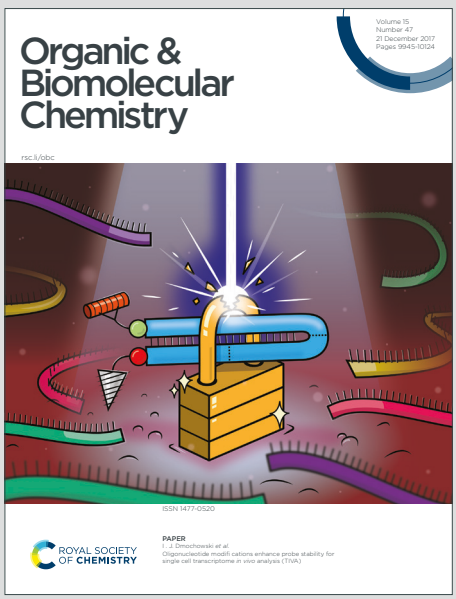
Check for updates

View Article Online
View Journal

Organic & Biomolecular Chemistry

Accepted Manuscript

This article can be cited before page numbers have been issued, to do this please use: K. Ikeda, K. Fujita, H. Ohno and N. Nakamura, *Org. Biomol. Chem.*, 2019, DOI: 10.1039/C9OB00900K.



This is an Accepted Manuscript, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this Accepted Manuscript with the edited and formatted Advance Article as soon as it is available.

You can find more information about Accepted Manuscripts in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this Accepted Manuscript or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

rsc.li/obc

View Article Online
DOI: 10.1039/C9OB00900K

Organic & Biomolecular Chemistry

ARTICLE

Effects of Charge Balance and Hydrophobicity of the Surface of Cytochrome *c* on the Distribution Behaviour in an Ionic Liquid/Buffer Biphasic System

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Kazuma Ikeda,[a] Kyoko Fujita,[b]* Hiroyuki Ohno [a] and Nobuhumi Nakamura [a]*

Factors contributing to the different distribution behaviour of cytochrome c were investigated in a biphasic tetrabutylphosphonium 2,4,6-trimethylbenzenesulfonate and potassium phosphate buffer system, which shows a lower critical solution temperature. To change charge balance and hydrophobicity of cytochrome c, surface modification with a few modifier molecules was applied. Surface charge and hydrophobicity affected the distribution behavior of chemically modified cytochrome c in the tetrabutylphosphonium 2,4,6-trimethylbenzenesulfonate and potassium phosphate buffer biphasic system. The distribution ratio into tetrabutylphosphonium 2,4,6-trimethylbenzenesulfonate decreased with decreasing isoelectric point of cytochrome c. Furthermore, cytochrome c possessing a low isoelectric point showed different distribution ratio depending on surface hydrophobicity. Taken together, these findings inidicate that isoelectric point and surface hydrophobicity of cytochrome c are important factors controlling the distribution behavior in temperature sensitive biphasic systems.

Introduction

Ionic liquids (ILs) have excellent physicochemical properties such as negligible vapour pressure and high thermal stability.[1] ILs have been gaining interest as new solvents in processes such as organic synthesis, catalysis and electrochemistry.[2] Recently, the application of ILs as media for biochemistry has received attention because ILs also possess interesting characteristics as enzyme solvents.[3]

IL-based aqueous two phase systems, such as hydrophobic IL/water systems and IL/inorganic salt solution systems, have been reported and have garnered much interest as solvent extraction media for many compounds including biopolymers.[4-7] Kragl *et al*. have investigated the partition coefficients of several proteins in IL/inorganic salt solution systems.[8] They have suggested that the charges on proteins are a major factor governing the dissolution of the proteins in the IL-rich phase.

Previously, we studied the remarkable properties of ILs upon mixing with water,[9] and reported the preparation of ILs with amino acid anions bearing a trifluoromethanesulfonyl group on the amino group. These ILs underwent a temperature sensitive phase change after mixing with water. For example, tetrabutylphosphonium *N*-trifluoromethanesulfonyl-leucine ([P_{4444}][Tf-Leu]) was found to be miscible with water; however, a clear phase separation was observed in the mixture upon heating, which became miscible again upon cooling. This reversible phase behaviour, classified as a lower critical solution temperature (LCST)-type phase change of the IL/water mixture, has been previously reported.[9, 10] The phase change between the homogeneous phase and the separated liquid/liquid biphasic system was controlled by temperature; thus, the LCST-type phase transition of the IL/water mixture permits applications such as the extraction of biopolymers including proteins.[11]

We have also reported that the surface charge of the proteins and their isoelectric point (p*I*) should influence the distribution value.[12] Proteins which have p*I* over around 7.0 showed extraction into IL phase, such as hemoglobin (p*I* 7.0), chymotrypsin (p*I* 8.6) and Lysozyme (p*I* 11.4). However, protein extraction in the biphasic system is not so simple. For example, horseradish peroxidase (HRP, p*I* 7.2) was expected to be extracted into the IL phase, but it was remaining in the water phase. On the other hand, albumin (BSA, p*I* 4.9) was transfer to IL phase. The distribution value of HRP and BSA cannot be explained only by p*I* value. Other factors could influence the partition coefficients of proteins including hydrophobic interactions[13] and the salting-out phenomenon.[14] However, the major driving force governing the extraction of proteins from the aqueous to the IL phase remains unclear. As previous studies utilised individual proteins, which have a distinct sequence, size and structure, the basic characteristics, such as hydrophobicity and p*I*, were also different. Thus, discussion about factors related to the protein distribution in IL/water systems was difficult in a systematic matter. Although there are some reports about distribution of proteins into ILs, these did not investigate the effects on distribution. In this study, we used surface modification on a single protein which is expected to enable an investigation of proteins with approximately the same size and basic features, except for surface modifier characteristics, in order to determine factors governing the

[a] *Department of Biotechnology, Tokyo University of Agriculture and Technology, 2-24-16 Koganei, Tokyo 184-8588, Japan.*
[b] *Department of Pathophysiology, Tokyo University of Pharmacy and Life Sciences, 1432-1 Horinouchi, Hachioji, Tokyo, 192-0392, Japan*
† Footnotes relating to the title and/or authors should appear here.
Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

Published on 17 July 2019. Downloaded by KEAN UNIVERSITY on 7/19/2019 8:50:09 PM.

Organic & Biomolecular Chemistry Accepted Manuscript

Please do not adjust margins

View Article Online
DOI: 10.1039/C9OB00900K

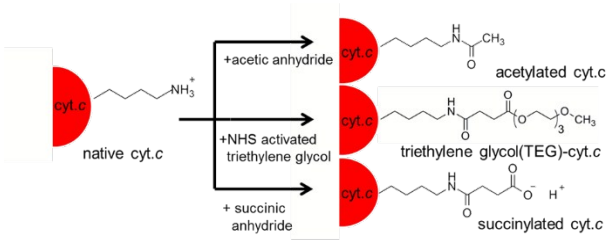


Fig. 1 Chemical modification of cyt.*c* for the preparation of acetylated, TEG and succinylated cyt.*c*.

extraction of proteins from the aqueous to the IL phase. We formulate analysed the effect of potential factor on the distribution value of the protein without influence by other factors.

In this study, cytochrome c (cyt. c) from *horse heart* is chosen as a model protein for study since it is one of the most thoroughly physicochemically characterized metalloproteins.[15] It is easy to confirm the effect of the surface modification on the redox state and coordination state of active site. Furthermore, modification technique of cyt.c by using lysine residue has been established.[16] Systematize examination is expected by surface modification to cyt.c with appropriate modifier. In addition, we have reported the extraction of proteins using such IL/water biphasic systems, and found that cyt.*c* was selectively transferred into the IL-rich phase.[7] Furthermore, the distribution behaviour of cyt.*c* differed with redox state[17]; oxidized cyt.*c* showed extraction from the aqueous phase to the IL phase via LCST transition, while reduced cyt.*c* remained in the aqueous phase even after LCST transition. With additional control of cyt.*c* redox state by applied electrochemical potential, phase transfer of cyt.*c* in an LCST type IL/ water biphasic system has been confirmed. We analysed controlling factors related to the distribution ratio of proteins using different chemical modifications of the protein, cyt.*c*. We investigated the effect of cyt.*c* surface charge and hydrophobicity on the distribution behaviour in a $[P_{4,4,4,4}][TMBS]$ / potassium phosphate buffer (PKB) biphasic system. The present study provides insight into the partition of proteins in IL-based aqueous two phase systems.

Results and discussion

The surface of cyt.*c* was modified using three different modifiers with different electric charge and hydrophobicity. Chemical modifications were targeted to the lysine residues on cyt.*c*, which has 19 lysine residues. The modifications included acetylation, triethylene glycol (TEG) modification and succinylation, resulting in the loss of a positive charge, the addition of an amphipathic unit and the exchange of a positive charge to a negative charge, respectively. The average chemical modification number and p*I* of the three types of synthesised chemically modified cyt.*c*s, acetylated cyt.*c*, succinylated cyt.*c* and methyl terminal TEG cyt.*c* (Fig. 1), were analysed as shown in Table 1. Modification numbers on cyt.*c* were related to the molar ratio of the modifier. Chemically modified cyt.*c* showed a decreasing p*I* with increasing modification number, as the positive charge of the lysine residues on cyt.*c* was used for modification, as shown in Fig. 1. By using these chemically modified cyt.*c*s and native cyt.*c*, the relationship between D_{IL} and p*I* were evaluated. The observed D_{IL} of native cyt.*c* was nearly 1.0, indicating that cyt.*c* transferred into the IL phase from the water phase following LCST biphasic behaviour. In contrast, the D_{IL} of chemically modified cyt.*c* decreased with decreasing p*I* (Fig. 2). This observation suggests that the p*I* of cyt.*c* is correlated with the distribution behaviour of cyt.*c* in the $[P_{4444}][TMBS]$/ PKB biphasic system. Interestingly, the D_{IL} of chemically modified cyt.*c* differed around p*I* 4 according to the modifier. All these chemically modified cyt.*c*s with a p*I* around 4 had modifications in over half of all lysine residues (10 – 14). This suggests that the positive lysine residues of cyt.*c*s were similarly used for modification. Of the modified cyt.*c*s, succinylated cyt.*c*s showed the lowest D_{IL} at around p*I* 4; the succinyl modifier may dissociate at pH 8. Succinylated cyt.*c* has been suggested to have a more hydrophilic surface than other modified cyt.*c*s with undissociated modifiers. Thus, the degree of cyt.*c* surface hydrophobicity could cause differences in D_{IL}. Therefore, the hydrophobicity order of chemically modified cyt.*c*s was compared.

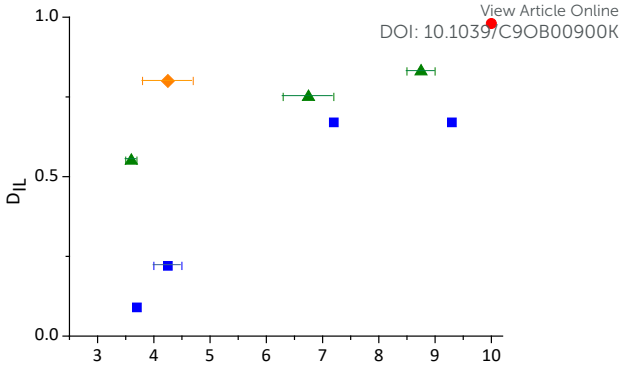


Fig. 2 Relationship between D_{IL} and p*I* for (●) native cyt.*c*, (◆) acetylated cyt.*c*, (▲) methyl-TEG-cyt.*c* and (■) succinylated cyt.*c*.

Table 1 Association between modifier molar ratio, average chemical modification number and p*I*

Sample	Molar ratio of modifier	Average chemical modification number	p*I*
native cyt. *c*	-	-	10
Acetylated cyt. *c*	*Purchased*	10.9	3.8-4.7
Succinylated cyt. *c*	X 0.5	6.5	7.2, 9.3
	X 0.7	9.5	4.0-4.5
	X 2.1	13.8	3.7
Methyl-TEG cyt. *c*	X 0.2	2.7	8.5-9.0
	X 0.5	8.7	6.3-7.2
	X 1.0	13.0	3.5-3.7

Table 2 Association between average chemical modification number and p*I* of TEG modified cyt.*c*

Sample	Average chemical modification number	pI
Ethyl-TEG cyt. c	2.7	8.5-8.8
	8.5	6.5-6.8
	12.5	3.8
Butyl-TEG cyt. c	2.4	8.1-9.3
	8.3	4.3-4.5, 5.9-6.3
	13.0	3.2-3.8

Published on 17 July 2019. Downloaded by KEAN UNIVERSITY on 7/19/2019 8:50:09 PM.

Organic & Biomolecular Chemistry Accepted Manuscript

This journal is © The Royal Society of Chemistry 20xx

Please do not adjust margins

View Article Online
DOI: 10.1039/C9OB00900K

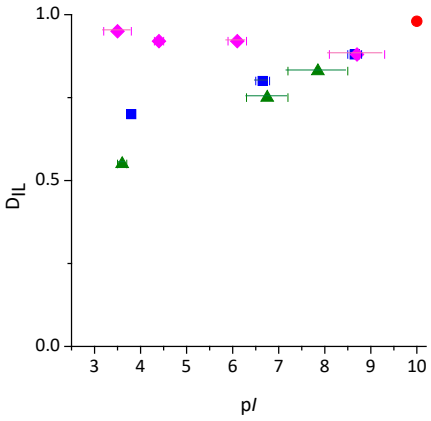


Fig. 3 Relationship between D_{IL} and p*I* for (●) native cyt.*c*, (◆) butyl-TEG cyt.*c*, (■) ethyl-TEG-cyt.*c* and (▲) methyl -TEG cyt.*c*.

The expected hydrophobicity order of the modified proteins was acetylated > TEG > succinylated cyt.*c*. Hydrophobic interaction chromatography was used to evaluate the degree of surface hydrophobicity of the modified cyt.*c*s. The chemically modified cyt.*c* was loaded onto a phenyl column, and the retention time was then measured by applying a concentration gradient of ammonium sulphate (0.8 ~ 0 M). The retention time of acetylated cyt.*c*, TEG cyt.*c* and succinylated cyt.*c* was 600 ~ 650 min, 400 ~ 450 min and 0 min (shortly after protein loading), respectively (Fig. S1 in Supporting information). Based on these results, the predicted hydrophobicity order was confirmed as acetylated cyt.*c*, TEG cyt.*c* and succinylated cyt.*c*. The predicted hydrophobicity order was quite consistent with the observed D_{IL} at p*I* 4_L. Succinylated cyt.*c* had the most hydrophilic surface of these chemically modified cyt.*c*s. Succinylated cyt.*c* with a p*I* of 4 showed a D_{IL} < 0.3. These cyt.*c*s remained in the aqueous phase even after LCST transition. The hydrophobicity derived from each modifier was suggested to affect the distribution ratio of cyt.*c* in the LCST biphasic system at the lower p*I* region.

Both the p*I* and surface hydrophobicity of cyt.*c* were suggested above as factors associated with the D_{IL} of cyt.*c* in the LCST biphasic system. The effect of these two factors, p*I* and surface hydrophobicity, on D_{IL} was further considered using TEG modifiers on cyt.*c*. Three different TEG-based modifiers with different terminal alkyl chain lengths, methyl, ethyl and butyl (Fig. S2 in Supporting information), were used to modify cyt.*c*. These TEG modifiers were expected to have a different hydrophobicity depending on the alkyl chain length. Table 2 shows the modification number and p*I* of the modified cyt.*c*s. p*I* decreased with increasing modification number because of the use of the positively charged lysine residues. Fig. 3 shows the observed relationship between D_{IL} and p*I* depending on the modifier. When methyl-TEG and ethyl-TEG were used to modify cyt.*c*, the D_{IL} decreased with decreasing p*I* of the modified cyt.*c*, which was based on the cyt.*c* modification number. In contrast, no change in D_{IL} was observed for cyt.*c* modified with butyl-TEG showed, regardless of p*I*, i.e. modification number. In particular, at the low p*I* region (p*I* 4 – 5) of TEG-modified cyt.*c*, D_{IL} differences dependent on terminal alkyl length were clearly observed. The D_{IL} value of methyl-TEG, ethyl-TEG and butyl-TEG modified cyt.*c* was 0.55, 0.80 and 0.92, respectively. Surface hydrophobicity based on TEG modifier was suggested as a preferential control factor of distribution behaviour when cyt.*c* has a certain p*I*. These findings strongly support the role of cyt.*c* surface hydrophobicity as a key factor affecting distribution behaviour at the low p*I* region (around 4) in the $[P_{4444}][TMBS]$/ PKB biphasic system.

Conclusions

Factors related to the different distribution behaviour of cyt.*c* were investigated in the $[P_{4,4,4,4}][TMBS]$ / PKB buffer biphasic system using chemical modification of cyt.*c*. By using the surface modification on a single protein, effect of focused factor i.e. charge balance and hydrophobicity of the surface on the distribution of protein was investigated. Influence from other factors were eliminated. Surface charge was controlled by changing the modification number of the 19 lysine residues of cyt.*c*. The p*I* of modified cyt.*c* was related to the modification number. Decreased p*I* was correlated with decreased D_{IL} values. Furthermore, chemically modified cyt.*c* with a similar low p*I* (e.g. a p*I* around 4) showed different D_{IL} values depending on the surface hydrophobicity based on the TEG modifier. The influence of the surface hydrophobicity of cyt.*c* on the distribution behaviour was observed more clearly with cyt.*c*s possessing a lower p*I*. The p*I* of chemically modified cyt.*c* and the surface hydrophobicity were revealed as important factors controlling the distribution behaviour of cyt.*c* in the $[P_{4,4,4,4}][TMBS]$ / PKB biphasic system. By tuning the surface charge and hydrophobicity, the distribution behaviour of a specific protein may be more easily controlled in the LCST biphasic system. This might provide a great advantage in the complete separation for recycling use of proteins and enzymes in reaction separation platforms in temperature sensitive aqueous and non-aqueous biphasic systems.

Experimental

Materials

Sodium 2,4,6-trimethylbenzenesulfonate (Na[TMBS]), succinic anhydride, triethylene glycol monomethyl ether, triethylene glycol monoethyl ether, triethylene glycol monobutyl ether, *N*-hydroxysuccinimide, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride were purchased from Tokyo Chem. Ind. Co. Hydrochloric acid (HCl) and pyridine were purchased from Wako Chem. Co. Cytochrome *c* and partially acetylated cytochrome *c* from *horse heart* were purchased from Sigma Aldrich. Tetrabutylphosphonium hydroxide ($[P_{4444}]OH$) were provided by Hokko Chem Co. A p*I* broad kit 3-10 was purchased from GE healthcare. All of the chemicals and proteins were used as received.

Synthesis of tetrabutylphosphonium 2,4,6-trimethylbenzenesulfonate ($[P_{4,4,4,4}][TMBS]$)

An aqueous solution of $[P_{4444}]OH$ was first neutralised with hydrochloric acid, to prepare $[P_{4444}]Cl$. Next, $[P_{4444}]Cl$ was dissolved in water, and a slight excess of Na[TMBS] was added. This solution was stirred for 24 h at room temperature. The product was extracted with dichloromethane, and the dichloromethane layer was washed repeatedly with water. The dichloromethane layer was evaporated

Published on 17 July 2019. Downloaded by KEAN UNIVERSITY on 7/19/2019 8:50:09 PM.

Organic & Biomolecular Chemistry Accepted Manuscript

Please do not adjust margins

View Article Online
DOI: 10.1039/C9OB00900K

Published on 17 July 2019. Downloaded by KEAN UNIVERSITY on 7/19/2019 8:50:09 PM.

and the product was dried under reduced pressure for 24 h. δ_H (400 MHz, $CDCl_3$) 0.95 (t, *J* 14.4, 12H, CH_2CH_3), 1.49–1.50 (m, 16H, CH_2), 2.30 (s, 3H, $ArCH_3$), 2.32–2.38 (m, 8H, PCH_2), 2.70 (s, 6H, $ArCH_3$), 6.79 (s, 2H, ArH)

Synthesis of N-Hydroxysuccinimide activated methyl, ethyl and butyl terminal triethylene glycol modifiers

Triethylene glycol monomethyl ether, triethylene glycol monoethyl ether and triethylene glycol monobutyl ether were dissolved in 1,2-dichloroethane under a Ar atmosphere each other. Next succinic anhydride was added into these solvents. To these reaction mixtures, pyridine was added dropwise. The reaction mixtures was then stirred at 40-50 °C for 24 h under Ar reflux. These were poured into water and neutralized with 1N concentrated HCl. The organic layer was then washed with brine solution. The 1,2-dichloroethane layer was evaporated and the product were dried under reduced pressure for 2h. These products were dissolved in acetonitrile. Next *N*-hydroxysuccinimide was added into these solvents. 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride was added drop wise to these reaction mixtures. The reaction mixtures were then stirred at r.t. for 48 h. The dichloromethane layer was then washed with brine solution. The dichloromethane layer was evaporated and the product were dried under reduced pressure for 2h. These were purified by passing through a silica gel column using methanol : chloroform, 1:9 (v/v).

N-Hydroxysuccinimide activated methyl terminal triethylene glycol
δ_H (400MHz,$CDCl_3$) 2.72-2.84(m,6H,OC(=O)CH_2,CH_2-C(=O)NC(=O)CH_2), 2.97 (t, *J* 14.2, 2H, C(=O)CH_2) ,3.38 (s, 3H, CH_3O),3.54-3.56(m,2H,OCH_2) ,3.63-3.72(m,8H,$CH_2OCH_2CH_2OCH_2$),4.73(t, *J* 9.6, 2H, CH_2C(=O))

N-Hydroxysuccinimide activated ethyl terminal triethylene glycol
δ_H (400 MHz,$CDCl_3$) 1.21(t, *J* 14.2,CH_3CH_2), 2.76-2.83(m,6H,OC(=O)CH_2,CH_2-C(=O)NC(=O)CH_2), 2.96 (t, *J* 14.2, 2H, C(=O)CH_2) ,3.38 (s, 2H, CH_2O)、3.50-3.54(m,2H,OCH_2) ,3.58-3.60(m,2H,OCH_2)、3.63-3.72(m,8H,$CH_2OCH_2CH_2OCH_2$),4.28(t, *J* 9.2, 2H, CH_2C(=O))

N-Hydroxysuccinimide activated butyl terminal triethylene glycol
δ_H (400 MHz,$CDCl_3$) 0.91(t,*J* 14.6, 3H,CH_3CH_2),1.31-1.60(m, 4H, CH_2CH_2O), 2.76-2.83 (m,6H,OC(=O)CH_2,CH_2-C(=O)NC(=O)CH_2), 2.96 (t, *J* 13.7, 2H, C(=O)CH_2), 3.38 (s, 2H, CH_2), 3.50-3.54(m,2H,OCH_2), 3.57-3.60(m,2H,OCH_2), 3.63-3.66(m,8H,$CH_2OCH_2CH_2OCH_2$), 4.28(t, *J* 9.6, 2H, CH_2C(=O))

Preparation of chemically modified cyt *c*

Lysine residues of cyt.*c* was modified by adding succinic anhydride, acetic anhydride and *N*-Hydroxysuccinimide activated triethylene glycols (succinylated cyt.*c*, acetylated cyt.*c*, triethylene glycol modified cyt.*c* (TEG cyt.*c*)) in boric acid buffer (100 mM, pH 9.5) at 35°C for 2 h. After the reaction, the solvents were replaced with pure water by using Amicon (Mw 5000) at 5000 g centrifugation. After centrifugation, these chemically modified cyt.*c*s were stored by using freeze dry procedure.

p*I* of chemically modified cyt.*c* was measured by isoelectric focusing (GE healthcare, phastsytem) and the number of chemical modification on lysine residues was determined by trinitrobenzosulfonate reaction.

Determination of the distribution ratio into $[P_{4444}][TMBS]$ phase (D_{IL}) of cyt.c

Tetrabutylphosphonium 2,4,6-trimethylbenzenesulfonate ($[P_{4,4,4,4}][TMBS]$) was used as the IL that undergoes the LCST-type phase transition. $[P_{4,4,4,4}][TMBS]$ was dissolved in 100 mM phosphate buffer (pH 8.0), and induced phase separation by heating. After phase separation, obtained bottom phase was used as an IL phase. cyt.*c* were dissolved in 100 mM phosphate buffer (pH 8.0), and the solution was added to the IL phase. The mixture formed a homogeneous phase. This homogeneous solution was then heated and left to stand until the phases were separated clearly. The absorbance of the buffer phase was measured by UV-vis spectroscopy. The D_{IL} of cyt.*c* was calculated using following equation: D_{IL} =($Abs_{buffer\text{-}before}$ -$Abs_{buffer\text{-}after}$) / $Abs_{buffer\text{-}before}$, where Abs_{buffer} denote the absorbance of the buffer phase. The maximum absorption of the Soret band around 408.0 nm was used for cyt.*c.*

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This research was supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (KAKENHI, No. 17H01225 and 18H01719)

Notes and references

1. P. Wasserscheid, T. Welton and Editors, *Ionic Liquids in Synthesis*, 2003.
2. M. Armand, F. Endres, D. R. MacFarlane, H. Ohno and B. Scrosati, *Nature Materials*, 2009, **8**, 621-629.
3. S. Oppermann, F. Stein and U. Kragl, *Applied microbiology and biotechnology*, 2011, **89**, 493-499.
4. A. Cizova, J. Korcova, P. Farkas and S. Bystricky, *Int J Biol Macromol*, 2017, **98**, 314-318.
5. C. P. Song, P. E. Liew, Z. Teh, S. P. Lim, P. L. Show and C. W. Ooi, *Frontiers in chemistry*, 2018, **6**, 529.
6. P. Xu, Y. Wang, J. Chen, X. Wei, W. Xu, R. Ni, J. Meng and Y. Zhou, *Talanta*, 2018, **189**, 467-479.
7. Y. Ito, Y. Kohno, N. Nakamura and H. Ohno, *International journal of molecular sciences*, 2013, **14**, 18350-18361.
8. S. Dreyer, P. Salim and U. Kragl, *Biochemical Engineering Journal*, 2009, **46**, 176-185.
9. K. Fukumoto and H. Ohno, *Angewandte Chemie*, 2007, **46**, 1852-1855.
10. Y. Kohno and H. Ohno, *Physical chemistry chemical physics : PCCP*, 2012, **14**, 5063-5070.
11. A. M. Ferreira, H. Passos, A. Okafuji, A. P. M. Tavares, H. Ohno, M. G. Freire and J. A. P. Coutinho, *Green chemistry,* 2018, **20**, 1218-1223.
12. Y. Kohno, S. Saita, K. Murata, N. Nakamura and H. Ohno, *Polym. Chem.*, 2011, **2**, 862-867.
13. Y. Pei, J. Wang, K. Wu, X. Xuan and X. Lu, *Separation and Purification Technology*, 2009, **64**, 288-295.
14. Z. Du, Y.-L. Yu and J.-H. Wang, *Chemistry – A European Journal*, 2007, **13**, 2130-2137.
15. G. W. Pettigrew, *Cytochromes C. Biological Aspects*, 1987.
16. K. Wada and K. Okunuki, *Journal of biochemistry*, 1969, **66**, 249-262.

Organic & Biomolecular Chemistry Accepted Manuscript

This journal is © The Royal Society of Chemistry 20xx

Please do not adjust margins

View Article Online
DOI: 10.1039/C9OB00900K

17. K. Ikeda, R. Ikari, N. Nakamura, H. Ohno and K. Fujita, *Journal of The Electrochemical Society*, 2018, **165**, G96-G100.

Published on 17 July 2019. Downloaded by KEAN UNIVERSITY on 7/19/2019 8:50:09 PM.

Organic & Biomolecular Chemistry Accepted Manuscript

Please do not adjust margins

View Article Online
DOI: 10.1039/C9OB00900K

Published on 17 July 2019. Downloaded by KEAN UNIVERSITY on 7/19/2019 8:50:09 PM.

Organic & Biomolecular Chemistry Accepted Manuscript

Surface charge and hydrophobicity affected the distribution behavior of chemically modified cyt.*c* in the $[P_{4,4,4,4}][TMBS]$/PKB biphasic system.