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Purification of monoclonal antibodies by chemical affinity mixed mode chromatography

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ABSTRACT

The application of several pyridine-based compounds as immobilised ligands has been investigated for the purification of monoclonal antibodies *via* mixed mode chromatography. The ligands employed were 4′-terpyridinysulfanylethylamine (4′-TerPSEA), 5-bromo-2-pyridinylsulfanylethylamine (5-Br-2-PSEA), 2-quinolinylsulfanylethylamine (2-QSEA) and 4-pyridinylsulfanylethylamine (4-PSEA). The performance attributes of adsorbents, derived from the immobilisation of these different ligands onto Sepharose 6 Fast Flow™, was evaluated from batch adsorption studies and from chromatographic experiments with humanised IgG1, IgG2 and IgG4 monoclonal antibodies produced by stable CHO cell lines cultured in chemical defined media. These results demonstrated that monoclonal antibodies of different subclasses can be efficiently purified from crude CHO cell culture supernatants using these new chemical affinity chromatographic systems. Moreover, the majority of the CHO host proteins could be eliminated during the chromatographic purification step with these resins, as monitored by a specific ELISA assay.

1. Introduction

Since their first production in 1975 [1], monoclonal antibodies (mAbs) have become powerful tools in basic research and increasingly have gained prominence in diagnosis and therapy [2,3]. For the latter purposes, large amounts of pure mAbs need to be purified to high stringency. However, current manufacturing and purification processes can place constraints on their production capacity [4]. In particular, the number of downstream unit operations required to achieve the required level of purity of the mAb is of great importance, not only for therapeutic purposes, but also to minimise interferences from background biologicals when the mAbs are used in detection of antigens or as agents in purification procedures [5].

Purification of monoclonal antibodies from crude cell culture feedstocks thus requires multi-step processes, which have in the past included membrane-based tangential flow filtration methods, aqueous two phase separation procedures and affinity, ion exchange and hydrophobic interaction chromatography [6–12]. Frequently, affinity chromatography has been employed as the preferred separation method at the laboratory or process scale [13] with the traditional ligands based on bacterial Fc receptors, such as Protein A from *Staphylococcus aureus*, or Protein G from *Streptococcus* sp, favoured [5,14,15]. The use of such biological ligands, however, has several drawbacks including their overall low productivity [12] and their relatively high cost [13]. In addition, the low pH conditions required to elute the antibody from Protein A resins can damage both the target and ligand proteins and can be incompatible with subsequent downstream steps [12,16]. Further disadvantages also include the inability of Protein A to bind strongly to antibodies from many mammalian species, some antibody subclasses and genetically engineered antibody domains [5,17].

In recent years, mixed mode chromatography has attracted considerable interest as a method for mAb purification with the objective to decrease cost associated with multiple chromatography steps, whilst still allowing high levels of protein purity to be achieved [12,18]. Mixed mode chromatography exploits the combined attributes of two or more physiochemical properties of a ligand. Generally, mixed mode ligands can be grouped into three subsets, based on the characteristics of the dominant functionalities: (1) those augmenting anion exchange behaviour with hydrogen bonding effects; (2) those augmenting metal coordination with electrostatic interactions; and (3) those augmenting hydrophobic interactions with dipole–dipole or van der Waals characteristics [6,12].

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In previous communications, we have described the synthesis of different libraries of pyridine-based compounds and evaluated some examples for their potential as ligands in the affinity and mixed mode chromatographic purification of recombinant proteins [19,20]. These ligands, comprising a pyridine core with a variety of different substituent moieties, have also included analogues with one or more additional aromatic ring structures which show increased protein binding attributes. These observations have parallels since, as reported previously [21], incorporation of aromatic substituents into the structures of ion exchange ligands also creates chromatographic resins with mixed mode properties with increased protein binding capacities. The structures of our pyridine-based ligands, when immobilised onto various support materials, allow similar mixed mode binding behaviour to be achieved, due to the participation of electrostatic and thiophilic-based interactions depending on the compositions and pH of the chosen loading, wash and elution buffers. Interestingly, in preliminary studies chromatographic resins generated from the immobilisation of several of these compounds showed high binding capacities for mAbs, which could be successfully eluted in a highly efficient manner through a step change in buffer pH [19,20].

In order to develop more efficient, cheaper and green chemical approaches for mAb purification, we have extended this work and report here these new results. Several pyridine-based ligands, varying in the nature of the pyridyl ring substitution, were investigated (Fig. 1). These include the un-substituted compound 4-PSEA, the bromo-analogue, 5-Br-2-PSEA, and two more hydrophobic ligands containing aromatic ring extensions, e.g. 2-QSEA and 4-TerPSEA. Based on batch adsorption results, which established the suitable binding conditions for the mAb–ligand interactions, chromatographic studies demonstrated that these new separation materials could be used to efficiently purify mAbs produced by engineered CHO cell lines cultures in chemical defined media, and thus provide alternative options to other affinity chromatographic resins.

2. Materials and methods

2.1. Synthesis and immobilization of pyridine-based ligands

The 4-pyridinylsulfanylethylamine (4-PSEA), 2-quinolinylsulfanylethylamine (2-QSEA) and 4′-terpyridinylsulfanylethylamine (4′-TerPSEA) (Fig. 1) were prepared from the reaction of cysteamine hydrochloride with the appropriate aromatic chloride with sodium hydride as base [19,20]. The 5-bromo-2-pyridinylsulfanylethylamine (5-Br-2-PSEA) was made in a similar manner from 2,5-dibromopyridine but with sodium ethoxide as the base in ethanol and microwave heating at 140 °C for 21 min [22]. The source and purity of the chemicals used in these syntheses have been described in detail previously [19,20,22].

Immobilisation of these ligands onto epichlorohydrin-activated Sepharose 6 FF™ was based on methods described previously [19,20]. The Sepharose 6 FF™ typically in aliquots of 500 mL was activated with epichlorohydrin according to described procedures [19,20]. For the immobilisation of 4-PSEA and 4′-TerPSEA, a 0.2 M solution of the ligand as the free base (20 mmol) was prepared by dissolving the ligand in an appropriate solvent (25% (*v*/*v*) MeOH:H_2O for 4-PSEA and 75% (*v*/*v*) MeOH:H_2O for 4′-TerPSEA). For the immobilisation of 5-Br-2-PSEA and 2-QSEA, a solution of the 2HCl salts was neutralised with 2 M NaOH and MeOH added to give a 0.2 M solution of the ligand (20 mmol) in 50% (*v*/*v*) MeOH:H_2O. Washed and suction-dried epoxy-activated Sepharose 6 FF™ gel (100 ml) was added to each ligand solution and the suspension gently mixed on a shaking platform at 28 °C for 21 h. The resulting adsorbents were collected by vacuum filtration, washed thoroughly with water and stored in 20% ethanol/H_2O (*v*/*v*) at 4 °C until required for use. The densities of the immobilised ligands on the matrix were determined by elemental nitrogen analysis (Dairy Tech. Services, Melbourne, Australia) and were as follows: 4′-TerPSEA: 296 μmol/g resin, 5-Br-2-PSEA: 290 μmol/g resin, 2-QSEA: 325 μmol/g resin and 4-PSEA: 371 μmol/g resin.

2.2. Determination of the batch binding characteristics of the pyridinyl adsorbents with mAbs under different pH and salt conditions using a robotic liquid handling system

Humanised murine monoclonal antibodies were expressed in the stable CHO cell line CHO-K1-SV under standard culture conditions with chemically defined media. The IgG1 (molecular mass 144,283 ± 22 daltons) and the IgG4 (molecular mass 143,853 ± 22 daltons) mAbs recognise the tumour-associated glycoprotein, TAG-72, whilst the IgG2 mAb (molecular mass 150,647 ± 23 daltons) recognised a proprietary antigen. Samples of each crude cell culture harvest supernatant was separately dialysed into one of four different buffers (5 × 1 L), (e.g., (a) 25 mM sodium acetate pH 5.0; (b) 25 mM Tris pH 7.0; (c) 25 mM Tris pH 8.0; and (d) 25 mM Tris pH 9.0) using cellulose membranes (Sigma Aldrich) with a 12.5 kDa molecular weight cut off at 4 °C. Following dialysis, the samples were collected and stored at 4 °C. The OD_{280} of each of the 12 samples was determined and protein concentrations of each solution adjusted to the same value (with OD_{280} = 3.5, corresponding to 2.6 mg/ml). Moreover, the protein concentrations for the crude supernatant, the purified mAb protein and the protein recovered in the chromatographic fractions were also determined on the basis of their OD_{280} values and confirmed by Bradford assays. Stock volumes of each of the four buffers, containing 1.2 M of various salts, were also made. The salts included in these studies were (i) sodium, ammonium and magnesium sulphate; (ii) sodium, ammonium, potassium and magnesium acetate; (iii) sodium, ammonium, potassium and magnesium chloride; (iv) di-ammonium, di-potassium and di-sodium hydrogen phosphate; (v) sodium and ammonium tartrate; (vi) sodium and potassium citrate; (vii) sodium succinate, (viii) sodium glycinate and (ix) potassium glutamate.

For the batch binding studies, samples of each resin (7.7 μg of suction dried resin suspended in the loading buffer (100 μl)) were dispensed using a ResiQuot™ aliquotation device (Atoll, Weingarten, Germany) into the wells of 96-DeepWell plates (1 mL, NalgeNunc, Roskilde, Denmark). A Tecan Freedom ECO200 robotic workstation (Tecan, Männedorf, Switzerland), equipped with an 8-pipette liquid handling arm, two gripping arms (ex-centric and

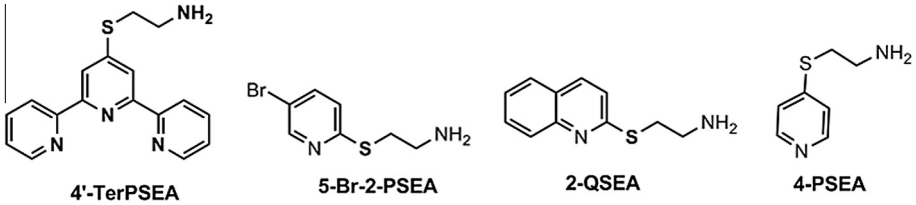

Fig. 1. Structures of the pyridine-based ligands, 4′-TerPSEA, 5-Br-2-PSEA and 2-QSEA and their relationship to the parent ligand, 4-PSEA, used in this investigation.