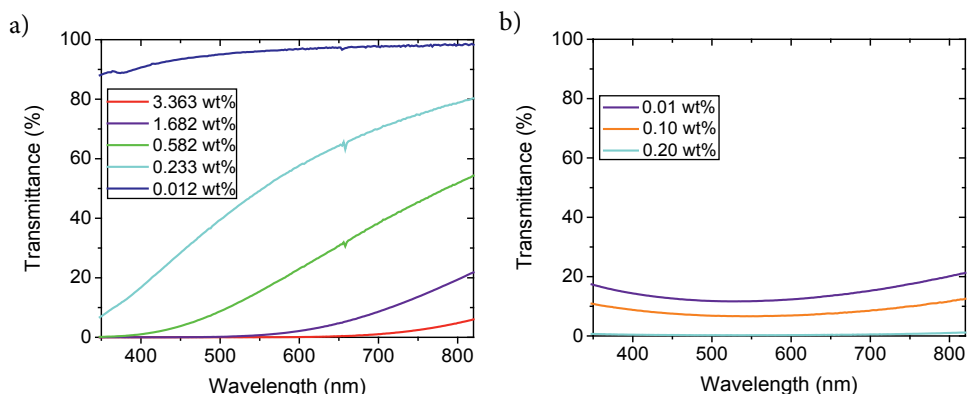


Figure 2.12: Transmission spectra at various concentrations of (a) white zein particles (diameter is 122 ± 49 nm) and (b) food grade TiO₂ particles (diameter is 376 nm).

As discussed earlier, the safety of titanium dioxide is under discussion and consumers prefer alternatives to this inorganic colorant. An example of a food product which contains titanium dioxide is mayonnaise. It was reported in the Dutch National Food Consumption Survey (DNFCS) that the oral intake of titanium dioxide originates for a relatively large part (7% in the age group 7-69 years old) from mayonnaise³⁸. Gu et al.¹⁹ proposed that unpurified zein particles could be used as a fat replacement in mayonnaise. They based their conclusion on the appearance, stability, and total colorimetric values; as well as on rheological, microstructure, and sensory analysis. This implies that purified zein particles are even better suited for use as titanium dioxide replacement in a product such as mayonnaise.

2.4 Conclusions

In this study, the possibility to use white zein particles as a replacement for titanium dioxide in liquid food systems was investigated. It was possible to purify zein protein and synthesize particles via anti-solvent precipitation to obtain stable white colloidal particles, in a variety of sizes (ranging from 35 to 135 nm). The particles were spherical in shape which creates the opportunity to investigate their optical properties further (see Chapter 5). Their scattering properties could be tuned by varying the concentration such that these particles could be used as white colorant or clouding agents replacing titanium dioxide, albeit at a higher concentration.

The combination of the food safe ingredients of this system and the simple anti-solvent precipitation technique together with dialysis, show great potential for usage of these particles as natural white colorant and clouding agent for application in wet food products and beverages.

2.5 Acknowledgements

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Chapter 3

Color-tunable particles through affinity interactions between water-insoluble protein and soluble dyes

Abstract

This research is focused on the delivery of color using zein particles as a carrier and contributes to understanding the interactions between dyes and zein. Co-precipitation of purified zein protein with anionic dyes such as sulfonate group containing azo-dyes (Patent Blue V sodium salt, azorubine, and Fast Green FCF) are used to prepare red, green, and blue colored nanoparticles. These dyes had electrostatic interactions with zein and they remained bound in the zein particles even after dialysis. This was not the case for purpurin, for which hydrogen bonding was expected as interaction. Upon increasing dye concentration, the particle sizes increased as a result of a change in the kinetics of precipitation, which was affected by the association between zein and the dye. However, the encapsulation efficiency continued to be very high and no plateau was reached for the used concentrations. The post-addition of dye on undyed zein particles was used to confirm that there is indeed a high affinity between the dye and the protein. The amount of free dye was determined and the affinity was again found to be high (dye adsorption >95%). The amount of dye incorporated into the particles and the resulting color were similar using both techniques. These results show that there is a high affinity between the zein protein and the dye, and may be exploited to produce bio-based and optically-functionalized nanoparticles using zein as a carrier.

3.1 Introduction

Nowadays, there is more and more a consumer demand for natural food products.^{1,2} This consumer pull has led to an enormous growth in research towards hydrocolloids, encapsulation and composite particle formation in which natural or functional ingredients are used for the application of food systems.^{3,4} Promising types of carriers for such hydrocolloid systems are proteins from both animal and plant origins. Examples include whey, casein, gliadin, and zein.⁵

Zein is a hydrophobic protein extracted from corn and is classified as a prolamin. It is generally recognized as safe (GRAS) and is biocompatible, biodegradable, non-toxic, and edible.^{6,7} Moreover, it is soluble in food safe solvents, such as water-ethanol mixtures. All of these properties make this protein a good candidate for many applications. Over the years, zein has been extensively researched for its use in forming coatings,^{8,9} fibers,^{10,11} and micro- and nanoparticles.^{12,13} Encapsulation with zein as a carrier has been investigated for many applications, such as drug delivery, delivery of essential oils, micro-nutrients, and color.^{14–17} The capability of zein, or any biopolymer for that matter, to form particles and to entrap colorants is directly influenced by different conditions such as medium polarity, steric and electronic parameters, pH and charge of the solute.^{18–20} On the other hand, colloidal particles can also be used to tune the coloring or optical properties of particle dispersions¹⁷ or even as a white colorant on their own without adding additional colorant.²¹

This research is focused on the delivery of color using zein particles as a carrier and contributes to understanding the interactions between dyes (soluble colorants) and zein. Food products are complex multi-component systems in which molecular interactions are present between macromolecules, small molecules and ions. These molecules have different interactivities including electrostatic interaction, hydrogen bonding, hydrophobic interaction, coordination force, π - π stacking and more. These interactions usually have dual effects on quality and functionality of foods. They can cause undesired properties such as reduced solubility and stability, but also can enhance the quality and functionality of foods.²² These interactions are important for the addition of colorants in food products, where colorants are used to make these products more attractive to consumers. Color has been shown to be of primary importance in the initial judgment of food, ultimately influencing the acceptance or rejection of this food product.^{23,24}

Four water-soluble four dyes were selected for this project, one of which is non-ionic: purpurin, which results in a red color. Three dyes are anionic, since they are the sodium salts

of the conjugate bases of strong acids: Patent Blue V sodium salt (E131), azorubine (E122), and Fast Green FCF (E143), which result in a blue, red, and green color, respectively. Molecular structures of these dyes are shown in Figure 3.1. Patent blue, azorubine and fast green are all used as synthetic food dyes. Purpurin is a naturally occurring dye not used for food coloring, but for cotton dyeing. To encapsulate colorants or create composite particles, it is important that the carrier and the functional ingredient have some affinity to each other. This affinity can be, for example, electrostatic interactions, a solubility difference of the pre-mix compared to the final medium, or hydrogen bonding.²⁵ Here, we demonstrate that electrostatic interactions are sufficient for water-soluble dyes to be encapsulated with zein. The same principle may therefore be employed to encapsulate other components, such as anionic natural dyes.

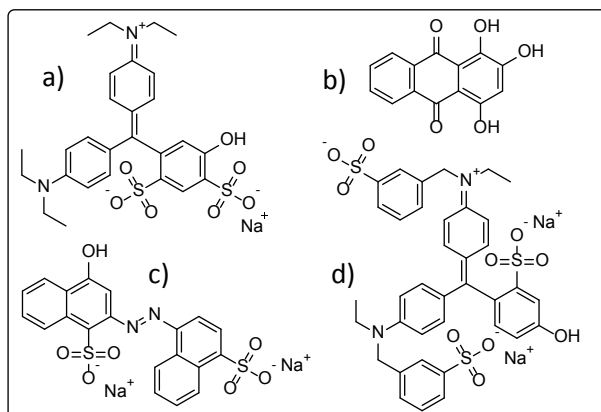


Figure 3.1: Structures of used dye molecules, a) Patent Blue V sodium salt, b) purpurin, c) azorubine and d) Fast Green FCF.

For all of these dyes it is investigated if it is possible to co-precipitate them with zein and stay inside of the particles after dialysis. After that, more specific research will be done for one selected sample. For patent blue V sodium salt, different particle sizes were synthesized and compared to white reference particles without dye. Also the effect of dye concentration on the final particle size was investigated. Then a comparison is made between two different ways to obtain colored particles: via co-precipitation of the zein together with the dye or using post synthesis addition of the dye, in which the dye was added to a white particle dispersion. Colorimetric analysis was performed on the resulting particles of both these techniques to map the observed differences between the two techniques.

3.2 Experimental

Food grade zein was purchased at Flo Chemical Corporation, type F4000C FG (lot nr. F40006011C6 (batch 1) and lot nr. F40006021C2 (batch 2), here batch 2 will be used unless otherwise stated). Ethanol (absolute, technisolv) was purchased at VWR. Hydrochloric acid (HCl), purpurin, Fast Green FCF, and Patent Blue V sodium salt were purchased at Sigma-Aldrich. Water was purified using a Millipore Direct-Q purification system.

3.2.1 Particle synthesis by co-precipitation of zein and water-soluble dye

Prior to all particle synthesis, most of the removable colored impurities were extracted from the zein powder to eliminate effects of these impurities on the particle synthesis and further characterization, by washing this in ethanol, as described in previous work.²¹

The purified stock solution was diluted with 85 wt% aqueous ethanol to prepare a range of different wt% zein solutions. The resulting solutions will be referred to as zein solutions. Water soluble dye (patent blue sodium salt, azorubine, fast green, or purpurin, see Figure 3.1) was dissolved in aqueous form in the zein solutions in different ratio's dye to zein (D:Z) prior to particle synthesis. To synthesize particles, 10 mL of colored zein solution was quickly added to a beaker with water (120 mL), while stirring at 280 rpm with a magnetic stirring bar. Directly after synthesis the encapsulation efficiency was determined (see section 2.2.4.) A three-day dialysis of the resulting dispersion against water adjusted to a pH of 4 with HCl was performed to remove the remaining ethanol and free dye. During dialysis the dispersions was kept in the dark to prevent possible color changes. The pH of the dialysis liquid was measured using pH paper, Whatman indicator 3.8-5.5 range. During this dialysis, the medium was replaced 3 times. Dialysis tubing membranes were purchased at Sigma-Aldrich, and had a molecular weight cut-off of 14,000 Da. The resulting colloidal dispersion was then centrifuged for 30 minutes at 222 rcf to remove possible large aggregates. A schematic overview of the synthesis procedure is represented in Figure 3.2. Finally, the samples were stored in the fridge at 7°C prior to DLS, zeta-potential and colorimetric experiments. During experiments we did not observe any color change of changes in peak positions during UV-measurements. Also all experiments were performed within two weeks of the particle synthesis, during which time we observed no color changes.

To investigate the effects of increasing concentration of dye, the initial zein concentration in the zein solution was kept the same, while increasing the dye concentration. D:Z weight ratios varied from 0.0025 to 0.02. No higher dye concentrations were prepared because of difficulties in dissolving these.

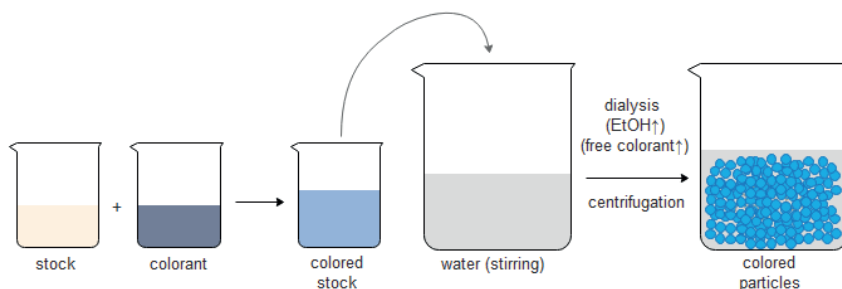


Figure 3.2: Schematic overview of the anti-solvent co-precipitation process. Zein stock and aqueous dye were mixed to form a colored stock, which was precipitated in water to form particles. Dialysis was performed to remove ethanol and free dye, and finally the dispersion was centrifuged to remove possible large aggregates.

3.2.2 Particle synthesis by post-addition of water-soluble dye

For post-addition of the dye, first particles were synthesized from the purified zein as described in section 2.1.1 but without any dye, also dialysis was performed to remove the ethanol. Then, aqueous solutions of different concentrations of dye were added to the zein particle dispersions while stirring. After addition of the dye, the samples were stirred for 30 minutes. Then the amount of adsorbed dye was determined spectrophotometrically (section 2.2.5). Finally, the samples were dialyzed against water adjusted to a pH of 4 with HCl to remove any non-adsorbed dye. A schematic overview of this procedure is presented in Figure 3.3. Then particle size, zeta-potential, and colorimetric experiments were performed. Samples were stored in the fridge at 7°C.

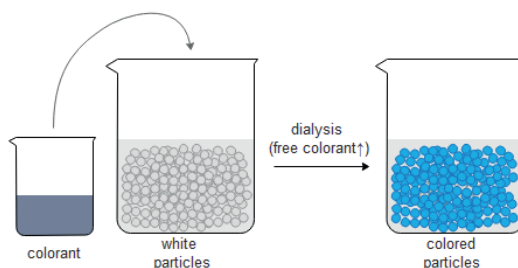


Figure 3.3: Schematic overview of the procedure of post-addition of dye. Aqueous dye was added to previously prepared white particle dispersion while stirring. Then dialysis was performed to remove free dye.

3.2.3 Particle shape, size and charge

Particle sizes and zeta potentials were measured by dynamic light scattering (DLS) and electrophoresis, using a Zetasizer Nano ZS series, Malvern Instruments. In DLS a CONTIN analysis was used to obtain the size distributions. Prior to the DLS and zeta potential measurements, the samples were synthesized as described in section 2.1.1 and dialyzed to remove ethanol and remaining free dye. Then samples were diluted to a suitable, low concentration to prevent multiple scattering, with water adjusted to pH 4 with HCl, which was checked using a Mettler Toledo FiveEasy pH meter, to prevent changes in pH from affecting the measurements. Scanning electron microscopy (SEM), Nova Nanolab, FEI, was used for determining the particle shape. To minimize changes in particle size and shape, samples were freeze-dried overnight using a lyophilizer (Virtis). The dry powder, which was loosely attached to the lyophilizing bottle, was collected and stored in a desiccator until SEM analysis. Prior to SEM analysis a platinum layer of about 4nm was sputtered onto these samples to prevent charging.

3.2.4 Encapsulation efficiency (EE) and adsorption

After co-precipitation or post-addition synthesis, a sample of the dispersion was filtered using a Vivaspin tube (Sartorius, equipped with a 100,000 MWCO membrane) using an applied pressure of 4 bar, to separate the particles from the medium. The encapsulation efficiency or adsorption was determined as follows:

$$EE = \frac{c_t - c_m}{c_t} 100\%$$

Here, EE is the encapsulation efficiency or adsorption, c_t is the concentration of dye added to the synthesis and c_m is the concentration of free dye in the medium. From this calculation also the total concentration of dye inside of the particles, c_p , can be calculated from $c_p = c_t - c_m$. UV-vis spectroscopy (HP 8953A spectrophotometer) was used to determine the concentration of free dye in the medium at a wavelength of 638 nm for patent blue, 522 nm for azorubine, 624 nm for fast green, and 256 nm for purpurin. Concentrations were calculated by comparison with an appropriate calibration curve using the same medium as the particle dispersions. All samples were measured within two weeks after synthesis.

3.2.5 Colorimetry

The color of the zein dispersions was determined by using the Digi-Eye (VeriVide). 5 mL of sample dispersion was pipetted into a 3.5 cm diameter petri-dish and placed in the Digi-Eye apparatus. The Digi-Eye is equipped with a D65 daylight lamp which was used as lighting for taking a photograph of colored samples. The Digi-Eye software was used to determine the CIE XYZ tristimulus values and the $L^*a^*b^*$ values. From this the CIE XYZ tristimulus values were calculated to x and y coordinates for representation in the CIE diagram via $x = X/(X + Y + Z)$ and $y = Y/(X + Y + Z)$.

3.3 Results & Discussion

3.3.1 Effects of type of dye on encapsulation

Since zein is extracted from a natural product, colored impurities such as β -carotene, zeaxanthin and lutein are in some quantity still present in the obtained zein powder.^{26,27} When performing research on encapsulating color with zein it is important to eliminate color effects from these yellow impurities. Therefore, efforts were made to purify this yellow zein to obtain so called white zein,²⁸ as described in the experimental section.

In this research, the encapsulation into zein nanoparticles of four dyes was investigated: patent blue V sodium salt, purpurin, azorubine, and fast green FCF, see Figure 3.1a, b, c and d, respectively. D:Z ratios used were 0.0025 for fast green FCF, patent blue V sodium salt, and azorubine, for purpurin a D:Z ratio of 0.0026 was used. Here, patent blue, azorubine, and fast green were used as examples to investigate whether electrostatic interactions play a role in the encapsulation in zein particles and purpurin was used as an example to investigate whether hydrogen bonding plays a role.

Directly after synthesis the dispersions with all used dyes were strongly colored (Figure 3.4a, b, c and d vial 1). After dialysis to remove free dye all dispersions retained their strong coloration, except the sample that was co-precipitated with purpurin (Figure 3.4a, b, c and d vial 2). After an extended dialysis time of 8 more days, this sample (Figure 3.4d vial 3) became less colored. Apparently, the affinity between zein and purpurin is lower than that between zein and the anionic dyes. However, the dialysis clamp used in the purpurin dialysis did get colored, which indicates that purpurin has a higher affinity with the material of the dialysis clamp than with the zein protein. These results suggest that a charge is more important for particle formation with zein than hydrogen bonding²⁹, which was the affinity between

purpurin and zein. This difference can be explained by the fact that the zein particles have a positive charge at a pH of 4. This means that anionic dye molecules will have electrostatic affinity with the zein protein, while purpurin does not. Since the isoelectric point of zein is in the range of 5.8-6.5,³⁰ samples were always prepared at a pH below this value. When the pH shifts from 4 to above the isoelectric point (6.2), the zein precipitates and the dye is released. This occurs for both co-precipitated and and post-addition samples.

This phenomenon, in which anions and therefore electrostatic interactions play a role in formation of complex structures of zein while adding a stabilizer or functional ingredient, has previously been discussed in literature.^{6,31-33} More specifically, zein nanoparticles have been recently proposed as a disperse solid-phase extraction adsorbent for determining trace amounts of azorubine in foodstuffs,³⁴ although the particle preparation was somewhat different. It was even possible to use this solid phase extraction technique to extract nitrite ions from environmental samples using zein,³⁵ which again shows that anions in solution can be successfully made into composite particles with zein. For the remaining part of the Chapter we will focus on patent blue sodium salt, as the behavior of this particular dye is expected to be similar to azorubine and fast green as water soluble dyes.

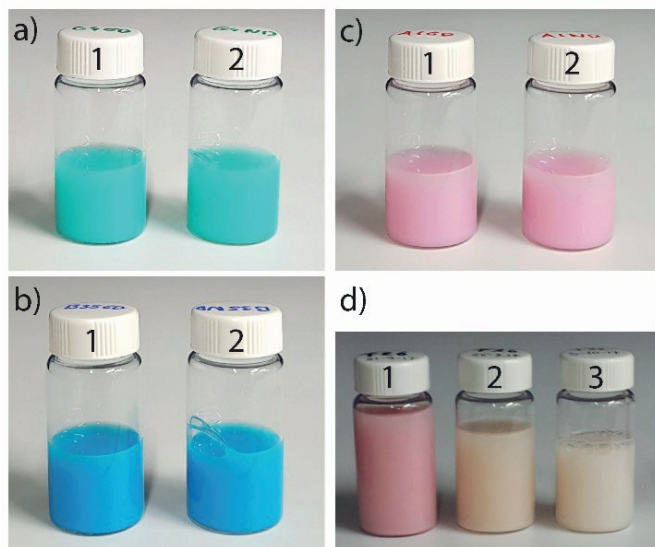


Figure 3.4: Zein particle dispersions with different dyes: (a) fast green FCF, (b) patent blue sodium salt, (c) azorubine, and (d) purpurin, before (1) and after (2) dialysis, and (3) after extended dialysis.

3.3.2 Influence of anionic dye on zein particles synthesized by co-precipitation

To determine the influence of the addition of dye on the particle size, reference particles without dye were synthesized and compared to colored particles synthesized via co-precipitation, using a dye to zein ratio of 0.0025. These samples were compared using DLS, see Figure 3.5a. Compared to the reference samples, colored particles differ a little in size which becomes more distinct for higher zein concentrations used. The particle shape was found to be spherical, which is similar to the reference samples (discussed in Chapter 2), and also here the polydispersity is high (see Figure 3.5b). To determine the reproducibility of the co-precipitated particles the particle sizes of samples created in two different batches of zein were compared, see Figure 3.6a. Here, for all particles the same D:Z ratio was used (0.0025). Similar to reference samples without dye (as discussed in Chapter 2), particle sizes can be tuned by using different starting concentrations of zein in the zein solution.²¹

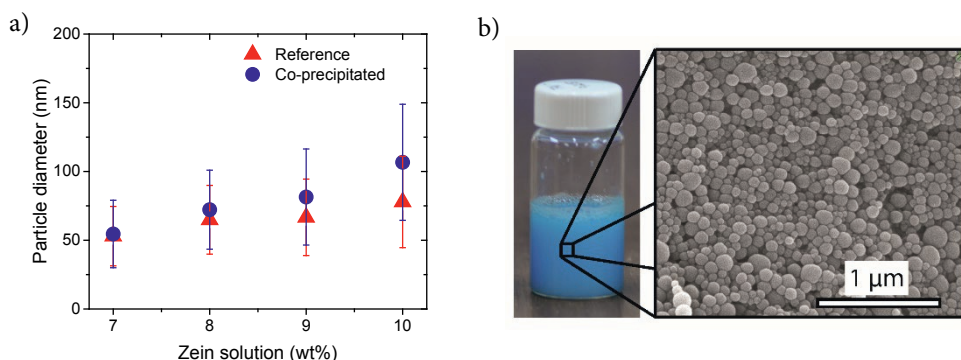


Figure 3.5: Particle sizes measured by DLS of colloidal dispersions synthesized with and without (reference) the co-precipitation using patent blue (D:Z ratio 0.00025) (a). The vertical bars show the standard deviation of the size distribution. In (b) a vial of a blue particle dispersion is shown including an SEM image, showing spherical particles with a high polydispersity.

As described before, there are big differences between the particles synthesized using batch 1 and batch 2. When looking at just one batch (batch 2) in Figure 3.6b, it is clear that the reproducibility seems to be lower than was found for particles synthesized without dye: a trend of increasing particle size with zein stock solution is much less visible. This indicates that it is more difficult to control the particle size when co-precipitating with dye compared to particles synthesized without a dye. Especially since the reproducibility of the reference

samples was found to be good (Chapter 2). A reason for this observation is that the kinetics of the precipitation is affected by the association between zein and the dye, and therefore the zein/dye solution forms particles in a different way than when no dye was added to the zein solution. It has been reported in literature that after encapsulation, particle sizes can increase or stay a similar size, depending on the used system.³⁶

For these zein to dye ratios, the encapsulation efficiency was 100% which is due to the relatively low amounts of dye used, and the expected high affinity of zein with the dye. Zeta-potentials were on average 47 ± 7 mV, which is similar to ones found for white zein particles and comparable to values found in literature.^{37,38}

Next, a series of zein dispersions was prepared by co-precipitation with varying patent blue concentration keeping the zein concentration constant. Results are shown in Table 3.1. The pH after precipitation was measured to test for differences between the colored and reference sample. The pH of the colored dispersion directly after synthesis was on average 4.4 ± 0.1 and was nearly similar for all concentrations of dye added to the synthesis, see Table 3.1. The pH of the colored samples was slightly higher than the pH of the reference sample (pH = 4.2).

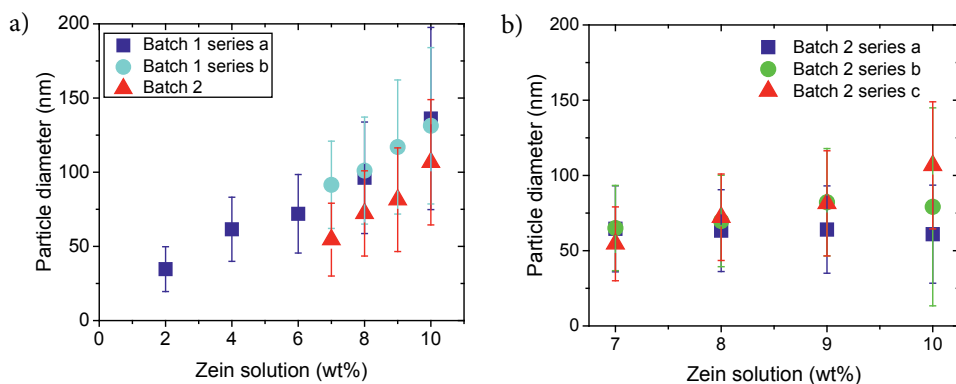


Figure 3.6: Particle sizes measured by DLS of colloidal dispersions in (a) a comparison between batch 1 and 2. In (b) reproducibility of batch 2. All particles are synthesized using co-precipitation with zein and patent blue. The vertical bars show the standard deviation of the size distribution.

The pH and ζ -potential of the reference sample precipitated without dye are comparable with values found in literature.^{37,38} A variation in zeta potential from 37 to 50 mV is observed for the colored particles. However, no obvious trend is observed (see Figure 3.8a). It was

expected that when incorporating anionic molecules, the zeta potential would decrease when the dye is encapsulated to a high extent. Overall, the zeta potential does seem to decrease upon increasing dye concentration. Further inspection reveals that any possible trend is masked by the spread between different preparations.

Interestingly, particle sizes increased upon increasing dye concentration, see Figure 3.8b. Probably, the kinetics of the precipitation are affected by the association between the zein and the dye, resulting in larger particles upon higher dye concentrations and a less clear trend in the zeta potential. It has been reported³⁹ that interactions between zein and anionic surfactant (sodium dodecyl sulfate) can result in an increase of the particle sizes of the zein-surfactant complex, as was observed here for zein and dye. Encapsulation efficiencies are all very similar and very high, even at the highest dye concentrations, see Table 3.1. When higher concentrations of dye are incorporated, the encapsulation efficiency seems to decrease slightly, however, not as much as expected when adding an excess of dye. No plateau is reached, even after addition of relatively high dye concentrations. This indicates that there must be a very high affinity between dye and zein protein, which is also indicated by the increase of particle sizes upon increasing dye concentration, see Figure 3.8 and Table 3.1. An increase of particle size upon higher loading has been described for a different system, in which anionic surfactant sodium dodecyl sulfate was added to zein particles,³⁹ for the case here we have an anionic dye which behaves in a similar manner. To show that there is indeed a high affinity between zein and the dye we will show that particles can also be dyed with post-precipitation addition of dye to white particles, which will be discussed in the next section.

Table 3.1: Encapsulation efficiency (EE), pH after precipitation, particle diameters measured by DLS, and zeta potential for each ratio zein to dye (patent blue). Particles were synthesized while keeping the initial zein solution weight percentage at 10wt%.

Zein (wt%)	Ratio D:Z	pH after precipitation	Particle diameter (nm)	Standard deviation of particle distribution (nm)	ζ -potential after dialysis (mV)	EE (%)
10	0	4.2	74.13	32.07	46.3 ± 7.37	-
10	0.0025	4.4	76.85	35.01	49.4 ± 7.75	99.9
10	0.003	4.3	81.12	35.67	49.1 ± 7.36	99.9
10	0.004	4.3	96.78	38.22	49.0 ± 7.46	99.7
10	0.005	4.2	89.19	37.48	50.6 ± 8.22	99.6
10	0.007	4.5	95.62	39.83	41.3 ± 7.21	98.6
10	0.007	4.5	95.47	37.10	42.3 ± 7.63	99.8
10	0.008	4.3	100.3	42.02	39.6 ± 6.67	99.4
10	0.008	4.4	78.05	39.69	42.2 ± 6.95	99.7
10	0.009	4.3	93.27	41.89	37.9 ± 5.77	99.2
10	0.009	4.4	86.62	48.03	40.3 ± 6.87	99.8
10	0.010	4.2	90.34	47.42	50.3 ± 7.71	99.8
10	0.010	4.3	101.8	41.47	39.2 ± 5.99	99.7
10	0.014	4.6	124.8	49.56	39.2 ± 6.23	99.1
10	0.016	4.4	131.6	52.75	42.1 ± 6.68	99.0
10	0.018	4.4	99.60	79.13	39.0 ± 6.32	98.1
10	0.020	4.6	130.7	56.49	44.9 ± 6.00	97.7

3.3.3 Co-precipitation versus post-addition of dye.

The nature of interaction was not only determined by using co-precipitation as described earlier in this Chapter, but also by using post-addition of the dye. Here, white particles were synthesized first, after which different concentrations of dye (ranging from a D:Z ratio of 0.0005 to 0.0417) were added to the dispersions. After dialysis to remove free dye, colored dispersions were again obtained, see Figure 3.7.

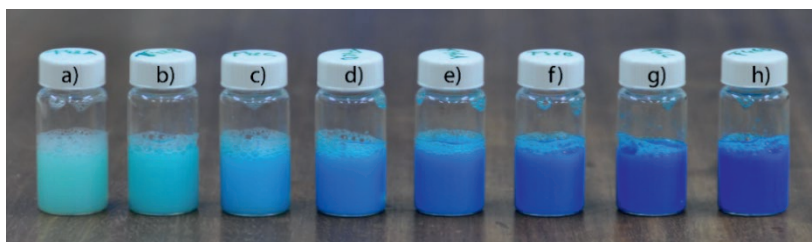


Figure 3.7: A range of dye concentrations, D:Z ratios (a) 0.0005, b) 0.0019, c) 0.0048, d) 0.0096, e) 0.0156, f) 0.0209, g) 0.0313, and h) 0.0417) were used in post-addition of dye in the same batch of particles. This resulted in dispersions with different shades of blue.

Particle sizes are constant upon increasing dye concentrations, see Figure 3.8b and Table 3.2. This is different compared to the co-precipitation technique but this is expected since the particles were synthesized prior to adding the dye. Zeta potentials start from a similar value as the reference (which is the original white particle dispersion). From this value the zeta potential gradually decreases as more dye is added, see Figure 3.8a and Table 3.2. This can be caused by the adsorption of the dye onto the surface of the particles, thus lowering the zeta potential. The zeta potential seems to follow a clearer trend for the post-addition samples than for the co-precipitated samples with increasing dye concentration. A reason for this might be that in the co-precipitated samples the zein was pre-mixed with the dye and therefore the dye is more homogeneously dispersed in the particle, while in the post-addition samples the surface of the particle is enriched with dye. On the other hand, the post-addition was performed on a single batch of white zein particles, therefore the samples are more similar to each other in shape and size and the only difference is the concentration of dye added. This may also have contributed to the clearer trend in zeta-potential drop compared to the co-precipitated samples.

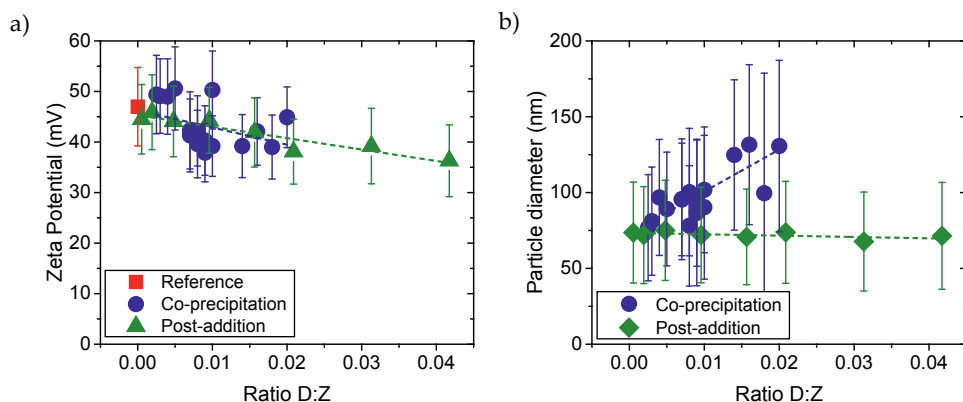


Figure 3.8: Zeta-potentials of co-precipitation and post-addition samples, upon increasing concentrations of patent blue used in the preparation (a). All particles were synthesized using an initial zein concentration of 10wt%. The vertical bars show the standard deviation of the zeta potential. Particle diameter, measured by DLS, upon increasing patent blue concentration in the particles while keeping the initial zein solution concentration at 10wt% (b). The vertical bars show the standard deviation of the size distribution. The dotted lines are present to guide the eyes.

Table 3.2: Different concentrations of dye were used in post-addition. The amount of adsorbed dye was determined and the dye concentration inside of the particles was calculated.

Zein (wt%)	Ratio D:Z	Particle diameter (nm)	Standard deviation of particle distribution (nm)	ζ -potential (mV)	Adsorption (%)
reference	0.0000	74.13	32.07	46.3 ± 7.37	-
A	0.0005	73.62	33.26	44.5 ± 6.88	95.8
B	0.0019	71.98	31.96	45.9 ± 7.40	98.8
C	0.0048	75.10	33.02	44.1 ± 7.00	99.3
D	0.0096	72.03	31.50	44.3 ± 6.53	99.3
E	0.0156	70.75	31.49	41.9 ± 6.84	99.2
F	0.0209	73.80	33.60	38.1 ± 6.43	99.3
G	0.0313	67.71	32.64	39.2 ± 7.46	99.0
H	0.0417	71.46	35.23	36.3 ± 7.10	98.9

When studying the affinity between the dye and the zein particles, it was found that the amount of free dye in the medium was very low and therefore the affinity was found to be high, >95% (see Figure 3.9). It is interesting that, similarly to the co-precipitation experiments, also here no plateau is reached, underlining the high affinity between zein and dye. When looking at D:Z ratios and concentration in the particles of Tables 3.1 and 3.2, it is clear that for both co-precipitation and post-addition comparable concentrations of dye end up bound to the particles. The high affinity makes it possible to reach equally high dye concentrations in zein particles by both co-precipitation and post-addition techniques.

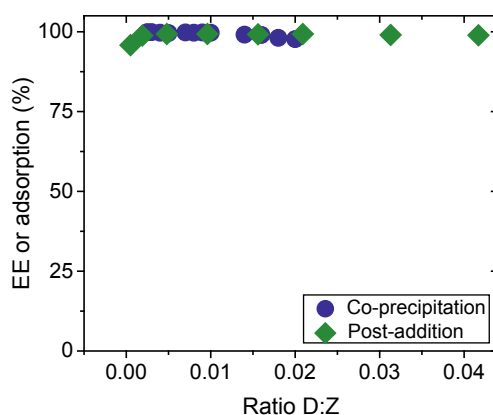


Figure 3.9: Encapsulation efficiencies of samples prepared using co-precipitation and post-addition.

Different shades of blue can be easily created by varying the concentration of dye added to the particle dispersion. This is similar to samples synthesized via co-precipitation. Color data of the post-addition samples, a reference, and co-precipitated samples are shown in Table 3.3 and are summarized after calculating their CIE x,y -values in the CIE diagram in Figure 3.10. The reference sample is observed as white, and upon increasing dye concentration, the color shifts to dark blue for both co-precipitated and post-addition samples.

Table 3.3: CIE and L*a*b* color data measured by the DigiEye for a reference sample without dye, post-addition samples A to H, and samples synthesized via co-precipitation (co-p 1 to 11).

Sample	Zein (wt%)	D:Z	X	Y	Z	L*	a*	b*
reference	10	0	78.63	83.32	83.12	93.15	-0.72	4.52
A	10	0.0005	45.09	59.50	71.47	81.56	-30.25	-6.45
B	10	0.0019	34.58	49.95	68.91	76.04	-39.50	-13.86
C	10	0.0048	20.68	33.81	60.56	64.81	-47.37	-25.95
D	10	0.0096	14.88	25.83	55.41	57.88	-48.71	-33.08
E	10	0.0156	12.89	21.37	51.67	53.35	-41.83	-37.19
F	10	0.0209	11.38	17.78	47.05	49.22	-34.46	-39.49
G	10	0.0313	10.21	14.77	41.99	45.32	-26.42	-40.57
H	10	0.0417	9.63	12.78	37.97	42.43	-18.60	-40.72
Co-p 1	10	0.0025	29.72	44.87	69.74	72.81	-43.14	-20.13
Co-p 2	10	0.0040	19.57	32.84	61.48	64.03	-49.48	-28.14
Co-p 3	10	0.0050	17.84	30.6	60.23	62.16	-50.41	-30.21
Co-p 4	10	0.0070	16.21	27.34	58.29	59.29	-47.02	-33.38
Co-p 5	10	0.0080	15.57	26.33	57.69	58.35	-46.67	-34.44
Co-p 6	10	0.0090	14.9	25.08	56.56	57.15	-45.49	-35.44
Co-p 7	10	0.0100	14.56	24.5	56.62	56.58	-45.09	-36.47
Co-p 8	10	0.0140	12.93	21.47	53.43	53.46	-41.99	-38.77
Co-p 9	10	0.0160	12.09	20.3	51.75	52.17	-42.17	-39.31
Co-p 10	10	0.0180	11.84	18.97	50.01	50.65	-37.4	-40.14
Co-p 11	10	0.0200	11.63	18.52	49.13	50.12	-36.59	-40.14

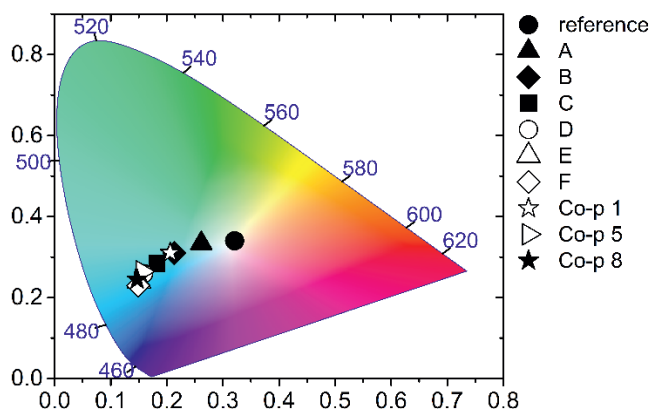


Figure 3.10: CIE diagram with CIE color data calculated from Table 3.1.

The first co-precipitated sample (Co-p 1) yields a color that is placed in the CIE-diagram between sample B and C. This is the expected location in the CIE-diagram for this sample when looking at the D:Z ratios, similar results are shown for other dye concentrations in Table 3.3 and Figure 3.11. For higher concentrations the values are much closer together, however, the same trend continues. Sample Co-p 5 should be between C and D, which is the case. It can even be observed that Co-p 5 is closer to D than to C, which is to be expected when viewing the D:Z ratio in Table 3.3. According to the D:Z ratio, sample Co-p 8 should be in between sample D and E, which also follows from the CIE diagram. This result shows that the observed color is independent of whether the particle was synthesized using co-precipitation or that the color is added to the particles via post-addition of the dye. Which means that similar colors can be achieved by using both techniques.

3.4 Conclusions

Summarizing, co-precipitation of purified zein protein with anionic dyes, such as patent blue sodium salt, azorubine and fast green FCF, were able to associate with the zein colloids, even after dialysis. This was not the case for the non-ionic dye, purpurin. Observing similar results for the three anionic dyes, the remaining experiments were performed with patent blue sodium salt. It is found that co-precipitation and post-addition techniques yield similar results in terms of concentration and adsorption or encapsulation, zeta potential, and color appearance. Particle sizes increased upon increasing dye concentration for co-precipitated samples, while for post-addition samples the particle size was similar to the original white particles. The results described in this Chapter show that there is high affinity between anionic dyes and the zein protein. It is expected that for the co-precipitation technique the mechanism is as follows: first, zein is dissolved in aqueous ethanol, then the dye is added which adsorbs on the zein proteins and then particles are formed by precipitation after mixing with the anti-solvent water. Colored particles synthesized via post-addition follow a different route. First particles are synthesized and then dye is added and adsorbs from the outside of the particle, indicating the high affinity between zein and dye. These results show that there is a high affinity between the zein protein and the dye. This may be exploited to produce bio-based and optically-functionalized nanoparticles using zein as a carrier.

3.5 Acknowledgements

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Chapter 4

Photo-stability of lutein in surfactant-free lutein-zein composite colloidal particles.

Abstract

Lutein suffers, just as many other natural colorants, from instability against light, heat, and oxygen. Several encapsulation approaches have been developed to prevent color degradation of natural colorants, however to maintain the natural character only natural encapsulating compounds should be used. In this research, the ability of nanoparticles from zein, a protein from corn, to protect lutein from light degradation was studied under various conditions. Lutein-zein nanoparticles were synthesized, after purification of zein, by anti-solvent precipitation. Particle sizes could be tuned by varying the zein concentration in the solvent phase and this was linked to the encapsulation efficiency, which increased upon increasing particle size. Zein nanoparticles showed the ability to increase the photo-stability of lutein when compared to lutein dispersions in water. The size of lutein-zein particles that were studied had no effect on lutein stability. To further promote the stability of the entrapped colorant, ascorbic acid was used as an antioxidant in the aqueous particle dispersion. The addition of ascorbic acid to zein and lutein-zein particles resulted in dispersions with similar particle sizes and zeta potentials. However, the photo-stability of lutein in nanoparticle dispersions stabilized with ascorbic acid improved significantly compared to samples without ascorbic acid or to pure lutein dispersions.

4.1 Introduction

There are many types of natural colorants of which one group is the carotenoids. These are natural pigments that are present in fruits, vegetables, other plants, algae, and photosynthetic bacteria.¹ Generally, carotenoids absorb wavelengths ranging from 400 to 550 nm (violet to green), which causes these compounds to be deeply colored yellow, orange, or red. These carotenoids can be roughly divided into two groups. The first consists of unoxxygenated carotenoids such as α -carotene, β -carotene, and lycopene and are called carotenes. The second are oxygen containing carotenoids called xanthophylls, examples are lutein and zeaxanthin. The carotenoid used in this research is lutein (see Figure 4.1a), a permitted food colorant (E161b). It is suggested that the lutein has two main roles: blue light filtration and an antioxidant function.^{2,3} In addition to lutein's use as a colorant, research indicates that it also has potential positive health benefits such as reducing the risk of eye diseases and improvement of cognitive functions^{4,5}, however additional research is needed.^{6,7}

Used as a colorant, lutein suffers from instability against light, heat, and oxygen, as do many natural (food) colorants.⁸ Because of these deficiencies the demand for super-pigments is raised by the food industry.⁹ A few solutions include patented processes to improve the stability of pigments by chemical modification; however, such processes are not consistent with the label natural. A natural colorant must be stabilized using natural methods, such as encapsulation, if it is to continue to be termed natural.⁹ Encapsulation of sensitive natural pigments can offer a solution by creating a barrier between the pigment and the environment.^{10,11} Additional benefits of encapsulation are that potential off-flavors may be masked by the biopolymer encapsulant^{12,13} and that other ingredients such as stabilizers (examples include: sodium caseinate, tween 80, pluronic F68, pluronic F127, span 80 and lecithin)^{14–17} can be co-encapsulated.

Lutein can be encapsulated using various methods¹¹ which can be subdivided in two main methods: surfactant-based encapsulation systems or biopolymer-based encapsulation systems. A surfactant-based encapsulation system was investigated by Davidov-Pardo et al. in which they prepared stable lutein-enriched emulsions using caseinate.¹⁸ Another research also describes surfactant-based system, using nanostructured lipid carriers (NLCs).¹⁹ These researchers conclude that NLCs have a better in vitro sustained release of lutein as compared to conventional nano-emulsions. A biopolymer-based encapsulation system was investigated by Qv et al. in which complex coacervation method was used²⁰ and gelatin and gum Arabic as encapsulating agents. The researchers conclude that their results suggest that

microencapsulation is an adoptable method to protect lutein against light effects. Hu et al. also use a biopolymer-based encapsulation system, preparing nanoparticles using the plant protein zein using solution enhanced dispersion by supercritical fluids.²¹ More recently, Chuacharoen et al. devised a simpler method using liquid-liquid dispersion to prepare lutein-zein nanoparticles²² using surfactants resulting in further improved stability against light. Cheng et al. even found that zein provides a degree of physical protection to encapsulated lutein in gastric conditions.²³

In this Chapter, we investigate the encapsulation of lutein using the biopolymer zein, and we study the effect of an antioxidant (ascorbic acid, see Figure 4.1b) on the light stability of lutein-zein particles.

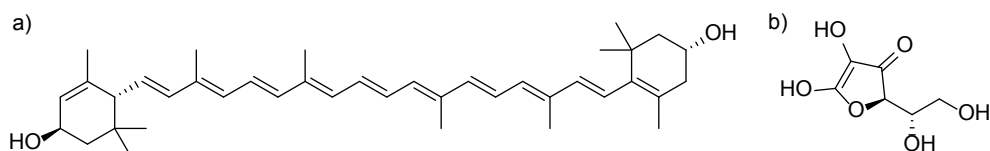


Figure 4.1: Chemical structure of lutein (a) and L-ascorbic acid (b).

Zein is a water-insoluble plant protein from corn. Zein has several beneficial properties for application in the food industry: it is edible, abundant, renewable, biodegradable and soluble in food safe solvents, such as water-ethanol mixtures.^{24,25} The mechanism of formation of zein colloidal particles is well established.^{26,27} Mostly, zein particles are synthesized using anti-solvent precipitation, often resulting in particles with a positive surface charge.²⁸ Thanks to these useful properties, zein has been well studied for the application of colloidal encapsulating agent focused on designing new food systems and functional ingredients such as delivery of color.^{29,30}

In this work, particles were synthesized from purified zein using anti-solvent precipitation. The effect of the zein concentration in the synthesis on the lutein-zein particles was studied first. Then, ascorbic acid, also known as vitamin C (E300), was added to improve the stability of the lutein-zein particles. Ascorbic acid (Figure 4.1b) is an antioxidant and forms the ascorbate anion when it is dissolved in water.^{31,32} Other research has confirmed the affinity between these anions and zein.^{33,34} Finally, the effect of ascorbic acid on the photochemical stability of the lutein-zein particles was investigated and compared to the light penetration depth of the dispersions.

4.2 Experimental

Food grade zein was purchased at Flo Chemical Corporation, type F4000C FG (lot nr. F40006021C2). Ethanol (absolute, technisolv) was purchased from VWR. L-Ascorbic acid (reagent grade), acetic acid and sodium acetate were purchased from Sigma-Aldrich and chloroform (HPLC grade, stab./amylene) from Biosolve. Food grade lutein (FloraGlo lutein, Crystalline dry) was kindly donated by DSM. Water was purified using a Millipore Direct-Q purification system.

4.2.1 Lutein-zein particle synthesis

Prior to particle synthesis, most of the colored impurities were extracted from the zein powder by washing this in ethanol³⁵ to eliminate color from the zein itself.^{36,37} Then a zein stock solution in aqueous ethanol (70 wt%) was made. Lutein-zein nanoparticles were synthesized using anti-solvent precipitation, based on the procedure of Chuacharoen et al.²² Briefly, 1 mL of 5 wt% purified zein solution in aqueous ethanol (70 vol%) was prepared. A lutein solution was prepared at 0.20 mg/mL with ethanol, where it is important to stay below the solubility limit (0.3 mg/mL)³⁸ of lutein in ethanol. This lutein solution was added dropwise to the zein solution at a volume ratio of 1:1 under light shaking. This was then added at once to 8 mL of milli-Q water under stirring (700 rpm). After preparation the sample was stirred for an additional 15 minutes. At this stage the samples are called “freshly-made”. To prepare lutein-zein particles in the presence of ascorbic acid (LZA), the lutein-zein solution was precipitated in 8 mL water including 0.20 mg/mL ascorbic acid.

Dialysis was used to remove the ethanol, using dialysis membranes with a molecular weight cut-off of 14,000 Da (Sigma-Aldrich). A two-day dialysis of the resulting dispersion against water adjusted to a pH of 4 with HCl was used and the medium was replaced 4 times. The dispersion was collected and kept in the fridge (at 7°C and in the dark) for further analysis.

Lutein dispersions without zein were prepared in parallel using the same method, with the exception that zein was not added.

4.2.2 Particle size, size distribution, and zeta-potential analysis

To determine size distributions, particle sizes were measured by dynamic light scattering (DLS) using a Zetasizer Nano ZS series (Malvern Instruments), which was also used to obtain zeta-potentials. In DLS, a CONTIN analysis was used to obtain the number averaged size

distributions. To prevent multiple scattering, samples were diluted five times using a buffer of pH 4 (acetic acid and sodium acetate), to a suitable concentration prior to the measurements. At least three separate measurements were performed. The pH was measured using a Mettler Toledo FiveEasy pH meter.

4.2.3 Encapsulation efficiency (EE)

To determine the encapsulation efficiency of the samples, 1 mL of the freshly-made Lutein-Zein particle dispersion was centrifuged at 21,000g for 3 hours and 15 minutes. The supernatant and the nanoparticle pellet were separated. The pellet was dissolved in 1mL aqueous ethanol (90 vol%) overnight and kept in the dark. The next morning lutein was extracted with chloroform (1:1 ratio), after which the lutein was diluted two times with the chloroform-ethanol-water solution. The concentration of lutein was measured using a UV-Vis spectrophotometer (HP 8953A spectrophotometer, or Perkin Elmer lambda 365 UV-vis) with quartz cuvettes of 1 cm path length recorded at a wavelength of 452 nm. The absorbance value was converted to lutein concentration using an appropriate calibration curve using the same medium as the particle dispersions and Equation 4.1.

$$EE(\%) = \frac{c_{\text{pellet}}}{c_{\text{theory}}} 100\% \quad (4.1)$$

Here, $EE(\%)$ is the encapsulation efficiency, c_{pellet} is the concentration of lutein present in the pellet and c_{theory} is the theoretical concentration of lutein added to the synthesis. The EE of all samples was measured within two days after synthesis.

4.2.4 Photo-chemical stability of lutein-zein particles

Nanoparticle and reference samples (10 mL) were placed in transparent glass vials (FIOLAKS clear, Schott) and stored in a dark room in the light beam of a UV-Vis lamp (Siemens HBO 75W - XBO 100W, see Figure 4.2 for its spectrum) for up to 20 days. A separate experiment was performed while placing the sample vials in the windowsill for several months (November until April). At exposure time intervals, 1 mL was withdrawn from each sample and then extracted as described in the preceding Section and analyzed for lutein concentration in the pellet using UV-vis spectroscopy (Perkin Elmer, lambda 365 UV-vis) at 452 nm. The experiments were performed in duplicate.

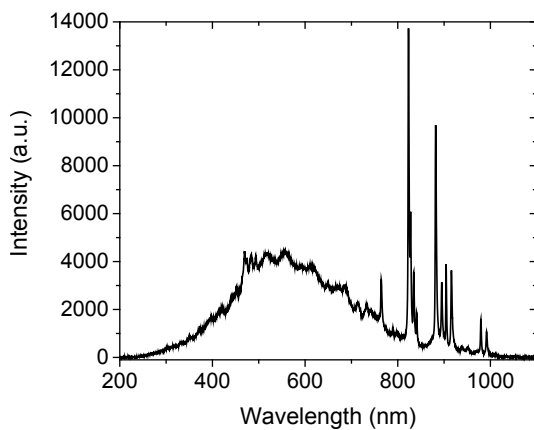


Figure 4.2: Spectrum of the Siemens HBO 75W – XBO 100W lamp, used for artificial illumination of lutein-zein particle dispersions.

4.3 Results & Discussion

4.3.1 Effect of zein concentration on lutein-zein particles

Zein-Lutein particles were synthesized using different amounts of zein while keeping the amount of lutein constant (see Table 4.1). By increasing the concentration of zein, bigger particles could be synthesized (see Figure 4.3), which corresponds to earlier findings.^{35,39} Note that due to the relatively small particle sizes and low concentration of zein, the dispersions are nearly transparent but colored (see Figure 4.8 for an example).

Table 4.1: Concentrations zein and lutein in stock before precipitation.

Zein (wt%)	Lutein (g/l)	Lutein wt%	Lutein:zein weight ratio
1.5	0.10	0.010	0.0067
2.5	0.10	0.010	0.0040
3.5	0.10	0.010	0.0029
4.5	0.10	0.010	0.0022
5.5	0.10	0.010	0.0018
6.5	0.10	0.010	0.0015

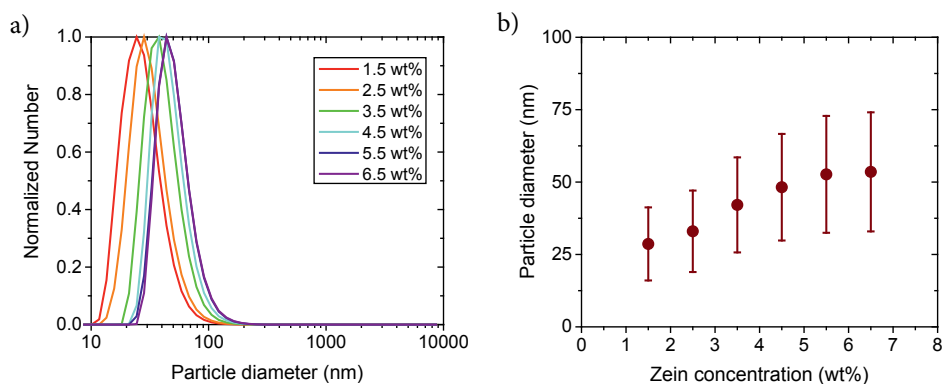


Figure 4.3: Tuning the particle size of zein-lutein particles by increasing the zein concentration in the stock solution, while keeping the lutein concentration constant. Particle sizes were measured using dynamic light scattering. (a) Shows the size distributions (number averaged) of the dispersions and (b) shows the average particle sizes with the standard deviations shown as error bars.

Upon increasing the zein concentration, the encapsulation efficiency also increased (Figure 4.4). This is according to expectations: since the amount of lutein did not change, there is more zein material available for encapsulation purposes and thus the encapsulation efficiency should increase.

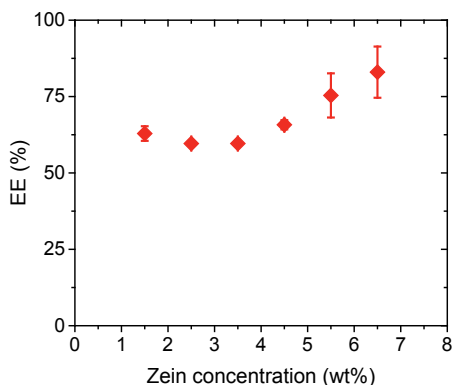


Figure 4.4: Effect of increasing zein concentration at constant lutein concentration on the encapsulation efficiency. Experiments were performed in duplicate.

Freshly made samples were used to determine the photochemical degradation of a selection of these lutein-zein particles with different zein wt% compared to a reference consisting of lutein in water. The samples were exposed to artificial light and analyzed spectrophotometrically at intervals for remaining lutein content, see Figure 4.5. Here, it is visible that the lutein concentration in the reference sample gradually decreased (Figure 4.5a and b), which is according to expectations.⁴⁰ The lutein-zein dispersions start at a lower concentration due to the different encapsulation efficiencies. For these lutein-zein dispersions, there first is a period of about 4 days during which the lutein content is constant. After that, the lutein concentration finally drops to a similar level as the reference sample without zein. This is also visible in the corresponding UV-vis spectra (Figure 4.5c, d, e, and f). Due to the transparency of the samples, differences in scattering and light penetration do not explain the difference in degradation. It was observed that neither the amount of zein, nor the particle size makes a significant difference in the degradation profile.

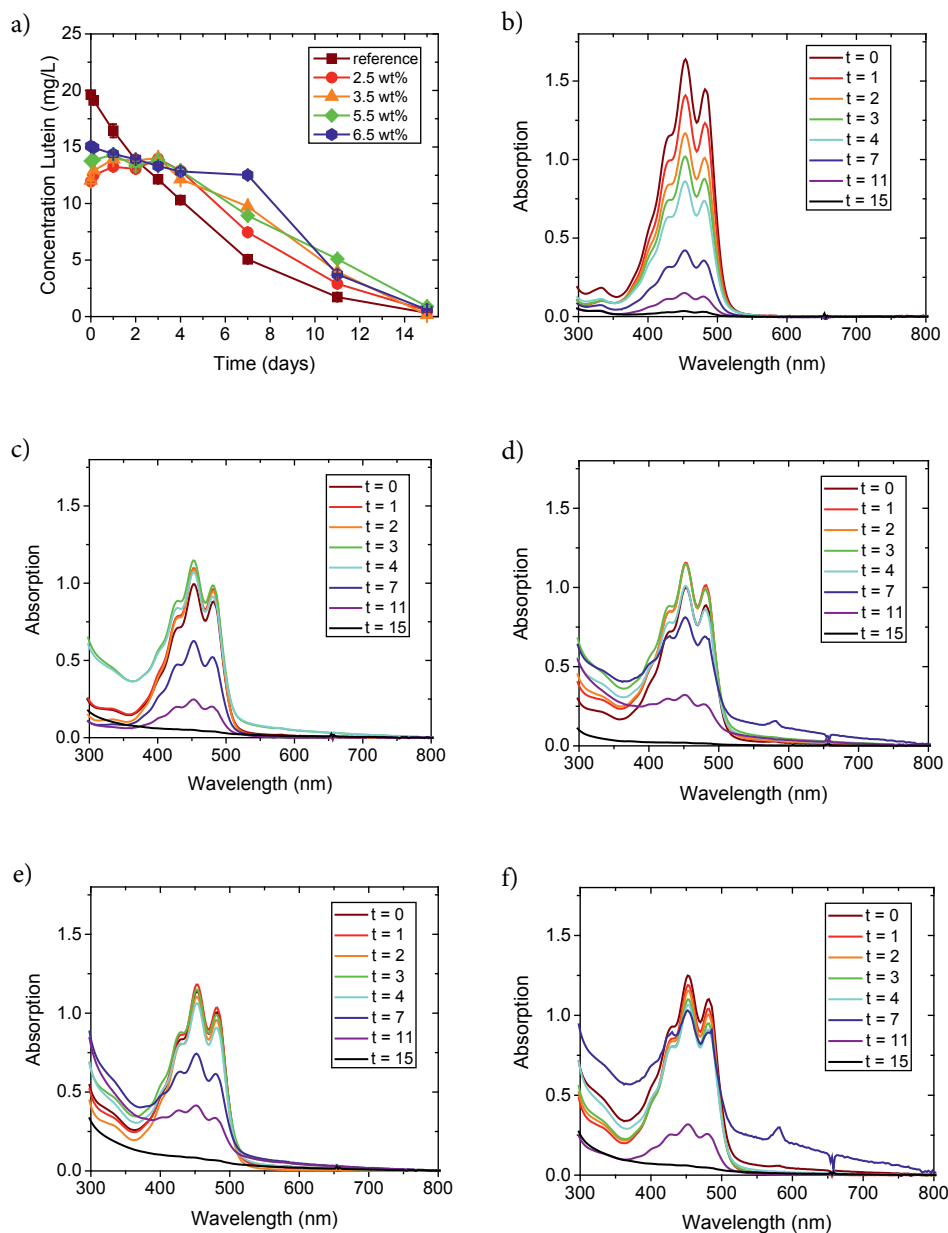


Figure 4.5: (a) Effect of exposure to light on the concentration of lutein. (b)-(f) UV-vis spectra over time for (b) the lutein reference, (c) a dispersion with 2.5 wt% zein concentration, (d) a dispersion with 3.5 wt% zein concentration, (e) a dispersion with 5.5 wt% zein concentration, and (f) a dispersion with 6.5 wt% zein concentration. All particle dispersions were prepared using an initial amount of 0.010 wt% lutein (including the reference).

4.3.2 The effect of ascorbic acid on the stability lutein-zein particles

Particle sizes and zeta potentials were measured after dialysis and after 7 days of storage, see Table 4.2. Here we can see that the particle sizes of the particles that were synthesized by precipitation in the presence of ascorbic acid (AA) are initially of similar size to the particles that were synthesized by precipitation in pure water. After having been stored for 7 days, a size increase was observed for samples that were synthesized by precipitation in ascorbic acid. This might be an indication of instability, however when measuring the zeta potentials of these samples no difference was observed. Interesting to note at this point is that compared to Chuacharoen et al., smaller particle sizes and positive instead of negative zeta-potentials are obtained. The difference in zeta-potentials are explained by the fact that here a pH of 4 is used⁴¹ while Chuacharoen et al used a pH of 7.²² The apparent difference in particle sizes is an effect of the averaging that was chosen. Table 4.2 shows number averaged particle sizes, this was chosen since for polydisperse samples the number averaged data is comparable to SEM data.⁴² While the intensity averaged data, see SI Table S4.1, corresponds much more closely to the results presented by Chuacharoen et al.²²

Table 4.2: Particle sizes (number averaged) and zeta-potentials of Z, LZ, ZA, and LZA dispersions measured directly after dialysis, and measured after 7 days after dialysis (Z = zein, LZ = lutein-zein, ZA = zein-AA, LZA = lutein-zein-AA). All particle dispersions were prepared using an initial amount of 0.010 wt% lutein, 2.5 wt% zein and, for AA samples, 0.016 wt% of ascorbic acid. The error is the standard deviation of the size distribution from DLS or zeta-potential.

Sample	Particle diameter (nm)	Particle diameter (nm)	Zeta potential (mV)	Zeta potential (mV)
	after dialysis	7 days after dialysis	after dialysis	7 days after dialysis
Z	38.1 ± 14.9	25.9 ± 12.1	27.5 ± 4.6	27.9 ± 4.1
LZ	41.8 ± 16.7	37.2 ± 14.9	28.9 ± 4.6	28.7 ± 4.9
ZA	24.7 ± 11.1	74.9 ± 43.0	27.4 ± 4.3	28.5 ± 4.3
LZA	34.8 ± 14.6	67.4 ± 37.5	29.4 ± 4.4	29.0 ± 4.7

The increase in particle size indicates that some aggregation occurs for particles synthesized in ascorbic acid. However, the zeta potentials are similar for all samples and do not seem to change over time (Table 4.2). Therefore, the pH was measured: samples prepared in water (Z and LZ) had a pH of 3.3 and samples prepared in AA (ZA and LZA) had a pH of 4.7. This is not close to the isoelectric point of zein, which is around 6.5.⁴³ Interestingly, this aggregation was not observed for samples synthesized in ascorbic acid which were freshly-made, which will be further discussed in Section 4.3.4.

4.3.3 Effect of ascorbic acid on the photochemical stability

Samples containing ascorbic acid were prepared according to Section 4.2.1, in which the milli-Q water, in which the lutein-zein mixture was precipitated, contained 0.20 mg/mL ascorbic acid. For these experiments only freshly made samples were used and reference samples were also made using this method. Following this, samples were illuminated by the UV-Vis lamp or by daylight, samples were taken at different time intervals, and the remaining lutein content inside of the particles was determined.

The degradation profiles of the samples illuminated by the lamp are represented in Figure 4.6a. Here it is visible that the reference samples (L and LA) show a gradual decrease in lutein content, which seems to be relatively similar and is comparable to earlier results (Section 4.3.1) as well as to literature⁴⁰. The presence of AA slows down the degradation slightly. This gradual decrease is also visible in the raw spectroscopy data (Figure 4.6b and c). The lutein-zein dispersion (LZ) and the lutein-zein-AA (LZA) dispersion both seem to have a plateau after which the lutein content decreases (see Figure 4.6a, d and e). The plateau is longer for LZA, which indicates that the presence of ascorbic acid in combination with zein improves the stability of lutein the most. Overall, these experiments again show that encapsulation of lutein in zein increases its photo-stability. Also, ascorbic acid has a positive effect on the lutein photo-stability compared to the lutein-zein particles without the addition of ascorbic acid.

Another batch of samples was synthesized and illuminated by daylight, of which the results are presented in Figure 4.7. Similar to previous results, the lutein reference (L) and the lutein reference in the presence of ascorbic acid (LA) seem to have similar degradation profiles. Note that the initial lutein content is different, however, the trend is the same (see also Figure 7b and c, the small jump in signal in Figure 4.7b and c is due to the changing of the source in the spectrophotometer at 400 nm). The lutein-zein dispersions (LZ and LZA) show a plateau before the concentration of lutein inside of the particles decreases, see also Figure 4.7d and e for the spectroscopy data. For LZA the lutein concentration first increases and later decreases (Figure 4.7a). This is probably due to zein particles aggregating or flocculating over time due to light and temperature. This could also explain the increase in particle size over time,⁴⁴ as further explained in Section 4.3.4.

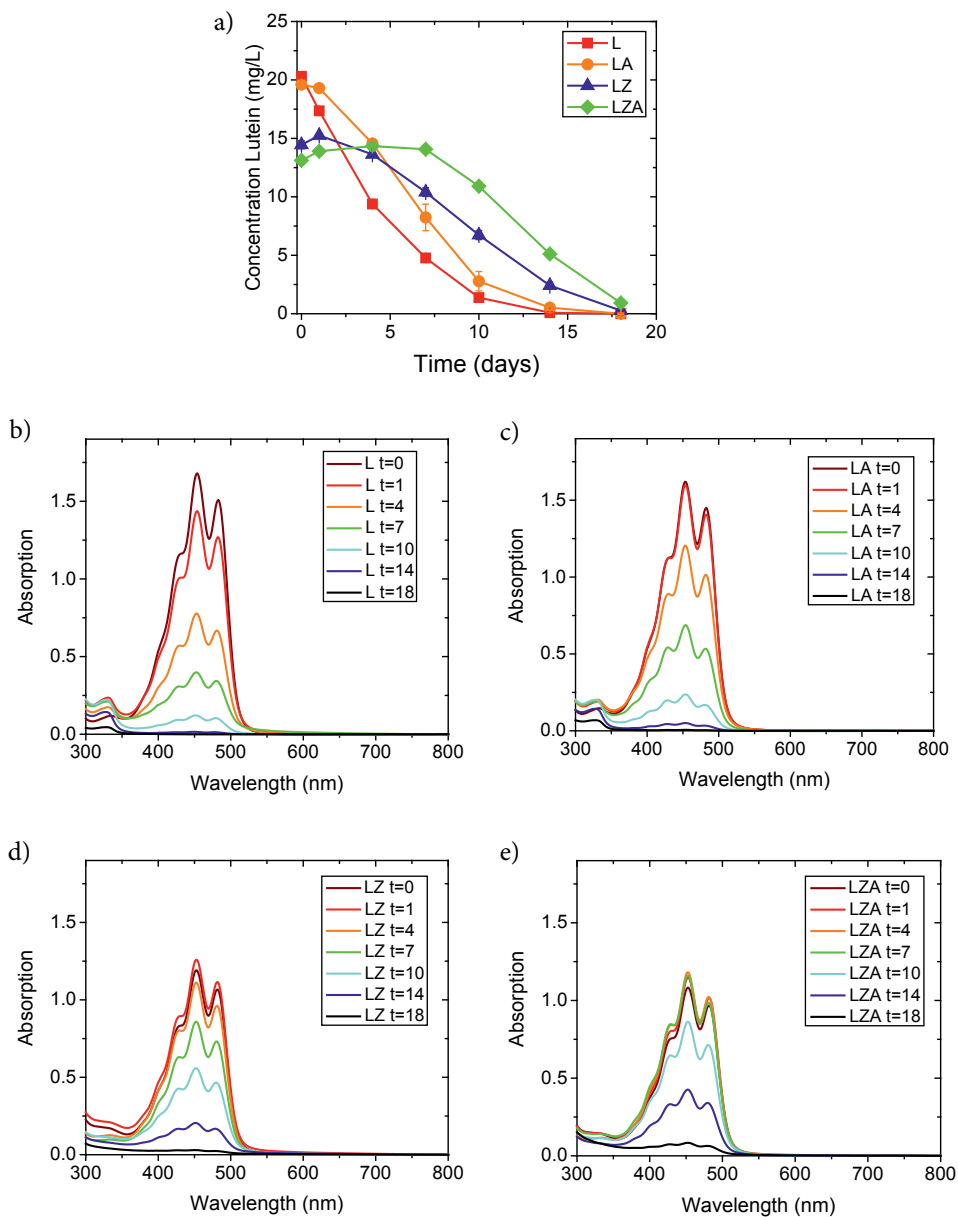


Figure 4.6: (a) Lutein content of Lutein-zein particle dispersions and reference placed in a dark room after illumination by a Siemens HBO 75W – XBO 100W lamp. (b)-(e) UV-vis spectra over time for (b) the 0.01 wt% lutein (L) reference, (c) 0.01 wt% lutein and 0.016 wt% AA (LA) reference, (d) a dispersion with 2.5 wt% zein concentration and 0.01 wt% lutein (LZ), and (e) a dispersion with 2.5 wt% zein concentration, 0.01 wt% lutein and 0.016 wt% AA (LZA).

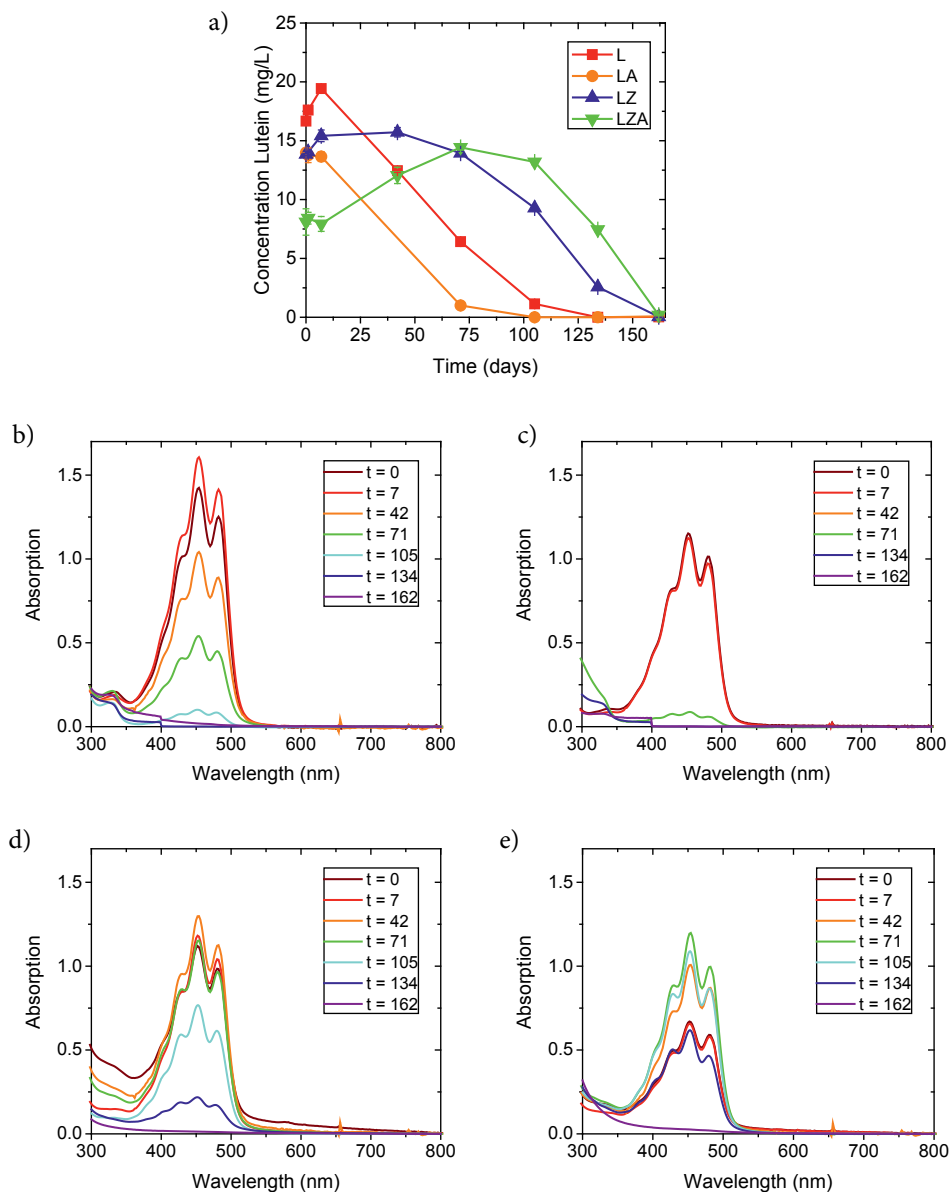


Figure 4.7: (a) Lutein-zein particle dispersions and references samples placed in the windowsill and illuminated by daylight (October until April). Including the UV-vis spectra over time for (b) the 0.01 wt% lutein (L) reference, (c) 0.01 wt% lutein and 0.016 wt% AA (LA) reference, (d) a dispersion with 2.5 wt% zein concentration and 0.01wt% lutein (LZ), and (e) a dispersion with 2.5 wt% zein concentration, 0.01 wt% lutein and 0.016 wt% AA (LZA).

4.3.4 Penetration depth and particle sizes upon illumination

After looking at the degradation profiles of lutein in lutein-zein particles and reference samples discussed in previous Sections, it is interesting to investigate the amount of light that reaches the lutein until it gets scattered or absorbed. One would expect for nearly transparent particle dispersions (see Fithat all particles are exposed to the light and lutein degradation takes place throughout the whole dispersion. This is opposed to strongly absorbing or scattering dispersions where a large portion of the light has interacted with only the first few millimeters of the sample dispersion. The last case will have an unwanted averaging effect on the lutein content that is measured using the method described in Section 4.2.4, since we want to know the effect of encapsulation and addition of an antioxidant on the photo-stability of lutein and not the effect of turbidity.

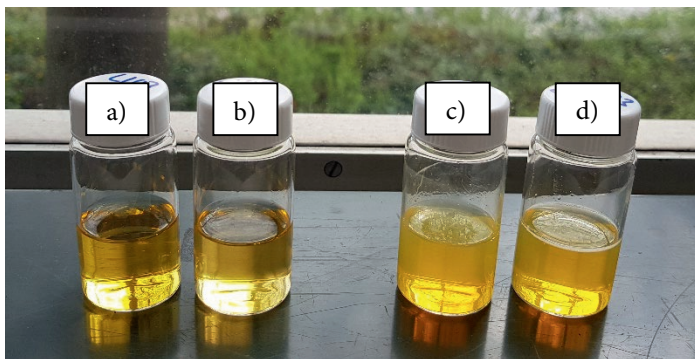


Figure 4.8: Freshly made lutein reference dispersions, (a) L and (b) LA, and lutein-zein dispersions, (c) LZ and (d) LZA. Due to the low concentration of zein not only the reference samples are colored but highly transparent, also the lutein-zein dispersions have a high transparency.

For this purpose, the optical penetration depth is determined. Absorption and scattering cause the intensity of light to decay with depth at a rate determined by the material's extinction, τ . In general, τ is a function of wavelength and temperature, but for constant τ , intensity I decays exponentially with depth z according to the Lambert-Beer's law as: $I(z) = I_0 e^{-\tau z}$. In which I_0 is the incident intensity. This can be used to determine the optical penetration depth $\delta = 1/\tau$, which is the depth at which the intensity of the transmitted light drops to $1/e$ of its initial value.⁴⁵ Penetration depths were determined by measuring direct transmission of which the results are presented in Figure 4.9.

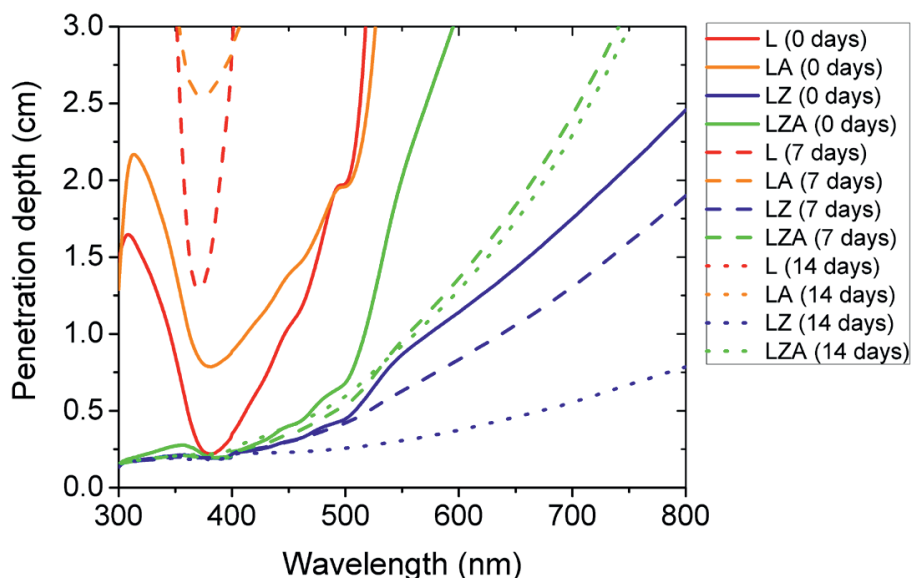


Figure 4.9: Optical penetration depth of L, LA, LZ and LZA dispersions measured at different time steps when illumination the samples using the Siemens HBO 75W – XBO 100W lamp (L = lutein, LA = Lutein-AA, LZ = lutein-zein, LZA = lutein-zein-AA).

As expected, not all wavelengths reach through the whole sample (of 1 cm) and the light gets absorbed or scattered, which explains the fact that L and LA have a faster lutein degradation than LZ and LZA. Interestingly, the results in Figure 4.6 and 4.7 show that LZ has a faster degradation than LZA, despite its having a lower penetration depth. This indicates that ascorbic acid in combination with zein has a significant positive effect on the stability of lutein, more than ascorbic acid alone or just the encapsulation with zein.

During the illumination experiments it was noted that the samples including zein became more opaque over time. This effect can be observed in Figure 4.9, the penetration depth for LZ and LZA decreases in time, which indicates that particle sizes are increasing, and the dispersion is slowly aggregating or flocculating, see Table 4.3. It seems that the stability of the zein colloids is affected during the experiment, causing the particle sizes to increase and the optical penetration depth to decrease for these samples. A possible cause is degradation of zein particles because of the light and temperature, since zein particle dispersions are known to be stable at low temperatures in the dark.^{46,47} In these experiments also degradation by bacteria cannot be excluded.

Table 4.3: Particle sizes of LZ and LZA dispersions measured at different time steps when illumination the samples using the Siemens HBO 75W – XBO 100W lamp (LZ = lutein-zein, LZA = lutein-zein-AA). The error is the standard deviation of the size distribution from DLS.

Illumination time (days)	LZ	LZA
	Particle diameter (nm)	Particle diameter (nm)
0	29.4 ± 14.4	24.0 ± 11.4
7	42.8 ± 22.6	46.5 ± 24.1
14	123.7 ± 62.6	48.2 ± 25.4

This information can be combined with that from the photo-stability experiments in Section 4.3.3, where it is observed that LZA dispersions lead to a slower lutein degradation than LZ dispersions. However, for LZA dispersions the penetration depth is constantly higher than for LZ dispersions. This is contradictory, since it would be expected that the sample with the lowest penetration depth has the highest lutein stability over time. These observations suggest that ascorbic acid has a strong effect on the stability of the particles and enhances the stability of the lutein. Possibly, by the flocculation and aggregation of the LZ particles lutein is released⁴⁸ which then degrades in the light without protection from either the zein or the ascorbic acid. Future experiments to prevent this aggregation and increase the stability could be in the direction of adding surfactants^{16,22,44,47} or crosslinking of the zein chains to form solid particles that are less eager to aggregate or flocculate.^{49–52} This could then result in a further increase in the lutein stability when this is necessary for a specific application.

4.4 Conclusion

In this study, lutein-zein colloidal particles were successfully synthesized using anti-solvent precipitation, following initial purification of zein. Also, ascorbic acid was successfully added to the synthesis as an antioxidant. Particle sizes could be tuned by changing the zein concentration during synthesis. When particle sizes increased, the encapsulation efficiency also increased, which is according to expectations. Zein nanoparticles showed a significant ability to protect lutein from degradation compared to lutein dispersions in water. However, no significant differences between particle sizes and photochemical stability were observed during photo-stability experiments. To promote the stability of the entrapped colorant even further, ascorbic acid was used as an antioxidant. The addition of ascorbic acid to lutein and lutein-zein colloidal particles did not lead to an increase in the particle size or to any changes in the zeta-potential, compared to dispersions without ascorbic acid. The photo-stability of the nanoparticle dispersions stabilized with ascorbic acid improved significantly compared to samples without ascorbic acid or lutein dispersions.

These results show that the degradation rate of natural colorant lutein against photo-degradation can be significantly decreased by encapsulation using zein as carrier material. Addition of an antioxidant, like ascorbic acid, has led to an even further improved photo-stability of this colorant. The simple anti-solvent precipitation technique offers further possibilities to improve the stability, by adding surfactants, crosslinkers, and other stabilizers to ease the implementation of such particle systems into an application matrix when this is necessary for the specific application.

4.5 Acknowledgements

I would like to thank Celine Carin-Cuissinat, Andrea Hitzfeld and Ralf Schweiggert (DSM) for their discussions on natural colorants in combination with food systems, and I gratefully thank DSM for the donation of the food grade lutein (FloraGlo lutein, Crystalline dry) used in this Chapter.

4.6 References

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4.6 Supplementary information

Table S4.1: Particle sizes (Intensity averaged) of Z, LZ, ZA, and LZA dispersions measured directly after dialysis, and measured after 7 days after dialysis (Z = zein, LZ = lutein-zein, ZA = zein-AA, LZA = lutein-zein-AA).

sample	Particle diameter (nm)	Particle diameter (nm)
	after dialysis	7 days after dialysis
Z	102.9 ± 44.6	127.1 ± 68.4
LZ	106.3 ± 46.2	111.6 ± 49.9
ZA	129.7 ± 72.3	231.5 ± 82.4
LZA	119.5 ± 61.2	237.1 ± 125.7

Chapter 5

Characterization and modeling of optical properties of white zein colloidal particles on the single particle level and in concentrated dispersions

Abstract

In this Chapter, a method to investigate the optical properties of white zein colloidal particles is presented in both diluted and concentrated dispersions. The white zein particles are synthesized by anti-solvent precipitation. The value of these particles as white colorant is examined by measuring their optical properties. Dilute dispersions were prepared to measure the extinction cross section of individual particles this was combined with Mie theory to determine a refractive index (RI) of 1.49 ± 0.01 for zein particles dispersed in water. This value was used to further model the optical properties of concentrated dispersions. The optimal size for maximal scattering efficiency was explored by modeling dilute and concentrated samples with RI's matching those of zein and TiO_2 particles in water. The transport mean free path of light was determined experimentally and theoretically and the agreement between the transport mean free path calculated from the model and the measured value was better than 30%. Such particles have the potential to be an all-natural edible alternative for TiO_2 as white colorant in wet food products.

5.1 Introduction

Colloidal particles offer many possibilities to control product appearance through the manipulation of particle size, composition, and shape. This originates from the ability of colloids to interact with light. Control over scattering and absorption of light by individual particles determines the appearance of consumer products. When organized in more complex structures, such as concentrated dispersion or even colloidal crystals, multiple scattering and interference of scattered light modify the appearance further.^{1,2}

The perceived quality of a product is primarily determined by its appearance, of which color is one of the main attributes.³⁻⁵ Color of particles in a dispersion, is determined by a combination of the sensitivity of the human eye, illumination effects, structure, shape and size of the particles, and actual color of the product. Titanium dioxide (TiO₂) is the best known and most widely used white food colorant. The whiteness that is observed for these TiO₂ particles is due its high RI, low absorption, and high scattering properties. TiO₂ is authorized for use in the European Union as E171 and is used in foods such as dairy products and candy. Despite this, concerns have been raised recently regarding the toxicity of nanosized TiO₂ following oral exposure.⁶ Since there is no conclusive results are available on this topic research on this topic ongoing.⁶⁻⁸ According to the current regulations TiO₂ is considered safe as food additive, but all natural fully edible alternatives are highly desirable.

Such a natural and fully edible additive is zein, a protein from corn, which has advantageous properties which make these proteins suitable for possible applications in the food industry such as edible, renewable, and biodegradable. Zein is soluble in a mixture of food safe solvents, such as ethanol/water mixtures; and it has good water barrier properties, both in particulate form and as films.⁹⁻¹⁴ Because of these advantageous properties towards food applications, many researchers have investigated the use of zein for these applications.^{15,16} As an example, zein has been proposed as a fat analogue for fats that are present in mayonnaise. These researchers investigated the rheological properties of zein but not the optical properties.¹⁷ Also, literature examples can be found in which spectral reflectance was used to determine and predict the color of emulsions. In these studies, the researchers use an optical reflectance spectroscopy technique which utilizes measurements of the wavelength dependence of the time-averaged back-scattered light which is then compared to the Kubelka-Munk theory.^{18,19}

In this Chapter another approach is used which is based on the diffusion of light waves through a concentrated colloidal dispersion. Using this, the optical properties of white zein

particles in dispersion are investigated. Similar to TiO_2 , the whiteness of the zein particles is due to low absorption and strong scattering by the particles. However, the RI is lower in the case of zein, which means that the concentration needs to be increased to obtain a similar opacity, which was already shown in Chapter 2, as was the synthesis. The value of these white zein nanoparticles as white colorant is quantified by measuring their scattering properties. The extinction cross section is measured on the single particle level; from this the refractive index (RI) of the zein particles in water can be determined by fitting Mie theory to the data. Transparent to opaque dispersions of zein nanoparticles are obtained by varying the concentration of particles in solution. The obtained dispersions are used to compare the zein particles to aqueous dispersions of TiO_2 , which has a much higher RI. The optimal particle size necessary for obtaining a maximal scattering efficiency is explored by modeling. This was done both for dilute and concentrated samples, using RI's that match those of white zein (1.49) and TiO_2 particles (2.5688) in water (1.330). Finally, and most importantly, the whiteness resulting from multiple scattering of light is quantified for more concentrated dispersions, and expressed in terms of the transport mean free path. The obtained results are then compared to multiple scattering theory. For future research it would be interesting to investigate the interaction between food components and zein particles after addition to real food systems.

5.2 Methods & Materials

5.2.1 Materials

Ethanol (100% pure) was obtained from Interchema. Hydrochloric acid (HCl) was purchased from Sigma Aldrich. Zein was purchased from Flo Chemical Corporation (Zein F4000C-FG, lot no.: F40006011C6) and water was purified using a Millipore Direct-Q purification system.

5.2.2 Preparation and characterization of white zein nanoparticles

The preparation and full characterization of white zein colloidal particles is described in detail in Chapter 2. Here the method is briefly summarized, since these particles were used for the experiments described in this chapter.

White particles were synthesized via anti-solvent precipitation after removal of colored impurities present in zein. Particle sizes were measured using DLS (Zetasizer Nano ZS series, Malvern Instruments). In DLS a CONTIN analysis was used to obtain the size distributions. Prior to the DLS measurements, the samples were diluted, with water adjusted to pH 4, to a suitable concentration, to prevent multiple scattering. The particle shape was determined by SEM (Nova Nanolab, FEI). To minimize changes in particle size and shape, samples were freeze-dried overnight using a lyophilizer (Virtis). The dry powder, which was loosely attached to the lyophilizing bottle, was collected and stored in a desiccator until SEM analysis. Prior to SEM analysis a platinum layer of about 4 nm was sputtered onto these samples to prevent charging. Then total reflectance spectroscopy was used to determine whether or not the particle dispersions are observed as white by a human observer. This is important since whiteness of the dispersion gives an indication that the absorption present in the sample will be negligible.

5.2.3 Optical properties of diluted dispersions

UV-vis spectroscopy (HP 8953A spectrophotometer) was used for determination of the extinction cross sections of the particles, which can later be used for the determination of the refractive index of the particles. Quartz cuvettes of 1 and 10 mm were used. The extinction cross sections of the zein particles were obtained by measuring absorption spectra of dilute dispersions of zein particles. Prior to the measurements, samples were diluted with water (pH 4), adjusted with HCl, to suitably low concentrations, where the samples appear almost completely transparent to the eye, to prevent multiple scattering. Final concentrations were,

depending on the particle size, between 0.06 wt% (for small particles) and 0.01 wt% (for large particles). The final concentration of each sample was measured by determination of the dry weight of the particles in the dispersion.

5.2.4 Optical properties of concentrated dispersions

The transport mean free path (I^*) was experimentally determined via total transmittance spectroscopy. Measurements were carried out with a spectrophotometer (HR4000, Ocean Optics) equipped with a 15 cm diameter integrating sphere (barium sulfate coated, Labsphere) with a sample opening of 18 mm, an opening for the detector port of 3 mm. As a light source, a Tungsten Halogen light source (HL-2000-FHSA-LL, Ocean Optics) was used and at the detection port a multimode fiber was used to couple the light into a spectrometer (HR4000, Ocean Optics). For an overview of the setup, see Figure 5.1. Concentrated samples were measured in cylindrical quartz cuvettes (series 120-QS, Hellma Analytics), with path lengths 1, 2, and 5 mm at wavelengths ranging from 400 to 800 nm. Total transmittance, $T(\%)$, data is obtained through equation 5.1:

$$T(\%) = \frac{S_\lambda - D_\lambda}{R_\lambda - D_\lambda} \cdot 100\% \quad (5.1)$$

In which S_λ is the measured spectrum of the sample at a certain path length, D_λ the dark spectrum when the shutter of the lamp is closed, and R_λ the reference measurement when there is no sample in front of the port and the lamp is on.

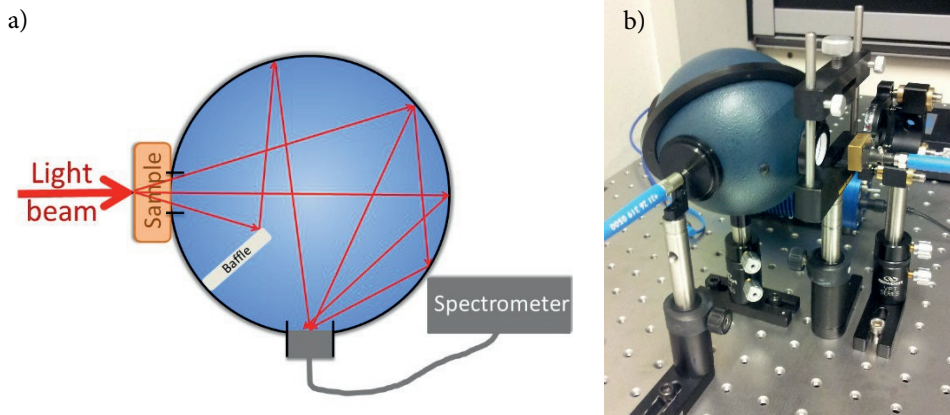


Figure 5.1: Schematic overview of the setup for total transmission measurements (a) and the actually built setup (b) including a sample holder between the light source and the port opening. An integrating sphere of 15 cm in diameter was used (Labsphere). The sample and light beam opening was 18 mm, the detector port opening was 3 mm.

5.3 Results and discussion

5.3.1 Preparation and Characterization of white zein nanoparticles

White zein colloidal particles were successfully synthesized via anti-solvent precipitation^{15,16} after removal of colored impurities present in zein, which is fully described in Chapter 2. It was possible to produce particle dispersions of different average sizes which could be tuned by changing the zein concentration in the stock. Obtained particles were highly polydisperse, however they were spherical in shape (see Figure 2.5). The volume distributions of particles with different average sizes (Figure 5.2), which were needed for the modeling of the transport mean free path (l^*), were obtained from DLS measurements.

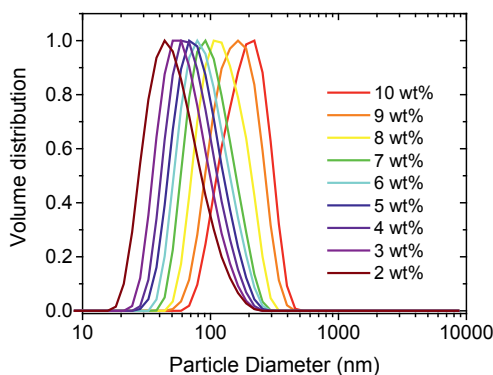


Figure 5.2 Normalized size distribution data (volume distribution) for particles was taken from DLS measurements, as an input for the polydispersity of the samples in the model to calculate the transport mean free path.

Total reflectance spectroscopy has shown that the particle dispersions are observed as white, and are therefore expected to have a negligible absorbance in the visible part of the spectrum.

5.3.2 Modeling and experiments on dilute dispersions

To obtain a colloidal dispersion with a white appearance it is important to use particles with optimal scattering efficiency. This can be modeled using Mie theory, which describes the scattering of light by spherical particles.²⁰ In a transmission measurement the extinction is measured, which is the sum of scattering and absorption. For modeling of the white zein particles, it is assumed that there is no absorption, so that the extinction efficiency is equal to

the scattering efficiency. However, since the particles may still absorb slightly, the term extinction will be used.

The quantity to consider is the extinction cross section per unit volume of scattering material. We will see shortly that this has a maximum for a particular, optimal, radius. To evaluate the theory, MiePlot software created by Philip Laven²¹ was used. To use this model for zein particles, the RI of the particles in water should be known in advance. The RI of dry zein fibers is reported^{22,23} to be 1.54 or 1.55. In this study there are no dry fibers, but particles dispersed in water. This makes it very likely that there is some water trapped in the colloidal particles which will lower the RI of the particles compared to dry zein. Common methods to determine the RI, such as refractive index matching measurements, were not an option, since it is impossible to predict the swelling behavior of the zein particles in other solvents. Moreover, zein is soluble in a large number of solvents²⁴. Therefore, experiments and theory are combined, by measuring the extinction on the single particle level and comparing this to the model in which the RI is a variable.

Lambert Beer's law was used to obtain the extinction efficiency. The transmitted intensity I is related to the extinction cross section C_{ext} per particle as:

$$I = I_0 e^{-\rho C_{ext} L} \quad (5.2)$$

Here, I_0 is the incident intensity and L the path length of the cuvette. The number density of particles ρ is obtained from the weight concentration of the dispersion $c_z = 4\pi r^3 \rho D_z / 3$, assuming spherical particles with radius r and mass density D_z (1.1 g.cm⁻³). Introducing the extinction $A = -\log(I/I_0)$, Equation 5.2 can be rewritten as:

$$\frac{Q_{ext}}{r} = A \ln(10) \frac{4D_z}{3c_z L} \quad (5.3)$$

Here, $Q_{ext} = C_{ext}/\pi r^2$ is the extinction efficiency. This Equation shows that the extinction is maximized by maximizing Q_{ext}/r . This quantity is essentially the scattering cross section per unit volume of scattering material: When a given volume of material is used to make spherical particles the extinction will be maximal when the particles are given the radius that maximizes Q_{ext}/r . Equation 5.3 was used to obtain Q_{ext}/r for synthesized zein particles. In Figure 5.3 the squares show these data plotted versus the average radius for several wavelengths ranging from 450 to 700 nm. As seen, Q_{ext}/r increases with r as all particle sizes are well below the optimal radius. Also, Q_{ext}/r increases as the wavelength decreases. For a

correct comparison of these measurements with theory, the latter should take into account the polydispersity of the corresponding experimental system. Since each particle scatters independently and in proportion to its extinction efficiency, Equation 5.2 should be replaced by:

$$I = I_0 e^{-\rho \sum_i f_i c_{ext,i} L} \quad (5.4)$$

where f_i is the fraction of particles of size r_i . Since now $c_z = \rho \sum_i 4\pi r_i^3 f_i D_z / 3$, this can be written as:

$$\left\langle \frac{Q_{ext}}{r} \right\rangle \equiv \frac{\sum f_i r_i^3 \frac{Q_{ext,i}}{r_i}}{\sum f_i r_i^3} = A \ln(10) \frac{4D_z}{3c_z L} \quad (5.5)$$

Thus, $\langle Q_{ext,i}/r_i \rangle$ should be averaged over the particle size distribution, using as weights the factors $f_i r_i^3$, which describe the distribution of particle volumes, rather than radii. This distribution is readily obtained from dynamic light scattering. The theoretical data (circles in Figure 5.3) were calculated in MiePlot according to Equation 5.5 using the volume distributions from DLS experiments on the corresponding samples (Figure 5.2). To achieve this, a RI of the medium water at 20°C was used, and for zein particles a RI of 1.49. This resulted in good agreement with experimental results. When a higher RI was used the theoretical points shifted up, and when a lower RI was used the theoretical points shifted down. Figures 5.3b and 5.3c show that the RI's of 1.48 or 1.50 result in a noticeably poorer agreement, from these results it can be concluded that the RI of these particles is 1.49 ± 0.01 .

Now it is possible to determine the optimal size for maximal extinction efficiency completely by the model, using 1.49 as input for the RI. Results, calculated using MiePlot, are shown in Figure 5.4a at a few wavelengths spanning the visible spectrum. This indicates that particles with an RI of 1.49 in water should have a diameter between 700 and 1000 nm to reach their optimal extinction efficiency, which is much larger than particles that were synthesized in this study. For comparison, Figure 5.4b includes the same model for TiO₂ particles (RI = 2.5688)²⁵ in water. Here the optimal particle size is much smaller, which has its origin at the higher RI ratio of the particles and the medium. Finally, comparison of the vertical scales of Figures 5.4a and 5.4b shows that TiO₂ scatters just about 10 times more strongly than zein on a per volume basis. Note that these Mie scattering calculations describe single scattering only. Multiple scattering will change the picture drastically, as will be shown next.

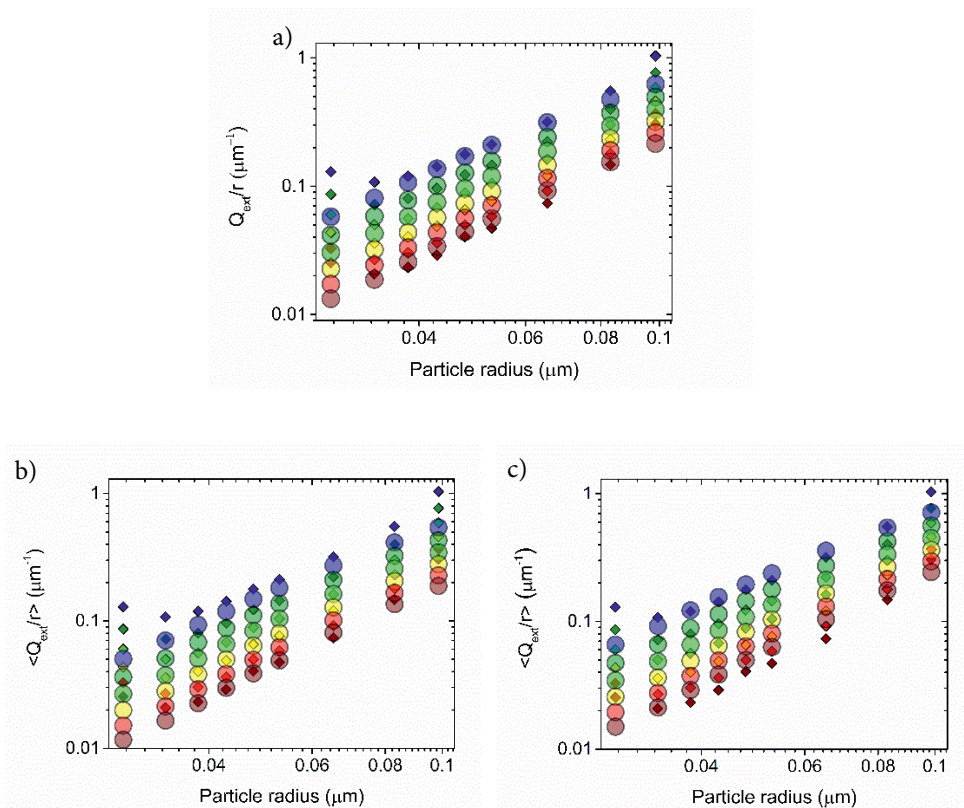


Figure 5.3: $\langle Q_{ext}/r \rangle$ versus average particle radius measured on dilute samples (small squares) and calculated from Mie theory (large circles). Input for the theory is: a particle refractive index of 1.49 (a), 1.48 (b), and 1.50 (c), a particle density of 1.1 g cm^{-3} , and the measured particle volume distribution from DLS for each sample. Data are shown for six wavelengths, shown in colors from blue to red: 450, 500, 550, 600, 650, 700 nm.

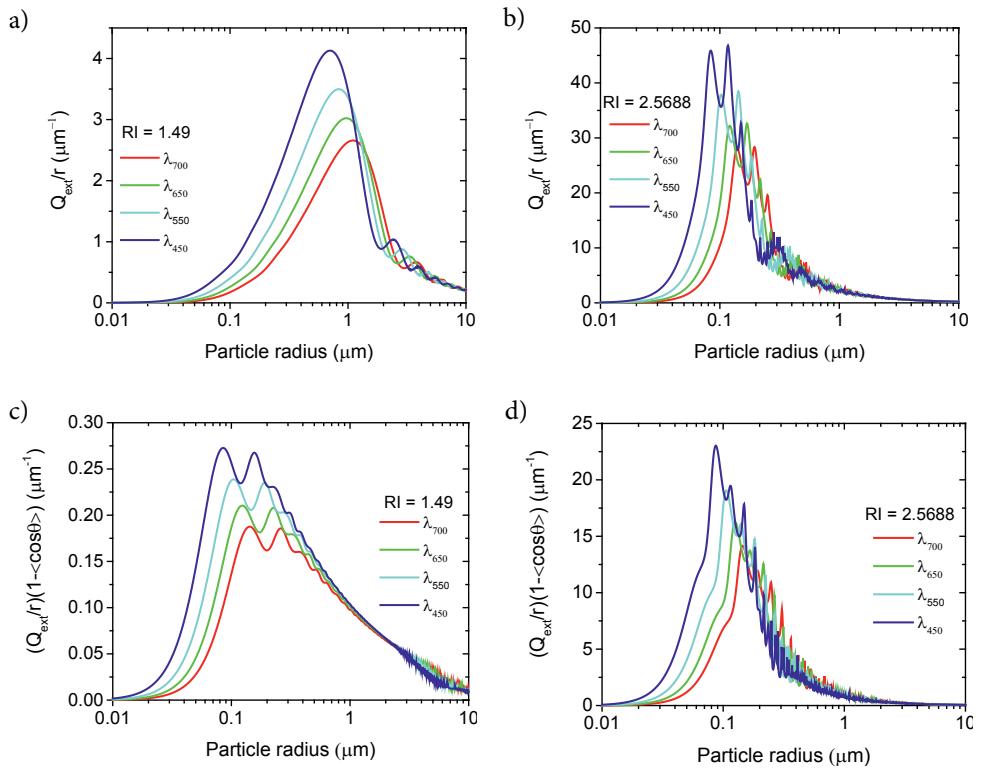


Figure 5.4: Single particle scattering model: Q_{ext}/r versus the particle radius for monodisperse particles with a refractive index (RI) of (a) 1.49 (zein) and (b) 2.5688 (TiO_2), with water as medium. Scattering model for concentrated dispersions: $(Q_{ext}/r)(1-\cos\theta)$ versus the particle radius for monodisperse particles of which the particles have a refractive index of c) 1.49 (zein) and d) 2.5688 (TiO_2). Calculations were done at four wavelengths in the visible spectrum.

5.3.3 Modeling and experiments on concentrated dispersions

In systems where the interest is in whiteness of the dispersion, the sample is concentrated. This means that light is scattered not once but multiple times before leaving the sample. The quantity $\rho C_{ext} = 1/l$ is called the inverse of the scattering mean free path (when absorption is absent). In this Equation l is a measure for the average distance that a light ray travels in the sample before it scatters. In the dilute samples discussed so far light will scatter at most once, and so $l \gg L$. In concentrated samples, the scattering strength is quantified by the transport mean free path, l^* , which describes the distance over which the direction of propagation of light is randomized. l^* is closely related to l , but in addition includes the average of the cosine of the scattering angle, or anisotropy factor, which can also be calculated from Mie theory²⁶:

$$\frac{1}{l^*} = \frac{1 - \langle \cos \theta \rangle}{l} \quad (5.6)$$

Using Equation 5.5, $\rho = 3c_z/(4D_z\pi r^3)$ and $Q_{ext} = C_{ext}/(\pi r^2)$, Equation 5.2 can be rewritten analogously to Equation 5.3:

$$\frac{Q_{ext}}{r} (1 - \langle \cos \theta \rangle) = \frac{1}{l^*} \frac{4D_z}{3c_z} \quad (5.7)$$

Thus, the left-hand side shows the quantity to maximize, in order to minimize the total transmittance (and maximize diffuse reflectance) of a multiply scattering sample at a fixed weight concentration. Note that here the assumption is made that the scattering particles are uncorrelated, which is the case for the relatively low concentrations (<3 wt%) used in this work. Again, MiePlot can be used for the calculation of Q_{ext} and $\langle \cos \theta \rangle$. Figure 5.5c and 5.5d show results of calculations from MiePlot for spheres of RI 1.49 and 2.5688 at several visible wavelengths in water. The optimal particle diameter now ranges from 100 to 250 nm in the case of zein particles, which is much smaller than for single scattering, and just within reach of the here presented synthesis. For TiO₂ particles, on the other hand, such a shift is not observed and the optimum of the scattering efficiency stays at a similar position. However, the absolute values are much higher. Again, when comparing the vertical scales of Figures 5.5c and 5.5d it is observed that TiO₂ scatters about 10 times more strongly than zein on a per volume basis. This indicates that by using a high enough concentration it is possible to achieve similar scattering properties for zein particles as for TiO₂ particles, as is also indicated in Figure 5.3b and c.

A common way to experimentally determine the transport mean free path is from the relation²⁷ between the total transmittance T , and the path length of the cell, L (Equation 5.8), which shows that total diffuse transmittance is low at small l^* . In this situation, most of the incident light is diffusely reflected (neglecting absorption). An example of total transmittance data for different path lengths is presented in Figure 5.5a, here the total transmittance ($T(\%)$) was calculated from experimental data using Equation 5.1.

$$T = (1 - R_s) \frac{1+z_e}{\frac{L}{l^*} + 2z_e}, \quad \text{with } z_e = \frac{2}{3} \frac{1+\bar{R}}{1-\bar{R}} \quad (5.8)$$

Here, $\bar{R}$ is the polarization and angle-averaged internal diffuse reflectivity of the sample, R_s is the specular reflectivity of the incident light beam from the front face of the sample, and z_e is the extrapolation length ratio that describes the boundary conditions of the diffuse intensity at the interface of the sample. R_s and $\bar{R}$ can be calculated via Fresnel's reflection coefficients as described by Vera and Durian²⁸ from the RI's of the sample medium, container wall, and external medium (i.e. 1.330, 1.458, and 1.000 respectively), which leads to $R_s = 0.0367$ and $z_e = 1.7365$. Finally, l^* is obtained from the slope of the linear fit of the $1/T$ vs. L plot, see Figure 5.5b for an example of such a plot.

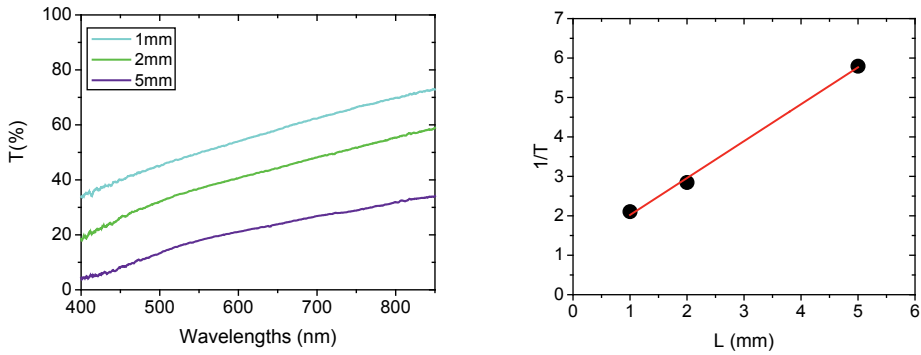


Figure 5.5: Total transmittance measurement data for path lengths of 1, 2 and 5 mm (a), and the linear fit of the $1/T$ vs. L plot for $\lambda = 550$ nm; here the R^2 of the fit is 0.99462 (b). The sample had a particle diameter of 115 ± 41 nm and a concentration of 1.9 wt%.

To compare experimental data to theory Equation 5.7 has to be averaged over the particle size distribution²⁹. For a multicomponent system l^* is given by:³⁰

$$\frac{1}{l^*} = \rho \sum_i f_i C_{ext,i} (1 - \langle \cos \theta \rangle_i) \quad (5.9)$$

In a similar way as before Equation 5.8 can be rewritten in a more useful form:

$$\left\langle \frac{Q_{ext}}{r} (1 - \langle \cos \theta \rangle) \right\rangle \equiv \frac{\sum_i f_i r_i^3 \frac{Q_{ext,i}}{r_i} (1 - \langle \cos \theta \rangle_i)}{\sum_i f_i r_i^3} = \frac{1}{l^*} \frac{4D_z}{3c_z} \quad (5.10)$$

The particle volume distribution $f_i r_i^3$ is again taken from DLS results of the corresponding samples. Results are shown in Figure 5.6.

Deviations between experimental results and theoretical calculations, as prescribed by Equation 5.10, are seen to be on average about 30%. This is still the case when different particle sizes or different volume fractions are used (sample A, B and C in Figure 5.6). All samples in Figure 5.6 show an increase in the transport mean free path when the wavelength increases. This is a trend which is consistent with that observed in literature for ensembles of highly polydisperse non-absorbing nanoparticle scatterers.^{27,31,32} In these works, a similar accuracy is obtained between experiment and theory. It can be observed that the values of the experimental results are always below the theoretical data, which indicates that the particles scatter more than expected. An explanation might be that part of the transmitted light was not captured by the integrating sphere, but escaped at the edges of the sample holders.

Despite this, the qualitative agreement between theory and experiment is good. Figure 5.6 shows that the models of samples B and C cross at a wavelength between 600 and 650; this is also visible for the experimental results. This indicates that experiments, synthesis, and total transmittance measurements are very sensitive, and therefore have a good agreement with theory.

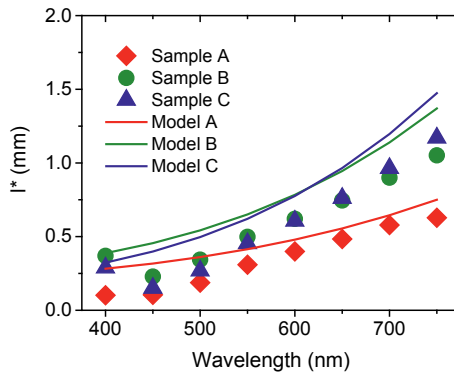


Figure 5.6: The transport mean free path, l^* , found experimentally by total transmittance spectroscopy (points) and calculated from theory (lines) of three zein particle dispersions with different mean particle sizes. Sample A had a particle diameter of 115 ± 41 nm and 1.9 wt%, sample B a diameter of 93 ± 32 nm and 1.6 wt%, and sample C a diameter of 76 ± 41 nm and 2.3 wt%.

5.4 Conclusions

In this study white zein colloidal particles were successfully synthesized via anti-solvent precipitation. The value of the zein nanoparticles as white colorant was examined by measuring their optical properties. Dilute dispersions were prepared to measure the extinction cross section on the single particle level. A method was devised to determine the RI of the white zein particles by fitting extinction measurements on dilute, but polydisperse, samples with Mie theory. It was found to be 1.49 ± 0.01 , which was then used to model optical properties and the transport mean free path for strongly scattering dispersions. The agreement between the transport mean free path calculated from the model and the measured value is better than about 30%.

These results show that it is possible to model the scattering behavior of dispersions of zein particles. This makes it possible to synthesize zein nanoparticles with specifically designed optical properties, which can be tuned towards a specific application.

5.5 Acknowledgements

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Chapter 6

Characterization of the scattering and absorption of colored zein colloids in optically dense dispersions

Abstract

The color of optically dense colloidal dispersions of dyed and undyed zein particles was modeled using results from multiple light scattering theory. These particles, as well as monodisperse silica colloids, were synthesized and characterized to obtain particle properties such as particle size, particle size distribution, refractive index, and absorption spectrum of the dye. This information was used to model the diffuse transmission of concentrated particle dispersions, which was measured using a specially designed variable path length quartz glass cuvette. For the non-absorbing silica dispersions, the transport mean free path throughout the visible range was obtained. Results differed less than 5% from the values calculated with a multiple scattering model using the single particle properties as input. For undyed zein particles, which are off-white, the deviation between model and experiment was about 30%, due to slight absorption at wavelengths below 550 nm, but <7% above this. From these results it was concluded that the model correctly describes diffuse transmission and that the measurements are sensitive to absorption. Finally, the method was applied to dispersions of dyed zein particles. Here, the transport mean free path was first determined for wavelengths at which there is no absorption, which agreed with theory better than 4%. The modeled transport mean free path was then used to extract the reciprocal absorption mean free path in the remaining parts of the visible spectrum and reasonable agreement with the absorption spectrum of the dye solution was obtained.

6.1 Introduction

In the observation of color, the interaction of a material with light, scattering and absorption play important roles. In colloids this interaction is important for understanding their appearance. Colloids are particles dispersed in a medium that have at least in one direction a dimension roughly between 1 nm and μm , or systems with discontinuities at distances of this order.¹ These particles are found in everyday life, where they are, for example, applied in foods,² pharmaceuticals,³ inks⁴ and coatings.⁵ Because many colloidal particles interact strongly with visible light, they have a color when observed. Contributing factors are their hierarchical order and scattering (structural colors),^{6,7} refractive index differences with the medium, their particle shape and size,^{8–10} absorption, and combinations of these.

Nowadays, it becomes clear that consumers prefer to buy products that contain natural and bio-based materials. An increasing amount of research has been done towards this bio-based and natural trend, which is ranging from polymers and additives^{11,12} towards foods and functional ingredients.^{13–15} A natural and bio-based class of materials to synthesize colloidal particles from is the class of proteins. Proteins have many advantageous properties, renewable and biodegradable and often even edible and soluble in food-safe solvents such as water-ethanol mixtures.^{16–18} These are properties that make these proteins suitable for many applications. For example, cellulose fibers¹⁹ were found to have enhanced light scattering properties. Other studies showed that dried cellulose films may exhibit tunable optical properties^{20,21} or stimuli-responsive optical properties.²² In our work we have chosen to work with zein, a protein from corn which has a GRAS (generally recognized as safe) status. Application areas for zein colloidal particles can be found in triggered-release mechanisms for drug delivery,²³ but also as a color carrier in food products²⁴ in which we continue this study.

Because of the importance of color in everyday life and the wide implementation of colloidal particles in a wide range of products, it would be beneficial if there was a simple way to predict optical properties from information about the constituent materials. This information consists primarily of the particle size and size distribution, the refractive indices of the medium and the particulate material, and the absorption spectrum of the colorant used. Different approaches to achieve this have been presented in literature already.²⁵ Examples include: full wave optical simulations suited for nanocomposites with extremely large filling fractions;²⁶ Density Functional Theory modeling for nano-diamond particles in a lattice structure;²⁷ and total differential effective medium theory for polymer nanocomposites,²⁸ all

of which are solid materials. Often, these are theoretical studies and are not compared to experimental results. Another approach, which was also applied on dispersions, is the Kubelka-Munk theory, which simplifies the description of diffuse reflection from a layer in terms of two parameters, related to scattering and absorption, of a continuous medium.^{29–31} A drawback of the Kubelka-Munk theory is that a direct connection with the optical properties of the individual particles is lost. Such a connection is retained by multiple light scattering, or photon diffusion, theory at the mild cost of moderately increased mathematical complexity. In this theory, diffusive light transport is quantified by the transport (l^*) and absorption (l_a) mean free paths, describing the effects of scattering and absorption. This theory has been highly successful in describing diffuse transmission and reflection of a wide variety of strongly scattering materials^{32–36} and living tissue.³⁷ Moreover, with Diffusing Wave Spectroscopy (DWS) particle dynamics in scattering, absorbing dispersions can be studied.³⁸ A limitation of photon diffusion theory is that it fails for strongly absorbing samples.³⁹ In this Chapter, we apply diffusion theory to polydisperse colloidal dispersions of absorbing (dyed) protein particles. Specifically, we attempt to relate the measured optical properties of the individual particles to the measured total transmission of opaque dispersions of these particles.

In this study, first the optical properties of non-absorbing and absorbing colloidal protein particle dispersions were determined experimentally. Second, the transport and absorption mean free paths were determined from total transmission measurements on optically dense dispersions. The mean free paths were also calculated from Mie theory and multiple scattering theory and compared with the data. To enable a comparison without adjustable parameters, information on particle shapes, sizes, size distributions, refractive indices, volume fractions, and an absorption spectrum of the colorant are necessary. To measure total transmittance at a wide enough range of path lengths of the sample, a quartz glass cuvette with a variable path-length was designed. To verify the set-up, non-absorbing, monodisperse silica particles were synthesized and measured using the newly designed set-up. Following this, the experimental results of these silica particles were compared to theory. When the setup was found to function as intended, this process was repeated for freshly-synthesized white zein particles. Finally, experiments and theory for absorbing blue zein particles were compared, resulting in a delimitation of the range of validity of the multiple scattering theory.

6.2 Experimental

Hydrochloric acid (HCl, Sigma), Tetraethyl orthosilicate (TEOS, 99%, Sigma), L-arginine (98%, Sigma), Patent Blue V sodium salt (Sigma), dimethylsulfoxide (DMSO, 99.99%, Sigma), 1-pentanol (99%, Sigma), and absolute ethanol (Merck) were used as received. Zein was purchased from Flo Chemical Corporation (Zein F4000C-FG, lot nr. F40006021C2) and water was purified using a Millipore Direct-Q purification system.

6.2.1 Particle synthesis

Three types of particles were synthesized for different purposes. First, silica particles were synthesized as monodisperse and non-absorbing particles to verify the performance of the transmittance set-up and to test the theory. Then, white zein colloidal particles were synthesized to check whether these particles are also non-absorbing and to test the sensitivity to polydisperse particles, and finally zein particles containing blue dye were synthesized to measure the scattering and absorption parameters of.

Silica synthesis

Silica particles were synthesized using the method of Shahabi et al.⁴⁰ First silica cores were prepared, which were grown larger in a second step. 183 mg L-arginine was dissolved in 169 mL water in a base bath cleaned 500 mL one-neck-flask. The mixture was heated to 70°C and stirred slowly (200 rpm) on a hotplate. After one hour, 11.2 mL of TEOS was added slowly via the wall. The resulting mixture was stirred for one day to complete the core synthesis; this is the core dispersion.

The cores were grown into larger particles in two synthesis steps. In the first step, 163 mg L-arginine was dissolved in 152 mL water, then 19.3 mL of the core dispersion were added. The mixture was heated to 70°C and stirred slowly (200 rpm). After one hour, 11.2 mL of TEOS was added slowly via the wall. The resulting dispersion was stirred for one day to complete the first growing step. In the second growing step 162 mg L-arginine was dissolved in 152 mL water. Then 19.3 mL of the particle dispersion from the first growth step was added. The mixture was again heated to 70°C and stirred slowly (200 rpm) and again after one hour, 11.2 mL of TEOS was added slowly via the wall. The resulting dispersion was stirred for one day to complete the synthesis. Now, the particles are at their target size and concentration.

White zein particles

White zein particles were synthesized using the following procedure.⁴¹ Prior to particle synthesis, most of the removable colored impurities were extracted from the zein powder by washing in ethanol. Then a stock solution was made in aqueous ethanol (85 wt%), 10 mL of stock solution was quickly added to a beaker with water (120 mL) while stirring (280 rpm). A two-day dialysis of the resulting dispersion against water adjusted to a pH of 4 with HCl was used to completely remove the ethanol; during dialysis, the medium was replaced four times. The resulting dispersion was centrifuged for 30 minutes at 222 rcf to remove possible large aggregates. Finally, the samples were stored in the fridge at 5°C for a maximum of two weeks before measurement. The dry weight of the zein stock solutions and colloidal dispersions were determined by weighing an amount of the solution or dispersion, letting the liquid evaporate at 120°C for two hours, and then measuring the dry weight. Prior to total transmission measurements, zein particles were concentrated using a centrifugal concentrator (Vivaspin 20, pore size 100,000 Da, Sartorius) at a pressure of 3 bar without centrifugation.

Blue zein particles

The synthesis of blue zein particles were synthesized using a co-precipitation method.^{14,24} Before particle synthesis, the zein was purified by washing in ethanol and a stock solution was prepared in 85 wt% aqueous ethanol. Patent Blue V sodium salt was dissolved in the zein solution at a dye to zein ratio of 0.0025 prior to particle synthesis. To synthesize particles, 10 mL of colored zein solution was quickly added to a beaker with water (120 mL), while stirring (280 rpm). Directly after synthesis the encapsulation efficiency was determined (see section 2.3). A three-day dialysis of the resulting dispersion against water adjusted to a pH of 4 with HCl was performed to remove the remaining ethanol and free colorant. During dialysis the dispersions was kept in the dark to prevent possible color changes. During this dialysis, the medium was replaced four times. The resulting colloidal dispersion was then centrifuged for 30 minutes at 222 rcf to remove possible large aggregates. Finally, the samples were stored in the fridge at 5°C for a maximum of two weeks before measurement. Prior to total transmission measurements, zein particles were concentrated using a centrifugal concentrator (Vivaspin 20, pore size 100,000 Da, Sartorius) at a pressure of 3 bar without centrifugation.

6.2.2 Particle shape, size, and size distributions

Transmission electron microscopy (TEM, Technai operating at 100 kV equipped with a tungsten filament) was used to determine particle sizes the cores and growth steps in the silica synthesis. Samples for analysis were prepared by first sonicating the silica dispersions for one minute to prevent aggregation of the particles on the TEM grid. Then the silica dispersion was drop-cast onto coated copper TEM grids at room temperature and the liquid was evaporated in air, leaving the silica particles on the grid.

Scanning electron microscopy (SEM), Nova Nanolab, FEI, was used for determining the particle shape of the samples prepared from zein. To minimize changes in particle size and shape, samples were freeze-dried overnight using a lyophilizer (Virtis). The dry powder, which was loosely attached to the lyophilizing bottle, was collected and stored in a desiccator until SEM analysis. Prior to SEM analysis a platinum layer of about 4 nm was sputtered onto these samples to prevent charging.

To determine size distributions, particle sizes were also measured by dynamic light scattering (DLS) using a Zetasizer Nano ZS series, Malvern Instruments. In DLS a CONTIN analysis was used to obtain the size distributions. Prior to the DLS measurements, the samples were diluted, with water adjusted to pH 4, to a suitable concentration in order to prevent multiple scattering.

6.2.3 Dye encapsulation efficiency

Directly after the co-precipitation in the blue particle synthesis, a sample of the dispersion was filtered using a Vivaspin tube (Sartorius, equipped with a 100,000 MWCO membrane) using an applied pressure of 4 bar, to separate the particles from the medium. The encapsulation efficiency or adsorption was determined as follows:

$$EE = \frac{c_t - c_m}{c_t} 100\% \quad (6.1)$$

Here, EE is the encapsulation efficiency or adsorption, c_t is the concentration of dye added to the synthesis and c_m is the concentration of free dye in the medium. From this calculation, the total concentration of dye inside of the particles, c_p , can be calculated from $c_p = c_t - c_m$. UV-vis spectroscopy (HP 8953A spectrophotometer) was used to determine the concentration of free dye in the medium at a wavelength of 638 nm. The concentration was calculated by comparison with an appropriate calibration curve using the same medium as the particle dispersions.

6.2.4 Particle refractive index

The refractive index (RI) of dispersed zein was determined from extinction measurements in a previous study,⁴¹ in which it was found to be 1.49 ± 0.01 ; this value is used in this study for both the white and blue zein particles.

The RI of silica was determined by refractive index matching. First, solutions are prepared by mixing two solvents with different refractive index ratios.⁴² The RI region used here is around $n_D = 1.45$, since for Stöber silica n_D values ranging from 1.43 to 1.462 are reported in literature^{43,44} and for these type of silica particles a similar range is expected, although a different synthesis route was used. For this purpose, the RI's of several ratios DMSO in 1-pentanol (vol%) were determined at 550 nm and 21°C (n_{550}^{21}). With a refractometer (Atago 3T) the n_D^{21} and the corresponding Cauchy's equations of these solutions were determined.⁴⁵ Then, the transmittance of DMSO and 1-pentanol mixtures containing particles was measured on a UV-Vis spectrophotometer (HP 8953A). A maximum is reached when the refractive indices match. To do this, 5 mL of a 2.17 wt% particle dispersion in water was transferred to a glass vial and the water was evaporated by placing it on a hot plate at 80°C for 16 hours. The particles were re-dispersed in 5 mL 1-pentanol by sonication, after which 0.5 mL of the stock dispersion of particles and various volumes of DMSO and 1-pentanol were transferred to separate vials to obtain a range of particle dispersions in different 1-pentanol/DMSO ratios. Absorption spectra of these solutions were measured at 21°C to determine the transmittance at 550 nm.

6.2.5 Optical properties of concentrated dispersions

The transport mean free path (l^*) and reciprocal absorption mean free path (α) were experimentally determined via total transmittance spectroscopy, which was performed on an in-house built set-up (Figure 6.1). Concentrated samples were measured at wavelengths ranging from 400 to 900 nm using a specially designed quartz glass cuvette (Figure 6.1a) with a variable path length, with an accuracy of <0.05 mm after initial calibration due to the use of an optical linear stage, Newport M-UMR5.16 with BM11.16, (see Figure 6.1b and c). Structural parts consist of a 90-degree bracket, Newport EQ50-E, and custom designed 3D printed parts printed in PLA on an Ultimaker2 FDM 3D-printer. The inner and outer quartz glass cylinders had diameters of 17 and 30 mm, respectively. Light from a Tungsten Halogen light source (HL-2000-FHSA-LL, Ocean Optics) was sent vertically down via a multimode optical fiber through the pair of circular optical flats that form the bottoms of the cylinders. Their separation was varied in the range 0.5 to 10 mm by raising and lowering the inner

cylinder. Transmitted light was collected by a 15 cm diameter integrating sphere (barium sulfate coated, Labsphere) with an entrance opening of 18 mm. The sphere had a detection port of 3 mm, at which light was collected by a multimode optical fiber and coupled into a spectrometer (HR4000, Ocean Optics). The modular design of the set-up enables quick and easy modifications for cleaning purposes and alternative configurations if required.

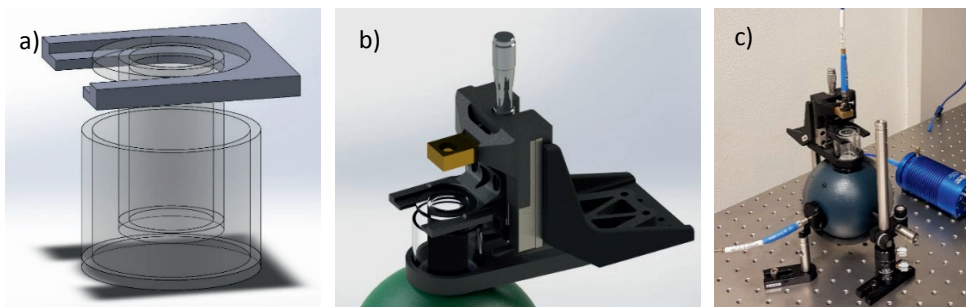


Figure 6.1: In-house built set-up for the variable path-length cell. The design of the quartz glass cuvettes (a), the design of the quartz glass cuvettes in 3D printed holder incorporating a linear stage and a separate holder for the light beam (b), and the final built set-up including integrating sphere and light source (c).

6.3 Results & Discussion

6.3.1 Particle characterization

The particle growth of the silica particles was followed by TEM, see Figure 6.2. First, seeds were grown with a size of about 26 nm (measured from TEM images), then the particles were grown to about 55 nm and finally to their final size of 128 nm. These silica particles are spherical and non-absorbing and can therefore be used to verify the set-up and theory.

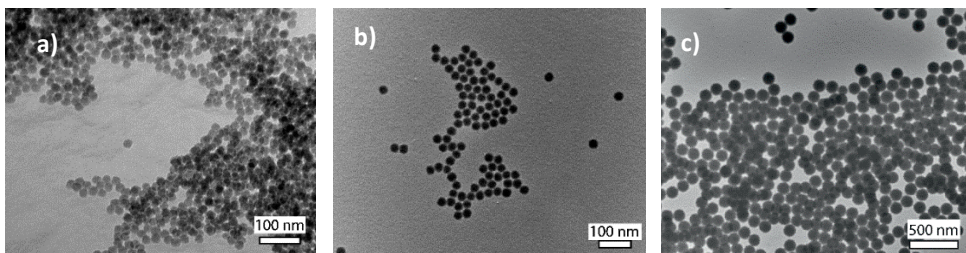


Figure 6.2: TEM images of silica particles. Particles resulting from the core synthesis (scale bar is 50 nm) particle size is approximately 26 nm (a), the first growth step (scale bar is 100 nm) after which the particle size was approximately 55 nm (b), and the final growth step (scale bar is 500 nm) after which the particles were approximately 128 nm, (c). Particles from the final growth step were used for further experiments.

The spherical shape of the zein particles (white and blue) was verified by SEM, see Figure 6.3. Compared to the silica particles, these zein particles are very polydisperse, which has been observed before for this type of particles in literature.^{46,47} There does not seem to be a difference in shape between the white and blue zein particles, as they both are spherical.

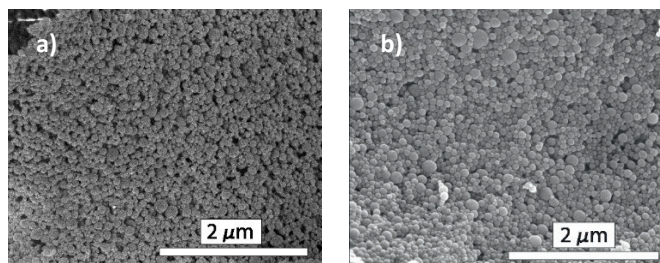


Figure 6.3: SEM images of white zein particles (a) and blue zein particles (b). After freeze drying, particles were spherical, although both samples were very polydisperse.

To exclude differences between sample preparations and microscopy techniques, DLS was used to determine the size and size distribution of the different particles. This resulted in particle sizes of silica: 140 ± 24 nm, white zein: 149 ± 60 nm, and blue zein: 156 ± 77 nm. Figure 6.4 presents the size distribution data that was obtained with DLS, which are used in the model for calculation of the transport mean free path (l^*).

Encapsulation efficiency of the blue zein particles was determined using Equation 6.1 and was found to be 99.9%, which is in agreement with previous experiments.¹⁴ A value of 1.49 ± 0.01 was found to describe the extinction of a large number of similarly prepared, polydisperse samples in the wavelength range of 450 to 700 nm; this value is used in this study for both the white and blue zein particles, regardless of the wavelength. The RI of silica was determined by refractive index matching as explained in the Supplementary Information S2 and was found to be 1.453, which is comparable to other values that can be found for silica particles in literature.^{48,49}

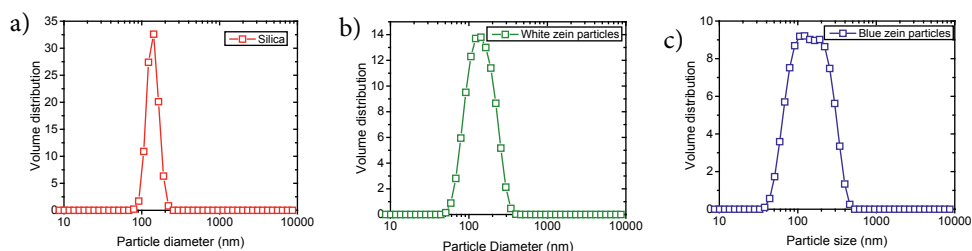


Figure 6.4: Size distribution (volume) data for silica particles (a), white zein particles (b), and blue zein particles (c). Data was obtained from dynamic light scattering.

6.3.2 Refractive index matching

In Figure 6.5a the refractive index at 550 nm and 21°C (n_{550}^{21}) was plotted as a function of the vol% DMSO in 1-pentanol and a linear fit was made. This relationship was used to find the refractive index of the silica particles. Then the transmittance at n_{550}^{21} of particle dispersions with media with different refractive indices was plotted and fitted using a quadratic equation, the fit had an R^2 of 0.9864. The refractive index was determined from the maximum of the transmittance: $n_{550}^{21} = 1.453$, see Figure 6.5b.

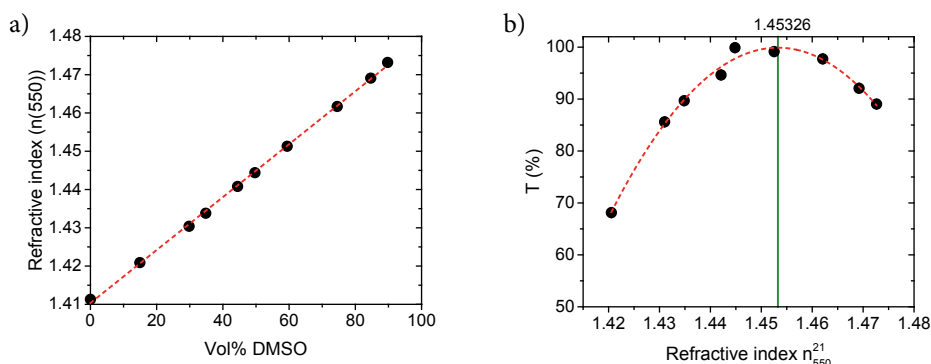


Figure 6.5: In (a) the refractive index of silica particles at n_{550}^{21} is plotted as a function of the vol% of DMSO in 1-pentanol. A linear equation was fitted to the data (red dotted line). In (b) the transmittance at n_{550}^{21} was plotted as a function of the refractive index in the medium. The black dots correspond to measurements of dispersions of particles in DMSO/1-pentanol mixtures with different refractive indices. A quadratic equation was fitted to the data (red dotted line) to determine the refractive index of maximum transmittance: $n_{550}^{21} = 1.453$.

6.3.3 Multiple scattering of optically dense dispersions.

Non-absorbing dispersions

The case of multiple scattering of non-absorbing polydisperse zein particles was described in our previous work.⁴¹ Here, the same phenomenon is described, however, now measured using the improved variable path-length cell set-up and using monodisperse silica to better compare theory with experiments. Since the mean free paths of our samples will turn out to be 0.2 - 2 mm the sample path lengths will be made several times that thick (up to 6 mm). At the same time the cell is wide enough (30 mm) to prevent any light leaking out the through side walls, even at the larger cell thicknesses. If this condition were not met, this light loss could masquerade as absorption. This improvement will allow us to include absorption into the model, as described in the next section. Only opaque dispersions are used, so light is scattered not once but multiple times before leaving the sample. With volume fractions of a

few percent these dispersions are dilute in the sense that particles interact only weakly. However, they are optically dense as no light is transmitted without scattering. The scattering strength is quantified by the transport mean free path, l^* , which describes the distance over which the direction of propagation of light is randomized:

$$\frac{1}{l^*} = \frac{1 - \langle \cos \theta \rangle}{l} \quad (6.2)$$

In this equation l is the scattering mean free path (the average distance between scattering events) and $\langle \cos \theta \rangle$ is the anisotropy factor, the average of the cosine of the scattering angle θ , both of which can be calculated from Mie theory.⁵⁰ In the absorption free-case, the transport mean free path can be experimentally determined from the relation³⁶ between the total transmittance T , and the path length of the cell, L (Equation 6.3), which shows that total diffuse transmittance is low at small l^* . Here, most of the incident light is diffusely reflected:

$$T = (1 - R_s) \frac{1 + z_e}{\frac{L}{l^*} + 2z_e}, \quad \text{with } z_e = \frac{2}{3} \frac{1 + \bar{R}}{1 - \bar{R}} \quad (6.3)$$

In this equation, $\bar{R}$ is the polarization and angle-averaged internal diffuse reflectivity of the sample-quartz glass-air interface; R_s is the specular reflectivity of the incident light beam from the front face of the sample; and z_e is the extrapolation length, which describes the boundary conditions of the diffuse intensity at the interfaces of the sample. R_s and $\bar{R}$ can be calculated via Fresnel's reflection coefficients as described by Vera and Durian⁵¹ from the RI's of the sample medium, container wall, and external medium (i.e. 1.330, 1.458, and 1.000 respectively, at 589 nm), which leads to $R_s = 0.0367$, $\bar{R} = 0.4452$, and $z_e = 1.7365$. Finally, l^* is obtained from the slope of the linear fit of the $1/T$ vs. L plot.

The experimentally obtained l^* values are collected in Figure 6. For silica particles this fit had a R^2 of 0.990 or higher for all wavelengths (between 400 and 750 nm), as shown in supplementary information (SI) Figure S6.1. From this result it can be concluded that the experimental set-up works according to expectations. The white zein particles give equally good fits at the longer wavelengths, but clearly deviate from a linear fit for wavelengths below 550 nm (see SI Figure S6.2). This could be a sign of absorption for these specific wavelengths, which will be considered in the next section.

As shown in our previous study⁴¹ the transport mean free path l^* for a polydisperse sample is related to the particle properties as:

$$\left\langle \frac{Q_{ext}}{r} (1 - \langle \cos \theta \rangle) \right\rangle \equiv \frac{\sum_i f_i r_i^3 \frac{Q_{ext,i}}{r_i} (1 - \langle \cos \theta \rangle_i)}{\sum_i f_i r_i^3} = \frac{1}{l^*} \frac{4}{3\varphi} \quad (6.4)$$

Here, φ is the total volume of particles and $Q_{ext,i} = C_{ext,i}/r_i$ with $C_{ext,i}$ the scattering cross section of a particle with size r_i . The summation accounts for the scattering contributions of particles of different sizes r_i , from a distribution f_i , which are calculated from Mie theory. The particle volume distribution $f_i r_i^3$ is taken from the size distribution data from DLS results of the corresponding samples (see Figure 6.4). Note that here the assumption is made that the scattering particles are uncorrelated, so that effects of the structure factor can be neglected. This is acceptable for the relatively low concentrations (<3 wt%) used in this work. The Mie theory software MiePlot⁵² were used for the calculation of Q_{ext} and $\langle \cos \theta \rangle$. The results are included in Figure 6.6.

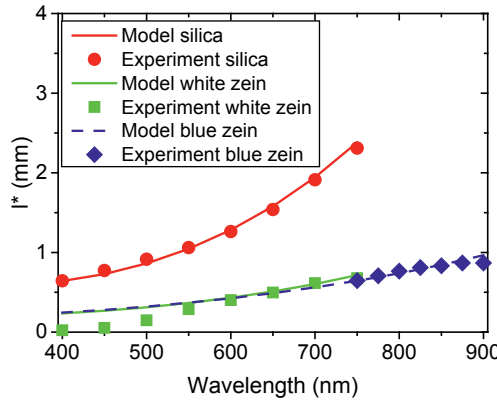


Figure 6.6: The transport mean free path, l^* , found experimentally by total transmittance spectroscopy (points) and calculated from theory (lines) of a silica particle dispersion (particle diameter 140 ± 24 nm and $\varphi = 0.014$) and a white zein particle dispersion (particle diameter 149 ± 60 nm and $\varphi = 0.024$), and a blue zein particle dispersion (particle diameter 156 ± 77 nm and $\varphi = 0.025$). In the blue zein case, only data are shown at wavelengths where absorption is absent, and the theory is used to extrapolate to wavelengths where absorption cannot be neglected. In the white zein case neglecting absorption below 550 nm underestimates l^* .

For both the silica and white zein particle dispersions the agreement between the model and theory is very good, especially when considering that no adjustable parameters were used. Deviations between experimental results and theoretical calculations, as prescribed by Equation 4, are seen to be $\leq 5\%$ for the silica particles. This is as expected, since silica is non-absorbing and will only scatter the light,⁴³ also the silica particles are spherical and monodisperse, as was observed in TEM and DLS measurements. For white zein particle

dispersions, deviations between experiments and theory are on average about 30%, which is very similar to the result obtained before with only fixed path length cells.⁴¹ However, when looking only at wavelengths of ≥ 550 nm, then the agreement is better than 7%, which is a large improvement compared to our previous work. It can also be observed that the values of the experimental results of white zein particles ≤ 550 nm are below the theoretical data line, which indicates that the samples transmit less light than expected based on their scattering cross sections. This is again an indication that these white zein particles still absorb some light at these wavelengths, resulting experimentally in too low values of l^* . The strong scattering of these samples increases the effective path length of transmitted light, strongly amplifying even low amounts of absorption. Therefore, we will now extend the theory to include absorption.

Absorbing dispersions

For the case in which particles absorb as well as scatter light the equations are in principle similar to the non-absorbing case as was described before. The analytical solution of the diffusion equation in the presence of absorption is, for the case of collimated normal-incident light, known to be:⁵³

$$T = (1 - R_s) \frac{1}{\alpha z_e} \frac{\sinh[\alpha(l^* + z_e)] \sinh[\alpha z_e]}{\sinh[\alpha(L + 2z_e)]} \quad (6.5)$$

Here, α is the reciprocal absorption mean free path, $\alpha = 1/l_a$. The absorption mean free path is the distance it takes light to be absorbed to a fraction $1/e$ while performing a random walk in the scattering sample. The extrapolation length, z_e , is also modified in the absorbing case:

$$z_e = \frac{1}{2\alpha} \ln \left(\frac{1 + \alpha z_0}{1 - \alpha z_0} \right), \quad \text{with } z_0 = \frac{2}{3} l^* \frac{1 + \bar{R}}{1 - R} \quad (6.6)$$

It can be checked that Equation 6.5 reduces to Equation 6.3 in the limit of low absorption ($\alpha \ll 1/l^*$). A direct two-parameter fit of l^* and α using Equations 6.5 and 6.6 unfortunately produced unreliable results, because the two parameters mainly enter the equations as their product. Therefore, we determined l^* first, after which α was fitted using Equation 6.5, as follows. Since Equation 6.4 has proven to be a reliable model we use it to obtain l^* at wavelengths where absorption becomes important. Figure 6.6 shows the predictions of Equation 4 and compares them with experimental results determined through Equation 6.3

for wavelengths without absorption (750 to 900 nm). It is seen that the agreement is quite good (better than 4%). The corresponding $1/T$ versus L plots are shown in Figure S6.5. The extrapolation is helped by the fact that the wavelength dependence of l^* is weak. It has been shown that in highly polydisperse systems, the transport mean free path is only weakly dependent on wavelength.^{53,54} This is also visible in the data in Figure 6.6, in which silica is reasonably monodisperse and indeed wavelength dependent. White zein particles, which are much more polydisperse, show much less wavelength dependency.

Extrapolation to wavelengths where there is absorption is therefore straightforward. Here, Equation 6.4 will be used as a reliable model to determine l^* for these wavelengths. Figure 6.6 compares the prediction of Equation 6.5 with experimental results that were determined through Equation 6.3 for wavelength without absorption (750 to 900 nm). It is seen that the agreement is good (better than 4%). The model was extended to wavelengths for which there is absorption (400 to 700 nm) and l^* was obtained from the model for these wavelengths. The corresponding $1/T$ versus L plots for wavelengths between 750 and 900 are shown in SI Figure S6.3.

Then, α was obtained from a fit of T versus L to Equation 6.5 in the absorbing region 400 to 700 nm. The corresponding fits are shown in SI Figure S6.4. The results are presented in Figure 6.7a. As expected, the resulting spectrum is similar in shape to the absorption spectrum of patent blue in water using the same dye concentration (i.e. without scattering, Figure 6.7b, blue line). The peaks and even the shoulder at 590 nm are well reflected in the measured spectrum. Since scattering is strongest at short wavelengths the absorption peak at 410 nm is strongly enhanced compared to the peak at 640 nm. To make a quantitative comparison between the absorption mean free path α measured on the dyed zein dispersion and the absorption coefficient $\mu_a = (1/L) \log T$ of the pure dye solution we calculate the absorption coefficient of the dispersion using:⁵⁵

$$\mu_a = \frac{l^* \alpha^2}{3} \quad (6.7)$$

Figure 6.7b shows that the agreement with the two datasets is reasonable. However, the absorption peaks from the dye in water are considerably higher than for the particle dispersion. There also seems to be a small shift in peak position. This is a phenomenon that can occur for Patent Blue V sodium salt when the dye is in a different medium or in a medium with a different pH.^{56–58}

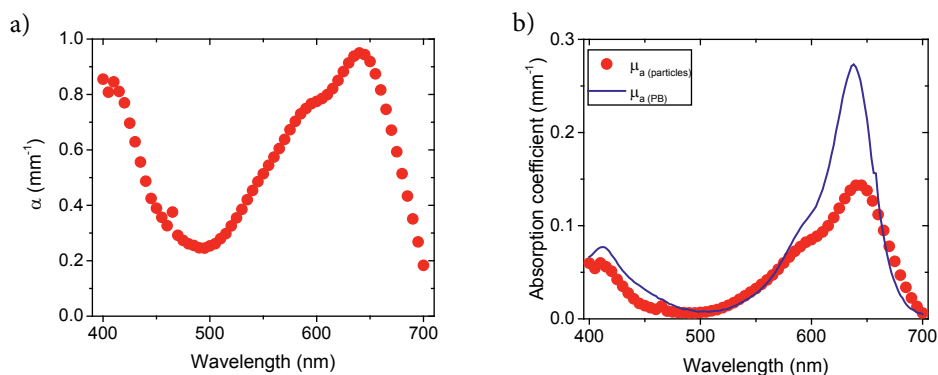


Figure 6.7: The reciprocal absorption mean free path, α , of the blue particle dispersion found experimentally by total transmittance spectroscopy (a), and calculated absorption coefficients using Equation 6.7 compared to the absorption spectrum of Patent Blue V sodium salt in water (b).

On the other hand, the difference between data sets can be explained by the quality of the fits (Figure S6.4), which is not as good as can be expected. A much-improved fit is obtained by plotting $1/T$ versus L , see Figure S6.5. Although the fit is better in wavelength regions with low to moderate absorption, it was not possible to fit the wavelength region with strong absorption (570–680 nm) using Equations 6.5 and 6.6 due to strong absorption of the sample for these wavelengths.

It has been shown by Leung et al. that Equation 6.5 becomes unphysical for samples with very strong absorption compared to scattering.⁵³ This is because the extrapolation length in the presence of absorption, z_e , diverges if the absorption mean free path, l_a , becomes similar to or smaller than the extrapolation length without absorption, z_0 . Then, in Equation 6.6, when $1 - \alpha z_0 > 1$, the logarithmic part of Equation 6.5 becomes divergent and cannot be used anymore.

For the wavelengths where it was possible to find a fit, α and the absorption coefficient was calculated (see Figure 6.8) and compared to the results from Figure 6.7. A clear difference in magnitude can be observed: the values found using the better fit are much higher. Figure 6.8b suggests that the absorption peak is much higher and possibly more comparable to the absorption spectrum of the dye in water. However, due to strong absorption the data are outside the range of applicability of diffusion theory.

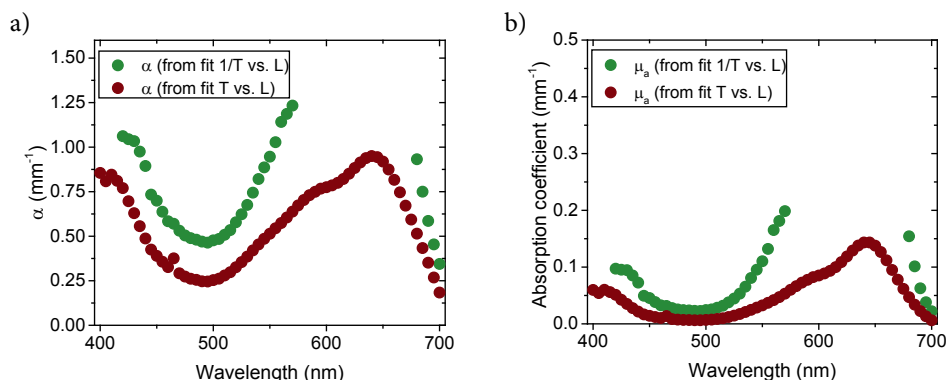


Figure 6.8: The reciprocal absorption mean free path, α , of the blue particle dispersion found experimentally by total transmittance spectroscopy (a), and calculated absorption coefficients using Equation 6.7 compared to the absorption spectrum of Patent Blue V sodium salt in water (b) using fits from both T vs. L and 1/T vs. L plots.

Finally, we return to the white zein particles to quantify their suspected absorption at wavelengths below 550 nm (see Figure 6.6). For these particles the absorption is not strong, and Equation 6.5 should be valid over the whole wavelength spectrum. We note that when α is obtained from T vs. L plots (figure S6.6), also here the results were not optimal, and so the fits were obtained by fitting α from plotting 1/T versus L, see Figure S6.7. since this dispersion is low in absorption, it was possible to find a good fit for all wavelengths of interest. From these fits α and the absorption coefficients were determined see Figure 6.9. Here, it is visible that there is indeed some absorption, mainly below 550 nm as was expected from the results in Figure 6.6.

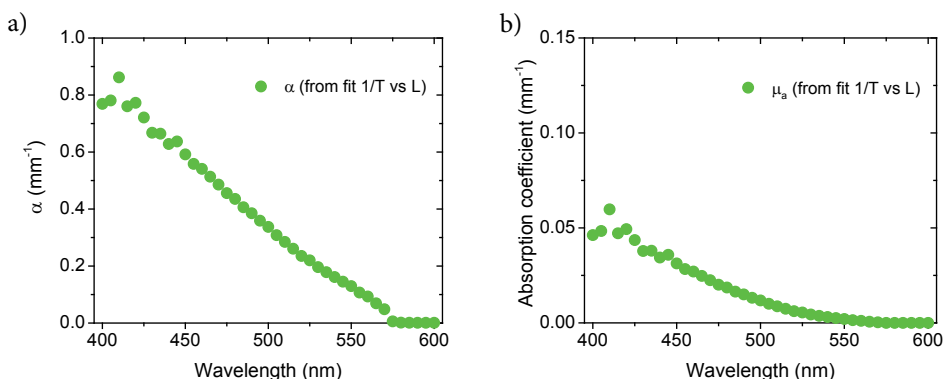


Figure 6.9: The reciprocal absorption mean free path, α , of the white zein particle dispersion of Figure 6.6 found experimentally by total transmittance spectroscopy and using fits from T versus L plots and 1/T versus L plots (a), and corresponding calculated absorption coefficients using Equation 6.7 (b).

6.4 Conclusions

In this study colloidal particles were synthesized and characterized to obtain particle properties such as particle size, particle size distribution, refractive index, and absorption spectrum of the dye. This information was used to model the optical properties of these concentrated particle dispersions, which were experimentally measured using a specially designed variable path length quartz glass cuvette.

Non-absorbing spherical and monodisperse silica particles were synthesized to verify the correct workings of the set-up and to check the applicability of multiple scattering theory. The agreement between experiment and theory in terms of the transport mean free path of these silica particles was better than 5%. The same experiment was performed on polydisperse white zein particles, which were slightly absorbing below wavelengths of 550 nm. Again good agreement between theory and experiments was obtained: better than 7% for wavelengths above 550 nm. Deviations at shorter wavelengths were attributed to some absorption by the zein and are indicative of the sensitivity of the method to absorption. Subsequently, absorbing and polydisperse blue zein particles were measured using the set-up. For these particles, the transport mean free path was determined for wavelengths where absorption is negligible, which gave a deviation between theory and experiment of $\leq 4\%$. The modeled transport mean free path was then used to find the reciprocal absorption mean free path and was compared to the absorption spectrum of the dye, which was found to have reasonable agreement. However, at the absorption peaks the agreement was less good, due to the fact that the absorption became stronger than the scattering. For a complete agreement a theory other than diffusion theory needs to be used to describe light transport. The diffusion theory worked best for the slightly absorbing white zein dispersion.

These results show that it is possible to predict the scattering and absorption behavior of opaque dispersions using as inputs the refractive index, volume fraction, dye absorption spectrum, particle size, and size distribution. This work is a step forward when particles with specifically pre-designed optical properties must be synthesized, which can be tuned towards a specific application.

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6.6 Supplementary Information

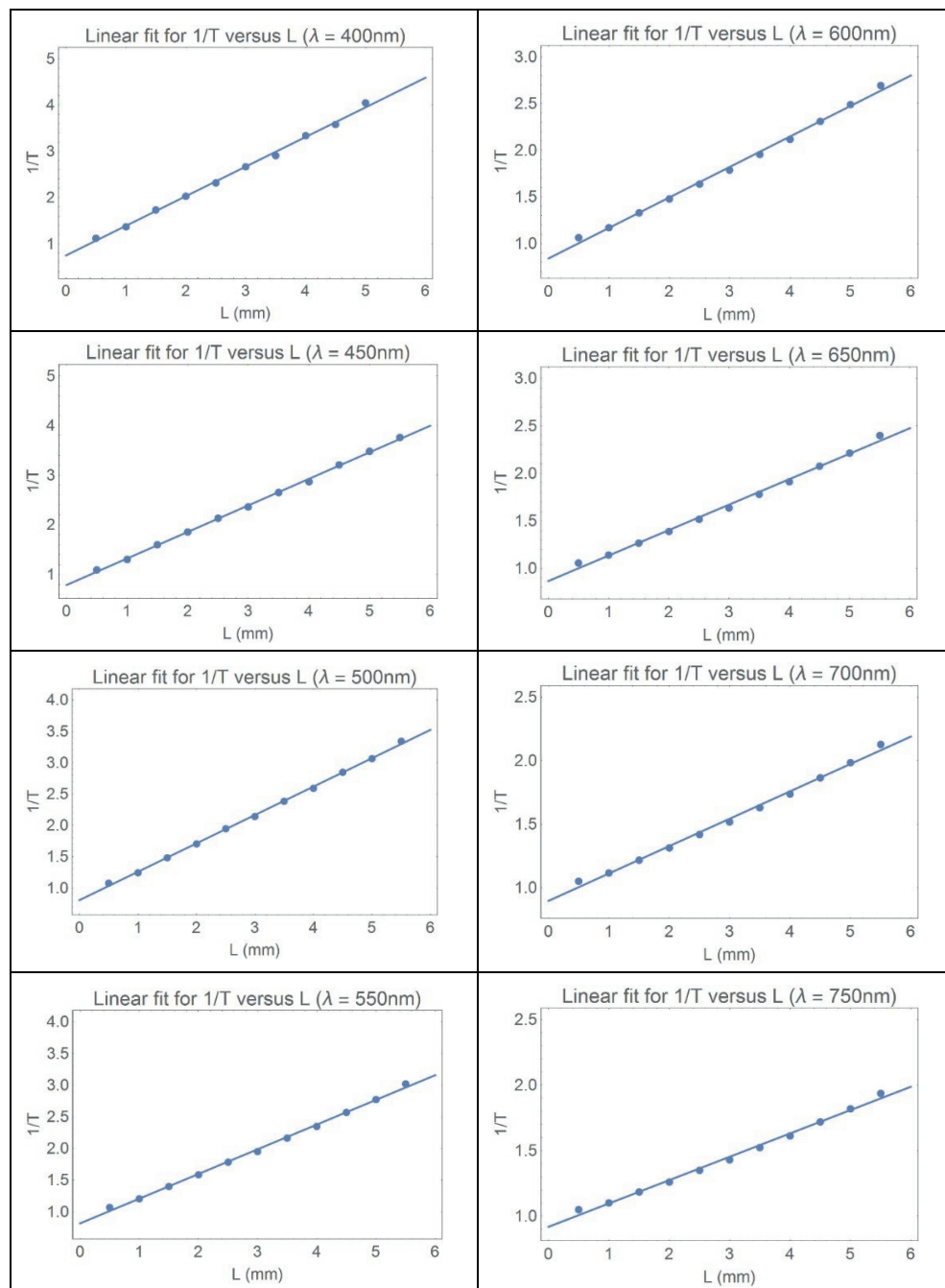


Figure S6.1: Linear fit of the $1/T$ vs. L plot for silica particle dispersions, path-lengths measured were ranging from 0.5 to 5.5 mm and wavelengths between 400 and 750 nm.

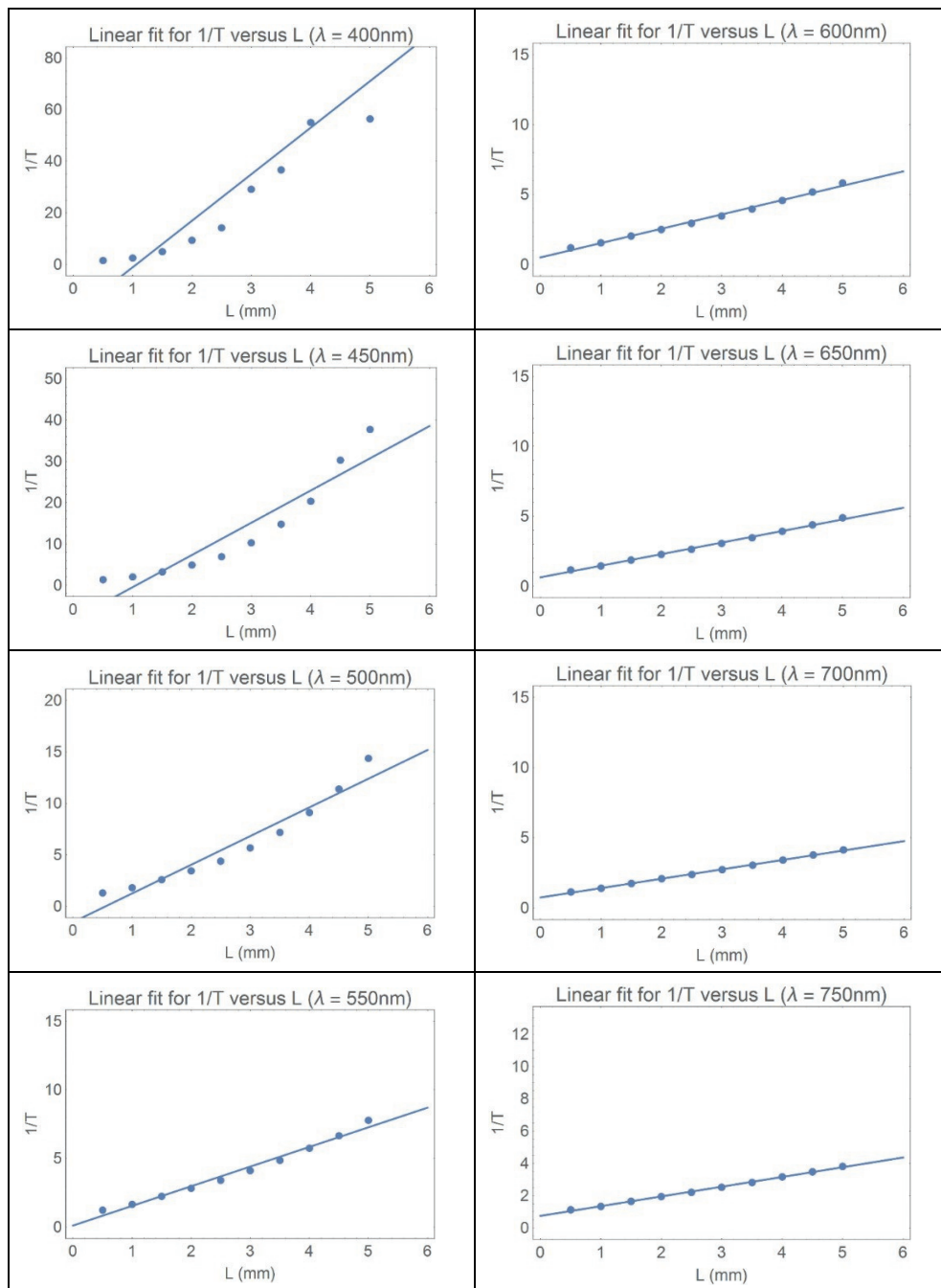


Figure S6.2: Linear fit of the $1/T$ vs. L plot for white zein particle dispersions, path-lengths measured were ranging from 0.5 to 5.0 mm and wavelengths between 400 and 750 nm. Deviation from the linearity is observed from 550 to 400 nm, this is a region where absorption starts for these white zein particles.

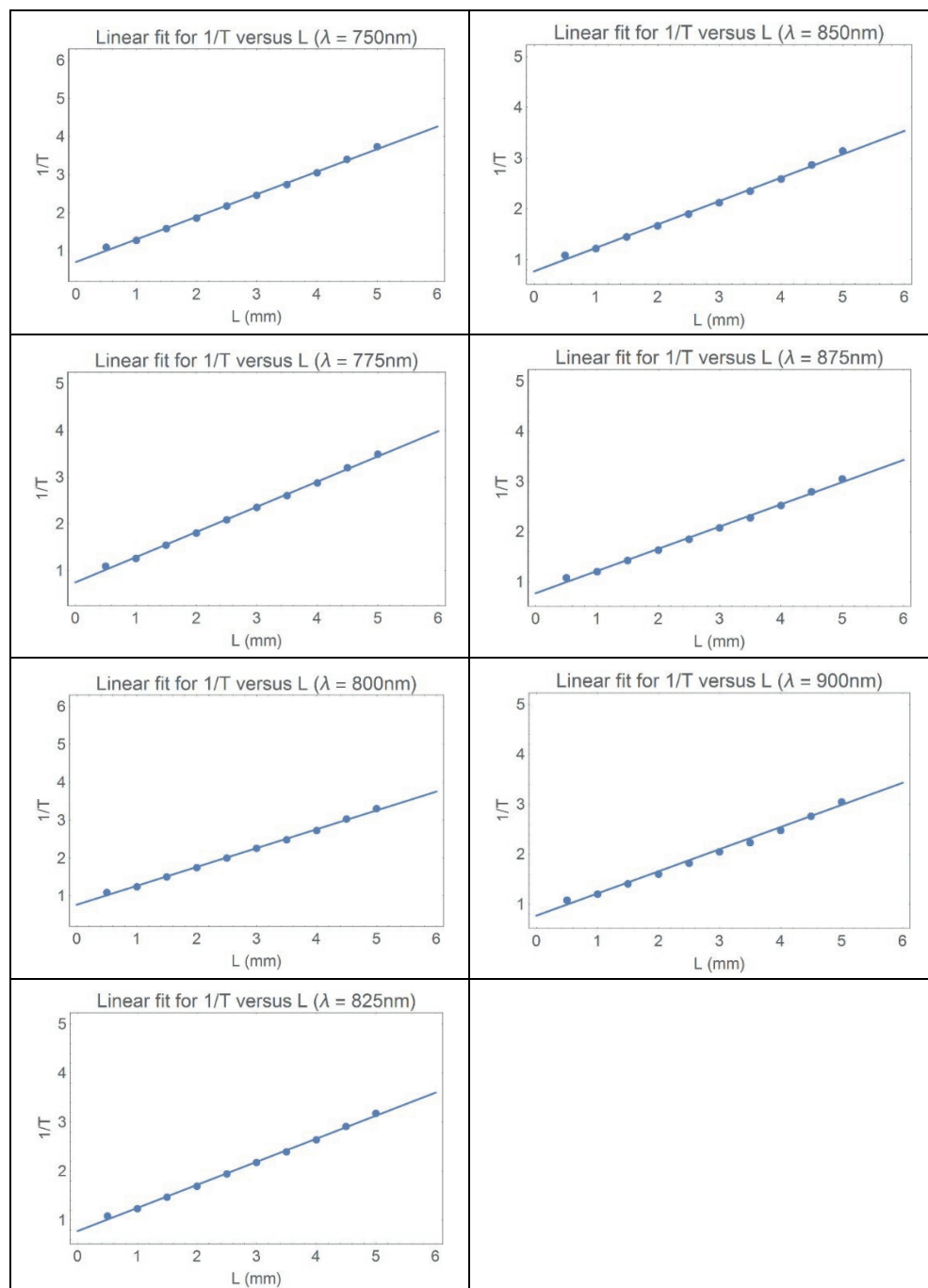


Figure S6.3: Linear fit of the $1/T$ vs. L plot for blue zein particle dispersions, path-lengths measured were ranging from 0.5 to 5.0 mm and wavelengths between 750 and 875 nm (a range in which there is no absorption).

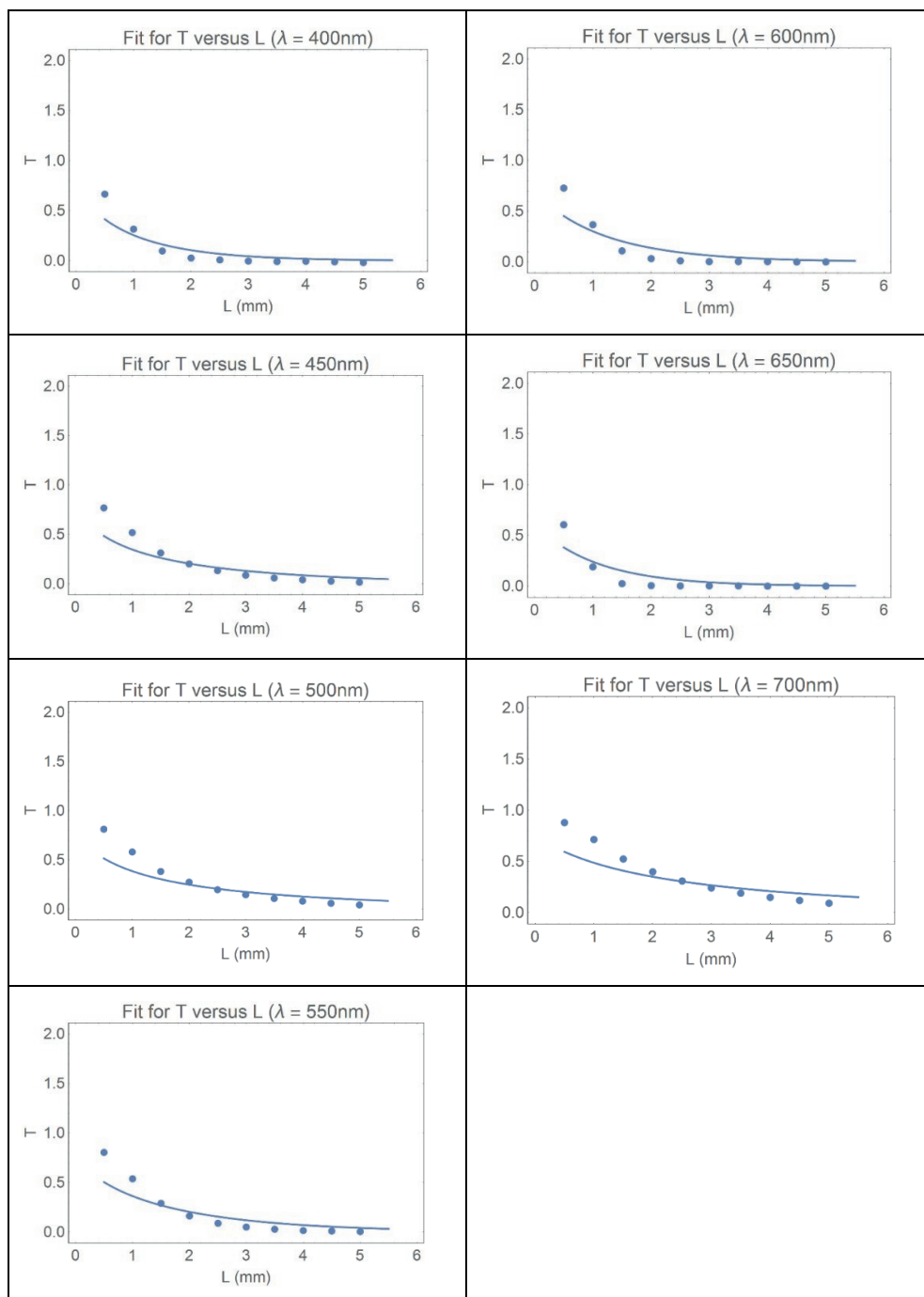


Figure S6.4: Fit of the T vs. L plot for blue zein particle dispersions using Equations 5 and 6, path-lengths measured were ranging from 0.5 to 5.0 mm and wavelengths between 400 and 700 nm (a range in which there is absorption).

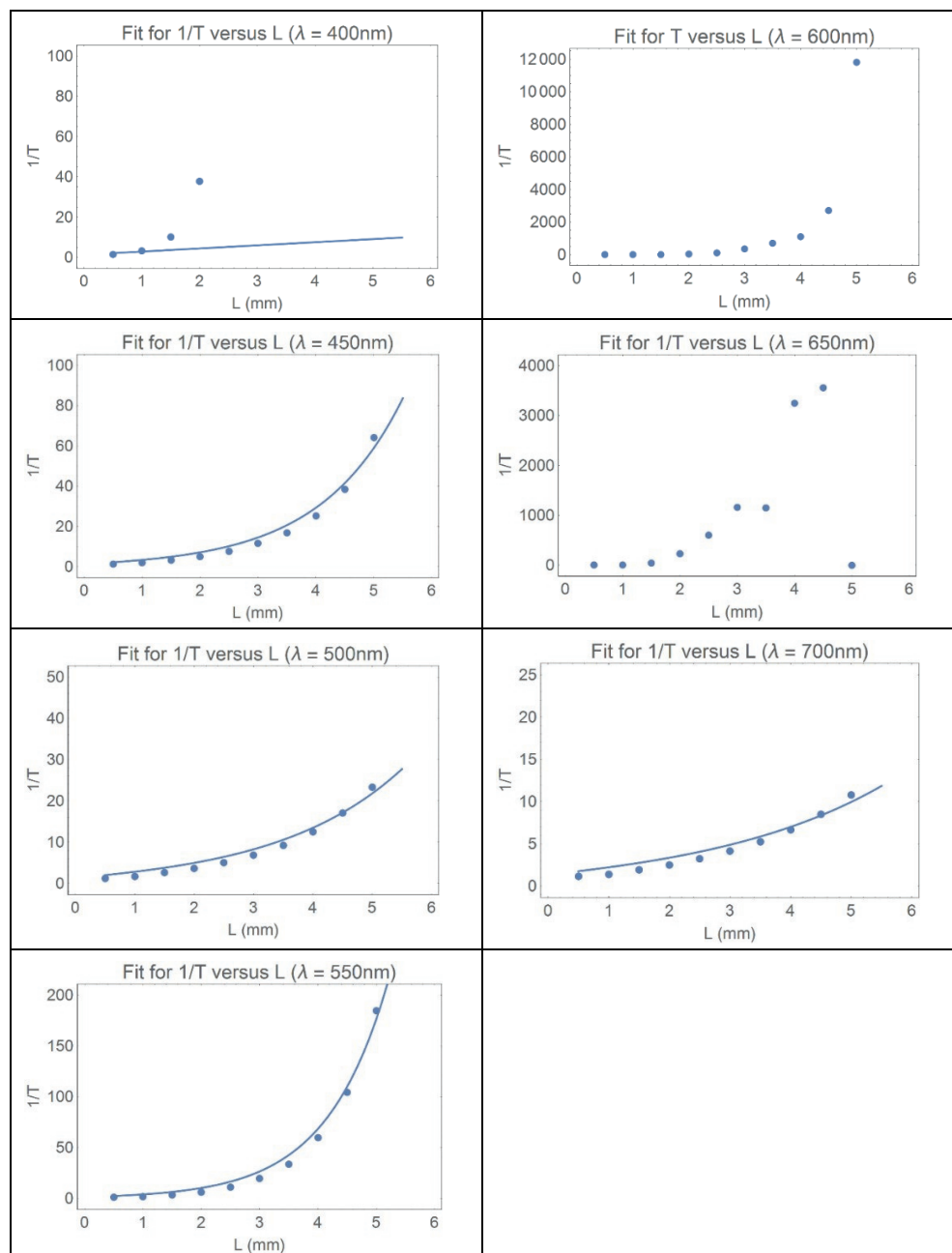


Figure S6.5: Fit of the $1/T$ vs. L plot for blue zein particle dispersions using Equations 5 and 6, path-lengths measured were ranging from 0.5 to 5.0 mm and wavelengths between 400 and 700 nm (a range in which there is absorption). A fit could not be obtained for below 420 nm due to noise and between 570 and 680 nm due to strong absorption of the sample for these wavelengths.

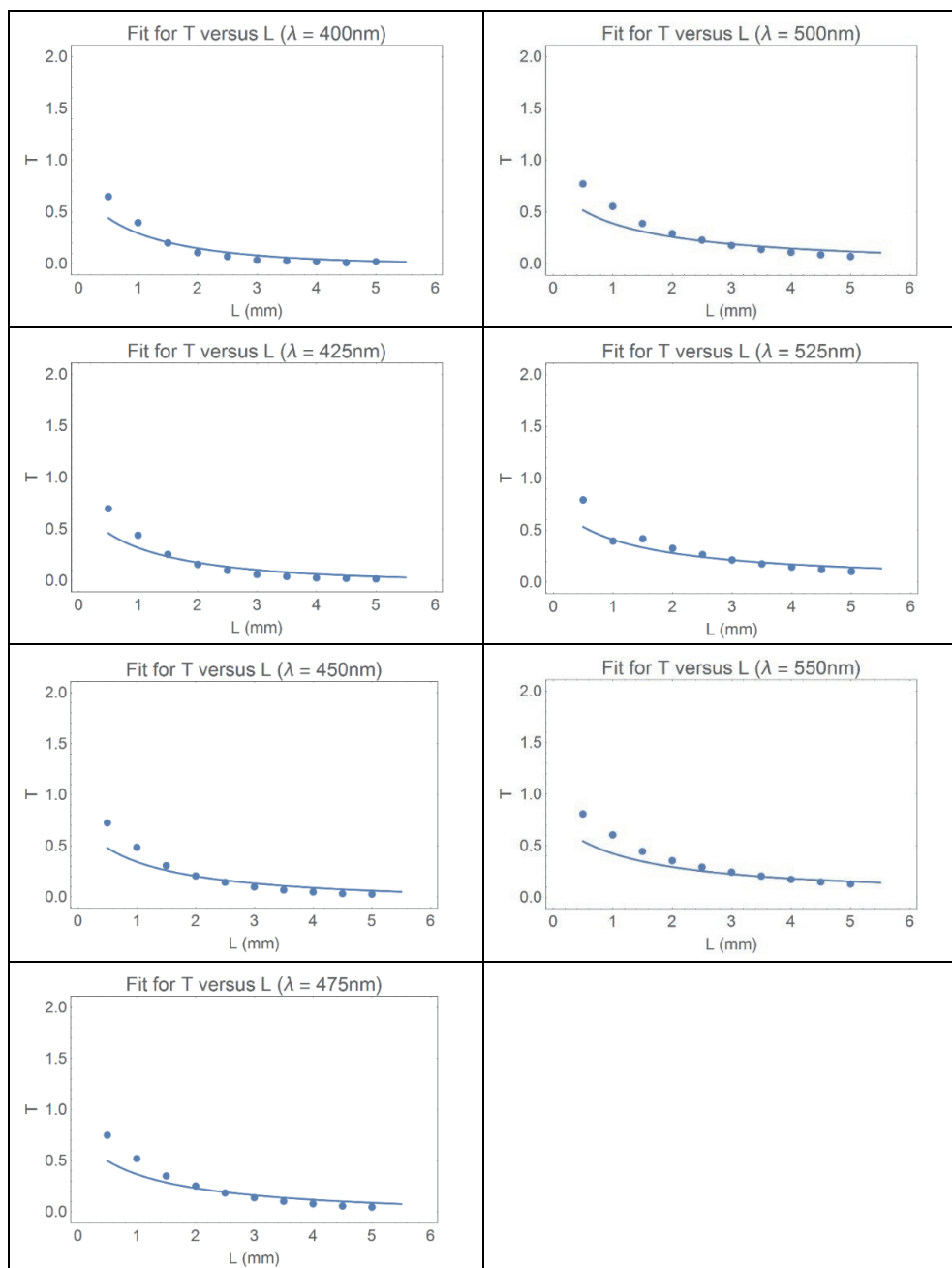


Figure S6.6: Fit of the T vs. L plot for white zein particle dispersions using Equations 5 and 6, path-lengths measured were ranging from 0.5 to 5.0 mm and wavelengths between 400 and 550 nm (a range in which there is absorption).

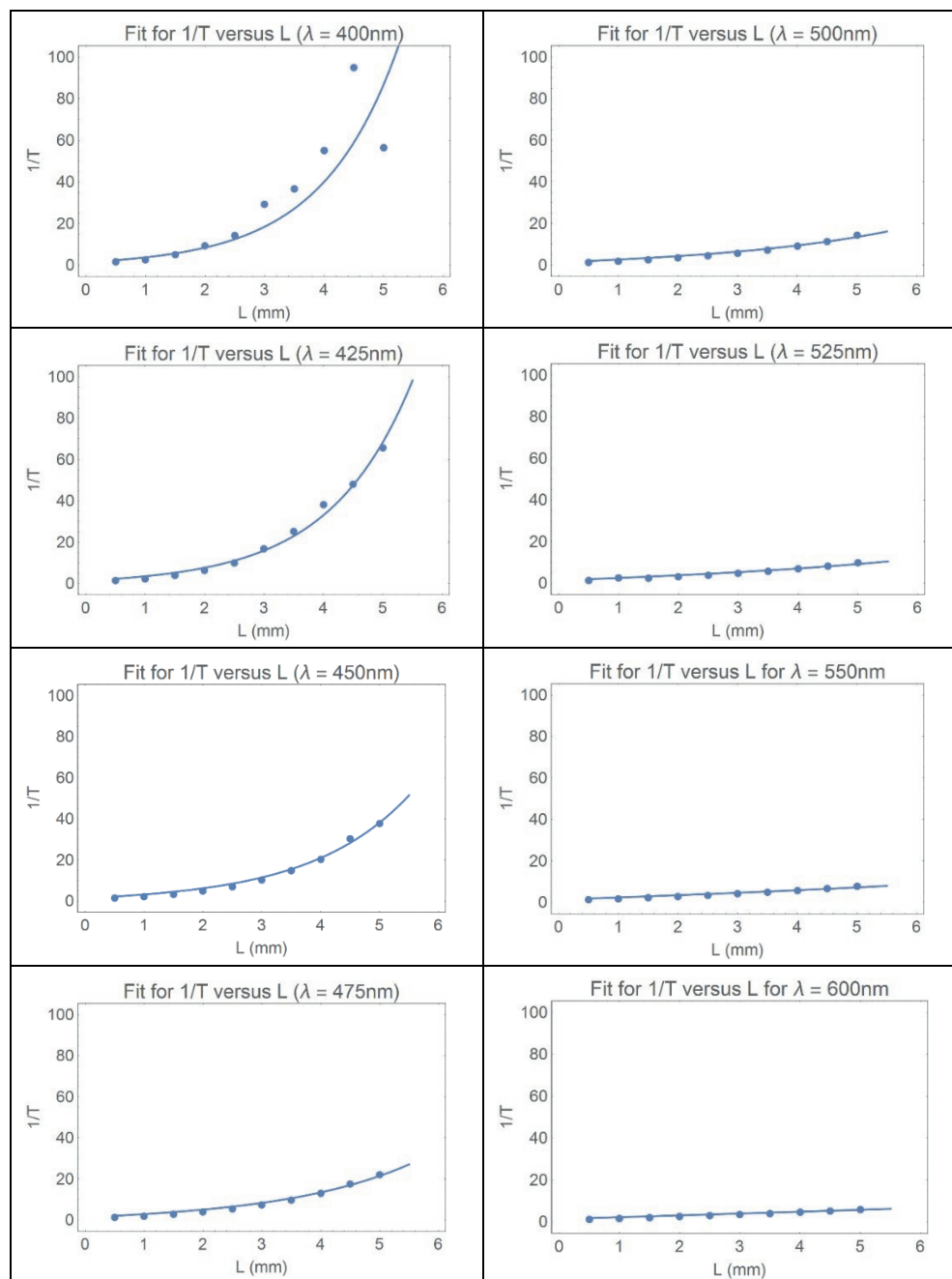


Figure S6.7: Fit of the $1/T$ vs. L plot for white zein particle dispersions using Equations 5 and 6, path-lengths measured were ranging from 0.5 to 5.0 mm and wavelengths between 400 and 550 nm (a range in which there is absorption).

Summary

Product appearance is an important factor for consumers to determine the quality of a product, and color is one of the most important factors that contribute to the product appearance. Currently, the safety and consumer acceptance of some colorants used in food products, such as titanium dioxide and some synthetic colorants, are under discussion. Therefore, new ways to use natural colorants as alternatives to these colorants for more applications are investigated. A promising method to increase the applicability for the often-sensitive natural colorants, is the encapsulation of these colorants in colloidal particles by natural polymers such as carbohydrates, lipids and proteins. In the work described in this Thesis we used the vegetable protein zein as a biopolymer and the simple anti-solvent precipitation technique as synthesis procedure. The scalability of this technique shows the possibility of industrial scale production of fully natural encapsulated particles. They can satisfy the consumers' wish for natural ingredients, i.e. the natural origin of both encapsulants and colorants. Moreover, their improved stability upon encapsulation, facilitates future implementation of these encapsulated colorants in consumer products. Not only in the food industry, but also in the pharmaceutical, cosmetic, textile, and other industries.

In this Thesis, we studied the particle formation and encapsulation properties of the vegetable protein zein without added surfactants. Following this, the optical properties of the resulting colloidal dispersions were studied and compared to theory in order to investigate whether or not it is possible to predict the color of such particle dispersions. The information described in this Thesis can roughly be divided into two parts. In the first part, Chapters 2-4 of this Thesis, we describe experimental studies on how to synthesize particles from the water insoluble protein zein and how to encapsulate colorants. The second part, Chapters 5 and 6, contains experimental studies that were compared to light scattering theory.

In Chapter 2 the synthesis of white zein colloidal particles as a possible replacement for titanium dioxide in liquid food systems was investigated. In this Chapter we discuss the purification of zein and a comparison was made between purified and unpurified zein. The purification method described here was used for all zein synthesis described in this Thesis. Following the purification, particles of a range of sizes were synthesized of which the dispersions have a white appearance and these dispersions are compared to a food grade titanium dioxide benchmark. The scattering properties of the zein particles could be tuned

by the concentration such that these particles could be used as colorant or clouding agents replacing titanium dioxide, albeit at a higher concentration.

Colored particle dispersions were synthesized using anionic dyes, in Chapter 3, which were found to have affinity with the zein protein. Co-precipitation of purified zein protein with anionic dyes, such as Patent Blue V sodium salt, azorubine and Fast Green FCF, were able to associate with the zein colloids, even after dialysis. This was not the case for the non-ionic dye, purpurin. Observing similar results for the three anionic dyes, the remaining experiments were performed with patent blue sodium salt. To illustrate the affinity of zein with an anionic dye, a comparison was made between colored particle dispersions that were synthesized using co-precipitation of the dye and dispersions that were synthesized using post-addition. It is found that co-precipitation and post-addition techniques yield similar results in terms of concentration and adsorption or encapsulation, zeta potential, and color appearance. Particle sizes increased upon increasing dye concentration for co-precipitated samples, while for post-addition samples the particle size was similar to the original white particles. These results show that there is a high affinity between the zein protein and the dye. This may be exploited to produce bio-based and optically-functionalized nanoparticles using zein as a carrier.

The encapsulation of a natural colorant, lutein, using zein as encapsulant was discussed in Chapter 4. Following encapsulation, experiments were performed on the ability of zein nanoparticles to protect lutein from light degradation under various conditions. Particle sizes were tuned by varying the zein concentration and this was linked to the encapsulation efficiency, which increased upon increasing particle size. Zein nanoparticles showed a significant ability to increase the photo-stability of lutein, when compared to plain lutein dispersions in water. To promote the stability of the entrapped colorant further, the use of ascorbic acid was studied as an antioxidant. The addition of ascorbic acid to lutein and lutein-zein particles resulted in dispersions with similar sizes and zeta potentials. However, the photo-stability of both nanoparticle and lutein dispersions stabilized with ascorbic acid improved significantly compared to samples without ascorbic acid. Encapsulation of lutein by zein in the presence of ascorbic acid is a viable strategy to improve photo-stability. The simple anti-solvent precipitation technique offers further possibilities to improve the stability, by adding surfactants, crosslinkers, and other stabilizers to ease the implementation of such particle systems into an application matrix.

The white zein particles that were first synthesized in Chapter 2 were further characterized in Chapter 5. Here, a method was developed to investigate the optical

properties of white zein colloidal particles in both diluted and concentrated dispersions. The value of these particles as white colorant is examined by measuring their optical properties. Dilute dispersions were prepared to measure the extinction cross section of individual particles this was combined with Mie theory to determine a refractive index (RI) of 1.49 ± 0.01 for zein particles dispersed in water. This value was used to further model the optical properties of concentrated dispersions. The optimal size for maximal scattering efficiency was explored by modeling both dilute and concentrated samples with RI's matching those of zein and TiO_2 particles in water. The transport mean free path of light was determined experimentally and theoretically and the agreement between the transport mean free path calculated from the model and the measured value was better than 30%. These results show that it is possible to model the scattering behavior of dispersions of zein particles. This makes it possible to synthesize zein nanoparticles with specifically designed optical properties, which can be tuned towards a specific application.

The method to measure concentrated dispersions experimentally was improved in Chapter 6. Colloidal particles were synthesized and characterized to obtain particle properties such as particle size, particle size distribution, refractive index, and absorption spectrum of the dye. This information was used to model the optical properties of these concentrated particle dispersions, which were experimentally measured using a specially designed variable path length quartz glass cuvette. Non-absorbing spherical and monodisperse silica particles were also synthesized to verify the correct workings of the set-up and to check the applicability of multiple scattering theory. An excellent agreement was found between experiment and theory in terms of the transport mean free path of these silica particles. The same experiment was performed on polydisperse white zein particles (see also Chapter 2). A detailed comparison with theory allows quantification of slight absorption < 550 nm of zein. Subsequently, absorbing and polydisperse blue zein particles (see also Chapter 3) were measured using the set-up. These dyed zein dispersions modeled well, except at the high absorption peaks. For a complete agreement a theory other than diffusion theory needs to be used to describe light transport. The diffusion theory worked best for the slightly absorbing white zein suspension. These results show that it is possible to predict the scattering and absorption behavior of opaque dispersions using as inputs the refractive index, volume fraction, dye absorption spectrum, particle size, and size distribution. This makes it possible to synthesize nanoparticles with specifically pre-designed optical properties, which can be tuned towards a specific application.

Samenvatting

Product-uitendijk is een van de belangrijkste aspecten voor consumenten om de product-kwaliteit te bepalen, waarvan kleur een van de belangrijkste factoren is. Tegenwoordig worden er vraagtekens gezet bij de veiligheid van sommige kleurstoffen die gebruikt worden in voedingsproducten, zoals titaniumdioxide en sommige synthetische kleurstoffen; deze stoffen worden niet als vanzelfsprekend meer geaccepteerd door consumenten. Daarom worden er voor steeds meer toepassingen manieren onderzocht om natuurlijke kleurstoffen te gebruiken als alternatieven hiervoor. Een veelbelovende methode om de toepasbaarheid van de vaak gevoelige natuurlijke kleurstoffen te vergroten, is de inkapseling van deze kleurstoffen in colloïdale deeltjes door natuurlijke polymeren zoals koolhydraten, lipiden en eiwitten. In het werk dat in dit Proefschrift wordt beschreven, hebben we het plantaardige eiwit zeïne als biopolymeer en de anti-solvent precipitatietechniek als de syntheseprocedure gebruikt. De schaalbaarheid van deze techniek creeërt de mogelijkheid van productie op industriële schaal van volledig natuurlijke ingekapselde deeltjes. Deze deeltjes kunnen voldoen aan de vraag van consumenten om natuurlijke ingrediënten, dat wil zeggen de natuurlijke oorsprong van zowel de inkapselingsmiddelen als de kleurstoffen. Bovendien vergemakkelijkt de verbeterde stabiliteit na inkapseling de toekomstige implementatie van deze ingekapselde kleurstoffen in consumentenproducten. Niet alleen in de voedings-middelenindustrie, maar ook in bijvoorbeeld de farmaceutische, cosmetische en textiel industrieën.

In dit Proefschrift hebben we de deeltjesvorming en inkapselings-eigenschappen bestudeerd van het plantaardige eiwit zeïne zonder extra toevoegingen, zoals bijvoorbeeld oppervlakte-actieve stoffen. Hierna werden de optische eigenschappen van de verkregen colloïdale dispersies onderzocht en daarna vergeleken met de theorie. Daarna werd onderzocht of het mogelijk is om de kleur van dergelijke deeltjesdispersies te voorspellen. De informatie die beschreven staat in dit Proefschrift kan ruwweg in twee delen worden verdeeld. In het eerste deel, Hoofdstukken 2-4 van dit Proefschrift, beschrijven we experimentele studies over hoe deeltjes te synthetiseren uit het, in water onoplosbare, eiwit zeïne en hoe kleurstoffen ingekapseld kunnen worden. Het tweede deel, Hoofdstukken 5 en 6, bevat experimentele studies die werden vergeleken met lichtverstrooiingstheorie.

In Hoofdstuk 2 wordt de synthese van colloïdale witte zeïne deeltjes als mogelijke vervanger van titaniumdioxide in vloeibare voedselsystemen onderzocht. In dit Hoofdstuk

bespreken we de opzuivering van zeïne en wordt een vergelijking gemaakt tussen de gezuiverde en de ongezuiverde zeïne. De opzuiveringsmethode die hier wordt beschreven, werd gebruikt voor alle zeïne syntheses in dit Proefschrift. Na de opzuivering werden colloïdale deeltjes van verschillende groottes gesynthetiseerd, waarvan de uiteindelijke dispersies dispersies een wit uiterlijk hebben. Deze dispersies werden vervolgens vergeleken met een voedingskwaliteit titaniumdioxide referentie. De verstrooiingseigenschappen van de zeïne deeltjes kunnen door variatie van de concentratie zodanig worden afgestemd, dat deze deeltjes kunnen worden gebruikt als kleurstof (of vertroebelingsmiddel) ter vervanging van titaniumdioxide, zij het bij een hogere concentratie.

Dispersies van gekleurde deeltjes werden gesynthetiseerd, gebruik makend van anionische kleurstoffen, die affiniteit bleken te hebben met het eiwit zeïne. Dit staat beschreven in Hoofdstuk 3. Co-precipitatie van gezuiverd zeïne-eiwit met anionische kleurstoffen, zoals Patent Blue V natriumzout, azorubine en Fast Green FCF, bond deze zelfs na dialyse met de zeïne colloïden. Dit was niet het geval voor de niet-ionische kleurstof purpurine. Omdat vergelijkbare resultaten voor de drie verschillende anionische kleurstoffen werden waargenomen, werden de overige experimenten uitgevoerd met Patent Blue V natriumzout. Om de affiniteit van zeïne met een anionische kleurstof aan te tonen, werd een vergelijking gemaakt tussen gekleurde dispersies die werden gesynthetiseerd met behulp van co-precipitatie van de kleurstof en gekleurde dispersies gesynthetiseerd met behulp van post-additie van de kleurstof. Het is gebleken dat zowel co-precipitatie en post-additie technieken vergelijkbare resultaten opleveren in termen van concentratie, adsorptie of inkapseling, zeta-potentiaal en kleur. De deeltjesgrootte nam toe bij het verhogen van de kleurstofconcentratie voor de co-precipitatie monsters, terwijl bij de post-additie monsters de deeltjesgrootte overeenkwam met de oorspronkelijke witte deeltjes. Deze resultaten tonen aan dat er een hoge affiniteit is tussen het zeïne-eiwit en de kleurstof. Dit kan worden gebruikt voor de productie van optisch-gefunctionaliseerde nanodeeltjes op biologische basis met zeïne als drager.

De inkapseling van een natuurlijke kleurstof, luteïne, met behulp van zeïne als inkapselingsmiddel wordt beschreven in Hoofdstuk 4. Na de inkapseling werden experimenten uitgevoerd naar het vermogen van zeïne nanodeeltjes om luteïne onder verschillende omstandigheden te beschermen tegen fotodegradatie. De deeltjesgroottes werden gevarieerd door de zeïne-concentratie te variëren en dit werd gekoppeld aan de inkapselingsefficiëntie, die toenam bij het vergroten van de deeltjesgrootte. Zeïne-nanodeeltjes vertoonden een aanzienlijk vermogen om de fotostabiliteit van luteïne

te verhogen in vergelijking met reguliere luteïne dispersies in water. Om de stabiliteit van de ingekapselde kleurstof verder te bevorderen, is het gebruik van ascorbinezuur als een antioxidant bestudeerd. De toevoeging van ascorbinezuur aan luteïne en luteïne-zeïne deeltjes resulteerde in dispersies met een vergelijkbare grootte en zeta-potentiaal. De fotostabiliteit van zowel de nanodeeltjes en de luteïne dispersies, beide gestabiliseerd met ascorbinezuur, verbeterde aanzienlijk in vergelijking met monsters zonder toegevoegd ascorbinezuur. Het inkapselen van luteïne door zeïne in de aanwezigheid van ascorbinezuur is dus een goede methode om de fotostabiliteit van luteïne te verbeteren. De anti-solvent precipitatietechniek biedt verdere mogelijkheden voor stabiliteitsverbetering, door toevoeging van bijvoorbeeld oppervlakte-actieve stoffen, crosslinkers en andere stabilisatoren. Dit zal de implementatie van dergelijke deeltjessystemen in een werkelijke (voedsel-) toepassing vergemakkelijken.

De witte zeïne deeltjes gesynthetiseerd in Hoofdstuk 2 worden verder onderzocht in Hoofdstuk 5. In dit Hoofdstuk staat een methode beschreven die is ontwikkeld om de optische eigenschappen van witte zeïne colloïdale deeltjes, in zowel verdunde als geconcentreerde dispersies, te onderzoeken. De waarde van deze deeltjes als witte kleurstof wordt onderzocht door het experimenteel bepalen van hun optische eigenschappen. Verdunde dispersies werden bereid om de extinctiedoorsnede van afzonderlijke deeltjes te meten. Dit werd gecombineerd met de Mie-theorie waarmee een brekingsindex (BI) van $1,49 \pm 0,01$ voor zeïne deeltjes gedispergeerd in water werd gevonden. Deze waarde werd gebruikt om de optische eigenschappen van geconcentreerde dispersies verder te modelleren. De optimale grootte voor maximale verstrooiingsefficiëntie werd onderzocht door het modelleren van zowel verdunde en geconcentreerde monsters met een BI overeenkomend met die van zeïne en titaniumdioxide deeltjes in water. De transport vrije weglengte van licht werd experimenteel en theoretisch bepaald. De overeenstemming tussen de transport vrije weglengte die berekend werd gebruikmakend van het model en de daadwerkelijk gemeten waarde, was beter dan 30%. Deze resultaten tonen aan dat het mogelijk is om het verstrooiingsgedrag van dispersies van zeïne deeltjes te modelleren. Dit maakt het mogelijk zeïne nanodeeltjes te synthetiseren met specifiek ontworpen optische eigenschappen, welke kunnen worden afgestemd op een specifieke toepassing.

De experimentele opstelling meten van geconcentreerde dispersies zoals uitgevoerd in Hoofdstuk 5, is verbeterd en staat beschreven in Hoofdstuk 6. Colloïdale deeltjes werden gesynthetiseerd en gekarakteriseerd om deeltjeseigenschappen te bepalen, zoals deeltjes-grootte, deeltjes-grootteverdeling, brekingsindex en het absorptiespectrum van de

kleurstof. Deze informatie werd gebruikt om de optische eigenschappen te modelleren van deze geconcentreerde dispersies, welke experimenteel werden gemeten met behulp van een speciaal ontworpen variabele-weglengthe kwartsglas cuvet. Niet-absorberende, ronde, monodisperse silicadeeltjes werden ook gesynthetiseerd en gemeten. Deze deeltjes werden gebruikt om de correcte werking van de opstelling te verifiëren en de toepasbaarheid van de meervoudige verstrooiingstheorie te controleren. Een uitstekende overeenkomst werd gevonden tussen experimentele en theoretische waarden met betrekking tot de transport vrije weglengte van deze silicadeeltjes. Hetzelfde experiment werd uitgevoerd met polydisperse witte zeïne deeltjes (zie ook Hoofdstuk 2). Een gedetailleerde vergelijking met de theorie maakt kwantificering van de geringe absorptie <550 nm van de zeïnedispersie mogelijk. Ten slotte werden de absorberende en polydisperse blauwe zeïne deeltjes (zie ook Hoofdstuk 3) gemeten met behulp van deze opstelling. Het bleek mogelijk om deze gekleurde zeïne dispersies goed te modelleren, behalve bij de hogere absorptiepieken. Voor een volledige overeenstemming is het nodig om een andere theorie dan de diffusietheorie te gebruiken om lichttransport door dit type monster te beschrijven. De diffusietheorie werkte het beste voor de licht absorberende witte zeïne suspensie. Deze resultaten laten zien dat het mogelijk is om het verstrooiings- en absorptiegedrag van ondoorzichtige dispersies te voorspellen met behulp van de brekingsindex, de volumefractie, het absorptiespectrum van de kleurstof, de deeltjesgrootte en de deeltjesgrootteverdeling als input. Dit maakt het mogelijk om nanodeeltjes te synthetiseren met vooraf specifiek ontworpen optische eigenschappen, die kunnen worden afgestemd op een specifieke toepassing.

Gearfetting

It uterlik fan in produkt is in wichtige faktor foar konsuminten om de sûnens fan in produkt te mjitten, kleur is ien fan 'e wichtichste eleminten fan it produktuterlik. Op it stuit binne minsken it net iens oer feiligens en it konsuminte-akseptearjen fan inkele kleuren dy't brûkt wurde yn itens produkten, lykas titium dioxide en inkele synthetiske kleuren. Dêrom wurdt it brûken fan natuerlike kleuren ûndersocht as alternatyf foar dizze kleuren yn mear applikaasjes. In metoade mei takomst foar in gruttere tapassing, fan de faaks-gefoelige natuerlike kleuren, is de ynkapsulaasje fan dy kleuren yn kolloidale dieltsjes fan natuerlike polymers lykas kohdhydraten, lipiden en proteins. Yn it wurk dat beskreaun is yn dizze dissertaasje, hawwe wy it planten protein zein, brûkt as in biopolymer en de ienfâldige anty-solvintyske precipitaasje technyk as synthesizipaasje. De skalberens fan dizze technyk toant de mooglikheid foar yndustriële skaalproduksje fan folslein natuerlik ynkapsulearre dieltsjes. De natuerlike komôf fan beide ynkapsulinten en kleuren, koe wol'ris it antwurd op'e winsken foar natuerlike yngrediïnten fan 'e konsuminten wêze. Boppedat makket har ferbettere stabiliteit op ynkapsulaasje, fasilitearret de takomstige ymplemintaasje fan dizze ynkapsulearre kleuren yn konsuminteprodukten. Net allinich yn 'e fiedingsindustry, mar ek yn' e farmazeutyske-, kosmetyske-, tekstyl- en oare bedriuwen.

Yn dizze dissertaasje ûndersykje wy op de partikelfoarming en ynkapsulearjende eigenskippen fan it planten protein zein sûnder tafoege tafanen. Hjirnei waarden de optyske eigenskippen fan 'e útkomsten kolloidale dispersjes ûndersocht en fergelike mei teory om te witten oft it mooglik is om de kleur fan soksoarte partikulêre dispersjes te foarsjen. De yn dizze dissertaasje beskreaune ynformaasje kin rûchwei ferdield wurde yn twa parten. Yn it earste part, chapter twa trije en fjouwer fan dizze dissertaasje, beskriuwe wy eksperimintele stúdzjes oer hoe't jo dieltsjes út it wetter ûnreplikke protein zein te meitsjen en hoe't jo kleuren ynkapsulearje. It twadde part, chapter fiif en seis, beskriuwt eksperimintele stúdzjes dy't mei de ljochtstreidingteorie fergelike binne.

Yn Chapter twa wurdt de synthesis fan wite kolloidale zein dieltsjes as in fakultative ferfanging foar titaniumdioxide yn floeistoflike itensystemen ûndersocht. Yn dit chapter behannelje wy de purgaasje fan sines en in fergelyk waard makke tusken purgere- en net purgere zein. De purgaasje metoade fan chapter twa is brûkt foar alle zein synthes dy't beskreaun binne yn dizze dissertaasje. Nei de purgaasje waarden dieltsjes fan in ferskaat oan grutte synthesized wêrfan de dispersjes in wyt utens hawwe, dizze dispersjes binne fergelike

mei in fiedingsgroep titium dioxide benchmark. De ferspriedingseigenskippen fan 'e zein dieltsjes kinne opnommen wurde troch de konsintraasje sadat dizze dieltsjes brûkt wurde kinne as kleur, of brûkt as bewolkins medium, inpleats fan Titan dioxide, mar dan yn in hegere konsintraasje.

Kleuriche dieltsjes dispersjes binne makke mei anionyske kleuren yn chapter trije, dy't fûn wurde om affiniteit te hawwen mei it zein protein. Coprecipitaasje fan purgearre zein proteïne mei anionyske kleuringen, lykas Patint "Blue V" sodium sâlt, azorubine en "Fast Green FCF", wiene ferbûn mei de zein kolloïden, ek nei dialysis. Dit wie net it gefal foar de non-ionyske kleur, purpurine. Om't fergelykbere resultaten foar de trije ferskate anionyske kleuren te sjen wienen, waarden de oerbleaun eksperiminten útfierd mei patintblauwe sodium sâlt. Om de affiniteit fan zein mei in anionyske foarkar te yllustrearjen, waard in fergelyking makke tusken kleurde partikulêre dispersjes dy't synthesized waarden mei co-precipitation fan de foarkleur en dispersjes dy't synthesized waarden mei post-addition. It blykt dat co-precipitation en post-oanfolling-technyske techniken fergelykbere útkomsten jouwe yn termen fan konsintraasje en adsorption of kapsulaasje, zeta potensjele en kleurferrein. Op 'e stevende Kleur-konzentraasje foar co-precipitation samples fegrutte de partikelgrutte, wylst foar post-tafoegings samples de partikelgrutte ferlykber wie mei de orizjinele wite partikels. Dizze útkomsten jouwe dat der in grutte kinskip tusken it zein protein en de Kleur is. Dit kin men verwurkje om bio-basearre en optysk-funksjonalisearre nano-partikels te brûken mei zein as drager.

De kapsulaasje fan in natuerlike kleur, lutein, mei zein as kapsulant waard beskreaun yn chapter fjouwer. Nei kapsulaasje waarden eksperiminten útfierd op 'e kapasiteit fan sane-nano-partikels om lutein tsjin ljocht degradaasje te beskermjen ûnder ferskate omstannichheden. Partikelgrutte waard opnij ôfstimd troch it feroarjen fan 'e zein-konsintraasje en dit waard ferbûn mei de kapsulaasje-effisjinsje, dy't mei ferheegjen fan de partikelgrutte ferhege. Zein-nanopartikels hawwe in wichtich potinsjeel om de foto-stabiliteit fan lutein te ferheegjen, ferlike mei lutein-dispersjonen yn wetter. Om de duorsumens fan 'e ynlutsen kleur fierder te befoarderjen, waard it gebrûk fan ascorbinsäure as in antioxidant ûndersocht. De tafoeging fan ascorbinsoal foar lutein en lutein-zein-partikels soarge foar ferspriedingen mei ferlykbere dimens- en zeta-potensjaasjes. Lykwols. De foto-stabiliteit fan sawol nano-partikels en lutein-dispersjonen stabilisearre mei ascorbinsäure waard wichtig ferbettere yn ferliking mei samples sûnder ascorbinsoal. Inkapsulaasje fan lutein troch zein yn 'e oanwêzigens fan ascorbinsäure is dêrom in goede metoade foar ferbettering fan foto-stabiliteit. De ienfâldige anty-solvintyske ôffektystechniek jout mear mooglikheden om de

stabiliteit te ferbetterjen, troch tafoegjende tafanen, crosslinkers en oare stabilisiers te foegejen. foar it útfieren fan soksoarte partikelsystemen yn in oanfraachmatrix.

De wite zein partikels dy't yn chapter twa synthesesearre waarden, wurde fierder ûndersocht yn chapter fiif. Yn dit chapter waard in metoade beskreaun om de optyske eigenskippen fan wite zein kolloidale partikels yn sawol ferâldere en konsintrearre dispersjes te ûndersikjen. De wearde fan dizze dieltsjes as wite kleur wurdt ûndersocht troch it mjitten fan har optyske eigenskippen. Dielste dispersjes waarden ree makke om de útstekingskrêft te mjitten fan yndividuele partikels. Dit waard kombinearre mei de Mie-teory om in refraktive yndeks (RI) fan $1,49 \pm 0,01$ te bepalen foar zein-partikels dy't yn wetter ferspraat waarden. Dizze wearde waard brûkt om de optyske eigenskippen fan konsintrearre dispersjes fierder te modellearjen. De optimale groei foar maksimale streek efektiviteit waard ûndersocht troch modeling fan beide ferlaten en konsintrearre problemen mei RI's oerienkomst fan zein- en TiO₂-partikels yn wetter. It ferfier frije paad fan ljocht waard eksperiminteel en teoretysk fêststeld en de oerienkomst tusken it ferfangen fan trochsnee frije paad is berekkene út it model en de mjitten wearde wie better as 30%. Dizze útkomsten bewize dat it mooglik is it streekgedrach fan 'e dispersjonearring fan zein-partikels te modellen. Dat is hoe't it mooglik is om zein-nanopartikels te meitsjen mei spesifike beëage optyske eigenskippen, dy't op in spesifike tapassing opnien wurde kinne.

De metoade foar mjittingen fan konsintrearre dispersjes as beskreaun in Chapter fiif waard eksperiminteel ferbettere yn chapter seis. Kolloidale partikels waarden synthesesearre en karakterisearre om partikeleare eigenskippen te krijen lykas partikelgrutte, partikelgrutteferbining, refraktive yndeks, en absorptyfe spektrum fan 'e kleur. Dizze ynformaasje waard brûkt om de optyske eigenskippen fan dizze konsintrearre partikel dispersions te modellearjen. Dy't eksperimenteel metten wurde mei in spesjaal ûntworpen plylk-kwartzglês- kuvette. Net-absorberjende rûne, monodisperse silica-partikels waarden ek synthesesearre en mjitten. Dizze partikels waarden brûkt om it krekte wurkjen fan 'e ynstellingen te kontrolearjen en de tapassing fan 'e mearfâldige scattering teory te kontrolearjen. In poerbêste oerienkomst waard fûn tusken eksperimint en teory yn 'e betsjutting fan de ferfangen fan trochsnee frije paad fan dizze silica-partikels. Itselde eksperimint waard útfierd op polydisperse wite zein partikels (sjoch ek chapter twa). In detaillearre fergeliking mei teory jout kwantifikaasje fan 'e lytse absorption <550 nm fan de zein-dispersje. Uiteinlik wurde absorptive en polydispearre blauwe zein partikels (sjoch ek chapter trije) mei dizze ynstellingen evaluerre. It die bliken dat it mooglik wie om dizze kleurige zein-dispersions goed te modellen, útsein by de hege absorptionske peaks. Foar in

folsleine oerienkomst moat in oare teory lykas diffusion teory brûkt wurde om it ljochtferfier te beskriuwen. De diffusion teory wurke it bêste foar de lyts absorberjende wite zein suspensjoniteit. Út dizze útkomsten blykt dat it mooglik is it ferspriedings- en absorptaasjegedrach fan ljochte dispersjes te foarsjen, dy't brûkt wurde as yngongen de refraktive yndeks, folume fraach, kleur-absorptyfespektrum, partikelgrutte, en grutteferdieling. Hjirtroch is it mooglik om nanotechnology te meitsjen mei spesifike foarôfsteld optyske eigenskippen, dy't op in spesifike tapassing opnien wurde kinne.

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“I don’t know half of you half as well as I should like; and I like less than half of you half as well as you deserve.”

Bilbo Baggins – The Fellowship of the Ring by J.R.R. Tolkien

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I have not extensively interacted with most group members. I attribute that to an introvert living far outside of Utrecht. However, I have had a focused patchy interaction! Interestingly, I even ended up in an office with Jantina and Sergey, who are also both not inhabitants of Utrecht. I remember a particularly stormy day during which the NS decided not to run any trains anymore for the rest of the day, where the three of us were frantically refreshing the NS-website in hopes of any resumption of service; though in the end we all had to be picked up by car anyway...

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About the Author

Frankjen Ynske de Boer (Frankje) was born in Tilburg, the Netherlands, on the 20th of September 1990. She finished secondary school at Markland College in Oudenbosch in 2008. Then started her bachelor program *Chemistry* at Avans University of Applied Science in Breda, within this program she participated in two internships, one at Fujifilm Manufacturing Europe B.V. and the other at TNO. Her Bachelor thesis was titled “Diels-Alder thermo-reversible adhesives” and was carried out at the Responsive Materials & Coatings group at TNO Eindhoven, under the supervision of dr. Herman Lenting. After graduating in 2012, she started the Pre-Master’s program in *Chemical Engineering* at the Eindhoven University of Technology. After completion of this program, she continued studying at the Eindhoven University of Technology to obtain her Master’s degree in *Chemical Engineering* with a specialization in *Polymers and Composites*. For her Master’s graduation project, Frankje worked in the Laboratory of Materials and Interfaces where she investigated the behavior of hydrophilic coatings in air and in water by atomic force microscopy. This was carried out under the supervision of prof. Gijsbertus de With and dr. Catarina Esteves. Attaining her diploma in 2015, she started her PhD project at Utrecht University in the Soft Condensed Matter & Biophysics group, with prof. Alfons van Blaaderen as promotor and prof. Krassimir Velikov and dr. Arnout Imhof as daily supervisors. During her PhD, she focused on the synthesis and characterization of colloidal colorants. The results of this project, of which parts are published in scientific journals and presented at national and international conferences, are described in this thesis.



List of publications

This thesis is partly based on the following publications:

- F. Y. de Boer, A. Imhof, K. P. Velikov, *Encapsulation of colorants by natural polymers for food applications*, Coloration Technology, 135, 183-194, 2019 (Chapter 1)
- F. Y. de Boer, R. N. U. Kok, A. Imhof, K. P. Velikov, *White zein colloidal particles: synthesis and characterization of their optical properties on the single particle level and in concentrated suspensions*, Soft Matter, 14, 2870-2878, 2018 (Chapters 2 and 5)
- F. Y. de Boer, A. Imhof, K. P. Velikov, *Color-tunable particles through affinity interactions between water-insoluble protein and soluble dyes*, Colloids and Surfaces A: Physicochemical and Engineering Aspects, 562, 154-160, 2019 (Chapter 3)
- F. Y. de Boer, A. Imhof, K. P. Velikov, *Photo-stability of lutein in surfactant-free lutein-zein composite colloidal particles*, (submitted) (Chapter 4)
- F. Y. de Boer, R. J. A. van Dijk-Moes, A. Imhof, K. P. Velikov, *Characterization of the scattering and absorption of colloids in optically dense dispersions*, (submitted) (Chapter 6)

Oral and Poster Presentations

Part of this thesis was presented at the following national and international conferences.

Poster presentations

- CHAINS2016, Veldhoven, December 2016 (audience award)
- Physics@Veldhoven 2017, Veldhoven, January 2017
- Physics@Veldhoven 2018, Veldhoven, January 2018
- 17th Food Colloids Conference: Application of Soft Matter Concepts, Leeds, United Kingdom, April 2018
- 4th International Conference on Bioinspired and Biobased Chemistry & Materials (N.I.C.E.) 2018, Nice, France, October 2018 (best poster prize)
- CHAINS2018, Veldhoven, December 2018
- Physics@Veldhoven, Veldhoven, January 2019

Oral presentations

- CHAINS2016, Veldhoven, December 2016
- CHAINS2017, Veldhoven, December 2017
- 16th Conference of the International Association of Colloid and Interface Scientists (IACIS2018), Rotterdam, May 2018
- 1st International BioColours Conference, Breda, May 2018
- 15th Coatings Science International Conference (CoSI) 2019, Noordwijk, June 2019

