

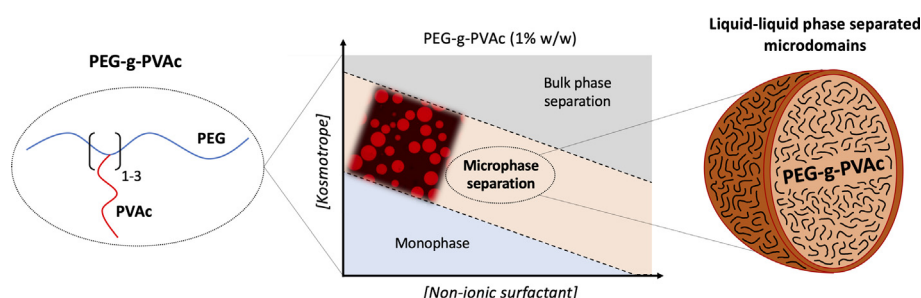
# Liquid-liquid phase separated microdomains of an amphiphilic graft copolymer in a surfactant-rich medium

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## GRAPHICAL ABSTRACT



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## ABSTRACT

The liquid-liquid phase separation (LLPS) of amphiphilic thermoresponsive copolymers can lead to the formation of micron-sized domains, known as simple coacervates. Due to their potential to confine active principles, these copolymer-rich droplets have gained interest as encapsulating agents. Understanding and controlling the conditions inducing this LLPS is therefore essential for applicative purposes and requires thorough fundamental studies on self-coacervation. In this work, we investigate the LLPS of a comb-like graft copolymer (PEG-g-PVAc) consisting of a poly(ethylene glycol) backbone (6 kDa) with ~2–3 grafted poly(vinyl acetate) chains, and a PEG/PVAc weight ratio of 40/60. Specifically, we report the effect of various water-soluble additives on its phase separation behavior. Kosmotropes and non-ionic surfactants were found to decrease the phase separation temperature of the copolymer, while chaotropes and, above all, ionic surfactants increased it. We then focus on the phase behavior of PEG-g-PVAc in the presence of sodium citrate and a C<sub>14-15</sub> E<sub>7</sub> non-ionic surfactant (N45-7), defining the compositional range for the generation of LLPS microdomains at room temperature and monitoring their formation with fluorescence confocal microscopy. Finally, we determine the composition of the microdomains through confocal Raman microscopy, demonstrating the presence of PEG-g-PVAc, N45-7, and water. These results expand our knowledge on polymeric self-coacervation, clarifying the optimal conditions and composition needed to obtain LLPS microdomains with encapsulation potential at room temperature in surfactant-rich formulations.

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## 1. Introduction

Responsive polymers, which reversibly change their properties in response to external stimuli or changes in the medium they are dissolved in [1–4], are increasingly used in several applicative

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areas, including drug delivery, sensors, and artificial actuators [5,6]. Thermosensitive polymers are probably the most explored class of smart polymers [7,8]; their thermally induced liquid-liquid phase separation depends on the polymer structure as well as on the medium composition, so that both the polymer and the medium can be designed to tune the transition temperature [9–11]. Thermoresponsive polymers that phase separate below a critical temperature are classified as UCST-type (Upper Critical Solution Temperature); conversely, if phase separation occurs above a critical temperature they are classified as LCST-type (Lower Critical Solution Temperature) [12–14]. LCST polymers can be soluble in aqueous media at room temperature, but their solubility decreases upon heating, and eventually phase separation into two liquid phases occurs [15]. This entropy-driven phase separation is marked by a cloudy appearance of the sample and is commonly termed as the Cloud Point Temperature (CPT) [16].

Poly(ethylene glycol) (PEG) is one of the most studied LCST-type homopolymers. Due to a thermally-activated conformational change of the oxyethylene groups [17], it becomes insoluble above the CPT, which depends on molecular weight [18,19]. Many classical studies have addressed the effect of additives in the aqueous medium on its CPT [20,21]. Moreover, PEG is commonly used in biomedical applications due to its biocompatibility, stealth effect, and steric stabilization properties [22]. The phase behavior of copolymers containing PEG blocks has also been extensively explored to understand self-assembly and sol-gel transition properties. In previous studies, PEG was conjugated with poly(propylene oxide) (PPO) to generate the Pluronic or poloxamer family [23–25], but also with poly(*N*-isopropylacrylamide) (PNIPAM) [26–28], polycaprolactone (PCL) [29–31], poly(*N*-vinylcaprolactam) (PVCL) [32,33], or poly(lactic-co-glycolic acid) (PLGA) [34,35], to name a few.

More recently, some reports have addressed the phase behavior of grafted poly(ethylene glycol)-*g*-poly(vinyl acetate) copolymers (PEG-*g*-PVAc) [36–38]. Graft copolymers, consisting of a backbone and side chains, present interesting properties in solution that can differ from linear diblock copolymers with the same composition [39,40]. They have been found to form a variety of self-assembled aggregates in water and organic solvents, which depend on the hydrophilic/hydrophobic features of the backbone and side chains [41–45]. Specifically, PEG-*g*-PVAc's self-assembly properties depend on the balance between the hydrophobic PVAc grafts and the hydrophilic PEG backbone, as well as in the number and length of the PVAc grafts.

The self-assembly properties of a PEG-*g*-PVAc (*M*<sub>w</sub> = 13,100 Da, 1–2 grafting points every ~100 units of ethylene glycol) in water have been reported in the recent past, showing the formation of single-chain nanoparticles (SCNPs) at concentrations below 10% w/w [36], and a progressive structuration of the SCNPs into hierarchically complex systems when increasing the concentration up to 90% w/w [37]. The LCST profile of this PEG-*g*-PVAc showed a critical temperature of 58 °C for a concentration of 1% w/w. The application of PEG-*g*-PVAc self-assemblies for the encapsulation of hydrophobic substances, and specifically fragrances, was also demonstrated [46,47], showing that the molecular properties of the guest molecule affect the structure of the copolymer assemblies and their phase behavior in ternary (i.e. PEG-*g*-PVAc/fragrance/water) systems. The interaction between a PEG-*g*-PVAc with a different grafting density (*M*<sub>n</sub> = 15,000 Da, one grafting point every ~20 units of ethylene glycol) and sodium dodecyl sulfate was recently reported too [48], highlighting the synergistic or competitive behavior at the air-water interface, depending on the concentration of anionic surfactant. In this contribution, we focus on a higher *M*<sub>w</sub> and more hydrophobic PEG-*g*-PVAc (*M*<sub>w</sub> = 18,100 Da) consisting of a comb-like macromolecular structure with a 6,000 Da PEG backbone and a grafting point every ~50 units of

ethylene glycol on average (~2–3 grafted PVAc chains per backbone), expected to exhibit a lower LCST. On the industrial side, this class of copolymers with a low degree of grafting has attracted interest as detergent additives for the formulation of home and beauty care products. Due to their adsorption properties, they promote a reduction of soil redeposition on garments, especially in hard water conditions [49–51].

PEG-*g*-PVAc's self-assembly was also investigated in formulations containing surfactant mixtures [38], identifying a region with liquid-liquid phase separation (LLPS) at room temperature. Interestingly, this LLPS gives rise to micron-sized domains, whose size and stability against coalescence depend on the composition of the aqueous medium. LLPS was not expected to take place in this kind of medium since normally the presence of surfactants and/or salts destabilizes conventional LLPS structures, such as coacervates and vesicles [52,53]. Moreover, these LLPS microdomains were able to confine different active principles to a larger extent than simple micelles, including hydrophobic (poly(dimethylsiloxane) bis(3-aminopropyl)) and hydrophilic ((*Z,Z*)-disodium distyryl-biphenyl disulfonate) molecules. The encapsulation of active principles with different water affinities in a surfactant-rich medium is a very interesting property, generally difficult to achieve with simple coacervation, making this copolymer a promising candidate for industrial formulations. Whether the use of PEG-*g*-PVAc is intended for fundamental studies on self-coacervation or for applicative purposes to form microcapsules in complex formulations, a thorough understanding and control of the compositions and conditions inducing the LLPS of this copolymer is needed.

In this study, we explore the effect of several water-soluble additives on the phase behavior of PEG-*g*-PVAc, namely kosmotropic and chaotropic compounds, as well as non-ionic, anionic, and cationic surfactants. We show that the copolymer's CPT displays a strong dependence on the chemical nature and concentration of these additives, in line with the reported CPTs of other LCST copolymers [23,54,55]. We then explore the effect of sodium citrate and a C<sub>14-15</sub> E<sub>7</sub> non-ionic surfactant (N45-7) on the phase behavior of PEG-*g*-PVAc, defining the phase boundaries for the generation of LLPS microdomains at room temperature and monitoring their formation with fluorescence confocal microscopy. Finally, we determine the composition of the LLPS microdomains through confocal Raman microscopy. The ensemble of results defines the optimal composition and medium conditions to obtain LLPS microdomains with encapsulation potential at room temperature in surfactant-rich formulations.

## 2. Materials & methods

### 2.1. Materials

Sodium chloride (NaCl), *M*<sub>w</sub> 58.4 g/mol, purity ≥ 99%; sodium bromide (NaBr), *M*<sub>w</sub> 102.9 g/mol, purity ≥ 99%; dodecyltrimethylammonium chloride (DTAC), *M*<sub>w</sub> 263.9 g/mol, purity > 98%; dodecyltrimethylammonium bromide (DTAB), *M*<sub>w</sub> 308.3 g/mol, purity > 98%; rhodamine B isothiocyanate, mixed isomers, *M*<sub>w</sub> 536.1 g/mol, were purchased from Sigma-Aldrich.

Trisodium citrate dihydrate (NaCit), *M*<sub>w</sub> 294.1 g/mol, purity > 98%, Jungbunzlauer; sodium cumene sulfonate (NaCS) 40%, *M*<sub>w</sub> 222.2 g/mol, Sasol; monopropylene glycol (MPG), *M*<sub>w</sub> 76.1 g/mol, INEOS Oxide; urea, *M*<sub>w</sub> 60.1 g/mol, VWR International; Neodol 45-7 (N45-7), primary alcohol ethoxylate, C14-15 EO7, *M*<sub>w</sub> 519–573 g/mol, Shell Chemical; Surfonic L24-9 (L24-9), primary alcohol ethoxylate, C12-14 EO9, *M*<sub>w</sub> 582–610 g/mol (theoretical), Huntsman; cetyltrimethylammonium bromide (CTAB), *M*<sub>w</sub> 364.5 g/mol, purity > 99%, Sigma-Aldrich; cetyltrimethylammo-

niium chloride (CTAC), Mw 320 g/mol, purity 96%, Alfa Aesar; Solfodac AC-3-H (HLAS), linear alkylbenzene sulfonate, C10-13, Mw 312–360 g/mol, Tellerini s.p.a.; Tensagex EOC970B (AE3S), sodium lauryl ether sulphate 70%, EO3, Mw 420 g/mol, KLK Tensachem S. A., were provided by Procter & Gamble.

Poly(ethylene glycol)-*graft*-poly(vinyl acetate) (PEG-g-PVAc) from BASF was also provided by Procter & Gamble. The copolymer has a PEG/PVAc weight ratio of 40/60,  $M_n = 10.3$  kDa,  $M_w = 18.1$  kDa (thus, with a polydispersity index (PDI) of 1.8). This copolymer has approximately one grafting point every 50 units of ethylene glycol, meaning that there are  $\sim 2$ – $3$  grafted PVAc chains per PEG backbone (which is of 6 kDa). PEG-g-PVAc was covalently labelled with rhodamine B isothiocyanate, according to a previously described procedure [38].

All reagents were used as received without any further purification. The water used throughout this work was of MilliQ grade (18.2 M $\Omega$ .cm at 25 °C)

## 2.2. Sample preparation

PEG-g-PVAc, a wax-like solid with a melting point above room temperature, was firstly warmed at 70 °C for better manipulation. Then, appropriate amounts of molten PEG-g-PVAc, water, and the selected components for the study were weighted in a glass vial with an analytical balance (Mettler Toledo XSR603S, accuracy of  $\pm 1$  mg). The samples were then mixed with a magnetic stirrer until a homogeneous dispersion was obtained.

PEG-g-PVAc concentration was kept at 1% w/w unless otherwise stated.

To map the phase behavior of PEG-g-PVAc with NaCit and N45-7 (Fig. 4A), five samples were prepared in glass vials containing a fixed amount of PEG-g-PVAc (1% w/w) and varying amounts of non-ionic surfactant N45-7 (1, 5, 10, 15 and 20% w/w) in water. After dissolving the copolymer and the non-ionic surfactant, the samples were titrated with sodium citrate while mixing at room temperature. All samples got cloudy after enough NaCit was added to induce LLPS (Table 1), and optical microscopy was then used to check the presence of LLPS microdomains (Fig. S5). Then, more NaCit was added to achieve a liquid-liquid bulk phase separation (Table 1).

## 2.3. Cloud point determination

The Cloud Point Temperature (CPT) was determined with turbidimetry using a Crystalline, from Technobis. The samples were analyzed in standardized glass vials of 8 mL, measuring transmittance at 623 nm between 20 °C and 90 °C, with a heating rate of 0.5 °C/min and a cooling rate of 5 °C/min, while mixed with a magnetic stirrer at 800 rpm. The CPT was considered as the temperature corresponding to 50% transmittance with respect to the

initial and final values. Each measurement was repeated at least three times.

To study the effect of ionic surfactants on the CPT, samples were prepared by adding dropwise a 0.1% w/w solution of ionic surfactant to the sample of 1% w/w PEG-g-PVAc and then mixed until homogenous. The CPT was measured after every addition. At higher concentrations, samples containing HLAS, AE3S, CTAB, or CTAC showed a marked Tyndall effect that prevented measuring properly the CPT at temperatures higher than 66 °C.

The measurement of the CPTs of aqueous solutions of PEG-g-PVAc at different concentrations (0.25, 0.5, 1, 2, 3, 4, 6, 10% w/w, see Fig. S1) revealed an LCST-type profile with a critical temperature of 51 °C, found for 1% w/w (approximately  $5.5 \times 10^{-4}$  mol/L).

## 2.4. Optical microscopy

The optical pictures were acquired using an optical microscope from ZEISS, model AXIO Imager A1, with an Axiocam 305 color camera from ZEISS as well, while using dry objectives of 10 $\times$ /0.30 and 40 $\times$ /0.75. Samples were placed between glass slides to delay water evaporation.

## 2.5. Fluorescence confocal microscopy

Fluorescence confocal microscopy imaging was carried out using a Leica TCS SP8 DMi 8 confocal microscope. Rhodamine B isothiocyanate was excited with an OPSEL laser at 552 nm, and the fluorescence emission was acquired using a hybrid SMD detector with a 572–612 nm spectral window. Dry objectives of 20 $\times$ /0.75 and 40 $\times$ /0.60 were used to image all samples, which were placed in an 8-well device (Lab-Tek Chambered 1.0 Borosilicate Coverglass System, Nalge Nunc International). All samples, containing a total of 0.01% w/w of rhodamine-labelled PEG-g-PVAc, were observed one day after preparation.

## 2.6. Confocal Raman microscopy

Raman spectra were acquired with a Renishaw inVia Qontor Confocal Raman Microscope, using a 532 nm excitation laser (Nd:YAG solid state type, 50 mW, 1800 l/mm grating, working at 50% intensity), and a Renishaw Centrus 1UTR57 detector. A dry objective 50Lx/0.5 was employed for all samples, which were previously deposited on a silicon substrate to avoid detecting Raman scattering from the background and covered with a glass slide to delay water evaporation. Samples were observed with the front-illuminated CCD camera (256  $\times$  1024 px). The exposure time was set to be 10 s for individual spectra acquisitions and 1 s for the two-dimensional intensity maps, which we rebuilt from spectra acquired every 0.5  $\mu$ m along both x and y directions. The confocal microscope (Leica DM 2700) was crucial to peak the regions of interest for acquiring the Raman spectra of the medium, the LLPS microdomains, and the precipitated copolymer. High-confocality mode was used for all measurements.

Since N45-7 and PEG-g-PVAc share some functional groups, their Raman profiles are similar (Fig. S8), with main peaks in the region between 2800 and 3100  $\text{cm}^{-1}$  (corresponding to C–H bonds' stretching). Therefore, the identification of N45-7 is challenging in an environment rich in PEG-g-PVAc. The largest difference between the two profiles is found on the shoulder of N45-7 at 2852  $\text{cm}^{-1}$ . On the other side, the relatively intense peak at 1730  $\text{cm}^{-1}$  arises from the C=O vibration of the ester group of PVAc, clearly not present for N45-7. Based on these differences, we were able to distinguish the presence of PEG-g-PVAc from N45-7.

A set of reference samples for PEG-g-PVAc in water (5, 10, 15, 20, 25% w/w) and N45-7 in water (3, 6, 9, 12, 15% w/w) were mea-

**Table 1**

Initial concentrations of the five samples used to study the phase behavior of PEG-g-PVAc (Fig. 4A), and corresponding concentrations of NaCit necessary to achieve LLPS and liquid-liquid bulk phase separation.

Initial concentrations (%) w/w)		NaCit for LLPS (%) w/w)	NaCit for bulk phase separation (%) w/w)
PEG-g-PVAc	N45-7		
1.0	1.0	4.8 $\pm$ 0.2	10.7 $\pm$ 0.5
1.0	5.0	3.8 $\pm$ 0.2	9.9 $\pm$ 0.5
1.0	10	2.4 $\pm$ 0.1	9.1 $\pm$ 0.5
1.0	15	1.0 $\pm$ 0.1	8.3 $\pm$ 0.4
1.0	20	/	7.4 $\pm$ 0.4

sured through confocal Raman microscopy to get the calibration curves and assess the PEG-g-PVAc/water and N45-7/water mass ratios, respectively. At least five spectra were acquired for each sample, focusing the beam on different spatial spots. To build the calibration curves, three peaks from the Raman profiles of the PEG-g-PVAc solutions (1730, 2893, and 2940  $\text{cm}^{-1}$ ) and three peaks from the N45-7 solutions (2852, 2887, and 2922  $\text{cm}^{-1}$ ) were considered. After normalizing all Raman spectra to the peak of water (at 3420  $\text{cm}^{-1}$ ), the intensities of each of the three peaks selected for PEG-g-PVAc and each of the three peaks selected for N45-7 were determined. The average intensity value of each sample, together with its standard deviation, was used to plot the calibration curves, reported in Fig. S9 (for PEG-g-PVAc) and Fig. S10 (for N45-7). These curves were then used to infer the PEG-g-PVAc/water and N45-7/water mass ratios of other samples. It is important to notice that the values obtained are not absolute but relative – ratios of PEG-g-PVAc/water and N45-7/water.

### 3. Results and discussion

#### 3.1. Effect of additives on the CPT of PEG-g-PVAc

The selected PEG-g-PVAc is characterized by a critical temperature of 51 °C, found for 1% w/w in water (Fig. S1). This concentration was chosen to investigate the effect of selected additives. Specifically, we addressed the effect of fourteen components, commonly used in home and beauty care products. These can be divided into four groups: (1) kosmotropes, (2), chaotropes, (3) non-ionic surfactants, and (4) ionic surfactants. The first group consists of sodium citrate (NaCit), sodium chloride (NaCl), and sodium bromide (NaBr). The second group covers sodium cumene sulfonate (NaCS), monopropylene glycol (MPG), and urea. Then, the third group includes Neodol 45–7 (N45-7) and Surfonic L24-9 (L24-9). Finally, the fourth group encompasses both cationic (CTAB, CTAC, DTAB, and DTAC), and anionic (HLAS, and AE3S) surfactants (see Fig. 1 for chemical structures). The evolution of the CPT of 1% w/w PEG-g-PVAc as a function of the w/w% concentration of these additives is plotted in Figs. 2 and 3.

The kosmotropic additives of group 1 decrease the CPT as their concentration raises, with their effectivity following the order NaCit > NaCl > NaBr. The same order is followed when their concentration is reported in mol/L (Fig. S2). It is known that both anions and cations are responsible for shifts in the CPT [55], but since the cation is shared among these salts, the different effect over the CPT is ascribed to the anions. According to the Hofmeister series and the salting-out effect [56], their different activity is related to their hydration. It is generally accepted that highly hydrated kosmotropes are “water structure-makers”, increasing water surface tension and decreasing the solubility of other solutes [57]. Indeed, it has been observed that kosmotropes affect the solvency of the ethylene oxide groups of Pluronics [58]. This is in line with our findings, with NaCit being the most effective additive to reduce the CPT. Some studies have justified the decreased solvency by a direct interaction of the anions with the solute and its hydration shell [55,59]. Other recent studies suggest that the effect of these anions should not be defined only by their influence on the structure of water, since the same effects have been observed in non-aqueous solvents [60,61]. Therefore, a more complex interplay between water, PEG-g-PVAc, and kosmotropic agent should be considered to understand our observations on the evolution of the CPT of PEG-g-PVAc. Assuming the salt effects over the CPT follow a linear trend, a concentration of 5.1% w/w (0.17 mol/L) of NaCit or 13.3% w/w (2.28 mol/L) of NaCl would be needed to phase separate 1% w/w of PEG-g-PVAc in water at room temperature.

Remarkably, the extrapolated concentration for NaCit agrees with the experimental results summarized later in Fig. 4.

On the other side, all the compounds from group 2 increase the CPT of PEG-g-PVAc as their concentration is raised, with their effectivity following the order of NaCs > urea > MPG. The same order is obeyed when measured in mol/L (Fig. S2). The different effect of these chaotropes on the CPT is due to a combination of both direct and indirect interactions with PEG-g-PVAc. At variance with kosmotropic additives, we observe a non-linear trend of the CPT for NaCS concentrations below 1% w/w (Fig. 2). This non-linear behavior, previously reported for other chaotropes on PNIPAM [59], can be related to a direct binding of the anion to the copolymer, which results in a solubility increase of the latter (ion-specific effect). After saturation of the available binding sites, the effect on the CPT would solely arise from anion-water interaction, in agreement with a linear dependence. For concentrations above 1% w/w, the chaotropic effect of NaCS can be explained by its low charge density, which translates into a reduced interaction with water and hence in a salting-in effect. Studies on the effect of NaCS over the CPT of non-ionic molecules are rather scarce, but some years ago a similar effect was observed on the CPT of an ethoxylated non-ionic surfactant (Neodol 25-9) [62].

The effect of urea on the water solubility of other species has been extensively described and discussed in the literature. It is reported that its effect arises from two main mechanisms: (i) a direct one, in which the formation of hydrogen bonds between urea and water around the hydrophobic portions of the solute cause the displacement of some water molecules from the hydration shell, resulting in a solubility increase; and (ii) an indirect one, whereby urea acts as a water structure breaker, facilitating the hydration of the solute [57,63,64]. Although the latter has been widely accepted, several studies have proved the direct interaction of urea with the solute to be the major contribution to its increased solubility. Some have suggested that urea binds to the hydrophobic regions of the copolymer, weakening the hydrophobic interaction [65], while others have proved that urea preferentially interacts with the PEG blocks of Pluronics, enhancing its solubility [66–68]. Regarding the effect of urea over PEG-g-PVAc, the increase of its CPT is likely due to a combination of both a direct interaction of urea with the copolymer and an indirect one coming from the effect of urea over water solubility.

MPG is a short-chain alcohol, whose effect on the CPT also arises from a combination of an indirect mechanism, making the medium less polar and decreasing the amphiphilicity of the copolymer, and a direct one, due to MPG's interactions with the hydrophobic blocks of the copolymer, which loosens the hydrophobic interaction between grafted chains [57,69,70].

The other components that decrease the CPT are the non-ionic surfactants N45-7 and L24-9. Their basic features are summarized in Table 2. In line with the components of group 1, the addition of either N45-7 or L24-9 causes a depression of the CPT (Fig. 2) since both promote copolymer-copolymer over copolymer-medium interactions. This can be attributed both to the effect of these surfactants in dropping water solvency and to the formation of copolymer-surfactant complexes due to hydrophobic interactions [77,78]. Indeed, previous works have justified this CPT depression with the presence of copolymer-surfactant mixed micelles interconnected through polymer chain bridging [79–82], also known as the “necklace and bead” model. In regard, the depression of the CPT of PEG-g-PVAc should be mainly coming from hydrophobic interactions between the non-ionic surfactant and the PVAc grafts of the copolymer. There is, however, some interaction between the non-ionic surfactant and the PEG backbone that is likely collaborating on the depression of the CPT too, as previously demonstrated [83].



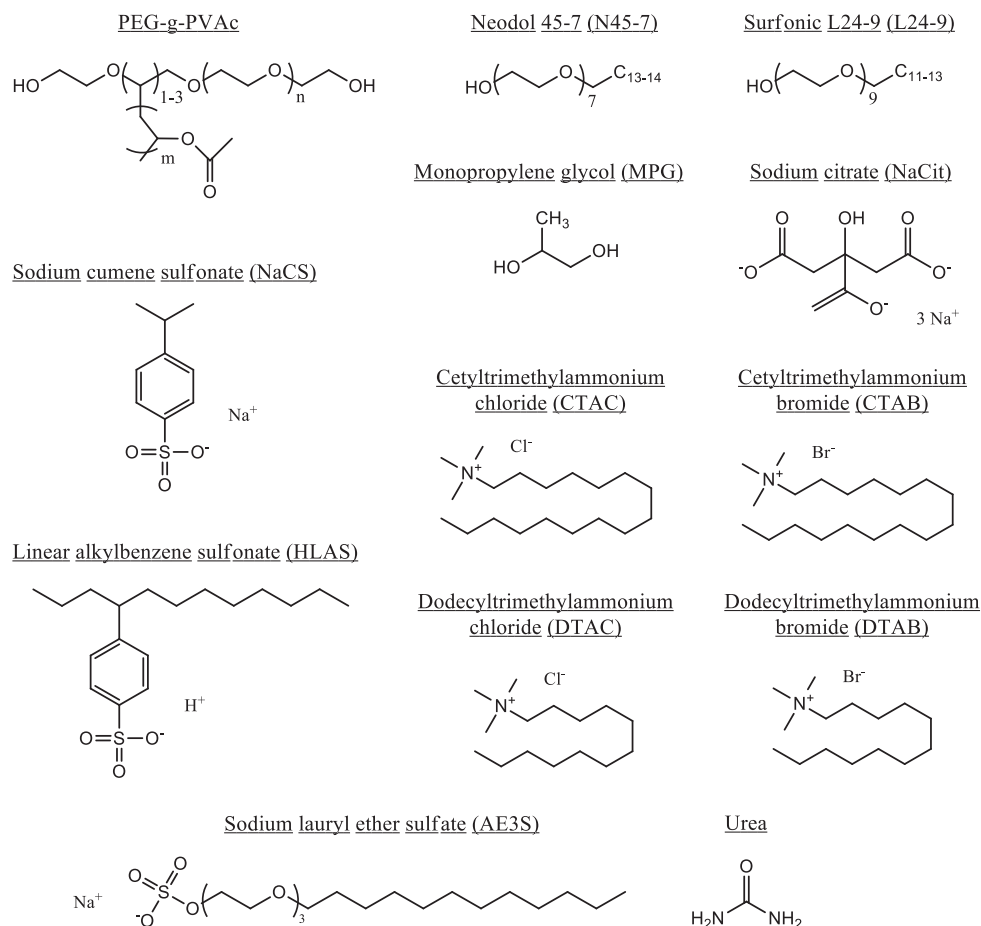


Fig. 1. Chemical structure of the components used throughout this study.

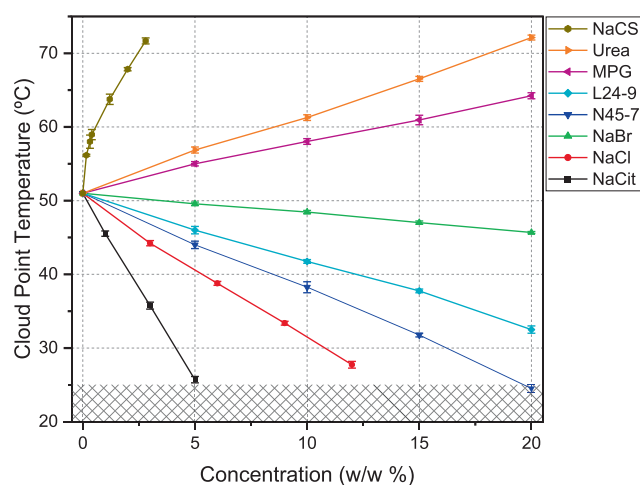


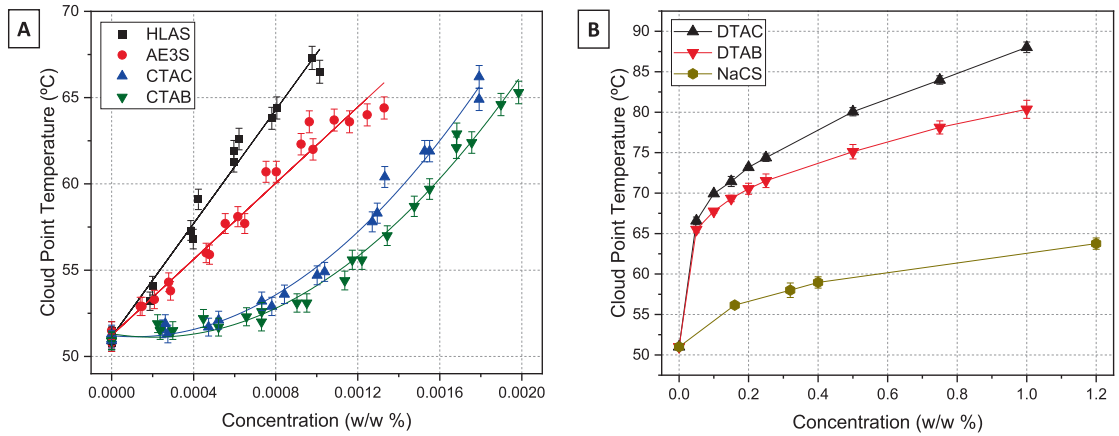
Fig. 2. Effect of kosmotropes, chaotropes, and non-ionic surfactants on the CPT of 1% w/w PEG-g-PVAc in water measured through turbidimetry. A patterned region has been added to indicate where LLPS at room temperature would be expected. Each point represents the average of at least three measurements. Some of the error bars are not visible because they fall within the data points drawn.

The higher effectiveness of N45-7 over L24-9 in decreasing the CPT of PEG-g-PVAc can be explained by its shorter ethoxy chain, resulting in a weaker interaction with water, thus a higher association with the copolymer and an enhanced stability of the copolymer-surfactant complex [84]. This lower hydrophilicity is consistent

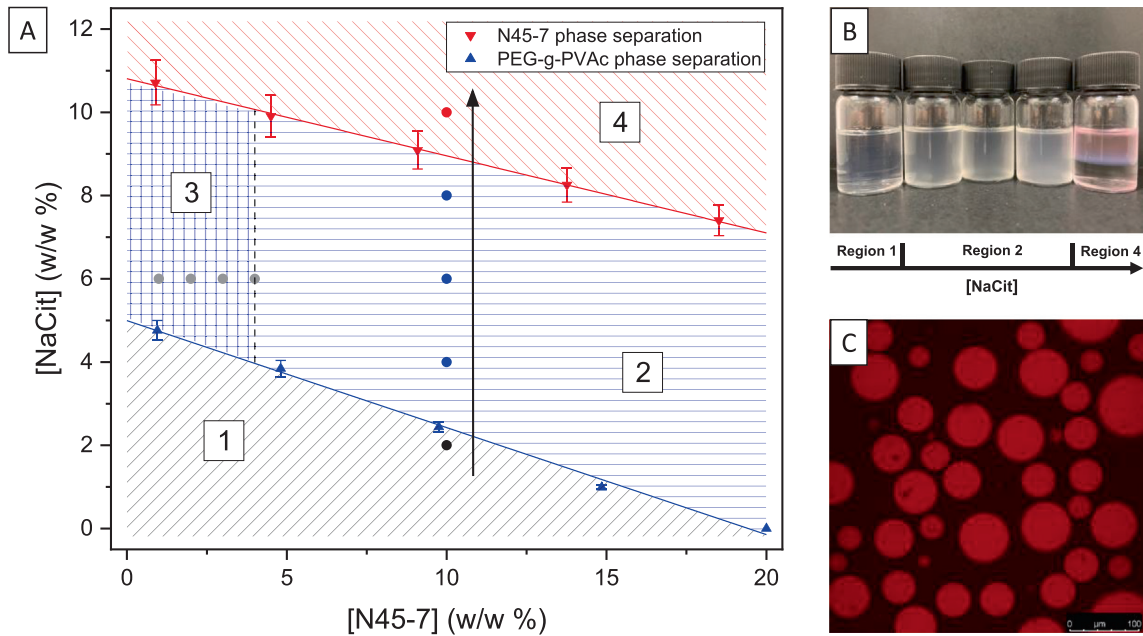
with the reduced CMC (Table 2) and LCST of N45-7, the latter being approximately 24 °C lower than that of L24-9 (Fig. S1). Additionally, the fact that the mixture of PEG-g-PVAc and either of these non-ionic surfactants presents CPT values below those of the two individual components (Fig. S1) proves the associative copolymer-surfactant interaction.

In some copolymer-surfactant systems, a low concentration of surfactant leads to the formation of mixed micelles, causing a decrease in the CPT, while a higher concentration of surfactant promotes the formation of surfactant micelles around single hydrophobic chains of the copolymer, increasing back the CPT [84–86]. This effect causes a minimum in the trend of the CPT as the concentration of surfactant increases. However, in the concentration range selected for this work, the CPT decreases linearly with the addition of the two non-ionic surfactants (Fig. 2). This behavior could be ascribed to a higher affinity between PVAc blocks than between PVAc and non-ionic surfactant. The extrapolation of their linear trend allows predicting that a concentration of 19.8% w/w (0.363 mol/L) of N45-7 or 28.9% w/w (0.485 mol/L) of L24-9 is needed to phase separate 1% w/w of PEG-g-PVAc in water at room temperature (see Fig. S2 for concentrations in mol/L). Remarkably, the extrapolated concentration for N45-7 agrees with the experimental results summarized later in Fig. 4.

The last group of additives includes six ionic surfactants, consisting of four cationics (CTAB, CTAC, DTAB, and DTAC), and two anionics (HLAS and AE3S). Among them, DTAB, DTAC, HLAS, and AE3S share the same alkyl chain length (12 carbon atoms) while CTAB and CTAC have a longer alkyl chain (16 carbon atoms). Their



**Fig. 3.** Effect of ionic surfactants on the CPT of 1% w/w PEG-g-PVAc in water, measured through turbidimetry, including (A) the two anionic surfactants (HLAS and AE3S) plus the two cationic surfactants with a longer alkyl chain (CTAC and CTAB), and (B) the two cationic surfactants with a shorter alkyl chain (DTAC and DTAB) plus NaCS. Each point in A corresponds to a single measurement due to the difficulty in obtaining the same low concentrations, while each point in B corresponds to the average of three measurements. Some of the error bars are not visible because they fall within the data points drawn.



**Fig. 4.** An aqueous solution of 1% w/w PEG-g-PVAc shows four different behaviors depending on the concentrations of both NaCit and N45-7 (A): in region 1, the copolymer is dissolved in the medium, showing a monophasic system; in region 2, LLPS of the copolymer takes place giving rise to kinetically stable microdomains; in region 3, LLPS microdomains are distinguished together with copolymer precipitation; finally, in region 4, liquid-liquid bulk phase separation takes place with the copolymer being found in the top phase. Error bars correspond to the cumulative instrumental error. In (B), five samples with constant concentrations of PEG-g-PVAc (1% w/w) and N45-7 (10% w/w), but different concentrations of NaCit, are shown to illustrate the optical difference between regions 1, 2, and 4. From left to right, the first sample shows a monophasic system, the next three show a cloudy system, while the last one shows a biphasic system. In the latter sample, rhodamine-labelled PEG-g-PVAc is used to prove its exclusive presence in the top phase. In (C), fluorescence confocal microscopy is employed to observe a sample with 1% w/w rhodamine-labelled PEG-g-PVAc, 10% w/w N45-7, and 5% w/w NaCit in water, presenting LLPS microdomains.

**Table 2**  
Basic characteristics of the eight surfactants used throughout this study. Their CMCs are expressed both in mol/L and % w/w for ease of comprehension.

Surfactant	Alkyl chain length	Ethoxy groups	Charge	CMC ( $10^{-3}$ mol/L)	CMC ( $10^{-3}$ % w/w)
N45-7	14–15	7	/	0.010 [71]	0.55
L24-9	12–14	9	/	0.03 [72]	2
AE3S	≈12	3	–	0.70 [73]	29
HLAS	≈12	/	–	1.2 [74]	40
DTAB	≈12	/	+	15.27 [75]	470.8
DTAC	≈12	/	+	23.00 [75]	607.0
CTAB	≈16	/	+	1.00 [76]	36.5
CTAC	≈16	/	+	1.30 [76]	41.6

CMCs are reported in Table 2. Fig. 3 shows that all six ionic surfactants increase the CPT of PEG-g-PVAc, and the effect is much stronger than that observed for the chaotropic additives (Fig. 2). The same increased solubility was previously reported for a PEG-g-PVAc with a  $M_w = 13,100$  Da [38], and a synergistic interaction has been recently observed at the air-water interface between an anionic surfactant (sodium dodecyl sulfate) and PEG-g-PVAc [48].

Fig. 3A includes the four ionic surfactants that induced the highest increase on the CPT of PEG-g-PVAc. The same graph with concentrations in mol/L can be found in the supplementary (Fig. S3). Their effect over the CPT is remarkable: to detect the CPT increase in a measurable temperature range, concentrations below their CMCs (Table 2) had to be used. The two anionic surfactants (HLAS and AE3S) were slightly more effective in increasing the CPT than the two cationic surfactants (CTAB and CTAC), even though these latter two have a longer alkyl chain. The increase of the CPT follows a linear trendline for anionic surfactants, while a lag phase followed by a rather linear trendline was observed for both CTAB and CTAC. This trendline evolution has been previously reported for other polymer-surfactant systems [87,88]. Unfortunately, a Tyndall effect was noticed once the CPT reached levels above 66 °C, which prevented properly studying the effect of higher concentrations of these ionic surfactants over the CPT.

Fig. 3B shows the effect of the other two cationic surfactants (DTAB and DTAC) over the CPT. These two ionic surfactants increased the CPT of PEG-g-PVAc to a minor degree, which allowed the comparison of their effect over the CPT with NaCS (see Fig. S4 for concentrations in mol/L). Even though the alkyl chain length of these two cationic surfactants is as long as that of HLAS and AE3S, there is a noticeable difference in their effect over the CPT – while around 0.0012% w/w ( $3 \times 10^{-5}$  mol/L) of anionic surfactant is enough to increase the CPT of PEG-g-PVAc to 65 °C, close to 0.05% w/w ( $2 \times 10^{-3}$  mol/L) of DTAB or DTAC is needed to obtain the same increase. It can be observed that the evolutions of the CPT of PEG-g-PVAc as a function of the concentrations of DTAB, DTAC, or NaCS follow a similar trend: parabolic at low concentrations and rather linear at higher ones. No Tyndall effect was observed when using these two cationic surfactants, which permitted assessing the effect of higher concentrations.

These results agree with the literature on the effect of ionic surfactants on the CPT of copolymers from the ethylene oxide family [23,70,89–91]. Generally, a significant increase on the CPT of water-soluble copolymers is observed after the addition of either anionic or cationic surfactants. This phenomenon was justified with the formation of a copolymer-surfactant complex, which can be originated from hydrophobic interactions, following the previously mentioned “necklace and bead” model [92,93], but also from electrostatic ones, considering the charged nature of the ionic surfactants’ headgroups [48,94,95]. This “charged” copolymer-surfactant complex, which could be considered as a polyelectrolyte [96], leads to both a better solvation of the copolymer and a higher electrostatic repulsion between complexes, thus hampering aggregation and consequently raising the CPT.

Following this explanation, the fact that the two anionic surfactants increase more effectively the CPT than any of the other four cationic surfactants suggests that the copolymer-surfactant complex of PEG-g-PVAc with anionic surfactants forms at lower concentrations and possesses a higher solubilizing effect than the complex formed with cationic surfactants, presumably due to a stronger interaction of the sulfate group than the trimethylammonium group with PEG-g-PVAc. Anionic surfactants have proved to be more effective than cationic surfactants in increasing the CPT of other systems as well [58,97,98]. Additionally, assuming again the formation of a copolymer-surfactant complex, a longer alkyl chain provides a higher hydrophobicity to the surfactant and reduces the repulsion factor between close polar head groups, thus

strengthening the interaction between the surfactant and the copolymer [99]. This explains why a longer alkyl chain brings a more noticeable increase on the CPT among the four cationic surfactants studied in this work. The same trend has been previously reported in other polymer-surfactant systems [23,91,97].

Regarding the effect of counterions, the difference between bromide and chloride shows a minimum influence on the effect of CTAB and CTAC over the CPT for the concentration range studied (clearly visible in Fig. S3), in agreement with the literature [97,100]. However, a more noticeable difference is discernible between DTAB and DTAC, which is accentuated at higher concentrations (Fig. 3B). Their different effectivity in increasing the CPT of PEG-g-PVAc can be explained by the different polarizability of bromide and chloride. It is known that chaotropic counterions bind more strongly to the micellar surface than kosmotropic counterions do, hence promoting micellar growth more effectively and reducing the CMC [101–103]. Indeed, the CMC of DTAB is lower than the one of DTAC (Table 2) because bromide interacts more favorably with the positively charged cationic surfactant than chloride does. A stronger binding of the counter-ion means a higher screening of the charged surfactant and thus a lower ionization degree, leading to a smaller solubilizing effect over the copolymer, hence a smaller increase on the CPT.

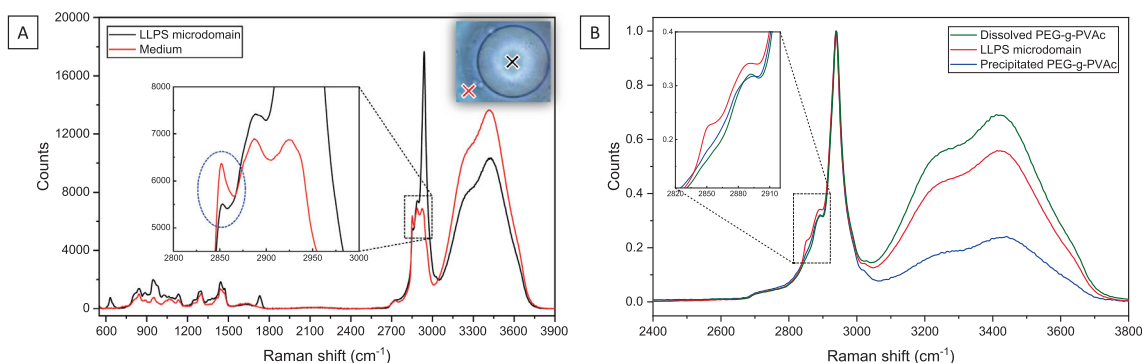
Even though it has not been investigated in the current work, other publications have proved that the addition of salts can counteract the strong effect of ionic surfactants over the CPT of copolymers, up to a point in which they behave like a non-ionic surfactant and decrease rather than increase the CPT [104,105].

### 3.2. LLPS microdomains of PEG-g-PVAc

As observed, kosmotropic salts and non-ionic surfactants decrease the CPT of PEG-g-PVAc. The most effective kosmotrope (sodium citrate) and non-ionic surfactant (Neodol 45–7) were selected to further explore PEG-g-PVAc LLPS when simultaneously present. Fig. 4A presents a mapping of the compositional space of an aqueous solution of 1% w/w PEG-g-PVAc. Four main scenarios occur, depending on the medium composition: homogeneous (monophasic) dispersion (1); formation of LLPS microdomains of PEG-g-PVAc with the absence (2) or presence (3) of precipitated copolymer; and liquid-liquid bulk phase separation (4). The experimental procedure used to assess the boundaries between regions of Fig. 4A is explained in the materials and methods section. In short, NaCit was stepwise added to five samples, each containing 1% w/w PEG-g-PVAc and varying concentrations of N45–7. The blue line separating regions 1 from 2 and 3 marks the appearance of a cloudy suspension, while the upper red line identifies the boundary from cloudy to biphasic.

Since PEG-g-PVAc is an LCST-type copolymer, a monophasic system was expected for low concentrations of NaCit and N45–7, where its CPT is still above room temperature, rendering the copolymer soluble in water. However, after adding enough NaCit and N45–7, liquid-liquid phase separation takes place, caused by the depression of the copolymer's CPT. In these conditions, optical microscopy showed the presence of micron-sized globular LLPS domains (Fig. S5). The difference between regions 2 and 3, both containing micron-sized LLPs domains, stems from the absence or presence of solid copolymer precipitates, respectively. The simultaneous presence of liquid-liquid and liquid-solid phase separation has also been detected and investigated for complex coacervates [106–108].

Interestingly, both additives lower the CPT in ternary systems (Fig. 2), but while the addition of N45–7 gives rise to LLPS microdomains, NaCit induces liquid-solid phase separation. We ascribe the different effects of these two additives to a higher degree of copolymer desolvation in the presence of NaCit, as later shown in



**Fig. 5.** The sample made of 1% w/w PEG-g-PVAc, 5% w/w NaCit, and 10% w/w N45-7 in water presented LLPS microdomains. In (A), Raman profiles of an LLPS microdomain (black) and the medium (red). The inset shows the three N45-7 diagnostic peaks, highlighting the different intensity of the  $2852\text{ cm}^{-1}$  one. An optical microscope picture of an LLPS microdomain is enclosed at the top right corner of this figure, marking the spots from where the two Raman spectra were acquired. In (B), the Raman profiles of different samples containing PEG-g-PVAc are normalized at  $2940\text{ cm}^{-1}$  for comparison. The Raman profile of the precipitated copolymer from a sample of 1% w/w PEG-g-PVAc and 6% w/w NaCit in water appears in blue, the averaged profile of several LLPS microdomains is in red, while the one of 25% w/w PEG-g-PVAc in water is in green. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

**Fig. 5B.** This also indicates that N45-7 is necessary to form LLPS microdomains. Moreover, if the concentrations of both PEG-g-PVAc (1% w/w) and NaCit (6% w/w) are kept constant and the dispersion is titrated with N45-7, a visible decrease of the amount of solid PEG-g-PVAc is observed (Table 3, plotted as grey dots on region 3 of Fig. 4A). The absence of PEG-g-PVAc precipitation is found from a concentration of 4% w/w N45-7.

According to the linear boundary separating region 1 from regions 2 and 3, 5% w/w of NaCit or 19.6% w/w of N45-7 is needed to phase separate PEG-g-PVAc. These two approximated concentrations are very close to the values obtained from the results of Fig. 2 (these being 5.1% w/w and 19.8% w/w, respectively). The fact that this transition boundary follows a linear regression suggests that the combined effect of NaCit and N45-7 over the CPT of PEG-g-PVAc is not synergistic but rather the sum of their individual effects.

To understand the role of NaCit to obtain the different regions of Fig. 4A, the phase behavior of five samples with constant concentrations of PEG-g-PVAc (1% w/w) and N45-7 (10% w/w), but different concentrations of NaCit (Table 3), was assessed. A day after sample preparation, three different scenarios could be distinguished in this set of samples (Fig. 4B). The sample with 2% w/w of NaCit was transparent (region 1), the samples with 4, 6, and 8% w/w of NaCit were cloudy (region 2), while the sample with 10% w/w of NaCit was showing a liquid-liquid bulk separation (region 4). Rhodamine-labelled PEG-g-PVAc was used in the sample presenting bulk phase separation with the aim to find out its distribution. As observable, PEG-g-PVAc could only be distinguished in the top phase. This phenomenon is attributed to the kosmotropic-induced phase separation of the non-ionic surfactant,

since a sample of 1% w/w PEG-g-PVAc without N45-7 did not present this liquid-liquid bulk phase separation at high NaCit concentrations (12% w/w), while a sample of N45-7 (10% w/w) and NaCit (10% w/w) without PEG-g-PVAc did present this liquid-liquid bulk phase separation.

To further study the role of the kosmotropic effect on the formation of LLPS microdomains, another set of five samples with a fixed concentration of PEG-g-PVAc (1% w/w) and different concentrations of NaCl was studied (Fig. S6). After adding enough N45-7 to induce LLPS, PEG-g-PVAc microdomains were successfully obtained in all five samples, which presented the same morphological features as the ones obtained with NaCit. These results demonstrate that NaCit is not specifically needed to obtain LLPS microdomains, but just a kosmotrope (such as NaCl) to decrease the CPT of PEG-g-PVAc and allow their formation at lower temperatures.

To demonstrate the presence of PEG-g-PVAc in these microstructures, an aqueous solution with 1% w/w PEG-g-PVAc (rhodamine-labelled), 5% w/w NaCit, and 10% w/w N45-7 was observed through fluorescence confocal microscopy (Fig. 4C). This instrument allowed the distinction of well-defined globular structures, with a typical diameter around  $50\text{ }\mu\text{m}$ . The strong red fluorescence of the LLPS microdomains indicates the enrichment of PEG-g-PVAc. Moreover, the copolymer appears to be homogeneously distributed within these microdomains, not following a core-shell structure. Due to the resolution of this technique, particles with a size below  $\sim 500\text{ nm}$  cannot be distinguished. However, considering previous studies [36], the presence of sub-micron structures made of PEG-g-PVAc and/or N45-7 is likely. It is relevant to add that these LLPS microdomains are kinetically stable, and thus sedimentation and coalescence will take place over time. For this specific sample, the microphase separation was stable for at least five days at room temperature. The kinetic stability of this system can be extended by either increasing the concentration of surfactant or adding a structuring agent, which consequently increases the sample's viscosity and slows down the diffusion of these microstructures. Additional fluorescence confocal microscopy pictures of these LLPS microdomains can be found in the [supplementary information](#) (Fig. S7).

### 3.3. Confocal Raman microscopy analysis of PEG-g-PVAc LLPS microdomains

Fluorescence confocal microscopy (Fig. 4B and Fig. S7) suggests that the LLPS microdomains contain PEG-g-PVAc. However, this technique cannot determine the distribution of N45-7 nor assess

**Table 3**

Composition of the two sets of samples used to determine the limit between regions 2 and 3 (minimum of N45-7), as well as to show the visual differences between regions 1, 2, and 4.

[N45-7] (% w/w)	[NaCit] (% w/w)	Sample state
1	6	Extended precipitation + LLPS microdomains
2	6	Precipitation + LLPS microdomains
3	6	Scarce precipitation + LLPS microdomains
4	6	LLPS microdomains
10	2	Monophasic
10	4	LLPS microdomains
10	6	LLPS microdomains
10	8	LLPS microdomains
10	10	Liquid-liquid bulk phase separation



the presence of water in these microstructures. Therefore, we selected a representable LLPS sample (1% w/w PEG-g-PVAc, 5% w/w NaCit, and 10% w/w N45-7 in water) for further investigation through confocal Raman microscopy. Reference Raman spectra of pure PEG-g-PVAc and N45-7 can be found in the [supplementary information](#) (Fig. S8). As expected, the Raman spectrum obtained from the LLPS microdomains differs with respect to the one from the suspending medium (Fig. 5A). The presence of PEG-g-PVAc (main peak at  $2940\text{ cm}^{-1}$ , C—H stretching), water (broad peak around  $3420\text{ cm}^{-1}$ , O—H stretching), and N45-7 (little shoulder around  $2852\text{ cm}^{-1}$ , C—H stretching) can be appreciated for LLPS microdomains, while the medium shows mainly the presence of N45-7 (with its three diagnostic peaks at 2852, 2887, and  $2922\text{ cm}^{-1}$ , originating from different C—H stretching modes), and water. We cannot rule out the presence of PEG-g-PVAc dissolved in the medium, but its occurrence does not emerge in this Raman analysis. From these results, we can qualitatively conclude that the LLPS microdomains show an enrichment of PEG-g-PVAc, in line with fluorescence confocal microscopy, while N45-7 is slightly more concentrated in the medium, as the comparison of the peaks' intensities at  $2852\text{ cm}^{-1}$  indicates (inset of Fig. 5A).

Since all the diagnostic peaks from N45-7 are in a spectral range where also PEG-g-PVAc presents Raman scattering, the accurate detection of the non-ionic surfactant in the microdomains is challenging. To address this issue, at least fifty Raman spectra from the LLPS microdomains, each normalized with respect to the main peak of PEG-g-PVAc ( $2940\text{ cm}^{-1}$ ), were averaged and compared to those of two reference samples: (i) 25% w/w PEG-g-PVAc in water, and (ii) precipitated copolymer from 1% w/w PEG-g-PVAc and 6% w/w NaCit (Fig. 5B). The spectrum of pure PEG-g-PVAc can be found in Fig. S8 for comparison. The Raman profiles of these three samples presented some obvious differences in the region of the O—H stretching of water (between  $3000$  and  $3700\text{ cm}^{-1}$ ) and C—H stretching of alkanes (between  $2800$  and  $3100\text{ cm}^{-1}$ ). Regarding the water region of the spectra, the LLPS microdomains still have a high content of water, as highlighted by a comparison with the reference samples (PEG-g-PVAc dissolved in water and precipitated). This result is expected and in line with other reports pointing that coacervates have a high content of water [109,110]. Moreover, the decreased water content of the precipitated copolymer sample suggests that this liquid-solid phase separation is ascribed to a higher desolvating effect of NaCit than N45-7, as previously commented for the results of Fig. 4. For the alkane region, the spectra from 25% w/w PEG-g-PVAc in water and that from precipitated PEG-g-PVAc are very similar. However, when comparing the averaged spectrum obtained from the LLPS microdomains to these two other profiles, we can distinguish a shoulder around  $2852\text{ cm}^{-1}$  and an increased intensity around  $2887\text{ cm}^{-1}$  (inset of Fig. 5B). These differences are attributed to the presence of N45-7 inside the LLPS microdomains.

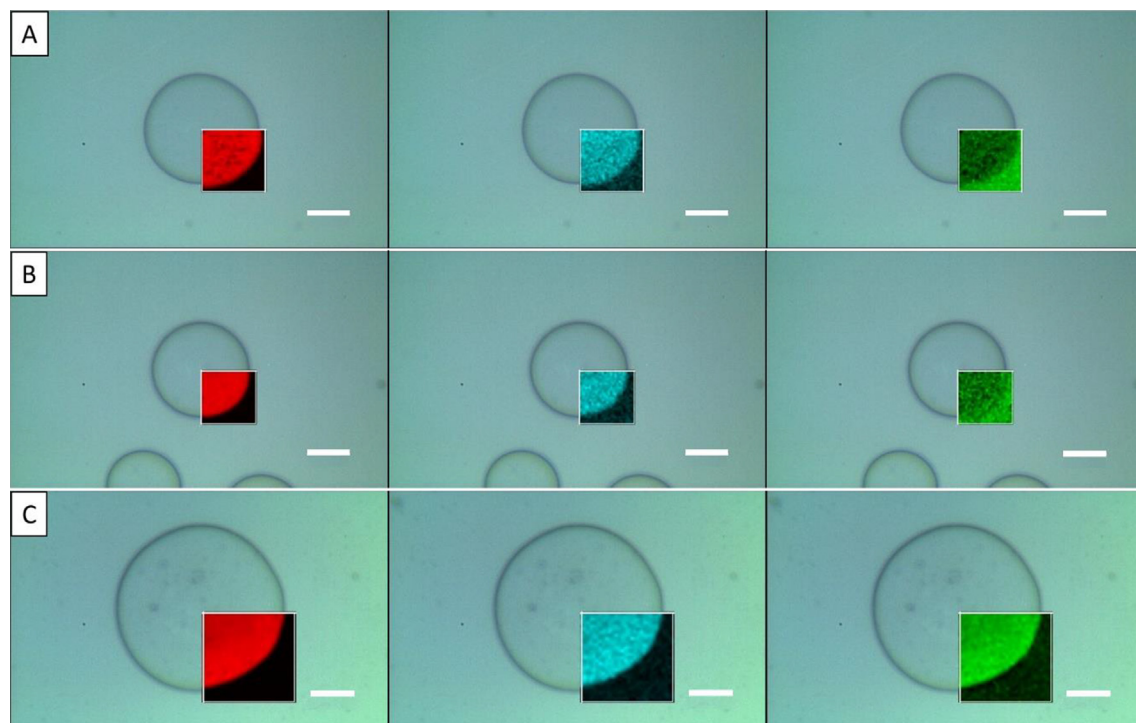
To better highlight the partition of N45-7 in the microdomains, a two-dimensional Raman intensity map was performed on the aqueous solution containing 1% w/w PEG-g-PVAc, 5% w/w NaCit, and 10% w/w N45-7 (Fig. 6A). The collection of Raman spectra was processed to obtain three different intensity distributions: one from the most intense Raman peak of PEG-g-PVAc ( $2940\text{ cm}^{-1}$ ), one from the ester peak of PEG-g-PVAc ( $1730\text{ cm}^{-1}$ ), and one from the N45-7 peak ( $2852\text{ cm}^{-1}$ ) – which appear in red, blue, and green, respectively, in Fig. 6A. The intensity measured for PEG-g-PVAc at  $2940\text{ cm}^{-1}$  can include a contribution from N45-7, which also shows Raman scattering in this region, while the intensity at  $1730\text{ cm}^{-1}$  is selective for PEG-g-PVAc. These intensity maps show clearly that the microdomains are rich in PEG-g-PVAc, in line with the fluorescence confocal microscopy images. On the other side, the intensity map at  $2852\text{ cm}^{-1}$  (Fig. 6A, green profile) indicates a higher concentration of N45-7

in the medium than in the LLPS microdomains, which agrees with the spectral profiles of Fig. 5A. It must also be noted that both PEG-g-PVAc and N45-7 have Raman scattering at  $2852\text{ cm}^{-1}$ . Therefore, the (green) intensity map of this emission wavelength originates both from the copolymer and the non-ionic surfactant. However, as seen from the distributions at  $2940$  and  $1730\text{ cm}^{-1}$  (in red and blue, respectively), PEG-g-PVAc is mainly concentrated inside the LLPS microdomains. Therefore, we can assume that the intensity contribution in the medium is mainly from N45-7.

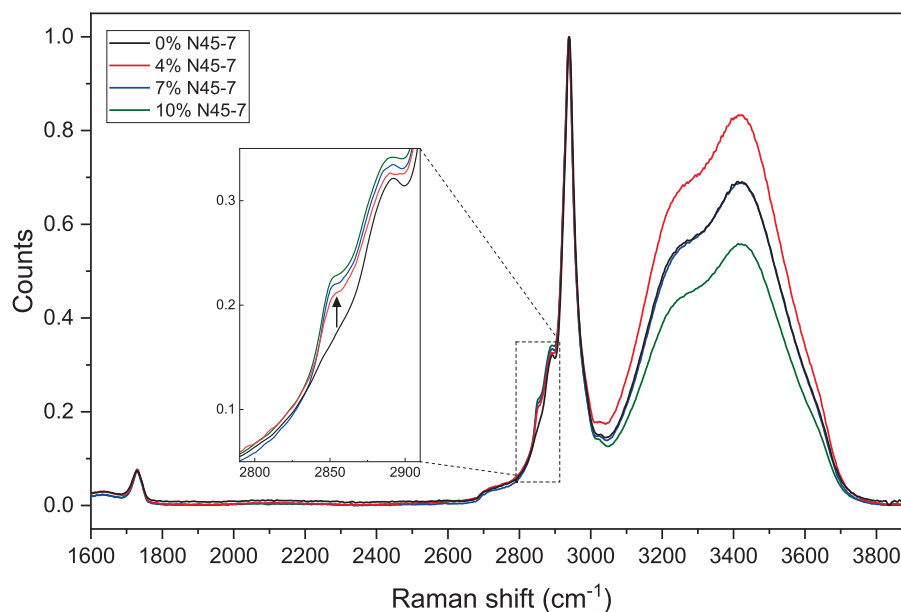
To better understand the effect of N45-7, we assessed two additional samples with 7% w/w and 4% w/w N45-7 (Fig. 6B and 6C, respectively), keeping unvaried the concentrations of PEG-g-PVAc (1% w/w) and NaCit (5% w/w). These intensity maps confirm again the localization of PEG-g-PVAc in the LLPS microdomains. Interestingly, the main difference between this series of samples is the intensity map at  $2852\text{ cm}^{-1}$  (green), presenting either a higher, similar, or lower intensity in the LLPS microdomains with respect to the medium for the samples with 4%, 7%, and 10% w/w of N45-7, respectively. Clearly, the more N45-7 in the formulation, the higher its presence in the medium rather than in the LLPS microdomains. This trend suggests that the N45-7/PEG-g-PVAc mass ratio might not vary much between LLPS microdomains, regardless of the bulk N45-7 concentration.

To address this point, the averages of at least fifty Raman spectra from the LLPS microdomains of the 7% and 4% w/w N45-7 samples, normalized with respect to the main peak of PEG-g-PVAc ( $2940\text{ cm}^{-1}$ ), were compared with: (i) the averaged spectrum of the LLPS microdomains in the 10% w/w N45-7 sample, and (ii) a reference spectrum of 25% w/w PEG-g-PVAc in water (Fig. 7). This comparison highlights once more the presence of the shoulder around  $2852\text{ cm}^{-1}$  in the microdomains, not present for 25% w/w PEG-g-PVAc in water (inset of Fig. 7). However, the intensity increase provided by N45-7 is small for all three concentrations, hence the amount of N45-7 in the LLPS microdomains must be low. Bearing this in mind, it can be assumed that the Raman intensity obtained from the inside of the LLPS microdomains in the intensity maps measured at  $2852\text{ cm}^{-1}$  is mainly coming from PEG-g-PVAc, not from the N45-7 (shown in green in Fig. 6). Furthermore, considering the closeness between the Raman profiles of these three samples in the alkane region (between  $2800$  and  $3100\text{ cm}^{-1}$ ), we can conclude that the N45-7/PEG-g-PVAc mass ratio inside the LLPS microdomains is very similar for the three different N45-7 concentrations. Therefore, an increased N45-7 concentration does not result in its noticeable enrichment inside the LLPS microdomains. The same behavior was described for coacervates made of  $\text{C}_{12}\text{E}_{10}$  non-ionic surfactant, where its concentration in the coacervate phase changed very little with increasing its initial concentration [111]. In addition, a semi-quantitative assessment of the N45-7/PEG-g-PVAc mass ratio in the LLPS microdomains yielded an approximate value between 0.1 and 0.2 (Fig. S9).

Interestingly, the most noticeable difference between the three samples of Fig. 7 is the water/PEG-g-PVAc content of the microdomains, which decreases as the total concentration of N45-7 increases. This indicates the non-ionic surfactant to be causing a decrease in the hydration within LLPS microdomains. A similar effect was reported for NaCl on the water content of simple coacervates [109]. This relates to the cloud point trend since, as previously shown in Fig. 2, the CPT decreases as the concentration of non-ionic surfactant increases. The lower the copolymer's CPT, the lower its affinity for water, which correlates with these Raman profiles showing a lower amount of water inside the PEG-g-PVAc LLPS microdomains as more N45-7 is in solution. This suggests that by controlling the amount of non-ionic surfactant in solution we can modulate the hydrophilicity of the LLPS microdomains and, thus, their affinity for actives with different hydrophobicity.



**Fig. 6.** Two-dimensional intensity maps obtained from the Raman intensity of 1% w/w PEG-g-PVAc, 5% w/w NaCit, and 10% (A), 7% (B), or 4% (C) w/w N45-7 in water. The red distribution represents the Raman intensity measured at  $2940\text{ cm}^{-1}$ , the blue represents the intensity at  $1730\text{ cm}^{-1}$ , and the green represents the intensity at  $2852\text{ cm}^{-1}$ . The scale bar is equivalent to  $20\text{ }\mu\text{m}$  in all pictures. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)



**Fig. 7.** Averaged Raman profiles of the LLPS microdomains from three different samples consisting of 1% w/w PEG-g-PVAc, 5% w/w NaCit, and 4 (red), 7 (blue), or 10% (green) w/w N45-7 in water. The reference Raman profile of 25% w/w PEG-g-PVAc in water (black) is added to notice the effect of N45-7, which is marked with a vertical black arrow in the inset. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Finally, aiming to roughly quantify the relative amounts of PEG-g-PVAc and N45-7 in the LLPS microdomains and the medium, a set of reference samples of PEG-g-PVAc in water (Fig. S10) and another set of N45-7 in water (Fig. S11) were used to obtain calibration curves for the PEG-g-PVAc/water and N45-7/water mass ratios, respectively. Taking advantage of their linear trend, the

PEG-g-PVAc/water and N45-7/water mass ratios from the references were extrapolated to the three studied samples with different N45-7 concentrations (Table 4). The extrapolation is described in detail in the materials and methods section. Even though these calibration curves cannot be used to obtain absolute concentration values, they allow determining that all studied LLPS microdomains

have a PEG-g-PVAc/water mass ratio of at least 0.25, which is equivalent to a sample made of 20% w/w PEG-g-PVAc in water. This outcome agrees with the results shown in Fig. 7, in which the sample containing 7% w/w N45-7 has an identical Raman profile with respect to 25% w/w PEG-g-PVAc in water, suggesting that the PEG-g-PVAc/water ratio of these two samples is very similar. Considering that all samples were prepared with an initial PEG-g-PVAc bulk concentration of 1% w/w, the concentration of copolymer in the LLPS microdomains is at least twenty times higher. The increase of the PEG-g-PVAc/water mass ratio in the LLPS microdomains at higher N45-7 bulk concentrations is attributed to the decreased hydration, as previously commented for the results of Fig. 7. The results of Fig. 5A and Fig. 6 let us assume that the amount of PEG-g-PVAc dissolved in the medium is negligible. Consequently, the extrapolation from these calibration curves also allows concluding that the N45-7/water mass ratio in the medium is practically the N45-7 initial bulk ratio in each of the three different samples (Table 4). The outcomes of the extrapolation are in line with previous observations: (i) the PEG-g-PVAc is highly concentrated in the LLPS microdomains, with a relative concentration over water much larger than the one set during sample preparation, and (ii) a higher N45-7 bulk concentration results in its higher presence in the medium rather than in the LLPS microdomains, as suggested by the intensity maps at  $2852\text{ cm}^{-1}$  (Fig. 6).

#### 4. Conclusions

In this contribution, we addressed the conditions for liquid-liquid phase separation (LLPS) of PEG-g-PVAc (poly(ethylene glycol)-g-poly(vinyl acetate)) in water, to understand and control the formation of LLPS microdomains at room temperature. We determined the effect of several additives over the cloud point temperature (CPT) of PEG-g-PVAc, showing that kosmotropes decrease the CPT ( $\text{NaCit} > \text{NaCl} > \text{NaBr}$ ), while chaotropes increase it ( $\text{NaCS} > \text{urea} > \text{MPG}$ ). The addition of non-ionic surfactants also reduces the CPT, with the most hydrophobic surfactant showing a higher effectivity ( $\text{N45-7} > \text{L24-9}$ ). Conversely, ionic surfactants markedly increase the CPT, with anionic surfactants being the most effective ( $\text{HLAS} \approx \text{AE3S} > \text{CTAC} \approx \text{CTAB} > \text{DTAC} > \text{DTAB}$ ). Similar trends were reported on the CPT evolution of other LCST (Lower Critical Solution Temperature) copolymers [54,55,57,91].

This set of results suggests that a combination of kosmotropes and non-ionic surfactants is optimal to promote the phase separation of PEG-g-PVAc at room temperature. In these conditions, we investigated the phase behavior of PEG-g-PVAc aqueous solutions in presence of N45-7 and NaCit to monitor the formation of LLPS microdomains. We observed that LLPS microdomains can be easily obtained at room temperature, provided that three conditions are met: (i) the CPT of PEG-g-PVAc is decreased below room temperature, (ii) enough N45-7 (at least 4% w/w) is added to avoid PEG-g-PVAc precipitation, and (iii) the concentration of NaCit is kept below a threshold value to prevent liquid-liquid bulk phase separation. These results add up to previous studies on the phase behavior of PEG-g-PVAc [36–38], demonstrating the easy forma-

tion of LLPS microdomains at room temperature in the presence of salts and surfactants. These features make PEG-g-PVAc an interesting candidate for industrial applications, even more considering its proven ability to confine and encapsulate hydrophobic active molecules, such as fragrances [38,46].

Finally, we used confocal Raman microscopy to define for the first time the composition of these LLPS microdomains, finding that their main component is PEG-g-PVAc, but they also contain water and N45-7. Interestingly, the amount of non-ionic surfactant in the microdomains did not significantly change when increasing its bulk concentration. This offers possible benefits for industrial formulations, where it is preferred to keep some surfactant in the medium for cleaning purposes. We also observed that the hydration of the LLPS microdomains decreases as the total concentration of N45-7 increases. This suggests that the amount of non-ionic surfactant can modulate the hydrophilicity of these microdomains, and thus their affinity for actives with different water affinities.

The results here gathered expand our knowledge on self-coacervation of grafted copolymers and define the conditions in which LLPS microdomains can be obtained in a surfactant-rich medium, which can be easily implemented in the body/home care, cosmetic, and food fields. Our findings sum up on the emerging interest to effectively control the formation of LLPS microdomains [112,113] and be used as possible *in vitro* models to mimic membrane-less organelles of living cells [114]. In addition, the comprehension of the formation of PEG-g-PVAc LLPS microdomains also brings directional insights on how to destabilize them, providing the first step towards a future study for the triggered release of any active molecule contained within them.

#### CRedit authorship contribution statement

**Xavier Castellvi Corrons:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Project administration. **Jeremie Gummel:** Conceptualization, Methodology, Resources, Writing – original draft, Supervision. **Johan Smets:** Resources, Funding acquisition. **Debora Berti:** Conceptualization, Methodology, Resources, Writing – original draft, Supervision, Funding acquisition.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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**Table 4**

PEG-g-PVAc/water and N45-7/water mass ratios of 1% w/w PEG-g-PVAc, 5% w/w NaCit, and 4, 7, or 10% w/w N45-7 in water. The initial ratios refer to the ones set during sample preparation for both PEG-g-PVAc and N45-7 over water. The measured ratios, which have been obtained from extrapolation of the calibration curves from Figs. S10 and S11, consist of both PEG-g-PVAc over water in the LLPS microdomains and N45-7 over water in the medium.

N45-7 (% w/w)	Initial ratios (%)		Measured ratios (%)	
	PEG-g-PVAc /water	N45-7 /water	PEG-g-PVAc/water (in LLPS)	N45-7/water (in medium)
4	1.1	4.4	$27 \pm 1$	$4.5 \pm 0.2$
7	1.1	8.0	$35 \pm 2$	$7.7 \pm 0.3$
10	1.2	11.9	$43 \pm 2$	$12.2 \pm 0.4$



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## Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jcis.2022.02.020>.

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