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Development of a displacer-immobilized ligand docking scheme for displacer screening for protein displacement chromatography

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ABSTRACT

Displacement chromatography is a powerful technique for protein purification, but the availability of high-efficacy displacers has greatly limited its applications. In this work, a displacer-immobilized ligand docking scheme was developed for the prediction of displacer efficacy and displacer screening for displacement chromatography of proteins. The structure of immobilized ligand was established by coupling a certain number of ligands to the 3D structure of agarose. A number of known cation, anion and hydrophobic displacers were docked to their respective immobilized ligands to verify the effectiveness of the scheme, and the Spearman ranking correlation coefficients of all cases were over 0.5. The scheme was then used to screen displacers for hydrophobic charge induction chromatography from over 1800 commercially available compounds. Column displacement experiments of several representative compounds showed that the identified displacers were efficacious in the displacement of single component and binary mixtures. It is expected that the combination of the docking scheme with the existing techniques for displacer discovery/design would greatly facilitate the discovery of high-affinity displacers for protein purification.

1. Introduction

Displacement chromatography is a powerful separation technique in which the solutes are sequentially displaced from the column in high concentration and purity by a high-affinity substance called displacer. It can be realized in a number of chromatographic methods based on different interaction mechanisms, i.e. ion-exchange, hydrophobic interaction, reversed phase, and hydroxyapatite chromatography. Compared with the commonly used elution chromatography, displacement chromatography can be performed at higher column loadings and product concentrations with comparable purity [1,2]. Research has also shown that displacement chromatography is especially suitable for "challenging" separations with small separation factors [3]. Therefore, it has been proved effective in various applications in bioscience, such as bioseparation [4–9], trace component amplification [10,11], and ligand–receptor interaction analysis [12–14].

Recently, a new displacement chromatography technique, hydrophobic charge induction displacement chromatography (HCIDC) [15], has been proposed. The displacers for HCIDC are specifically selected with a hydrophobic moiety and a charge-able moiety. After hydrophobic adsorption and displacement, the displacer can be easily removed from the column by pH-induced electrostatic repulsion between the displacer and the chromatographic ligand. HCIDC has thus not only incorporated the advantages of high capacity, low salt operation of hydrophobic charge induction chromatography (HCIC) [16–19] but also addressed the common problem of column regeneration in other displacement chromatography techniques. Therefore, it is expected that HCIDC can facilitate the applications of displacement chromatography in broader areas.

Despite the various advantages, the selection and development of suitable displacers still remains an issue that hampers the application of displacement chromatography. Cramer and coworkers have made tremendous contributions to the screening and rational design of displacers for ion-exchange chromatography. Various displacers consisting of multiple charge groups were synthesized [20–23], and a high-throughput screening and quantitative structure–efficacy relationship (QSER) technique had been developed for the screening of high-affinity and chemically selective displacers [24–33]. Chemically selective displacers with high specificity had also been designed by combining a stationary-phase-binding moiety with a protein-binding moiety [34,35]. Effective as they are, most of these approaches are based on the knowledge of existing displacers and their efficacy, which are not so readily perceivable for displacers based on hydropho-

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bic interaction. Experimental methods can give reliable efficacy information but are limited in scale by time, labor and materials. Moreover, synthesis of many novel displacers reported requires multiple steps and complex procedures, making them unsuitable for real applications. Therefore, it is still necessary to develop a method for displacer efficacy prediction, which would facilitate the discovery of high efficacy displacers for chromatographic methods based on hydrophobic interaction from commercially available compounds.

Molecular docking [36–42] is a fast computational method to predict the binding conformation and strength of two molecules. It is one of the most commonly used strategies in drug discovery, which can identify novel lead compounds from a large library of candidate molecules within a relatively short period of time. Although the docking calculation is a simplified and empirical process compared with more sophisticated simulation methods like molecular dynamics, it is advantageous in its high speed, low computational resource and reasonable accuracy. Thus, it has been proved effective in drug discovery by numerous successful applications [38]. In recent years, docking has also been successfully used for the virtual screening of ligands for affinity chromatography [43–47]. In this article, a displacer-immobilized ligand docking scheme was proposed for the prediction of displacer efficacy in protein chromatography. The scheme was first verified by experimental data of known displacers in literatures and then applied for a preliminary screening of displacers for HCIDC from commercially available compounds. Finally, the displacers screened were tested by column displacement experiments. It is expected that the scheme can not only identify novel HCIDC displacers but also expand its applications to the discovery of displacers for other forms of chromatography.

2. Experimental

2.1. Materials

Cetyl dimethyl benzylammonium chloride (CDBAC), tributyl tetradecyl phosphonium chloride (TTPC), Janus Green B (JGB), benzyl tributylammonium chloride (BTBAC), 5-aminoindole and lysozyme were purchased from Sigma (St. Louis, MO, USA). Acetonitrile and trifluoroacetic acid were from Merck (Darmstadt, Germany). Other reagents were analytical grade from local sources. Superose 12 prep grade, HR 5/10 column (100 mm × 5 mm i.d.) and Tricorn 5/200 column (200 mm × 5 mm i.d.) were from GE Healthcare (Uppsala, Sweden). Zorbax 300-SB C18 reversed phase column was purchased from Agilent (Santa Clara, CA, USA).

2.2. Immobilized ligand structure

The chromatographic matrices involved in this work, Sepharose and Superose, were both agarose-based media that consist of 6% crosslinked agarose and 94% water (as stated by the manufacturer). Agarose is a polymer consisting of multiple repeating units of D-galactose and 3,6-anhydro-α-L-galactopyranose (Fig. 1a). In biochromatography, ligands of different properties are coupled to the hydroxyl groups of agarose to make adsorbents of different modes. Although there are several hydroxyl groups in the repeating unit, the most active one is the 6-hydroxyl group on D-galactose. Therefore, it is assumed that all ligands are coupled to the 6-hydroxyl groups of D-galactose.

The three-dimensional structure of an agarose chain segment [PDB ID: 1aga, see Fig. 1b] was obtained from PDB database (Protein Data Bank, www.rcsb.org), which is a double-helix structure consisting of two chains with three repeating units in each chain. The structures of ligands used in the establishment of immobilized

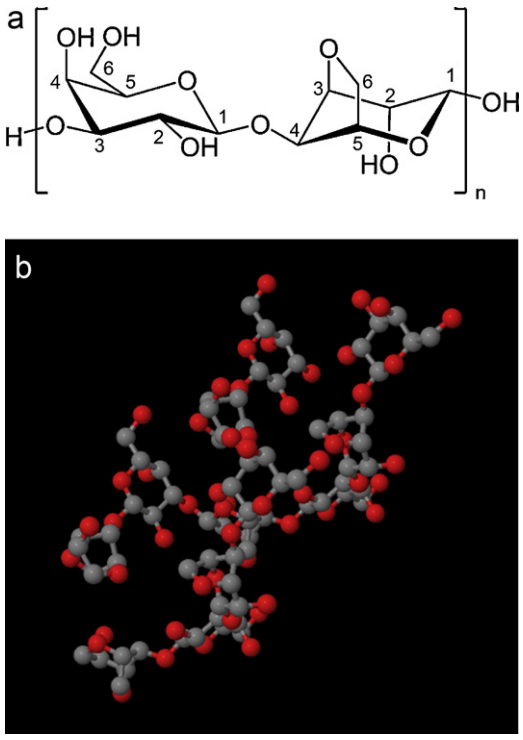

Fig. 1. Structure of agarose. (a) The D-galactose and 3,6-anhydro-α-L-galactopyranose repeating unit and (b) three-dimensional structure of an agarose chain segment.

ligand structure are listed in Fig. 2. The structure of immobilized ligands was obtained by coupling the corresponding ligand structure to the 6-hydroxyl group of the agarose structure in Sybyl 6.9.2 (Tripos, St. Louis, MO, USA). The actual number of ligands was calculated according to the molar ratio of ligand and the repeating unit, with the ligand density data from the manufacturer and the

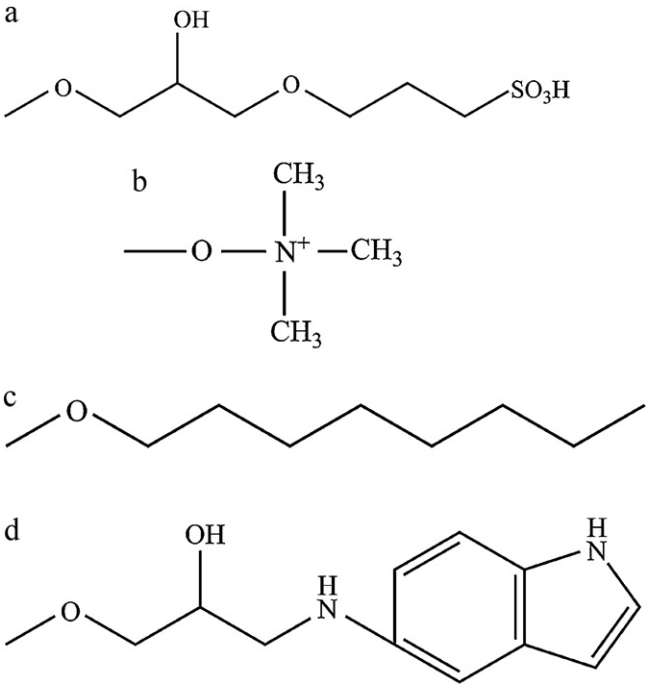

Fig. 2. Structure of ligands used in docking. (a) Sulphopropyl (SP), (b) quaternary ammonium (Q), (c) Octyl, and (d) aminoindole.

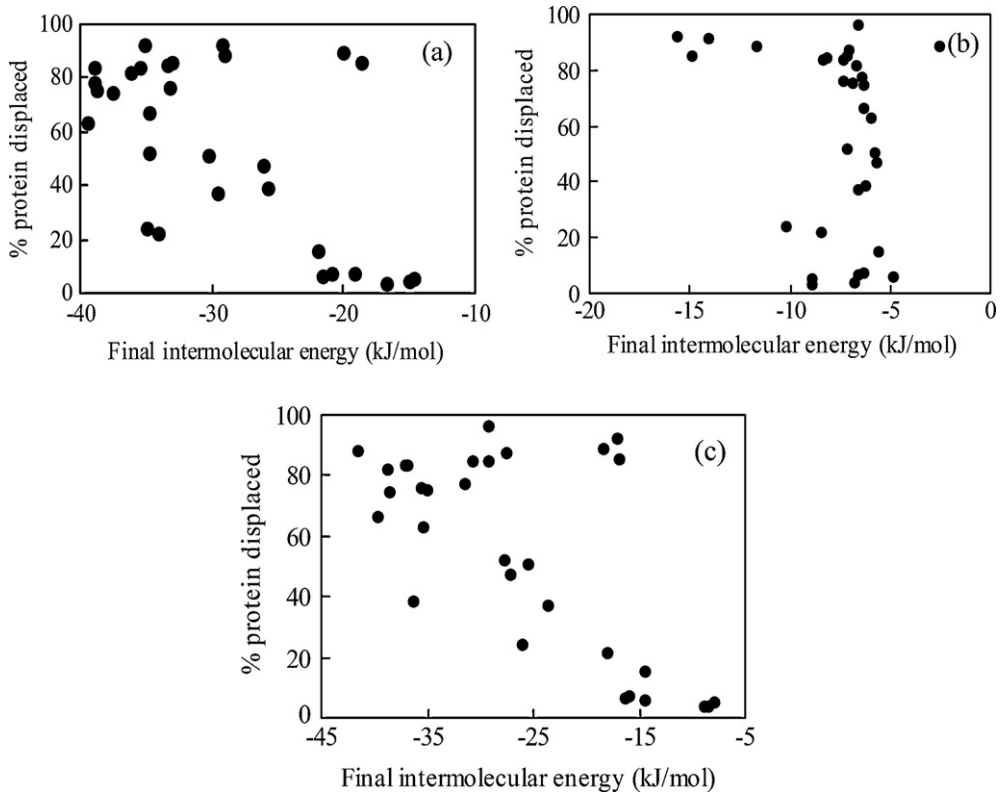

Fig. 3. Docking results with different ligand–receptor relationships versus percentage of protein displaced. (a) Displacers to immobilized ligand, (b) free ligand to displacers, and (c) displacers to free ligand. Ligand structure established from Fig. 2a according to the procedures described in Section 2.2. The experimental values of percentage of protein displaced are from Ref. [24] as listed in its tables.

mass density of the medium taken as 1 g/mL. As has been observed by many researchers with confocal laser scanning microscopy [48–52], the distribution of ligands is even for various chromatographic adsorbents with different ligands and matrices. Therefore, the substituting ligands in this immobilized ligand model were located on the agarose backbone as evenly as possible.

2.3. Molecular docking

The three-dimensional structures of displacers were obtained from PubChem Compound database (http://pubchem.ncbi.nlm.nih.gov). For the compounds whose three-dimensional structures were not available in the database, three-dimensional structures were calculated by MarvinSketch 5.0.6 (ChemAxon Kft., Budapest, Hungary) from their two-dimensional structures obtained from the database or drawn by ourselves. All salt ions were removed prior to docking. Docking was performed by Autodock4 (http://autodock.scripps.edu/) on a dual-core 3.0 GHz Levono PC. In all cases, all rotatable bonds on the docking ligand were set flexible while the docking receptor was regarded as rigid for the balance of accuracy and speed, which is consistent with the common practice in current docking-based virtual screenings [38,53]. Lamarckian genetic algorithm (LGA) was used for conformational searching. The number of GA runs was set to 20 and the mean final intermolecular energy of the 20 runs was taken as the final result.

2.4. Preparation of AI-Superose

5-Aminoindole Superose (AI-Superose) was prepared according to the method reported previously [54] with minor modifications. Superose beads were solvated by dimethyl sulphoxide (DMSO) and suction-dried, and 2 g of the drained beads were mixed with 1 mL epichlorohydrin, 4 mL dimethyl sulphoxide (DMSO) and 3 mL of 0.8 mol/L NaOH. The suspension was placed in a 50 °C water-bath and shaken at 170 rpm for 3 h. The activated Superose gel was washed with DMSO and deionized water, drained and reacted with 0.5 g 5-aminoindole in 2 mL DMSO and 2 mL of 0.8 mol/L NaOH for 48 h at 49 °C and 170 rpm. After that, the gel was washed, dispersed in 30 mL of 0.5 g/L $NaBH_4$ and shaken for 12 h to reduce the residual epoxy groups to hydroxyl groups. The reduced gel was washed subsequently with DMSO and deionized water until the absorbance of the eluent at 226 nm was zero. The coupling density of the medium obtained was 70 μmol/mL.

2.5. Displacement chromatography

Displacement chromatography was performed with an HR 5/10 or Tricorn 5/100 column packed with AI-Superose on an ÄKTA Basic liquid chromatograph system (GE Healthcare, Uppsala, Sweden). The column was equilibrated with 50 mmol/L sodium phosphate buffer (pH 7.0) and protein sample was loaded by a 1 mL or 2 mL sample loop. Displacer solution was continuously applied by a 50 mL Superloop till its breakthrough. The column was then regenerated by applying 10 column volumes of 50 mmol/L glycine–HCl buffer (pH 3.0) and re-equilibrated for the next operation. Fractions of the column effluent were continuously collected during loading and displacement, with sizes of 0.2 mL or 0.5 mL.

2.6. Reversed-phase liquid chromatography (RPLC)

Fractions of the column effluent collected above were analyzed with a Zorbax 300-SB C18 reversed-phase column on an Agilent 1100 high-performance liquid chromatograph (Agilent, Santa Clara, CA, USA). An Alltech 2000 Evaporative Light Scattering Detector (ELSD) (Grace Davison Discovery Sciences, Deerfield, IL, USA) was used for the quantification of all components. The UV–vis detector on the HPLC was also used in conjugation with ELSD at

210 nm to ensure the accuracy of detection. Eluent A was 0.1% TFA in deionized water and Eluent B was 0.1% TFA in acetonitrile, and a linear gradient of 30% B to 100% B in 20 min was used. The sample loading volume was 20 μL for each analysis.

3. Results and discussion

3.1. Determination of ligand–receptor relationship

Displacement chromatography is a separation technology based on the competitive adsorption of target molecules and the displacer to the chromatographic adsorbent [55,56]. Therefore, compounds with high affinity to the chromatographic ligands are expected to be potential displacers. Docking is a fast computational method that can predict the binding strength of two molecules. Therefore, it is expected that docking of the displacer and the corresponding chromatographic ligand can provide insight to its efficacy.

AutoDock4 (http://autodock.scripps.edu/) is a set of automated docking tools to predict the binding of small molecules to a known 3D structure. In the calculation of AutoDock4, the receptor is represented by a set of pre-calculated three-dimensional affinity grids, and the binding conformation of the ligand to the receptor is searched by a Lamarckian genertic algorithm (LGA). The affinity grids are calculated for each type of atom in the ligand as well as grids of electrostatic and desolvation potentials. Binding conformations are evaluated by a semiempirical free energy force field, including dispersion/repulsion, hydrogen bonding, electrostatics, desolvation and conformational entropy lost upon binding. These have covered most of the decisive interactions in chromatographic separations. In this work, AutoDock4 is used for the estimation of binding energy between the displacer and the chromatographic ligand.

As can be seen from the context above, the ligand and the receptor are treated differently in docking simulations. Therefore, a correct ligand–receptor relationship is essential for correct estimation of the binding energy. Conventionally, in virtual screening of chromatographic ligands, the candidate molecules are used as the docking ligand and the target protein is used as the docking receptor [43–47]. This is consistent with the underlying principles of the docking programs and the common practice in drug discovery, in which small molecules are regarded as ligands while the target protein is regarded as the receptor. However, in modern displacement chromatography, small-molecule displacers are commonly used due to their low cost and ease of separation from the product [57–60]. This means that both the displacer and the ligand are small molecules, which make the common docking scheme not so readily implantable to the displacement system. Therefore, we have proposed a displacer-immobilized ligand docking scheme to simulate the interactions between the displacer and the chromatographic ligands. In this scheme, displacers are taken as the docking ligand while immobilized chromatographic ligands are taken as the docking receptor. This is a more realistic model of the actual situation in which chromatographic ligands are immobilized while displacers can move freely around the ligands to search for a stable binding conformation. As there is usually more than one ligand on the immobilizing medium structure and the binding of displacers to the ligands is non-specific, it is conceivable that there will be many different binding conformations. Visual inspection of the docked conformations by AutoDockTools (http://autodock.scripps.edu/), the graphical user interface of AutoDock, has also confirmed that the displacer molecule can bind to different ligands on the agarose backbone in different conformations (data not shown). Thus, the mean final intermolecular energy of different docking runs was averaged as a statistical indicator of the overall binding strength while the binding conformations obtained from docking were not further investigated.

Cramer and his co-workers [24,26,33] have studied the efficacies of various displacers by high-throughput screening. In their work, chromatographic adsorbents with adsorbed proteins were mixed with displacer solutions, and the equilibrium concentrations of the proteins displaced to the liquid phase were determined. The efficacy of displacers was indicated by the percentage of protein displaced at a certain displacer concentration. The higher the percentage is, the more efficacious the displacer is. However, when the percentage of protein displaced approaches 100%, the efficacy of displacers cannot be well discriminated by the data. Therefore, another index of displacer efficacy, DC-50, was proposed by the same group [28]. DC-50 is the displacer concentration required to displace half of the adsorbed protein on the chromatographic medium. The DC-50 of a displacer can be determined by a series of high-throughput experiments at different displacer concentrations. The lower the DC-50 is, the more efficacious the displacer is. Compared with the percentage of protein displaced, DC-50 can give a better discrimination of displacer efficacy. In this work, the percentages of proteins displaced and the DC-50 values of displacers reported in the above-mentioned references were used to verify the effectiveness of the docking scheme.

The efficacy data of various cationic displacers on SP Sepharose in Ref. [24] were used to verify the effectiveness of the displacer-immobilized ligand docking scheme. Three ligand–receptor relationships were investigated, i.e. displacer to immobilized ligand, free ligand to displacer and displacer to free ligand. The results are shown in Fig. 3.

Spearman rank correlation coefficient (R_s) has been used for the evaluation of the accuracy of docking programs in ranking the affinity of the ligand to the receptor [61,62]. It is a non-parametric statistical measure of the correlation between two sets of variables, with the definition of Eq. (1):

$$R_s = 1 - \frac{6\sum_i (R_i - S_i)^2}{n^3 - n} \quad (1)$$

where R_i is the rank of the ith data in the first set of variables, S_i the rank of the ith data in the second set of variables, and n the number of data in each set.

An R_s greater than 0.5 shows that there are significant correlation between the two sets of variables. This is also what most docking programs can achieve in ligand–protein docking ($R_s \sim 0.5$–0.6) [61,62]. In this work, the displacer efficacy data were ranked from high to low while the final intermolecular energy values were ranked from high absolute values to low absolute values, from which Spearman rank correlation coefficient was calculated.

Fig. 3a shows the correlation between the experimental data and displacer-immobilized ligand docking results. It can be seen from the figure that the absolute values of final intermolecular energy and the percentage of protein displaced by most displacers are positively correlated. The Spearman rank correlation coefficient of Fig. 3a is 0.51, which is close to the values of effective ligand–protein docking programs obtained by other researchers [61,62]. Though some outliers with medium efficacies can still be found in the figure (it may be due to the limited accuracy of the docking program for such a diverse set of displacers), the overall correlation coefficient is good enough with regard to the high throughput of the docking scheme. This means that the displacer-immobilized ligand docking scheme can effectively predict the efficacy of displacers.

The free ligand–displacer docking results are shown in Fig. 3b. It can be seen that the correlation of the calculated and experimental results is poor. The calculated R_s value is 0.30, indicating that there are great deviations from the free ligand–displacer docking results and the actual experimental ranking.

The displacer-free ligand docking results are shown in Fig. 3c. It can be seen from the figure that the correlation between the

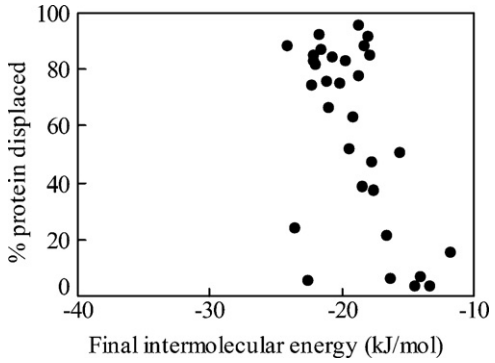

Fig. 4. Docking of displacers to blank agarose structure. The experimental values of percentage of protein displaced are from Ref. [24] as listed in its tables.

docking and experimental results has almost the same degree of significance as that of displacer-immobilized ligand docking (R_s = 0.55). This indicates that ligand–receptor relationship is essential for a relatively accurate estimation of the binding energy in docking. Although the immobilized and free ligand structures are different in the number of ligands and the presence of the agarose backbone, the treatment of them as the docking receptor and the displacer as the docking ligand gives comparable results. Nevertheless, the displacer-immobilized ligand docking scheme is more analogous to the actual situation in structure and mode of binding. The inclusion of the agarose medium is also advantageous for more precise estimations of displacer–ligand binding by soft docking in future studies, where the immobilized ligands will also be treated as flexible. Therefore, the displacer-immobilized ligand docking scheme was selected for all following calculations.

In order to investigate the effect of the immobilizing medium on the docking results, the displacers were also docked to a blank agarose medium (Fig. 4), i.e. the original structure of agarose without any ligands coupled. It can be seen from Fig. 4 that the final intermolecular energy values between the displacers and agarose are much smaller than those between the displacers and immobilized ligand. Moreover, the energy values of different displacers are close to each other. This is consistent with the fact that ligand is the primary factor in the binding of target molecules to the chromatographic medium, which has further proved the rationality and feasibility of the displacer-immobilized ligand docking scheme.

3.2. Further verification of the displacer-immobilized ligand docking scheme

The displacer-immobilized ligand docking scheme was further verified by the efficacy data of anionic [25] and hydrophobic [33] displacers with known structures, and the results are given in Fig. 5.

As is shown in Fig. 5a, there are also significant correlation between the docking and experimental results of anionic displacers (R_s = 0.56). This indicates that the docking scheme can also effectively predict the efficacy of anionic displacers.

On the other hand, the docking results of hydrophobic displacers (Fig. 5b) are not so well-correlated with the experimental results (R_s = 0.03). This can be partly attributed to the distribution of the original experimental set, which contains relatively small amount of displacers with similar efficacy data (about 30% or 70%). It can also be seen from the figure that there are four significant outliers (as indicated by the circle), which correspond to amaranth, sunset yellow, brilliant black and chenodeoxycholic acid. The structures of these four displacers are shown in Fig. 6.

As can be seen from Fig. 6, there are several aromatic rings in the structure of amaranth, sunset yellow and brilliant black and

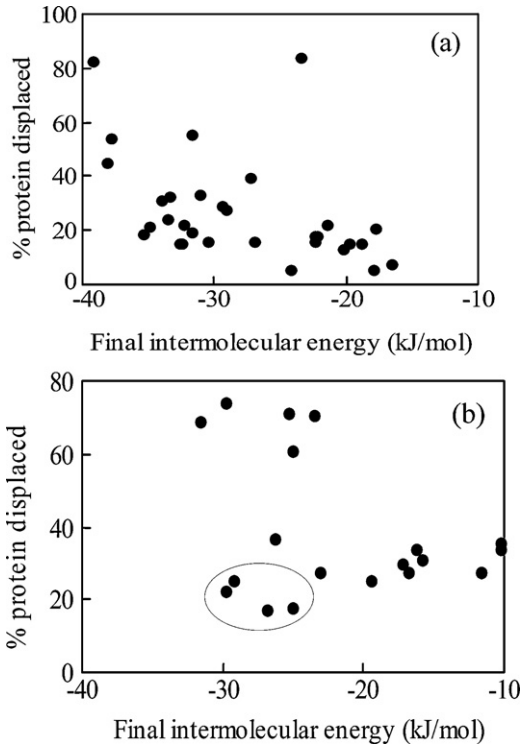

Fig. 5. Displacer-immobilized ligand docking results versus percentage of proteins displaced. (a) Anionic displacers to Q Sepharose and (b) hydrophobic displacers to Octyl Sepharose. Ligand structure established from Fig. 2b and c according to the procedures described in Section 2.2. The experimental values of percentage of protein displaced are from Refs. [25,33] as read from their figures.

aliphatic rings in chenodeoxycholic acid. These structures are significantly hydrophobic moieties, which are expected to be effective in displacing the proteins adsorbed by hydrophobic interactions. It is also worth noting that many of the efficacious hydrophobic displacers in Ref. [33] also have aromatic or aliphatic rings (structures not shown). On the contrary, the efficacy data of these displacers on hydrophobic adsorbents are even lower than the buffer control (see Ref. [33]), which is not a reasonable phenomenon. This may be caused by the possible interactions between the protein and these displacers. As many dyes are known to bind or precipitate proteins [63,64], the low percentage of protein detected in the liquid phase in the cases of these outlying displacers may be caused by precipitation of the protein by the displacers. In other words, these outlying displacers may have effectively displaced the protein from the adsorbent, but the protein displaced was subsequently precipitated by these displacers, resulting in low protein concentrations in the liquid phase and thus low apparent efficacy. As the effect of protein precipitation cannot be covered by the displacer-immobilized ligand docking scheme, it is justified that the experimental result cannot be well predicted. Removal of these four data gives an R_s value as high as 0.57. This indicates that the displacer-immobilized ligand scheme can also effectively predict the efficacy of hydrophobic displacers when unreasonable data are excluded.

As mentioned above, although the percentage of protein displaced can reflect the efficacy of displacers, it cannot give a good discrimination of them when the percentage is close to 100%. On the other hand, DC-50 can give a finer discrimination of efficacy between different displacers. Therefore, the DC-50 data of various cationic displacers [28] were used to further verify the effectiveness of the docking scheme, and the results are shown in Fig. 7.

It can be seen that the data in high efficacy region (around 90% displacement) of Fig. 3a are dense and not so well-correlated, while those in the corresponding region (low DC-50 values) of Fig. 7 are

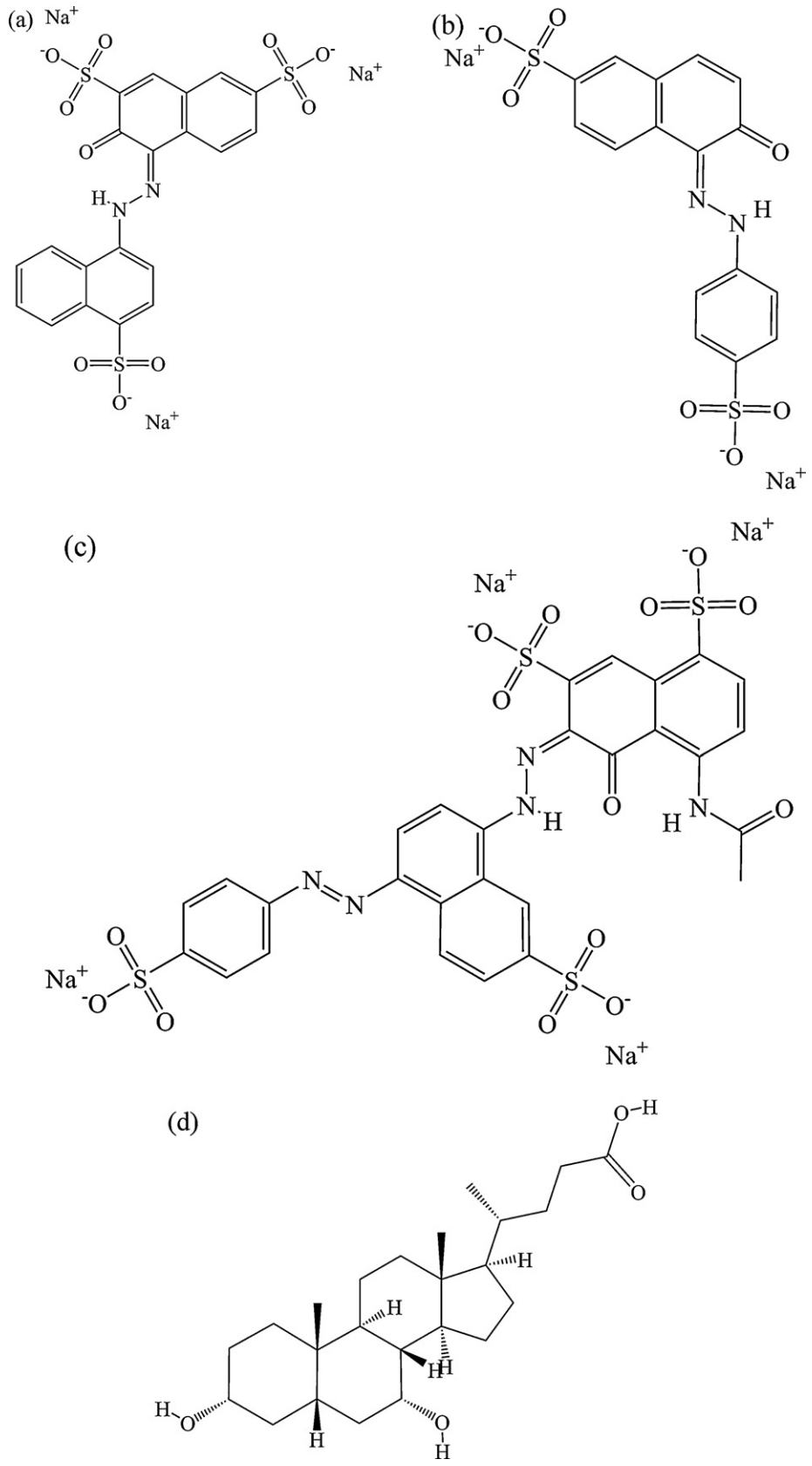

Fig. 6. Structure of the outlying hydrophobic displacers. (a) Amaranth, (b) sunset yellow, (c) brilliant black, and (d) chenodeoxycholic acid.

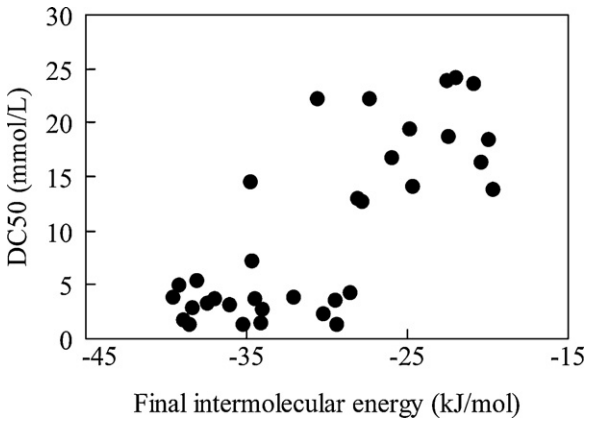

Fig. 7. Displacer-immobilized ligand docking results versus DC-50 values. Ligand structure established from Fig. 2a according to the procedures described in Section 2.2. The experimental DC-50 values are from Ref. [28] as listed in its tables.

better discriminated. The R_s for Fig. 7 is 0.67, indicating the effectiveness of the docking scheme we proposed.

3.3. Displacer screening for HCIDC

3.3.1. Establishment of a displacer candidate library

As has been mentioned in Section 1, HCIDC [15] is advantageous in its low-salt operation and facile column regeneration. In order to achieve these advantages, however, the displacers for HCIDC should meet the following requirements:

(a) *Moderate to high hydrophobicity*. The displacer must be hydrophobic enough to displace the target proteins by hydrophobic interaction.
(b) *Proper dissociating characteristics*. The displacer should take the same kind of charge as the HCIC ligand within certain range of pH and thus can be repulsed from the adsorbent by adjusting pH value.
(c) *Water solubility*. The displacer must have sufficient solubility for aqueous operations.
(d) *Availability*. The displacer should be inexpensive and biocompatible (low toxicity, etc.).

Though several displacers for hydrophobic-based chromatography, i.e. hydrophobic interaction chromatography (HIC) [33,65] and reversed phase chromatography (RPLC) [65,66], have already been reported, these displacers may not serve as effective displacers for HCIDC. As the ligand density of HIC adsorbent is much lower than that of HCIC, the displacers for HIC are usually weaker in hydrophobicity, making it incapable of displacing proteins from HCIC adsorbents. Displacers for RPLC have higher hydrophobicities but are usually not so soluble in aqueous mobile phases. Moreover, the dissociation properties of these displacers may not meet the requirements for charge-induced regeneration. Therefore, it is still necessary to develop novel displacers for HCIDC.

As the purpose of this preliminary screening is to demonstrate the effectiveness of the docking scheme in screening applications, the scale is limited to commonly used chemical reagents. Chemicals in the Sigma–Aldrich product catalog are selected for this purpose. Taking into account the advantages of small-molecule displacers and the capacity of the docking program, only compounds with molecular weights from 200 to 3000 are considered. The number of carbon atoms is limited from 1 to 35 for the concerns of solubility.

Dissociating properties are essential for HCIDC displacers in column regeneration. As most docking programs (including AutoDock4) assume a neutral pH for both the ligand and the receptor in the assignment of partial charges, it is not easy to investigate the binding of the displacer and immobilized ligand at the pH of regeneration directly by docking. Therefore, dissociation constants (pK_a) of the displacers are investigated as a reflection of their regeneration characteristics. The HCIC ligand used in this study, 5-aminoindole ($pK_a = 3.9$), can take on a proton and be positively charged at low pH. In order to be repulsed from the ligand in pH-induced regeneration, the displacer should also be positively charged around pH 3. Both the compounds with permanent positive charges and the compounds with appropriate pK_a or p*I* can suffice this requirement. In this work, the pK_a or p*I* cutoff is set as not lower than 4.

All the requirements can be used as search criteria in Pubchem Compound database except pK_a or p*I*. Thus, the pK_a or p*I* values of the searched compounds were estimated by MarvinSketch 5.0.6 (ChemAxon Kft., Budapest, Hungary). Finally, a candidate library of 565 compounds with permanent positive charges and 1270 compounds with appropriate pK_a or p*I* values was obtained.

3.3.2. Virtual screening by the displacer-immobilized docking scheme

The candidate molecules in the library were docked to immobilized 5-aminoindole ligands, and the corresponding final intermolecular energy data were obtained. Final intermolecular energy < −23.0 kJ/mol was set as the criterion for the screening, from which 152 compounds with permanent positive charges and 152 compounds with appropriate pK_a or p*I* values were obtained (see the Supplementary material). These compounds were further examined in price, availability and solubility, from which the promising ones were selected for column displacement. Note that the −23.0 kJ/mol criterion was set arbitrarily to reduce the scope of investigation to displacers with relatively higher affinities, and it does not mean that compounds with final intermolecular energies higher than −23.0 kJ/mol cannot be effective displacers at all. In fact, some of these compounds have also been proved effective in our studies (data not shown). Due to the complex nature of real separation processes and the accuracy limits of docking programs, a clear cutoff between “displacers” and “non-displacers” may be difficult to find. On the other hand, the compounds with energies lower than −23.0 kJ/mol may also fail to be effective displacers due to solubility, stability or cost problems, and further investigation is still needed. Nevertheless, the ranking provided by docking is still of great help for the discovery of novel displacers in reducing the scale of further investigation and experimental studies.

Four representative compounds, which are all commonly used chemicals, were selected from the list of identified displacers for experimental verification. None of these displacers have been reported previously in protein purification. The first three of these displacers are permanently charged with a positive charge while the last one, tetracycline, does not have permanent charges. However, it was found in later experiments that tetracycline was insoluble at neutral pH and thus cannot be used as a displacer. Further examination of the candidate molecules without permanent charges (Table S2) did not lead to any potential displacers with reasonable solubility and price. Therefore, only displacers with permanent positive charges were used in the following experiments. A compound with small final intermolecular energy, benzyltributylammonium chloride (−19.7 kJ/mol), was also selected as a negative control. The structures of these compounds are shown in Fig. 8.

3.4. Experimental verification of screened displacers

The displacers selected above were first used for the displacement of lysozyme on the 70 μmol/mL AI-Superose column, and the results are shown in Fig. 9. It can be seen from Fig. 9 that the three displacers of high intermolecular energy values (cetyldimethylben-

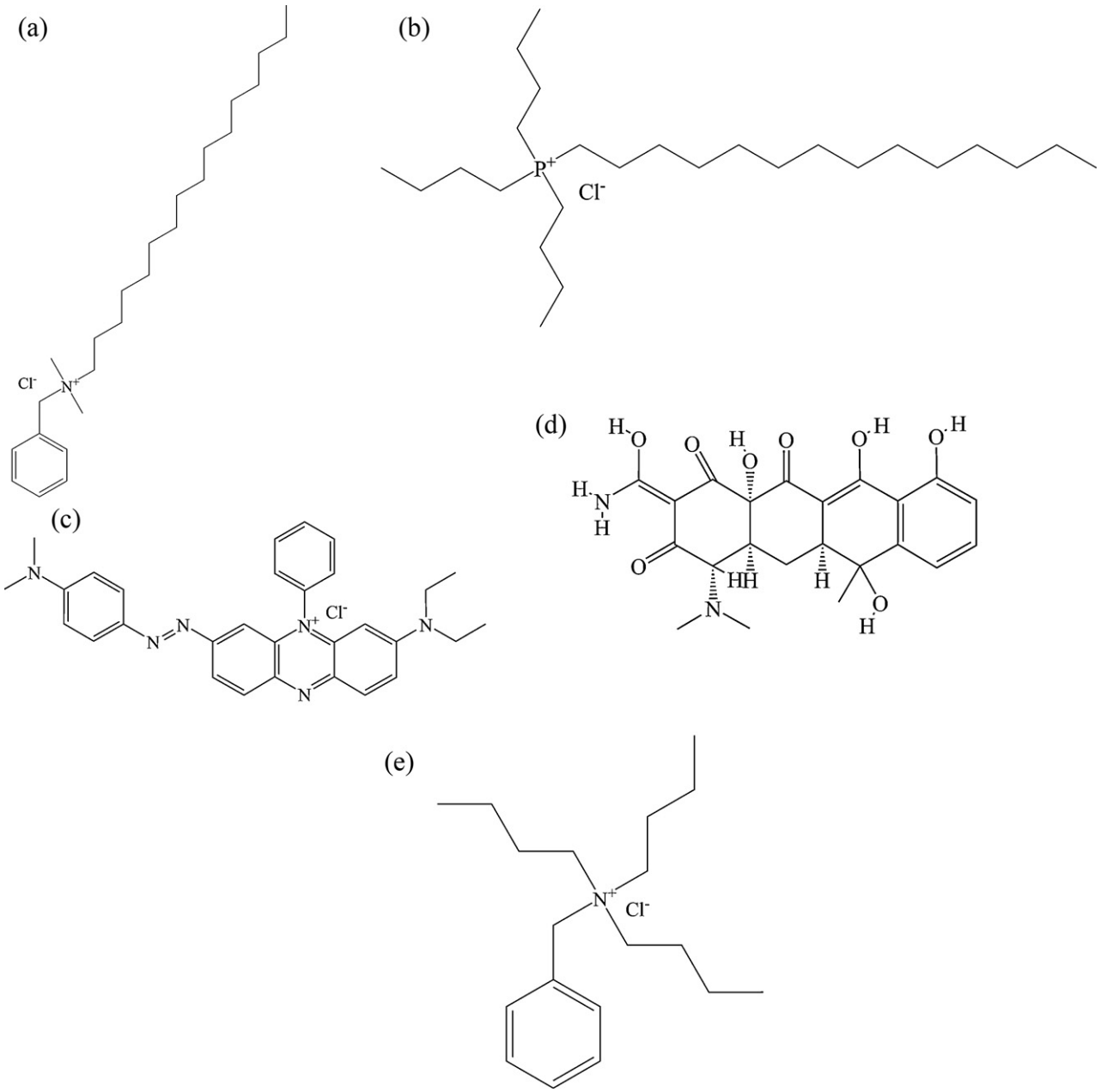

Fig. 8. Selected displacers for column displacement experiments. (a) Cetyldimethylbenzylammonium chloride, −25.03 kJ/mol; (b) tributyl-tetradecylphosphonium chloride, −25.15 kJ/mol; (c) Janus Green B, −29.32 kJ/mol; (d) tetracycline, −24.53 kJ/mol; (e) benzyltributylammonium chloride, −19.71 kJ/mol.

zylammonium chloride, tributyl-tetradecylphosphonium chloride and Janus Green B) can effectively displace lysozyme, while the displacer of small intermolecular energy (benzyltributylammonium chloride) can only elute the protein at the same concentration. The successful displacement (i.e., minimal overlap of the protein band and the displacer) by the three displacers with permanent charges indicates that the positive charge on the displacer molecules did not interfere with the displacement via hydrophobic interactions. Further experiments with benzyltributylammonium chloride at higher concentrations did not lead to any successful displacement (data not shown). This has proved that the docking scheme can indeed provide an effective aid to the identification and discovery of displacers.

In order to further investigate the effectiveness of the displacers screened, displacement of a binary protein mixture was performed with tributyl-tetradecylphosphonium chloride as the displacer.

As shown in Fig. 10, the displacer has successfully displaced the proteins with a certain degree of separation. However, a considerable portion of the three components are still overlaid, especially for the two proteins. Changing the loading volume and displacer concentration did not lead to any significant improvement in resolution. As the particle size of the adsorbent used in this study is relatively large (30 μm) compared with the most adsorbents used in displacement chromatography (∼10 μm) with columns of similar dimensions [8,11,20,57–59,66–70], the low resolution is considered due to the relatively large size of the adsorbent. The molecular size of proteins is much larger than that of the displacer, leading to lower rate of mass transfer and poorer resolution of proteins. As HCIC adsorbents of small particle sizes are not available at present, the preparation of specially designed adsorbents for high-performance HCIDC separations would be a subject of future research. Further optimization of experimental parameters is also needed to make full use of the potential of HCIDC.

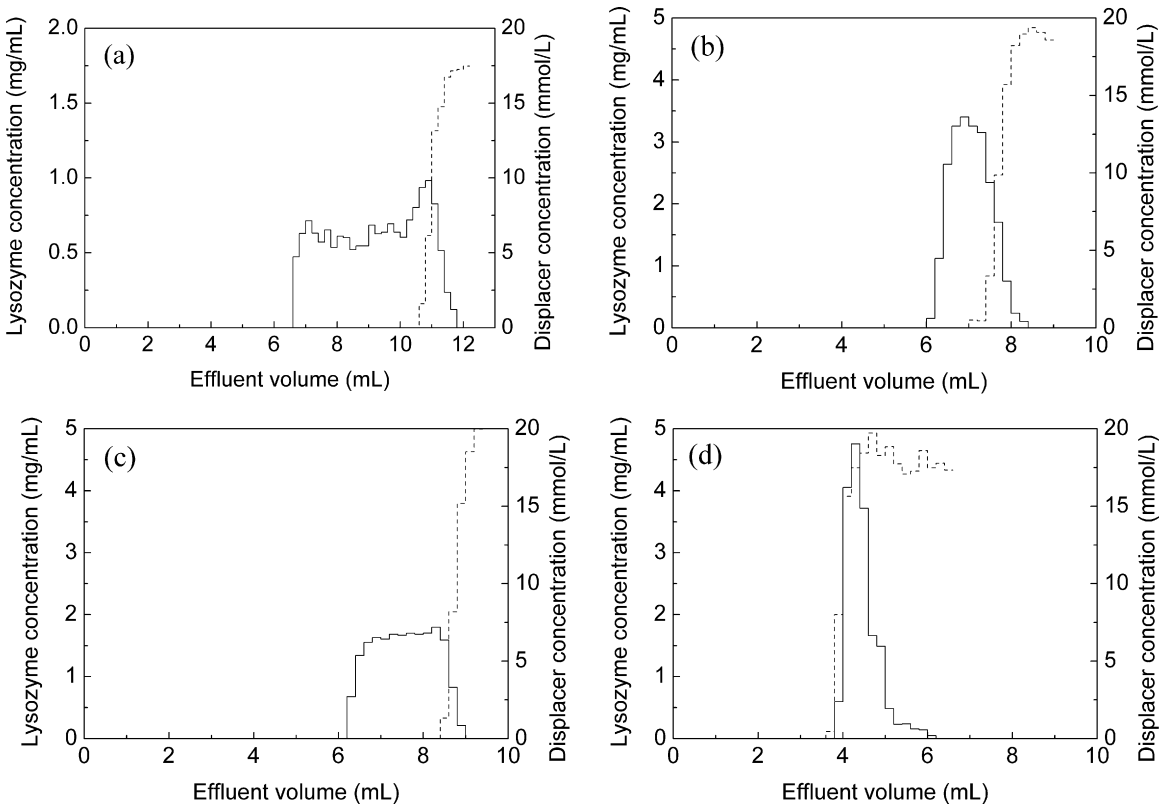

Fig. 9. Hydrophobic charge induction displacement chromatography with selected displacers. (—) Lysozyme and (- - -) displacer. (a) Cetyldimethylbenzylammonium chloride, (b) tributyl-tetradecylphosphonium chloride, (c) Janus Green B, and (d) benzyltributylammonium chloride. HR5/10 column (4.6 mm I.D. × 100 mm length), stationary phase: 5-Aminoindole Superose, 70 μmol/mL; mobile phase: 50 mmol/L phosphate buffer; displacer concentration: 20 mmol/L; loading: 5 mg/mL lysozyme, 1 mL; flow rate: 0.1 mL/min; fraction size: 0.2 mL.

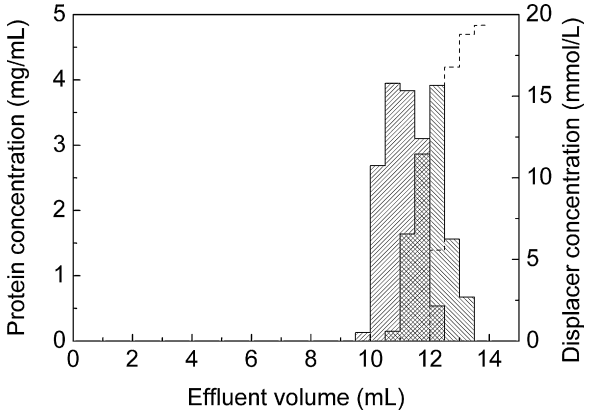

Fig. 10. Displacement of a binary protein mixture. (▨) Lysozyme; (▧) α-chymotrypsinogen A; (- - -) displacer. Tricorn 5/200 column (4.6 mm I.D. × 200 mm length), stationary phase: 5-Aminoindole Superose, 70 μmol/mL; mobile phase: 50 mmol/L phosphate buffer; displacer: tributyl-tetradecylphosphonium chloride; displacer concentration: 20 mmol/L; loading: 5 mg/mL lysozyme + 3 mg/mL α-chymotrypsinogen A, 2 mL; flow rate: 0.1 mL/min; fraction size: 0.5 mL.

4. Conclusions

Discovery of high-efficacy displacers is of great significance for the development and application of displacement chromatography. In this work, a displacer-immobilized docking scheme was proposed for the prediction of displacer efficacy in protein chromatography. The docking scheme was verified by the efficacy data of a number of cationic, anionic and hydrophobic displacers reported in the literature [24,26,28,33]. The scheme was the n used for the screening of displacers for hydrophobic charge induction displacement chromatography (HCIDC) from commercially available compounds. Column displacement experiments with representative identified displacers showed that these displacers are efficacious in HCIDC. These results indicate that the displacer-immobilized scheme is a feasible, fast and convenient method for displacer screening. While only a limited number of compounds are subjected to the screening process as well as the experimental test, the high efficiency and reasonable accuracy of the docking scheme shows great potential in the discovery of more novel displacers. The development of novel high-efficacy displacers from a larger chemical space as well as from combinatorial compound libraries with the assistance of the docking scheme will be a subject of future research.

In this work, the docking scheme was used only for the screening of HCIDC displacers. However, the consistency of docking results with experimental data in different modes of displacement chromatography suggests that this scheme can be readily extended to other displacement chromatography systems. The development of an adsorbent-based (i.e., immobilized ligands-based) virtual screening methodology has great significance for the studies of displacement chromatography. Although the displacer-based screening strategies (such as quantitative structure–efficacy relationships) are also capable of screening large number of candidate molecules within a small time scale, adsorbent-based virtual screening has eliminated the need for efficacy data of known displacers, which determines not only the accuracy but also the scope of displacer-based strategies. In other words, as an approach based on structure similarity, displacer-based method is more likely to give compounds that are more or less analogous to the known lead compounds, while the adsorbent-based method has enabled the discovery of completely new displacers. However, as can be seen from the results of this work, the docking strategy

can only give a crude estimation but not a precise ranking of displacer efficacy. It has also been documented by many authors that the major weakness of docking is the commonplace of false-positives and false-negatives [36–42]. Therefore, it is necessary to combine docking with other strategies in displacer screening and design. For example, in order to develop high-affinity displacers for a certain mode of chromatography, docking may be used first to conduct an extensive and thorough search of potential displacers from commercially available compounds. The potential displacers identified may be further subjected to high-throughput screening experiments, which can reveal the true lead compounds. The experimental data can then be used to establish quantitative structure–efficacy relationships, which enable finer screening and structure-based displacer design. It is expected that the combination of these methodologies would greatly facilitate the discovery of high-efficacy displacers for protein purification.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bej.2011.03.004.

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