



Novel chitosan-based Pickering emulsions as green encapsulation systems and delivery vehicles of bioactive agents

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Abstract

The increase in consumers' awareness towards safe and eco-friendly products continuously motivates researchers to provide products with higher sustainability and safety. Surfactants used in conventional emulsions, which represent a wide variety of food, cosmetic and pharmaceutical products, are reported to have negative effects on the environment and health. In contrast, Pickering emulsions (PEs), which are emulsions stabilized by solid particles, are considered eco-friendly alternatives due to their surfactant-free nature. The wettability of the particles is an important criterion in controlling the stability of the formed PE. Among a wide range of biopolymers that are used in the production of Pickering stabilizers, chitosan is considered a promising candidate due to its biodegradable, biocompatible and non-toxic properties. However, chitosan is highly hydrophilic and, thus, requires modification to tune its wettability, and enhance its surface activity and emulsification properties.

This thesis focuses on the development of novel, surfactant-free, and stable chitosan-based PEs. The work comprises five main studies that contribute to the ongoing research in the field of PEs which have the potential to be used in cosmetic and food applications. In the first study, chitosan/gum Arabic (CH/GA) nanoparticles with near-neutral wettability were successfully produced and employed as novel stabilizers for PEs exhibiting high storage stability. The produced CH/GA PEs were then assessed for the encapsulation and topical delivery of resveratrol (RSV). The PEs resulted in high cutaneous retention and low permeation of RSV, indicating their potential as green cosmetic vehicles. The developed PEs remarkably enhanced RSV photostability. In the third phase of the research, novel Pickering emulsions stabilized with chitosan/collagen peptides (CH/CP) nanoparticles were developed. The CH/CP nanoparticles have shown enhanced wettability and a higher ability to reduce the interfacial tension compared to that of chitosan alone, which led to the production of highly stable PEs. Moreover, skin tracking of the CH/CP nanoparticles provided new insights into their fate following the skin application of PEs. In the fourth study, the developed CH/CP PEs were evaluated as green topical delivery vehicles of cannabidiol (CBD). These novel systems demonstrated superior CBD protection after five

months of storage. Additionally, the skin absorption and cell viability studies have shown that the produced formulations acted as effective non-toxic vehicles for the topical delivery of CBD. The final study of the thesis investigated the effect of the degree of deacetylation (DDA) of chitosan on the stability of CBD-loaded CH/GA PEs and the topical delivery of CBD. The DDA of chitosan was found to have no significant effect on the amount of CBD deposited in the skin. However, it highly influenced the stability of the PEs. Hence, the DDA is an important parameter that should be carefully considered to guarantee a long shelf-life, which is an essential objective for emulsions intended for cosmetic and food applications.

Drawing on interdisciplinary approaches, this thesis aspires to contribute to the existing knowledge and advance the ongoing research in the areas of product engineering, biopolymeric dispersions, characterization of interfaces and colloids, drug encapsulation, and sustainable cosmetic and food applications. The novel chitosan-based PEs developed in this work can open new avenues in cosmetic and food applications to meet the consumers' demands and the industrial requirements for safer and sustainable products.

Keywords: Pickering emulsions; Chitosan; Wettability; Emulsion stability; Encapsulation; Topical delivery

Resumo

O aumento da consciencialização dos consumidores para o uso de produtos seguros e mais ecológicos tem vindo a motivar os investigadores para o desenvolvimento de soluções mais sustentáveis e seguras. Os surfactantes convencionalmente usados nas emulsões, que representam uma grande variedade de produtos alimentares, cosméticos e farmacêuticos, têm efeitos negativos no meio ambiente e na saúde. Contrariamente, as emulsões Pickering (EPs), que consistem em emulsões estabilizadas por partículas sólidas, são consideradas alternativas ecológicas devido à sua natureza livre de surfactantes. A molhabilidade das partículas é um critério importante no controle da estabilidade da EP formada. Entre a ampla gama de biopolímeros que são utilizados na produção de estabilizantes Pickering, o quitosano é considerado um candidato promissor devido às suas propriedades biodegradáveis, biocompatíveis e atóxicas. No entanto, o quitosano é altamente hidrofílico, requerendo a sua modificação para ajustar a molhabilidade, melhorar a atividade superficial e propriedades de emulsificação.

Esta tese centra-se no desenvolvimento de novas EPs à base de quitosano estáveis e livres de surfactantes. O trabalho compreende cinco estudos principais que visam contribuir para a investigação em curso na área das EPs com potencial uso em aplicações cosméticas e alimentares. No primeiro estudo, foram produzidas com sucesso nanopartículas de quitosano/goma arábica (QT/GA) com molhabilidade praticamente neutra e empregadas como novos estabilizantes em EPs que demonstraram elevada estabilidade ao armazenamento. As EPs QT/GA produzidos foram avaliados quanto à capacidade de encapsulamento e administração tópica de resveratrol (RSV). As EPs conduziram a uma alta e elevada retenção cutânea e uma baixa permeação do RSV, indicando o seu potencial para atuar como veículos cosméticos “verdes”. As EPs desenvolvidas melhoraram substancialmente a fotoestabilidade do RSV. Na terceira fase deste trabalho, foram desenvolvidas novas emulsões Pickering estabilizadas com nanopartículas de quitosano/peptídeos de colágeno (QT/CP). As nanopartículas de QT/CP mostraram uma molhabilidade superior e uma maior capacidade de reduzir a tensão interfacial, comparativamente ao uso individual do quitosano, o que levou à produção de EPs altamente estáveis. Adicionalmente, o rastreamento das nanopartículas de QT/CP na pele

forneceu novas pistas sobre seu destino após a aplicação das EPs na pele. No quarto estudo, as emulsões baseadas em QT/CP foram avaliadas como veículos “verdes” de entrega tópica para o canabidiol (CBD). Estes novos sistemas demonstraram uma proteção superior para o CBD após cinco meses de armazenamento. Adicionalmente, os estudos de absorção na pele e de viabilidade celular mostraram que as formulações atuaram como veículos não tóxicos eficazes para a entrega tópica de CBD. O estudo final da tese focou-se na avaliação do efeito do grau de desacetilação (GD) do quitosano na estabilidade das EPs de base QT/GA carregadas com CBD e na entrega tópica de CBD. O GD do quitosano não teve efeito significativo sobre a quantidade de CBD depositado na pele. No entanto, influenciou significativamente a estabilidade das EPs. Assim, o GD é um parâmetro importante que deve ser considerado para garantir uma vida de prateleira longa dos produtos desenvolvidos, que é um objetivo essencial para emulsões destinadas a aplicações cosméticas e alimentares.

Tendo por base abordagens interdisciplinares, esta tese visa contribuir para o conhecimento existente e avanços na investigação em curso nas áreas de engenharia de produto, dispersões biopoliméricas, caracterização de interfaces e coloides, encapsulamento de drogas e aplicações sustentáveis em cosméticos e alimentos. As novas EPs à base de quitosano desenvolvidas neste trabalho podem abrir novos caminhos no campo das aplicações cosméticas e alimentares atendendo às necessidades dos consumidores e aos requisitos industriais cada vez mais focados na obtenção de produtos mais seguros e sustentáveis.

Palavras-chave: Emulsões Pickering; Quitosano; Molhabilidade; Estabilidade da emulsão; Encapsulação; Entrega tópica

Dedication

To my mother, thank you for all the support, encouragement, love, and prayers. ***To my father***, you will always be my academic role model, my favorite thinker, and problem solver.

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List of Abbreviations

ANOVA	Analysis of variance
ATR	Attenuated total reflectance
CBD	Cannabidiol
CH	Chitosan
CLSM	Confocal laser scanning microscopy
CP	Collagen peptides
DDA	Degree of deacetylation
EE	Encapsulation efficiency
FTIR	Fourier transform infrared spectroscopy
G'	Storage modulus
G''	Loss modulus
GA	Gum Arabic
GRAS	Generally regarded as safe
h	hour
HIPEs	High internal phase emulsions
HIU	High intensity ultrasonication
HLB	Hydrophilic-lipophilic balance
HPLC	High performance liquid chromatography
Hz	Hertz
LP	Liquid paraffin
µm	Micrometer
nm	Nanometer
NPs	Nanoparticles
o/w	Oil-in-water
OECD	The Organisation for Economic Co-operation and Development
OL	Olive oil
OSA	Octenyl succinic anhydride
Pa.s	Pascal.second
PE	Pickering emulsion

PEG	Polyethylene glycol
PLGA	Poly lactic-co-glycolic acid
rpm	Rotation per minute
RSV	Resveratrol
SC	<i>Stratum corneum</i>
SD	Standard deviation
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
TPP	Tripolyphosphate
UV	Ultraviolet
v	Volume
VED	Viable epidermis and dermis
w	Weight
w/o	Water-in-oil

1 Introduction

This chapter presents a general overview of the relevance of my thesis topic and the motivation for conducting this research. It further states the overall objectives of the thesis and outlines how the next chapters are organized.

I first started to develop an interest in Pickering emulsions, the principal theme of this thesis, after attending a presentation at the 25th International Conference on Bioencapsulation (Neufeld et al., 2017), where I presented the research work I conducted for my MSc studies (Sharkawy et al., 2017). I took an interest specifically in the surfactant-free nature and eco-friendliness of these emulsion systems. This motivated me to venture on an exciting journey of extensive study and reading on the topic, followed by conducting experimental research whose outcome is an original contribution to the field of Pickering emulsions.

Worth noting is that the research conducted in this thesis draws on interdisciplinary approaches involving different intersecting research areas, namely, engineering of biopolymeric dispersions, characterization of interfaces and colloids, drug encapsulation, and food and sustainable cosmetic applications. It, therefore, aspires to advance the existing knowledge and ongoing research in the aforementioned areas, as well as engage an interdisciplinary readership.

1.1 Relevance and motivation

Emulsions have numerous applications in the food, pharmaceutical and cosmetic industry (Xiao et al., 2016). They are widely used as encapsulation systems of active agents for delivery and protection purposes (Matos et al., 2018; Wilson et al., 2021; Zillich et al., 2013). Emulsions are liquid preparations formed of two immiscible phases; a dispersed phase and a continuous (dispersing) phase. They are thermodynamically unstable systems, and hence, the interface between the droplets (dispersed phase) and the continuous phase requires stabilization by using emulsifiers. An emulsifier is a surfactant that decreases the

energy at the liquid-liquid interfaces (interfacial tension) and delays the emulsion phase separation owing to its amphiphilic structure (Yang et al., 2017).

Emulsifiers that are incorporated in emulsions and many emulsion-based products have been reported to exhibit adverse effects on health. For example, many of the emulsifiers that are used in cosmetic or pharmaceutical topical products have demonstrated undesirable effects such as skin barrier impairment, irreversible changes in the structural components of the skin, skin irritation and cytotoxicity (Bárány et al., 2000; Venkataramani et al., 2020). Furthermore, emulsifiers that are commonly present in food emulsions, such as polysorbate 80 (Tween[®] 80), have been reported to interfere with the host-microbiota interactions and cause intestinal inflammation (Chassaing et al., 2017). They can also promote the absorption of some food pollutants (e.g. phthalic acid esters) that happen to exist accidentally in food (Zhu et al., 2021). Moreover, some dietary emulsifiers have recently been linked to cardiovascular diseases (Rhodes, 2018). Emulsifiers have also been reported to have adverse environmental impacts, such as aquatic contamination and toxicity (Rodríguez-López et al., 2018). Hence, reducing the use of emulsifiers in food and cosmetic applications is of great importance.

Emulsions stabilized by solid particles, also known as Pickering emulsions, provide a safer and sustainable alternative than the conventional emulsions which are stabilized with the classical emulsifiers (Xiao et al., 2016; Yang et al., 2017). They were first reported by Ramsden, and then by Pickering in the past century in 1903 and 1907, respectively (Chevalier & Bolzinger, 2013; Rayner et al., 2014). But, they only regained attention during the past two decades because of the environmental and safety questioning of the classical emulsifiers and owing to the consumers' demands which have changed over the years, as well as the immense development in particle chemistry synthesis and characterization techniques (Rayner et al., 2014), which all led to the current “neo-Pickering” era (Dickinson, 2020). However, the application of Pickering emulsions for the encapsulation and delivery of bioactive agents is still limited (Zhang et al., 2021).

Many types of inorganic particles have been frequently studied for the stabilization of Pickering formulations (Lam et al., 2014). Nevertheless, due to low consumer acceptance and regulatory issues, the use of these particles in food and cosmetic products has shown

limited applications. In fact, the potential use of biopolymeric-based particles as Pickering stabilizers has only recently become recognized (Dickinson, 2020). The same trend has also been described for Pickering emulsions designed for cosmetic and skin applications (Marto et al., 2016). Therefore, there is a persistently increasing interest in developing novel particles from naturally occurring polymers for the formulation of new Pickering emulsion systems.

Chitosan has been widely used as – and still considered – a perfect candidate for the delivery of bioactive agents and drugs due to its biocompatibility and biodegradability, together with its cationic nature that allows it to interact with oppositely charged materials and mucosal membranes (Mohammadi et al., 2021). Nowadays, chitosan is also receiving increasing recognition in the food and cosmetic industries. Being produced chiefly from seafood waste, chitosan is considered a sustainable and environmentally-friendly material that contributes to creating and maintaining a “circular economy” model (Bellich et al., 2016). Additionally, chitosan-based products indirectly provide ecological benefits and solutions that help to overcome problems arising from the absence of acceptable waste management procedures for the waste produced globally from the shellfish processing industries (Bellich et al., 2016; Rizzi et al., 2019).

1.2 Thesis objectives and outline

The main objective of this thesis is to develop novel, surfactant-free, and stable chitosan-based Pickering emulsions that have the potential to be used in cosmetic and food applications. The thesis also aims to investigate the applicability and utilization of the produced Pickering emulsions as green encapsulation systems and topical delivery carriers of bioactive agents.

The thesis is composed of eight chapters, five of which have recently been published as peer-reviewed journal articles. The thesis chapters are organized according to their objectives as follows:

Chapter 1 (the present chapter) provides a general introduction for the thesis topic, describes the relevance and motivation, and highlights the thesis objectives and outline.

Chapter 2 reviews the recent knowledge and advances in chitosan-based Pickering emulsions. It comprehensively discusses the different production methods of chitosan-based Pickering particles. It also addresses the factors that influence the formation of Pickering emulsions stabilized with chitosan-based particles. In addition, the chapter discusses the utilization of these Pickering emulsion systems in food and cosmetic applications. The final section of the chapter highlights the knowledge gaps and challenges related to chitosan-based Pickering emulsions. This chapter was published as:

Sharkawy, A., Barreiro, M. F., & Rodrigues, A. E. (2020). Chitosan-based Pickering emulsions and their applications: A review. *Carbohydrate Polymers*, 250, 116885.

Chapter 3 aims to produce chitosan/gum Arabic nanoparticles with appropriate properties for their utilization to develop novel stable surfactant-free polysaccharide-based Pickering emulsions. The work entails detailed physicochemical characterization of the produced chitosan/gum Arabic nanoparticles and the Pickering emulsions developed thereof. This is the first study in the literature that reported the formulation of Pickering emulsions using chitosan/gum Arabic nanoparticles. The results of this chapter aim to open new research avenues for using these novel Pickering emulsions in cosmetic and food applications. This chapter was published as:

Sharkawy, A., Barreiro, M. F., & Rodrigues, A. E. (2019). Preparation of chitosan/gum Arabic nanoparticles and their use as novel stabilizers in oil/water Pickering emulsions. *Carbohydrate Polymers*, 224, 115190.

Chapter 4 investigates the potential use of the Pickering emulsions stabilized with chitosan/gum Arabic nanoparticles (that were successfully developed in Chapter 3) for the encapsulation and topical delivery of resveratrol. The work also examines the ability of the developed biopolymeric-based Pickering emulsions to protect trans-resveratrol against UV degradation. This is the first study in the literature that investigates the use of Pickering emulsions as topical delivery carriers of resveratrol. This chapter was published as:

Sharkawy, A., Casimiro, F. M., Barreiro, M. F., & Rodrigues, A. E. (2020). Enhancing trans-resveratrol topical delivery and photostability through entrapment in chitosan/gum Arabic Pickering emulsions. *International Journal of Biological Macromolecules*, 147, 150–159.

Chapter 5 introduces a new stable Pickering emulsion system stabilized with chitosan/collagen peptides nanoparticles. It then investigates the microstructure, stability, and rheological properties of the produced Pickering formulations, which can be used in food and cosmetic applications. Moreover, this chapter provides new insights into the fate of the nanoparticles used to stabilize Pickering emulsions following their skin application by tracking their skin distribution, which is a research point scarcely addressed by studies that focus on the use of Pickering emulsions in cosmetic applications. This chapter was published as:

Sharkawy, A., Barreiro, M. F., & Rodrigues, A. E. (2021). New Pickering emulsions stabilized with chitosan/collagen peptides nanoparticles: Synthesis, characterization and tracking of the nanoparticles after skin application. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 616, 126327.

Chapter 6 aims to contribute to the ongoing research in the area of sustainable cosmetics. It mainly explores the potential of the Pickering emulsions stabilized with chitosan/collagen peptides nanoparticles (that were introduced and studied in Chapter 5) as green non-toxic topical delivery vehicles of cannabidiol (CBD), a trending ingredient in the cosmetic market. It also investigates the physicochemical properties and encapsulation efficiencies of the produced Pickering emulsions, and the effect of using different oils and different oil volume fractions on these properties, as well as on the skin deposition of the encapsulated CBD. This is the first study in the literature that reports the encapsulation of CBD in Pickering emulsions and their utilization as potential eco-friendly cosmetic vehicles for CBD. This chapter was published as:

Sharkawy, A., Silva, A. M., Rodrigues, F., Barreiro, F., & Rodrigues, A. (2021). Pickering emulsions stabilized with chitosan/collagen peptides nanoparticles as green topical delivery vehicles for cannabidiol (CBD). *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 631, 127677.

Chapter 7 investigates the effect of the degree of deacetylation of chitosan on the properties of chitosan/gum Arabic complex particles, as well as on the properties and stability of the produced Pickering emulsions. The work also studies the impact of the degree of deacetylation of chitosan on the encapsulation efficiency and topical delivery of

CBD, which was used as the model bioactive agent. This is the first study that evaluates the impact of chitosan degree of deacetylation on the physicochemical properties of Pickering emulsions stabilized with chitosan-based complex particles and compares their potential as dermal delivery vehicles of lipophilic active agents.

Chapter 8 states the main conclusions of the thesis and provides suggestions for future work.

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2 Chitosan-based Pickering emulsions and their applications: State of the art

Pickering emulsions, which are emulsions stabilized by solid particles, have gained increased research interest owing to their eco-friendliness and high stability. Among a wide range of solid particles, chitosan particles and chitosan-based particles have become attractive candidates as Pickering stabilizers due to their biodegradable, biocompatible and nontoxic properties. This chapter aims to provide an insight into the recent advances in the production and physicochemical properties of these systems. Moreover, it highlights the research progress in employing chitosan-based Pickering emulsions in different application areas, such as food and cosmetic applications, and environmental research. Chitosan-based Pickering emulsions have opened new avenues for the designing and production of innovative materials. The chapter also sheds light on the novel materials that are synthesized from these Pickering emulsion systems. Future research directions and opportunities on these promising Pickering systems are also addressed.

This chapter is based on:

Sharkawy, A., Barreiro, M. F., & Rodrigues, A. E. (2020). Chitosan-based Pickering emulsions and their applications: A review. *Carbohydrate Polymers*, 250, 116885.
<https://doi.org/10.1016/j.carbpol.2020.116885>

2.1 Introduction

Chitosan is a linear polysaccharide obtained by the deacetylation of chitin, naturally occurring in the exoskeletons of crustaceans, insect cuticles and cell walls of some fungi. Chitosan is constantly inspiring industrial and academic sectors to develop novel chitosan-based formulations and materials (Bernkop-Schnürch & Dünnhaupt, 2012). It is a biopolymer with antibacterial, antifungal, mucoadhesive and gelling properties. It has been known as a promising candidate in the areas of tissue engineering and drug delivery due to its non-toxicity, biodegradability, and biocompatibility. Its diverse properties and cationic nature make it unique in comparison with other polymers. The cationic nature of chitosan has allowed its use in several applications, taking advantage of the interaction with the negatively charged moieties in proteins, enzymes, cell membranes and various other polymers (Bernkop-Schnürch & Dünnhaupt, 2012; de Farias, Sant'Anna Cadaval Junior, et al., 2019). Chitosan has been used in several materials such as aerogels, hydrogels, electrospun fibers, microcapsules and nanoparticles.

With regard to the food industry, chitosan has been used in many countries as a dietary food additive and as a food processing co-adjuvant agent (Bakshi et al., 2019; Costa et al., 2018). Chitosan has dietary health benefits owing to its ability to bind with fat, and therefore it is used to control obesity (Bakshi et al., 2019). Additionally, chitosan, as a dietary fiber, has been reported to have hypocholesterolemic activity as it is capable of reducing total cholesterol and low-density lipoprotein cholesterol (van der Gronde et al., 2016). Its film-forming properties and potent antimicrobial activity allowed its use in food packaging applications. Moreover, it is used in food coating and protection to enhance the sensory properties of food (Mujtaba et al., 2019).

Chitosan is of great interest for the cosmetic industry due to its anti-oxidant and antibacterial properties (Aranaz et al., 2018). It has been reported to have superior skin hydration effects due to its high “water-holding” capacity (Chen & Heh, 2000). Chitosan is also used as a viscosity controlling agent in cosmetic formulations since product viscosity can be modified by changing polymer concentration and molecular weight (Aranaz et al.,

2018). It has also been employed to preserve the sensory properties of fragrances and prolong their release (Lopes et al., 2019).

Emulsions, such as creams, gels, pastes, ointments, and lotions are widely used in cosmetic, food and pharmaceutical applications. Simple conventional emulsions are colloidal dispersions that are formed of two phases, the oil phase and the aqueous phase. Emulsions are thermodynamically unstable systems as they are susceptible to phase separation, coalescence and Ostwald ripening (McClements & Jafari, 2018). Therefore, stabilizers, such as emulsifiers and texture modifiers are included in emulsion formulations to avoid these undesirable phenomena which interfere with stability.

Pickering emulsions are defined as emulsions stabilized by solid particles instead of the classical emulsifiers (Chevalier & Bolzinger, 2013). Their surfactant-free nature makes them promising candidates to overcome the adverse effects of the classical emulsifiers on the environment and health. They are named after S.U. Pickering who reported them in 1907 (Pickering, 1907). Stabilization of Pickering emulsions occurs by reducing the interfacial energy between the two immiscible phases through the adsorption of the solid particles at the surface of the emulsion droplets. The wettability of the particles determines the type of the formed emulsion; whether oil-in-water (o/w) or water-in-oil (w/o) emulsion (Binks, 2002). The biocompatibility and high stability of chitosan-based Pickering emulsions make them advantageous for food and cosmetic applications (Costa et al., 2020; Sharkawy et al., 2020).

The main objective of this chapter is to review the recent knowledge and advances in chitosan-based Pickering emulsions. The production of Pickering particles made of chitosan solely, by complexing it with other polymers, or through the hydrophobic modification of its structure has been comprehensively reviewed and discussed. The work also addresses the factors affecting the formation of Pickering emulsions by chitosan-based particles. The production methods and physicochemical properties of chitosan-based Pickering emulsions are discussed. The chapter then focuses on the use of these Pickering emulsion systems in food and cosmetic applications, and environmental research. The utilization of chitosan-based Pickering emulsions in the production of novel microcapsules

and materials is also covered. The final section addresses the challenges and future directions related to chitosan-based Pickering emulsions.

2.2 Chitosan structure and properties

Chitosan consists of repeated β -(1 $\rightarrow$ 4) *D*-glucosamine and *N*-acetyl-*D*-glucosamine units (Atarian et al., 2019; Mwangi, Ho, Tey, et al., 2016). It is a water-insoluble polysaccharide at neutral pH. However, it is readily soluble in dilute acid aqueous solutions. At pH values lower than its pKa (< 6.5), the amino groups become protonated (as illustrated in Figure 2.1), allowing chitosan to interact with negatively charged compounds, such as fatty acids, proteins and anionic polysaccharides. Chitosan is obtained from the partial deacetylation of chitin. The degree of deacetylation (DDA) reflects the amount of the *D*-glucosamine units present per molecule (Klinkesorn, 2013). Chitosan DDA varies between 60 and 100% (Croisier & Jérôme, 2013). Chitosan with a higher DDA possesses a higher number of amino groups, which increases the amount of positive charges in acidic media, and thus its solubility. This subsequently leads to a higher capacity to form complexes with anionic substances (Klinkesorn, 2013). The protonation of the amino groups at acidic pH results in the expansion of the polymeric chains (volume increase) due to electrostatic repulsion. This phenomenon becomes more prominent as the DDA increases. In contrast, a lower DDA, i.e. the existence of a greater number of acetylated groups, increases the rigidity of the polymeric chains since it favors hydrogen bonding (Klinkesorn, 2013).

Apart from the DDA, the molecular weight (MWt) of chitosan has also a great influence on its solubility and viscosity. The MWt of chitosan depends on its source and preparation method. The fungal chitosan has a relatively lower MWt than that from an animal source (Pochanavanich & Suntornsuk, 2002). Low molecular weight chitosan has a MWt < 50 kDa, while medium molecular weight chitosan is in the range of 50-250 kDa, and high molecular weight chitosan has a MWt > 250 kDa (de Farias, Grundmann, et al., 2019). Chitosan of higher molecular weight gives solutions of higher viscosity, whereas lower molecular weight chitosan has better solubility (Klinkesorn, 2013).

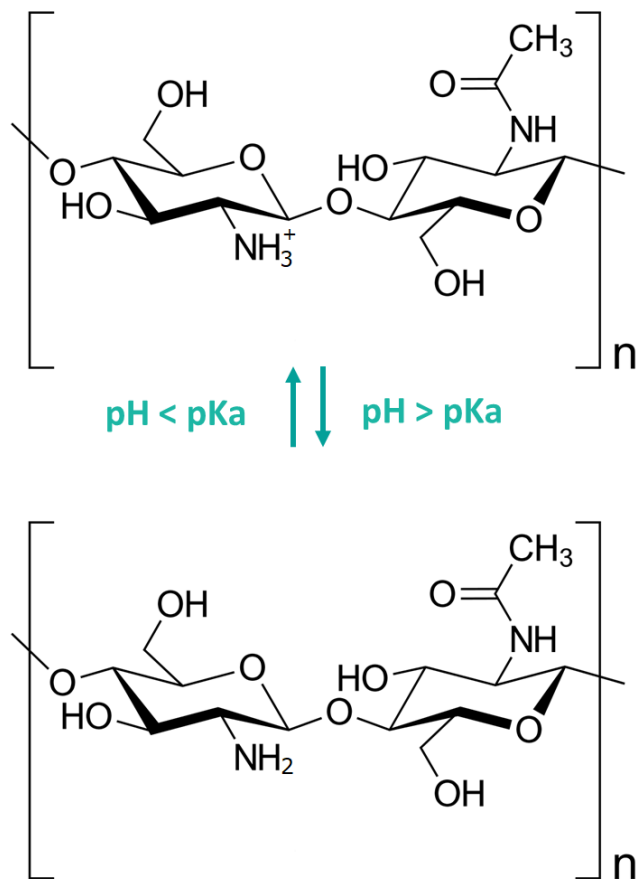


Figure 2.1. The protonated and deprotonated forms of chitosan as a function of the pH of the medium.

2.3 Chitosan in classical (conventional) emulsions

Emulsions consist of two immiscible phases, a dispersed phase comprising spherical droplets which are distributed in a continuous phase. The process of emulsification entails the disruption of the dispersed phase into small droplets of a high surface area via homogenization. This step is subsequently followed by the stabilization of the generated droplets' interfaces by means of emulsifiers. The role of the emulsifiers is to adsorb rapidly at the surface of the formed droplets and create a protective layer around them, which reduces the interfacial tension between the two immiscible phases and guards against the coalescence of the formed droplets that tend to revert to larger droplets (Dickinson, 2009; Klinkesorn, 2013). Chitosan has been applied to stabilize classical emulsions through

increasing the viscosity of the continuous phase, or by forming a layer at the surface of the dispersed oil droplets alongside with other adsorbed surface-active agents, such as anionic surfactants or proteins (X. Y. Wang & Heuzey, 2016a).

Emulsifiers are generally given a value called the hydrophilic-lipophilic balance (HLB). This value expresses the emulsifier compatibility with the water and oil phases indicating its suitability to stabilize (o/w) or (w/o) emulsions. The HLB scale typically ranges from 0-20 (Martins et al., 2011). Conventional emulsifiers with HLB values in the range of 9.6-17.6 stabilize o/w emulsions, whereas those with values ranging from 4.7-6.7 stabilize w/o emulsions (Sharkawy et al., 2017; L. Zhang & Que, 2008). The HLB scale was originally developed for non-ionic emulsifiers (Housaindokht & Nakhaei Pour, 2012). However, it is now used in a wider way for other compounds. For instance, oleic acid has a value of 0.1 and sodium lauryl sulfate, which is an anionic surfactant, has a value of 40 (Klinkesorn, 2013). The HLB value of chitosan has been reported to be 34-36.7 (Klinkesorn, 2013; Schulz et al., 1998). This value indicates that chitosan has an emulsifying capacity. Nevertheless, it is not as high as the other conventional emulsifiers. It is postulated that the structural heterogeneity of chitosan is the reason for its emulsifying capacity. The D-glucosamine units which are the major component of chitosan represent the hydrophilic moieties, whereas the N-acetyl groups represent the hydrophobic parts. So, it is expected that chitosan with a high degree of deacetylation (i.e., with a low number of N-acetyl groups) would be more hydrophilic. This heterogeneity allows chitosan to be adsorbed at the oil/water interface providing mechanical and electrostatic hindrance and preventing droplets coalescence (Schulz et al., 1998). However, the emulsifying capacity of chitosan is considered “a matter of debate” (Klinkesorn, 2013). It has been reported that chitosan has no surface activity at low concentrations but its emulsification capability was enhanced at a concentration of 0.75% (X. Li & Xia, 2011). It has also been reported that the emulsification properties of chitosan are influenced by the DDA. Solutions of chitosan with DDA between 73% and 95% were investigated for their emulsification properties. It was found that the optimal DDA values lay between 80%-89%, and the best ones were namely 81% and 88% as they have resulted in complete emulsification without showing any residual oil in the preparation of o/w emulsions. It was also observed that highly

deacetylated chitosan has poor emulsification properties. However, the authors noted that the changes in the type/nature of oil can influence the obtained emulsion characteristics and may require chitosan with different DDA (Agulló et al., 2003; Del Blanco et al., 1999).

2.4 Chitosan-based particles as Pickering emulsion stabilizers

Chitosan nanoparticles have long been used as effective carriers for controlled drug delivery (Ali & Ahmed, 2018), and more recently, they have been successfully employed as Pickering stabilizers. Chitosan-based particles used in Pickering emulsion stabilization can be made of chitosan solely, by complexing with other polymers, or through the hydrophobic modification of its structure. The preparation methods of chitosan-based Pickering particles can be classified into four main methods (Figure 2.2), and are discussed in the following subsections.

2.4.1 Self-aggregation

The most common methodology to prepare chitosan nanoparticles is self-aggregation. It occurs through the deprotonation of the amino groups at alkaline pH, which reduces the surface charge, leading to the self-assembly of chitosan into colloidal particles. Several studies have reported the utilization of chitosan nanoparticles prepared by self-aggregation in the preparation and stabilization of Pickering emulsions (Asfour et al., 2017; Liu et al., 2012; Mwangi, Ho, Tey, et al., 2016). The presence of amino groups within chitosan structure makes it a pH-responsive polymer. Chitosan nanoparticles are obtained at a pH slightly higher than chitosan pKa (pKa ~6.5) (Ho et al., 2016). At low pH, chitosan has a cationic character due to the protonation of its amino groups, which results in high transmittance and a transparent solution. At pH close to 6.5 (near the pKa of chitosan), the amino groups of chitosan become deprotonated leading to the formation of interpolymer interactions/associations which result in the formation of nanoparticles. The formation of the nanoparticles turns the solution opalescent. At higher pH (e.g., pH 9), aggregates/precipitates are formed, and hence the transmittance significantly decreases. By decreasing the pH, the aggregates become dissolved, and the solution gets transparent again, reflecting the pH-tunable sol-gel transition character of chitosan.

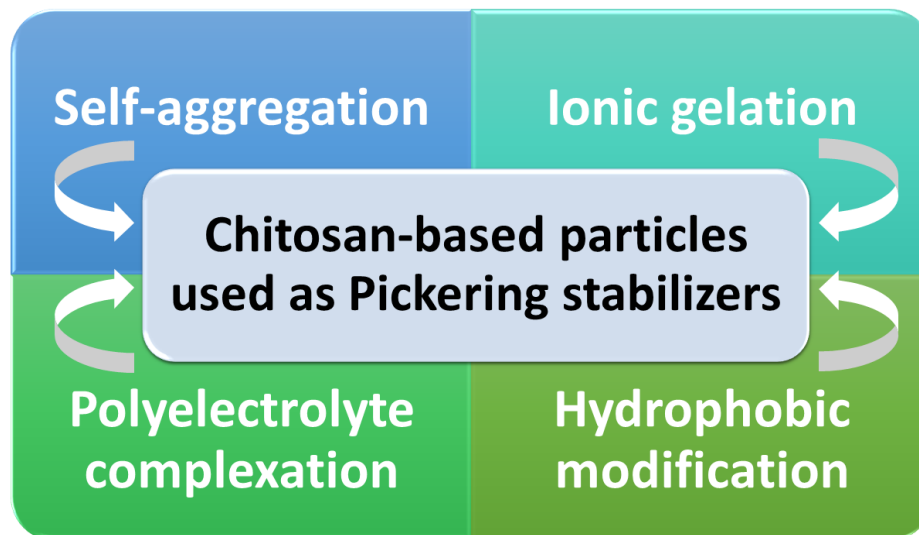


Figure 2.2. Production methods of the chitosan-based particles used in the stabilization of Pickering emulsions.

The stability of Pickering emulsions produced by self-aggregated chitosan particles depends on the initial pH of the aqueous phase. Liu et al. observed that no emulsions were formed at low pH (4.1) (chitosan solution); which indicates that “chitosan itself is not a surfactant” at this pH. This was confirmed by determining the interfacial tension of chitosan solution at this pH, which was found to be similar to that of water. In contrast, the nanoparticles produced at a pH close to 6.5, and the aggregates (precipitate) produced at pH > 9 were able to form Pickering emulsions. The formed Pickering emulsions stabilized by chitosan particles produced at pH > 6.5 maintained their stability for 2 months, whereas the Pickering emulsions stabilized by the particles produced at pH < 6.5 were unstable as they rapidly demulsified after 2 days of preparation. The results reflected that chitosan nanoparticles produced at pH 6-6.5 have a high hydrophilic surface due to the partial deprotonation of the amino groups, making them weak emulsifiers for o/w Pickering emulsion stabilization. The chitosan aggregates formed at pH > 9 have a relatively hydrophobic surface due to the complete deprotonation of the amino groups making them suitable emulsifiers for o/w Pickering emulsions (Liu et al., 2012).

2.4.2 Ionic gelation

The production of chitosan nanoparticles by the ionic gelation method is attracting increasing attention as it is simple and economic. This method is promoted by the ionic attraction between the positively charged amino groups of chitosan (at $\text{pH} < \text{pKa}$ (6.5)) and the negatively charged groups of a polyanion. In this context, the polyanion triphosphosphate (TPP) has been extensively used for the ionic gelation of chitosan and the production of chitosan-based particles. These particles have been used for the encapsulation of bioactive agents (S. F. Hosseini et al., 2013; Luo et al., 2010), and recently as Pickering emulsion stabilizers (Shah, Li, et al., 2016; Tian et al., 2019). Pickering emulsions stabilized by chitosan-based particles produced through the ionic-gelation method have been employed to control the rate of lipid digestion and as vehicles for the successful oral delivery of different bioactive agents (as will be discussed in Section 2.8.1).

2.4.3 Polyelectrolyte complexation

The formation of chitosan-based nanoparticles through polyelectrolyte complexation between chitosan and an oppositely charged polymer has been frequently reported for the encapsulation and delivery of bioactive agents (M. F. Li et al., 2018; Mumuni et al., 2020). Complexation between chitosan and other polymers is mainly promoted by intermolecular electrostatic attraction between the positive charges of chitosan at acidic pH and the negatively charged polymers at the same pH. Besides, it involves intermolecular hydrogen bonding formation, Van der Waals forces and hydrophobic interactions (D. Wu et al., 2020). Sometimes polyelectrolyte complexation is referred to as ionic gelation (Avadi et al., 2010); since the charged polymers act as polyions. Chitosan-based complex nanoparticles have opened new avenues for the development of various novel Pickering emulsion systems. Complexation of chitosan with other polymers renders it more surface-active (Murray, 2019), and thus facilitates the tuning of its wettability. This subsequently leads to increasing the stability of the produced Pickering emulsions. The polyelectrolyte complexation method allows the formation of various chitosan-based Pickering particles through the electrostatic attraction of chitosan with negatively charged materials, such as organic and inorganic polymers, surfactants, polymeric particles and some bacteria.

Chitosan-based complex particles can be obtained by the electrostatic complexation of chitosan with either proteins or polysaccharides as long as the two polymers become oppositely charged at the same pH range. The majority of the studies on Pickering emulsions stabilized by chitosan-based complex nanoparticles involved the complexation of chitosan with proteins, such as chitosan/gelatin (X. Y. Wang & Heuzey, 2016b), chitosan/gliadin (M.-F. Li et al., 2019) and zein/chitosan (L.-J. Wang et al., 2016) complex particles. However, very few studies reported the production of Pickering emulsions stabilized by complex nanoparticles formed by chitosan and other polysaccharides, e.g. chitosan/gum Arabic (Sharkawy et al., 2019), and chitosan/octenyl succinic anhydride (OSA) starch (Yan et al., 2019) complex particles.

Most of the chitosan-based complex particles are used solely to stabilize Pickering emulsions. However, in some cases, an emulsifier is also incorporated in the system to offer a synergistic stabilization effect. For example, zein/chitosan complex particles were used along with RH40 (a non-ionic emulsifier) to enhance the stability of the developed Pickering emulsions (Y. Zhang et al., 2017).

Chitosan-based complex Pickering particles can be also formed by the complexation of chitosan with a surfactant. Complex particles of chitosan/sodium dodecyl sulfate (SDS) (an anionic surfactant) were formed by non-covalent electrostatic attraction. The complexation of chitosan with SDS, even at trace amounts of SDS, was found to enhance the hydrophobicity and surface activity of chitosan (Ren & Zhang, 2020). The produced Pickering emulsions presented switch-ability behavior; as they were reversibly destabilized by the introduction of hexadecyltrimethylammonium bromide (CTAB), which promotes the desorption of SDS from chitosan and initiates its combination with CTAB.

Chitosan-based Pickering stabilizers can be also produced by the coating of polymeric particles with chitosan through complexation. For example, the coating of alginate particles with chitosan reduced the high hydrophilicity of alginate giving rise to stable Pickering emulsions. Chitosan was adsorbed onto alginate particles by electrostatic interactions (Nan et al., 2014). Another example is the complexation between chitosan and polystyrene (PS) particles, which has been reported to enhance the stability of Pickering emulsions in comparison with systems produced by only PS particles or chitosan (S. Zhang et al., 2015).

The emulsification mechanism of the complexes depended on the adsorption of chitosan onto the PS particles which subsequently induced their flocculation, enabling them to be anchored at the oil-water interface. When chitosan was added at low concentrations (0.05-0.1 wt%), the flocculation of the particles improved the emulsifying capacity. However, complexes became more hydrophilic with the increase of chitosan concentration above this range, which led to lower emulsification ability (S. Zhang et al., 2015). In the same context, chitosan-modified silica nanoparticles were investigated to produce stable Pickering emulsions (Alison et al., 2016, 2018). The hydrophobicity of the negatively charged inorganic silica nanoparticles was increased by the adsorption of the positively charged chitosan through electrostatic modification. The obtained food-grade emulsions were found to be stable for more than 3 months. The microstructure of the produced emulsions was influenced by chitosan concentration. At a low concentration of chitosan (1 wt %, silica-basis), a film of particles was formed at the oil/water interface, while at a higher concentration (5 wt %, silica-basis) the droplets became surrounded by a percolating network of the particles responsible for the stabilization of the formed Pickering emulsions (Alison et al., 2016).

Chitosan-based complex particles can be also obtained by the complexation of chitosan derivatives with other polymers. Glycidyltrimethylammonium chloride (GTMAC)-chitosan, which is a water-soluble chitosan derivative, was prepared by the reaction between the amino groups of chitosan and the epoxy group GTMAC (a quaternary ammonium compound containing a glycidyl group). The produced GTMAC-chitosan was complexed with phosphorylated cellulose nanocrystals to produce complex particles that were successfully used to stabilize Pickering emulsions (Baek et al., 2019).

In a slightly different approach, bacteria/chitosan self-assembled networks were produced by complexation between negatively charged bacterial cells and positively charged chitosan. The produced particles gave rise to highly stable o/w Pickering emulsions, which can be considered as sustainable microreactors. They depend on the bacterial cells and the biodegradability of chitosan and can find use in various organic synthesis assisted by microorganisms (Wongkongkatep et al., 2012). In that study, chitosan significantly modified the wettability of the bacterial cells (*E. coli*) as the value of the contact angle was

found to change from $15.8 \pm 1.0^\circ$ to $54.5 \pm 2.0^\circ$. Thus, chitosan had an important role in the stability of the obtained Pickering emulsions.

2.4.4 Hydrophobic modification

The fourth method to obtain chitosan-based particles for Pickering stabilization involves the hydrophobic modification of chitosan. The modification usually takes place through the reaction of chitosan amino groups with the carboxyl groups of compounds possessing alkyl groups, which leads to the addition of a hydrophobic segment to chitosan, and thus results in improving its emulsifying properties. In this context, Atarian and co-workers prepared chitosan/stearic acid nanogel particles through amide bond formation between the amino groups of chitosan and the carboxylic groups of stearic acid. This covalent cross-linking reaction is mediated through EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) that activates the carboxyl groups. The resulting chitosan/stearic acid nanogel particles were used to develop sunflower o/w Pickering emulsions (Atarian et al., 2019). The authors reported that more stable emulsions were obtained at higher stearic acid to chitosan ratios, and alkaline pH. Similarly, chitosan/myristic acid nanogel particles were prepared through the formation of amide bonds between chitosan and myristic acid by an EDC-mediated reaction (E. Hosseini et al., 2019). The most stable Pickering emulsions were obtained at a myristic acid to chitosan ratio of 0.75:1 and pH 8.

It is noteworthy that chitosan-based Pickering particles can be formed by the combination of the hydrophobic modification method and another method. For instance, particles formed of dihydrocaffeic acid modified chitosan and tripolyphosphate were prepared through amidation reaction and ionic gelation (R. Zhang et al., 2019). The dihydrocaffeic acid/chitosan copolymer amidation synthesis reaction was also mediated by EDC, followed by the formation of particles through ionic gelation with sodium tripolyphosphate. These particles produced Pickering emulsions that were stable over a wide pH range (2-10) (R. Zhang et al., 2019).

The formation of amide bonds can also take place between chitosan derivatives and carboxyl groups through the same EDC-mediated reaction. An example of this reaction is the amide bond formation between the water-soluble chitosan hydrochloride and

carboxymethyl starch (X.-M. Li et al., 2019). The developed nanogel particles were employed to produce Pickering emulsions, where they formed a densely packed layer around the emulsion droplets which significantly contributed to maintaining emulsion stability. It is worth mentioning that in this study, and in contrast to the previous ones on amide linkage formation, carboxymethyl starch was used to decrease the hydrophobic character of chitosan hydrochloride and tune the contact angle to near-neutral wettability (as further discussed in Section 2.5.1).

Figure 2.3 shows examples of chitosan-based particles that are used in the stabilization of Pickering emulsions. It can be observed that they could be designed and modulated to have different morphologies and sizes, depending on their composition and preparation method.

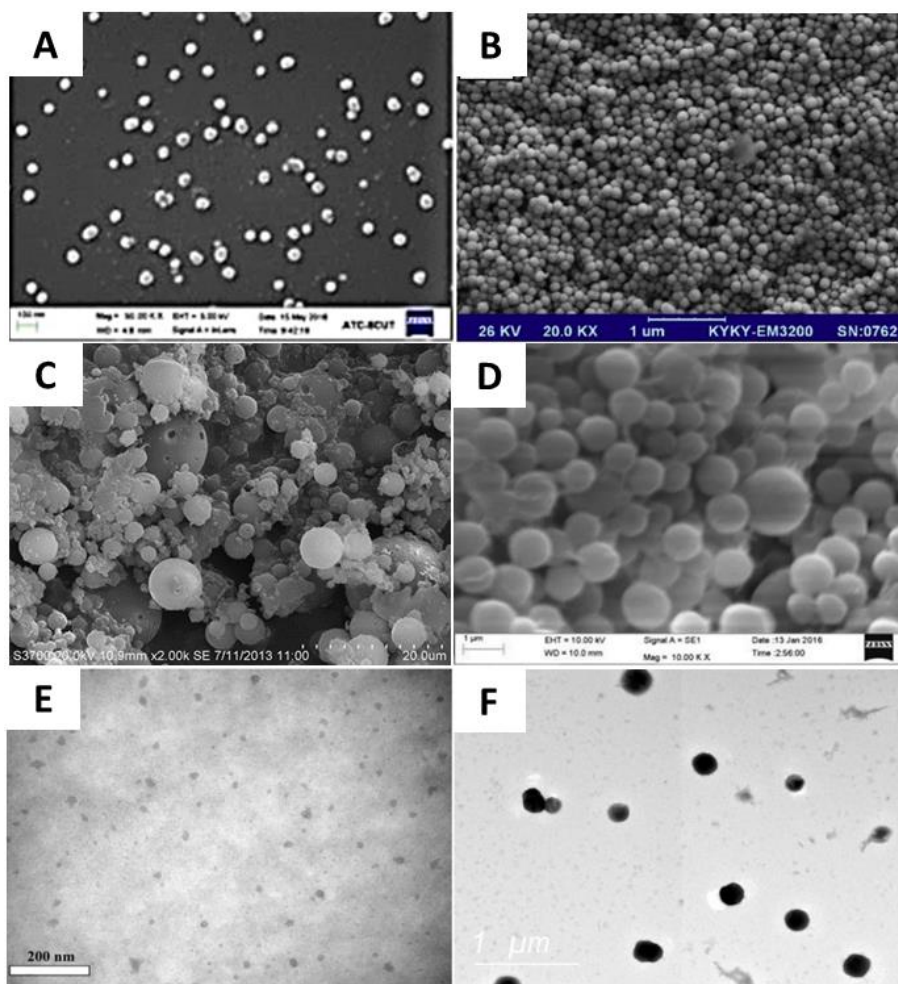


Figure 2.3. Electron microscope images of some chitosan-based nanoparticles used for Pickering emulsions stabilization: A) SEM image of chitosan/caseinophosphopeptides

complex particles. Reprinted from (Huang et al., 2019), Copyright (2019), with permission from Elsevier; B) SEM image of chitosan/stearic acid nanogels prepared through the hydrophobic modification of chitosan. Reprinted from (Atarian et al., 2019), Copyright (2019), with permission from Elsevier; C) SEM image of zein/chitosan complex particles. Reprinted from (L.-J. Wang et al., 2016), Copyright (2016), with permission from Elsevier; D) SEM image of gliadin/chitosan hybrid complex particles. Reprinted from (Zeng et al., 2017), Copyright (2017), with permission from Elsevier; E) TEM image of chitosan/tripolyphosphate (TPP) particles prepared by ionic gelation. Reprinted from (Shah, Li, et al., 2016), Copyright (2016), with permission from Elsevier, and F) TEM image of self-aggregated chitosan nanoparticles. Reprinted from (Asfour et al., 2017), Copyright (2017), with permission from Elsevier.

2.5 Factors influencing the formation of Pickering emulsions by chitosan-based particles

2.5.1 Wettability

The wettability of the particles is an important criterion to prepare Pickering emulsions as it controls the type of the formed Pickering emulsion, as well as its stability. The wettability of the particles is determined by the value of the contact angle (θ) (Figure 2.4). Particles with contact angle (θ) of less than 90° are hydrophilic and tend to form o/w emulsions, whereas particles with contact angle higher than 90° are hydrophobic and form w/o emulsions. The most stable Pickering emulsions are formed when the contact angle is close to 90° (Binks, 2002). Consequently, developing particles of an amphiphilic nature or with an appropriate wettability is one of the most important steps in formulating Pickering emulsions (Yang et al., 2017). The suitable wettability enhances particles adsorption at the water/oil interface and subsequently provides a steric hindrance that guards against emulsion droplets coalescence. The amount of energy required to remove (desorb) a solid particle from the oil/water interface is given by equation (1):

$$\Delta E = \pi r^2 \gamma_{o/w} (1 \pm \cos \theta)^2 \quad (1)$$

where r is the particle radius, $\gamma_{o/w}$ is the oil/water interfacial tension (Binks, 2002; Binks & Yin, 2016). The plus sign refers to the particle desorption into the oil phase, whereas the

minus sign refers to that in the case of the water phase (Binks & Yin, 2016). The value of ΔE for sub-micron particles is always greater than the thermal energy of the particle. Therefore, when solid particles are adsorbed at the interface, they can be considered as being irreversibly anchored (Binks & Yin, 2016; Tang et al., 2015).

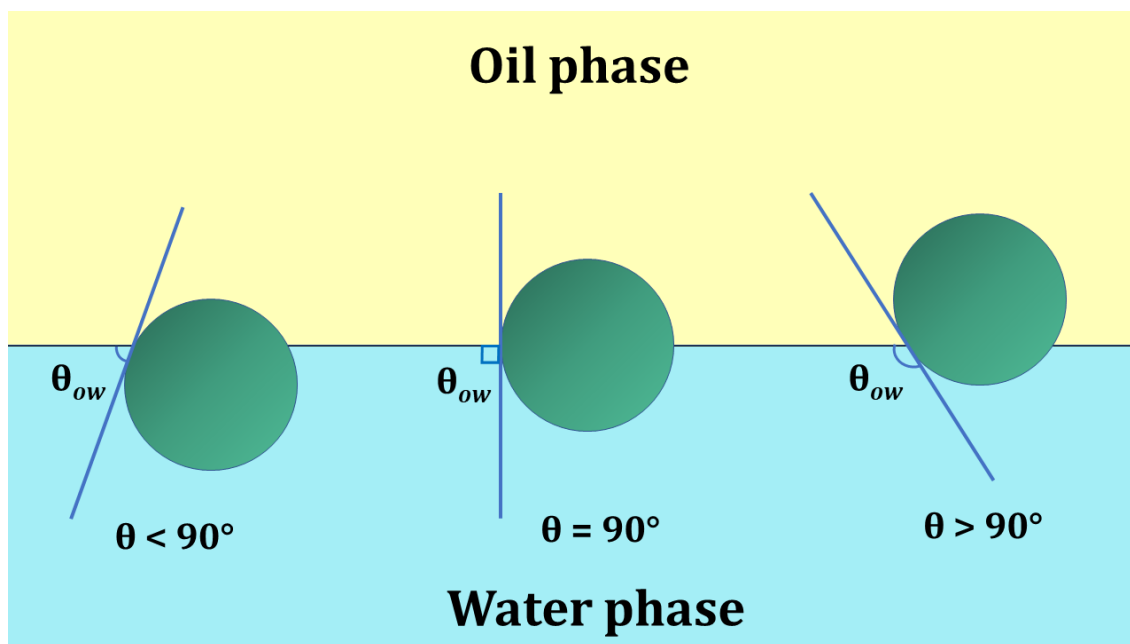


Figure 2.4. Schematic representation of the wettability of solid particles at the oil/water interface as determined by the value of the contact angle (θ_{ow}) (Binks, 2002); (left) hydrophilic particle, (right) lipophilic particle and (center) equally hydrophilic and lipophilic particle (i.e. particle with an intermediate wettability).

Chitosan nanoparticles used as Pickering stabilizers have been reported to be hydrophilic. Asfour et al. reported that chitosan nanoparticles produced by self-aggregation with NaOH had a contact angle of $42.47 \pm 1.19^\circ$ (Asfour et al., 2017). To enhance the wettability of chitosan-based nanoparticles, Pickering stabilizers formed of chitosan and other polymers have been developed. Moreover, the modification of the wettability of chitosan-based particles (hybrid particles) to obtain a near-neutral wettability has been achieved by some strategies that were reported in several studies. Figure 2.5 summarizes these strategies. The first strategy involves controlling the pH. For example, the contact angle of gliadin/chitosan complex nanoparticles was reported to increase as the pH increases. At a pH of 3, the contact angle of the particles was 37.88° which indicates their weak adsorption capacity at the oil/water interface. The contact angle increased to 82.74° and 96.32° by increasing the

pH to 5 and 7, respectively, suggesting enhanced lipophilicity of the particles with pH increase. The increased lipophilicity is attributed to the increased interaction between gliadin and chitosan by the electrostatic attraction and hydrogen bonding (M.-F. Li et al., 2019). It was also observed that the wettability of octenyl succinic anhydride (OSA) starch was enhanced by the addition of chitosan (Yan et al., 2019). The contact angle of OSA starch was reported to be 36.83° indicating that it is extremely hydrophilic and has a weak adsorption capacity at the oil/water interface. In the same study, the contact angle of the chitosan solution was 136.50° . Perhaps this hydrophobicity is related to a low degree of DDA, although the DDA was not mentioned in that study. The contact angle of the hybrid particles increased by increasing the concentration of chitosan, indicating the modification of the wettability of OSA starch by the presence of chitosan. It was also observed that the contact angle of the particles increased from 56.67° to 95.70° when the pH value increased from 3 to 7 due to the stronger interaction between OSA starch and chitosan through electrostatic interactions (Yan et al., 2019). In another study, chitosan hydrochloride was reported to be predominantly hydrophobic; as it showed a contact angle of 116.20° (with a DDA% of 85.47%), whereas carboxymethyl starch had a contact angle of 37.45° , which reflects its high hydrophilicity. Chitosan hydrochloride/carboxymethyl starch nanogel particles that were produced through amide bond formation between the two polymers had a contact angle close to 90° , indicating wettability tuning to a near-neutral value, which helped in acquiring stable Pickering emulsions (X.-M. Li et al., 2019).

Aside from the pH, the wettability of chitosan-based complex particles can be also tailored through using different mass ratios of chitosan to the other polymer. Sharkawy et al. developed chitosan/gum Arabic nanoparticles with different mass ratios (4:1, 2:1, 1:1, 1:2 and 1:4) and observed that the contact angle of the particles increased by increasing the amount of gum Arabic (Sharkawy et al., 2019). The increase in the contact angle value is attributed to the amphiphilic structural moieties of gum Arabic. It was concluded that nanoparticles formed using a chitosan/gum Arabic mass ratio of 1:1 had a contact angle of 89.2° , which was the closest to 90° among the tested mass ratios, suggesting that they are more suitable Pickering stabilizers than the ones formed using other chitosan/gum Arabic mass ratios. In another study, Wang et al. reported that the contact angle of zein/chitosan complex particles could be tuned through using different mass ratios of chitosan and zein

(L.-J. Wang et al., 2016). It was shown that the interaction between zein (which is highly hydrophobic) and chitosan introduces hydrophilic moieties (the amino and hydroxyl groups of chitosan) to the nanoparticles, and thus the hydrophobicity of the particles decreases to a suitable value. In this study, it was found that the contact angle closest to 90° was obtained at a chitosan-to-zein ratio of 1:10 and 1:20. Although the ratios of chitosan-to-zein protein in these complex particles are low, the role of chitosan was important in tuning the wettability of the particles, which is a key prerequisite in the formation of Pickering emulsions. This was proved by the enhanced interfacial adsorption of the complex particles that promoted the formation of stable emulsions. Similarly, chitosan in gliadin/chitosan complex Pickering particles, despite its low amount, had an important role in modulating the surface properties of gliadin to produce complex particles with enhanced wettability (Zeng et al., 2017). In another recent study, it was found that changing the mass ratios of chitosan to casein peptides from 3:1 to 1:3 highly influenced the value of the contact angle of the produced Pickering particles, raising it from 37.2° to 78.4° through the hydrophobic groups provided by casein peptides (Meng et al., 2022).

The third method for tuning the wettability of chitosan-based particles involves ultrasonication pre-treatment. The ultrasonication of chitosan, before particle production, decreases the contact angle of the produced particles in comparison with particles formed from a chitosan solution not subjected to ultrasonication (Ho et al., 2016). This increase in the hydrophilicity (reduced hydrophobicity) of chitosan nanoparticles by ultrasonication of the initial chitosan solution can be attributed to the fact that ultrasonication reduces chitosan polymer chains length, leading to the production of a higher number of shorter chains with a lower number of N-acetyl-D-glucosamine units. These units are considered the hydrophobic moieties of chitosan, and when they decrease in number, chitosan hydrophilicity increases. The ultrasonicated chitosan solution (i.e., the solution with a higher number of shorter chains, but with fewer hydrophobic units) results in the production of a higher number of particles, smaller in size, but with higher hydrophilicity levels. Therefore, ultrasonication pretreatment of chitosan can be considered as a solution to produce chitosan nanocomplexes with polymers of high hydrophobicity.

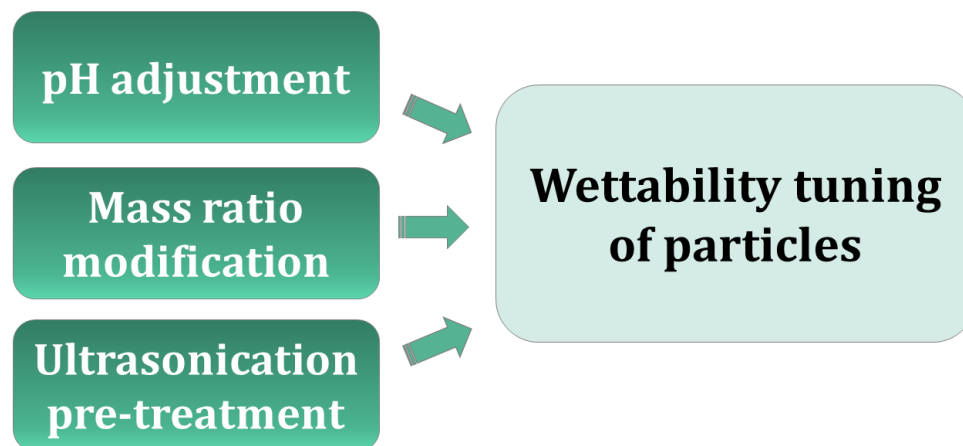


Figure 2.5. Wettability tuning strategies for chitosan hybrid nanoparticles used as Pickering stabilizers.

2.5.2 Size and surface charge of particles

In general, for successful Pickering emulsion formation, the particles should be smaller than the targeted emulsion droplets so that the particles can be properly adsorbed on the surface of the droplets (Tavernier et al., 2016). It should be taken into consideration while designing particles for Pickering emulsions that very small sizes (< 0.5 nm) result in poor emulsion stabilization. This fact can be explained in light of the previous equation (1) (Section 2.5.1). Particles of very small size act similarly to surfactant molecules as they easily become detached from the oil/water interface, and thus cannot be considered as effective stabilizers, whereas larger particles require greater desorption energy (Binks, 2002).

Chitosan-based particles that are used as Pickering stabilizers have different sizes; most of them are in the nanoscale, but some are of micro-size. The size of chitosan-based complex particles has been reported to be influenced by pH. It was observed that gliadin/chitosan nanoparticles exhibited a bigger size by increasing the pH which is attributed to the increase in the electrostatic interactions between gliadin and chitosan (M.-F. Li et al., 2019). The same particles resulted in emulsions of high stability since the wettability was also enhanced by increasing the pH. Similarly, the particle size of OSA starch/chitosan particles was reported to increase by increasing the pH. This is also attributed to the

increased electrostatic interactions between chitosan and OSA starch as pH increases (Yan et al., 2019).

Ultrasonication treatment affects the size and the polydispersity of chitosan particles used as Pickering stabilizers. The untreated particles have shown a unimodal size distribution and a large size (~ 1000 nm), while when ultrasonic treatment was applied, smaller and bimodal size distributions were obtained. The study suggested that smaller particles are more easily adsorbed producing a more homogenous layer around the emulsion droplet, which enhances emulsion stability (Costa et al., 2018).

The value of the surface charge of chitosan-based complex particles can be modulated by changing the pH and ionic strength, and by using different mass ratios of chitosan to the other complexing polymer. The impact of the particle charge on the emulsion stability is a matter of debate/dispute. Theoretically, it has been suggested that highly charged particles cannot produce Pickering emulsions as they will experience strong repulsion between the charges on the particle surface and similar charges at the oil/water interface, inhibiting their adsorption (Low et al., 2020; H. Wang et al., 2012). This was reported in several studies related to chitosan-based Pickering emulsions. For example, in the case of gliadin/chitosan nanoparticles, it was observed that the lower the particles' surface charge, the higher the obtained interfacial absorption (M.-F. Li et al., 2019). In the same study, zeta-potential of the particles was measured at different pH values (3, 5 and 7), and it was observed that the surface charge of gliadin/chitosan particles decreases as pH increases, leading to greater accumulation of the particles at the oil/water interface due to the weakening of the electrostatic repulsion force between the particles, and also due to the improved wettability (as discussed previously in Section 2.5.1) (M.-F. Li et al., 2019). However, this is not always the case since chitosan-based Pickering emulsions stabilized with highly charged particles have also been reported. Zein/chitosan micro-sized complex particles with a high surface charge (+65 mV) were able to produce Pickering emulsions of high stability (L.-J. Wang et al., 2016).

2.5.3 Particle concentration

The stability of chitosan-based Pickering emulsions improves with increasing the concentration of the particles, regardless of their type. The increase of particle concentration is linked to the increase of the available number of particles to cover a larger interfacial area and hence increases emulsion stability. Furthermore, at high chitosan particle concentration, particles were found to form networks in the continuous phase which helped in reducing the droplet collisions and guarded against eventual coalescence (Mwangi, Ho, Tey, et al., 2016).

2.6 Preparation methods of chitosan-based Pickering emulsions

Most of the chitosan-based Pickering emulsion formulations reported in the literature are prepared using rotor-stator homogenization. This is due to the availability, simplicity and low energy requirement of the rotor-stator homogenizers (Albert et al., 2019). Recently, ultrasound-assisted emulsification has been applied to form different emulsions. However, it has been reported that chitosan can be depolymerized by ultrasonication since the high mechanical forces can break the low energy glycosidic linkages. Moreover, it has been reported that the hydrophobicity of chitosan nanoparticles decreases when ultrasonication pre-treatments are used prior to their utilization in Pickering emulsions formation by rotor-stator homogenization. This ultrasonication pretreatment of the chitosan nanoparticles eventually resulted in Pickering emulsions with a high creaming index and low stability (Ho et al., 2016).

The pH of the medium used during the preparation by the ultrasonication process affects the type of the resulting chitosan-based emulsion; whether a Pickering emulsion or a conventional emulsion. High-intensity ultrasonication has a dual effect in the production of chitosan-based emulsions, including Pickering emulsions, as it aids in the homogenization and also depolymerization. The emulsions produced by ultrasonication at different pH values, without the use of any emulsifiers, were found to be stable up to 5 months. The ultrasonication treatment led to the breaking and dispersing of chitosan agglomerates, especially at $\text{pH} \geq 4.5$, resulting in a decrease of its molecular weight. The emulsion

stability and type (whether conventional or Pickering emulsion) are controlled by the pH of the chitosan solution. It was observed that by increasing the pH from 3.5 to 5.5, conventional emulsions with stability from a few days to 2 months were formed. This could be explained by the change of the conformation of chitosan molecules from an extended structure to a more flexible one. At a pH of 6.5, ultrasonication causes chitosan molecules to self-assemble and form nanoparticles leading to the formation of Pickering emulsions with up to 5 months of stability. These results suggest that chitosan emulsification ability is highly influenced by the pH value and ultrasonication treatment (X. Y. Wang & Heuzey, 2016a).

A one-step ultrasound process has been recently used to produce Pickering emulsions stabilized with chitosan nanoparticles. The method involved only an ultrasonication step that was not preceded by rotor-stator homogenization as in the conventional ultrasound-assisted methods. The work studied the effect of the ultrasonication power and time on the stability of the formed Pickering emulsions. It was shown that the droplet size decreased as the ultrasonication power increased. It was also observed that at high ultrasonication power, the emulsion droplets demonstrated bridging (particle sharing), which led to an increase in the viscosity and hence to the stability of the formed Pickering emulsions (Costa et al., 2018).

Table 2.1 shows examples of Pickering emulsions stabilized by chitosan-based particles. The table summarizes the composition of the particles, their size and wettability, and the composition of the dispersed phase. It can be noticed that most of the developed emulsions are of the o/w type. This is because most of the natural biopolymers, including chitosan, are more compatible with water than with oil (Murray, 2019).

2.7 Physicochemical properties of chitosan-based Pickering emulsions

2.7.1 Morphology and interfacial structure

Almost all the reported chitosan-based Pickering emulsion droplets have spherical morphology. This is a common case with other Pickering emulsions, as the spherical morphology results in the minimizing of the surface energy (Yang et al., 2017). Confocal laser scanning microscopy (CLSM) aids in the visualization of the interfacial structure of

the Pickering emulsions and confirms the type of the formed emulsion. It involves the use of two different fluorescent stains to distinguish between the oil phase and the adsorbed nanoparticles positioned at the oil/water interface. Nile Red is commonly used to stain the oil phase, while chitosan insoluble complexes and nanoparticles have been reported to be stained by either fluorescein 5(6)-isothiocyanate (FITC) (Huang et al., 2019; Nan et al., 2014; L.-J. Wang et al., 2016), Acridine Orange (Dammak & José do Amaral Sobral, 2018) or Nile Blue (Calero et al., 2013; Sharkawy et al., 2019). Figure 2.6 gives an insight into the morphology and fluorescent staining of chitosan-stabilized Pickering emulsion droplets. The morphology and the particle adsorption of chitosan-based Pickering emulsions have been also investigated and visualized using scanning electron (SEM) microscopy (S. Zhang et al., 2015) and cryo-SEM (Sharkawy et al., 2020).

2.7.2 Pickering emulsion droplet size

The droplet size of Pickering emulsions is an important parameter to be determined since it strongly influences the properties, stability and applications of the obtained Pickering emulsion (Low et al., 2020). Chitosan-based Pickering emulsions have a wide range of droplet diameters (as can be seen in Table 2.1). It has been reported that the droplet size is influenced by the emulsion preparation method and used parameters (Costa et al., 2018), namely particle concentration, oil volume fraction, pH, ionic strength (Mwangi, Ho, Tey, et al., 2016) and oil type (Shah, Zhang, et al., 2016).

Increasing the concentration of chitosan particles (Mwangi, Ho, Tey, et al., 2016), and chitosan-based complex particles (Sharkawy et al., 2019), was found to decrease the droplet size of the formed Pickering emulsion. This phenomenon is ascribed to the increase in the number of the available particles as the particle concentration increases, which eventually leads to larger interfacial area coverage and the formation of small droplet sizes (Mwangi, Ho, Tey, et al., 2016).

The droplet size of chitosan-based Pickering emulsions is also influenced by the pH. It has been reported that increasing the pH (in the range of 3-8) results in the reduction of the chitosan Pickering emulsion droplet size (obtained at low chitosan concentration 0.1% w/v). This is attributed to the deprotonation of the amino groups with the pH increase,

leading to a weak electrostatic repulsion between the polymer chains of chitosan, a higher adsorption at the interface, and eventually, to a larger interfacial coverage (Mwangi, Ho, Tey, et al., 2016). On the contrary, in another study, it has been reported that the increase in pH, namely in the range 6.4-7, resulted in the increase of the emulsion droplet size (Figures 2.6E and 2.6F) (X. Y. Wang et al., 2020). This finding is perhaps related to the higher chitosan concentration (1% w/v) used in the latter study, comparatively to the former one (0.1% w/v) (Mwangi, Ho, Tey, et al., 2016). Higher chitosan concentration leads to the formation of a greater number of particles that saturate the interface. Eventually, after the interface gets saturated with particles, and as the pH increases, the unadsorbed chitosan particles from the continuous phase start to get deprotonated, which leads to a decrease in the repulsion force and increase in the intra- and inter-particle interactions. This subsequently leads to their clustering and accumulation at the oil/water interface, and thus results in an apparent increase of the emulsion droplet size, which can be observed in the CLSM images presented in Figure 2.6F (X. Y. Wang et al., 2020). Similarly, the droplet size of Pickering emulsions stabilized by gliadin/chitosan complex particles significantly increased as the pH increased from 3 to 7 due to the decrease in the electrostatic repulsion between the particles, and their subsequent increased adsorption and accumulation at the oil/water interface (M.-F. Li et al., 2019).

The type and amount of oil also affect the emulsion droplet size. Chitosan Pickering emulsions prepared with corn oil (it contains long-chain fatty acids) had smaller emulsion droplet sizes, in comparison with those prepared with MCT (medium-chain triglyceride) (Shah, Zhang, et al., 2016). Additionally, at a fixed particle concentration, it was observed that the droplet size of chitosan-based emulsion increases by increasing the oil volume fraction (Atarian et al., 2019; Sharkawy et al., 2019). This increase in the droplet size is attributed to the decrease in the number of chitosan particles (relative to the oil amount) available to become adsorbed at the oil/water interface (Mwangi, Ho, Tey, et al., 2016).

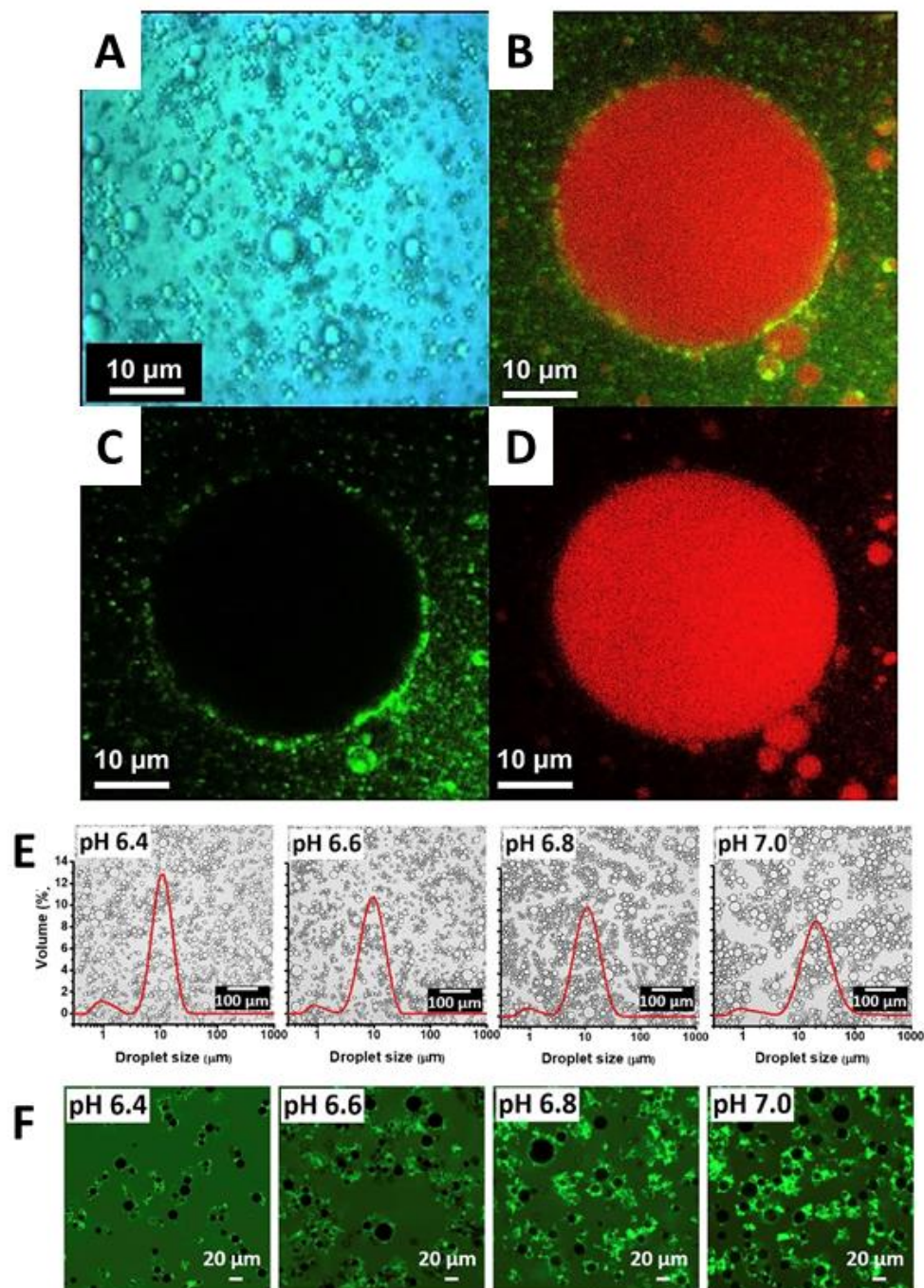


Figure 2.6. A) Optical microscope image of Pickering emulsions stabilized with chitosan nanoparticles and lecithin; B) CLSM image of a Pickering emulsion droplet with the oil phase stained with Nile Red (appears in red) and chitosan nanoparticles stained with Acridine Orange (appear in green); C) CLSM image of the emulsion droplet showing the adsorbed chitosan nanoparticles at the oil/water interface; D) CLSM image of the emulsion

droplet showing the oil phase with red fluorescence. Reprinted from (Dammak & José do Amaral Sobral, 2018), Copyright (2018), with permission from Elsevier; E) Optical microscope images and droplet size distribution of Pickering emulsions stabilized with chitosan nanoparticles at a pH 6.4-7, and F) CLSM images of the same formulations in E. Images E and F are reprinted from (X. Y. Wang et al., 2020), Copyright (2020), with permission from Elsevier.

Table 2.1. Examples of Pickering emulsions stabilized by chitosan-based nanoparticles available in the literature.

Composition of particles as Pickering stabilizers	Average size and wettability* of the particles	Oil phase	Type of emulsion	Emulsion droplet size	Reference
Chitosan nanoparticles obtained via self-aggregation	287.3 nm 42.47°	Rutin loaded in propylene glycol/oleic acid	o/w	5.8- 18.7 μm	(Asfour et al., 2017)
Chitosan nanoparticles obtained via self-aggregation	~350 nm at pH 6.7 *NA	Medium-chain triglycerides (MCT)	o/w	~40-80 μm	(Mwangi, Ho, Tey, et al., 2016)
Chitosan nanoparticles obtained via self-aggregation	474.6 nm *NA	Roasted coffee oil	o/w	8.7 μm (oil phase 50% w/w of emulsion)	(Ribeiro et al., 2019)
Zein/chitosan complex particles	< 1-2 μm , 80.98 ° (chitosan/zein ratio= 1:20)	n-tetradecane	o/w	42.61-76.75 μm (ϕ = 0.2-0.6)	(L.-J. Wang et al., 2016)

Chitosan/gum Arabic complex particles	108.6 nm, 89.2° (chitosan/gum Arabic ratio= 1:1)	Olive oil	o/w	7-32 μm	(Sharkawy et al., 2019)
Chitosan/ lactic acid bacteria/ hydroxypropyl methylcellulose	*NA	Soybean oil	o/w	0.04- 0.12 mm	(Rattanaburi et al., 2019)
Gliadin/chitosan particles	$190.3 \pm 16.5 \text{ nm}$ - $588.8 \pm 18.5 \text{ nm}$	Corn oil ($\phi = 0.1\text{-}0.8$)	o/w	20-90 μm	(Yuan et al., 2017)
Gliadin/chitosan particles	$\sim 600 \text{ nm}$ (pH 5) 82.74° (pH 5) 96.32° (pH 7)	Corn oil loaded and unloaded with curcumin	o/w	$\sim 10\text{-}90 \mu\text{m}$ $\sim 50 \mu\text{m}$ (pH 5) $\sim 90 \mu\text{m}$ (pH 7)	(M.-F. Li et al., 2019)
Gliadin/chitosan particles, and curcumin-loaded gliadin/chitosan particles	$588.8 \pm 13.1 \text{ nm}$ (pH 5). $84.0 \pm 6.0^\circ$	Corn oil	o/w	$93.5 \pm 0.5 \mu\text{m}$ - $25.5 \pm 0.4 \mu\text{m}$	(Zeng et al., 2017)
Octenyl succinic anhydride (OSA) starch/ chitosan complex particles	88.83° (pH 6) 912.9 nm (pH 6)	DHA-rich algae oil (DHA: Docosahexaenoic acid) Oil phase 75% w/w of emulsion	o/w	$16.70 \pm 0.24 \mu\text{m}$ (pH 6)	(Yan et al., 2019)

Hyaluronic acid/chitosan complex colloidal particles,	420.9 nm at a hyaluronic acid/chitosan ratio of 2:1 (non-loaded particles)	White oil	o/w	42.92–56.16 μm and 101.15–125.05 μm	(Zhao et al., 2015)
Chitosan/ tripolyphosphate (TPP) particles	1.54 μm *NA	Citral-loaded in medium chain triglycerides (MCT)	o/w	14.4 μm (for fresh emulsions)	(Tian et al., 2019)
Chitosan/ caseinophosphopeptides nanocomplexes	20 nm at pH 5 *NA	Corn oil, Linseed oil ($\phi = 0.8$)	o/w	31.42 -52.48 μm	(Huang et al., 2019)
Chitosan/myristic acid nanogels	< 200 nm *NA	Flaxseed oil	o/w	~10-75 μm (for fresh emulsions at pH 8)	(E. Hosseini et al., 2019)
Chitosan/ stearic acid nanogels	< 200 nm *NA	Sunflower oil	o/w	Mean~10-60 μm (for fresh emulsions at pH 8)	(Atarian et al., 2019)

Chitosan hydrochloride/ carboxymethyl starch (CHC-CMS) complex nanogels	378.2 nm, 89.30° for complex nanogels at CHC: CMS ratio of 2:1. 116.20° for chitosan hydrochloride alone	Corn oil	o/w	8 μm (for an oil phase $\phi = 0.5$, mean size was constant for 3 months)	(X.-M. Li et al., 2019)
Chitosan/cellulose nanocrystals (CH/CNC) particles crosslinked with glutaraldehyde	505.6 ± 22.3 nm for a weight ratio of 1:4 (CH/CNC), 53.7° and 73.1° values measured with water and oil	Camellia oil	o/w	Ranging between 10 μm and 100 μm depending on the oil phase volume fraction	(Z. Li et al., 2022)

*Value of the contact angle if available.

2.7.3 Rheological properties

The rheological properties of the emulsions are important criteria to be studied as they control the stability of the emulsions (Yuan et al., 2017). They are also critical attributes for the successful application of the developed emulsions in food and cosmetics. Pickering emulsions stabilized by chitosan nanoparticles exhibited shear-thinning behavior; which means that they have high viscosity at low shear rates that decreased as the shear rate increased (Asfour et al., 2017). Besides, the viscosity of Pickering emulsions stabilized with self-aggregated chitosan nanoparticles increases with increasing chitosan concentration. The same phenomenon was observed in Pickering emulsions stabilized by chitosan complex particles, such as chitosan/gum Arabic Pickering emulsions (Sharkawy et al., 2019) and zein/chitosan Pickering emulsions (L.-J. Wang et al., 2016). In these formulations, it was observed that the storage modulus (G') and the loss modulus (G'') increase as the concentration of nanoparticles increases. It was also observed that the storage modulus (G') values of these formulations were higher than the loss modulus (G'') values (at oil volume fraction (ϕ) > 0.5), which suggests the presence of elastic gel networks between the nanoparticles and emulsion droplets. It is worth mentioning that the storage modulus (G') reflects the elastic solid-like character of the material, whereas the loss modulus (G'') indicates the viscous behavior of the material. This indicates that the formed chitosan-based Pickering emulsions have predominant elastic properties and that, rheologically, they are similar to gels. The values of (G') and (G'') were also reported to depend on the pH of the produced Pickering emulsions. In a recent study (M.-F. Li et al., 2019), it was observed that Pickering emulsions stabilized by gliadin/chitosan nanoparticles have shown higher values of (G') and (G'') as the pH increased from 3 to 5 and 7, which indicates that the pH value has a significant impact on the elasticity of the developed Pickering emulsions. To summarize, it can be concluded that the rheology of the chitosan-based Pickering emulsions can be readily tuned by changing their composition and pH.

2.7.4 Emulsion stability

Generally, Pickering emulsions show high stability against coalescence and Ostwald ripening (Chevalier & Bolzinger, 2013; Tavernier et al., 2016). The stability of Pickering

systems is influenced by the particle wettability, particle concentration, particle size, type and amount of the dispersed phase oil and environmental conditions (such as the pH and ionic strength) (Linke & Drusch, 2018; Tavernier et al., 2016).

Increasing the oil volume fraction, with the use of a fixed concentration of chitosan complex particles, reduces phase separation and enhances emulsion stability. This observation was reported when the oil fraction was increased from 0.2 to 0.7 in Pickering emulsions stabilized by chitosan/gum Arabic particles (Sharkawy et al., 2019), and chitosan/zein particles (L.-J. Wang et al., 2016), above which deformation of emulsion droplets occurred due to the presence of an insufficient amount of particles to stabilize the oil/water interface.

The influence of pH on the stability of Pickering emulsions stabilized by chitosan nanoparticles was studied in the pH range of 2 to 8 (Mwangi, Ho, Tey, et al., 2016). The emulsions have shown high stability against coalescence and creaming as the pH increased due to the decrease in the electrostatic repulsion which enhanced the non-adsorbed particles to aggregate at the oil/water interface and thicken the interfacial film. The enhancement of emulsion stability by increasing the pH was also reported for Pickering emulsions stabilized with chitosan complex particles, e.g. chitosan/gliadin complex particles (M.-F. Li et al., 2019).

The coalescence and creaming stability of chitosan Pickering emulsions are enhanced by the addition of salts. NaCl reduces the electrostatic repulsion between the adsorbed and non-adsorbed particles, which allows more particles to be adsorbed at the interface. The particles also tend to form a network in the continuous phase that retards the movement of emulsion droplets and prevented their coalescence (Mwangi, Ho, Tey, et al., 2016). This phenomenon of stability enhancement was observed at a salt concentration up to 200 mM, above which the emulsion stability was affected due to non-adsorbed particle precipitation.

The effect of increasing the temperature on the stability of chitosan Pickering emulsions was studied by Mwangi and co-workers. Chitosan Pickering emulsions stabilized by self-aggregated chitosan particles were reported to be stable with increasing temperature up to 50 °C, above which the mean droplet size started to significantly increase, and the emulsions started to become unstable. High temperature increases the Brownian motion of

the particles and may result in the desorption of the particles from the oil/water interface. Subsequently, the emulsion droplet's surface becomes uncovered with particles and the droplets start to coalesce (Mwangi, Ho, Tey, et al., 2016).

In general, chitosan-based emulsions have been reported to exhibit high storage stability while maintaining the physicochemical properties of the fresh emulsions. Pickering emulsions stabilized by zein/chitosan complex particles have shown remarkable stability over prolonged storage. The creaming and phase separation after 9 months of storage decreased with the increase of the zein/chitosan particles concentration and was found to be reversible with gentle shaking (L.-J. Wang et al., 2016). In contrast, Pickering emulsions stabilized by zein nanoparticles (without complexation with chitosan) exhibited a significant instability and volume reduction after 2 weeks of preparation (De Folter et al., 2012). Moreover, Pickering emulsions stabilized by zein/chitosan particles have shown high stability against any microbial growth over 9 months without the addition of any antimicrobial additive. The authors attributed this microbiological stability to the presence of the chitosan at the oil/water interface, and also in the continuous phase (L.-J. Wang et al., 2016). These results reflect the important role of chitosan (present in the produced zein/chitosan complex particles) in enhancing the storage and microbiological stability of the developed Pickering emulsions.

2.8 Applications

2.8.1 Chitosan-based Pickering emulsions for food applications

2.8.1.1 Encapsulation and delivery of bioactive compounds

The encapsulation of nutraceuticals is one of the most important applications of chitosan-based Pickering emulsions in the food area. They have been reported to protect and enhance the bio-accessibility of the encapsulated active agents. In this context, Shah et al. have successfully encapsulated curcumin in Pickering emulsions stabilized by chitosan/tripolyphosphate nanoparticles. The developed Pickering emulsions enhanced the stability of the encapsulated curcumin (Shah, Li, et al., 2016). Their findings indicate that chitosan/tripolyphosphate Pickering emulsions can be used as an effective oral delivery

system for highly lipophilic bioactive agents. In another study, hesperidin was encapsulated in Pickering emulsions stabilized with chitosan and lecithin (a food-grade emulsifier) (Dammak & José do Amaral Sobral, 2018). The emulsions have shown high stability and were reported to have the potential to be utilized for the encapsulation of other active agents. In a recent study, β -carotene was encapsulated in Pickering emulsions stabilized by chitosan hydrochloride/carboxymethyl starch nanogel particles (X.-M. Li et al., 2020). The encapsulation of β -carotene in the developed Pickering emulsions protected β -carotene from photodegradation by UV (365 nm) and enhanced its thermal stability and bio-accessibility. Furthermore, in another recent study, it was found that the encapsulation of d-limonene in chitosan-based Pickering emulsions highly protected it against thermal decomposition (Alehosseini et al., 2021).

Rattanaburi et al. produced Pickering emulsions stabilized by chitosan/lactic acid bacteria/hydroxypropyl cellulose, which combines the health benefits of chitosan and the antimicrobial activity of bacteriocin produced by the lactic acid bacteria. Stabilization of the emulsion was aided by the hydroxypropyl cellulose, which was able to fill the gaps between chitosan/lactic acid bacteria network existing at the oil interface, and thus was considered as a co-emulsifying agent. These Pickering emulsions acted as producing and delivery vehicles for bacteriocin (Rattanaburi et al., 2019).

Pickering high internal phase emulsions (Pickering HIPEs) stabilized with gliadin/chitosan hybrid particles and encapsulating curcumin have been reported to be a promising substitute for margarine to reduce the use of partially hydrogenated oils that increase the risk of cardiovascular diseases (Zeng et al., 2017). In this study, the formed Pickering HIPEs have shown viscoelastic properties contributing to the formation of stiff solid-like materials (similar to margarine) owing to the percolating network structure of the emulsion droplets. The encapsulation of curcumin in the produced Pickering emulsions was found to protect it against UV-induced degradation compared to the curcumin present in bulk oil.

2.8.1.2 Controlling lipid digestibility

Controlling lipid digestibility is considered a unique feature for some colloidal food systems/products; as it renders them suitable for reducing obesity, boosting satiety and

reducing caloric intake (Costa et al., 2020; Yao et al., 2013). Furthermore, it assists in improving the bioavailability of the lipophilic bioactive agents and their delivery (Tzoumaki et al., 2013). Chitosan conventional emulsions, when administered orally, have been reported to help in reducing body weight in rats, and thus, could be used as a low-calorie functional food to enhance satiety (Wani et al., 2016).

Some Pickering emulsions have been reported to be able to delay lipid absorption. This property is attributed to the irreversible adsorption of particles at the oil/water interface of the emulsion droplets, which subsequently restricts the access of lipase enzymes and bile salts to the dispersed phase, and hence delays its digestibility. The *in vitro* lipid digestion rate of Pickering emulsions stabilized by three different food-grade polysaccharide particles (chitosan, cellulose crystals and cellulose nanofibers) was compared in a recent study (Costa et al., 2020). It was found that Pickering emulsions stabilized with cellulose crystals and cellulose nanofibers were capable of inhibiting lipid digestion due to the strong adsorption of the particles at the oil/water interface that prevented the action of lipase enzymes on the dispersed phase. On the other hand, Pickering emulsions stabilized by chitosan particles delayed the lipid hydrolysis because of the interactions between chitosan chains at the near-neutral pH of the intestine and the intestinal digestive fluids at the emulsion droplet surface (Costa et al., 2020). Therefore, it could be concluded that Pickering emulsions with cellulose particles have the potential to be used as lipid digestion inhibitor systems, whereas chitosan-based Pickering emulsions can be used as oral delivery systems of nutraceuticals with delayed lipid hydrolysis

In another report, the digestion rate and bioaccessibility of curcumin-loaded Pickering emulsions stabilized by chitosan/tripolyphosphate particles were studied (Shah, Zhang, et al., 2016). The emulsions were prepared with either corn oil, as a long-chain triglyceride (LCT), or medium-chain triglyceride (MCT). Pickering emulsions have shown a slower rate of digestion in comparison with nanoemulsions, and subsequently, the amount of the released free fatty acids was lower from Pickering emulsions. Also, Pickering emulsions have shown lower bioaccessibility values of curcumin which is dependent on the slower digestion rate. The slow bioaccessibility is due to the deprotonation of chitosan at high pH value. At pH 7, the pancreatic lipase had little access to the lipids entrapped in the chitosan aggregates. These results show that chitosan Pickering emulsions reduce lipids digestibility.

In a different approach, some studies have shown that the incorporation of chitosan in Pickering emulsions stabilized by non-chitosan particles influences their lipid digestion pattern. For example, chitosan-coating of emulsions stabilized by octenyl succinic anhydride (OSA)-modified maize starch nanocrystals was found to decrease the lipolysis of the emulsions and enhance their storage stability under acidic conditions (Jo et al., 2019). The lipolysis rate of the emulsions decreased with the increase of the thickness of the chitosan layer, which suggests that the incorporated chitosan in the emulsions has the potential to control lipid digestion.

2.8.1.3 Retardation of lipid oxidation in food formulations

Lipid oxidation is a problematic issue for food product manufacturers since it negatively affects the sensorial properties of food formulations, produces harmful substances, and thus decreases the shelf-life of the products.

Atarian et al. reported that the rate of lipid oxidation was remarkably reduced in sunflower oil-in-water Pickering emulsions stabilized by chitosan/stearic acid in comparison to sunflower oil-in-water emulsion stabilized with Tween 80. The enhanced lipid oxidative stability is attributed to the positively charged surface of the emulsion droplets owing to the presence of free amino groups of chitosan in the chitosan/stearic acid nanogels. Subsequently, the positively charged transition metals (pro-oxidants present in the aqueous phase) were repelled away from the surface of the oil droplets in the emulsion, retarding the lipid oxidation (Atarian et al., 2019).

Food-grade Pickering emulsions stabilized by chitosan grafted with caffeic acid have been reported to have enhanced free radical scavenging properties (İlyasoğlu et al., 2019). The synthesis of caffeic acid grafted chitosan was promoted by ascorbic acid. The produced particulate emulsifier based on chitosan modification has improved the solubility of chitosan and was also reported to retard lipid oxidation as it provided excellent antioxidant properties.

2.8.1.4 Enhancing the properties of edible films

Biopolymeric films have recently attracted increasing attention owing to their biodegradable, biocompatible and non-toxic properties, which make them an environmentally friendly alternative for plastic films/packaging. The edible films and coatings have been used to extend the shelf life of food products and improve their quality (Agulló et al., 2003). Interestingly, Pickering emulsions have been used in the formation of edible films that have excellent oxygen barrier properties. Shi et al. produced Pickering emulsions stabilized by zein/chitosan colloidal particles and subsequently incorporated the produced Pickering emulsions into a chitosan-based matrix under ultrasonication to form self-supported emulsion films as shown in Figure 2.7 (Shi et al., 2016). In another recent study, chitosan Pickering emulsions encapsulating hesperidin were incorporated in gelatin-based films (Dammak et al., 2019). The films were produced by incorporating the chitosan Pickering emulsions at different concentrations (5, 10, 20, 30 and 50 g oil/100 g gelatin). The films have shown high flexibility but less resistance to tension. Furthermore, the water vapor barrier activity of the films was enhanced by increasing the Pickering emulsion incorporation levels. The films possessed good antioxidant activity owing to the encapsulated hesperidin.

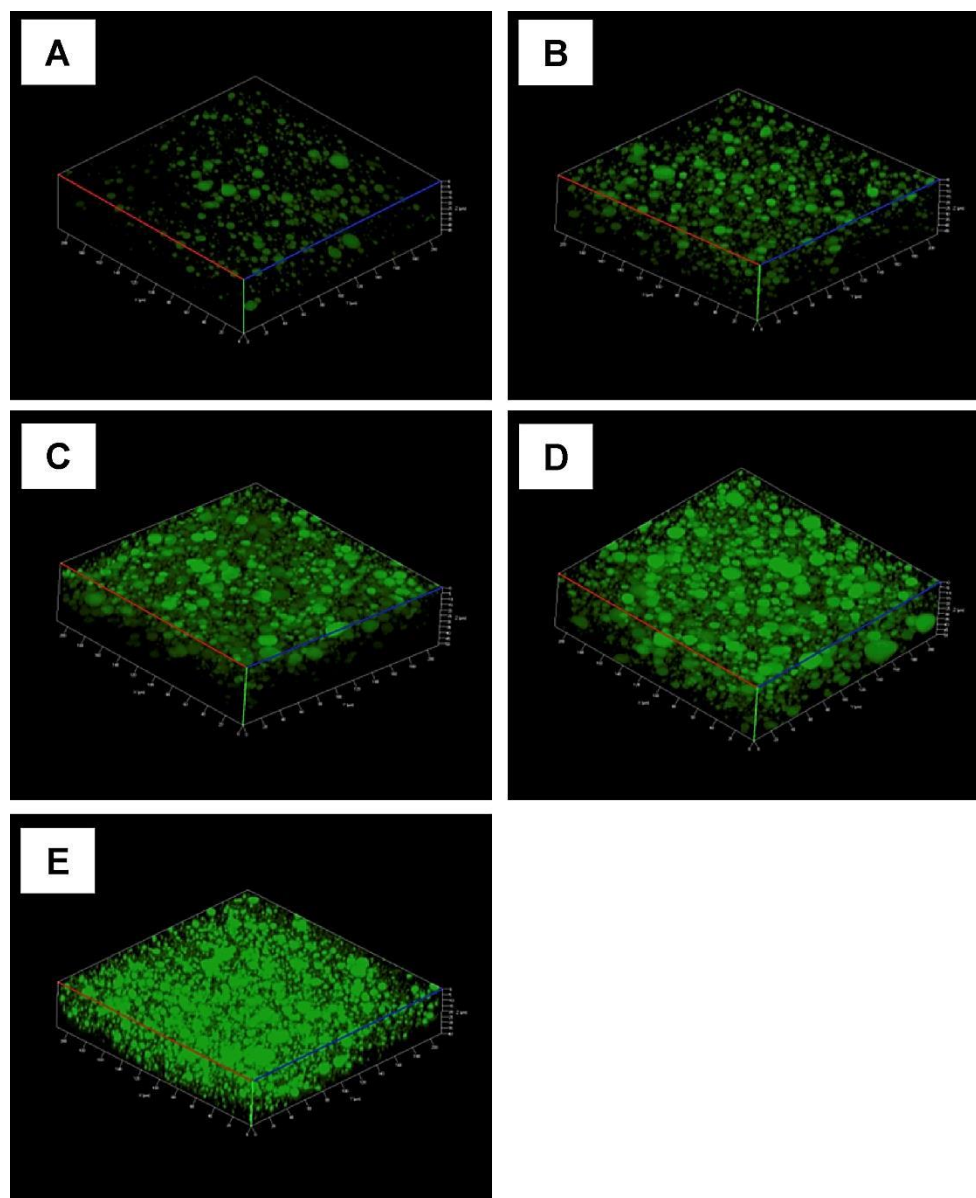


Figure 2.7. 3D CLSM images of chitosan films loaded with Pickering emulsions stabilized with zein/chitosan colloidal particles as a function of oil-to-chitosan ratios: A) 5%; B) 10%; C) 20%; D) 30% and E) 50%. The green fluorescence from Nile Red indicates the spherical emulsion droplets. Reprinted from (Shi et al., 2016), Copyright (2016), with permission from Elsevier.

2.8.1.5 Incorporation in complex food systems

The incorporation of Pickering emulsions in complex food matrices is an innovative research area that has been explored just recently. Hosseini and Rajaei investigated the

incorporation of fish oil-in-water Pickering emulsions stabilized with chitosan/stearic acid nanogels containing clove essential oil in mayonnaise (R. S. Hosseini & Rajaei, 2020). It was observed that the introduction of fish oil in an emulsified form resulted in enhancing the oxidative stability and the rheological properties of mayonnaise in comparison to non-emulsified (free) fish oil. The study also showed that the presence of clove essential oil in the nanogels significantly improved the elasticity and, subsequently, the textural properties of mayonnaise. This study presents a new fortification strategy of food systems with bioactive compounds based on the incorporation of Pickering emulsions.

2.8.2 Chitosan-based Pickering emulsions for skin applications

The use of Pickering emulsions in dermal formulations has only emerged recently due to the increasing consumers' demands for safer and green products, as well as the need to adopt green chemistry principles and environmentally friendly processes by the industrial sector (Marto et al., 2016). Most of the research on Pickering emulsions for dermal applications focused on the use of inorganic particles, such as silica, titanium dioxide. Recently, the use of biodegradable synthetic particles (e.g., polylactide-based particles) (Laredj-Bourezg et al., 2017), and particles of natural origin (e.g., starch and cyclodextrins) as Pickering stabilizers for dermal formulations have been reported (Marku et al., 2012; Marto et al., 2016).

The delivery of active agents to or across the skin is a challenging issue since the skin functions as a natural barrier. The active agent needs to overcome the *stratum corneum* (the outermost layer of the skin) in order to reach its delivery site, either to the inner skin layers (viable epidermis and dermis) or to the systemic circulation. The use of chitosan in topical and transdermal delivery systems has gained much interest due to its ability to enhance skin penetration and permeation. Chitosan, due to its positive charge, has been reported to depolarize the negative charges of the *stratum corneum* cell membrane, and subsequently leads to the driving of the drug, or active agent, within and/or through the skin (Ammala, 2013). Moreover, it was found that chitosan increases the water content of the *stratum corneum*, modifies the structure of its keratin content and decreases the HaCaT cells membrane potential, which results in a transdermal penetration enhancement (He et al., 2009).

Despite the importance of chitosan in dermal and transdermal delivery systems, very few studies focused on the formulation of Pickering emulsions stabilized by chitosan and chitosan-based particles. For example, it has been recently reported that rutin-loaded Pickering emulsions stabilized by chitosan nanoparticles, and containing 20% (v/v) of oleic acid, enhanced wound healing in rat skin (Asfour et al., 2017). The findings of this study suggest that the successful dermal delivery of rutin from Pickering emulsions, as well as the presence of chitosan and oleic acid, had a synergetic healing effect for the cutaneous wounds present in rats.

In another study, it has been reported that trans-resveratrol delivery to the viable epidermis and dermis was remarkably enhanced when it was incorporated in Pickering emulsions stabilized by chitosan/gum Arabic nanoparticles (Sharkawy et al., 2020). It was observed that chitosan presented in the developed particles played an important role in the improvement of the dermal delivery of resveratrol. The dermal delivery enhancement is attributed to the cationic nature of chitosan which allows it to open the tight junctions of the *stratum corneum* cells that are negatively charged, permitting resveratrol to cross the *stratum corneum* and accumulate in the viable epidermis and dermis where it would perform its cosmetic and skin repair effect (Sharkawy et al., 2020).

The dermal delivery of resveratrol by Pickering emulsions-chitosan hydrogel beads carrier system has been investigated in a recent work by Wu and co-workers who reported that the produced Pickering emulsions resulted in high skin retention and low permeation of resveratrol (B. Wu et al., 2021). Moreover, these resveratrol-loaded Pickering emulsions demonstrated high cell viability (B. Wu et al., 2021).

2.8.3 Chitosan-based Pickering emulsions for environmental applications

Chitosan and chitosan-based materials (such as nanoparticles, hydrogels, electrospun fibers and membranes) have long been used in different environmental applications because of their ability to remove pollutants owing to the adsorption capacity of chitosan (Chauhan et al., 2014; Mohammadzadeh Pakdel & Peighambaroust, 2018; Riegger et al., 2018; Salehi et al., 2016).

Some studies have investigated the production of novel materials, based on Pickering emulsions stabilized by chitosan particles, to remove the environmental pollutants. In a recent study (Q. Li et al., 2019), w/o chitosan-based Pickering emulsions were produced and used to prepare a chitosan/hydrophobic-modified montmorillonite composite via Pickering emulsion polymerization with glutaraldehyde. The resultant microcomposite has shown a high capability of adsorbing iodide ions. It is worth mentioning that the radioactive isotopes of iodine (I^{131} and I^{129}) represent high environmental hazards due to their high volatility and solubility. It was observed that the developed composite could adsorb iodide ions efficiently because of the simultaneous adsorption from both chitosan and the hydrophobically modified montmorillonite. Nevertheless, it was found that increasing the chitosan content in the nanocomposite led to a better adsorption performance. The experiments were conducted with I^{127} instead of the radioactive iodine for safety concerns, yet this ion has similar properties. The eco-friendliness of this composite which is produced from the polymerization of chitosan-based Pickering emulsion distinguishes it as a novel alternative for the metal-based composites that have been previously employed for the removal and separation of radioactive iodine isotopes (Q. Li et al., 2019).

Magnetic imprinted polymers (MIPs) are in general produced by the polymerization of suitable functional monomers in the presence of the targeted template molecules. Upon removal of the template molecules, specific binding sites are formed within the polymer matrix. These sites have a high binding affinity to the template molecules. MIPs were produced by Pickering emulsion polymerization and were used to adsorb erythromycin (an antibiotic that has hazardous effects when it accumulates in the ecosystem). The o/w Pickering emulsion was stabilized by chitosan nanoparticles and contained Fe_3O_4 nanoparticles as the hydrophobic magnetic carrier. The MIP system was produced by polymerization with the functional monomer in the oil phase. The produced MIP adsorbent system has shown high adsorption capacity and ability to recognize erythromycin (Ou et al., 2015). This system, which is based on a chitosan Pickering emulsion, provides an effective way to remove erythromycin from aqueous media and guards against its accumulation in the ecosystem.

2.8.4 Production of different structural materials from chitosan-based Pickering emulsions

2.8.4.1 Microcapsules produced from chitosan-based Pickering emulsions

Microcapsules prepared from Pickering emulsions are advantageous in terms of their eco-friendliness owing to the absence of surfactants in their composition. The formation of microcapsules from Pickering emulsions stabilized by inorganic particles has been frequently reported in the literature, whereas studies on microcapsules derived from Pickering emulsions stabilized by biopolymeric nanoparticles (including chitosan derived particles) are scarce. Table 2.2 summarizes some microcapsule systems developed from chitosan-based Pickering emulsions available in the literature. The production of microcapsules from Pickering emulsions involves the adoption of shell formation techniques to anchor (lock) the particles at the oil-water interface (Bago Rodriguez & Binks, 2019). The formation of microcapsules from Pickering emulsions has been reported to significantly increase the mechanical stability of the emulsion oil droplets, which led to retaining the microcapsules' round shape after drying (Zou et al., 2019).

Degradable microspheres have been produced from chitosan-based Pickering emulsions by photopolymerization (Liu, Wei, et al., 2014). The photopolymerization of the monomer trithiol (trimethylolpropane tris(3-mercaptopropionate)), and multifunctional acrylates, in Pickering emulsions (containing chloroform-in-water and chitosan as a green stabilizer), was initiated by UV irradiation. The microspheres were loaded with ibuprofen whose release rate was increased by pH and temperature increase. Additionally, the produced microcapsules have shown high degradability in weak alkaline media. The results of this study demonstrated the potential of chitosan-based Pickering emulsions in the production of green degradable microspheres.

The effect of high-intensity ultrasonication (HIU) on the production of composite nanospheres from chitosan-based Pickering emulsions was recently studied (Jiaxin Wang, Chen, et al., 2017). In this study, Pickering emulsions were stabilized with chitosan particles (produced from HIU pretreated chitosan solutions) and contained poly(lactic-co-glycolic) acid (PLGA) in CH_2Cl_2 as the dispersed phase. It was observed that the HIU

assisted Pickering emulsions resulted in the formation of monodisperse composite nanospheres. In contrast, the non-HIU assisted Pickering emulsions gave rise to the production of microcapsules (not nanospheres) with wide distribution. The authors concluded that the Pickering emulsions HIU treatment generated a strong shear force that successfully reduced the emulsion droplet size and thus improved the mono-dispersity. The produced monodisperse composite nanospheres based on biodegradable polymers, such as chitosan, have outstanding applications as drug delivery systems (Jiaxin Wang, Law, et al., 2017).

Table 2.2. Examples of microcapsules synthesized from chitosan-based Pickering emulsions available in the literature.

Particles composition	Organic phase in Pickering emulsion	Microcapsule's shell composition	Reference
Chitosan	Tocotrienol in refined palm oil	Chitosan/sodium tripolyphosphate	(Mwangi, Ho, Ooi, et al., 2016)
Chitosan	Dichloromethane (CH ₂ Cl ₂)/poly-lactic-co-glycolic acid (PLGA)	Chitosan/poly-lactic-co-glycolic acid (PLGA)	(Wei et al., 2012)
Chitosan-coated colloidal lignin particles (CLPs)	Ciprofloxacin and Tall oil fatty acid in olive oil	Chitosan-coated CLPs crosslinked with sodium tripolyphosphate	(Zou et al., 2019)

The studies mentioned in Table 2.2 involve microcapsules developed from Pickering emulsions originally stabilized with chitosan particles, in which the microcapsules were obtained by crosslinking the particles to form the microcapsule's shell. In a different approach, the synthesis of microcapsules from Pickering emulsions stabilized by poly(ethyleneimine) surface modified Laponite particles was documented. The shells of the microcapsules were then developed by the deposition of chitosan and alginate on the

Pickering emulsion droplets. The produced nanocomposite microcapsules contain natural polymers, and hence have great potential for applications in the food and medical areas (Liu, Gu, et al., 2014). In another recent study (Palamarchuk et al., 2018), Pickering emulsions stabilized by hydrophobically modified silica nanoparticles were used as templates for the formation of microcapsules through the deposition of chitosan and alginate polyelectrolyte layers. The use of chitosan and alginate in the microcapsule shell formation enhanced the biocompatibility of the produced microcapsules, which enables their use in biomedical applications (Palamarchuk et al., 2018). Similarly, composite microcapsules loaded with citronella oil were developed by citronella oil/water Pickering emulsions stabilized by hydroxyapatite nanoparticles. The shells of the composite microcapsules consisted of hydroxyapatite/quaternary ammonium salt of chitosan/sodium alginate. They were formed by the electrostatic adsorption of the quaternary ammonium salt of chitosan and sodium alginate, followed by the chelation of alginate with the calcium ions released from the hydroxyapatite nanoparticles (Jingguang Wang et al., 2018). The produced microcapsules have shown sustained release and effective antibacterial properties and are suitable for food and agricultural applications.

2.8.4.2 Chitosan-based Pickering emulsion hydrogels

Hydrogels are three-dimensional networks of polymeric chains that swell when they come in contact with water but maintain their structure and resist dissolution (Qu & Luo, 2020). They have been widely used in drug delivery and tissue engineering. In the swollen state, the mass of water in the hydrogel is much higher than the mass of the polymer. The ability of hydrogels to absorb water is due to the presence of hydrophilic functional groups in the polymer chains, at the same time they resist dissolution because of the cross-linking between the network chains (Ahmed, 2015).

An innovative research trend has been applied recently to immobilize Pickering emulsions in hydrogels, especially Pickering emulsions stabilized by protein-based particles (Xiao et al., 2017). This immobilization permits higher stability of the produced Pickering emulsions at severe processing conditions, such as high temperature and high alkaline media. In contrast, this trend of Pickering emulsions immobilization in hydrogels has been recently applied to chitosan-based Pickering emulsions for increasing the stability of the

developed hydrogels. It has been reported that the stability of alginate hydrogels was highly improved by the immobilization of chitosan Pickering emulsions in them (Lim et al., 2020). In this study, it was observed that the chitosan nanoparticles that are adsorbed at the oil-water interface of the oil droplets, as well as the excess non-adsorbed nanoparticles, enhanced the stability and strength of the produced hydrogels through electrostatic attraction. Moreover, these systems have shown higher stability in comparison to similar alginate hydrogels stabilized by Tween 80.

2.8.4.3 Highly porous materials from chitosan-based Pickering emulsions

The development of highly porous water-insoluble materials with large surface area and interconnected porous structures finds increasing interest in various applications such as biomedical devices, packaging, sorption and separation science, electronic materials and sensors (Stein, 2003; Zhou et al., 2019).

Biocompatible and biodegradable highly interconnected porous structures obtained from high internal phase emulsions (HIPEs) have received high research interest in particular due to their surfactant-free properties and high microporosity. The production of such porous structures for biomedical applications (e.g., scaffolds) based on HIPEs involves the polymerization, or the cross-linking, of the continuous phase of the HIPEs followed by extraction/removal of the dispersed phase (Tan et al., 2017).

In a recent study, Zhou et al. developed, for the first time, a simple and non-toxic highly porous material by the freeze-drying of Pickering high internal phase emulsions (HIPEs) that are stabilized by gliadin-chitosan complex particles. The porous materials developed from the HIPEs (PM-HIPEs) (Figure 2.8) were used as oil absorbents and have shown high oil absorption capacity (Zhou et al., 2019). These materials are of high importance in the mitigation of oil spills owing to their high porosity which provides them with high absorption capacity.

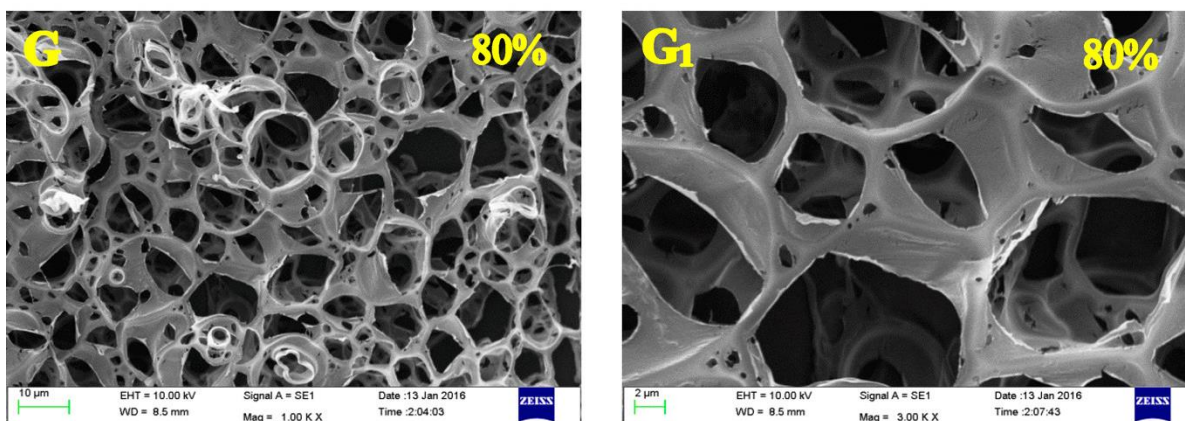


Figure 2.8. SEM images of water-insoluble porous materials obtained from the freeze-drying of high internal phase Pickering emulsions stabilized by 2% chitosan/gliadin particles with $\phi = 0.8$. Image G1 is a magnification of image G. Reprinted with permission from (Zhou et al., 2019), Copyright (2019) American Chemical Society.

2.9 Conclusions and future directions

There has been considerable increasing interest in Pickering emulsions stabilized with biopolymeric particles. Chitosan-based particles are promising candidates as Pickering stabilizers, owing to their biodegradable and biocompatible character, which allows them to replace the low molecular weight surfactants that have negative effects on the environment and health. Novel Pickering emulsions based on chitosan are often obtained using hydrophobically modified chitosan or chitosan complex particles rather than using chitosan itself. The role of chitosan in the particles used as Pickering stabilizers can be related to tuning the wettability and surface charge, aiming at improving the stability of the resulting Pickering emulsions. Furthermore, in some cases, it can be directly associated with the improvement of the functionality of Pickering emulsions targeting specific applications, such as retardation of lipid oxidation in food formulations, control of lipid digestion rate, or enhancement of the skin uptake of cosmeceuticals.

Certainly, there is a significant research advancement in utilizing chitosan-based Pickering emulsions in different areas, such as food and skin applications, environmental research and novel materials development (e.g., microcapsules, hydrogels, films and porous materials). However, there are still some challenges and research opportunities to be addressed.

Despite the advanced ongoing studies in Pickering emulsions, in general, it should be noted that the molecular-based emulsifiers are still the most commonly used in industry (McClements & Jafari, 2018). Therefore, the main challenge is to find the most studied Pickering emulsions utilized in real products in areas, such as food and beverages, pharmaceuticals and cosmetics (Murray, 2019). This can be successfully achieved by scaling up studies, namely for the production of the particles and the emulsions (Albert et al., 2019), together with conducting a cost-benefit analysis for the particles and the Pickering emulsions production method (Marto et al., 2016).

Another major challenge for Pickering emulsion formulators is to further improve the stability of emulsions (Gonzalez Ortiz et al., 2020). For instance, more research is required to investigate the stability of the food-grade Pickering emulsions in real food systems. The stability of many chitosan-based Pickering emulsions has been investigated under different environmental conditions (e.g., temperature, pH and ionic strength changes). However, research that explores the possible interactions of chitosan Pickering emulsions with food systems scarcely exists.

Moreover, there is a lack of information on the contact angle values of some chitosan-based particles that are being proposed as novel Pickering stabilizers. A better understanding of the wettability of the developed particles would, undoubtedly, enable improving the stability of the developed chitosan-based Pickering emulsion systems. Furthermore, the identification and utilization of new natural biopolymers, that can complex with chitosan, to tune the wettability of chitosan particles are of great importance for exploratory future studies. Also, most of the reported preparation methods of chitosan-based Pickering emulsions are limited to the rotor-stator homogenization and ultrasonication methods. Other preparation methodologies, such as microfluidic emulsification and membrane emulsification should be explored in future studies, and investigated for improved emulsion stability.

The studies on using chitosan-based Pickering emulsions, and materials thereof, in skin applications are very few, which represents a valuable research opportunity in this area in order to leverage chitosan non-toxic and biodegradable properties, as well as its skin benefits. The sensorial perception of cosmetic Pickering emulsions stabilized with chitosan

particles has never been studied. The type of particles remarkably influences the sensory properties of the developed cosmetic Pickering emulsions as they determine the level of greasiness, whitening residues, ease of spreading and glossiness (Terescenco et al., 2020). Although these aspects are crucial for the commercialization of Pickering emulsions intended for cosmetic use, such studies were only performed for Pickering emulsions stabilized by inorganic particles. Therefore, future efforts should be also directed towards the study of the sensory properties of Pickering emulsions stabilized by different chitosan particles.

Finally, it must be highlighted in this review that almost all the chitosan-based Pickering emulsions that are developed for food and cosmetic applications were tested only by *in vitro* studies. However, it is recommended that the *in vitro* examination should be complemented by *in vivo* studies. The combination of both types of tests provides a comprehensive and thorough evaluation of the developed emulsion for the validation of the potential application.

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3 Preparation of chitosan/gum Arabic nanoparticles and their use as novel stabilizers in O/W Pickering emulsions

Recently, there has been a renewed interest in Pickering emulsions owing to their surfactant-free nature, and the use of natural-based particles as stabilizers became a priority due to the applications they can enable. In this work, chitosan/gum Arabic (CH/GA) nanoparticles were synthesized and tested as novel stabilizers. Among the tested CH/GA weight ratios, the particles prepared using 1:1 ratio exhibited near-neutral wettability, an average size of 108.6 nm and a zeta potential of 56.3 mV. Pickering emulsions prepared from these particle dispersions (1.5% w/v), and high oil volume fractions ($\phi = 0.6, 0.7$), have shown high storage stability. Moreover, confocal laser scanning microscopy confirmed the o/w type and the effective adsorption of the nanoparticles at the oil/water interface forming a barrier against droplets coalescence. The emulsions have shown shear-thinning and elastic-like behavior. These findings open new avenues for using these emulsions as novel delivery systems, e.g., in cosmetic and food applications.

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3.1 Introduction

Emulsion-based systems are widely used as food and cosmetic products, and pharmaceutical dosage forms owing to their facile formulation and enhancement of active agents and drug solubilization (Harman et al., 2019). These systems are typically stabilized by emulsifiers, which are being increasingly associated with environmental and health concerns in the long term. Alternatively, emulsions stabilized by solid particles, also known as Pickering emulsions, are emerging as promising substitutes in the areas of food, cosmetics and pharmaceuticals (Chevalier & Bolzinger, 2013; Dickinson, 2017). They are named after S.U. Pickering who reported them in 1907 (Pickering, 1907); however, they gained relevance only recently because of the rising environmental public awareness, and also due to the advancements in the development and characterization of nanoparticles. The surfactant-free nature of Pickering emulsions makes them an excellent choice to overcome the adverse effects exhibited by the chemically synthesized emulsifiers used to prepare conventional emulsions (Chevalier & Bolzinger, 2013).

Until the past few years, most of the research conducted on Pickering emulsions focused on the use of inorganic particles, such as silica and clay, as stabilizers (Lam et al., 2014). However, at present, the development of organic and biopolymeric particles as Pickering stabilizers has become an important field of research; in order to achieve both biodegradability and biocompatibility with the aim of utilizing them in food, cosmetic and pharmaceutical applications (Lam et al., 2014; Laredj-Bourezg et al., 2017; Marto et al., 2019). Nevertheless, the production of stable Pickering emulsions based on biopolymers is relatively challenging; since polysaccharides and some proteins are naturally very hydrophilic hindering the achievement of an intermediate particle wettability and a suitable contact angle at the oil/water interface (Xiao, Wang, et al., 2016). In this context, recent studies have successfully employed cellulose nanocrystals (Errezma et al., 2018), chitin nanocrystals (Perrin et al., 2014) and chitin nanofibers (Larbi et al., 2018) as Pickering stabilizers.

Chitosan is a linear polysaccharide, obtained by the deacetylation of chitin, a natural abundant polymer present in the exoskeleton of crustaceans. Chitosan has antibacterial, antifungal and gelling properties. It has been known as a promising material in the areas of

tissue engineering, drug delivery systems, food and cosmetic applications due to its non-toxicity and biodegradability (Joye & McClements, 2014; Muxika et al., 2017). Chitosan possesses amino groups in its polymeric backbone, making its solubility pH-dependent. At low pH (<6-6.5), the amino groups become protonated and acquire positive charges, rendering chitosan soluble. At higher pH values, they are deprotonated, making chitosan uncharged and able to aggregate and precipitate. Chitosan nanoparticles have been used extensively as drug delivery systems, but recently greater attention was given to the use of these particles as Pickering emulsion stabilizers (Asfour et al., 2017; Mwangi et al., 2016). Being a highly hydrophilic biopolymer, chitosan requires hydrophobic modification, or complexation with other polymers, to modify its wettability, and consequently its emulsification properties (Atarian et al., 2019; Elsabee et al., 2009). Complexation of chitosan with other biopolymers is considered a facile alternative to the currently used chemical surface modification strategies.

Gum Arabic (GA), also called Acacia gum, is a dried exudate obtained from the stems and trunk of *Acacia* tree. It is an amphiphilic branched polysaccharide that has good emulsifying properties and low viscosity (Dickinson, 2003). It is used in different applications, such as food, pharmaceutical, biomedical, cosmetic and textile industries (Ali et al., 2009). The use of gum Arabic in the formulation of Pickering emulsions has been reported, recently, in very few studies, with a focus mainly on food applications. In this context, (Dai, Sun, et al., 2018) and (Li et al., 2018) produced Pickering emulsions stabilized by gum Arabic/zein protein particles. The particles were formed through electrostatic attraction between the two oppositely charged polymers and hydrogen bonding between them. In another study, (Wei & Huang, 2019) stabilized edible Pickering emulsions using gum Arabic/ovotransferrin glycoprotein nanoparticles. However, no studies documented the complexation of gum Arabic with another polysaccharide biopolymer to produce nanoparticles with near-neutral wettability, which is a crucial prerequisite required to achieve high Pickering stabilization.

The main objective of this work is to produce chitosan/gum Arabic nanoparticles by a facile method, and subsequently utilize these novel biopolymeric nanoparticles to develop stable surfactant-free polysaccharide-based Pickering emulsions. The study also aims to investigate the effect of the oil phase volume fraction and nanoparticles concentration on

the stability, microstructure and rheological properties of the developed emulsions. To the best of our knowledge, no previous work reported the formulation of Pickering emulsions using chitosan/gum Arabic nanoparticles.

3.2 Materials and methods

3.2.1 Materials

Chitosan (ChitoClear[®]) with a degree of deacetylation of 96% and MW 100000-200000 Da was a gift from Primex ehf, Iceland. Gum Arabic (MW= 250,000 Da), olive oil (highly refined, low acidity), Nile Red and Nile Blue were purchased from Sigma Aldrich. Hydrochloric acid (0.1 N) and isopropyl alcohol were obtained from Merck, and acetic acid (0.1N) was purchased from Alfa Aesar.

3.2.2 Chitosan/gum Arabic (CH/GA) nanoparticles

3.2.2.1 Synthesis procedure

The nanoparticles were prepared following the method of (Tan et al., 2016) with slight modifications. The process was applied without the addition of a hydrophobic core or emulsifier. Nanoparticles of different CH/GA weight ratios (4:1, 2:1, 1:1, 1:2 and 1:4) were prepared at a fixed concentration of 1.5% (w/v). Briefly, and considering the example of CH/GA 1:1, chitosan was dissolved in 0.1N acetic acid at a concentration of 1.5% w/v (0.75 g chitosan in 50 ml of acetic acid), followed by the dropwise addition of 50 ml of gum Arabic solution of the same concentration. The mixture was kept under constant magnetic stirring (800 rpm) for 30 minutes to allow complexation between chitosan and gum Arabic. An opalescent dispersion was obtained indicating a successful formation of the particles. The pH of the final dispersion was 4.

3.2.2.2 Wettability measurements

The wettability of the produced CH/GA particles, conducted using an optical contact angle measuring device (OCA15 Plus, Dataphysics, Germany), was determined to evaluate their hydrophilic/hydrophobic character. The prepared CH/GA nanoparticles dispersions were

placed on smooth glass slides separately and allowed to dry at room temperature. The dried film was placed on the device platform, and then 4 μl of deionized water was deposited on the surface using a high-precision injector at 25°C. Images of the formed drops were acquired using a digital camera coupled to the equipment and the measurements were averaged over at least 4 drops. The contact angle values were calculated automatically by the equipment software by fitting the profile data of the formed droplets to the Laplace–Young equation.

3.2.2.3 Transmission electron microscopy (TEM)

The morphology of the particles was examined by TEM. Briefly, 10 μl of the freshly prepared nanoparticles dispersion was diluted (1 ml of the nanoparticles dispersion was diluted to 10 ml using deionized water) and mounted on Formvar/carbon film-coated mesh nickel grids (Electron Microscopy Sciences, Hatfield, PA, USA) and left standing for 2 min. The liquid in excess was removed with filter paper. Visualization was carried out by a JEOL JEM 1400 TEM at 120kV (Tokyo, Japan). Images were digitally recorded using a CCD digital camera Orious 1100W (Tokyo, Japan).

3.2.2.4 Particle size and zeta potential

The average diameter, zeta potential and polydispersity index (PDI) were determined by a dynamic light scattering particle size analyzer (ZetaSizer Nano ZS ZEN3600 from Malvern Instrument). For that, the samples were diluted to an appropriate concentration (100-fold serial dilution) with deionized water. All measurements were carried out at 25°C, and the results were reported as averages of three consecutive readings.

3.2.3 Pickering emulsions

3.2.3.1 Preparation procedure

To prepare the oil/water Pickering emulsions from the CH/GA nanoparticles, olive oil was slowly added to the nanoparticles dispersion with continuous stirring at 13,500 rpm for 7 minutes, using an Ultraturrax DI 25 basic IKA homogenizer. The total volume of the emulsion was fixed to 100 ml. To study the effect of the oil volume fraction on emulsion

properties, emulsions of different oil volume fractions ($\phi = 0.3, 0.4, 0.5, 0.6, 0.7$ and 0.8) were prepared from CH/GA 1:1 nanoparticles at a fixed concentration of 1.5% (w/v). To study the effect of nanoparticles concentration, emulsions were prepared using dispersions with different concentrations (0.5%, 1%, 1.5% and 2% w/v) of CH/GA 1:1 nanoparticles (synthesized following the procedure described in section 3.2.2.1), and a fixed oil volume fraction of $\phi = 0.6$.

3.2.3.2 Microstructure of Pickering emulsions

The morphology of the formed emulsions was examined by optical microscopy using a Leica DM 2000 optical microscope equipped with a Leica Application Suite Interactive Measurement imaging software.

Moreover, confocal laser scanning microscopy (CLSM) was used to examine the interfacial microstructure, and to visualize the location of CH/GA nanoparticles. These assays were conducted using a Leica TCS SP5 CLSM (Leica Microsystems Inc., Germany). Briefly, after their preparation, the emulsions were stained with a mixture of fluorescent dyes in isopropyl alcohol consisting of Nile red and Nile blue (0.1% w/v each). Nile red was used to stain the oil phase and Nile blue for staining the nanoparticles. The CLSM was operated using two laser excitation wavelengths; 488 nm for the Nile red and 633 nm for the Nile blue.

3.2.3.3 Droplet size of Pickering emulsions

The average droplet size was measured using a laser diffraction particle size analyzer (LS 230, Beckman Coulter, USA). The refractive indexes for olive oil and water were 1.46 and 1.33, respectively.

3.2.3.4 Creaming index

The creaming index (CI %) determination was used as an indicator for the stability of the emulsions against separation. It was calculated according to the following equation (Li et al., 2018; Mwangi et al., 2016):

$$CI (\%) = \frac{H_s}{H_t} \times 100$$

where H_s is the height of the serum layer and H_t is the total emulsion height.

3.2.3.5 Rheological studies

The rheological studies were conducted to investigate the effect of the oil volume fraction and the nanoparticles concentration on flow behavior and viscoelastic properties of the formulations. The measurements were done using a Kinexus Pro Rheometer (Malvern, UK). All the measurements were conducted at 25°C. The steady flow measurements were performed using a cone plate of angle 4° with a diameter of 40 mm and a fixed gap of 0.15 mm. The emulsions were placed on the plate and allowed to stand 5 minutes for temperature equilibrium before measurements. The apparent viscosity (η) was determined over a shear rate range from 0.1 to 100 s⁻¹. The frequency sweep measurements were done using a parallel plate (diameter 20 mm) with a fixed gap of 1mm. All the dynamic tests were performed within the linear viscoelastic region. The frequency was oscillated from 0.01 to 10 Hz and the strain was made at 1%. The elastic modulus (G') and loss modulus (G'') were recorded versus frequency.

3.3 Results and discussion

3.3.1 Production and characterization of CH/GA nanoparticles

The production of CH/GA nanoparticles was based on polyelectrolyte complexation; which occurs chiefly due to the electrostatic attraction between oppositely charged polyelectrolytes (Tan et al., 2016). The same complexation process has been referred to as “ionic gelation” (Avadi et al., 2010). The degree of electrostatic attraction between the positively charged CH and the negatively charged GA is dependent on the pH and also on the used CH/GA weight ratio (Butstraen & Salaün, 2014). It has been reported that the coacervate yield of CH and GA is maximized at pH 3.5-5, and that at pH 4 the two polymers form homogenous particles (Tan et al., 2016). Hence, this pH was used in the present work.

3.3.1.1 Wettability of nanoparticles

An important prerequisite for particles to be used as Pickering stabilizers is that they should be partially wetted by both the dispersed and the continuous phase in order to achieve the required emulsion stabilization. The wettability of the particles reflects their degree of hydrophilicity/hydrophobicity, similarly to how the hydrophilic-lipophilic balance (HLB) scale is used to describe the amphiphilic properties of the conventional surfactants (Xiao, Li, et al., 2016). To assess this behavior, the contact angle (θ) is used to quantify the surface wettability of the solid particles. The value of the contact angle determines the type of the formed Pickering emulsion. Thus, hydrophilic particles have a low contact angle ($<90^\circ$), and stabilize preferentially o/w emulsions, whereas hydrophobic particles form w/o emulsions and have high values of contact angle ($>90^\circ$) (Dickinson, 2017). The maximum adsorption energy of a particle at the oil-water interface is achieved when the contact angle equals 90° , which contributes to maximizing emulsion stability as the particles will be strongly adsorbed at the oil/water interface forming a layer that sterically hinders the coalescence of the emulsion droplets (Binks & Clint, 2002).

Figure 3.1 shows the measured contact angle for the nanoparticles prepared with different CH/GA weight ratios (4:1, 2:1, 1:1, 1:2 and 1:4). It was observed that the contact angle increases as the amount of gum Arabic in the nanoparticles increases. This behavior could be attributed to the amphiphilic moieties present in the structure of gum Arabic, namely arabinogalactan-protein fraction (AGP), which contains both hydrophobic polypeptide chains and hydrophilic polysaccharide blocks which provide it with excellent interfacial properties (Hu et al., 2019; McClements & Gumus, 2016). On the other hand, chitosan is known to have weak surface activity due to the absence or limited number of hydrophobic moieties in its structure (Elsabee et al., 2009). It has been reported that chitosan nanoparticles have a contact angle of $42.47 \pm 1.19^\circ$ (Asfour et al., 2017). Thus, the results obtained in the present work suggest that GA played an important role in enhancing the wettability of the produced particles and tuning the value of the contact angle close to 90° . The results show that the hydrophilic character of the nanoparticles decreased with increasing the amount of GA due to its hydrophobic moieties. By analyzing the produced nanoparticles, it can be observed that the contact angle for the particles formed using a CH/GA weight ratio of 1:1 at pH 4 was 89.2° , indicating their hydrophilic nature and thus

the preferable tendency to stabilize o/w emulsions. The value of the contact angle of the CH/GA nanoparticles prepared with a weight ratio of 1:1 was the closest to 90° which suggests, among the tested nanoparticles, that they have higher potential for Pickering emulsion stabilization. Therefore, in light of this conclusion, this study proceeds with the use of the particles produced with a CH/GA weight ratio of 1:1 (CH/GA 1:1).

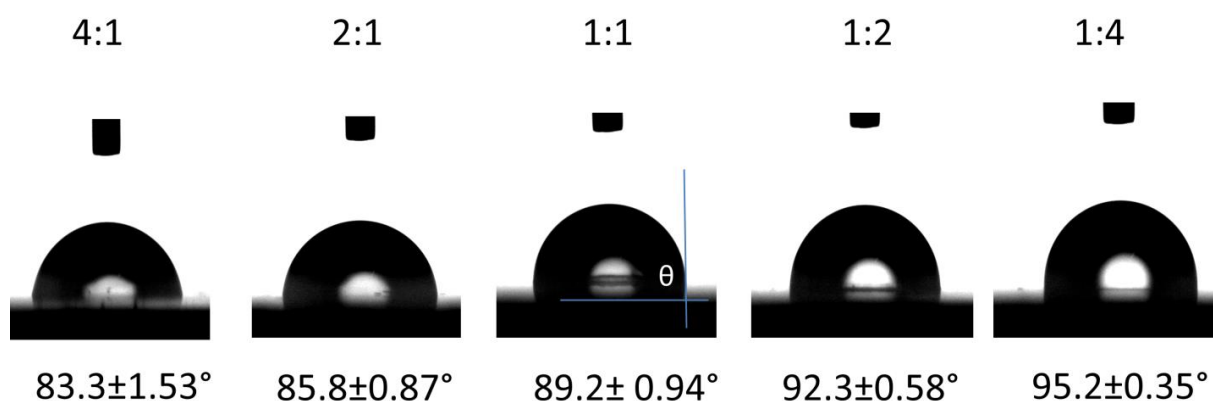


Figure 3.1. Contact angles of nanoparticles formed using different CH/GA weight ratios measured by dropping a water droplet onto films of the respective nanoparticles in air environment.

3.3.1.2 Size, zeta potential and morphology of CH/GA nanoparticles

The freshly prepared CH/GA 1:1 nanoparticles had an average size (in number) of 108.6 nm, a polydispersity index (PDI) of 0.218 and a zeta potential of +56.3 mV. The measurement was repeated for the same nanoparticles dispersion after a storage period of 8 days, showing an increase in the average size to 183.07 nm, whereas the PDI was roughly maintained (0.221), pointing out only minor particles agglomeration during storage. This is attributed to the high zeta potential value of the formed nanoparticles (> 30 mV), which is an indication of high stability. The results are in agreement with the monodisperse nanoparticles produced at pH 4 using CH/GA weight ratio of 1:1 that had a high positive zeta potential value (Tan et al., 2016).

TEM analysis revealed that the CH/GA 1:1 nanoparticles are predominantly spherical, but also some are oval in shape (Figure 3.2). The non-homogeneity in the particles morphology could be attributed to the lack of a hydrophobic core, and hence the lack of a spherical

template where the two biopolymers could deposit on. By comparing the size of the particles observed in the TEM images with the one previously determined by dynamic light scattering (DLS), it was observed that the one obtained by TEM is lower. This can be due to the fact that particles in the DLS measurements are in wet conditions (hydrated), while in the case of TEM the particles are dehydrated (Yang et al., 2017).

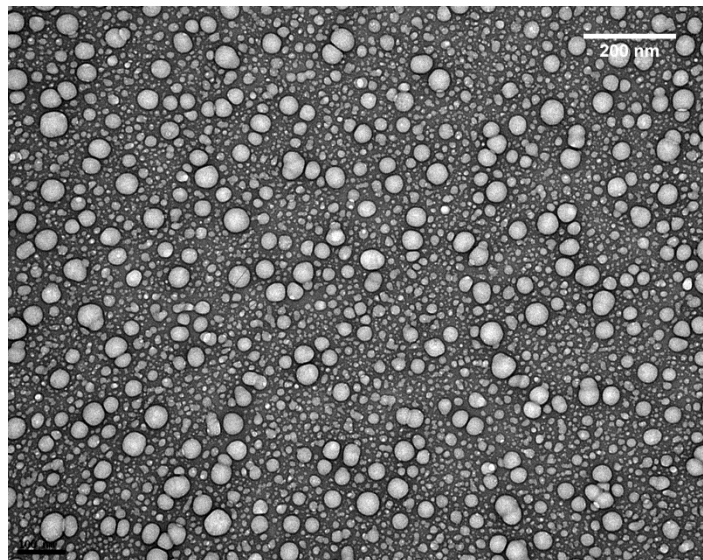


Figure 3.2. TEM image of CH/GA 1:1 nanoparticles (1.5% w/v), under a magnification of 100000X.

3.3.2 Production and characterization of Pickering emulsions

3.3.2.1 Effect of the oil volume fraction on morphology and stability

To study the effect of the oil volume fraction, Pickering emulsions with different oil fractions ($\phi = 0.3, 0.4, 0.5, 0.6, 0.7$ and 0.8) were produced using the nanoparticles dispersion CH/GA 1:1 nanoparticles with a fixed concentration of (1.5% w/v). The morphology of the produced Pickering emulsions was examined by optical microscopy, and the obtained results are shown in Figure 3.3. As expected, since the preparation conditions (total emulsion volume, stirring rate and time) were the same for all samples, the emulsion droplet size increased as the oil volume fraction increased. This observation is consistent with the decreased total amount of nanoparticles available to be adsorbed at the oil/water interface, which results in the formation of droplets of larger size. These results are in agreement with the findings reported in previous studies (Atarian et al., 2019; Dai,

Sun, et al., 2018; Li et al., 2018). Moreover, when the oil volume fraction increased above 0.7 ($\phi = 0.8$), oiling off and droplet flocculation were noticed, and the droplets started to deform by losing their spherical shape; indicating that the number of the available nanoparticles was not enough to stabilize the Pickering droplets against coalescence. A similar observation was noticed in zein/gum Arabic Pickering emulsions with an oil fraction above 0.6 (Li et al., 2018). For this reason, this sample ($\phi = 0.8$) was discharged from subsequent characterizations.

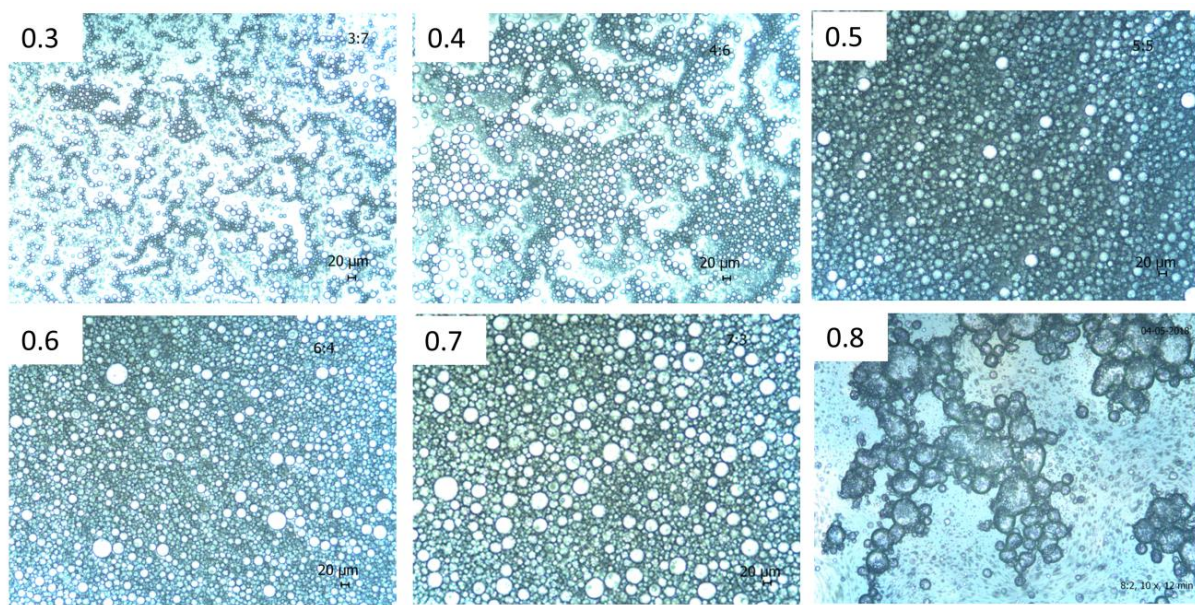


Figure 3.3. Optical microscope images of Pickering emulsions prepared with different oil volume fractions and a fixed concentration of CH/GA 1:1 nanoparticles (1.5% w/v).

Figure 3.4A shows the digital photographs of the freshly produced Pickering emulsions for the tested volume fractions in the range 0.3-0.7, and Figure 3.4B shows their aspect after a two-month storage period showing the corresponding creaming index (CI%). It can be observed that formulations with lower olive oil fractions underwent faster separation and had higher values of CI%, indicating poor storage stability. However, when the oil fraction increased from 0.3 to 0.5, the value of the CI% decreased from 50.7% to 17.8%, implying that more stable Pickering emulsions are formed as the oil volume fraction increases. Additionally, formulations with high oil volume ($\phi = 0.6$ and 0.7) have shown no gravitational phase separation during the tested storage period of two months (CI%=0), suggesting their high stability. Moreover, the formulation with $\phi = 0.7$ exhibited the

densest/thickest appearance/texture (gel-like appearance) (Figure 3.4C), as noticed by visual inspection and was confirmed further by the performed rheological studies (Section 3.3.2.4). The results of the evolution of the average size (obtained by laser diffraction) of the emulsion droplets as the oil volume fraction increases (Figure 3.4D) were consistent with the observations made by the optical microscopy.

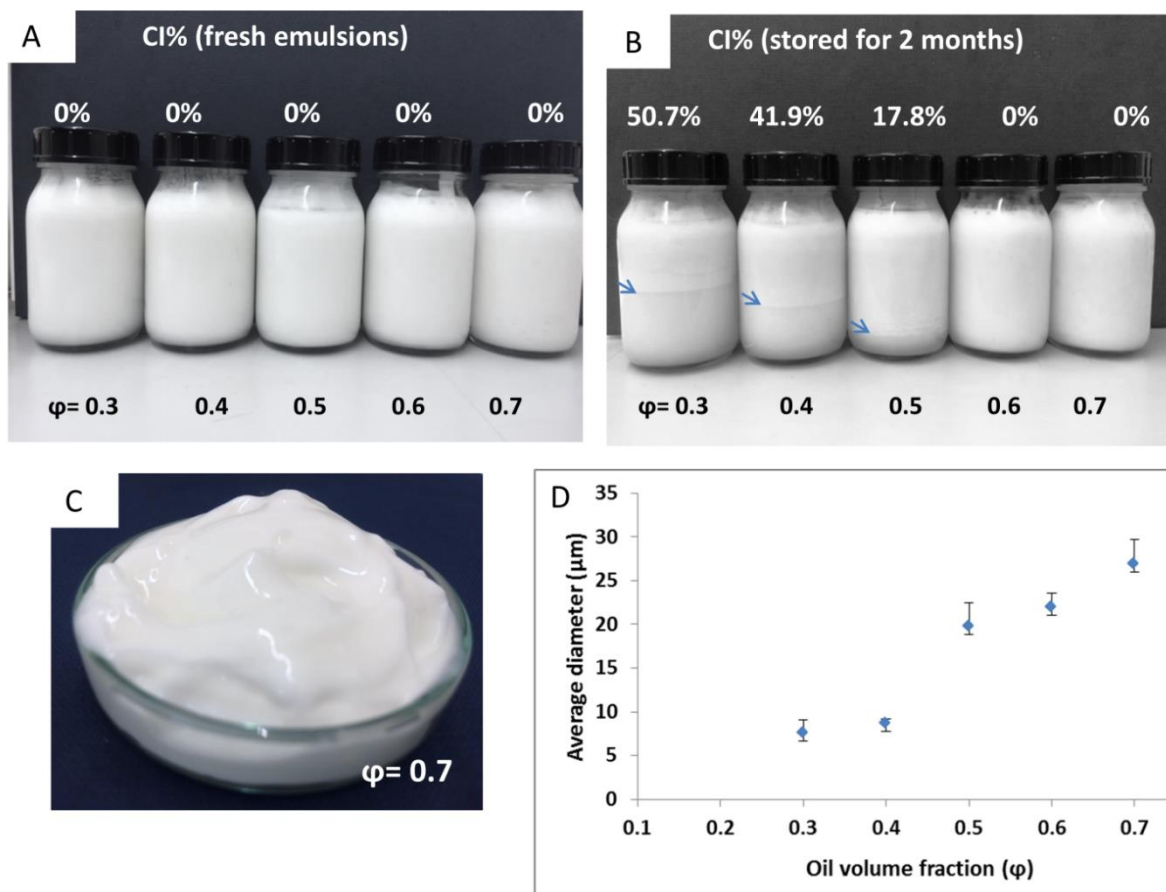


Figure 3.4. Visual appearance and creaming index % (CI%) values of Pickering emulsions prepared with oil volume fractions of 0.3, 0.4, 0.5, 0.6 and 0.7, and a dispersion of CH/GA 1:1 nanoparticles (1.5% w/v) right after preparation (A); after 2 months at room temperature (the arrows point to separation if present) (B); visual appearance of the formulation with $\phi = 0.7$ (C); and average droplet size for the tested formulations (D).

3.3.2.2 Effect of nanoparticles concentration on morphology and stability

To assess the effect of nanoparticles concentration, the oil volume fraction was fixed ($\phi = 0.6$) and dispersions of CH/GA 1:1 nanoparticles with concentrations of 0.5%, 1%, 1.5%

and 2% (w/v) were used. Figure 3.5 shows the optical microscopy images taken for each prepared formulation. From the observation of the images, it can be perceived that the sample prepared with the dispersion of low nanoparticles concentration (0.5 %w/v) gave rise to a Pickering emulsion with non-uniformly spherical pattern. However, Pickering droplets consistently acquired a uniform spherical morphology at higher CH/GA nanoparticles concentrations. In addition, the average droplet size was found to decrease as the nanoparticles concentration increases. This behavior is supported by the fact that a higher CH/GA nanoparticles concentration induces larger interfacial area coverage, limiting droplets coalescence, which results in smaller droplet sizes (higher stabilized interfacial area). These results indicate that the emulsification efficiency of the produced Pickering emulsions is directly linked to the ability of CH/GA nanoparticles to cover the oil droplets at the interface. Similar observations were noticed with chitosan (Mwangi et al., 2016) and kafirin (Xiao, Wang, et al., 2016) nanoparticles-stabilized Pickering emulsions.

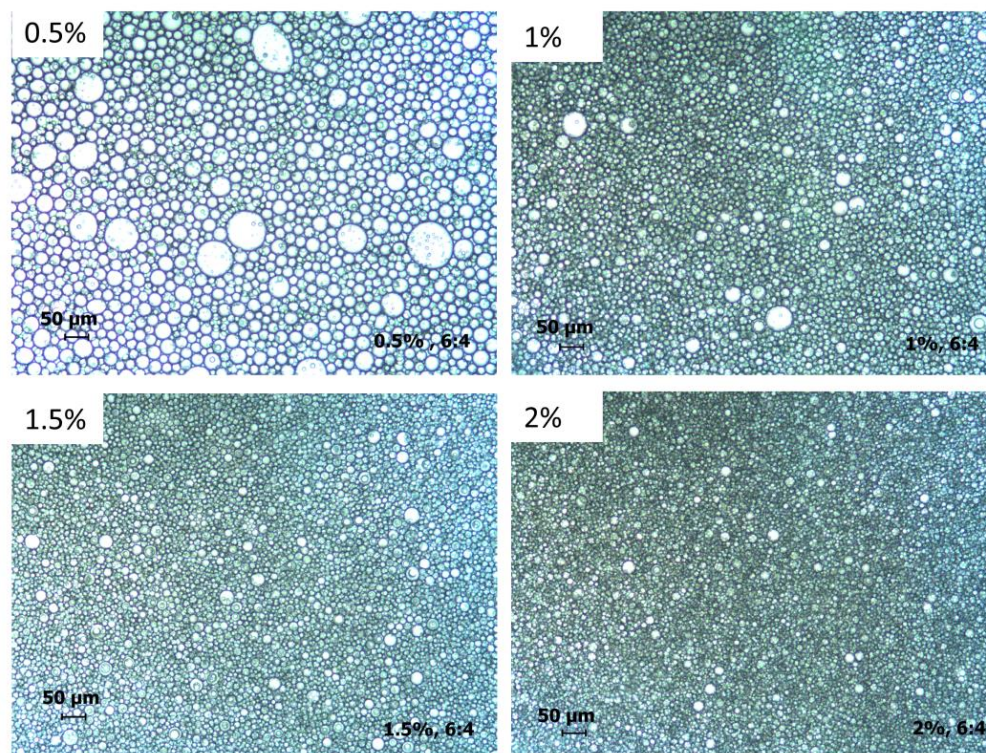


Figure 3.5. Optical microscope images of Pickering emulsions prepared with different CH/GA 1:1 nanoparticles concentrations (0.5%, 1%, 1.5% and 2% w/v) and a fixed oil volume fraction ($\phi = 0.6$).

Concerning the storage stability of the produced emulsions, it was observed that it increased as the CH/GA 1:1 nanoparticles concentration increased. Figures 3.6A and 3.6B show that the samples prepared with nanoparticles concentrations of 1.5% and 2% w/v did not develop any phase separation after the studied storage period of two months. The determined CI% values remained 0%, indicating that the presence of higher CH/GA 1:1 nanoparticles concentration promoted a higher degree of coverage of the oil droplets, which inhibited their aggregation and subsequently prevented the creaming and separation phenomena. Furthermore, it was observed that when the nanoparticles concentration was increased, the formed Pickering emulsions exhibited thicker gel-like texture. Figure 3.6C shows the formulation prepared with nanoparticles concentration of 2 %w/v which had the densest appearance among the tested concentrations. This was further assessed and confirmed by the rheological measurements. It was also found that the emulsion droplet average size decreased as the nanoparticles concentration increased (Figure 3.6D), which is in agreement with the average sizes observed previously by the optical microscopy.

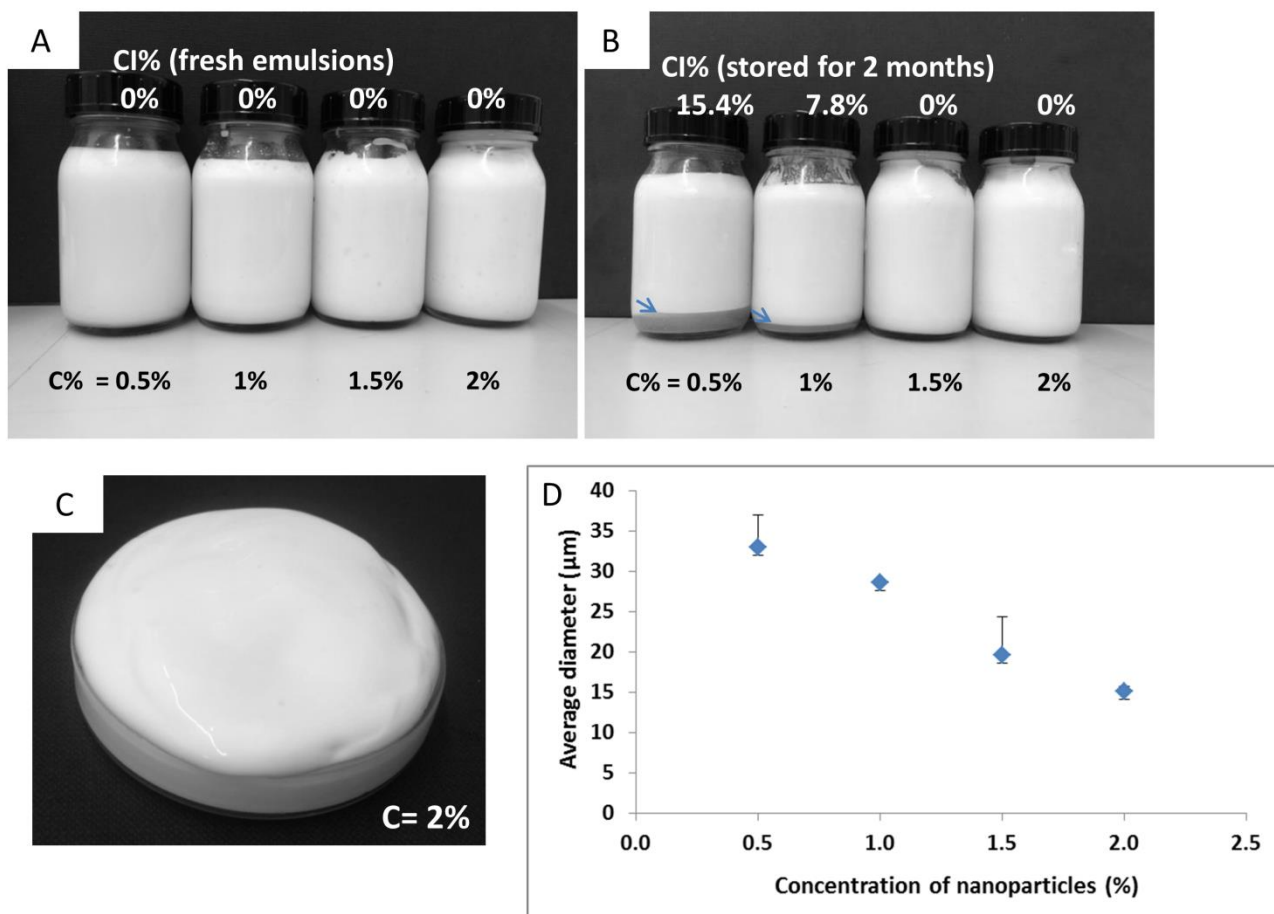


Figure 3.6. Visual appearance and creaming index (CI %) values of Pickering emulsions produced with different concentrations of CH/GA 1:1 nanoparticles ($C = 0.5\%$, 1% , 1.5% and 2% w/v) using a fixed oil volume fraction ($\phi = 0.6$) right after preparation (A); and after 2 months at room temperature (the arrows point to separation if present) (B); visual appearance of formulation with $C = 2\%$ w/v (C); and average droplet size for the tested formulations (D).

3.3.2.3 Interfacial structure of Pickering emulsions

CLSM was used to examine the interfacial structure and to confirm the morphology and type of the formed Pickering emulsions. CLSM images (Figure 3.7) reveal that the nanoparticles form a distinct layer (seen as red fluorescent halos) at the boundary of the oil droplets which act as a barrier against coalescence. The images also show that the oil phase (seen as green spheres) is the dispersed phase confirming the formation of an o/w emulsion, and that no phase inversion took place. It could be observed that when the oil volume

fraction increased from 0.5 (Figure 3.7A) to 0.7 (Figure 3.7B), using a fixed CH/GA 1:1 nanoparticles concentration (1.5% w/v), the oil droplets maintained their spherical shape and increased in size. A similar observation was noticed in o/w emulsions stabilized with zein/gum Arabic nanoparticles, which at a high oil fraction (0.7) showed no phase inversion (Dai, Sun, et al., 2018). However, deformation and merging of emulsion droplets were observed in emulsions stabilized with chitosan/zein nanoparticles when a similar oil volume was used (Wang et al., 2016).

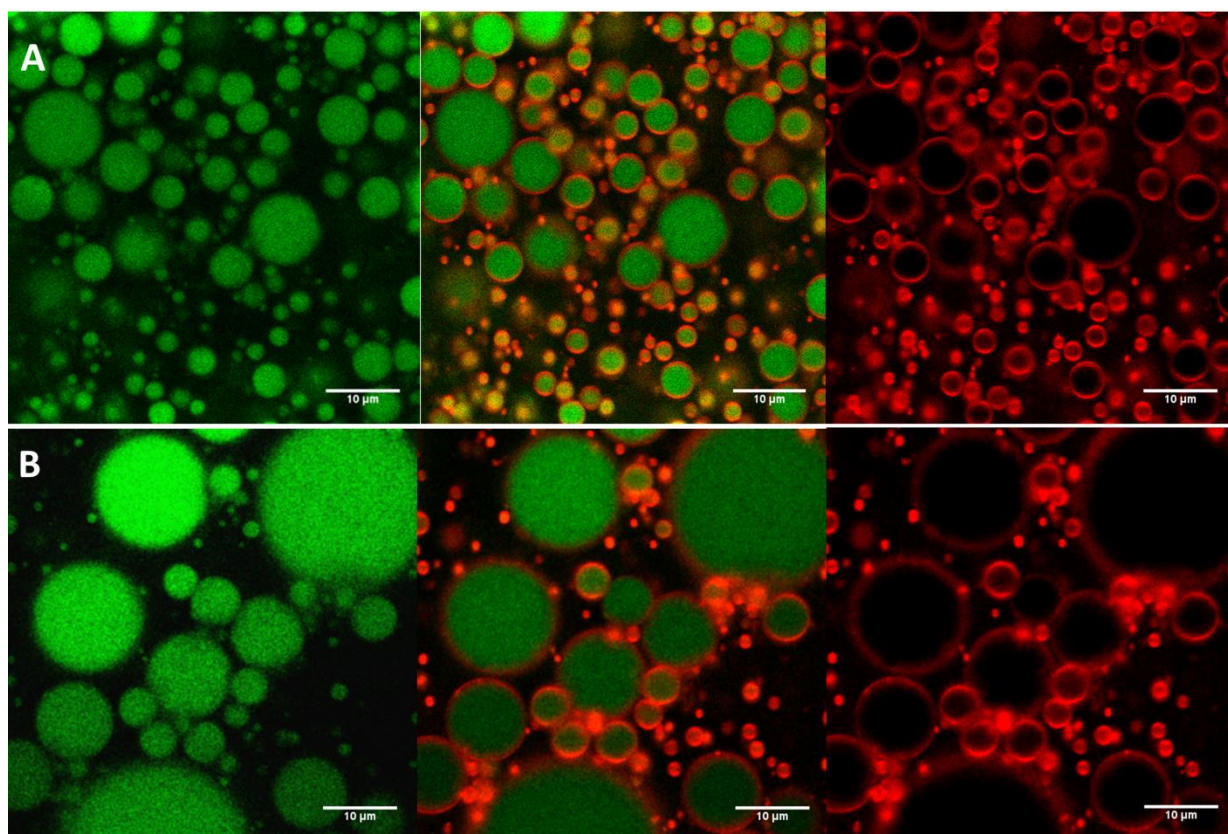


Figure 3.7. Confocal laser scanning microscopy images of formulations prepared with a fixed concentration of CH/GA 1:1 nanoparticles dispersion (1.5% w/v) and an oil volume fraction of (A) $\phi = 0.5$; and (B) $\phi = 0.7$. The emulsion oil phase appears in green (on the left), whereas the adsorbed nanoparticles appear in red (on the right). The images in the middle are an overlay of these two images (right and left).

3.3.2.4 Rheology of Pickering emulsions

The rheological properties of the emulsions are significantly linked to their properties and performance, as well as their storage stability (Liu et al., 2019). The effect of the oil volume fraction on the rheological properties of the prepared Pickering emulsions was studied considering the formulations prepared with $\phi = 0.5, 0.6$ and 0.7 , and using CH/GA 1:1 nanoparticles dispersion with a concentration of 1.5% (w/v). Figure 3.8A shows that, for the three tested formulations, the apparent viscosity decreased as the shear increased from 0.1 to 100s^{-1} , indicating a shear-thinning and a non-Newtonian fluid behavior. The reduction in viscosity reflects the deflocculation of the emulsion droplets and the breaking of the internal structure upon increasing the shear rate (Dai, Sun, et al., 2018; Li et al., 2018). In addition, the formulations have shown an increase in viscosity as the oil volume fraction increased. These results are in agreement with previous reports on the rheological properties of Pickering emulsions (Dai, Sun, et al., 2018; Li et al., 2018; Xiao, Wang, et al., 2016).

Frequency sweep measurements were done to determine the storage modulus (G') and the loss modulus (G'') *versus* frequency increase. The storage modulus reflects the elastic solid-like character of the material, whereas the loss modulus indicates the viscous response and measures the dissipated energy. It was observed that G' and G'' values increased as the oil fraction increased (Figure 3.8B). Additionally, it was observed for an oil volume fraction of 0.5 , G'' was slightly higher than G' ; indicating the liquid nature of the emulsion, whereas for the formulations with high oil volume fraction (0.6 and 0.7), it was observed that $G' > G''$. This reflects that the rheological behavior of formulations with higher oil volume fraction is predominantly elastic, i.e. they are composed of elastic gel solid-like networks. Figure 3.8B also shows that the values of G' and G'' rose with oil volume fraction increase. Recently published studies also reported similar behavior for G' and G'' as the oil volume fraction in Pickering emulsions increases (Dai, Sun, et al., 2018; Dai, Zhan, et al., 2018; Wang et al., 2016). These results can be explained by the fact that the oil droplets in highly concentrated emulsions become more densely packed, resulting in high G' values and high resistance to deformation by shear stress. It can be concluded from both assays that the oil volume fraction had a significant impact on the rheological properties of the Pickering emulsions. Furthermore, it is generally accepted that the presence of gel-like structure

stabilizes the emulsion droplets by hindering their free movement, and thus prevent the occurrence of phase separation (Wei & Huang, 2019). In addition, according to Stokes' law, gravitational separation rate is related to the system viscosity; as phase separation increases when viscosity decreases. Therefore, the higher the viscosity of the formulation, the higher is the expected stability (Li et al., 2018; Mwangi et al., 2016).

The effect of the nanoparticles concentration on the rheological properties of the produced Pickering emulsions was tested by using the samples prepared from dispersions with 1%, 1.5% and 2% (w/v) CH/GA 1:1 nanoparticles, and a constant oil volume fraction of 0.6. It can be observed from Figure 3.9A that the emulsions exhibited shear-thinning behavior as their viscosity decreased with shear rate increase. It can be also inferred that the apparent viscosity of the emulsions increased as the nanoparticles concentration increases, indicating the formation of a stronger structural network. This behavior was described in studies using other Pickering stabilizers, such as kafirin (Xiao, Wang, et al., 2016), ovotransferrin/gum Arabic (Wei & Huang, 2019), and octyl-grafted alginate amide gel particles (Yang et al., 2017). This can be attributed to the fact that increasing the concentration of Pickering particles causes the emulsion droplets to be in closer proximity, and thus packed in a denser way. This dense distribution of emulsion droplets results in higher viscosity and makes them more resistant to deformation (Wei & Huang, 2019). Furthermore, all the formulations have shown higher G' values in comparison with G'' (Figure 3.9B), which indicates that they have predominantly elastic properties, namely due to the formation of elastic gel networks between the nanoparticles and oil droplets. Taken together, these findings show that the rheology of Pickering emulsions stabilized by CH/GA nanoparticles can be tuned by altering the composition of the emulsion; by either changing the oil volume fraction or the nanoparticles concentration.

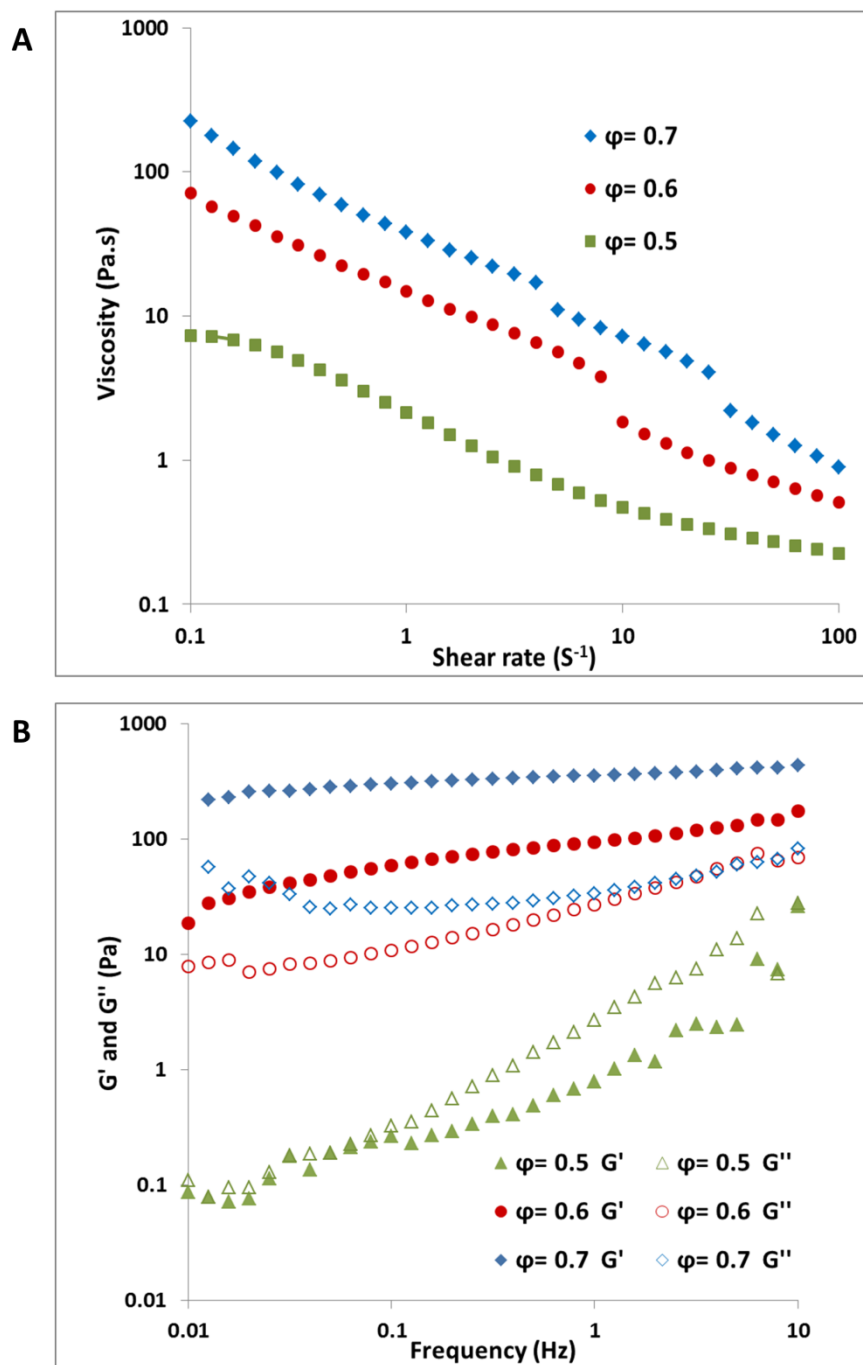


Figure 3.8. Effect of oil volume fraction on rheological properties; A) Apparent viscosity versus shear rate; B) Oscillatory frequency sweep curves. G' : storage modulus; and G'' : loss modulus.

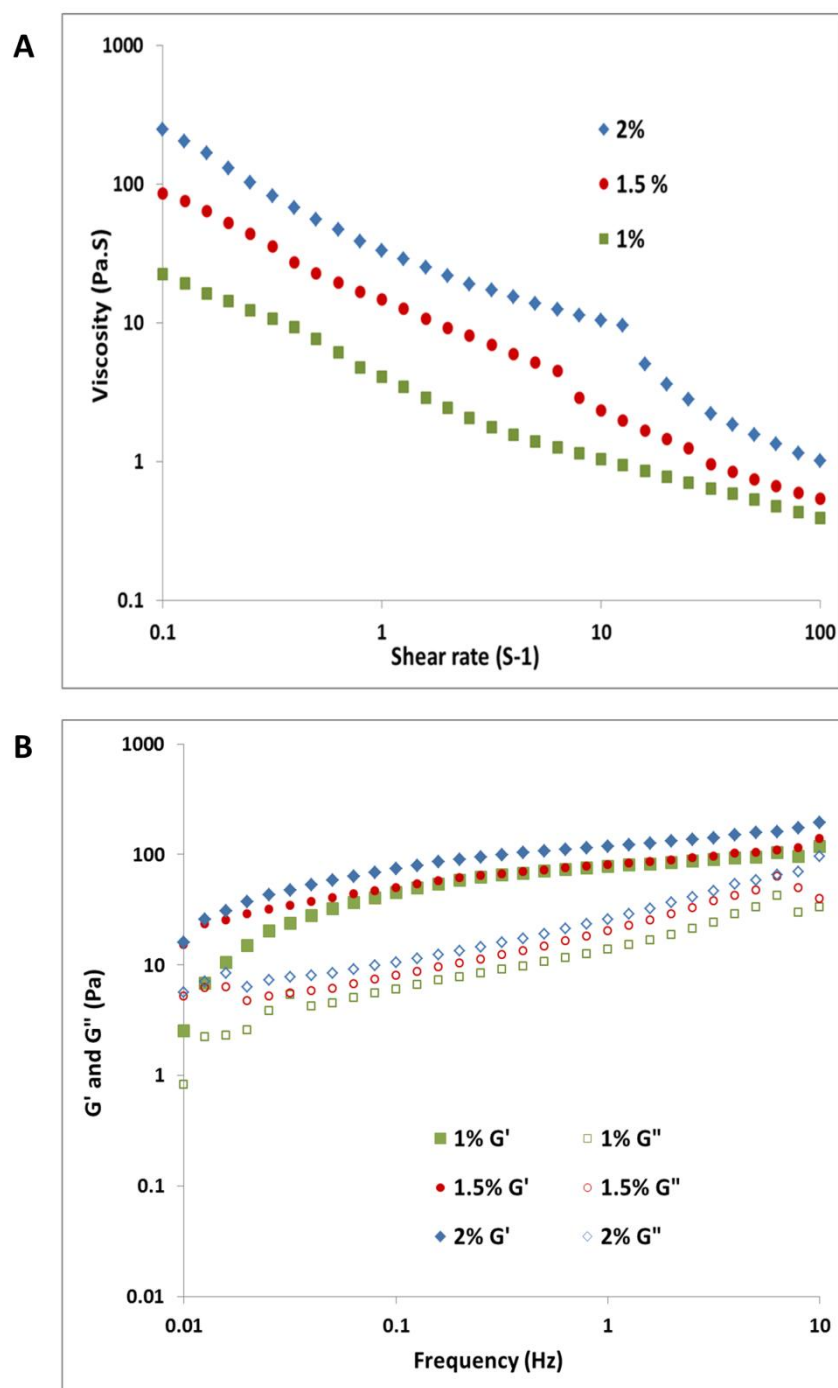


Figure 3.9. Effect of the concentration of nanoparticles on rheological properties; A) Apparent viscosity versus shear rate; B) Oscillatory frequency sweep curves. G': storage modulus; and G'': loss modulus.

3.4 Conclusions

In this work, CH/GA nanoparticles have been synthesized and their use as novel stabilizers for Pickering emulsions was tested. This strategy ensures the production of surfactant-free emulsions, an interesting topic in the context of sustainable and green chemistry principles. GA with its hydrophobic moieties had an important function in adjusting the wettability of the developed particles. The nanoparticles produced using a CH/GA weight ratio of 1:1 demonstrated near-neutral wettability (contact angle close to 90°). CLSM analysis revealed that the nanoparticles formed a very perceptible layer around the oil droplets, which prevents droplets from coalescence assuring emulsion stability. Moreover, it was shown that gravitational separation on long-term storage was inhibited by increasing the oil volume fraction and the nanoparticles concentrations in the produced Pickering emulsions. The formulations were found to be stable during the tested storage period of two months. The rheological properties of the formulations were shown to be tunable; as they depended on the oil volume fraction and the nanoparticles concentration in the emulsions. In addition, they have shown shear thinning and elastic-like behavior. This work provides new insights for CH/GA based Pickering emulsions as novel surfactant-free biocompatible emulsion systems. Future efforts will explore using these Pickering emulsions for the encapsulation and delivery of bioactive compounds in cosmetic or food formulations.

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4 Enhancing trans-resveratrol topical delivery and photostability through entrapment in chitosan/gum Arabic Pickering emulsions

The surfactant-free nature and higher stability of Pickering emulsions make them preferable solutions over conventional emulsions for skin applications. In this work, Pickering emulsions stabilized by chitosan/gum Arabic (CH/GA) nanoparticles were tested as vehicles for trans-resveratrol topical delivery. Skin absorption was examined *ex vivo* using Franz diffusion cells and porcine skin. Pickering emulsions allowed higher cutaneous retention and lower permeation of resveratrol, in comparison with a control solution based on a 20% v/v ethanol. The total amount of resveratrol retained in the skin, 24 hours after the application, was 11.60% and 10.82% of the applied dose for the tested Pickering emulsion-based formulations prepared with 0.5% and 1.5% w/v CH/GA nanoparticles, respectively. In contrast, resveratrol skin retention from the control solution was only 2.86%. Confocal laser scanning microscopy revealed enhanced skin deposition of Nile Red to deeper layers from the Pickering emulsion-based formulations. Moreover, Pickering emulsions led to trans-resveratrol photostability enhancement, as measured after exposure to UV for 4 hours. These results show that the CH/GA Pickering emulsions are promising solutions for the topical delivery of trans-resveratrol and have the potential to be used as green cosmetic products.

This chapter is adapted from:

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4.1 Introduction

Research recognition has recently been given to natural-based cosmetics to overcome the side effects and toxicity-related issues of synthetic ingredients in cosmetics and skincare products. Furthermore, there has been an increasing commitment from the cosmetic and pharmaceutical industry sectors to adopt green chemistry principles and to meet the consumers' demands for healthier and safer products, thus moving towards embracing Corporate Social Responsibility (CSR) by prioritizing respect for the environment and biodiversity, and giving back to the community (Philippe et al., 2012). Thus, the market for green cosmetics is quickly growing as these environmental and consumers' demands are rising competitive advantages (Liobikienė & Bernatoniene, 2017). This can be corroborated by the increasing dependence of the cosmetic industry on renewable plant/animal ingredients, which are gradually substituting petrochemical ones and reinforcing the renewable carbon content in the final product (Philippe et al., 2012).

Pickering emulsions are emulsions stabilized by solid particles instead of the classical surfactants (Chevalier & Bolzinger, 2013). The surfactant-free nature of Pickering emulsions makes them an emerging choice for topical formulations to overcome the adverse effects exhibited by the chemically synthesized emulsifiers on the skin (such as irritation and eczema), and on the environment (Chevalier & Bolzinger, 2013; Seweryn, 2018). The majority of the research conducted on Pickering emulsions for topical application reported the use of inorganic particles as stabilizers, such as silica and TiO₂ (Marto et al., 2016). However, at present, the use of organic and biopolymeric particles as Pickering stabilizers has acquired relevance over the synthetic and inorganic particles due to their sustainable and biocompatible character (Laredj-Bourezg et al., 2017; Marku et al., 2012; Marto et al., 2016, 2019).

Chitosan is a polysaccharide obtained from the deacetylation of chitin, which is a natural polymer present in the exoskeleton of crustaceans. Chitin is insoluble in aqueous solutions, whereas chitosan is soluble in acidic media (Aranaz et al., 2018). Chitosan has been reported to have skin moisturizing, humectant, antioxidant, anti-inflammatory and UV protective properties (Afonso et al., 2019; Aranaz et al., 2018). Gum Arabic is a natural polysaccharide obtained from Acacia tree that, according to *in vitro* studies, is able to

protect the skin against lipid peroxidation caused by the continuous exposure to UV light, and guard against premature skin aging (Trommer & Neubert, 2005). Despite these skin benefits, the use of gum Arabic in Pickering emulsion formulations has been only recently reported in very few studies that focused on food applications.

Resveratrol is a plant polyphenol with diverse therapeutic and prophylactic properties. It has been reported to have anti-inflammatory, antimicrobial, chemopreventive and strong antioxidant activities (Chedea et al., 2017; Pannu & Bhatnagar, 2019; Summerlin et al., 2015). Studies have shown that it protects the skin against aging by neutralizing pro-oxidant species directly, as well as by stimulating endogenous antioxidant defense pathways in the skin (Chedea et al., 2017). It has been also demonstrated that it positively affects the expression of specific genes in human skin, in a way that stimulates the production of collagen and elastin, and hence, enhances skin elasticity and thickness, and reduces wrinkles (Lephart et al., 2014). Resveratrol has poor water solubility and exists in the “cis” or “trans” configuration, with the trans form reported to have higher biological activity than the cis form. However, the trans form is photosensitive and transforms into the cis form when exposed to direct sunlight or UV radiation (Chedea et al., 2017; Summerlin et al., 2015). To enhance the solubility and stability of resveratrol, and to leverage the previously mentioned biological potentials, its encapsulation in different systems designed for topical delivery, such as liposomes, lipid nanoparticles and emulsions has been tested (J. Chen et al., 2017; Park et al., 2014; Zillich et al., 2013).

The use of Pickering emulsions as vehicles of hydrophobic drugs has been recently studied aiming at enhancing the absorption and bioavailability of the active agents (Lima Cardial et al., 2019; Lu et al., 2019). In what concerns resveratrol, the examples involving the use of Pickering emulsions are scarce. Matos and co-workers (Matos et al., 2018) developed resveratrol-loaded Pickering emulsions stabilized by modified quinoa starch particles. Also, Zhang and co-workers (Yali Zhang et al., 2017) encapsulated resveratrol in Pickering emulsions stabilized by zein/chitosan particles, proving higher stability and better sustained release of resveratrol in comparison with nanoemulsions developed in the same study. These resveratrol-loaded Pickering systems focused only on food applications, and no

studies to date examined resveratrol entrapment in Pickering emulsions for skin applications.

In a recent study, our research group developed biopolymeric chitosan/gum Arabic nanoparticles with near-neutral wettability and successfully used these nanoparticles to produce novel biodegradable o/w Pickering emulsions, which have presented high storage stability and excellent rheological properties for cosmetic applications (Sharkawy et al., 2019). Following these advancements, the main objective of the present work is to investigate the potential use of Pickering emulsions stabilized with chitosan/gum Arabic nanoparticles as novel surfactant-free delivery vehicles for the topical application of trans-resveratrol. Moreover, the work also aims to examine the ability of the developed biopolymeric-based Pickering emulsions to protect trans-resveratrol against UV degradation. To the best of our knowledge, this is the first study that investigates the use of Pickering emulsions for the topical delivery of resveratrol.

4.2 Materials and methods

4.2.1 Materials

Chitosan (ChitoClear[®]) with a degree of deacetylation of 96% and MW of 100-200 kDa was a kind gift from Primex ehf, Iceland. Gum Arabic, olive oil (highly refined, low acidity), trans-resveratrol (grade $\geq 99\%$), Tween 20[®] and Nile Red were purchased from Sigma Aldrich. Methanol and formic acid (gradient grade for HPLC) were supplied by Merck. Ethanol and acetic acid (0.1N) were purchased from Carlo Erba Reagents and Alfa Aesar, respectively. The porcine skin was obtained from pig ears which were supplied by a local slaughterhouse in Portugal.

4.2.2 Methods

4.2.2.1 Preparation of chitosan/gum Arabic (CH/GA) nanoparticles and Pickering emulsions

The nanoparticles were prepared through ionotropic gelation following a method developed in our research group (Sharkawy et al., 2019). Briefly, CH/GA nanoparticles (with 1:1

weight ratio) were prepared at two different concentrations, namely 0.5% and 1.5% w/v. The nanoparticle dispersions were obtained by dissolving the required amount of chitosan in 50 ml of 0.1N acetic acid, followed by the dropwise addition of the corresponding weight of gum Arabic dissolved in 50 ml of deionized water. The addition was done under continuous magnetic stirring at 800 rpm for 30 minutes. The final pH of the dispersions was maintained at 4.

CH/GA nanoparticles stabilized Pickering emulsions were prepared by slowly adding the olive oil to the nanoparticle dispersions. The process was conducted under continuous homogenization (using Ultraturrax DI 25 basic IKA) at a speed of 13500 rpm for 5-7 minutes. Pickering emulsions were prepared using the two produced CH/GA nanoparticles dispersions (with concentrations 0.5% and 1.5% w/v), and a constant oil volume fraction ($\phi = 0.6$). The total volume of the formed emulsions was 100 ml.

Resveratrol-loaded Pickering emulsions were prepared by incorporating trans-resveratrol in the used olive oil phase. The dissolution was aided by applying ultrasonication for 30 minutes, avoiding light exposure during this step. Then, the emulsions were prepared as previously described. Resveratrol concentration in the prepared emulsions was 0.1 % w/v (100 mg in 100 ml total emulsion volume). The emulsions were prepared using 0.5% and 1.5% w/v CH/GA nanoparticles to assess the effect of low and high nanoparticles concentration on resveratrol skin uptake and photostability.

4.2.2.2 Characterization of the CH/GA nanoparticles and Pickering emulsions

Transmission electron microscopy (TEM) was used to examine the morphology of the produced nanoparticles. The freshly prepared nanoparticles dispersion (prepared with a concentration of 1.5% w/v) was mounted on Formvar/carbon film-coated mesh nickel grids (Electron Microscopy Sciences, Hatfield, PA, USA). Visualization was conducted by a JEOL JEM 1400 TEM at 120 kV (Tokyo, Japan), and the images were acquired using a CCD digital camera Orious 1100 W (Tokyo, Japan).

The average size of the formed nanoparticles was measured by dynamic light scattering (DLS) using a Zetasizer Nano ZS ZEN3600 (Malvern Instruments, UK). The sample was

diluted to 100-fold with deionized water (serial dilution). The results were reported as the average of three consecutive readings.

The dynamic interfacial tension between CH/GA nanoparticles dispersions (with concentrations of 0.1%, 0.5% and 1.5% w/v) and olive oil was determined as a function of time (over 4000 seconds). The test was conducted using a Dataphysics OCA15 Plus device (Dataphysics, Germany), by forming a drop of the CH/GA nanoparticles dispersion inside a cuvette filled with olive oil with a submerged syringe tip. The interfacial tension was calculated by the pendant drop shape with the Young-Laplace equation. The measurements were performed at room temperature (25 °C).

The interfacial structure of CH/GA nanoparticles-stabilized Pickering emulsions was visualized by a cryo-scanning electron microscope (JEOL JSM 6301F/ Oxford INCA Energy 350/ Gatan Alto 2500). This characterization was performed for the formulations prepared with nanoparticles with a concentration of 1.5% w/v (with and without resveratrol). Briefly, the specimen was rapidly cooled by plunging it into sub-cooled nitrogen (slush nitrogen) and transferred under vacuum to the cold stage of the preparation chamber. The specimen was fractured, sublimated for 60 seconds at -90°C, and coated with Au/Pd by sputtering for 50 seconds. The sample was then transferred into the SEM chamber. The sample was analyzed at a temperature of -150°C, and the images were taken at different magnifications (10000x and 20000x).

Morphology comparison of the resveratrol-loaded Pickering emulsions prepared with 0.5% and 1.5% w/v nanoparticle concentrations was examined by optical microscopy using a Leica DM 2000 optical microscope. The average droplet size was measured using a laser diffraction particle size analyzer (LS 230, Beckman Coulter). The refractive indexes for the water and the oil phase were 1.33 and 1.46, respectively. The morphology and the average droplet size were examined for the freshly prepared resveratrol-loaded Pickering formulations and after storage for 2 months at room temperature.

4.2.2.3 Resveratrol encapsulation efficiency

The encapsulation efficiency (EE %) was determined to assess the amount of resveratrol retained in the core oil droplets and know its distribution between the oil and aqueous

phases of the Pickering emulsion. It was conducted by accelerated phase separation achieved by centrifugation in accordance with a previously reported method, but with slight modifications (Shao et al., 2019). Briefly, 1 ml of the Pickering emulsion was diluted with 10 ml of methanol and placed in a sonication bath for 10 minutes in order to de-emulsify the formed emulsion and extract the total resveratrol (RSV) amount. Thereafter the sample was filtered through a 0.2 μm nylon syringe filter, and RSV concentration (Total RSV) was determined by HPLC (as described in Section 4.2.2.8). Additionally, another 1 ml of the Pickering emulsion was centrifuged at 10000 rpm for 15 minutes. The obtained serum layer, which represents the aqueous phase, was collected, filtered, and analyzed by HPLC for RSV quantification (Free RSV). EE% was then calculated by the following equation:

$$EE \% = \frac{\text{Total RSV} - \text{Free RSV}}{\text{Total RSV}} * 100$$

4.2.2.4 *In-vitro* release studies

Resveratrol release was studied using jacketed Franz diffusion cells (PermeGear, USA) with an effective diffusion area of 0.64 cm^2 (contact area between donor and receptor compartments), and a receptor chamber volume of 5 ml. A schematic representation of the Franz cell is provided in Figure 4.1. A cellulose acetate membrane with a pore size of 0.2 μm (Sartorius Stedim Biotech GmbH, Germany), placed between the donor compartment and the receptor compartment, was used. The receptor chamber contained an ethanol/water solution (1:1) containing 5g/L of Tween 20[®]; to ensure sink conditions during the experiment period (Scalia et al., 2015). The receptor medium was kept under magnetic stirring at 600 rpm, and the temperature was maintained at 32 °C using a thermostatic water bath. The donor chamber was filled with 300 μl of the Pickering emulsion sample or the RSV control solution (RSV in hydroalcoholic solution (20% v/v ethanol)). Both samples contained an equivalent amount of RSV (1mg/ml). At predefined time intervals, 400 μl of the receptor fluid was withdrawn and immediately replaced by an equal volume of fresh receptor fluid medium. All the assays were conducted in triplicate. The collected samples were then characterized by HPLC for RSV quantification. The cumulative RSV release % was calculated by the following equation (Dai et al., 2019):

$$\text{Cumulative RSV release\%} = \frac{\text{Amount of RSV released at time } t}{\text{Total amount of encapsulated RSV}} * 100$$

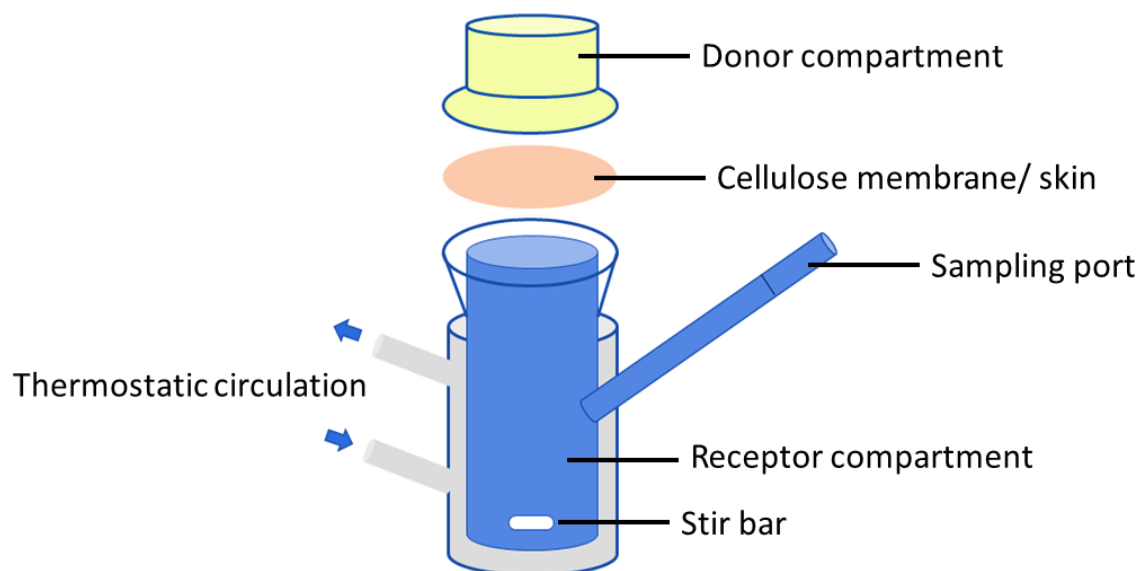


Figure 4.1. Schematic representation of Franz diffusion cell.

4.2.2.5 *Ex-vivo* skin retention and permeation studies

Resveratrol skin absorption and permeation were evaluated using porcine ear skin mounted in the previously described Franz diffusion cells, according to the OECD guidelines (OECD, 2004). The full-thickness skin was excised, and the hair was shaved off with a razor. The subcutaneous fat and hypodermis tissue were removed with forceps and surgical scissors. The skin membrane was placed with the *stratum corneum* side facing the donor compartment and the dermis facing the receptor medium compartment. The receptor medium (ethanol and water solution (1:1) and Tween 20[®] (5 g/L)) was kept under magnetic stirring at 600 rpm at 32 ± 1 °C. For the assays, 300 µl of the Pickering emulsion, or of the control sample (RSV in 20% v/v ethanol) was added to the donor chamber and uniformly spread over the skin. The concentration of RSV in the control sample was the same as in the tested Pickering formulations (i.e., 0.1% w/v). The experiment was performed under occlusive conditions by covering the donor chambers with Parafilm[®] to avoid water evaporation. Aliquots of 400 µl were collected from the receptor fluid at predetermined time intervals (0, 2, 4, 6, 8 and 24 h) to quantify the permeated resveratrol. The collected volume was replaced by an equal volume of fresh receptor medium to keep sink conditions.

The collected samples were protected from light to avoid RSV degradation and isomerization before quantification by HPLC.

Total mass recovery of RSV was checked by considering the mass balance of RSV distributed among the skin, and the donor and receptor compartments (Laredj-Bourezg et al., 2017; Y. Zhang et al., 2019). At the end of the assay, the Franz diffusion cells were disassembled, and the residual Pickering emulsion (or control sample) in the donor compartment was diluted with 30 ml of fresh receptor fluid solution. Moreover, the skin surface was washed with 10 ml of fresh receptor fluid, then the resulting washing solution was added to the previously obtained solution. This final solution was then filtered and analyzed by HPLC for RSV quantification. Afterward, the skin was dried with a wipe, and then stripping was done using Scotch® Crystal adhesive tape (3M, Minnesota, USA). Eighteen tape-stripping were applied consecutively to remove the *stratum corneum*. The tapes were collected, cut into small pieces, added to 40 ml of methanol in a sealed glass bottle and placed in an ultrasonic water bath for 30 minutes to extract RSV from the removed *stratum corneum*. The extraction liquid was then filtered and analyzed by HPLC to quantify RSV. The remaining skin membrane consisting of the viable epidermis and dermis (VED), was first cut into small pieces, then put in 40 ml of methanol and placed in an ultrasonic water bath for one hour to extract RSV. The sample was then filtered and analyzed by HPLC. All the samples were protected from light to prevent RSV degradation. Each test was performed, at least, in triplicate ($n \geq 3$). This mass balance is important to validate the performed studies.

4.2.2.6 Examination of skin retention by confocal laser scanning microscopy (CLSM)

CLSM was used to visualize the retention and distribution in the skin layers by using Nile Red as a fluorescent probe. This procedure has become a well-recognized technique to detect the distribution of hydrophobic drugs in the different skin layers (Hu et al., 2018; Laredj-Bourezg et al., 2017). The Pickering emulsion formulations were prepared using 0.01% w/v of Nile Red (instead of RSV) following the base procedure described in Section 4.2.2.1. The control sample was prepared by dissolving the same amount of Nile Red in deionized water. The assay was performed by applying 300 μ l of the Pickering emulsion (or the control sample) on the porcine skin in the Franz cells, as described in Section

4.2.2.5. After 6 hours, the skin samples were removed and washed thoroughly with deionized water and phosphate buffer solution to remove any excess of undiffused Nile Red. The skin samples were placed in a tissue freezing medium and frozen overnight at -20°C . On the second day, the samples were cryo-sectioned by a cryostat (Cryostat Leica CM 3050S) to skin sections with a thickness of $16\text{ }\mu\text{m}$. The sections were covered by a drop of mounting medium and glass coverslips, and visualized using a Leica TCS-SP5 AOBS confocal microscope (Leica Microsystem Inc., Germany) at an excitation wavelength of 561 nm and emission wavelengths of 570 nm and 691 nm.

4.2.2.7 Photostability of resveratrol

The photostability studies were conducted following the method of Khan et al. (Khan et al., 2019), with some modifications. Resveratrol-loaded Pickering emulsion formulations prepared with 0.5% and 1.5% (w/v) CH/GA nanoparticle concentrations, and a control sample of RSV in methanol were exposed to UV light (365 nm) using an HQ Power VDL 15 W ultraviolet lamp (Velleman[®], Belgium) for 4 hours. 15 ml of each sample were placed in glass round plates (with a diameter of 6 cm) and positioned 20 cm away from/below the lamp. Irradiated samples were collected after 0.5, 1, 2, 3 and 4 hours, and analyzed by HPLC for trans-resveratrol quantification.

4.2.2.8 Resveratrol quantification

Trans-resveratrol was quantified by high performance liquid chromatography (HPLC) using a reversed phase column ACE 5 C18-pentafluorophenyl group (250 mm x 3 mm, $5\mu\text{m}$). The eluents consisted of solution A (water/methanol: 95% /5 % (v/v)) and solution B (water/methanol: 5%/ 95 % (v/v)). Both solutions were acidified with 0.1% (v/v) formic acid. The analysis was conducted at 30°C using isocratic flow at a flowrate of 0.5 ml/min, and UV detection at 306 nm.

4.3 Results and discussion

4.3.1 Characterization of the produced CH/GA nanoparticles and Pickering emulsions

TEM images (Figure 4.2) show that the developed CH/GA nanoparticles are spherical, semi-spherical and oval. The irregular morphology of the nanoparticles is due to the absence of an oil core template for the deposition of the CH/GA coacervates (Sharkawy et al., 2019).

The obtained size distribution in number of the produced CH/GA nanoparticles (1.5% w/v) is characterized by an average size of 109 nm, and a polydispersity index (PDI) of 0.218. Previous studies on this system indicated that only minor agglomeration occurred during storage (Sharkawy et al., 2019).

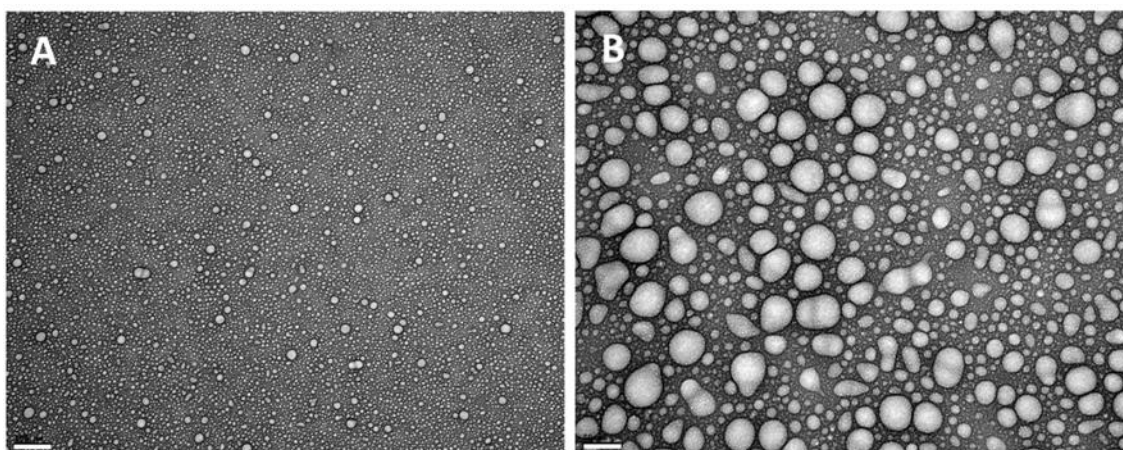


Figure 4.2. TEM images of CH/GA nanoparticles (1.5% w/v); Magnification A) 50000X, scale bar: 100 nm and B) 200000X, scale bar: 50 nm.

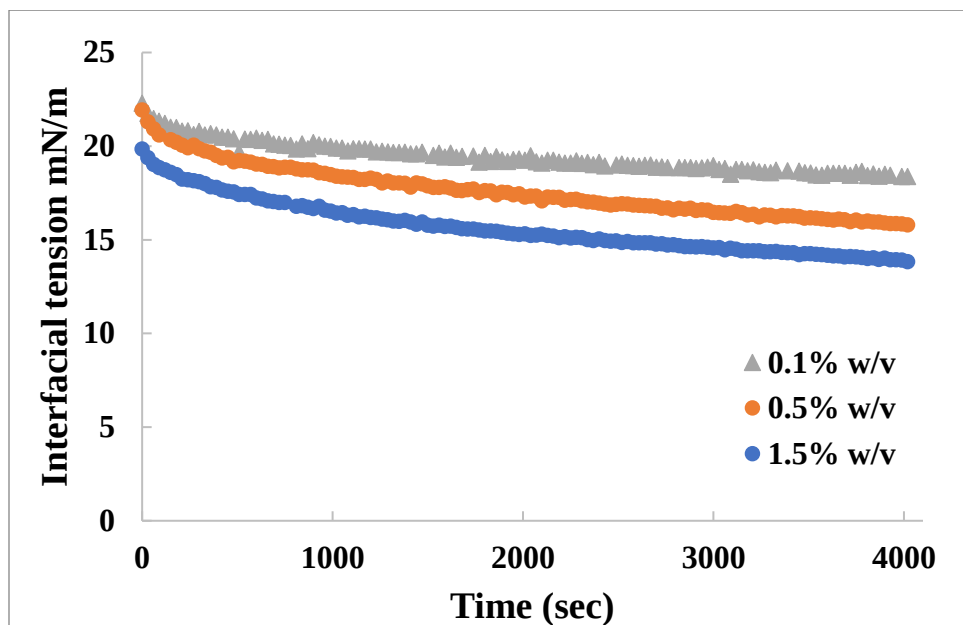


Figure 4.3. Dynamic interfacial tension between CH/GA nanoparticles with different concentrations (0.1%, 0.5% and 1.5% w/v) and olive oil *versus* time.

The interfacial tension between CH/GA nanoparticles and olive oil decreased with the increase in the concentration of the nanoparticles, as shown in Figure 4.3. Moreover, it can be noted that the rate of this decrement was initially high but became slower after 1500 seconds. This could be attributed to the penetration and initial adsorption of the nanoparticles at the oil-water interface leading to interfacial tension decrease. This phenomenon was followed by the packing and rearrangement of the nanoparticles at the interface. The saturation of the oil-water interface with nanoparticles decreases its ability to adsorb more particles, which subsequently led to a near-equilibrium state (J. Li et al., 2018; Wei & Huang, 2019). The results show that CH/GA nanoparticles of higher concentrations (0.5% and 1.5% w/v) possess better emulsification properties in comparison to the nanoparticles of lower concentrations (0.1% w/v).

Cryo-SEM technique is currently applied to investigate the interfacial structure of emulsion systems, including Pickering emulsions. As can be observed from Figure 4.4A, CH/GA nanoparticles form a dense layer around the oil droplet. The presence of such layer or sheath of nanoparticles, at the oil-water interface (i.e. around the oil core), results in emulsion stabilization; as it provides steric hindrance and electrostatic repulsion that act

against the coalescence of the droplets (X. Li et al., 2016; Zhu et al., 2019). Figures 4.4B and 4.4C show the surface of intact Pickering emulsion droplets, evidencing their rough and non-smooth appearance. At higher magnification (Figure 4.4D), the surface reveals the presence of depressions or “dimpled-like” texture, similar to that of a golf ball surface. This might be explained by the irregular deposition and non-uniform stacking of the nanoparticles onto the droplet surface. Similar indentations and rough surfaces were reported for Pickering emulsions stabilized by zein/corn fiber gum colloidal particles (Zhu et al., 2019) and zein/pectin nanoparticles (Jiang et al., 2019).

Cryo-SEM images of the resveratrol-loaded Pickering emulsion (Figures 4.4E and 4.4F) have shown that the emulsion droplets surface maintained its rough texture and possessed the same dimpled-like depressions observed in the base Pickering formulation (Pickering emulsion without RSV). This suggests that the incorporation of RSV did not influence the morphology and the deposition pattern of CH/GA nanoparticles at the oil-water interface, indicating that the stability of the emulsion droplets against coalescence was not affected.

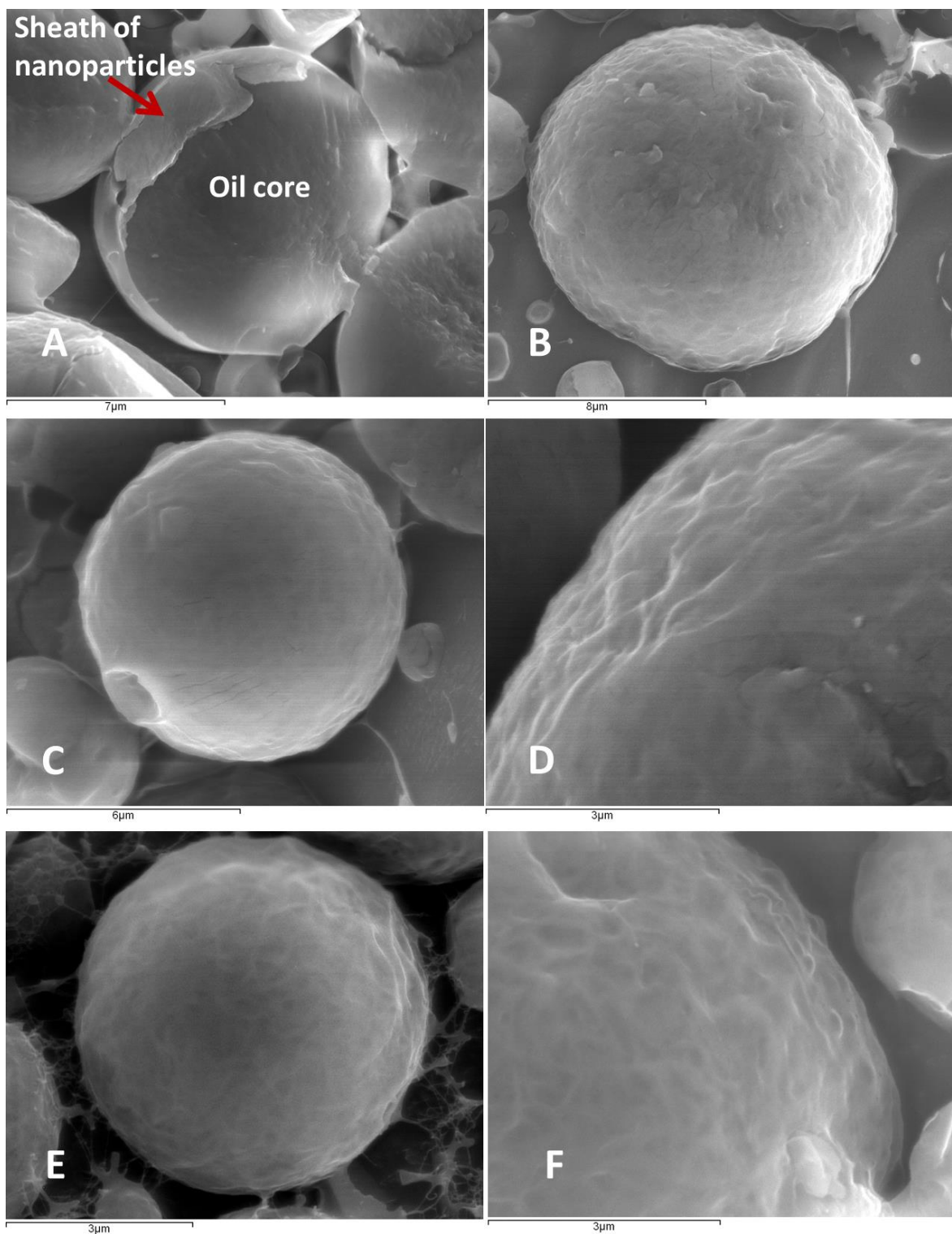


Figure 4.4. Cryo-SEM images of A) Pickering emulsion stabilized with CH/GA nanoparticles (1.5% w/v) showing a fractured droplet; B) Intact emulsion droplet of the same formulation showing at the surface the adsorbed CH/GA nanoparticles; C) Intact

emulsion droplet of the same formulation showing the rough indented surface at the edge; D) Surface of an intact Pickering emulsion droplet of the same formulation; E) Intact emulsion droplet of the resveratrol-loaded Pickering emulsion stabilized with CH/GA nanoparticles (1.5% w/v), and F) Surface of an intact droplet of resveratrol-loaded Pickering emulsion of the same formulation.

The optical microscopy images of freshly prepared RSV-loaded Pickering emulsions stabilized with 0.5% w/v and 1.5% w/v CH/GA nanoparticles are shown in Figures 4.5A and 4.5C, respectively. The images show that the emulsion droplets possess well-defined spherical morphology for both concentrations. The droplet size distribution profiles of both formulations have shown a bimodal distribution (Figure 4.6). It can be also observed that the size of the emulsion droplets decreased as the concentration of the nanoparticles increased, which is in accordance with the optical microscopy images (Figures 4.5A and 4.5C). The optical microscope images of the two Pickering formulations after storage for two months at room temperature (Figures 4.5B and 4.5D) show that the emulsion droplets maintained their spherical morphology, and only a slight increase in the droplet size was observed.

Laser diffraction analysis has shown that the RSV-loaded Pickering formulation prepared with 0.5% w/v CH/GA nanoparticles has an average diameter of 23.98 μm , while the average diameter of the one prepared with 1.5% w/v is 17.11 μm . This is related to the fact that higher nanoparticle concentration causes the coverage of a larger interfacial area, thus decreasing the droplets size. After storage for 2 months, the average diameter was 30.47 μm and 20.88 μm for the formulations prepared with 0.5% w/v and 1.5% w/v CH/GA nanoparticles, respectively. These values are in agreement with what was observed previously by optical microscopy, which pointed out for a minor coalescence of the droplets with time. Moreover, the values follow the pattern of our previous study concerning the development of the base Pickering emulsions (prepared without RSV) (Sharkawy et al., 2019). These base formulations maintained their stability for the studied period of two months.

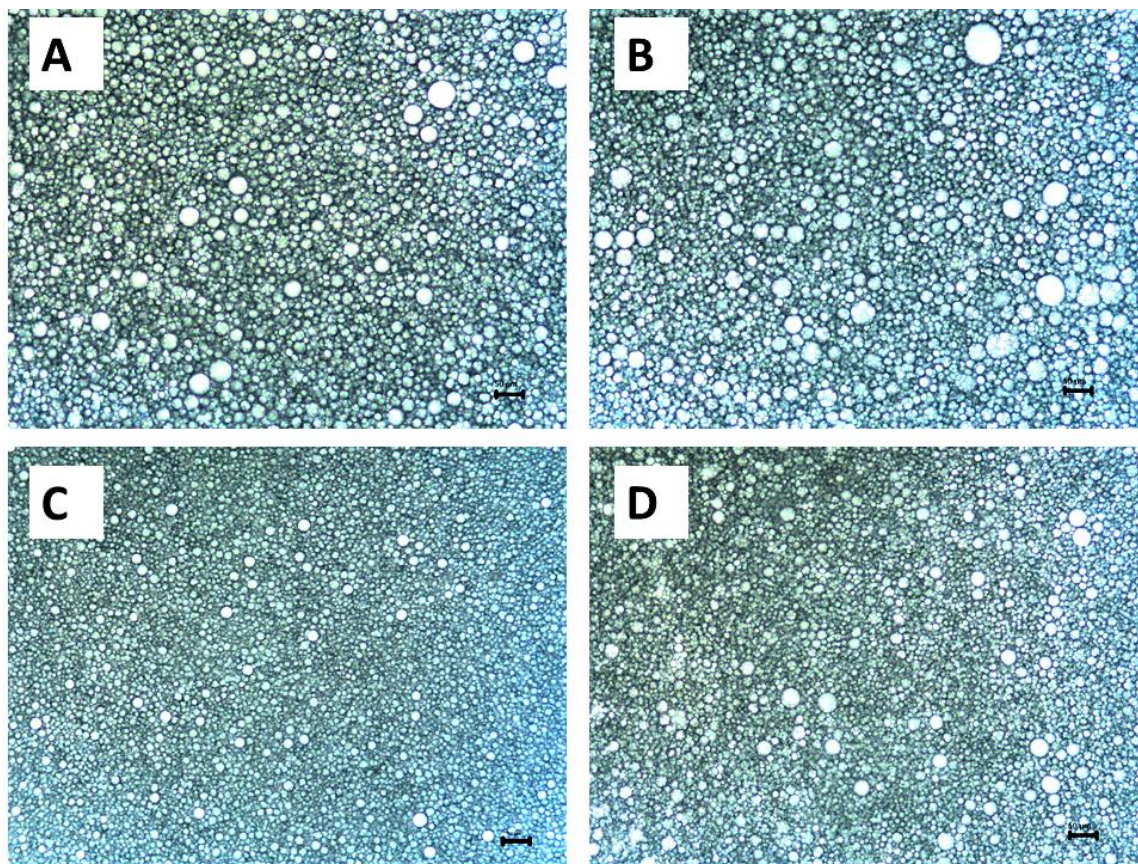


Figure 4.5. Optical microscope images of fresh and stored resveratrol-loaded Pickering emulsions; (A) stabilized with 0.5% w/v CH/GA nanoparticles after preparation; (B) stored for 2 months; (C) stabilized with 1.5% w/v CH/GA nanoparticles after preparation; and (D) stored for 2 months. The scale bar corresponds to 50 μm .

The RSV encapsulation efficiency (EE%) for the formulation prepared with 0.5% w/v CH/GA nanoparticles was $92.81\% \pm 1.76$, whereas it was $94.50\% \pm 0.23$ for the emulsion prepared with 1.5% w/v CH/GA nanoparticles. Similar high values of RSV encapsulation efficiency were reported for Pickering emulsions stabilized by octenyl succinic anhydride (OSA) modified starch particles (Matos et al., 2018). In the present study, the slight difference in the values of EE% of the two formulations demonstrates that the increase in the CH/GA nanoparticle concentration had no influence on resveratrol retention. This suggests that the presence of the nanoparticles at a low concentration (0.5% w/v) was sufficient to form a mechanical barrier at the water-oil interface, which successfully achieved resveratrol entrapment in the oil phase.

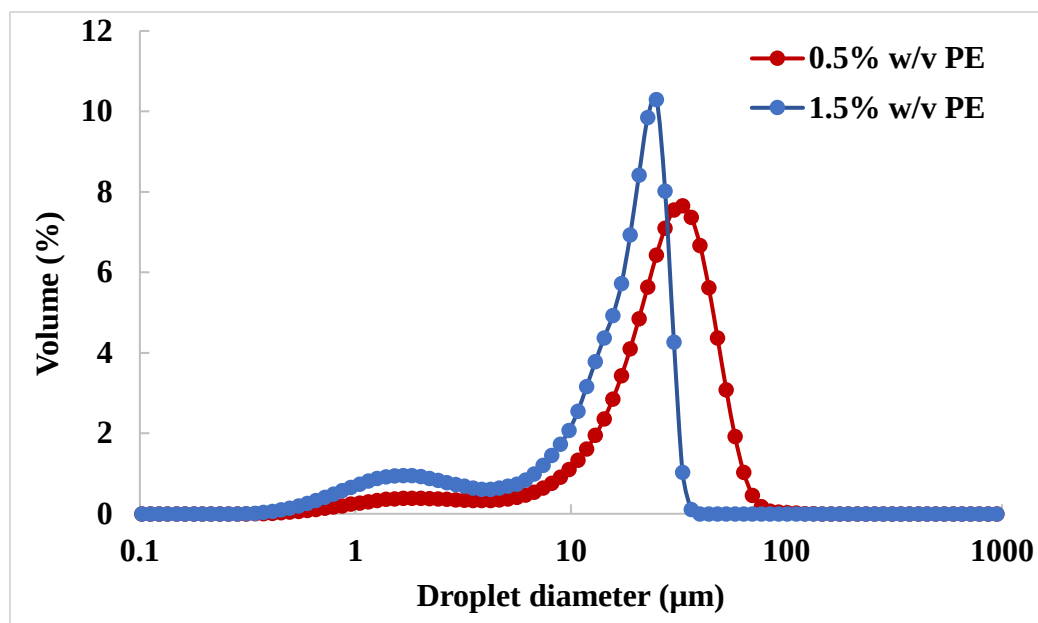


Figure 4.6. Droplet size distribution profiles of RSV-loaded Pickering emulsions stabilized with 0.5% and 1.5% w/v CH/GA nanoparticles.

4.3.2 Resveratrol *in vitro* release and diffusion

The release profiles of RSV from the Pickering emulsions prepared with 0.5% and 1.5% w/v CH/GA nanoparticles concentrations, and the control solution are shown in Figure 4.7. It can be seen that after 24 hours, 53.7% of the total RSV was released from the Pickering formulation prepared with 0.5% w/v CH/GA nanoparticles, whereas only 38.5% was released from the formulation stabilized with 1.5% w/v nanoparticles. The slower release from the second formulation could be explained by the presence of a higher amount of nanoparticles. These particles being adsorbed at the emulsion oil droplet surfaces have eventually reduced the rate of RSV release from the oil core.

The cumulative release profile of RSV from diluted ethanol was faster in the beginning (during the first 4 hours) reflecting the free passage of RSV through the cellulose acetate membrane. This behavior was expected along the entire test time, nevertheless, the experiment was discontinued after 4 hours due to the drying of the vehicle and the membrane (despite applying occlusive conditions; i.e. covering the donor compartment

with Parafilm®). Accordingly, the experimental points for the control solution represented in Figure 4.7 only relate to the first four hours.

The release of an active agent from the Pickering emulsions is controlled by its ability to cross and diffuse through the nanoparticles shell covering the oil droplets. It is also dependent on its solubility and partition between the oil and aqueous phases of the emulsion (Hu et al., 2018). Therefore, the slow release of RSV from Pickering emulsion formulations, in comparison with the control solution, could be explained based on RSV solubility difference in the aqueous and oil phases of the emulsion. The solubility of RSV in water is relatively low (0.05 mg/ml) compared to its solubility in the oil phase. Therefore, it is expected that RSV would be predominately distributed in the oil phase of the emulsion. This results in reducing the RSV release rate from the oil core, which subsequently decreases its passage through the membrane.

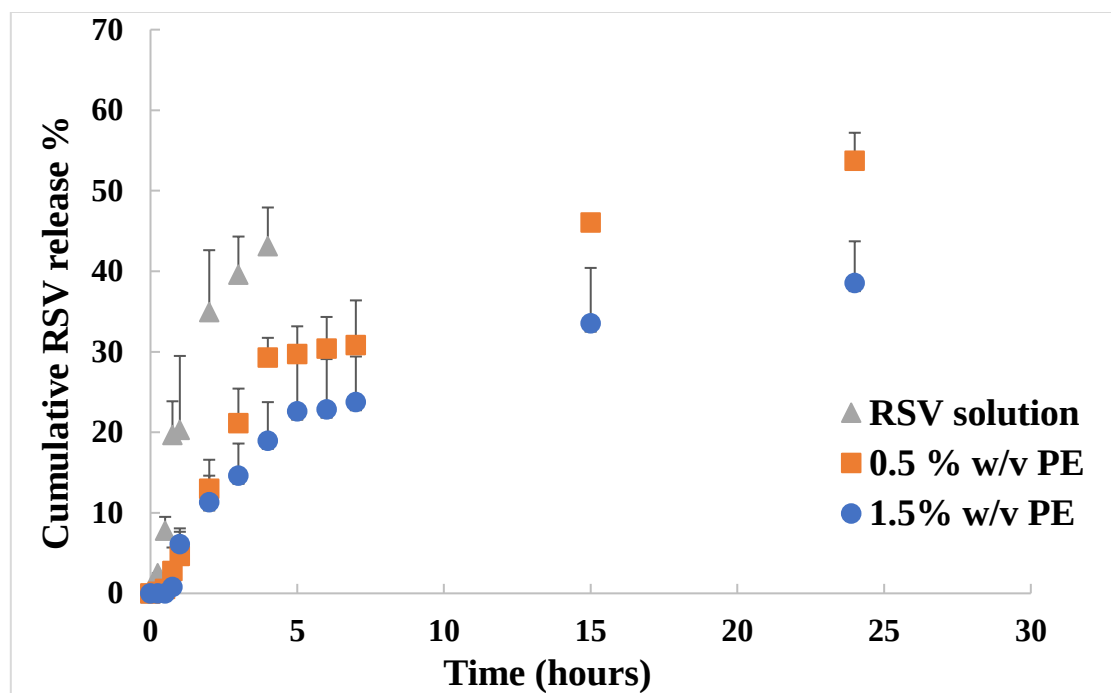


Figure 4.7. In vitro RSV release profiles of Pickering emulsion formulations prepared with 0.5% w/v and 1.5% w/v CH/GA nanoparticles, and RSV solution in diluted alcohol (20% v/v) through cellulose acetate membrane.

4.3.3 *Ex-vivo* skin retention and permeation

The *stratum corneum* which is the outermost layer of the skin is considered the main barrier for the permeation of the active agent. It consists of corneocytes fixed in an intercellular lipid matrix (Sapra et al., 2008). In the case of the topical route and cosmetics, it is important to enhance the permeation of the active agent through the *stratum corneum* in order to be retained in the other skin layers. The active agent should penetrate inner skin layers moderately, and not deeply to avoid reaching the blood circulation and making a systemic/therapeutic effect (Müller et al., 2002). Avoiding systemic absorption is a crucial issue to enhance the skin benefits of the active agent intended for topical delivery (Marto et al., 2016, 2019). Therefore, efficient delivery of a cosmetic ingredient should achieve higher skin retention rather than permeation (Kong et al., 2011). In general, the degree of skin penetration depends on the formulation, the chemical nature of the active compound and the skin hydration state (Marto et al., 2016). It has been reported in previous studies that cosmetic formulations with microspheres smaller than 3 μm penetrate the skin by diffusion and through the hair follicles, whereas formulations with microspheres of sizes ranging from 3 μm to 10 μm penetrate through the hair follicles (Schaefer et al., 1994; Marto et al., 2019). Formulations with microspheres greater than 10 μm tend to remain on the skin surface and the active agent eventually diffuses from them to the skin (Schaefer et al., 1994; Marto et al., 2019). Therefore, in the present study, it is expected that the Pickering emulsion droplets of sizes exceeding 10 μm would remain on the skin, allowing RSV to be released with time from them to the skin, whereas the emulsion droplets of sizes less than 10 μm are expected to penetrate the skin.

As shown in Figure 4.8 and Table 4.1, after 24 hours of skin exposure to the Pickering formulations, only low amounts of RSV were detected in the receptor fluid. The percentage of the permeated RSV was 2.20% and 3.16% of the total applied dose, respectively for the Pickering emulsions stabilized by 0.5% and 1.5% w/v CH/GA nanoparticles. However, the skin absorption percentages were 11.60% and 10.82% of the total applied dose for Pickering emulsions stabilized by 0.5% and 1.5% w/v CH/GA nanoparticles, respectively. These results suggest that RSV-loaded Pickering emulsions retarded the diffusion of the RSV from the skin to the receptor fluid. In contrast, the control solution resulted in the

permeation of a high amount of RSV through the skin (11.62% of the total applied dose), suggesting its unsuitability as a cosmetic vehicle for RSV.

The concentration of RSV in the final preparation is restricted by its solubility in olive oil. In general, the solubility of RSV in oils is low (Matos et al., 2018). The used strategy concerning the amount to be added to the formulation takes into consideration the dose of RSV required to achieve a cosmetic effect and the final dose that is successfully taken up by the skin. RSV has been reported to enhance the firmness of the skin and stimulate the synthesis of hyaluronic acid when incorporated at a concentration ranging between 0.01% to 1% by weight of the topical formulation composition (Thomas et al., 2015). Additionally, the reported results in the current study have shown relatively high skin uptake amounts of RSV in the viable epidermis and dermis (46.24-50.41 $\mu\text{g}/\text{cm}^2$), in comparison with other recent studies (Chedea et al., 2017).

Pickering emulsions have been reported to enhance the permeation of hydrophilic drugs such as caffeine readily to the receptor compartment (Frelichowska, Bolzinger, Valour, et al., 2009), while they favor the retention of lipophilic drugs in the *stratum corneum* (Frelichowska, Bolzinger, Pelletier, et al., 2009; Laredj-Bourezg et al., 2017). CH/GA nanoparticles (with a 1:1 weight ratio) are positively charged and have a zeta potential of +56.3 mV (Sharkawy et al., 2019). In this work, resveratrol showed a tendency toward higher retention in the viable epidermis and dermis (VED) than in the *stratum corneum*. The skin penetration enhancing effect of chitosan and its derivatives has been reported in some studies. It has been postulated in previous studies that chitosan, based on its cationic nature, has the ability to open the tight junctions between the *stratum corneum* cells which carry a negative charge (Y. Chen et al., 2013; Sapra et al., 2008). Thus, it increases the intercellular spaces and detaches/loosens the corneocytes by disordering the lipid bilayer structure (Sapra et al., 2008). This penetration enhancement effect possibly permitted RSV to cross the *stratum corneum* causing its accumulation in the viable epidermis and dermis. At the same time, Pickering formulations acted as a barrier against the transfer and diffusion of RSV to the receptor chamber, thus successfully achieving the topical delivery of RSV.

The findings of the present work demonstrate the important role of CH in the produced nanoparticles and derived emulsions in enhancing the skin delivery of RSV. As such it is worth noting that the presence of GA in CH/GA nanoparticles is crucial in tuning the wettability of the produced nanoparticles, and hence improving the stability of the developed Pickering emulsions (Sharkawy et al., 2019).

The overall mass recovery % was quite satisfactory for a highly photosensitive compound like resveratrol. It was observed that more than 85% of the applied dose of resveratrol was recovered. This is in agreement with the OECD guidelines which states that a mass recovery of $100 \pm 20\%$ is acceptable for chemically unstable substances (OECD, 2004), which validates the obtained results.

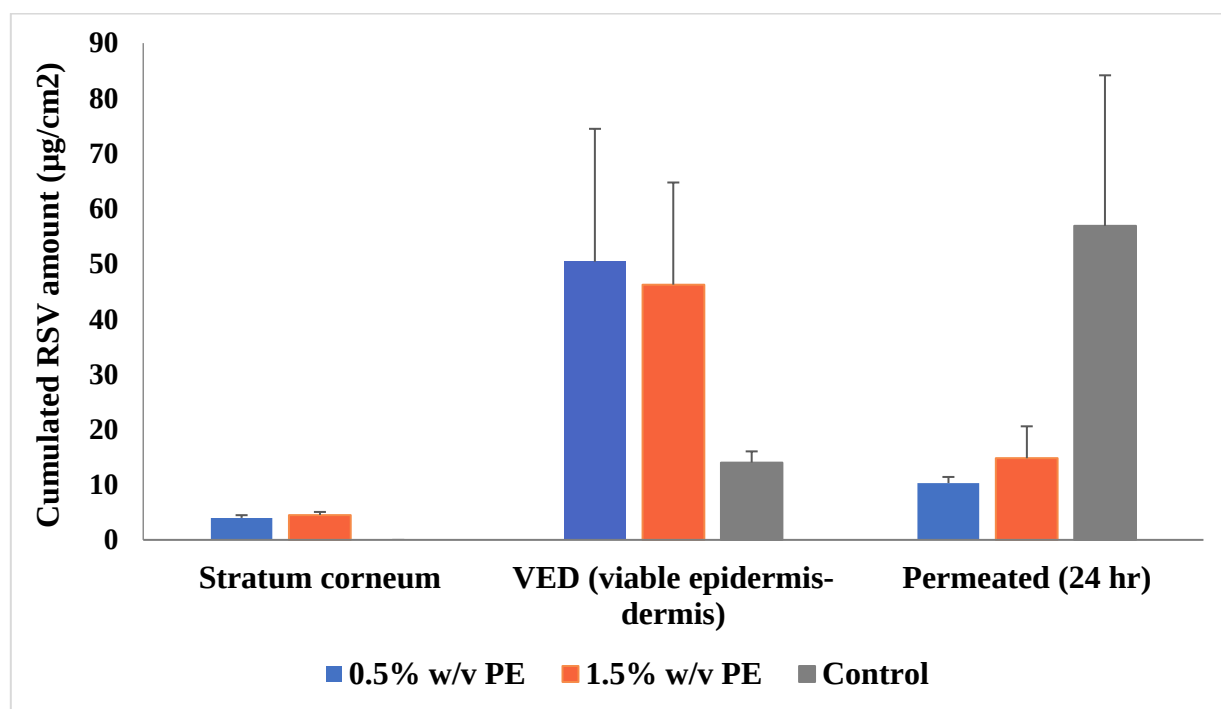


Figure 4.8. RSV distribution in the skin layers and receptor fluid after 24 hours of exposure to Pickering emulsion formulations stabilized by CH/GA (0.5%w/v and 1.5%w/v), and the resveratrol control solution. Values are represented as mean $\pm$ SD ($n = 3$). Note: No RSV was detected in the *stratum corneum* for the control solution.

Table 4.1. Distribution of resveratrol within skin layers (*Stratum corneum* and VED), receptor medium (permeated RSV), and donor compartment (skin surface) after 24 hours in contact with the Pickering emulsions (stabilized with 0.5% and 1.5% w/v CH/GA nanoparticles) and the control sample. The results are represented by the quantified drug amount ($\mu\text{g}/\text{cm}^2$) $\pm$ SD and the percentage relative to the total applied dose.

Formulation	Total applied dose	<i>Stratum corneum</i> (SC)	Viable epidermis and dermis (VED)	Permeated RSV (24 hours)	Skin surface*	Mass recovery
0.5% w/v PE						
$\mu\text{g}/\text{cm}^2$	468.75 $\pm$ 0.00	3.98 $\pm$ 0.50	50.41 $\pm$ 24.07	10.30 $\pm$ 1.10	341.23 $\pm$ 196.46	405.92 $\pm$ 180.40
% of total applied dose		0.85	10.75	2.20	72.80	86.60
1.5% w/v PE						
$\mu\text{g}/\text{cm}^2$	468.75 $\pm$ 0.00	4.51 $\pm$ 0.55	46.24 $\pm$ 18.51	14.79 $\pm$ 5.78	444.66 $\pm$ 22.08	510.21 $\pm$ 35.34
% of total applied dose		0.96	9.86	3.16	94.86	108.84
Control (RSV solution)						
$\mu\text{g}/\text{cm}^2$	489.64 $\pm$ 36.17	0	14.02 $\pm$ 2.01	56.88 $\pm$ 27.29	398.74 $\pm$ 8.01	469.64 $\pm$ 32.26
% of total applied dose		0	2.86	11.62	81.44	95.92

* represents the amount of RSV quantified on the skin surface and the residual sample in the donor compartment.

4.3.4 Determination of skin retention by CLSM

CLSM was used to visualize the skin retention through Nile Red. The use of fluorescent dyes as a substitute for the active agents and drugs to study the permeation of new formulations is a common technique applied to study topical applications (Hu et al., 2018; Laredj-Bourezg et al., 2017). Nile Red is a lipophilic dye with a high octanol-water partition coefficient ($\log P_{o/w} > 3$) (Hu et al., 2018). Therefore, it was chosen in this study to substitute RSV ($\log P_{o/w}$ of 3.1) (Summerlin et al., 2015), and visualize its skin retention and distribution by the produced Pickering formulations.

The studied Pickering emulsions (stabilized by 0.5% and 1.5% w/v CH/GA nanoparticles) were representative of low and high nanoparticles concentration. It must be highlighted that according to previous studies of our group, lower concentrations did not allow the preparation of stable emulsions (Sharkawy et al., 2019), and higher concentrations will give rise to smaller droplet size, and could potentiate skin permeation which was not the objective of this work.

Figure 4.9 shows CLSM images of cross-sectioned skin treated with Nile Red-loaded Pickering emulsions stabilized by 0.5% w/v and 1.5% w/v CH/GA nanoparticles and the control solution containing Nile Red. It can be observed that the fluorescence intensity in the skin sections treated with the Pickering formulations (Figures 4.9B and 4.9C) is higher than the one treated with the control sample, which indicates higher skin penetration and deposition of the lipophilic dye from the Pickering formulations. These results are in agreement with the overall retained RSV mass values obtained in Table 4.1, which shows that the Pickering formulations led to greater cutaneous deposition of the lipophilic active agent. The images also show that the Pickering formulation stabilized by 0.5% w/v CH/GA nanoparticles has higher fluorescence intensity in the viable epidermis layer than in the dermis (Figure 4.9B), while the fluorescence of the viable epidermis was less intense in the case of the formulation stabilized by 1.5% w/v nanoparticles (Figure 4.9C). However, it can be observed that the fluorescence in the dermis for the formulation stabilized with 1.5% w/v nanoparticles is more intense (Figure 4.9C) than that for the formulation stabilized with 0.5% w/v nanoparticles. The images again confirm that the presence of a greater number of

the cationic CH/GA nanoparticles enhances the penetration of the active agent (Nile Red in this case) through the *stratum corneum*, and subsequently its deposition in deeper skin layers.

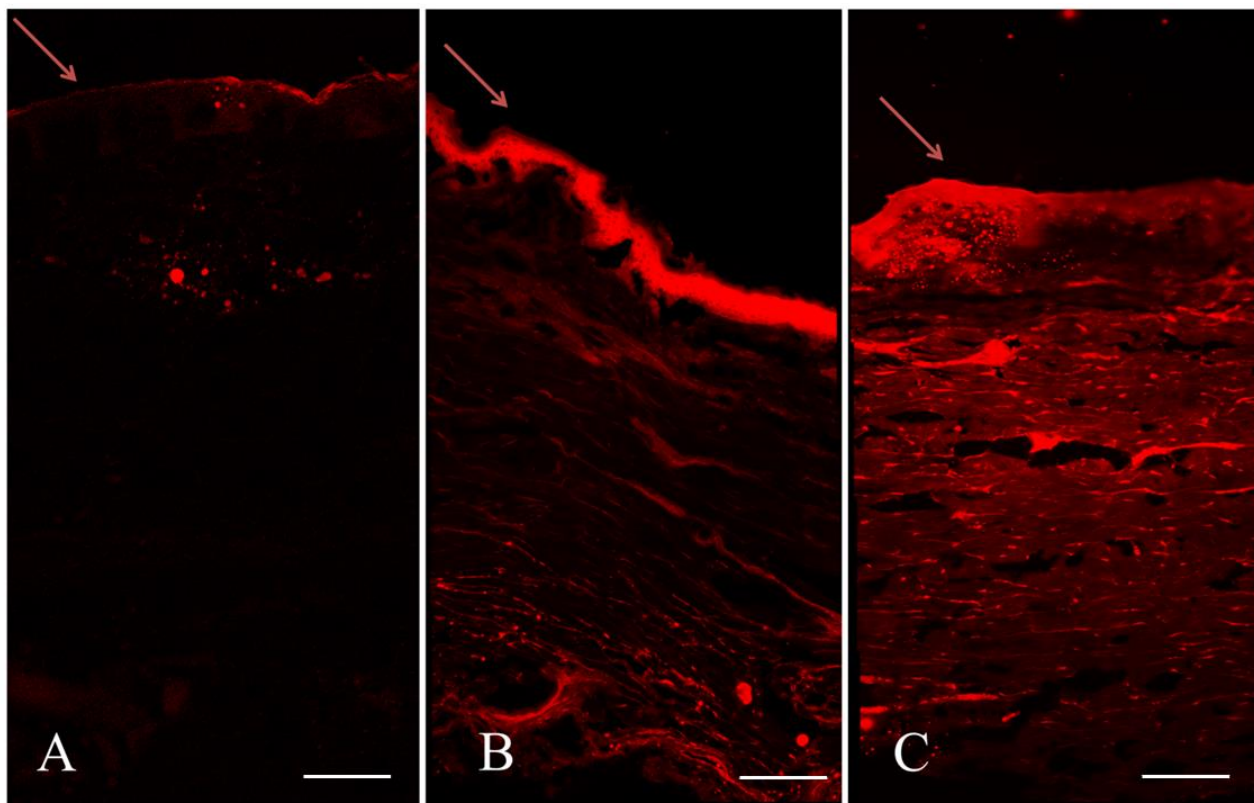


Figure 4.9. Confocal laser scanning microscopy images showing the distribution of Nile Red in vertical sections of porcine skin after being exposed for 6 hours to A) Control solution (Nile Red in water), B) Pickering emulsions stabilized with 0.5% w/v CH/GA nanoparticles, and C) Pickering emulsions stabilized with 1.5% w/v CH/GA nanoparticles. The arrows point to the skin surface (the *stratum corneum* side). Scale bar corresponds to 100 μm .

4.3.5 Photostability

Trans-resveratrol is known to have strong antioxidant activity. However, being unstable, it converts to the less bioactive isomer cis-resveratrol upon exposure to UV radiation (Silva et al., 2013). Therefore, it is important to maintain its photostability during storage, and subsequently during its application on the skin. The photodegradation profiles (Figure 4.10)

show that only 68.15% of RSV in the control solution was remaining after 4 hours of UV exposure, whereas the amount of RSV in the Pickering emulsion formulations remained unchanged. This protection from degradation is mainly attributed to the polymeric nanoparticles that surround the emulsion droplets and provide a shielding effect and physical barrier against the destructive UV radiation. Additionally, the presence of olive oil at high concentration ($\phi=0.6$) in the developed Pickering emulsions helped in the phytostabilization of resveratrol. It has been reported that the nanoparticles present at the surface of the droplets in Pickering emulsions scatter and attenuate the incident UV radiation leading to the phytostabilization of trans-resveratrol (Dai et al., 2019). Olive oil and other vegetable oils have been reported to efficiently protect photosensitive compounds from photodegradation in oil solutions and in cosmetic creams (Brisaert & Plaizier-Vercammen, 2007). The results in the present work show that Pickering emulsion formulations stabilized with 0.5% and 1.5% w/v CH/GA nanoparticles have protected resveratrol against UV degradation after 4 hours of exposure.

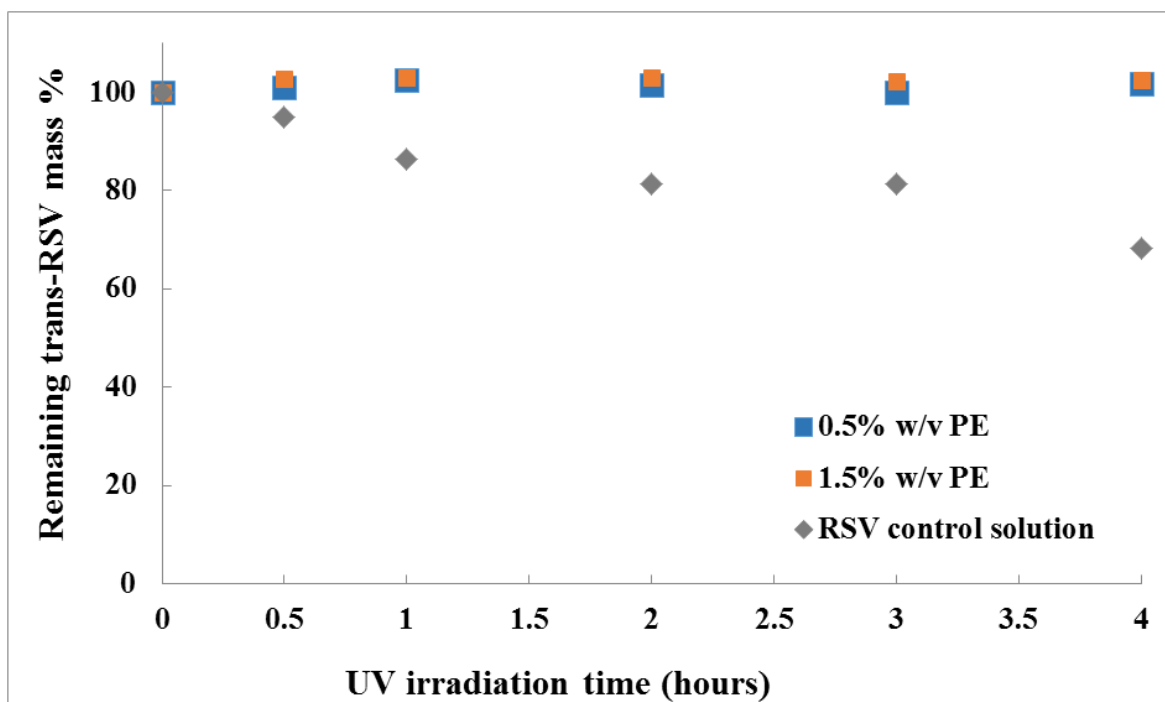


Figure 4.10. Percentages of remaining RSV mass in Pickering emulsions stabilized by 0.5% and 1.5% w/v CH/GA nanoparticles, and in RSV control solution upon exposure to UV radiation.

4.4 Conclusions

Chitosan/gum Arabic Pickering emulsions were successfully used as biodegradable surfactant-free vehicles for the skin application of trans-resveratrol. The skin permeation results of resveratrol from the developed Pickering emulsions have shown that resveratrol was retained in higher levels in the viable epidermis and dermis, which is advantageous to give the required cosmetic effect without the need of using chemical penetration enhancers. Photostability tests have shown that Pickering formulations protected trans-resveratrol against degradation by UV radiation. To our knowledge, this is the first study that investigated the skin uptake of resveratrol from Pickering emulsions. The results obtained in this work suggest that the produced chitosan/gum Arabic nanoparticles-stabilized Pickering emulsions enhanced the cutaneous delivery of resveratrol, thus they demonstrate a potential contribution towards green and sustainable cosmetics.

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5 New Pickering emulsions stabilized with chitosan/collagen peptides nanoparticles: Synthesis, characterization and tracking of the nanoparticles after skin application

Pickering emulsions based on biopolymeric particles are gaining increasing research recognition in numerous applications due to their biocompatibility and eco-friendliness. In this chapter, chitosan/collagen peptides nanoparticles were applied to stabilize Pickering emulsions for potential cosmetic applications. The nanoparticles had a contact angle of $78.02^\circ \pm 2.04^\circ$, reflecting their hydrophilic nature. Their average size and zeta potential were 32.27 nm and +59.7 mV, respectively. Confocal laser scanning microscopy (CLSM) showed that the nanoparticles become well-adsorbed at the oil-water interface. The emulsions displayed shear-thinning viscosity and gel-like texture. Their average droplet size ranged between 7.63 μm and 15.72 μm . CLSM skin tracking of the nanoparticles, after *ex vivo* skin application of the Pickering emulsions, revealed the ability of the emulsion droplets to penetrate the *stratum corneum* and deposit in deeper skin layers. The penetration degree depends on the concentration of the nanoparticles in the emulsion and on the contact time with the skin. These results provide new insights into the fate of the nanoparticles used to stabilize Pickering emulsions following their skin application.

This chapter is based on:

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5.1 Introduction

Sustainable cosmetics are gaining increasing interest in the industrial and academic sectors due to consumer demands towards safe and environmentally-friendly products with a “clean-label” (Bom et al., 2019). Cosmetic emulsions, in the form of creams, lotions and gels are manufactured on a wide scale. These formulations contain surfactants that are used to disperse and emulsify the oil phase. Among the most commonly used surfactants in cosmetic emulsions are sodium lauryl sulfate, sodium dodecyl sulfate, sodium laureth sulfate, polyethylene glycol ethers, polysorbate 80, and sorbitan oleate (Span 80) (Venkataramani et al., 2020). Although these surfactants are very popular in cosmetic formulations, recent studies have shown that they have adverse effects on both the environment and human health as they have been reported to cause cytotoxicity and haemolysis (Cserháti et al., 2002; Venkataramani et al., 2020). Moreover, many surfactants that are used as emulsifiers or detergents can induce/evoke skin adverse reactions, such as inflammation and contact dermatitis (Lémery et al., 2015).

Pickering emulsions are emulsions stabilized by solid particles that become adsorbed at the oil/water interface (L. Chen et al., 2020; Chevalier & Bolzinger, 2013; Dickinson, 2012). They are considered eco-friendly alternatives to the classical emulsion-based systems due to their emulsifier-free nature (Chevalier & Bolzinger, 2013; Marto, Ascenso, Simoes, et al., 2016). Pickering emulsions are finding increasing attention in the food and cosmetic applications (Hosseini & Rajaei, 2020; Marto et al., 2020; Sharkawy, Casimiro, et al., 2020; F. Wu et al., 2020; Yang et al., 2020). Pickering emulsions based on biopolymeric particles, in particular, are increasingly becoming attractive vehicles in cosmetic and skin applications due to their non-toxic, biocompatible, and biodegradable properties (Asfour et al., 2017; Bao et al., 2020; Marto et al., 2015; Sharkawy, Barreiro, et al., 2020).

Chitosan is a linear polysaccharide obtained from the deacetylation of chitin, which is a natural polymer that occurs in the exoskeleton of crustaceans. It is a copolymer that consists of D-glucosamine (deacetylated units) and N-acetyl-D-glucosamine (acetylated units) linked by β -(1,4) glycosidic bonds (Hamed et al., 2016). Chitosan is classified as GRAS (Generally Recognized As Safe) (Bellich et al., 2016). Recently, chitosan-based particles have been used as successful Pickering emulsion stabilizers in various applications

(Asfour et al., 2017; Atarian et al., 2019; Mwangi et al., 2016; Ribeiro et al., 2020; Shah et al., 2016; Sharkawy, Barreiro, et al., 2020). Chitosan has been used widely in cosmetic and skin formulations owing to its antibacterial, antioxidant, and skin regenerative properties (Cinelli et al., 2019; Hilmi et al., 2013), and also to enforce the circular economy principles for a sustainable cosmetic market (Cinelli et al., 2019). It has also been used to enhance the stability of cosmetic formulations by acting as a stabilizer and a viscosifier (rheology modifier) (Aranaz et al., 2018). Additionally, chitosan, when in the protonated form, has been reported to improve the cutaneous penetration of drugs and cosmeceuticals due to its ability to interact with the negative charges in the tight junctions of the *stratum corneum* (the outermost layer of the skin) (Abdel-Hafez et al., 2018; Al-Kassas et al., 2016; Y. Chen et al., 2013; Sharkawy, Casimiro, et al., 2020). Studies have also shown that chitosan changes the structure of keratin in the skin, decreases the cell membrane potential, and promotes cell membrane fluidity which leads to improving the cutaneous penetration (Abdel-Hafez et al., 2018).

Pickering emulsions stabilized with polysaccharide/protein complex particles have gained increasing research recognition as they generally demonstrate high stability and long shelf life (Z. Li et al., 2020). Collagen peptides (collagen hydrolysates) are produced by the enzymatic, acidic or alkaline hydrolysis of collagen, the main structural protein present in the skin, bones and connective tissues (Hong et al., 2019). Their low molecular weight has been reported to increase their bioavailability compared to their parent proteins. The utilization of collagen peptides in food supplements, cosmetic and pharmaceutical products is rapidly increasing (Hong et al., 2019; Zhang et al., 2020). The incorporation of collagen peptides in commercial cosmetic formulations has recently become exceptionally trendy due to their anti-aging properties. Collagenase enzyme is known to hydrolyze the collagen present in the skin, leading to dehydration and gradual loss of skin elasticity, and, subsequently, to the formation of wrinkles. Collagen peptides have been reported to have anti-collagenase activity, and thus help in preserving the endogenous skin collagen (Park & Jo, 2019). They have also been reported to possess anti-inflammatory and antioxidant properties (Offengenden et al., 2018).

Recent studies on Pickering emulsions developed for cosmetic and skin applications focused chiefly on tracking the fate of the active agent (the drug/cosmeceutical compound) that is incorporated/encapsulated in the dispersed phase (Asfour et al., 2017; Frelichowska, Bolzinger, Pelletier, et al., 2009; Marku et al., 2012; Sharkawy, Casimiro, et al., 2020). However, to date, studies that explore the fate of the nanoparticles that stabilize Pickering emulsions intended for dermal and/or transdermal delivery are very scarce in the literature. Such studies are relevant to show how deep the nanoparticles of the Pickering emulsions can penetrate the skin. Additionally, these studies can open new dimensions for the use of the nanoparticles; as they can be tailored/designed to provide skin healing and therapeutic effects, alongside their main function which is the stabilization of the emulsion. Unlike the case of Pickering emulsions, tracking of the nanoparticles and investigating their skin penetration and distribution has been tackled by several studies in which the particles acted individually as dermal and/or transdermal delivery vehicles (Abdel-Hafez et al., 2018; Alvarez-Román et al., 2004; Khabir et al., 2019; Opanasopit et al., 2012). In these studies, confocal laser scanning microscopy (CLSM) has proved to be a successful technique for the tracking of the particles.

The objective of this work is to develop novel stable Pickering emulsions stabilized with chitosan/collagen peptides nanoparticles for potential cosmetic applications. The work aims to investigate the microstructure, stability, and rheological properties of the produced Pickering formulations. Besides, since both polymers used in the production of the nanoparticles have skin benefits, the work aims to investigate the fate of the nanoparticles after skin application by tracking their skin distribution following the penetration of the emulsion droplets. To the best of our knowledge, this is the first study that explores the fate of chitosan-based particles after the topical application of Pickering emulsions.

5.2 Materials and methods

5.2.1 Materials

Chitosan (ChitoClear[®]) with a molecular weight of 100-200 kDa, and a degree of deacetylation of 96% was a kind gift from Primex ehf, Iceland. Collagen peptides (the hydrolysed form of collagen type I), with a molecular weight of 5000 Da and an isoelectric

point of 5.2, were obtained from HiMedia Laboratories, India. Olive oil (highly refined, low acidity), sodium tripolyphosphate (technical grade, 85%), Nile Red and Nile Blue A (certified by the Biological Stain Commission) were purchased from Sigma Aldrich. Acetic acid (0.1N standardized solution) was supplied by Alfa Aesar. The porcine skin samples were a kind gift from Grupo Primor, Portugal.

5.2.2 Preparation of the chitosan/collagen peptides (CH/CP) nanoparticles

The CH/CP nanoparticles were produced by polyelectrolyte complexation followed by ionic gelation as described by Anandhakumar and co-workers (Anandhakumar et al., 2017), with some modifications. Dispersions with different concentrations of the nanoparticles were prepared; namely with 1%, 1.5%, 2% and 2.5% (w/v). Briefly, and taking the 1.5% w/v nanoparticles dispersion as an example, the preparation method comprised the next steps. Firstly, 0.75 g of chitosan was left overnight to dissolve in 50 ml of 0.1 N acetic acid with magnetic stirring at 800 rpm. Thereafter, a solution of collagen peptides (pH= 6-6.2) was prepared by dissolving 0.75 g (the same amount as chitosan) in 15 ml of deionized water and added to the chitosan solution (pH= 4.2) under continuous stirring to prepare chitosan/collagen peptides (CH/CP) complex mixture (pH= 4.7). Finally, 0.014 g of sodium tripolyphosphate (TPP) was dissolved in 35 ml of deionized water and added dropwise over 30 minutes to the CH/CP mixture under continuous stirring at 600 rpm. The mixture changed into an opalescent dispersion indicating the formation of the nanoparticles through the ionic gelation of the CH/CP complex mixture by TPP (Anandhakumar et al., 2017). The produced opalescent nanoparticle dispersion was kept under the same stirring rate for one hour. The pH of the produced CH/CP nanoparticle dispersion was 5.08. All the steps were carried out at room temperature (25 °C). The amount of TPP was always kept at a content of 1.8 % relative to the chitosan amount in all the produced CH/CP nanoparticle dispersions (1%, 1.5%, 2%, and 2.5% w/v). This low concentration of TPP was enough to form opalescent dispersions of nanoparticles. Higher concentrations of TPP relative to chitosan resulted in undesired precipitation.

5.2.3 Preparation of the Pickering emulsions

The emulsions stabilized with CH/CP nanoparticles of different concentrations (1%, 1.5%, 2%, and 2.5% w/v) were prepared by emulsifying 40 ml of the nanoparticle dispersion with 60 ml of olive oil using a high-speed homogenizer (Ultra-Turrax Digital T25, IKA, Germany) at 13500 rpm for 6 minutes. The oil was added slowly in a portion-wise manner.

5.2.4 Characterization of the produced CH/CP nanoparticles

5.2.4.1 Morphology and size of the nanoparticles

Transmission electron microscopy (TEM) was used to examine the morphology and size of the produced nanoparticles. The sample was prepared by mounting 10 μ L of the nanoparticle dispersion on Formvar/carbon film-coated mesh nickel grids (Electron Microscopy Sciences, Hatfield, PA, USA) and left standing for 2 min. Afterward, negative staining was done by adding 10 μ L of 1% uranyl acetate onto the grids and left standing for 10 s. Visualization was carried out with a JEOL JEM 1400 TEM at 120 kV (Tokyo, Japan). Images were digitally recorded using a CCD digital camera Orious 1100W (Tokyo, Japan). The average size of the nanoparticles was determined by using ImageJ software.

5.2.4.2 Zeta potential of the nanoparticles

The zeta potential of the produced CH/CP nanoparticles (1.5% w/v), chitosan solution (0.75g/50 ml), CP solution (0.75g/15 ml), and CH/CP complex mixture (obtained by mixing chitosan and collagen peptides solutions) was determined using a Zetasizer Nano ZS ZEN3600 (Malvern Instruments Ltd., UK). The concentration of the samples was the same as the concentration of their solutions used in the production of the nanoparticle dispersion. The results were reported as the average of three runs for each sample.

5.2.4.3 Wettability of the nanoparticles

The wettability of the nanoparticles was determined using Dataphysics OCA15 Plus equipment (Dataphysics, Germany). All the measurements were conducted at room temperature (25 °C). For the wettability experiments, homogenous films of CH/CP

nanoparticle dispersion were prepared by depositing the dispersion (1.5% w/v) on glass slides, which were left to dry at room temperature. The contact angle of the nanoparticles was determined by injecting deionized water droplets (4 μ l each) onto the surface of the dried films. The shape of the droplet was photographed after 30 seconds for equilibrium (Lv, Wang, Chen, et al., 2020; Meng et al., 2020) using a digital camera coupled to the equipment, and the Young-Laplace equation was applied by the software to fit the profile data of the droplet. Measurements were taken as the average of at least 3 droplets on 2 different films of the same nanoparticle dispersion. The contact angle of chitosan and collagen peptides was also measured using the same method.

5.2.4.4 Dynamic interfacial tension

The interfacial tension was measured by the pendant drop method using the Dataphysics OCA15 Plus device (Dataphysics, Germany). Briefly, a drop of the CH/CP nanoparticle dispersion (1.5% w/v) was formed by a submerged syringe inside a cuvette filled with olive oil. The interfacial tension was measured between CH/CP nanoparticle dispersion and olive oil over 2400 seconds and was calculated by the pendant drop shape with the Young-Laplace equation using the equipment software. The measurements were also conducted between CH/CP complex mixture and olive oil, as well as between chitosan solution and olive oil, and between collagen peptides solution and olive oil.

5.2.5 Characterization of the Pickering emulsions

5.2.5.1 Droplet size

The Pickering emulsion average droplet size was measured by a laser diffraction particle size analyzer (LS 230, Beckman Coulter, USA). The refractive indexes were set as 1.46 for olive oil and 1.33 for water. The measurements were done in triplicate for each sample.

5.2.5.2 Creaming index

The Creaming Index (CI%) was determined for freshly prepared emulsions and after storage for two months to investigate the level of any potential phase separation. The

emulsions were kept in sealed bottles to prevent water evaporation. CI% was calculated using the following formula (Lv, Wang, Dai, et al., 2020; Singh et al., 2021):

$$CI\% = \frac{H_s}{H_t} \times 100$$

where H_s is the height of the serum layer and H_t is the total height of the emulsion.

5.2.5.3 Microstructure

The microstructure of the Pickering emulsions produced with different nanoparticle concentrations was analyzed using a Leica DM 2000 optical microscope equipped with a Leica Application Suite Interactive Measurement imaging software. The images were acquired at a magnification of 10x.

Confocal laser scanning microscopy (CLSM) was also used to examine the interfacial microstructure and confirm the type of the obtained Pickering emulsions. A fluorescent dye solution in isopropyl alcohol was prepared by mixing Nile Blue (0.1% w/v) and Nile Red (0.1% w/v) dyes. The Pickering emulsion samples were stained after their preparation by mixing 1 ml of the fluorescent dye mixture with 35 ml of the emulsion. Nile Blue was used to stain the CH/CP nanoparticles, whereas Nile Red was used to stain the oil phase. The analysis was conducted using a Leica TCS SP5 CLSM (Leica Microsystems Inc., Germany). The applied excitation wavelengths were 633 nm and 488 nm for Nile Blue and Nile Red, respectively.

5.2.5.4 Rheological properties

The rheological properties of the Pickering emulsions were investigated using a Kinexus Pro Rheometer (Malvern, UK). For the steady flow assays, the apparent viscosity (η) was measured *versus* shear rate (ranging from 0.1 to 100 s⁻¹). These measurements were conducted using a cone plate with a diameter of 40 mm, an angle of 4°, and a fixed gap of 0.15 mm. The frequency sweep measurements were conducted at a constant strain amplitude of 1 % (within the linear viscoelastic region) using a parallel plate of a diameter of 20 mm with a fixed gap of 1mm. The storage modulus (G') and loss modulus (G'') of the

produced Pickering emulsions were measured over a frequency range of 0.01-10 Hz. All the measurements were conducted at 25 °C.

5.2.6 *Ex vivo* skin application studies and tracking the fate of the nanoparticles

5.2.6.1 Skin preparation and Pickering emulsions application

Dorsal porcine skin samples were obtained from pigs slaughtered at a local slaughterhouse with permission from the General Directorate of Food and Veterinary Medicine (DGAV, Portugal). The skin samples were shaved by an electric shaver (LS7140 Grundig, Grundig Intermedia GmbH, Germany). The subcutaneous fat layer was carefully removed with a surgical scalpel according to the OECD guidelines (OECD, 2004). The skin samples were then rinsed with water, wrapped in parafilm and aluminum foil, and stored at -20 °C until used.

The produced Pickering emulsions stabilized with 1% and 2% w/v CH/CP nanoparticles were stained by Nile Blue (0.1% w/v) by mixing 1 ml of the dye solution with 35 ml of the emulsions. The stained emulsion samples were then applied to the skin using Franz diffusion cells (PermeGear, USA). Briefly, the skin samples were left to thaw at room temperature for 1 hour before the experiments. They were then mounted between the donor and the receptor compartments of the Franz diffusion cells, with the *stratum corneum* side facing the donor compartment. Afterward, a sample of 300 µl of Pickering emulsion formulation was added to the donor compartment and spread uniformly on the skin. The receptor fluid consisted of a 1:1 mixture of ethanol and PBS (pH 7) and was kept under magnetic stirring. The addition of ethanol to the receptor fluid is in accordance with the OECD guidelines for the *ex vivo* skin permeation experiments (OECD, 2004), and with previously reported methodologies (Abdel-Hafez et al., 2018). The experiment was conducted under occlusive conditions to prevent water evaporation from the formulations (Laredj-Bourezg et al., 2017; Marto, Ascenso, Gonçalves, et al., 2016), and the temperature was kept at 32°C using a thermostatic water bath. The contact time of the formulations with the skin (the duration of the experiment) was set as 6 hours, as well as 24 hours for each formulation. At the end of the experiment, the skin samples were collected, rinsed with water thoroughly (to remove any residual formulation remaining on the surface), placed in

tissue freezing medium (Killik[®], Bio-Optica), and kept in an ultra-low temperature freezer (-78 °C) until cryo-sectioning.

5.2.6.2 Skin distribution and tracking of the CH/CP nanoparticles

Confocal laser scanning microscopy (CLSM) was used to track the nanoparticles and explore their distribution in the skin after the topical application of the emulsion formulations. For this purpose, the skin samples treated with Pickering emulsions stabilized with 1% and 2% w/v CH/CP nanoparticles (labeled with Nile Blue) were cryo-sectioned using a cryostat (Cryostat Leica CM 3050S) to sections with a thickness of 20 µm. The skin sections were visualized with CLSM at an excitation wavelength of 633 nm.

5.2.7 Statistical analysis

The results were presented as the mean $\pm$ standard deviation using Microsoft Excel 365. The statistical comparisons were performed using one-way analysis of variance (ANOVA) and the independent sample *t*-test (with the level of significance at *p* values < 0.05) (Wang et al., 2021).

5.3 Results and discussion

5.3.1 Production and characterization of CH/CP nanoparticles

5.3.1.1 Morphology, size and zeta potential of the nanoparticles

CH/CP nanoparticles were prepared by complexation between the positively charged chitosan polymer chains and the negatively charged collagen peptides, followed by ionic gelation between the positively charged CH/CP complex mixture and the polyanions of sodium tripolyphosphate (TPP) (Anandhakumar et al., 2017). TEM images (Figure 5.1) have shown that the produced CH/CP nanoparticles had an average size of 32.27 nm $\pm$ 10.14 nm, with a minimum measured size of 14.40 nm and a maximum one of 59.39 nm. The TEM images also show that the nanoparticles are irregularly spherical and oval.

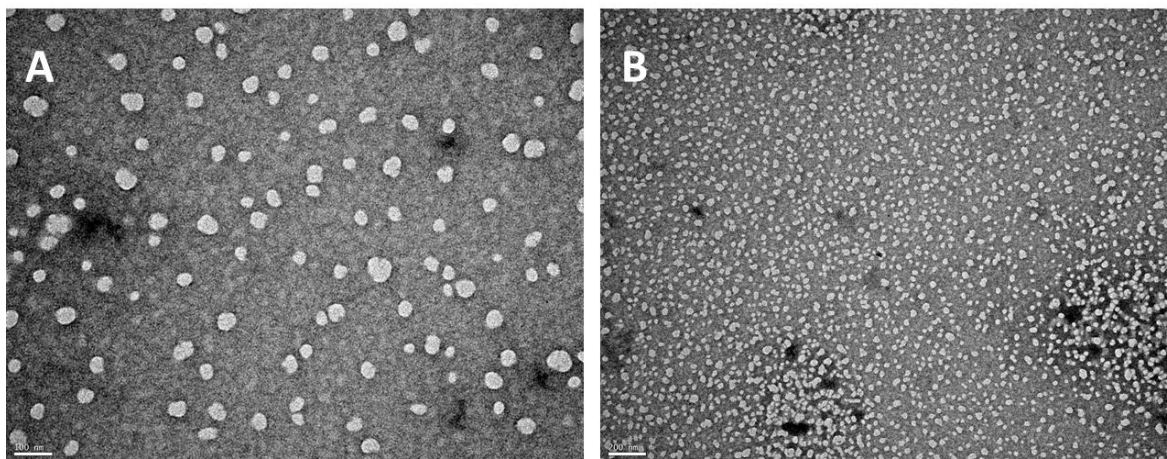


Figure 5.1. TEM images of chitosan/collagen peptides nanoparticles at a magnification of (A) 100000X, scale bar = 100 nm, and (B) 50000X, scale bar = 200 nm.

The produced CH/CP nanoparticles are positively charged and showed an average zeta potential of $+59.7 \pm 2.66$ mV as shown in Table 5.1. It was also observed that the collagen peptide solution had a negative zeta potential (-16.1 ± 0.361 mV) which allowed the polyelectrolyte complexation with the positively charged chitosan. The complexation between the two polymers occurs mainly through hydrogen bonding between chitosan and collagen peptides (Anandhakumar et al., 2017), as well as via the electrostatic interaction between the oppositely charged chitosan and collagen peptides which was evident in the observed decrease in the zeta potential of chitosan solution from $+79.4 \pm 3.71$ mV to $+62.3 \pm 2.1$ mV during the CH/CP complex mixture formation. The CH/CP nanoparticles were then obtained by the ionic gelation between the polyanions TPP and CH/CP complex mixture which decreased the zeta potential slightly from $+62.3 \pm 2.1$ mV to $+59.7 \pm 2.66$ mV. Although the amount of the used TPP is small, which is reflected in this slight decrease in the zeta potential value, the step of the ionic gelation by TPP was important for the production of the nanoparticles. The addition of TPP resulted in the transition of the transparent CH/CP complex mixture into an opalescent nanoparticle dispersion.

Table 5.1. Zeta potential values of chitosan and collagen peptides solutions, complex mixture, and nanoparticles.

Sample	Zeta potential (mV)
Chitosan (CH) solution	$+79.4 \pm 3.71$
Collagen peptides (CP) solution	-16.1 ± 0.361
CH/CP complex mixture	$+62.3 \pm 2.1$
CH/CP nanoparticles	$+59.7 \pm 2.66$

5.3.1.2 Wettability of the nanoparticles

Solid particles that are used as emulsion stabilizers should have adequate wettability to allow them to be available at the oil/water interface. The wettability of the particles is calculated by the contact angle value which determines their hydrophilic or lipophilic nature, and hence the type of the Pickering emulsion they can stabilize. Particles that have a contact angle less than 90° are hydrophilic and produce o/w Pickering emulsions, while particles with a contact angle higher than 90° are hydrophobic and form w/o emulsions (Binks, 2002; Dickinson, 2017; J. Wu & Ma, 2016). Particles with a contact angle of 90° are wetted equally by the oil and water phases, and theoretically, result in the formation of emulsions with optimized stability. However, in practical terms, it has been reported that emulsion stability is enhanced when a slightly greater part of the particle is placed in the continuous phase, i.e., when the particle has a slightly higher affinity to the external phase (Dickinson, 2017). In this work, it was observed that the contact angle of the CH/CP nanoparticles was $78.02 \pm 2.04^\circ$ (Figure 5.2), which reflects their hydrophilic nature. It was also observed that the contact angle of the produced CH/CP nanoparticles was higher than that of chitosan ($63.89 \pm 1.08^\circ$) and also higher than that of collagen peptides ($33.67 \pm 2.92^\circ$). These values suggest that the interaction between collagen peptides and chitosan to form the nanoparticles decreased the hydrophilicity of both polymers, which would enhance their ability to be adsorbed at the oil/water interface and hence produce more stable emulsions. It has been reported that during the formation of complex chitosan/collagen peptides nanoparticles, the long chitosan chains fold around the collagen

peptides and form a new phase complex having properties differing from the ones of the individual components (Anandhakumar et al., 2017). This process was reported to occur mainly *via* hydrogen bonding. In this work, the decreased hydrophilicity of the produced CH/CP nanoparticles could be attributed to the preferential orientation of the hydrophobic units of chitosan (N-acetyl-D-glucosamine units) towards the nanoparticle surface since the hydrophilic groups become internally involved in the hydrogen bonding with the collagen peptides.

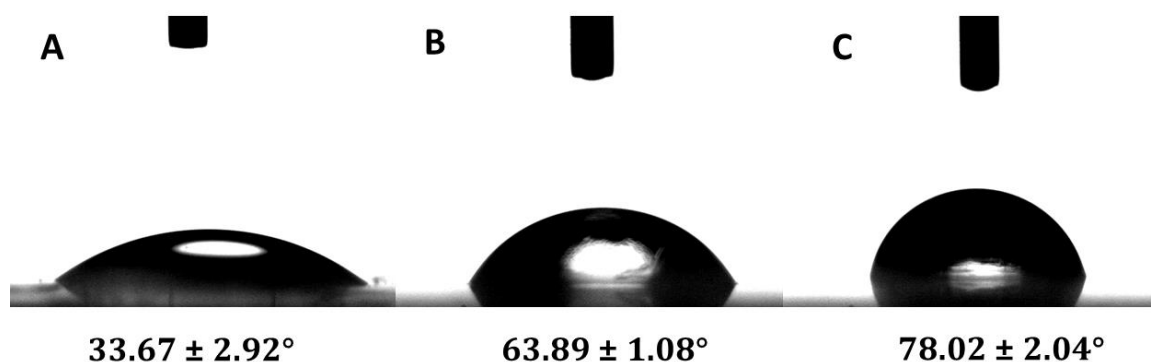


Figure 5.2. Contact angle in the air environment of A) Collagen peptides, B) Chitosan, and C) Chitosan/collagen peptides nanoparticles.

5.3.1.3 Interfacial tension

The dynamic interfacial tension measures the degree of the assembly/adsorption of the nanoparticles at the oil/water interface (Ho et al., 2016; Nikbakht Nasrabadi et al., 2019; Zhou et al., 2019). Figure 5.3 shows that the pure collagen peptides (CP) solution has lower interfacial tension than the chitosan (CH) solution, indicating that the CP solution is more interfacially active than the CH solution. It can be also observed that the CH/CP nanoparticles and the CH/CP complex mixture have lower interfacial tension than the chitosan solution, reflecting that the interaction between collagen peptides and chitosan enhanced the interfacial properties relative to chitosan. This observation suggests that the interaction between the two polymers exposed more hydrophobic domains/moieties facilitating the adsorption at the oil/water interface (as discussed previously in Section 5.3.1.2). It has been reported that the exposure (uncovering) of the non-polar N-acetyl-D-glucosamine units of chitosan confers some hydrophobic features to the chitosan particles

and improves their surface activity (Costa et al., 2018). Figure 5.3 also shows that the CH/CP nanoparticles exhibited slightly lower interfacial tension than that of the CH/CP complex mixture throughout the experiment indicating that CH/CP nanoparticles have higher adsorption ability at the oil/water interface than the CH/CP complex mixture.

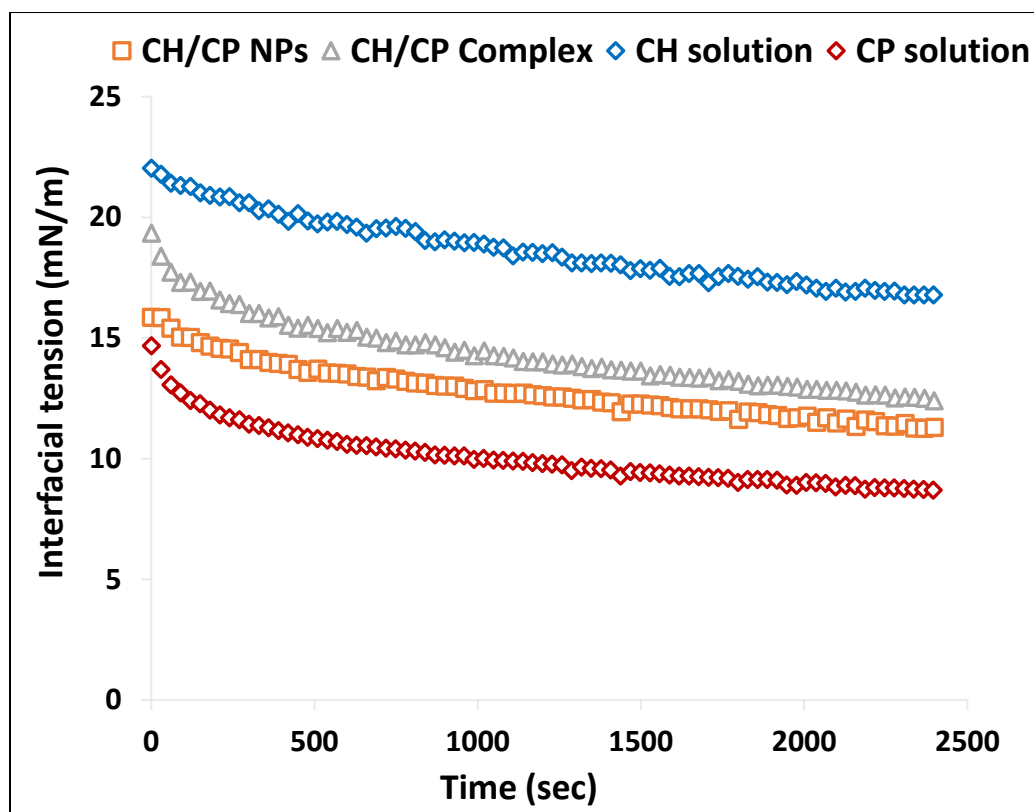


Figure 5.3. Dynamic interfacial tension at the olive oil/water interface with the adsorption of chitosan (CH) solution, chitosan/collagen peptides complex mixture (CH/CP complex), chitosan/collagen peptides nanoparticles (CH/CP NPs), and collagen peptides (CP) solution.

5.3.2 Production and characterization of the Pickering emulsions

5.3.2.1 Visual appearance and average droplet size of Pickering emulsions

The formulation of cosmetic products containing sustainable and biodegradable materials can be extremely challenging due to instability constraints (Bom et al., 2019). The stability of emulsions is determined by the creaming index (CI%) and the size variation of the

emulsion droplets during storage (Du & Wang, 2020). Pickering emulsions are generally known to have higher stability than classical emulsions. Digital photographs of freshly prepared and stored Pickering emulsions stabilized by CH/CP nanoparticles with concentrations of 1%, 1.5%, 2%, and 2.5% w/v are shown in Figure 5.4A. No phase separation was observed in the freshly prepared formulations (CI% =0), which was maintained after two months of storage except for the formulation stabilized with nanoparticle concentration of 1% w/v. Unchanged CI% after storage indicates excellent Pickering emulsion stability (Lv, Wang, Chen, et al., 2020). The formulation stabilized with 1% w/v CH/CP nanoparticles showed a CI% of 9.09% after a storage period of two months, implying low water retention ability due to the weak structural network of the formed Pickering emulsion. However, CI% values were unchanged (=0%) as the nanoparticle concentration increased due to the presence of a stronger and more compact network structure, leading to better water retention, and hence to higher stability (Feng et al., 2020). It is noteworthy that the used chitosan to collagen peptides mass ratio of 1:1 was used since it resulted, based on preliminary studies, in the formation of both stable CH/CP nanoparticles and stable Pickering emulsions.

Figure 5.4B shows the average droplet size of the produced Pickering emulsions measured directly after preparation, and after a storage period of 2 months. The freshly prepared emulsions were characterized by an average droplet diameter ranging between 7.63 ± 0.20 μm and 15.72 ± 0.19 μm , which increased slightly to 10.19 ± 0.46 μm and 17.19 ± 0.98 μm after storage. It can be noticed that the size of the emulsion droplets is much higher than the size of the nanoparticles (as shown previously in the TEM images in Figure 5.1), which is in agreement with the prerequisite that the size of the particles should be at least one order of magnitude smaller than the oil droplets for the Pickering emulsions to be formed (Du & Wang, 2020). Additionally, Figure 5.4B shows that the droplet size of the produced Pickering emulsions significantly decreased as the nanoparticle concentration increased. At a fixed oil fraction, the dispersed phase is initially broken into small droplets by homogenization to the same extent. These droplets tend to coalesce to reduce the interfacial area. At higher particle concentrations, more particles are available to adsorb at the oil/water interface preventing the droplets from coalescence and resulting in the decrease of the droplet size (Lv, Wang, Chen, et al., 2020). Figure 5.4B also shows a significant

difference in the average droplet size of some formulations after storage ($p < 0.05$). However, this variation is noticeably low which suggests that the produced Pickering emulsions are less susceptible to aggregation or coalescence (Abdul Hadi et al., 2020), and implies good storage stability as evidenced by the absence of phase separation after storage.

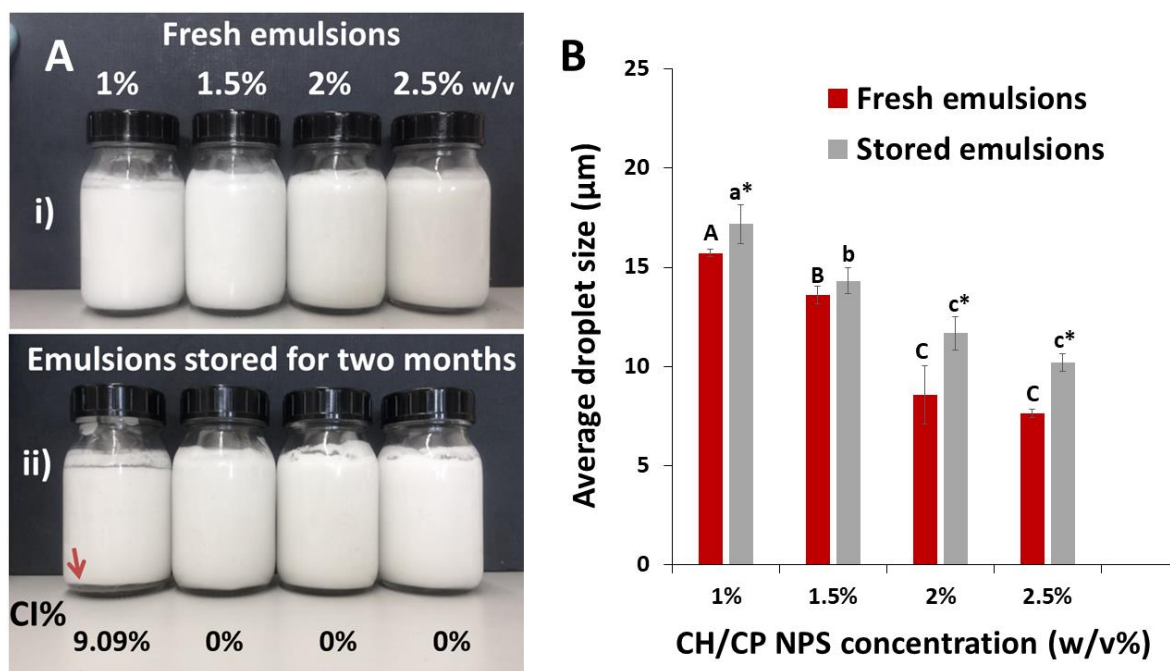


Figure 5.4. (A) Photographs of Pickering emulsions stabilized with chitosan/collagen peptides nanoparticles prepared with concentrations of 1%, 1.5%, 2%, and 2.5% (w/v): (i) Freshly prepared emulsions and (ii) Emulsions stored for two months at room temperature with the corresponding creaming index value (CI%). The arrow (if present) shows phase separation. (B) The average emulsion droplet size of the produced Pickering emulsions. Different uppercase letters (A-C) indicate significant differences in the average droplet size between the fresh emulsions prepared with different CH/CP NPs concentrations ($p < 0.05$), different lowercase letters (a-c) indicate significant differences in the stored emulsions with different CH/CP NPs concentrations ($p < 0.05$), and * (if present) indicates significant differences between the fresh and stored emulsions with the same CH/CP NPs concentration ($p < 0.05$).

5.3.2.2 Morphology and interfacial structure of Pickering emulsions

Optical microscopy images (Figure 5.5) show that the droplets of the produced Pickering emulsions were all intact and spherical. It can be observed that the droplet size decreases as the CH/CP nanoparticle concentration increases, which is in agreement with the results of the average droplet size shown in Section 5.3.2.1. Moreover, the optical microscopy images show that the microstructure of the formulations becomes more compact as the nanoparticle concentration increases. This could be explained by the fact that by the increase of the nanoparticle concentration, more nanoparticles were available to become adsorbed at the oil/water interface and form a dense network structure resulting in the decrease of the emulsion droplet size (Feng et al., 2020; Sharkawy et al., 2019). Additionally, this compact/dense network structure hinders the collision of the droplets, preventing their coalescence and Ostwald ripening, and hence enhances the emulsion stability (Feng et al., 2020).

The interfacial structure of Pickering emulsions stabilized with CH/CP nanoparticles (1.5% w/v) was investigated by the CLSM. The images (Figure 5.6) show that the nanoparticles form a dense layer (appears in red) around the oil droplets (which appear in green) indicating efficient nanoparticle adsorption at the oil/water interface. The CLSM images also confirm that produced Pickering emulsions stabilized with CH/CP nanoparticles are o/w emulsion type.

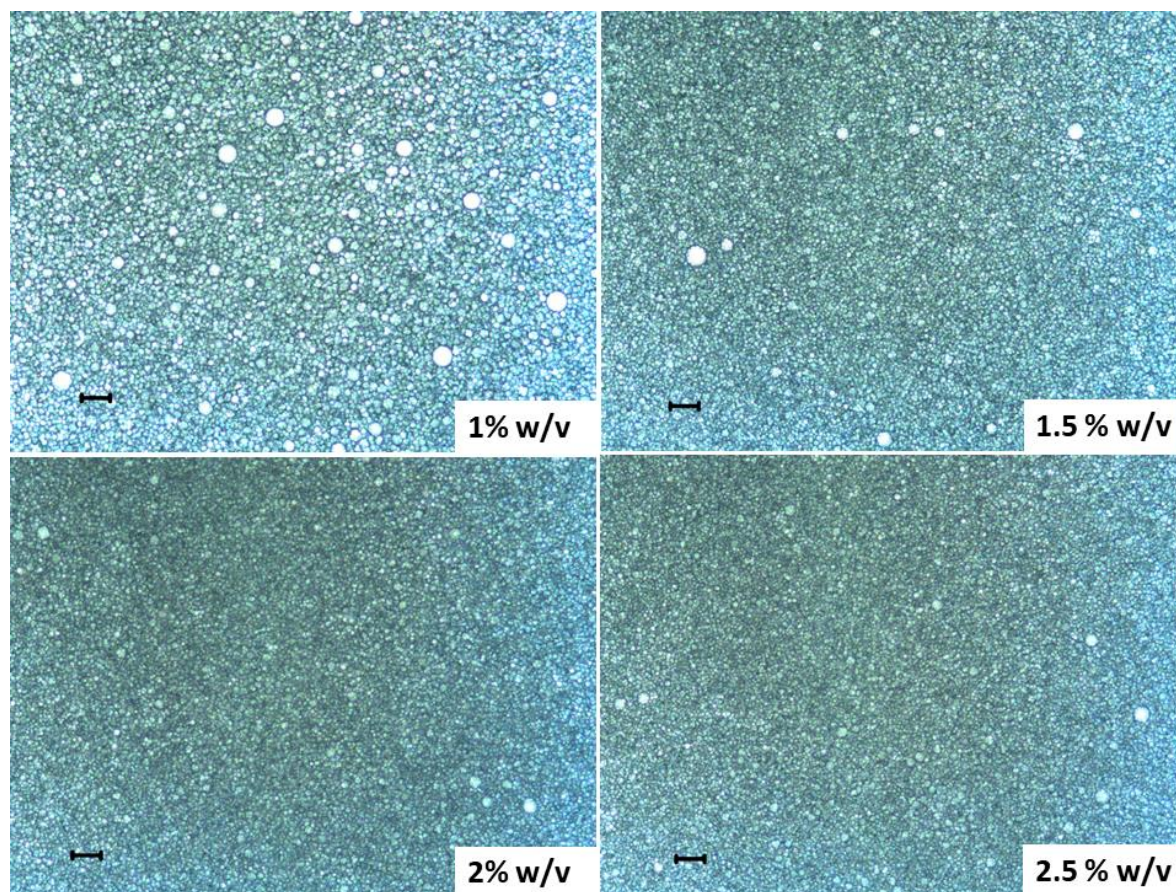


Figure 5.5. Optical microscopy images of Pickering emulsions prepared with chitosan/collagen peptides nanoparticles at concentrations of 1%, 1.5%, 2% and 2.5% w/v. Scale bar = 50 μm .

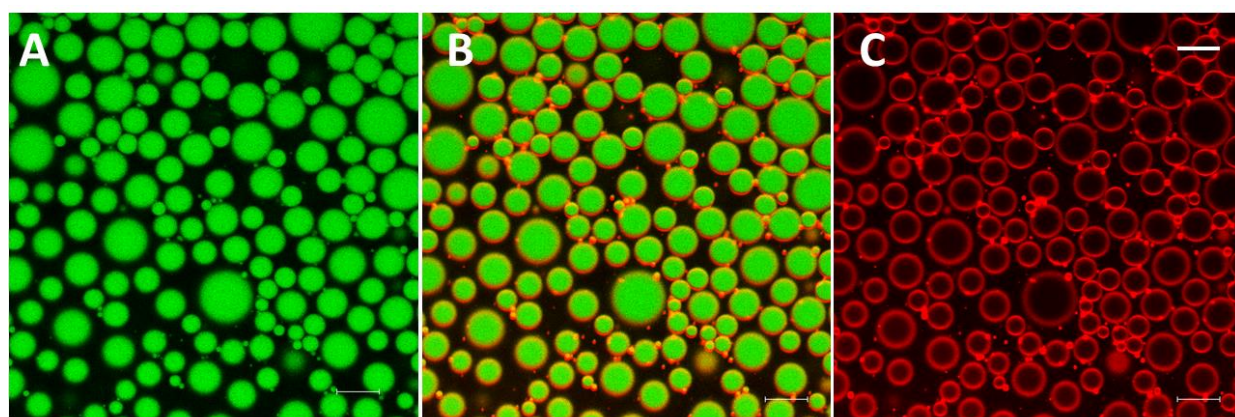


Figure 5.6. Confocal laser scanning microscopy images of Pickering emulsions stabilized with chitosan/collagen peptides nanoparticles (1.5% w/v); (A) The emulsion droplets stained with Nile Red, (C) The adsorbed nanoparticles stained with Nile Blue appearing as

red rings, and (B) An overlay of images (A) and (C) combined using LAS X software. Scale bar = 10 μm .

5.3.2.3 Rheological properties

The rheology of topical Pickering formulations is an important quality parameter (Wahlgren et al., 2014). In this study, the apparent viscosity of the produced Pickering emulsions decreased with the increase of shear rate from 0.1 to 100 s^{-1} as shown in Figure 5.7A, indicating that the emulsions demonstrate a shear-thinning and non-Newtonian fluid behavior (F. Li et al., 2021). The shear-thinning behavior is a desirable feature for topical formulations as it facilitates the application of the product on the skin (Özcan et al., 2013), and leaves a smooth sensorial feeling (Venkataramani et al., 2020). It can be also observed that the apparent viscosity of the produced emulsions increased with the increase of CH/CP nanoparticle concentration from 1% w/v to 2% w/v. This increase in the apparent viscosity subsequently leads to higher stability due to the formation of a network structure that restricts/limits the emulsion droplet movement and hence prevents aggregation, coalescence, and creaming (Feng et al., 2020).

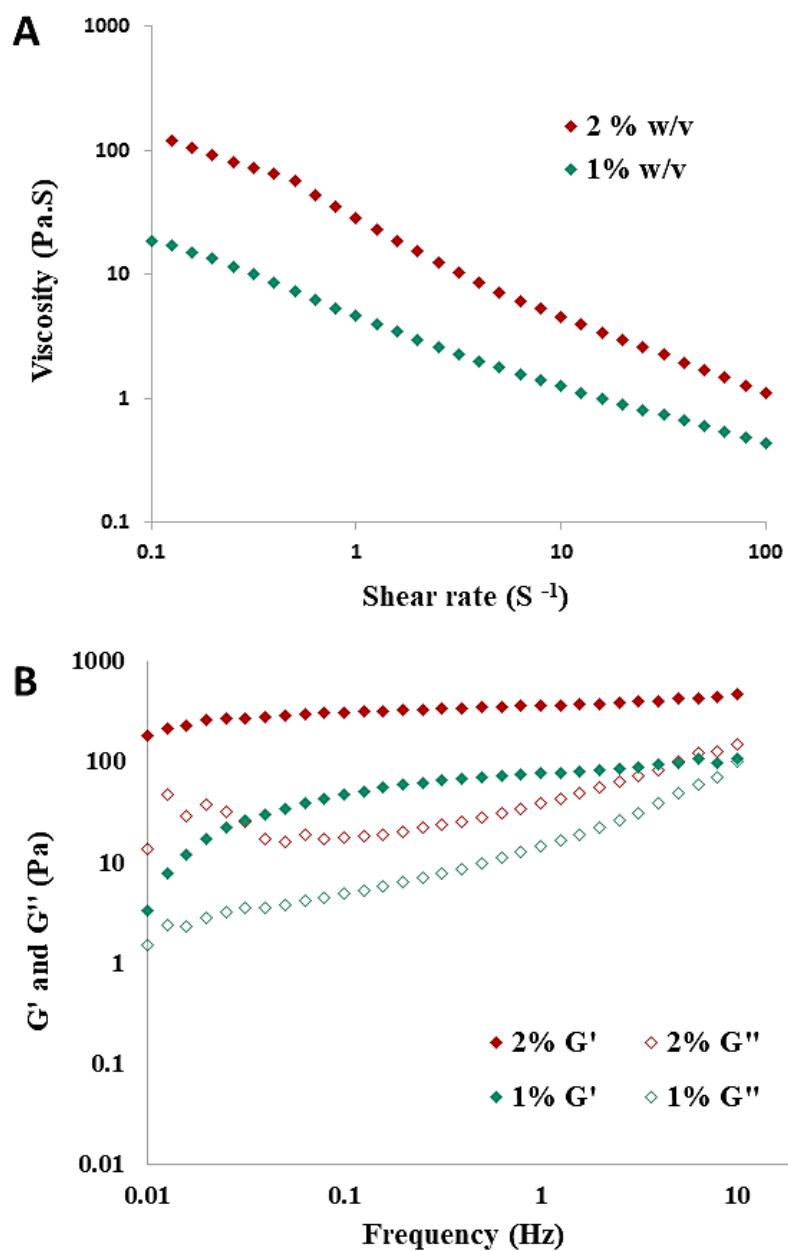


Figure 5.7. The rheological profiles of Pickering emulsions stabilized with 1% w/v and 2% w/v chitosan/collagen peptides nanoparticles: (A) Apparent viscosity versus shear rate, and (B) Frequency sweep curves showing values of the storage modulus (G') and loss modulus (G'').

The strength of the network structure can be determined from the storage modulus (G') which indicates the elastic properties of the emulsion, and the loss modulus (G'') that indicates the viscous properties of the emulsion (Yegya Raman & Aichele, 2020). The results of the dynamic oscillatory measurements of the Pickering emulsions with the frequency ranging from 0.01 to 10 Hz are shown in Figure 5.7B. It can be noted that the values of G' and G'' increased by increasing the CH/CP nanoparticle concentration, which can be attributed to a higher particle adsorption rate leading to the formation of a stronger network structure (Feng et al., 2020). Additionally, it can be observed that the values of G' were higher than G'' for both formulations, implying the formation of an elastic gel-like network in the formed Pickering emulsions. Higher values of G' than G'' also indicate that the reversible deformation of the emulsion droplets is greater than the irreversible deformation because of the dominating elastic gel-like behavior (Yang et al., 2020).

5.3.3 Skin distribution and tracking of the CH/CP nanoparticles

The skin distribution of the nanoparticles stabilizing the Pickering emulsion formulations was investigated after 6 and 24 hours of skin application. Figure 5.8 shows the CLSM images of porcine skin sections treated with Pickering formulations stabilized with 1% and 2% w/v CH/CP nanoparticles. Higher fluorescence intensity was observed in samples treated with the Pickering formulation stabilized with 2% w/v CH/CP nanoparticles (Figures 5.8C and 5.8D) compared to the samples treated with the formulation stabilized with 1% w/v nanoparticles (Figures 5.8A and 5.8B). Moreover, it is noticeable that the highest fluorescence intensity was observed through the entire skin layers (*stratum corneum*, viable epidermis, and dermis) for the formulation stabilized with 2% w/v CH/CP nanoparticles after 24 hours of skin exposure. This observation indicates that a higher degree of penetration was achieved with prolonged skin contact time.

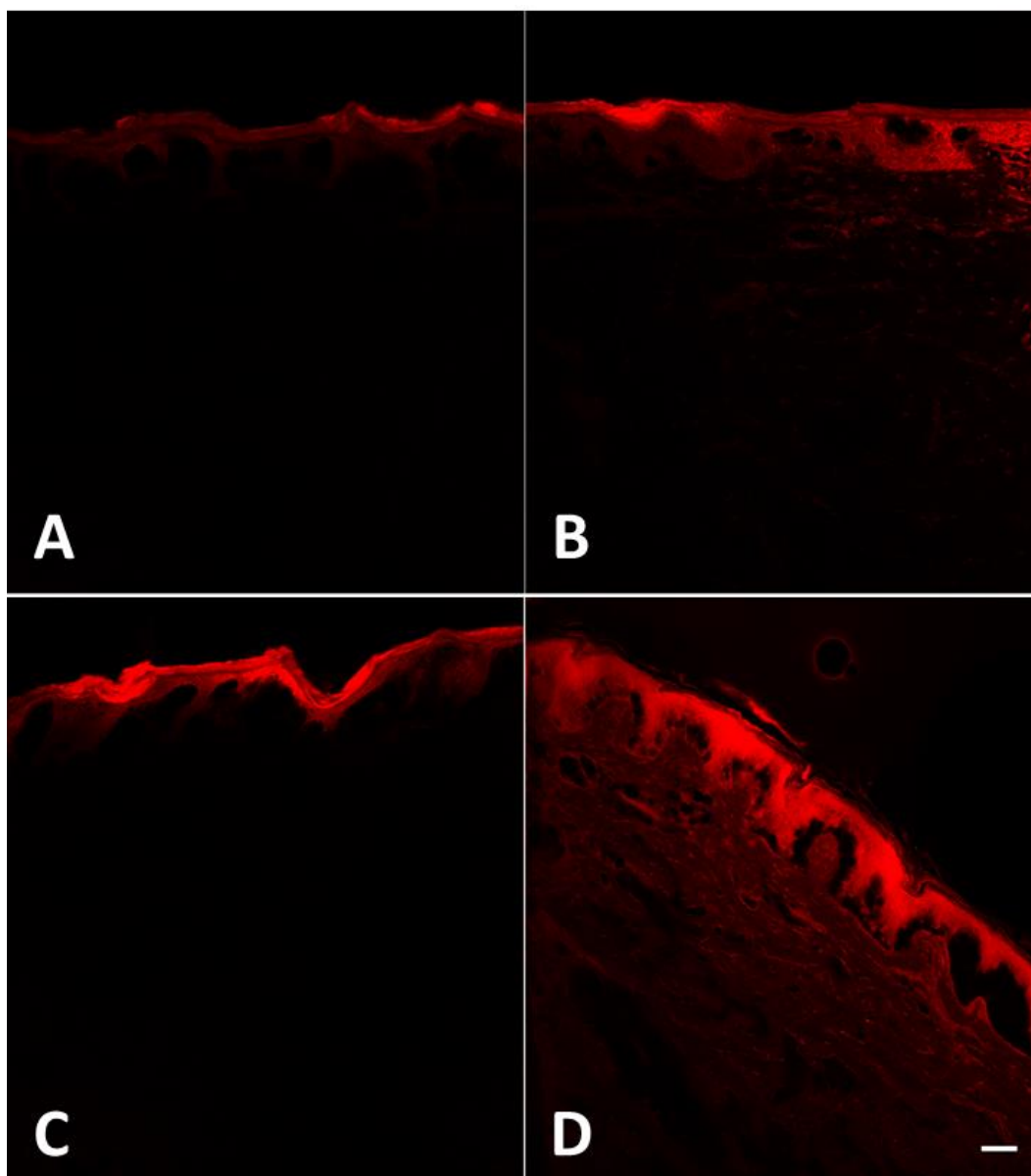


Figure 5.8. CLSM images of skin sections showing the distribution of chitosan/collagen peptides nanoparticles from Pickering emulsions: (A) Formulation stabilized with 1% w/v nanoparticles after 6 hours; (B) Formulation stabilized with 1% w/v nanoparticles after 24 hours; (C) Formulation stabilized with 2% w/v nanoparticles after 6 hours, and (D) Formulation stabilized with 2% w/v nanoparticles after 24 hours. Scale bar = 50 μm .

It has been reported that polymeric microparticles/microspheres made of natural and synthetic polymers with a diameter ranging between 3 μm and 10 μm penetrate the skin mainly through hair follicles and to a less extent via diffusion through the *stratum corneum*. Microparticles with sizes smaller than 3 μm penetrate easily by diffusion through the *stratum corneum* in a high concentration as well as the hair follicles, while those with sizes larger than 10 μm remain on the skin surface (Schaefer et al., 1994). However, that study did not discuss the other physicochemical properties of the particles that can influence their penetration at non-follicular regions of the *stratum corneum*, and it only dealt with aqueous dosage forms as carriers of the particles. In fact, aside from the size, other particle properties such as the surface charge and hydrophobicity have been reported to play an important role in their penetration through the *stratum corneum* and deposition in the skin tissue (Alvarez-Román et al., 2004). The size that allows the penetration of the particles through the *stratum corneum* can be different for particles dispersed in an oil vehicle (such as the case of Pickering emulsions) since the surface of such particles becomes more hydrophobic than the particles dispersed in an aqueous vehicle. This increased hydrophobicity of particles permits them to easily penetrate the *stratum corneum* through its lipid extracellular pathway (Frelichowska, Bolzinger, Valour, et al., 2009).

Studies on the skin distribution of the nanoparticles in the viable epidermis and dermis layers resulting from topical Pickering emulsions penetration are very scarce in the available literature. The skin penetration of the Pickering emulsion droplets stabilized with modified cellulose nanocrystals was evaluated in a very recent study (Hiranphinyophat et al., 2020). The presence of the nanocrystals in the viable epidermis and dermis was detected 24 hours after skin application. It was concluded that the Pickering emulsion droplets penetrated through the *stratum corneum* with the nanocrystals adsorbed on their surface due to the small average size of the droplets ($< 3 \mu\text{m}$).

The skin penetration of the particles from Pickering emulsions is either achieved through the penetration of the intact emulsion droplets (with the particles being adsorbed on their surface) and/or through the possible breaking up of the emulsion droplets on the skin surface during application, which leads to the releasing of the previously adsorbed particles that eventually penetrate the skin easily (Frelichowska, Bolzinger, Valour, et al., 2009).

In the present study, the ability of the emulsion droplets stabilized with chitosan/collagen peptides nanoparticles to penetrate the *stratum corneum* and reach deeper skin layers is attributed mainly to the dominating positive charge of the adsorbed particles (as was discussed in Section 5.3.1.1). The positively charged chitosan has been reported to open the epidermal tight junctions and disorder the intercellular lipid bilayer as a result of interacting with the negatively charged cells of the *stratum corneum*, which was evident in the slight loosening of the surface layer and the creation of minute microscopic pores (cavitations) in the *stratum corneum* (Sapra et al., 2008). Therefore, in the present work, it is suggested that the charge of the cationic chitosan/collagen peptides nanoparticles adsorbed on/stabilizing the Pickering droplets facilitated the passage of the small emulsion droplets through the *stratum corneum* to deeper layers. Increasing the concentration of the nanoparticles provided a higher positive charge on the surface of the emulsion droplets, and hence led to a deep penetration which was evident in the higher fluorescence intensity in the viable epidermis and dermis (that can be observed in Figures 5.8C and 5.8D). Furthermore, higher nanoparticle concentration results in a decrease in the droplet size of the produced Pickering emulsion (as discussed previously in Section 5.3.2.1), and hence allows higher penetration of the emulsion droplets that are smaller in size. Additionally, free particles that can be present in excess in the external phase of Pickering emulsions easily penetrate the *stratum corneum* due to their small size (Frelichowska, Bolzinger, Valour, et al., 2009). The higher concentration of the CH/CP nanoparticles (2% w/v) results in the presence of a greater number of available nanoparticles. Some of these nanoparticles could have existed freely in the external phase, and eventually penetrated the *stratum corneum* and reached the deeper skin layers.

It is noteworthy that the higher viscosity of the Pickering emulsion formulation stabilized with 2% w/v CH/CP nanoparticles, comparatively with the formulation stabilized with 1% w/v, had no impact/did not hinder the penetration of the droplets to deep skin layers. It is suggested that the influence of the positive charge and the smaller droplet size predominated over the higher viscosity effects that could have hindered the penetration.

It is also important to mention that no fluorescence was detected in samples withdrawn from the receptor fluid for both formulations (1% and 2% w/v) indicating that no

nanoparticles or emulsion droplets permeated the skin, but they were rather retained/localized in the skin. A similar observation was reported by the CLSM tracking of chitosan nanoparticles designed for transdermal delivery (Abdel-Hafez et al., 2018). Moreover, it has been reported that nanoparticles designed for topical applications require to be dispersed in a semisolid carrier/vehicle such as hydrogels to ensure the adherence of the nanoparticles to the skin. At the same time, the chosen semisolid vehicle should not interfere with the characteristics of the dispersed nanoparticles system (Al-Kassas et al., 2016). Therefore, the present study suggests that Pickering emulsions could act as effective platforms for the nanoparticles dermal delivery since the CH/CP nanoparticles were able to penetrate the *stratum corneum* and deposit in the viable epidermis and dermis.

5.4 Conclusions

Chitosan/collagen peptides nanoparticles acted as effective biopolymeric stabilizers for Pickering emulsions. The produced nanoparticles have shown enhanced wettability and a higher ability to reduce the interfacial tension compared to that of chitosan alone, suggesting that the interaction of chitosan with collagen peptides introduced and/or exposed more hydrophobic moieties. The produced Pickering emulsions demonstrated excellent storage stability, which increased as the nanoparticle concentration increased. No phase separation was observed in the Pickering emulsions stabilized with CH/CP nanoparticles at concentrations above 1% w/v during the storage evaluation period of 2 months. The produced Pickering emulsions have shown a shear-thinning behavior and a gel-like structure. Tracking of the CH/CP nanoparticles by CLSM after the skin application of the Pickering emulsions confirmed the presence of the nanoparticles in deeper skin layers, and not just in the *stratum corneum*. The degree of skin penetration increased as the concentration of the nanoparticles stabilizing the Pickering emulsions increased. The higher viscosity of the Pickering emulsions, related to the higher nanoparticle concentration, did not influence the degree of skin penetration. Moreover, the degree of skin penetration increased as the contact time of the emulsions with the skin increased. These findings reveal, for the first time, the effective deposition of the chitosan-based nanoparticles in skin layers, which was achieved through the developed Pickering emulsions formulations. These results will be helpful for future studies to expand the application of the produced Pickering

emulsions as prospective cosmetic vehicles/candidates, or as dispersion medium/carriers for the topical delivery of functionalities after evaluating the toxicological profile of the formulations.

5.5 References

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6 Pickering emulsions stabilized with chitosan/collagen peptides nanoparticles as green topical delivery vehicles for cannabidiol (CBD)

In this chapter, PEs stabilized by chitosan/collagen peptides (CH/CP) nanoparticles were assessed as green surfactant-free vehicles for the topical delivery of cannabidiol (CBD), a highly lipophilic unstable drug that is finding an increasing appeal in the cosmetic market. The influence of the oil phase volume fraction (ϕ) and the oil type on the emulsion properties, stability, rheological properties, as well as on the *ex-vivo* skin absorption of CBD was evaluated. The PE prepared with olive oil ($\phi = 0.6$) exhibited elastic gel-like properties and demonstrated long-term stability after 5 months of storage, with a CBD content of 99.45 % of the initially added amount. The skin absorption studies showed that CBD was retained in high amounts in the *stratum corneum*, while the CBD skin permeation was extremely low, indicating that the produced formulations are suitable as topical delivery vehicles. ATR-FTIR examination of the treated skin samples confirmed that the produced PEs were able to overcome the *stratum corneum* barrier. These findings suggest that the PEs stabilized with CH/CP nanoparticles provide an effective surfactant-free alternative for the topical delivery of CBD.

- This chapter is based on:

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- The cell viability assays were conducted by Dr. Francisca Rodrigues and Ms. Ana Margarida Silva (at ISEP, Portugal), who produced Figures 6.9 and 6.10 in this chapter.

6.1 Introduction

Cannabidiol (CBD) is a non-psychoactive phytocannabinoid naturally found in *Cannabis sativa* L. During the last decade, CBD has attracted a growing interest in medicine due to its various therapeutic activities (Bruni et al., 2018; Kamali et al., 2019; Ladha et al., 2020; Salami et al., 2020). Recently, cannabis extracts, and CBD in particular, have also gained tremendous attention in the cosmetic industry sector owing to their moisturizing, anti-aging, anti-inflammatory, antioxidant and cytoprotective properties (Atalay et al., 2021; Jhawar et al., 2019). Moreover, recent studies have shown that cannabis extracts do not lead to cytotoxic effects on keratinocytes and skin fibroblasts, proving the safety and non-irritancy of cannabis active ingredients (Di Giacomo et al., 2021; Zagórska-Dziok et al., 2021). CBD is a highly lipophilic drug ($\log P = 6.3$) and, therefore, it is frequently incorporated in alcohol-based formulations or oils (Millar et al., 2020). Additionally, CBD has been reported to be readily degraded by light, temperature and oxidation (Fraguas-Sánchez et al., 2020; Millar et al., 2020). Hence, the development of novel and safe formulations is important for enhancing the stability, solubility and bioavailability of CBD.

Emulsions stabilized by solid particles, also known as Pickering emulsions, provide an eco-friendly alternative to the conventional emulsions stabilized by classical molecular emulsifiers (Chevalier & Bolzinger, 2013; Rayner et al., 2014). The particles used as Pickering emulsions stabilizers possess intermediate wettability, which allows them to be irreversibly anchored at the oil-water interface providing steric hindrance and preventing coalescence, and subsequently resulting in high emulsion stability (Binks, 2002). Inorganic particles have been frequently used in Pickering emulsion stabilization (Binks, 2002; Marto et al., 2016). However, due to the consumers' demands and industrial preference for natural and label-friendly ingredients, recent research has been directed towards developing particles of natural origin as Pickering stabilizers (Atarian et al., 2019; Carriço et al., 2019; Huan et al., 2021; Ribeiro et al., 2020; Tiong et al., 2020).

The use of Pickering emulsions in dermal applications is finding increasing interest due to the consumers' demands and industry regulations to switch to green and safe formulations and to overcome the skin sensitization and irritation effects provoked by surfactants (Bordes et al., 2021; Marto et al., 2016; Y. shan Wei et al., 2020; Wu et al., 2020).

Pickering emulsions stabilized with particles of biological origin have not been frequently reported for cosmetic applications. However, this area is expected to be addressed more in future research owing to the large scale of the cosmetic market and to fulfill the biocompatibility and safety issues (Jiang et al., 2020).

Among the various biopolymers utilized in the development of particles employed as Pickering stabilizers, chitosan has emerged as a promising candidate in various applications due to its biocompatibility, biodegradability and non-toxic properties (Alehosseini et al., 2021; Atarian et al., 2019; F. Li et al., 2021). Chitosan has been widely used in topical and transdermal formulations as it has been reported to have many skin benefits, such as moisturizing, antiaging, antioxidant, antimicrobial and wound healing properties (Aranaz et al., 2018; Casadidio et al., 2019). It also enhances the skin delivery of active agents due to its cationic nature that allows its adhesion to the negatively charged skin surface (Ta et al., 2021). Nevertheless, to date, very few studies investigated the use of chitosan-based Pickering emulsions in dermal and cosmetic applications despite the advancements these formulations proved to achieve (Sharkawy, Barreiro, et al., 2020). For example, Pickering emulsions stabilized with chitosan nanoparticles have exhibited a sustained release of rutin and demonstrated an enhanced *in vivo* wound healing effect (Asfour et al., 2017). In another recent study, chitosan-based Pickering emulsions were reported to enhance the dermal bioavailability of resveratrol and improve its photostability upon exposure to ultraviolet radiation (Sharkawy, Casimiro, et al., 2020).

It is noteworthy that chitosan-based Pickering emulsions are mainly produced by particles solely made of chitosan through self-aggregation, particles formed via the hydrophobic modification of chitosan, or by particles that are formed through the complexation of chitosan with other polymers (Sharkawy, Barreiro, et al., 2020). In general, it has been reported that the latter methodologies demonstrated improved wettability of the nanoparticles, and hence enhanced Pickering emulsion stability (Atarian et al., 2019; Sharkawy et al., 2019). Additionally, the complexation of chitosan with other polymers enhances the functionality of the produced chitosan-based Pickering emulsions when the other polymer contributes with an added value to the resulting formulation, such as adding skin benefits by complexing with collagen peptides (Sharkawy et al., 2021) or providing

nutritious advantages as in the case of chitosan/caseinophosphopeptides nanocomplexes (Huang et al., 2019).

In a recent study, we developed Pickering emulsions stabilized with chitosan/collagen peptides nanoparticles that demonstrated high stability and enhanced ability to penetrate the *stratum corneum* (Sharkawy et al., 2021). Following these achievements, the main objective of the present work is to explore the potential of these biodegradable Pickering emulsions as green non-toxic cosmetic vehicles of CBD. The work also aims to investigate the physicochemical properties and encapsulation efficiencies of the developed CBD-loaded Pickering emulsions, and the effect of using different oils and different oil volume fractions on these properties, as well as on the skin penetration and deposition of the encapsulated active agent. To the best of our knowledge, this is the first study that reports the encapsulation of CBD in Pickering emulsions and the utilization of Pickering emulsions as potential cosmetic vehicles for CBD.

6.2 Materials and methods

6.2.1 Materials

Chitosan with a molecular weight of 100-200 kDa, and a degree of deacetylation of 96% was a kind gift from Primex ehf, Iceland. Collagen peptides (the hydrolyzed form of collagen type I), with a molecular weight of 5000 Da were supplied by HiMedia Laboratories, India. Cannabidiol (CBD) was purchased from THC Pharm (Frankfurt, Germany). Olive oil (highly refined, low acidity), Nile Red, Nile Blue, and sodium tripolyphosphate were obtained from Sigma Aldrich. Acetic acid (0.1N standardized solution) was purchased from Alfa Aesar. Liquid paraffin (with the European pharmacopeia specifications) was purchased from LabChem, Portugal. Acetonitrile and methanol (HPLC grade) were supplied by Carlo Erba Reagents. HaCaT cells were obtained from American Type Culture Collection (ATCC, USA). Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), Hank's Balanced Salt Solution (HBSS), non-essential amino acids, penicillin, streptomycin and trypsin-EDTA were obtained from Invitrogen Corporation (Life Technologies, S.A., Madrid, Spain). DMSO and Triton X-100 were

purchased from Sigma-Aldrich (Steinheim, Germany), respectively. The dorsal porcine skin samples were received as a gift from Grupo Primor, Portugal.

6.2.2 Preparation of chitosan/collagen peptides nanoparticles and Pickering emulsions

The chitosan/collagen peptides (CH/CP) nanoparticles were prepared following a method reported by Anandhakumar and co-workers (Anandhakumar et al., 2017), with some modifications described previously by our research group (Sharkawy et al., 2021). In brief, CH/CP nanoparticles dispersion with a polymers' concentration of 2 % w/v was prepared by dissolving 1 g of chitosan in 50 ml of 0.1 N acetic acid with continuous overnight magnetic stirring at 800 rpm. A solution of collagen peptides was prepared by dissolving 1 g in 15 ml of deionized water and was then added dropwise to the chitosan solution under magnetic stirring at 600 rpm. Thereafter, 0.018 g of sodium tripolyphosphate was dissolved in 35 ml of deionized water and added in a dropwise way to the chitosan/collagen peptides mixture over 30 minutes and constant magnetic stirring at 600 rpm to obtain an opalescent dispersion of CH/CP nanoparticles.

The cannabidiol-loaded Pickering emulsions were prepared by dissolving 0.6 g of cannabidiol (CBD) in the oil. Then, the oil phase with the dissolved CBD was added slowly and portion-wise to the CH/CP nanoparticle dispersion at 13500 rpm for 6 minutes using a high-speed homogenizer (Ultra-Turrax Digital T25, IKA, Germany). Two different oils were used separately in the formulations; olive oil and liquid paraffin. Each oil was incorporated at an oil volume fraction (ϕ) of 0.4 and 0.6. The final volume of the emulsion was always equal to 100 ml.

Table 6.1 summarises the composition and the volume fraction of the oil phase and aqueous phase in the prepared formulations. The pH of the Pickering emulsion formulations prepared with olive oil was 5.03 ± 0.01 and 5.11 ± 0.01 for oil volume fractions of 0.6 and 0.4, respectively, whereas it was 5.15 ± 0.00 and 5.16 ± 0.00 for their counterparts prepared with liquid paraffin. These values are suitable for skin applications as they are compatible with the natural pH of the skin. The pH of healthy skin has been reported to be in the range of 4.1 – 5.8 (Proksch, 2018), which varies with age, gender and body site (Proksch, 2018; Schreml et al., 2012), and has an average value of 4.7 as reported by Lambers et al. (2006)

(Lambers et al., 2006). The concentration of the CBD in the final produced emulsions was 6 mg/ml in all the formulations, which was selected based on the concentration of CBD in topical formulations present in the market.

Table 6.1. Composition of the produced Pickering emulsions describing the oil type and oil volume fraction (ϕ) in each formulation. The total volume of each formulation is 100 ml.

Formulation	Oil type	Oil volume fraction (ϕ)	CH/CP nanoparticles dispersion volume fraction
1	Olive oil	0.6	0.4
2	Liquid paraffin	0.6	0.4
3	Olive oil	0.4	0.6
4	Liquid paraffin	0.4	0.6

6.2.3 Characterization of the chitosan/collagen peptides (CH/CP) nanoparticles and the CBD-loaded Pickering emulsions

The dynamic interfacial tension between the CH/CP nanoparticles dispersion and liquid paraffin, as well as between the CH/CP nanoparticles dispersion and olive oil was measured by the pendant drop method over 2400 seconds, using a Dataphysics OCA15 Plus device (Dataphysics, Germany). In brief, a drop of the CH/CP nanoparticles dispersion was formed inside a cuvette filled with liquid paraffin (or olive oil) with a submerged syringe tip. The interfacial tension was calculated by the drop shape analysis with the Young-Laplace equation.

The microstructure of the produced Pickering emulsions was assessed by optical microscopy using a Leica DM 2000 optical microscope equipped with a Leica Application Suite Interactive Measurement imaging software.

The interfacial microstructure of the produced Pickering emulsions was investigated by confocal laser scanning microscopy (CLSM). The analysis was performed using a Leica TCS SP5 CLSM (Leica Microsystems Inc., Germany). Briefly, a fluorescent dye mixture was prepared by mixing Nile Red and Nile Blue solutions in isopropyl alcohol (0.1 %w/v

each). The freshly prepared Pickering emulsion samples were stained by mixing 0.5 ml of the fluorescent dye mixture with 18 ml of the emulsion. The excitation wavelengths were 488 nm and 633 nm for Nile Red and Nile Blue, respectively.

The mean droplet size and droplet size distributions of the Pickering formulations were measured by laser diffraction using a Beckman Coulter LS 230 particle size analyzer. The refractive index was set as 1.33 for the aqueous phase, and 1.46 and 1.48 for olive oil and liquid paraffin, respectively.

The creaming index (CI %) was used to express the emulsion stability and was calculated according to the following equation (Lv et al., 2020):

$$CI\% = \frac{H_s}{H_t} * 100$$

where H_s is the height of the serum layer that could appear at the bottom of the emulsion during storage, and H_t is the height of the total volume of the emulsion. Lower CI% values indicate higher stability.

The rheological properties of the produced Pickering emulsions were investigated using a Kinexus Pro Rheometer (Malvern, UK). The apparent viscosity (η) was measured versus the shear rate ranging from 0.1 to 100 s^{-1} , using a cone plate with a diameter of 40 mm, an angle of 4°, and a fixed gap of 0.15 mm. The frequency sweep measurements were conducted within the linear viscoelastic region at a constant strain amplitude of 1 %, using a parallel plate of a diameter of 20 mm with a fixed gap of 1mm. The storage modulus (G') and loss modulus (G'') of the produced Pickering emulsions were measured versus a frequency range of 0.01-10 Hz.

6.2.4 Determination of the encapsulation efficiency of cannabidiol (CBD)

The encapsulation efficiency was measured to determine the amount of CBD that was encapsulated in the emulsion droplets and to investigate its distribution between the oil and water phases of the produced Pickering emulsions following previously reported methods (X. Chen et al., 2019; Sharkawy, Casimiro, et al., 2020). Briefly, 1 ml of the emulsion was centrifuged at 10000 rpm for 15 minutes. The liquid layer at the bottom was then collected with a syringe and filtered (representing the aqueous phase of the emulsion). The filtrate

was analyzed by HPLC (as will be described in Section 6.2.6) to quantify the non-encapsulated amount of CBD which will be denoted as the “Free amount of CBD”. Another 1 ml of the Pickering emulsion was diluted with 10 ml methanol and put in a sonication bath for 10 minutes to extract the total CBD amount (encapsulated and non-encapsulated). This procedure was done to guarantee that all the added CBD remained in the formulation. The encapsulation efficiency (EE %) was calculated based on the non-encapsulated CBD through the following equation:

$$EE \% = \frac{\text{Total amount of CBD} - \text{Free amount of CBD}}{\text{Total amount of CBD}} * 100$$

6.2.5 *Ex vivo* skin application studies

6.2.5.1 Skin preparation

The preparation of the skin samples was performed in accordance with the OECD guidelines outlined for the *in-vitro* skin absorption studies (OECD, 2004). Porcine skin samples were used in this study as a practical alternative to human skin due to the limited supply of the latter (Diembeck et al., 1999), and owing to the similarities in the permeability and morphological properties between both skin types (Diembeck et al., 1999; OECD, 2004). Briefly, the dorsal porcine skin samples, obtained from pigs slaughtered at a local slaughterhouse, were shaved by an electric shaver. The subcutaneous fat was removed by a scalpel to obtain an appropriate thickness (Hiranphinyophat et al., 2021; Laredj-Bourezg et al., 2017); since excessive thickness (i.e. thickness > 1 mm) should be avoided (OECD, 2004). The skin samples were washed thoroughly with water, wrapped in parafilm and aluminum foil, and stored at -20 °C until used. It is noteworthy to mention that the process of storing and freezing the skin samples at this temperature (-20 °C) does not cause alterations in the permeability characteristics of the skin after thawing (Harrison et al., 1984), and is permitted as per the OECD skin preparation protocol (OECD, 2004). The skin samples were taken out of the freezer and allowed to thaw for one hour before being used in the following experiments.

6.2.5.2 Evaluation of the skin retention and permeation of CBD

The skin retention and permeation of CBD from Pickering emulsion formulations stabilized with CH/CP nanoparticles prepared with olive oil or liquid paraffin at different oil volume fractions (ϕ) were studied and compared. The experiments were performed according to the OECD guidelines for the conduct of skin absorption studies (OECD, 2004), and following methodologies described previously in the literature for the determination of the skin absorption of active agents from Pickering emulsions (Frelichowska et al., 2009; Laredj-Bourezg et al., 2017). In brief, the formulations were applied to the skin samples mounted in Franz diffusion cells (PermeGear, USA), with a diffusion area of 0.64 cm² and receptor compartment volume of 5 ml. The skin samples were placed with the *stratum corneum* (uppermost layer of the skin) facing the donor compartment. An amount of 300 μ l of each Pickering emulsion formulation was placed in the donor compartment and spread on the skin using a small plastic spatula. The receptor fluid consisted of a mixture of ethanol and ultrapure water (50:50 v/v) (Casiraghi et al., 2020), and was kept under constant magnetic stirring at 600 rpm. The temperature of the Franz cells was controlled by a thermostatic water bath and kept at 32 $\pm$ 1 °C according to the OECD guidelines (OECD, 2004). The experiment was conducted under occlusive conditions. A sample of 400 μ l was collected from the receptor fluid after 0, 2, 4, 8, and 24 hours. The sample vials were wrapped in aluminum foil to protect the CBD against potential degradation by light. After each sample withdrawal, the collected volume was immediately replaced with an equal fresh volume of the receptor medium to keep sink conditions.

The total mass recovery of CBD was determined by investigating the mass balance of CBD in the skin, as well as in the donor and the receptor compartments according to previously reported methods on the mass recovery of active agents from topically applied Pickering emulsions (Frelichowska et al., 2009; Laredj-Bourezg et al., 2017). At the end of the experiment (after 24 hours), the residual Pickering emulsion formulation present in the donor compartment was collected and diluted with 25 ml of methanol. Additionally, the skin surface was washed with 25 ml of methanol and the resultant washing solution was added to the previously collected solution (to ensure that all the residual Pickering formulation present on the skin surface is collected). The final solution was filtered and

analyzed for CBD quantification using HPLC, representing the “residual sample” remaining on the skin surface. The skin surface was then dried with a wipe. Thereafter, the stripping of the *stratum corneum* was carried out using Scotch[®] adhesive tape (3M, USA). Twenty tape-stripping were applied. The tapes were then cut into small pieces, added to 60 ml of methanol, and placed in an ultrasonic water bath for 30 minutes to extract the CBD from the stripped *stratum corneum*. The solution was then filtered and analyzed by HPLC to determine the amount of CBD absorbed by the horny layer of the skin (*stratum corneum*). The skin membrane remaining after the stripping of the *stratum corneum* (representing the viable epidermis and dermis) was cut into small pieces, added to 50 ml of methanol, and placed in an ultrasonic water bath for 2 hours to extract the CBD present in the viable epidermis and dermis of the skin. Afterward, the solution was filtered and analyzed by HPLC. Each test was conducted four times ($n = 4$).

6.2.6 Quantification of CBD

CBD was quantified by high performance liquid chromatography (HPLC) using a reversed phase column ACE 5 C18-pentafluorophenyl group (250 mm × 3 mm, 5 μm). The mobile phase consisted of a mixture of (7:1:2 v/v) acetonitrile/methanol/water following a previously reported method (Lodzki et al., 2003). The analysis was conducted at room temperature at a flow rate of 0.8 ml/min, and UV detection at 220 nm.

6.2.7 ATR-FTIR skin examination

ATR-FTIR spectroscopy was used to determine the potential variations in the structure of the *stratum corneum* after the application of the formulations according to methods described in the literature (Prasch et al., 2000; Schwarz et al., 2013). Briefly, the produced Pickering emulsions were first applied to the skin samples mounted in Franz diffusion cells. The skin samples and Franz diffusion cells were prepared and arranged as per the experimental conditions previously described in Sections 6.2.5.1 and 6.2.5.2. After 24 hours of skin exposure to the formulations, the skin samples were removed and rinsed with water to remove the residues of the formulation present on the surface and air-dried for 15 minutes. Afterward, the ATR-FTIR measurements of the surface of the *stratum corneum*

treated with the Pickering emulsion formulations were carried out using a Perkin Elmer FTIR spectrometer equipped with a Universal ATR Sampling Accessory. The spectra were recorded at 32 scans with a resolution of 2 cm⁻¹.

6.2.8 Cell viability assessment

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to evaluate the effect of the different Pickering emulsion formulations (with and without CBD) on HaCaT cell line (passages 89–90). Briefly, cells (25 × 10³ cells per ml) were incubated for 24 h with a fresh medium in the absence or presence of the formulations (0.1, 1, 10, 100 and 1000 µg/ml) dissolved in cell culture medium. Following the formulations removal from each well, cells were washed with HBSS. The number of viable cells was determined by adding MTT reagent and incubating for 3 h at 37 °C. DMSO was used to solubilize the crystals. The positive control used was DMEM and the negative control was 1% (w/v) Triton X-100. Cells were grown according to a method previously described in the literature (Pinto et al., 2021). The absorbance was read at 590 nm with background subtraction at 630 nm. The results were expressed as percentages of cell viability.

6.2.9 Statistical analysis

The results were presented as the mean ± standard deviation using Microsoft Excel 365. The statistical comparisons were performed using the Student's *t*-test, and one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison test (with *p* values < 0.05 denoting significant differences). The cell viability results were analyzed using one-way ANOVA followed by Tukey's HSD test for the post hoc comparisons of the means (with *p* values < 0.05 denoting significant differences) through the IBM SPSS Statistics 26.0 software (SPSS Inc., Chicago, IL, USA).

6.3 Results and discussion

6.3.1 Characterization of the chitosan/collagen peptides (CH/CP) nanoparticles

The CH/CP nanoparticles were prepared by the polyelectrolyte complexation method followed by ionic gelation, and were reported to have hydrophilic properties leading to the formation of O/W Pickering emulsions as discussed in our previous work (Sharkawy et al., 2021). In the present study, the CH/CP nanoparticles are used to stabilize Pickering emulsions containing olive oil or paraffin oil as oil phases. The dynamic interfacial tension was used to assess the degree of the adsorption of the CH/CP nanoparticles at the oil/water interface using the two different oils. Figure 6.1 shows that the CH/CP nanoparticles are capable of decreasing the interfacial tension at the oil/water interface of both liquid paraffin and olive oil in a time-dependent way, indicating that the nanoparticles become adsorbed at the oil/water interface, and form an interfacial layer (T. Feng et al., 2021). It can be also observed that the interfacial tension between the CH/CP nanoparticles and liquid paraffin decreased at a higher rate at the initial phase (compared to olive oil), and then slowed down after 500 seconds, reflecting an initial higher adsorption rate of the nanoparticles, which is followed by their packing and rearrangement at the oil/water interface (Z. Wei & Huang, 2019). Figure 6.1 also shows that the initial interfacial tension between the nanoparticles and liquid paraffin equals 24.16 mN/m and decreased to 14.61 mN/m after 2400 seconds, whereas the initial one recorded between the nanoparticles and olive oil equals 16.62 mN/m and reached 9.95 mN/m after the same period, suggesting that the CH/CP nanoparticles have a better interfacial activity towards olive oil than liquid paraffin (Jiang et al., 2021).

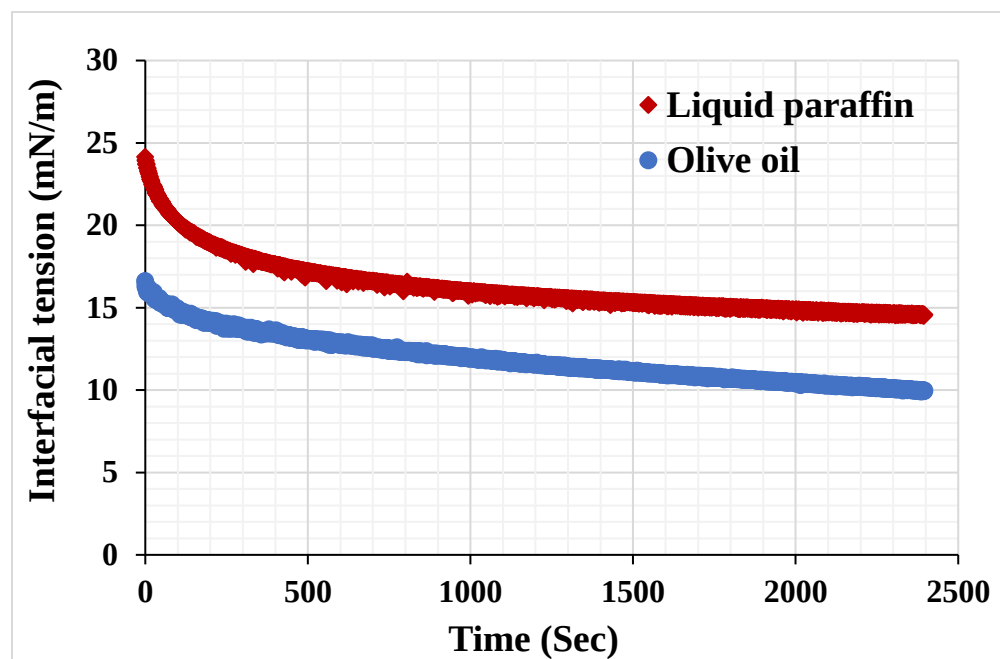


Figure 6.1. Dynamic interfacial tension at the oil/water interface measured over 2400 seconds between CH/CP nanoparticles and liquid paraffin, and CH/CP nanoparticles and olive oil.

6.3.2 Characterization of the CBD-loaded Pickering emulsions

6.3.2.1 Microstructure

The microstructure of the Pickering emulsions stabilized with chitosan/collagen peptides nanoparticles (at a constant nanoparticles' concentration of 2% w/v) was examined by optical microscopy. It was observed that the oil volume fraction influences the morphology and microstructure of the formulations. According to Figure 6.2, the Pickering formulations prepared with an oil volume fraction (ϕ) of 0.6 had a denser microstructure than those prepared with a ϕ of 0.4. The microstructure of the latter is less compact (Figure 6.2C and 6.2D). The optical microscope images also reveal that all the formulations have spherical droplet morphology.

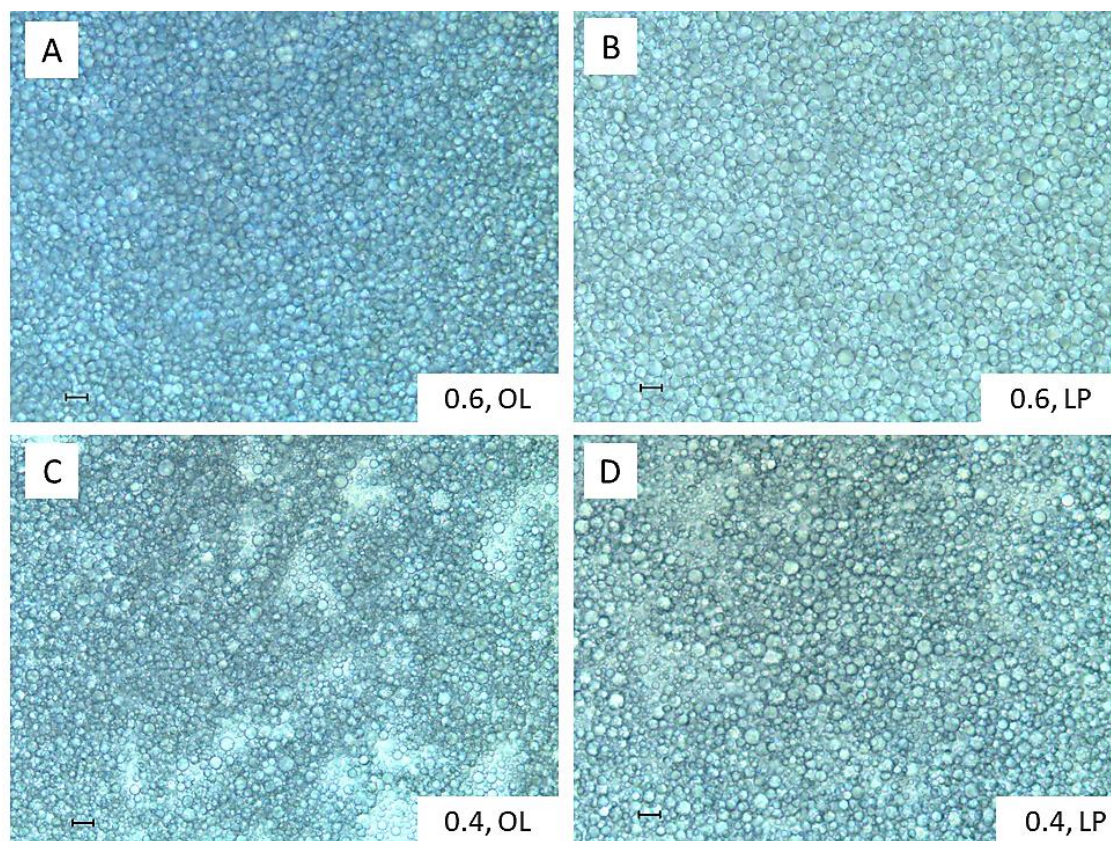


Figure 6.2. Optical microscopy images of CBD-loaded chitosan/collagen peptides Pickering emulsion formulations prepared with different oils and oil volume fraction at a magnification of 20x (A) olive oil ($\phi= 0.6$), (B) liquid paraffin ($\phi= 0.6$), (C) olive oil ($\phi= 0.4$) and (D) liquid paraffin ($\phi= 0.4$). Scale bars = 20 μm .

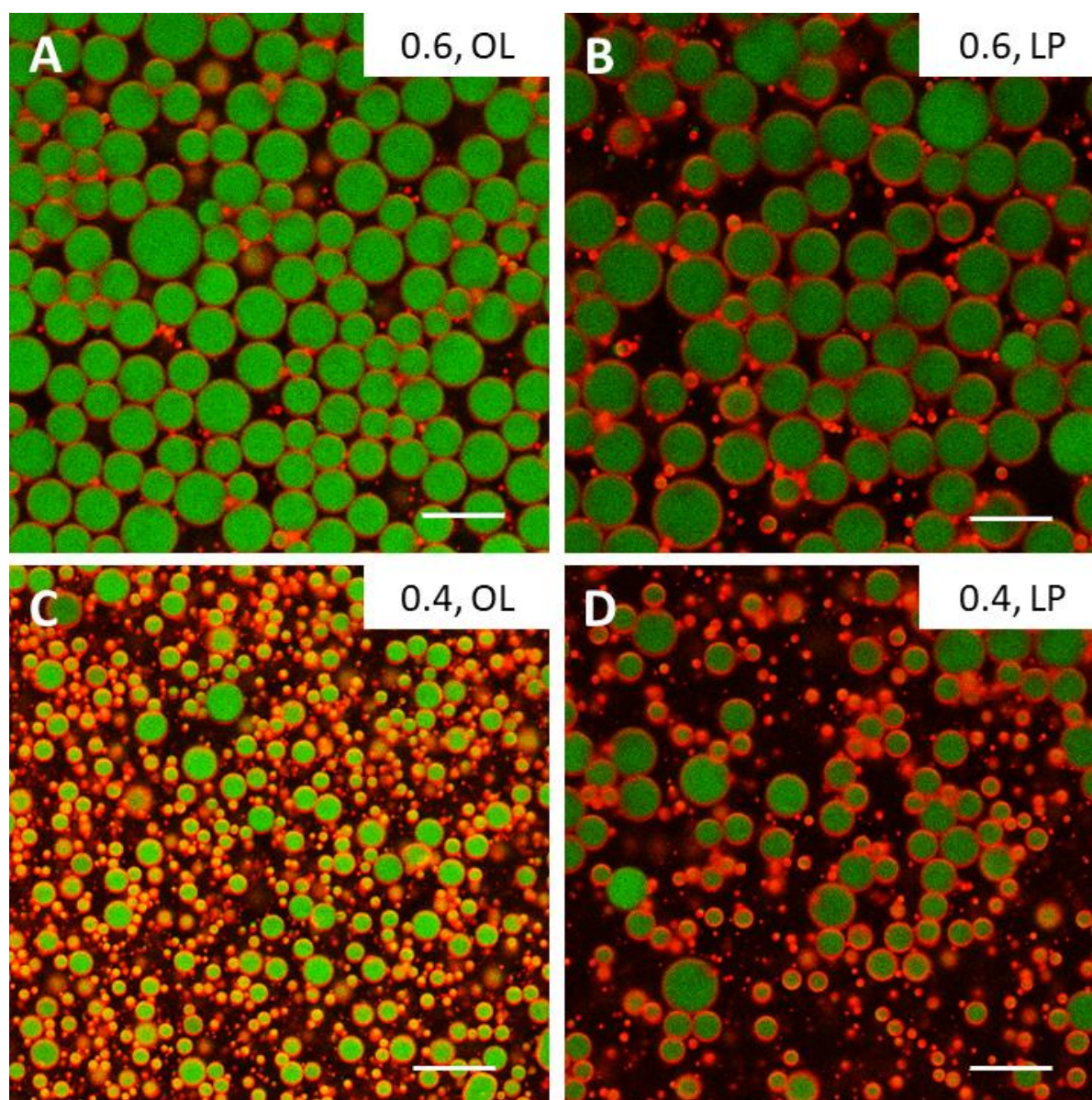


Figure 6.3. CLSM images of CBD-loaded chitosan/collagen peptides Pickering emulsion formulations prepared with different oils and oil volume fraction (A) olive oil ($\phi = 0.6$), (B) liquid paraffin ($\phi = 0.6$), (C) olive oil ($\phi = 0.4$) and (D) liquid paraffin ($\phi = 0.4$). The oil phase is stained with Nile Red appearing as green spheres, while the CH/CP nanoparticles are stained with Nile Blue and appear as red rings surrounding the oil droplets. Scale bars = 20 μm .

The interfacial microstructure of the produced Pickering emulsions was visualized by the CLSM. The images (Figure 6.3) reveal that the nanoparticles are well-adsorbed at the oil/water interface. The images also confirm that all the formed emulsions are o/w

emulsions. It can be also observed that the Pickering formulations produced with an oil volume fraction of 0.6 (Figures 6.3A and 6.3B) have larger droplet sizes and a more compact microstructure than the Pickering emulsions prepared at lower oil volume fractions (Figures 6.3C and 6.3D). Moreover, it can be noticed that the formulations containing a lower volume fraction have denser interfacial layers of adsorbed nanoparticles.

6.3.2.2 Visual appearance, creaming index (CI %), and mean droplet size

Figure 6.4A shows that all the freshly prepared Pickering formulations had no phase separation (CI% = 0). However, it can be observed that the CI% of the formulations after storage for 30 days was affected by the type of oil present in the emulsion as well as by the oil volume fraction (Figure 6.4B). The formulations prepared with olive oil exhibited a lower phase separation (lower CI% values) than those prepared with liquid paraffin, contributing to higher emulsion stability. Figure 6.4B also shows that the formulations with higher oil volume fraction ($\phi = 0.6$) presented a lower phase separation and higher emulsion stability than the formulations prepared with an oil volume fraction of 0.4. The formulation prepared with olive oil ($\phi = 0.6$) possessed the highest storage stability (CI% = 0). Besides, it can be noticed that the bottom serum layer in the formulations with lower oil volume fraction ($\phi = 0.4$) has a more opaque appearance, which is likely due to the presence of a higher amount of nanoparticles in the formulation that separated from the emulsion to the bottom layer during storage. A similar observation was reported for Pickering emulsions prepared with kafirin colloidal nanoparticles (Xiao et al., 2016).

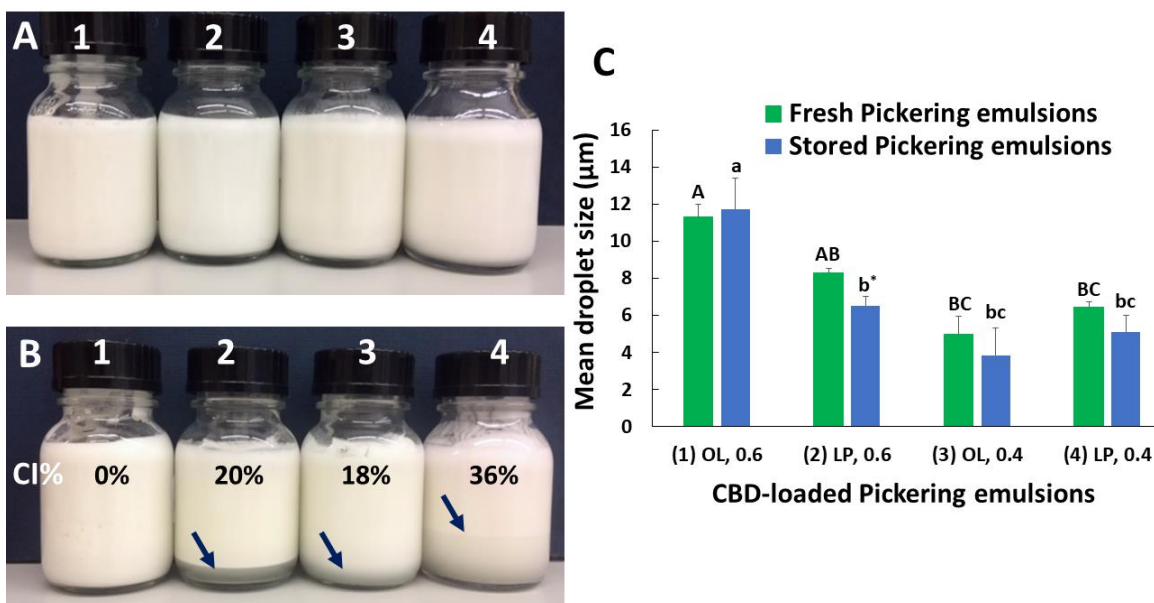


Figure 6.4. (A) Visual appearance of the freshly prepared CBD-loaded Pickering emulsions (1: olive oil ($\phi=0.6$), 2: liquid paraffin ($\phi=0.6$), 3: olive oil ($\phi=0.4$), 4: liquid paraffin ($\phi=0.4$)) (B) Visual appearance of the same Pickering emulsion formulations stored for 30 days at room temperature showing the values of the calculated CI%. The arrow (if present) points to the phase separation, and (C) The mean droplet sizes of the freshly prepared formulations and those stored for 30 days (OL: olive oil, LP: liquid paraffin). Different uppercase letters (A-C) indicate significant differences in the mean droplet size between the fresh Pickering emulsions ($p < 0.05$), different lowercase letters (a-c) indicate significant differences between the stored emulsions ($p < 0.05$), and * (if present) denotes significant differences between the fresh and stored emulsion (prepared with the same oil and the same oil volume fraction).

The mean emulsion droplet size of the fresh formulations versus the stored ones is shown in Figure 6.4C. It can be observed that formulations with higher oil volume fractions exhibited larger droplet sizes than the ones prepared with low oil volume fractions. This observation is attributed to the decreased amount of the nanoparticles present during emulsification due to the decreased volume of the aqueous phase, which subsequently results in decreasing the number of the nanoparticles that are expected to be adsorbed at the oil/water interface of the droplets leading to the formation of larger emulsion droplets (Dai et al., 2018). Figure 6.4C also shows that the oil volume fraction (ϕ) had a more dominant effect on the mean droplet sizes than the oil type. It can be observed that the mean droplet sizes of the

formulations prepared with an oil volume fraction of 0.6 (containing either olive oil or liquid paraffin) are not significantly different. Additionally, no significant difference was observed between the mean droplet size of formulations prepared with low oil volume fraction ($\phi = 0.4$). These results are in alignment with the optical microscopy results (Figure 6.2), and the CLSM images (Figure 6.3) which demonstrated that the morphology of the produced formulations was influenced by the oil volume fraction more than the oil type. Although the difference in interfacial tension and viscosities between oils is expected to result in significant variations in the emulsion droplet sizes, high particle concentrations lead to insignificant differences in the droplet size among the emulsions prepared with different oils (Tan et al., 2021). In the current study, it was observed that CH/CP nanoparticles had a higher dynamic interfacial tension with liquid paraffin than olive oil (as was shown in Figure 6.1). It is also noteworthy that the viscosity of liquid paraffin equals 148 mPa.s at 20 °C (as specified by the manufacturer), while the viscosity of olive oil is 74.1 mPa.s at 22 ± 1 °C (Sahasrabudhe et al., 2017). Therefore, it was expected that formulations prepared with liquid paraffin would have larger droplet sizes than their counterparts prepared with olive oil. However, in the present work, the CH/CP nanoparticles have been incorporated at a high enough concentration (2% w/v) in all the prepared formulations which allowed them to sufficiently cover the produced emulsion droplets at a specific oil volume fraction giving rise to similar droplet sizes when different oils were used (Tan et al., 2021).

Figure 6.4C shows that the formulations that exhibited phase separation after storage demonstrated a reduction in the mean droplet size. The decrease in the mean droplet sizes after storage of Pickering emulsions has been reported in some studies (Hosseini et al., 2020; Marefati & Rayner, 2020; Zafeiri et al., 2017). It usually occurs when the original dimension distribution of the emulsion droplets is not monomodal (Marefati & Rayner, 2020; Zafeiri et al., 2017). Zafeiri et al. (2017) suggested that it could be linked to the potential desorption of some particles from the oil-water interface during storage for 30 days, which eventually causes a drop in the overall mean sizes of the emulsion droplets (Zafeiri et al., 2017). In another study, Marefati et al. (2020), stated that the decrease in the mean droplet sizes could be due to the possible dissociation of aggregated emulsion droplets after storage for 2-8 years (Marefati & Rayner, 2020).

6.3.3 Rheological properties of the produced Pickering emulsions

The steady flow curves of the formulations are shown in Figures 6.5A and 6.5B. It can be observed that the formulations demonstrated a non-Newtonian fluid behavior, where the viscosity decreased by the increase in the shear rate. This shear-thinning behavior was more prominent in the formulations prepared with a higher oil to nanoparticles ratio (i.e., $\phi = 0.6$). This observation is consistent with previous studies on Pickering emulsions stabilized with xanthan gum/lysozyme nanoparticles (Z. Li et al., 2020) and kafirin nanoparticles (Xiao et al., 2016). This phenomenon happens because when the oil content increases, the Pickering formulation obtains a higher ability to overcome the Brownian movement when subjected to shear force, and hence the viscosity decreases more rapidly (Z. Li et al., 2020). It can be also noticed from the steady flow curves (Figures 6.5A and 6.5B) that the Pickering emulsions prepared with olive oil had higher viscosities than the ones prepared with liquid paraffin and formulated with the same oil volume fractions. It has been reported that the apparent viscosity of the produced emulsion depends on the hydrodynamic inter-particle interactions between the oil droplets and the solid particles, and not just on the viscosity of the oil (Yan & Masliyah, 1993). Therefore, it is suggested that the higher viscosity of the emulsions prepared with olive oil than those prepared with liquid paraffin (at the same oil volume fraction) is related to the greater interaction existing between the CH/CP nanoparticles and olive oil, than that present between the nanoparticles and liquid paraffin due to the structural differences of the two oils. Olive oil is mainly composed of triacylglycerols and free fatty acids (such as oleic, linoleic and linolenic acids), which has been reported to be able to interact with chitosan directly through the N-acetyl-D-glucosamine units, or indirectly by the aid of the free fatty acids through electrostatic binding to the amino groups of the D-glucosamine units of chitosan (Dimzon et al., 2013). In contrast, liquid paraffin is a mixture of high molecular weight alkane derivatives, which makes it incapable of any electrostatic binding with the amino groups of chitosan or collagen peptides in the CH/CP nanoparticles. Therefore, the interaction of the CH/CP nanoparticles and liquid paraffin was relatively low which resulted in lower apparent viscosities.

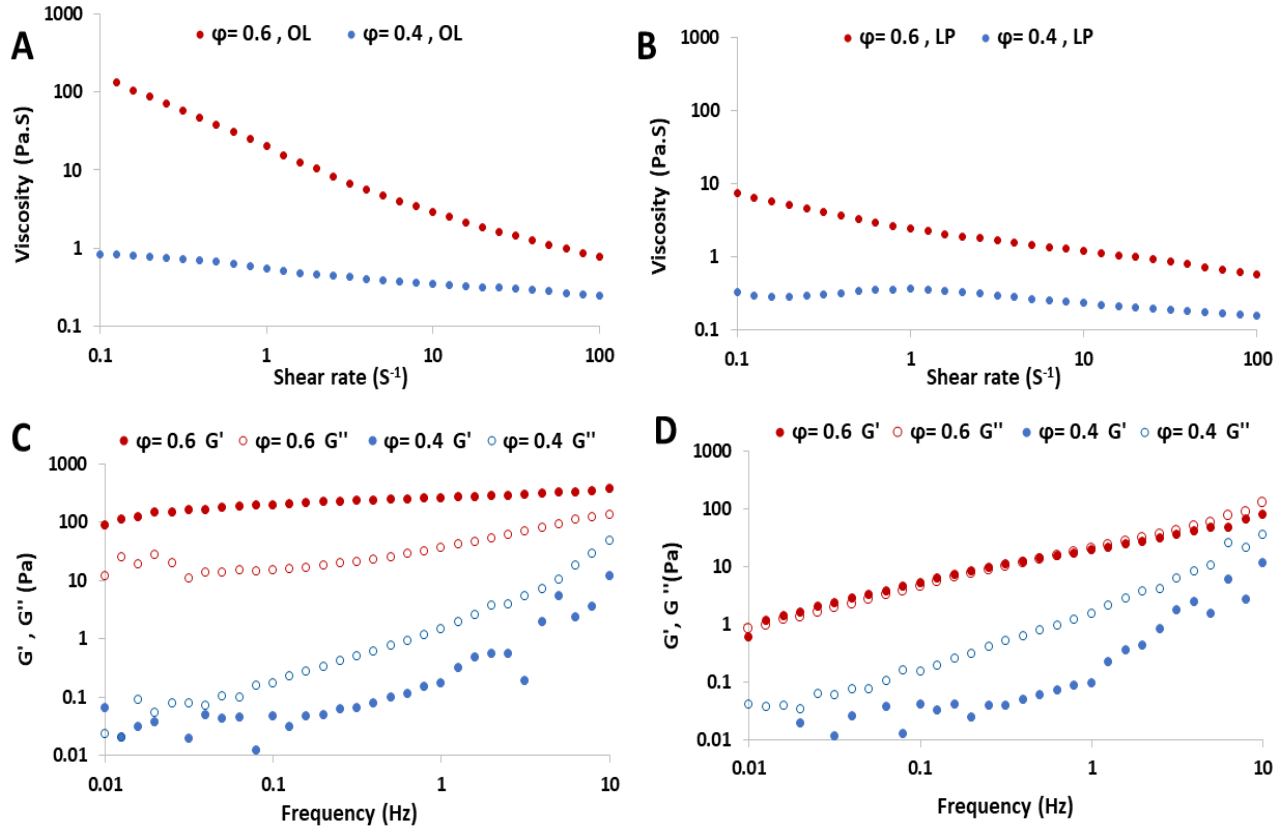


Figure 6.5. Effect of the oil type and oil volume fraction on the rheological profiles of CBD-loaded Pickering emulsions stabilized with chitosan/collagen peptides nanoparticles; (A) Viscosity versus the shear rate of formulations prepared with olive oil (OL); (B) Viscosity versus shear rate of formulations prepared with liquid paraffin (LP); (C) Frequency sweep curves of formulations prepared with OL, and (D) Frequency sweep curves of formulations prepared with LP (G' denotes the storage modulus, while G'' is the loss modulus).

The oscillatory frequency sweep curves of the Pickering formulations prepared with olive oil (Figure 6.5C) show that the emulsion containing a higher oil fraction had higher storage modulus (G') values than the loss modulus (G''), indicating the elastic gel-like characteristics of the formulation with $\varphi=0.6$, while the one produced with $\varphi=0.4$ showed a liquid behavior as G'' was always greater than G' over the tested range of frequency (0.01-10 Hz). This observation points to the presence of the emulsion droplets in a tightly packed way and close proximity to one another in the formulation with $\varphi=0.6$, resulting in higher storage modulus (Sharkawy et al., 2019; Xiao et al., 2016). The predominant liquid

character of the formulations produced with lower oil content is insufficient to inhibit the creaming process, resulting in lower emulsion stability (Santos et al., 2020). The dense and compact packing of the formulation with $\phi = 0.6$ is in agreement with its optical microscopy image and CLSM image (previously shown in Figures 6.2A and 6.3A, respectively). Regarding the frequency sweep curves of the formulations prepared with liquid paraffin (Figure 6.5D), it was observed that the G' and G'' values of the formulation with $\phi = 0.6$ were very close to each other, suggesting that the formulation does not possess the elastic gel properties as its counterpart formulated with olive oil. However, it is still not purely liquid-like compared to the formulation prepared with liquid paraffin but with lower oil content. It has been reported that the formation of an elastic gel network structure in Pickering emulsions (which is indicated by higher values of G') is mainly related to the interaction of the particles on the oil droplets (X. Feng et al., 2020), droplet-droplet interaction, droplet-bridging and partial aggregation (Ben Cheikh et al., 2021). Therefore, a higher interaction leads to the formation of a more compact three-dimensional gel-like structure that restricts (or lowers) the droplet movement and migration rate, preventing creaming (i.e., reducing CI%) and coalescence, and thus results in higher emulsion stability (Ben Cheikh et al., 2021; X. Feng et al., 2020). In the present work, it is suggested that the structural differences between olive oil and liquid paraffin (that were discussed in the previous paragraph) resulted in variations of such interactions (at the same oil volume fraction), which resulted in a lower CI% and thus enhanced emulsion stability in the case of the Pickering emulsions prepared with olive oil (as was shown in Figure 6.4B).

6.3.4 Encapsulation efficiency (EE %) of CBD

The EE% of CBD in the Pickering emulsions prepared with oil volume fraction (ϕ) of 0.6 was found to be 99.66 ± 0.12 % and 99.04 ± 0.10 %, for olive oil and liquid paraffin formulations, respectively. For Pickering emulsions prepared with lower oil volume fraction ($\phi = 0.4$), the EE% values were 99.40 ± 0.05 % and 99.28 ± 0.13 % for olive oil and liquid paraffin formulations, respectively. The high values of EE% of all formulations, which were above 99%, are due to the high hydrophobicity of CBD, as well as the formation of a strong barrier of chitosan/collagen peptides nanoparticles surrounding the oil

droplets, which guarded against the leakage of CBD to the surrounding phase during the centrifugation step.

6.3.5 Effect of long-term storage on emulsion properties, and on the total amount of CBD

The long-term stability (after 5 months of storage) was evaluated for the formulation prepared with olive oil ($\phi = 0.6$) only as it did not exhibit any phase separation after the initial short storage period of one month. Figures 6.6A and 6.6B show that there is almost no difference in the microstructure of the freshly prepared formulation and after being stored for 5 months. The figures show that the formulation maintained its dense/compact microstructure after storage. Moreover, no phase separation occurred (CI% = 0) during this long storage period as shown in Figure 6.6C. Nevertheless, there was a slight change in the emulsion droplet size distribution for the stored formulation compared to the freshly prepared one as could be seen in Figure 6.6D. However, this change did not affect the overall stability in terms of the visual appearance and creaming index. This high stability is attributed to the high viscosity of the formulation and the remarkable gel-like structure which guarded against the movement and potential coalescence of the emulsion droplets.

Interestingly, the total amount of CBD in this formulation after 5 months of storage (protected from light), was found to be 99.45 % of the initial incorporated amount (i.e., 5.996 mg/ml) compared to the initial amount of 6.03 mg/ml. In a recent study (Mazzetti et al., 2020), it has been reported that many of the CBD products available in the market show a significant decline in the amount of CBD in contrast to the amount claimed on the label after storage. Our present study suggests that the Pickering emulsion stabilized with CH/CP nanoparticles is an excellent vehicle for the encapsulation and protection of CBD.

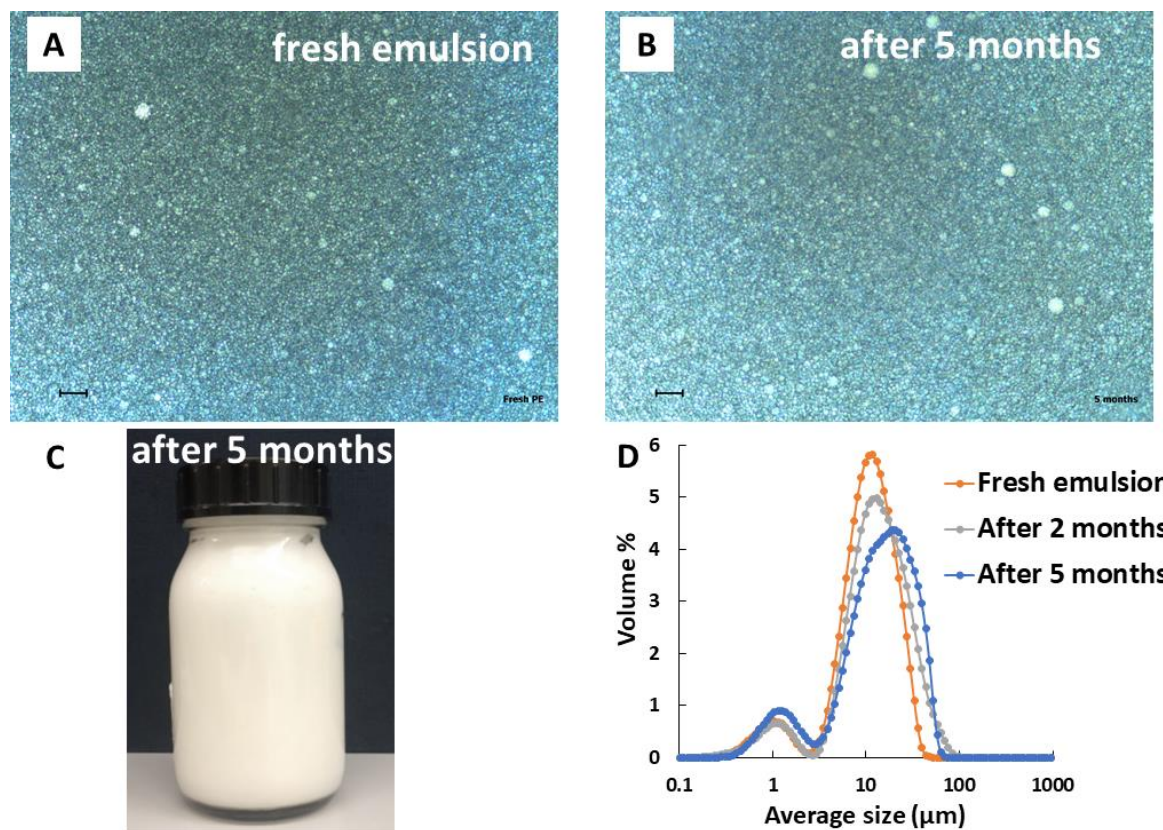


Figure 6.6. (A) Optical microscopy image of the freshly prepared Pickering emulsion formulated with olive oil ($\phi = 0.6$) (B) Optical microscopy image of the same formulation stored for 5 months at a magnification of 10x. Scale bars = 50 μm , (C) Visual appearance of the formulation stored for 5 months, and (D) Size distribution in volume of the fresh formulation, and after 2 months and 5 months of storage.

6.3.6 *Ex-vivo* skin absorption and permeation of CBD

Formulations designed for the dermal (topical) delivery of cosmetic and cosmeceutical active agents involve targeting the skin sites, ensuring minimal or no systemic absorption (Brown et al., 2006). Figure 6.7 shows that CBD permeation after 24 hours of exposure to the formulations is very low in comparison to the amount of CBD absorbed in the *stratum corneum*, and the viable epidermis and dermis. It can be also observed that the highest amount of CBD was retained in the *stratum corneum* rather than the viable epidermis and dermis, owing to the high lipophilicity of CBD ($\log P = 6.3$). It has been reported that highly lipophilic agents have high affinity to the skin horny layer (*stratum corneum*) than the

hydrophilic ones, and thus they have a lower ability to achieve high permeation to the receptor fluid (Frelichowska et al., 2009). Therefore, the *stratum corneum* is considered as a “reservoir” for highly lipophilic drugs encapsulated in Pickering emulsions, which allows their sustained release from the *stratum corneum* to deeper layers of the skin (Frelichowska et al., 2009; Laredj-Bourezg et al., 2017).

The effect of the volume fraction of the oil phase (ϕ) on the skin penetration of active agents from Pickering emulsions is scarcely reported in the literature. Figure 6.7 shows that the amount of penetrated CBD is enhanced by the increase of oil volume fraction from 0.4 to 0.6. Moreover, it was observed that the total amount of CBD that was absorbed in the skin (in the *stratum corneum*, viable epidermis and dermis) was 4.42 % and 4.78 % of the total applied dose for Pickering emulsions with an oil volume fraction of 0.6, whereas it was found to be 3.11 % and 2.58 % for the ones prepared with an oil volume fraction of 0.4 (as summarized in Table 6.2).

The total amount of CBD absorbed in the *stratum corneum*, viable epidermis, and dermis after 24 hours of application was $143.98 \mu\text{g}/\text{cm}^2$ and $158.78 \mu\text{g}/\text{cm}^2$ from Pickering emulsion formulations prepared with higher oil volume fraction ($\phi = 0.6$), while it was $97.26 \mu\text{g}/\text{cm}^2$ and $80.16 \mu\text{g}/\text{cm}^2$ for their counterparts prepared with lower oil volume fraction ($\phi = 0.4$) as can be calculated from Table 6.2. The amounts reported in the current study are comparable with the amounts of CBD absorbed from topical formulations that have been recently reported in the literature. For example, in a recent study, Vanti et al. (2021) reported that the amount of topically absorbed CBD was $115.30 \pm 16.99 \mu\text{g}/\text{cm}^2$ after 24 hours of applying novel microemulsion gels loaded with 1% (w/w) CBD to an artificial skin membrane, while the amount absorbed was low when rabbit ear skin was used (Vanti et al., 2021). In another work, Casiraghi et al. (2020) investigated, for the first time, the influence of different vehicles and formulation composition on the skin permeation and absorption of CBD (Casiraghi et al., 2020). In that study, the amount of CBD delivered to the skin after 24 hours of topical administration of 1% (w/w) solutions of CBD in liquid paraffin and virgin olive oil was reported to be $15.15 \pm 4.02 \mu\text{g}/\text{cm}^2$ and $6.45 \pm 3.23 \mu\text{g}/\text{cm}^2$, respectively, while it increased to $37.81 \pm 9.20 \mu\text{g}/\text{cm}^2$ by incorporating CBD in a mixture of propylene glycol/water (80/20, v/v) (Casiraghi et al.,

2020). The present work shows that the encapsulation of CBD in Pickering emulsions has enhanced its topical delivery.

The type of oil has been described to have a significant effect on the dermal delivery of lipophilic active agents incorporated in cyclodextrin-based Pickering emulsions (Hu et al., 2018), but it did not impact the transdermal diffusion of active agents from starch-based Pickering emulsions (Marku et al., 2012). Furthermore, it has been reported that the solution of CBD in liquid paraffin results in higher skin permeation and higher skin retention of CBD than when it is dissolved in olive oil (Casiraghi et al., 2020). However, in the present work, the oil type does not seem to have a remarkable impact on the total amount of CBD delivered to the skin. The results presented in Figure 6.7 show that the oil phase volume fraction has a predominant effect on the skin absorption of CBD more than the oil type. These results suggest that the chitosan/collagen peptides nanoparticles (2% w/v) present in high amounts in the formulations prepared with an oil volume fraction of 0.4 retarded the diffusion of the CBD from the oil droplets to the surroundings, and hence hampered its availability in the skin. This observation also indicates that the presence of smaller droplet mean size of the produced Pickering emulsions is not a crucial factor in enhancing the amount of CBD delivered to the skin. In the present study, the highest amount of CBD delivered to the skin was achieved by the formulations with larger mean droplet size (the formulations prepared with olive oil and liquid paraffin, having $\phi=0.6$).

The overall mass recovery % of CBD ranged between 91.91% and 84.70% (as shown in Table 6.2) which is a satisfactory percentage for active agents that are susceptible to degradation and oxidation during handling and conducting the experiments (Laredj-Bourezg et al., 2017; OECD, 2004).

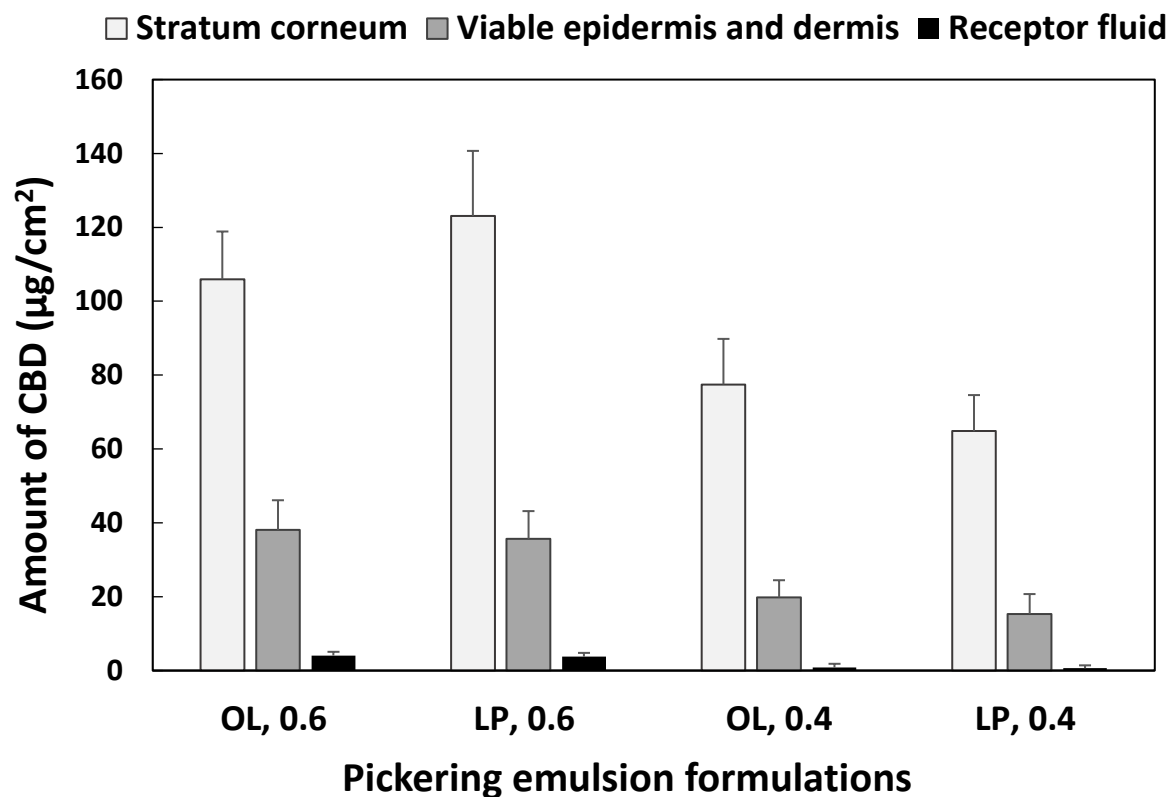


Figure 6.7. The amounts of CBD ($\mu\text{g}/\text{cm}^2$) distributed in the skin layers and receptor fluid after 24 hours of the skin exposure to Pickering emulsion formulations stabilized with chitosan/collagen peptides nanoparticles and formulated with olive oil (OL) or liquid paraffin (LP) at different oil volume fractions ($\phi = 0.4$ or 0.6). The amount of CBD is shown in $\mu\text{g}/\text{cm}^2 \pm \text{SD}$ ($n=4$).

Table 6.2. Distribution of CBD in skin layers (*Stratum corneum*, viable epidermis (VE) and dermis (D)), receptor fluid, and skin surface after 24 hours of exposure to the formulations. The amount of CBD is shown in $\mu\text{g}/\text{cm}^2 \pm \text{SD}$ ($n=4$).

	Applied dose	<i>Stratum corneum</i>	VE and D	Receptor fluid	Residual sample (skin surface)	Mass recovery
Formulation 1 ($\phi=0.6$, olive oil)						
$\mu\text{g}/\text{cm}^2$	3257.30 ± 117.29	105.92 ± 12.95	38.06 ± 8.05	4.06 ± 1.01	2846.02 ± 104.41	2994.06 ± 98.30
% of total applied dose		3.25	1.17	0.12	87.37	91.91 %
Formulation 2 ($\phi=0.6$, liquid paraffin)						
$\mu\text{g}/\text{cm}^2$	3319.68 ± 99.39	123.11 ± 17.59	35.67 ± 7.50	3.78 ± 1.00	2785.30 ± 139.75	2947.86 ± 137.28
% of total applied dose		3.71	1.07	0.11	83.90	88.79 %
Formulation 3 ($\phi=0.4$, olive oil)						
$\mu\text{g}/\text{cm}^2$	3120.29 ± 20.64	77.47 ± 12.33	19.79 ± 4.67	0.80 ± 1.03	2579.72 ± 55.07	2677.77 ± 62.78
% of total applied dose		2.48	0.63	0.03	82.68	85.82 %
Formulation 4 ($\phi=0.4$, liquid paraffin)						
$\mu\text{g}/\text{cm}^2$	3101.37 ± 15.576	64.82 ± 9.770	15.34 ± 5.37	0.64 ± 0.77	2546.36 ± 104.93	2627.17 ± 102.62
% of total applied dose		2.09	0.49	0.02	82.10	84.70 %

6.3.7 ATR-FTIR skin analysis

ATR-FTIR spectroscopy is a non-invasive technique normally used to provide insight into the molecular structure and the barrier function of the *stratum corneum* (Ge et al., 2014; Qurt et al., 2018). The main bands associated with the alkyl chain of the *stratum corneum* lipids vibration include the asymmetric ($\sim 2900\text{ cm}^{-1}$) and symmetric (2850 cm^{-1}) stretching CH_2 vibrations (as shown in Figure 6.8). It has been reported that even slight changes (slight shift to higher wavelength) in the asymmetric and symmetric CH_2 stretching vibrations (caused by vehicles or permeation enhancers) have a profound impact on the conformation of the lipids present in the *stratum corneum* which affects the drug penetration through the skin (Obata et al., 2010). Additionally, the amide I and amide II bands at $\sim 1640\text{ cm}^{-1}$ and $\sim 1540\text{ cm}^{-1}$ (denoted by numbers 3 and 4 in Figure 6.8) provide information about the secondary structure of proteins present in the *stratum corneum*. For instance, wavenumbers of amide I band ranging between 1638 and 1645 cm^{-1} indicate a random coil structure of keratin in the horny layer (Schwarz et al., 2013). Furthermore, the two amide bands have also been reported to be sensitive to new hydrogen bond formation (Dufton, 2018).

Figure 6.8 highlights the main structural changes in the molecular vibrations of the components of the *stratum corneum* after the application of Pickering emulsion formulations. It can be observed that there are changes in the asymmetric and symmetric vibrational wavelength values between the control skin samples and the other skin samples treated with the Pickering emulsion formulations. These shifts are indicative of changes in the lipid order which contribute to increasing the fluidity of the *stratum corneum* lipids and consequently, increasing skin penetration (Ge et al., 2014). Nevertheless, the wavenumbers of the amide I band reveal that the keratin in the *stratum corneum* maintained its coil structure with all formulations.

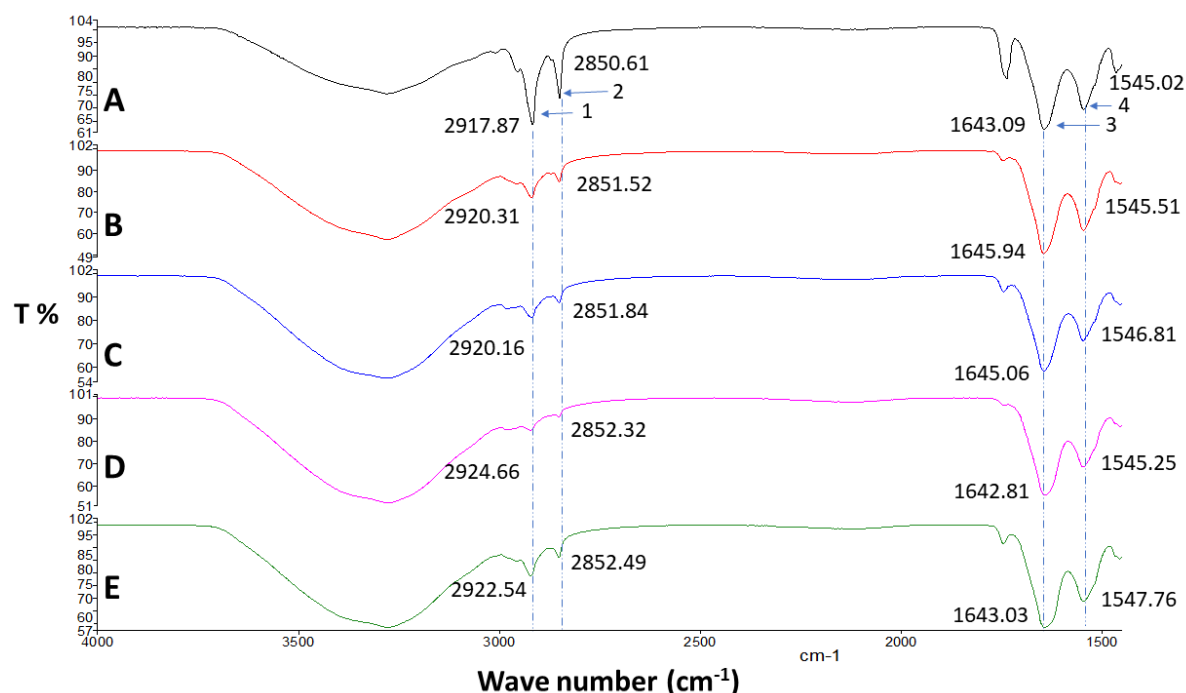


Figure 6.8. FTIR spectra of porcine skin (A) control (no formulation was applied), (B) after application of formulation 1 (olive oil, $\phi=0.6$), (C) after application of formulation 2 (liquid paraffin, $\phi=0.6$), (D) after application of formulation 3 (olive oil, $\phi=0.4$), and (E) after application of formulation 4 (liquid paraffin, $\phi=0.4$). The arrows point to (1) the asymmetric CH_2 stretching, (2) symmetric CH_2 stretching, (3) amide I, and (4) amide II bands.

The FTIR spectra also show that the changes in the stretching vibrations were greater as the oil volume fraction decreased from 0.6 to 0.4. This could be attributed to the presence of greater amounts of chitosan/collagen peptides nanoparticles in the formulations with $\phi=0.4$. Chitosan and chitosan-based nanoparticles have been reported to promote the skin absorption of drugs in topical formulations owing to their positive charges which allow their adhesion to the skin surface which is naturally negatively charged (Y. Chen et al., 2013; Sapra et al., 2008; Ta et al., 2021). Chitosan is also known to increase the water content in the *stratum corneum* enhancing the cell membrane fluidity (Ta et al., 2021). Chitosan/collagen peptides nanoparticles have been reported to have a high positive zeta potential (59.7 mV) (Sharkawy et al., 2021). In the present work, it is suggested that the presence of cationic chitosan/collagen peptides particles in higher amounts (when $\phi=0.4$) resulted in greater interactions with the lipids of the *stratum corneum*. Nevertheless, these

interactions did not result in increased skin penetration of the CBD (compared to formulations having $\phi = 0.6$) since high amounts of nanoparticles present at the oil/water interface surrounding the emulsion droplets (as was revealed by the CLSM images in Figure 6.3) retard the diffusion of the active agent from the oil phase to the surrounding medium as has been discussed in Section 6.3.6.

6.3.8 Effects of the produced Pickering emulsions towards keratinocytes

HaCaT cells are considered a good model for the evaluation of skin toxicity since they possess the functional and morphological properties of the normal epidermal keratinocytes (Taofiq et al., 2019). This cell line is the most common one used to assess the cytotoxicity of topically applied Pickering emulsions. For instance, it has been used to evaluate the cytotoxicity of Pickering emulsions stabilized with *Quercus suber* bark (i.e., cork) particles, poly(lactide-co-glycolide)-based nanoparticles, and calcium carbonate particles (Carriço et al., 2019; Marto et al., 2020; Y. Shan Wei et al., 2020). In this work, the cell viability of chitosan/collagen peptides Pickering emulsions (prepared with olive oil or paraffin oil) was tested on HaCaT cells. To our knowledge, this is the first study that assesses the effect of chitosan-based Pickering emulsions towards HaCaT cells. The results of the cell viability % of the formulations are presented in Figures 6.9 and 6.10. It can be observed that the cells maintained a high survival rate for the formulations loaded and unloaded with the CBD, suggesting that the produced Pickering emulsions are non-toxic to skin keratinocytes.

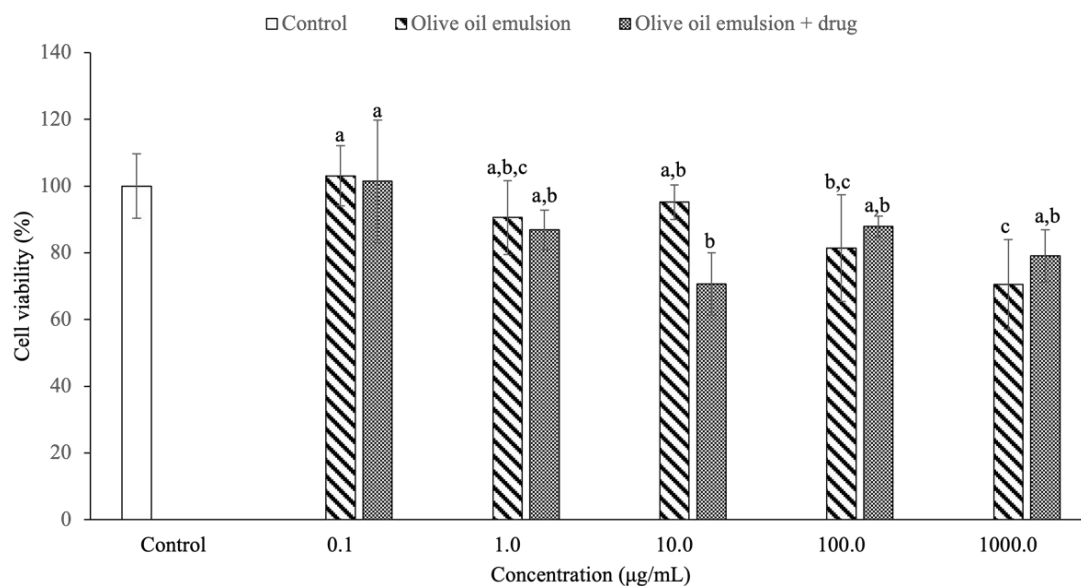


Figure 6.9. Effect of chitosan/collagen peptides Pickering formulations prepared with olive oil ($\phi = 0.6$, with and without CBD) on the viability of keratinocytes (HaCaT) cells at different concentrations, as measured by the MTT assay ($n=3$). Different letters mean significant differences between concentrations of the same formulation ($p < 0.05$).

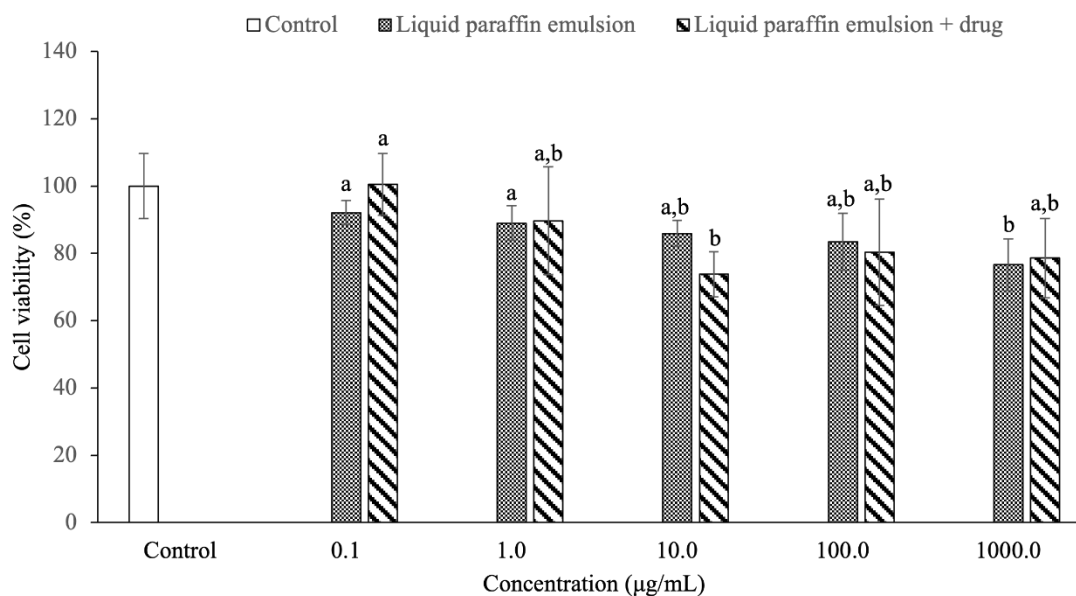


Figure 6.10. Effect of chitosan/collagen peptides Pickering formulations prepared with liquid paraffin ($\phi = 0.6$, with and without CBD) on the viability of keratinocytes (HaCaT)

cells at different concentrations, as measured by the MTT assay ($n=3$). Different letters mean significant differences between concentrations of the same formulation ($p < 0.05$).

6.4 Conclusions

Pickering emulsions stabilized with the biocompatible and biodegradable chitosan/collagen peptides nanoparticles were efficiently assessed as green biodegradable topical vehicles of CBD. The physicochemical properties and stability of the produced CBD-loaded Pickering emulsions were influenced by the amount of the oil and water phases, as well as the oil type. The formulation produced with olive oil ($\phi=0.6$) has shown long-term stability during the testing period that lasted for 5 months. The *in vitro* skin absorption studies revealed high skin deposition of CBD in the *stratum corneum*, while very low amounts permeated to the receptor fluid. The skin deposition was enhanced by increasing the oil volume fraction in the formulations regardless of the oil type. The ATR-FTIR results have shown that the Pickering emulsion formulations caused changes in the microstructure and molecular vibrations of the *stratum corneum* which enabled them to overcome its barrier function. The findings obtained in this work prove the successful utilization of Pickering emulsions stabilized by biopolymeric nanoparticles, namely chitosan/collagen peptides nanoparticles, as environmentally-friendly and non-toxic topical delivery vehicles of a highly lipophilic active agent and, thus contribute to the ongoing research in the area of sustainable cosmetics. Future work will focus on comparing the skin delivery of the CBD-loaded Pickering emulsions developed in the current study with different skincare CBD-based formulations that are available in the market.

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7 Pickering emulsions stabilized with chitosan/gum Arabic complex particles: Effect of chitosan degree of deacetylation on the physicochemical properties and topical drug delivery

In this chapter, the effect of the degree of deacetylation (DDA) of chitosan on the properties of chitosan/gum Arabic (CH/GA) complex particles and PEs was investigated. The hydrophilicity of the CH/GA particles increased with lower DDA chitosan, whereas the emulsification properties increased with higher DDA chitosan. The encapsulation efficiency of CBD was 99.60% and 95.76%, and the mean emulsion droplet size was $15.57 \pm 4.25 \mu\text{m}$ and $21.14 \pm 2.61 \mu\text{m}$ for PEs prepared with high and low DDA chitosan, respectively. The high DDA PE formulation exhibited higher stability after two months of storage than its counterpart prepared with low DDA chitosan. Both PE formulations demonstrated elastic gel-like properties, with a slightly higher apparent viscosity for the high DDA PE than the low DDA one. The amount of CBD absorbed by the *stratum corneum*, viable epidermis and dermis was significantly higher in both PE formulations than the permeated amount, but no significant difference was observed between the amounts absorbed from both formulations.

7.1 Introduction

Pickering emulsions are emulsions stabilized with solid particles instead of the classical surfactants (Dickinson, 2012). They were first reported by Ramsden in 1903, and then, they were named after S.U. Pickering who described them in 1907 (Chevalier & Bolzinger, 2013; Low et al., 2020; Rayner et al., 2014). Pickering emulsions are finding tremendous research interest nowadays due to the increasing awareness towards the environmental hazards and adverse health issues related to the small molecular weight surfactants, as well as the ongoing advances in the production and characterization techniques of the nanoparticles (Rayner et al., 2014).

Polymers of natural origin are considered attractive particulate emulsion stabilizers owing to their sustainability and eco-friendliness which makes them perfect candidates for the development of clean-label products (Ribeiro et al., 2021). Pickering emulsions stabilized with polysaccharide-based particles, aside from their cost-effective and affordable characteristics, have been reported to offer various functional properties such as, controlling lipid digestibility, reducing saturated fat and calorie content, and enhancing the delivery of bioactive agents (Cui et al., 2021).

Chitosan is a natural polymer obtained by the partial deacetylation of chitin (de Alvarenga et al., 2010). It consists of β -(1-4)-linked D-glucosamine units and N-acetyl-D-glucosamine units (Wu et al., 2020). It has been widely used in drug delivery due to its biodegradable, biocompatible and bioadhesive properties (Mohammadi et al., 2021; Wu et al., 2020). Furthermore, there has been an increasing interest in the use of chitosan-based particles as Pickering stabilizers for Pickering emulsions designed for food, pharmaceutical and cosmetic applications (Asfour et al., 2017; Atarian et al., 2019; Sharkawy, Barreiro, et al., 2020; H. Yang et al., 2020).

The particles that are used as Pickering stabilizers should have a proper partial wettability and amphiphilic nature in order to achieve high emulsion stability (Rayner et al., 2014). Chitosan particles have been reported to be highly hydrophilic (Asfour et al., 2017; Sharkawy, Barreiro, et al., 2020). Therefore, to enhance the wettability of chitosan, chitosan-based Pickering stabilizers are often formed through the interaction of chitosan with another polymer through the polyelectrolyte complexation method (Sharkawy et al.,

2019) or the polyelectrolyte complexation together with the ionic gelation method (Sharkawy, Barreiro, et al., 2021), or *via* the hydrophobic modification of chitosan (Atarian et al., 2019).

Gum Arabic is a branched biopolymer obtained from Acacia trees (Isobe et al., 2020). It consists of polysaccharide units called arabinogalactans (which presents ~90% of the total mass), arabinogalactan-protein (AGP) units (~10% of the total gum mass), and glycoprotein (~1% of the total mass) (Hu et al., 2019; Isobe et al., 2020). It has excellent emulsification properties over a wide range of pH and a good ability to tolerate high temperatures (Li et al., 2018). The emulsification properties of gum Arabic are attributed to its amphiphilic nature that is conferred by the presence of the hydrophilic polysaccharide domains and the hydrophobic peptides moieties (present in AGP) (Hu et al., 2019; Isobe et al., 2020). Gum Arabic has been incorporated in several Pickering emulsion systems due to its ability to modify the wettability of the particles that are used as Pickering stabilizers, and achieve high emulsion stability (Li et al., 2018; Sharkawy et al., 2019; Wei & Huang, 2019).

The degree of deacetylation (DDA) of chitosan is controlled by an alkaline hydrolysis process of chitin by changing the exposure time and temperature (Foster et al., 2015). The acid hydrolysis process has been reported to result in the hydrolysis of the polysaccharide as it affects the glycosidic linkage between the polymer units, and therefore it is not used (Meyers & No, 1995; S. Zhang et al., 2020). The proportion of the acetylated and non-acetylated glucosamine units in chitosan affects the extent of its protonation in dilute acids, and subsequently, affects its solubility, reactivity, adsorption and bio-adhesiveness (de Alvarenga et al., 2010). Chitosan degree of deacetylation has been reported to influence the physicochemical and rheological properties of chitosan-based particles (Tavares et al., 2020). To date, few studies investigated the effect of chitosan degree of deacetylation on emulsions prepared with chitosan-based complexes (F. Zhang et al., 2021), but no studies have focused on the impact of chitosan degree of deacetylation on the properties and stability of chitosan-based Pickering emulsions.

The use of chitosan-based Pickering emulsions as topical delivery vehicles for bioactive agents has been investigated in very few studies in the literature (Asfour et al., 2017;

Sharkawy, Casimiro, et al., 2020). In a previous study, we reported for the first time the production of Pickering emulsions stabilized with chitosan/gum Arabic nanoparticles that possess biodegradable nature and high stability (Sharkawy et al., 2019). The main objective of the present work is to investigate the influence of the degree of deacetylation of chitosan on the properties of chitosan/gum Arabic complex particles, as well as on the microstructure, rheology and stability of the produced Pickering emulsions. The work also explores the impact of the degree of deacetylation of chitosan on the encapsulation efficiency and topical delivery of cannabidiol (CBD), which was used as a model lipophilic active agent due to its high lipophilicity and skin benefits (Casiraghi et al., 2020). To the best of our knowledge, this is the first study that evaluates the impact of chitosan degree of deacetylation on the physicochemical properties of Pickering emulsions stabilized with chitosan-based complex particles, and compares their potential as dermal delivery vehicles of lipophilic active agents.

7.2 Materials and methods

7.2.1 Materials

Chitosan with a high (96%) and low (78%) degree of deacetylation and a molecular weight of 120-135 kDa was a kind gift from Primex ehf, Iceland. Gum Arabic (Molecular weight = 250 kDa), olive oil, Nile Red and Nile Blue were obtained from Sigma Aldrich. Cannabidiol (CBD) was obtained from THC Pharm (Frankfurt, Germany). Methanol and acetonitrile (HPLC grade) were supplied by Carlo Erba Reagents. Acetic acid (0.1 N) was purchased from Alfa Aesar. The porcine skin samples were a kind gift from Grupo Primor, Portugal.

7.2.2 Production of chitosan/gum Arabic (CH/GA) particles

Two types of CH/GA particle dispersions were produced depending on the used degree of deacetylation (DDA %) of chitosan; high DDA CH/GA particles in which the DDA% of chitosan is 96% and low DDA CH/GA particles in which the DDA% is 78%. The particles were prepared through polyelectrolyte complexation between chitosan and gum Arabic following a method reported by Tan and co-workers (Tan et al., 2016), and modified by our

research group (Sharkawy et al., 2019). In brief, 1 g of chitosan was dissolved in 50 ml of 0.1 N acetic acid, followed by the addition of gum Arabic solution (consisting of 1 g of gum Arabic dissolved in 50 ml of deionized water) in a dropwise manner, with constant magnetic stirring (at 800 rpm) for 30 minutes. The resultant CH/GA particle dispersion (2% w/v) had a pH of 5 and 4.8 when chitosan of high and low DDA was used, respectively.

7.2.3 Production of CBD-loaded Pickering emulsions stabilized with CH/GA particles

The cannabidiol-loaded Pickering emulsions were prepared by dissolving 0.6 g of cannabidiol (CBD) in 60 ml of olive oil. The oil phase was then added portion-wise to the CH/GA particle dispersion with continuous homogenization at 13500 rpm for 7 minutes using a high-speed homogenizer (Ultra-Turrax Digital T25, IKA, Germany). The final volume of the emulsion was 100 ml, with an oil volume fraction (ϕ) of 0.6. Two types of Pickering emulsions were produced; high DDA and low DDA Pickering emulsions in which the DDA% of the chitosan used in the preparation of the particle dispersion is 96% and 78%, respectively.

7.2.4 Characterization of the produced CH/GA particles

The wettability of the particles was determined by measuring the contact angle using Dataphysics OCA15 Plus equipment (Dataphysics, Germany). Briefly, homogenous films of the particles were prepared by the deposition of CH/GA particles dispersion (2% w/v) onto glass slides that were dried at room temperature. The contact angle was measured by injecting water droplets (of a volume of 4 μ l each) on the surface of the particles films. The droplets were photographed at zero seconds and after 30 seconds to allow equilibrium to take place (Lai et al., 2021). The Young-Laplace equation was used to fit the profile data of the imaged droplets to calculate the contact angle. The measurements were conducted at least three times.

The size and zeta potential of the produced CH/GA particles dispersions were measured using a Zetasizer Nano ZS ZEN3600 (Malvern Instruments, UK). The results were reported as the average of three consecutive runs.

The morphology of the particles was examined by transmission electron microscopy (TEM) using a JEOL JEM 1400 TEM at 80 kV (Tokyo, Japan). Samples of the particles dispersions prepared with high and low DDA were both diluted with deionized water (1/1000). Afterward, 10 μ l of each sample was mounted on Formvar/carbon film-coated mesh nickel grids (Electron Microscopy Sciences, Hatfield, PA, USA). The negative staining was then performed by the addition of 10 μ l of 1% uranyl acetate on the prepared grids. The images were obtained using a CCD digital camera Orious 1100 W (Tokyo, Japan).

The dynamic interfacial tension between the CH/GA particles dispersions and olive oil was determined using a Dataphysics OCA15 Plus device (Dataphysics, Germany). The experiments were conducted by the pendant drop method, which entailed the formation of a drop of the CH/GA particle dispersion by a syringe submerged inside a cuvette filled with olive oil. The dynamic interfacial tension was measured over 2400 seconds and was calculated using the Young-Laplace equation according to the pendant drop shape (Sharkawy, Barreiro, et al., 2021).

7.2.5 Characterization of the produced Pickering emulsions

The morphology and microstructure of the high and low DDA Pickering emulsions were examined by optical microscopy using a Leica DM 2000 optical microscope.

The interfacial microstructure of the produced Pickering emulsions was investigated by confocal laser scanning microscopy (CLSM) using a Leica TCS SP5 CLSM (Leica Microsystems Inc., Germany). Nile Red was used to stain the oil droplets of the emulsions, while Nile Blue dye was used to stain the CH/GA particles. The excitation wavelengths were 488 nm for Nile Red and 633 nm for Nile Blue.

The mean droplet diameter was determined by laser diffraction using LS230 Beckman Coulter equipment. The refractive index was set as 1.46 and 1.33 for the oil phase (olive oil) and water phase, respectively.

The creaming index (CI%) was used to evaluate the stability of the produced PEs and was calculated according to the following equation (Li et al., 2018):

$$CI\% = \frac{H_s}{H_t} * 100$$

where H_s is the serum layer height and H_t is the total emulsion height.

The encapsulation efficiency (EE%) was investigated to determine the distribution of CBD within the oil and aqueous phases through accelerated phase separation via centrifugation according to a previously reported method (Sharkawy, Silva, et al., 2021). The CBD concentration was determined by HPLC (as will be described in Section 7.2.7). The EE% was calculated using the following equation:

$$EE\% = \frac{Total\ CBD - Free\ CBD}{Total\ CBD} * 100$$

The rheological properties of the produced Pickering emulsions were examined using a Kinexus Pro Rheometer (Malvern, UK). The apparent viscosity was measured versus the shear rate using a cone plate (with a diameter 40 mm, an angle of 4° and a fixed gap of 0.15 mm). The storage modulus and loss modulus were determined over a frequency of 0.01-10 Hz using a parallel plate (with a diameter of 20 mm and a fixed gap of 1 mm). All measurements were conducted in triplicate at 25 °C.

7.2.6 Ex-vivo skin absorption studies

The dorsal porcine skin samples were prepared according to the OECD guidelines (OECD, 2004). In brief, the skin samples were carefully shaved with an electric shaver (Grundig Intermedia GmbH, Germany), and the subcutaneous layer was removed. The samples were then rinsed with water and stored at -20 °C until the time of the experiment.

The skin membranes were left to thaw at room temperature for one hour before their use. They were then placed between the donor and the receptor compartments of the Franz diffusion cells (PermeGear, USA), with the *stratum corneum* (uppermost skin layer) facing the donor compartment. The receptor medium consisted of ethanol and ultrapure water (1:1), and was kept under continuous magnetic stirring (600 rpm) at 32 ± 1 °C using a thermostatic water bath. An amount of 250 µl of the Pickering emulsion sample was added to the donor compartment and was uniformly spread on the skin. The experiment was performed under occlusive conditions to prevent water evaporation. Thereafter, samples of

400 µl were collected from the receptor compartment at predetermined time intervals (0, 2, 4, 8 and 24 hours), and were protected from light until analysis to prevent any possible degradation of CBD by light. The collected samples were replaced immediately by equal volumes of fresh receptor medium to keep sink conditions.

The total mass recovery of CBD was determined based on the mass balance of CBD distributed in the skin and the donor and receptor compartments following a previously reported method (Sharkawy, Silva, et al., 2021). Briefly, at the end of the experiment, the residual emulsion sample was collected from the donor compartment. The skin surface was washed with methanol and the washing solution was added to the residual sample. The final solution was filtered and analyzed by HPLC to determine CBD in the residual emulsion sample. Tape stripping of the *stratum corneum* was then performed with twenty adhesive tapes (Scotch[®], 3M, USA), which were cut into small pieces to which methanol was added, and placed in an ultrasonic water bath for CBD extraction from the cut tapes. The solution was then analyzed to determine the amount of CBD present in the *stratum corneum*. The skin sample representing the viable epidermis and dermis (after stripping off the *stratum corneum*) was cut into small pieces, added to methanol, and placed in an ultrasonic water bath to extract the CBD from the viable epidermis and dermis. The experiment was performed four times ($n = 4$) for each formulation.

7.2.7 Quantification of CBD

CBD was quantified by HPLC using a reversed-phase column ACE 5 C18-pentafluorophenyl group (250 mm × 3 mm, 5 µm). The mobile phase consisted of acetonitrile/methanol/water (7:1:2 v/v) (Lodzki et al., 2003). The analysis was performed at room temperature at a flow rate of 0.4 ml/min, and UV detection at 220 nm.

7.2.8 Statistical analysis

The results of the measurements were presented as the mean ± standard deviation using Microsoft Excel 365. The statistical comparisons were performed by the Student's *t*-test, and one-way analysis of variance (ANOVA) (with the level of significance at p values < 0.05).

7.3 Results and discussion

7.3.1 Effect of chitosan degree of deacetylation (DDA) on the physicochemical properties of the produced CH/GA particles

7.3.1.1 Wettability of the CH/GA particles

The wettability of the particles was assessed by measuring the contact angle. It has been reported that the degree of deacetylation of chitosan influences its surface wettability. However, the results on this issue are somehow contradictory. Some studies reported that the hydrophilicity increases with the increase in DDA of chitosan (Tomihata & Ikada, 1997), while other studies showed that the hydrophilicity of chitosan decreased with the increase of DDA (Foster et al., 2015). These differences could be related to the chitosan film preparation methods, and also to the changes in the surface availability of the chitosan amine groups (Foster et al., 2015). In the present work, it was observed that the hydrophilicity of the produced CH/GA particles was higher when the low DDA chitosan was used (as shown in Figure 7.1). It is suggested that the higher degree of polyelectrolyte complexation between the positively charged amino groups of chitosan and the negatively charged groups of gum Arabic in the case of high DDA chitosan (due to the presence of a higher number of amino groups) resulted in greater surface exposure of the hydrophobic groups (of both polymers), leading to a higher contact angle value.

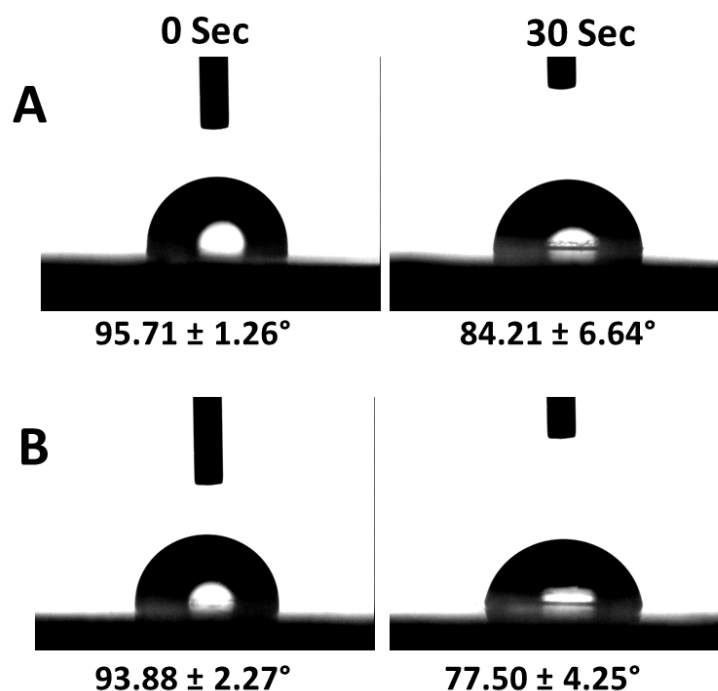


Figure 7.1. Contact angle of CH/GA particles prepared with chitosan of (A) High DDA (96%), and (B) Low DDA (78%). The measurements were conducted in the air environment at zero seconds and after 30 seconds.

7.3.1.2 Morphology of the CH/GA particles

The TEM examination of the particles revealed that they have spherical and oval morphology (as shown in Figure 7.2). The TEM images also show that the DDA of chitosan affected the number of the produced CH/GA complex particles. It can be observed that the particles produced with the higher DDA chitosan were greater in number than their counterparts produced with the lower DDA chitosan. This observation is attributed to the presence of a greater number of positively charged amino groups on the high DDA chitosan chains providing higher charge density (Mun et al., 2006), which facilitated the attraction of the negatively charged gum Arabic chains leading to the production of more particles.

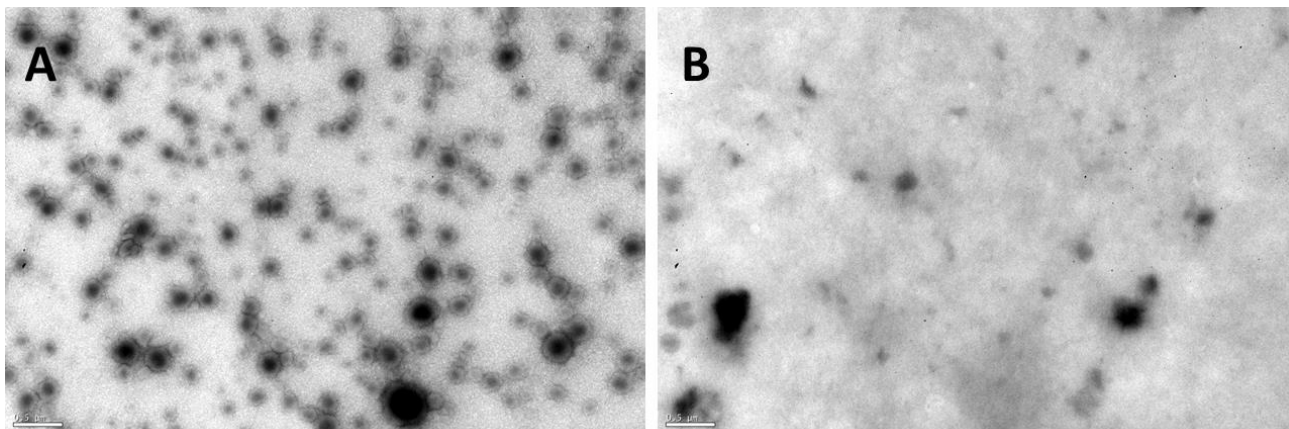


Figure 7.2. TEM images of CH/GA particles (2% w/v) prepared with chitosan of (A) high DDA, and (B) low DDA at a magnification of 25000x and same dilution (1/1000). Scale bars= 500 nm.

7.3.1.3 Average size and zeta potential

Figure 7.3 shows that the CH/GA particles prepared with chitosan of high DDA exhibited a monomodal size distribution, whereas their counterparts produced with chitosan of low DDA had a bimodal size distribution. A similar observation was described by Yang and co-workers who reported that chitosan nanoparticles prepared using chitosan with DDA of 75% had a bimodal size distribution due to the presence of a lower number of amino groups resulting in uneven interactions with tripolyphosphate (TPP) ions (H. C. Yang & Hon, 2010). The hydrodynamic average particle size of the particles prepared with the high DDA chitosan was reported to be 787.7 ± 36.75 nm, while the particles prepared with the low DDA chitosan had average sizes of 45.02 ± 8.59 nm and 433.7 ± 30.64 nm according to the peaks in Figure 7.3.

The zeta potential of the produced CH/GA particles was $+68.5 \pm 4.94$ mV and $+46.7 \pm 1.72$ mV for the particles formulated with chitosan of high DDA and low DDA, respectively. Previous studies showed that CH/GA complex particles had a positive zeta potential (Sharkawy et al., 2019; Tan et al., 2016). It is suggested that the particles produced with the high DDA chitosan exhibited higher zeta potential due to the presence of a greater number of cationic amino groups compared to those available in particles prepared with chitosan of low DDA.

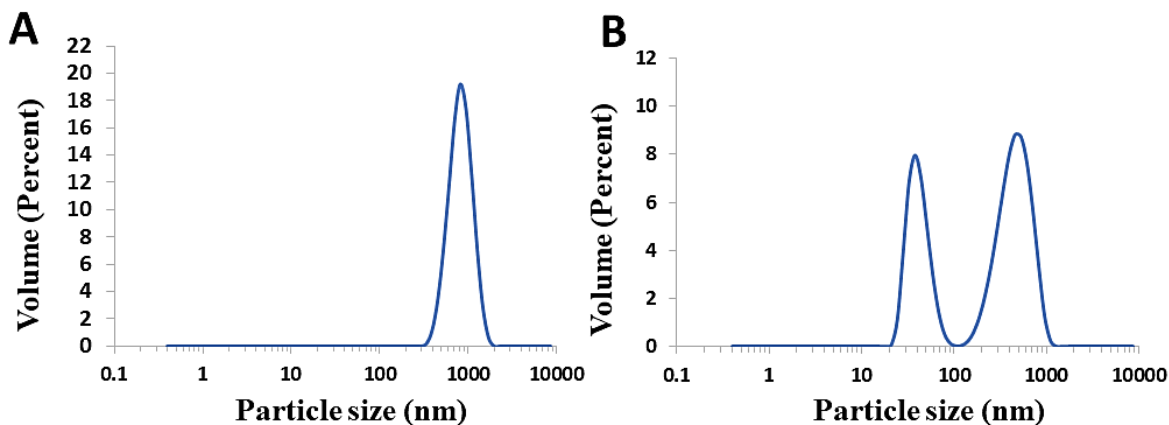


Figure 7.3. Size distribution in volume of CH/GA particles prepared with chitosan of (A) high DDA, and (B) low DDA.

7.3.1.4 Dynamic interfacial tension

The dynamic interfacial tension was assessed to compare the emulsification ability of CH/GA particles prepared with chitosan of high and low DDA. Figure 7.4 shows that both types of particles decreased the interfacial tension in a time-dependent manner. The results also show that the particles prepared with high DDA chitosan have slightly better emulsification properties than their counterparts prepared with chitosan of low DDA. This observation could be related to the presence of a greater number of particles in the first sample which allowed higher adsorption of the particles at the interface. Moreover, it is suggested that the higher positive charge of the particles prepared with chitosan of high DDA, due to the presence of a greater number of amino groups (as discussed in Section 7.3.1.3), resulted in better interaction with the free fatty acids present in olive oil. This electrostatic interaction between olive oil and chitosan has been discussed in previous studies (Dimzon et al., 2013; Sharkawy, Silva, et al., 2021).

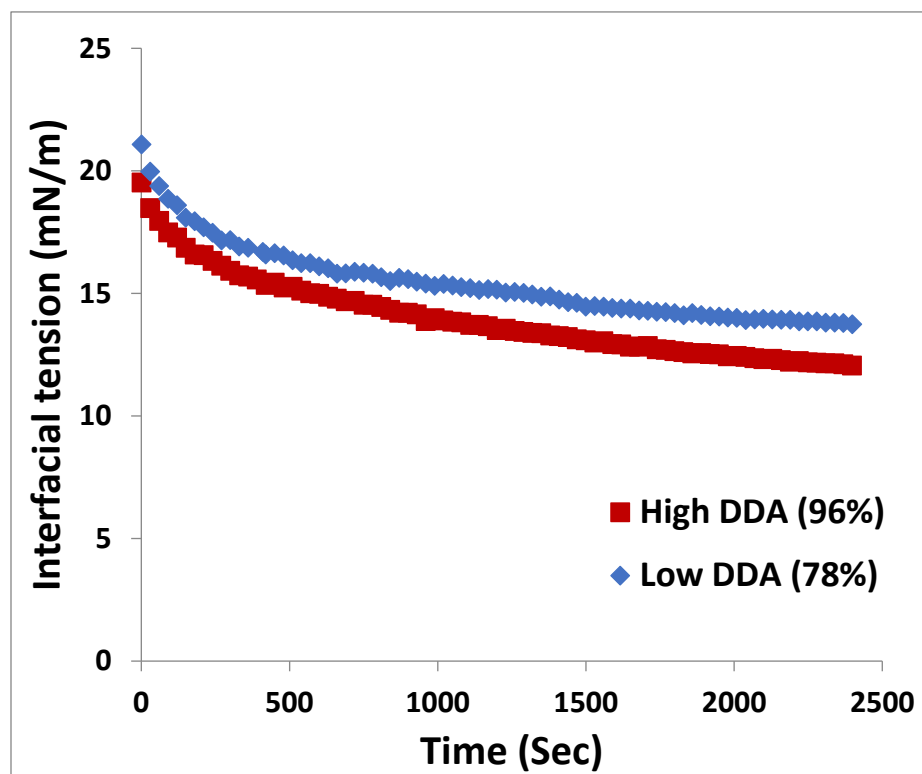


Figure 7.4. Dynamic interfacial tension between chitosan/gum Arabic particles (prepared with chitosan of high and low DDA) and olive oil.

7.3.2 Effect of chitosan degree of deacetylation on the physicochemical properties of the produced Pickering emulsions (PEs)

7.3.2.1 Microstructure

Optical microscopy and CLSM were both used to visualize the microstructure of the produced PEs. The optical microscopy images of the emulsions (Figure 7.5) show that the emulsion droplets are intact and spherical. The images also reveal that the emulsion droplets of the PE prepared with chitosan of high DDA (high DDA PE) were smaller in size than the droplets of the emulsion prepared with chitosan of low DDA (low DDA PE).

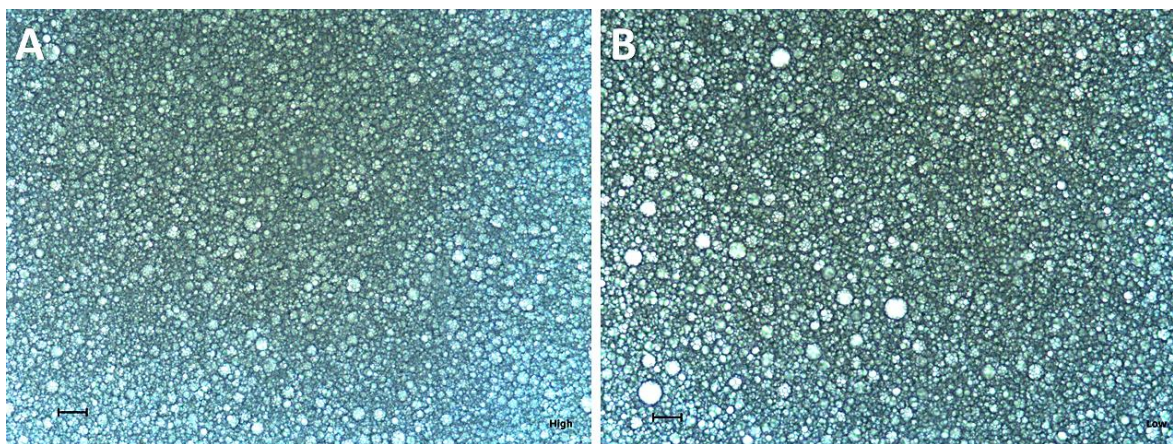


Figure 7.5. Optical microscopy images of CBD-loaded Pickering emulsions stabilized with CH/GA particles (2% w/v) formulated with chitosan of (A) High DDA, and (B) Low DDA. Scale bars= 50 μm .

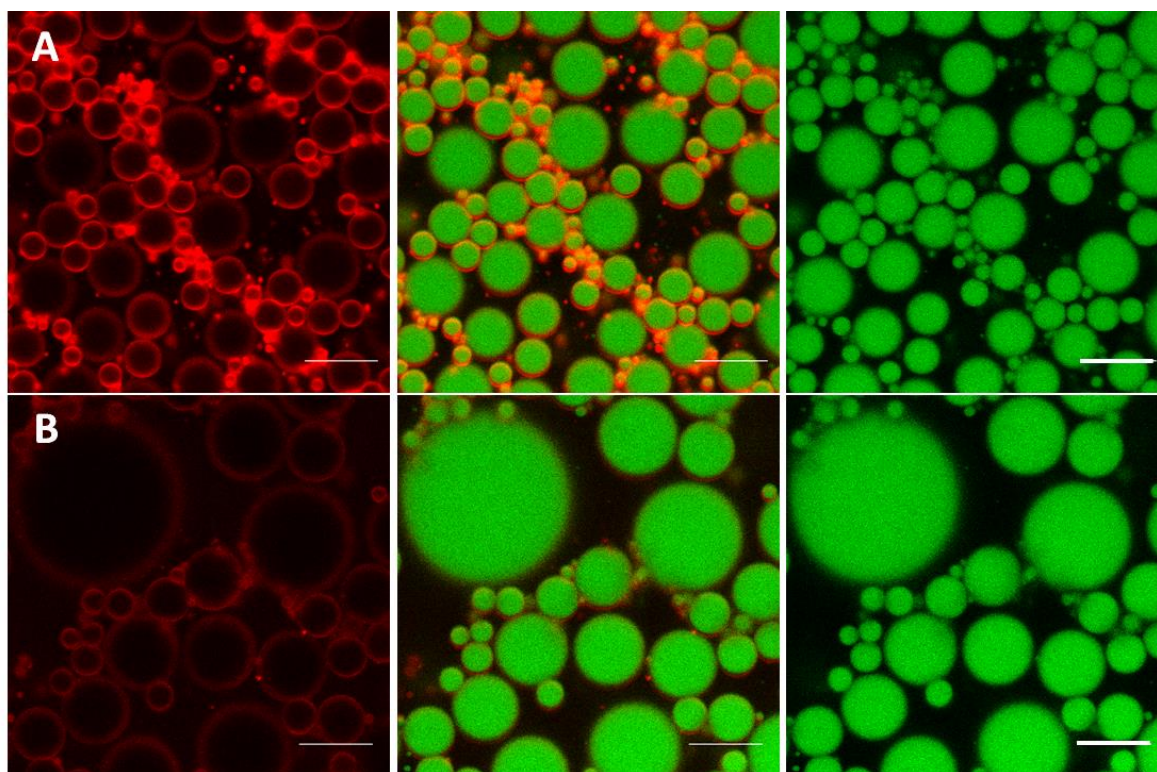


Figure 7.6. CLSM images of CBD-loaded chitosan/gum Arabic Pickering emulsions formulated with chitosan of (A) High DDA (96%), and (B) Low DDA (78%). The images on the left show the particles as red rings stained with Nile Blue, whereas the images on the right represent the emulsion oil droplets stained with Nile Red, and the images in the middle are an overlay of these images. Scale bars= 10 μm .

CLSM images (Figure 7.6) confirm the adsorption of the particles which appear as red halos on the surface of the oil droplets containing the encapsulated CBD (stained in green). Figure 7.6 also shows that the high DDA PE had smaller droplet sizes than the low DDA one, which is in agreement with the optical microscopy images. It can be also observed that the fluorescence intensity of the adsorbed CH/GA particles is higher in the case of the high DDA PE formulation (Figure 7.6A) than that of the low DDA PE one (Figure 7.6B), owing to the presence of a greater number of particles. This observation is in alignment with the TEM images of the produced particles (Figure 7.2) which showed that the number of particles produced with high DDA chitosan was higher than the ones produced with low DDA chitosan.

7.3.2.2 Emulsion droplet size, storage stability and encapsulation efficiency (EE%)

The emulsion droplet size was measured for both high and low DDA Pickering emulsions directly after preparation and after storage for 60 days. Table 7.1 shows that the formulation prepared with high DDA chitosan had a smaller droplet size than its counterpart prepared with low DDA chitosan. This observation could be explained by the presence of a higher amount of CH/GA particles prepared with high DDA chitosan (as was confirmed by the TEM images in Figure 7.2) resulting in larger interfacial area coverage and smaller droplets (Sharkawy et al., 2019). There was also a slight increase in the droplet size of both formulations after storage for 60 days, with a noticeably less change in the high DDA formulation. Moreover, Table 7.1 shows that the high DDA formulation did not exhibit phase separation after storage (the CI% remained 0), whereas the low DDA formulation demonstrated a small phase separation after 60 days with the CI% reaching 10% reflecting the higher stability of the high DDA formulation.

Table 7.1. The mean droplet diameter, creaming index (CI%) for fresh formulations (d0) and after 60 days of storage (d60), and encapsulation efficiencies of the PE formulations prepared with chitosan of high and low DDA.

Formulation	Mean diameter (day 0)	Mean diameter (day 60)	CI% (d0)	CI% (d60)	EE %
High DDA	15.57±4.25 μm	17.01±3.70 μm	0%	0%	99.60%
Low DDA	21.14±2.61 μm	25.13±2.27 μm	0%	10%	95.76%

Table 7.1 also shows that the EE% of CBD in both Pickering formulations was high. This is attributed to the high lipophilic nature of CBD favoring its presence in the oil phase (emulsion droplets) rather than in the external phase (Sharkawy, Silva, et al., 2021). It was also observed that the EE % of CBD in the formulation prepared with chitosan of high DDA was slightly higher than the low DDA formulation, which is related to the presence of a denser layer of particles surrounding the emulsion droplets (as was revealed by the CLSM images in Figure 7.6). This denser/thicker layer guarded against the diffusion of CBD to the emulsion external phase which led to a higher value of EE%.

7.3.2.3 Rheological properties

The apparent viscosity of the CBD-loaded PEs prepared with high and low DDA chitosan exhibited a gradual decrease with the increase of shear rate (from 0.1 to 100 s^{-1}) as shown in Figure 7.7A, reflecting a non-Newtonian and shear-thinning behavior (Sharkawy et al., 2019). This observation is attributed to the breaking/disruption of the internal microstructure of the Pickering emulsions with increasing the shear rate, leading to a decrease in the apparent viscosity (Dai et al., 2018). Figure 7.7A also shows that the viscosity of the high DDA PE is slightly higher than the low DDA formulation throughout the tested range of shear rate, which is suggested to be due to the presence of a stronger internal three-dimensional network structure owing to the higher degree of interactions between the particles formed with high DDA chitosan and olive oil (as has been discussed earlier in Section 7.3.1.4). It has been reported that the apparent viscosity fundamentally

increases with the increase in the inter-particle interactions between the solid particles and the oil droplets (Yan & Masliyah, 1993), which is in agreement with the findings in the current study. It is also suggested that the higher apparent viscosity of the high DDA formulation led to higher stability and no phase separation after storage (CI% = 0) as shown in Section 7.3.2.2.

The frequency sweep curves of the formulations (Figure 7.7B) show that the values of the storage modulus (G') were always higher than the values of the loss modulus (G'') over the tested frequency range. This observation reflects the elastic gel-like composition of the produced PEs (Sharkawy et al., 2019), and their tendency to store energy rather than lose it upon applying strain (Ribeiro et al., 2020).

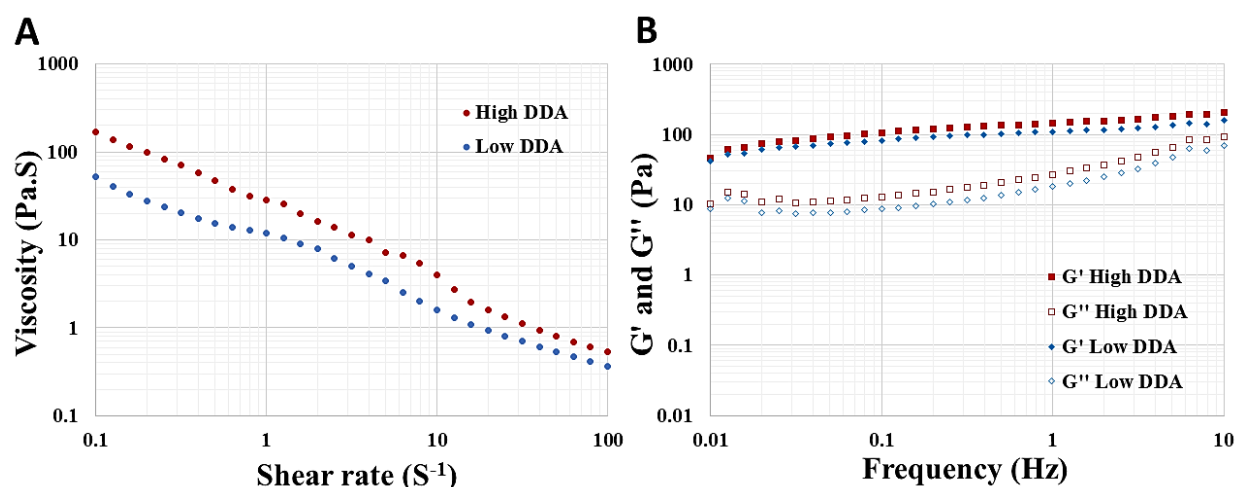


Figure 7.7. The rheological profiles of CBD-loaded Pickering emulsions stabilized with CH/GA particles prepared with chitosan of high and low DDA; (A) Apparent viscosity versus shear rate, and (B) Storage modulus (G') and loss modulus (G'') versus frequency..

7.3.3 Effect of chitosan DDA on the *ex-vivo* skin absorption of CBD from PEs

Pickering emulsions have been reported to promote the accumulation of highly lipophilic compounds in the *stratum corneum* which acts as a reservoir for the sustained release of the active agent to deeper skin layers (Frelichowska et al., 2009; Marto et al., 2019). Chitosan has been reported to enhance the dermal delivery of bioactive agents because of its cationic nature that allows it to interact with negatively charged skin cells (Chen et al., 2013). The

DDA is an important parameter that has been reported to influence the biological properties of topically applied chitosan, such as wound healing, antibacterial activity and hemostasis (Song et al., 2021). However, the effect of the DDA on the dermal bioavailability of bioactive agents is scarcely reported in the literature.

Figure 7.8 shows that the amount of CBD absorbed by the *stratum corneum*, viable epidermis and dermis was significantly higher in both PE formulations in comparison with the permeated amount ($p < 0.05$), suggesting that both formulations are suitable for CBD cosmetic delivery. It can also be observed that the amount of CBD retained in the skin layers is slightly higher in the case of low DDA PE than the high DDA PE. However, this difference is not statistically significant ($p > 0.05$). It is suggested that the presence of a denser layer of CH/GA particles surrounding the emulsion droplets (containing the encapsulated CBD) in the high DDA PE did not hinder the diffusion of the CBD from the emulsion droplets to the skin. This could be explained by the higher zeta potential of the CH/GA particles prepared with chitosan of high DDA that allowed good interaction and adhesion to the skin cells.

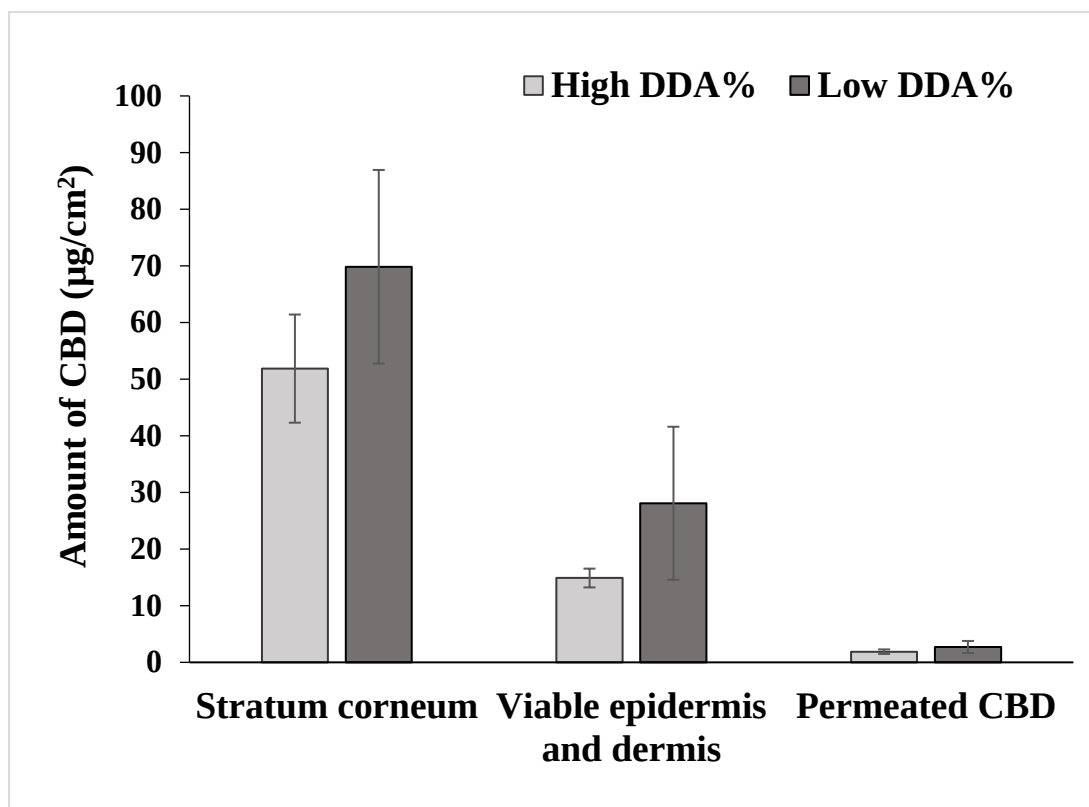


Figure 7.8. Distribution of CBD in skin layers and receptor fluid (permeated CBD) from Pickering emulsions stabilized with CH/GA particles formulated with chitosan of high and low DDA.

Table 7.2. Distribution of CBD in the *stratum corneum*, viable epidermis and dermis, receptor fluid, and residual sample on the skin surface after 24 hours of exposure to the formulations prepared with chitosan of high and low DDA%. The amount of CBD is shown in $\mu\text{g}/\text{cm}^2 \pm \text{SD}$ ($n=3$), as well as percentages of the applied dose.

	Applied dose	<i>Stratum corneum</i>	Viable epidermis & dermis	Permeated CBD	Residual sample	Mass recovery
High DDA% (96%)						
$\mu\text{g}/\text{cm}^2$	2328.81 $\pm$ 18.07	51.86 $\pm$ 9.55	14.89 $\pm$ 1.66	1.89 $\pm$ 0.40	2187.31 $\pm$ 152.87	2255.95 $\pm$ 161.64
% of the applied dose		2.23%	0.64%	0.08%	93.92%	96.87%
Low DDA% (78%)						
$\mu\text{g}/\text{cm}^2$	2278.36 $\pm$ 248.08	69.84 $\pm$ 17.09	28.09 $\pm$ 13.51	2.71 $\pm$ 1.07	2134.38 $\pm$ 330.80	2235.03 $\pm$ 327.39
% of the applied dose		3.07%	1.23%	0.12%	93.68%	98.10%

Table 7.2 shows that the mass recovery percentage of CBD from both formulations had high values as more than 95% of CBD was recovered, which is considered a very satisfactory value according to the OECD guidelines for the *ex-vivo* skin absorption testing of unstable compounds (OECD, 2004). Table 7.2 also shows that the total amount of CBD absorbed by the skin is 2.87% and 4.30% of the total applied dose for the high and low DDA PEs, respectively. These results are comparable with the amounts of highly lipophilic drugs delivered by o/w topical PE formulations (Frelichowska et al., 2009). Moreover, it is noteworthy that the amounts of CBD absorbed by the skin from the PEs in the current study are in the range of the amounts that have been reported in recent studies on CBD cosmetic delivery (Casiraghi et al., 2020; Sharkawy, Silva, et al., 2021), reflecting the suitability of the PE formulations stabilized with CH/GA particles to act as potential eco-friendly cosmetic carriers for CBD.

7.4 Conclusions

The effect of chitosan DDA on the physicochemical properties of CH/GA complex particles and the produced PEs was evaluated. The DDA had an impact on the wettability, interfacial tension, average size and zeta potential of the produced particles. CLSM revealed that the PE formulation produced with high DDA had a denser layer of CH/GA particles around the emulsion droplets. Moreover, the PE formulation produced with high DDA chitosan has demonstrated higher stability (no phase separation) and higher CBD encapsulation efficiency compared with its counterpart produced with low DDA chitosan. The *ex-vivo* skin absorption assessment showed high CBD retention in the *stratum corneum* and very low permeation to the receptor fluid. It was also observed the CBD deposition in the *stratum corneum*, viable epidermis and dermis was not significantly different between the PE formulations prepared with high and low DDA chitosan. The results obtained in this study are particularly useful in the preparation of chitosan-based PEs. The findings also unveil the effect of the chitosan DDA on the stability, physicochemical properties and dermal delivery of drugs encapsulated in chitosan-based PEs.

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8

Conclusions and Future Prospects

8.1 General conclusions

This thesis focused chiefly on developing new, surfactant-free, and stable chitosan-based Pickering emulsions that have the potential to be used in cosmetic and food applications. Furthermore, the thesis investigated the utilization of the produced Pickering emulsions as green encapsulation systems and topical delivery carriers of bioactive agents, namely resveratrol (RSV) and cannabidiol (CBD), which are known to have cosmetic and dermatological benefits. The main findings of the thesis and its original contribution to knowledge can be summarized as follows:

- A. Novel polysaccharide-based Pickering emulsions stabilized with chitosan/gum Arabic complex nanoparticles were developed. The stable emulsions were produced by tuning the wettability of the chitosan/gum Arabic nanoparticles to an appropriate value by changing the weight ratios between the two biopolymers. The work focused on understanding the properties of the produced emulsions, such as the effect of the oil phase volume fraction and nanoparticles concentration on the stability, microstructure, and rheology. To the best of my knowledge, no previous studies reported the formulation of Pickering emulsions using chitosan/gum Arabic nanoparticles. These new Pickering emulsions are expected to open new doors to a wide array of food and cosmetic applications.
- B. The developed chitosan/gum Arabic Pickering emulsions were successfully utilized as biodegradable surfactant-free vehicles for the skin application of trans-RSV. The topical delivery of bioactive agents is generally considered a challenging target owing to the structural and functional barrier of the *stratum corneum* (the outermost layer of the skin). Herein, the results have shown that RSV was retained in higher levels in the viable epidermis and dermis, which is highly desirable to give the required cosmetic effect without the need of using chemical penetration enhancers. Furthermore, the produced Pickering formulations protected trans-RSV against degradation by UV

radiation. These findings suggest that the produced chitosan/gum Arabic nanoparticles-stabilized Pickering emulsions enhanced the cutaneous delivery of RSV, thus they demonstrate a potential contribution towards green and sustainable cosmetics. To my knowledge, this is the first study that investigated the skin uptake of RSV from Pickering emulsions.

- C. The second novel chitosan-based Pickering emulsion system that was successfully developed in this thesis was obtained using chitosan/collagen peptides nanoparticles which acted as effective Pickering stabilizers. The produced nanoparticles demonstrated enhanced wettability and a higher ability to reduce the dynamic interfacial tension compared to that of chitosan alone, indicating better emulsification properties. Moreover, the produced Pickering emulsions possessed excellent storage stability during the storage evaluation period of two months. These stable Pickering emulsions can be used in food and cosmetic applications.
- D. Tracking of the chitosan/collagen peptides nanoparticles by CLSM after the skin application of the Pickering emulsions confirmed the presence of the nanoparticles in deeper skin layers, and not just in the *stratum corneum*. It was found that the degree of skin penetration increased as the concentration of the nanoparticles stabilizing the Pickering emulsions increased. It also increased as the contact time of the emulsions with the skin increased. The findings obtained in this thesis reveal, for the first time, the effective deposition of the chitosan-based nanoparticles in skin layers through Pickering emulsions. The nanoparticles designed for topical applications have generally been reported to require dispersion in a semisolid vehicle (e.g., hydrogels) to guarantee proper adherence and improved availability of the nanoparticles when applied onto the skin. Therefore, the present study suggests that the produced Pickering emulsions could act as new effective platforms or dispersion vehicles for the nanoparticles. Additionally, these findings can open new dimensions for the utilization of the nanoparticles; as they can be tailored to impart skin healing and therapeutic effects, together with their primary function which is the stabilization of the emulsion.
- E. Pickering emulsions stabilized with chitosan/collagen peptides nanoparticles proved their effectiveness as a stable surfactant-free alternative for the topical delivery of CBD.

The skin deposition was improved by increasing the oil volume fraction in the formulations, but it was not influenced by the oil type. The results obtained in this thesis showed that CBD was deposited in high amounts in the skin in comparison with other CBD delivery vehicles that are reported in the literature, indicating that the encapsulation of CBD in Pickering emulsions has enhanced its topical delivery. It was also found that the encapsulation of CBD in Pickering emulsions provides superior protection for CBD after five months of storage, which is considered a remarkable finding since many of the commercially available CBD products have been reported to show a significant decrease in the amount of CBD after storage.

- F. Pickering emulsions stabilized with chitosan/gum Arabic complex particles have shown that they are suitable environmentally-friendly candidates for the topical delivery of CBD as well. Although the degree of the deacetylation of chitosan did not show a significant impact on the amount of CBD deposited in the skin, it was found that it highly influences not just the physicochemical properties of the chitosan-based Pickering emulsions, but also their stability. Hence, it can be concluded that the degree of deacetylation is a crucial parameter that should be taken into consideration when a long shelf-life is required for the product, which is an important objective for emulsion-based applications in the food and cosmetic industries.

8.2 Suggestions for future work

- A. It is suggested to use other Pickering emulsion preparation methods, such as ultrasonic emulsification and high-pressure homogenization, and compare them with the method used in this thesis (i.e., rotor-stator homogenization) in order to study the effect of different preparation methods on the stability and physicochemical properties of the developed chitosan-based Pickering emulsions.
- B. Further investigations are needed for the developed Pickering emulsions in areas related to skin applications, such as the anti-inflammatory and anti-oxidant efficacies of the emulsions loaded with the bioactive agents. It will be also extremely useful to compare the skin uptake of resveratrol and cannabidiol from the formulations developed in this

thesis with the skin uptake of resveratrol and cannabidiol from commercially available products.

- C. It is recommended to study the stability of the produced Pickering emulsion systems at different environmental conditions, such as different pH, temperature, and ionic strengths in order to extend the utilization of these systems to various applications.
- D. The developed Pickering emulsions could be utilized in synthesizing eco-friendly surfactant-free microcapsules by adding a natural hardening agent that anchors or cross-links the particles to form the microcapsules' shells.
- E. Another interesting area for future work is the use of the developed Pickering emulsions in active packaging which is finding increasing recognition in the food industry to prolong the shelf-life of packaged food products. It is worth mentioning that the Pickering emulsion system stabilized with chitosan/gum Arabic nanoparticles that was developed in this thesis has recently been utilized by researchers at the National Research Center in Cairo to produce novel active packaging films (Sultan et al., 2022). The films were produced by incorporating chitosan/gum Arabic Pickering emulsions containing coconut oil in gelatin films. The results have shown that the Pickering emulsions granted the films higher scavenging and antimicrobial properties compared to the control films.
- F. Despite the injectability of chitosan, and the possibility of incorporating an injectable oil phase such as squalene (Fox, 2009), chitosan-based Pickering emulsions have never been explored for their potential use as vaccine adjuvants. This topic was recently tackled by a study on Pickering emulsions stabilized with PLGA nanoparticles which showed promising results in modulating the immune response (Xia et al., 2018). In addition, using Pickering emulsions as vaccine adjuvants could possibly contribute to solving the problem of allergic reactions and anaphylaxis triggered by emulsifiers and stabilizers which are widely used in the production of vaccines (Stone et al., 2019). Perhaps the most recent example of these negative effects is the reported allergies towards the polyethylene glycol (PEG 2000) which is present in the covid-19 vaccines produced by Moderna and Pfizer (to stabilize the lipid nanoparticles), and polysorbate which is present in the covid-19 vaccine produced by Johnson & Johnson (Carpenter et

al., 2021). The current year certainly proved that the benefits of the vaccines outweighed their potential adverse reactions, yet current research remains directed towards improving the vaccine adjuvants to minimize or prevent the potential adverse effects. Chitosan-based Pickering emulsions could be promising adjuvants which makes them worth exploring. This calls for future collaborations with researchers in the immunology, medicine and pharmaceutical formulation engineering fields.

8.3 References

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