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Nanohydroxyapatite Pickering emulsions: from product development to functional ingredients in food applications

PhD Dissertation
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by

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To my Family.

"Education is the most powerful weapon which
you can use to change the world."

Nelson Mandela

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"Love is the strongest force the world possesses." *Mahatma Gandhi*

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ABSTRACT

Pickering emulsions, which are stabilised by solid particles, are known as highly stable products. They can act as carriers of active compounds, offering the possibility to generate greener alternative solutions. Thus, Pickering emulsions can be the basis of functional foods protecting active compounds over storage and ingestion conditions, contributing to improving bioaccessibility after digestion, complying with consumers' demand for safe and healthy products. In this context, the present work addresses the study of nanohydroxyapatite (n-HAp) particles as Pickering stabilisers focusing on stability, production, and application issues in order to give new insights into food applications. In particular, emulsions' stability aimed at developing vitamin E-loaded Pickering emulsions and fortified foods was a central point of the work.

Oil-in-water (O/W) Pickering emulsions were firstly produced in batch mode using a rotor-stator device, and the parameters influencing the stability of the emulsions were studied in terms of n-HAp concentration (0.5 to 15 wt%) and O/W ratio (10/90 to 60/40). Complementary, a model to predict emulsion type and stability (unstable, stable and *gel-like*) considering the nanohydroxyapatite (n-HAp) concentration and dimensions, O/W ratio and droplet diameter was developed.

Afterwards, the feasibility of applying the NETmix technology to produce, in continuous mode, Pickering emulsions was attempted. In this case, the effect of the Reynolds number (200 to 500) and the number of recirculation cycles (2 to 35) needed to achieve a specific emulsion droplet size was evaluated. The produced Pickering emulsions (batch and continuous) were fully characterised concerning droplet size and morphology. Stable Pickering emulsions were achieved using the rotor-stator and NETmix devices; however, NETmix enables easy control of Pickering emulsion droplet size. The results also confirmed the role of the n-HAp particles as effective Pickering stabilisers, giving rise to emulsions with oil core – n-HAp shell morphology.

The stability of Pickering emulsions with different droplet sizes, produced in batch (~18 μm) and continuous mode (~7, 11 and 18 μm), was studied against environmental stresses, such

as temperature (5-90 °C), pH (2-10) and ionic strength (0-500 mM). This study aimed to check the effect of the emulsion droplet size and the production method on stability conditions. Except for pH2 and high temperatures (70-90 °C), which led to emulsion instability phenomena, the n-HAp Pickering emulsions showed to be stable independently of the size and production method.

Following these achievements, vitamin E-loaded Pickering emulsions (1mg/mL, ~7, 11 and 18 μm) were produced and used to fortify gelatine and milk foods. Pickering emulsions were subjected to *in vitro* gastrointestinal digestion (mouth, gastric and intestinal stages), where a quite similar behaviour was observed, namely a complete disruption of n-HAp particles under gastric conditions and aggregates formation under intestinal conditions. This behaviour was found to impact vitamin E bioaccessibility, which was generally low. However, vitamin E bioaccessibility was improved after incorporating the Pickering emulsions in food matrices (gelatine and milk). This fact was mainly related to the macronutrients presence and the lower content of n-HAp particles in formulation.

Finally, the modification of the n-HAp surface using sodium oleate was tested, aiming at producing water-in-oil (W/O) stable Pickering emulsions, solutions less studied in literature. The n-HAp particles were modified by applying different sodium oleate layers (0.5, 1 and 2), and a shift in the hydrophilic/hydrophobic character depended on the used number of sodium oleate layers was observed. The modified particles with hydrophobic nature were successfully used to stabilise W/O emulsions (a 15-days period was tested). These emulsions can be the basis of hydrophilic compounds carriers.

KEYWORDS: Nanohydroxyapatite, Pickering emulsions, Stability, NETmix reactor, Vitamin E, Fortified foods

RESUMO

As emulsões Pickering são estabilizadas por partículas sólidas e conhecidas pela sua alta estabilidade. Estas emulsões podem ser usadas para o transporte de compostos ativos, oferecendo a possibilidade de criar soluções alternativas mais ecológicas. Desta forma, as emulsões Pickering podem integrar alimentos funcionais para proteger os compostos ativos das condições de armazenamento e de ingestão, contribuindo para melhorar a bioacessibilidade após a digestão. Podem ainda responder à alta procura dos consumidores por produtos seguros e saudáveis. Nesse contexto, o presente trabalho estuda partículas de nano-hidroxiapatite (n-HAp) como estabilizadores de emulsões com foco nas questões de estabilidade, produção e aplicação. A estabilidade das emulsões Pickering foi o ponto principal do trabalho para desenvolver emulsões enriquecidas com vitamina E e alimentos fortificados.

Emulsões Pickering do tipo óleo-em-água (O/A) foram inicialmente produzidas em *batch* utilizando um homogeneizador do tipo *rotor-stator* e os parâmetros que afetam a estabilidade estudados em termos de concentração de n-HAp (0.5 – 15 m%) e rácio O/A (10/90 – 60/40). Complementarmente, foi desenvolvido um modelo para prever o tipo e a estabilidade das emulsões Pickering tendo em conta fatores como concentração e dimensão das partículas n-HAp, rácio O/W e diâmetro de gotícula.

Posteriormente, procurou-se viabilizar a aplicação da tecnologia NETmix para produzir, em contínuo, as emulsões Pickering. Neste caso, foi avaliado o efeito do número de Reynolds (200 - 500) e do número de ciclos de recirculação (2 - 35) necessários para atingir um tamanho de gotícula específico. As emulsões produzidas (*batch* e contínuo) foram caracterizadas quanto ao tamanho e morfologia das gotículas. Foram obtidas emulsões Pickering estáveis utilizando os dois equipamentos de produção; no entanto, o NETmix permite maior facilidade no controlo do tamanho da emulsão. Os resultados também confirmaram a eficácia das partículas de n-HAp como estabilizadoras dando origem a emulsões com morfologia similar a uma cápsula, nomeadamente núcleo de óleo protegido por partículas de n-HAp.

A estabilidade de emulsões Pickering com diferentes tamanhos de gotícula produzidas em *batch* (~18 μm) e contínuo (~7, 11 e 18 μm) foi estudada em diferentes condições ambientais: temperatura (5-90 °C), pH (2 -10) e força iónica (0-500 mM). O principal objetivo foi verificar o efeito do tamanho das gotículas e o método de produção nas condições testadas. De modo geral, as emulsões Pickering estabilizadas por partículas de n-HAp mostraram-se estáveis independentemente do tamanho e método de produção, com exceção para pH2 e altas temperaturas (70-90 °C) que levou ao desenvolvimento de instabilidades.

De seguida, foram produzidas emulsões Pickering enriquecidas com vitamina E (1 mg/mL, ~7, 11 e 18 μm) e usadas para fortificar gelatina e leite. Estas emulsões foram submetidas à digestão gastrointestinal *in vitro* (oral, gástrica e intestinal), onde foi observado comportamento semelhante entre as emulsões de diferentes tamanhos, nomeadamente uma completa degradação das partículas de n-HAp em condições gástricas e formação de agregados em condições intestinais. Este último comportamento teve impacto na bioacessibilidade da vitamina E, que foi geralmente baixa. No entanto, a bioacessibilidade da vitamina E foi melhorada após a incorporação das emulsões Pickering nos alimentos (gelatina e leite). Esta melhoria foi relacionada, sobretudo, com a presença de macronutrientes e ao menor teor de partículas de n-HAp incorporada nos alimentos.

Por último, foi testada a modificação de superfície das partículas de n-HAp utilizando oleato de sódio, onde o principal objetivo era a produção de emulsões Pickering estáveis do tipo água-em-óleo (A/O). As partículas de n-HAp foram modificadas pela aplicação de diferentes camadas de oleato de sódio (0.5, 1 e 2) e dependendo do número de camadas usadas foi observada uma mudança no caráter hidrofílico / hidrofóbico. As partículas modificadas com natureza hidrofóbica foram usadas com sucesso para estabilizar emulsões A/O (durante 15 dias).

PALAVRAS-CHAVE: Nano-hidroxiapatite; Emulsões Pickering; Estabilidade; Reator NETmix; Vitamina E; Alimentos fortificados

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LIST OF SYMBOLS

Nomenclature

d_h	Channel hydraulic diameter
A/O	Água-em-óleo
G'	Storage modulus
G''	Loss modulus
O/A	Óleo-em-água
O/W	Oil-in-water
O/W/O	Oil-in-water-in-oil
Re	Reynolds number
v/v	Volume/volume
W/O	Water-in-oil
W/O/W	Water-in-oil-in-water
w/v	Weight/volume

Greek Letters

v	Average velocity
μ	Viscosity
ρ	Density

Indices

Au	Gold
C	Carbon
Ca	Calcium
Cl	Chloride
HCl	Hydrochloride acid
K	Potassium
KCl	Potassium chloride
KH_2PO_4	Monopotassium phosphate

$MgCl_2(H_2O)_6$	Magnesium chloride hexahydrate
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
$(NH_4)_2CO_3$	Ammonium carbonate
O	Oxygen
P	Phosphorous
Pd	Palladium
SiO_2	Silica

Acronyms and Abbreviations

2D	Two dimensional
3D	Three dimensional
CFD	Computational Fluid Dynamics
CIMO	Centro de Investigação de Montanha
CLSM	Confocal Laser Scattering Microscopy
Cryo-SEM	Cryo-Scanning Electron Microscopy
CTAB	Cetylmethylammonium bromide
DLS	Dynamic Light Scattering
DME	Direct Membrane Emulsification
EDS	Energy Disperse X-ray Spectroscopy
EFSA	European Food Safety Authority
FDA	Food and Drug Administration
FM	Fluorescence Microscopy
GI	Gastrointestinal
GIT	Gastrointestinal Tract
HAp	Hydroxyapatite
HIPEs	High Internal Phase Emulsions
HLB	Hydrophilic-Lipophilic Balance
LSRE-LCM	Laboratory of Separation and Reaction Engineering and Laboratory of Catalysis and Materials

n-HAp	Nanohydroxyapatite
OM	Optical Microscopy
OSA	Octenyl Succinic Anhydride
PCL	Poly-ε-caprolactone
PG	Propyl gallate
PGMA-PS	Poly(glycerol monomethacrylate)-polystyrene
PgPr	Polyglycerol Polyricinoleate
PLLA	Poly-L-lactic acid
PME	Premix Membrane Emulsification
PMF	Poly(melanine-formaldehyde)
PS	Polystyrene
RME	Rotational Membrane Emulsification
SCME	Stirred-Cell Membrane Emulsification
SD	Standard Deviation
SDS	Sodium-dodecylsulfate
SGF	Simulated Gastric Fluid
SIF	Simulated Intestinal Fluid
SO	Sodium Oleate
SSF	Simulated Salivary Fluid
STY	Space-Time Yield
UPLC	Ultra-Performance Liquid Chromatography
UVC	Ultraviolet C
XME	Cross-flow membrane emulsification

1 INTRODUCTION

Food is the primary source of energy for the human body. To preserve the attractive aspect and increase the shelf-life of food products, the food industry needs to use additives such as preservatives, flavours, colourants, and emulsifiers. According to Regulation (EC) no 1333/2008 of the European Parliament and Council of the European Union, a food additive can be defined as:

> "…any substance not normally consumed as a food in itself and not normally used as a characteristic ingredient of food, whether or not it has nutritive value, the intentional addition of which to food for a technological purpose in the manufacture, processing, preparation, treatment, packaging, transport or storage of such food results, or maybe reasonably expected to result, in it or its by-products becoming directly or indirectly a component of such foods."

The food additives are used to produce a desired effect on foodstuffs, including increasing shelf-life (Branen & Haggerty, 2002). They have been used since prehistoric times, but their application raised with the technological advances in food processing (Branen & Haggerty, 2002). Nowadays, more than 2500 synthetic food additives are used in the food industry (Branen & Haggerty, 2002). Furthermore, they are usually used for technical and/or commercial reasons, not adding nutritional value (Branen & Haggerty, 2002).

Many food products are emulsions, products that combine two immiscible liquids, oil and water. Emulsions are the basis of various daily consumed products, e.g., milk, ice cream, dressings, mayonnaise, margarine, soups, and beverages (Marefati, 2018). Emulsions are thermodynamically unstable systems undergoing phase separation (Marefati, 2018). Emulsifiers, which are food additives, improve stability by reducing the surface tension at the oil-water interface (Mahungu, 2002). Apart from stabilising emulsions, emulsifiers can assume other roles such as dough conditioner and fat crystallisation modifier (Mahungu, 2002). According to Regulation (EC) no 1333/2008 of the European Parliament and Council of the European Union, food emulsifiers can be defined as:

> "…substances which make possible to form or maintain a homogenous mixture of two or more immiscible phases such as oil and water in foodstuff."

The extensive use of emulsifiers in processed food leads to their increased intake. Although the emulsifiers are limited in content (≤ 2% by weight), several scientific studies have shown that the excessive use of food additives can be related to some harmful effects (Ceyhan *et al.*, 2013; Di Sotto *et al.*, 2014; Ngwabebhoh *et al.*, 2018). Moreover, the use of emulsifiers has been recently associated with the undesirable taste of some foodstuffs. In fact, Coughlan and Kellow (2015) reported that some additives have received the "generally recognised as safe (GRAS)" status improperly, and Chassaing *et al*. (2015) reported that relatively low concentrations (1% w/v) of two commonly used food emulsifiers, carboxymethylcellulose and polysorbate-80, induced low-grade inflammation and obesity/metabolic syndrome in wild-type hosts and promoted robust colitis in mice. Also, the intake of polysorbate-80 emulsifier may impact dietary factors effects on Crohn's disease pathogenesis (Roberts *et al.*, 2010).

Regarding the reported constraints associated with the use of traditional emulsifiers, namely the health problems deriving from their intake, it becomes important to develop healthier and safer alternative approaches for food applications. To highlight that these new approaches must guarantee the stability requirements of emulsion-based food products, and, preferably, be prepared using industrially transferable processes.

1.1 Motivation and Relevance

In the 21st century, with modern lifestyles, specific needs and especially eating habits are changing. Consumers search for a healthy and balanced diet to maximise physiological functions and ensure health and well-being (Roberfroid, 2002). The statement "Let food be thy medicine and medicine be thy food", espoused by Hippocrates nearly 2500 years ago, is receiving renewed attention and put into practice. In this regard, consumer requirements have changed considerably since they have realised that food contributes directly to their health (Caleja *et al.*, 2017). Food is not intended only to satisfy hunger and provide essential nutrients but also to reduce nutrition-related diseases and improve physical and mental well-being (Bigliardi & Galati, 2013). Thus, the major challenge of food science is to progress from improving life expectancy to enhance life quality/wellness.

Functional food is a recent, interesting, and stimulating concept. It emerged for the first time in the '80s from a study where the relationship between nutrition, sensory satisfaction, fortification and modulation of physiological systems was analysed (Bigliardi & Galati, 2013). Functional food is a wide concept that can be affected by local characteristics or cultural traditions, becoming hard to be defined (Roberfroid, 2002). Thus, given that functional food is a concept rather than a well-defined group of foods, one possible definition, and the most accepted, is the one proposed by Diplock *et al*. (1999):

> "A food can be regarded as functional if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to either improved stage of health and well-being and/or reduction of risk of disease. A functional food must remain food and it must demonstrate its effects in amounts that can normally be expected to be consumed in

> the diet: it is not a pill or a capsule, but part of the normal food pattern. A functional food can be a natural food, a food to which a component has been added, or a food from which a component has been removed by technological or biotechnological means. It can also be a food where the nature of one or more components has been modified, or a food in which the bioavailability of one or more components has been modified, or any combination of these possibilities."

Summarising, functional foods may be defined as food products enriched with different specific or multiple compounds (e.g., fatty acids, antioxidants, proteins, vitamins, minerals, among others), having beneficial physiological effects on the organism (Caleja *et al.*, 2017). It refers to foods containing ingredients that aid specific bodily functions and are nutritious (Caleja *et al.*, 2017). Dias *et al.* (2015) introduced functional foods as products at the frontier between nutrition and health.

Functional foods have received significant attention and are one the most challenging, and active research and innovative fields in the food industry sector (Annunziata & Vecchio, 2011). The functional food market is in continuous expansion, and its global revenue was estimated to be around 161 billion dollars in 2018 (Research, 2019). This demand leads to an increased incorporation of functional compounds such as ω-3 fatty acids, fibres, vitamins, minerals, and other active compounds to improve the nutritional content of foodstuffs (Caleja *et al.*, 2015; Ribeiro *et al.*, 2015; Stratulat *et al.*, 2015). Thus, it is expected that the worldwide functional food market will increase substantially, from 175 billion dollars in 2019 to over 276 billion dollars in 2025 (Shahbandeh, 2019b). In Europe, the functional foods sector generated a revenue of approximately 20 billion dollars in 2016, and is forecast to reach about 30 billion dollars in 2023 (Shahbandeh, 2019a). Since functional foods are a relevant topic, numerous studies were developed aiming at obtaining better and more effective products. In most cases, incorporating active compounds in food matrices is not enough, needing the development and application of stabilisation technologies, such as microencapsulation or emulsion-based technologies (Dias *et al.*, 2015).

In response to the high demand for new alternatives to substitute the conventional food additives, namely emulsifiers, sometimes reported as harmful to human health (Ceyhan *et al.*,

2013; Chassaing *et al.*, 2015; Di Sotto *et al.*, 2014; Ngwabebhoh *et al.*, 2018), the development of Pickering stabilisers and their emulsions can emerge as a solution. In this context, the development of new stabilisers for the food industry have been extensively promoted, with considerable efforts directed to the design and fabrication of nano- and micro-structured materials to be used as Pickering stabilisers (Zoldesi *et al.*, 2007). The development of edible Pickering emulsions based on food-grade particles is becoming a need and a challenge for the food industry, aiming at obtaining food emulsions with enhanced performance, namely stability (Chevalier & Bolzinger, 2013). These emulsions can be used as food products itself or incorporated in food matrices to confer added value and promote health and wellness (Skelhon *et al.*, 2012; Stratulat *et al.*, 2015). Pickering emulsions can substitute conventional emulsions (Berton-Carabin & Schroën, 2015; Dickinson, 2012), acting also as encapsulation systems due to the protective layer formed around the dispersed phase, which might include the active principle (Diarrassouba *et al.*, 2015; Frelichowska *et al.*, 2009a).

Face to this demand, it is also important to implement more efficient and reproducible technologies to produce Pickering emulsions. In this context, the adoption of processes enabling large volume scale, namely in continuous mode, becomes important to promote the industrialization of Pickering emulsions production.

1.1.1 PICKERING EMULSIONS

The first mention to Pickering emulsions dates from the 1900s by Ramsden and Pickering (Pickering, 1907; Ramsden, 1903). Since then, Pickering emulsions formulation has gained considerable interest from the scientific community. The term 'Pickering' means colloidal species that can act as mechanical barriers against emulsion instability. Pickering emulsions refer to emulsions that are stabilised by solid particles. These particles stabilise the multi-phase structure through their adsorption at the oil or water droplet's surface, forming a layer preventing coalescence and phase separation (Rousseau, 2013).

Figure 1.1 shows the increasing interest and relevance of Pickering emulsions over the last years (data collected from ISI Web of Knowledge in September 2020). Although Pickering emulsions are known for over a century, their interest has increased only in the past decade

due to the high physical stability of these emulsions, which potentiate several applications. Studies on food applications are still limited due to the relatively low number of food-grade particles, even a clear investment in this field is clearly perceived from recent literature analysis. However, development of food applications from Pickering emulsions is an important topic due to the high demand for *clean-label* products.

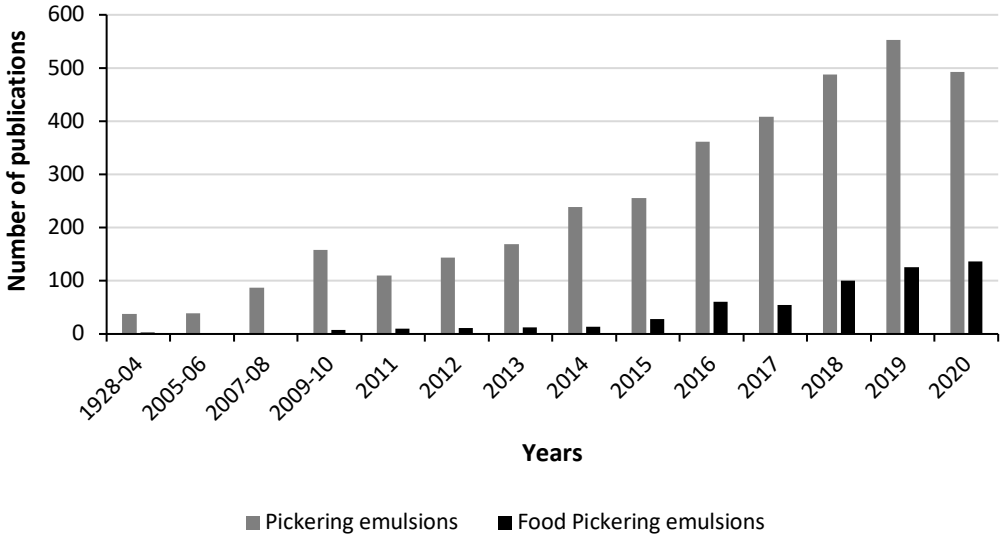

Figure 1.1: Evolution of Pickering emulsion interest analysed in terms of publications. Keywords: "Pickering emulsions" and "food Pickering emulsions". Data collected from ISI Web of Knowledge in September 2020.

Pickering emulsions emerged as an important research area in emulsion and colloidal science. This trend has aroused interest for food engineering, allowing a route for long-term stability and functional foods development. Emulsifier-free emulsions meet the demand for *clean-label* requirements, creating environmentally friendly solutions, a need for commercial purposes (Linke & Drusch, 2018). Furthermore, Pickering emulsions have been receiving attention due to their potential for drugs and bioactive compounds encapsulation, enabling protection and controlled release (Rousseau, 2013).

A main constraint related to Pickering emulsions is the emulsification process, which requires a substantial amount of energy. Namely, most of the reported works use emulsification processes applying mechanical energy via vigorous stirring using rotor-stator homogenisers or ultrasonic devices (Mahungu, 2002). These conventional devices need large amounts of energy and dissipate high heat amounts, causing emulsion over-heating (Albert *et al.*, 2019). Thus, a device capable of producing a Pickering emulsion efficiently with low energy

requirements and able to easily control temperature is important to achieve. Furthermore, a continuous process should bring industrial advantages over batch processes.

1.1.2 NETMIX TECHNOLOGY

NETmix is a new static mixer and a reactor developed and patented at the Laboratory of Separation and Reaction Engineering – Laboratory of Catalysis and Materials (LSRE-LCM) (Lopes *et al.*, 2005), enabling continuous mode operation.

NETmix consists of a network structure that combines two main elements, chambers and channels (Lopes *et al.*, 2005). The chambers are described as perfectly mixing zones, while the channels are parallel flow segregation zones (Laranjeira *et al.*, 2009). Networks are generated by the repetition of unit cells, where each unit cell comprises one chamber, and two inlet and two outlet half-channels (Laranjeira *et al.*, 2009). The mixing can only be achieved above a critical channel's Reynolds number. The flow inside the chambers evolves to a self-sustained oscillatory laminar flow regime inducing strong local dynamic mixing (Fonte *et al.*, 2013; Laranjeira *et al.*, 2009). This occurs due to the geometric characteristics of the NETmix network, and the local hydrodynamic instabilities induced by the immerging jets interactions at the chambers (Fonte *et al.*, 2013).

The NETmix network can be easily scaled up or numbered up depending on the production objectives. The controlled mixing inside the chambers helps to control the emulsion production and the droplet size distribution, as a result of the chaotic and oscillatory flow regime observed inside the chambers where mixture at micromixing scale occurs. At the same time, NETmix provides optimal heat transfer properties allowing the production of Pickering emulsions without temperature rising, as currently observed in traditional homogenisers such as rotor-stator devices, which is an advantage when using active compounds. In conclusion, NETmix reactor may enable the production of Pickering emulsions with more controllable production parameters, namely without temperature rising, lower energy consumption, high efficiency and reproducibility.

1.2 THESIS OBJECTIVES AND LAYOUT

Figure 1.2 presents, schematically, the main objectives of this work. One of the main goals is to develop Pickering emulsions from nanohydroxyapatite (n-HAp) particles. For this purpose, commercial n-HAp provided by a Portuguese company (Fluidinova) was used. These particles are produced with high quality and reproducibility using the NETmix technology. These n-HAp particles have as main advantages the biocompatibility and the absence of solvents, avoiding emulsion contamination issues. As a first approach, Pickering emulsions were developed in batch mode, followed by the implementation of their production in continuous mode using the NETmix technology. The produced Pickering emulsions were fully characterised aiming at achieving the best process parameters leading to stable products. The most promising Pickering emulsions formulations were then used as carriers for lipophilic active compounds, namely vitamin E. The main objective was to protect/stabilise this active compound, ensuring a better performance along storage and improve bioaccessibility upon ingestion. Finally, the development of fortified foods through the incorporation of the Pickering emulsions enriched with vitamin E is another objective.

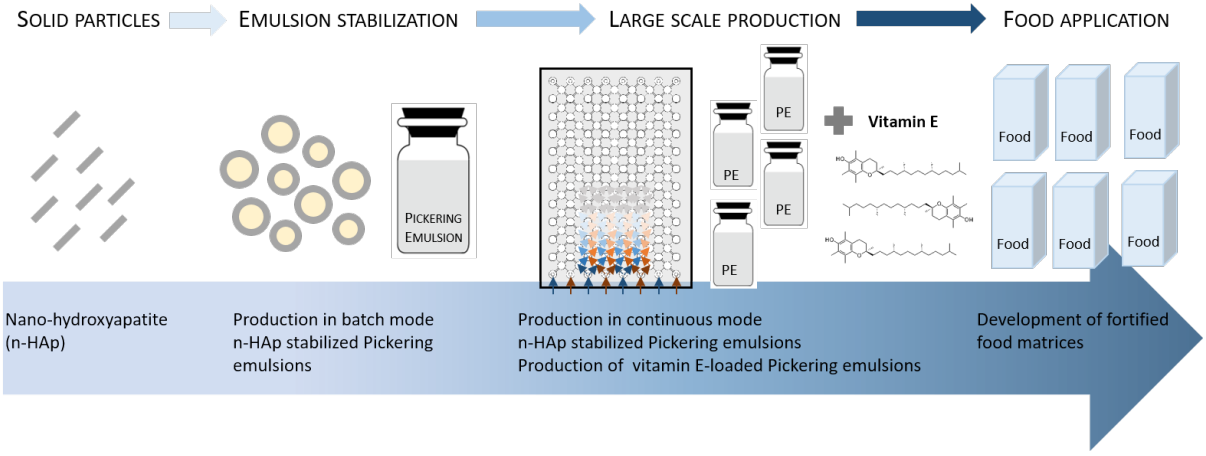

Figure 1.2: Schematic representation of the main goals of the present work and their time sequence.

To achieve the objectives abovementioned, the following tasks were carried out:

- Optimisation of Pickering emulsions batch production. For that, parameters such as oil-water ratio and n-HAp content were studied aiming at obtaining stable O/W Pickering emulsions formulations. The characterisation of the obtained emulsions considered size and morphology analysis evaluated using laser diffraction and

different microscopy techniques. A model to predict emulsion stability was developed and unstable, stable and *gel-like* regions identified in the ternary phase diagrams.

- Pickering emulsion NETmix production using a stable formulation achieved at batch mode. The effect of the Reynolds number and the number of recirculation cycles needed to achieve a certain emulsion droplet size was evaluated. The produced Pickering emulsions were characterised following the same methodologies used for batch mode. In the scope of this work, NETmix was tested as a continuous process alternative to the use of conventional high shear devices.
- Production in batch and continuous mode (using a rotor-stator homogeniser and NETmix, respectively) of Pickering emulsions of different droplet sizes. The objective was to study their stability against different environmental stresses (temperature, pH, and ionic strength), which might impact food manufacture and digestion conditions.
- Production in continuous mode of vitamin E-loaded Pickering emulsions and their use to functionalise food matrices. Both vitamin E-loaded Pickering emulsions and fortified food matrices were subjected to *in vitro* digestion (via gastrointestinal model that comprises the oral, gastric and intestinal phases) and vitamin E bioaccessibility evaluated after gastrointestinal tract.
- Modification of n-HAp surface using sodium oleate to achieve water-in-oil (W/O) stable Pickering emulsions. This work intended to extend the use of n-HAp particles to other fields of application. As such n-HAp particles tend to stabilise O/W emulsions.

Following this line of thought, the present thesis is structured in 8 chapters approaching the general and specific concepts, experimental results and discussion, and main conclusions. To highlight that the thesis is organised based on publications outputs (accepted or under submission), namely one review article and five experimental-based articles.

The 8 chapters are organized as follows:

- This chapter, **Chapter 1 (Introduction)**, is oriented to describe the main problematic of food additives, namely emulsifiers, and the main challenges to be overcome. The aim was to contextualise the main motivation leading to the definition of the main objectives of the present work and used strategies for their completion. Moreover,

some relevant concepts and definitions have been also introduced. Finally, the layout of the document is also presented.

- **Chapter 2** – This chapter entitled "**Inorganic solid particles as safe stabilisers for Pickering emulsions**", follows a review structure providing detailed information about Pickering emulsions, including stability and instability mechanisms and the parameters influencing them. The role of hydroxyapatite in Pickering emulsions stabilisation and the main methods used to produce Pickering emulsions are highlighted. The description and importance of static mixers, more specifically NETmix technology, is provided. Finally, the prospectus and potential of using Pickering emulsions in food applications, including for the delivery of active compounds or for the development of functional foods, is documented.
- **Chapter 3 (Nanohydroxyapatite as a Pickering stabiliser in oil-in-water emulsions)** focuses on batch production of n-HAp stabilised O/W Pickering emulsions and respective stability studies in terms of n-HAp concentration and O/W ratio. Furthermore, based on solid particles content, O/W ratio, droplet diameter and n-HAp dimensions, a model to predict the emulsion stability (unstable, stable, and *gel-like* regions) was developed and validated.
- **Chapter 4 (Continuous production of nanohydroxyapatite Pickering emulsions)** presents a new alternative method to produce O/W Pickering emulsions, namely NETmix technology. Different parameters were tested and their impact in the Pickering emulsion formation and stability evaluated. This methodology was applied to a previously validated batch formulation. Concluding, this chapter presents a new route to produce Pickering emulsions in continuous mode using low energy devices, becoming an important achievement in light of Pickering emulsion industrial production.
- **Chapter 5** entitled "**Effect of environmental stresses in nanohydroxyapatite Pickering emulsions**" presents an experimental study concerning the performance evaluation of the n-HAp Pickering emulsions at different environmental stresses (temperature, pH, and ionic strength). This study also addresses the effect of the Pickering emulsion droplet size and the effect of using different production methodologies (rotor-stator device *versus* NETmix technology) on the evaluated stability conditions.

- **Chapter 6 (Nanohydroxyapatite Pickering emulsions as vitamin E carriers for food applications)** reports the results achieved in the production of vitamin E-loaded Pickering emulsions and their incorporation in food matrices (gelatine and milk) to produce fortified foods. The vitamin E-loaded Pickering emulsions and fortified foods behaviour was tested using simulated digestion to check the impact of using this encapsulation strategy on vitamin E bioaccessibility.
- **Chapter 7 (Water-in-oil nanohydroxyapatite Pickering stabilisers through surface modification)** focuses the development of sodium oleate modified n-HAp particles to create stable W/O Pickering emulsions. The modified particles were characterised concerning wettability, being verified a shift on the hydrophilic/hydrophobic character dependent on the applied number of sodium oleate. To validate the successful modification of the particles, they were used to stabilise W/O emulsions.
- **Chapter 8 (Conclusions and Future work)** summarises the main conclusions of this work. Suggestions for future work are also provided in this chapter.

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2 INORGANIC SOLID PARTICLES AS SAFE STABILISERS FOR PICKERING EMULSIONS

This chapter is based on: Andreia Ribeiro, Yaidelin A. Manrique, Maria Filomena Barreiro, José Carlos B. Lopes, Madalena M. Dias. *Inorganic solid particles as safe stabilisers for Pickering emulsions.* Under submission.

Pickering emulsions, defined as emulsions stabilised by solid particles, have attracted attention in different fields such as the food, pharmaceutical and cosmetics industry, mainly due to good physical emulsion stabilisation properties. Additionally, Pickering emulsions can also be a promising strategy for developing functional interfaces, since the particles can play a role as active compound carriers and can also answer important sustainability and green chemistry issues arising as a new alternative to conventional emulsifier-based systems. This review is focused on the applicability of the safe-inorganic solid particles on emulsion stabilisation, discussing the main involved stabilisation mechanisms at the water-oil interfaces, and the main parameters that govern Pickering emulsions stability. Additionally, this work aims to focus on hydroxyapatite as Pickering stabilisers discussing its lasted advances as a Pickering stabiliser. The main technologies used to produce Pickering emulsions are also presented, and their advantages and disadvantages are discussed. From an industrialization perspective, an effort was made to research new productive technologies used at laboratorial scale but considering the feasibility for industrial implementation and energy requirements. Finally, the advantages and potential applications of Pickering emulsions in the food industry are also described. Overall, this review describes developments in the formulation, production and properties of food-grade Pickering emulsions based on safe inorganic solid particles.

2.1 INTRODUCTION: CONVENTIONAL EMULSIONS *VS* PICKERING EMULSIONS

Emulsions are considered one of the most important systems since they are widely used in many industries, including the food industry (McClements, 2016). Emulsions are defined as a mixture of two immiscible liquids, where typically one liquid is dispersed in droplets form in another liquid (McClements, 2016). The phase that forms droplets is called a dispersed phase, and the phase in which the droplets are dispersed is called a continuous phase (McClements, 2016; Niakousari *et al.*, 2018). Depending on the liquid that forms the dispersed and continuous phases, two different types of emulsions (Figure 2.1) can be obtained:

- Oil-in-water – O/W - oil droplets are dispersed in a continuous aqueous phase;
- Water-in-oil – W/O - water droplets are dispersed in a continuous oil phase.

More complex emulsions, so-called emulsions of emulsions or double-emulsions, can also be formed, such as: oil-in-water-in-oil (O/W/O) and water-in-oil-in-water (W/O/W) (Figure 2.1) (Koubaa *et al.*, 2018; McClements, 2016).

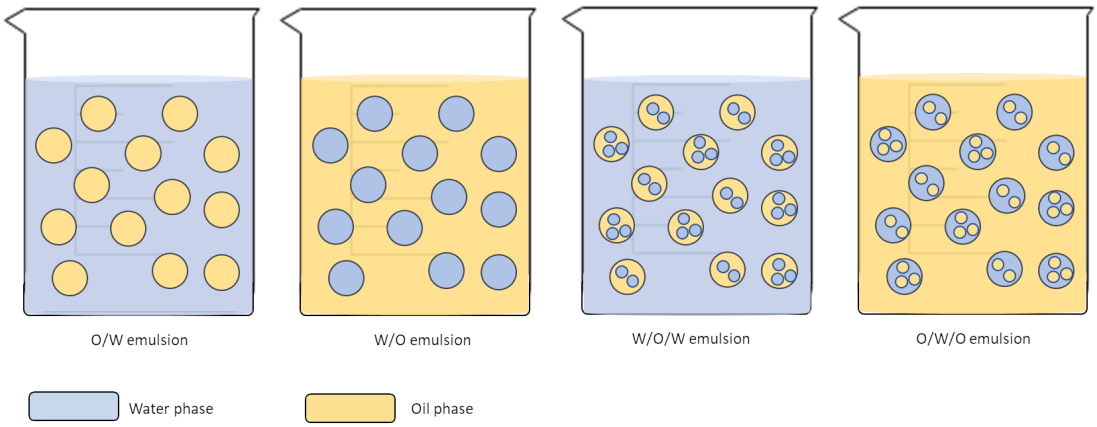

Figure 2.1: Schematic representation of different types of emulsions.

Emulsions are usually formed by intense mixture of the two phases, however, shortly after the mixing mechanism stops, a rapid phase separation occurs between the two immiscible phases, in order to minimize the contact area. Thus, emulsions are thermodynamically unstable systems (Wu & Ma, 2016), and surface-active agents, known as emulsifiers, are generally used to prevent the rapid phase separation and achieve the desired stability.

Emulsions are widely used in various fields of application, for example in cosmetics, for creams and lotions production (Faria-Silva *et al.*, 2020; Nastiti *et al.*, 2017) and in pharmaceutical products for pastes, transdermal patches and vaccines production (Albert *et al.*, 2019; Khurana *et al.*, 2013; Shah *et al.*, 2015). In the food industry emulsions are used for the production of dairy drinks, soups, mayonnaise, salad dressings, ice cream and chocolate as O/W emulsions; and in margarine, butter and spread as W/O emulsions (Berton-Carabin & Schroën, 2015). In the food sector, some of emulsions are final products in themselves such as mayonnaise; however, in most cases, emulsions are used as an ingredient for the development of more complex products such as yoghurts (Dickinson, 2010; Pichot *et al.*, 2010).

2.1.1 CONVENTIONAL EMULSIONS

Emulsions stabilised with emulsifiers are usually referred as conventional emulsions. An emulsifier is a general term that describes any molecule with an active surface, which can interact with oil and water phases preventing the emulsion destabilisation (Niakousari *et al.*, 2018). Both phases should adsorb the surface-active molecule, in order to decrease the interfacial tension created by the two immiscible liquids in an emulsion (Berton-Carabin & Schroën, 2015; Rayner, 2015). The capacity and the high speed that emulsifier has to adsorb the oil-water interface makes it ideal to stabilise the emulsion, preventing the rapid emulsion destabilisation through creation of a layer that eliminates the interfacial tension (Niakousari *et al.*, 2018). Various types of emulsifiers are used to kinetically stabilise the emulsion and ionic and non-ionic surfactants and amphiphilic biopolymers are the most used. The use of emulsifiers in the food industry is regulated by the Food and Drug Administration (FDA) in the United States (US) and by the European Food Safety Authority (EFSA) in European Union (EU) (Carocho *et al.*, 2014). However, in the last years, the use of emulsifiers has been the subject of concern, since some of these emulsifiers have been associated with harmful health effects such as irritations or inflammatory responses (Chassaing *et al.*, 2015; Di Sotto *et al.*, 2014).

Nevertheless, emulsifiers are essential and mandatory to increase the shelf-life of the products, such as foodstuffs, for market distribution (Pichot *et al.*, 2010; Timgren *et al.*, 2011).

In this sense, and in order to satisfy the concerns that consumers have shown regarding conventional emulsifiers, new alternatives have been researched by both academic institutions and industry.

Pickering emulsions, a particular type of these alternative emulsions, and the main focus of this review, are introduced next.

2.1.2 PICKERING EMULSIONS

Pickering emulsions are stabilised by solid particles instead of conventional emulsifiers (Berton-Carabin & Schroën, 2015; Dickinson, 2010). The ability of solid particles to physically stabilise emulsions was discovered by the pioneering work of Ramsden (Ramsden, 1903). A few years later, Pickering (Pickering, 1907) confirmed the previous work through experimental data originating an article that reported the formation of paraffin O/W emulsions. Even though Pickering did acknowledge Ramsden's work, the term "Pickering particle" is now applied for colloidal solid particles that can physically stabilise emulsions, that in turn are called Pickering emulsions (Schröder *et al.*, 2018b). And, although the Pickering emulsions concept emerged at the beginning of the twentieth century, substantial interest has been rising only in the last two decades.

Figure 2.2 shows a scheme of oil and water droplets stabilised by solid particles as well as an actual image of Pickering droplets.

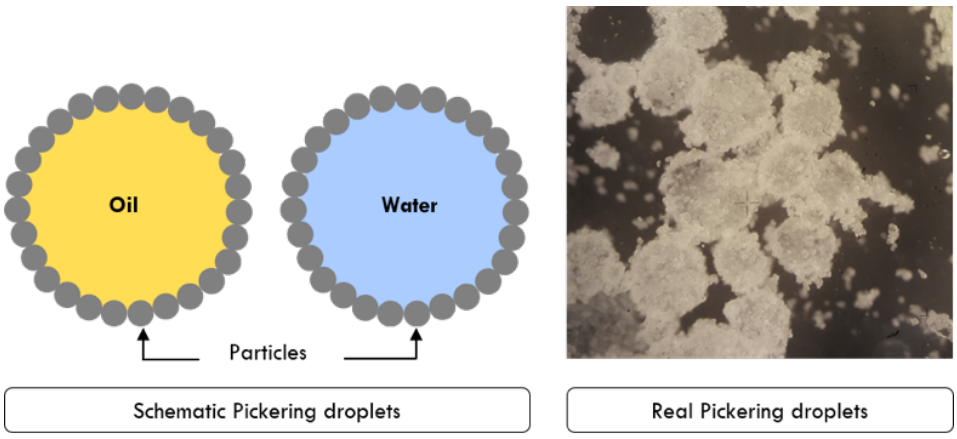

Figure 2.2: Schematic representation of oil or water Pickering droplets (left). Real optical microscope image of the Pickering emulsion stabilised with calcium carbonate (right).

The solid particles, which form a physical barrier, reduce the energy of the emulsion system through the reduction of contact between the immiscible liquids similar to the role of emulsifier (Schröder *et al.*, 2018b). Contrarily to the conventional emulsifier, the solid particles are generally not amphiphilic, and their absorption at the interface is not spontaneous (Reed, 2011). Solid particles should be insoluble in both disperse and continuous phases but need to present wettability for both. By fulfilling these prerequisites, the particles can be adsorbed at the oil-water interface and then a physical barrier is formed that prevents the contact between the droplets of the disperse phase. The role of each stabiliser, emulsifiers for conventional emulsions and particles for Pickering emulsions, are compared in Table 2.1.

Table 2.1: Comparison between emulsifiers and particles used to stabilise conventional and Pickering emulsions, respectively.

	EMULSIFIER	PARTICLES
Size	~0.4 to 1 nm	10 nm to 10 μm
Active surface	Yes	Not usually
Emulsion type (O/W or W/O)	Influenced by HLB	Influenced by contact angle
Adsorption kinetics	Fast	Slow
Desorption energy	Low	High
Stabilisation mechanism	Steric hindrance, electrostatic repulsion	Adsorption of the oil-water interface, mechanical barrier
Stability to coalescence	Low - high	High

Similar to conventional emulsions, Pickering emulsions may also be classified as O/W or W/O (Huang *et al.*, 2019) and double emulsions can also be obtained with solid particles (Marefati *et al.*, 2015). Pickering emulsions can also be prepared with traditional emulsification techniques such as high-speed, high-pressure and ultrasound homogenisation.

The main goal in the research of the Pickering emulsions is to improve the physical stability of emulsions, mainly against coalescence (Schröder *et al.*, 2018a). Moreover, the development of functional emulsions with biocompatible materials (Marefati *et al.*, 2017; Yuan *et al.*, 2017) and enhancement of chemical stability (Dokić *et al.*, 2012; Kargar *et al.*, 2012; Osborn & Akoh, 2004) are advantages that have been attributed to Pickering emulsions. Another positive

characteristic of Pickering emulsions is the possibility to create emulsions with an emulsifier-free label (Chevalier & Bolzinger, 2013). Pickering emulsions may be an attractive alternative for the food industry as well as other fields such as pharmaceutical and cosmetic products (Frelichowska *et al.*, 2009b), oil recovery (Son *et al.*, 2015) and catalysis (Hiebler *et al.*, 2018).

The following sections present an overview of Pickering Emulsions, addressing stability and degradation mechanisms, parameters that influence stability, types of solid particles used, methods for preparation and production, and finally the application of Pickering emulsions in the food industry.

2.2 Stability mechanisms of the Pickering emulsions

Since solid particles are known to be suitable for stabilising both O/W and W/O emulsions, various works have studied the mechanisms that govern the formation of Pickering emulsions and the respective stability (Albert *et al.*, 2019; Ortiz *et al.*, 2020; Tavernier *et al.*, 2016; Wu & Ma, 2016).

Such as referred above, emulsions are known as thermodynamically unstable systems, due to an imbalance of molecular forces at the interface, since the droplets of the dispersed phase tend to coalesce with their neighbours, eventually leading to total phase separation (McClements, 2016). The phase separation occurs to reduce the area in which interfacial tension between oil and water occurs. The excess free energy, ΔG, at the interfacial area between two immiscible liquids is given by (Schröder *et al.*, 2018b)

$$\Delta G = \gamma_{OW} \Delta A_{OW} \tag{2.1}$$

where γ_{OW} represents the interfacial tension and ΔA_{OW} the total interfacial area, between the two phases, oil and water.

However, it is possible to form kinetically stable emulsions during a reasonable period (days, weeks or years) introducing, in their formulation, stabilisers (McClements, 2016). For Pickering emulsions, where the solid particles guarantee the stabilisation, the stabilisation mechanism is based on the adsorption of particles at the oil-water interface, resulting in the reduction of ΔA_{OW}, and conferring distinctive physical and mechanical properties compared with

conventional emulsions (Albert *et al.*, 2019; Berton-Carabin & Schroën, 2015). The main advantage of Pickering emulsions is their high ability to resist coalescence phenomena.

The physical stability of the Pickering emulsions is conferred through different mechanisms, including:

- Interactions between solid particles and the dispersed and continuous phases that lead to high desorption energy;
- Attractive interactions between solid particles at the oil-water interface (resulting from capillary forces), inducing the formation of a strong interfacial shell;
- Attractive interactions between solid particles in the continuous phase, that induce the formation of a network.

The main mechanism by which solid particles stabilise Pickering emulsions is related to their interaction energy with the oil-water interface. Thus, particle desorption energy, also known as detachment energy, is a main factor that theoretically links how well particles, with different characteristics (discussed in section 2.2.1), stabilise emulsions (Hunter *et al.*, 2008; Schröder *et al.*, 2018b).

2.2.1 DESORPTION ENERGY

Desorption energy is related to the free energy involved in removing an adsorbed particle from an oil-water interface (Hunter *et al.*, 2008). The main characteristic of Pickering emulsions is the high energy required to remove the particles adsorbed at the interface. The amount of energy needed to remove a spherical particle, that is the desorption energy, can be expressed by (Berton-Carabin & Schroën, 2015)

$$\Delta G_d = \pi r^2 \gamma_{OW} (1 - |cos\theta|)^2 \quad (2.2)$$

where r is the radius of the solid particle and γ_{OW} is the interfacial tension between oil and water.

Figure 2.3 shows the dependency of the desorption energy on the contact angle, particle size, and interfacial tension. The desorption energy required to remove the spherical particle from the interface is highest for θ = 90 ° and for $\gamma_{OW} = 25\ \mathrm{mN/m}$ (interfacial tension between water and vegetable oil) varies by 4 orders of magnitude (Figure 2.3 A). Considering the case

for θ = 90 ° (similar wettability by both phases) the desorption energy is directly proportional to the particle radius and, less significantly, to the interfacial tension. Furthermore, the desorption energy is always higher than the thermal energy ($K_B T$, black dashed line), even for a particle of 1 nm, which is close to the typical dimensions of conventional emulsifiers (Albert *et al.*, 2019; Berton-Carabin & Schroën, 2015; Schröder *et al.*, 2018b).

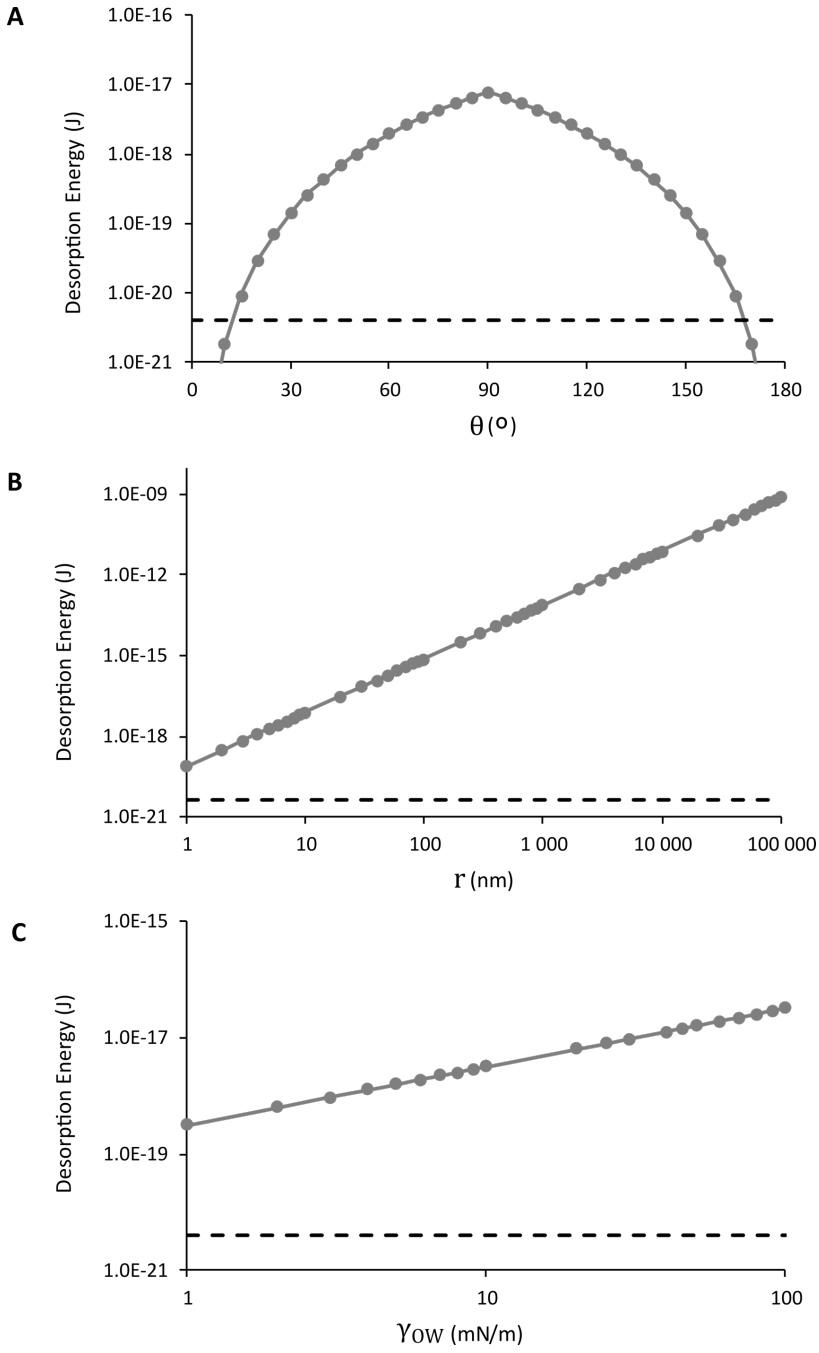

Figure 2.3: Desorption energy of the particle at oil-water interface as a function: A) contact angle vs θ (with γ_{OW} = 25 mN/m and r = 10 nm), B) particle radius vs r (with γ_{OW} = 25 mN/m and θ = 90 °), and C) interfacial tension at oil-water interface vs γ_{OW} (with r = 10 nm and θ = 90 °). In all graphs the dashed line represents thermal energy at 298 K ($k_B T$).

Therefore, the energy required for desorption of solid particles with appreciable wettability is higher than the thermal energy, and hence the particles are considered as irreversibly adsorbed at the interface. As a result, the particles have the ability to form a steric barrier that efficiently prevents droplet coalescence (Albert *et al.*, 2019; Schröder *et al.*, 2018b).

2.2.2 CAPILLARY FORCES

Apart to the desorption energy, the solid particles can interact with each other, developing different structures in Pickering emulsions that can contribute to physical stability over storage. These interactions can be localized at the interface or outside, i.e., in the continuous phase. Thus, particle-particle forces such as capillary forces are of great importance to overall emulsion stability (Hunter *et al.*, 2008).

Capillary forces, which are attractive, result from interactions between particles at the interface (Figure 2.4), and are present in any system where the solid particles cause local perturbations at the interface, and usually, the overlap of interfacial perturbations between two close particles causes an attraction (Hunter *et al.*, 2008; Schröder *et al.*, 2018b). The origin of interfacial perturbations is dependent on the solid particle size and wettability. In the first case, the capillary force increases with the increase of particle size. Particles larger than 5 μm can disturb the interface inducing capillary forces by their weight (buoyancy forces); however, for particles smaller than 5 μm gravity effects are negligible (Hunter *et al.*, 2008). In the second case, the interfacial perturbations are induced by particle confinement in the liquid film. This is related to particle wettability and affects, mainly, small particles, as long as they are larger than the solvent molecules (Schröder *et al.*, 2018b).

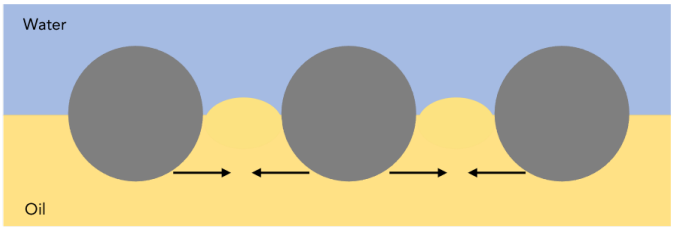

Figure 2.4: Representation of capillary attractive forces between solid particles at the oil-water interface of a Pickering emulsion. Adapted from (Schröder *et al.*, 2018b).

The use of shape-anisotropic particles is associated to non-uniform perturbation at the interface; it can increase capillary forces between particles and consequently the emulsion stability is better when anisotropic particles are used instead of spherical particles (Dugyala *et al.*, 2013; Lou *et al.*, 2016). Anisotropic particles deform the interface, inducing capillary attractions between them, and consequently, forcing particles to approach each other and pack more orderly (Lou *et al.*, 2016).

Capillary forces induce the formation of a cohesive interfacial network with higher surface shear and dilatational moduli than interface stabilised by emulsifiers. In this way, capillary forces contribute positively to stabilisation of Pickering emulsions (Schröder *et al.*, 2018b).

2.2.3 PARTICLE-PARTICLE NETWORKS

A typical representation of the Pickering droplets is a dense particle monolayer at the oil-water interface. However, due to the properties of the particles, particle-particle interactions can occur, forming unique and flocculated network structures (Hunter *et al.*, 2008). These network structures can be created due to attractive interactions between solid particles, and their arrangement on the Pickering emulsions can influence, positively, the emulsion physical stability (Schröder *et al.*, 2018b).

The network in Pickering emulsions increases mainly due to two factors:

- *Particle bridging* occurs when a particle has a contact angle significantly smaller than 90º, and each single particle adsorbed onto one droplet can interact with a neighbouring droplet interface, i.e., one particle can be adsorbed at two droplet interfaces simultaneously (Figure 2.5 A). It leads to the creation of bridges between the droplets causing stable droplet flocs (Schröder *et al.*, 2018b).
- *Particle aggregation state* occurs when the particles have a state of (weak) aggregation creating a disordered network between particles stabilising all particles simultaneously (Figure 2.5 B). This situation contributes to better emulsion stability against coalescence since it permits the formation of a viscoelastic network. However, such condition is only achieved when an excessive amount of the solid particles is available (Schröder *et al.*, 2018b).

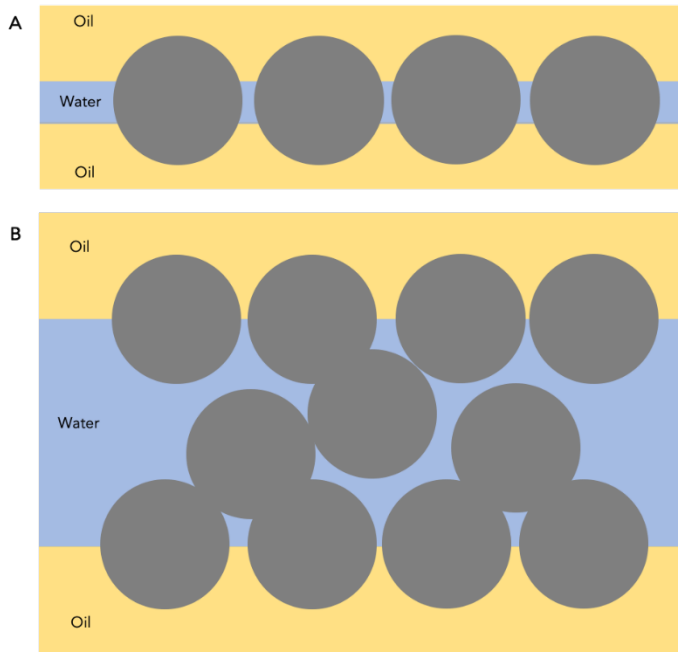

Figure 2.5: Representation of network structure developed between solid particles. Adapted from (Schröder *et al.*, 2018b).

According to Hunter *et al.*, 2008 the developed network structures can be categorized into four different levels of the particle aggregate state: (i) hexagonal close-packed monolayer; (ii) partially flocculated 'islands' networks; (iii) partially flocculated *meso*-chain networks; and (iv) high concentration multilayer flocculation.

Particle-particle interactions can form unique structural arrangements on Pickering emulsions leading better physical stability. However, it should be noted that these networks may set the whole emulsion structure and induce yield stress (Schröder *et al.*, 2018b).

Changes in environmental conditions (e.g., pH or electrolyte concentration) may affect the physicochemical characteristics of the particles as well as the electrostatic and steric interactions. This changes also affect the contact angle and hence the desorption energy. These parameters are presented and discussed in section 2.4.

2.3 Degradation mechanisms of the Pickering emulsions

Emulsions are considered stable as long as the number and the size distribution of the droplets remain constant over the storage time period (Reed, 2011). Thus, emulsion stability is defined as the capacity of an emulsion to resist to changes maintaining their initial

properties over time (Niakousari *et al.*, 2018). However, physical and chemical processes can lead to the formation of an unstable emulsion (McClements, 2016). Flocculation, creaming, sedimentation, coalescence and Ostwald ripening are the main physical instabilities, whereas oxidation and hydrolysis are the primary examples of chemical instabilities (McClements, 2016). The most common physical mechanisms that are responsible for the instability of emulsions (conventional or Pickering) are schematically shown in Figure 2.6. All destabilisation mechanisms contribute to the formation of unstable emulsions since they lead to the breakdown of the emulsion, and can occur individually or simultaneously (Niakousari *et al.*, 2018; Reed, 2011). Environmental conditions such as temperature, pH, ionic strengths, humidity and light exposure, mechanical agitation and storage time, as well as the composition and microstructure of each emulsion are the main factors that affect the rate of breakdown of the emulsion and the mechanism by which the instability occurs (McClements, 2016; Niakousari *et al.*, 2018).

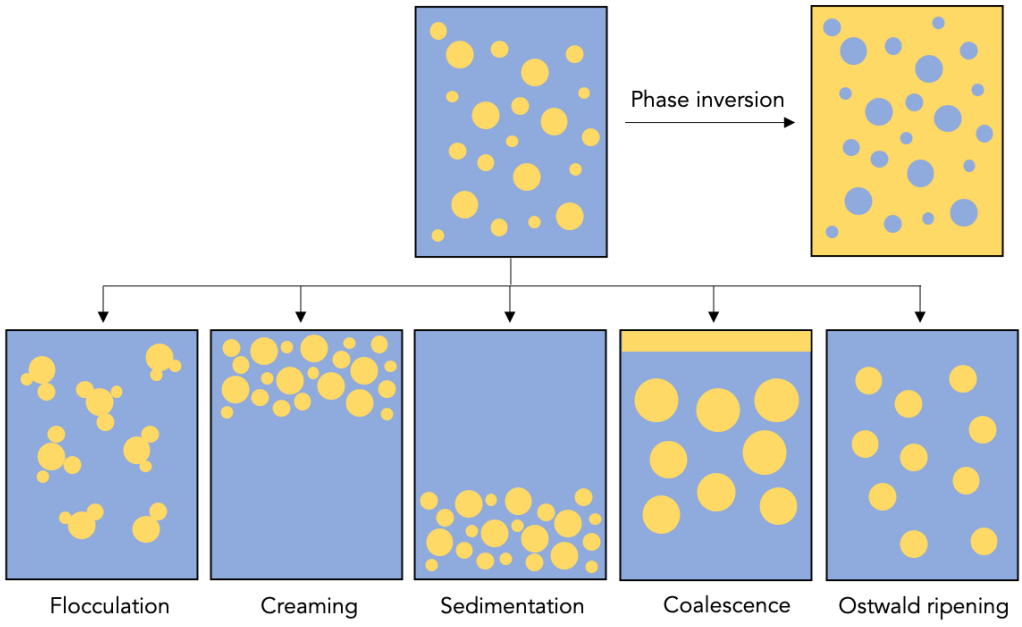

Figure 2.6: Schematic representation of the most common physical destabilisation mechanisms of the emulsions.

A brief description of each destabilisation mechanism responsible for changing the properties of the emulsion is given next:

- *Flocculation* – This process occurs when two or more droplets associate with each other, while maintaining their individual integrity. In its case, the number of droplets

remains constant. Flocculation process can accelerate the gravitational separation in diluted emulsions reducing the shelf-life of emulsions (McClements, 2016).

- *Creaming/sedimentation* – These processes occur mainly due to gravitational forces caused by differences in density between continuous and dispersed phases. Traditionally, creaming represents the migration of droplets to the upper surface in case of O/W emulsions, while, sedimentation represents the migration of the droplets to the bottom in case of the W/O emulsions (Reed, 2011). The density of most oils is lower than water, existing a tendency for the accumulation of oil at the top and water at the bottom of an emulsion (McClements, 2016). This mechanism can be prevented or delayed by increasing the viscosity of the continuous phase or reducing the density difference (Reed, 2011).
- *Coalescence* – This process occurs when two or more droplets merge in order to produce a single larger droplet (Reed, 2011). With the size increase, creaming or sedimentation process can occur more rapidly. On the other hand, creaming, sedimentation and flocculation mechanics, where the close packing of droplets is promoted, increase coalescence (Reed, 2011). In this situation, the thin film of the material that separates the liquids, is prone to rupture (McClements, 2016). In O/W and W/O emulsions, the destabilisation is associated with the formation of an oily layer on top (also often referred to as oil off) and water layer at the bottom, respectively (McClements, 2016). Coalescence promotes a decrease in the contact area between the immiscible liquids leading, in most cases, to total phase separation. When this occurs, the emulsion stays in its most thermodynamically stable state (McClements, 2016). For these reasons, the coalescence is considered an irreversible process.
- *Ostwald ripening* – This process is characterised by large droplets growing at the expense of smaller ones (McClements, 2016). The smaller oil droplets are more soluble in the continuous phase that larger oil droplets; this leads to the oil dissolving from the smallest droplets and diffusing through the continuous phase to then condense onto the larger droplets (McClements, 2016; Reed, 2011). Ostwald ripening causes an increase in droplet diameter and a decrease in the number of droplets (Reed, 2011). Ostwald ripening can be avoided through the use of additives or by using a disperse phase with a low solubility in the continuous phase (Reed, 2011).

- *Phase inversion* - This is the process by which an O/W emulsion is converted into an O/W emulsion type, or vice-versa (McClements, 2016). Usually, phase inversion is prompted by alteration in the emulsion composition or by environmental conditions, such as the disperse phase volume fraction, emulsifier type and concentration, solvents, temperature, and mechanical agitation (McClements, 2016).

Physical processes are the main and most common destabilisation mechanisms; nevertheless, there are other types of destabilisation such as chemical or biochemical reactions that can have adverse effects on the quality of emulsions (McClements, 2016). For example, emulsion properties can be modified by lipid oxidation, leading to the development of undesirable and potentially toxic reaction products. The emulsion stability can also be affected when proteins are cleaved by enzyme hydrolysis leading to their denaturation. Other mechanisms include flavour and colour degradation. On the other hand, and in the case of the O/W emulsion, Pickering emulsions can act positively in chemical protection due to the physical tightly packed particle barrier conferred by solid particles (Dokić *et al.*, 2012; Kargar *et al.*, 2012; Kargar *et al.*, 2011; Osborn & Akoh, 2004).

Furthermore, it is important to understand which factors influence the destabilisation of the emulsions, as well as, understanding the methods for controlling and monitoring these destabilisation mechanisms; this information is useful to formulate emulsions with enhanced shelf-life and has been the subject of intense study (McClements (2016)).

As previously referred, solid particles play an important role in emulsion stabilisation. However, the solid particles must include characteristics such as wettability, size, shape and surface (Pawlik & Norton, 2014; Schröder *et al.*, 2018b). Furthermore, other parameters can aid govern the stability of the Pickering emulsions, such as oil type and oil/aqueous phase ratio (Ortiz *et al.*, 2020). The characteristics of the solid particles and of both aqueous and oleic phases that determine Pickering emulsion stability and, in some cases, the emulsion formation, are discussed in the following section.

2.4 PARAMETERS THAT INFLUENCE THE PICKERING EMULSIONS STABILITY

The stabilisation of emulsions from solid particles is different than those stabilised by conventional emulsifiers. However, concepts and relationships developed for conventional emulsions can be used to predict the formation and stabilisation of the Pickering emulsions. For example, when particles are used to stabilise emulsions, wettability is equivalent to the hydrophilic-lipophilic balance (HLB), since they are related to the affinity of the particles or the emulsifier for oil and water (Grodzka & Pomianowski, 2006).

The effectiveness of solid particles as emulsions stabilisers depends mainly on their wettability; however, other factors such as particle size, particle shape and particle charge are also important and must be considered. Electrolyte concentration and pH of the continuous phase, oil type and volume fraction are examples of other critical parameters that influence the correct stabilisation of the oil-water interface (Pawlik & Norton, 2014). Figure 2.7 summarises the main parameters that influence the stability of Pickering emulsions, and are discussed in following sub-sections.

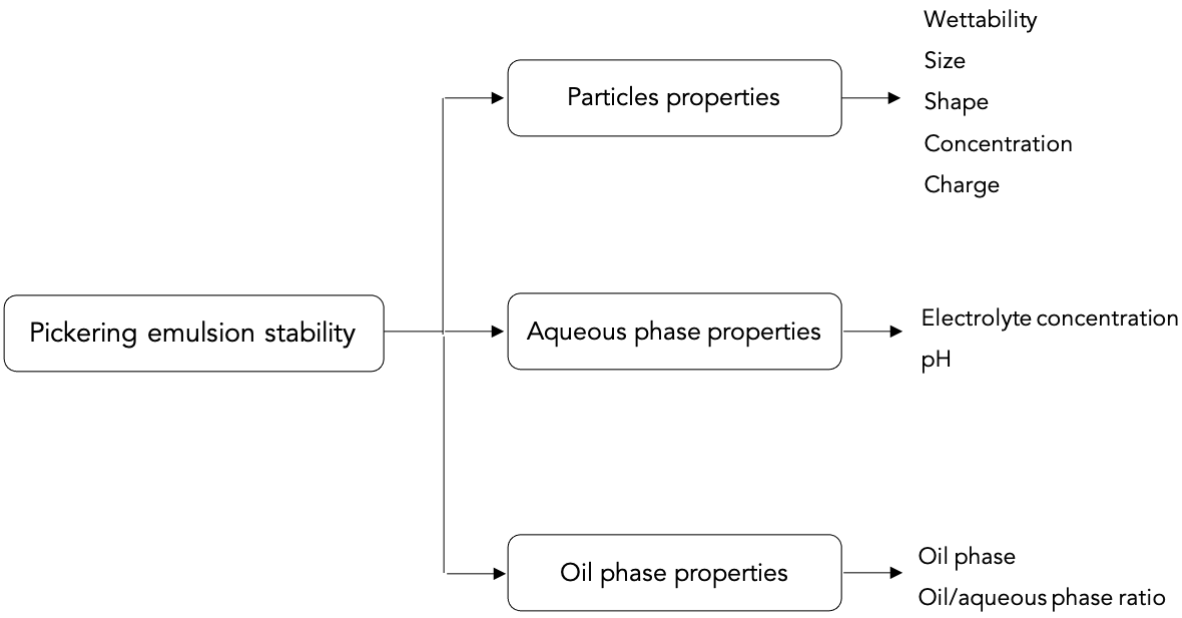

Figure 2.7: Parameters that influence the Pickering emulsion stability.

2.4.1 PARTICLES PROPERTIES

2.4.1.1 Solid particle wettability

The adsorption of the particles at the oil-water interface is strongly influenced by their hydrophobicity, which is in turn related to wettability, that is the tendency of a particle to adhere to either phase at the oil-water interface (Schröder *et al.*, 2018b). However, an important prerequisite is that the solid particles should be wetted by both oil and water phases (Berton-Carabin & Schroën, 2015).

The wettability of a solid particle is characterised by the three-phase contact angle (θ) (Aveyard *et al.*, 2003; Berton-Carabin & Schroën, 2015; Ortiz *et al.*, 2020). The contact angle is a result of the balance of the interfacial tensions at the water-oil, particle-water, and the particle-oil interfaces, as expressed by Young's equation

$$\cos(\theta) = \frac{(\gamma_{po} - \gamma_{pw})}{\gamma_{ow}} \quad (2.3)$$

where, γ_{po}, γ_{pw} and γ_{ow} are the interfacial tensions of particle-oil, particle-water and oil-water interfaces, respectively.

The emulsion type follows the empirical Finkle rule that admitted that θ is directly linked to the type of the stabilised emulsion, O/W or W/O (Figure 2.8) (Finkle *et al.*, 1923). When the contact angle is $\theta < 90°$ the particles are preferentially wetted by water (hydrophilic characteristics) and therefore suitable for forming O/W emulsions, whereas when $\theta > 90°$ the particles are preferentially wetted by oil (hydrophobic characteristics) and suitable for forming W/O emulsions. In some cases, the solid particles have identical affinity for both water and oil, in this case the contact angle of the particle is $\theta = 90°$, and theoretically these particles are able to stabilise O/W or W/O emulsions (Albert *et al.*, 2019; Berton-Carabin & Schroën, 2015; Pawlik & Norton, 2014; Xiao *et al.*, 2018).

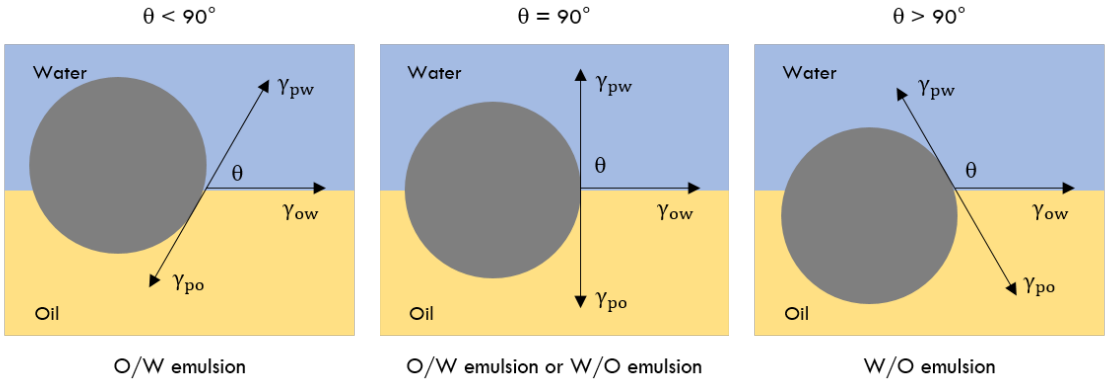

Figure 2.8: Influence of contact angle, θ, on the location of a solid particle at oil-water interface. Adapted from Schröder *et al.* (2018b).

Particle wettability is important to be considered since it determines the type of emulsion and ensures the appropriate anchoring of the solid particles at the interface as well as their efficiency on stabilisation (Albert *et al.*, 2019). However, the solid particles can be modified at the surface in order to change the contact angle, and subsequently improve the original wettability of the native particles. For example, for an O/W emulsion, enhancement of the particle/oil affinity, decreases the initial repulsion force due to the decrease in the repulsive hydration force; additionally, increasing particle/oil affinity also promotes the attachment through the capillary force, which increases with the increasing hydrophobicity of the particles (Tsabet & Fradette, 2015).

Thus, particles modifications are performed to enable better anchoring of the particles at the interface, to control emulsion type as well as to control the emulsion stability (Monteillet *et al.*, 2014; Wang & Wang, 2016; Xiao *et al.*, 2018). Various authors have been worked on the development of new particles through surface modifications from native particles. These studies contemplate both inorganic and organic particles through physical or chemical methods using small molecules or polymers (Xiao *et al.*, 2016; Xiao *et al.*, 2018). Palmitic and oleic acids were added at aqueous dispersions to modify silica particles, improving the adhesion of the particles at the droplet surface, and increasing the stability time (Sadeghpour *et al.*, 2013; Santini *et al.*, 2014). In the work developed by Björkegren *et al.* (2017), the surface of the silica particles was modified with hydrophilic (methyl poly(ethylene glycol) silane) or hydrophobic (organosilanes) compounds. The type and stability of the emulsions were strongly dependent on the amount of the hydrophilic and hydrophobic compounds. $CaCO_3$

particles were modified by fatty acids (sodium carboxylates) resulting in the formation of O/W or W/O emulsions (Cui *et al.*, 2012).

Feng and Lee (2016) modified the surface of zein (a protein extracted from maize) particles, which have low wettability, to improve the stability of the Pickering emulsions. The surface modification was carried out with sodium caseinate, and the subsequent emulsions exhibited greater centrifugal stability comparatively to plain zein particles. Another material that is commonly modified is starch because the native granule is not suitable for creating stable Pickering emulsions (Zhu, 2019). In this case, the most common practice is increasing the hydrophobicity through esterification with octenyl succinic anhydride (OSA), acetic anhydride or phthalic anhydride or by heat treatments (Xiao *et al.*, 2016; Zhu, 2019).

Li and Zhu (2018) modified micro-sized quinoa starch granules with OSA treatments for Pickering emulsion production. Marefati *et al.* (2015) modified quinoa starch particles also via OSA treatment to stabilise W/O/W Pickering emulsions. Recently, (Marefati *et al.*, 2017) stabilised Pickering emulsions with quinoa starch particles after heat treatments.

Although surface modification offers ample opportunities for industry-related applications, in some cases, their application should be cautious mainly if the final product is for the food industry. For example, although starch is a natural material with GRAS recognition, the degree of substitution by OSA cannot exceed 3% (maximum amount recommended by the FDA) to be used in food-related applications (Xiao *et al.*, 2016).

2.4.1.2 Solid particle concentration

A direct relationship between particle concentration and emulsion stability has been reported (Frelichowska *et al.*, 2010; Kargar *et al.*, 2012), where the increase of solid particle concentration available results in more stable Pickering emulsions over time. More particles available have the capacity to form a tighter packed layer around the emulsion droplets, and hence, coalescence (main destabilisation mechanism) can be prevented for a longer time (Aveyard *et al.*, 2003). Furthermore, the increase of solid particle concentration can aid in droplet size decreasing.

Frelichowska *et al*. (2010) observed the passage of an unstable to a stable emulsion with increased concentration of silica particles. Furthermore, the authors claim control of emulsion droplet size with increasing of silica content. Kargar *et al*. (2012) found that when microcrystalline cellulose or modified starch concentration increase from 0.1% to 2.5% the size of the droplets decreases, and the physical stability of a sunflower oil/water emulsion was enhanced. In general, both authors report that the increase of solid particle concentration was associated with the improvement of Pickering emulsion stability, mainly preventing coalescence.

2.4.1.3 Solid particle size

Solid particle size also affects the emulsion formation and stability (Schröder *et al.*, 2018b). A relationship between particle and droplet size has been developed and currently, it is considered that particles should be substantially smaller than the emulsion droplet size (Albert *et al.*, 2019; Dickinson, 2012; Pawlik & Norton, 2014). Gould *et al*. (2013) found that solid particles should be, at least, one order of magnitude smaller than the emulsion droplet. This difference is recommended for enabling the formation of a structured interface layer around the droplets, and for improving the adsorption energy which is proportional to the contact area (Gould *et al.*, 2013; Schröder *et al.*, 2018b).

Lou *et al*. (2016) reported that hexane/water Pickering emulsions stability was strongly dependent on the aspect ratio of the silica rods. For higher aspect ratios, the emulsion stabilisation was improved, which was attributed to higher steric hindrance, interface adsorption energy and capillary forces. Similar results were also obtained by Madivala *et al*. (2009) with ellipsoidal hematite particles.

2.4.1.4 Solid particle shape

The shape of the solid particles used to stabilise Pickering emulsions can influence the emulsion stability. Usually, stabilisation performance is studied for spherical particles (Figure 2.9 A) assuming that it is the ideal shape, and for which models have been developed (e.g., desorption energy – section 2.2.1) (Schröder *et al.*, 2018b). However, most solid particles do not have a spherical shape. Figure 2.9 shows examples of non-spherical shape particles used

to stabilise O/W or W/O Pickering emulsions: rods (Lou *et al.*, 2016), ellipsoidal (Madivala *et al.*, 2009), fibres (Sanchez-Salvador *et al.*, 2019), cubes (Folter *et al.*, 2014), peanut-shaped (Folter *et al.*, 2014), microbowl-like (Nonomura *et al.*, 2011), discs (Bippus *et al.*, 2009) and deformable-gels (Destribats *et al.*, 2011)). In the food industry, particle shape is an important factor since food-grade or food compatible particles usually have irregular or anisotropic shape; their impact on Pickering emulsion stabilisation should be assessed.

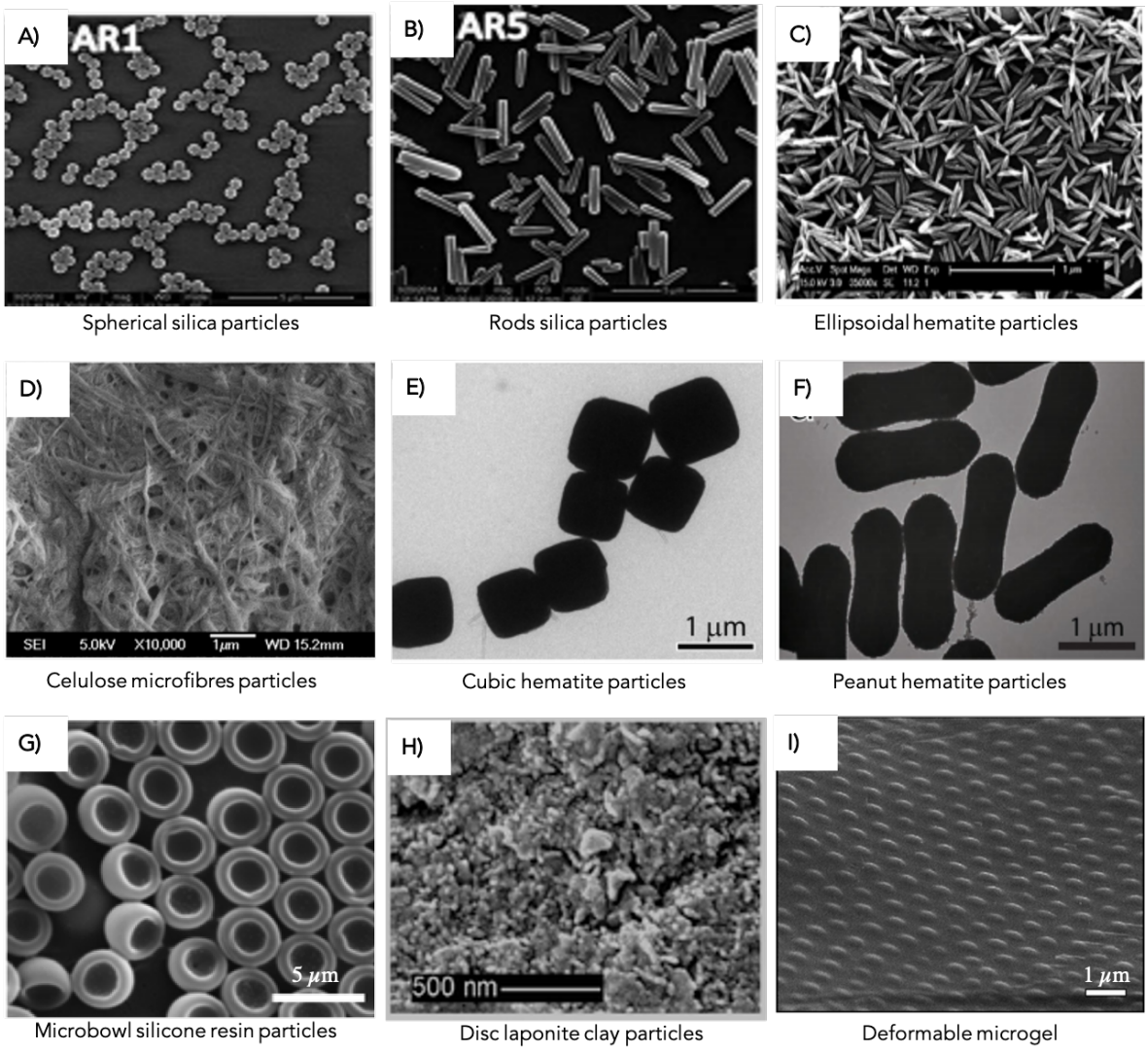

Figure 2.9: Different solid particle shape. A) spherical (Lou *et al.*, 2016), B) rods (Lou *et al.*, 2016), C) ellipsoidal (Madivala *et al.*, 2009), D) fibres (Sanchez-Salvador *et al.*, 2019), E) cubic (Folter *et al.*, 2014), F) peanut *(Folter et al.*, 2014), G) microbowl (Nonomura *et al.*, 2011) and H) disc (Bippus *et al.*, 2009) I) deformable-gels (Destribats *et al.*, 2011).

Although the stabilisation mechanisms of non-spherical solid particles are not yet fully elucidated, it is recognized that such particles can improve emulsion stability since it allows better interfacial coverage as well as unique properties in the interfacial network (Albert *et al.*,

2019). When anisotropic particles, such as rods and ellipsoids, are used there is an enhanced network between the particles around emulsion droplets surface, resulting in stable Pickering emulsions over time (Dugyala *et al.*, 2013). Thus, it is important to chart shape anisotropy effects on interfacial packing and orientation, as well as on capillary interactions (Schröder *et al.*, 2018b). Creighton *et al.* (2014) stabilised Pickering emulsions using ultrathin plate (similar to a disc shape), graphene oxide particles. These authors developed a thermodynamic model to find the most suitable material surface chemistry and geometry as well as to explain the Pickering emulsion stabilisation behaviour. Figure 2.10 shows a schematic representation of plate particles (represented as two-dimensional materials) on an oil droplet surface, comparing the effectiveness of stabilisation between 2D materials and spherical particles.

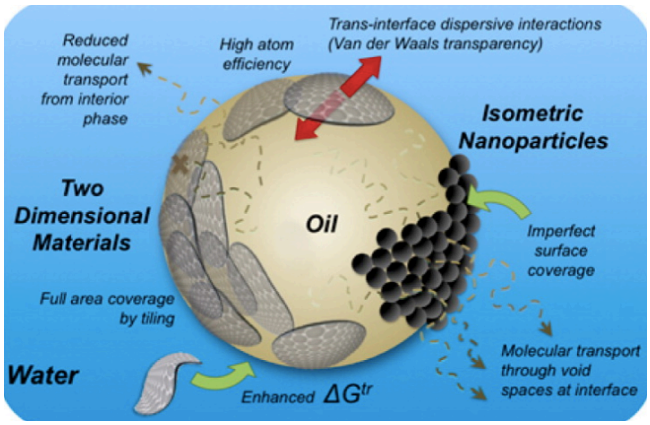

Figure 2.10: Schematic representation of plate particles on oil surface and their advantages in comparison to spherical particles (Creighton *et al.*, 2014).

Folter *et al.* (2014) reported good emulsions stability against coalescence, up to a one year period, using hematite cubic- or peanut-shaped (Figure 2.9 E and F, respectively) particles since its revealed unique interfacial packings and orientations that improved the irreversible attachment of the particles on the droplet surface. Lou *et al.* (2016) reported in their work that the Pickering emulsion stability is improved when silica rod-like particles are used instead of spherical particles (Figure 2.9 B and A, respectively), achieving longer (various months) stabilisation time. The utilization of the disc particles (Figure 2.9 H) allows the formation of stable Pickering emulsion for a long storage time, preventing creaming and coalescence (Ashby & Binks, 2000; Dinkgreve *et al.*, 2016). Furthermore, Pickering emulsion stability can also be enhanced using deformable materials (Destribats *et al.*, 2011); these materials can adapt to the surface of the droplets inducing efficient coverage.

2.4.1.5 Solid particle charge

Most solid particles in the aqueous phase are charged and thus particle surface charge is another factor that can influence Pickering emulsion stability (Aveyard *et al.*, 2003). When the surface of the particles is charged there are electrostatic repulsions between particles and droplets by which it is induced slow adsorption of the particles at the oil-water interface; consequently, the stability of the Pickering emulsion is lower (Schröder *et al.*, 2018b).

2.4.2 AQUEOUS PHASE PROPERTIES

2.4.2.1 Electrolyte concentration and pH

Due to the solid particle surface change, electrolyte concentration and pH of the continuous phase is often required to overcome the repulsions between particles and droplets. Addition of electrolytes and pH changes to the continuous phase leads to a decrease, or even suppression, of the electrostatic repulsion between the solid particles and the oil-water interface. Moreover, these modifications alter particle-particle interactions that can lead to the passage of repulsive to attractive forces between them. This can induce the aggregation of the particles into flocs, which in turn influences emulsions properties and stability, as long as there is weak flocculation between the particles (Albert *et al.*, 2019; Aveyard *et al.*, 2003).

Various authors have reported that the presence of electrolytes can be a factor in achieving stable Pickering emulsions (Ashby & Binks, 2000; Xiao *et al.*, 2016a; Yang *et al.*, 2006). Partial flocculation, i.e., moderately prevalent attraction between the particles, seems to provide better adsorption of the particles at the interface, improving the stability of the Pickering emulsion (Aveyard *et al.*, 2003; Rayner, 2015).

2.4.3 OIL PHASE PROPERTIES

2.4.3.1 Oil type and volume fraction

The oil type, as well as the oil/aqueous phase ratio, have some influence on emulsion stability, and sometimes also in the type of formed emulsion (Ortiz *et al.*, 2020).

The oil type, which can range from non-polar hydrocarbons, with relatively high γ_{OW}, to polar alcohols and esters with relatively low γ_{OW}, has an important role because it determines the interfacial tension at the oil-water interface, and it influences the interactions with solid particles (three-phase contact angle – see eq.1). Oil properties, such as polarity and viscosity, have substantial importance since they directly affect the θ value (Albert *et al.*, 2019; Aveyard *et al.*, 2003; Ortiz *et al.*, 2020). Binks and Lumsdon (2000b) studied the effect of different oils containing silica particles with intermediate hydrophobicity. They found that the emulsion type is O/W for non-polar oils and W/O for polar oils. Bai *et al.* (2019) studied the influence of oil type on stabilisation of Pickering emulsions with cellulose nanocrystals, choosing different oil types such as corn, fish, sunflower, medium-chain triglycerides, flaxseed and orange oils. They reported that stable Pickering emulsions were produced using either oil type at 0.75 wt.% of cellulose nanocrystals. After 14 days of storage, all emulsions appeared to have good stability to droplet coalescence (without droplet size modifications) with the exception of the orange oil emulsion; in this formulation, an increase of droplet size was observed due to Ostwald ripening destabilisations. This situation was associated with the high polarity of orange oil.

The oil viscosity influences the efficiency of breakage droplets during the emulsification process. Tsabet and Fradette (2015) noticed an increase of droplet size when silicone oil viscosity increases above the 486 mPa·s. For higher oil viscosities, the authors reported that the emulsification process and the adsorption ability of the particles (glass beads) at the interface were affected. In terms of stabilisation, the oil viscosity retards the particles adsorption at the oil-water interface that allows the coalescence between oil droplets.

Emulsion type is greatly influenced by the volume of the dispersed phase (Ortiz *et al.*, 2020). When the oil phase is increased up to a limit, it can improve the emulsion stability since the phase separation can be retarded due to the high viscosity of all system (McClements, 2016). However, when the oil volume fraction exceeds this limit, the phase inversion of the emulsion is favoured. Binks and Lumsdon (2000a) report results for water-in-toluene Pickering emulsions stabilised with either hydrophobic or hydrophilic silica particles. The authors found that Pickering emulsions stabilised with hydrophobic silica can be inverted from W/O to O/W upon increasing the water volume fraction; inversely, Pickering emulsions stabilised with

hydrophilic silica can be inverted from O/W to W/O upon increasing the oil volume fraction. The point where the catastrophic inversion occurs, was similar for both systems, around 0.7 (volume fraction of the dispersed phase). Additionally, Binks and Lumsdon (2000b) studied the water volume fraction needed for phase inversion to occur for different oil types.

2.5 SOLID PARTICLES AS PICKERING STABILISERS

Solid particles must present a set of characteristics in order to govern or control Pickering emulsions stabilisation. Wettability, size, concentration, among others, are some examples and have been discussed above.

This section describes the main solid particles used as Pickering stabilisers for emulsions. The principal focus will on food-grade particles, and a special attention is given to hydroxyapatite particles.

2.5.1 TYPES OF SOLID PARTICLES

The available types of solid particles that can be used to adsorb liquid interfaces and stabilise emulsions have a tendency to grow. The solid particles commonly used to stabilise Pickering emulsions are commonly divided into safe inorganic particles and food-grade organic particles as listed in Table 2.2.

Table 2.2: Examples of safe inorganic and organic solid particles used as food-grade Pickering stabilisers (Chevalier & Bolzinger, 2013; Schröder *et al.*, 2018b).

PARTICLES	GROUP	EXAMPLES
Organic	Polysaccharides	Modified starch; Microcrystalline cellulose; Chitin nanocrystals; Chitosan particles; Fibres
	Proteins	Zein; Soy protein; Lactoferrin; Whey protein; Caseins
	Lipids	Fats; Waxes; Sterols; Phospholipids; Glycerides; Glycolipids
Inorganic		Silica (E551-559); Calcium carbonate (E170); Hydroxyapatite;

Table 2.2 summarises organic particles used as Pickering stabilisers. Polysaccharides are natural polymeric carbohydrates that can be used as Pickering stabilisers, with starch being the most used. Proteins are natural amphiphilic polymers, which can be obtained from plants and animal sources (Berton-Carabin & Schroën, 2015). Although proteins have successfully been used for Pickering stabilisation, these have been generally not considered solid particles as they unfold, disassociate, or rearrange upon adsorption at oil-water interface (Schröder *et al.*, 2018b). Lipids are a large and diverse group of naturally occurring organic compounds and waxes and fat crystals have been used as Pickering stabilisers (Rousseau, 2013; Rousseau & Hodge, 2005; Yusoff & Murray, 2011). To achieve a desired Pickering stabilisation, these organic particles are subjected to different treatments. For example, native starch particles do not stabilise Pickering emulsions without chemical treatment to increase their hydrophobicity; octenyl succinic anhydride (OSA) (Marefati *et al.*, 2015; Yusoff & Murray, 2011) sulfuric acid hydrolysis (Azfaralariff *et al.*, 2020; Li *et al.*, 2012) and acetic anhydride and phthalic anhydride esterification (Tan *et al.*, 2012; Tan *et al.*, 2014) are some examples that have been applied to modify starch and make it more hydrophobic. Proteins are usually treated by anti-solvent methods (Dai *et al.*, 2018; Folter *et al.*, 2014; Liu & Tang, 2014; Xiao *et al.*, 2016a). These types of treatments are mostly applied to achieve the desired particle wettability; however, problems may arise, since non-food grade solvents are used, implying possible particle contamination. More information on Pickering emulsion using organic particles can be found in (Albert *et al.*, 2019; Sharkawy *et al.*, 2020). The focus of this review is on inorganic particles.

Within the safe inorganic particles group (Table 2.2), silica (SiO_2) particles are the most studied since their surface can be modified (through chemical or thermal treatments) in order to obtain particles with different/desired characteristics, mainly wettability (Destribats *et al.*, 2014). For example, White *et al.* (2011) found that the elimination of water from the surface of silica particles or condensation in siloxane bridges increased the hydrophobicity of these particles. Silica has been previously reported as food-grade particles (Dickinson, 2012; Pichot *et al.*, 2010; Skelhon *et al.*, 2012). EFSA - European Parliament and Council Directive No 95/2/EC approved the use of the silica and silicates (E551-E559) as food additives; however, in some foods, the utilization is limited up to a maximum content (10 g/kg for dried powdered

foodstuffs and 30 g/kg for greasing forms products) (European_Parliament & Council_Directive, 1995). In contrast, in dietary food supplements and foodstuffs in the form of tablets with or without coating silicates can be used in "*quantum satis*" (European_Parliament & Council_Directive, 1995), this term is applied by EFSA – Regulation 1333/2008 and is defined as (Regulation, 2008):

> "No maximum numerical level specified and substances shall be used in accordance with good manufacturing practice, at a level not higher than necessary to achieve the intended purpose and provided the consumer is not misled."

Calcium carbonate (E170) is also a safe inorganic particle, and has been used as a Pickering stabiliser (Binks *et al.*, 2017), authorized as a food additive by EFSA – Commission Regulation 1129/2011 (Commission_Regulation, 2011).

The pursuit of solid particles with an ideal character to be applied in food has been gaining increased interest, which is evidenced by the large number of the scientific publications that appeared in the last years (Figure 2.11).

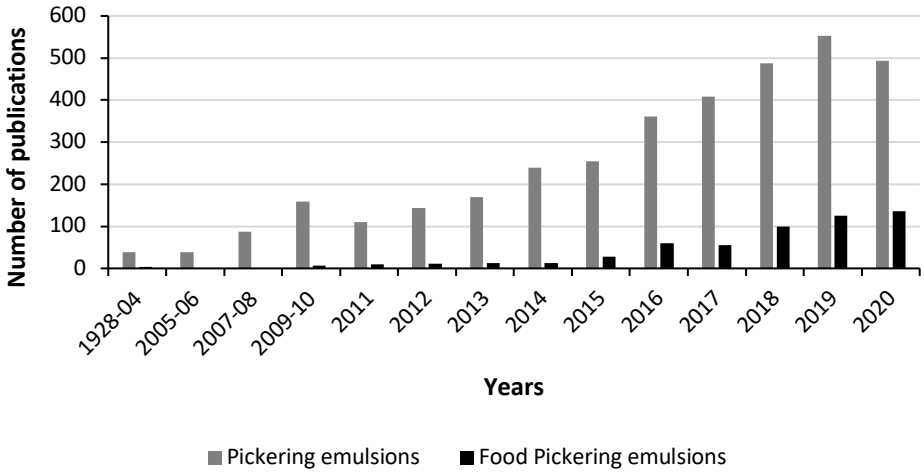

Figure 2.11: Number of publications in last hundred years in Pickering emulsions scope. Data collected in ISIweb of Science site in September 2020 with key words: Pickering emulsions e Food Pickering emulsions.

The capacity of different materials to develop new particles has been investigated, and the respective potential safety risks must be examined (Schröder *et al.*, 2018b). Their use in the food industry still involves some scepticism because there is not assertive legislation on the safety of their application (Schröder *et al.*, 2018b). Nevertheless, several Pickering emulsions

have been developed and studied to investigate and understand the behaviour of particles in the stabilisation of emulsions. Only in this way, it is possible to collect essential data to make a cautious and scientifically correct decision. Table 2.3 summarises previous works using safe inorganic food-grade Pickering particles. The table describes, when data is available, the type, shape, and size of the solid particles, parameters that influence Pickering emulsions stabilisation, use aqueous and oleic phases, as well as the production method.

A first conclusion from Table 2.3 is that most common Pickering emulsions are O/W type, with fewer works on W/O emulsion. In some cases, the solid particles surface was modified to obtain better performance in emulsion stabilisation, but also to obtain W/O emulsions. In some works, W/O emulsions were achieved after surface modification of the solid particles with other molecules such as fatty acids. Solid lipid particles are one of the few that stabilise W/O emulsions with no need for previous treatments; due to their wettability, these particles prefer to disperse in the oil phase, resulting in O/W emulsions. Thus, lipids can help in the modification of other solid particle wettability. Calcium carbonate and zirconium phosphate were also modified with fatty acids and octadecyl isocyanate, respectively, to promote longer stabilisation time of O/W Pickering emulsions (Cui *et al.*, 2012; Wang & Wang, 2015).

Eskandar *et al.* (2007) studied the synergistic effect on formation and long-term stability of O/W Pickering emulsions using silica particles modified with lecithin or oleyamine. Hydrophilic silica particles were modified with sodium caseinate, tween 20, tween 60, or different fatty acids to stabilise O/W Pickering emulsions (Binks & Yin, 2016; Björkegren *et al.*, 2017; Drelich *et al.*, 2010; Pichot *et al.*, 2009, 2010, 2012; Sadeghpour *et al.*, 2013; Santini *et al.*, 2014; Whitby *et al.*, 2009; Zhu *et al.*, 2015).

Skelhon *et al.* (2012) stabilised water-in-sunflower oil using fumed silica particles with chitosan. Björkegren *et al.* (2017) used silica particles covalently modified with organosilanes containing mainly propyl and methyl groups working as a hydrophobic group mimicking the properties of surfactants, to stabilise W/O emulsions. Cui *et al.* (2012) controlled emulsion type (O/W or W/O) by the amount of the fatty acids that calcium carbonate adsorbs at their surface.

Table 2.3: Examples of Pickering emulsions stabilised with inorganic food-grade solids.

Particle characterisation				Emulsion characterisation			Production		Reference
Solid particle	Surface modification	Shape	Size (width x length)	Water phase	Oil phase	Emulsion type	Homogeniser	Speed/Time or pressure/cycles	
Silica	-	Spherical	30 nm	Water	Tricaprylin	O/W	Microfluidizer	-	Xu et al. (2005)
Silica	Lecithin or oleylamine	Spherical	7 nm	Water	Miglyol	O/W	High pressure	500–1000 bar/5 cycles	Eskandar et al. (2007)
Silica	Monoolein	Spherical	150 nm	Water	Vegetable oil	O/W	High shear	8000 rpm/5 min	Pichot et al. (2009)
Silica	Sodium dodecyl sulphate	Spherical	12 nm	Water	*n*-dodecane	O/W	Rotor-stator	13000 rpm/2 min	Whitby et al. (2009)
Silica	-	Spherical	80 nm or 800 nm	Water	Ethyl acetate	O/W	XME; RME	-	Yuan et al. (2010)
Silica	Tween 60; sodium caseinate; lecithin	Spherical	150 nm	Water	Vegetable oil	O/W	High shear	8000 rpm/5 min	Pichot et al. (2010, 2012)
Silica	-	Spherical	145 nm	Water	Hexadecane	O/W	Hand shaking	n.d.	Gautier et al. (2007)
Silica	-	Spherical	100 nm	Water	Toluene	O/W	Ultrasonic	40% amplitude/n.d.	Frelichowska et al. (2010)
Silica	-	Spherical	12 nm or 200nm	Water	Corn oil	O/W	High pressure	350-1000bar/ 1cycle	Kohler et al., 2010
Silica	-	Spherical	12 nm	Water	Sunflower oil	O/W	Rotor-stator	n.d./7 min	Kargar et al. (2011)
Silica	-	Spherical	800 nm	Water	Tricaprylin oil	O/W	RME	-	Manga et al. (2012)
Silica	-	Spherical	10-12 nm	Water	Tricaprylin oil	O/W	SCME	-	Manga and York (2017)
Silica	-	Spherical	15 nm	Water	*n*-dodecane	O/W	Rotor-stator	13000 rpm/3 min	Kumar et al. (2020)
Silica	-	n.d.	8 nm	Water	Canola oil	O/W	High pressure	600 bar/3 cycles	Tikekar et al. (2013)
Silica	Sorbitan monooleate	Spherical	12 nm	Water	Paraffin	O/W	Rotor-stator	25000 rpm/5 min	Drelich et al. (2010)
Silica	mPEG silanes; organosilanes	Spherical	13 – 70 nm	Water	Exxsol D60	O/W; W/O	Rotor-stator	10000–20000 rpm/ 4 min	Björkegren et al. (2017)
Silica	Palmitic acid	Spherical	15 nm	Water	Hexane	O/W	Rotor-stator	10000 rpm/10 min	Santini et al. (2014)
Silica	Oleic acid	Spherical	5-10 nm	Water	Paraffin oil	O/W	Magnetic stirrer	2500 rpm/2 min	Sadeghpour et al. (2013)

Table 2.3 (*Continued*): Examples of Pickering emulsions stabilized with inorganic food-grade solids.

Particle characterisation				Emulsion characterisation			Production		Reference
Solid particle	**Surface modification**	**Shape**	**Size** (width x length)	**Water phase**	**Oil phase**	**Emulsion type**	**Homogeniser**	**Speed/Time or pressure/cycles**	
Silica	CTAB	Spherical	20 nm	Water	*n*-dodecane	O/W	Rotor-stator	7000 rpm/2 min	Zhu et al. (2015)
Silica; hydroxyl methyl cellulose	Tween 20; whey protein	n.d.	n.d.	Water	Sunflower oil	O/W	RME	-	Arkoumanis et al. (2019)
Silica + PS latex	SDS; HTAB; Tween 20	n.d.	n.d.	Water	Paraffin oil; ethyl acetate; sunflower oil	O/W	XME; RME	-	Yuan and Williams (2016)
Silica (1) or Zirconia (2)	Dipropyl adipate	Spherical; n.d.	5–30 nm (1); 5–10 nm (2)	Water	*n*-dodecane	O/W	Rotor-stator	13000 rpm/2 min	Binks and Yin (2016)
Clay (1); silica (2); Fe_2O_3 (3); Oleic acid-coated Fe_2O_3 (4); microgel (5)	-	Platelets (1); spherical (2,3,4); microgel (5)	1x30 nm (1); 5-30 nm (2); 5 nm (3,4); 220 nm (5)	Water	Styrene; toluene	W/O/W; O/W/O	Ultrasonic Hand shaking	n.d./2 min n.d./2 min	Zou et al. (2013)
Silica/ chitosan	-	n.d.	n.d.	Water	Sunflower oil; cocoa butter	W/O	Rotor-stator	11000 rpm/2 min	Skelhon et al. (2012)
Silica/ chitosan	-	n.d.	n.d.	Water	Corn oil	O/W	High-pressure	1380 bar/7 cycles; 2760 bar/1 cycle	Alison et al. (2016)
Clay	SDS; DTAB; Pluronic	Spherical	9–50 nm	Water	Mineral oil	O/W	Rotor-stator	11000 rpm/5 min	Zheng et al. (2020)
Calcium carbonate	-	Cubic	~1 μm	Buffer solution	Sunflower oil	O/W	Rotor-stator	6000 rpm/2 min	Wang et al. (2012)
Calcium carbonate	-	Spherical; Cube; Rod-like	~5 μm	Water	Soybean oil	O/W	Hand shaking	30 s	Huang et al. (2019)
Calcium carbonate	Fatty acids	Spherical	80–100 nm	Water	Toluene	O/W; W/O	Rotor-stator	5000 rpm/2 min	Cui et al. (2012)
Silicone resin	-	Microbowl	2-2.5 μm	Water	*n*-dodecane	O/W	Vortex mixer	n.d./2 min	Nonomura et al. (2011)
Zirconium phosphate	Octadecyl isocyanate	Spherical	200–300 nm	Water	Nonadecane	O/W	Hand shaking	30 s	Wang and Wang (2015)
PGMA-PS	-	n.d.	230 nm	Water	Sunflower oil	O/W	SCME	-	Thompson et al. (2011)

CTAB - Cetyltrimethylammonium bromide; DTAB - dodecyltrimethylammonium bromide; HTAB – hexadecyl trimethyl ammonium bromide; mPEG – poly(ethylene glycol) silane; n.d. – not defined; PGMA-PS poly(glycerol monomethacrylate)- polystyrene; RME – Rotational membrane emulsification; SCME – stirred cell membrane emulsification; SDS - Sodium-dodecylsulfate; XME – cross-flow membrane emulsification.

Recently, Pickering emulsions have also been applied in the development of double emulsions. In the food industry, these emulsions can provide additional protection and controlled release of the bioactive compounds, and they may also mask flavour or unpleasant taste of some nutritional components (Frasch-Melnik, 2011; Xiao *et al*., 2016); additionally, these emulsions can reduce fat content in a processed food product (Frasch-Melnik, 2011). However, these systems are still a challenge due to the different nature of stabilisers (hydrophilic and lipophilic) which tend to diffuse between the interfaces. Different authors have worked on double Pickering emulsions, nevertheless, polyglycerol polyricinoleate (PgPr) is used as a surfactant for stabilising at least one emulsion and solid particles just for another one (Frasch-Melnik *et al*., 2010; Marefati *et al*., 2015; Matos *et al*., 2013; Xiao *et al*., 2017). An exception was observed in the work of Zou *et al*. (2013). A W/O simple or primary Pickering emulsion was stabilised through hydrophobic particles (silica and oleic acid-coated Fe_2O_3), and the respective double emulsion (W/O/W) was stabilised with hydrophilic particles (clay, Fe_2O_3 or microgel). The reverse emulsion (O/W/O) was also obtained where a O/W simple emulsion was firstly stabilised by hydrophilic particles, and the respective double emulsion was achieved with hydrophobic particles.

The main concern to produce emulsions, and in particular Pickering emulsions, is their stability over storage time. Their integrity over time is essential to ensure, in case of the food industry, the product quality during shelf-life. Hence, most of the works shown in Table 2.3 were developed to verify the emulsion stability over time. As discussed in section 2.4, parameters such as particle size or concentration, volume fraction of the aqueous or oil phase and pH of the emulsion were taken into account and adjusted to improve the emulsion stability, and hence obtain a product with long stability.

Recently, Pickering emulsions have also attracted attention since they can act as an enhanced system to avoid oil oxidation (when oil is a dispersed phase), or to improve encapsulation systems of various compounds. The development of more stable emulsions both physically and chemically, enabled the development of enhanced or new approaches for active compounds delivery. Kargar *et al*. (2011) studied lipid oxidation in O/W Pickering emulsions stabilised with silica particles and compared their performance with conventional emulsions stabilised with sodium caseinate and Tween 20. They reported that emulsions stabilised by

silica particles reduce the oxidation rate when compared with the conventional emulsifier. Tikekar *et al*. (2013) encapsulated curcumin using silica particles to stabilise the canola oil/water emulsions. Results showed that silica stabilised Pickering emulsion can maintain stability of encapsulated curcumin, which was approximately 100-fold higher than the stability of curcumin suspended in water.

Furthermore, with the perspective of developing food-grade emulsions, and considering the data available in the table, it should be noted that many of the reported processes use non-food elements. Some emulsions contain non-food-grade oil phases, such as toluene, hexane or silicone oil, as well as non-food-grade emulsifiers such as sodium-dodecylsulfate (SDS). Hence, although the studies have been carried out with food-grade solid particles, further work must be done to develop more realistic systems using food-grade oils, and if necessary, food-grade molecules/emulsifiers to modify the surface of the particle to change the wettability.

2.5.2 HYDROXYAPATITE AS PICKERING STABILISER

Calcium phosphates are well-established biomaterials and that have been used in biomedical products (Canillas *et al.*, 2017), cosmetics (Coelho *et al.*, 2019) and toothpastes (Enax & Epple, 2018). Calcium phosphates are common minerals encountered in nature; they are produced in different environments such as geological (igneous apatite), geochemical (phosphorite) and biological (biological apatite) (Omelon *et al.*, 2013). The apatite found in sedimentary rocks is essentially carbonate fluorapatite, whereas the one found in mammalians bones and teeth is mainly (based on crystallography and chemical composition comparison) hydroxyapatite (HAp) (Chan, 2016).

HAp is a double salt of tricalcium phosphate and calcium hydroxide with a stoichiometric formula $Ca_{10}(PO_4)_6(OH)_2$. It can be found in two different crystal forms, monoclinic (Figure 2.12 A) and hexagonal (Figure 2.12 B), the latter being the most often found in crystal form. The significant difference between them is reflected by the ordering of the hydroxyl ions (Ma & Liu, 2009). While in hexagonal HAp, the hydroxyl ions are similarly arranged in columns in 2-fold disorder, monoclinic HAp has both the intracolumn and intercolumn ordering of hydroxyl

ions (Ma & Liu, 2009). From the theoretical point of view, the monoclinic HAp structure is thermodynamically more stable than the hexagonal one; however, the hexagonal form allows an easier exchange of OH groups with other ions, which is essential for bone tissue functions (Verezhak *et al.*, 2018). Hydroxyapatite has a Ca/P ratio of 1.67; different ratios in Ca/P can lead to phase impurities in HAp originating other calcium phosphates such as tricalcium phosphate, dicalcium phosphate, among others (Chan, 2016; Epple, 2018). HAp solubility depends on factors such as shape, size, porosity and crystallinity (Chan, 2016). HAp is poorly soluble or even almost insoluble at basic and neutral pH solutions; but soluble in acid solutions, typically at pH≤4 (Epple, 2018).

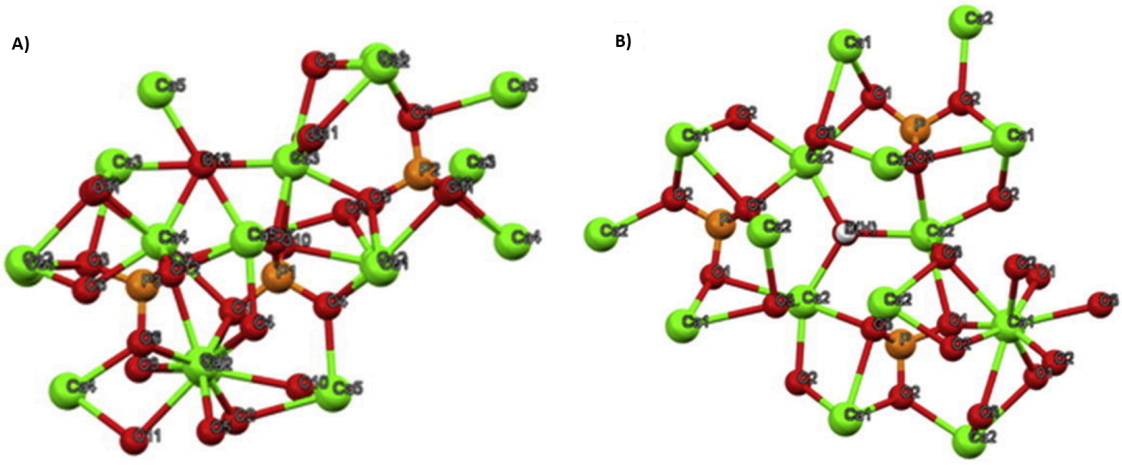

Figure 2.12: Schematic representation of A) monoclinic and B) hexagonal HAp forms (Verezhak *et al.*, 2018).

Nano sized HAp and other calcium phosphates are emerging for application in the biomedical field. Various HAp-based products, such as Ostim®, OsSatura®, NAnOss®, and EqivaBone® have been developed in last years, and are nowadays commercialized and used with FDA approval to mimic bone structure allowing cell adhesion and growth (Bobo *et al.*, 2016). NanoXIM-HAp paste, a nanocrystalline hydroxyapatite with high quality and reproducibility is produced by Fluidinova S.A (www.fluidinova.com), a Portuguese spin-off company, using the NETmix technology. In general, calcium phosphate is used as bone substitution material, enhancing the growth of bone, and ensuring an excellent mechanical interlock between implant and bone (Epple, 2018). Furthermore, different sized calcium phosphates have also been used in various kinds of toothpaste to enhance tooth repair (Enax & Epple, 2018). These applications are due to good biocompatibility, absorbability and osteoinductivity.

Calcium phosphates, including HAp, is also used as a calcium source, an essential element for all biological organisms, in food supplements (Schoepf *et al.*, 2017). Calcium phosphates received the GRAS statement by the FDA, and their use in food was recognized as safe by the same identity (FDA, 1975). However, the utilization of HAp in food was put into question when in 2016 was discovered needle-shaped HAp in the baby formulas (Epple, 2018). Epple (2018) revised the potential health risks associated with nanoscopic calcium phosphates, including HAp. The author reported that when HAp particles are ingested, will pass by different digestive organs, namely stomach and gut. In the stomach, the complete dissolution of the HAp is achieved because they enter in contact with very low stomach pH (around 1-2). Such as referred above, in these high acid conditions, the HAp particles are dissolved, losing their original chemical structure and shape. Ramis *et al*. (2018) report that nanoparticulate HAp is rapidly or even instantly dissolved when added to a simulated gastric fluid (pH around 1.2) in Ca^{2+} and PO_4^{2-} ions. Afterwards, the recently dissociated HAp enters the gut where the pH becomes basic. The small intestine absorbs calcium ions, but recrystallization of HAp may occur locally. Under these new physiological conditions and in the presence of a high concentration of phosphate and calcium ions, HAp may precipitate if its solubility is exceeded and the high concentration of biomolecules does not inhibit the nucleation; i.e., when the pH increases, ions can have a tendency to precipitate again, but not necessarily in the same form and size (Epple, 2018). Generally, precipitation occurs in amorphous calcium phosphate forms (Epple, 2018). After concerns have been raised about the use of HAp in food, other studies have been conducted to evaluate the safety of their use in oral and cosmetic products. For example, Schoepf *et al*. (2017) evaluated and studied the presence of HAp in six different infant formula samples. Although n-HAp crystalline needle-shaped were detected in half the samples, these authors also documented rapid dissolution of the HAp for low pH conditions as present in the simulated biological acidic gastric fluids. Furthermore, Ramis *et al*. (2018) also reported similar behaviour for the rod-like HAp particles in acidic fluids; these authors also reported that HAp has not any effect on contact with human gingival epithelium tissue. These works are of significant importance in order to understand HAp behaviour (with different shapes but in the nano-scale range) in the human organism. Safety assessment is reinforced with these works as they report the instantaneous dissolution of calcium

phosphates in their ions. This conclusion is in agreement with the Epple (2018) statement that rules out any adverse health effect by oral exposition to calcium phosphate.

Recently, HAp has been described as a promising material for stabilisation of Pickering emulsions (Fujii *et al.*, 2012). Due to good interaction with various compounds, HAp has already been used in the formation of Pickering emulsions, which can be applied to a variety of applications. In the food sector, the use of particles instead of conventional emulsifiers presents advantages in terms of physical stability and improvement of the functionality and performance of the food emulsions (Berton-Carabin & Schroën, 2015). Furthermore, it will be possibly the production of emulsions with a *clean-label*.

The HAp tends to form O/W emulsions due to hydrophobicity, but if the oil has an ester group or if polymers are added to the oil phase, the formation of the emulsion is facilitated. Some works reported the capacity of HAp to produce Pickering emulsions or products derived from Pickering emulsions. However, their use for stabilising food-grade emulsions is still scarce. Table 2.4 summarises works that use HAp to produce Pickering emulsions. In most cases, different surface modifiers or polymers dissolved in the oil phase are used to achieve the desired final goal through Pickering emulsions template. The most used polymer is poly(*ε*-caprolactone) (PCL) and poly(L-lactic acid (PLLA).

Fujii *et al*. (2012) and Fujii *et al*. (2013) produced microspheres prepared via the Pickering emulsion solvent evaporation method with HAp and PCL. In these works, HAp was an adequate Pickering stabiliser to produce PCL coated HAp particles only when the interaction of carbonyl/carboxylic acid groups of the polymer and solid particles is possible. This played a crucial role to obtain stable and well-defined droplets and subsequent microspheres. The effect of HAp and PCL concentration and homogenisation rate on droplet size distribution was studied.

Table 2.4: Main Pickering emulsions produced with HAp as solid stabiliser.

Particle characterisation				Emulsion characterisation			Production		Purpose	Reference
Solid particle	Surface modification	Shape	Size (width x length)	Water phase	Oil phase	Emulsion type	Homogeniser	Speed/time or pressure/cycles		
HAp	PCL*	Rod-like	30 nm	Water	DCM	O/W	Rotor-stator	20500 rpm / 1 min	Pickering emulsion stabilisation	Fujii et al. (2012)
HAp	PCL*	Rod-like	30 nm	Water	DCM	O/W	Rotor-stator	14500–30000 rpm / 1 min	Pickering emulsion stabilisation	Fujii et al. (2013)
HAp	PCL*	Fibril	23x140 nm	Water	DCM and DMF	W/O	Rotor-stator	15000 rpm/n.d.	Scaffolds fabrication	Samanta et al. (2017)
Hap	PCL*	Rod-like	20-50x80-220 nm	Water	DCM	W/O	Vortex mixer	3500 rpm/n.d.	Scaffolds fabrication	Hu et al. (2019)
HAp	P(LLA/CL)*	Spherical	50 nm	Water	DCM	O/W	Rotor-stator	20450 rpm / 3 min	Scaffolds fabrication	Liu et al. (2011)
HAp	PLLA*	Spherical	30–70 nm	Water	DCM	W/O	Rotor-stator	12000 rpm / 1 min	Scaffolds fabrication	Hu et al. (2014)
HAp	Alginate+PLLA*	Spherical	20–70 nm	Water	DCM	O/W	Rotor-stator	12000 rpm / 1.5 min	Scaffolds fabrication	Hu et al. (2016)
HAp	Stearic acid + PLLA*	n.d.	n.d.	Water	DCM	O/W; W/O	Rotor-stator	17000 rpm/1 min	Pickering emulsion stabilisation	Zhang et al. (2017)
HAp	PLLA*	n.d.	n.d.	Water	DCM	O/W; W/O	Rotor-stator	200–20000 rpm / 0.2–3 min	Pickering emulsion stabilisation	Zhang et al. (2017b)
HAp	CTAB and PG + PLLA*	n.d.	0.2–1.2 µm	Water	DCM	O/W	Ultrasonic	250 W/5 min	Pickering emulsion stabilisation	Tham and Chow (2017)
HAp	Stearic acid; PLLA+Span 80*	n.d.	n.d.	Water	DCM	O/W; W/O	Rotor-stator	10000-20000 rpm / 0.5-4 min	Pickering emulsion stabilisation	Song et al. (2018)
HAp	PS*	Spherical	40 nm	Water	DCM	O/W	Vortex mixer	3200 rpm/1 min	Pickering emulsion stabilisation	Okada et al. (2012)
HAp	Sodium oleate	Rod-like	23x70 nm	Water	Cy	W/O; O/W	Ultrasonic	300 W/6 cycles	Pickering emulsion stabilisation	Hu et al. (2015)
HAp	Stearic acid	n.d.	30 nm	Water	n.d.	W/O	Magnetic stirrer	12000 rpm / n.d.	Pickering emulsion stabilisation	Rodríguez et al. (2019)
HAp	PMF	Spherical	30-70 nm	Water	Artemisia argyi oil	O/W	Rotor-stator	10000 rpm / 2 min	Pickering emulsion stabilisation	Hu et al. (2013)
HAp	DBP	Rod-like	n.d.	Water	Hexanol	O/W	Ultrasonic	n.d. / n.d.	Protocells fabrication	Zhang et al. (2019)
HAp	-	Rod-like	50 nm	Water	Sunflower oil	O/W	Rotor-stator	11000 rpm / 6 min	Pickering emulsion stabilisation	Ribeiro et al. (2020)
HAp	-	Rod-like	50 nm	Water	Sunflower oil	O/W	NETmix	200-500 Reynolds number / 1-35 cycles	Pickering emulsion stabilisation	Ribeiro et al. (2021)

* - Dispersed in oil phase to modify the HAp surface during emulsification; CTAB - cetylmethylammonium bromide; Cy – cyclohexane; DBP - dibutyl phosphate; DCM – dichloromethane; DMF – dimethylformide; HAp – hydroxyapatite; O/W – oil-in-water; PCL - poly(ε-caprolactone); PG - propyl gallate ; PLLA - poly(L-lactic acid); P(LLA/CL - poly(L-lactide–co-ε-caprolactone); PMF - Poly(melamine formaldehyde); PS – Polystyrene; W/O – water-in-oil.

Another polymer often combined with HAp is PLLA (poly(*L*-lactic acid). HAp stabilised Pickering emulsions were produced with PLLA dissolved in dichloromethane, which is used as the oil phase; emulsion type and stability were studied by Zhang *et al*. (2017b). They showed that the interaction between HAp and PLLA has an essential role in Pickering emulsion stabilisation since it promotes the adsorption of the HAp particles at the oil-water interface. The formation of microspheres through O/W and porous materials through W/O Pickering emulsions were obtained after cured Pickering emulsion via in situ solvent evaporation method.

Zhang *et al*. (2017) studied the effect of HAp modified by stearic acid dispersed in different solvents (water, ethanol, or dichloromethane) and phase oil containing PLLA on Pickering emulsion stability and their ability to originate different structures after solvent evaporation. The authors noted that surface modification of HAp in different solvents played an important role in Pickering emulsion stabilisation and microstructure of the cured materials. HAp modified with stearic acid in the presence of ethanol increased the emulsion stability and allowed the obtainment of cured materials with uniform pore size. Song *et al*. (2018) followed the previous line of research and studied the factors that influence the stability of Pickering emulsions stabilised by HAp and non-ionic surfactants. In this work, the hydrophobicity of HAp particles was enhanced by surface modification using stearic acid dispersed in ethanol; PLLA and span 80 were dissolved in the oleic phase. For moderated span 80 concentrations, emulsion stability was improved, and porosity of the cured materials was increased; however, with increasing span 80 concentration, the emulsion undergoes a phase inversion from O/W to W/O type.

The formation of Pickering emulsions stabilised via interaction between HAp particles and polystyrene (PS), dispersed in water and oil phase respectively, was studied by Okada *et al*. (2012). The authors investigated the influence of polymer end groups on formation of HAp stabilised droplets/microspheres, reporting that the end groups have an important role in emulsion stabilisation and in controlling the morphology of the microspheres prepared by evaporating solvent through the emulsion. Tham and Chow (2017) modified the HAp surface properties through incorporation of salt and amphiphilic agents such as cetylmethylammonium bromide (CTAB) and propyl gallate (PG); the pH was also varied to

study the effect on HAp interfacial adsorption. In this way, the authors can control the droplets morphologies, which range from spheres, dumbbell, plated-shape and deflated-sphere, changing the interfacial adsorption behaviour of HAp solid particles.

In the works of Hu *et al*. (2015) and Rodríguez *et al*. (2019), Pickering emulsions were produced with modified HAp particles by sodium oleate and stearic acid, respectively; however, no polymers or surfactants were added to the oil phase. Hu *et al*. (2015) observed a double phase inversion of the Pickering emulsion, that is an initial inversion from O/W to W/O and then back to O/W type, induced by increasing surfactant concentration. Rodríguez *et al*. (2019) produced W/O Pickering emulsions that can successfully be used as a venom treatment substituting the traditional Freund's emulsion (vaccine therapy, widely used in these treatments), which has limited use due to tissue damage issues. The produced n-HAp Pickering emulsions have similar rheological behaviour to Freund's emulsions with lower oil content and surfactant concentration. The n-HAp Pickering emulsions resulted in similar adjuvant activity with minimized negative side effects compared to the traditional ones.

Pickering emulsions with modified HAp with poly(melamine-formaldehyde) (PMF) were used to stabilise and protect an antibacterial oil such as artemisia argyll oil (Hu *et al.*, 2013). The authors produced hybrid spherical and rough microcapsules, through in situ polymerization of PMF at the oil-water interface and reported capsules with good thermal stability and long-term antimicrobial activity.

In addition to the production of microspheres through the Pickering emulsion route, production of scaffolds has also been attempted. In this case, polymers dissolved in the oil phase to improve the interaction of the HAp particles were also used, and the scaffolds were prepared using solvent evaporation from templating Pickering emulsion. Composite PCL-HAp scaffolds produced via Pickering emulsions were developed for implementation on osteoblast cell proliferation by Samanta *et al*. (2017), while Hu *et al*. (2019) studied the production of scaffolds from the combination of HAp modified by PCL. In this last case, scaffolds with adjustable grid-like structure were fabricated by solvent evaporation of 3D printed W/O high internal phase emulsions (HIPEs), revealing a potential for anti-inflammatory drug release as well as efficient support for cell adhesion and proliferation. Hu *et al*. (2014)

fabricate scaffolds with HAp and PLLA also for anti-inflammatory drug release. Liu *et al*. (2011) investigated the combination of HAp with PLLA and PCL for fabricating scaffolds, and HAp modified with alginate and coated PLLA scaffolds were developed by Hu *et al*. (2016).

HAp modified by dibutyl phosphate (DBP) were employed to prepare protocells via Pickering emulsion (Zhang *et al*., 2019). The developed study provides a novel and environment-friendly approach for enrichment and removal of trace organic pollutants in wastewater.

In recent studies, stable O/W Pickering emulsions using HAp solid particles as Pickering stabilisers without polymers or emulsifiers requirements were produced by Ribeiro *et al*. (2020) and Ribeiro *et al*. (2021). The authors reported that for HAp content above 5 wt% there are available enough solid particles to stabilise the oil surface and to develop the particle network in the continuous phase, enabling the emulsion stabilisation for at least a 2-month storage period.

Different production processes are applied in Pickering emulsion preparation. A summary of the different methods used is given below.

2.6 Preparation of Pickering emulsions – production processes

The process by which two immiscible liquids are converted in an emulsion is known as emulsification, while homogenisation is the act that makes an emulsion homogenous, that is with the emulsion droplets uniformly distributed. Emulsification and the subsequent homogenisation are usually carried out using mechanical devices known as homogenisers (McClements, 2016). The production of emulsions requires external mechanical energy in order to break up the dispersed phase in small droplets, homogenising this phase throughout the continuous phase (Lee *et al*., 2018). The most common equipment's used to prepare Pickering emulsions are high shear mixers, high-pressure and ultrasonic homogenisers (see Table 2.3 and Table 2.4). These types of devices allow the preparation of small amounts of Pickering emulsions, and subsequently, the production is often performed in batch mode. However, techniques such as membrane homogenisers and microfluidizers have been applied to prepare Pickering emulsions. A brief description of each technique is given next.

2.6.1 HIGH SHEAR MIXERS

High shear mixers, which are a type of rotor-stator device, are the most used devices for homogenising oil and aqueous phases (McClements, 2016); in Pickering emulsions production, this device is the most used one (Albert *et al.*, 2019). It consists of a rotor and stator with blades and openings, respectively; usually, the emulsification takes place in a suitable container (Figure 2.13 A). When the rotor rotates, a depression is created, drawing the liquid in and out, resulting in liquid circulation (Albert *et al.*, 2019). The shear force occurring between rotor and stator and the high liquid acceleration causes the reduction of droplet size of the dispersed phase (Albert *et al.*, 2019). For production of the Pickering emulsions, the oil and water phases can be added at the beginning of the process or added sequentially in order to improve the emulsification in terms of the droplet size (Albert *et al.*, 2019; McClements, 2016).

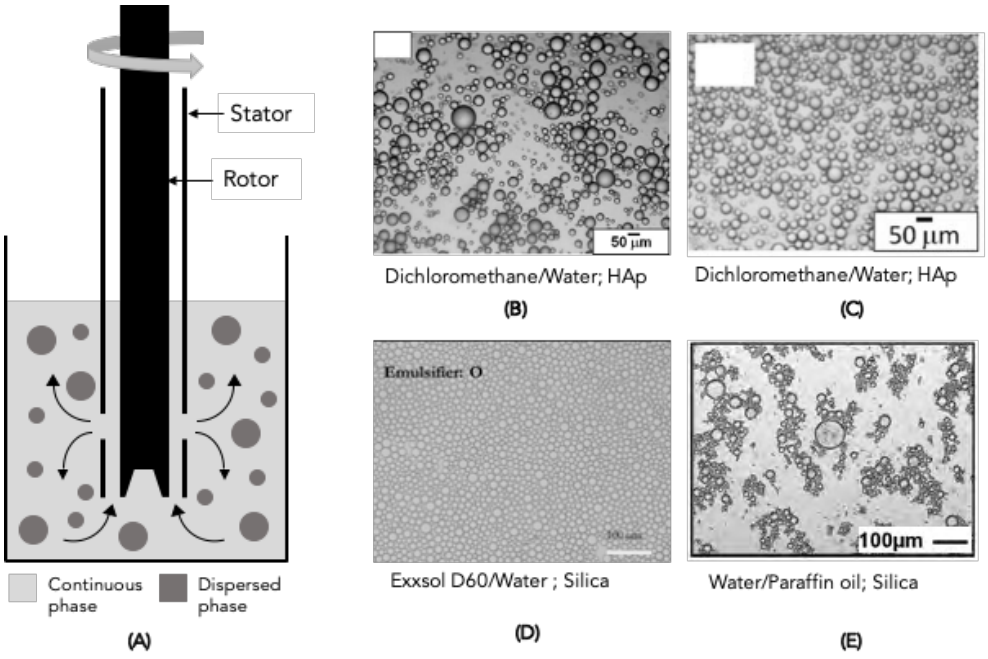

Figure 2.13: Schematization of rotor stator device (A), and some examples of Pickering emulsions (B – Fujii *et al.* (2012), C – Fujii *et al.* (2013), D - Björkegren *et al.* (2017), E – Drelich *et al.* (2010)).

According to the literature, high shear mixers are the most applied devices in order to obtain Pickering emulsions. However, different operating conditions such as rotation speed and time are used by different authors to produce the Pickering emulsions with different oil and aqueous phases. In general, the rotation speed ranges from 2500 to 30000 rpm while the

rotation time range from 30 seconds to a few minutes. The lack of control during emulsification is the main disadvantage of this device, leading to the obtention of Pickering emulsions with broad droplet size distributions that can range from a few to hundreds of microns. For example, Björkegren *et al*. (2017) investigated the energy input during the emulsification process and concluded that it is an essential parameter to decrease the Pickering emulsion droplet size. The increase of stirring speed from 10000 rpm to 20000 rpm led to the decrease of droplet mean diameter from 10 μm to 4 μm. Cui *et al*. (2012), Santini *et al*. (2014), Binks and Yin (2016) and Song *et al*. (2018) are other examples of production of Pickering emulsions with a rotor-stator device with different solid particles. Recently, Ribeiro *et al*. (2020) produced Pickering emulsions stabilised by n-HAp solid particles also using a rotor-stator device. Pickering emulsions were produced at 11 000 rpm during 6 min, using a thermostatic bath to control temperature during emulsification overcoming temperature rising constraints.

Figure 2.13 B, C, D and E show images of Pickering emulsions stabilised with different solid particles where is observable the droplet size range. However, a problem with the increase of stirring speed is the temperature rise, and thus other techniques have been used to produce Pickering emulsions with the desired droplet size.

2.6.2 ULTRASONIC HOMOGENISER

Ultrasonic technology is another method used to produce Pickering emulsions and, the ultrasonic probe is the most commonly used (Albert *et al.*, 2019). It consists of a titanium probe that vibrates due to a transducer that contains a piezoelectric crystal, which converts electric energy to very high-frequency mechanical motion (Figure 2.14 A). The probe transmits ultrasonic energy to the surrounding sample, inducing emulsification mainly through cavitation and turbulent effects (Albert *et al.*, 2019; McClements, 2016). Ultrasonic devices use high power to interact with the materials; however, in Pickering emulsions that use sensitive particles, the use of ultrasonic homogenisers requires some caution, since these homogenisers expose the emulsion at high-intensity ultrasound powers promoting physical and/or chemical degradation of the particles (McClements, 2016). For Pickering emulsions production, the emulsions are subjected to amplitudes that range from tens to hundreds of

watts and a few minutes is usually applied to prepare the emulsion. In general, these are the main parameters that affect the emulsion droplet size and using optimized parameters the minimum droplet size can be achieved.

Previous works reported Pickering emulsions produced by ultrasonic emulsification with various solid particles, including silica and HAp. Some of the optical images of these emulsions are shown in Figure 2.14 B and C (Frelichowska *et al.*, 2010; Hu *et al.*, 2014; Tham & Chow, 2017; Zhang *et al.*, 2019; Zou *et al.*, 2013). However, according to the data present in Table 2.3 and Table 2.4 few works produce Pickering emulsions using this emulsification method, when compared with other methods such as rotor-stator. For this reason, most of the works reported in the literature do not make an intensive study about the influence of ultrasonic emulsification parameters on emulsion characteristics and stability. Nevertheless, when data is available, the operation parameters were reported in Table 2.3 and Table 2.4.

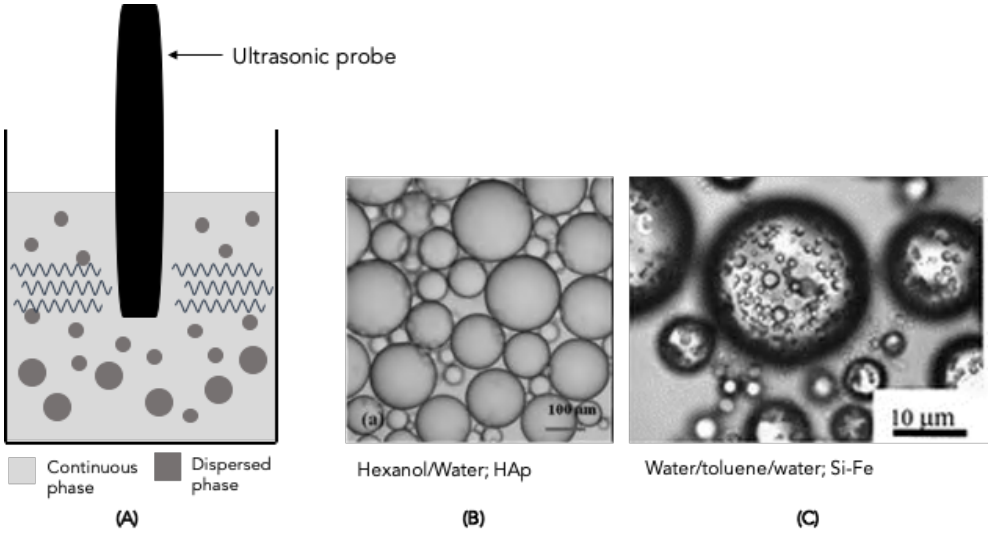

Figure 2.14: Schematization of ultrasonic device (A), and some examples of Pickering emulsions (B – Zhang *et al.* (2019); C – Zou *et al.* (2013)).

2.6.3 HIGH-PRESSURE HOMOGENISER

High-pressure homogenisers are the most common device in the food industry to prepare conventional emulsions (McClements, 2016), however, according to the Table 2.3 and Table 2.4, this homogeniser is seldom used in Pickering emulsions preparation. Before the production of the Pickering emulsion in this homogeniser, it is recommended to obtain a

coarse emulsion (McClements, 2016). A schematic diagram of a high-pressure homogeniser is shown in Figure 2.15 A.

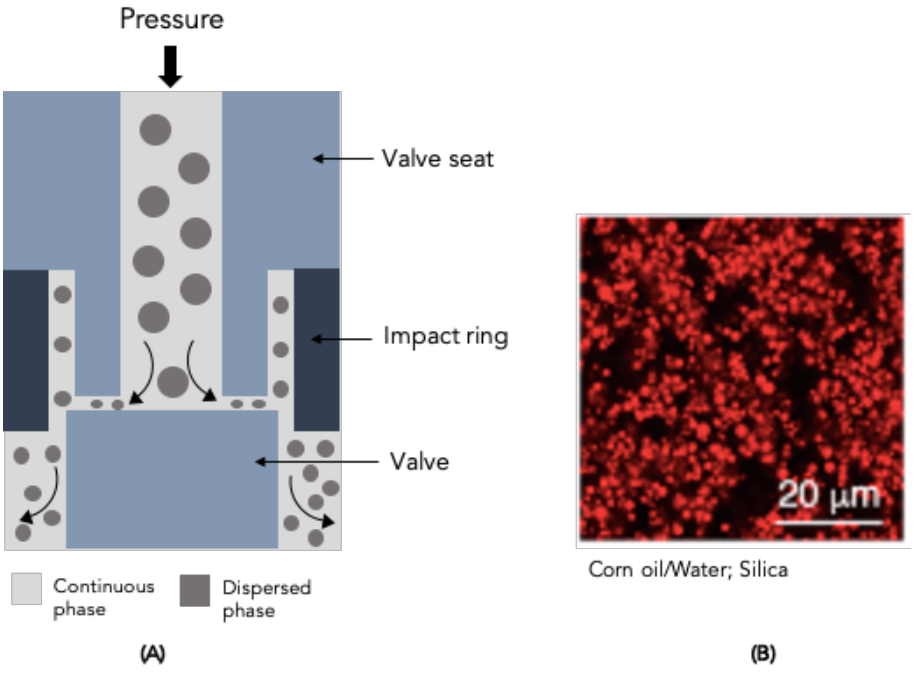

Figure 2.15: Schematization of high-pressure device (A), and example of Pickering emulsion (B, Alison *et al.* (2016)).

The pressure increases due to a high-pressure pump and the coarse emulsion is injected in a homogenising nozzle of small size which disrupts the drops (breaking down the larger droplets into smaller ones), inducing emulsification and producing a fine emulsion (Albert *et al.*, 2019). The fluids characteristics and nozzle design are the main parameters responsible for the disrupting of the droplets (McClements, 2016). Different types of nozzles have been designed and fabricated to increase the efficiency of droplet disruption; moreover, the emulsion droplet size can also be reduced through repeated cycles of the Pickering emulsion in the homogeniser (Albert *et al.*, 2019; McClements, 2016). According to the literature, Pickering emulsions with lower droplets size can be obtained ranging the pressure from tens to hundreds of MPa or/and repeatedly recirculating the emulsion through the device. Köhler *et al.* (2010) studied the impact of pressure on emulsion formation with silica. They reported a reduction of ~40 µm to ~10 µm of the droplet size with an increase of pressure from 350 to 800 bar, after which no further droplet break-up was observed. Eskandar *et al.* (2007) and Alison *et al.* (2016) also reported the use of a high-pressure device to produce Pickering

emulsions; in both works distinct operation parameters were used, suggesting that the pressure and number of cycles have a crucial role on emulsion droplet size. These parameters can be adjustable according to particles and oil type used. Figure 2.15 B shows a fluorescent image of the dyed Pickering emulsion, where it is possible to observe the oil droplets (red regions), produced with this method.

2.6.4 MEMBRANE HOMOGENISER

In the membrane emulsification technique the dispersed phase is pressed through a porous membrane, that contains well-defined pores, into a continuous phase that usually contains the stabiliser, solid particles in case of the Pickering emulsions (Manga & York, 2017). According to the composition of the dispersed phase, the production of Pickering emulsions through porous membranes can be divided in direct membrane emulsification (DME) and premix membrane emulsification (PME) (Figure 2.16) (Albert *et al.*, 2019). In DME the emulsion is formed from initially separated oil and water phases where the dispersed phase is pressed through the porous membrane into the continuous phase; in PME the coarse emulsion is pressed through the membrane and involves mainly the reduction of droplet size (Albert *et al.*, 2019; McClements, 2016). DME techniques have been upgraded, and three main types can be considered: crossflow membrane emulsification (XME), stirred-cell membrane emulsification (SCME) and rotational membrane emulsification (RME). Both XME and SCME are similar to DME, but while in XME the fluid flows along a channel formed by the membrane, in SCME, the membrane is placed inside a stirred vessel. For RME, the main characteristic is that the membrane is not stationary. For SCME and RME, agitation improves the droplets detachment from the membrane inducing a smaller droplet size formation (Albert *et al.*, 2019). The membranes can be designed and manufactured with different pore sizes that will induce different droplet size in the emulsion, and with a different polarity that should be carefully selected since it determines the type of emulsion (McClements, 2016). However, this technique has been only used in research; its use is not common in the food industry due to low volume throughputs (McClements, 2016).

Yuan *et al*. (2010) used two types of silica particles as O/W emulsion stabilisers and compared XME and RME Pickering emulsions production, obtaining highly stable emulsion with narrow

droplet size distributions. Yuan and Williams (2016) produced co-stabilised Pickering emulsions (emulsions stabilised by particles and emulsifiers), which can be used to guide the formulation of complex particulates of multi-functional products, also using XME and RME techniques. In both cases, depending on the oil flux rate and membrane speed, the droplet mean diameter ranged from 10 to 200 μm. Furthermore, the authors reported that the co-stabilised emulsion has a lower mean diameter than surfactant only stabilised emulsion.

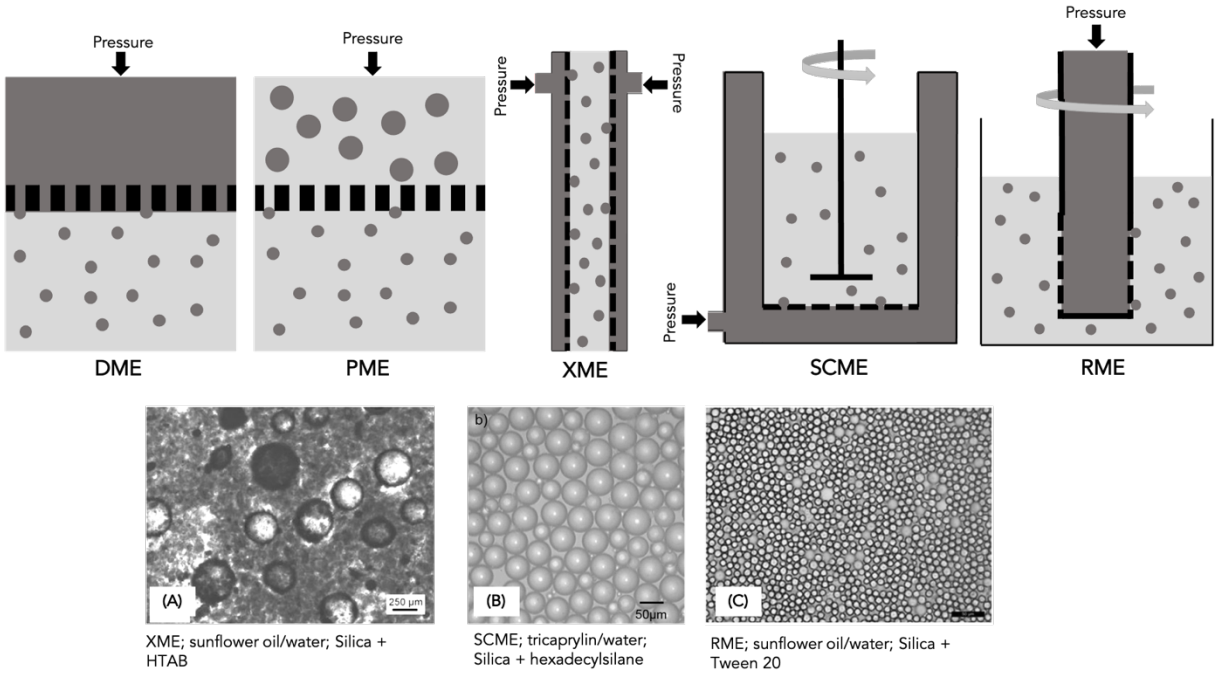

Figure 2.16: Schematization of membrane emulsification. Some examples of Pickering emulsions obtained from crossflow membrane emulsification (XME) – A (Yuan & Williams, 2016), stirred-cell membrane emulsification (SCME) - B (Manga & York, 2017) and rotational membrane emulsification (REM) – C (Arkoumanis *et al.*, 2019).

Oil-in-water Pickering emulsions with relatively narrow size distributions have been prepared by SCME using poly(glycerol monomethacrylate)-polystyrene (PGMA-PS) latex as stabiliser of the sunflower oil, where droplets with 44-78 μm were obtained through a membrane with 5 μm of pore size (Thompson *et al.*, 2011). The authors observed that higher stirring speed aided the droplet breakup. Manga and York (2017) also used SCME to prepare O/W Pickering emulsions solely stabilised by silica particles. The authors achieved optimal conditions, in terms of oil flux rates (6 L/m^{2}h), paddle stirrer speeds (1250 rpm) and oil volume fraction, to control droplet size and size distribution. The droplet size increased with oil volume fraction increase and ranged from 40 to 170 μm. According to the oil characteristics, the oil flux rates can assume different maximum values to obtain a minimum droplet size, thus having a critical

flow rate value below which the emulsions are very stable with constant droplet size, and above which the emulsion droplets are larger due to coalescence (Albert *et al.*, 2019; Yuan *et al.*, 2010).

RME was used to produce silica stabilised tricaprylin/water Pickering emulsions with controlled droplet size with narrow polydispersity and highly stable emulsion (Manga *et al.*, 2012). The effect of the membrane rotational speed was tested, and the reported data showed a crucial influence on decreasing droplet size since it induces an easier detachment of the droplets from the membrane. Sun *et al*. (2014) and Arkoumanis *et al*. (2019) produced O/W stable Pickering emulsions with small droplet size and narrow distribution via RME.

2.6.5 MICROFLUIDIZERS

Microfluidic devices consist of microchannels with specific and well-defined geometry in which fluids circulate (Albert *et al.*, 2019). Fluids are introduced into the device, which is accelerated to a high velocity inside the channels through a pump, promoting an impingement with each other. When the two fluid streams collide, high disruptive forces are generated, causing the mixture of the disperse and continuous phases, as well as the break-up of the droplets, upon which, the Pickering emulsion is produced (McClements, 2016). There are two main types of the microfluidizers, the single-inlet (e.g., T-junction) and the double-inlet microfluidizers (e.g., flow-focusing). In T-junction devices (Figure 2.17 A), the dispersed phase is forced to flow through a small orifice into the perpendicularly flowing continuous phase.

In contrast, in the flow-focusing devices (Figure 2.17 B), the dispersed phase is focused on two perpendicular streams of the continuous phase from both sides (Albert *et al.*, 2019). Through this method, the Pickering emulsion droplet size can be controlled, changing the flow rate or by changing the channel geometry. However, changing the channel geometry is often unfeasible and/or expensive. This method has been commonly applied to conventional emulsions, but only recently applied for production of Pickering emulsions, and very few scientific works are encountered.

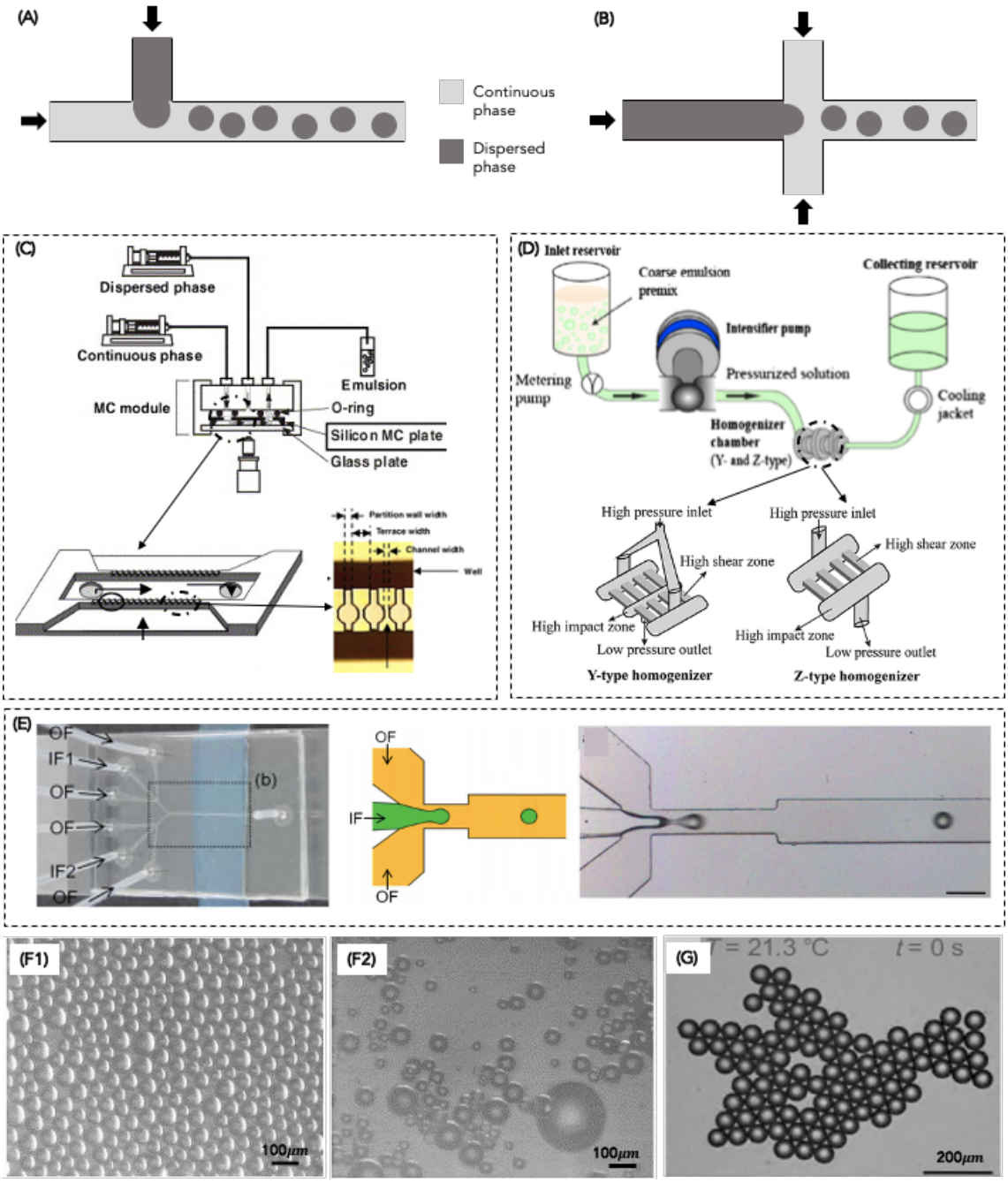

Figure 2.17: Schematization of microfluidizers (A - T-junction; B - flow-focusing) and different microfluidic technologies (C – Xu *et al*. (2005); D –Bai *et al*. (2016), Bai and McClements (2016); E – Sun *et al*. (2016)). Some optical images of Pickering emulsions F1 and G produced by microfluidizers C and E, respectively. F1 and F2 compares emulsions produced by microfluidic device, and rotor-stator, respectively.

Xu *et al*. (2005) produced O/W Pickering emulsions with silica through a microfluidizer (Figure 2.17 C). The emulsions produced through a microchannel method (Figure 2.17 F1) allowed the obtention of well-defined droplets and emulsions with high stability when compared with the ones produced by the traditional rotor-stator method (Figure 2.17 F2). Other microfluidic devices have been reported in the literature for producing Pickering emulsions without high

shear forces (Bai *et al.*, 2016; Bai & McClements, 2016; Sun *et al.*, 2016). This technique has been gaining interest in the scientific community to produce emulsions since it offers a simple preparation and precise control of emulsion droplets.

2.6.6 STATIC MIXERS

High-shear mixers, high-pressure and ultrasonic homogenisers, by using high shear mechanical forces, require high-energy inputs to produce emulsions. The main limitation is the lack of control, mainly in the droplet size, during the emulsification process (Manga & York, 2017). However, they are the most used devices to achieve an adequate emulsification step. Recently, and as an alternative to these devices, other techniques have been gradually developed, such as membrane and microfluidic devices and subsequent application to produce Pickering emulsions have been studied (Arkoumanis *et al.*, 2019; Manga & York, 2017; Schröder *et al.*, 2018a). The need to increase production scales has led to the pursuit of other types of devices.

The use of static mixers has been increasing in industrial applications mainly due to their unique advantages such as mixing, heat transfer capabilities and operation in semi- or continuous mode (Ghanem *et al.*, 2014). These devices appear as an alternative to the traditional mechanical mixers for mixing immiscible liquids, homogenisation of solid particles and/or for heat and mass transfer improvement. Static mixers divide and redistribute streamlines in a sequential form and mixing between the fluids is ensured by the flow energy; thus, static mixers do not require external power, just the power for pumping the fluids through the mixer (Ghanem *et al.*, 2014).

Muruganandam *et al.* (2018) produced O/W emulsions using a SMX static mixer (nine perpendicular Teflon elements assembled against each other) and studied the impact of the dispersed phase concentration, flow rate and operating time on emulsion droplet size. The authors reported a decrease of the Sauter average diameter of oil droplet from 8 to 4 μm with increasing Reynolds number (Re) and a constant diameter around 4 μm when increasing the concentration of the dispersed phase from 1:100 to 1:25.

More recently, NETmix, a mesostructured static mixer (Figure 2.18 A) was applied to produce Pickering emulsions products. Ribeiro *et al*. (2021) developed Pickering emulsions in continuous mode considering an industrial perspective; n-HAp stabilised Pickering emulsions were successfully produced in continuous mode, and their size can be controlled depending on the used Re and number of cycles. The authors showed reduction of average droplet size increasing the Re or the number of cycles; the minimum average droplet size obtained was around 7 µm when using 17 cycles and Re = 400 (Figure 2.18 B). For Pickering emulsions production, NETmix ensures easy control of the parameters affecting mixing providing good reproducibility between assays, does not limit the production volume, can reduce the production time, and allows production in continuous mode.

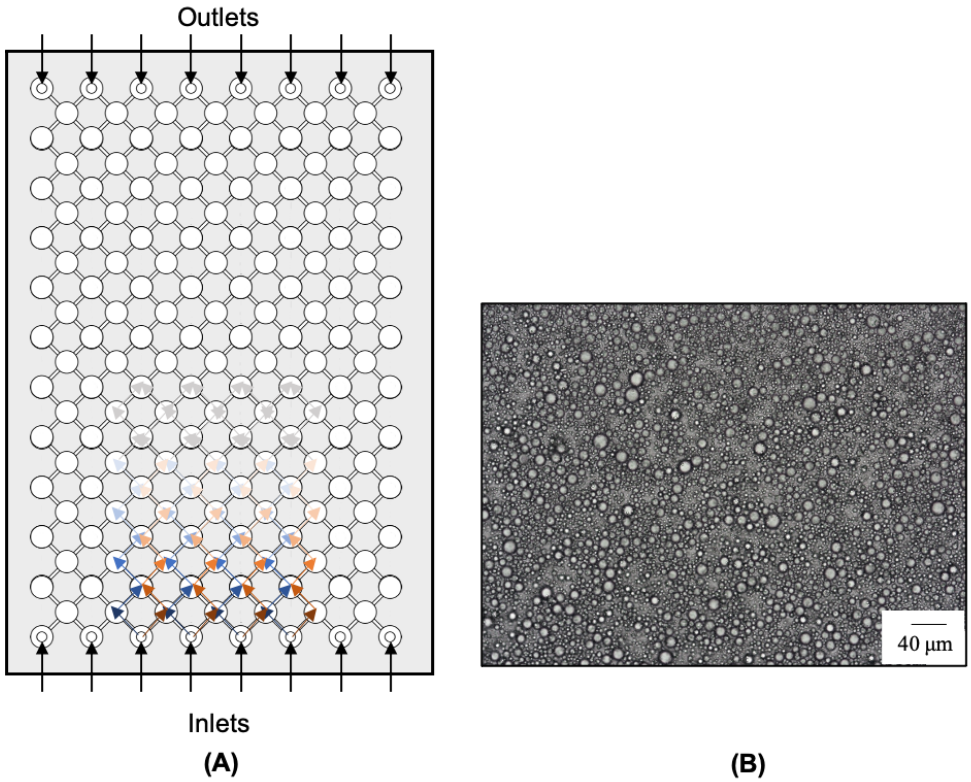

Figure 2.18: Schematization of mesostructured static mixer - NETmix (A) with representation of the fluid streams inside of the reactor, and example of Pickering emulsion produced in NETmix (B, Ribeiro *et al*. (2021)).

The higher mixing capacity of static mixers compared to currently commercially available devices enable a high potential for mixing two immiscible liquids (Ghanem *et al.*, 2014; Muruganandam *et al.*, 2018; Ribeiro *et al.*, 2021), and achievement the desired objective, mainly in terms of droplet size, with lower production costs. Static mixers such as SMX and

NETmix can offer a high mixing efficiency when compared to the traditional mechanical stirred vessels, which in most cases is a decisive criterion for overall process performance. However, in the Pickering emulsions field the number of publications in literature concerning this type of devices is still rather low.

Table 2.5 lists and describes the main production processes used for Pickering Emulsions. Overall, the choice of the device used for emulsification depends on various factors including the scale and the volume of the emulsion, the physicochemical properties of the phases, the desired droplet size distribution, and the purchasing costs, as well as their specific advantages and disadvantages (McClements, 2016) as listed in Table 2.5.

2.7 PICKERING EMULSIONS FOR FOOD APPLICATIONS

Food has an essential role in the nutrition and health of humans. It represents the primary source of energy and essential nutrients such as vitamins, minerals and bioactive phytochemicals (Caleja *et al.*, 2017). Currently, there is a growing demand for food safety and healthy products, leading to research and development of new processes and applications in food in order to satisfy the consumer's concerns.

Pickering emulsions popularity for food applications has increased in recent years; this is mainly due to their high stability compared with conventional systems, as well as to diverse range of the stabilising particles (Tavernier *et al.*, 2016). These advantages make Pickering emulsions good candidates for delivery systems since stimuli-responsive Pickering emulsions can function as a route to the triggered release ensuring the protection and/or delivery strategies of the various bioactive compounds (Winuprasith *et al.*, 2018). For food applications, the choice of the emulsifier is crucial; it should be biocompatible, non-toxic, and biodegradable or easy excreted from the body after intake; this is the principal issue and the major challenge for the food applications. To overcome this, recently and motived by the high interest of Pickering systems, several types of solid particles are being developed and/or tested.

Table 2.5: Summary of characteristics, advantages and disadvantages of main emulsification devices used for Pickering emulsions production.

Homogeniser type	Throughput	Efficiency	Droplet size		Advantages	Disadvantages
			Control	Minimum		
High shear	Batch	Low	Rotation speed Emulsification time	2 μm	Easy set-up Quick processes Low operating cost Small amounts of the liquids Different apparatus available	Particle disruption Temperature increase Broad droplet size Limited energy input
Ultrasonic	Batch	Low	Ultrasound frequency Amplitude Emulsification time	0.1 μm	Easy set-up Quick processes Small amounts of the liquids	Particle disruption Temperature increase Broad droplet size Probe degradation
High-pressure	Batch or continuous	High	Pressure value Number of homogenizing cycles	0.1 μm	Quick processes Narrow droplet size	Particle disruption Temperature increase High energy consumption Difficult cleaning
Membrane	Batch or continuous	Very high	Membrane pore size Injection rate Agitation speed	0.3 μm	Particle integrity Temperature control Narrow droplet size Low energy consumption	Set-up Slow process Viscosity of the fluids
Microfluidizers	Continuous	High	Flow rate Microchannel geometry Number of cycles Phase viscosities	0.1 μm	Particle integrity Temperature control Droplet size control Narrow droplet size Multiple emulsion production Low energy consumption	Viscosity of the fluids Set-up Slow process
Static mixers	Continuous	High	Flow rate Number of cycles	0.3 μm	Particle integrity Mixing control Temperature control Droplet size control Low energy consumption	Viscosity of the fluids

Most research performed on Pickering emulsions has been conducted with safe inorganic particles such as silica, which is used as model Pickering stabilisers for design and development of new Pickering emulsions (Schröder *et al.*, 2018b). This knowledge allows the development of potential applications for different fields, including the food industry. However, there are some challenges related to the implementation of Pickering emulsions in food that must be taken into account to ensure preservation and robustness of the Pickering emulsion. For example, electrolyte concentration, pH and other food ingredients are some examples which could affect the Pickering emulsion structural integrity.

2.7.1 SUBSTITUTION OF EMULSIFIERS IN FOOD

Such as already discussed in this work, some traditional emulsifiers commonly used in the food industry to stabilise emulsions have been related to some harmful health problems. Intoxications or inflammatory process are some examples as the body reactions to the continuous intake of emulsifiers. Pickering emulsions arise in an attempt to develop new alternatives to the conventional emulsifiers and response to consumers' issues and/or concerns.

In most works, solid particles are used just to stabilise Pickering emulsions. However, the use of solid bioactive particles can also, by themselves, improve the emulsion functionality. Example of these particles are flavonoids, polyphenols and proteins which can attribute some beneficial health effect after consumption.

Flavonoids are naturally present in fruit and vegetables, and their consumption has been associated with antioxidant and anti-inflammatory functionalities in the body (Funakoshi-Tago *et al.*, 2011). Recently, Luo *et al.*, 2012 investigated the use of three types of flavonoids (tiliroside, rutin and naringin) as stabilisers of Pickering O/W emulsions. Stabilisation was dependent on pH, increasing for higher pH. Rutin was found to improve the oxidative stability of whey protein stabilised O/W emulsions during 1 month of storage at 50 °C, and the presence of rutin was also related to the improvement of stability against coalescence (Atarés *et al.*, 2012). The authors also reported that a significant proportion of rutin was adsorbed at the oil-water interface, either partially replacing the protein or co-adsorbing with it, forming

a densely adsorbed layer at the interface that can be antioxidant and hence protect the emulsion against chemical degradation.

Polyphenols are abundant micronutrients in fruit and vegetables, and evidence an important role in the prevention of degenerative diseases such as cancer and cardiovascular diseases (Manach *et al.*, 2004). Recent studies have shown that polyphenols can act as Pickering stabilisers at the water-oil interface (Zembyla *et al.*, 2019). Zembyla *et al*. (2019) propose a novel way to stabilise water droplets via interfacial complex formation through water-insoluble polyphenol crystals and protein. The authors observed that polyphenol crystals (curcumin or quercetin) adsorb at the interface and provide stabilisation of water droplets for several days; the formation of the complex polyphenol-whey protein at the water-oil interface revealed a pronounced improvement in the stabilisation.

Proteins in emulsion systems also entail potential health benefits which may arise for the consumption of bioactive proteins or the formation of bioactive peptides post-ingestion (Shimoni *et al.*, 2013). Lactoferrin has aroused interest for its various implications to biological functions, such as antioxidant and antimicrobial activities (Nagpal *et al.*, 2011). Shimoni *et al*. (2013) studied the ability of lactoferrin to stabilise O/W emulsions. They found that the use of protein nanoparticles increased the stability of coarse emulsions but not of fine emulsions produced by high-pressure homogenisation. The combination of lactoferrin with the alginate and i-carrageenan improved emulsion stability against proteolysis during in vitro gastric digestion compared to conventional emulsifiers.

Although bioactive particles are promising as functional Pickering stabilisers, their chemical stability should also be considered. For example, since particles adsorb at oil-water interface, they become more susceptible to oxidation.

2.7.2 REDUCTION OR SUBSTITUTION OF FAT

Obesity is a worldwide health problem, which in most cases is related to excessive intake of saturated fatty acids commonly encountered in the processed foods. Ingestion of a considerable amount of saturated fatty acids can lead to severe health problems such as cardiovascular diseases (Briggs *et al.*, 2017). In this way, there is an increased interest to

substitute the saturated fatty acids by healthier alternatives. A possible approach is the use of Pickering emulsions as templates to develop low-viscosity liquid oil into soft gels *(Gao et al.*, 2014) or high internal phase emulsions (HIPEs) (Okuro *et al.*, 2019; Yuan *et al.*, 2017). Gao *et al*. (2014) developed a zein protein sodium stearate complex-based O/W Pickering emulsion, a suitable process for producing oil gels. Pickering emulsions with sodium stearate and zein (10 mM and 0.5 wt%, respectively) revealed to be a homogeneous and translucent gel without oil leakage. Water-in-oil HIPEs can provide interesting textures and can be used to reduce trans- and/or saturated fat content in food products. HIPEs are characterised by their high disperse phase volume ratio (ϕ) of 0.74 or higher, showing droplets tightly packed and the continuous phase acting as a liquid film giving to these emulsions a highly viscous characteristic (Okuro *et al.*, 2019). In this way, HIPEs can serve as a direct substitute for the oil in order to decrease the fat intake satisfying the consumer's demand for healthier products. Yuan *et al*. (2017) and Okuro *et al*. (2019) studied the production of HIPEs with gliadin/chitosan complex particles and PgPr and lecithin, respectively. Both particles can be used to produce surfactant-free O/W Pickering emulsions and Pickering HIPEs.

Another alternative is the replacement of saturated fats by polyunsaturated fats, which are known to be healthier. In this line of thought, Pickering emulsions are an advantage due to high physical and chemical stability, since polyunsaturated fats are highly susceptible to lipid oxidation. Kargar *et al*. (2011) and later Kargar *et al*. (2012) are some examples of works that studied the oxidative Pickering emulsion stability with silica and microcrystalline cellulose and modified starch. Particles demonstrated the ability to separate pro-oxidants present in the continuous phase from hydroperoxides located at the droplet interface (Tavernier *et al.*, 2016).

The high particle concentrations in the continuous phase may have a filler effect or function as a fat substitute (Tavernier *et al.*, 2016). As an example, Skelhon *et al*. (2012) created healthier chocolate which was infused by fruit juice via W/O Pickering emulsion; the emulsion was produced with silica-chitosan particles. The authors replaced ~50 wt% of the chocolate fat with fruit juice in the form of emulsion droplets.

2.7.3 ENCAPSULATION OF ACTIVE COMPOUNDS AND DEVELOPMENT OF FUNCTIONAL FOODS

Functional foods refer to those foods that have an active or functional compound, which is not naturally present. It should add benefits or functions that otherwise, the food would not have (Caleja *et al.*, 2017). Vitamins, including the oil-soluble vitamins (A, E, D and K), fatty acids ($\omega - 3$), dietary fibres, proteins or natural bioactive compounds such as polyphenols are some examples of the active compounds that are usually used to fortify the final food product (Tavernier *et al.*, 2016). These active compounds, which have mostly antioxidant capabilities, are incorporated in the food matrix in order to provide physiological benefits preventing some diseases such as heart diseases, hypertension, inflammatory processes among others (Carocho *et al.*, 2018; Heleno *et al.*, 2015).

Lipophilic bioactive compounds, specifically vitamins, have reduced solubility in water and can also be unstable in adverse conditions (Winuprasith *et al.*, 2018; Yang & McClements, 2013). Thus, O/W emulsions are among the most relevant and versatile encapsulating and delivery systems of these compounds for food applications. Pickering emulsions, which are known as excellent physical-chemical stability properties, can serve as encapsulating paths for hydrophobic compounds, and at the same time can improve the stability process and bioaccessibility for target delivery and controlled release (Tavernier *et al.*, 2016). The encapsulation technique allows the production of a barrier that protects sensitive compounds from the hostile environment; in this way, it can lead to effective absorption of the active compound by the body (Leal-Calderon & Schmitt, 2008).

In recent years, various works report the role of Pickering emulsions on emulsion stability, encapsulation, and release of bioactive compounds. For example, Tikekar *et al*. (2013) produced O/W Pickering emulsions through silica particles and used the emulsions as controlled-released vehicles for curcumin. Curcumin, which is a hydrophobic polyphenol, has significant antioxidant and anti-inflammatory properties but it is quickly unstable under unfavourable environment conditions (Anand *et al.*, 2007) and has to be encapsulated to maintain its bioactivity. Tikekar *et al*. (2013) reported that silica stabilised Pickering emulsions have ~80% of curcumin retention after gastric digestion and ~60% of curcumin release after two hours of intestinal digestion.

Zhou *et al*. (2018) studied the use of oregano essential oil in Pickering emulsions stabilised by cellulose nanocrystals for the delivery of antimicrobial essential oils in the food industry. Results showed that the oregano essential oil Pickering emulsions inhibited the growth of four microorganisms (*Escherichia coli, Staphylococcus aureus, Bacillus subtilis* and *Saccharomyces cerevisiae*) by destroying the integrity of the respective cells membrane.

Vitamin D_3 is a fat-soluble vitamin and essential for humans but its synthesis is only achieved after sun exposure (Winuprasith *et al.*, 2018). Pickering emulsions emerge as a good approach to increase the use of this vitamin. Winuprasith *et al*. (2018) studied the encapsulation of vitamin D_3 using Pickering emulsions through mangosteen cellulose particles. The authors reported that for relatively low levels of solid particles, the vitamin can be digested and absorbed in the gastrointestinal tract. However, when large amounts of solids particles were used, the particles function as a "protective shell" inhibiting the release of the vitamin in the gastrointestinal tract.

Furthermore, as an example of Pickering emulsions applied in a food matrix, Stratulat *et al*. (2015) used this approach for fortifying cheese with vitamin D_3. Their results showed a recover level of vitamin D_3 around 84% maintaining its stability during 3 months of storage. Overall, the results indicate that the encapsulation of vitamin D_3 in cheese in the form of emulsified particles increased retention and stability of the vitamin in the curd and improved the chemical stability of fortified chess against oxidation.

Pickering emulsions have been shown their ability to stabilise and facilitate the release of lipophilic bioactive compounds. These studies suggest that particle-stabilised interfaces allow for excellent physicochemical stability during storage and targeted release in the gastrointestinal tract.

2.8 CONCLUSIONS

Emulsions stabilised by solid particles, Pickering emulsions, offer attractive advantages compared to conventional emulsions, stabilised by emulsifiers. The generally good inherent stability of Pickering emulsions is the most important advantage. Pickering emulsions have

attracted attention in different research and industrial fields over the last 10 years, leading to an increase in emulsions applicability in a wide range of products.

Various kinds of solid particles with different properties have been investigated as Pickering stabilisers. However, a wide range of parameters must be in consideration to develop a stable Pickering emulsion. Parameters such as particles properties (wettability, size, shape, concentration, and charge), aqueous phase properties (electrolyte concentration and pH) and oil phase properties (oil phase and oil/aqueous ratio) have an important role in the government of Pickering emulsion stability. These parameters are often related, and changing one parameter, may lead to changes in other parameters, altering the emulsion system. Thus, these parameters should be considered in the design and development of stable Pickering emulsions.

Pickering emulsions are recognised as long-term stable emulsions; the mechanisms that govern the Pickering emulsion stability are different from conventional emulsions. Interactions between solid particles and the dispersed and continuous phases, attractive interactions between solid particles at the oil-water interface, and attractive interactions between solid particles in the continuous phase are the most important mechanisms that enable emulsion stabilisation. These confer unique stabilisation properties to the Pickering emulsions.

Safe-inorganic solid particles can be used as Pickering stabilisers. Usually, they are well-stabilised particles with specific and constant size. Silica particles are the most studied, but others can be used, such as hydroxyapatite.

Pickering emulsions are predominantly prepared using high shear techniques such as rotor-stator, high-pressure homogenisers, and ultrasonic homogenisers, since these technologies are also the most applied and available methods to produce conventional emulsions, both at laboratory and industrial scales. Although these technologies enable relative fast production, they are characterised by the lack of control during production. Membranes and microfluidic devices appear to overcome the disadvantages of high-shear technologies producing Pickering droplet emulsions in a controlled manner and with a lower polydispersity. However, to answer the demand of Pickering emulsions and industrialisation, devices such as static

mixers have been used to produce the emulsions stabilised by solid particles. These devices enable high performances since continuous production is allowed; additionally, the emulsion droplet size control and reproducibility are also characteristics of static mixers.

Overall, this review focus on safe inorganic solid particles as Pickering stabilisers, reporting the main stabilisation mechanisms and the main parameters that govern the Pickering emulsion stability. A review of the used methods to produce Pickering emulsions was also given. Finally, the relevance of Pickering emulsions on the food industry was described.

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3 NANOHYDROXYAPATITE AS A PICKERING STABILISER IN OIL-IN-WATER EMULSIONS

This chapter is based on: Andreia Ribeiro, Yaidelin Manrique, Isabel C.F.R. Ferreira, Filomena Barreiro, José Carlos B. Lopes, Madalena M. Dias (2020). Nanohydroxyapatite (n-HAp) as Pickering stabilizer in oil-in-water (O/W) emulsions: a stability study. *Journal of Dispersed Science and Technology*. DOI: 10.1080/01932691.2020.1845199

Surfactant-free emulsions, such as Pickering emulsions, have been gaining an increased interest. They constitute green alternatives to the current industrial practices in emulsion technology finding diverse technological uses. In this work, nanohydroxyapatite (n-HAp) was tested as an oil-in-water (O/W) Pickering stabiliser using a chemical system comprising sunflower oil at different O/W ratios (10/90 to 60/40) and n-HAp at concentrations ranging from 0.5 to 15 wt%. The produced emulsions were characterised, and stability evaluated over a two-month period. Based on the results achieved for the 20/80 series, a model to predict the emulsion stability taking into account the O/W ratio, total solids content, droplet diameter, and n-HAp dimensions, was developed. Cryo-SEM evidenced the attachment of n-HAp particles at the oil surface (oil core-n-HAp shell morphology), corroborating their role as Pickering stabilisers. The experimental results, versus the predicted results were compared using a ternary phase diagram, which evidenced the formation of three zones (unstable, stable and gel) depending on the used O/W ratio and n-HAp concentration. Moreover, the comparison of the predicted with the obtained experimental data validated the model predictability.

3.1 INTRODUCTION

Emulsions are mixtures of two immiscible liquids, oil and water, traditionally stabilised by one or more synthetic emulsifiers. However, some of these emulsifiers have been associated with allergic reactions and carcinogenicity, leading to health problems (Yang *et al.*, 2017). In this context, Pickering emulsions, which are based in non-toxic solid particles, have started to appear as alternatives (Berton-Carabin & Schroën, 2015). In Pickering emulsions, solid particles get attached at the interface between the two immiscible liquids, stabilising the emulsified droplets against coalescence (Dickinson, 2012).

The first mention to Pickering emulsions dates from the 1900s (Pickering, 1907; Ramsden, 1903), and since then an increased interest has been observed, both at academic and industrial level, due to the non-toxic nature of the particles and the possibility to add functional properties (Berton-Carabin & Schroën, 2015; Hu *et al.*, 2015). Pickering emulsions have found applications in areas such as pharmaceutical, agrochemical, and food, among others (Albert *et al.*, 2019; Berton-Carabin & Schroën, 2015; Son *et al.*, 2015). This process allows the formation of a protective shell around the oil or aqueous core, enabling their use as encapsulation systems for drug and bioactive compounds (Berton-Carabin & Schroën, 2015; Frelichowska *et al.*, 2009; Hu *et al.*, 2015).

In Pickering emulsions, the type of formed emulsions depends on the particle's hydrophobicity. More specifically, predominantly hydrophilic particles, with water contact angles between 60° and 80°, are adequate to stabilise oil-in-water (O/W) emulsions, whereas predominantly hydrophobic particles (contact angle between 100° and 130°) are suitable to stabilise water-in-oil (W/O) emulsions (Dickinson, 2012). However, some works also reported that the type of formed emulsions, and associated stability, depends on other parameters, such as the oil-water ratio and solids content (Zhang *et al.*, 2017b). The assortment of studied Pickering stabilisers comprises organic and inorganic solid particles, such as modified silica (Björkegren *et al.*, 2017), protein and polysaccharide-based particles (Barkhordari & Fathi, 2018; Meshulam & Lesmes, 2014), and fat crystals (Schröder *et al.*, 2018).

Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) is widely used in the biomedical and biotechnological fields, due to its excellent biocompatibility, since it is the main component of both human bones and teeth (Zhang *et al.*, 2017). Recently, this material started to be described as a suitable Pickering stabiliser, which due to its hydrophilicity tends to stabilise O/W emulsions (Zhang *et al.*, 2017; Zhang *et al.*, 2017b). In this scope, some authors have developed Pickering emulsions stabilised by HAp particles, in most cases combined with other polymers. Fujii *et al*. (2012), Zhang *et al*. (2017b) and Okada *et al*. (2012) produced microspheres via Pickering emulsion by using n-HAp dispersed in the aqueous phase and poly-**ε**-caprolactone (PCL), poly-L-lactic acid (PLLA) and polystyrene dissolved in the oil phase. Zhang *et al*. (2017) and Hu *et al*. (2015) evaluated the effect of HAp surface modification with stearic acid and sodium oleate, respectively, aiming at improving Pickering emulsions stability. Samanta *et al*. (2017) and Hu *et al*. (2014) prepared nanocomposite scaffolds using Pickering emulsions as templates. In these two works, HAp was functionalised with PCL and PLLA in order to ensure emulsion stability during the scaffold's development.

To stabilise a Pickering emulsion, the solid particles should be irreversibly anchored at the oil or water surface to form a steric barrier (Liu & Tang, 2016). In this regard, some authors explained the Pickering emulsion stabilisation mechanism through theoretical approaches and thermodynamic calculations based on the nature of the interactions between the liquids and the solid surface. For that Young equation was applied using surface energy considerations in order to interpret the interactions between the solid particles and the two liquid phases, and, consequently, to predict the contact angles (Binks & Clint, 2002; Binks & Olusanya, 2017; Hodge & Rousseau, 2005; Menon & Wasan, 1988).

Formation and stability of Pickering emulsions depends on various factors such as particle wettability, particle concentration, particle size and shape, coverage density and pH of the aqueous phase (Linke & Drusch, 2018). Previous studies have shown that there is a correlation between droplet size and particle concentration, and, consequently, with emulsion stability (Albert *et al.*, 2019; Rayner, 2015), and a model to predict particle's concentration at the core surface was developed. This model is based on the simple mass-balance, based on the argument that particles show similar behaviour to surfactants when electrostatic repulsion is suppressed (Linke & Drusch, 2018; Tcholakova *et al.*, 2008) and considering that Pickering

emulsions undergo "limited coalescence" during emulsification (Arditty *et al.*, 2003; Kalashnikova *et al.*, 2011; Tcholakova *et al.*, 2008). Thus, the interfacial area, created during emulsification, should be larger than the area that can be covered by the particles, so that when stirring stops, in order to reduce the interfacial area, the droplets coalesce until the oil/water interface is adequately covered by the particles (Albert *et al.*, 2019; Arditty *et al.*, 2003; Tcholakova *et al.*, 2008; Xiao *et al.*, 2016). When this phenomenon occurs, the emulsion has a unimodal droplet size distribution that is directly related to the particle mass and droplet coverage by (Albert *et al.*, 2019)

$$\frac{1}{D} = \frac{m_p a_p}{6C\rho_p V_d v_p} \tag{3.1}$$

where D is the final drop diameter, m_p is the mass of particles, C is the surface coverage (the fraction of the interfacial area covered by the particles), ρ_p is the particle density, V_d is the volume of dispersed phase, a_p is the particle area projected on the interface and v_p is the particle volume. However, this equation can be applied only if: (i) the particles are completely and irreversibly attached at the surface; (ii) the emulsification process produces more oil/water interface than what the particles can cover; (iii) the emulsion is produced with intermediate particle concentration; and (iv) electrostatic repulsion is suppressed (Albert *et al.*, 2019; Arditty *et al.*, 2003; Linke & Drusch, 2018).

At low solid particles concentration, instability is often observed due to a lack of particles to stabilise the droplets (Albert *et al.*, 2019). At high solid particles concentration, there is an excessive number of particles compared to the oil/water interfacial area created during the emulsification process. In this case, the stabilisation can occur in different ways: (i) the droplets formed during the emulsification process are stabilised right away inducing size heterogeneity, or (ii) constant droplet sizes are obtained but particles are in excess in the continuous phase leading to a network in the continuous phase that can improve the emulsion stability (Albert *et al.*, 2019; Wu & Ma, 2016). Arditty *et al.* (2003) have shown that when a low SiO_2 solid particle concentration is used, the emulsion droplet surface is not covered completely and droplet coalescence occurred, destabilising the emulsion system. Aveyard *et al.* (2003) found that with an increase of the SiO_2 particle concentration, up to 3%, the droplet

size of the emulsion decreases; however, further increase in particle concentration does not change the droplet diameter, and the particles in excess stay dispersed in the continuous phase. Recently, Liu and Tang (2016) also determined the percentage of soy glycinin particle's surface coverage and particle centre-to-centre distance. The extent of surface coverage was obtained directly from the ratio between the potential coverage area of the soy glycinin particles, and the total interfacial area of the emulsion droplets. Additionally, and in order to describe the packing of the particles at the interface, the distance between them was estimated using two models (square and regular hexagonal array models).

The aim of this work was to develop stable O/W Pickering emulsions using n-HAp particles as stabilisers. The impact of process parameters, such as oil-water ratio and solids (n-HAp) content, on emulsion stability through the evaluation of phase separation by creaming or sedimentation occurrence, was studied. Pickering emulsions were characterised in terms of particle's size and shape by laser diffraction techniques and microscopy and studied concerning their rheological behaviour. The emulsion stability as function of the oil-water ratio and n-HAp concentration was evaluated after 2 days, 2 weeks, and 2 months. In order to predict the formation and stability of n-HAp Pickering emulsions a model was developed and compared with the experimental results.

This paper aims at the development of Pickering emulsions using a chemical system based on nanohydroxyapatite (n-HAp). The work reports experimental data on emulsions preparation and characterisation covering a wide range of conditions and introduces a model to explain the observed stability behaviour. To the best of our knowledge this is the first work reporting a model where n-HAp was used as Pickering stabilisers and that emulsion stability can be predicted with a model that takes into account the oil-water ratio, total solids content, droplet diameter, and n-HAp dimensions. Thus, this work is a step forward in the field of synthetic surfactants substitution contributing for the sustainable development of emulsions, which might find use in a wide range of applications, namely, to act as vehicles to support the delivering of functionalities.

3.2 MATERIALS AND METHODS

3.2.1 STANDARDS AND REAGENTS

N*ano*XIM-CarePaste used in this work was supplied by Fluidinova S.A. (www.fluidinova.com) (Silva *et al*., 2008). According to the technical information provided by Fluidinova, *Nano*XIM-CarePaste is composed by 15.5 ± 0.5 wt% of n-HAp particles with typical size below 50 nm in a rod-like shape, and 4.5 ± 0.5 wt% KCl. The water content is ≤ 81.0 (wt%). This n-HAp paste was chosen due to its readily commercial availability. The presence of KCl in the *nano*XIM-CarePaste is a result of its synthesis process, and in this work this paste was used as such.

Distilled water, treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA), was used as the aqueous phase. Sunflower oil was purchased from a local supermarket and used as the oil phase. It is a food grade vegetable oil obtained from sunflower seeds and mainly constituted by polyunsaturated fatty acids.

3.2.2 PREPARATION OF THE PICKERING EMULSIONS

Emulsions preparation comprised two steps, namely the preparation of the n-HAp dispersions, followed by oil addition to form the final Pickering emulsion. The experimental set-up is similar to the one developed by Ruphuy *et al*. (2016) (Figure 3.1). In the first step, the n-HAp aqueous paste was dispersed in water at different concentrations using a rotor-stator homogeniser (Miccra RT D-9, Mülheim, Germany) to ensure nanoparticles dispersion. Briefly, the n-HAp paste was injected into the reaction vessel (C) containing the water, through a feeding channel (D) using a peristaltic pump at 240 rpm (43 mL/min, E). The rotor-stator homogeniser device (B) was set-up to operate at 39000 rpm during 4 min. Additionally, a thermostatic bath (A) was coupled to the system to ensure temperature control of the reaction vessel (20 °C).

In the second step, the sunflower oil was fed dropwise into the previously prepared n-HAp aqueous dispersion using a peristaltic pump (E) operating at 240 rpm. The emulsification step was carried out using a rotor-stator homogeniser (B); after the oil addition, the system was

kept under stirring, set to operate at 11000 rpm during 4 min and constant temperature (20 °C).

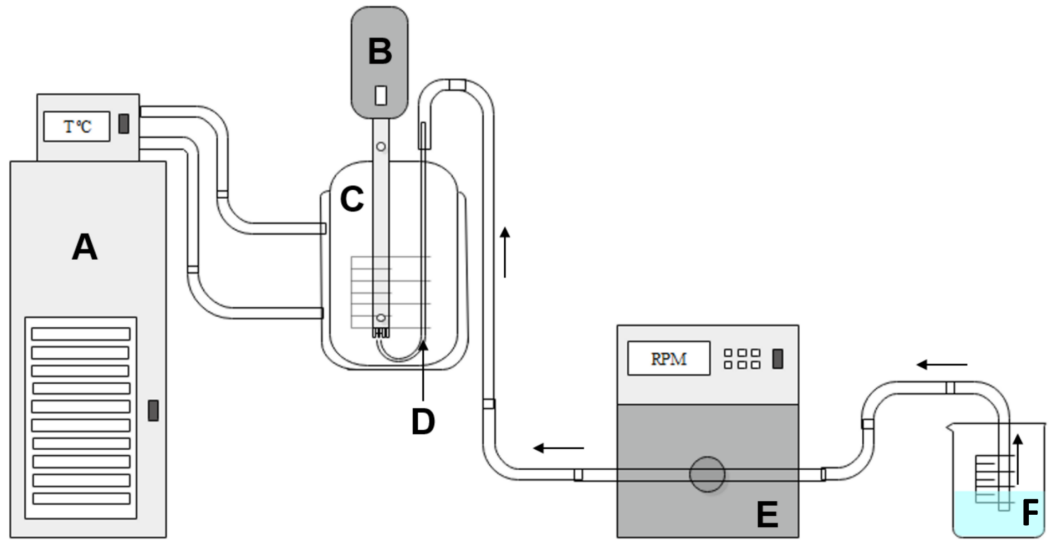

Figure 3.1: Experimental setup used to prepare n-HAp dispersions and Pickering emulsions. A) thermostatic bath, B) rotor-stator homogeniser, C) mixing reactor, D) feeding channel, E) peristaltic pump, and F) beaker with n-HAp paste or oil.

Various n-HAp concentrations (water-basis, wt%) and oil-water ratios (defined as the ratio between the oil and the water phases, *v/v*) were tested. The concentration of n-HAp ranged from 0.5 wt% to 15 wt% (water phase-basis), with 15 wt% corresponding to the original paste, and the lower concentrations were obtained by dilution. The oil-water ratio ranged from 10/90 to 60/40 *(v/v)*. Table 3.1 summarises the tested formulations.[1] The experimental assays were based on a 400 ml total volume scale of the aqueous phase. All emulsions were prepared in duplicate.

3.2.3 Characterisation of the Pickering emulsions

The produced emulsions were classified as O/W or W/O type by using the conventional emulsion drop test (Zhang *et al.*, 2017). This test evaluates the emulsion behaviour upon in contact with the two used phases (oil and water). If the Pickering emulsion drop is rapidly dispersed in water and remains agglomerated in the oil phase, it is O/W type; otherwise, it is

[1] After publication, further assays were performed to confirm the model results. These results are shown in Appendix A.

a W/O emulsion. To perform this test, 2-3 emulsion drops were added to the water and oil phases followed by gentle stirring before visual inspection.

Table 3.1: Pickering emulsions: n-HAp concentrations and oil-water ratio.

TYPE OF HAP PASTE	OIL-WATER RATIO (*v/v*)	n-HAP CONCENTRATION (wt%)
*nano*XIM-CarePaste	10/90	1.5, 5.0, 10
	20/80	0.5, 1.5, 2.0, 2.5, 3.0, 5.0, 7.0, 10, 15
	40/60	1.5, 5.0, 10
	60/40	1.5, 5.0, 10*

*Not used due to preparation difficulties.

The stability of the produced Pickering emulsions was checked immediately after preparation (0 days) and monitored along the storage time (a total time-frame of two months was used with sampling at 2 days, 2 weeks, and 2 months). Instability occurrence (creaming or sedimentation) was visually checked and photographically registered with a Nikon digital camera (Nikon D3400, Tokyo, Japan). In parallel, emulsion's morphology was checked by optical microscopy (OM) using a Carl Zeiss Axiotech 100 HD microscope (Zeiss Instruments, Jena, Germany) equipped with a digital camera (AxioCam 105 colour; Zeiss Instruments, Jena, Germany). To perform these analyses, a sample aliquot was placed on a microscope slide, then gently covered with a coverslip. Image acquisition and processing were performed using Zen software (2.3; blue edition). Both transmitted and reflected light modes were used. Complementary, size distributions were determined from fresh Pickering emulsions using laser diffraction through coulter (LS230 Series instruments, Brea, California) and the corresponding average size (in volume) was calculated.

Cryo-SEM (Cryo-Scanning Electron Microscopy) was used to inspects the morphology of the produced Pickering emulsions. The analyses were carried out in a high-resolution scanning electron microscope (JEOL JSM 6301F, Washington, USA), with X-ray microanalysis (Oxford INCA Energy 350, Wiesbaden, Germany), and cryo-SEM experimental facilities (Gatan Alto 2500, München, Germany). The sample was rapidly cooled by plunging it into sub-cooled nitrogen and then transferred under vacuum to the cold stage of the preparation chamber. Thereafter, the sample was fractured, sublimated (180 seconds at -90 °C), and coated with

Au/Pd (45 seconds under 12 mA current). Finally, the sample was transferred into the SEM chamber and observed at -150 °C. This characterisation was intended to check the successful formation of the emulsion droplets and their coverage with the n-HAp Pickering particles. This analysis was performed with the sample prepared with an O/W ratio of 20/80 and 5 wt% n-HAp.

Rheological studies were carried out to understand the impact of the n-HAp concentration and the oil volume fraction on the viscous and viscoelastic behaviour of the produced emulsions. The tests were done using an Anton Paar rheometer (Anton Paar, GmbH, Austria) with a parallel plate. All measurements were made with a fixed gap of 0.5 mm and at 20 °C. The viscosity measurements of the Pickering samples were determined as a function of the shear rate in a range from 500 to 4500 1/s. The frequency sweep was oscillated from 0.3 to 100 rad/s, and all measurements were performed within the determined linear viscoelastic region (LVE) and made at 0.1% strain. Data is reported as elastic or storage modulus (G') or loss modulus (G'') as a function of frequency.

3.3 Results and discussion

3.3.1 Morphology of the formed Pickering emulsions

All the produced emulsions were of O/W type, as checked by the drop test (i.e., all the emulsions droplets dispersed in water medium). Moreover, Figure 3.2 shows cryo-SEM images obtained for a fractured cross-section of the frozen Pickering emulsion using an oil-water ratio of 20/80 and 5 wt% of n-HAp. Cryo-SEM is one of the few techniques available to visualise the interfacial structure of emulsions stabilised by solid particles (Binks & Kirkland, 2002). During sample preparation, namely during the sublimation step, the water phase is evaporated while the oil phase remains.

Figure 3.2 A and B show frozen Pickering droplets at 1000x and 3000x magnification, respectively. Some non-spherical shape and incomplete Pickering droplets were observed, which can be explained by the freezing and fracturing of the sample, which in some cases, also implies the fracturing of the n-HAp layer, resulting in the exposure of the oil core. Additionally, the continuous phase is characterised by the presence of striations, which can

be related with the excess of n-HAp remaining free in the continuous phase or due to slow freezing, which may lead to the formation of salt crystals since the n-HAp paste has KCl. Previously, Binks and Kirkland (2002) documented the presence of striations in cryo-SEM images of the Pickering emulsions and related their presence with the use of excessive solid particles. Griffith and Daigle (2018) also showed the presence of the striations (referred as a bridged network) on the continuous phase of the Pickering emulsion and related it as excessive solid particles. They also concluded that these solid particles were responsible for inhibiting long term emulsion creaming and coalescence.

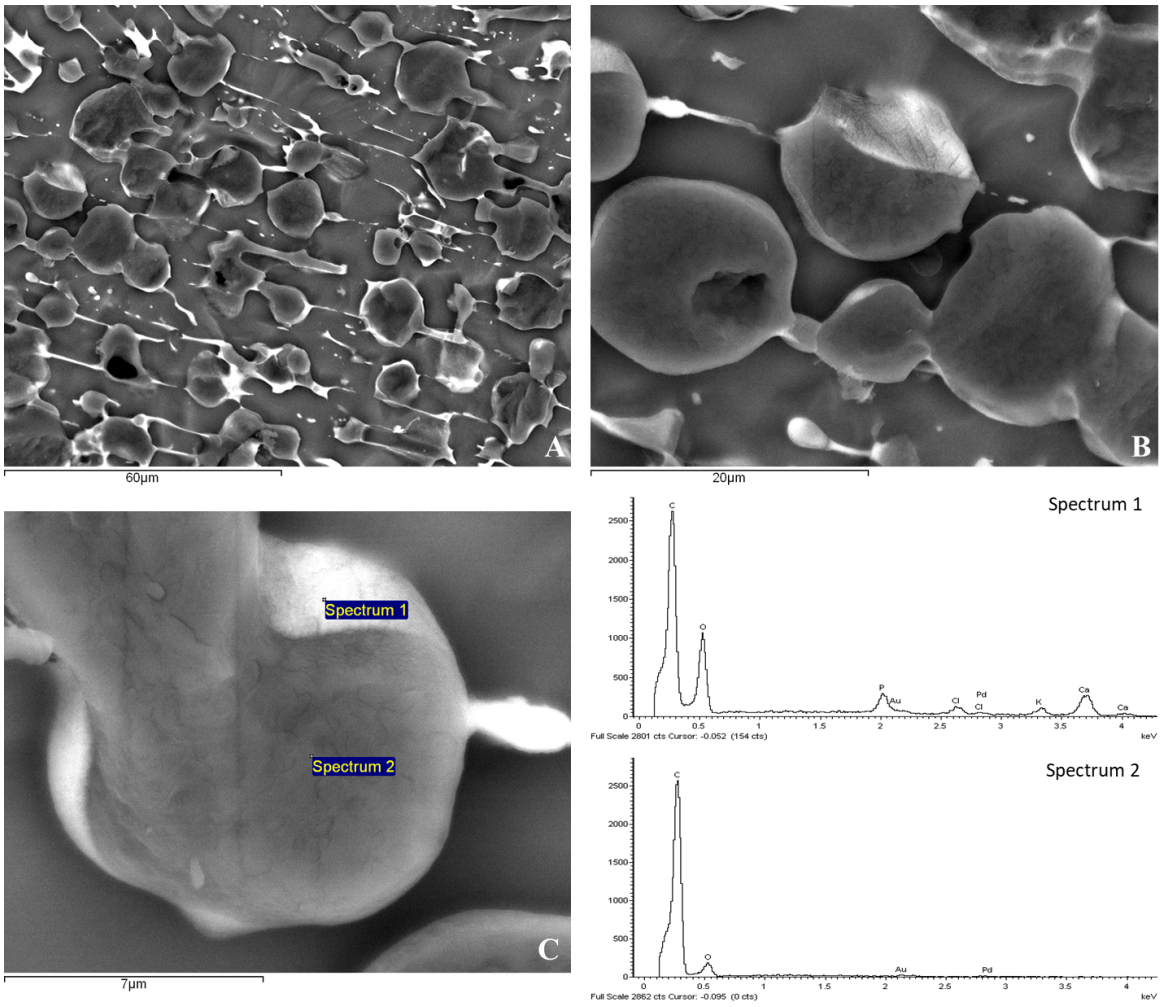

Figure 3.2: Cryo-SEM micrographs for fractured cross-section of a Pickering emulsion using a 20/80 oil-water ratio and 5 wt% of n-HApCare (with KCl): A) 1000x, B) 3000x and C) 8000x. The two EDS spectra correspond to the identified points in image C (spectrum 1 and spectrum 2). All micrographs were obtained with 15 Kv.

Figure 3.2 C details a single Pickering droplet and the respective EDS (Energy Dispersive X-Ray Spectroscopy) analysis acquired in two different points – outside (spectrum 1) and inside (spectrum 2) of the droplet. From the droplet observation, it is possible to view a white layer

around the oil droplet, evidencing that the n-HAp particles were attached to the oil droplet surface. In agreement with paste composition, spectrum 1 shows the presence of n-HAp and KCl salt in the layer structure through the presence of oxygen (O), phosphorus (P), calcium (Ca), potassium (K) and chloride (Cl). Spectrum 2, taken inside the droplet, indicates the presence of oil through at the existence of just carbon (C), and oxygen (O) the major elements in the oil (triacylglycerol). Thus, this analysis confirmed that the particles had an oil core covered by a n-HAp shell (shell-like morphology). The presence of the gold (Au) and palladium (Pd) elements result from the used coating process. An estimate of the n-HAp shell thickness was obtained through the measurement of the white border of a Pickering droplet resulting on an average value of 200 nm. This value indicates a thickness higher than one individual n-HAp (4-fold the dimensions of a n-HAp particle, which is around 50 nm), meaning that deposition of multiple n-HAp layers has occurred.

3.3.2 ANALYSIS OF PHASE SEPARATION

Phase separation is a catastrophic phenomenon for any emulsified product, compromising its shelf-life. Thus, stability under storage conditions is a key parameter to study since it determines, to some extent, if the product is suitable for a given application (Hu *et al.*, 2016).

3.3.2.1 Effect of n-HAp concentration

To evaluate the effect of n-HAp concentration in the stability of Pickering emulsions, the oil-water ratio was fixed at 20/80 (*v/v*) with n-HAp concentration varying between 0.5 to 15 wt% (0.5, 1.5, 2.0, 2.5, 3.0, 5.0, 7.0, 10, and 15 wt%). The stability of the prepared Pickering emulsions was monitored along a two-month period under storage at room temperature and natural light exposure. Production of emulsions in the absence of n-HAp particles (control emulsions) was also performed in the testing of the mixing system, leading to the expected phase separation within a short timeframe (data not shown).

Figure 3.3 shows the optical microscopy images of the Pickering emulsions produced with different n-HAp concentrations as a function of storage time. All images were obtained with a magnification of 20x. Right after the emulsification step (0 days), all the produced Pickering emulsions presented a morphology characterised by spherical shape and individualised

droplets with a size ranging from 10-25 μm (values corroborated by laser diffraction from the average size in volume). Moreover, the data obtained by laser diffraction (Table 3.2) puts in evidence the decrease trend of the mean droplet size as the n-HAp content increases. However, at low n-HAp concentration (1.5 wt%), the emulsions become unstable after a short period of time, as can be observed in the OM image acquired after 2 days (Figure 3.3 – 1.5 wt% n-HAp), where larger and irregular shaped droplets can be observed, pointing out to the occurrence of coalescence. For this n-HAp concentration, this phenomenon increased along storage time, becoming highly noticeable after the two-month period, together with n-HAp particles agglomeration which is visually associated with the presence of darker regions in the microscopy images. On the other hand, stable Pickering emulsions were obtained for higher n-HAp concentrations. For example, when 5 wt% was used, individual and spherical droplets were observed along the examined storage time, i.e., particles retained their initial size and shape. In these cases, the absence of coalescence was noticed, meaning that higher n-HAp concentrations, in this study up to 10 wt%, favoured Pickering emulsion stability. *Gel-like* Pickering emulsions were achieved when n-HAp was used at 15 wt% concentration. The stability of the Pickering emulsions can also be verified through the evaluation of the data in Table 3.2, where it is possible to observe the maintenance of the mean droplet size for the stable Pickering emulsions along the timeframe of storage.

Macroscopically, initially all the prepared Pickering emulsions were homogenous, i.e., no phase separation was observed. However, after two days, phase separation started to be observed for emulsions prepared with 1.5 wt% of n-HAp, and after 2 weeks for the ones prepared with 2.5 wt%. In these cases, a layer at the top of the emulsion (creaming) was observed.

The presence of KCl in the used n-HAp, which is associated with its synthesis process, might also help to promote emulsion stabilisation, as previously reported (Ashby & Binks, 2000; Yang *et al.*, 2006). Nevertheless, the concentration of the used solid particles was also reported to significantly affect Pickering emulsions stability, as well as the obtained droplet size, as was also verified in the present work. For example, Zhang *et al*. (2017b) showed that the n-HAp concentration had a fundamental role in their stabilisation since stable emulsions were only achieved when high concentrations were used. The same behaviour was verified

with studies using other solid particles such as soy glycinin, starch nanocrystals and hemp powder (Liu & Tang, 2016; Ou *et al.*, 2019; Yang *et al.*, 2018). Moreover, the droplet size of Pickering emulsions decreased with the increase of particle's concentration with a consequent improvement of the emulsion stability (Liu & Tang, 2016; Ou *et al.*, 2019; Yang *et al.*, 2018).

Table 3.2: Effect of n-HAp concentration and oil-water ratio in the mean droplet size right after preparation and at the end of the study period (2 months).

		MEAN DROPLET SIZE *(μm)*	
	n-HAp concentration	0 days	2 months
Fixed oil-water ratio 20:80	1.5 wt%	20.46	-
	2.5 wt%	19.41	-
	5.0 wt%	18.42	18.61
	10 wt%	15.94	15.37
	15 wt%	14.73	14.49
	Oil-water ratio	0 days	2 months
Fixed n-HAp concentration 5 wt%	10:90	12.71	-
	20:80	18.42	18.61
	40:60	19.50	19.67
	60:40	23.08	23.02

- Not evaluated since the emulsion become unstable (phase separation).

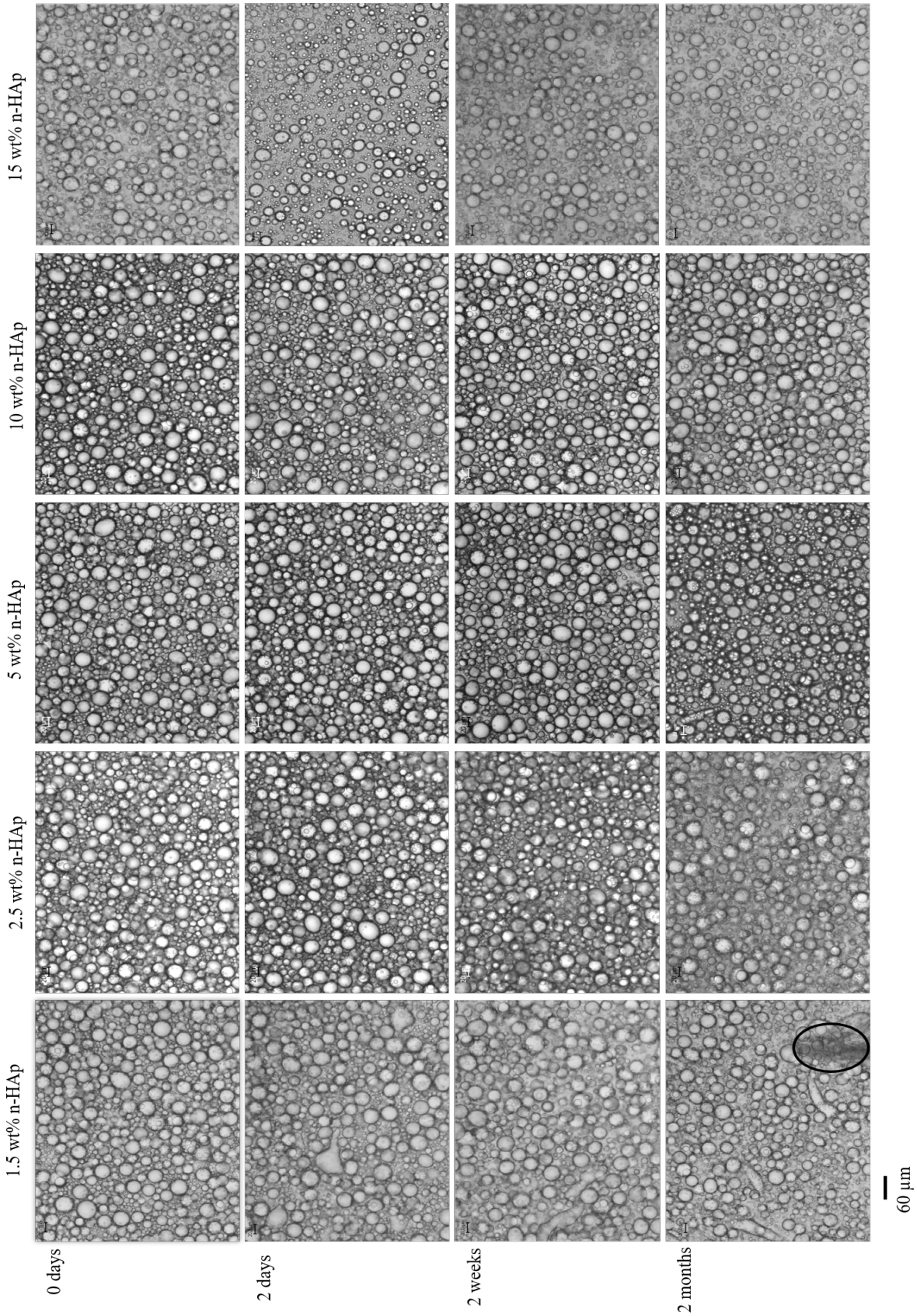

Figure 3.3: OM images, acquired in transmitted light mode, of Pickering emulsions stabilised with different n-HAp concentrations and fixed oil-water ratio (20/80, *v/v*). All images were registered at 20x magnification. The circle on the 2-month 1.5 wt% image shows regions of n-HAp agglomeration.

3.3.2.2 Effect of oil-water ratio

The effect of the oil-water ratio on the Pickering emulsion stability was evaluated by studying oil-water ratios of 10/90, 20/80, 40/60 and 60/40 at a fixed n-HAp concentration of 5 wt%.

Figure 3.4 shows the obtained OM images along the two-month period evaluation. For the whole range of tested oil-water ratios, well-defined droplets right after emulsification (0 days) were observed. Comparing different oil-water ratios, and as expected, it is possible to observe that higher values lead to a higher number of droplets. Namely, in the 60/40 ratio images, dense emulsions with tightly packed droplets are perceived. This effect was also observed by Yang *et al*. (2018) for Pickering emulsions with other solid particles (starch nanocrystals). Concerning the size of the droplets, a range between 13-23 μm was observed (corroborating by laser diffraction from the average size in volume). Through the analysis of mean droplet size obtained by laser diffraction (Table 3.2), it is possible to corroborate that the increase of the oil-water ratio leads to an increase in mean droplet size. Moreover, for the stable Pickering emulsions, the mean droplet size was observed to remain unaltered along the 2-months period study.

Regarding emulsion stability, Zhang *et al*. (2017b), who produced Pickering emulsions with HAp particles and PLLA, and dichloromethane as the oil phase, reported that emulsion stability increased with the decrease of oil-water ratio, however, the emulsions did not exceed a stability of 6 days. The authors justified this fact with the hydrophilicity of HAp since it is easier to prepare stable O/W (from 10 to 60 oil volume fraction) instead of W/O emulsions (from 70 to 90 oil volume fraction). In this work, all emulsions (from 10 to 60 oil volume fraction) were stable for up to one-month. However, the emulsions prepared using 10/90 oil-water ratio showed signs of destabilisation after two months. This could be observed by visual inspection (creaming appearance) and also by the appearance of n-HAp agglomerates and oil segregation, as identified in the OM images of Figure 3.4 through the presence of darker regions and large oil droplets, respectively.

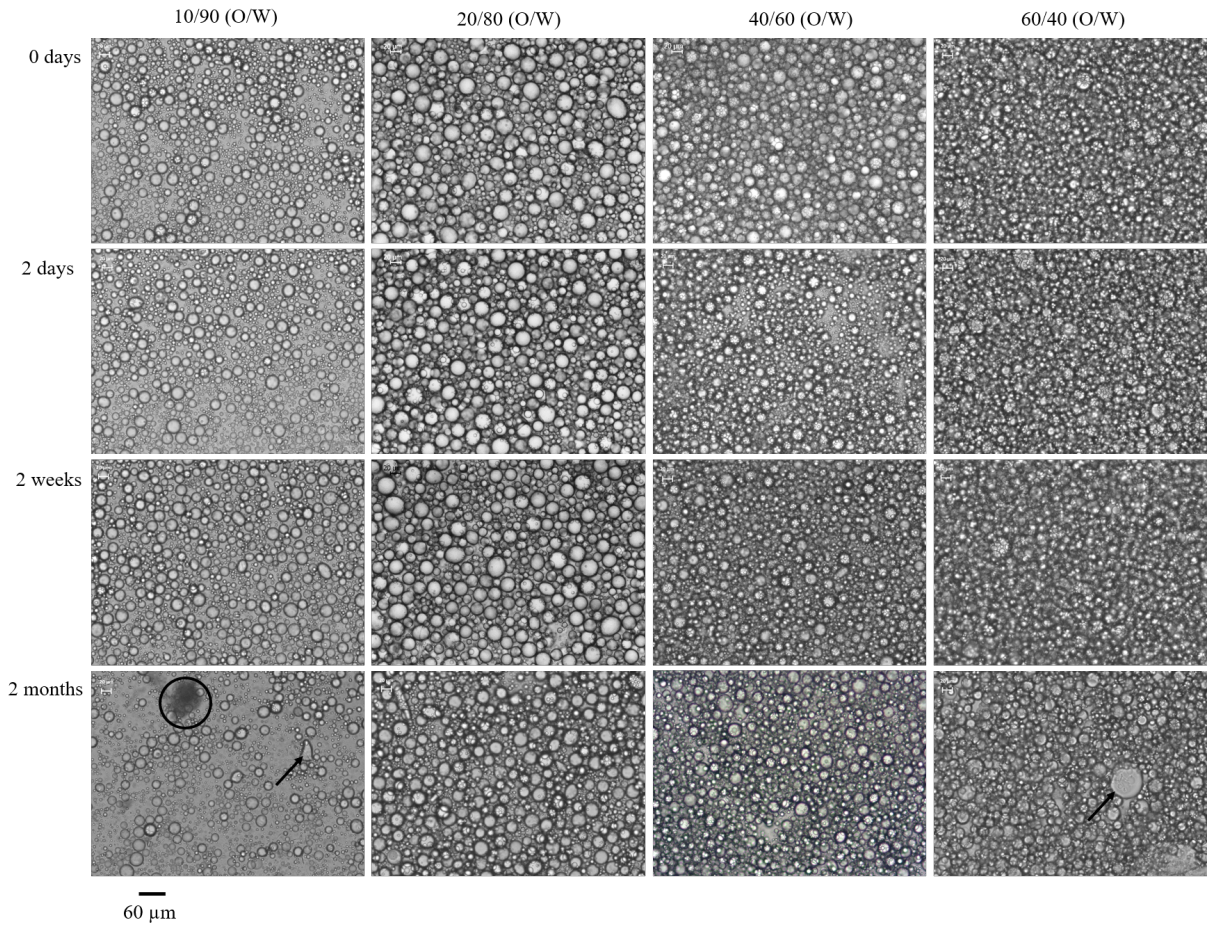

Figure 3.4: OM images, acquired in transmitted light mode, of Pickering emulsions produced with different oil-water ratios at a fixed n-HAp concentration (5 wt%). All images were registered at 20x magnification. The arrows indicate large oil droplets and the circles indicate dark regions associated with n-HAp agglomeration.

Although the emulsion prepared with a 20/80 as well as 60/40 oil-water ratios showed some coalescence, in particular of small droplets, this occurrence was not enough to destabilise the emulsion. Samples were also analysed through OM in reflected light mode. Figure 3.5 compares two different Pickering emulsions, namely the ones with 10/90 and 20/80 oil-water ratios, at times 0 days and 2 months. A white layer around the droplets can be observed, evidencing the presence of n-HAp at the oil droplet surface. At 0 days, images for both emulsions are similar, but after 2 months the emulsion using 10/90 ratio presents signs of instability and evidence of n-HAp agglomeration. The emulsion prepared with a 20/80 ratio, even after 2 months, retains droplet's shape and n-HAp remains at the oil surface.

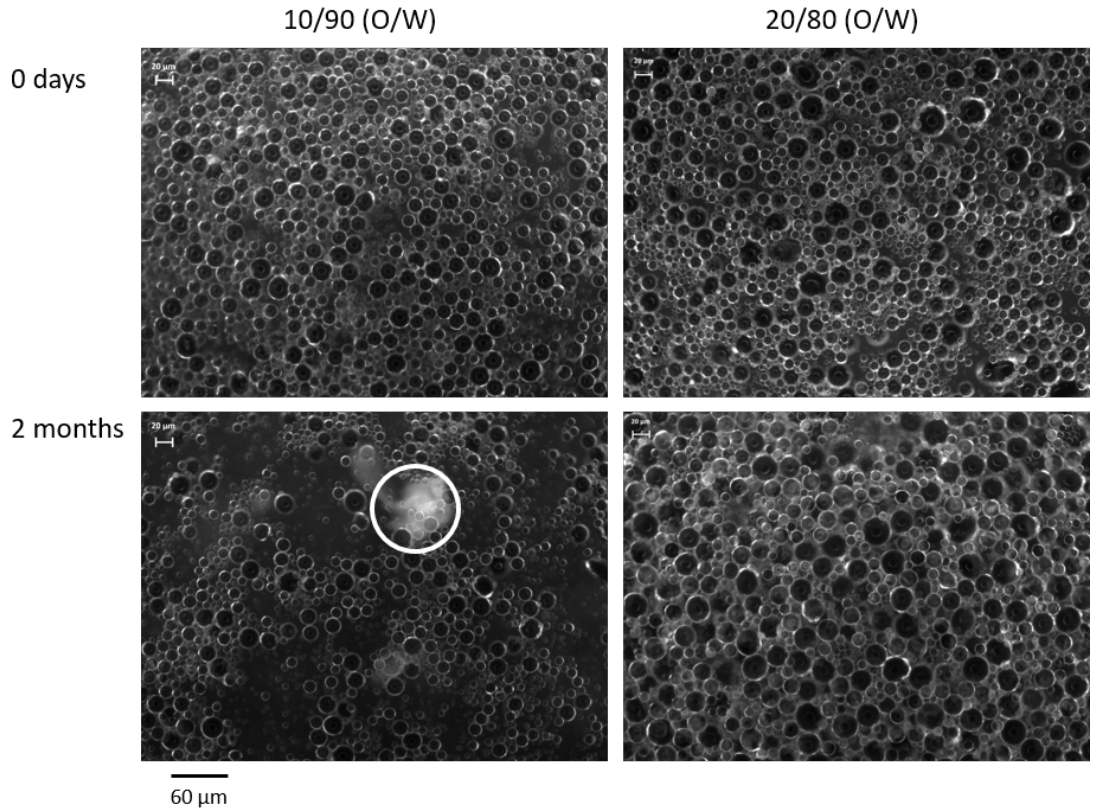

Figure 3.5: OM images, acquired in reflected light mode, of Pickering emulsions produced with different oil-water ratios and n-HAp concentration of 5 wt%. Magnification of 20x. Circumference indicates white region associated with n-HAp agglomeration (observed as dark regions in the transmitted light mode).

3.3.3 PICKERING EMULSION STABILITY PREDICTION

3.3.3.1 Model development

The objective here is to develop a model to estimate the concentration of n-HAp required to produce a Pickering emulsion given the oil/water ratio, and also to predict the stability of these emulsions given the suspension concentration and the solid particles diameter. This model takes into account factors such as oil-water ratio, total solids concentration, droplet diameter and solid particles dimensions.

Considering the schematic representation of a Pickering emulsion shown in Figure 3.6, this model considers that:

- the emulsion is composed of an aqueous suspension of free solid particles, and an oleic phase of oil droplets surrounded by one or more layers of solid particles (Figure 3.6 A);

- one oil droplet surrounded by one or more layers of solid particles is called a Pickering droplet (Figure 3.6 B);
- free n-HAp particles and Pickering particles are in constant interaction through collisions creating a state of equilibrium;
- the oil droplets diameter, d_o, is much larger than the solid particle characteristic dimension, namely d_s, which is the typical length of n-HAp rod-like particles (at least 3 orders of magnitude);
- the volume occupied by the solid layer(s) in a Pickering particle is negligible compared with the volume of the oil droplet;
- the solid particles (n-HAp) are rod like structures (Figure 3.6 C), approximated by a cylinder with height, d_s, as the characteristic dimension, and a shape factor of 5:1 (Gomes *et al.*, 2009).

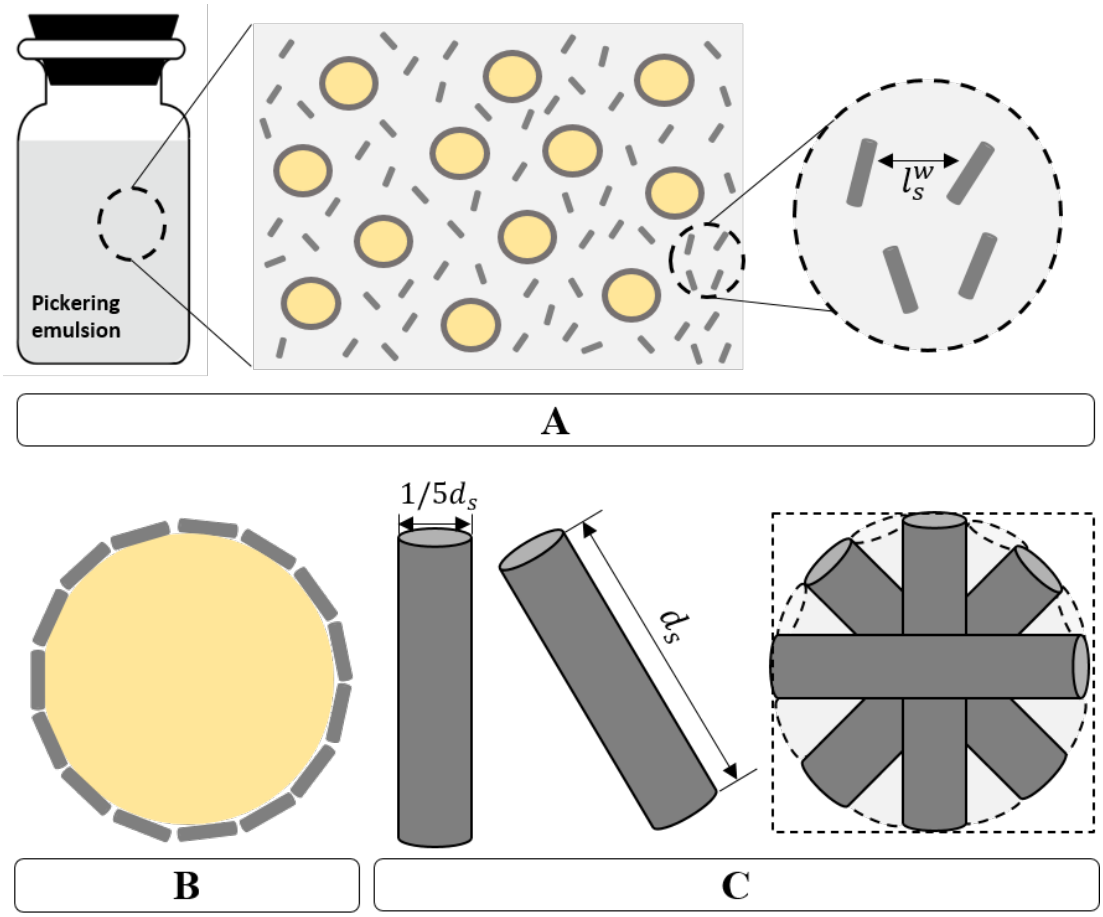

Figure 3.6: Schematic representation of a Pickering emulsion: A) Pickering emulsion with oil droplets surrounded by n-HAp, and aqueous phase composed with n-HAp suspended, B) Pickering droplet and C) represents the n-HAp structure as well as their dimensions.

The model considers that the stability of a Pickering emulsion depends on the degree of mobility of the solid particles, that is, on the ratio l_s^w/d_s, where l_s^w is the mean distance between solid particles in suspension (aqueous phase), given by

$$l_s^w = \left(\frac{m_s}{C_s^w}\right)^{1/3} \tag{3.2}$$

where m_s is the mass of one solid particle and C_s^w is the concentration of solid particles in suspension (aqueous phase).

The mass of a single n-HAp particle is given by $m_s = \rho_s v_s$, where ρ_s is the solid density (3160 kg/m^3), and v_s is the volume of a solid particle. Assuming a cylindrical shape for the n-HAp particle with 5:1 as shape factor, then

$$v_s = \frac{\pi}{4}\left(\frac{d_s}{5}\right)^2 d_s = \frac{\pi}{100} {d_s}^3 \tag{3.3}$$

The concentration of solid particles in the Pickering droplets, i.e., the particles that surround the oil droplets is given by

$$C_s^\alpha = \frac{m_s N_s^\alpha}{V_w} \tag{3.4}$$

where N_s^α is the minimum number of solid particles required to form α layers around the oil droplets and V_w is the total volume of water. Since the solid particles are assumed to be much smaller than the oil droplets

$$N_s^\alpha = \alpha n_s \, N_o \tag{3.5}$$

where $N_o = 6V_o/\pi {d_o}^3$ is the number of oil droplets, α the number of layers and V_o the total oil volume. The number of solid particles necessary to form a single layer is given by $n_s = a_o/a_s$, where $a_o = \pi {d_o}^2$ is the surface area of an oil droplet with diameter d_o, and a_s is the area covered by a one solid particle. For a n-HAp particle and taking into account that each particle can take a random orientation at the oil surface, a_s can be estimated assuming that a single solid particle covers a projected area equivalent to a square with side equal to d_s (Figure 3.6 C), and thus $a_s = {d_s}^2$.

Combining all the previous relationships results in

$$C_s^{\alpha} = \alpha \rho_s \frac{6\pi}{100} \frac{d_s}{d_o} \frac{V_o}{V_w} = \alpha \rho_s \frac{6\pi}{100} \frac{d_s}{d_o} \frac{X_o}{1 - X_o} \tag{3.6}$$

where X_o and is the volume fraction of oil in the emulsion. In this relationship all values are known, except d_o the diameter of the oil droplet and α the number of layers.

Finally, the concentration of solid particles in suspension (aqueous phase) can be obtained by

$$C_s^w = C_s^{in} - C_s^{\alpha} \tag{3.7}$$

where C_s^{in} is the initial formulation concentration of solid particles.

The model was applied to the O/W ratio of 20/80 (see Table 3.1) experimental data (2 days storage period) and the following limits were obtained

$$\begin{cases} l_s^w/d_s > 1.7 & \text{unstable} \\ 1 \leq l_s^w/d_s \leq 1.7 & \text{stable} \\ l_s^w/d_s < 1 & \text{gel formation} \end{cases} \tag{3.8}$$

In these calculations $d_o = 18\ \mu m$ was taken as the average droplet diameter obtained through laser diffraction analysis. The parameter number of layers was assumed as constant, $\alpha = 4$, in agreement with an estimated shell thickness of 200 nm, as reported in section 3.3.1 Morphology of the formed Pickering emulsions.

The three types of formed emulsions (unstable, stable, and gel) are shown in Figure 3.7. The gel region should be understood as a stable region, but due to its high viscosity it may find constraints for some applications. Nevertheless, this type of formulations can find diverse uses, and thus the identification of the range of compositions at which it occurs is of interest.

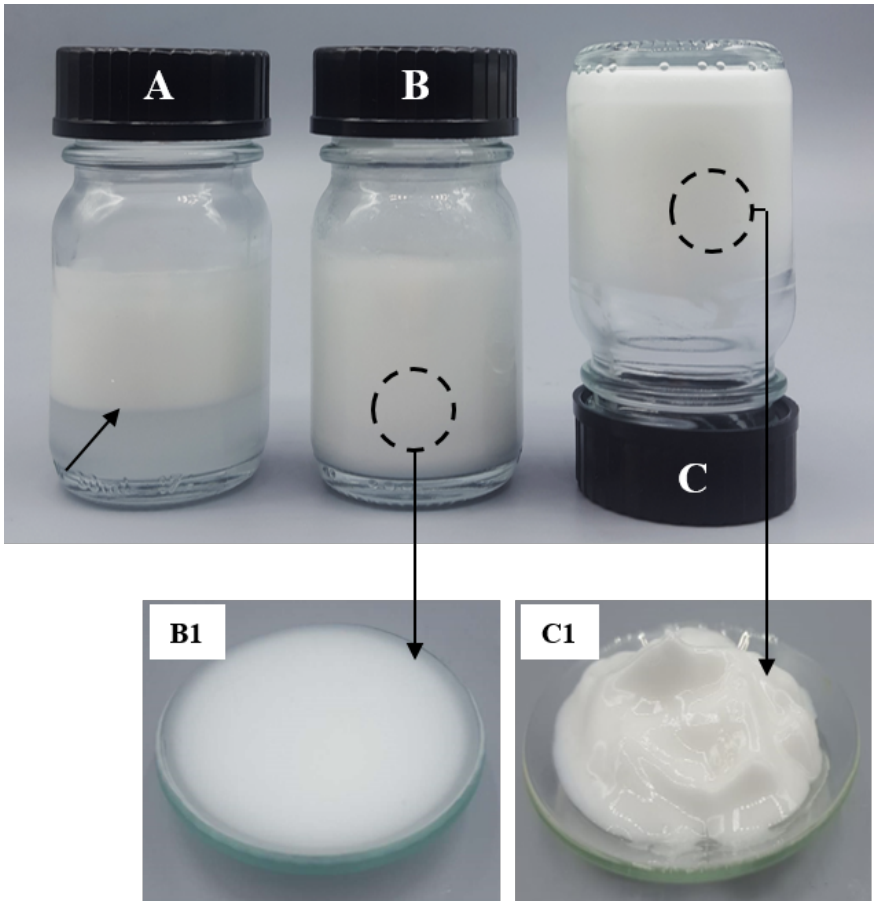

Figure 3.7: Comparison between different Pickering emulsions: A) unstable, B) and B1) stable and C) and C1) gel stabilised with different n-HAp concentrations (1.5, 5.0 and 15 wt%, respectively) and fixed oil-water ratio (20/80, *v/v*).

3.3.3.2 Model validation

Ternary diagrams are convenient tools to represent three-component systems, as it is the case of emulsions (oil, water, and stabiliser). They have been used to represent systems besides thermodynamic equilibrium data, including emulsions (Kumar & Kumar, 2018), and more recently Pickering emulsions (Rincón-Fontán *et al.*, 2019).

The three stability regions – unstable, stable, gel - are identified in the ternary diagram of Figure 3.8 A, where it is clear that the model is in good agreement with the data for other O/W ratios. A single exception was observed for $X_o = 0.6$ with the formation of a gel where a stable emulsion should have been formed. However, this result is in accordance to the results obtained by Xiao *et al.* (2016a) and Zhu *et al.* (2018), where the emulsion with higher oil ratio has a higher apparent viscosity compared with other ones with lower ratios. Furthermore,

others previous works showed also a similar behaviour of the viscosity increase with oil ratio increased (Dai *et al.*, 2018; Shao & Tang, 2016).

Figure 3.8 B and Figure 3.8 C show the stability evolution of the Pickering emulsions after two weeks and two months, respectively. After the two-week period the stability of the emulsions was not changed, except for the emulsion with 20/80 oil-water ratio and 2.5 wt% n-HAp concentration. After two months, the stability of the 20/80, 40/60 and 60/40 oil-water ratio emulsions was unchanged, with the exception of the one prepared with an oil-water ratio of 10/90 and an n-HAp content of 5%, which became unstable. For low oil content, the observed phase separation may be mostly due to the density difference between the two phases (oil and water) combined with a lower droplet concentration. The use of high oil-water ratios increases the droplets concentration, that become more tightly together delaying phase separation by preventing their movement. In case of low oil-water ratios, n-HAp content increase can also promote stabilisation, i.e., n-HAp particles not participating in the stabilisation (they might be in excess) can act as an external thickener. This might be the case of the 10/90 formulation using 10% n-HAp.

3.3.4 PICKERING EMULSION RHEOLOGY STUDY

The rheological properties of Pickering emulsions can be related with their performance and storage stability. The effect of the n-HAp concentration was studied, maintaining the oil-water ratio as 20/80 (*v/v*), in terms of the viscosity and elastic (G') and loss (G'') modulus through steady-state flow and dynamic oscillatory measurements, respectively. The obtained results are presented in Figure 3.9. Figure 3.9 A shows the viscosity of the Pickering emulsions stabilised with different n-HAp concentrations. It can be perceived that the Pickering emulsions have a n-HAp concentration dependent behaviour since the viscosity increases as the n-HAp concentration increases. Similar results were obtained by Xiao *et al.* (2016a) that also observed an increase of the Pickering emulsion viscosity as the concentration of kafirin particles increased.

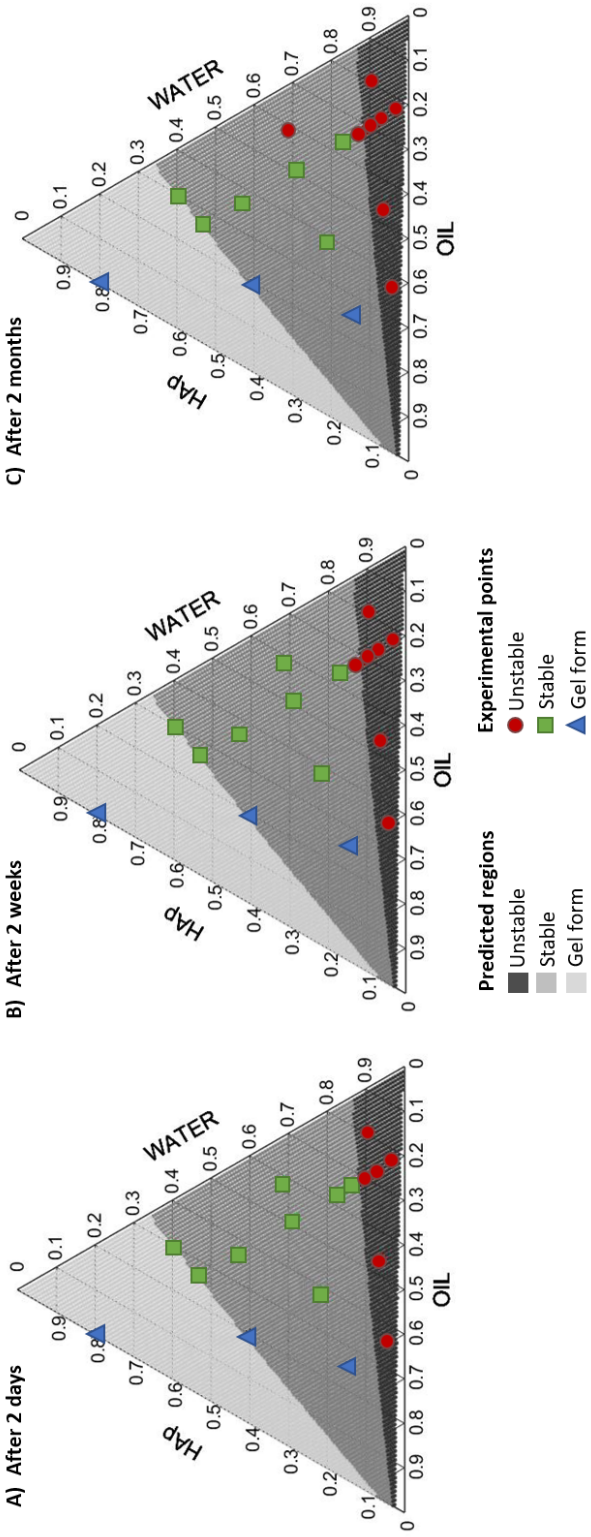

Figure 3.8: History of experimental point (2 days - 2 moths). Ternary diagram of O/W Pickering emulsions with n-HAp and experimental points. Assumption: $l_s^w/d_s > 1.7$ – unstable, $l_s^w/d_s < 1$ – gel formation and $1 \leq l_s^w/d_s \leq 1.7$ – stable.

The formation of the *gel-like* structures in Pickering emulsions was evaluated through oscillatory measurements. Figure 3.9 B shows G' and G'' as a function of frequency. For all measurements, it is observed that both moduli were nearly independent of the frequency (for range 0.3-100 rad/s), but G' is always higher than G'' (in magnitude) over the tested frequency range. This result indicates that the rheological behaviour of the Pickering emulsions was predominantly elastic, which is compatible with the development of *gel-like* networks. The development of the *gel-like* networks on Pickering emulsions is influenced by the n-HAp concentration. This behaviour was previously reported in studies dealing with other Pickering stabilisers such as kafirin (Xiao *et al.*, 2016a) and cellulose (Li *et al.*, 2018).

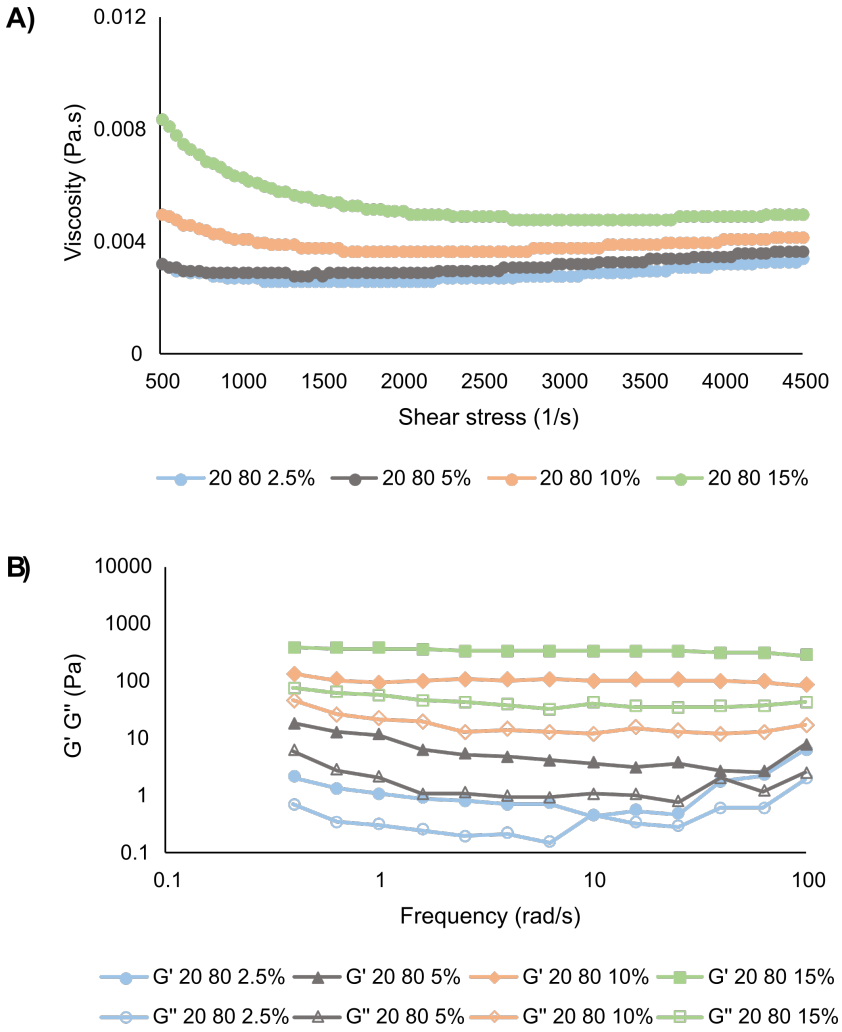

Figure 3.9: Rheological properties of Pickering emulsions stabilised with different n-HAp concentrations (2.5, 5, 10 and 15 wt%) and fixed oil-water ratio (20/80, *v/v*): A) Pickering emulsion viscosity versus shear rate and B) oscillatory frequency sweep curves: G' storage modulus and G'' loss modulus.

In general, the increase of the n-HAp concentration promotes the interactions between the free-particles leading to the formation of a structural network, avoiding phase separation. The results were reproducible for the other tested oil-water ratios (data not shown). These findings show that the stability of the Pickering emulsions can be improved by increasing the n-HAp concentration, or oil volume fraction, which is in agreement with the model predictions.

3.4 CONCLUSIONS

Hydroxyapatite is a biomaterial that has been used in different fields. Recently it has been applied in Pickering emulsions production, mostly following a strategy of combining it with polymers such as poly-ε-caprolactone and poly-L-lactic acid (Zhang *et al.*, 2017; Zhang *et al.*, 2017b). In this study, a commercial n-HAp paste (*Nano*XIM-CarePaste) was used in the preparation of the Pickering emulsions. Emulsion stability was studied as a function of processing parameters, such as oil-water ratio, and n-HAp content. According to the drop test, independently of the used oil-water ratio or n-HAp content, all the produced Pickering emulsions were of O/W type. The microscopy evaluation by optical and cryo-SEM confirmed the formation of spherical Pickering droplets, and the presence of n-HAp attached at the oil droplet's surface (through cryo-SEM), corroborating an oil core–HAp shell morphology, similarly to a microcapsule.

In this work, a wide range of compositions, namely oil-water ratios (10/90-60/40, *v/v*) and n-HAp concentrations (0.5-15, wt%) were studied. In a general way, it was observed that when the n-HAp content increases a decrease of the mean droplet size was observed, and that the oil-water ratio increase favoured the increase of the mean droplet size.

According to the developed model, three regions in a ternary phase diagram were defined (unstable, stable and gel), and the experimental data was generally in good agreement with the model. By using this model, it is possible to predict the type of emulsion to be formed based on the used formulation, as well as to predict its stability. Face to the analysed timeframe the model is reliable up to two weeks; only after 2 months differences were found. Rheological studies show that the stability of the Pickering emulsions can be improved by

increasing the n-HAp concentration, or the oil volume fraction, which is in agreement with the model predictions.

This work is a step forward in the field of synthetic surfactants substitution contributing to the sustainable development of emulsions. Pickering particles emerge as an alternative to traditional emulsifiers, which have been related with harmful health effects (Chassaing *et al.*, 2015; Di Sotto *et al.*, 2014). The use of a commercial n-HAp is an important achievement since the stabilisation is guaranteed by a native n-HAp that was not processed with solvents, conventional emulsifiers, or combined with other compounds to improve particle's wettability, avoiding issues such as emulsion contamination. Further studies are under development in order to produce these Pickering emulsions in continuous mode (increasing production scale) through the use of low energy devices. These Pickering emulsions can be used as versatile food-grade vehicles for the encapsulation of hydrophobic compounds such as vitamins, which is, technologically, an important issue, namely to increase their bioavailability.

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4 CONTINUOUS PRODUCTION OF NANOHYDROXYAPATITE PICKERING EMULSIONS

This chapter is based on: Andreia Ribeiro, Yaidelin Manrique, Filomena Barreiro, José Carlos B. Lopes, Madalena M. Dias (2021). Continuous production of hydroxyapatite Pickering emulsions using a Mesostructured reactor. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 616, 126365-77. https://doi.org/10.1016/j.colsurfa.2021.126365

Emulsions are used in a wide range of applications, including food and cosmetics. Nowadays, the demand for sustainable products has increased with Pickering emulsions emerging as clean alternatives. To achieve the industrial implementation of Pickering emulsions, continuous production and less intensive energy devices are required. In this context, a mesostructured reactor based on an innovative static mixer, NETmix, was tested to produce Pickering emulsions from a previously developed formulation in batch mode. The effect of the number of cycles (2-35) and Reynolds number (200-500), parameters that influence the residence time and the quality of mixing, on the Pickering emulsion properties (average droplet size, droplet morphology, and stability) was studied. The obtained results pointed out the feasibility of using NETmix to produce Pickering emulsions. It is a versatile technique to control in a tailor-made way the droplet size and generate small droplets at short times. Results show a decrease of the droplet size with increasing number of cycles and Reynolds. Under the tested conditions, stable dispersions with droplet size of ~7 μm were produced using 17 cycles and Re=400. Microscopy images show an oil core and nanohydroxyapatite shell morphology.

4.1 INTRODUCTION

The concept of Pickering emulsions was introduced by Ramsden and Pickering at the beginning of the 20th century (Pickering, 1907; Ramsden, 1903). They are characterised by using solid particles that once positioned at the oil-water interface form a thick barrier able to act as a stabiliser (Fujii *et al.*, 2012; Rincón-Fontán *et al.*, 2019; Rousseau, 2013; Rousseau & Hodge, 2005). The stability and emulsion type (oil-in-water (O/W) or water-in-oil (W/O) are affected by several factors like particle's concentration, shape, size and wettability, and the pH and ionic strength of the aqueous phase (Linke & Drusch, 2018).

Pickering emulsions, due to the formation of the protective shell around the oil or aqueous core can act as encapsulation systems for drugs and/or bioactive compounds (Asabuwa Ngwabebhoh *et al.*, 2018; Winuprasith *et al.*, 2018), where the use of non-toxic particles together with the surfactant-free character makes them advantageous for various applications, such as food, pharmaceutical and agrochemical (Albert *et al.*, 2019; Linke & Drusch, 2018; Rayner, 2015). Particularly, in the food area, Pickering emulsions can help to reach the *clean-label* trend by ensuring environmentally friend solutions (Green *et al.*, 2013; Linke & Drusch, 2018).

Pickering emulsions can be prepared by the typical methodologies for conventional emulsions, namely rotor-stator homogenisation and sonication, which are the most commonly used batch mode processes (Shimoni *et al.*, 2013; Tan *et al.*, 2014; Tzoumaki *et al.*, 2011). These technologies enable droplet break-up due to the applied high-shear rate (rotor-stator homogeniser), or mechanical vibrations (ultrasonic devices). In rotor-stator homogenisation, a pressure differential drawing the liquid in and out is created due to the rotor rotation, promoting liquid circulation. The droplet size is reduced due to the high liquid acceleration and shear force occurring between the rotor and stator (Albert *et al.*, 2019). In the case of ultrasonic devices, the probe vibration transmits the ultrasonic energy to the surrounding medium, inducing emulsification mostly by cavitation and ultrasonic forces (Albert *et al.*, 2019). The two techniques have similar advantages, namely low cost and ease setting-up, quickness of the emulsification process, offering also the possibility to work with small volumes. Production of large-size droplets, high energy requirements, high heat release,

difficult industrial scale-up, and contamination with metals, are among their main disadvantages (Albert *et al.*, 2019).

Despite the rising interest for Pickering emulsions at scientific and technological level, their preparation methods rely mostly in batch-mode processes, highlighting the importance to propose and test novel alternatives, which should improve the control of the emulsification processes as well as enable continuous production. For this propose, some membrane devices (Arkoumanis *et al.*, 2019) and microfluidizers (Bai *et al.*, 2019) have gather recent interest to be applied in the production of Pickering emulsions.

NETmix, a technology developed at LSRE-LCM, Faculty of Engineering University of Porto (FEUP) (Lopes *et al.*, 2005), can offer several advantages for the production of Pickering emulsions. Briefly, it comprises a network of unit cells, each cell composed by one chamber and four channels (two inlet and two outlet half channels) oriented at 45° from the main flow direction (Figure 4.1). The network size is given by the number of rows (along the main flow direction) and the number of columns (perpendicular to the flow) (Laranjeira *et al.*, 2009).

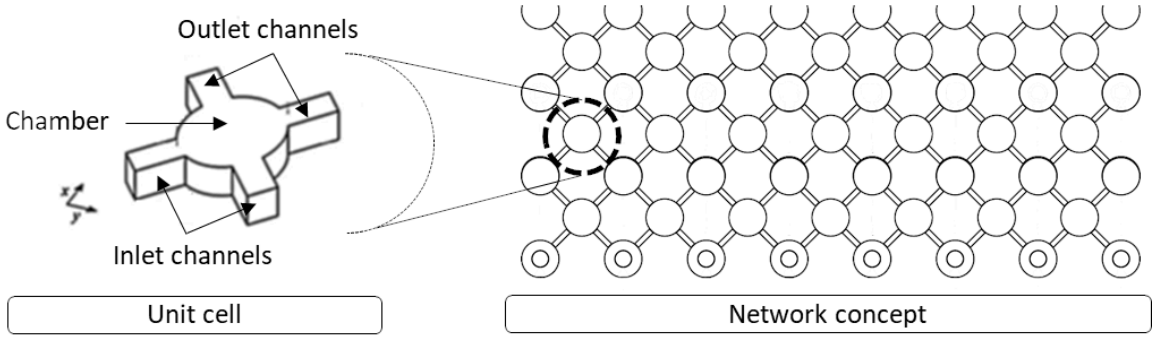

Figure 4.1: Example of one-unit cell with one chamber and channels, and schematization network concept of NETmix.

The chambers operate as mixing zones and the channels as plug flow zones (Laranjeira *et al.*, 2009). In this way, a chamber contacts with fluids from two chambers of the previous row, splitting and feeding two chambers in the following row. NETmix operation is based on this flow pattern, promoting successive contacts and separations from channels to chambers. It has a considerable potential to generate interfacial area between two miscible fluids (Fonte *et al.*, 2013) and with immiscible fluids (Fernandes, 2018; Fernandes *et al.*, 2021).

In NETmix, the major parameter influencing mixing is the Reynolds number (Re), and proper mixing can only be achieved above the critical channel's Re which can be determined by

$$Re = \frac{\rho\, v\, d_h}{\mu} \tag{4.1}$$

where ρ is the mixture density (kg/m^3), v the average velocity in the channels (m/s), $\mathrm{d_h}$ the channels hydraulic diameter (m) and μ the emulsion viscosity (Pa·s).

Previous works have shown that for Re > 150, the flow evolves to a self-sustained dynamic and chaotic oscillatory flow regime inducing strong laminar mixing (Fonte *et al.*, 2013; Laranjeira *et al.*, 2009). This occurs due to the geometric characteristics of the NETmix network, and the local hydrodynamic instabilities induced by the immerging jet interactions at the chambers. Due to this reason, NETmix has significant advantages over other emulsification devices since it induces successive repeated and well-localized mixing inside the chambers throughout the reactor, promoting an easily reproducible emulsification step.

NETmix has various advantages; namely, it consists on a simple structure enabling fast mixing, Re can be adjustable at different flow rates, it facilitates temperature control due to the large surface area favouring heat exchange, and it offers an easy scale-up or numbered-up depending on the target process and productive objectives (Gomes *et al.*, 2009).

NETmix technology has been applied for different products. Examples include the production of nanocrystalline hydroxyapatite (Gomes *et al.*, 2009), this process already at industrial level (Fluidinova, 2019), metal-organic frameworks (Fonte, 2013), magnetic iron oxide nanoparticles (Lima *et al.*, 2014), and calcium carbonate nanoparticles (Almeida *et al.*, 2018). Furthermore, this technology has also been employed for the continuous production of nanohydroxyapatite (n-HAp)/chitosan aqueous dispersions (Ruphuy *et al.*, 2018) and microencapsulated systems (Moreira *et al.*, 2020).

For Pickering emulsions production, NETmix ensures easy control of the parameters affecting the mixing providing good reproducibility between assays, it does not limit the production volume, can reduce the production time, and allows production in continuous mode. In the present study, the NETmix technology was tested, for the first time, as a novel process to produce Pickering emulsions in continuous mode, namely O/W Pickering emulsions stabilised

by n-HAp. The effect of the number of cycles (2 - 35) and the Re number (200 - 500) on Pickering emulsion droplet size and stability (60-days period), evaluated by laser diffraction and optical microscopy, was studied. The effective coverage of the oil droplets by the n-HAp particles was checked by confocal laser microscopy and cryo-scanning microscopy. Having in view the importance of continuous methods at industrial level, this work is a contribution to the field by testing the potential of NETmix, a novel network static mixer and reactor, in the development of Pickering emulsions. The produced emulsions can find use in a wide range of applications, including food applications due to the use of food-grade components, including the n-HAp.

4.2 MATERIALS AND METHODS

4.2.1 STANDARDS AND REAGENTS

The hydroxyapatite aqueous paste, *nano*XIM-CarePaste, was supplied by Fluidinova S.A. It is composed by 15.5 ± 0.5 (wt%) of HAp nanoparticles (n-HAp) with particle size < 50 nm, 4.5 ± 0.5 (wt%) KCl and a water content ≤ 81.0 (wt%). The sunflower oil (rich in oleic acid) was purchased from a local supermarket and used as the oil phase. Distilled water, treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA), was used for diluting the n-HAp paste, and also as the aqueous phase. Nile red and Nile blue A, used in the confocal laser scanning microscopy, were obtained from Sigma-Aldrich, and isopropyl alcohol was purchased from Riedel-de Haen.

4.2.2 EXPERIMENTAL SET-UP AND PROCEDURE FOR PICKERING EMULSIONS PRODUCTION

Pickering emulsions were produced in continuous mode using the best formulation according to the results obtained in batch mode, as previously reported by Ribeiro *et al*. (2020), namely concerning the good stability within a 2-month analysis period. The formulation corresponds to an oil/water ratio of 20/80 (*v/v*) and an n-HAp concentration of 5 wt%. This emulsion is characterised by individualised droplets of spherical shape with an average diameter of 18 μm.

The used NETmix, constructed in stainless steel, comprises 25 rows and 8 columns. It has chambers of 3.3 mm diameter, and channels with a width and depth of 0.5 mm, making a total volume of 1 mL. To obtain the pre-Pickering emulsion (i.e., the emulsion corresponding to the first pass in the NETmix), the feed comprised two solutions designated by "Solution A" and "Solution B". Solution A, the aqueous phase, is composed of nano-XIM-HApCare dispersed in water at a concentration of 5 wt%; and Solution B, the oil phase, corresponds to sunflower oil. Solutions A and B were fed to the NETmix reactor in pre-mixed mode (input configuration) with the total flow rate adjusted according to the desired Reynolds number (Re) using two pumps. The mixture occurs inside the NETmix chambers, and the pre-Pickering emulsion was collected at the top of the reactor (Figure 4.2 A). Then, in order to increase the residence time, NETmix was let to operate in recirculation mode (2, 5, 17 and 35 cycles were tested). For this mode, the experimental set-up was partially modified, namely the NETmix reactor was fed, firstly with the produced pre-Pickering emulsion, then with the emulsions produced after each pass, using just one pump (Figure 4.2 B).

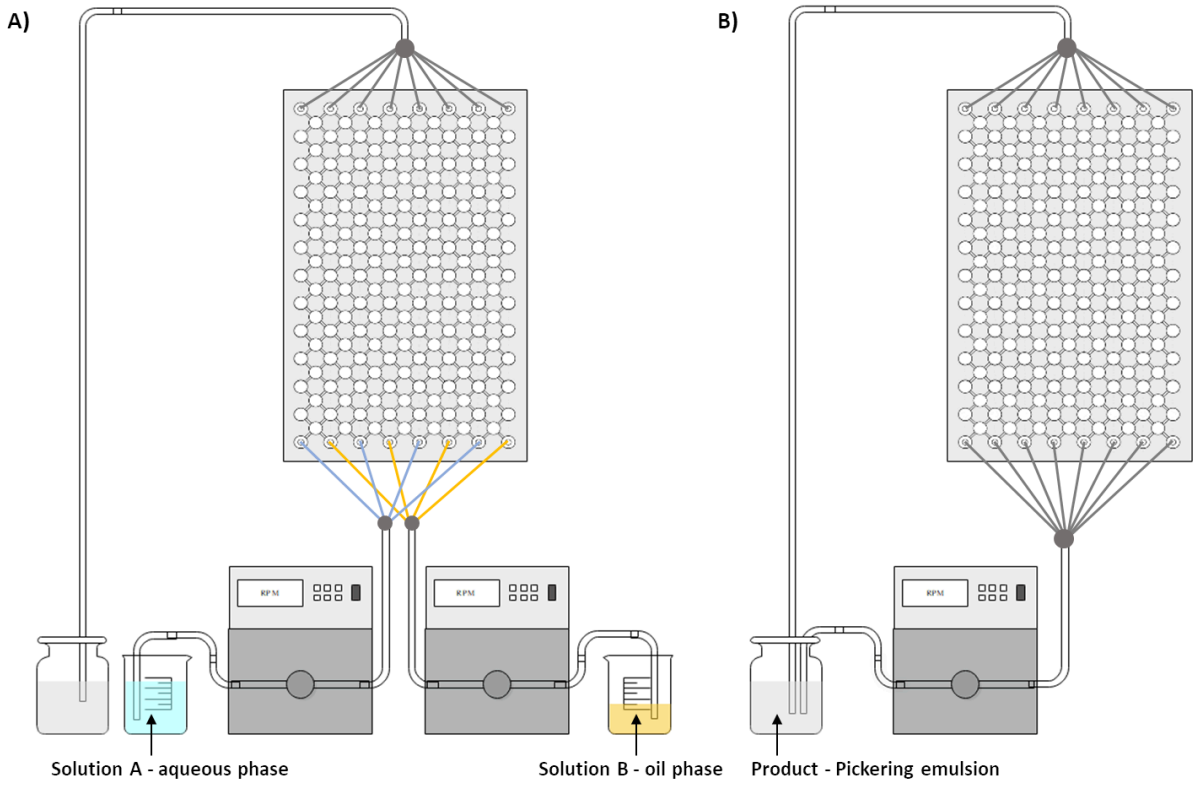

Figure 4.2: Experimental set-up used to produce the Pickering emulsions in continuous mode. A) configuration for the first pass i.e., pre-mixed mode injection scheme (blue – solution A – aqueous phase; yellow – solution B – oil phase), B) configuration for the recirculation mode.

According to the desired Re, a peristaltic pump (Re ≤ 300) or a diaphragm pump (Re ≥ 400) was used. In order to determine the needed number of cycles to a certain residence time, the cycle time was determined according to Eq. 4.2.

$$\text{Cycle time (s)} = \frac{\text{Emulsion volume}}{\text{Flow rate}} \tag{4.2}$$

In order to test the reproducibility of the continuous process, the Pickering emulsions were prepared twice under the same conditions.

4.2.3 CHARACTERISATION OF THE PICKERING EMULSIONS

The obtained Pickering emulsions were characterised in terms of morphology using different microscopic techniques, namely optical microscopy, confocal laser scanning microscopy and cryo-scanning electron microscopy. Moreover, size distributions were determined by laser diffraction analysis. This analysis, combined with optical microscopy and visual inspection, is useful to check for instability phenomena. Additionally, the emulsion type (O/W or W/O) was checked by the drop test and by confocal laser scanning microscopy.

4.2.3.1 Optical Microscopy (OM) analysis

OM was used to monitor the morphology of the Pickering emulsions during the productive process, and along a two months-period under storage (sampling times: 2, 7, 14, 30 and 60 days) to check for the occurrence of instability phenomena. For this, an aliquot of the sample was placed on a microscope slide, then gently covered with a coverslip. A Carl Zeiss Axiotech 100 HD optical microscope (Zeiss Instruments, Jena, Germany) fitted with a digital camera (AxioCam 105 colour) was used, and the image acquisition and processing was done using Zen (2.3 blue edition) software.

4.2.3.2 Confocal Laser Scanning Microscopy (CLSM)

CLSM was used to check for the effective coverage of the oil droplets by the n-HAp particles in order to confirm their successful role as O/W Pickering stabilisers. These experiments were performed using a Leica TCS-SP5 AOBS (Leica Microsystems Inc. Heidelberg, Germany). For the analysis, Pickering emulsion samples were stained with a mixture of fluorescent dyes in

isopropyl alcohol, namely Nile red at 0.1% *w/v* and Nile blue A at 0.1% *w/v*. The stained Pickering emulsion sample was put on concave slides, and the fluorescent dyes excited by an argon laser at 488 nm for Nile red or a helium-neon laser at 633 nm for Nile blue A.

4.2.3.3 Cryo-Scanning Electron Microscopy (Cryo-SEM)

Cryo-SEM was used to determine the morphology of the produced Pickering emulsions, as well as to verify, complementary to CLSM, the successful attachment of solid particles at the oil surface. The analysis was carried out using a JEOL JSM 6301F/ Oxford INCA Energy 350/ Gatan Alto 2500. Briefly, the sample was rapidly cooled, by plunging it into sub-cooled nitrogen and then transferred under vacuum to the cold stage of the preparation chamber. After, the sample was fractured, sublimated (180 seconds at -90 °C), and coated with Au/Pd (45 seconds and with a 12 mA current). Finally, the sample was transferred into the SEM chamber and observed at -150 °C.

4.2.3.4 Laser diffraction analysis

Emulsion droplet size distributions, in volume, were determined using a laser diffraction particle size analyser (Beckman Coulter LS230, California). The evaluations were performed with the freshly prepared Pickering emulsions, and with samples taken along the two months period, namely at 2, 7, 14, 30 and 60 days. From the obtained volume distributions, the average size was determined and reported as the average diameter in volume, $d_{4,3}$. These evaluations allow to inspect for coalescence phenomena since it leads to a diameter increase, thus complementing the optical microscopy analysis. The data is expressed as average$\pm$SD.

4.2.3.5 Drop test

The emulsion type (O/W or W/O) was confirmed with the drop test, which can be related with the medium at which the emulsion become dispersed, i.e., if the Pickering emulsion drop rapidly disperses in water, and remain agglomerated in the oil, the emulsion is considered as the O/W type. If the opposite occurs, it is of W/O type. To perform this test, two or three emulsion drops were added to the water and the oil phases. After a gentle stirring, the emulsion was classified according to the observed behaviour.

4.2.3.6 Visual inspection

The produced emulsions were examined visually, and the macroscopic aspect registered using a Nikon digital camera. The time-frame for this evaluation was two months with images registered at 2, 7, 14, 30 and 60 days.

4.3 RESULTS AND DISCUSSION

Having in view the testing of NETmix technology for the continuous production of the n-HAp Pickering emulsions, the impact of the number of cycles (2, 5, 17 and 35) and of the Re number (200, 300, 400 and 500) in their morphology and stability was evaluated. The effect of the number of cycles was studied by fixing the Re number at 400, whereas the effect of the Re number was studied by fixing the number of cycles at 17. The Reynolds number was varied by changing the flow rate (viscosity, density, and geometry of NETmix was constant). A cycle corresponds to the passage of the total emulsion volume in NETmix.

4.3.1 EFFECT OF THE CYCLES NUMBER ON EMULSIONS MORPHOLOGY AND STABILITY

The number of times that the emulsion passes inside the NETmix reactor (cycles number) increases the residence time and reduces the size of the emulsion droplets. The first pass allows the mixing of the oil and water phases (pre-Pickering emulsion), which is subsequently recirculated in the NETmix to promote size reduction. The use of Re above a critical value (Re = 150) maximizes the mixing of the two phases inside the chambers, as previously reported (Gomes, 2011; Laranjeira *et al.*, 2009). Namely, in the study of Gomes (2011), which performed tracer studies on NETmix for 50 ≤ Re ≤ 400, a state of complete mixing between the streams within the chambers at Re = 400 was observed. In this study the effect of 2, 5, 17 and 35 cycles on the droplet size was studied at Re = 400, which from a productive point of view is enough to ensure a fast process, since it allows the fast reduction of emulsion droplet size.

OM images of the obtained Pickering emulsions after the first pass (pre-Pickering emulsion) and after recirculation (2, 5, 17 and 35 cycles) are shown in Figure 4.3. It can be observed that the pre-Pickering emulsion corresponds to a coarse emulsion with droplets of large size and high size dispersity, maintaining the stability just for a couple of hours.

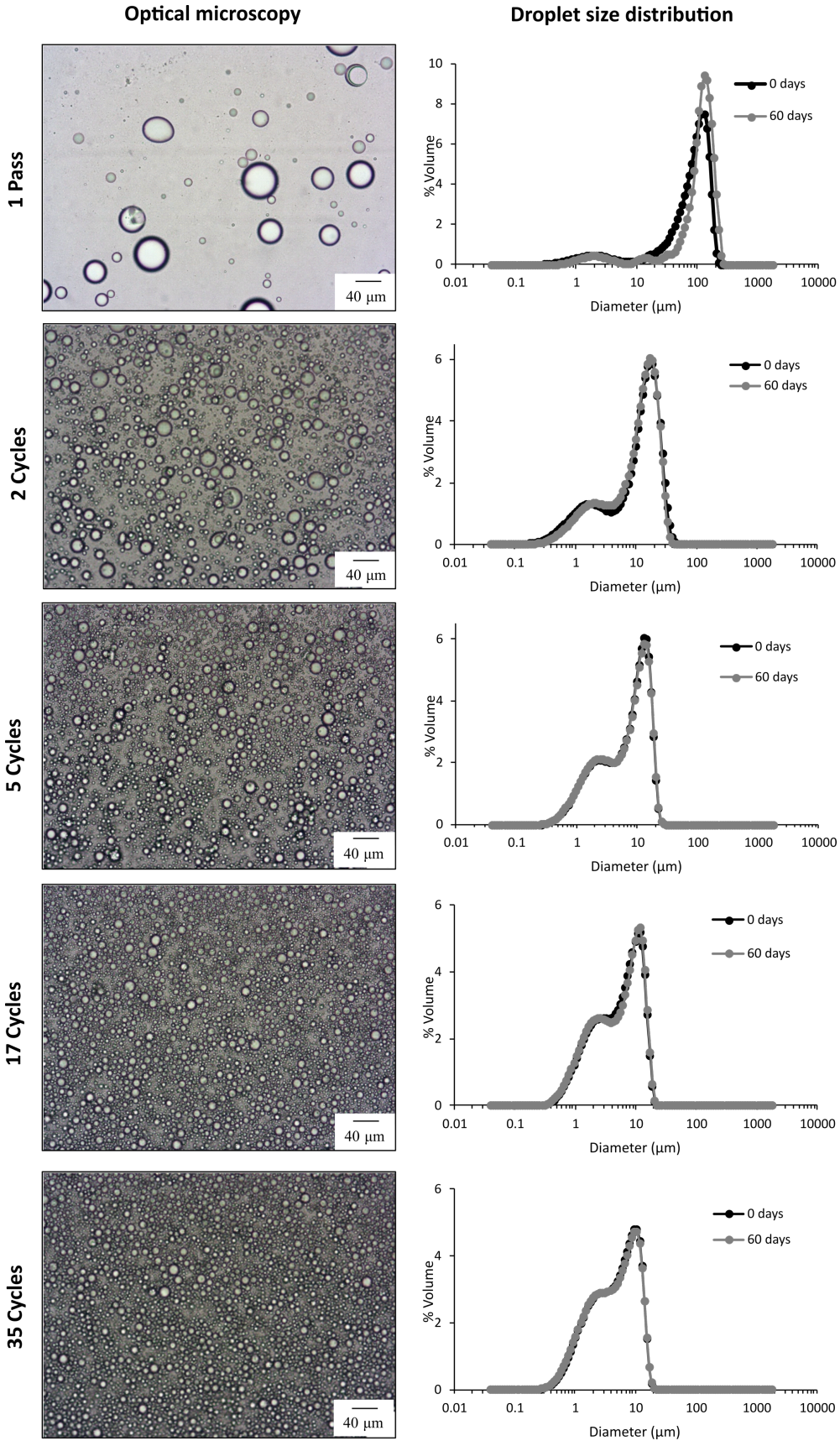

Figure 4.3: Optical microscopy images and size distributions for 0 and 60 days of the produced Pickering emulsions (Re = 400) as a function of the applied number of cycles. All images were taken with magnification of 20x.

After 2 cycles, Pickering droplets retain the spherical shape, and droplet size was substantially reduced, although some dispersity continued to be observed. This effect (size and dispersity reduction) was intensified as the number of cycles increased up to 17, after which, no further significant changes were detected.

The droplet size distributions of the produced Pickering emulsions as a function of the applied number of cycles are also included in Figure 4.3. The analysis was made immediately after preparation and along a specific storage period (0, 2, 7, 14, 30 and 60 days); however, to facilitate the analysis, only the results obtained after preparation and after 60 days of storage are shown. As the number of cycles increases (1 pass to 2 cycles), it is possible to observe the displacement of the size distribution to lower sizes. In the subsequent cycles, an increase in the volume associated to smaller sizes was observed, indicating an effective reduction of size. Moreover, the difference between the obtained distributions (0 and 60 days) become negligible as the number of cycles increases, corroborating the achieved good stability. To complete the data the corresponding average sizes after production and after 60 days under storage are presented in Table 4.1. The analysis of the obtained values corroborates the decrease of the average droplet size as the number of cycles increased, with no significant change after 17 cycles. These results are consistent with the observations made by optical microscopy analysis. With the exception of the pre-Pickering emulsion, where the droplet size increases along the storage time, indicating coalescence (unstable system), the other emulsions maintain their droplet size over the analysed 60 days period (stable systems).

Table 4.1: Mean droplet size (μm) of the produced Pickering emulsions for the different number of cycles at constant Re of 400 for 0 and 60 days of storage time. Represented as average ± SD (μm).

	0 DAYS	60 DAYS
1 pass (pre-Pickering emulsion)	97.76±4.05	119.65±2.85
2 cycles	12.83±0.19	12.66±0.04
5 cycles	8.61±0.01	8.55±0.01
17 cycles	6.83±0.01	6.89±0.01
35 cycles	6.14±0.01	6.07±0.01

The increasing number of cycles through recirculation, i.e., the increase of the residence time, submits the emulsion to higher shear rates for longer times leading to the formation of low-size droplet emulsions with also narrower size dispersity. These two combined effects contribute to improve stability and the shelf-life of the product (Zanatta *et al.*, 2017). Other authors also reported that the emulsion stability depends on the droplet size. Namely, Saari *et al.* (2019) showed that an emulsion stabilised with modified starch, which presented a droplet size of approximately 17 μm, evolves to 85 μm after a 30-days period under storage. Zanatta *et al.* (2017) showed that an emulsion stabilised with Tween 20 maintained their droplet structure and size after a 60-days storage period when the diameter of the droplets is around 3–5 μm, and if preserved in the fridge.

Figure 4.4 shows the digital photographs of the Pickering emulsion produced with Re = 400 and 17 cycles. In Figure 4.4 A the freshly produced emulsion is shown, while in Figure 4.4 B the same emulsion after 60 days under storage is presented. Comparing both samples, it is possible to observe that the Pickering emulsion did not present changes perceptible to visual observation. No phase separation, and macroscopic characteristics preservation (mainly colour) was noted. Furthermore, by the drop test, this Pickering emulsion revealed to be O/W type, with no change over time.

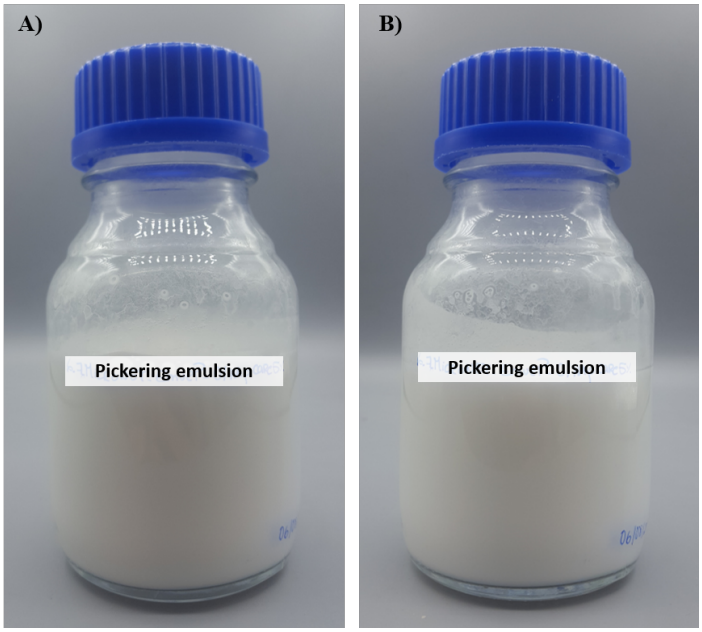

Figure 4.4: Images of the Pickering emulsion produced with Re=400 and subjected to 17 cycles. A) after production (0days) and B) after 60-days of storage.

4.3.2 EFFECT OF RE NUMBER ON EMULSION MORPHOLOGY AND STABILITY

To study the effect of Re, and the advantages of using different conditions, Pickering emulsions were produced using Re of 200, 300, 400 and 500, by changing the flow rate, and fixing the number of cycles at 17, since previous results indicate that this value gives rise to the maximum reduction in the droplet size. The effect of the Re number on the droplet shape and size was monitored by optical microscopy and laser diffraction, respectively, by analysing the sample along a 60-days period under storage (2, 7, 15, 30 and 60 days).

Table 4.2 shows the flow rates for each one of the used solutions (A, and B) in order to achieve the desired Re in the pre-Pickering emulsion formation, and for the Pickering emulsion once entering the recirculation phase. Viscosity of solutions A, and B, and the pre-Pickering emulsion were determined at 0.002, 0.060 and 0.004 Pa·s, respectively. This last value, along with the mixture's density of 1006 kg/m^3 was used for the calculation of Re. After the preparation of the pre-Pickering emulsion from solutions A and B the mixture was recirculated in the system and the Re number changed by modifying the flow rate. The use of different Re will influence the time needed to reach the chosen number of cycles (i.e., the residence time). This time decreases as higher Re values are used. The time values were calculated for a Pickering emulsion volume production of 230 mL; however, this volume can be modified as long as the total recirculating time is adjusted to achieve the desired number of cycles.

Table 4.2: Flow rates of the solution A and B, and of the Pickering emulsion to achieve a Re number of 200, 300, 400, and 500.

	200	300	400	500
Flow rate for Solution A (mL/s)	4.9	7.4	9.9	12.4
Flow rate for Solution B (mL/s)	1.2	1.9	2.5	3.1
Flow rate for Pickering emulsion (mL/s)	6.1	9.3	12.4	15.4
Time for 17 cycles (min)	10.6	7.0	5.3	4.2

Figure 4.5 shows the optical microscopy images taken for the Pickering emulsions produced with the evaluated Re numbers (200, 300, 400, and 500). Pickering emulsions produced at lower Re (Re = 200) gave rise to a product with a non-spherical droplet pattern. This emulsion

has oil droplets with irregular shape and large size. As a consequence, a broad size distribution was obtained, and the emulsion became unstable after 7 days (visual observation of phase separation). In fact, broad size distributions facilitate coalescence and Ostwald ripening phenomena where larger droplets grow by diffusion of oil coming from the smaller ones (Zanatta *et al.*, 2017).

The Pickering emulsions produced with higher Re values (Re > 200), characterised by a uniform spherical shape and small size, have maintained the stability over the tested storage time. In Figure 4.5, the obtained size distributions of the produced Pickering emulsions, right after production and after 60-days under storage, are also shown. It is possible to observe a narrowing trend of the distributions, as well as an increase in the volume associated with smaller sizes as the Re increases. In Table 4.3, the average diameter of the Pickering droplets after 0 and 60 days under storage are shown. The rise of the Re number leads to a decrease of the average droplet size, corroborating the data obtained by optical microscopy. When the Re increases from 200 to 400, the droplet size decreases from ~60 to ~7 μm. However, for Re = 500 no significant changes in the droplet size were observed in comparison with Re = 400.

Table 4.3: Mean droplet size (μm) of the produced Pickering emulsions for the different Re at constant number of cycles (17) for 0 and 60 days of storage time. Represented as average±SD (μm).

	0 DAYS	60 DAYS
200	60.76±1.17	65.93±1.50
300	21.83±0.09	21.73±0.09
400	6.83±0.01	6.89±0.01
500	6.12±0.02	6.00±0.01

The results shown in the present section pointed out that Re played an important role in emulsion formation process, namely in the obtained droplet size. Muruganandam *et al.* (2018) claim that the oil droplet size distribution in a static mixer is influenced by the viscous shear force, frictional force, which tends to deform the drops. The relative magnitude of these forces

on the droplet break-up is described by Reynolds number and Weber number (Muruganandam *et al.*, 2018).

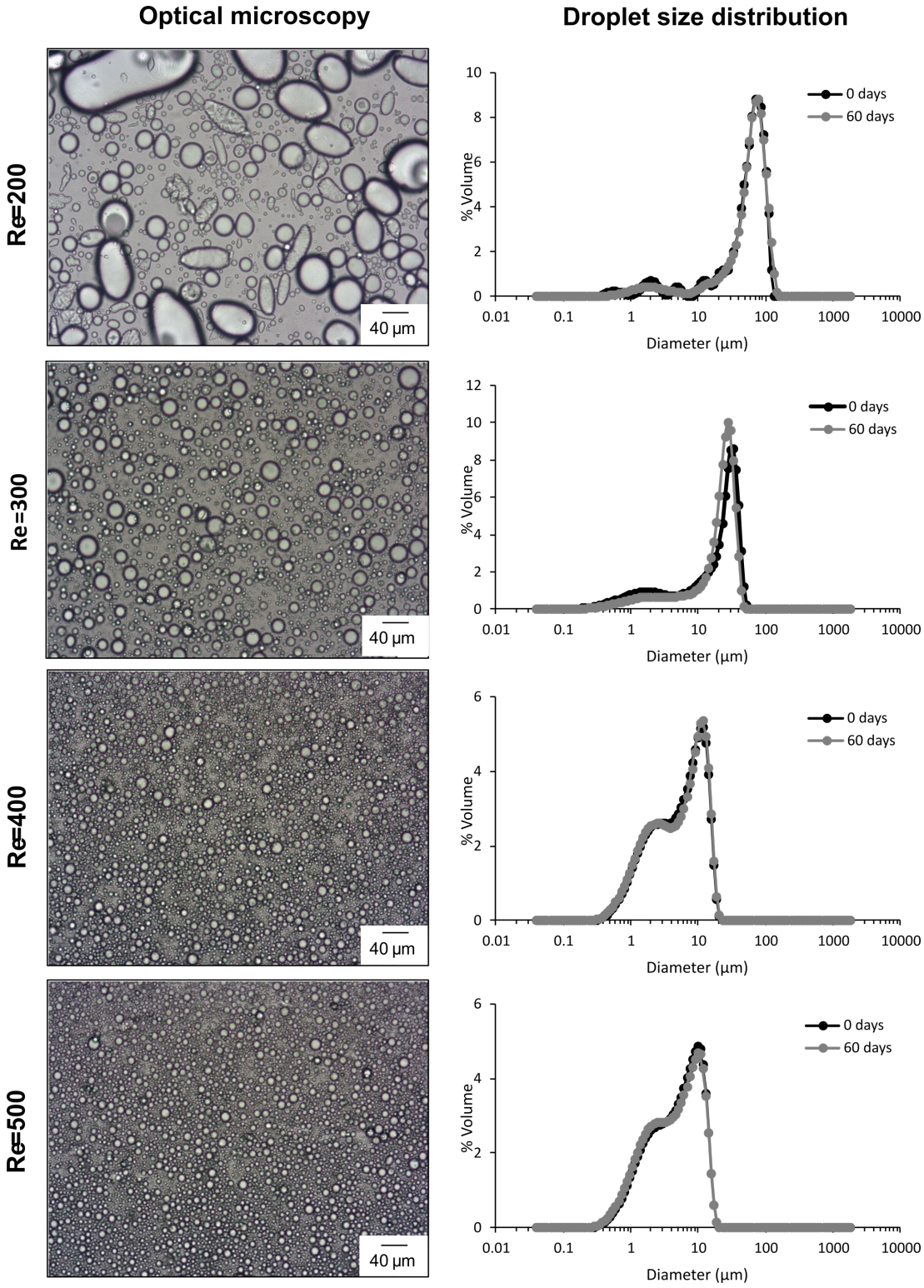

Figure 4.5: Optical microscopy images and size distributions for 0 and 60 days of the produced Pickering emulsions (17 cycles) as a function of Re. All images were taken with magnification of 20x.

In previous works, various models have been proposed for the prediction of the droplet size. For example, Boxall *et al*. (2012) estimate the emulsion droplet size according to Weber and Reynolds numbers. In this respect, Muruganandam *et al*. (2018) showed that when a O/W emulsion is prepared in a SMX static mixer the Sauter mean diameter of the oil droplets decreases from 8 μm to 4 μm as the Reynolds number increases. Furthermore, in cellulose acetate microcapsules produced by NETmix reactor, a size reduction was also observed as the Re increases, namely when Re increases from 200 to 500, the average diameter decreased from 30 to 5 μm (Moreira *et al.*, 2020). The findings reported by these authors are in agreement with the ones obtained in this work.

Additionally, it was also verified that the droplet shape, emulsion stability and experiment duration strongly depend on Re number. By increasing the Re value, the Pickering emulsion needs less time to be prepared (i.e., to achieve the desired number of cycles). Furthermore, when Re increased up to 400 a decrease of the droplet size and an increase of particle's sphericity is observed. Above 400 no further significant size reduction was observed, and the gain of time was insignificant. The use of Re higher than 500 was not considered due to equipment constraints.

All produced Pickering emulsions revealed to be of O/W type by the drop test.

4.3.3 Interfacial structure of Pickering emulsion droplets

The microstructure, especially the interfacial structure, of the Pickering droplets was examined by CLSM and cryo-SEM. These techniques allow to observe the existent solid particles at the droplet's surface and thus check their effective role as Pickering stabilisers.

In the case of CLSM, the Pickering emulsion sample was stained using two dyes. Nile red was used to stain the oil phase and Nile blue A to stain the n-HAp particles. Figure 4.6 show CLSM images of the Pickering emulsion obtained for Re = 400 and 17 cycles. Figure 4.6 A shows the Pickering emulsions after production (0 days) and Figure 4.6 B shows the emulsion after 60 days of storage. The green fluorescence in the images puts in evidence the oil droplets, while the red fluorescence indicates the positioning of the n-HAp particles. In the overlapped images (3rd image in each row) both oil and n-HAp phases can be observed. From the confocal

images it was possible to observe well-defined oil droplets, and in the overlaid images a clear red halo was perceptible corroborating the presence of n-HAp particles surrounding the oil core, i.e., forming a layer at the boundary of the oil droplets. This interfacial structure provides a physical barrier for Pickering emulsions, which improved the storage stabilisation against coalescence. Previous works reported that octenyl succinic anhydride-modified gliadin nanoparticles, zein/gum arabic complex nanoparticles and β-cyclodextrin particles also form a dense layer at the surface of the oil droplets acting as steric barriers against coalescence and Ostwald ripening phenomena (Dai *et al.*, 2018; Li *et al.*, 2019; Xi *et al.*, 2018). The authors reported the long-term storage stability of their emulsions; however, 30 days was the best mark. In this work, it is shown that the n-HAp Pickering emulsions maintaining their droplet size and n-HAp particles covering the oil surface for at least 60 days. The sample subjected to a 60 days storage period (B), presented a quite similar morphology to the one achieved for the freshly prepared emulsion (A) indicating none or minimal coalescence phenomenon.

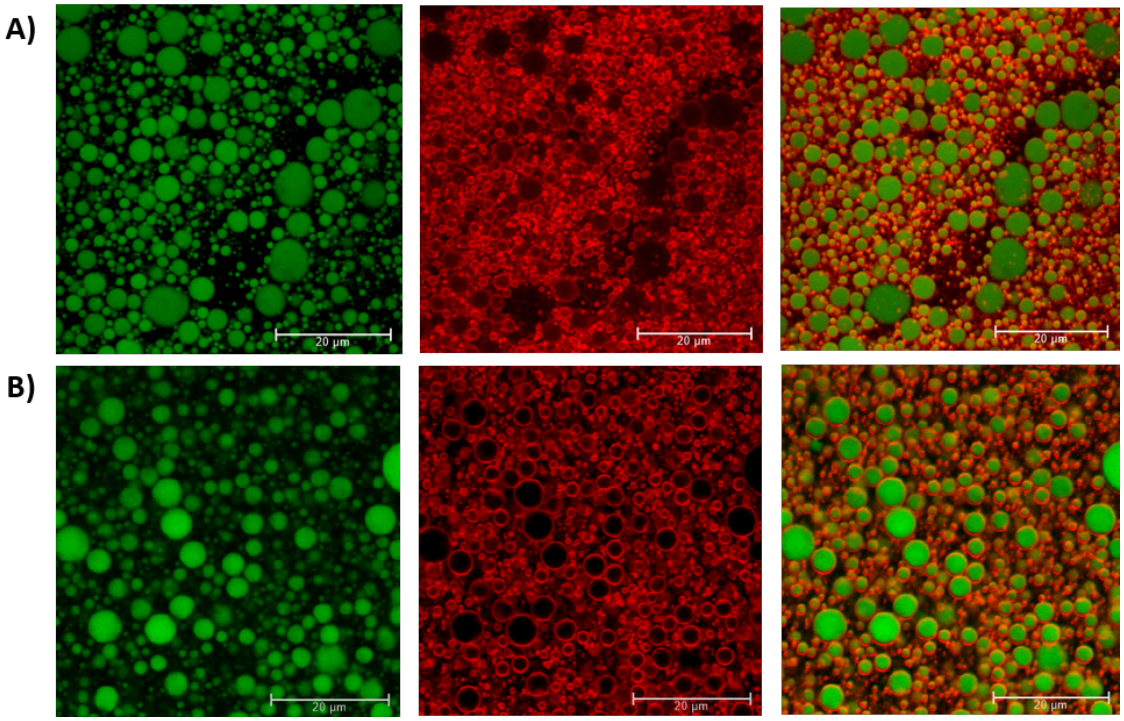

Figure 4.6: Confocal laser scanning microscopy (CLSM) images of the Pickering emulsion produced at Re = 400 and 17 cycles and stabilised with n-HAp particles: A) after production and B) after 60 days of storage. Oil core was stained with Nile red and n-HAp particles stained by Nile blue A. The fluorescent dyes excited at 488 nm for Nile red (green fluorescence) and at 633 nm for Nile blue A (red fluorescence). For interpretation of the references to the colour in this figure legend, the reader is referred to the web version of this article. Scale bar corresponds to 20 µm, all images were acquired with lens of 40x and 6x zoom.

Figure 4.7 shows cryo-SEM images where it is possible to observe individualised Pickering droplets covered by n-HAp. The lack of the white layer on the top of the droplets surface is a consequence of the sample preparation procedure for the analysis, which is also a useful feature since it allows to expose the droplet core thus facilitating their examination and identification. Different phases can be distinguished due to their different texture and colour after freezing. The sunflower oil has a texture relatively smoother than the n-HAp particles, which are whiter than the oil and water phases.

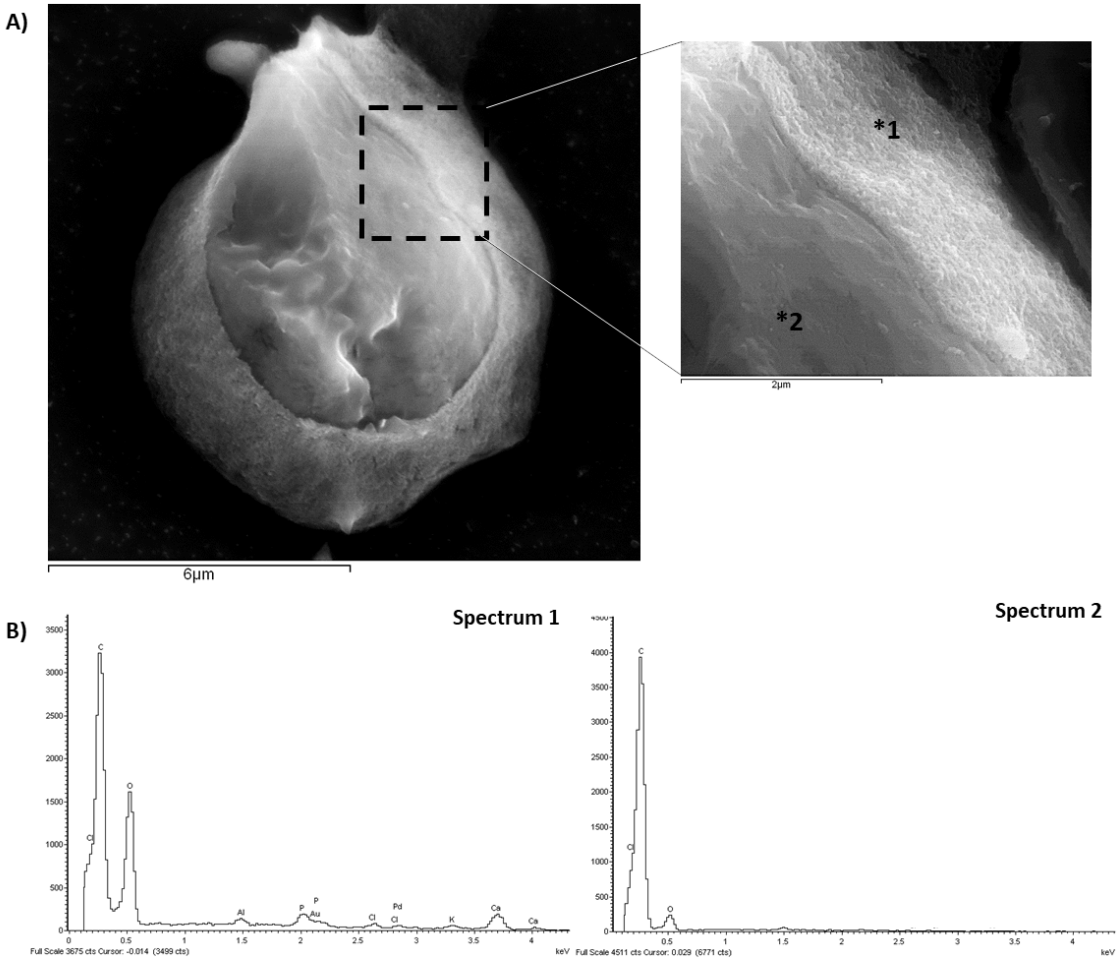

Figure 4.7: Cryo-scanning electron microscopy image (cryo-SEM) of the Pickering emulsion stabilised with n-HAp particles. A) micrograph and B) EDS analysis.

Furthermore, from Figure 4.7 A it is also clear the hydroxyapatite layer around the oil core, and in the amplified image it is possible to depict the individualised n-HAp particles, which indicates the clear ability of the n-HAp particles to act as Pickering stabilisers. The EDS analysis (Figure 4.7 B) confirms the presence of the main elements of each phase. Phosphorus (P), calcium (Ca), potassium (K) and chloride (Cl) are the main elements of the n-HAp particles

(spectrum 1) and are only detected in the layer covering the oil droplet. Inside the Pickering droplet, carbon (C) was the main detected element corresponding to the oil core (spectrum 2). Other authors also analysed Pickering emulsions through cryo-SEM. Griffith and Daigle (2018) have confirmed through this technique that silica nanoparticles modified with hydrophilic silane were adsorbed at the oil droplets surface, and Binks and Tyowua (2016) showed that silica particles are entirely covering the interface of the droplets.

The two used microscopy techniques, CLSM and cryo-SEM, also allowed to confirm the O/W emulsion type, and the hydroxyapatite shell morphology.

4.3.4 Advantages of NETmix production over batch processes

This work shows a new alternative to produce Pickering emulsions in continuous mode using the NETmix technology, and this section aims to discuss the advantages of using this process over the previously tested batch-mode (Ribeiro *et al.*, 2020). Thus, the base batch formulation (oil/water ratio of 20/80 (*v/v*), n-HAp content of 5 wt%), produced using a rotor-stator homogeniser at 11000 rpm and 6 minutes, was compared with the emulsion produced with 17 cycles at Re = 400. These two assays conducted the maximum size reduction in each of the tested techniques.

Figure 4.8 shows the comparison between the two emulsions, namely by presenting the data obtained by optical microscopy, and size distribution by laser diffraction, right after production (0 days). From the comparison it can be perceived the lower size obtained by the continuous process, which was not possible to achieve with batch mode, respectively ~7 μm and ~18 μm (~60% lower in the case of NETmix). This effect was also accompanied by the narrowing of the size distribution for the NETmix system. In both cases, the produced Pickering emulsions remained stable for the evaluated 60 days period, but due to the lower size obtained for the NETmix based dispersions, a higher long-term stability is expected for this last case.

NETmix allows an easy size reduction control by adjusting the number of cycles and Re number, giving rise to the achievement of low particle size emulsions at short times (an advantage from a productive point of view). Moreover, it offers the advantage to tailor-made

dispersions according to target droplet sizes, which is an advantage for several applications where particle size is an important parameter.

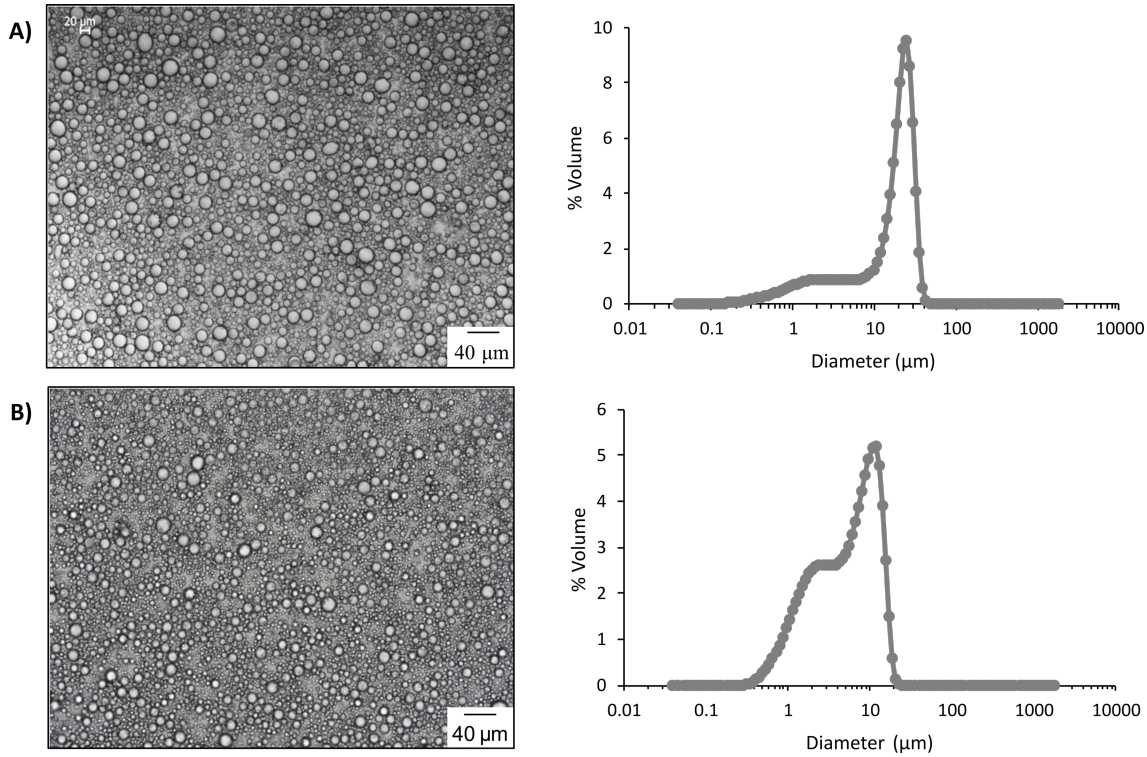

Figure 4.8: Optical images and size distributions comparison obtained from batch using rotor stator homogeniser (A) and continuous using NETmix (B). The Pickering emulsions were obtained with 20:80 (*v/v*) as oil-water ration and with 5 wt% of n-HAp particles.

The space-time yield (STY), defined as the mass flow rate per reactor volume, was used to compare the two devices in terms of production. For the rotor-stator, STY = 2.4×10^5 kg/m^3/day was estimated as the produced emulsion mass divided by reactor volume and stirring time. For NETmix, STY = 3.0×10^7 kg/m^3/day was obtained using the mass flow rate, the reactor volume and total recirculation time. NETmix, a low energy device, is capable of productions two orders of magnitude higher.

By using NETmix the overheating of the sample is avoided, which is not easy to achieve when using rotor-stator homogeniser systems, demanding extra investment in effective cooling systems. In fact, NETmix due to the micro-mixing level inside of the chambers, offers an easy control of temperature, which can assist processes where this factor is relevant. Table 4.4 summarises the main advantages using NETmix over rotor stator homogenisers in Pickering

emulsion production. These analyses were produced according to the tested setups, and the findings in our research team.

Table 4.4: Advantages comparison of NETmix over to rotor-stator in Pickering emulsion production.

	CONTINUOUS NETMIX	BATCH ROTOR-STATOR HOMOGENISER
Installation assembly		
Easy set-up	+/-	+/-
Emulsion Production		
Submicrometric droplets	+	+/-
Risk of solid particles disruption	-	+/-
Risk of temperature rise	-	+
Energetic point and industrialization		
Energy consumption	-	+
Industrialization possibilities	+	+/-

+ = positive; - = negative: +/- = intermediate.

4.4 CONCLUSIONS

In this work, Pickering emulsions stabilised with n-HAp were successfully produced in continuous mode using NETmix, a mesostructured static mixer and reactor. This strategy ensures the production of surfactant-free emulsions overcoming constraints associated with batch mode production. Stable O/W Pickering emulsions were produced, and the used number of cycles and Re influenced droplet size, shape and stability, parameters that can be used to tailor-made emulsions according to size. Using 17 cycles and Re = 400, an emulsion with an average size of 7 μm was obtained, also proving the suitability of NETmix to achieve, easily, small size droplets, which responds to requisites of some applications, and promotes stability (under the tested conditions stability was proved till 60-days). CLSM and cryo-SEM microscopy techniques showed the effective role of n-HAp particles, which covered the oil droplet surface confirming the oil core nature of the droplets. The achieved results in this work

are quite promising and point out the feasibility to use the NETmix technology to produce Pickering emulsions and control droplet size. Additionally, NETmix is a low energy device and can assure an effective temperature control, avoiding the overheating occurring with rotor-stator homogenisers. Moreover, considering that Pickering emulsions can be applied in a wide range of the products, their production in continuous mode supports high-volume scale production, which is more compatible with industrial production.

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5 EFFECT OF ENVIRONMENTAL STRESSES IN NANOHYDROXYAPATITE PICKERING EMULSIONS

This chapter is based on: Andreia Ribeiro, Yaidelin A. Manrique, José Carlos B. Lopes, Madalena M. Dias, Maria Filomena Barreiro. *Effect of temperature, pH and ionic strength on hydroxyapatite stabilised Pickering emulsions produced in batch and continuous mode.* Under submission.

Oil-in-water (O/W) Pickering emulsions are attracting attention as carriers of lipophilic active compounds with clear advantages over traditional systems. Having in view their effective use it is important to study their stability against environmental stresses impacting manufacture, storage, and final application conditions. In this work, Pickering emulsions produced in continuous mode using a mesostructured reactor (average size ~7, 11 and 18 μm) and in batch mode using a rotor-stator device (average size ~18 μm) were studied concerning their behaviour at different temperatures (5-90 °C), pH (2-10) and ionic strength (0-500 mM). Droplet size, morphology, and zeta-potential were analysed after 1 and 7 days under storage. In general, and despite the droplet size, the n-HAp Pickering emulsions were stable within the tested ionic strength range, at relatively high pH environments (6-10), and at temperatures up to 70 °C. Pickering emulsions undergo complete phase separation at very low pH (2) due to n-HAp particle's disruption. Nevertheless, a clear tendency to aggregation and coalescence was observed for high temperatures (70-90 °C). Results indicate no significant differences related to the used production method since the produced emulsions had similar stability. From an industrial perspective, this work also corroborates that the scale-up to a continuous process using a mesostructured reactor, NETmix, from a batch laboratorial process is feasible without impacting stability.

5.1 INTRODUCTION

Emulsification is considered a suitable method for preserving the innate characteristics of bioactive compounds over time, similarly to an encapsulation technique. Moreover, it facilitates the combination of highly lipophilic and hydrophilic components in the same product, which is an important strategy in the manufacture of important commercial products (McClements, 2016; Yang & McClements, 2013). In this field, Pickering emulsions, due to their surfactant-free nature, are greener alternatives to conventional emulsions, offering advantages to protect bioactive compounds (Hategekimana *et al.*, 2015; Mitbumrung *et al.*, 2019). Their stabilisation is ensured by solid particles, which due to their high attachment energy, bind almost irreversibly to the droplet's surface, ensuring great stability, particularly against coalescence during storage. In fact, Pickering emulsions are recognised to have good physical and chemical stability (Albert *et al.*, 2019; Chevalier & Bolzinger, 2013; Ortiz *et al.*, 2020), which is important for applications in food, cosmetics and pharmaceuticals, fulfilling sustainability and green label issues (Albert *et al.*, 2019).

Oil-in-water (O/W) Pickering emulsions are suitable systems to encapsulate lipophilic active compounds (Winuprasith *et al.*, 2018), bringing advantages over traditional ones. They can improve the compatibility between components in a final product (Albert *et al.*, 2019; Frelichowska *et al.*, 2009b), enhancing the stability, bioaccessibility and absorption of the lipophilic compounds in the human body (Albert *et al.*, 2019; Frelichowska *et al.*, 2009a).

The advantages and extensive applicability of Pickering emulsions, mainly in the development of fortified foods, functional cosmetics, and pharmaceuticals, is rising the interest for these systems. Of high interest are stability studies against environmental stresses such as temperature, pH, and ionic strength, aiming at getting insights on their behaviour under stress or stringent conditions. Most of the reported data on Pickering emulsions stability focus factors influencing their production, such as the solid particles concentration, droplet size, and oil content, and the stability to storage under controlled conditions. In this perspective, knowing if the Pickering emulsion remains physically and chemically stable throughout manufacture, storage, and final application conditions (e.g., consumption) by simulating relevant conditions through environmental stress tests is of high relevance (Winuprasith *et al.*,

2018). Also, the incorporation of Pickering emulsions into final products should not adversely impact product's final physicochemical and organoleptic properties.

In this context, Mitbumrung *et al*. (2019) showed that Pickering emulsions stabilised by nanofibrillated mangosteen cellulose were stable under a wide range of ionic strength conditions and temperatures, becoming sensitive to low pH by showing droplet's aggregation and vitamin D_3 encapsulation efficiency loss. Zhou *et al*. (2020) evidenced that relatively high pH values (from 5 to 8) favour the aggregation of the nanochitin-stabilised Pickering emulsions. In another study, Bai *et al*. (2019) reported that Pickering emulsions stabilised by cellulose nanocrystals had quite good stability to pH changes (3 to 10), to the presence of electrolytes (100 mM NaCl), and to temperature (30 to 90 °C). However, cellulose nanocrystals-based Pickering emulsions showed droplet flocculation under acidic conditions (pH 2) and at high ionic strength media (200–500 mM NaCl). These observations corroborate the need to conduct these stability studies, which are significantly influenced by the nature of the solid particles. Furthermore, these studies can help to establish effective encapsulation procedures using emulsion-based carriers, e.g., for the protection and delivery of lipophilic vitamins in food products.

Most of the reported studies in the literature focus Pickering emulsions production in batch mode using rotor-stator or ultrasonic devices (Ribeiro *et al.*, 2020; Samanta *et al.*, 2017; Zhang *et al.*, 2019), and in continuous mode through microfluidic devices (Thompson *et al.*, 2011; Yuan & Williams, 2016). However, these techniques have disadvantages associated with the used high shear conditions and limited production volumes (Albert *et al.*, 2019). To surpass these constraints, NETmix, a mesostructured reactor based on a static mixer enabling production in continuous mode, was recently proposed for Pickering emulsions production (Ribeiro *et al.*, 2021), showing to be a promising alternative. In this context, the purpose of the present work was to study the impact of different environmental stresses (temperature (5-90 °C), pH (2-10), and ionic strength (0-500 mM) on the stability of nanohydroxyapatite (n-HAp) stabilised Pickering emulsions produced by traditional (rotor-stator) and innovative (NETmix) methods. In this study, the morphology, droplet size, and zeta potential, were evaluated over a storage period of 7 days (sampling at 1 and 7 days) to understand Pickering emulsions' behaviour after the applied stresses, expanding the applicability of Pickering

emulsions stabilised by n-HAp. To the best of our knowledge, this work reports, for the first time, a systematic study analysing the influence of environmental stresses on the n-HAp Pickering stability, which might be useful considering production, storage and final application scenarios of these products.

5.2 MATERIALS AND METHODS

5.2.1 MATERIALS

An hydroxyapatite aqueous paste, *nano*XIM-CarePaste, was supplied by Fluidinova S.A. N*ano*XIM-Care Paste is composed of 15.5 ± 0.5 (wt%) of HAp nanoparticles (n-HAp) with particle size < 50 nm, 4.5 ± 0.5 (wt%) KCl and water content ≤ 81.0 (wt%). Sunflower oil was purchased from a local supermarket. Sodium oleate and fluorescent dyes (Nile red and Nile blue A) were obtained from Sigma-Aldrich. Isopropyl alcohol was purchased from Riedel-de Haen. Distilled water, treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA), was also used. All other chemicals were of analytical grade.

5.2.2 PICKERING EMULSION PRODUCTION

Oil-in-water (O/W) Pickering emulsions were prepared according to two previously reported procedures of the group, in batch mode using a rotor-stator device (Ribeiro *et al.*, 2020), and in continuous mode using the static mixer NETmix (Ribeiro *et al.*, 2021). The Pickering emulsions were obtained using an oil to water ratio of 20/80 (*v/v*). The aqueous phase corresponds to n-HAp (5 wt%) dispersed in water, and the oil phase to sunflower oil. In the batch procedure, the aqueous phase was poured into the reactor and the sunflower oil injected dropwise using a peristaltic pump. The rotor-stator device was setup to operate at 11000 rpm for 6 min. In the NETmix procedure, the aqueous and oil phases were firstly mixed to form a coarse emulsion. Then, the Pickering emulsion with the desired droplet size was obtained by recirculating the coarse emulsion inside the NETmix reactor during the needed number of cycles at the required Reynolds number (Re). The production conditions in terms of Re were 300-400 and number of cycles 5-17. Three samples were produced with NETmix (average size ~7, 11 and 18 μm; to study the effect of the droplet size), and one with the rotor-

stator device (average size ~18 μm; to compare with the NETmix and evaluate the effect of the used preparation method).

5.2.3 PICKERING EMULSIONS ENVIRONMENTAL STABILITY TESTS

The produced Pickering emulsions were evaluated concerning the stability against typical stresses with interest for the production of commercial products and upon ingestion (gastrointestinal tract passage). The range of the selected average droplet sizes took into account the typical sizes used in emulsion food products. Thus, all Pickering emulsions were submitted to different temperature conditions (5, 22, 30, 50, 70 and 90 °C), pH (2, 4, 6, 8 and 10) and electrolyte (NaCl) concentrations (0, 100, 200, 300, 400 and 500 mM) aiming at evaluating their effect on emulsion properties (droplet size, shape, and surface charge). Additionally, the stability of the Pickering emulsions' subjected to the applied stresses was evaluated considering the importance of these parameters also during storage. For that, emulsions were stored for 7 days at room temperature and further evaluated. Each condition was tested in triplicate (independent assays).

5.2.3.1 Temperature effect

To evaluate the temperature effect, 10 mL of the Pickering emulsion were transferred into test tubes. The procedure was adapted according to the tested temperature. Namely, for low temperatures, the Pickering emulsions were stored in the fridge (5 °C), and at room temperature (22 °C). For higher temperatures, the Pickering emulsions were incubated in a water bath at the required value (30 to 90 °C). After reaching the temperature equilibrium, samples were left under the chosen conditions for 30 min, then cooled to room temperature and stored for 1 and 7 days before analysis.

5.2.3.2 pH effect

To evaluate the pH effect, 10 mL of the Pickering emulsion were transferred to test tubes. The pH was adjusted to the required value (2, 4, 6, 8 and 10) with NaOH (0.1 M) or HCl (1 or 2.5 M) solutions, as needed. The pH was measured with a pH probe accompanied by gentle stirring. The emulsions were stored for 1 and 7 days, at room temperature, before analysis.

5.2.3.3 Ionic strength effect

An appropriated amount of anhydrous NaCl powder, namely the one needed to achieve the desired ionic strength (100 to 500 mM), was added directly into the freshly prepared Pickering emulsions that were then gently stirred to ensure NaCl solubilisation. The samples were stored at room temperature for 1 and 7 days until analysis.

5.2.4 PICKERING EMULSIONS CHARACTERISATION

After submitting the Pickering emulsions to the different environmental stresses, the emulsion properties (droplet size, morphology, and surface charge) were evaluated. For that, diffraction particle size analyser, fluorescence optical microscopy and zeta potential analysis were conducted.

5.2.4.1 Droplet size analysis

Droplet size distributions (in volume) were obtained using a laser diffraction particle size analyser (Beckman Coulter LS230). The average size was determined from the obtained volume distributions and reported as the average volume diameter, $d_{4,3}$. These evaluations allow to inspect for possible coalescence and/or aggregation phenomena, reflected as an average diameter increase, which indicates signs of instability. The analysis was done in duplicate for each sample triplicate, and the data expressed as average ± standard deviation (SD).

5.2.4.2 Zeta potential analysis

The zeta potential, evaluated through the droplet surface charge, is an indicative of the emulsion stability. Thus, the sample can be considered stable for high positive (≥ 30 mV) or high negative (≤ -30 mV) values. In magnitude, zeta potential higher than 30 mV is obtained for stable emulsions meaning that, within this range, droplets develop enough electrostatic repulsion to avoid aggregations. The zeta potential was determined using an electrophoresis instrument, Zetaziser Nano ZS90 (Malvern Instruments). For the analysis, Pickering emulsions were diluted with distilled water as needed to avoid multiple scattering effects. The diluted sample was put into a folded capillary Zeta cell (ref: DS7010), and the determination carried

out at 25 °C. The analysis was performed in triplicate of each sample triplicate, and the data expressed as average±SD.

5.2.4.3 Fluorescence microscopy analysis

Fluorescence microscopy was used to monitor the evolution of the oil droplets morphology and size after subjecting the Pickering emulsion to the environmental stresses. This technique enables a better observation of the oil droplets allowing to easily inspect the presence of the oil, namely the free oil, within the emulsion. Nile red stains the oil phase (individual oil droplets and lipid rich regions) that will appear as bright areas, whereas the aqueous phase (not dyed) will appear as dark regions in the micrographs. For fluorescence microscopy analysis, 100 µL of the Pickering emulsion was stained with 10 µL of a Nile red solution at 0.1% *w/v*. Then, an aliquot of each sample was placed on a slide and gently covered with a coverslip. The analysis was performed using a Nikon optical microscope fitted with a TRITC filter and a digital camera (Nikon DS-Q_i2). The image acquisition and processing was done using NIS-Elements Nikon software.

5.2.5 STATISTICAL ANALYSIS

The experiments were performed in triplicate and expressed as average ± SD. The statistical analysis was performed using the commercial software IBM SPSS statistics (version 27.0, SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was used to analyse the data of the environmental stresses' studies, and significant differences between the average values ($p < 0.05$) evaluated using Tukey's test. Additionally, *T*-test was used to analyse size significant differences ($p < 0.05$) between 1 and 7 days under storage for each environmental stress.

5.3 RESULTS AND DISCUSSION

5.3.1 INITIAL PICKERING EMULSIONS

The Pickering emulsions produced according to the selected average sizes using the mesostructured reactor NETmix and the rotor-stator device are listed in Table 5.1. It puts in evidence the production conditions (Re and number of cycles) to achieve the required

dimensions using the NETmix, and stirring rate (rpm) for the rotor-stator device, together with the performed characterisations (average diameter and zeta potential). The influence of the production parameters in the chosen system, (O/W n-HAp stabilised Pickering emulsions prepared with sunflower oil) was previously studied by Ribeiro *et al*. (2021), where the feasibility of the NETmix technology to control the droplet size was reported. NETmix Pickering emulsions are referred as NET-low, NET-middle and NET-high, respectively for the samples holding proximate dimensions of 7, 11, and 18 μm. The sample produced with the rotor-stator, with proximate dimension of 18 μm, is referred as Batch. Their typical size distributions are shown in Figure 5.1 A. The produced Pickering emulsions were characterised by strong positive values of zeta potential (all over 30 mV), indicating stable emulsions with strong electrostatic repulsion inhibiting droplet aggregation. Pickering emulsions macroscopic appearance was registered photographically after 1 day under storage for the samples not subject to any environmental stress (Figure 5.1 B). For all cases, independently of the used productive method and droplet size, samples had a white appearance and good resistance to gravitational separation (data not shown), in accordance with the data previously reported by Ribeiro *et al*. (2020) and Ribeiro *et al*. (2021) which indicated a 2-months stability for this Pickering emulsion formulation.

Table 5.1: NETmix and rotor-stator device productive conditions and respective Pickering emulsions characterisation (average diameter and zeta potential).

SAMPLE NAME	PRODUCTION CONDITIONS			CHARACTERISATION	
	Re	CYCLES	STIRRING (RPM)	AVERAGE DIAMETER (μm)	ZETA POTENTIAL (mV)
NET-low	400	17	-	7.11±0.05	30.97±0.41
NET-middle	400	5	-	11.34±0.27	32.72±1.09
NET-high	300	10	-	17.87±0.58	32.00±0.91
Batch	-	-	11000	17.58±0.11	31.53±1.01

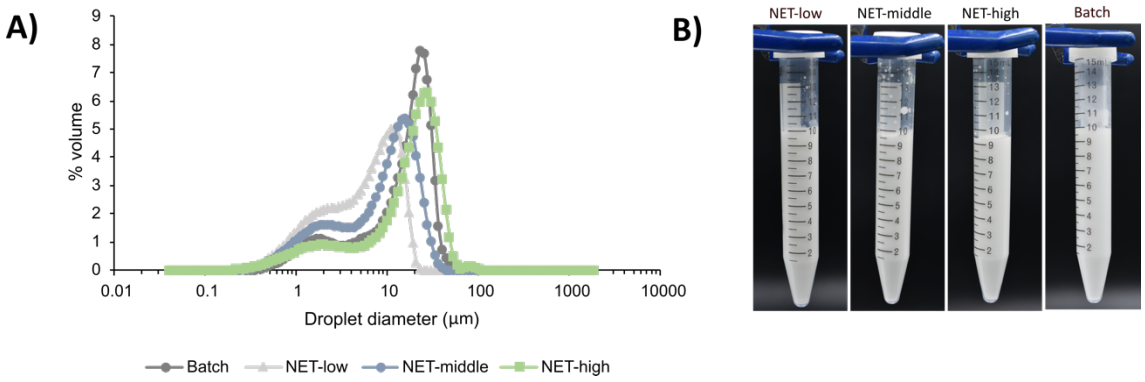

Figure 5.1: Droplet size distribution (A) and photographic register after 1 day (B) of the Pickering emulsions produced by NETmix and rotor-stator device. The shown samples were not subjected to any environmental stress.

5.3.2 ENVIRONMENTAL STRESS STABILITY STUDIES

The produced Pickering emulsions were submitted to different environmental stresses, namely temperature (5–90 °C), pH (2-10) and ionic strength (100-500 mM) to evaluate their stability under these conditions. The study was carried out using the three Pickering emulsions produced with NETmix and the one produced in Batch as listed in Table 5.1. The aim was to evaluate the effect of the droplet size, and the effect of using two distinct productive technologies. All analyses were performed at 1 and 7 days after production to perceive also the occurrence of destabilisation phenomena with storage time.

5.3.2.1 Temperature effect

The produced n-HAp Pickering emulsions (NET-low, NET-middle, NET-high and Batch) were incubated at different temperatures, namely at 5, 22 (room temperature), 30, 50, 70 and 90 °C, and the effect on the average diameter, zeta potential and morphology evaluated considering two storage times: 1 and 7 days.

Figure 5.2 shows the size expressed as average diameter and the zeta potential of the Pickering emulsions produced using the NETmix technology and the rotor-stator device, respectively, as a function of temperature for 1 day (filled columns) and 7 days (empty columns). The statistical analysis was done for the following 3 cases: (i) for each size category (NET-low - 7 µm, NET-middle - 11 µm, NET-high – 18 µm and batch – 18 µm) comparison of emulsion sizes for all temperatures after 1 day, (i) for each size category (NET-low - 7 µm, NET-

middle - 11 μm, NET-high – 18 μm and batch – 18 μm) comparison of emulsion sizes for all temperatures after 7 days, and (iii) for each tested temperature (5, 22, 30, 50, 70 and 90 °C) comparison of the emulsion size after 1 and 7 days of storage. From the analysis of Figure 5.2 it is possible to observe the impact of temperature on the Pickering emulsions with different sizes and produced by the two different technologies. Analysing the emulsion size after 1 day, there are changes for emulsions incubated at 90 °C ($p < 0.05$), independently of the studied emulsion category. However, after 7 days of storage, differences start to be perceived for emulsions treated at 70 °C (or higher temperatures), with exception of the NET-low ($p < 0.05$). The emulsions start to develop instability under storage mainly those incubated at high temperatures. Additionally, it is possible to perceive that, temperatures in the range 5-50 °C do not negatively impacted emulsion stability, which was preserved; the average diameters for these samples were similar for both sampling times (1 and 7 days) without statistical difference ($p < 0.05$). However, for higher temperatures (70 and 90 °C) some differences with time were registered, mainly for the NET-middle, NET-high and Batch samples. For the NET-low sample the size at high temperatures (70 and 90 °C) was maintained constant with time.

Analysing the zeta potential, as registered in Figure 5.2, it can be concluded that emulsions preserved the value higher than 30, which is an indicative of stability. The more relevant changes in the zeta potential values were observed for 90 °C. In this case, the NET-low and NET-middle samples maintained the zeta potential value within the examined time period. However, the batch and NET-high samples revealed a significant decrease in the zeta potential, which agrees with the observed average diameter increase for these samples. In fact, samples holding droplets of large size, are more prone to destabilisation at high temperatures (90 °C).

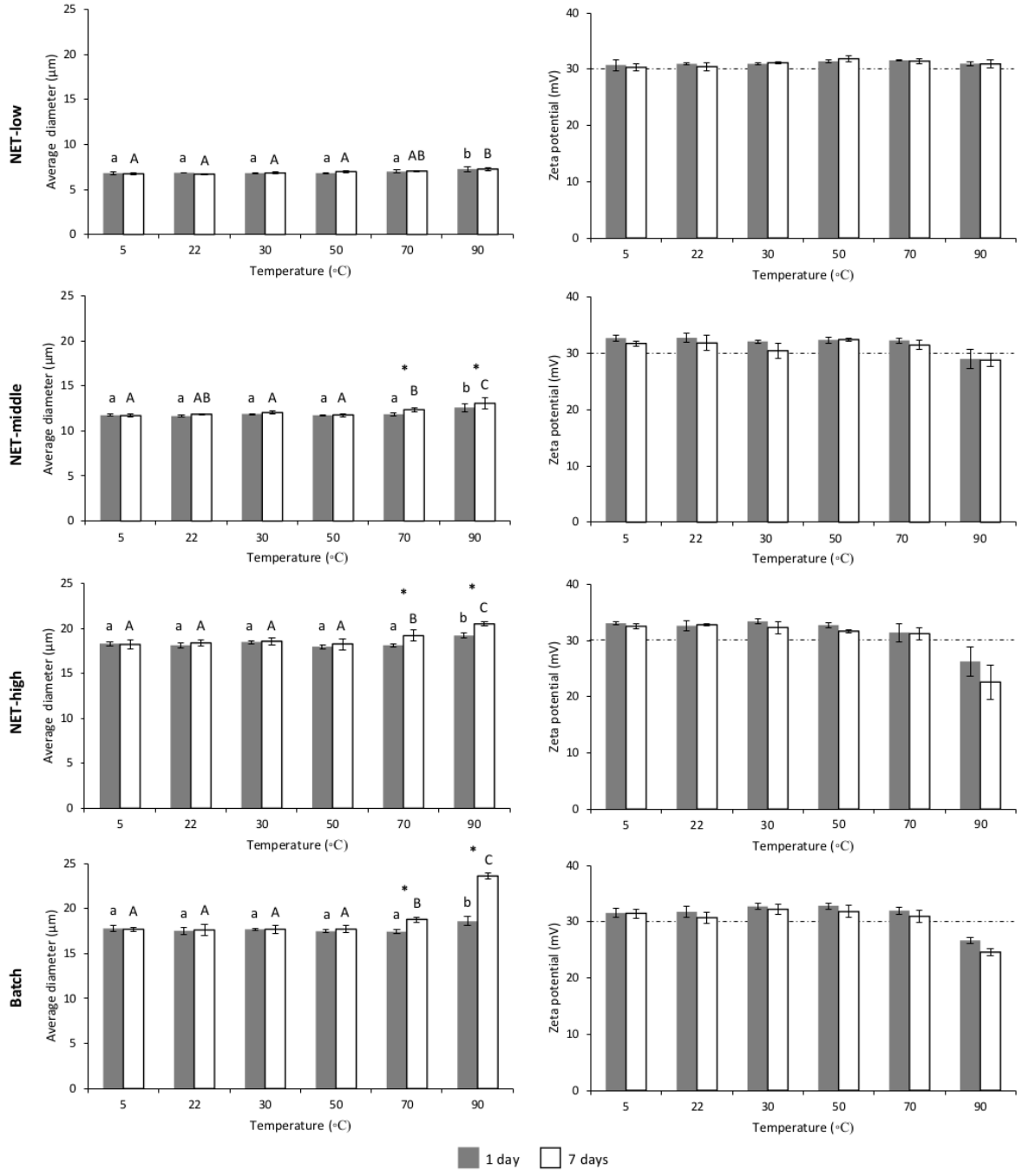

Figure 5.2: Effect of temperature (5, 22, 30, 50, 70 and 90 °C) on size and zeta potential of the n-HAp stabilised Pickering emulsions with different average diameters (NET-low - 7 µm, NET-middle - 11 µm, NET-high - 18 µm, and batch - 18 µm). Different lowercase and uppercase letters represent, for each size category, significant differences in emulsion size for all temperatures after 1 day and 7 days, respectively ($p < 0.05$). * indicates significant differences, for each tested temperatures, between 1 and 7 days of storage ($p < 0.05$).

Figure 5.3 shows the fluorescence microscopy images of the studied Pickering emulsions, as a function of temperature, after 7 days under storage. The oil phase was stained with Nile red dye, and the droplets are visualised through emitted fluorescence in red colour. This technique enables the observation of the oil phase. After 1 day, the emulsions are

characterised by a regular and uniform morphology, at different tested temperatures (see Figure B.1 of Appendix B). Although the statistical differences of the size after 1 day at 90 °C, in terms of optical observations no significant changes on emulsion morphology were observed. However, after 7 days (Figure 5.3), particularly for the Pickering emulsions with large sizes (NET-middle, NET-high and batch) some droplet aggregations were observed at high temperatures. Namely, the Pickering emulsions subjected to 70 and 90 °C presented clear signs of instability evidenced by small groups of droplet's aggregation and coalescence (indicated with an arrow). However, it is important to highlight that NET-middle, NET-high and batch emulsions heated at 70 °C present fewer aggregates, comparatively with the emulsion treated at 90 °C. These aggregates occurring at 70 °C agree with the slight, but significantly higher average diameter, as reported in Figure 5.2 ($p < 0.05$). However, the recorded data for zeta potential (Figure 5.2) indicates that they are, probably, in a reversible and weak state of aggregation since the achieved value indicated a stable emulsion.

The increase of the average diameter at higher temperatures can be related to Brownian motion, promoting particles' redistribution and detachment from the interface (Mwangi *et al.*, 2016). Thus, the increase in temperature leading to kinetic energy increase, could cause the exposure of the droplets' surface promoting droplet's collision and coalescence phenomena (Mwangi *et al.*, 2016). For example, Mwangi *et al.* (2016), who studied emulsions stabilised by chitosan with an average diameter of around 75 μm reported stable emulsions up to 50 °C. Further temperature increase led to a considerable rise of the average droplet diameter and phase separation. On contrary, Bai *et al.* (2019) that studied emulsions stabilised by cellulose nanocrystals with an average diameter of around 1.2 μm reported emulsion stability up to 90 °C (only minor average diameter and zeta potential changes were observed). Thus, previous reported literature results agree with the observed for the studied n-HAp stabilised Pickering emulsions. The temperature had adverse effects on emulsions with large droplet diameters, comparatively with the lower ones.

Comparing the behaviour of the n-HAp Pickering emulsions holding the same particle size but produced by different technologies (NET-high and Batch), it can be concluded that the productive method does not influences the emulsion behaviour in the range of studied temperatures since similar responses were detected.

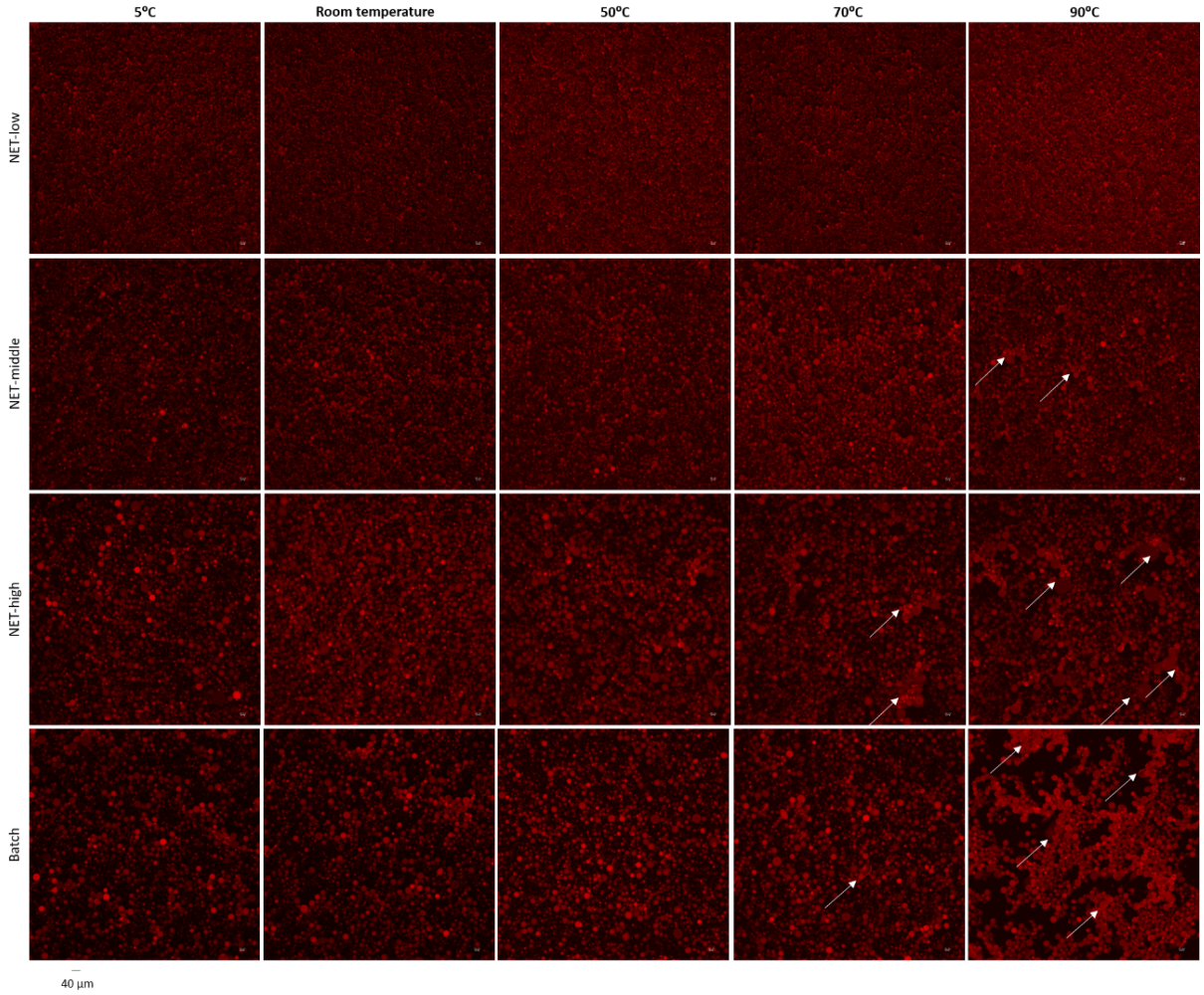

Figure 5.3: Fluorescence microscopy images of the Pickering emulsions with different average diameters (NET-low - 7 µm, NET-middle - 11 µm, NET-high - 18 µm, and batch - 18 µm) at different temperatures after 7 days of storage. The oil phase was stained with Nile red. Magnification: 10x.

5.3.2.2 pH effect

The influence of pH media on Pickering emulsions stability was studied. The pH ranged from 2 to 10, interspersed 2 in 2. The effect of pH on the average diameter and zeta potential of the produced emulsions is shown in Figure 5.4. To complement this study, Figure 5.5 shows the fluorescence microscopy images of the Pickering emulsions collected after 7 days under storage.

Figure 5.4 shows the average diameter size and the zeta potential as a function of pH for 1 day (filled columns) and 7 days (empty columns). The statistical analysis was performed considering the following 3 cases: (i) for each size category (NET-low - 7 µm, NET-middle - 11 µm, NET-high – 18 µm and batch – 18 µm) comparison of all pH values after 1 day, (ii) for

each size category (NET-low - 7 μm, NET-middle - 11 μm, NET-high – 18 μm and batch – 18 μm) comparison of all pH values after 7 days, and (iii) for each tested pH (2, 4, 6, 8 and 10) comparison for 1 and 7 days of storage. Figure 5.4 evidences that all Pickering emulsions were sensitive for very low pH (pH 2), with no Pickering emulsion remaining due to the complete degradation of the n-HAp particles (detected as a transparent aqueous phase), accompanied by the oil phase at the top of the test tube; i.e. a complete phase separation (water and oil) have occurred, as shown in the photographic record. Accordingly, zeta potential and fluorescence microscopy were not determined.

At pH 4, an increase of the average droplet diameter was perceived for all the studied samples, when compared with the originally reported size in Table 5.1. Furthermore, the statistical analysis indicates that the emulsion at pH 4 had significant differences in size comparatively with the emulsions subjected to other pH values (6-10) after 1 day ($p < 0.05$). This fact can be related with the droplet aggregations observed in Figure 5.5 (indicated by an arrow). After 7 days, the emulsions subjected to pH 6 showed slight differences from the other ones. Comparing the storage time (1 and 7 days), the statistical analysis indicates that the pH has a prompt effect, and no instabilities were further developed along the analysed storage period ($p < 0.05$). Additionally, through zeta potential analysis (Figure 5.4), it is possible to conclude that the pH highly impacts n-HAp Pickering emulsions stability. An increase of Pickering emulsion stability is verified at high pH values, which was accompanied by a zeta potential rise. Intermediate pH values (pH 4-6) originate Pickering emulsions with lower zeta potential, which may be ascribed to the formation of Ca^{2+} and PO_4^{2-} ions derived from the n-HAp solubilisation at acidic pH, reducing droplets' electrostatic repulsion. Pickering emulsions are considered stable for high pH (10) since the droplets' electrostatic repulsions were kept. Nevertheless, Zhou *et al*. (2020), which also reported significant changes in nanochitin stabilised Pickering emulsions with pH variation, reported significant morphology changes with droplet's size increasing with pH rising.

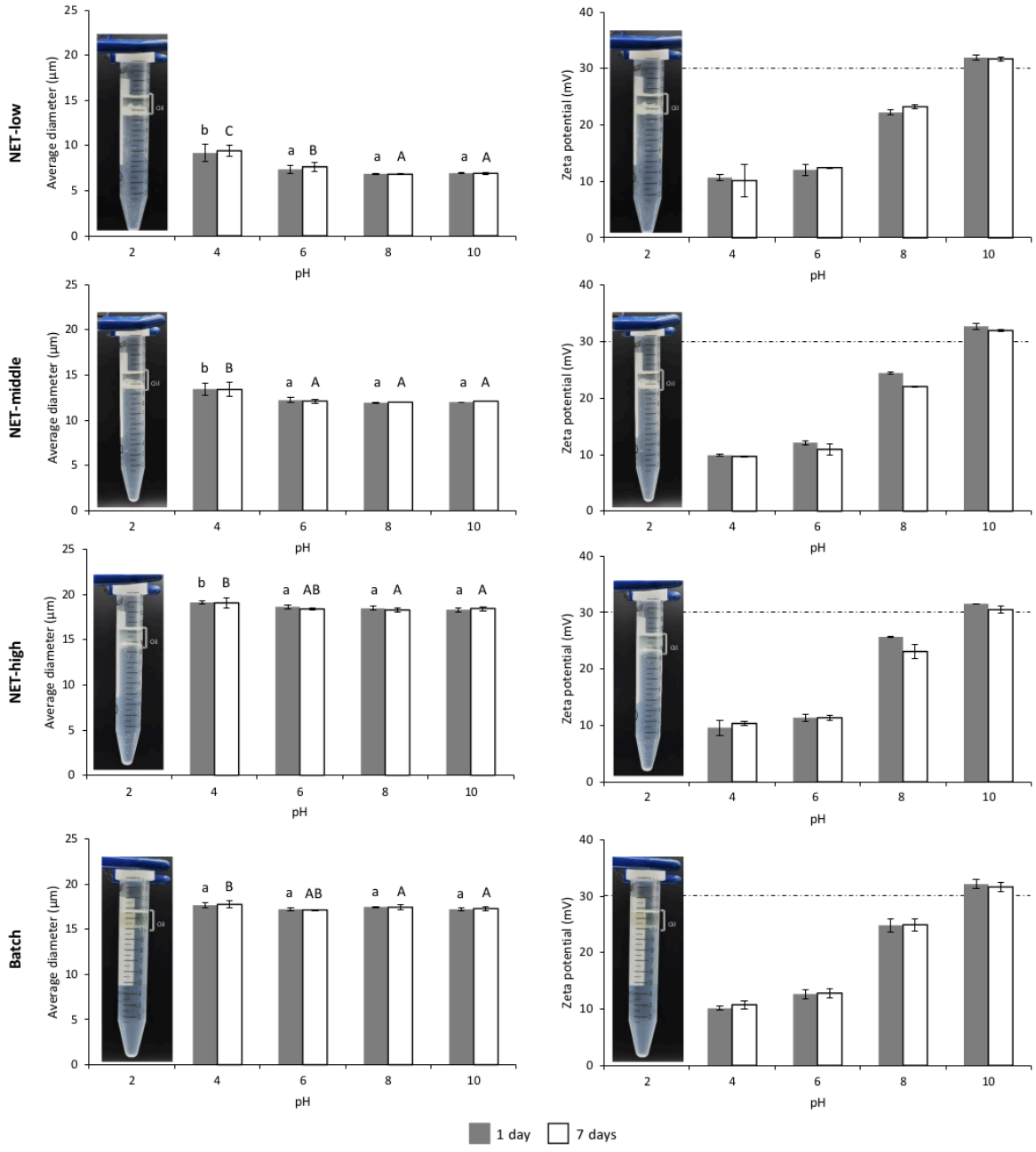

Figure 5.4: Effect of pH (2, 4, 6, 8 and 10) on size and zeta potential of the n-HAp stabilised Pickering emulsions with different average diameters (NET-low - 7 µm, NET-middle - 11 µm, NET-high - 18 µm, and batch - 18 µm). Different lowercase and uppercase letters represent, for each size category, significant differences in emulsion size for all pH values after 1 day and 7 days, respectively ($p < 0.05$). * indicates significant differences, for each tested pH values, between 1 and 7 days of storage ($p < 0.05$). Photographic register acquired after 1 day of the n-HAp Pickering emulsion at pH 2.

Analysing the fluorescence microscopy images (Figure 5.5), which shows the NET-low, NET-middle, NET-high and Batch emulsions, it is possible to observe the maintenance of the droplets shape from pH 6 to 10, meaning that they were stable against coalescence and

aggregation phenomena within this pH range and during 7 days under storage. Figure B.2 (Appendix B) shows the fluorescence microscopy images of the Pickering emulsions after 1 day of storage. The emulsions subjected to low pH (4) tend to develop instabilities, manly, aggregations. Although zeta potential values are lower for emulsions at pH 4, the observed instabilities and increased diameter were most noted for Net-low and NET-middle. In this case, there was a weaker electrostatic repulsion between the oil droplets; therefore, the repulsion generated by the adsorbed n-HAp layer was no longer enough compared to the van der Waals attractions, promoting droplet aggregation (Bai *et al.*, 2019). For the NET-high and batch emulsions, the increase of the droplet size and aggregation formation was less noted. This fact may be related to the smaller size bar difference (in magnitude) registered for pH4 for these samples. For the other tested pH values (6 and 8), the fluorescence microscopy images evidenced a well-defined morphology without changes in the droplet size. Despite the zeta potential value being below 30, no instability phenomena were optically observed. This can be justified by the fact that pH 6 promotes partial solubilisation of the n-HAp particles forming other calcium phosphate (with different Ca/P ratios), which promoted the O/W emulsion stabilisation. Solid particles such as n-HAp are stable at very specific conditions. However, the released ions decrease the zeta potential of the medium. Taking into account the achieved zeta potential value, it is expectable that emulsions at pH 6 are able to develop instability faster than emulsions at pH 8.

Results demonstrate that the stability of the n-HAp stabilised emulsions is strongly affected by pH, being these emulsions more stable at high pH environments. Thus, the pH has a significant influence on Pickering emulsions stability, mainly due to the nature of the solid particles (n-HAp).

Comparing the behaviour of the two n-HAp Pickering emulsions produced by different technologies (NET-high and Batch), no difference derived from the used productive method was observed. The two emulsions presented a similar behaviour at the different pH values.

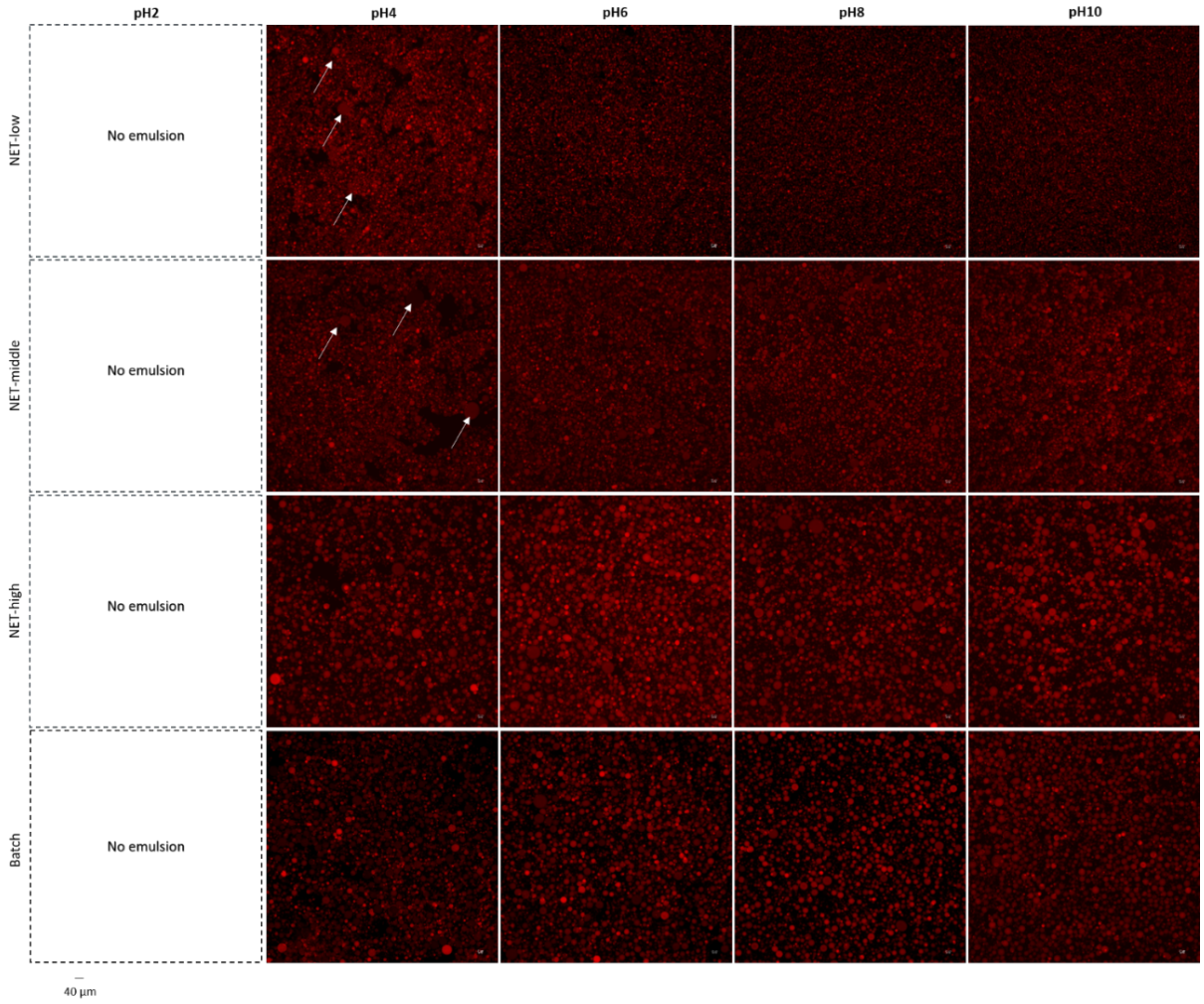

Figure 5.5: Fluorescence microscopy images of the Pickering emulsions with different average size diameters (NET-low - 7 µm, NET-middle - 11 µm, NET-high - 18 µm, and batch - 18 µm) at different pH after 7 days of storage. The oil phase was stained with Nile red. Magnification: 10x.

5.3.2.3 Ionic strength effect

The ionic strength was tested on the produced emulsions (NET-low, NET-middle, NET-high, and Batch) by varying the electrolyte (NaCl) concentration from 0 to 500 mM. Figure 5.6 shows the average diameter and the zeta potential of the Pickering emulsions as a function of the ionic strength for 1 day (filled columns) and 7 days (empty columns). The statistical analysis was performed considering the following 3 cases: (i) for each size category (NET-low - 7 µm, NET-middle - 11 µm, NET-high – 18 µm and batch – 18 µm) comparison of all ionic strengths after 1 day, (ii) for each size category (NET-low - 7 µm, NET-middle - 11 µm, NET-high – 18 µm and batch – 18 µm) comparison of all ionic strengths after 7 days, and (iii) for

each ionic strength (0, 100, 200, 300, 400 and 500 mM) comparison for 1 and 7 days of storage.

Pickering emulsions stabilised by n-HAp solid particles were stable in terms of droplet size without significant changes after 1 day for all tested NaCl concentrations ($p < 0.05$); however, very slight changes were statistically observed after 7 days (Figure 5.6; $p < 0.05$). Except for the Batch Pickering emulsion subjected to 300 mM of NaCl, which showed differences between 1 and 7 days, it is possible to conclude that no significant changes occurred in the average droplet diameter for all the other tested emulsions (Figure 5.6), within the studied NaCl concentration range when comparing 1 and 7 days of the storage time. Overall, the emulsions maintained their size even at high electrolyte concentrations within the tested time-frame of 7 days. According to literature, different behaviours were observed for the Pickering emulsions face to NaCl adding. For example, similar results were reported by Xu *et al*. (2019), where protein stabilised emulsions were stable at high ionic strength. On the contrary, Mwangi *et al*. (2016) reported droplet's aggregation for the complete range of tested NaCl concentrations, when studying Pickering emulsions stabilised with chitosan particles, whereas Bai *et al*. (2019) reported emulsion creaming formation for concentrations higher than 200 mM.

In terms of zeta potential (Figure 5.6), which indicates the emulsions' stability, it is possible to observe that all the emulsions revealed strong positive values (higher than 30), and therefore can be considered stable after 1 and 7 days under storage; compatible with the existence of enough electrostatic repulsions between droplets ensuring emulsion's stability. Mitbumrung *et al*. (2019), who studied the influence of NaCl on nanofibrillated cellulose and whey protein Pickering emulsions, reported that the NaCl concentration increase (100 - 500 mM) promoted the accumulation of Na^+ ions at the negatively droplet surface, leading to a decrease of the zeta potential value.

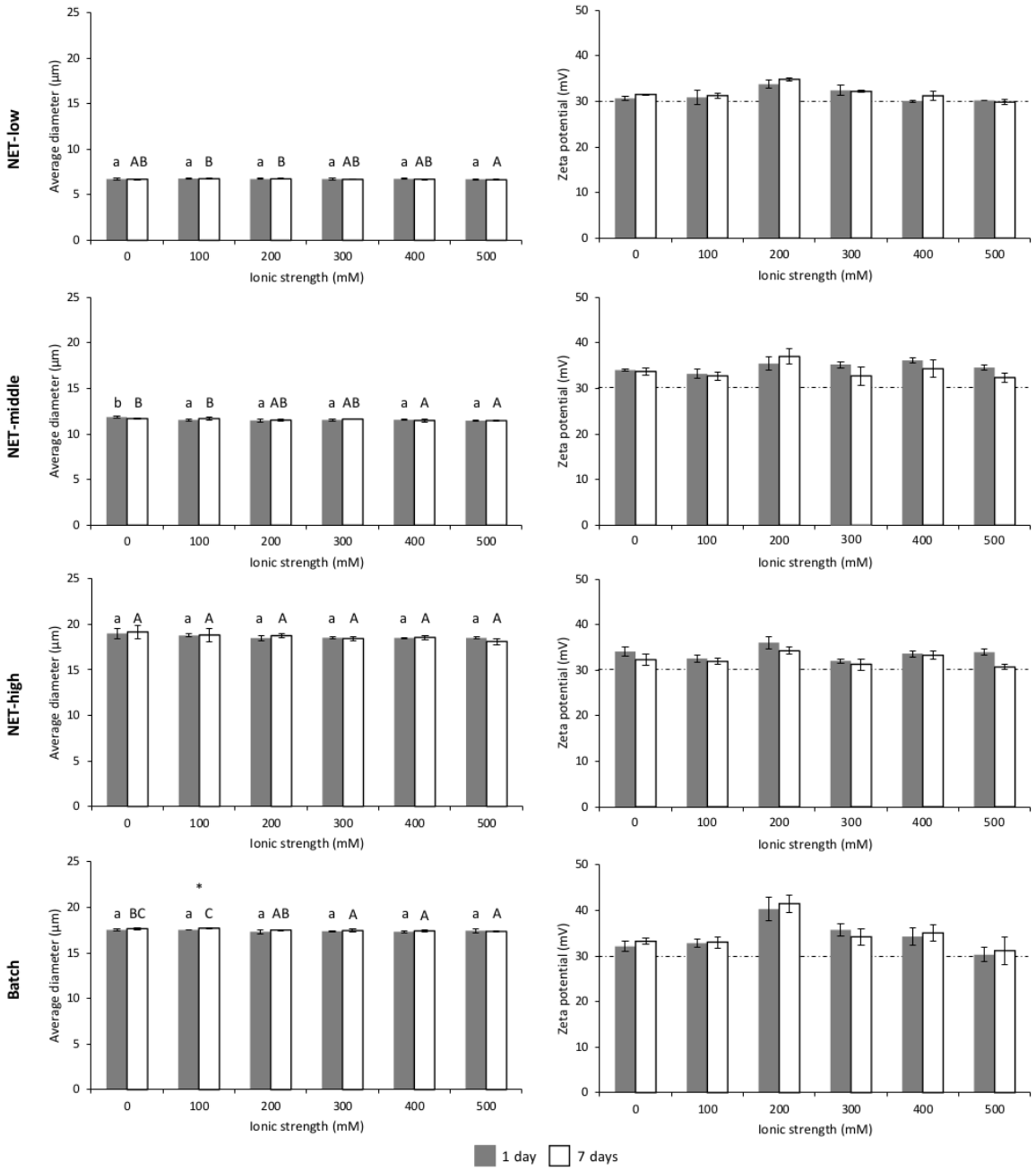

Figure 5.6: Effect of ionic strength (100, 200, 300, 400 and 500 mM, NaCl) on size and zeta potential of the n-HAp stabilised Pickering emulsions with different average diameters (NET-low - 7 µm, NET-middle - 11 µm, NET-high - 18 µm, and batch - 18 µm). Different lowercase and uppercase letters represent, for each size category, significant differences in emulsion size for all electrolyte concentrations after 1 day and 7 days, respectively ($p < 0.05$). * indicates significant differences, for each tested electrolyte concentrations, between 1 and 7 days of storage ($p < 0.05$).

The n-HAp stabilised Pickering emulsions were resistant to the presence of high NaCl concentrations. No coalescence and aggregation phenomena were observed after 7 days according to the fluorescence microscopy images, as shown in Figure 5.7. The presence of

NaCl does not compromise the O/W stabiliser role of the n-HAp particles within the time-frame of 7 days. The results for 1 day are presented in Figure B.3 of the Appendix B. The excellent stability of the n-HAp Pickering emulsions at different ionic strength can be attributed to the existing electrostatic repulsion between the droplets that overcome van der Waals attractions.

Similarly to the other studied environmental stresses, no significant differences were observed between the two used techniques (NETmix and batch), corroborating the advantages of using the NETmix technology in the production of Pickering emulsions, as already discussed in a previous work of the group (Ribeiro *et al.*, 2021).

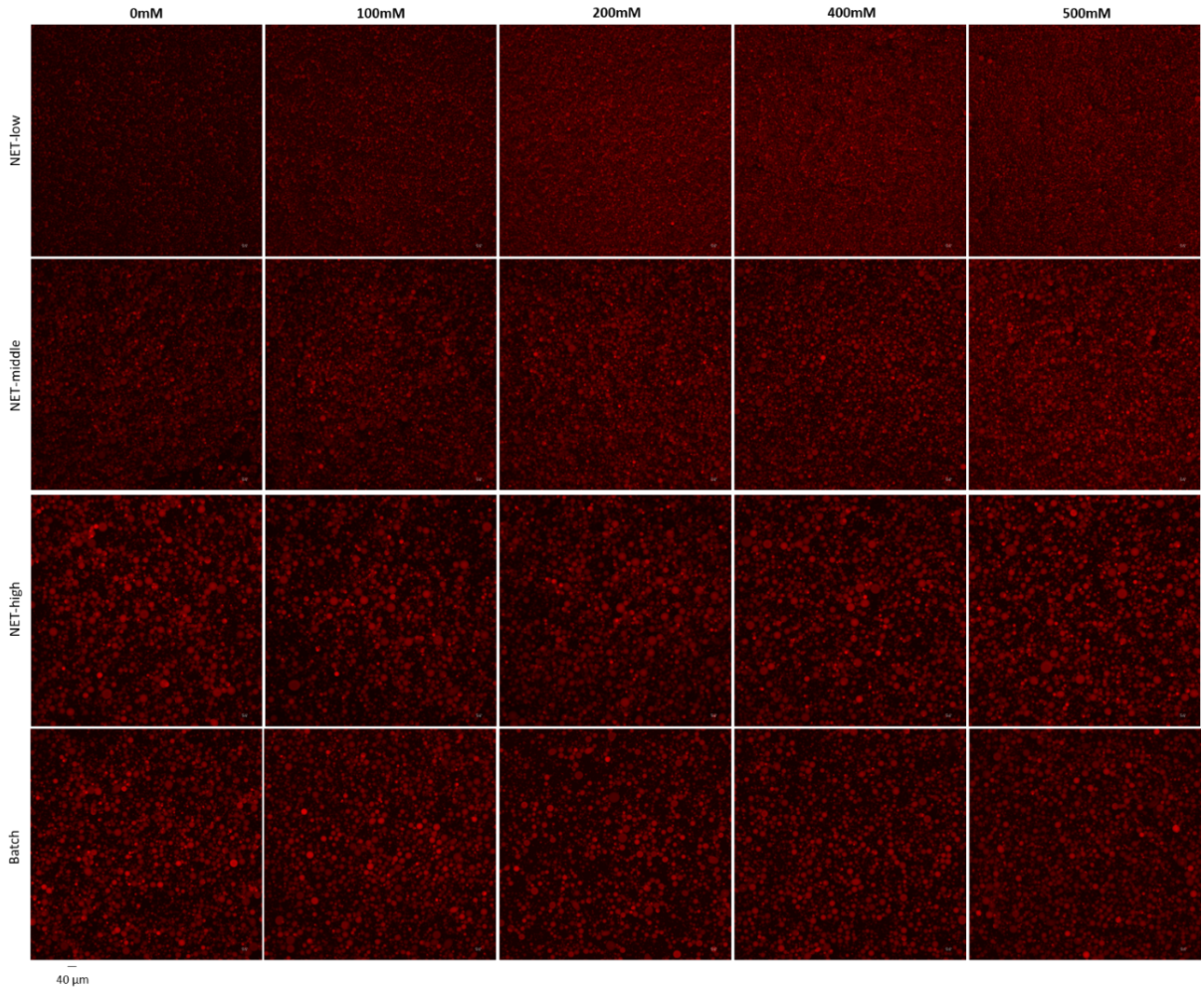

Figure 5.7: Fluorescence microscopy images of the Pickering emulsions with different average size diameters (NET-low - 7 µm, NET-middle - 11 µm, NET-high - 18 µm, and batch - 18 µm) at different ionic strength after 7 days of storage. The oil phase was stained with Nile red. Magnification: 10x.

5.4 CONCLUSIONS

In this work, Pickering emulsions with different average droplet diameters were produced using the NETmix reactor (continuous mode; ~7, ~11, ~18 μm). For comparison of the applied technique, a rotor-stator device was also used (batch mode; ~18 μm). In the NETmix reactor, the droplet size of the Pickering emulsions was controlled by the Reynolds number and applied number of recirculation cycles. NETmix is an attractive low-energy and versatile technique enabling continuous production, ensuring industrial scale-up of the process. The stability of the produced emulsions against different environmental stresses (temperature, pH and ionic strength) was studied due to their importance for Pickering emulsion's manufacture, storage, and final application conditions. In this study, the temperature (5, 22, 30, 50, 70 and 90 °C), pH (2, 4, 6, 8 and 10), and ionic strength (0, 100, 200, 300, 400 and 500 mM) were varied to cover a wide range of stress conditions.

Regarding the effect of the environmental stresses, the n-HAp Pickering emulsions were stable within the full tested ionic strength range (0 to 500 mM), between 5 to 70 °C, and in the pH interval 6-10. The emulsions have shown a similar behaviour regardless of the droplet size; however, the ones with higher droplet sizes were more unstable at high temperatures (70-90 °C), mainly after 7-days storage time. In this case, droplet's aggregations and coalescence were observed. The emulsions with lower diameters (~7 μm) were resistant to all assayed temperatures, being more stable than the other tested samples. Additionally, all Pickering emulsions undergo phase separation at pH 2 due to n-HAp particle's disruption. Moreover, at low pH values ($4 \leq pH \leq 6$), the emulsions were characterised by low zeta potential values pointed out for droplet aggregation or instability development, justified by a potential partial solubilisation of the n-HAp particles at acidic environment. At these pH values the samples with lower average diameters tend to develop a prompt aggregation; for the ones with higher droplet sizes a tendency to develop instabilities, mainly after the 7-days of storage, was also observed.

Comparing the two emulsions produced by different techniques (NET-high and Batch; ~18 μm), identical behaviours under the tested stresses were observed, i.e., the used productive method have not influenced the emulsion behaviour, meaning that the scale-up

by NETmix from a batch laboratorial scale is feasible, also reinforcing the previous achievements of this group (Ribeiro *et al.*, 2021) were NETmix technology was firstly introduced to produce Pickering emulsions.

Overall, the results presented in this work provide new insights concerning n-HAp based Pickering emulsions stability. It was observed that n-HAp solid particles provide good stability to the emulsions under strong adverse conditions (with the exception of pH 2 and 90 ºC), maintaining their droplet size, morphology, and surface charge. This is an important achievement aiming at predicting the emulsion behaviour, e.g., during manufacturing or after ingestion throughout the gastrointestinal tract. It can also support and consolidates the prospectus of using n-HAp Pickering emulsions as carriers of bioactive principles, more specifically lipophilic ones.

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6 NANOHYDROXYAPATITE PICKERING EMULSIONS AS VITAMIN E CARRIERS FOR FOOD APPLICATIONS

This chapter is based on: Andreia Ribeiro, Raquel F. S. Gonçalves, Ana C. Pinheiro, Yaidelin A. Manrique, Maria Filomena Barreiro, José Carlos B. Lopes, Madalena M. Dias (2022). *In vitro* digestion and bioaccessibility studies of vitamin E-loaded nano*hydroxyapatite Pickering emulsions and derived fortified foods. LWT – Food Science and Technology*, 154, 112706-15. https://doi.org/10.1016/j.lwt.2021.112706

Vitamin E is a lipophilic vitamin playing an important role in human health, but its oxidative instability leads to its fast degradation and loss of bioactivity. In this study, vitamin E-loaded Pickering emulsions stabilised by nanohydroxyapatite (n-HAp) were produced using a static mixer, NETmix, enabling continuous production and tailoring of the droplet size. The emulsions containing vitamin E at a content of 1 mg/mL were produced with different droplet sizes (~7, 11 and 18 μm) using an O/W ratio of 20:80. Their behaviour throughout an *in vitro* gastrointestinal system and vitamin E bioaccessibility were investigated, showing that several physical and chemical modifications occur; namely, the disruption of the n-HAp particles in the stomach and the formation of aggregates in the small intestine, these later associated with the observed low vitamin E bioaccessibility due to oil entrapment. The emulsions showing the highest vitamin E bioaccessibility (~3%) (sample with higher average size) were used to produced fortified gelatine and milk, resulting in increased bioaccessibility (~11% and 18%, respectively), fact associated to macronutrients presence and lower content of n-HAp particles. Overall, n-HAp Pickering emulsions offer advantages for vitamin E encapsulation directed to fortified foods development, with the possibility to extend these results to other lipophilic vitamins.

6.1 INTRODUCTION

Vitamin E is a lipophilic vitamin comprising eight compounds, namely: α-, β-, γ- and δ-tocopherol and tocotrienol. Among them, α-tocopherol is the most abundant and biologically active form (Borel *et al.*, 2013; Burton, 1994; Yang & McClements, 2013). This vitamin can be naturally found in various foods, with fresh fruits (kiwi, mango, tomato), vegetables (broccoli) and dry fruits (sunflower seeds, hazelnuts, peanuts, almonds) as the most representative sources.

Vitamin E is an essential vitamin for humans. Its strong antioxidant activity (Burton, 1994; Wolf, 2005) avoids cellular ageing, reducing the occurrence of diseases such as dementia, cancer and cardiovascular disorders (Burton, 1994; Katouzian & Jafari, 2016; Wolf, 2005; Yang *et al.*, 2015). Preserving vitamin E correct levels in the body helps health maintenance, increasing well-being (Katouzian & Jafari, 2016). Moreover, and also due to the high antioxidant activity, vitamin E can protect food lipids against oxidation (Yang *et al.*, 2015).

Vitamin E is typically added in commercial fortified foods, beverages and supplements (Lv *et al.*, 2019). However, the effectiveness of vitamin E in food products is not easy to achieve due to manipulation and storage constraints associated to its high lipophilic character, and thus poor solubility in water. Vitamin E cannot be effectively incorporated into aqueous systems, and presents oxidative instability to external environmental stresses, degrading fast and losing bioactivity (Hategekimana & Zhong, 2015; Katouzian & Jafari, 2016). For these reasons, vitamin E is often encapsulated to increase stability, bioavailability, and compatibility with hydrophilic food matrices (Hategekimana *et al.*, 2015; Lv *et al.*, 2019; Mujica-Álvarez *et al.*, 2020; Yang & McClements, 2013).

Pickering emulsions, which due to the surfactant-free nature are greener alternatives to conventional emulsions, offer advantages as encapsulation systems, namely of vitamins (Hategekimana *et al.*, 2015; Mitbumrung *et al.*, 2019). Moreover, Pickering emulsions are recognized to have good physical and chemical stability (Albert *et al.*, 2019; Chevalier & Bolzinger, 2013; Ortiz *et al.*, 2020), and thus more adequate for applications such as foods,

cosmetics and pharmaceutical, answering to sustainability and green label issues (Albert *et al.*, 2019).

Water-in-oil (O/W) Pickering emulsions can be used to encapsulate lipophilic active compounds, from omega fatty acids to lipophilic vitamins (e.g., vitamin E), bringing advantages such as: (i) improve the level of lipophilic vitamins' incorporation of in food and pharmaceutical products (Albert *et al.*, 2019; Frelichowska *et al.*, 2009b), (ii) improve the stability and bioaccessibility of the lipophilic compounds in controlled and targeted delivery applications (Albert *et al.*, 2019; Frelichowska *et al.*, 2009a), and (iii) improve the absorption of lipophilic compounds in the human body (Albert *et al.*, 2019).

The performance of vitamin-loaded emulsions has been reported in some recent works. In this context, Yang and McClements (2013) studied the effect of the oil composition (medium-chain *versus* long-chain fatty acids) on vitamin E bioaccessibility using emulsions stabilised with quillaja saponin. The influence of using different solid particles as Pickering stabilisers on lipid digestibility and vitamin E bioaccessibility was studied by Lv and co-workers (Lv *et al.*, 2019). Furthermore, Zhou *et al*. (2020) prepared Pickering emulsions stabilised with nanochitin particles, aiming at studying the bioaccessibility of vitamin D_3 after *in vitro* gastrointestinal digestion. Winuprasith *et al*. (2018) checked the *in vitro* digestion and bioaccessibility of vitamin D_3 encapsulated in Pickering emulsions stabilised with nanofibrillated cellulose. In general, these works have shown that the bioaccessibility of lipophilic vitamins encapsulated in Pickering emulsion-based systems depends on various factors, including the solid particles type, oil phase composition, O/W ratio, and O/W interfacial properties. Furthermore, the presence of calcium ions, bile salts, and phospholipids can also affect (positively or negatively) the bioaccessibility of the vitamins (Yang *et al.*, 2015); however, these studies are less explored in the literature. Recently, Zhou *et al*. (2021) introduced nanochitin and titanium dioxide particles in milk to study the behaviour of these nanoparticles inside the human gut after digestion, particularly to assess their impact on vitamin D_3 bioaccessibility. The authors showed that the titanium dioxide and nanochitin did not significantly affect the vitamin bioaccessibility, but contributed to increase the whiteness and viscosity of the milk, respectively. In a different study, Tan *et al*. (2020) showed that chitosan reduced vitamin D_3 bioaccessibility in food emulsions by binding to the mixed micelles.

The purpose of the present work was to produce E-loaded Pickering emulsions using NETmix technology, a continuous process recently introduced by our group to produce Pickering emulsions (Ribeiro *et al.*, 2021) and study vitamin E digestibility and bioaccessibility. In this context, a systematic study aiming at understanding the effect of n-HAp Pickering emulsions tailored with different droplet sizes on the digestion and bioaccessibility of encapsulated vitamin E, before and after incorporated into food matrices (gelatine and milk), has been conducted. To the best of our knowledge, this work reports, for the first time, the use of n-HAp Pickering emulsions as carriers of vitamin E and their use to produce fortified food products. Moreover, n-HAp Pickering emulsions digestibility under gastrointestinal conditions was not previously addressed. These results can be further extended to the encapsulation of other lipophilic vitamins.

6.2 MATERIALS AND METHODS

6.2.1 MATERIALS

The *nano*XIM-CarePaste, hydroxyapatite aqueous paste, was supplied by Fluidinova S.A. N*ano*XIM-CarePaste is composed of 15.5 ± 0.5 (wt%) of HAp nanoparticles (n-HAp) with particle size < 50 nm, 4.5 ± 0.5 (wt%) KCl and a water content ≤ 81.0 (wt%). Sunflower oil, milk (semi-skimmed) and neutral gelatine (in powder) were purchased from a local supermarket. Fluorescent dyes (Nile red and Nile blue A) and α-tocopherol (98%), referred in the text as "vitamin E", were obtained from Sigma-Aldrich. Isopropyl alcohol was purchased from Riedel-de Haen. The nutritional declaration of the neutral gelatine refers <0.1 g of fat, 0 g of carbohydrates, 88 g of protein, and 0.17 g of salt per 100 g of the product. The semi-skimmed milk contained 1.6 g of fat (of which 0.9 are saturated fat), 4.8 g of carbohydrate (representing a total of sugars), 3.2 g of protein, and 0.13 g of salt per 100 mL of milk.

Pepsin from porcine gastric mucosa (≥ 2500 U.mg-1), bile extract porcine, pancreatin from porcine pancreas (8x USP), Pefabloc® SC and the salts used for the preparation of oral, gastric and intestinal electrolyte solutions were purchased from Sigma-Aldrich. Ethanol and methanol were purchased from Fisher Scientific, and hexane was obtained from VWR Chemicals.

Distilled water, treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA), was used. All other chemicals were of analytical grade.

6.2.2 VITAMIN E-LOADED PICKERING EMULSIONS PRODUCTION

Oil-in-water (O/W) Pickering emulsions were prepared, in continuous mode, according to a previously reported technology, NETmix (Ribeiro *et al.*, 2021). Briefly, the Pickering emulsions were obtained using an O/W ratio of 20:80 (*v/v*). The aqueous phase (water phase) corresponds to n-HAp (5 wt%) dispersed in water, and the oil phase corresponds to a mixture of sunflower oil and vitamin E. The bioactive compound, vitamin E, was mixed directly in the sunflower oil at a content of 5 mg/mL (oil phase).

The procedure in the NETmix comprised a first step where the aqueous and oil phases were mixed to form a coarse emulsion designated by pre-emulsion. Then, droplet size reduction was achieved by recirculating the pre-emulsion in the NETmix reactor using a diaphragm pump. The production conditions in terms of Reynolds number (Re, 300 and 400) and the number of cycles (5, 10 and 17) varied according to the required droplet size, as described in Table 6.1. Moreover, a cooling bath (15 °C) was added to control NETmix temperature and prevent vitamin E degradation. In this work, Pickering emulsions with a proximate droplet size of 7, 11 and 18 μm were produced and named as NET-low, NET-middle and NET-high, respectively.

Table 6.1: NETmix conditions used to produce the n-HAp stabilised Pickering emulsions.

SAMPLE NAME	Re	CYCLES
NET-low	400	17
NET-middle	400	5
NET-high	300	10

6.2.3 VITAMIN E-FORTIFIED FOODS PRODUCTION

To produce the vitamin E-fortified foods, vitamin E-loaded Pickering emulsions were incorporated in two different food matrices (neutral gelatine and semi-skimmed milk). A ratio

of 1:2 of Pickering emulsion to food matrix was used. The final concentration of vitamin E in the food matrix was 0.33 mg/mL, higher than the daily reference intakes established by Regulation (EU) no 1169/2011 (1.8 mg of vitamin E per 100 g of food or 0.9 mg of vitamin E per 100 mL of beverages). This value was used to enable an easy quantification of the vitamin E by UPLC after digestion. For the fortified milk, 25 mL of Pickering emulsion were added to 50 mL of milk. The mixture was gently stirred for 1 min and thereafter stored in the fridge. For the fortified gelatine, 1.5 g of powder gelatine were hydrated with 25 mL of distilled water under stirring, thereafter completed with 25 mL at 50 °C (total water 50 mL). Then, 25 mL of the Pickering emulsion were added to the gelatine preparation under gently stirring for 1 min and the final mixture stored in the fridge to solidify.

6.2.4 *IN VITRO* GASTROINTESTINAL (GI) DIGESTION STUDIES

The produced vitamin E-loaded Pickering emulsions (NET-low, NET-middle and NET-high) and fortified foods (milk and gelatine with vitamin E – loaded Pickering emulsions) were digested *in vitro* to check emulsions' behaviour in case of vitamin E-loaded Pickering emulsions; and vitamin E bioaccessibility for both the emulsions and fortified food samples. The harmonized static *in vitro* digestion model described by Minekus *et al*. (2014) was used for the digestion simulation. This is a three-stage model comprising the simulation of the mouth, stomach and small intestine conditions. The composition of the different simulated digestion fluids is presented in Table 6.2. All fluids and samples (vitamin E-loaded Pickering emulsions, fortified milk and gelatine matrices) were pre-heated at 37 °C during 5 min. For the oral stage, simulated salivary fluid (SSF), $CaCl_2(H_2O)_2$ 0.3 M (to achieve 0.75 mM at the final mixture) and Milli-Q water were added to 5 mL of tested sample. The mixture was incubated at 37 °C for 2 min under orbital stirring at 120 rpm. A final ratio of food to SSF of 1:1 (*v/v*) was targeted. Since the samples did not have starch, α-amylase was not used (Brodkorb *et al.*, 2019). In the gastric stage, simulated gastric fluid (SGF), $CaCl_2(H_2O)_2$ 0.3 M (to achieve 0.075 mM at the final mixture) and pepsin solution (with the final activity of 2000 U/mL in the final mixture) were added to the previous mixture. The pH was adjusted to 3.0 with HCl 1 M, and Milli-Q water was added to make up the final volume, and the mixture

incubated for 2 h at 37 °C under orbital stirring at 120 rpm. A final ratio of oral sample to SGF of 1:1 (*v/v*) was targeted.

Finally, the intestinal stage consisted of simulated intestinal fluid (SIF), $CaCl_2(H_2O)_2$ 0.3 M (to achieve 0.3 mM at the final mixture), bile salts (to reach 10 mM at the final mixture) and pancreatin solution (with the final activity of 100 U/mL in the final mixture). The pH was adjusted to 7.0 with NaOH 1 M or HCl 1 M, and then Milli-Q water was added to achieve the final volume. The samples were incubated for 2 h at 37 °C under orbital stirring at 120 rpm. A final ratio of gastric sample to SIF of 1:1 (*v/v*) was targeted. After each stage, samples were collected. The samples collected at the end of the gastric stage were maintained in an ice bath to decrease the pepsin activity until analysis. After complete digestion, the reaction was stopped by adding the enzyme inhibitor Pefabloc® (1 mM) (10 µL for each 1 mL of the sample). All the samples were tested at least in triplicate.

Table 6.2: Composition of simulated digestion fluids.

SALT SOLUTION	SSF (mM)	SGF (mM)	SIF (mM)
KCl	15.1	6.9	6.8
KH_2PO_4	3.7	0.9	0.8
$NaHCO_3$	13.6	25	85
NaCl	-	47.2	38.4
$MgCl_2(H2O)_6$	0.15	0.12	0.33
$(NH_4)_2CO_3$	0.06	0.5	-
HCl	1.1	15.6	8.4

SSF: simulated salivary fluid; SGF: simulated gastric fluid; SIF: simulated intestinal fluid.

During digestion, an in order to check the morphology and stability of vitamin E-loaded Pickering emulsions along the simulated gastrointestinal tract (GIT), a sample was collected in each stage (i.e., mouth, stomach and small intestine) for confocal laser scanning microscopy (CLSM) and zeta potential analyses. The sample provided after the intestinal stage was used to analyse bioaccessibility and stability, for both vitamin E-loaded Pickering emulsions and fortified foods.

6.2.5 PICKERING EMULSIONS DIGESTA CHARACTERISATION

Zeta potential value gives an indication concerning emulsion stability. The emulsion can be considered stable for high positive (≥ 30) or high negative (≤ -30) values. Zeta potential was determined using a Zetaziser Nano ZS90 (Malvern Instruments). For that, the Pickering emulsions were diluted with distilled water to avoid multiple scattering effects, then placed in a folded capillary Zeta cell (ref: DS7010), and the measurement was conducted at 25 °C. Each sample was measured in triplicate and the data expressed as average ± standard deviation (SD).

To understand the impact of digestion on Pickering emulsions morphology, the samples collected at each GIT stage were analysed using CLSM. CLMS is a microscopic technique allowing the observation of emulsions' solid particles and oil phase. These experiments were performed using a Leica TCS-SP5 AOBS (Leica Microsystems). The digesta sample (100 µL) was stained with a mixture of Nile red at 0.1% (*w/v*) and Nile blue A at 0.1% *w/v* dissolved in isopropyl alcohol (10 µL). Nile blue and Nile red are able to dye the solid particles and the oil phase, respectively. Then, the dyed digesta Pickering emulsion was placed on a slide, and the fluorescent dyes excited at 488 nm (Nile red) and at 633 nm (Nile blue A). The images were digitally recorded and processed with a LasX software. The initial Pickering emulsion was also analysed for comparison purposes.

6.2.6 VITAMIN E BIOACCESSIBILITY, STABILITY AND BIOAVAILABILITY EVALUATION

After passing through the three stages of the simulated GIT assay, the concentration of vitamin E was determined, according to the procedure described by Lv *et al*. (2019) with some modifications. Briefly, the digesta obtained after the small intestine stage was centrifuged (Allegra 64R, Beckman Coulter Inc.) at 18700 *g* and 4 °C for 30 min to obtain the micellar phase. Before ultra-performance liquid chromatography (UPLC) analysis, the oil-soluble vitamin was extracted from digesta and micellar phase samples. For that, 3 mL of the sample were mixed with 3 mL of a hexane/ethanol mixture (1/1, *v/v*), then centrifuged at 4000 rpm for 2 min (Multifuge X3R, Heraeus) to obtain a supernatant layer. This extraction was repeated three times, and the obtained supernatant layers mixed and dried under nitrogen. Thereafter,

the dried samples were dissolved in methanol and filtered through a 0.22 μm filter. The vitamin E concentration was determined using a UPLC (Nexera X2, Shimadzu) with a C18 column (150×2.1 mm, 1.7 μm, Kinetex®) using a mixture of 95% methanol and 5% double-distilled water as the mobile phase. An isocratic elution mode at 0.4 mL/min, with a column temperature of 40 °C and a 10 μL injection volume were used. The detection wavelength for vitamin E was 295 nm.

Bioaccessibility corresponds to the vitamin E fraction released by the Pickering emulsion and solubilized in the mixed micelle phase after digestion and absorbed by the small intestine. Vitamin bioaccessibility was determined by measuring the concentration of vitamin E in the mixed micelle ($C_{micelle}$) and digesta phases ($C_{digesta}$) using the following equation:

$$Bioaccessibility = \frac{C_{micelle}}{C_{digesta}} \times 100 \quad (6.1)$$

Stability is measured as the vitamin E fraction remaining untransformed in the small intestine and calculated as

$$Stability = \frac{C_{digesta}}{C_{initial}} \times 100 \quad (6.2)$$

where, $C_{initial}$ represents the vitamin E concentration in the initial stage, i.e., the vitamin E concentration of the Pickering emulsion (1 mg/mL).

The bioavailability of the vitamin E can be thus estimated as the product of bioaccessibility and stability

$$Bioavailability = Bioaccessibility \times Stability \quad (6.3)$$

The calculated bioavailability corresponds to an estimative of vitamin E absorption.

6.2.7 STATISTICAL ANALYSIS

The digestion and bioaccessibility studies of Pickering emulsions and fortified foods were done in triplicate and duplicate, respectively. Data is presented as average ± SD. The statistical analysis was performed using the commercial software IBM SPSS statistics (version 27.0, SPSS Inc., Chicago, IL, USA). One-way analysis of variance of independent variables followed by Tukey's test was carried out on the digesta Pickering emulsions measurements to

establish significant differences ($p < 0.05$). For fortified foods measurements, a *t*-test was used to determine the significant difference among the tested samples ($p < 0.05$).

6.3 Results and discussion

6.3.1 Vitamin E-loaded Pickering emulsions characterisation

The vitamin E-loaded Pickering emulsions with tailored droplet average sizes of 7, 11, and 18 µm, respectively NET-low, NET-middle, and NET-high, were produced using the mesostructured reactor NETmix according to the parameters described in Table 6.1. The influence of these parameters for the studied system (n-HAp sunflower oil Pickering emulsions) in the average droplet size was previously reported by Ribeiro *et al*. (2021). The used vitamin E concentration did not modified viscosity of the sunflower oil (0.06 Pa·s), enabling the direct adoption of the conditions reported in the study.

Figure 6.1 shows the optical microscopy images of NET-low, NET-middle and NET-high samples with their average droplet size (7.53, 11.56 and 17.72 µm, respectively) and zeta potential (+31.18, +32.20, and +32.05 mV, respectively) characterisations. In terms of the zeta potential, similar values were obtained for the 3 samples; namely, the produced Pickering emulsions were characterised by strongly positive zeta potential values, indicating that the droplet aggregations are inhibited and stable emulsions were formed due to electrostatic repulsion mechanisms.

6.3.2 Vitamin E-loaded Pickering emulsions gastrointestinal digestion

After ingestion, the Pickering emulsion passes through different zones of the upper GIT, promoting physicochemical and physiological changes (McClements, 2016). In the mouth (1st stage) the emulsion contacts with saliva (SSF, neutral pH), which contains biopolymers, salts, and enzymes, promoting emulsion changes and enabling bolus formation. In the stomach (2nd stage) the bolus is mixed with gastric juices (SGF, highly acidic pH). This step facilitates the breakdown of the emulsion for further nutrient absorption, forming the chime. In the small intestine (3rd stage) the chyme is mixed with the alkaline small intestinal fluids (SIF, basic pH), composed of various bile salts, digestive enzymes, and phospholipids, which facilitates the

conversion of macronutrients such as lipids, proteins and starch into small peptides, free amino acids and fatty acids, among others. In this stage, maybe the most important stage of digestion, several chemical and physical processes occur, leading to the formation of mixed micelles enhancing the absorption of lipophilic micronutrients and digested macronutrients.

The vitamin E-loaded Pickering emulsions were digested *in vitro* through a simulated GIT. After each stage (mouth, gastric and intestine phases), a sample was collected for analysis, namely concerning sample's microstructure (CLSM), zeta potential, and vitamin E bioaccessibility and stability.

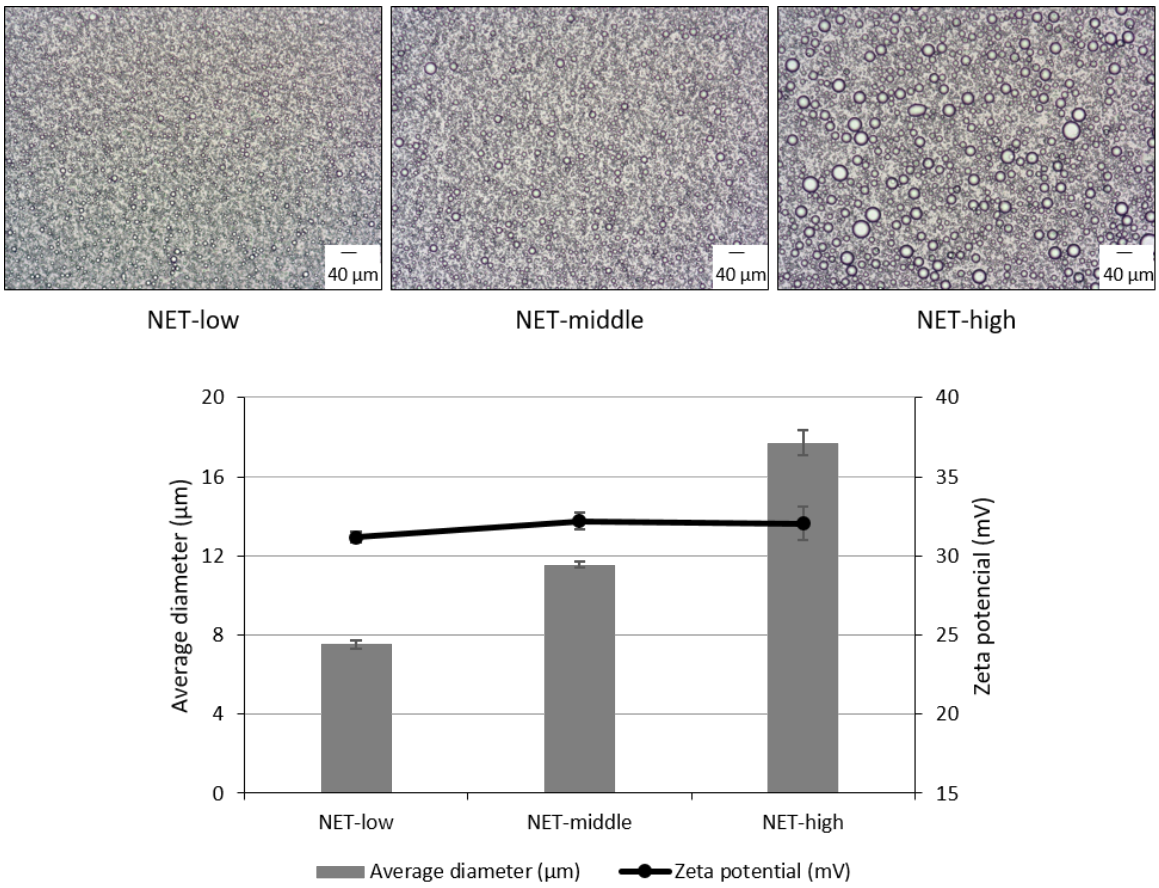

Figure 6.1: Optical images, average diameter (µm) and zeta potential (mV) of the produced vitamin E-loaded Pickering emulsions produced using NETmix technology.

6.3.2.1 Influence on microstructure morphology

Figure 6.2 shows the CLSM analysis of the studied Pickering emulsions (NET-low, NET-middle, and NET-high) in the initial stage and throughout the simulated GIT. The red fluorescence

colour represents n-HAp particles (dyed by Nile blue), whereas the green fluorescence colour represents the oil phase (stained by Nile red).

At the initial stage, a packed n-HAp layer is observed around the oil droplets for all tested Pickering emulsions, confirming the efficient stabilisation role of the nanoparticles. After exposure to the mouth stage, the number of oil droplets decreased, justified by the dilution factor applied in the digestion model. However, in this stage, no changes in the n-HAp stabilising role were perceived, i.e., the n-HAp particles maintained their tight position at the oil surface. Also, for all the tested samples, no evident changes in the droplet size were observed, as well as no agglomerates were detected.

In the stomach, the Pickering emulsions face stringent conditions, namely they contact with gastric fluids characterised by a highly acidic pH (around 1-3), relatively high ionic strength (around 150 mM), and a high enzymes content medium (McClements, 2016). The acquired CLSM images (Figure 6.2) revealed that this is the first local in GIT where the Pickering emulsions undergo strong physicochemical changes. Namely, it is evident that the n-HAp particles are not covering the oil droplets, i.e., they are not acting anymore as Pickering stabilisers. The n-HAp particles seem to have disrupted, becoming dissolved in the aqueous medium, fact compatible with the observation of a quite uniform red background. In fact, the Nile blue, which dyes the hydrophilic compounds, is possibly staining the Ca^{2+} and PO_4^{2-} species of the disrupted n-HAp particles, as well as other molecules resulting from the two digestive stages. These results are in agreement with the work reported by Ramis *et al*. (2018), which study the behaviour of a n-HAp paste in simulated gastric fluid, identifying its rapid and complete dissolution in the gastric fluids after 7.5 min at 37 ºC. Concomitantly with the particles disruption, in the present work, a slight increase in the size of the oil droplets was observed after exposure to gastric conditions, which may also be justified due to the n-HAp detachment from the oil droplets facilitating droplets coalescence.

After passing the stomach, medium in which the n-HAp particles become completely dissolved, the digestion proceeds to the small intestine. Within this phase, the environment conditions change, namely the pH turn to neutral. Under these conditions, and as shown in the CLSM images taken after the intestine phase (Figure 6.2), appreciable changes were

noticed. The material of the dissolved n-HAp particles coming from the stomach phase forms large and random calcium phosphates clusters, upon in contact with neutral pH medium. This result corroborates the observations made by Epple (2018) that reported the HAp precipitation in random calcium phosphate clusters at neutral pH after contacting acidic pH.

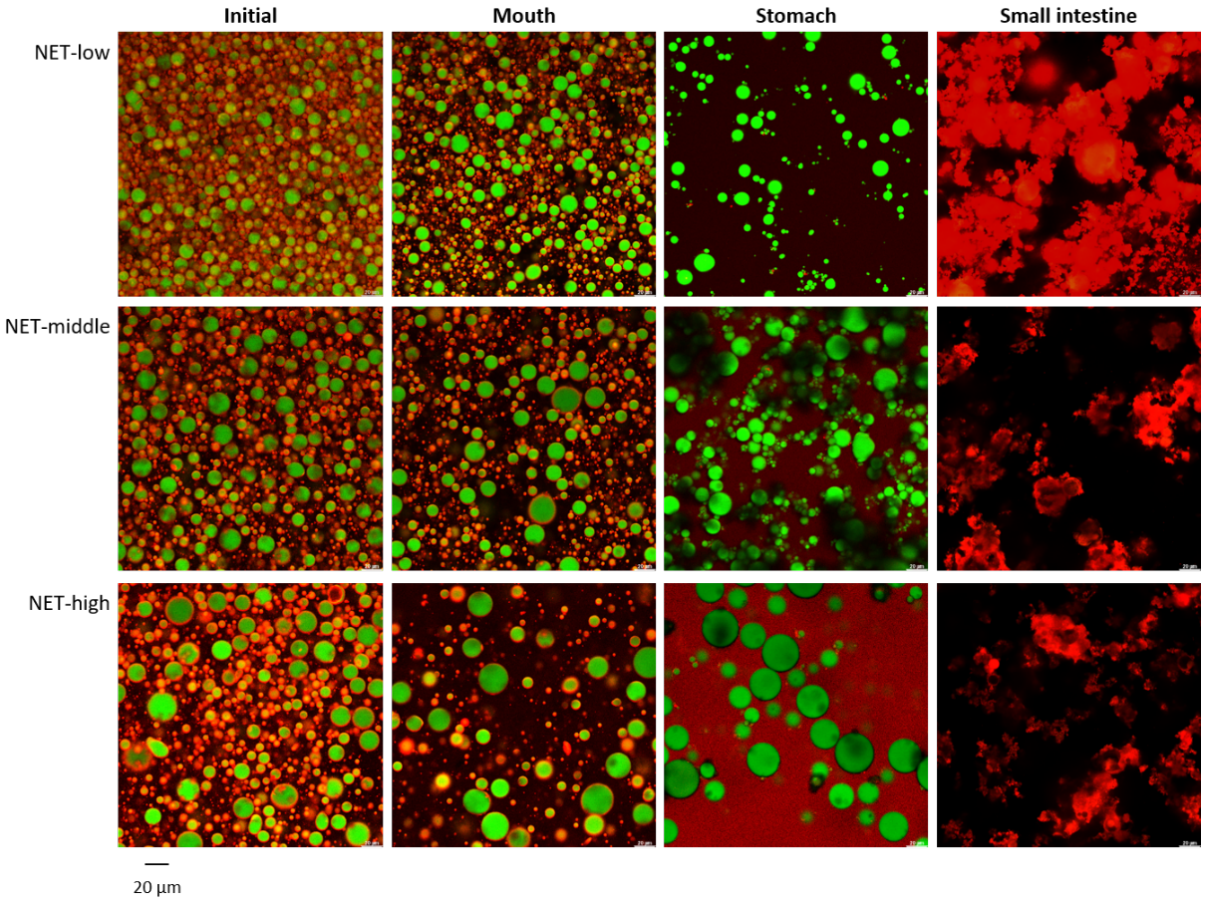

Figure 6.2: Overlapped CLMS images of the vitamin E loaded Pickering emulsions with different sizes (NET-low= ~7 µm, NET-middle: ~11 µm; NET-high: ~18 µm) after each stage of digestion (mouth, stomach and small intestine), comparatively with the initial emulsion. The n-HAp particles and the oil droplets are presented as red and green colour, respectively. The images were taken with a magnification of 20x and zoom of 3x.

The Pickering emulsions tailored with different sizes showed similar behaviour during GI digestion with apparent stability in mouth stage, n-HAp disruption under gastric conditions and formation of aggregates under intestinal conditions. However, in NET-low Pickering emulsion has bigger aggregates than other after intestinal phase (Figure 6.2).

6.3.2.2 Influence on zeta potential

Zeta potential analysis can give indications concerning the emulsions' interfacial properties. Figure 6.3 shows the zeta potential of the studied Pickering emulsions (NET-low, NET-middle, NET-high) throughout the simulated GIT. For comparison purposes, the initial zeta potential of the emulsions is also included. It is possible to observe that the value changes throughout the GIT, including the surface charge shifts from positive to negative between the mouth and stomach. The three emulsions presented similar behaviour for all GIT stages ($p < 0.05$).

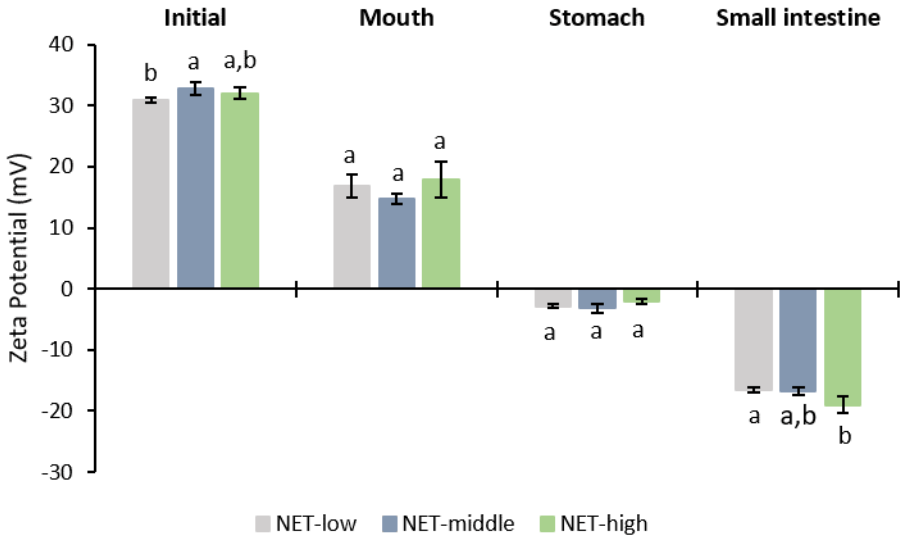

Figure 6.3: Zeta potential of the vitamin E-loaded Pickering emulsions (NET-low= ~7 µm, NET-middle: ~11 µm; NET-high: ~18 µm) along the gastrointestinal tract (mouth, stomach and small intestine), comparatively with the initial emulsion. In each GIT stage, different letters represent significant differences between the Pickering emulsions ($p < 0.05$).

The contact with the mouth has promoted an appreciable decrease in the zeta potential magnitude (from approximately from 32 mV to 16 mV), probably associated with the neutral pH of this stage and the adsorption of ionic species at the droplets' surface. These results suggest that the Pickering emulsions become more prone to instabilities after the mouth stage. In accordance, other authors working with Pickering emulsions stabilised with nanofibrillated cellulose particles observed small changes in the droplet size and morphology after this first phase of the digestion, which was related to mucin presence (Winuprasith *et al.*, 2018).

Upon in the stomach, the Pickering emulsions zeta potential becomes low in magnitude and negative (around -3 mV). This change can be attributed to the highly acidic pH of the gastric fluids together with the presence of enzymes (i.e., pepsin), which can lead to the adsorption of negatively charged molecules neutralizing the positively charge (Zhou *et al.*, 2020). Also, the high ionic strength medium can have contributed to the destabilisation of the emulsion system. These observations are in agreement with the observed CLSM images acquired after the stomach stage (Figure 6.2).

In the small intestine, all the tested Pickering emulsions presented a relatively strong negative zeta potential, which can be attributed to the presence of bile acids, phospholipids, and free fatty acids generated from the digestion of the emulsified oil (triglycerides). These molecules are negatively charged, leading to a zeta potential decrease. This observation is in accordance with the work of Zhou *et al*. (2020) that reported a strong zeta potential reduction for emulsions after the small intestine stage, fact associated with the presence of bile acids and peptides.

6.3.2.3 Influence on vitamin E bioaccessibility and stability

In this section, the influence of the GI digestion on vitamin E bioaccessibility and stability of the studied Pickering emulsions (NET-low, NET-middle, NET-high) was evaluated (Figure 6.4). For that, after the intestinal stage, the digesta was collected and centrifuged forming the micellar phase and a sedimented layer. The micellar phase and the whole digesta samples were collected to determine the vitamin E bioaccessibility and stability. In general, all Pickering emulsions presented a high vitamin E stability. The NET-high sample showed the lowest vitamin E stability followed by NET-low and NET-middle, respectively, 28.9 ± 2.59%, 32.1 ± 1.43% and 33.8 ± 1.53% ($p < 0.05$). However, all emulsions exhibited small vitamin E bioaccessibility values, with NET-high sample presenting a slightly higher bioaccessibility, comparatively with NET-low and NET-middle, respectively, 3.29 ± 0.57%, 2.15 ± 0.15% and 2.03 ± 0.12% ($p < 0.05$). Therefore, NET-high sample presented the highest value of calculated bioavailability, followed by NET-middle and NET-low, 0.94 ± 0.15%, 0.70 ± 0.03% and 0.69 ± 0.03%, respectively ($p < 0.05$).

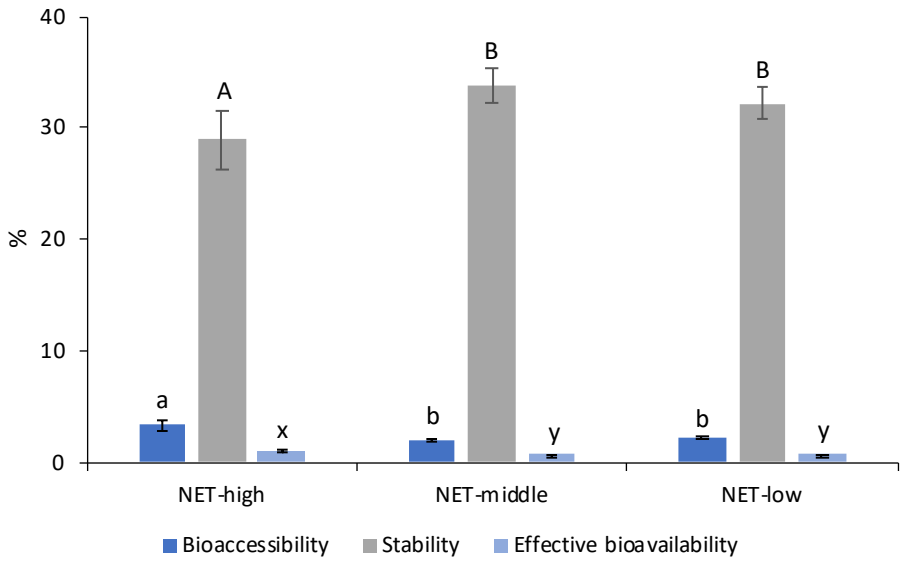

Figure 6.4: Bioaccessibility, stability and effective bioavailability of vitamin E loaded Pickering emulsions with different particle's sizes (NET-low= ~7 µm, NET-middle: ~11 µm; NET-high: ~18 µm) after *in vitro* digestion. Different letters in each evaluated parameter represent significant differences between the tested Pickering emulsions (p<0.05; one-way ANOVA): $^{a-b}$ for bioaccessibility, $^{A-B}$ for stability, and $^{x-y}$ for effective bioavailability.

The low bioaccessibility observed for all the tested Pickering emulsions can be attributed to the high amounts of free Ca^{2+} ions derived from the n-HAp particles disruption in the gastric phase. Ca^{2+} ions can form insoluble aggregates with anionic species, such as bile salts and free fatty acids, decreasing the mixed micelle's formation. Additionally, Ca^{2+} can also form aggregates with PO_4^{2-} species (random calcium phosphates). These aggregates can entrap the lipidic phase, hindering its digestion and, consequently, the micellar phase formation. The CLSM images taken after the intestinal phase (Figure 6.5), can support this hypothesis. In fact, the insoluble aggregates (stained in red) and the lipidic phase (stained in green) are superimposed, meaning that some lipidic phase remains entrapped inside these structures. In addition, the insoluble aggregates can also entrap the mixed micelles containing the vitamin E, fact in accordance with the observed high stability and low bioaccessibility, as also reported by (Yang *et al.*, 2015) in a work also addressing vitamin E. Zhou *et al.* (2020), which compared the effect of using Pickering emulsions produced with nanochitin particles with emulsions produced with Tween 80 on vitamin D_3 bioaccessibility, reported a lower bioaccessibility when Pickering emulsions were used. The authors justified these results by the precipitation of the mixed micelles in the presence of the nanochitin together with the presence of the non-digested vitamin D_3 lipid phase carrier. Additionally, Tan *et al.* (2020)

examined the impact of using chitosan on vitamin D_3 bioaccessibility with results showing that this biopolymer promoted severe droplet flocculation in the small intestine, reducing the amount of free fatty acids and leading to a decrease in the vitamin D_3 bioaccessibility. This effect was justified due to the fact that chitosan bounds to the vitamin-loaded mixed micelles promoting their precipitation.

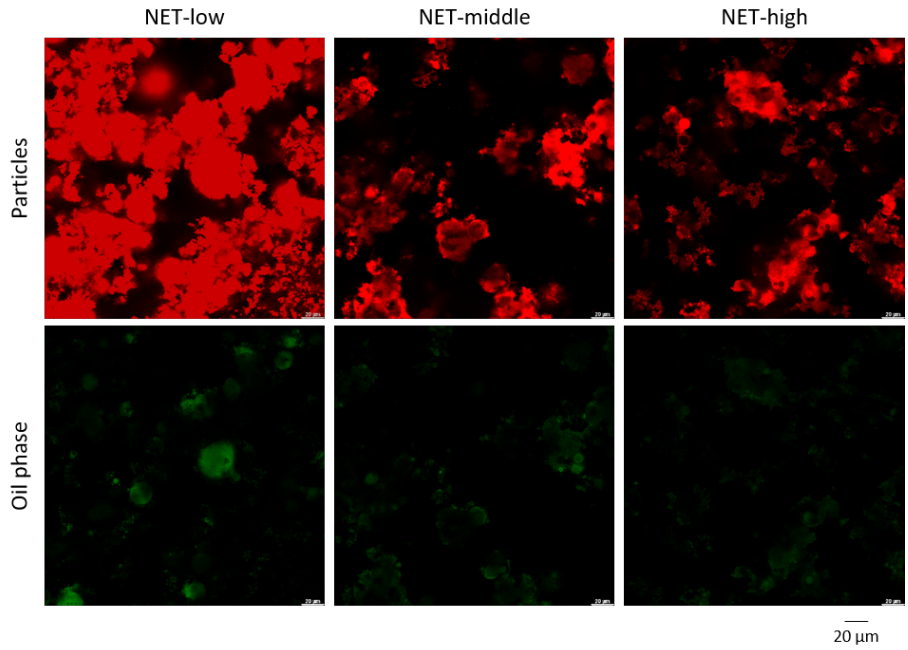

Figure 6.5: CLSM images Pickering emulsions (NET-low, NET-middle and NET-high) after intestinal phase.

The NET-high sample presented a slightly, but significantly higher vitamin E bioaccessibility, comparatively with the other tested emulsions ($p < 0.05$). This can be associated to the observed droplet size for this emulsion after the gastric phase. Once the NET-high sample had a higher droplet size, the lipid digestion rate at the intestinal phase was, probably, slower than the ones observed for the other emulsions, meaning that the formation of the mixed micelles delayed the Ca^{2+} ions binding to other ionic species. Therefore, the presence of Ca^{2+} ions showed to have a high impact on vitamin E bioaccessibility and stability. Moreover, the Pickering emulsion produced with the larger particle size (NET-high) lead to better results in terms of vitamin E bioaccessibility.

6.3.3 VITAMIN E FORTIFIED FOODS

The Pickering emulsion presenting the best vitamin E bioaccessibility after digestion (NET-high) was incorporated in gelatine and milk to study the effect of the food matrix in this parameter. Gelatine is constituted only by proteins and milk comprises fat, proteins and carbohydrates. Gelatine is a food matrix easy to prepare, and milk, a more complex food matrix, is a widely consumed food product. Solid particles, including inorganic solid particles, are usually used in the food industry to modify properties such as viscosity, brightness, and whiteness (Weir *et al.*, 2012; Zhou *et al.*, 2021). In this work, the n-HAp solid particles are used as stabilisers in Pickering emulsions, which can contribute to the organoleptic properties of foods and add functional properties, when used as carriers of bioactive compounds.

Figure 6.6 shows the photos of the used food matrices, gelatine (Figure 6.6 A) and milk (Figure 6.6 B) without incorporation (control samples) and after the incorporation of the vitamin E-loaded Pickering emulsion (fortified foods). In gelatine, the addition of the emulsion changes the sample from transparent to white, effect clearly observed. Regarding milk, no significant macroscopic changes in terms of colour were observed. In Figure 6.6 A1 and A2, and Figure 6.6 B1 and B2 the optical microscopy images of gelatine and milk (control sample and fortified food, respectively) are shown. It is possible to observe a clean and homogeneous image for the control food matrices, whereas for the fortified foods the presence of the Pickering emulsion is noticeable. To note that the Pickering droplets remained with their typical morphology and size after incorporation.

After emulsion incorporation, the fortified foods (fortified milk and fortified gelatine) were digested to access vitamin E bioaccessibility and stability. For that, the fortified food was subjected to the GIT assay (mouth, stomach, and small intestine). After the intestinal stage, the vitamin E bioaccessibility and stability were determined by analysing both the micellar and whole digesta samples. To check for any food matrix or Pickering emulsion materials effects, control samples (foods without incorporation, and foods added with non-loaded Pickering emulsions) were also *in vitro* digested to determine vitamin E presence.

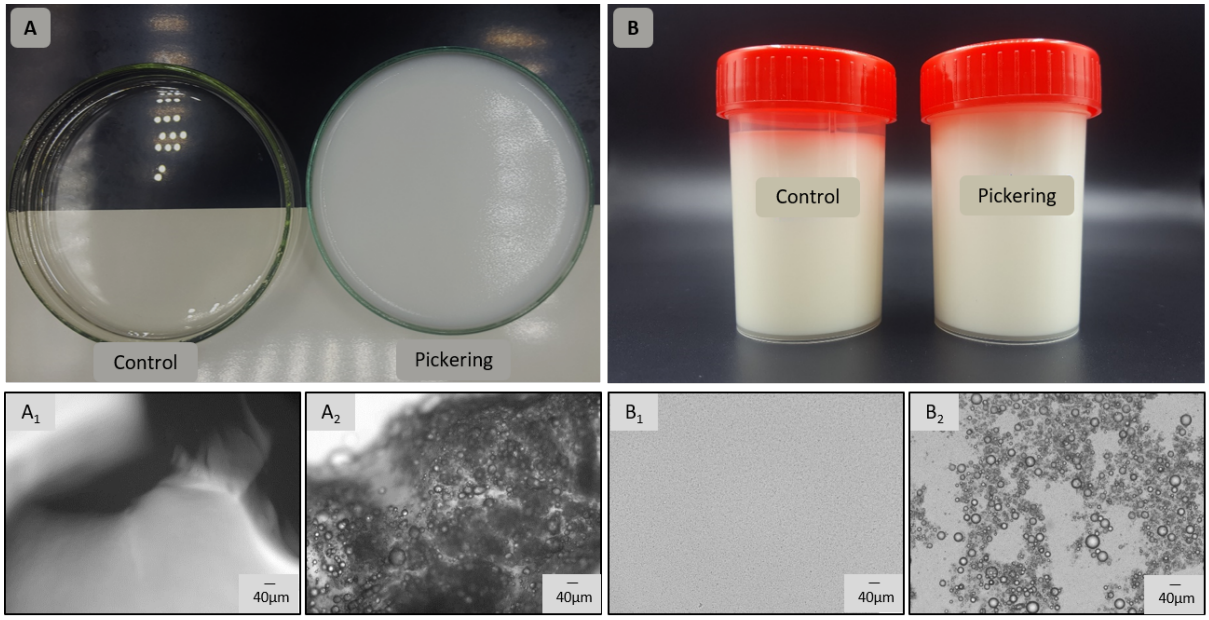

Figure 6.6: Photos of control samples and fortified foods: gelatine (A) and milk (B). Optical microscopy images for gelatine and milk controls, (A_1 and B_1, respectively), and fortified gelatine and milk (A_2 and B_2, respectively). Images were acquired at a magnification of 10x.

Figure 6.7 shows the bioaccessibility, stability and bioavailability of the vitamin E after fortified gelatine and fortified milk digestion. In general, the two fortified foods lead to good vitamin E stability, with fortified gelatine (gelatine_VitE-PE) showing higher vitamin E stability, comparatively with fortified milk (milk_vitE-PE), namely 49.2 ± 2.28% and 38.4 ± 1.10%, respectively ($p < 0.05$). Comparing the two fortified foods, the milk_vitE-PE sample presented higher bioaccessibility (18.1 ± 2.90% in comparison with 10.9 ± 1.04% for gelatine_VitE-PE) ($p < 0.05$). In addition, milk_vitE-PE presented higher bioavailability than gelatine_VitE-PE (6.93 ± 1.09% and 5.33 ± 0.36%, respectively) ($p < 0.05$). To note that the control samples, namely the foods without incorporation presented no vitamin E (data not shown). In the case of foods added with non-loaded Pickering emulsions, only a very residual vitamin E content (data not shown), attributed to the sunflower oil, was detected. Thus, the quantified vitamin E after digestion of the fortified foods was assumed as proceeding from the incorporated vitamin E-loaded Pickering emulsions.

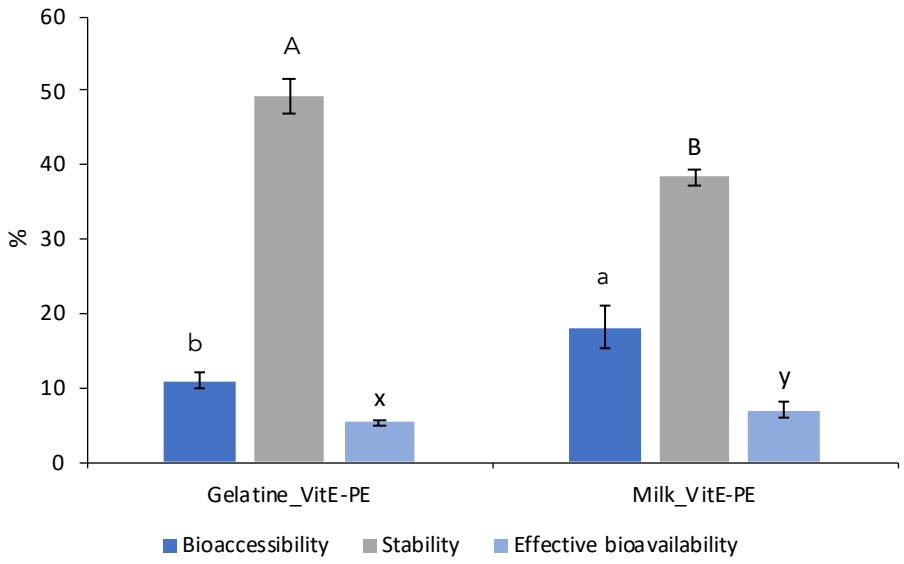

Figure 6.7: Bioaccessibility, stability and adequate bioavailability of fortified food with vitamin E loaded Pickering emulsions (NET-high: ~18 µm) after *in vitro* digestion. Different letters represent significant differences between fortified food matrices in each parameter ($p<0.05$; *t*-test): $^{a\text{-}b}$ for bioaccessibility, $^{A\text{-}B}$ for stability, and $^{x\text{-}y}$ for adequate bioavailability.

One important point to highlight is the fact that fortified foods lead to better vitamin E bioaccessibility and stability in comparison with the non-incorporated vitamin E-loaded Pickering emulsions. Namely, for vitamin E-loaded Pickering emulsions, the maximum achieved bioaccessibility and stability values were 3.29 ± 0.57% and 28.9 ± 2.59%, receptively, for the sample NET-high, which is much lower than the values obtained with fortified milk (18.1 ± 2.90% and 38.4 ± 1.10%for bioaccessibility and stability, receptively) and fortified gelatine (10.9 ± 1.04% and 49.2 ± 2.28% for bioaccessibility and stability, respectively). The natural presence of the macronutrients in the fortified foods led to the vitamin E bioaccessibility increase, possibly associated with their ability to displaced a fraction of the n-HAp particles from the oil droplet's surface, facilitating lipid digestion, vitamin E release and solubilization, and mixed micelles formation (Zhou *et al.*, 2020). Furthermore, fortified milk presented a slightly higher bioaccessibility than gelatine, which can be related to the presence of fat in this food matrix, which can contribute to a more efficient micellar phase formation. This can be explained because the mixed micelles generated during the lipid digestion had more free-fatty acids available for their formation. Therefore, if a higher amount of mixed micelles can be formed, more vitamin E can be incorporated leading to an increased bioaccessibility. Another fact contributing to the better vitamin E bioaccessibility is

the lower amount of n-HAp particles presented in the fortified food. This leads to the formation of less Ca^{2+} species, decreasing the amount of formed aggregates. Additionally, the incorporation of vitamin E-loaded Pickering emulsion in gelatine and milk also lead to an increase vitamin E stability, which indicates lower vitamin E loss during GI digestion.

Although considering different lipophilic vitamins, the results reported in the present work for bioaccessibility agree with those published by Zhou *et al*. (2021). The plant-based milk fortified with a vitamin D_3-loaded emulsion together with nanocellulose or titanium dioxide particles showed a vitamin D_3 bioaccessibility around 20% (Zhou *et al.*, 2021).

Overall, the reported results showed that the presence of macronutrients can help to increment the bioaccessibility of vitamin E by avoiding or delaying the formation of precipitates entrapping the vitamin and oil, i.e., the vitamin and oil phase remain more accessible for micellar phase formation. Consequently, an improvement of vitamin E bioaccessibility was achieved, and more efficient functional foods were developed.

6.4 CONCLUSIONS

Nowadays, there has been an increased interest in using Pickering emulsions as carriers of lipophilic vitamins, foreseeing their use in fortified foods more effectively. To comply with this objective, studying the digestion of both Pickering emulsions and respective fortified foods throughout the GIT and vitamin bioaccessibility is needed.

In this study, Vitamin E was incorporated in the oil phase of O/W Pickering emulsions stabilised by n-HAp particles. Emulsions with tailored droplet size (~7, 11 and 18 μm) were produced by NETmix. The main objective was to check the effectiveness of the produced Pickering emulsions and derived fortified foods in vitamin E bioaccessibility aiming at contributing to functional foods development.

In terms of digestion, the vitamin E-loaded Pickering emulsions presented similar behaviour over the GIT, with complete disruption of the n-HAp particles under gastric conditions followed by the formation of aggregates under intestinal environment. The droplet average diameter impacted vitamin E bioaccessibility, with the NET-high sample, i.e., the sample with

the higher droplet size (18 μm), leading to the higher vitamin E bioaccessibility results. The formation of the aggregates was associated with the complexation of the Ca^{2+} ions derived from the n-HAp disruption in the gastric stage, with anionic species, namely PO_4^{2-}, but also bile salts and free fatty acids. As a result, a reduced number of mixed micelles was formed. Concomitantly, aggregates can have also entrapped the lipid phase and/or micelles. These phenomena can justify the low vitamin E bioaccessibility observed for the vitamin-loaded Pickering emulsions.

Fortified gelatine and milk produced by incorporating the vitamin E-loaded Pickering emulsions were evaluated regarding vitamin E bioaccessibility, revealing better bioaccessibility performances (around 10% for gelatine and 18% for milk), comparatively with the non-incorporated emulsion (NET-high, 3.29%). This fact can be explained by the lower amount of n-HAp particles in the final formulation, as well as to the presence of macronutrients naturally existing in foods (e.g., proteins and fat), which might have circumventing and/or delaying the formation of aggregates avoiding vitamin E entrapment together with the oil phase during the intestinal stage.

The results presented in this work offer new insights about n-HAp based Pickering emulsions digestion and incorporation in food matrices. The results contribute to a better understanding of emulsions' behaviour in food systems aiming at achieving more effective functional foods.

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7 WATER-IN-OIL NANOHYDROXYAPATITE PICKERING STABILISERS THROUGH SURFACE MODIFICATION

This chapter is based on: Andreia Ribeiro, Yaidelin A. Manrique, José Carlos B. Lopes, Madalena M. Dias, Maria Filomena Barreiro. *Surface modified sodium oleate nanohydroxyapatite as a Pickering stabiliser for water-in-oil emulsions*. Under submission.

The stabilisation of water-in-oil (W/O) Pickering emulsions remains a challenge for the scientific community. In this work, hydroxyapatite nanoparticle's (n-HAp) surface was modified with sodium oleate (SO) to tune particle's wettability and enable their use as W/O stabilisers. The approach considers the amount of SO covering the n-HAp particles, evaluated as the number of layers, and their impact on particle's size, zeta potential, and wettability. The obtained results pointed out that the wettability depends on the number of applied SO layers leading to the switching from hydrophobic to a hydrophilic character. The particle size decreased with the number of SO layers, compatible with an electrostatic repulsion increasing, preventing aggregates formation. The produced Pickering emulsions based on the SO modified n-HAp holding one SO layer (contact angle close to 90º) revealed good stability (only at 30 days, slight signs of instability started to be observed), with particles tending to be adsorbed at the water-oil interface (confocal analyses). Thus, the present work contributes to the field of W/O emulsions development, a topic scarcely addressed in the literature, opening new avenues for several industrial needs, increasing the applicability of Pickering emulsions.

7.1 INTRODUCTION

Several food, pharmaceutical, and agrochemical products, among others, are emulsions (Berton-Carabin & Schroën, 2015; McClements, 2016). Emulsions can be classified according to the disperse and continuous phase nature. When oil droplets are dispersed in water it is an oil-in-water emulsion (O/W), whereas if the opposite occurs, it is a water-in-oil emulsion (W/O) (McClements, 2016). To ensure the stabilisation of this bi-phasic system, a third component, called surfactant or emulsifier, is needed (McClements, 2016). The emulsifier molecules can be adsorbed at the oil-water interface decreasing the interfacial tension, thus preventing emulsion droplets against instability phenomena (McClements, 2016; Petersen & Ulrich, 2013).

Pickering emulsions, identified by Ramsden (Ramsden, 1903) and Pickering (Pickering, 1907), started to raise interest at the beginning of the 20th century, then increasing throughout the years. Pickering emulsions use solid particles as stabilisers, providing high physical and chemical stability due to their irreversible adsorption at the oil-water interface (Li *et al.*, 2019; Ortiz *et al.*, 2020). They have unique stability characteristics, and satisfy the lack of alternatives for *clean-label* emulsifiers in the food, cosmetic and pharmaceutical areas (Zembyla *et al.*, 2019). Moreover, they can act as encapsulation systems for bioactive compounds such as nutrients or drugs (Frelichowska *et al.*, 2009b; Frelichowska *et al.*, 2009a; Marefati *et al.*, 2017; Mitbumrung *et al.*, 2019). Most of the reported Pickering particles are adequate to stabilise O/W emulsions, and a lack of solutions to stabilise W/O is noted. In accordance, studies addressing O/W Pickering emulsions are predominant in literature (Zembyla *et al.*, 2019).

Hydroxyapatite (HAp), which is naturally presented in the human tooth and bones, is a surface-active material with chemical composition $Ca_{10}(PO_4)_6(OH)_2$ (Enax & Epple, 2018). It is recognised as a biocompatible material, widely used in the biomedical field (Enax & Epple, 2018; Epple, 2018), and, recently, in tooth care products (Coelho *et al.*, 2019; Vano *et al.*, 2018). HAp started to enter novel fields of application, namely in the stabilisation of emulsions, which due to their predominant hydrophilic characteristics, is appropriate to stabilise O/W emulsions (Zhang *et al.*, 2017). Namely, the results reported in our previous works showed that O/W Pickering emulsions prepared with n-HAp have good physical

stability and interesting rheological properties, pointing out promising application prospects in several application (e.g., food, pharmaceutical, and cosmetics) (Ribeiro *et al.*, 2020). Moreover, the continuous production of n-HAp based emulsions was also demonstrated in a recent work of the group using NETmix technology, thus approaching the industrial viability of these systems (Ribeiro *et al.*, 2021).

Pickering emulsions based on n-HAp are also being proposed as templates for microspheres or porous materials production through combination with different polymers, with reported improvements on particle's adsorption at droplet's surface (Fujii *et al.*, 2013; Song *et al.*, 2018; Tham & Chow, 2017; Zhang *et al.*, 2017). For example, Fujii *et al*. (2013) and Liu *et al*. (2011) used O/W Pickering emulsions as templates to obtain HAp microspheres coated with PCL and PLLA, respectively. Also, W/O templates based on HAp/PLLA, HAp/PCL particles were tested in the fabrication of porous materials (scaffold materials through oil phase evaporation), as reported by Zhang *et al*. (2017) and Hu *et al*. (2019), respectively.

The wettability, which can be determined by the contact angle (θ), is an important parameter to consider when preparing Pickering emulsions. If θ is lower than 90°, the particle is hydrophilic and tend to stabilise O/W emulsions. If θ is higher than 90°, the particle is considered hydrophobic and tend to stabilise W/O emulsions. In case θ is equal to 90°, the particles are wetted equally by the oil and water phases giving rise to the maximum particle's adsorption at the interface (Ortiz *et al.*, 2020). This parameter can be modulated by particle's surface chemistry. Two main approaches can be used, namely physical adsorption and chemical anchoring of small molecules or polymers to the particle's surface (Xiao *et al.*, 2018). Reported studies focus the combination of the solid particles with emulsifier molecules to improve the physical stabilisation of the emulsions. Namely, Skelhon *et al*. (2012) concluded that the addition of chitosan to silica particles favoured the adsorption of the particles at the W/O interface, and Sakazaki *et al*. (2019) demonstrated that the wettability of silica-coated titanium dioxide particles can be tuned by soaking the particles in cyclic silicone oil, adjusting the particles to the stabilisation of O/W and W/O emulsions.

In this context, the objective of this work was to tune the wettability of the n-HAp particles to obtain suitable stabilisers for W/O emulsions. Comparatively with O/W, studies addressing

W/O Pickering emulsions are scarcely reported in the literature, thus this work aims to contribute to overcome this constraint. In fact, W/O systems are interesting solutions for the encapsulation and delivery of hydrophilic active principles, e.g., polyphenolic extracts. The envisaged approach uses sodium oleate (SO) to modify the n-HAp surface and improve the hydrophobicity of the pristine particles. SO is an ionic emulsifier with high hydrophobicity and a critical micelle concentration in water of 2.15 mM (Hu *et al.*, 2015). The modification was carried out taking into consideration an estimated number of layers covering the n-HAp particles. Their effect on physical (size and structure), surface (surface charge) and wettability (contact angle) properties were studied. Then, the produced SO modified n-HAp particles were validated to produce W/O Pickering emulsions, which were characterised by optical (OM) and confocal laser scanning microscopy (CLSM) to check the droplet's morphology and particle's adsorption at the W/O interface. Furthermore, emulsion's stability was studied along one month of storage, and the emulsion type confirmed by the drop test and CLMS. Overall, this study provides new insights into the production of W/O Pickering emulsions through modified n-HAp particles, which are biocompatible systems with potential to be used in the delivery of hydrophilic active principles.

7.2 MATERIALS AND METHODS

7.2.1 MATERIALS

The *nano*XIM-HAp102, hydroxyapatite aqueous paste, was supplied by Fluidinova S.A. *Nano*XIM-HAp102 is solely composed of 15.0 ± 1.0 (wt%) of n-HAp with particle sizes < 50 nm and water. This n-HAp was chosen due to absence of the salts or other materials in the formulation, making it more suitable to study the effect of the SO in the surface modification. Sunflower oil was purchased from a local supermarket. Sodium oleate and fluorescent dyes (Nile red and Nile blue A) were obtained from Sigma-Aldrich. Isopropyl alcohol was purchased from Riedel-de Haen. Distilled water, treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA), was used. All the other chemicals were of analytical grade.

7.2.2 PRODUCTION AND CHARACTERISATION OF MODIFIED N-HAP PARTICLES

7.2.2.1 Production of the modified n-HAp particles

The n-HAp (*nano*XIM-HAp102) particle's modification was done with sodium oleate (SO). Firstly, the SO powder was dissolved in distilled water at a predefined concentration aiming at achieve the desired number of layers to cover the n-HAp particles (0.5, 1, and 2), which was 0.09 g, 0.18 g and 0.35 g, considering 1 g of n-HAp102 particles. The methodology used to estimate the SO amount can be consulted in the Appendix C (C.1 section). Briefly, SO solution was prepared under stirring using a magnetic bar until complete dissolution in water was reached. In parallel, the n-HAp paste was dispersed in water using a rotor-stator homogeniser (Miccra RT D-9) operating at 11000 rpm for 2 min. Finally, the SO solution was injected to the dispersed n-HAp paste with a peristaltic pump at 240 rpm. The mixture continued to be stirred with the rotor-stator homogeniser at 11000 rpm for 2 min. The influence of the number of layers on the particle size, zeta potential, morphology (SEM analysis), and wettability of the modified particles was evaluated.

7.2.2.2 Characterisation of the modified n-HAp particles

7.2.2.2.1 Particle size and zeta potential

The particle size and the zeta potential of the SO modified n-HAp dispersions were determined by dynamic light scattering (DLS) and particle electrophoresis, respectively, using a Nano-ZS Zetasizer instrument (Malvern Instruments Ltd.). For the assays, the samples were diluted to an appropriate concentration (0.05 wt%, n-HAp basis) with distilled water. All measurements were carried out in triplicate at 25 °C using the value of 1.650 for the refractive index. The results are reported as average ± standard deviation (SD).

7.2.2.2.2 Particle wettability

The wettability measurements were carried out with an optical contact angle device (Biolin Scientific - Theta Lite 100). To carry out the measurements, pellets of SO modified n-HAp particles were prepared, after drying the dispersion in an oven at 80 °C for at least 3 h to obtain the powders. Pellets with a diameter of 13 mm and an approximate thickness of 1 mm were prepared using a hydraulic press (PerkinElmer) at 10 tonnes for 2 min. Measurements

were made right after pellets preparation by pouring a sessile drop of distilled water (5 µL) at their surface using a high-precision injector. The droplet was recorded with a high-speed video camera coupled to the equipment, and the contact angle values between the droplet and compressed particles (left and right) automatically calculated by the software using the Laplace-Young equation. The measurements were performed in triplicate using different pellets, and the results are reported as average ± SD.

7.2.2.2.3 Scanning electron microscopy analysis

Scanning electron microscopy (SEM) was used to check the morphology of the SO modified n-HAp particles. For the analysis, the dried SO modified n-HAp was mounted in a carbon adhesive, followed by coating with an Au/Pd thin film by sputtering, using the SPI Module Sputter Coater equipment. Then, the SEM/EDS analysis was performed using a high resolution (Schottky) environmental scanning electron microscope with X-ray microanalysis and electron backscattered diffraction analysis: Quanta 400 FEG ESEM/EDAX Genesis X4M.

7.2.3 PRODUCTION AND CHARACTERISATION OF THE PICKERING EMULSIONS STABILISED WITH THE MODIFIED N-HAP PARTICLES

7.2.3.1 Production of the Pickering emulsions

The Pickering emulsions were prepared in batch mode using a rotor-stator homogeniser. For that, the SO modified n-HAp particle's dispersion, as prepared in section 7.2.2.1, was added dropwise to the continuous phase (sunflower oil) using a peristaltic pump operating at 240 rpm. The emulsification was carried out using a rotor-stator homogeniser running at 11000 rpm for 1 min. To avoid the temperature rise generated by the homogeniser, during the emulsification process the vial used for the preparation was submerged in an ice bath. Emulsions with a W/O ratio of 20/80, and 30/70, using respectively 1.6%wt, and 2.7%wt of SO modified n-HAp particles were prepared. The methodology to estimate the amount of required particles can be consulted in the Appendix C (C.2 section).

7.2.3.2 Characterisation of the Pickering emulsions

7.2.3.2.1 Optical microscopy

Optical microscopy (OM) was used to monitor the morphology of the Pickering emulsions right after the emulsification process (0 days), and along a storage period of 30 days (sampling at 2, 7, 14 and 30 days) to check for instability development. For that, an aliquot of the sample was placed on a slide and gently covered with a coverslip. A Carl Zeiss Axiotech 100 HD optical microscope (Zeiss Instruments) fitted with a digital camera (AxioCam 105 colour) was used. The image acquisition and processing was made using Zen (2.3 blue edition) software.

7.2.3.2.2 Confocal Laser Scanning Microscopy

Confocal Laser Scanning microscopy (CLMS) was used to the check the interfacial structure of the Pickering droplets. For that, the Pickering emulsion was stained with a mixture of fluorescent dyes in isopropyl alcohol, namely Nile red at 0.1% *w/v* and Nile blue A at 0.1% *w/v*. The two dyes were chosen to dye differently the continuous oil phase (Nile red, green colour) and the particles (Nile blue A, red). These experiments were performed using a Leica TCS-SP5 AOBS (Leica Microsystems Inc. Heidelberg, Germany). The stained emulsion was put on a slide, and the fluorescent dyes excited by different wavelengths; namely 488 nm for Nile red and 633 nm for Nile blue A. The images were digitally recorded and processed with LasX software.

7.2.3.2.3 Emulsion type

The drop test is a rapid test to check the formed emulsion type (O/W or W/O). This test is based on the fact that the emulsion will disperse when put in contact with the respective continuous phase. Briefly, if the Pickering emulsion drop rapidly spreads in water and remain agglomerated in the oil phase, the emulsion is of O/W type. If the Pickering emulsion drop rapidly disperses in oil and remain agglomerated in the water phase, the emulsion is of W/O type. This test is carried out by adding two or three emulsion drops in water and oil phases, followed by slight stirring and observation.

7.3 RESULTS AND DISCUSSION

7.3.1 SODIUM OLEATE MODIFIED N-HAP PARTICLES

In this study, the effect of sodium oleate (SO) on the n-HAp particles surface was studied. The main objective is to increase the hydrophobicity of the solid particles modifying their wettability to render them appropriate for W/O Pickering emulsions stabilisation. For that, the SO was added at an amount according to the number of desired layers covering the n-HAp solid particles (see Appendix C – C.1 section). The produced SO modified n-HAp particles were characterised in terms of size, potential zeta, morphology (SEM) and wettability, and the effect of the applied number of layers evaluated.

7.3.1.1 Effect of the layers number in particle's size and zeta potential

The particle size and zeta potential of the SO modified n-HAp particles were evaluated as a function of the used number of layers. These parameters can impact on Pickering emulsions development, namely, particles with smaller sizes can be better adsorbed at the droplet interface, as stated by Xi *et al*. (2018). In this study, a commercial n-HAp formulation (n-HAp102) was modified with SO (0.5, 1, and 2 layers). Table 7.1 shows the results obtained with the particles right after production. It is possible to verify that, as the number of layers increases, a significant impact on both size and zeta potential parameters was observed, namely the size decreases and the zeta potential increases (in magnitude), in accordance with an effective surface modification. The magnitude of the zeta potential gives an indication concerning particles stability in suspension. Namely, values higher, in magnitude to 30 mV, i.e., ≤-30 and ≥30 give rise to particles with enough electrostatic repulsion forces to prevent the formation of aggregates. In HAp102 a change from -25 mV to -46 mV, was observed; the particles range from low stability to high stability as the number of layers increased from 0.5 to 2 (values lower than -30 mV). The measured apparent lower size can be justified by the increase of the electrostatic repulsion, preventing the formation of aggregates.

Other previous works reporting the influence of surface modifications on particle size and zeta potential have been published. In the work of Zhu *et al*. (2015) zeta potential of silica particles was improved by increasing cetyltrimethylammonium bromide (CTAB) concentration, also

reporting the adsorption of CTAB molecules at the particle–water interface at these concentrations. Li *et al*. (2019) that studied gliadin nanoparticle's modification with octenyl succinic anhydride (OGNPs) reported changes in the particle size and zeta potential of OGNPs, comparatively with GNPs. Namely, an increase of the zeta potential (stability improvement) was observed as the octenyl succinic anhydride content increased; which was accompanied also by the production of larger size particles.

Table 7.1: Particle size and zeta potential of different SO modified n-HAp102 according to the number of layers.

SO LAYERS	SIZE (μm)	ZETA POTENTIAL (mV)
0.5	1.72±0.49	-25.2±0.6
1	0.50±0.03	-35.8±0.1
2	0.12±0.01	-45.5±0.5

7.3.1.2 Effect of the layers number in the particle's wettability

To achieve Pickering emulsion stabilisation, particles with a suitable wettability must be selected (Binks *et al.*, 2013; Dickinson, 2012; Ortiz *et al.*, 2020), similarly to traditional emulsifiers that are ruled by the hydrophilic-lipophilic balance (HLB) scale. The wettability measured by the contact angle determination, indicates the hydrophilic or hydrophobic character of the solid particles (Ortiz *et al.*, 2020). Thus, when solid particles have a contact angle <90°, the particles are considered more suitable to stabilise O/W emulsions; if the contact angle is >90°, the particles tend to stabilise W/O emulsions (Dickinson, 2012). The wettability of the particles can be tuned through thermal (Marefati *et al.*, 2017; Zhu, 2019), and chemical treatments (Li *et al.*, 2019), or through the combination with emulsifiers or polymers (Binks *et al.*, 2013; Zhang *et al.*, 2017). This can be advantageous for emulsion stabilisation when the original particles are highly hydrophilic or hydrophobic (Berton-Carabin & Schroën, 2015).

The contact angle of the n-HAp102 particles, untreated and SO surface modified (0.5, 1 and 2), was determined and the results are shown in Figure 7.1, accompanied with a schematic representation reflecting the rearrangement of the SO molecules with the number of layers. For the untreated sample, the contact angle was around 20°, indicating their higher suitability

to stabilise O/W Pickering emulsions. As the number of SO layers increased, the contact angle of n-HAp particles increased until 1 layer, decreasing for 2 layers. Namely, when 1 layer was applied the contact angle increased until 92°, and when 2 layers were applied the value decrease to 38°. This behaviour is compatible with the fact that the second layer is formed over an existing layer (Figure 7.1), switching the hydrophobic character achieved with 1 layer to hydrophilic, namely by exposing the emulsifier head (hydrophilic). This effect was also reported by Binks *et al*. (2013) using silica particles and a di-chain cationic surfactant as the modifying agent. The authors reported a successive transition from hydrophilic, to hydrophobic, and again to hydrophilic as the concentration of the surfactant increased.

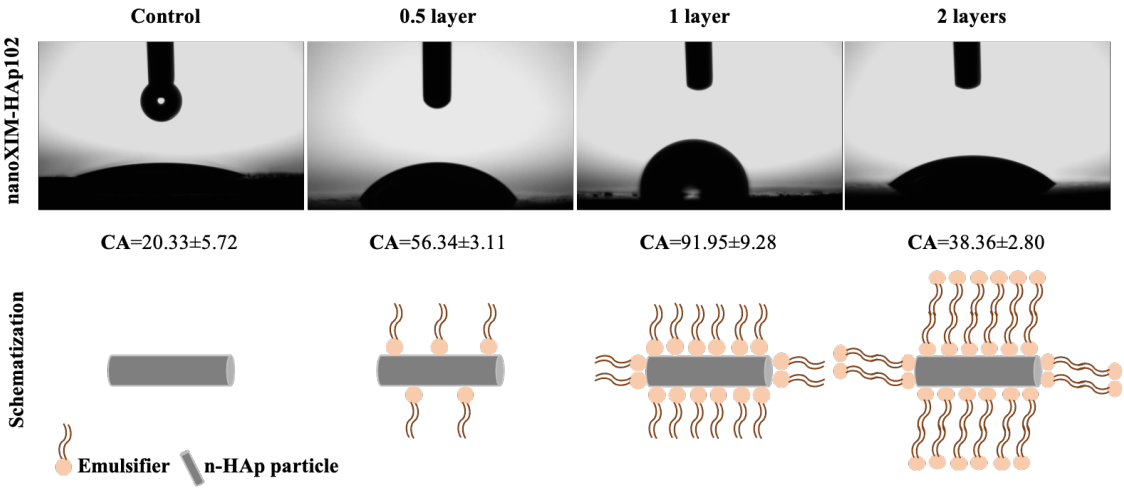

Figure 7.1: Registration of the contact angle measurements of the original n-HAp (control) and SO modified n-HAp at different contents to achieve different number of layers (0.5, 1, and 2). Schematic representation reflecting the rearrangement of the SO molecules with the number of layers.

7.3.1.3 Effect of the layers number in the particle's morphology

The effect of SO in the particle's morphology was investigated by scanning electron microscopy (SEM), and the obtained images shown in Figure 7.2 for the n-HAp102 particles without treatment (control), and the SO modified n-HAp102 particles with 0.5, 1 and 2 layers.

The n-HAp102-control particles exhibited the typical rod-like shape and the reported dimensions by Fluidinova (Fluidinova, 2019a, b), namely a typical length of 50 nm. With SO addition the morphology was modified; namely as the number of layers increased from 0.5 to 2, the n-HAp surface become smother. This effect evidences the successful adsorption of the SO at the n-HAp surface.

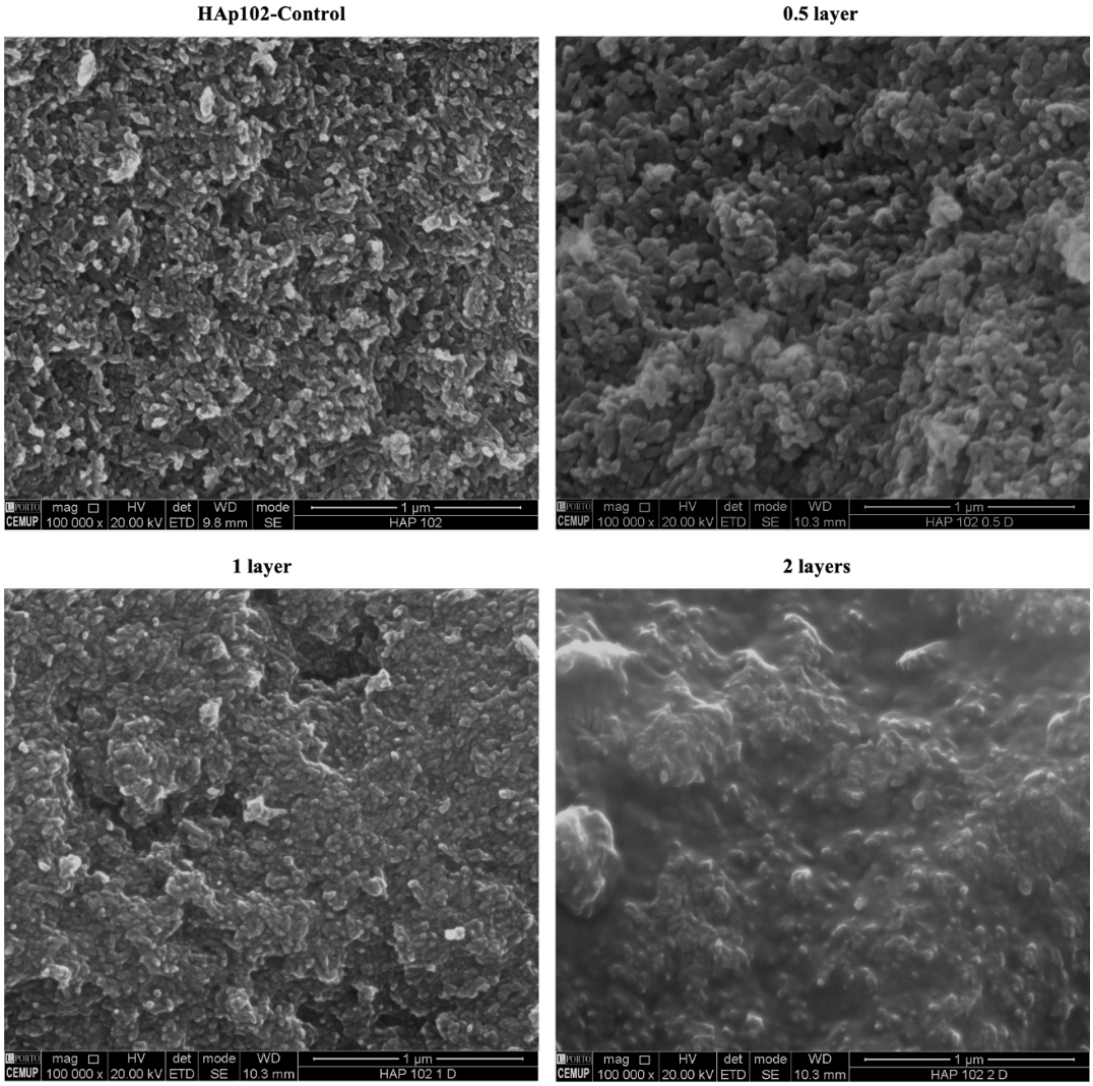

Figure 7.2: SEM images of the control n-HAp particles and SO modified n-HAp particles with different layers (0.5, 1 and 2). The scale bar represents 1 μm.

7.3.2 PICKERING EMULSIONS STABILISED WITH THE MODIFIED N-HAP PARTICLES

The production of O/W n-HAp based Pickering emulsions was previously reported by our group in batch (Ribeiro *et al.*, 2020), and continuous mode (Ribeiro *et al.*, 2021). In order to increase the range of applications, namely for the loading of hydrophilic active principles the interest for the production of n-HAp based W/O Pickering emulsion was identified. Based on the previous section results the SO modified n-HAp102 with one layer, i.e., the particles characterised by a contact angle slightly higher than 90°, were used. According to evidences reported in literature, particles with a contact angle around 90° facilitate their adsorption at the droplet's interface. They can also produce a steric hindrance against droplet's aggregation (Li *et al.*, 2019).

To produce stable Pickering emulsions, it is important to know the needed amount of n-HAp particles to stabilise the droplet's surface. In this work, the required amount of SO modified n-HAp particles was determined based on the findings of a previous work (Ribeiro *et al.*, 2020). Briefly, the amount of particles was given by the product between the density, volume and the number of solid particles needed to cover droplets surface. Details of the used methodology can be seen in Appendix C (C.2 section). The W/O Pickering emulsions were produced according to the estimated mass of SO modified n-HAp that cover the total droplets. Two W/O ratios were used, namely 20/80 and 30/70 (W/O, *v/v*) and the emulsification carried out with a rotor-stator homogeniser. The 20/80 and 30/70 were produced with particles concentration of 1.6%wt and 2.7%wt, respectively. The optical images of both W/O Pickering emulsions, examined right after production, are shown in Figure 7.3. It can be perceived the spherical shape of droplets, some level of aggregation, and size dispersity.

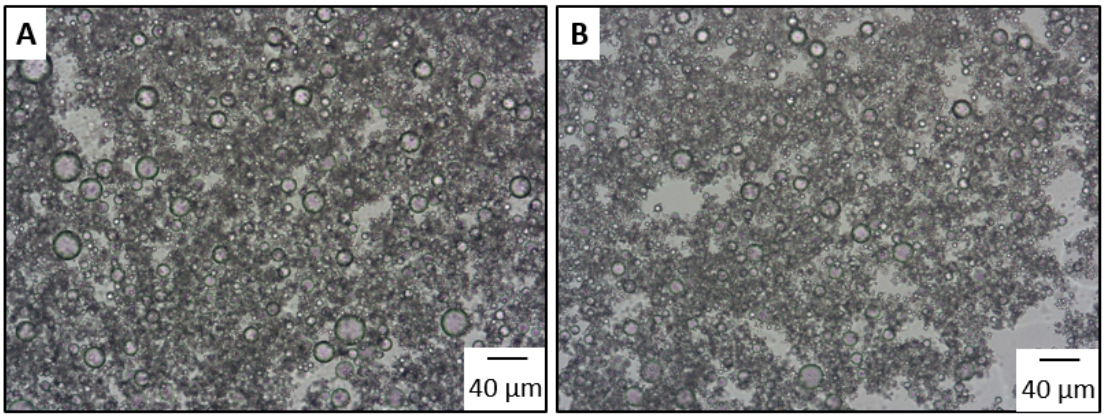

Figure 7.3: Optical images of the W/O Pickering emulsions stabilised with the SO modified n-HAp: A) 20/80 and B) 30/70, acquired after production. Magnification: 20x.

7.3.2.1 Type and storage stability evaluation of the W/O Pickering emulsions

After production, the emulsion type was determined through the drop test, and the stability evaluated along a storage period of 30 days (sampling at 0, 7 and 30 days) at room temperature. Figure 7.4 shows the drop test made with the produced 20/80 Pickering emulsion. It is possible to observe that the drops of the Pickering emulsion spread in the oil phase and agglomerated in the water phase thus conforming the W/O type. This behaviour was also achieved for the Pickering emulsion produced with a ratio of 30/70.

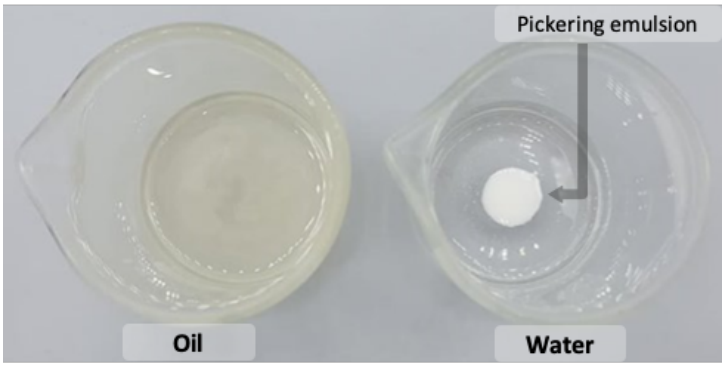

Figure 7.4: Drop test behaviour for sample 20/80 (*v/v*).: Pickering emulsion dispersed in the oil phase (left beaker) and Pickering emulsion agglomerated in the water phase (right beaker).

Figure 7.5 shows representative images of the produced W/O Pickering emulsions (20/80 and 30/70) at different storage times (0, 15 and 30 days). The Pickering emulsions remained stable (without any phase separation) over 15 days at room temperature, as can be inspected in Figure 7.5. However, some sedimentation phenomenon started to occur after this storage time (assigned by the black arrow). The stability of the W/O n-HAp based Pickering emulsions was also reported by Hu *et al*. (2019) that prepared high internal phase Pickering emulsions (HIPEs). In this work, the authors reported a storage stability of 30 days. However, in this study, the emulsions had a *gel-like* structure, thus a higher viscosity, which can justify their increased stability over time. High viscosities in emulsions create mechanical rigidity helping emulsion stabilisation (McClements, 2016). For W/O emulsions produced out of this range of compositions, instability was observed at early stages. This is the case of Zhang *et al*. (2017b) that produced Pickering emulsions based in n-HAp and PLLA achieving a stability not surpassing the 24 hours. Zhang *et al*. (2017) in their study addressing the effect of stearic acid modified n-HAp in Pickering emulsions and HAp/PLLA composites, reported stabilities of only 4 hours. Furthermore, Hu *et al*. (2015) reported rapid destabilisation of W/O Pickering emulsions stabilised with sodium oleate modified HAp. The destabilisation was associated with the antagonistic effect caused by high SO concentrations; i.e., under the used conditions the particles and surfactants lose their ability to stabilise the oil/water interface. Compared to the literature results, this work gave rise to satisfactory stability levels, which indicates that the Pickering emulsions produced with SO modified n-HAp particles with a monolayer results in an improved stability during storage time. This result also evidences the importance to analyse the effect of the used number of layers.

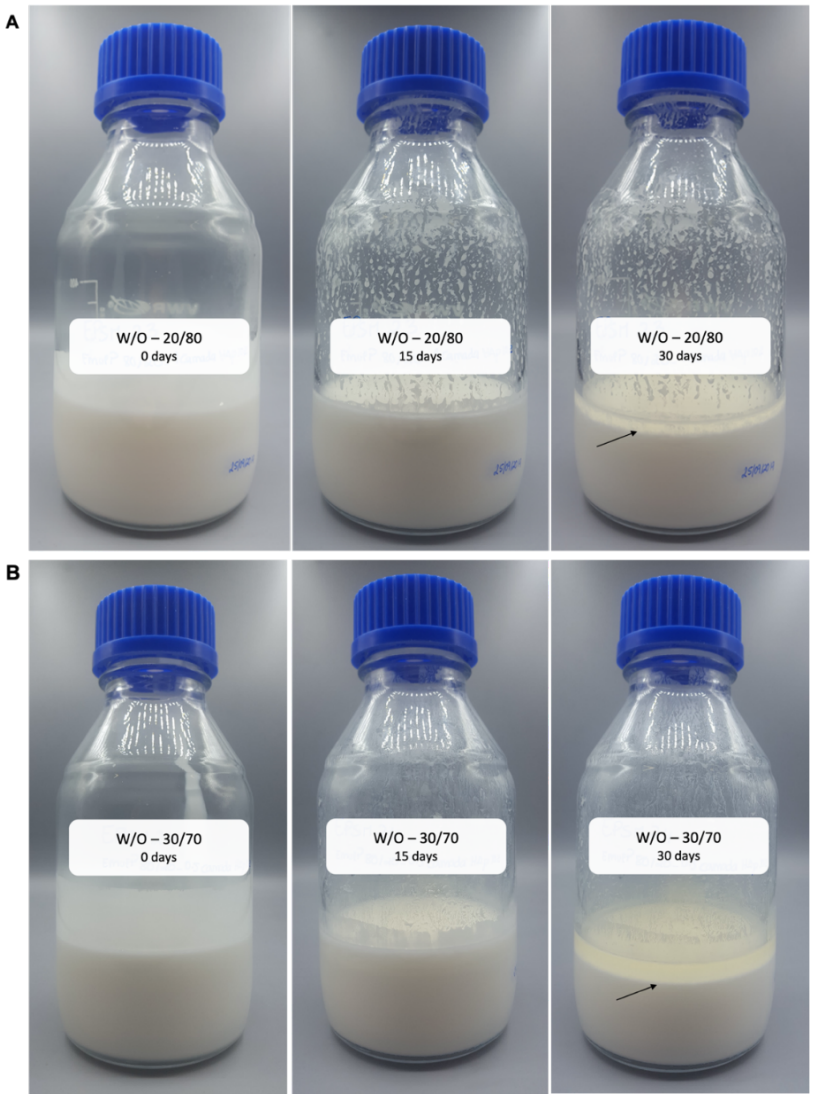

Figure 7.5: Representative photographs of the W/O Pickering emulsions A) 20/80 (*v/v*) and B) 30/70 (*v/v*) as a function of the storage time (0 days – right after production, 15 and 30 days). The arrow indicates the emulsion phase separation.

CLSM allows the visualisation of the droplet's interface, letting also to confirm the emulsion type. The fluorescence microscopy images of the 20/80 Pickering emulsion are shown in Figure 7.6 and Figure 7.7. Both figures are composed of three images, which correspond to red fluorescence (showing the SO n-HAp modified particles), green fluorescence (showing the sunflower oil), and the overlap of the two fluorescence images. Figure 7.6 shows the Pickering emulsions obtained after 1 day of production, where it is possible to identify the sunflower oil as the continuous phase (green fluorescence) and the modified n-HAp particles at the boundary of the droplets (red fluorescence). This observation is in agreement with the results given by the application of the drop test (Figure 7.4). The higher magnification (bottom line)

confirms, more clearly, the presence of the SO modified n-HAp particles covering the surface of the water droplet (evidenced as a red ring).

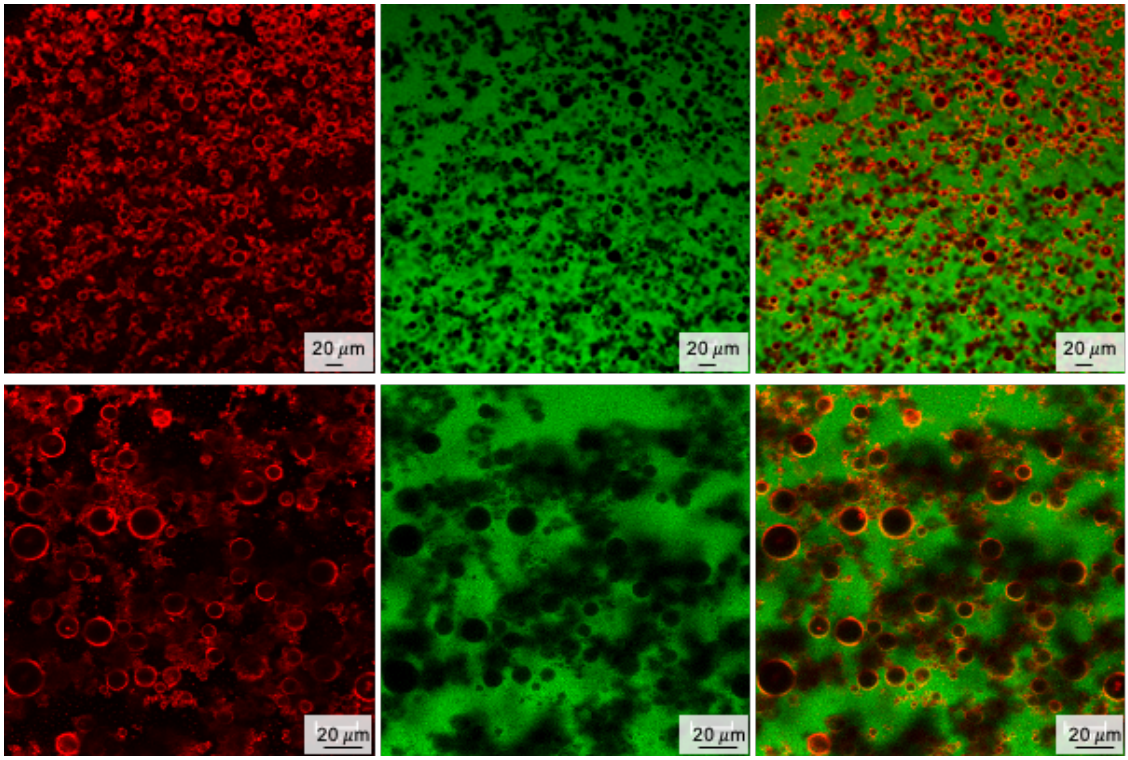

Figure 7.6: CLSM images of W/O Pickering emulsions (20/80, *v/v*) after 1 day of production. The scale bar is equal to 20 μm.

In Figure 7.7 a comparison between the Pickering emulsion produced after 1 day (top line) and 30 days (bottom line) is made. From the set of images, it is possible to verify that there was no phase inversion, although the loss of the droplet's spherical shape is perceived. Furthermore, after 30 days of storage, some aggregation among the droplets is observed (coalesce phenomenon). This observation is in accordance with the observations of Figure 7.6 that put in evidence the occurrence of phase separation after 30 days of storage.

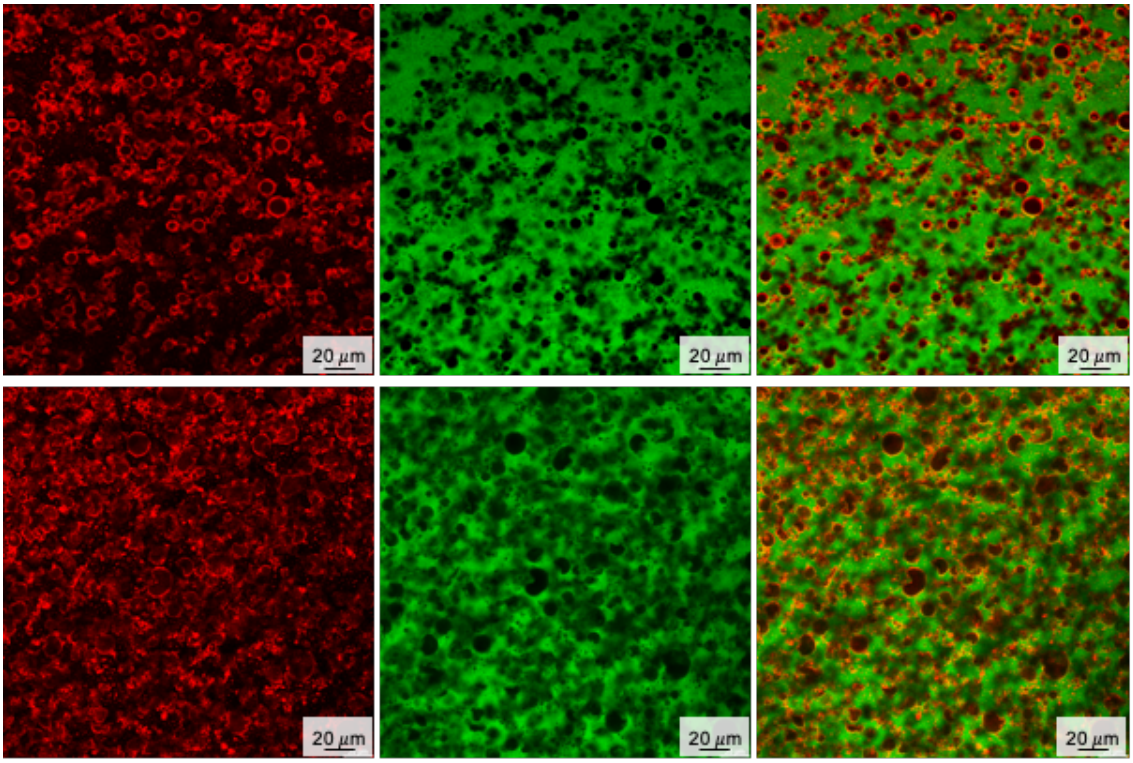

Figure 7.7: CLSM images of W/O Pickering emulsions (20/80, *v/v*) after 1 day (top line) and 30 days (bottom line) of production. The scale bar is equal to 20 µm.

7.4 Conclusions

This work addressed nanohydroxyapatite (n-HAp) modification aiming at achieve suitable Pickering stabilisers for W/O emulsions, which was motivated by the lack of solutions in the field and their potential for several applications. The n-HAp surface was modified with sodium oleate (SO) to tune the wettability of the pristine particles, which are characterised by a natural hydrophilic character. It was verified that the number of SO layers impacts the wettability of the n-HAp particles, which varies from hydrophilic (0.5 layers) to hydrophobic (1 layer), and again to hydrophilic (2 layers). Additionally, the presence of SO at the surface of n-HAp particles affects their physical characteristics, mainly the size and surface charge. As the number of layers increases, more stable the particles become in suspension, as evidenced by the measured zeta potential (< -30 mV). The particle size decreased as a result of the electrostatic repulsion increase, which presents advantages avoiding aggregates formation. The modified n-HAp particles with contact angle close to 90° were chosen to test in the preparation of W/O Pickering emulsions, corresponding to the ones treated with one SO layer, i.e., a saturated monolayer conferring a hydrophobic character. It was possible to

confirm the ability of the particles to adsorb at the water droplets surface and their W/O typology. Besides, it was possible to achieve good emulsion stability over 15 days of storage with only slight signs of instability observed for 30 days.

Overall, the work presents a systematic approach to carry out surface modifications of solid particles. The wettability of the n-HAp particles can be tuned according to the number of applied SO layers. By following this strategy, W/O emulsions were obtained opening new avenues for products based on n-HAp particles, which by nature stabilise O/W emulsions.

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8 CONCLUSIONS AND FUTURE WORK

The field of Pickering emulsions is presently a hot topic for several scientific areas (e.g., food, pharmaceutical, biomedical and cosmetics) due to their surfactant-free nature, answering important sustainability and green chemistry issues. Furthermore, Pickering emulsions have high stability, mainly against coalescence, due to the formation of a steric and mechanically robust interfacial barrier by the irreversibly adsorbed particles. Due to these relevant advantages, Pickering emulsions emerged as great alternatives to conventional emulsions where particles (Pickering stabilisers) can replace synthetic emulsifiers that may cause undesired physiological responses. Additionally, Pickering emulsions can be used as carriers of active compounds, acting as a microencapsulation systems, contributing to the development of more effective functional foods.

This work was structured in five experimental chapters corresponding to different study objectives. Each chapter includes the main conclusions about the treated topic. In this chapter an overview of the main findings of the present work are summarised. Finally, a list of suggestions for future work is also provided.

8.1 OVERALL CONCLUSIONS

Nanohydroxyapatite is widely used in the biomedical and biotechnological fields due to its excellent biocompatibility. In this work, commercial food-grade nanohydroxyapatite (n-HAp) particles were used to stabilise Pickering emulsions. These particles are produced using a low energy device, having high quality and reproducibility, which is an asset to ensure quality control. This fact is advantageous since particle's production is most often a complex, energy-intense, unsustainable, and expansive process, e.g., using complexation or heating. The use of n-HAp in Pickering emulsions started to be reported only recently, mainly following a strategy where it is combined with polymers such as PCL and PLLA. In Chapter 3, n-HAp particles alone demonstrated to act as suitable Pickering stabilisers giving rise to stable oil-in-water (O/W) emulsions, depending on the used n-HAp concentration (0.5 to 15 wt%) and O/W ratio (10/90 to 60/40, *v/v*). These initial tests were made in batch mode using a rotor-stator device for emulsification.

Regarding the obtained results, it was possible to verify that the n-HAp content increase led to a decrease in the mean droplet size, whereas the O/W ratio increase favoured mean droplet size increase. Independently of the used O/W ratio or n-HAp content, all the produced Pickering emulsions were of O/W type, as verified by the drop-teste. Furthermore, the microscopy analysis showed spherical Pickering droplets (optical microscopy) and n-HAp particles tightly adsorbed at the oil droplet's surface (cryo-SEM), corroborating an oil core–HAp shell morphology, similarly to microcapsules, validating the effective role of n-HAp particles as Pickering stabilisers. Chapter 3 was also complemented with a model to predict emulsion stability. This model considers factors such as O/W ratio, n-HAp concentration, droplet diameter and n-HAp dimensions. According to the developed model, three regions (unstable, stable and *gel-like*) were defined in a ternary phase diagram. The experimental data was generally in good agreement with the model, which can be used to predict the emulsion

type from the used formulation, as well as its stability. The model was consistent up to two weeks; only after 2-months differences were found (some emulsions become unstable).

Additionally, rheological studies were performed, showing that Pickering emulsions stability could be improved by increasing the n-HAp concentration or the O/W ratio. With the oscillatory measurements it was verified that Pickering emulsions had, predominantly, an elastic behaviour, which was related to the structural network developed among the free n-HAp particles in suspension. These results are in agreement with model predictions. Summarising, Chapter 3 reports, for the first time, the production of O/W Pickering emulsions stabilised by pristine n-HAp particles, which is an important achievement since it avoids problems of emulsion contamination (e.g., by solvents, chemical emulsifiers etc.). Furthermore, a model predicting emulsion stability according to their composition was introduced.

Pickering emulsions are typically produced in batch mode using high shear devices. Regarding the achievement of large production scale in view of a potential industrialisation, new devices must be used. In this context, Chapter 4 proposes NETmix technology, a low-intensive energy device, for the continuous production of the O/W n-HAp stabilised Pickering emulsions. This objective was approached using a formulation previously developed in batch mode and by studying the influence of process parameters like Reynolds number (200-500) and the number of cycles (2-35) (parameter related to the NETmix residence time) in the emulsion morphology and stability. The results showed a decrease in the droplet size as the number of cycles and Reynolds number increased. Regarding the effect of the number of cycles (determined for Re=400), and except for the emulsion obtained with 2 cycles, the emulsions showed to be stable over a 2-months period. For the Re effect (determined using a number of cycles=17), the Pickering emulsions presented small size droplets, except the ones produced at Re=200, which presented a broad size distribution becoming unstable after 7 days. Although the tested formulation gives rise to stable emulsions by batch mode (data of Chapter 3), the droplet size plays a crucial role on Pickering emulsions stability since large droplets (~60 μm) tend to develop coalescence and Ostwald ripening phenomena. Using NETmix, the maximum average size reduction for the tested conditions was ~7 μm (17 cycles and Re = 400). For higher number of cycles and Re, no significant changes were detected.

Summarising, the production of n-HAp stabilised O/W Pickering emulsions in continuous mode by NETmix technology was introduced for the first time.

Overall, comparing the batch process (**Chapter 3**) with NETmix (**Chapter 4**) production, NETmix enables the production of lower size droplets and narrow size distributions. A higher emulsification control can be achieved using the NETmix, enabling tailor-made dispersions according to target droplet sizes. Moreover, in terms of production (space-time yield value), NETmix has a productivity of two orders of magnitude higher than the batch mode. These results, where the feasibility of a continuous mode was demonstrated, can be a driving force for the industrial implementation of Pickering emulsion production.

Although Pickering particles show many advantages in emulsion stabilisation, some challenges need to be overcome before the fully application of these products in foods. Food products have a complex structure and different characteristics, becoming challenging to predict how Pickering emulsions would behave under food processing conditions, even they are stable under storage. In this context, **Chapter 5** reports the behaviour of the n-HAp stabilised Pickering emulsions, produced in batch (~18 μm sample) and continuous modes (~7, 11 and 18 μm samples), at different temperatures (5, 22, 30, 50, 70 and 90 °C), pH (2, 4, 6, 8 and 10) and ionic strength (0, 100, 200, 300, 400 and 500 mM). Droplet size, morphology, and zeta-potential were analysed after 1 and 7 days under the storage. Regarding the tested environmental stresses, the n-HAp Pickering emulsions were stable within the full range of ionic strength, between 5 and 70 °C, and within the pH interval of 6-10. No significant differences were detected in the droplet size. The more severe destabilisation was registered at very low pH (2), where complete phase separation occurred, fact associated to the complete disruption of the n-HAp particles. At $4 \leq pH \leq 6$, aggregation and coalescence phenomena were observed, mainly attributed to the surface charge of the n-HAp particles caused by the partial ion dissolution related to the low pH. Additionally, the Pickering emulsions with larger droplet sizes were more unstable at high temperatures (70-90 °C), particularly after 7-days. In this case, droplet's aggregations and coalescence were observed. Overall, pH produces immediate instabilities, temperature produces immediate instabilities, also evolving during storage, and the electrolyte concentrations do not affect the Pickering emulsions stability. Following a previous work, where NETmix was firstly introduced for

continuous production of n-HAp Pickering emulsions (**Chapter 4**), **Chapter 5** reinforced the suitability and interest of using this technique. Pickering emulsions with similar stability to the batch process have been produced (rotor-stator device, **Chapter 3**), corroborating that the scale-up for NETmix from a batch laboratory scale is feasible. Summarising, the results of **Chapter 5** can contribute to expand the applicability of n-HAp Pickering emulsions. A systematic study analysing the influence of environmental stresses on emulsion stability was conducted for the first time. The results are useful considering production, storage, and final application scenarios of these products, namely to predict the emulsion behaviour after ingestion.

Although emulsion stability is an essential parameter, the evaluation of its behaviour under digestion conditions allows obtaining important information to produce more efficient food solutions. Namely, to address questions of bioaccessibility and stability of encapsulated active compounds. Recently, literature started to report works dealing with the digestion of Pickering emulsions. These studies pointed out the need to invest further in systematic studies, namely the ones including the analysis of Pickering emulsions added foods. In this context, in **Chapter 6**, n-HAp Pickering emulsions loaded with vitamin E were obtained and used to produce two fortified foods (milk and gelatine). The main objective was to evaluate vitamin E bioaccessibility in Pickering emulsions and respective fortified foods, contributing to functional foods development. Vitamin E-loaded Pickering emulsions with tailored droplet size (~7, 11 and 18 μm) were produced using NETmix. These Pickering emulsions were subjected to a simulated gastrointestinal digestion comprising the following stages: mouth, stomach, and small intestine. Pickering emulsions presented similar behaviour in each stage, with complete disruption of the n-HAp particles under gastric conditions (such as predicted by pH environmental stress studies in **Chapter 5**) and formation of aggregates under intestinal conditions. Moreover, the work pointed out that the formation of aggregates can be ascribed to the Ca^{2+} ions (derived from n-HAp disruption in the gastric stage) complexation with PO_4^{2}, bile salts or free fatty acids. As a consequence, a reduced number of micelles is formed, together with lipid phase and/micelles entrapment. This fact leads to low vitamin E bioaccessibility such as verified for n-HAp vitamin-loaded Pickering emulsions. Nevertheless, the emulsion with higher average diameter showed a slightly higher bioaccessibility value

(~3%). Moreover, the incorporation of this emulsion into food matrices resulted in an increased vitamin E bioaccessibility, namely ~11% for fortified gelatine and ~18% for fortified milk. This result can be justified by the lower amount of n-HAp particles present in the formulation and to the macronutrients naturally presented in food, contributing to avoid and/or delay the formation of aggregates in the intestinal stage. Summarising, this study is a step forward to understand the n-HAp Pickering emulsions behaviour along the different digestion stages, providing also new insights on the development of fortified foods with desired functionality. It can also supports and consolidates the possibility of using n-HAp Pickering emulsions as carriers of bioactive principles, more specifically the lipophilic ones.

Finally, in Chapter 7 an approach dealing with nanohydroxyapatite (n-HAp) particle's surface modification is proposed aiming at develop suitable n-HAp based stabilisers for W/O Pickering emulsions. The limited number of available solutions for W/O Pickering emulsion stabilisers is a constraint and a need for several industrial fields. In this context, in Chapter 7 the controlled modification of the pristine n-HAp particles according to the number of SO layers (0.5, 1 and 2), is presented as a systematic approach to carry out the surface modification. The obtained results indicate that wettability depends on the number of applied SO layers, which switched from hydrophilic (0.5 coatings) to hydrophobic (1 layer) and again to hydrophilic (2 layers). Additionally, the particle size decreased with the number of SO layers, compatible with an electrostatic repulsion increase, preventing aggregates formation. The produced W/O Pickering emulsions based on the SO modified n-HAp holding one SO layer (contact angle close to 90°, hydrophobic character) revealed good stability with n-HAp particles adsorbed at the water-oil interface. Slight signs of instability started to be observed only after 30 days under storage. Summarising, with this strategy, n-HAp particles with suitable wettability were obtained and used in W/O emulsions, opening new avenues for developing new functional products, e.g., based on hydrophilic active principles.

To conclude, this thesis combines five interlinked research articles. First, the use of n-HAp solid particles as a Pickering stabiliser was demonstrated and emulsion stability predicted based on a developed model. Second, in perspective of a large-scale production, the developed n-HAp Pickering emulsions in batch mode were transposed to NETmix, a continuous process. Third, stability studies against different environmental stresses

(temperature, pH and ionic strength) were performed to know their behaviour under conditions important to processing, and final applications development. Fourth, vitamin E loaded Pickering emulsions were produced by NETmix and incorporated in food matrices to develop fortified foods, which revealed to ensure better vitamin E bioaccessibility, comparatively with the former emulsion. Lastly, n-HAp surface modification with sodium oleate was performed to extend the use of n-HAp based stabilisers to W/O emulsions, whose success was proved by producing stable W/O Pickering emulsions.

8.2 FUTURE WORK

Following this work, some suggestions for future activities are presented in this chapter:

1. Model: A model to predict the stability of Pickering emulsions was developed. However, it takes into account only the specifications of n-HAp particles. It would be interesting to extend this model to the other solid particles in order to become more general.
2. Cytotoxicity studies: Although the environmental stability testes and the digestion study indicates a rapid disruption of the n-HAp solid particles at acid pH, it is recommended to conduct cytotoxicity studies of the produced n-HAp Pickering emulsions.
3. Oxidative studies: The O/W interface can be a critical local for lipid oxidation. In this way, it would be interesting to perform oxidative analyses to perceive if oxidation impacts n-HAp stabilised Pickering emulsions.
4. Antioxidant properties: After incorporating the vitamin E-loaded Pickering emulsions into the food matrices (fortified foods), it would be interesting to evaluate vitamin E role as an antioxidant by *in vitro* studies.
5. n-HAp modifications: n-HAp particles have a natural tendency to stabilise O/W emulsions. Surface modifications are needed to obtain stable W/O Pickering emulsions. Thus, it would be interesting to carry out studies utilising natural-based modifiers (e.g., polyphenols or sterols). It would be an asset to increase the potential and functionality of the emulsions. Additionally, the development of W/O Pickering emulsions in the NETmix is also an area of interest to be investigated.

Appendix A Ternary diagram of Pickering emulsions with additional experimental points

This appendix shows additional experimental points that were performed to confirms the developed model showed in **Chapter 3**.

Table A.1 shows, in bold type, the tested additional points. The Pickering emulsions were produced with 2, 3 and 15 wt% of n-HAp concentration and 10/90 or 40/60 O/W ratios. In Figure A.1 are shown the additional experimental points in ternary diagram. The orange, dark green and dark blue colours symbolise the unstable, stable and *gel-like* emulsions, respectively.

Table A.1: Pickering emulsions: n-HAp concentrations and oil-water ratio (additional points shown in bold type).

TYPE OF HAP PASTE	OIL-WATER RATIO *(v/v)*	n-HAP CONCENTRATION (wt%)
*nano*XIM-CarePaste	10/90	1.5, **2.0, 3.0**, 5.0, 10, **15**
	20/80	0.5, 1.5, 2.0, 2.5, 3.0, 5.0, 7.0, 10, 15
	40/60	1.5, **2.0, 3.0**, 5.0, 10, **15**
	60/40	1.5, 5.0, 10*

* Not used due to preparation difficulties.

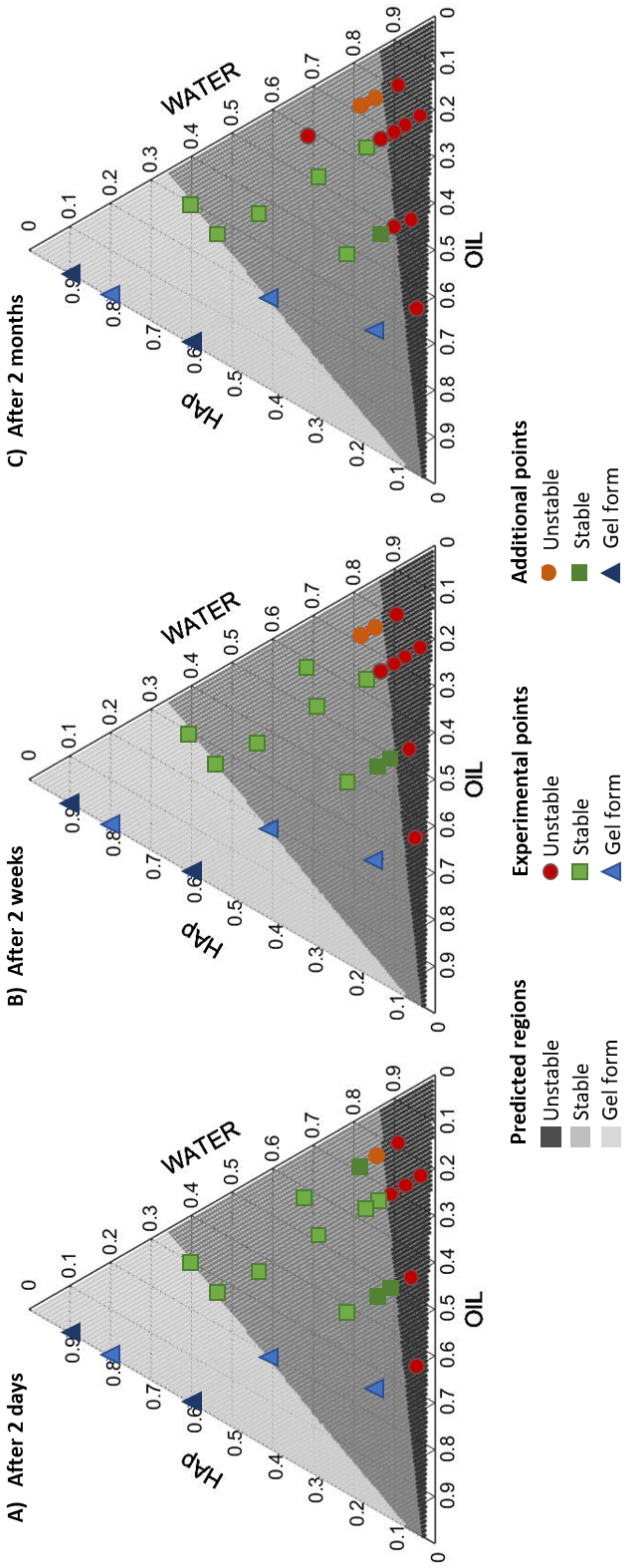

Figure A.1: History of experimental point (2 days - 2 moths). Ternary diagram of O/W Pickering emulsions with n-HAp and experimental points. Assumption: $l_s^w/d_s > 1.7$ – unstable, $l_s^w/d_s < 1$ – gel formation and $1 \leq l_s^w/d_s \leq 1.7$ – stable.

Appendix B Environmental stresses – Fluorescence microscopy images after one day

This appendix shows the results of the fluorescence microscopy images of the Pickering emulsions subjected at different environmental stresses (temperature, pH and ionic strength) for 1 day of storage, and supports results presented in Chapter 5.

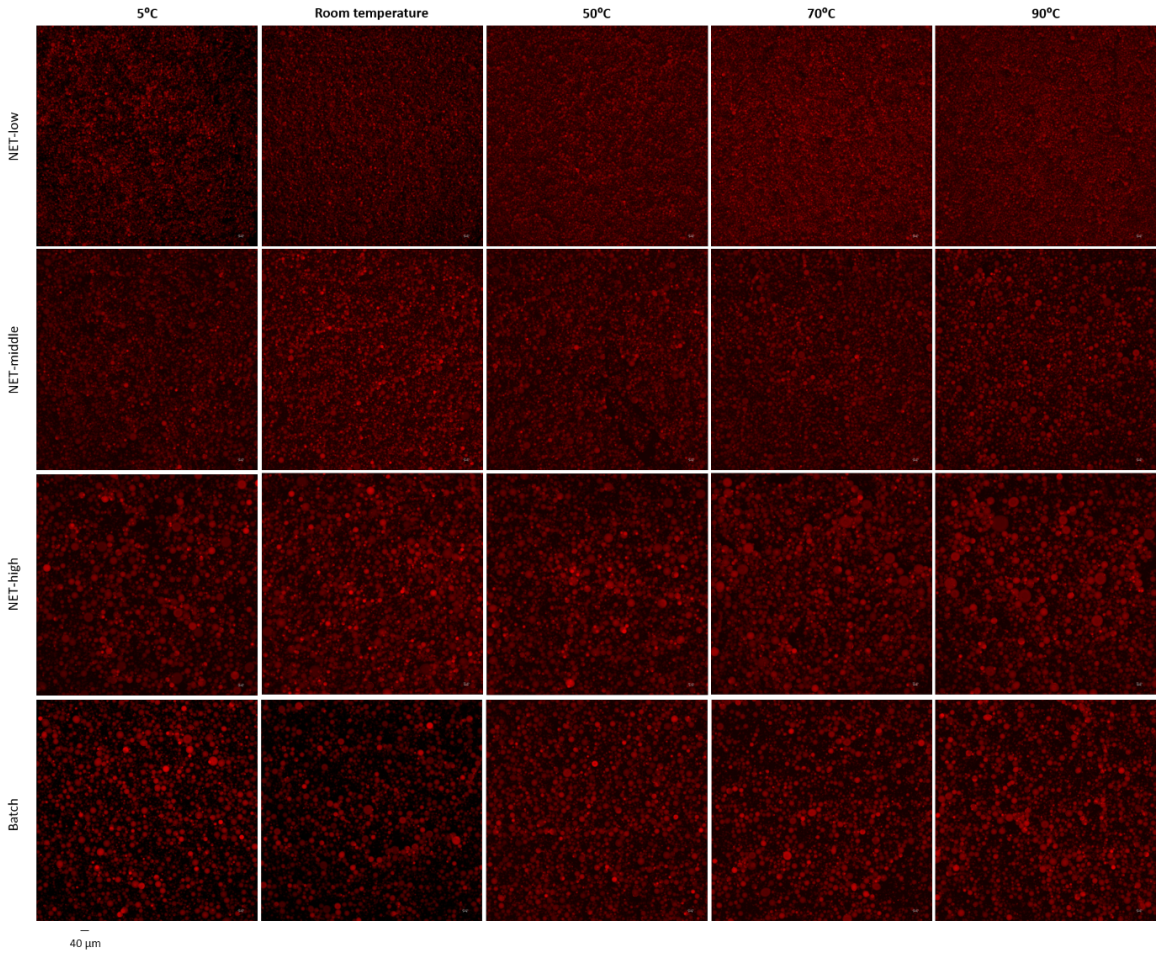

Figure B.1: Fluorescence microscopy images of the Pickering emulsions with different average size diameters (NET-low - 7 µm, NET-middle - 11 µm, NET-high - 18 µm, and batch - 18 µm) at different temperatures after 1 day of storage. The oil phase was stained with Nile red. Magnification: 10x.

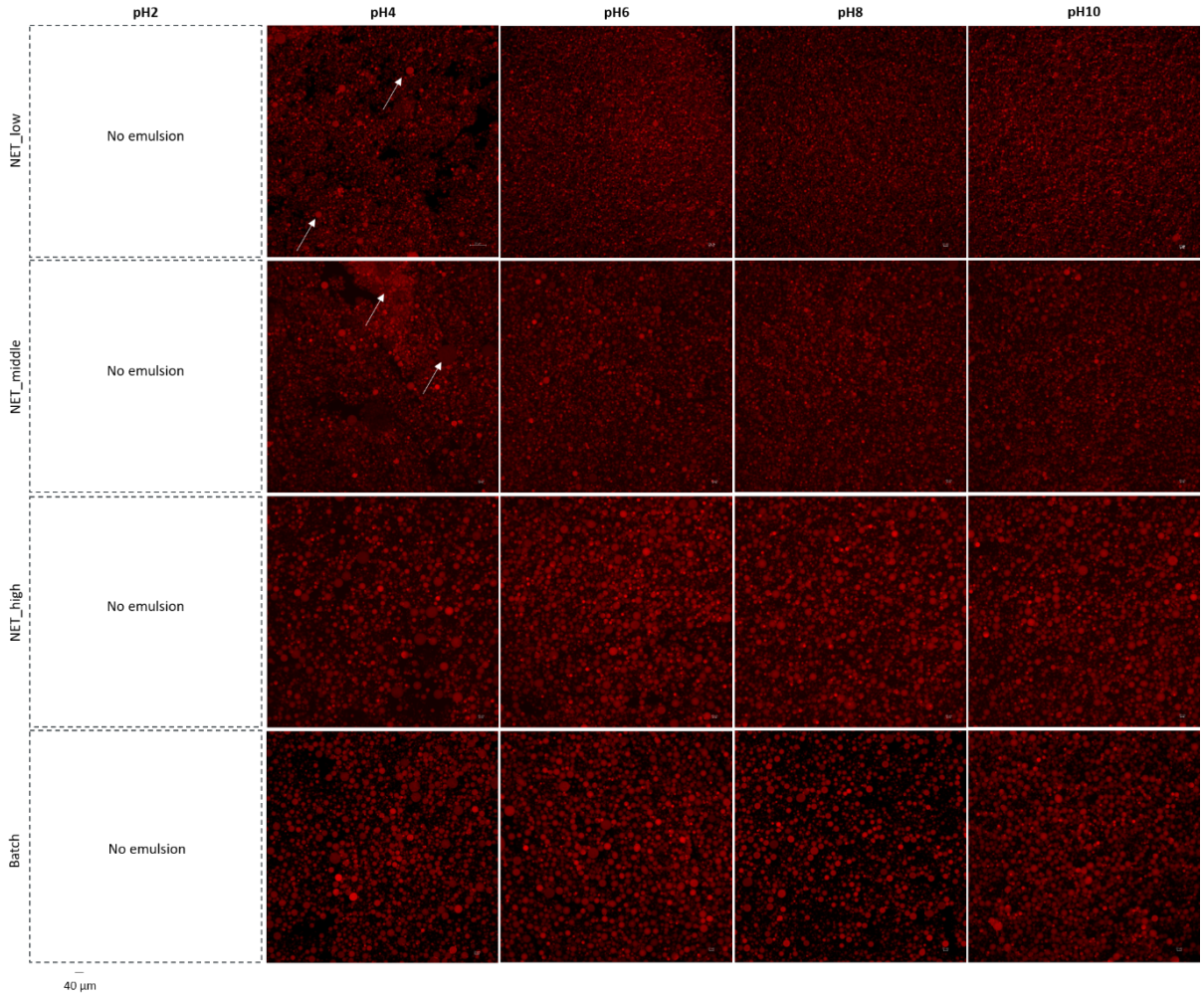

Figure B.2: Fluorescence microscopy images of the Pickering emulsions with different average size diameters (NET-low - 7 µm, NET-middle - 11 µm, NET-high - 18 µm, and batch - 18 µm) at different pH after 1 day of storage. The oil phase was stained with Nile red. Magnification: 10x.

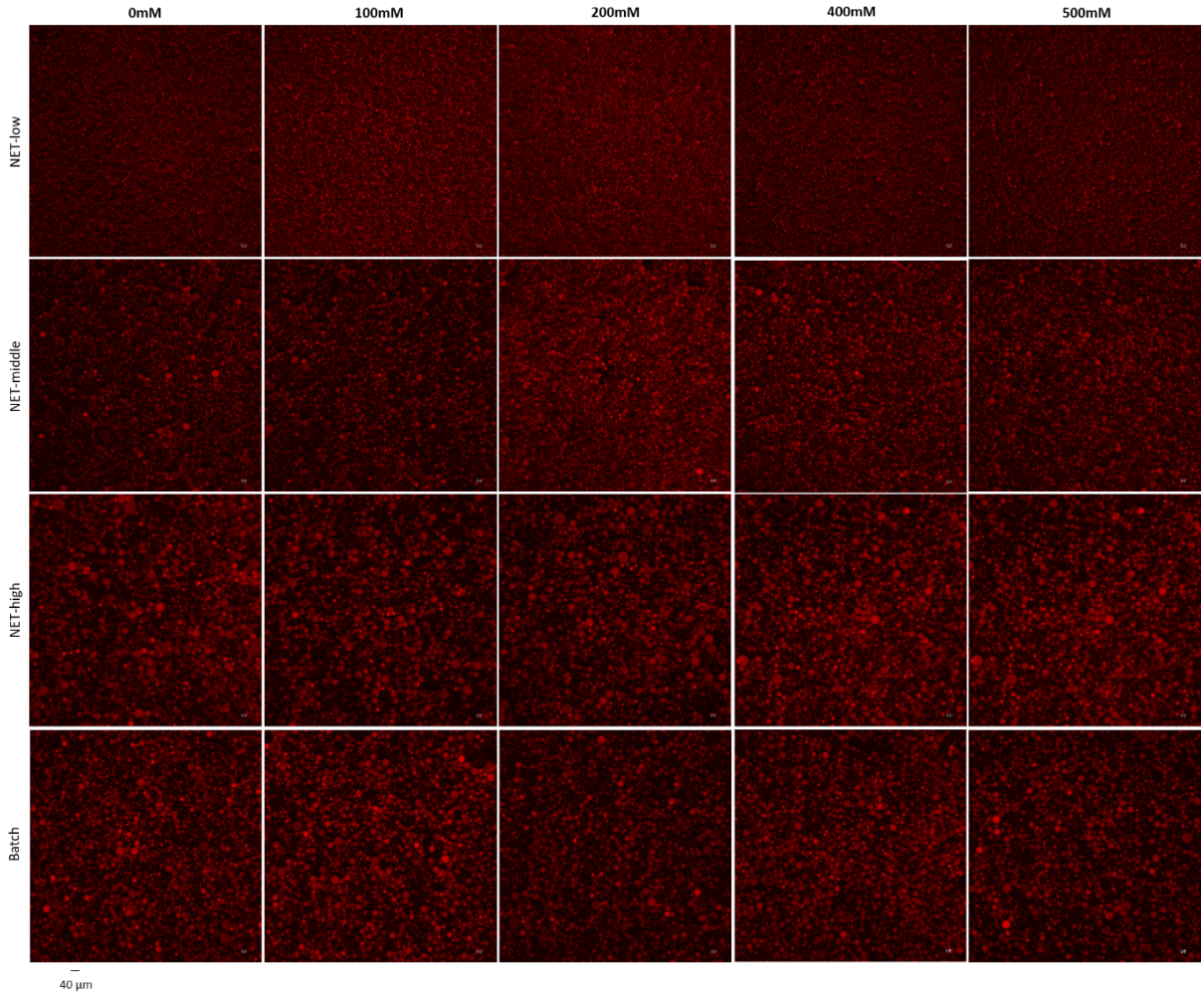

Figure B.3: Fluorescence microscopy images of the Pickering emulsions with different average size diameters (NET-low - 7 µm, NET-middle - 11 µm, NET-high - 18 µm, and batch - 18 µm) at different ionic strength after 1 day of storage. The oil phase was stained with Nile red. Magnification: 10x.

Appendix C Estimation of the SO number layers and n-HAp modified particles

This appendix shows supplementary material that supports the Chapter 7. Here, the calculations performed to estimate the sodium oleate (SO) number layers to modify n-HAp particles and SO modified n-HAp particles to cover droplets emulsion are presented.

C.1 Estimation of the SO number layers

The number of SO molecules covering one n-HAp particle ($N_{SO/HAp}$) is given by:

$$N_{SO/HAp} = \frac{A_{HAp}}{TPSA_{SO}} \tag{C.1}$$

where A_{HAp} is the surface area of a n-HAp particle and $TPSA_{SO}$ the topological polar surface area (TPSA) of a SO molecule, 0.401 nm^2 (PubChem, 2020).

The n-HAp particles have a rode-like shape, approximate to a cylinder with a 5:1 (length:width) ratio. Assuming that all n-HAp particles are individualised and have similar size, the surface area, A_{HAp}, is given by:

$$A_{HAp} = 2\pi rh + 2\pi r^2 \tag{C.2}$$

where r ($r = l_{HAp}/10$) and h ($h = l_{HAp}$)are the width and length of the n-HAp, respectively.

Knowing that l_{HAp} corresponds to 50 nm, equation 2 can be re-written as:

$$A_{HAp} = \frac{\pi}{5} l_{HAp}^2 + \frac{\pi}{50} l_{HAp}^2 \tag{C.3}$$

Additionally, the number of SO molecules needed to cover all n-HAp particles (N_{SO}) can be estimated as:

$$N_{SO} = \alpha N_{HAp} N_{SO/HAp} \quad \text{(C.4)}$$

where α represents the number of desired SO layers and N_{HAp} the number of n-HAp particles present in solution, which can be estimated as:

$$N_{HAp} = \frac{m_{HAp}}{m_{HAp}^1} \quad \text{(C.5)}$$

where m_{HAp} is the total mass of n-Hap particles present in solution and m_{HAp}^1 is the mass of one n-HAp particle.

The total mass of n-HAp can be obtained by:

$$m_{HAp} = C_{HAp} V_{sol} \quad \text{(C.6)}$$

where C_{HAp} is the concentration of n-HAp and V_{sol} the total volume.

The mass of one n-HAp particle can be obtained by:

$$m_{HAp}^1 = \rho_{HAp} V_{HAp}^1 \quad \text{(C.7)}$$

where ρ_{HAp} represents the density of n-HAp particle and V_{HAp}^1 the volume of one particle.

Finally, using the Avogadro constant ($N_A,\ 6.022 * 10^{23}\ mol^{-1}$) and the molecular weight of the sodium oleate ($M_{SO},\ 304.4\ g/mol$), the mass of SO (m_{SO}) to be added can be calculated as:

$$m_{SO} = \frac{N_{SO}}{N_A} M_{SO} \quad \text{(C.8)}$$

This approach allows the estimation of the SO mass needed to form the desired number of layers around the n-HAp particles. Namely, to achieve 0.5, 1 and 2 layers, and taking as reference 1 g of n-HAp, the following amounts of SO were used: 0.09, 0.18 and 0.35 g, respectively.

C.2 Estimation of SO modified n-HAp content to cover emulsion droplet's surface

To estimate the amount of the SO modified n-HAp particles to stabilise a W/O emulsions the following assumptions were considered:

- the size distribution obtained for the O/W emulsion can be used to estimate the one of the corresponding W/O emulsion;
- the SO molecules remain at the n-HAp surface (Figure C.1 A);
- the mean size of the SO modified n-HAp particles, measured by DLS, was assumed to represent the size of all particles in suspension ($d_{SO.HAp}$~0.5 μm);
- the n-HAp particles with contact angle close to 90° are irreversibly adsorbed at droplet interface (Figure C.1 B);
- all water droplets, assumed to have spherical shape, are covered by just one layer of the SO modified n-HAp particles ($\alpha = 1$).

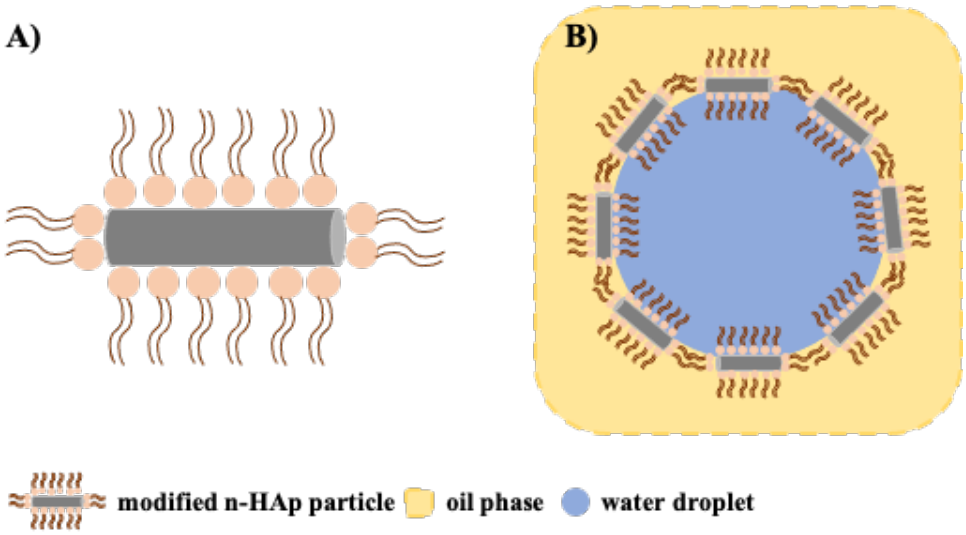

Figure C.1: Representative image of n-HAp particle modified with one layer of sodium oleate (A) and water droplet covered with modified n-HAp (B).

The number of droplets in emulsion (N_o) can be given by the ratio between the total volume of the dispersed phase, V_o, and the volume of the droplet with diameter d_o :

$$N_o = \frac{6V_o}{\pi d_o^3} K \tag{C.9}$$

where K represents the percentage of the droplets within a diameter range.

To know the number of droplets for each droplet's diameter range it is necessary to consider the number of solid particles ($N_{SO.HAp}$) needed to cover the droplets that is given by:

$$N_{SO.HAp} = \alpha N_o n_{SO.HAp} K \tag{C.10}$$

where α represents the number of layers covering the droplet and $n_{SO.HAp}$ the number of solid particles needed to form a single layer that is given by:

$$n_{SO.HAp} = \frac{a_o}{a_{SO.HAp}} \quad \text{(C.11)}$$

where $a_o = \pi d_o^2$ is the surface area of an oil droplet with diameter d_o and $a_{SO.HAp} = d_{SO.HAp}^2$ is the area covered by one particle, which can assume a random orientation at the water surface, and thus may be estimated as a projected area equivalent to a square with a side equal to $d_{SO.HAp}$.

Considering all parameters, the mass of SO modified n-HAp particles ($m_{SO.HAp}$) can be estimated by:

$$m_{SO.HAp} = \sum_{d_o=0}^{max} \rho_{HAp} v_{SO.HAp} N_{SO.HAp} \quad \text{(C.12)}$$

where ρ_{HAp} represents the HAp density (3160 kg/m^3), $v_{SO.HAp}$ is the volume of modified HAp particle assuming a cylindrical shape with a 5:1 shape factor ($v_{SO.HAp} = \frac{\pi}{4}\left(\frac{d_{SO.HAp}}{5}\right)^2 d_{SO.HAp} = \frac{\pi}{100} d_{SO.HAp}^3$).

This approach allows the estimation of the SO modified n-HAp particles needed to stabilise the emulsions as 1.6%wt, and 2.7%wt, respectively for the emulsions using a W/O ratio of 20/80, and 30/70.