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ARTICLE TYPE

Ionic Liquid Poly(3-n-dodecyl-1-vinylimidazolium) Bromide as Adsorbent for the Sorption of Hemoglobin

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A novel polymeric ionic liquid, poly(3-n-dodecyl-1-vinylimidazolium) bromide (poly(C_{12}vim)Br), is prepared via solution polymerization. The poly(C_{12}vim)Br exhibits excellent adsorption performance toward hemoglobin in the presence of other protein species. It is shown that favorable adsorption of hemoglobin is achieved at pH 8.0, and the variation of ionic strength poses virtually no effect on the adsorption of hemoglobin at a NaCl concentration up to 0.4 mol L^{-1}. A maximum adsorption capacity of 205.4 mg g^{-1} is derived for hemoglobin. An adsorption efficiency of 93.8% is obtained by processing 80 μg mL^{-1} of hemoglobin in 1.0 mL of solution with 2 mg of poly(C_{12}vim)Br, and afterwards the use of 1.0 mL of sodium dodecyl sulfate (SDS, 0.5%, m/v) provides a recovery of ca. 86.3%. It is indicated the process of adsorption/desorption causes a slight conformational change for hemoglobin, while its structure remains predominantly as α-helix. The poly(C_{12}vim)Br has been used for the adsorption of hemoglobin from complex biological sample matrixes, e.g., human whole blood, and the favorable separation and purification performance is demonstrated by SDS-PAGE assay.

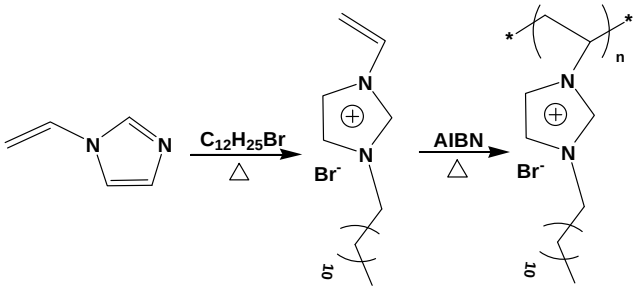
Introduction

Ionic liquids (ILs) possess unique physic-chemical properties as well as excellent solubility in aqueous medium.[1] They are generally recognized as green solvents and they represent adequate replacement for traditional deleterious and toxic organic solvents.[2] Nowadays, the applications of ILs in the field of analytical sciences have attracted extensive attentions, e.g., solvent/solid phase extraction,[3,4] electroanalytical chemistry,[5,6] chromatography[7,8] and chemical sensors.[9,10] Liquid-liquid extraction based on ionic liquid is widely concerned, especially for the extraction and separation of metal cations,[11,12] biomacromolecules[13-16] and organic pollutants.[17] As a favorable separation medium, ILs have been favorably applied for the extraction of proteins, which can be used in both conventional liquid-liquid extraction,[18-21] aqueous two-phase extraction[22,23] and micro-emulsion extraction systems.[24,25] In practice, however, liquid-liquid extractions are usually not favorable due to the complicated operation and the use of relatively large amount of reagents.

In this respect, solid phase extraction schemes are much more welcome by use of ionic liquid modified solid supports as sorption media. There are generally two main approaches for the preparation of solid adsorbent, e.g., dip-coating and covalent-bonding, and the latter is frequently used. The selectivity for the adsorption of proteins could be improved through anchoring of the ionic liquid,[26-31] and this generally results in a dramatic improvement on the adsorption capacity to proteins. Fe_3O_4@SiO_2 exhibits selectivity for the adsorption of bovine serum albumin (BSA),[32] while the product Fe_3O_4@SiO_2-[O_3SiMIM]$^+Cl^-$ (Fe_3O_4@SiO_2@IL) after two-steps modification regulated the selectivity for the retention of hemoglobin,[30] and offered an ultra-high adsorption capacity of 2.15 mg mg^{-1}. The porous nano-TiO_2 particles provide selectivity to cytochrome c (cyt-c),[33] the functionalization with ionic liquid gave rise to a N,N-bis[2-methylbutyl] imidazolium hexafluorophosphate-TiO_2 nanocomposite (PPimPF_6/TiO_2), which turns its selectivity to hemoglobin,[27] exhibiting an adsorption capacity of 122.3 mg g^{-1}. The ionomer PVC-[NMIM]$^+Cl^-$ generated by immobilization of N-methylimidazole onto polyvinyl chloride has been demonstrated to be much more selective toward hemoglobin.[28] These practices have clearly illustrated that the functionalization of solid supports with certain kind of ionic liquid moiety significantly changed the adsorption performance. However, it should be emphasized that the functionalization or modification processes are unable to completely cover the surface of the supporting materials. Therefore, the non-specific adsorption behavior of the solid support surface usually contributes to the adsorption of the targets to certain extent. In this respect, polymeric ionic liquids (PILs) should be a better choice. On one hand, PILs as polymers are generally solid materials at room temperature with functional groups of ionic liquid moiety,[34] PIL-based composites and PIL-co-polymers are widely used in separation sciences.[35-39]

In the present work, a novel linear polymeric ionic liquid poly(1-vinylimidazolium-3-n-dodecyl) bromide (poly(C_{12}vim)Br) is prepared via solution polymerization, as shown in scheme 1. The poly(C_{12}vim)Br exhibits highly adsorption towards hemoglobin under controlled condition at pH 8.0, and the retained protein can be readily recovered by using a 0.5% (m/v) sodium dodecyl sulfate (SDS). The practical applicability of poly(C_{12}vim)Br is demonstrated by isolation of hemoglobin from

human whole blood.



Scheme 1 The pathway for the preparation of poly(C_{12}vim)Br

Experimental

Chemicals and Materials

All the reagents used are at least of analytical reagent grade unless otherwise stated and 18 MΩ cm deionized water is used throughout. Vinylimidazole is purchased from Sigma-Aldrich (Shanghai, China).The protein molecular weight marker (low, D530A, TaKaRa Biotechnology Co., Dalian, China) is a mixture of six purified proteins (Mr in kDa: phosphorylase B 97.2, serum albumin 66.4, ovalbumin 44.3, carbonic anhydrase 29.0, trypsin inhibitor 20.1 and lysozyme, 14.3). Methanol, ether, 1-bromododecane, azobis(2-methylpropionitrile) (AIBN), hemoglobin (Hb), bovine serum albumin (BSA), sodium dodecyl sulfate (SDS) and other reagents are obtained from Sino-pharm Chemical Reagent Co. (Shanghai, China) and used as received.

pH of the aqueous medium is controlled by a 10-fold diluted Britton-Robinson (B-R) buffer within a range of pH 4.0-10.0 (100 mL of acid mixture with 0.04 mol L^{-1} phosphoric acid, acetic acid and boric acid, to which different volumes of 0.2 mol L^{-1} sodium hydroxide is added).

Human whole blood samples from healthy volunteers are provided by the hospital at Northeastern University, Shenyang, China. After adding appropriate amount of anticoagulant, the blood samples are stored in a refrigerator at -20°C for future use.

Preparation and polymerization of the ionic liquid monomer 3-n-dodecyl-1-vinylimidazolium bromide

Typically, 1-vinylimidazole (10 mL, 0.11 mol) and an excessive amount of 1-bromododecane (27 mL, 0.112 mol) are loaded into a 100 mL round-bottom flask containing 20 mL of methanol. The mixture is stirred at 70°C for overnight. After cooling down to room temperature, the mixture is added drop-wisely into 250 mL of diethyl ether to eliminate the residues of 1-vinylimidazole and 1-bromododecane. The product, i.e., white solid of 3-n-dodecyl-1-vinylimidazolium bromide (C_{12}vimBr), is filtered off and dried under vacuum until a constant weight is obtained, giving rise to a yield of 85.1%.

1.0 g of C_{12}vimBr and 0.02 g of AIBN are added into 20 mL of chloroform. The solution is then heated at 70°C under N_2 atmosphere for 3 h. Afterwards, the chloroform is removed from the reaction system on a rotary evaporator and the product is washed by deionized water for three times. The final product, poly(C_{12}vim)Br, is dried in a vacuum oven until a constant weight is achieved and it is then ground for further use.

Characterizations

FT-IR spectra are obtained with a Nicolet 6700 FT-IR spectrophotometer within a range of 500-4000 cm^{-1} (Thermo Electron, USA). UV-Vis spectra are recorded by using a U-3900 UV spectrophotometer (Hitachi, Tokyo, Japan). The morphology of PIL is studied with a Scanning Electron Microscope S-3400N (Hitachi, Tokyo, Japan). The conformational change of protein is investigated by evaluating the circular dichroism (CD) spectra recorded by using a MOS-450 spectrometer/polarimeter (Biologic Science Instrument, France). pH value is measured by PB-10 Standard pH meter (Sartorius, Germany).

Protein adsorption studies

2.0 mg of the poly(C_{12}vim)Br is applied for the adsorption of two model protein species in 1.0 mL of sample solution, i.e., Hb and BSA. The adsorption experiments are performed in 1.5-mL centrifuge tubes at room temperature. The mixture is shaken vigorously for 20 min to facilitate adsorption of proteins. UV-Vis absorptions are used to measure the concentration of the proteins in the aqueous phase at their characteristic absorption wavelengths, i.e., 406 nm for Hb and 595 nm for BSA (after BSA has been stained with Coomassie Brilliant Blue dye by using the Bradford method). The adsorption efficiency for proteins is calculated on the basis of the protein concentration in the aqueous medium before and after undergoing the adsorption process. After adsorption, poly(C_{12}vim)Br is mixed with 1.0 mL of SDS solution (0.5%, m/v) and the mixture is oscillated for 5 min to facilitate the desorption of the absorbed protein from the adsorbent. The supernatant containing the recovered protein is finally collected by centrifugation for 5 min at 8000 rpm for performing the ensuing investigations.

Isolation of hemoglobin from human whole blood

For practical application, the poly(C_{12}vim)Br has been applied for the selective isolation of hemoglobin from complex sample matrixes, e.g., human whole blood. For this purpose, 10 μL of the anticoagulated blood is diluted for 700-fold with B-R buffer. After centrifugation at 5000 rpm for 3 min, 1.0 mL of the supernatant is collected and then subject to the adsorption process as described in the experimental section. The protein retained on poly(C_{12}vim)Br is thereafter recovered by using a 0.5% SDS solution as stripping reagent, followed by performing a standard SDS-PAGE assay to evaluate the practical efficiency for hemoglobin isolation. Before the SDS-PAGE assay, the sample solutions are boiled for 5 min and then subject to electrophoresis on 5% polyacrylamide stacking gels at 90 V, 12% polyacrylamide resolving gels at 180 V and in standard discontinuous buffer systems. The protein bands are visualized by staining using 0.2% Coomassie Brilliant Blue G250 for 60 min, and then de-stained with a solution containing 7.5% (v/v) acetic acid and 5% (v/v) methanol.

Results and discussion

Characterization of the polymeric ionic liquid poly(C_{12}vim)Br

Fig. S1 shows the FT-IR spectra of 1-vinylimidazole, C_{12}vimBr and poly(C_{12}vim)Br. The C-N stretching vibration of imidazole at 1500 cm^{-1} is obviously red-shifted to 1560 cm^{-1}. In addition, an enhancement on the characteristic absorption of alkyl at 2800-

3000 cm^{-1} confirms the formation of the ionic liquid monomer. In the FT-IR spectra for poly(C_{12}vim)Br, the two absorption bands at 937 cm^{-1} and 962 cm^{-1} corresponding to the C-H bending in C=C-H of 1-vinylimidazole were not observed. Meanwhile, the disappearance of the C=C absorption band at 1650 cm^{-1} further indicates the formation of the polymer. The ^{1}H NMR and ^{13}C NMR (600MHz, $CDCl_3$) of IL monomer and PIL (Fig. S2 and S3) consistent with that reported in previous literature.[40] In the mass spectrum of IL monomer(Fig. S4), there is an obvious peak at m/z 263.25, which is the molecular ion peak of IL monomer cation. The low peaks at m/z 264.25 and 265.25 are ascribed to the isotope of N in the imidazole ring. The SEM image in Fig. S5 illustrates the morphological characters of the polymeric ionic liquid, showing amorphous nature of the poly(C_{12}vim)Br.

Protein adsorption behavior by the poly(C_{12}vim)Br

The adsorption behaviors of poly(C_{12}vim)Br towards BSA and Hb within a pH range of 4-10 are illustrated in Fig. 1. It is clear that favorable adsorption for Hb is achieved at pH 8.0 giving rise to a sorption efficiency of 93.8%. Meanwhile, however, the adsorption of BSA is greatly suppressed within the same pH range. The adsorption mechanism for Hb is assumed to be associated with the coordination between the cationic $C_{12}vim^{+}$ and iron atom in heme group of Hb. The iron atom is coordinated with four pyrrole nitrogen atoms of protoporphyrin IX and nitrogen atom in imidazole of histidine[41] and also provides a sixth vacant coordinating position, which offers a potential to coordinate with an extra nitrogen atom of imidazole or from other molecules.[42] Previous studies have shown that imidazole is a strong coordinating ligand with iron atom in heme group[43-45] and the covalent coordination of imidazole with iron atom in Hb plays a dominant role in the adsorption process.[20,30] Hb generally has two states, i.e., relaxed state[46] and tense state,[47] with characteristic adsorption wavelengths at 406 nm and 417 nm, respectively. This feature could be applied for the discrimination of the state of the sixth vacant coordinating position. For the elucidation of Hb adsorption mechanism by poly(C_{12}vim)Br, UV-Vis absorption spectra of Hb solution, Hb-poly(C_{12}vim)Br mixture solution and poly(C_{12}vim)Br solution are recorded and illustrated in Fig. 2. For the Hb-poly(C_{12}vim)Br mixture system, an obvious red-shift for the maximum absorption wavelength of Hb is observed, i.e., from 406 nm to 417 nm. This observation indicates that Hb exists in relaxed state before extraction, while it turns to tense state after shaking with the polymeric ionic liquid, whose UV-Vis absorption spectrum shows no peaks at all within a range of 380-430 nm. For the adsorption of other proteins by ionic liquids, hydrophobic interaction plays a predominant role as demonstrated in previous studies for the extraction of proteins by ionic liquid two aqueous phase systems.[22,48] It is known that pH value has significant influence on the conformation of protein species,[49,50] which might affect the coordination and hydrophobic interactions between protein and the ionic liquid moiety. Therefore, the variation of pH value can be useful for regulating such interactions.

Our experiments have indicated that the increase of adsorption time improves the sorption efficiency for hemoglobin at <10 min, and an equilibrium is achieved at ca. 20 min, a further increase of the adsorption time contributes nothing to the sorption efficiency of hemoglobin.

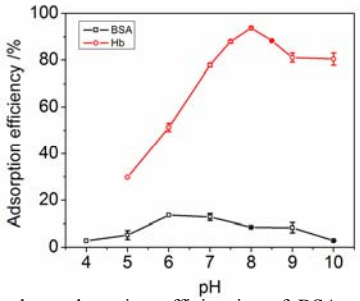


Fig. 1 pH-dependent adsorption efficiencies of BSA and Hb onto the poly(C_{12}vim)Br surface. Protein solution: 80 μg mL^{-1}, 1.0 mL; poly(C_{12}vim)Br: 2.0 mg; adsorption time: 20 min; Replicate number of each test:3.

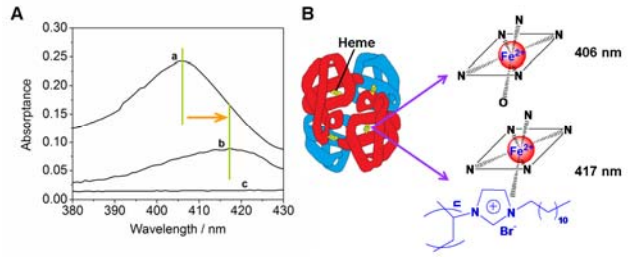


Fig. 2 (A) UV-Vis absorption spectra of a) Hb solution, b) poly(C_{12}vim)Br solution containing Hb, and c) poly(C_{12}vim)Br solution; (B) The interaction mode of poly(C_{12}vim)Br with iron in hemoglobin. Hb: 40 μg mL^{-1}, 1.0 mL; pH 8.0; poly(C_{12}vim)Br: 2.0 mg; shaking time: 5 min.

In the practice of protein adsorption by adsorbents, the ionic strength of the sample solution generally plays a key role in governing the adsorption performance. In the present case, the adsorption of hemoglobin by varying the ionic strength within a certain range is investigated by using a Hb solution of 80 mg mL^{-1}, and the results are given in Fig. 3. It is obvious that a very small variation of the adsorption efficiency for hemoglobin is observed with the increase of ionic strength, i.e., the concentration of NaCl, up to 0.4 mol L^{-1}. As discussed in the previous section that a complex is formed between Hb and the poly(C_{12}vim)Br during the adsorption process based on the coordination of imidazole with iron atom in Hb. As is known that such complexes are categorized into inner sphere surface complex (ISSC)[51] and outer sphere surface complex (OSSC)[52] according to the existing forms of water molecules. In the present case, the coordination between cationic $C_{12}vim^{+}$ and iron atom in heme group is in accordance with the OSSC formation mode, where the adsorption efficiency is basically not affected by the variation of ionic strength.

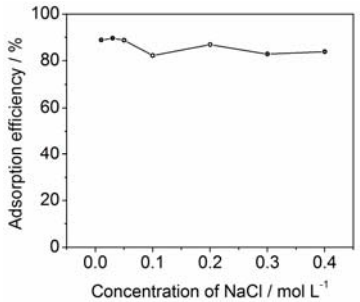


Fig. 3 The effect of ionic strength on the adsorption efficiencies of Hb. Hb: 80 μg mL^{-1}, 1.0 mL; pH 8.0; poly(C_{12}vim)Br: 2.0 mg; adsorption time: 20 min; Replicate number of each test:3.

The dynamic adsorption isotherm of Hb onto poly(C_{12}vim)Br at room temperature within a concentration range of 25-550 mg L^{-1} is illustrated in Fig. 4, showing a Langmuir adsorption. The maximum adsorption capacity for Hb is derived to be 205.4 mg g^{-1}. As a comparison, Table 1 summarizes the sorption capacity for hemoglobin by some previously reported ionic liquid related materials. It is seen that poly(C_{12}vim)Br offers a favorable sorption capacity for Hb with respect to the given materials, i.e., imidazolium-modified polystyrene (PS-CH_2-[MIM]$^+Cl^-$), ionic liquid-polyvinyl chloride ionomer (NmimCl-PVC) and PPimPF_6/TiO_2 nanocomposites. As an exception, the ionic liquid modified magnetic microspheres (Fe_3O_4@SiO_2@IL) provided an ultra-high sorption capacity attributed probably to the very large surface area. On the other hand, however, it should be emphasized that the poly(C_{12}vim)Br in the present study avoids non-specific adsorption to proteins, which is frequently problematic for the previously reported IL-modified solid supports, where non-specific adsorption is generally caused by the exposed surface functional groups on the solid support surface due to incomplete coverage by the IL moiety during the modification or functionalization process.

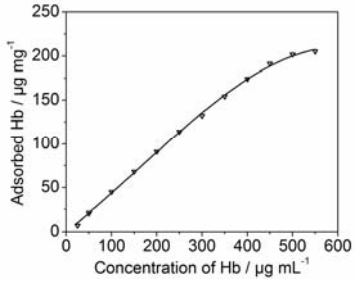


Fig. 4 The adsorption isotherm for Hb. pH 8.0; poly(C_{12}vim)Br: 2.0 mg; adsorption time: 20 min.

Table 1. A comparison on the adsorption capacity of Hb by various ILs-modified materials reported in the literatures.

Materials	Adsorption capacity /mg g^{-1}	Ref.
PS-CH_2-[MIM]$^+Cl^-$	23.6	53
PVC-[NMIM]$^+Cl^-$	26.5	28
SPEEK-Bmim	31.6	54
PPimPF_6/TiO_2	122.3	27
Fe_3O_4@SiO_2@IL	2150	30
Poly(C_{12}vim)Br	205.4	This work

The recovery of adsorbed proteins from poly(C_{12}vim)Br

Generally, the transfer of the adsorbed protein by the poly(C_{12}vim)Br into aqueous phase is necessary in order to facilitate further biological investigations. As discussed in the above section, Hb is retained on poly(C_{12}vim)Br via coordination between the cationic $C_{12}vim^+$ and iron atom in heme group of Hb. For the recovery of the adsorbed hemoglobin, a series of potential eluents, i.e., Tris-HCl, B-R buffer, NaCl and SDS solutions have been employed. The experimental results indicated that except for SDS solution, no obvious recovery of Hb is observed by the other media. Considering that coordination between the cationic $C_{12}vim^+$ and iron atom is a strong interaction, thus the recovery of Hb should be better performed by an appropriate replacing ligand or surfactant, e.g., SDS. Further studies have shown that SDS is effective in the stripping of hemoglobin, as demonstrated in Fig. 5, showing the dependence of Hb recovery upon the concentration of SDS solution. By taking into account the fact that SDS tends to induce denaturation or conformational change for protein,[55] its concentration should be kept at a minimum. In the present case, a 0.5% (m/v) SDS solution gives rise to an elution efficiency of ca. 86.3%.

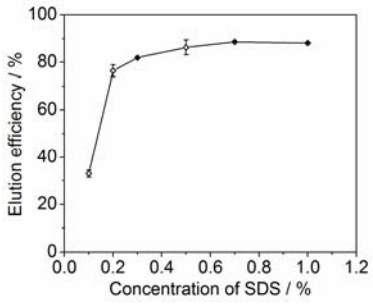


Fig. 5 The effect of SDS concentration on the elution efficiencies. Hb: 80 μg mL^{-1}, 1.0 mL; pH 8.0; poly(C_{12}vim)Br: 2.0 mg; adsorption time: 20 min; elution time: 5 min; Replicate number of each test:3.

The conformation change of hemoglobin has been carefully evaluated, by recording circular dichroism (CD) spectra for native Hb solution prepared in B-R buffer and 0.5% SDS solution, and that recovered from the poly(C_{12}vim)Br surface are also prepared in 0.5% SDS solution. The results are shown in Fig. 6. It is seen that CD spectra of the three Hb solutions exhibit two negative bands in the far-UV region at 210 nm and 220 nm, which are assigned to α-helical proteins structure. A slight decrease of CD signal for the recovered Hb solution is observed with respect to native Hb solution in B-R buffer and SDS solution. The structure change of Hb is evaluated by calculating the content of α-helix using the method described by Greenfield.[56] The native Hb contains 37.5% of α-helix in B-R solution and 36.6% in 0.5% SDS solution, while that in the recovered Hb solution has decreased to 31.9%. These results are in agreement with a previous report,[57] where it indicated that the secondary structure would be slightly changed when protein is adsorbed onto the surface of solid supports. The CD data indicates that the adsorption by poly(C_{12}vim)Br followed by elution with SDS solution causes a slight conformational change for hemoglobin, while its structure remains predominantly as α-helix.

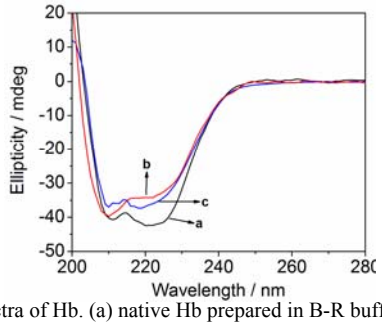


Fig. 6 CD spectra of Hb. (a) native Hb prepared in B-R buffer at pH 8.0; (b) native Hb prepared in 0.5% SDS solution; (c) the Hb recovered from the poly(C_{12}vim)Br with 0.5% SDS solution.

Isolation of hemoglobin from human whole blood

The practical applicability of poly(C_{12}vim)Br as adsorbent for protein isolation is investigated by performing adsorption of Hb from complex matrix sample, i.e., human whole blood. The

results for the standard SDS-PAGE assay are illustrated in Fig. 7. It is obvious that a few protein bands are observed for human whole blood (Lane 2), which might be attributed mainly to albumin, hemoglobin and other proteins. After treatment by the poly(C_{12}vim)Br followed by elution with 0.5% SDS solution, a clear and pure band for hemoglobion at ca. 14.3 kDa is identified (Lane 3). As a comparison, the electrophoretogram for 200 mg mL^{-1} hemoglobin is recorded, it is shown that a clear band is observed in the same region (Lane 4). These results well illustrate the effective isolation of Hb from human whole blood with other coexisting protein species and the presence of complex sample matrixes.

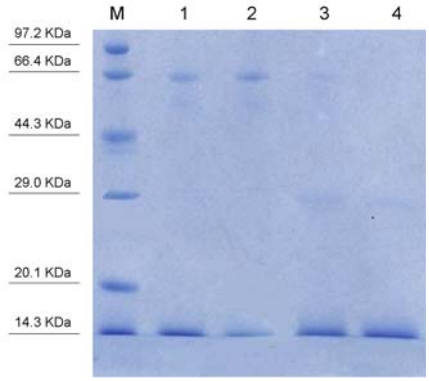


Fig. 7 The standard SDS-PAGE of the samples. Lane M: protein marker; lane 1: 700-fold diluted human whole blood without any pretreatment; lane 2: 700-fold diluted human whole blood after adsorption by 2.0 mg of poly(C_{12}vim)Br ; lane 3: Hb recovered by the present procedure; and lane 4: a standard Hb solution of 250 μg mL^{-1}.

Conclusions

In the present study, a novel polymeric ionic liquid is prepared to extract hemoglobin, which provides a favorable sorption capacity of 205.4 mg g^{-1}. The mechanism studies indicate that the covalent coordination of imidazole with iron atom in Hb is the main driving force for the adsorption of Hb. The PIL provides an efficient adsorbent for the isolation/purification of Hb from human whole blood in the presence of other coexisting proteins. In comparison with the previously reported IL-modified sorbent materials, the present PIL eliminates non-specific adsorption attributed to the exposed surface functional groups on the solid support surface due to incomplete coverage by the IL moiety, offering a favorable sorption capacity towards Hb. The present study provides an approach for the development of selective adsorbents for protein isolation.

Acknowledgments

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Notes and references

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