Supramolecular biosolvents made up of self-assembled rhamnolipids: synthesis and characterization

Encarnación Romera-García[1], Ana Ballesteros-Gómez[1]*, Soledad Rubio[1]

[1]Department of Analytical Chemistry, University Institute of Nanochemistry, Faculty of Science, University of Córdoba, Marie Curie Annex Building, Campus of Rabanales, 14071 Córdoba, Spain

∗Corresponding author. Tel.: +34 957 218 644; fax: +34 957 218 644. E-mail address: ana.ballesteros@uco.es.

Abstract

Simple coacervation of surfactants constitutes a powerful bottom-up strategy for the production of tailored supramolecular solvents (SUPRASs), which feature outstanding properties in extraction processes. In this study, we develop for the first time SUPRASs made up from biosurfactants (produced by microorganisms) as a greener alternative to synthetic surfactants. Rhamnolipds (RLs) were selected for this purpose due to their green properties and their high potential for industrial applicability. BioSUPRASs were spontaneously produced at room temperature from aqueous solutions of rhamnolipids (RLs) by salt-induced coacervation (NaCl, Na_2SO_4 or $NH_4CH_3CO_2$). RLs quantitatively incorporated into the bioSUPRAS phase, so that the process had high atom economy. The boundaries for the coacervation region were delimited as a function of RL and salt concentration and equations were derived to predict the volume of bioSUPRAS from the composition of the synthesis mixture. The composition of bioSUPRASs could be tailored by modifying the concentration of the coacervation-inducing salt. BioSUPRAS aggregates were characterized by dynamic light scattering and cryo-scanning electron microscopy and consisted of vesicles in a size range from nm to µm. These aggregates offer a variety of interactions for solute solubilisation (dispersion, ionic, dipole-dipole and hydrogen bonding), different polarity microenvironments (RL head group, RL hydrocarbon chains, vesicle aqueous cavity) and a huge number of binding sites (RL concentration varied from 205 to 444 $g \cdot L^{-1}$). The potential of bioSUPRASs for efficient extraction was illustrated by the recovery of highly polar ionic dyes from water with yields above 94%. The compliance of RL-based bioSUPRASs with the twelve principles of green chemistry is discussed.

Keywords: supramolecular solvent (SUPRAS), biosurfactant, rhamnolipid, salt-induced coacervation, vesicles, extraction.

1. Introduction

The design and production of green solvents with properties that match specific chemical objectives (extraction or purification processes, catalysis, etc.) constitute a strategic priority within the framework of green chemistry.[1] The global market of green solvents, valued at $7 billion in 2018, is estimated to grow at an annual rate of 7.5% in the next decade.[2] Key drivers for this demand are the stringent regulations on VOC emissions, the toxicity of conventional solvents and the volatility of petrochemical prices.[3,4]

Green solvents are commonly defined as those that do not exhibit health, safety, and environmental concerns and that are characterized by a reduced life cycle impact.[5,6] They are expected to meet twelve criteria,[7] although unfortunately, there is not any solvent that fulfils all of them. Intensive research efforts over the two last decades have enabled the synthesis of innovative green solvents (e.g. bio-based solvents, ionic liquids, deep eutectic mixtures, supercritical fluids) and the development of breakthrough applications in organic synthesis, catalysis, biotransformations and/or separations.[8-10] However, there is still a long way to go and many issues need to be satisfactorily resolved (solvent performance, energy-saving synthesis processes, availability, etc.).[7]

Supramolecular solvents (SUPRASs)[11] constitute other suitable alternative to conventional organic solvents for extraction processes. SUPRASs are nanostructured liquids synthesized from the self-assembly and coacervation of amphiphiles through a bottom-up approach (**Figure ESI1)**.[12] SUPRAS synthesis involves, first, the spontaneous *self-assembly* of amphiphiles into three-dimensional aggregates (e.g. micelles or vesicles) above a critical aggregation concentration (*cac*) to generate a colloidal system (**Figure ESI1**). Aggregation of amphiphiles at *cac* is considered a *start-stop* process,[13-15] being the *start* driven by the solvophobic effect[16] and the *stop* arising from the repulsions among amphiphile head groups.[17,18] Secondly, *coacervation* (i.e. separation into two liquid phases in colloidal systems)[19,20] must be produced by the growth of the aggregates in the colloid, and this involves reducing the repulsions among the amphiphilic head groups that stopped aggregation at *cac*.[17] How to achieve this aim mainly depends on amphiphile structure. For ionic amphiphiles, coacervation is accomplished by adding an organic[21] or inorganic[22] counterion or fixing the pH below the pKa of the ionic group.[23] The growth of non-ionic aggregates is mainly driven by increasing the temperature[24] or by adding a poor solvent for the amphiphile that is miscible with the solvation solvent.[25] In all cases,

oily coacervate droplets are spontaneously produced and form clusters that separate as a new colloid-rich phase (coacervate phase or SUPRAS). The coacervate droplets keep as individual entities and are in equilibrium with the bulk solution containing the amphiphile at the *cac* (**Fig. ESI1**). The overall process can be considered an essentially energy-saving synthesis, with 100% selectivity for SUPRAS formation and amphiphile conversions above 90%[26] that can approach 100% by applying strategies to reduce the *cac*.[27]

SUPRASs have long proved unique features for the simultaneous, efficient and fast extraction of organic compounds in a wide polarity range,[28,29] metal ions[30] and proteins[31,32] from both liquid and solid samples. The superior performance of SUPRASs in extraction processes compared to molecular solvents mainly arise from three characteristics.[29] First, the *different polarity microenvironments* present in SUPRAS aggregates (e.g. polar at the head groups and nonpolar at the hydrophobic moieties), which allows the simultaneous extraction of both polar and nonpolar compounds from aqueous media. Secondly, *the multiple binding sites* owing to the huge concentration of amphiphile in SUPRAS (0.1-1 $mg \cdot \mu L^{-1}$). This characteristic, along with the mixed mechanisms available for solute solubilization, allows efficient extractions at low SUPRAS/sample ratios. Thirdly, the *large surface area* of SUPRASs arising from the coacervate droplets that make them up, which enables fast solute mass transfer in extraction processes.

These characteristics have been long exploited for the development of innovative sample treatments in chemical analysis,[11,28,29,33] and, more recently, for the extraction of bioactives from vegetal biomass and agrifood residues[34,35] and for wastewater treatment.[36,37] The reversible character of SUPRAS nanostructures, which are formed through non-covalent interactions, constitutes an excellent opportunity for the production of environment-responsive SUPRASs. This property has allowed the synthesis of SUPRASs with restricted access properties that are able to exclude major matrix interferents[25] and it has been exploited to produce carotenoid oleoresins at a much lower cost than those produced with supercritical fluids.[38]

SUPRAS meet some outstanding green criteria[7] (e.g. use of energy-saving and high atom-economy synthesis processes, exhibition of remarkable performances for some chemical objectives, low volatility and flammability, etc.). However, surfactants used up to date are petrochemical-based (a non-renewable resource) and only partially biodegradable (sometimes producing toxic degradation products), e.g. alcohol and alkylphenyl

ethoxylates, alkanols, alkyl sulphate and sulphonate salts, gemini surfactants, alkyl ammonium salts, etc.).[11] Furthermore, SUPRAS synthesis often requires the use of organic co-solvents, such as methanol or tetrahydrofuran,[26] highly acidic conditions (3-5 M HCl)[23] or high temperature,[24] this compromising their sustainability and hindering large-scale application. Some recent developments have been made by our research group to produce low toxicity SUPRAS which were made up of (synthetic) alkyl-carboxylic acids and fatty alcohols in mixtures of ethanol and water.[35,39,40] However, the use of organic solvent and surfactants from not renewable sources compromised their green properties.

In this study, we propose for the first time the production of supramolecular biosolvents (bioSUPRASs) by coacervation of biosurfactants with green agents. The developed bioSUPRASs are expected to better meet the criteria set for green solvents.[7] Biosurfactants are amphiphilic compounds mainly produced by bacteria, yeasts and fungi.[41] They have interesting properties to be used as SUPRAS ingredients, including low toxicity, biodegradability, high stability in a wide range of pH, temperature and salinity, low *cac*, production from renewable resources and scale-up capacity.[42]

Rhamnolipids (RLs) are produced by bacteria of the genus *Pseudomonas* or *Burkholderia* and consist of one or two L-rhamnose (Rha) residues linked to one or two 3-hydroxyfatty acids of various chain lengths, typically ranging from eight to sixteen.[43,44] Among biosurfactants from microbiological sources, e.g. glycolipids (rhamnolipids, sophorolipids , trehalose lipids and mannosylerythritol lipids) and lipopeptides (surfactin and lichenysin), RLs have been recognized as the best alternatives to synthetic surfactants with a market value of $2.8 billion in 2023.[45] They stand out because of their eco-friendly properties and use in a broad range of products and applications, such as food, pharmaceutical, cosmetics, detergents and cleaning agents, bioremediation, enhanced oil recovery and agriculture.[46,47]

RLs self-assemble into micelles and vesicles in aqueous solutions,[48-50] so they produce colloidal systems above the *cac* and, in principle, they have the potential to undergo coacervation. The liquid-liquid phase separation of biosurfactants in colloidal systems remains virtually unexplored, and to the best of our knowledge, only the coacervation of the glycolipid manosyl-erythritol lipid-A in water has been reported so far.[51] In this paper, the production of bioSUPRASs from RLs under the action of several salts (NaCl, Na_2SO_4 and $NH_4CH_3CO_2$) was investigated. Coacervation regions for the different

bioSUPRASs were delimited and prediction equations for the generated volume under different synthesis conditions were proposed. The chemical composition and physicochemical properties of bioSUPRASs were determined and their nanostructures were characterized. Extraction properties of bioSUPRASs were evaluated using anionic and cationic dyes as model compounds.

2. Material and methods

2.1 Chemicals

All chemicals were used as supplied. The rhamnolipid (RL) employed for bioSUPRAS synthesis (CAS number: 869062-42-0, 90% purity) was purchased from Sigma-Aldrich (Madrid, Spain). According to product specifications, it contains a mixture of decanoic acid, 3-((6-deoxy-2-O-(6-deoxy-α-L-mannopyranosyl)-α-Lmannopyranosyl)oxy)-, 1-(carboxy methyl)octyl ester (Rha-Rha-C_{10}-C_{10}) and 1-(carboxymethyl)octyl 3-((6-deoxy-α-L-mannopyranosyl)oxy)decanoate (Rha-C_{10}-C_{10}), **Figure ESI2**. Type II water was obtained from an Elix® Essential 3 water purification system (Merck Millipore, Madrid, Spain). Sodium chloride (NaCl, ACS reagent, ≥ 99.0% purity), sodium sulphate anhydrous (Na_2SO_4, tested according to Ph. Eur., 99.5% purity) and ammonium acetate ($NH_4CH_3CO_2$, ≥ 98.0% purity) were supplied by Sigma-Aldrich (Madrid, Spain). Methanol (CH_3OH, gradient grade for HPLC, Reag. Ph. Eur., ≥ 99.8% purity) was purchased from VWR (Barcelona, Spain). Trypan blue ($C_{34}H_{24}N_6O_{14}S_4Na_4$) was obtained from Fluka (Madrid, Spain) and malachite green oxalate salt ($C_{23}H_{25}N_2 \cdot C_2HO_4 \cdot 0.5C_2H_2O_4$, certified by BSC, ≥ 90% purity) was supplied by Sigma-Aldrich (Madrid, Spain).

2.2 Apparatus

BioSUPRAS synthesis required a vortex mixer and a centrifuge. The following devices were used for the synthesis of the whole range of bioSUPRASs: a Reax Top vortex mixer equipped with an attachment for centrifuge microtubes from Heidolph (Schwabach, Germany), a Vortexer vortex mixer equipped with an attachment for different size tubes from Heathrow Scientific (Vernon Hills, IL, USA), a MPW-350R high speed brushless centrifuge equipped with an angle rotor 36×2.2/1.5 mL from MPW Med. Instruments

(Warsaw, Poland) and a Mixtasel BLT digitally regulated centrifuge equipped with an angle rotor 16×15 mL from JP Selecta (Barcelona, Spain). A 831 KF Coulometer with generator electrode without diaphragm from Metrohm (Herisau, Switzerland) and a EA3000 elemental analyzer from EuroVector Srl (Milan, Italy) were respectively used for the determination of water and rhamnolipid contents in the bioSUPRASs. A 848 Titrino plus from Metrohm (Herisau, Switzerland) and a LP 2000 Turbidity Meter from Hanna Instruments (Guipúzcoa, Spain) were respectively employed for the quantification of Cl^- and SO_4^{2-} in the equilibrium solution. The electron micrographs were acquired with an EVO LS 15 scanning electron microscope from Zeiss (Oberkochen, Germany) and the size of the RL aggregates was measured using a Zetasizer NANO ZSP from Malvern Panalytical (Madrid, Spain). An UV-Vis spectrophotometer (model 99-90287) from BioTek Instruments (Winooski, VT, USA) was used for quantifying remaining dyes in water samples after extraction with bioSUPRASs.

2.3 Phase diagrams for ternary mixtures of rhamnolipid/water/salt

Phase diagrams were constructed in order to define the rhamnolipid/water/salt ratios required for bioSUPRAS production. For this purpose, the biosurfactant was dissolved in water into 15 mL centrifuge tubes and, then, the salt was added in order to promote coacervation. The mixture was vortex-shaken for 5 min to favour the contact between their components and then centrifuged (3,500 rpm, 30 min) to accelerate phase separation. Rhamnolipid and salt (NaCl, Na_2SO_4 and $NH_4CH_3CO_2$) concentrations were varied in the intervals of 0.09-9% (w/v) and 0-3 M, respectively. All experiments were performed in duplicate and the temperature was kept constant at 25 ºC. Boundaries of phase diagrams were defined through visual observation. The formation of two immiscible liquid phases was the criterion used to determine the formation of bioSUPRASs, otherwise homogeneous liquid phases or liquid-solid phases were observed.

2.4 BioSUPRAS volume and density

The volume of solvent that was formed within the coacervation region was measured for bioSUPRASs induced by NaCl and Na_2SO_4. It was calculated by measuring its height in the cylindrical tube with a digital calliper. The statistics package Statgraphics Centurion XVI.II was used to fit a model, through non-linear regression, that could predict the

volume of bioSUPRAS as function of the composition of the ternary mixture. The density of bioSUPRASs synthesized under different conditions was calculated by weighting a given volume of coacervate in an analytical balance. The experiments were conducted in duplicate.

2.5 Chemical composition of bioSUPRASs

The concentration of water, rhamnolipid and salt in the bioSUPRASs (%, w/w) was determined as function of the concentration of rhamnolipid and salt in the synthesis mixture. Coulometric Karl Fischer titration was used to determine the water content. For that, an aliquot of bioSUPRAS (50 µL) was weighted and dissolved in methanol up to 2 mL in a centrifuge microtube. After it was vortex-shaken (2 min) and centrifuged (15,000 rpm, 5 min), 100 µL of the supernatant was injected into the titration cell. All experiments were made in duplicate.

The concentration of rhamnolipid in the bioSUPRASs (and in the equilibrium solutions) was estimated from the carbon content through elemental microanalysis. For this purpose, an aliquot of 1-5 mg of bioSUPRAS was weighted in a tin capsule and then sealed and placed into the autosampler. The sample was combusted in a reactor at 1020 ^{o}C for 4.4 sec, in a temporarily enriched oxygen atmosphere (7 mL, ΔPO_2=25 kPa). The combustion products were carried by a helium stream (110 kPa) through an oxidation catalyst and a copper reducer. Finally, the gases were separated in a stainless steel packed GC column at 90 ^{o}C and detected using a thermal conductivity detector. The run time was 120 sec.

The concentration of NaCl and Na_2SO_4 incorporated into the bioSUPRAS were calculated as the difference among the initial salt concentration added to the synthesis mix and the concentration measured in the equilibrium solution after the coacervation process. Cl^- was determined by the classic precipitation titration with $AgNO_3$ (0.1 M) in acid medium which was monitored by potentiometric measurement with a silver sensor (method AOAC 963.05). SO_4^{2-} was measured by the classic turbidimetric method based on addition of $BaCl_2$ and a stabilizing solution to measure the barium sulfate turbidity (method EPA 9038).

2.6 Characterization of the bioSUPRAS structure

The hydrodynamic diameter of RL aggregates in bioSUPRASs produced from different salt concentrations was measured by dynamic light scattering (DLS). The measurements were carried out in 12 mm square polystyrene cuvettes placed in a thermostatic holder (25 ^{o}C), and data were collected at 173^{o} scattering angle. The intensity-based size distribution was calculated through non-negative least squares (NNLS) analysis. Each bioSUPRAS was prepared in duplicate and each sample was analysed three times.

The morphology of the aggregates was visualized through cryo-scanning electron microscopy (cryo-SEM). The preparation of the samples started by pouring a drop of bioSUPRAS between two rivets and plunging it in liquid nitrogen. Then, the sample was inserted into the cryogenic ante-chamber (-120 ^{o}C, $3.2 \cdot 10^{-6}$ mbar), where it was fractured to expose a cross section of the drop. The superficial ice was removed by sublimation and the aggregates were then revealed. For this purpose, the temperature varied (5 ^{o}C/min) up to -90 ^{o}C, where it kept constant for 15 minutes, and then, once again lowered to -120 ^{o}C. Finally, the sample was transferred to the microscope where the electron micrographs were acquired at -120 ^{o}C.

2.7 BioSUPRASs-based extraction of dyes

The extraction capacity of bioSUPRASs was investigated by extracting two dyes (trypan blue and malachite green) from water. For this purpose, different bioSUPRASs (0.9 and 4.5% of RL (w/v), 1 and 1.5 M of NaCl) were synthesized directly in tap water samples (4 mL) containing the dyes at 7 mg·L^{-1}. The mixture was vortex-shaken (10 min) to favour the extraction and centrifuged (3,500 rpm, 30 min) to accelerate the separation of the bioSUPRAS. The remaining concentrations of trypan blue and malachite green in the equilibrium solution were monitored at 607 and 617 nm, respectively. All experiments were conducted in duplicate. Calibration was carried out by preparing aqueous solutions containing the dyes in the concentration range of 0.2-10 mg·L^{-1}.

3. Results and discussion

3.1 Salt-induced synthesis of bioSUPRASs from rhamnolipids

RLs are produced, mostly by *Pseudomonas Aeruginosa*, as a mixture of mono-Rha and di-Rha homologues whose composition depends on the bacterial strains, substrates and culture conditions.[46] The fermentation broth contains RL homologues and a mixture of unfermented substrates, polysaccharides, salts, amino acids, proteins and other metabolic products.[43] Purification of RLs can contribute up to 50-80% of the total production cost.[44]

The commercially available RL used in this study (90% purity) consisted of a mixture of anionic Rha-C_{10}-C_{10} and Rha-Rha-C_{10}-C_{10} (**Figure ESI2)** and it was obtained using the fermentation of canola oil and/or vegetable oil by *Pseudomonas Aeruginosa*. It has been long proved that both single and mixed Rha-C_{10}-C_{10} and Rha-Rha-C_{10}-C_{10}, with purities in the range of 60-100%, are able to give colloidal systems, so they were considered excellent candidates for producing bioSUPRASs.[48,49,52-54] Given that the RL cost greatly increases with the level of product purity, and that common RL impurities are not amphiphilic and consequently are not expected to give coacervates, we decided to investigate the production of bioSUPRASs using a non-highly purity RL product (90% purity).

Table ESI1 shows the reported critical aggregation concentration (*cac*) of colloidal systems produced from Rha-C_{10}-C_{10} and Rha-Rha-C_{10}-C_{10} at different pH values, electrolyte concentration and product purity. [48,49,52-54] The reported *cac* for RLs (pK_a 5.6-5.9 for the carboxylic groups present in these biosurfactants) is higher for the anionic form compared to the non-ionic one, owing to the greater repulsion between anionic RL molecules (e.g. *cac* values were 1.6-50 times higher at pH 7.4 or 9 than at pH 4 or in ultra-high quality water, **Table ESI1**).[49] On the other hand, electrolytes such as NaCl had a negligible or limited effect on the *cac* of non-ionic RLs but they considerably reduced the *cac* of anionic RLs.[48,52-54] This reduction is the consequence of the shielding of the negative charge and dehydration of carboxylate groups by Na^+ ions, which results in the formation of a close-packed aggregate.[48] *Cac* values changed similarly against pH and with the presence and concentration of electrolytes independently of the type of RL homologue and product purity (**Table ESI1**).

Taking into account the aggregation behaviour in colloidal systems of RLs, the formation of bioSUPRASs was tried from colloidal dispersions of anionic RLs in the presence of electrolytes. Three salts were investigated for this purpose, namely sodium chloride, sodium sulphate and ammonium acetate. **Figure 1** shows the phase diagrams obtained at 25 ^oC from the three different rhamnolipid/water/salt ternary mixtures. They were plotted

as the concentration of salt (M) versus the percentage of RL (w/v) in the colloidal system. The study was restricted to biosurfactant concentrations in the range of 0.09-9% (w/v) because, as it will be commented later, the most interesting applications of SUPRASs in extraction processes involve a low concentration of this ingredient.

Three regions were always observed in the phase diagrams as the concentration of salt increased; an isotropic solution, two immiscible liquid phases (i.e. the region for bioSUPRAS formation) and a liquid-solid phase region where the biosurfactant precipitated. Thus, the three salts were able to induce the coacervation of RLs, although both the minimum concentration required for liquid phase separation, that is an indicator of their coacervation strength, and the extension of the coacervation region, depended on the nature of the salt. The ordering of salts in terms of coacervation strength was $NH_4CH_3CO_2 > Na_2SO_4 > NaCl$. The formed bioSUPRASs separated from the equilibrium solution as an upper (Na_2SO_4-induced) or bottom ($NH_4CH_3CO_2$- and NaCl-induced) phase.

Although the microscopic origins of coacervation still remain elusive and there are only few precedents of electrolyte-inducing coacervation of ionic amphiphiles,[55,56] it is widely accepted that addition of salt to ionic colloidal systems causes destruction of the hydration layer of surfactant head groups and decreases electrostatic repulsions.[17] In this way, the effective area per molecule at the interface diminishes and surfactant monomers can be packed closer together leading to aggregate growth and liquid phase separation.[57] Each salt is expected to have a specific influence on the coacervation of the ionic amphiphile, whether it tends to adsorb in the interface between the amphiphile aggregate and water or remains strongly hydrated in the bulk.[57] In addition, the effects of salts are concentration-dependent; electrostatic interactions dominate at concentrations below 0.1 M and dehydration is prevailing at intermediate concentration (0.1-2 M). At the highest concentrations, most of the water is captured at the ion hydration spheres and salting-out usually occurs.[58]

Regarding the coacervation of RLs, the binding of RL carboxylate groups to Na^+ and NH_4^+ will diminish electrostatic repulsions. Counterion binding to surfactant head groups has been recently rationalised by the *law of matching water affinities* (LMWA), which asserts that ion specificity to form contact ion pairs is favoured when their water affinities match, this meaning that they share similar water hydration enthalpies ($\Delta H_{hydration}$).[59] As a consequence, kosmotropic (highly hydrated) ions tend to pair together and chaotropic

(poorly hydrated) ions tend to form tight ion pairs. The sign of the Jones-Dole viscosity coefficient (B) is a measure of ion hydration (positive for kosmotropic and negative for chaotropic).[59] Carboxylate head groups are strongly hydrated (hydration number from 5 to 7)[60] and they are considered to be kosmotropic. So, they are expected to bind more strongly to Na^+ (kosmotropic, B: 0.086) than to NH_4^+ (chaotropic, B: -0.007).[57]

Considering that the coacervation strength of salts was in disagreement with the binding strength of cations to RL carboxylate groups, the dehydration of head groups could be the dominant mechanism for RL coacervation. RL headgroups count with big polar non-ionic rhamnosyl groups (**Figure ESI2**). These groups are expected to be strongly hydrated and, consequently, they could also be dehydrated by salt anions. In this respect, the water *withdrawing power* of anions follows the sequence CH_3COO^- (kosmotropic, B: 0.250) > $SO_4^=$ (kosmotropic, B: 0.208 > Cl^- (chaotropic, B:-0.007). This trend was in agreement with the coacervation strength of the salts ($NH_4CH_3CO_2$ > Na_2SO_4 > NaCl). Furthermore, salting-out effects in the bulk solution can help to coacervation. This study shows that the selection of both cations and anions are of primary importance for the coacervation of ionic amphiphiles.

The formation region of the ammonium acetate-induced bioSUPRAS was very small (**Figure 1**), which could hinder its production from low purity RLs. It was only formed from RL percentages above 2.7%, which hampers its applicability in extraction processes where high concentration factors are required. Consequently, we did not further investigate this system. Both NaCl and Na_2SO_4, were selected as coacervation-inducing agents for further study. They are nontoxic and have low cost and reactivity and high stability, which makes them suitable for the scale-up of bioSUPRAS production.

3.2 BioSUPRAS volume and density

The volume of bioSUPRAS that was produced in the colloidal system (expressed as µL of bioSUPRAS per mL of synthesis mixture) was a function of both the concentration of rhamnolipid and of salt. This volume linearly increased with the concentration of biosurfactant (**Figure 2 A, B**). The slopes and correlation coefficients of the linear regression lines as a function of biosurfactant and at different concentrations of NaCl and Na_2SO_4, are shown in **Table ESI2**. This linear dependence is common in SUPRAS

production since SUPRAS composition usually keeps constant as the experimental conditions leading to coacervation (e.g. salt concentration) remain unchanged.[11]

Regarding the effect of salts, results in **Figure 2 C, D** and **Table ESI2** clearly show that the volume of bioSUPRAS decreased as the concentration of NaCl and Na_2SO_4 increased. This behaviour suggests that bioSUPRAS composition is dependent on the concentration of the coacervation-inducing agent and consequently, they are environment responsive. **Figure ESI3** illustrates how the slopes of the linear regression lines decreased in the presence of NaCl and Na_2SO_4. Slopes were lower for Na_2SO_4 than for NaCl, so we measured smaller increments of bioSUPRAS volumes for Na_2SO_4 as the concentration of biosurfactant increased. In general, as illustrated in **Figure ESI4**, a lower volume of bioSUPRAS will be produced under the action of NaCl, except at the highest tested concentrations of biosurfactant.

Non-linear regression was used to fit a model (n_{NaCl}=55, n_{Na2SO4}=47) which predicts the volume of solvent produced as a function of the composition of the colloidal system:

$$V_{\text{bioSUPRAS}} = \frac{\text{Rhamnolipid}}{(0.0200\pm0.0007)\cdot NaCl - (0.010\pm0.001)} - \frac{(292\pm21)}{NaCl} + (117\pm13) \qquad [1]$$

$$V_{\text{bioSUPRAS}} = \frac{\text{Rhamnolipid}}{(0.0319\pm0.0006)\cdot Na_2SO_4 - (0.0123\pm0.0007)} - \frac{(94\pm6)}{Na_2SO_4} + (112\pm4) \qquad [2]$$

The dependent variable, $V_{bioSUPRAS}$, is the volume of bioSUPRAS ($\mu L \cdot mL^{-1}$), and the independent variables, *rhamnolipid* and $NaCl/Na_2SO_4$, are the initial concentrations of biosurfactant (%, w/v) and salt (M) in the colloidal system. Equation 1 is valid within the range: 2.7-9.0% (w/v) RL and 1.25-2.25 M NaCl; while equation 2 has the following boundaries: 1.8-9.0% (w/v) RL and 1-1.75 M Na_2SO_4. The good capability of prediction of these models was proved by their determination coefficients: $R^2_{equation1}$=0.9951, $R^2_{equation2}$=0.9991 (**Figure ESI5).** These equations are of interest for application of bioSUPRASs in extraction processes. Thus, for the extraction of contaminants, bioactives, metabolites etc. from liquid samples, where the bioSUPRAS is generated in the sample, the most favorable fractional bioSUPRAS phase volume (i.e. bioSUPRAS volume/sample volume) will be obtained at the lowest and highest concentrations of RL and salt, respectively, within the coacervation region. The fractional bioSUPRAS phase volume could reach values down to ~0.03 (concentration factor of ~30) by using NaCl. On the other hand, these equations also predict that for a given bioSUPRAS composition, the highest solvent volumes will be produced at the highest concentrations of RL within

the coacervation region. This is interesting for the extraction of organic compounds from solid samples, where the bioSUPRAS is previously generated in an aqueous medium, and then separated from the equilibrium solution and stored until use. It was checked that bioSUPRASs, once separated from the equilibrium solution, were stable at room temperature in closed bottles for at least one month.

Tables ESI3 and ESI4 show representative values for the density of the bioSUPRASs produced from different percentages of RL and varying concentrations of NaCl and Na_2SO_4, respectively. No significant differences in density were observed for each type of bioSUPRAS under the different synthesis conditions. The mean values were 1.08±0.02 $g \cdot mL^{-1}$ and 1.11±0.04 $g \cdot mL^{-1}$ for bioSUPRASs formed with NaCl and Na_2SO_4, respectively. The density values found in the literature for aqueous solutions of NaCl (1-2.25 M, 20 ºC) and Na_2SO_4 (1-1.75 M, 20ºC) varied in the ranges of 1.04-1.08 and 1.13-1.21 $g \cdot mL^{-1}$, respectively. So, the bioSUPRAS formed as an upper (Na_2SO_4) or bottom (NaCl) phase from the colloidal system depended on the salt used for its formation. Depending on the particular application it may be operationally more advantageous that the solvent remains either in the lower or upper part of the container.

3.3 Chemical composition of bioSUPRASs

Table 1 shows representative results about the bioSUPRAS composition within the whole region of coacervation. These results indicate that bioSUPRASs were primarily made of RL and salty water, and that their composition was independent of RL concentration in the synthesis mixture but significantly depended on salt concentration. Thus, as the concentration of salt in the synthesis mixture raised, the water content in the bioSUPRASs progressively decreased while the solvent gradually became more and more concentrated with amphiphile. The reduction in water content fitted a negative linear relationship with both NaCl and Na_2SO_4 (**Figure ESI6**).

These results confirm that both types of bioSUPRASs are environment responsive and that their composition can be tuned according to the concentration of salt added to the colloidal system. On the other hand, the same range of bioSUPRAS composition (i.e. RL: 19-40%, w/w and salty water: 81-62%, w/w, **Table 1**) can be obtained from both NaCl and Na_2SO_4. However, the concentration of salt required to obtain a specific bioSUPRAS will be dependent on the type of electrolyte. The percentage of RL in the bioSUPRASs

was in the same order of magnitude than that reported in bibliography for the synthetic surfactant 9-methyl dodecanoate (20-33%, w/v), which coacervates from 0.86% (w/v) of amphiphile and 1 M of salt (NaCl, KCl, NaSCN, KSCN) at 70 ºC.[61]

RL residues were not detected in the equilibrium solutions above the quantitation limit of the employed technique (~0.1% C, equivalent to ~3 mM rhamnolipid). As it has been widely reported in coacervation-induced liquid phase separation processes,[11,61] the concentration of amphihile in the equilibrium solution is expected to be near the critical aggregation concentration (e.g. 0.03-0.05 mM in presence of 0.5-1 M NaCl for Rha-C10-C10, see **Table ESI1**). Thus, the incorporation of RL to the bioSUPRAS was around 100% under all the experimental conditions and, consequently, the synthesis of RL by coacervation at room temperature can be considered a high atom-economy process, in addition to be energy-saving.

Finally, the water fraction in the bioSUPRAS kept the same salt concentration (± 0.05 M) as that initially employed for the formation of the bioSUPRAS (~0.5-2.25 M in water), which support the key role of the salt in the coacervation process.

3.4 Characterization of bioSUPRAS structure

The morphology and size of the RL aggregates in colloidal systems at different pHs and concentrations of biosurfactant and salt have been widely investigated by electron microscopy and DLS. **Table ESI5** shows representative results for anionic RLs in the presence and absence of NaCl.[48,52,54,62] In general, RL aggregates within several size ranges (i.e. bimodal or multimodal distribution) co-exist in colloidal systems and become bigger with increasing RL and salt concentration. Reported RL morphologies include a broad variety of aggregates (e.g. micelles, vesicles, cubic lamellar phases, hexagonal phases, etc.). Studies with RL concentrations as high as those found in bioSUPRASs (e.g. 205-444 $g \cdot L^{-1}$) have not been undertaken so far (e.g. RL concentrations in **Table ESI5** are within the range 0.07-3.6 $g \cdot L^{-1}$).

The hydrodynamic diameters of the RL aggregates in bioSUPRASs were calculated by DLS. **Figure 3** shows, as an example, the well-separated multimodal distribution obtained for bioSUPRASs generated by NaCl. We observed aggregates within three size ranges of 5-14 nm, 42-400 nm and 500-4500 nm that shifted towards bigger sizes at NaCl concentrations higher than 1.5 M (e.g. 23-170/200 nm, 300-1500/2000 nm; 2500/3000-

6500 nm). Results were in agreement with studies on RL aggregates in colloidal systems, [48,52,54,62] which reported the coexistence of different self-assembled structures and bigger sizes at increasing salt concentrations.

The morphology of bioSUPRAS aggregates was investigated with cryo-SEM. The sample was fractured and the surface water was removed by controlled sublimation. **Figures 4** and **5** show representative images for bioSUPRASs formed with Na_2SO_4 and NaCl, respectively. They clearly show the formation of relatively big spherical structures and internal cavities can be observed, thus confirming the formation of vesicles. The size of the structures (from nm to µm) was in accordance with the results predicted by DLS measurements. Micelles could be also present at the lowest size ranges observed by DLS (e.g. 5-14 nm in **Figure 3 A**).

The same type of structures were observed for bioSUPRASs promoted by $NH_4CH_3CO_2$ as investigated by optical microscopy (**Figure ESI7**). This indicates that vesicles seems to be the most energetically favourable structures in bioSUPRASs made up of RLs.

3.5 Potential of bioSUPRASs for extraction processes

BioSUPRASs made up of RL vesicles meet the characteristics to be excellent extractants of organic compounds in a wide polarity range from both liquid and solid samples. They provide microenvironments of different polarity (RL polar groups (-OH, $-COO^-$), RL hydrocarbon chains and vesicular aqueous cavities), a huge number of binding sites (RL in the bioSUPRASs was in the range of 205-444 $g \cdot L^{-1}$), different types of interactions (ionic, polar, donor/acceptor hydrogen bonds and dispersion), and a broad vesicle size range (from nm to µm). Combination of these properties enables the efficient extraction of compounds in a wide polarity and size range through mixed-mode extraction mechanisms.

Two highly water soluble synthetic dyes (trypan blue and malachite green) were extracted from spiked tap water in order to prove the extraction capacity of bioSUPRASs. Trypan blue is an anionic dye (**Figure ESI8 A**) with high molecular weight (868.85 $g \cdot mol^{-1}$), water solubility (up to 10 $g \cdot L^{-1}$) and 4/20 donor/acceptor hydrogen bonds. Malachite green is a cationic dye (**Figure ESI8 B)** with moderate molecular weight (329.46 g mol^{-1}), high water solubility (up to 110 $g \cdot L^{-1}$) and only one acceptor hydrogen bond.

Table 2 shows the results for the extraction of the two dyes, expressed as percent recovery. Three synthesis conditions were selected in order to study the effect of bioSUPRAS composition on recoveries. Excellent results were obtained for malachite green under all the conditions investigated, that suggesting that ionic attractive interactions were an effective mechanism for the extraction of this highly water-soluble dye. On the other hand, bioSUPRAS composition was determinant in the extraction of trypan blue and the recovery increased from 53 to 94 % for bioSUPRAS 1 and 2, respectively. As shown in **Table 2**, a higher concentration of RL was present in bioSUPRAS 2 (31%, w/w) compared to bioSUPRAS 1 (19%, w/w), thus favouring the partition of trypan blue. The dye was extracted by mixed mode mechanisms, so driving extraction forces involved hydrogen bonding, dispersion and polar interactions at bioSUPRAS phase and probably salting-out by NaCl too. The extraction with a higher volume of bioSUPRAS (e.g. compare results for bioSUPRASs 2 and 3) did not improve further the extraction of trypan blue.

These results illustrate how tailoring of bioSUPRAS composition provides a simple strategy to improve extraction efficiencies of highly polar compounds.

3.6 Compliance of bioSUPRASs with green solvent criteria

The RL-based bioSUPRASs are fully or partially compliant with the twelve criteria set for green solvents.[7] Thus, regarding their *performance* they have shown potential to be advantageous to conventional solvents employed in extraction processes in terms of scope, efficiency and tailoring for different application strategies. As an example of this potential we have discussed the efficient extraction of two highly water soluble compounds from water, an application that would not be affordable with conventional water immiscible organic solvents. Likewise, bioSUPRAS *synthesis* is carried out through an energy-saving process (spontaneous coacervation at room temperature) that has a high-atom economy (RL is virtually completely incorporated into the bioSUPRAS).

On the other hand, there are several criteria (*toxicity, biodegradability, stability and flammability)* for which, bioSUPRAS characteristics should be closely related to their components (RL and water). The low toxicity and high biodegradability under aerobic, anoxic and anaerobic conditions of RLs have been widely confirmed.[47] Also, RLs are

thermally stable (boiling point around 170 ºC) and non-flammable. So RL-based SUPRASs are expected to be fully compliant with these criteria.

With respect to market criteria (*grade, price, availability and renewability)*, we must focus on RL since it is the main ingredient and determinant factor in the cost of bioSUPRAS production. RLs are still in need of an economically available mass production scheme,[44] and currently they are not economically competitive ($20-25/kg) compared to synthetic surfactants (e.g. $1-3/kg).[43] The costs involved in RL production originate from the raw materials to serve as carbon and nitrogen sources for the microorganisms, the fermentation procedures and subsequent purification processes.[43] Many strategies have been developed to reduce the cost of each of these steps.[43,44] Specifically, RLs of different technical grade are available and the product purification cost can be significantly lowered if cell-free fermentation broth or less purified RLs can be used in place of purified RLs. Here, we have proved that bioSUPRASs are generated from 90% purity RLs and future investigation should be conducted to study the formation of bioSUPRASs from less purified RLs.

BioSUPRASs are formed in situ when they are applied to liquid samples, so the criterion *storage* mainly applies to applications involving solid samples. Because of their composition, bioSUPRASs fulfil all legislations to be safely transported and we verified that they were stable in closed bottles for at least one month at room temperature.

Finally, regarding *recyclability*, we should consider the recovery of RL from the bioSUPRASs and the salt from the synthesis equilibrium solution. In general, reported purification/reuse strategies with non-volatile alternative solvents (deep eutectic solvents and ionic liquids) are based on back-extraction of the target compounds with anti-solvents for the extractant, evaporation/reconstitution steps and, in a lesser extent, solid-phase extraction with macroporous resins (e.g. ME-2 polystyrene matrix, XAD-16 styrene–divinylbenzene).[63] In this sense, RLs could be recovered from the final SUPRAS extracts by precipitation in acidic medium (pK_a 5.6-5.9 for the carboxylic groups), by the addition of a poor solvent (anionic RLs are poorly soluble in organic solvents as acetone or acetonitrile), by increasing salt concentration (**Figure 1**) or by using ion exchange resins. Regarding the leaching of SUPRAS components into treated liquid samples, since we measured that ~100% of the surfactant was incorporated into the SUPRAS phase, only traces of RLs would remain in the treated water and this should not be of concern due to their eco-friendly properties. Nevertheless, since salty water is needed to promote

SUPRAS formation, when dealing with water samples, these processes would be advantageous for treatment of seawater or salty industrial wastewater (e.g. textile and oil mill wastewater). When solid samples would be treated, the bioSUPRAS would be first generated and then separated from its equilibrium salty solution, before adding it to the solid sample as it has been reported with SUPRAS made up of synthetic surfactants.[61] The salty equilibrium solution could be used for the synthesis of new bioSUPRASs.

Table ESI6 compares different SUPRAS that have reported for extraction processes[64-69] in terms of environmental, health and sustainability concerns and the market price of the surfactant.

4. Conclusions

To the best of our knowledge, bioSUPRASs produced from aqueous solutions of rhamnolipids through salt-induced coacervation are described for the first time. These biosolvents exhibit all the intrinsic properties of SUPRASs: versatile nanostructured liquids, high efficient extractants and simple and quick procedures of synthesis, but they are greener since synthetic surfactants, organic co-solvents, high concentration of acids or high temperatures are not necessary for their production. That turns bioSUPRASs into a green alternative to conventional solvents due to their biodegradability, low toxicity and sustainability. This study revealed the first characterization of bioSUPRASs in terms of composition, structure, and extraction capacity. It is expected that a greater knowledge of these solvents helps to broaden their application in different fields, including the treatment of wastewater with high saline concentration (e.g. brine in food industry), sample treatment (extraction and clean-up) for analytical methods or the enrichment and encapsulation of bioactive compounds.

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Figure 1. Phase diagrams of ternary mixtures of rhamnolipid/water/salt (A: NaCl; B: Na_2SO_4; C: $NH_4CH_3CO_2$) at 25 ^oC. Concentration of salt (M) is plotted versus concentration of rhamnolipid (%, w/v) in the synthesis mixture

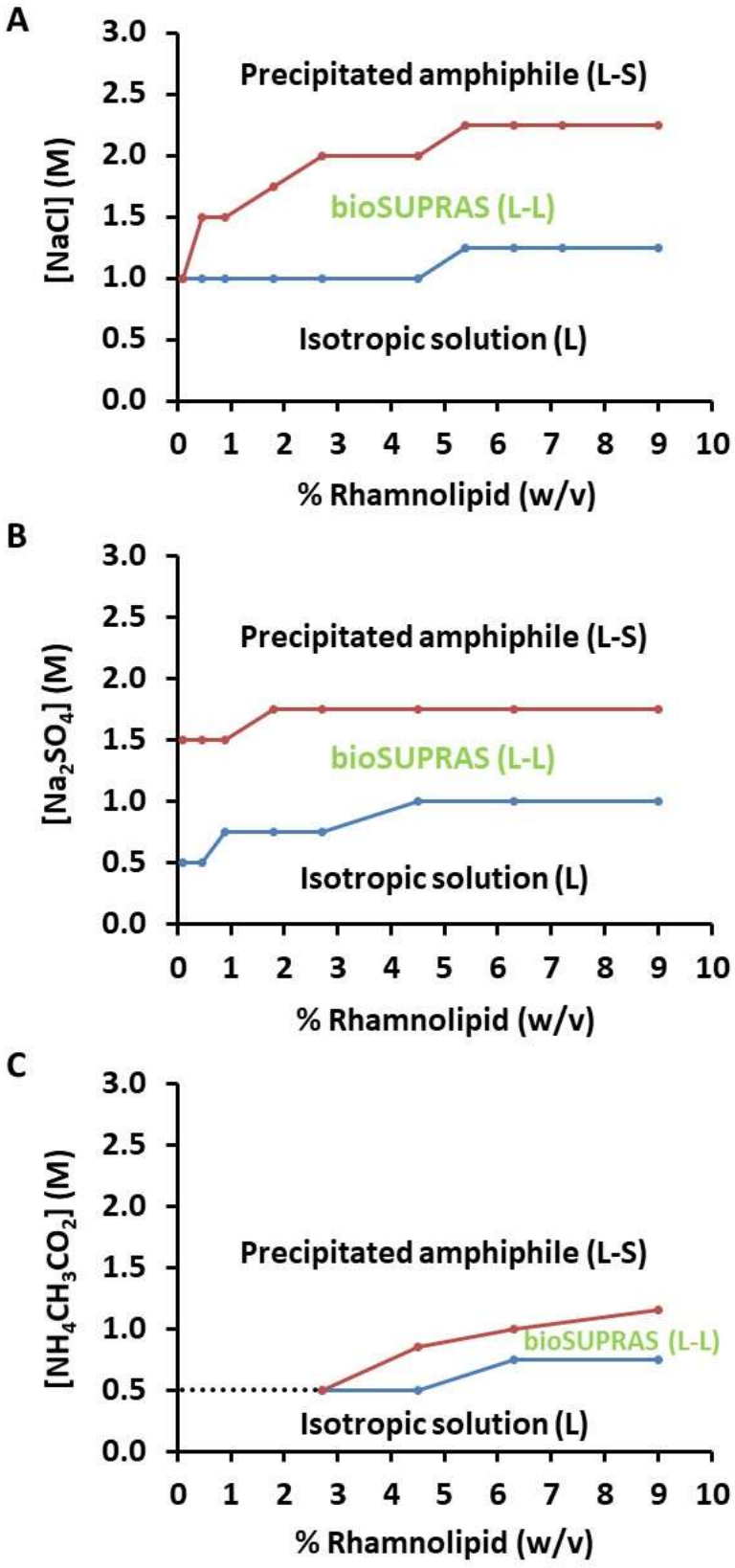

Figure 2. Volume of bioSUPRAS (μL·mL^{-1} mixture) as a function of the initial concentration of rhamnolipid (%, w/v) (A: NaCl; B: Na_2SO_4) and salt (M) (C: NaCl; D: Na_2SO_4)

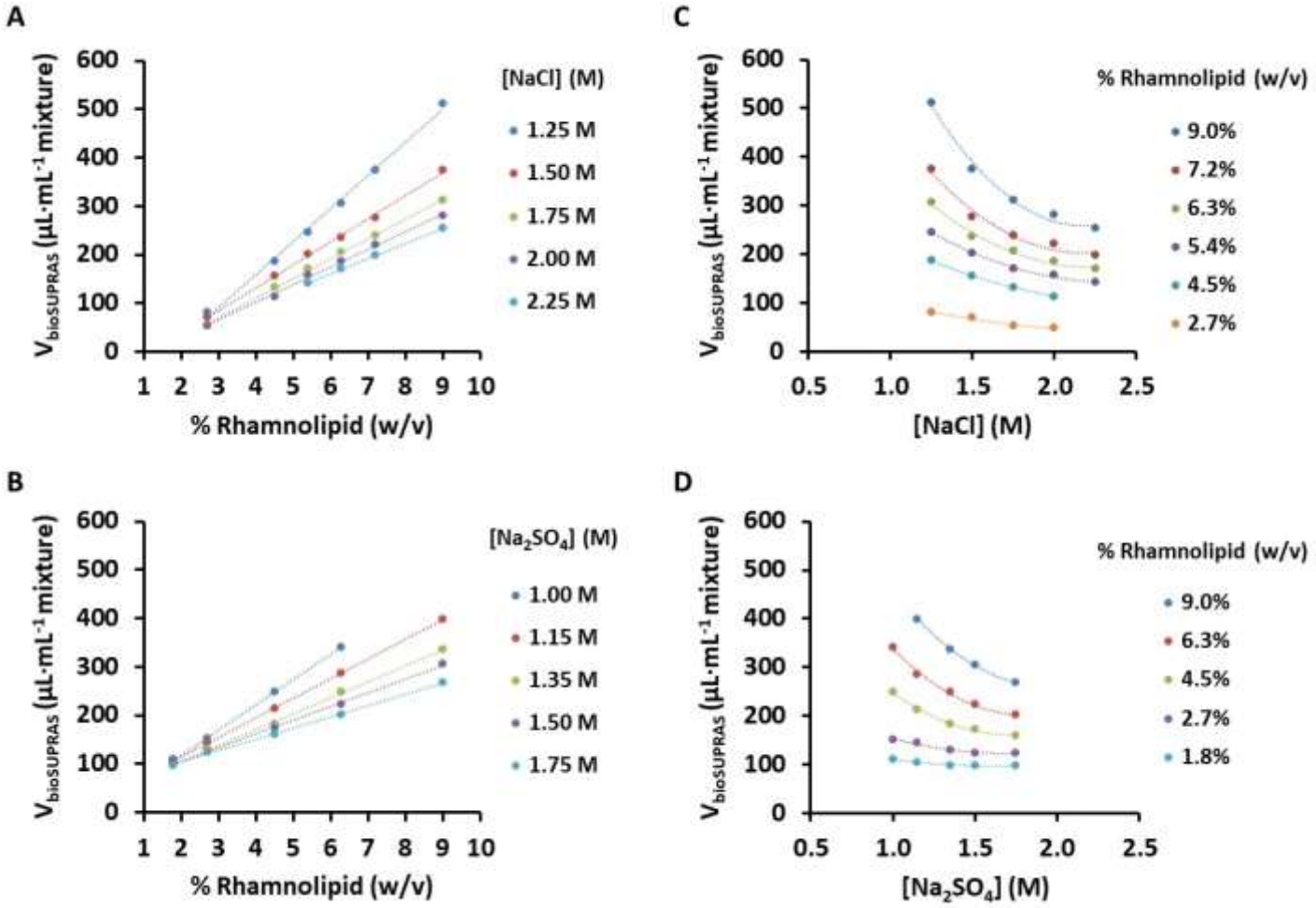

Figure 3. Intensity-based aggregate size distribution of bioSUPRASs synthesized from mixtures containing 4.5% of rhamnolipid (w/v) and different concentrations of NaCl (A: 1.25 M; B; 1.50 M; C: 1.75 M). Measurements were carried out by DLS at 173º scattering angle and 25 ºC

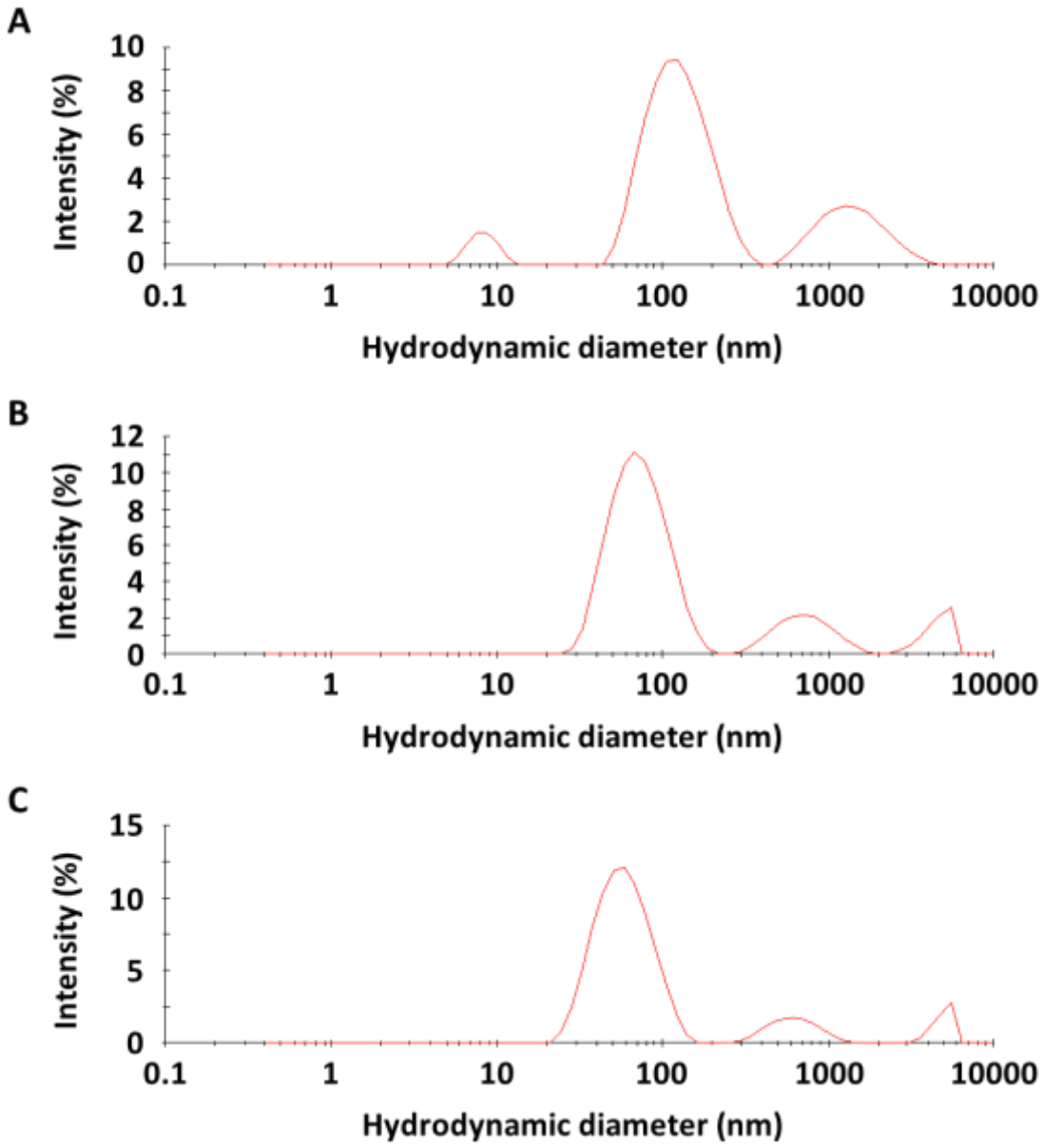

Figure 4. Cryo-SEM micrographs of a bioSUPRAS synthesized from a mixture containing 4.5% of rhamnolipid (w/v) and 1.5 M of Na_2SO_4

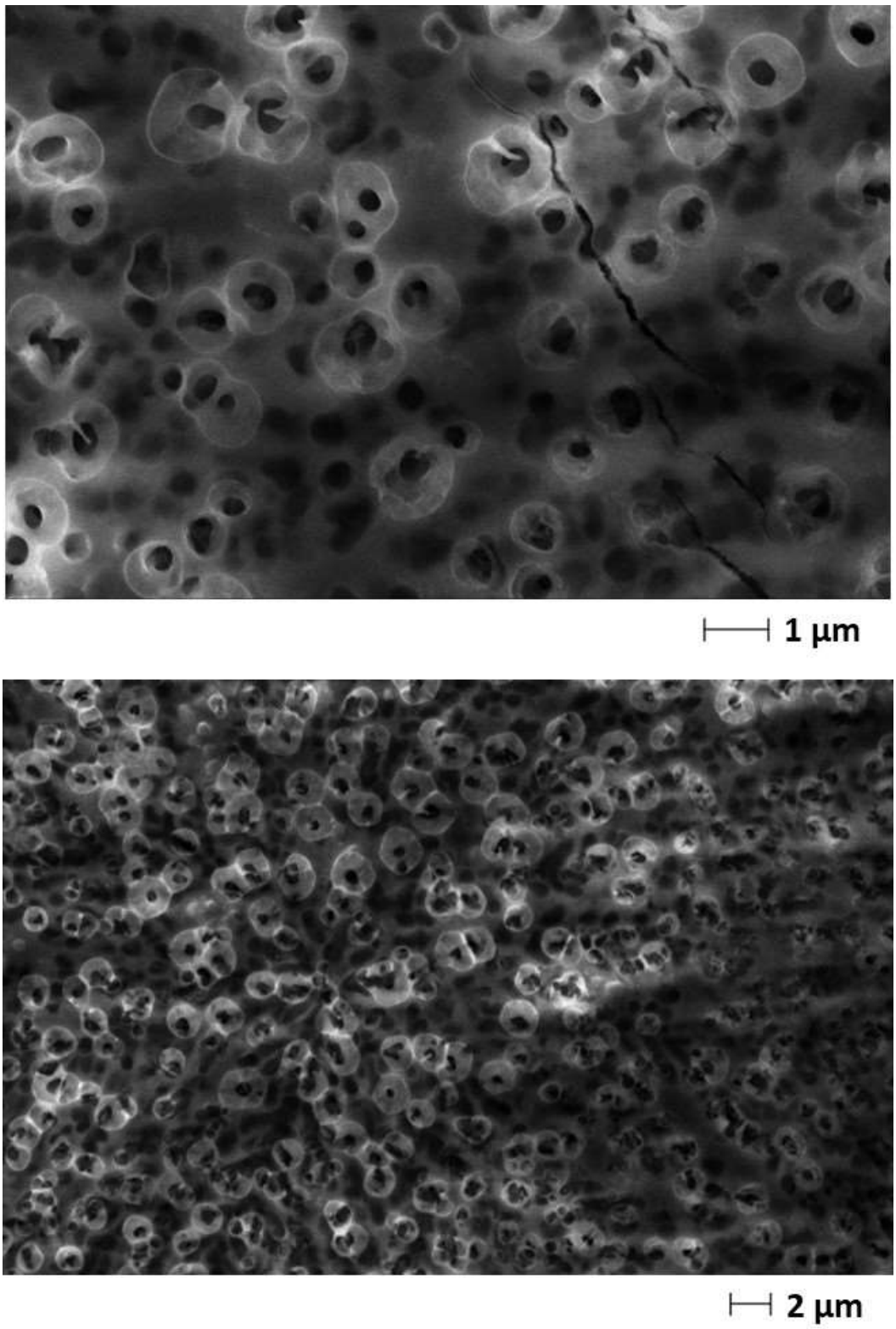

Figure 5. Cryo-SEM micrographs of a bioSUPRAS synthesized from a mixture containing 4.5% of rhamnolipid (w/v) and 1.5 M of NaCl

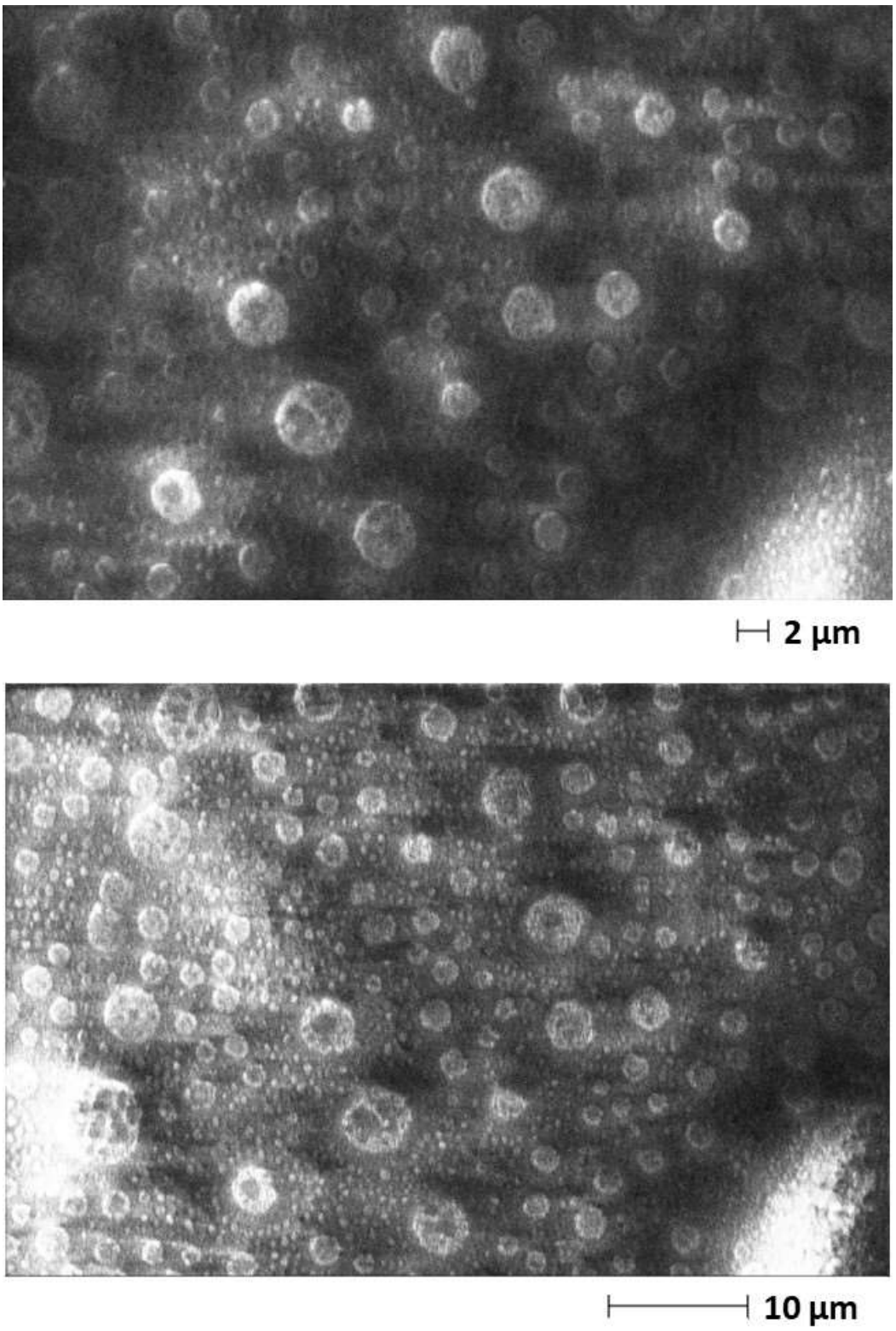

Table 1. Composition of bioSUPRASs formed from different coacervation conditions

Synthesis conditions	***bioSUPRAS composition***		
[1][NaCl] (M)	**% H_2O±SD (w/w)**	**% Rhamnolipid±SD (w/w)**	**% Salt±SD (w/w)**
1.00	77±2	19±3	4.5±0.3
1.25	73±5	24±5	5.3±0.2
1.50	68±4	31±4	6.0±0.1
1.75	64±5	33±4	6.5±0.1
2.00	60±3	37±3	7.0±0.4
2.25	55±3	40±2	7.2±0.6
[1][Na_2SO_4] (M)	**% H_2O±SD (w/w)**	**% Rhamnolipid±SD (w/w)**	**% Salt±SD (w/w)**
1.00	71±7	19±2	10.1±0.4
1.15	65±3	26±1	10.7±0.7
1.35	60±4	29±1	11.4±0.7
1.50	56±5	36±3	12±1
1.75	50±6	40±2	12±1
[2]% Rhamnolipid±SD (w/v)	**% H_2O±SD (w/w)**	**% Rhamnolipid±SD (w/w)**	**% Salt±SD (w/w)**
2.7	58±4	38±1	6.8±0.4
4.5	59.9±0.8	37±3	7.0±0.2
5.4	60±4	38±2	7.0±0.3
6.3	60.7±0.4	38±4	7.1±0.1
9.0	60.9±0.9	37±1	7.1±0.7
[3]% Rhamnolipid±SD (w/v)	**% H_2O±SD (w/w)**	**% Rhamnolipid±SD (w/w)**	**% Salt±SD (w/w)**
1.8	59±4	30±3	11.3±0.7
2.7	60±4	28±2	11.4±0.7
4.5	59±3	28±2	11.3±0.3
6.3	60±3	29±3	11±1
9.0	59±2	29±2	11±1

[1]Percentages of H_2O and rhamnolipid in the bioSUPRAS are mean values for RL concentrations in the interval of 1.8-9 % (w/v); [2]NaCl: 2 M; [3]Na_2SO_4: 1.35 M.

Table 2. Mean percent recoveries obtained for the extraction of trypan blue and malachite green in spiked tap water with different bioSUPRASs

	Synthesis conditions		*bioSUPRAS*	Recovery±SD (%)	
	% Rhamnolipid (w/v)	[NaCl] (M)	% Rhamnolipid (w/w)	Trypan blue	Malachite green
1	0.9	1	19	53±3	100.3±0.3
2	0.9	1.5	31	94.2±0.1	100.1±0.4
3	4.5	1.5	31	90.8±0.5	92±2