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ABSTRACT

This communication presents a simple yet straightforward method for preparing water-in-water-in-water particle-stabilized double emulsions, also known as Pickering double emulsions. The approach involves using oppositely charged nanoparticles (OCNPs) in two distinct fluid phases, promoting self-assembly and the formation of aggregates with varying sizes and compositions. By enhancing the interfacial area through the adsorption of aggregates at the interface, this method increases the Gibbs detachment energy of particles between the two aqueous phases, forming stable double emulsions. Furthermore, we investigated the impact of the molecular weight of polyethylene oxide and dextran in the respective fluid phases and the mass ratio (M) of the OCNPs on double emulsion formation. The results demonstrate that the molecular weight of the polymers used in the aqueous phase is a critical parameter influencing the structural formation of the emulsion and the generation of double emulsions. Consequently, double emulsions are formed when equal molecular weight polymer mixtures are employed at an appropriate M , with the dispersed phase placed in the highly viscous continuous phase. The proposed method offers a one-step synthesis process, enabling easy preparation, and exhibits excellent stability for at least 30 days. This study represents the first reported approach for the one-step synthesis of multiple emulsions in an aqueous two-phase system utilizing a Pickering emulsion template.

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An emulsion is a heterogeneous mixture of two immiscible liquids, where one liquid is dispersed throughout the other in the form of droplets. The recent advances in emulsion droplet generation have led to the development of multiple emulsions, which have found diverse applications in various fields, including food industries, drug delivery, oil extraction, cosmetics, emulsion explosives, and reaction media. These multiple emulsions are complex systems that consist of droplets of one liquid dispersed within another liquid, which in turn is dispersed in a third liquid, resulting in a water-in-oil-in-water (w/o/w) or oil-in-water-in-oil (o/w/o) multiple emulsion. These emulsions show improved stability, making them highly attractive for various applications, such as the controlled release of active ingredients. Recently, Akram *et al.* demonstrated the formation of double emulsions in an oil-in-water system, creating micro and nano double emulsions using a double emulsion approach.¹ They employed silica particles as stabilizers for the inner droplets and surfactants for the outer droplets. However, long-term stability remained a challenge. In 2020, Khadem *et al.* investigated the release and swelling phenomena during preparation and storage.² In that work, they reported various parameters affecting the preparation of double emulsions and the stability of double emulsions during storage. Furthermore, they examined

the evolution of the outer droplet size and release rate in double emulsions, highlighting the impact of overswelling-breakdown on the quality of these emulsions. Consequently, there has been a significant interest in developing new and innovative methods for synthesizing and characterizing multiple emulsions.^{3–9} This type of system is characterized by the presence of two interfaces, one between the inner droplets and the surrounding droplets and another between the outer droplets and the external environment. Multiple emulsions can be further classified based on the number and size of the dispersed droplets. The most common types are double emulsions, where one set of droplets is dispersed within another set, and triple emulsions, where two sets of droplets are dispersed within the same continuous phase.^{5,8,10,11} Multiple and double emulsions in oil–water systems, such as water-in-oil-in-water (w/o/w), water-in-water-in-oil (w/w/o), and oil-in-water-in-oil (o/w/o) emulsions, exhibit excellent stability and are used for a wide range of applications in biomedical and biological screening assays. They are also utilized in fabricating liposomes and colloidosomes capsules for drug delivery. These emulsions have unique structural and physical properties, such as high surface area and controlled release characteristics, which make them attractive for these applications. In addition, they can be easily modified by incorporating various

functional additives, such as drugs or biomolecules, to tailor their properties for specific applications.^{12–14} The formation of double emulsions is a complex process, often requiring two steps, which can pose challenges for large-scale production. Currently, available techniques for generating double emulsions include microfluidics, double-step mixing, and phase inversion. Microfluidic methods involve using microchannels to generate emulsions, but they require sophisticated equipment and are often not scalable to larger production volumes. Double-step mixing methods involve the formation of primary emulsions followed by a second mixing step to generate the double emulsion, which can be time-consuming and may result in low yields. Phase inversion methods rely on manipulating the interfacial tension between the two immiscible phases to form the double emulsion, but the resulting emulsions may be unstable and difficult to control. As such, there is a need to develop more efficient and scalable methods for forming double emulsions for use in various applications.^{15–17} The current literature has grown significantly with numerous examples of the various morphologies observed in oil/water multiple emulsion systems, including *o/w/o*, *w/o/w*, and others. However, despite their inherent stability and effective stabilization, there has been a dearth of research on water-in-water-in-water emulsions, which may offer new opportunities in various fields, such as drug delivery, food science, and materials engineering.^{11,18,19}

An aqueous two-phase system (ATPS) is a thermodynamically equilibrated system combining two incompatible hydrophilic polymers in two phases. In a classical ATPS, a water-in-water emulsion is formed by dispersing one aqueous phase as droplets within the other, which serves as the continuous phase. The interfacial tension between the two aqueous phases in this emulsion type is extremely low, typically between 1 and 10 $\mu\text{N/m}$. This low interfacial tension results from the similar chemical nature of the two aqueous phases, which results in weak intermolecular forces and minimal thermodynamic barriers at the interface between the two phases. The unique properties of ATPS, such as their biocompatibility, tunable selectivity, and mild processing conditions, have led to their use in various applications, including bio-separation, biocatalysis, and drug delivery.^{20–22} To date, there have been relatively few studies on the formation of water–water–water (*w/w/w*) emulsions, and most of the reported methods involve either microfluidic techniques or two-step processes. The pioneering work in applying microfluidics with flow focusing for generating monodispersed droplets, particles, and bubbles was carried out by Gañán-Calvo.^{23–26} While microfluidic methods are highly efficient and enable precise control over droplet size and morphology, they may not be scalable for larger production volumes. Two-step methods involve generating a primary water-in-water emulsion, which is then subjected to a second emulsification step to generate the *w/w/w* emulsion. Although these methods are relatively simple and do not require specialized equipment, they may result in low yields and are often time-consuming. Norton and Frith have demonstrated the formation of multiple water-in-water-in-water (*w/w/w*) emulsions at or near 50/50 volume fraction using the phase transition technique in a biopolymer system comprising gelatin and methyl dextran.²⁷ This method involves exploiting the thermodynamic properties of the polymer system to induce a phase transition that results in the formation of emulsions. The multiple *w/w/w* emulsions were observed to have stable morphologies and were found to be tunable by varying the polymer concentration and emulsion volume fraction. Nonomura *et al.* have proposed an

excellent method for forming multiple emulsions using micro bowls.²⁸ They have reported that anisotropic particles can stabilize multiple emulsions in a single step, and they have demonstrated the formation of oil-in-water-in-oil (*o/w/o*) emulsions using this approach. They observed that the particle shape and surface chemistry played a critical role in stabilizing the emulsions. Anisotropic particles are particles with a non-spherical shape, exhibiting unique surface properties that enable them to act as effective stabilizers for emulsions. Several researchers have also reported using microfluidic techniques to prepare *w/w/w* emulsions, which provide excellent control over the structure and morphology of the formed droplets.^{11,29} In 2020, Jeyhani *et al.* studied the stabilization of double and triple emulsions in an aqueous two-phase system (ATPS) consisting of polyethylene glycol (PEG) and dextran.²⁹ They utilized a microfluidic device to generate the emulsions and employed lysozyme as a stabilizer for the formed emulsions. However, the stability of the emulsions remained a subject of uncertainty or a lack of understanding. However, there has been little exploration of the single-step process for forming these double emulsions as it involves the simultaneous formation of multiple emulsion droplets in a mixture. Therefore, research is needed to optimize the process parameters and understand the mechanisms underlying the formation and stabilization of these emulsions.

This communication presents a simple yet straightforward single-step mixing approach for synthesizing *w/w/w* double emulsions with high stability for up to 30 days. Our approach involved the use of polyethylene oxide (PEO) and dextran bio-polymer with varying molecular weights, as well as oppositely charged silica nanoparticles, to induce *in situ* hetero-aggregate formation in an aqueous phase. Our work primarily focuses on synthesizing double emulsions in aqueous two-phase systems (ATPS) consisting of two immiscible aqueous phases in thermodynamic equilibrium. The results of our study demonstrate the potential of our single-step approach for the scalable and efficient production of *w/w/w* double emulsions with controlled morphology and stability.

To generate double emulsions, we used polyethylene oxide (PEO) and dextran biopolymer with varying molecular weights, including 1×10^5 (1E5), 2×10^5 (2E5), 3×10^5 (3E5), 6×10^5 (6E5) Da and 6×10^5 (6E3), 4×10^5 (4E4), 1×10^5 (1E5), 2×10^5 (2E5) Da, respectively. We employed Ludox HS-40 (40 wt. %) and CL-30 (30 wt. %) silica nanoparticles, which had a diameter of 16 ± 2 and 16 ± 2 nm (as determined by transmission electron microscopy) (see Fig. S1 in the supplementary material), and zeta potential of -55 ± 1.8 and 51 ± 2.1 mV, obtained through electrophoretic light scattering, respectively. Figure 1 depicts a schematic representation of the emulsion preparation process. Each aqueous phase was formulated separately, and an equivalent quantity of both polymers (5 wt. %) was included along with the de-ionized water. The mixture was allowed to soak overnight and then subjected to 2 min of mixing using a vortex shaker to achieve a homogeneous solution. Following this, a single type of particle (CL-30 in PEO or HS-40 in dextran solution) was added to one of the phases while ensuring that the total concentration of the nanoparticles did not exceed 1 wt. % of the entire solution. Notably, all experiments were performed in triplicate to ensure the reproducibility and reliability of the results. The two solutions were combined using a homogenizer (Make: IKA, Model: Ultra Turrax T25) operating at 10 000 rpm for 2 min and then incubated at a constant temperature of 25 °C for 48 h. To discern the type of emulsion

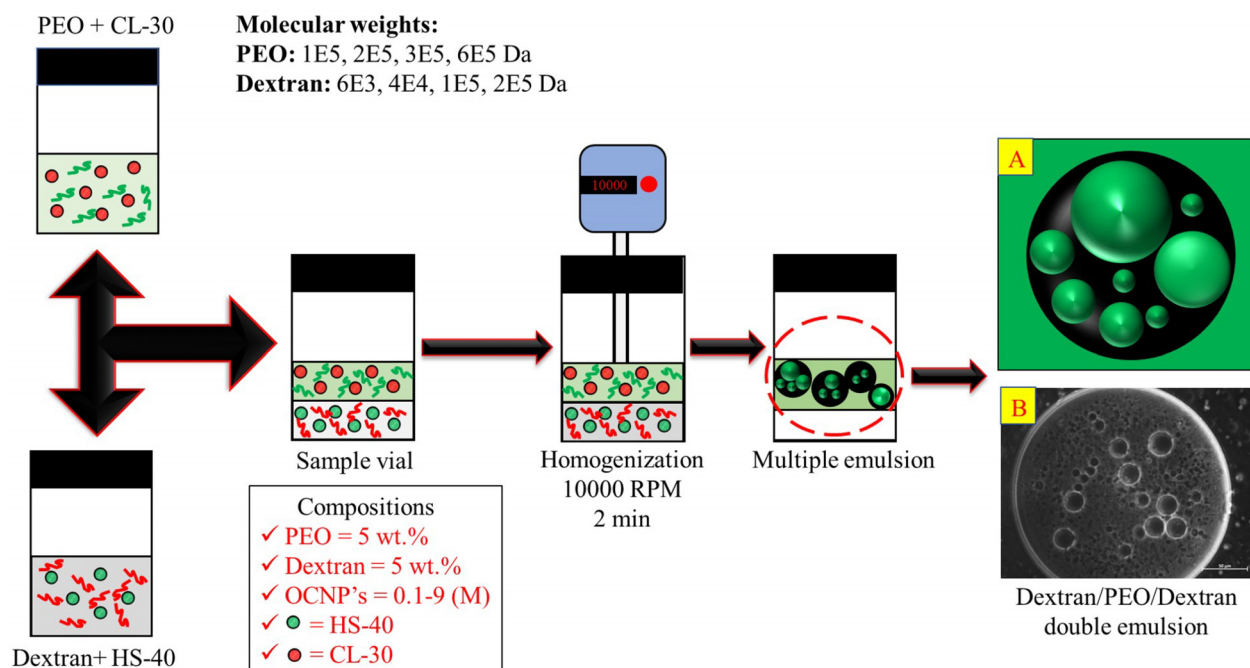


FIG. 1. Schematic description of the double emulsion formation: (a) schematic of D/P/D emulsion and (b) microscopic image of D/P/D emulsion (note: scale bar corresponds to 50 μm).

created, we fluorescently labeled the dextran with FITC (Fluorescein-5-isothiocyanate). De-ionized water (18.2 M Ω), obtained from the Barnstead™ Smart2Pure™ water purification system (Make: Thermo Fisher Scientific), was used for all experiments. The viscosity of the polymers was measured using a twin-drive rheometer (Make: Anton Paar, Model: MCR-702) at a steady temperature of 25 °C. Notably, all samples were examined after 48 h, which serves as a reference point, i.e., $t = 0$ days, for comparison.

First, we present a simple approach for synthesizing multiple water-in-water emulsions. By preparing an aqueous phase with polymers of equal molecular weight and choosing a particular weight ratio of positively charged nanoparticles to negatively charged nanoparticles (referred to as M), the w/w/w Pickering emulsion template was created to gain a deeper understanding of the double emulsion formation. Previous research by Norton and Frith²⁷ has shown that multiple/bicontinuous emulsions can be formed at the phase volume line concentration on the binodal curve of the polymer system. Beyond this concentration, phase inversion occurs. Our research has found that high-speed mixing of the two polymers leads to the initial separation of the polymers within the droplets rather than by diffusion to already-formed droplets due to the differences in solution viscosity. Once the primary droplets have formed, they behave as if they were isolated phases, and a second separation occurs within these microenvironments, resulting in the formation of included droplets. Our findings contribute to a deeper understanding of double emulsion formation's physical mechanisms and practical implications for developing water-in-water emulsions. The stability of the system that we have formed is a concern, as the low surface tension between the phases in APTS can lead to instability. To address this, we have

utilized polymer concentrations based on the binodal curve of PEO and dextran, as previously reported by our research group.²⁰

Our research observed the phase line at a 50:50 phase volume ratio of PEO and dextran biopolymer. Additionally, Nonomura *et al.*²⁸ have reported that anisotropic particles can synthesize double emulsions in a single step in the O/W emulsion system. To investigate the effect of anisotropic particles on the structural state and stability of the emulsion, we used hetero-aggregates formed using the oppositely charged nanoparticles (OCNPs). Through a systematic study, we varied the molecular weight of the polymer and M (the ratio of charged particles) and observed changes in the structure of the synthesized emulsion. Notably, we could produce double emulsions in a single step without using a microfluidic approach. Based on the experimental observation, we constructed a state diagram demonstrating the structural formation and transition from a single to double emulsion by varying the molecular weight and composition of OCNPs of a two-phase mixture stabilized by the homogenizer at a programmed speed of 10 000 rpm. This state diagram is illustrated in Fig. 2.

In our study, we made an interesting observation that the double emulsion formation occurred at M values of 4 and 9 corresponding to positively charged hetero-aggregates of nanoparticles²⁰ when the equal molecular weight of the polymers was employed. We attribute this phenomenon to the wettability of the aggregate particles in the Pickering emulsion, which is responsible for the emulsion formation. Furthermore, we envisage that the formation of multiple emulsions could be linked to the contact angle distribution of the anisotropic particles. This trend suggests that the anisotropy of the particles is a crucial factor in the formation of double emulsions, as it influences the contact angle and, thus, the stability of the emulsion.²⁸ On the other

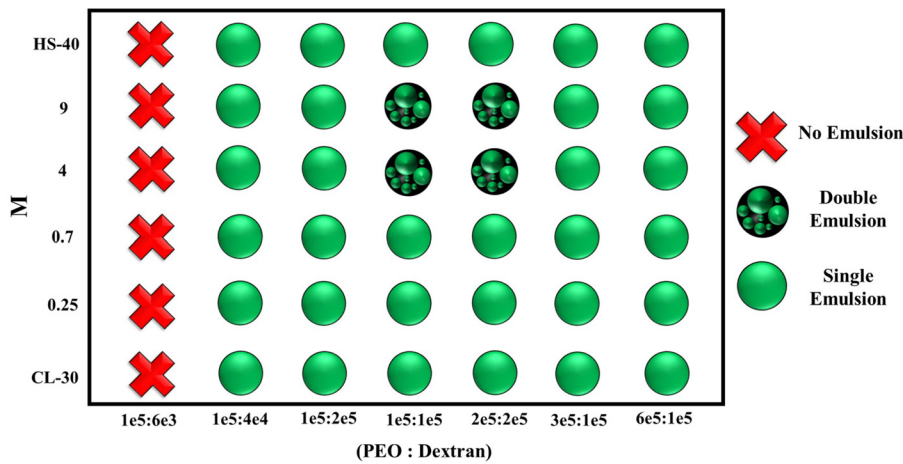


FIG. 2. State diagram demonstrated the double emulsion formation with respect to the molecular weight ratios of the biopolymers and M .

hand, it can be envisaged that the steric stabilization of polymer on silica particles would result in a modification of the wettability characteristics of the particles.³⁰ However, the spatial distribution and positioning of the particles across various interfaces in multiple emulsions still need to be fully elucidated.³¹

The stabilization study revealed that dextran, with a molecular weight of 6E3 Da, did not exhibit the emulsion formation as the viscosity of the polymer solution was similar to that of water (refer to Fig. S2 in the supplementary material). In contrast, other molecular weights of dextran showed the single emulsion formation in all mass ratios (M) of the particles, except for equal molecular weight (1E5:1E5 and 2E5:2E5 Da) of polymers at $M = 4$ and 9 due to changes in the viscosity of the phases and arrangements of particles at the interface of the system. The formation of the emulsion structure was confirmed using a microscope (see Fig. 3), and the type of emulsion formed was determined using an FITC-labeled dextran. The microscopic images of the emulsion droplets were utilized to assess the stability of formed

emulsions. The rectangular box highlighted in red indicates the stable morphology of the double emulsion formation observed for up to 30 days. Conversely, in the case of the 1E5:6E3 (PEO:Dextran) polymer ratio, no emulsion droplets were observed, suggesting the formation of a complete solution or the absence of a two-phase system. The stability of emulsions comprising 1E5:4E4 (PEO:Dextran) Da polymers and either CL-30 or HS-40 single-type charged particles was found to be stable for up to 7 days; however, after 30 days, no droplets were discernible under microscopy, indicating short-lived stability. The study also established that the stability of the emulsions formed was positively correlated with an increase in the molecular weight of the polymers in the two-phase system. This trend was confirmed through microscopic images and emulsion index measurements of the formed emulsion. Specifically, the experimental results consistently demonstrate that the formulation comprising higher molecular weight polymers exhibits superior stability characteristics. Figures 3(a) and 3(b) illustrate the morphological characteristics of the formed

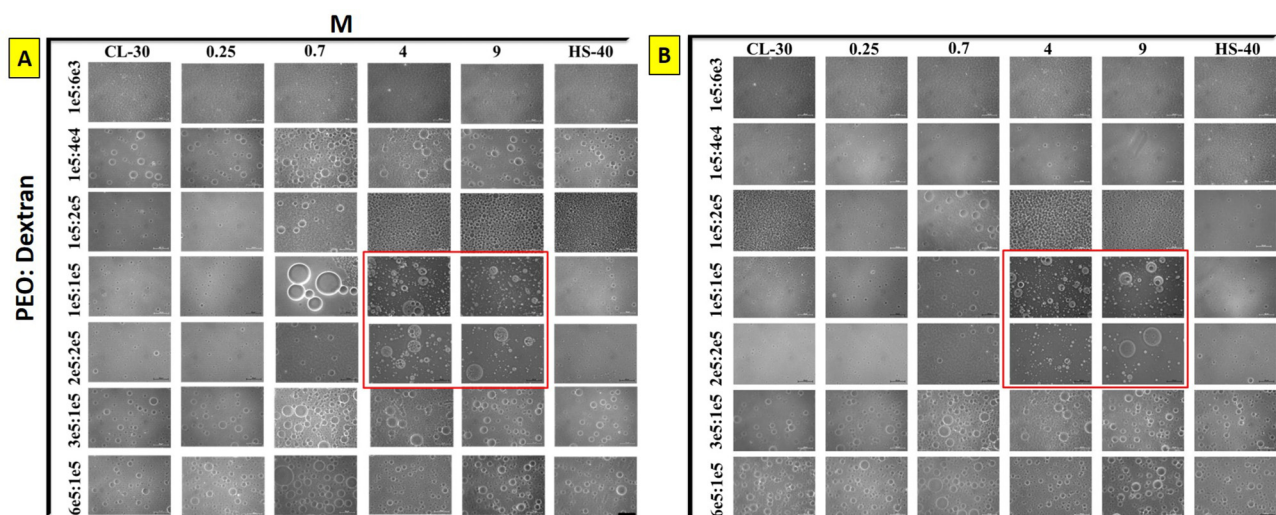


FIG. 3. Representative microscopic images of the formed emulsions: (a) 0th day and (b) after 30th day. The scale bars given in the images correspond to 50 μm .

emulsion in the molecular weight system at $t = 0$ and 30 days. These images were obtained using an inverted microscope (Model: Axio Vert A1; Make: Carl Zeiss, India) with brightfield mode at $40\times$ magnification to cover the large surface area of the sample, providing a visual representation of the stability of the emulsions over time. The samples were carefully prepared on the cleaned glass slide and covered with a coverslip to enhance the viewing and minimize the evaporation rate of the emulsion sample. Specifically, the images allow for assessing changes in the emulsion morphology, such as the droplets' size, shape, and distribution. Such information is valuable in determining the stability of the emulsions and evaluating the effectiveness of the stabilizing agents used in the system. The formed emulsion was identified as Dextran/PEO/Dextran (D/P/D) emulsion, as shown in Fig. 4, using fluorescent microscopy (Model: DM IL LED Fluo, Make: Leica Microsystems).

To investigate the influence of mixing on the formation of double emulsion, we prepared emulsions with varying mixing speeds at a 50:50 volume ratio of polymer with 1E5 Da molecular weight, where both polymers have equal molecular weight, at $M=4$, as indicated in the state diagram shown in Fig. 2. The results depicted in Fig. 5 demonstrate that the double emulsion formation occurred at all mixing speeds, indicating that the process depends on the viscosity of both phases. Specifically, the polymers with equal molecular weights exhibited a one-order magnitude difference in the viscosity of the phases (see Fig. S2 in the supplementary material). This finding suggests that once primary droplets are formed, they behave like an isolated phase in the highly viscous medium and exhibit the emulsion's double structure morphology. A recent study conducted by Sabri *et al.*³² demonstrated that multiple emulsions could be generated in a single step by a phase inversion mechanism using silica particles in a highly viscous oil-in-water emulsion system.

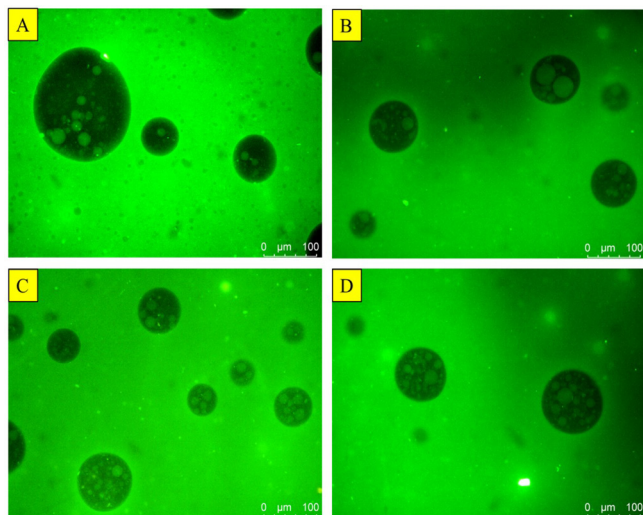


FIG. 4. Representative fluorescent images of formed double emulsion (a) and (b) corresponds to the 1E5:1E5 Da (c and d) corresponds to 2E5:2E5 Da molecular weight ratios of the polymer at $M=4$ and 9, respectively. The scale bar corresponds to the $100\ \mu\text{m}$. In the image, green corresponds to the fluorescently labeled dextran phase, while black corresponds to the non-fluorescent PEO phase.

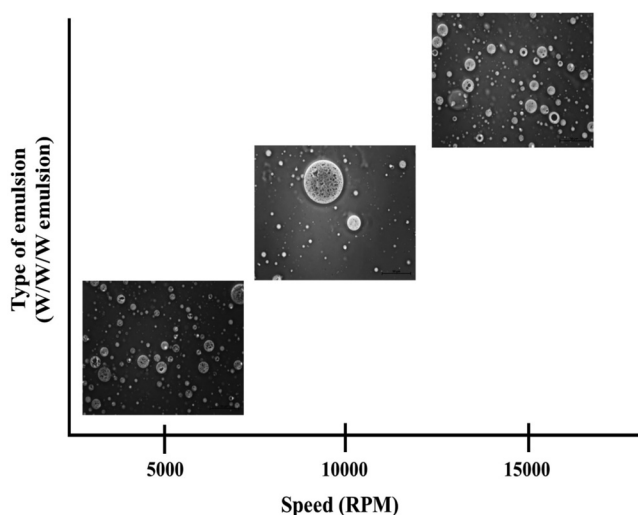


FIG. 5. Mixing effect on the emulsion formation for 1E5 (PEO):1E5 (Dextran) Da, at $M=4$. The scale bar corresponds to $50\ \mu\text{m}$.

We also investigated the scalability of the proposed technique for the double emulsion formation. For the study, a separate set of experiments was conducted at different batch sizes, measured in 5, 10, and 15 g of the total solutions, using polymers with an equal molecular weight of 1E5 Da and 1 wt. % of the OCNPs at all mass ratios (M). To ensure meaningful comparisons, we focused on the experiments at one set of favorable conditions for the double emulsion formation, namely, equal molecular weight at 1E5 Da. Intriguingly, the double emulsion formation consistently occurred across all batch sizes, as depicted in Fig. 6. However, except for $M=4$ and 9, the experiments conducted under other conditions resulted in the formation of single emulsions. Our findings indicate that the formation of double emulsions is independent of the size (volume) of the experiment. However, we were prepared for the possibility that the nanoparticles' stabilization capacity may affect the emulsions' quality. To gain deeper insight into the effect of the nanoparticles, the samples with different particle concentrations were prepared at a suitable mass ratio (M). The results revealed a dependency on the concentration of OCNPs to form double emulsions. It comes to say that the concentrations exceeding 1 wt. % led to the formation of single emulsions. These findings are consistent with the work of He *et al.*³³

The stability of the formed emulsion was assessed for 30 days. The emulsion stability was quantified by calculating the emulsion index (%), which was determined based on the following equation [see Eq. (1)],³⁴ as shown in Fig. 7, for various mass ratios (M) of nanoparticles using vial images displayed in Fig. S4 of the supplementary material:

$$EI = \frac{h}{H} \times 100, \quad (1)$$

where h and H refer to the height of the emulsion phase and total sample height, respectively. To calculate this index, we measured the percentage ratio of the height of the emulsion (height up to which the emulsion spanned) to the total height corresponding to the mixture after emulsification using Image-J software, an open-

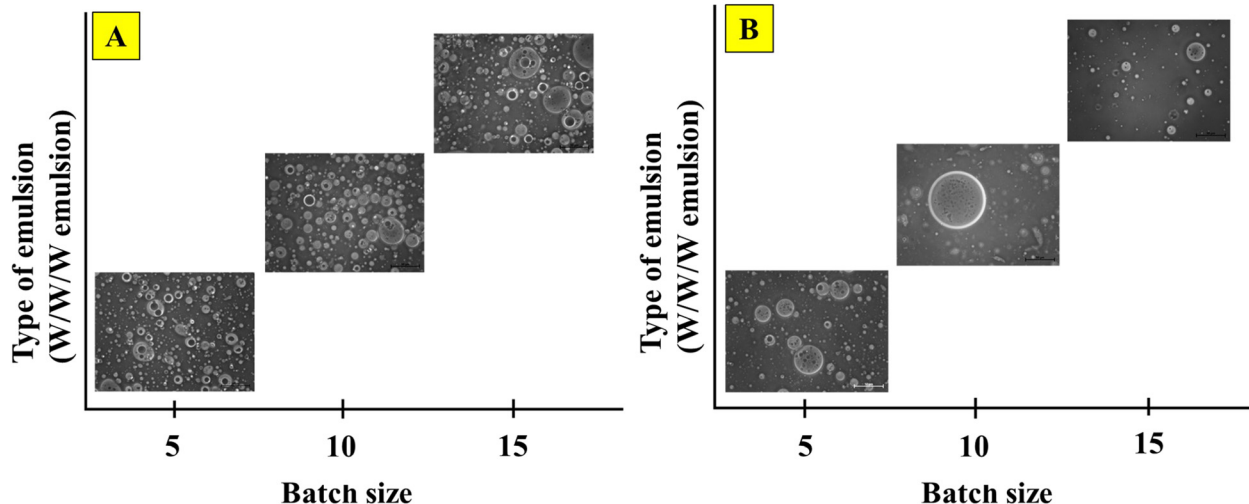


FIG. 6. Plot showing the effect of scaling by batch size on the formation of double emulsion: (a) for $M=4$ and (b) for $M=9$ for 1E5 (PEO):1E5 (Dextran) Da in an equal amount of polymers (5 wt. %).

source software tool. The results indicated that emulsions formed with single particles, such as HS-40 and CL-30, in low molecular weight PEO (4E4 Da) were destabilized after 48 h. A quick overview of the state of formed emulsions at different values of M can be obtained by referring to the vial images in Fig. S4 of the supplementary material. The stability of the formed emulsion starts to increase with the increase in the molecular weight of the continuous phase (PEO), in the case of pure HS-40 or CL-30, due to the strong adsorption of the PEO in the continuous phase on silica nanoparticles. According to the work of Liu and Xiao,³⁵ the zeta potential decreases with an increase in the molecular weight at the same concentration of PEO due to the configuration change of PEO segments to loops, which eventually contributes significantly

to the net electrostatic attraction between the particles and polymers. The stability of the emulsion was confirmed through the microscopic analysis using an inverted microscope, as evidenced by the presence of stable droplets observed for up to 30 days after the emulsion formation. The high viscosity of the polymers, in conjunction with particulate barriers at the interfaces within the aqueous phase, contributed to the stabilization of the water sub-inclusions and prevented their transfer into the continuous aqueous phase during processing. This mechanism is similar to the formation of sub-inclusions in highly viscous melt-processed polymer blends, wherein phase inversion during processing results in the kinetic trapping of sub-inclusions within the dispersed phase, leading to the formation of multiple emulsions.

In this Letter, we report a single-step approach that enables the generation of double emulsions at a suitable experimental regime. We have demonstrated that the molecular weight of the polymers used to form the aqueous phase is a critical factor in the structural formation of the emulsion, resulting in the formation of double emulsions. Our proposed strategy is easy to implement and can offer excellent throughput over the fabrication of double emulsions with precise control of the structural qualities. Since the proposed technique involves stabilizing the droplets using the aggregate particles formed due to the self-assembly of oppositely charged nanoparticles, the required increment in Gibbs detachment energy is naturally gained without adding any unconventional stabilizers. This study is the first report to demonstrate the formation of a w/w/w emulsion using a Pickering emulsion template in an ATPS system through a single-step process.

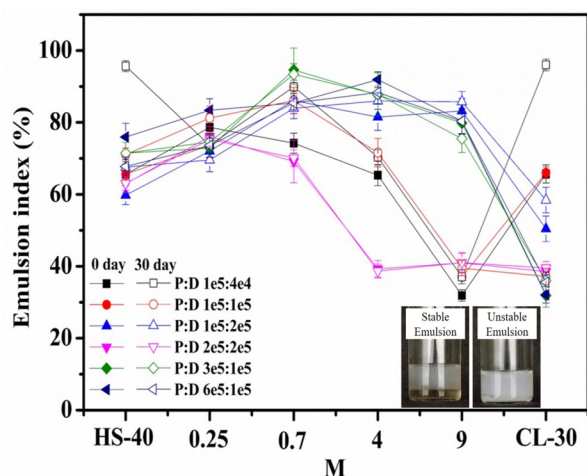


FIG. 7. Plot showing the stability of the formed emulsion. The filled symbol indicates 0th day and open symbol represents 30th day, respectively.

SUPPLEMENTARY MATERIAL

See the supplementary material for referring to transmission electron microscopic images of OCNPs, viscosity measurement of the polymers, and vial images of the formed emulsion.

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AUTHOR DECLARATIONS

Conflict of Interest

The authors have no conflicts to disclose.

Author Contributions

Chandra Shekhar: Conceptualization (equal); Formal analysis (equal); Investigation (equal); Experimental methodology (equal); Validation (equal); Writing – original draft preparation (lead). **Vishwajeet Mehandia:** Formal analysis (supporting); Resources (supporting); Investigation (supporting); Validation (equal); Writing – review & editing (equal). **Manigandan Sabapathy:** Conceptualization (equal); Writing – original draft preparation (Supporting); Writing – review & editing (equal); Funding acquisition; Project administration; Supervision.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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