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Short communication

Towards an electrochemically modulated chromatographic stationary phase

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Abstract

We have identified heme as a novel stable functional group for an electromodulated chromatographic stationary phase targeted for bioseparations. Preliminary experiments with a heme–agarose column show that β-lactoglobulin exhibits differential binding towards the two redox states of heme. We have also developed a tentative chemical coupling procedure suitable for covalent immobilization of heme onto a conductive glassy carbon electrode via a polyethylene glycol spacer arm as a necessary first step towards the development of an electrochemical chromatography system.

1. Introduction

The application of an electric field to a chromatographic stationary phase can provide a convenient means for manipulating protein adsorptive properties in situ and on the fly. Using this technique, two major modes of operation are available. The first, denoted electrochromatography, consists of the application of an axial electric field across the chromatographic column in order to induce electrophoretic motion as exemplified by Rudge et al. [1]. The second method, termed electrochemical chromatography, relies on the application of the electrical field across the solvent–sorbent interface in order to induce electrosorption–repulsion or to selectively modify the sorbent material itself. The later will be the approach discussed in the following sections.

Roughly a dozen different types of electrochemical chromatography media have been studied during the past several decades, a brief review of which is given by Ge et al. [2]. Most systems that have been investigated run from unfunctionalized metallic beds to the more recent conducting polymer coatings, such as polypyrrole, bioactive peptide–polypyrrole copolymer, ferrocene-based polymers and their derivatives, on diverse conductive particles [3]. Although there have been abundant reports of successful electrochemical modulation of conducting polymer based stationary phases, those studies have mainly dealt with small organic and inorganic molecules [4]. Application of this technology to bioseparations has been only marginally successful [5]. This is perhaps due to the complex interactions between the biomolecules and the ill-characterized surface of conducting

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polymers which are prone to air oxidation, as is the case for reduced polypyrrole, or degradation by various aqueous buffer components, as observed for ferrocene-based systems [6,7].

A metalloporphyrin-based stationary phase has been described by Kibbey and Meyerhoff [8] for use in a reversed-phase chromatography mode for the separation of small organic molecules. However, the use of metalloporphyrin functional groups in an *electrochemical* mode has not been exploited. We report here, as an exploratory study, experiments involving the binding of a model protein, β-lactoglobulin, to both redox states of chloroprotoporphyrin IX iron (heme) immobilized on an agarose gel. We also propose a chemical immobilization procedure for the covalent attachment of heme to a conductive glassy carbon electrode surface as a potential monolithic electrochemical stationary phase.

2. Experimental

For the chromatography experiments, heme–agarose was purchased from Sigma (St. Louis, MO, USA) and gravity packed in a Pharmacia HR 5/10 column. Sodium dithionite (sodium hydrosulfite) was obtained from Aldrich (Milwaukee, WI, USA), HEPES buffer, β-lactoglobulin (mixture of A and B forms), sodium nitrite and sodium chloride were from Sigma. For the chemical coupling, hemin (chloroprotoporphyrin IX iron III) was procured from Sigma, dicyclohexylcarbodiimide (DCC), dimethylaminopyridine (DMAP), 1000-dalton polyethylene glycol (PEG), potassium dichromate and dioxane were all from Aldrich.

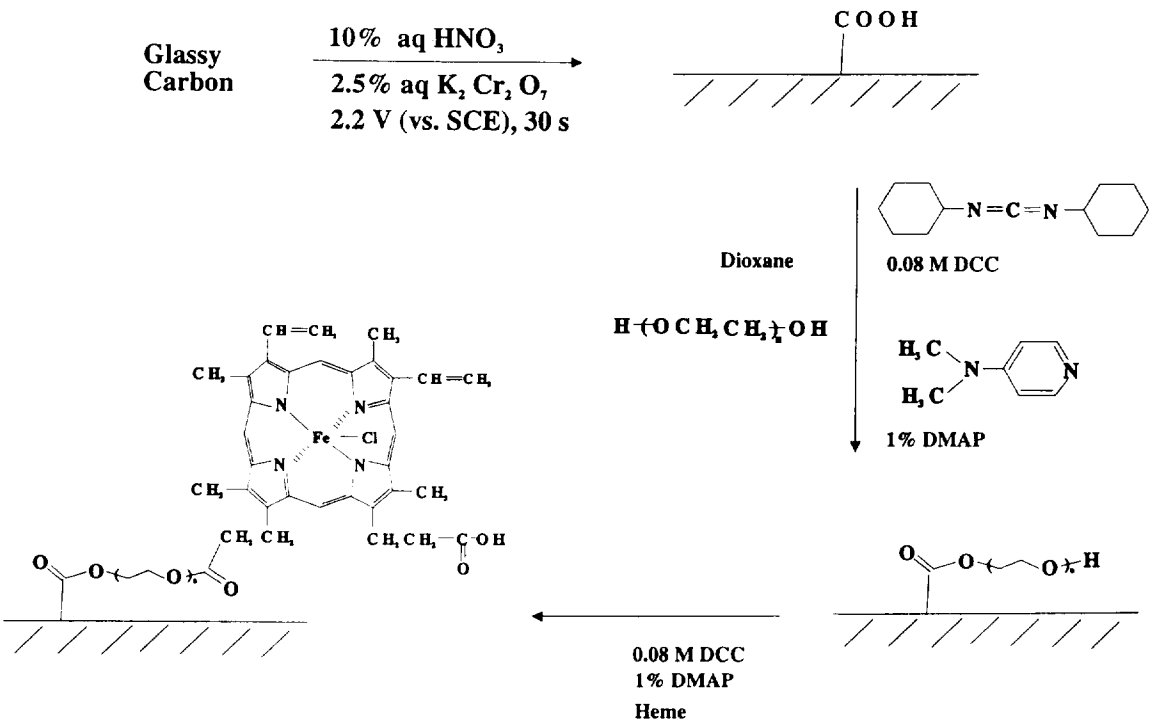
Chromatographic studies were performed using a Pharmacia P-50 pump and an ABI Analytical Kratos Spectroflow 757 detector. The detector signal was collected via an A/D board on a personal computer.

A chemical coupling based on standard DCC coupling procedures, shown in Scheme 1, was developed to immobilize heme onto glassy carbon via a spacer arm [9]. As the porphyrin ring is rather hydrophobic, polyethylene glycol was chosen as a spacer arm in order to minimize further non-specific interactions. A glassy carbon electrode ($7.85 \cdot 10^{-3}$ cm^2) is connected as the working electrode in a three-electrode system with reference to a saturated calomel electrode (SCE). The derivatization electrolyte solution is composed of 10% aqueous nitric acid and 2.5% aqueous potassium dichromate. The working electrode is subjected to a potential of 2.2 V vs. SCE for approximately thirty seconds. This procedure is known to oxidize the glassy carbon surface to carboxylate groups; other oxidation products may also be present to a lesser extent [10]. The surface-oxidized glassy carbon electrode is then rinsed with deionized water and buffed dry prior to immersion in a solution of 0.08 *M* DCC, approx. 1% DMAP and approx. 100 m*M* 1000-dalton PEG in dioxane. The reaction is allowed to proceed overnight at room temperature to couple one end of the PEG to the glassy carbon carboxylate group generated in the previous step. A large excess of PEG ensures that a bridging reaction due to the coupling of both ends to the glassy carbon surface is inhibited by mass action. The electrode is once again rinsed with fresh dioxane and allowed to stand in a fresh dioxane solution composed of 0.08 *M* DCC, approx. 1% DMAP and saturated with heme. The reaction is again allowed to proceed overnight at room temperature to couple the free end of the PEG to a heme carboxylate group. After this final step, the electrode is washed and soaked in warm dioxane to remove any non-covalently bound heme from the surface.

Cyclic voltametry of heme immobilized on a glassy carbon surface was conducted with an EG&G Princeton Applied Research Model 173 potentiostat equipped with a Model 175 programmer. The scans were recorded on a Houston Instruments Omnigraphic 2000 XY plotter.

3. Results

Fig. 1 is a chromatogram of the β-lactoglobulin mixture on a heme–agarose bed with heme in both the oxidized and reduced states. Since the support matrix is non-conductive, the heme reduction and oxidation steps were performed



Scheme 1. Chemical immobilization of heme onto glassy carbon via PEG spacer arm.

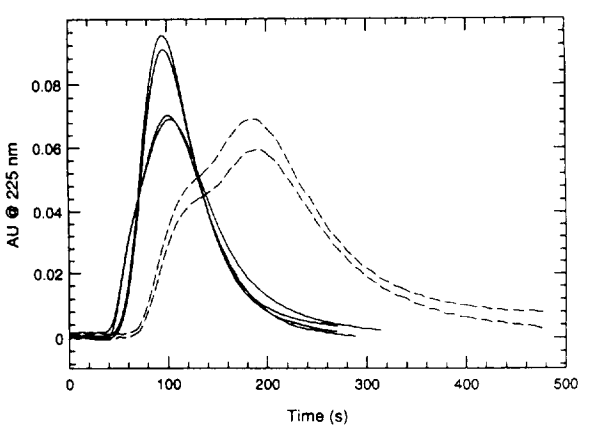


Fig. 1. β-lactoglobulin chromatograms on a 8.4 × 0.5 cm column. Solid line: oxidized heme; dashed line: reduced heme. Flow-rate: 1 ml/min, mobile phase: 50 m*M* HEPES, pH 6.9, 0.5 *M* NaCl and injections were 20 μl pulses of 1 mg/ml β-lactoglobulin (mixture of A and B). Reduction done with 3 ml of 100 m*M* $Na_2S_2O_4$, re-oxidation with 3 ml of 100 m*M* $NaNO_2$, both at 0.5 ml/min [9,16]. Duplicate runs were performed in each redox state.

chemically. As evidenced by the isocratic elution profiles, the β-lactoglobulin mixture is retained to a greater extent on a reduced heme–agarose bed as compared with the oxidized counterpart. The re-oxidation step confirms that heme can be cycled between the oxidized and reduced forms. We believe that metal coordination, primarily by histidine side chains, is the mechanism of protein–sorbent interaction. These results appear to be in concordance with reported binding equilibrium constants for imidazole with Fe(III) heme which are an order of magnitude less than for imidazole with Fe(II) heme [11]. Rassi and Horvath have also reported that Fe(II) had a very different selectivity than Fe(III) towards proteins as studied on an iminodiacetic acid (IDA) stationary phase [12]. The very weak interactions displayed by our heme–agarose sorbent with the protein as compared with conventional metal affinity systems such as Cu–IDA

bonded stationary phases can be rationalized as follows. Firstly, the molecular nature of the iron–porphyrin system only affords two possible coordination sites to other ligands and these sites are located on opposite sides of the porphyrin ring. Thus, statistically, only one site is accessible for binding at a time, in contrast to a metal–IDA system which displays all possible binding sites on the same face. Secondly, the ligand density for this commercial resin is quoted by Sigma to be 4.7 μmoles/ml of gel and is at least an order of magnitude lower than that for a typical chelating gel [13]. Although the column used herein is of comparable dimensions to commercial columns, the capacity of this heme–agarose gel is much lower than would be expected for a commercial metal affinity media. The low resolution of the separation can be attributed in part to the heterogeneity of the agarose particles which range from roughly 90–500 μm (data from Sigma). The presence of a shoulder on the elution profile for the reduced heme case (dashed lines in Fig. 1) may reflect a partial separation of β-lactoglobulins A and B. No further attempt was made to identify the peak components. Upon reoxidation, the peak size was reduced as compared to the pre-reduction case but the amount of tailing is more important (not fully shown on the figure) and reflects incomplete oxidation of the heme.

In order to conduct in situ electrochemical chromatography, the ligand must be attached to a conductive support. The coupling procedure described above can be used to covalently link heme onto glassy carbon. A PEG spacer arm is introduced in order to improve accessibility and minimize protein non-specific interactions with the glassy carbon surface. The success of the immobilization and electrochemical activity of the resulting heme–glassy carbon is assessed by cyclic voltametry. Fig. 2 contrasts cyclic voltamograms of a heme-derivatized glassy carbon electrode with that of an underivatized clean electrode. The cyclic voltamogram of the underivatized electrode is rather featureless except for the registry of some charging current caused by the potential ramp. In comparison, the cyclic voltamogram of the heme-derivatized electrode

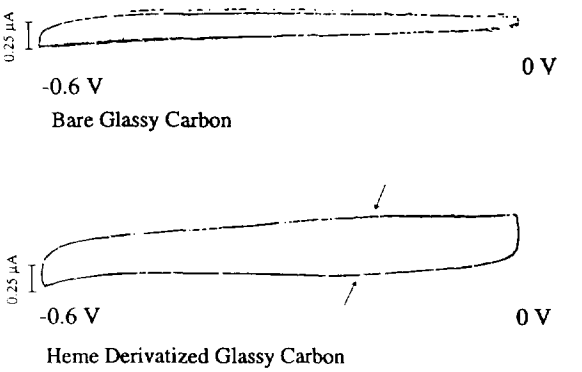


Fig. 2. Cyclic voltamogram of clean glassy carbon and heme–glassy carbon in 0.5 *M* NaCl electrolyte, SCE reference and Pt mesh counter electrode, scanned at 50 mV/s.

shows a noticeably higher overall current. The generation of carboxylic groups on the surface of the glassy carbon electrode by oxidative treatment causes the charging current to be significantly increased as observed by cyclic voltametry [14]. This process tends to make the location of small peaks difficult as their signal is swamped by the background. After the attachment process, weak oxidative and reductive currents peaks are registered, the relative location of which corresponds well with that of heme in solution (data not shown) suggesting the presence of a redox active heme on the surface of the electrode. Because the covalent attachment procedure results in monolayer coverage at most, the total number of heme groups present on the surface is quite low (approx 1 heme per 1000 square Å as measured electrochemically), explaining the small magnitude of the peak current.

4. Conclusions

Our preliminary experiments suggest that heme can be used as a functional group for an electrochemically modulated chromatographic stationary phase. We have also demonstrated a synthetic strategy for preparation of such a stationary phase. An electrochemically modulable system may prove to be quite useful as selectivity may be changed on the fly in order to either bind or release a ligand. Another advantage of metal–porphyrins is their stability; the

metallic ion is not leachable under standard chromatographic conditions [15]. A monolithic device consisting of a piece of vitreous carbon matrix can be derivatized with a metalloporphyrin and can be used to conduct electrochemical separations. Future work will involve the investigation of other metalloporphyrins, the development of a chemical coupling route for higher ligand density on other conducting supports such as platinum, and the construction of suitable chromatographic hardware for conducting in situ electrochemical chromatography.

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