ChemComm

COMMUNICATION

View Article Online
View Journal

Cite this: DOI: 10.1039/c9cc03831k

Received 17th May 2019,
Accepted 3rd July 2019

DOI: 10.1039/c9cc03831k

rsc.li/chemcomm

Temperature-responsive extraction of violacein using a tuneable anionic surfactant-based system†

Nicolas Schaeffer, [a] Mariam Kholany, [a] Telma L. M. Veloso, [a] Joana Luísa Pereira, [b] Sónia P. M. Ventura, [a] Jean-Marc Nicaud [c] and João A. P. Coutinho *[a]

A tuneable thermoresponsive system based on combinations of sodium dodecyl sulfate surfactant with tetrabutylammonium chloride salt is presented as an integrated process for the solid–liquid extraction of violacein from bio-engineered *Yarrowia lipolytica* yeast and its purification by cloud-point separation from contaminant proteins.

The concept of bio-refinery, in which petroleum resources are substituted by biological feedstocks for the production of value-added products, is critical to the emergence of a sustainable economy.[1] However, biomass are complex matrices, with the viability of biorefinery processes limited by their separation operations due to the difficulties in achieving high purities and yields of the target compounds.[2] Integrated separation processes capable of combining multiple unit operations through changes in external stimuli appear as more economical and sustainable. Temperature-responsive systems that undergo a reversible phase separation by temperature variation are one of the most relevant approaches to these separation platforms. In thermoreversible systems, extraction or catalysis can occur under homogeneous conditions prior to the inducement of a biphasic regime by temperature adjustment leading to the separation of products and reactants.[3,4] However, systems capable of undergoing such phase transitions are for the most part restricted to nonionic surfactants and polymers and rarely occur in ionic systems due to electrostatic interactions.[5] One exception is the combination of the anionic surfactant sodium dodecylsulfate (SDS) with tetrabutylammonium ionic liquids (ILs) ($[N_{4444}]A$, where A is a halogenated anion) in aqueous solutions. Whilst this system was studied by experimental[6–8] and computational techniques,[9] applications remain lacking. In some cases, SDS has shown increased potential compared to nonionic surfactants for biomass disruption and the extraction and enhanced solubilization of hydrophobic biomolecules, cationic dyes and heavy metals.[10] Multiple approaches are available for cell disruption including mechanical, ultrasound, microwave, super critical carbon dioxide and organic solvents to name but a few. However, these options suffer from high capital and operational costs and/or toxicity.[11] Thermo-responsive aqueous surfactant-based systems appear as a more benign alternative. In this work, aqueous solutions containing variable molar ratio of $[N_{4444}]Cl$ and SDS are investigated as integrated platforms for the cell disruption, extraction and purification of violacein from bio-engineered *Yarrowia lipolytica* (*Y. lipolytica*) yeast. Violacein is a hydrophobic violet-hued bisindole pigment [$\log(K_{OW}) = 3.34$] of scientific and economical interest due to its reported anticancer and antibiotic properties.[12] The experimental methodology followed and the biomass used in this work are described in the Experimental section of the ESI.†

By tuning the $[N_{4444}]Cl$ to SDS ratio, task-specific solutions are obtained with designed properties for a more selective purification of violacein from contaminants. Fig. 1 shows the variation in the cloud point (CP) as a function of $[N_{4444}]Cl$ to SDS molar ratio at a fixed [SDS] = 0.2 M, with the system presenting a non-linear lower critical temperature (LCST) response. Neither SDS, $[N_{4444}]Cl$ nor $[N_{4444}]Cl$:SDS mixtures for a molar ratio ≤ 0.5 display any clouding below 373 K. Above $[N_{4444}]Cl$:SDS > 0.5, the CP sharply decreases reaching 304.9 K for equimolar $[N_{4444}]Cl$:SDS aqueous solutions. Further addition of $[N_{4444}]Cl$ has a lesser impact on the CP has it reaches a plateau at higher $[N_{4444}]Cl$: SDS ratio. Dynamic light scattering (DLS) measurement of equimolar $[N_{4444}]Cl$:SDS solutions with temperature (Fig. S1, ESI†) indicates the exponential growth of the aggregate size close to the CP, shifting from a micellar solution to the formation of $[N_{4444}]DS$ coacervate aggregates with temperature increase. A similar CP behavior was reported for ILs based on the tetrabutylphosphonium cation ($[P_{4444}]^+$),[13] the phosphonium analogue to the $[N_{4444}]^+$ cation used in this work. This suggests that $[N_{4444}]^+$-induced aggregation drives the LCST transition.

[a] CICECO – Aveiro Institute of Materials, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal. E-mail: jcoutinho@ua.pt
[b] Department of Biology and CESAM – Centre for Environmental and Marine Studies, University of Aveiro, 3810-193 Aveiro, Portugal
[c] Micalis Institute, INRA, AgroParisTech, Université Paris-Saclay, 78350 Jouy-en-Josas, France

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c9cc03831k

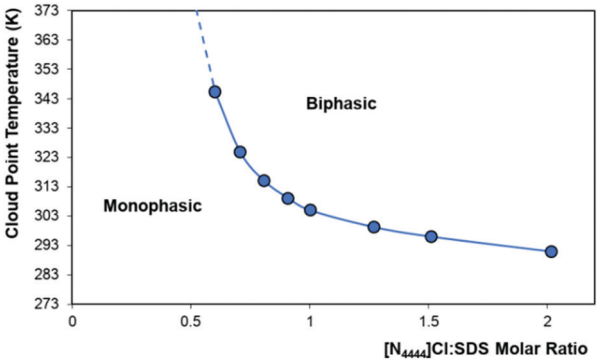

Fig. 1 Cloud point of aqueous mixtures for varying $[N_{4444}]Cl$ to SDS molar ratios ([SDS] = 0.2 M).

$[N_{4444}]^+$ possesses a symmetrical structure with a large hydrophobic volume and sterically shielded charge. Due to the dispersive contribution of the butyl chains, $[N_{4444}]^+$ cations can overcome electrostatic repulsion to self-assemble in aqueous solutions.[14] $[N_{4444}]^+$ can strongly interact with both itself and the $[DS]^-$ micelle surface by insertion of one or two of its alkyl chains into the hydrophobic core of the micelle, thereby minimizing the charge repulsion between the anionic surfactants and dehydrating the micelle surface.[9] Dehydration of the micelle surface is further accentuated with temperature increase, favoring van der Waals forces and promoting aggregate–aggregate interaction, ultimately resulting in an entropically-driven phase separation. For equimolar systems ([SDS] = 0.2 M), substitution of $[N_{4444}]Cl$ by the more hydrophilic $[N_{xxxx}]Cl$ (x = 1–3) did not result in any experimentally observable CP below 373 K, whilst the CP of the more hydrophobic $[N_{5555}]Cl$–SDS solution is below 273 K. With respect to the applicability of the studied system as an integrated purification platform, an important transition occurs in the range $0.5 < [N_{4444}]Cl:SDS < 0.6$, as observed by the shift in CP. Conductivity and DLS measurements (Fig. S2 and S3, ESI†) confirm a transition in the system behavior at $[N_{4444}]Cl:SDS > 0.5$. SANS measurement of the $[N_{4444}]Br$–SDS system indicated that this ratio corresponds to the change in system behavior from one characteristic of ionic surfactants to one typically associated with nonionic surfactants.[6] The physical properties below the CP of four $[N_{4444}]Cl$–SDS systems relevant to biomolecule extraction are presented in Table 1. An increase in the zeta potential (ZP) and viscosity (η) is observed as the amount of $[N_{4444}]Cl$ is increased from pure SDS to $[N_{4444}]Cl:SDS = 1$. A molar excess of $[N_{4444}]Cl$ ($[N_{4444}]Cl:SDS = 1.5$) results in a decrease in η and ZP of the system assigned to a phase transition of the $[N_{4444}]Cl$–SDS aggregate.[8] In all cases viscosity is low, an important factor when considering the potential applicability and scale-up of such systems.

Table 1 Cloud point, viscosity (η) and zeta potential (ZP) of the various $[N_{4444}]Cl$–SDS systems used for the extraction of violacein ([SDS] = 0.2 M). η and ZP were measured below the CP at T = 288 K

IL : SDS ratio	CP (K)	η (mPa s)	ZP (mV)
0.0	—	1.63 ± 0.05	−64.1 ± 2.4
0.5	>373	4.57 ± 0.06	−40.6 ± 4.6
1.0	304.9	5.16 ± 0.06	−25.2 ± 1.2
1.5	295.5	4.95 ± 0.07	−35.2 ± 2.7

The extraction of violacein and contaminant proteins from *Y. lipolytica* yeast at various $[N_{4444}]Cl$: SDS ratios, extraction temperatures and SDS concentrations are presented in Fig. 2 and compared to that using the traditional organic solvent ethanol (dashed line). Standard conditions of $[N_{4444}]Cl:SDS = 1$, [SDS] = 0.2 M and 293 K were used unless otherwise stated. With the exception of aqueous $[N_{4444}]Cl$ solutions, all other systems achieve greater extraction yields compared to ethanol suggesting that the tensioactive property of SDS is critical for *Y. lipolytica* cell disruption and violacein liberation. It must be highlighted that the SDS concentration in all systems is far above its cmc value of 8.2 mM.[15] Extraction results in Fig. 2 indicate that violacein and protein extraction can be tuned by changes in the $[N_{4444}]Cl$–SDS molar ratio, a tunability not available for solutions solely containing surfactants. Furthermore, the violacein extraction selectivity using $[N_{4444}]Cl$–SDS is notably superior to that achieved using the commercial nonionic surfactant Triton X-114 (Fig. S4, ESI†).

The addition of $[N_{4444}]Cl$ suppresses the extraction of contaminant proteins due to the competing nature of the $[N_{4444}]Cl$–SDS interaction, decreasing their concentration by 168.6 μg mL^{-1} from aqueous SDS solution to one containing $[N_{4444}]Cl:SDS$ = 1.5. Systems at conditions within 5 K of their CP, namely $[N_{4444}]Cl:SDS = 1.5$, 303 K and 0.3 M of $[N_{4444}]Cl$–SDS, displayed a decrease in violacein extraction. This is attributed to the increased intra $[N_{4444}]Cl$–SDS interaction at the expense of SDS–biomass interaction. The highest violacein extraction was achieved for $[N_{4444}]Cl:SDS = 1$, $[N_{4444}]Cl$–SDS = 0.2 M and 298 K, attaining an extraction yield of 103.1 μg mL^{-1} or 4.1 mg (g wet biomass)$^{-1}$. This represents 58.4 wt% recovery of the total violacein content in just one extraction step, with optimization of the solid–liquid ratio likely to improve the extraction yield. The enhanced violacein extraction at this $[N_{4444}]Cl$: SDS molar ratio is most likely due to the greater nonionic-like character of

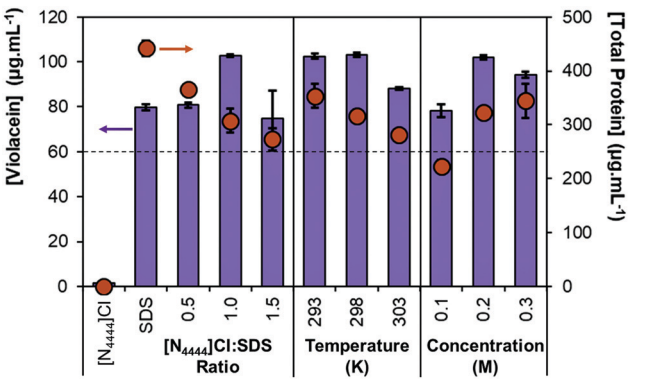

Fig. 2 Final violacein and total protein concentration in the final extraction solution as a function of $[N_{4444}]Cl$: SDS ratio, extraction temperatures and concentration. Standard conditions of $[N_{4444}]Cl:SDS = 1$, $[N_{4444}][DS]$ = 0.2 M and 293 K were used unless otherwise stated. Dashed line represents the extraction of violacein using ethanol.

the system (Table 1). However, when standardized per gram of SDS used, a maximum extraction of 2.72 mg (g SDS)$^{-1}$ was obtained for [N_{4444}]Cl–SDS = 0.1 M.

Taking advantage of the LCST transition shown in Fig. 1, the potential for the selective cloud point extraction of violacein was assessed. Phase separation was induced in the system's displaying a CP by heating to 323 K. The partition of violacein and proteins to the surfactant-rich phase after phase separation is presented in Fig. 3. In all cases, quantitative extraction of violacein was observed highlighting its preference for the most hydrophobic phase; the water content of the surfactant-rich phase for a starting concentration of [N_{4444}]Cl–SDS = 0.1 M is 26.7 wt%. The [N_{4444}]Cl : SDS ratio does not have a significant influence on the subsequent separation of violacein from contaminant proteins in contrast to the [N_{4444}]Cl–SDS concentration. Lower concentrations favor the selective extraction of violacein due to the saturation of the surfactant-rich phase. For [N_{4444}]Cl–SDS = 0.1 M, the protein extraction was reduced to 30.6 ± 1.5 wt% yielding a surfactant-rich phase highly concentrated in violacein (1065 ± 19 μg mL^{-1}). Successive CP extraction cycles ([N_{4444}]Cl–SDS = 0.1 M) did not result in improved violacein purity. The separation of violacein from impurities using [N_{4444}]Cl–SDS = 0.1 compares favorably to that obtained using Triton X-114 (Fig. 3). The [N_{4444}]Cl–SDS system achieved a greater selectivity for violacein over proteins both in the extraction and CP separation stages compared to Triton X-114, confirming the tuneable nature of the proposed system.

The addition of [N_{4444}]Cl to SDS results in an *in situ* metathesis reaction due to the differences in the entropy of hydration of the respective anion and cation combinations, yielding a final [N_{4444}][DS] IL and NaCl salt. This mechanism was verified by chloride analysis of the surfactant-rich phase after phase separation at 323 K. A final chloride concentration of 27.4 ± 2.1 mM chloride was obtained, confirming [N_{4444}][DS] IL as the primary constituent of the surfactant-rich phase. Quantitative ^{1}H-NMR analysis of the aqueous phase under the same conditions for the [N_{4444}]Cl–SDS = 0.1 M system in the absence of violacein (Fig. S5, ESI†) indicates that approximately 85 mol% of [N_{4444}]$^+$ and 95 mol% [DS]$^-$ partition to the surfactant-rich phase. Complete recovery of the system components was achieved by addition of 0.5 M NaCl, an important consideration from a recycling perspective to maximize the benefits of the proposed process.

Recovery of violacein from the surfactant-rich phase by conventional methods (dilution below the cmc, addition of cold acetone or solvent extraction using ethyl acetate) yielded a final product heavily contaminated in surfactant. Most surfactants, both ionic and non-ionic, display a high solubility in many organic solvents, rendering the selective back-extraction of biomolecules a challenge.[16] A 1 : 1 menthol : thymol hydrophobic eutectic solvent (HES) based on natural terpene precursors was used for the back-extraction of violacein. HES were shown to extract a range of biomolecules[17] as well as being common excipients in pharmaceuticals,[18] an important criterion to valorize the reported antibacterial properties of violacein.[12]

Following dilution of the isolated violacein-rich extract to 1 mL, addition of the HES resulted in the complete back-extraction of violacein (organic : aqueous phase ratio = 0.5). An additional benefit of the HES is the precipitation of the protein content at the solvent interface (Fig. S6, ESI†), facilitating violacein purification. Quantitative ^{1}H-NMR analysis of the HES phase before and after back-extraction, Fig. 4, indicates a low final [DS]$^-$ concentration of 21 ± 5 mM. This could be further reduced below the detection limit (5 mol%) by scrubbing the HES phase with a 0.5 M KCl solution at 293 K (organic : aqueous phase ratio = 0.2), precipitating residual [DS]$^-$ anions as K[DS].[19] The overlap of HES and [N_{4444}]$^+$ peaks prevented the latter's quantification but its presence is identifiable in Fig. 4. The peaks at δ = 4.3 ppm and δ = 9.2 ppm suggest the presence of additional undefined impurities. The emergence of the band

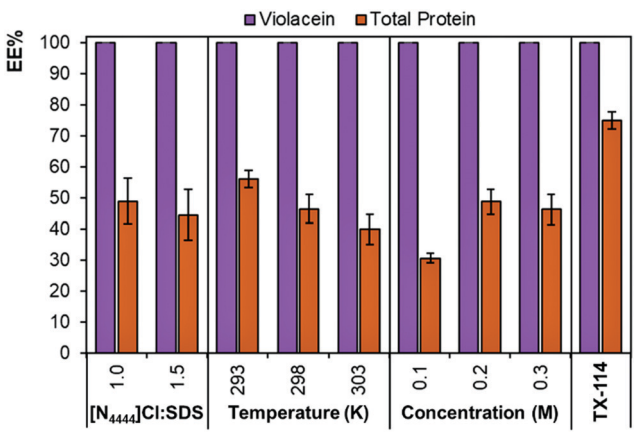

Fig. 3 Cloud point extraction of violacein and total proteins after heating to 323 K for the systems described in Fig. 2 (TX-114 – Triton X-114, 0.1 M).

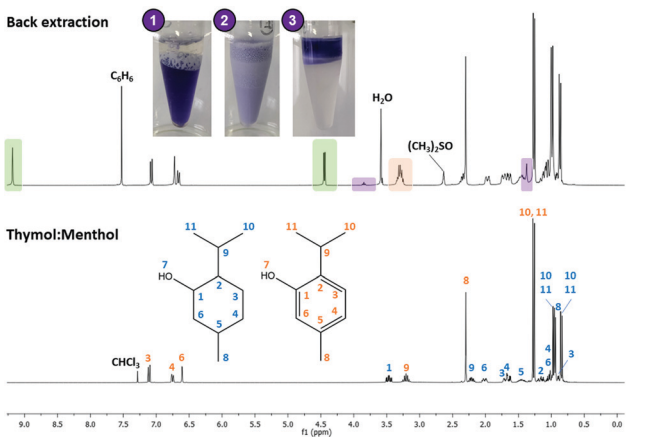

Fig. 4 Bottom: ^{1}H-NMR with peak identification of the synthesised 1 : 1 menthol : thymol HES. Top: Quantitative ^{1}H-NMR analysis of the HES phase after violacein back extraction with benzene as internal standard (δ = 7.8 ppm – [benzene] = 3.19 × 10^{-5} mol). Peaks associated with [N_{4444}]$^+$ and [DS]$^-$ are highlighted in yellow and purple, respectively. Peaks corresponding to unidentified compounds are highlighted in green. The [DS]$^-$ concentration was estimated from its CH_2–OSO_3 peaks at δ = 3.8 ppm. Inset: Picture of before, during and after back-extraction of violacein from the isolated surfactant-rich phase using HES.

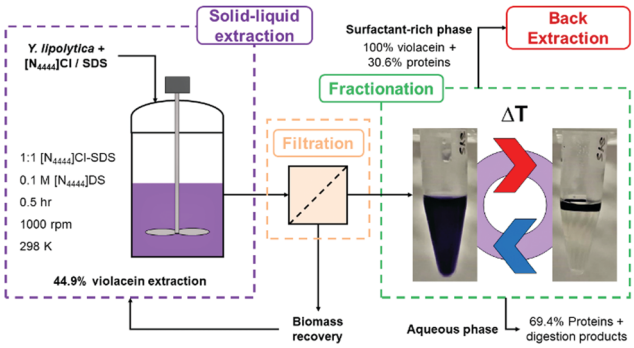

Fig. 5 Schematic of the process for the integrated extraction and cloud point separation of violacein from bioengineered *Y. lipolytica* strain JMY7019.

δ = 9.2 ppm, characteristic of acidic functional groups, is unsurprising as terpene–fatty acids HES were previously characterized.[20] ^{1}H-NMR analysis of the aqueous following back-extraction in Fig. S7 (ESI†), did not detect the presence of the HES constituent. Fig. 4 suggests that sustainable HES are viable alternatives for the back-extraction of pigments from complex matrices and surfactants. Furthermore, preliminary results indicate that the HES could be recovered by rotary evaporation for 2 h at 333 K under vacuum (<20 mbar). Alternatively, a resin adsorption method was previously shown to efficiently recover bioactive compounds from HES.[21]

This work successfully demonstrated the potential of aqueous solutions of $[N_{4444}]$Cl–SDS as an integrated platform for the solid–liquid extraction of violacein from biomass and its subsequent cloud-point extraction and separation from contaminant proteins. A schematic of the proposed process is presented in Fig. 5. Through variations in the $[N_{4444}]$Cl to SDS ratio, the character of the system could be selectively tuned from anionic to pseudo-nonionic. This greater degree of flexibility was applied to enhance the extraction and purification of violacein from real biomass. This approach can be further extended to other high value compounds, including hydrophobic dyes and metals,[10] by adapting the apparent surface charge of the $[N_{4444}]$Cl–SDS aggregates. Finally, a HES based on menthol:thymol was used to back-extract violacein and separate it from the dodecyl sulfate anion.

This work was developed in the scope of the project CICECO – Aveiro Institute of Materials, POCI-01-0145-FEDER-007679 (FCT Ref. UID/CTM/50011/2019). S. P. M. Ventura and M. Kholany acknowledge FCT for the contract IF/00402/2015 and PhD grant SFRH/BD/138413/2018, respectively. Thanks are due to FCT/MCTES for the financial support to CESAM (UID/AMB/50017/2019), through national funds; J. L. Pereira is funded by national funds (OE), through FCT, in the scope of the framework contract foreseen in article 23 of the Decree-Law 57/2016, changed by Law 57/2017.

Conflicts of interest

There are no conflicts to declare.

References

1 F. Cherubini, *Energy Convers. Manage.*, 2010, **51**, 1412–1421.
2 J. Humphrey and G. Keller, *Separation Process Technology*, McGraw-Hill, New York, 1997.
3 A. M. Ferreira, H. Passos, A. Okafuji, A. P. M. Tavares, H. Ohno, M. G. Freire and J. A. P. Coutinho, *Green Chem.*, 2018, **20**, 1218–1223; M. Gras, N. Papaiconomou, N. Schaeffer, E. Chainet, F. Tedjar, J. A. P. Coutinho and I. Billard, *Angew. Chem., Int. Ed.*, 2018, **57**, 1563–1566.
4 F. H. Quina and W. L. Hinze, *Ind. Eng. Chem. Res.*, 1999, **38**, 4150–4168.
5 P. Mukherjee, S. K. Padhan, S. Dash, S. Patel and B. K. Mishraa, *Adv. Colloid Interface Sci.*, 2011, **162**, 59–79; A. Z. Naqvi and Kabir-Ud-Din, *Colloids Surf., B*, 2018, **165**, 325–344.
6 S. Kumar, V. Aswal, A. Z. Naqvi and P. S. Goyal, *Langmuir*, 2001, **17**, 2549–2551.
7 S. Kumar and D. Sharma, *Langmuir*, 2003, **19**, 3539–3541; M. Benrraou, B. L. Bales and R. Zana, *J. Phys. Chem. B*, 2003, **107**, 13432–13440; B. L. Bales and R. Zana, *Langmuir*, 2004, **20**, 1579–1581; D. Mitra, I. Chakraborty, S. C. Bhattacharya and S. P. Moulik, *Langmuir*, 2007, **23**, 3049–3061; S. Kumar and A. Bhadoria, *J. Chem. Eng. Data*, 2012, **57**, 521–525; S. Kumar, A. Bhadoria, H. Patel and V. K. Aswal, *J. Phys. Chem. B*, 2012, **116**, 3699–3703; J.-H. Lin, W.-S. Chen and S.-S. Hou, *J. Phys. Chem. B*, 2013, **117**, 12076–12085; S. K. Yadav, R. B. Yadav and S. Kumar, *J. Surfactants Deterg.*, 2016, **19**, 1063–1069.
8 J. Mata, D. Varade, G. Ghosh and P. Bahadur, *Colloids Surf., A*, 2004, **245**, 69–73.
9 G. Liu, H. Zhang, G. Liu, S. Yuan and C. Liu, *Phys. Chem. Chem. Phys.*, 2016, **18**, 878–885.
10 Y. S. Lai, F. De Francesco, A. Aguinaga, P. Parameswaran and B. E. Rittmann, *Green Chem.*, 2016, **18**, 1319–1326; F. A. Vieira, R. J. R. Guilherme, M. C. Neves, A. Rego, M. H. Abreu, J. A. P. Coutinho and S. P. M. Ventura, *Sep. Purif. Technol.*, 2018, **196**, 300–308; S. Shariati, M. Faraji, Y. Yamini and A. Asghar Rajabi, *Desalination*, 2011, **270**, 160–165; E. Ranjbari, M. R. Hadjmohammadi, F. Kiekens and K. De Wael, *Anal. Chem.*, 2015, **87**, 7894–7901; C. K. Ahn, D. Park, S. H. Woo and J. M. Park, *J. Hazard. Mater.*, 2009, **164**, 1130–1136.
11 K.-Y. Show, D.-J. Lee, J.-H. Tay, T.-M. Lee and J.-S. Chang, *Bioresour. Technol.*, 2015, **184**, 258–266.
12 S. Y. Choi, K.-H. Yoon, J. I. Lee and R. J. Mitchell, *BioMed Res. Int.*, 2015, 465056.
13 S. Saita, Y. Kohno and H. Ohno, *Chem. Commun.*, 2013, **49**, 93–95.
14 D. Dong, J. B. Hooper and D. Bedrov, *J. Phys. Chem. B*, 2017, **121**, 4853–4863.
15 P. Mukerjee and K. J. Mysels, National Standard Reference Data System, 1971, NSRDS-NBS 36, Washington, DC, USA.
16 J. M. Pollard, A. J. Sh and K. E. Göklen, *J. Chem. Eng. Data*, 2006, **51**, 230–236.
17 D. J. G. P. Van Osch, C. H. J. T. Dietz, J. van Spronsen, M. C. Kroon, F. Gallucci, M. van Sint Annaland and R. Tuinier, *ACS Sustainable Chem. Eng.*, 2019, **7**, 2933–2942.
18 I. M. Aroso, J. C. Silva, F. Mano, A. S. Ferreira, M. Dionísio, I. Sá-Nogueira, S. Barreiros, R. L. Reis, A. Paiva and A. R. Duarte, *Eur. J. Pharm. Biopharm.*, 2016, **98**, 57–66.
19 J. R. Lu, A. Marrocco, T. J. Su, R. K. Thomas and J. Penfold, *J. Colloid Interface Sci.*, 1993, **158**, 303–316.
20 M. A. R. Martins, E. A. Crespo, P. V. A. Pontes, L. P. Silva, M. Bülow, G. J. Maximo, E. A. C. Batista, C. Held, S. P. Pinho and J. A. P. Coutinho, *ACS Sustainable Chem. Eng.*, 2018, **6**, 8836–8846.
21 J. Cao, M. Yang, F. Cao, J. Wang and E. Su, *J. Cleaner Prod.*, 2017, **152**, 399–405.

