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Continuous production of hydroxyapatite Pickering emulsions using a mesostructured reactor

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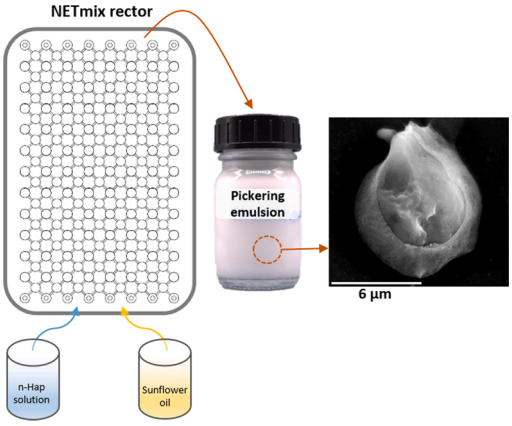
HIGHLIGHTS

- Continuous production of stable Pickering emulsions using nano-hydroxyapatite.
- Tailor-made and small size droplet emulsions obtained.
- High-volume scale production using a mesostructured reactor (NETmix).

GRAPHICAL ABSTRACT



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ABSTRACT

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 nano-hydroxyapatite shell morphology.

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1. Introduction

The concept of Pickering emulsions was introduced by Ramsden and Pickering at the beginning of the 20th century [1,2]. They are characterized by using solid particles that once positioned at the oil-water interface form a thick barrier able to act as a stabilizer [3–6]. 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 [7].

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 [8,9], 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 [7,10,11]. Particularly, in the food area, Pickering emulsions can help to reach the "clean label" trend by ensuring environmentally friend solutions [7,12].

Pickering emulsions can be prepared by the typical methodologies for conventional emulsions, namely rotor-stator homogenization and sonication, which are the most commonly used batch mode processes [13–15]. These technologies enable droplet break-up due to the applied high-shear rate (rotor-stator homogenizer), or mechanical vibrations (ultrasonic devices). In rotor-stator homogenization, 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 [11]. 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 [11]. 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 [11].

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 [16] and microfluidizers [17] 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) [18], 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 (Fig. 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) [19].

The chambers operate as mixing zones and the channels as plug flow zones [19]. 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 [20] and with immiscible fluids [21,22].

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

$$Re = \frac{\rho\, v\, d_h}{\mu} \tag{1}$$

where ρ is the mixture density (kg/m^3), v the average velocity in the channels (m/s), 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 [19,20]. 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 [23].

NETmix technology has been applied for different products. Examples include the production of nanocrystalline hydroxyapatite [23], this process already at industrial level [24], metal-organic frameworks [25], magnetic iron oxide nanoparticles [26], and calcium carbonate nanoparticles [27]. Furthermore, this technology has also been employed for the continuous production of nano-hydroxyapatite (n-HAp)/chitosan aqueous dispersions [28] and microencapsulated systems [29].

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 stabilized 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.

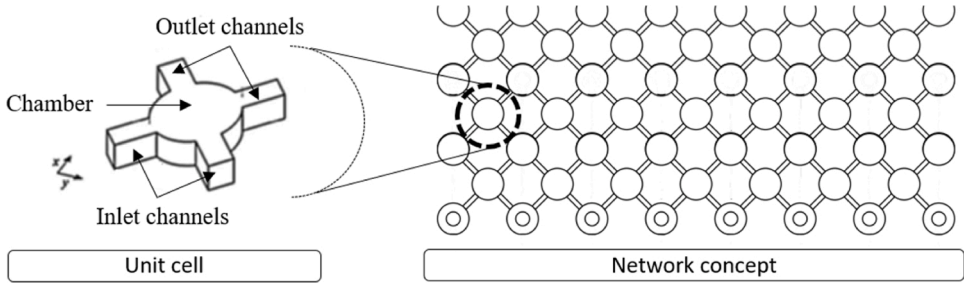

Fig. 1. Example of one-unit cell with one chamber and channels, and schematization network concept of NETmix.

2. Experimental methods

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.

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, Manrique, Ferreira, Barreiro, Lopes and Dias [30], 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 characterized by individualized 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 (Fig. 2A). 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 (Fig. 2B). 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. 2

$$Cycle\ time\ (s) = \frac{Emulsion\ volume}{Flow\ rate} \quad (2)$$

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

2.3. Characterization of the Pickering emulsions

The obtained Pickering emulsions were characterized 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.

2.3.1. Optical Microscopy analysis (OM)

OM was used to monitor the morphology of the Pickering emulsions

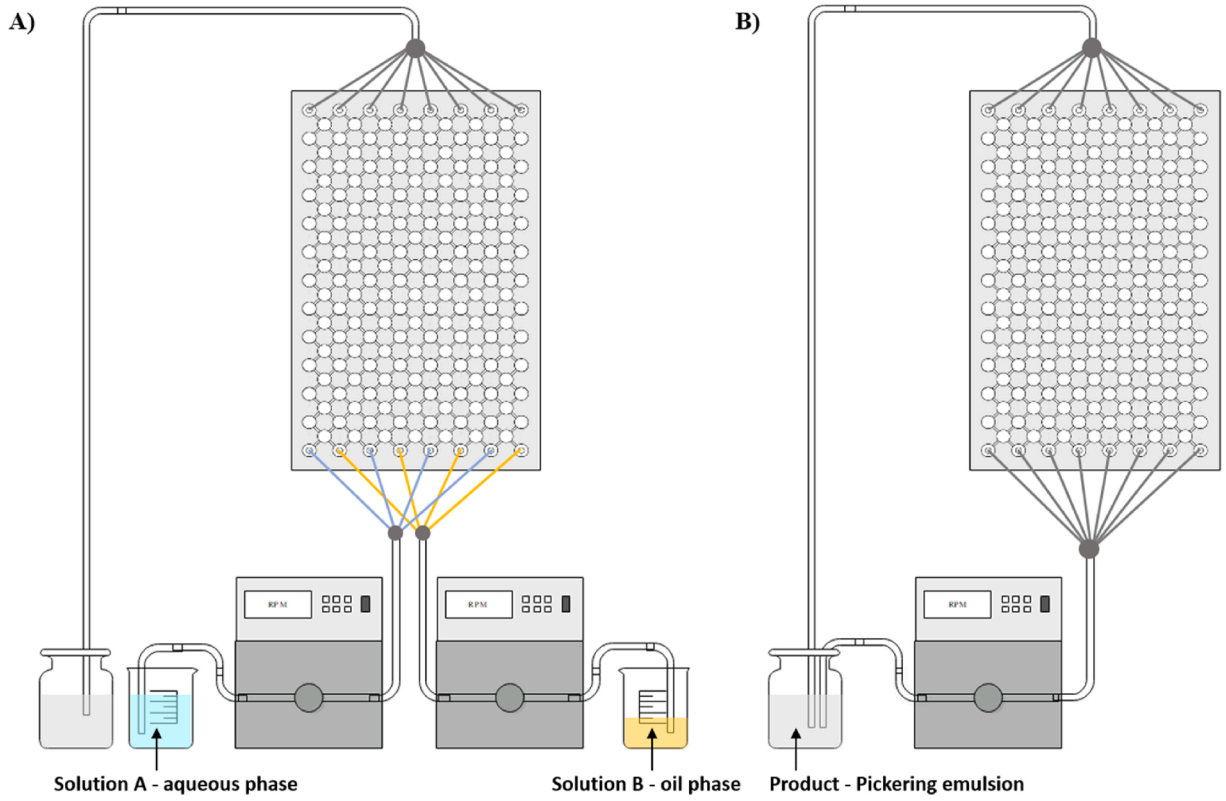

Fig. 2. Experimental set-up used to produce the Pickering emulsions in continuous mode. A) configuration for the first pass i.e., pre-mixed mode injection scheme (blue – solution A – aqueous phase; yellow – solution B – oil phase), B) configuration for the recirculation mode.

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 color) was used, and the acquisition and image processing was done using Zen (2.3 blue edition) software.

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 stabilizers. 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.

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 6301 F/ 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 s at −90 °C), and coated with Au/Pd (45 s and with a 12 mA current). Finally, the sample was transferred into the SEM chamber and observed at −150 °C.

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 ± SD.

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.

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.

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 fix 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.

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 [19,31]. Namely, in the study of Gomes [31], which performed tracer studies on NETmix for $50 \geq Re \leq 400$, 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 Fig. 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. 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 Fig. 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 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 (instable system), the other emulsions maintain their droplet size over the analysed 60 days period (stable systems).

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 [32]. Other authors also reported that the emulsion stability depends on the droplet size. Namely, Saari, Wahlgren, Rayner, Sjöö and Matos [33] showed that an emulsion stabilized with modified starch, which presented a droplet size of approximately 17 μm, evolves to 85 μm after a 30-days period under storage. Zanatta, Rezzadori, Penha, Zin, Lemos-Senna, Petrus and Di Luccio [32] showed that an emulsion stabilized 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.

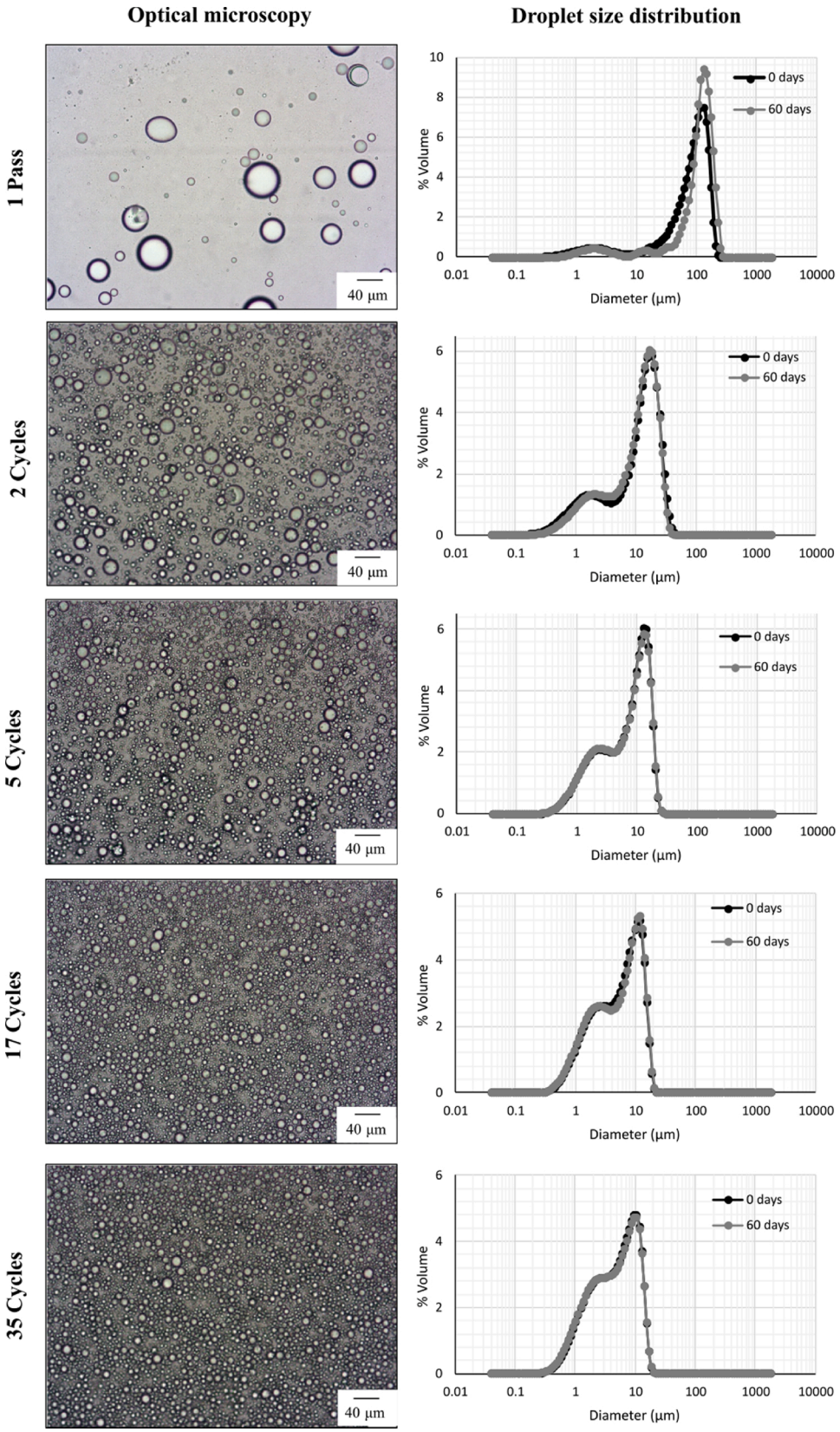

Fig. 3. Optical microscopy images and size distributions for 0 and 60 days of the produced Pickering emulsions (Re = 400) as a function of the applied number of cycles. All images were taken with magnification of 20 × .

Table 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

Fig. 4 shows the digital photographs of the Pickering emulsion produced with Re = 400 and 17 cycles. In Fig. 4A the freshly produced emulsion is shown, while in Fig. 4B 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.

3.2. Effect of Re number on emulsions 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 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.

Fig. 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 [32].

The Pickering emulsions produced with higher Re values (Re > 200), characterized by a uniform spherical shape and small size, have maintained the stability over the tested storage time. In Fig. 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

Table 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

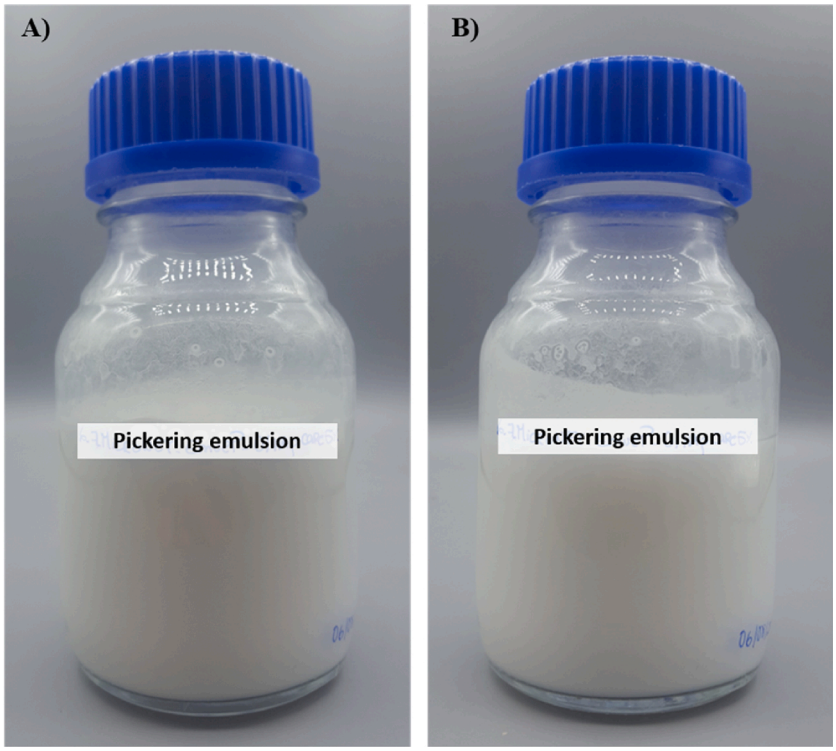

Fig. 4. Images of the Pickering emulsion produced with Re = 400 and subjected to 17 cycles. A) after production (0days) and B) after 60-days of storage.

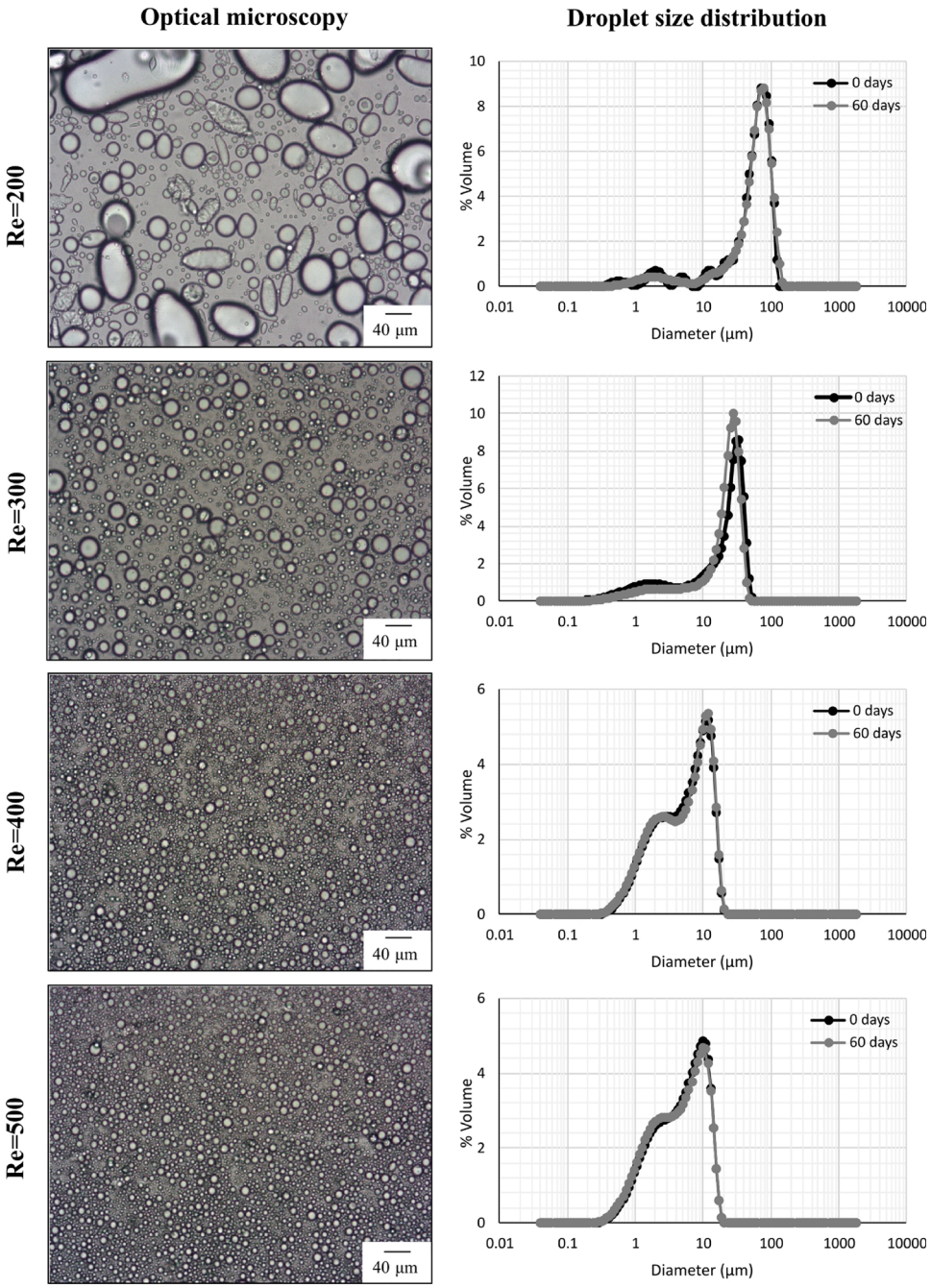

Fig. 5. Optical microscopy images and size distributions for 0 and 60 days of the produced Pickering emulsions (17 cycles) as a function of Re. All images were taken with magnification of 20 × .

the volume associated with smaller sizes as the Re increases. In Table 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.

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, Kunal and Melwyn [34] 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 [34].

Table 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).

Re	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

In previous works, various models have been proposed for the prediction of the droplet size. For example, Boxall, Koh, Sloan, Sum and Wu [35] estimate the emulsion droplet size according to Weber and

Reynolds numbers. In this respect, Muruganandam, Kunal and Melwyn [34] showed that when a O/W emulsion is prepared in an 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 [29]. 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.

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 stabilizers.

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. Fig. 6 show CLSM images of the Pickering emulsion obtained for Re = 400 and 17 cycles. Fig. 6A shows the Pickering emulsions after production (0 days) and Fig. 6B 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 stabilization 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 [36–38]. 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.

Fig. 7 shows cryo-SEM images where it is possible to observe individualized 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

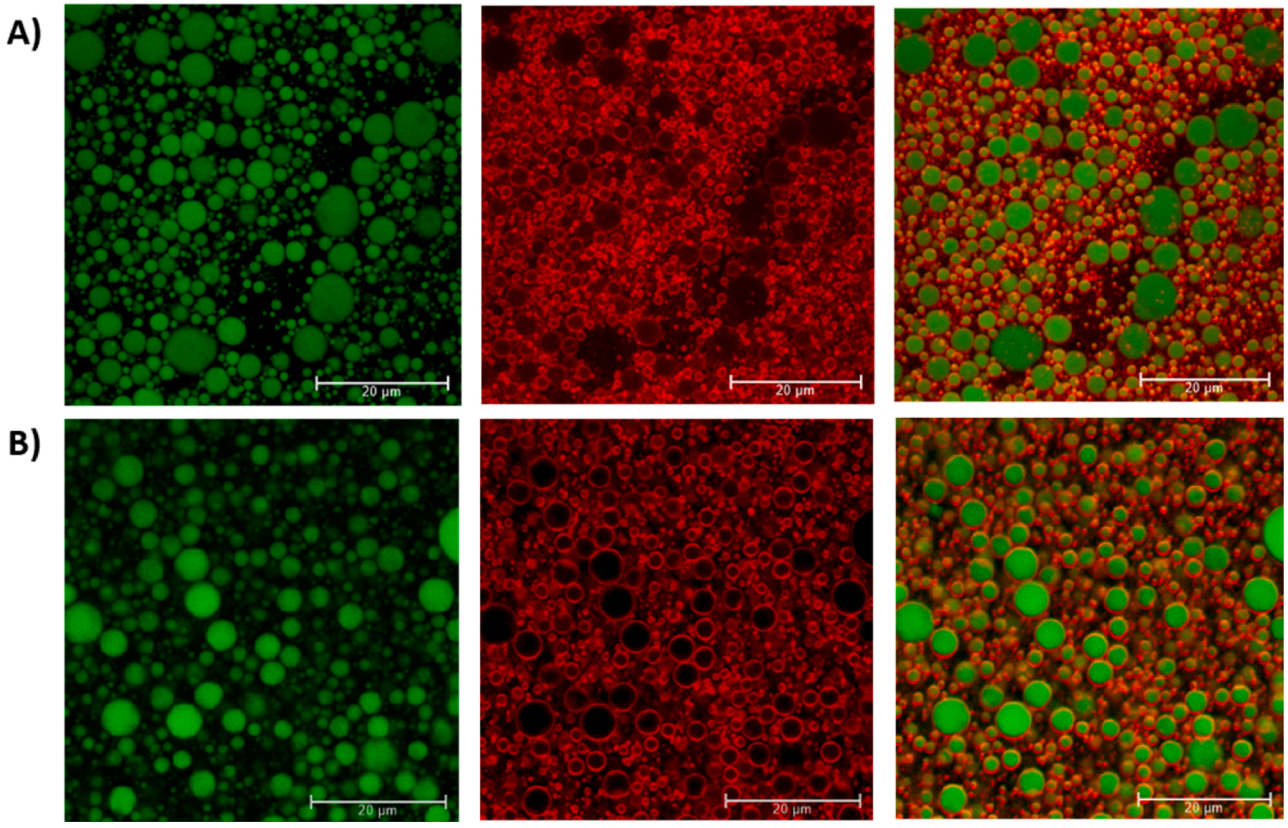

Fig. 6. Confocal laser scanning microscopy (CLSM) images of the Pickering emulsion produced at Re = 400 and 17 cycles and stabilized with n-HAp particles: A) after production and B) after 60 days of storage. Oil core was stained with Nile red and n-HAp particles stained by Nile blue A. The fluorescent 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 color 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.

texture relatively smoother than the n-HAp particles, which are whiter than the oil and water phases. Furthermore, from Fig. 7A it is also clear the hydroxyapatite layer around the oil core, and in the amplified image it is possible to depict the individualized n-HAp particles, which indicates the clear ability of the n-HAp particles to act as Pickering stabilizers. The EDS analysis (Fig. 7B) 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 analyzed Pickering emulsions through cryo-SEM. Griffith and Daigle [39] have confirmed through this technique that silica nanoparticles modified with hydrophilic silane were adsorbed at the oil droplets surface, and Binks and Tyowua [40] 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.

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 [30]. Thus, the base batch formulation (oil/water ratio of 20:80 (*v/v*), n-HAp content of 5 wt%), produced using a rotor-stator homogenizer at 11,000 rpm and 6 min, 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.

Fig. 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

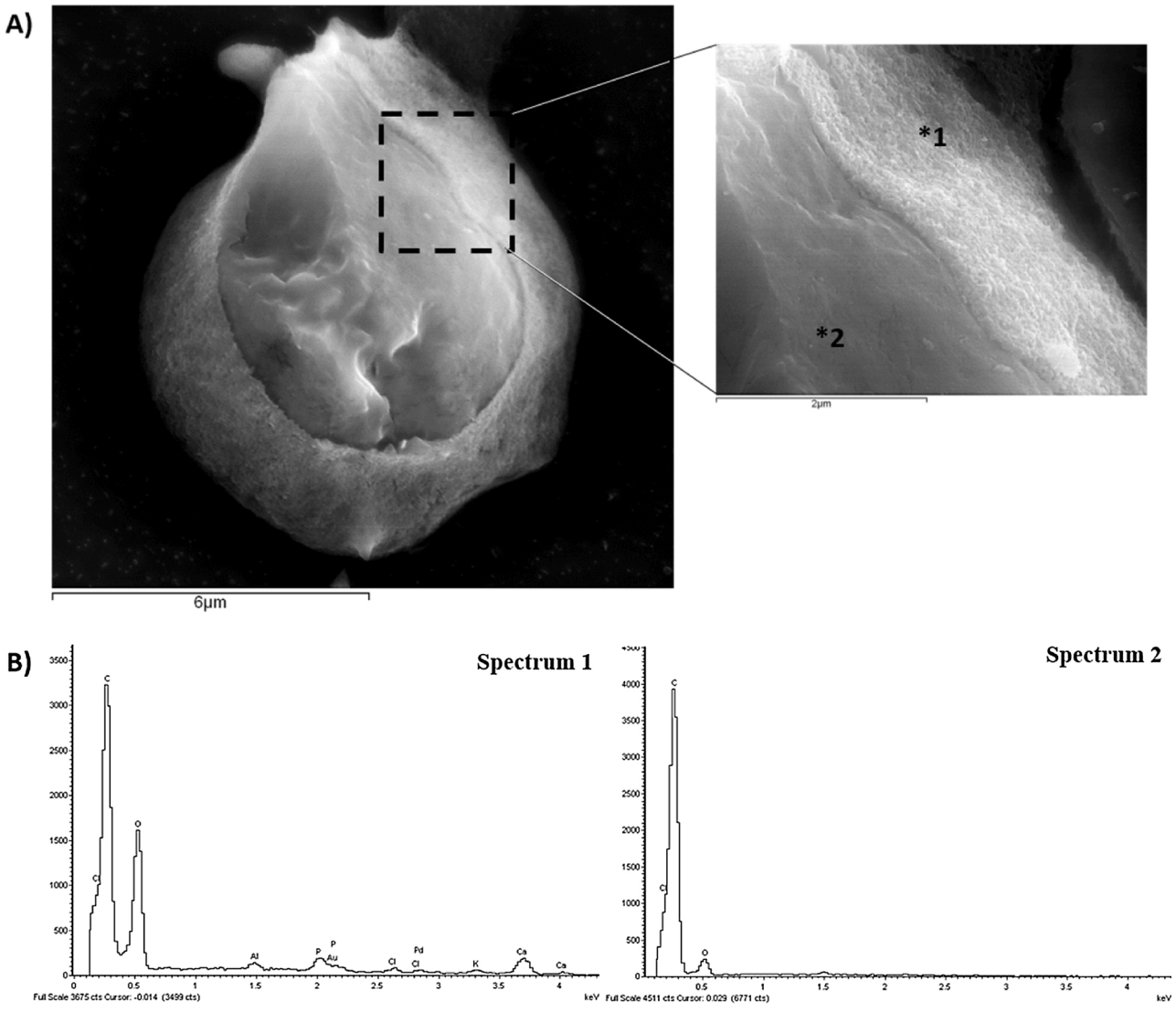

Fig. 7. Cryo-scanning electron microscopy image (cryo-SEM) of the Pickering emulsion stabilized with n-HAp particles. A) micrograph and B) EDS analysis.

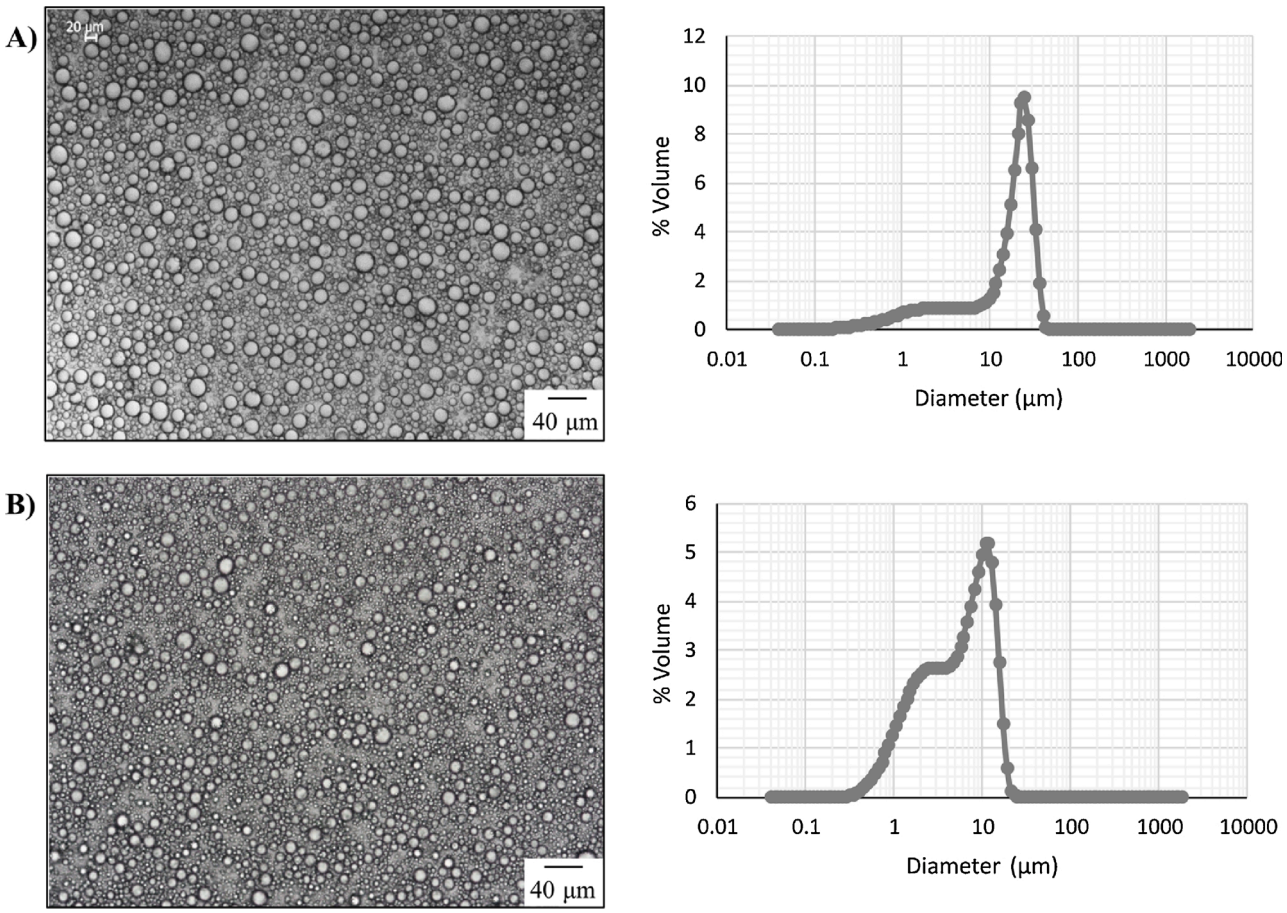

Fig. 8. Optical images and size distributions comparison obtained from batch using rotor stator homogenizer (A) and continuous using NETmix (B). The Pickering emulsions were obtained with 20:80 (*v/v*) as oil-water ratio and with 5 wt% of n-HAp particles.

several applications where particle size is an important parameter.

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 homogenizer 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 summarizes the main advantages using NETmix over rotor stator homogenizers in Pickering emulsion production. These analyses were produced according to the tested setups, and the findings in our research team.

Table 4
Advantages comparison of NETmix over to rotor-stator in Pickering emulsion production.

	Continuous NETmix	Batch rotor-stator homogenizer
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. Conclusions

In this work, Pickering emulsions stabilized 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 homogenizers. 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.

CRediT authorship contribution statement

Andreia Ribeiro: Methodology, Investigation, Validation, Writing - original draft. **Yaidelin A. Manrique:** Methodology, Writing - review & editing. **Filomena Barreiro:** Conceptualization, Supervision, Writing - review & editing. **José Carlos B. Lopes:** Conceptualization, Methodology. **Madalena M. Dias:** Conceptualization, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors report no declarations of interest.

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