Affinity ionic liquid†

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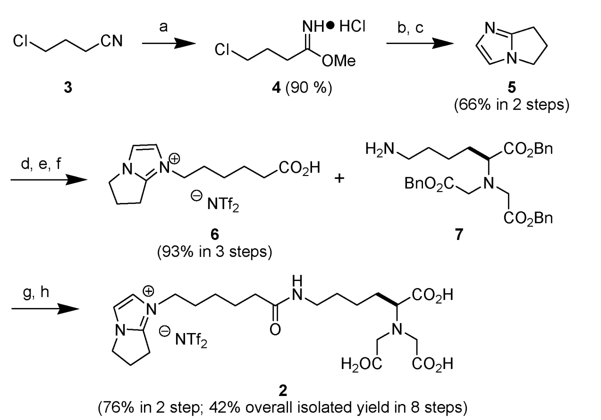
An affinity ionic liquid, based on biomolecular recognition, was developed and found to be capable of quantitative partitioning of biomacromolecules from aqueous buffer to ionic liquid.

This paper reports the use of an affinity ionic liquid (AIL) for the study of biomolecular recognition in pure ionic liquid. Ionic liquids have attracted much attention in recent years and carry many desirable properties, such as wide liquid range, thermal stability, high ionic conductivity, enhanced solubility with many organic compounds, attractive recyclability, very low flammability, and negligible vapor pressure, that are well suited for numerous innovative applications, including high temperature lubricants, re-writeable image surfaces, and reaction media for synthesis and catalysis, and novel electrolytes for solar and fuel cells.[1] Ionic liquids are also considered as environmentally friendly substitutes to conventional organic solvents and often outperform molecular solvents in chemical reactions and applications.[1] Recently, significant progress has been made to explore functionalized ionic liquids through incorporation of functional groups as a part of developing ionic liquids possessing tailored properties.[2] Here, we are reporting our initial development of AILs for use to investigate biomolecular interactions in ionic liquids, with a direct and immediate aim to facilitate the one-step affinity isolation and purification of recombinant fusion proteins in bacterial cells such as *E. coli*.

Ionic liquids possess remarkable solubility with many small molecules and, in the literature, have shown their superior applicability toward extraction of metal ions and amino acids.[3] Moreover, ionic liquids are polar ionic solvents and have been demonstrated as excellent media for peptide synthesis.[4] In this paper, we illustrate our AIL methodology using a fluorescein-labeled hexahistidine peptide FITC-(His)$_6$-NH$_2$ (**1**), a His-tag green fluorescent protein (GFP) and an AIL **2** as the model system. This system of biomolecular recognition has been extensively studied using other techniques and a substantial amount of literature concerning its interaction is available (footnote 1, ESI†). Peptide **1** could be prepared in a straightforward manner by solid-phase peptide synthesis using the standard Fmoc chemistry. In this work, the recombinant His-tag GFP was used as a model protein with which to demonstrate the technology. As a popular reporter of gene expression, GFP is a well known protein routinely used in molecular biology as the proof-of-concept that a gene can be expressed throughout a given organism.[5] The AIL **2** was readily synthesized by standard organic chemistry protocols.

The synthesis of AIL **2** is outlined in Scheme 1. Based on our previous report on the synthesis of a series of new bicyclic imidazolium ionic liquids that are far more chemically stable than the common [bmim][PF$_6$] and [bdmim][PF$_6$],[6] it prompted us to incorporate this structural core for the development of AILs. In short, the synthesis of AIL **2** began with commercially available 4-chlorobutyronitrile **3** and, having the imidate **4** as the intermediate compound, the key bicyclic imidazole **5** was prepared using the protocol previously developed by us (59% isolated yield in three steps) (Scheme 1).[6] A direct alkylation of the resulting imidazole **5** with benzyl 6-bromohexanoate under heated conditions followed by deprotection using catalytic hydrogenation and subsequently an ion exchange in water readily afford the precursor ionic liquid acid **6** with a high 93% isolated yield in three steps. Finally, the EDC coupling of **6** with a protected NTA amine **7** in dichloromethane followed by the deprotection using catalytic hydrogenation optimally achieved the preparation of AIL **2** as a viscous liquid at room temperature (76% yield in 2 steps). In our hands, the overall isolated yield of AIL **2** synthesis was high: 42% with a total of eight steps starting from **3** (Scheme 1). Details of the synthetic procedures are summarized in the ESI.†

Results in Fig. 1 clearly show that, upon association with Ni(II), the AIL **2** dissolved in [bdmim][NTf$_2$] ionic liquid (**8**)



Scheme 1 Synthesis of AIL **2**. Experimental conditions: (a) HCl, MeOH, ether, −20 °C, 24 h; (b) $H_2NCH_2CH(OMe)_2$, Et_3N, CH_2Cl_2, reflux, 2 h; (c) HCO_2H, reflux, 20 h; (d) $Br(CH_2)_5CO_2Bn$, 80 °C, 2 h; (e) H_2, $Pd(OH)_2/C$, MeOH, rt, 1 h; (f) $LiNTf_2$, H_2O, rt, 12 h; (g) EDC, DIEA, CH_2Cl_2, rt, 5 h; (h) H_2, $Pd(OH)_2/C$, MeOH, rt, 1 h.

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specifically recognized the fluorescent peptide **1** in PBS buffer (upper layer) and, as a result, effectively extracted it into the ionic liquid phase (bottom layer). In these affinity extraction experiments, the ionic liquid phase was prepared by dissolving AIL **2** in ionic liquid **8** and the aqueous solution was separately prepared by dissolving peptide **1** in PBS buffer. Equal volumes of the ionic liquid **8** and buffer solution were mixed and the resulting mixture was then gently shaken at ambient temperature to reach the completion of extraction. As shown in Fig. 1 (tube a), without AIL **2** the bright green fluorescence remained in the upper layer, indicating that the peptide **1** was retained in the PBS buffer. Also, in the absence of Ni(II) cation, the partitioning of the peptide **1** into ionic liquid phase containing AIL **2** was negligible (tube b). Furthermore, the affinity extraction experiment performed in tube c (Fig. 1) unambiguously proved that the transfer of the peptide **1** from the aqueous phase into the ionic liquid **8** was solely due to affinity recognition by the Ni(II)-chelated AIL **2**. This quantitative extraction of peptide **1** was also apparent to the naked eye, since the bright green fluorescence of the peptide visually indicated its distribution between two layers (Fig. 1B). It is worth mentioning that no precipitation of peptide at the ionic liquid–water interface was observed; that is, **1** was completely soluble in the ionic liquid **8**. Fig. 1 also demonstrated that the total recovery of the peptide **1** from ionic liquid phase into the buffer layer could be readily achieved by competitive binding with AIL **2** using a high concentration of imidazole (tube d).

For the result shown in tube c of Fig. 1, we noted that the fluorescence of peptide **1** in ionic liquid phase was diminished completely. This experimental observation is in agreement with previous literature reports that ionic liquids could act as quenchers for fluorescence probes.[7] We have also experimentally confirmed by UV-vis spectroscopy that, upon affinity extraction by the Ni(II)-chelated AIL **2**, the peptide **1** partitioned entirely in the bottom ionic liquid layer and underwent a major blue shift with significant decrease in absorption intensity in its spectrum: λ_{max} = 495 nm in buffer *vs.* 445 nm in ionic liquid **8** (Fig. S1A, ESI†). Moreover, the fluorescence measurement further verified that the steady-state fluorescence of the fluorophore FITC itself in ionic liquid **8** was essentially quenched (Fig. S1B, ESI†). Therefore, it appears likely that, once in the ionic liquid phase, the close contact between the fluorophore and the ionic liquid molecules result in an efficient static fluorescence quenching (tube c in Fig. 1B).

For a receptor–ligand interaction, the stoichiometry of the binding is valuable to measure the concentration of active receptor and to probe ligand specificity. To determine the stoichiometry of our binding system, samples having a fixed concentration of the AIL **2** in ionic liquid **8** and various concentrations of the Fmoc-(His)$_6$-NH$_2$ peptide (**9**) in buffer were prepared to perform affinity extractions. Results in Fig. 2 showed that, as the ratio of [**9**]$_{total}$/[Ni(II)-chelated AIL **2**] increased, the concentration of **9** extracted into the ionic liquid phase increased (*i.e.*, peptide was transferred to the ionic liquid and its concentration in buffer phase was minimal) until the ratio reached its binding stoichiometry; beyond this value no more peptide **9** could be extracted because all Ni(II)-chelated AIL **2** in the ionic liquid phase has been fully titrated and associated with **9**, and therefore excessive **9** remained unbound in the buffer layer. An abrupt change in the slope, in a plot of the concentration of free peptide **9** in buffer phase *vs.* the ratio of [**9**]$_{total}$/[Ni(II)-chelated AIL **2**] in samples, corresponded to the binding stoichiometry of the system studied; that is, an expected 1 : 1 stoichiometry of this model binding system was obtained (Fig. 2). Furthermore, a similar experiment of the [**9**]$_{buffer}$ *vs.* the ratio of [**9**]$_{total}$/[Ni(II)] clearly indicated that, with Ni(II) alone but without AIL **2**, the peptide **9** was mostly retained in aqueous phase, suggesting that both AIL **2** and Ni(II) are required for efficient extraction of peptide **9** into the ionic liquid phase (Fig. S2, ESI†). Overall, we were notably pleased that the Ni(II)-chelated AIL **2** not only dissolved and delivered the peptide **9** into ionic liquid **8**, but also maintained its binding integrity (to form a stable 1 : 1 complex) and high specificity to peptide in pure ionic liquid.

Encouraged as we were by the peptide result, we then turned our attention to the green fluorescent protein (GFP), a powerful imaging tool, of the jellyfish *Aequorea aequorea*[5] and investigated it as a model protein with which to develop an effective platform for the one-step affinity isolation and

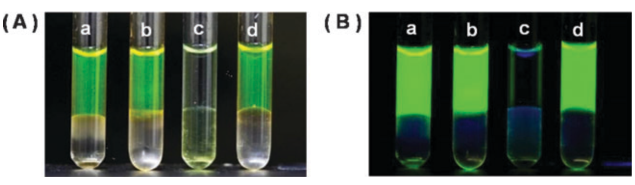

Fig. 1 Extraction of the fluorescent peptide **1** in PBS buffer (upper layer) by AIL **2** solubilized in ionic liquid **8** (bottom layer). (A) and (B) are optical and fluorescence photographic images, respectively, of peptide extraction into ionic liquid: (a) extraction of peptide **1** (100 μM) in PBS buffer into ionic liquid **8** containing no AIL **2**; (b) extraction of peptide **1** (100 μM) in PBS buffer into ionic liquid **8** having AIL **2** (20 mM) but without NiCl$_2$; (c) extraction of peptide **1** (100 μM) in PBS buffer layer into ionic liquid layer containing Ni(II)-chelated **2** (20 mM); (d) competitive extraction of peptide **1** (100 μM) in ionic liquid layer into the PBS buffer layer using imidazole (500 mM). Note that fluorescence of the peptide **1** was, in (c), quenched in ionic liquid (bottom layer) and, in (d), re-established in aqueous buffer (upper layer).

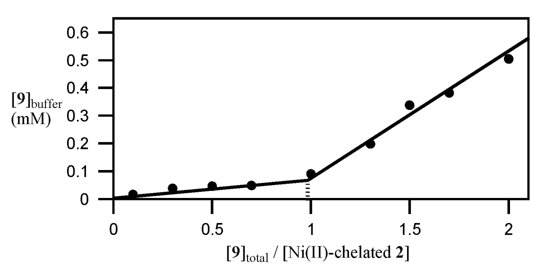

Fig. 2 Determination of the binding stoichiometry of the Ni(II)-chelated AIL **2** (1.00 mM) dissolved in ionic liquid **8** to peptide **9** (0.067–1.33 mM) in PBS buffer. The Fmoc-(Gly)$_6$-NH$_2$ peptide **10** was used as the internal standard in buffer. After each extraction, the molar ratios of [**9**]/[**10**] in the aqueous layer were quantitatively determined at 280 nm by C$_{18}$-HPLC. Peptides **9** and **10** were used in this experiment, primarily for the reason that they could be readily separated and quantified by HPLC.

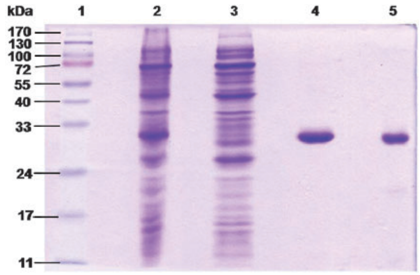

Fig. 3 SDS-polyacrylamide gel electrophoresis of direct extraction of the His-tag GFP in crude *E. coli* cell lysate by the Ni(II)-chelated AIL **2**. Lane 1, protein molecular weight markers (170, 130, 100, 72, 55, 40, 33, 24, 17, and 11 kDa); lane 2, total cell lysate; lane 3, the cell lysate after affinity extraction by Ni(II)-chelated AIL **2**; lane 4, competitive extraction of His-tag GFP in ionic liquid into aqueous buffer by imidazole; lane 5, purified GFP (2 g). Electrophoresis was performed under reduced conditions on 12% polyacrylamide gel.

purification of proteins by AILs (footnote 2, ESI†). Because GFP is fluorescent, the recombinant His-tag GFP is well-suited for our AIL experiments.

Our GFP (26.9 kDa) was expressed in *E. coli* as His-tag fusion to the N terminus of GFPmut1 that contains the double-amino-acid substitution (F64L and S65T), and reportedly has been optimized for brighter fluorescence and higher expression in *E. coli*.[8] With this protein in hand, we embarked upon direct affinity extraction of His-tag GFP in crude *E. coli* lysate by Ni(II)-chelated AIL **2** onto ionic liquid.

It was reported in literature that if GFP is denatured its fluorescence is quenched, and GFP emits fluorescence only when it has the correct tertiary structure of the native form.[9] Results of affinity extraction of Ni(II)-chelated AIL **2** with His-tag GFP shown in Fig. 3 and 4 and S3 (ESI†) clearly indicate that, because of the presence of fluorescence in the ionic liquid layer, this His-tag GFP likely maintains its native conformation with the ionic liquid (footnote 3, ESI†). No precipitation of GFP at the ionic liquid–water interface was observed. Both denaturing and native polyacrylamide gel electrophoresis experiments illustrated that His-tag GFP could be effectively extracted from the crude cell lysate onto the ionic liquid and then competitively back-extracted using imidazole to a newly prepared buffer solution (lane 4 in Fig. 3; lane 3 in Fig. S3, ESI†). We noted that, upon affinity extraction, the protein-bound AIL **2** was not totally miscible with ionic liquid **8** but uniformly dispersed in the ionic liquid phase (but clearly not at all partitioning in aqueous layer) (Fig. 4B) (footnote 3, ESI†). This phenomenon may be explained by the fact that Ni(II)-chelated AIL **2** has a strong affinity for His-tag GFP and therefore tightly coordinates with it to enable the complex formation, ultimately leading to the complete departure of the protein from the aqueous layer and quantitative transfer to suspension in the ionic liquid phase.

This ionic liquid effect on proteins at the molecular level is highly complex and understanding is far from complete, what can be said is that in hydrophobic ionic liquid **8** this His-tag GFP has shown its high stability (Fig. 4B). Furthermore, the hydrophobic solvent usually has a lesser tendency to take away the essential water from the protein surface and the

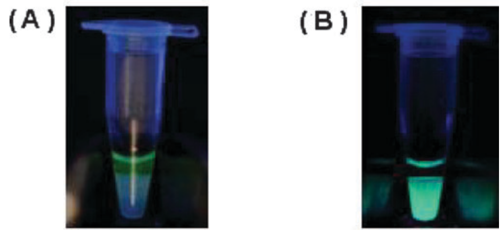

Fig. 4 Direct affinity extraction of the His-tag GFP from cell lysate: (A) before extraction and (B) after extraction. The upper layer was aqueous phase containing cell lysate (5 μL) in PBS (25 μL) and the bottom layer was Ni(II)-chelated AIL **2** (89 mM) solubilized in ionic liquid **8** (30 μL). Note that, after extraction, GFP remains fluorescent in the suspended ionic liquid layer.

hydrogen bonds and other interactions within the protein are not significantly perturbed by suspending in ionic liquid, leading to the conservation of the overall stability in protein. Though unlike the peptide case that is completely soluble in ionic liquid (Fig. 1), our affinity extraction of proteins evidently shows that the GFP maintains fluorescence (Fig. 4 and S2, ESI†) and can be isolated and purified using the protocol developed in this work.

In conclusion, we described our initial development of AILs and demonstrated that Ni(II)-chelated AIL **2** is eminently capable of performing binding interactions with His-tag peptides and proteins in ionic liquid. Based upon biospecific molecular recognition, this is the sole binding system of receptor–ligand interactions in pure ionic liquid to date. As the advance in research of protein compatible ionic liquids are of great interest, the affinity protocol developed in this work is practical and, most significantly, the GFP protein studied is fluorescent and remains active. The results presented in this report hold compelling possibilities for advancing biosensors targeting a new range of analytes and applications in biotechnology. Quantitative binding measurements of Ni(II)-chelated AIL **2** with His-tag peptides and proteins in pure ionic liquids are actively being pursued and the result will be reported in due course.

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