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Studies with an immobilized metal affinity chromatography cassette system involving binuclear triazacyclononane-derived ligands: Automation of batch adsorption measurements with tagged recombinant proteins

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ABSTRACT

This study describes the determination of the adsorption isotherms and binding kinetics of tagged recombinant proteins using a recently developed IMAC cassette system and employing automated robotic liquid handling procedures for IMAC resin screening. These results confirm that these new IMAC resins, generated from a variety of different metal-charged binuclear 1,4,7-triaza-cyclononane (tacn) ligands, interact with recombinant proteins containing a novel N-terminal metal binding tag, NT1A, with static binding capacities similar to those obtained with conventional hexa-His tagged proteins, but with significantly increased association constants. In addition, higher kinetic binding rates were observed with these new IMAC systems, an attribute that can be positively exploited to increase process productivity. The results from this investigation demonstrate that enhancements in binding capacities and affinities were achieved with these new IMAC resins and chosen NT1A tagged protein. Further, differences in the binding performances of the *bis*(tacn) xylenyl-bridged ligands were consistent with the distance between the metal binding centres of the two tacn moieties, the flexibility of the ligand and the potential contribution from the aromatic ring of the xylenyl group to undergo π/π stacking interactions with the tagged proteins.

1. Introduction

Immobilized metal affinity chromatography (IMAC) is a popular technique for the purification of recombinantly-tagged proteins particularly at the laboratory scale, with iminodiacetic acid (IDA) [1–3] and nitrilotriacetic acid (NTA) [4,5] the two most commonly used metal ion chelators. Despite their popularity, both of these chelating systems have a number of limitations. The structurally unconstrained tridentate metal chelator, IDA, exhibits comparatively low metal ion stability constants with most 'borderline' transition group metal ions [6], such as Cu^{2+}, Ni^{2+}, Co^{2+} or Zn^{2+}, with $\log K_{sc}$ (or β-values) between 7.2 and 10.7 [7]. Since the affinity of histidine (as well as several other amino acid residues) for these metal ions falls into a similar range ($\log K_{assoc}$ values between 6.5 and 10.2), a frequently observed phenomenon with IDA-based IMAC systems is metal ion transfer (MIT) from the resin to the protein. This behavior leads to loss of metal ions from the IMAC adsorbent, and thus reduced binding capacity, with product contamination occurring due to the presence of leached metal ions [8]. On the other hand, the functional entities of the tetradentate ligand, NTA, occupy four coordination sites of borderline metal ions and result in larger β-values, but typically leave only one or two metal coordination sites available for interaction with e.g. histidyl groups of a protein, depending on the coordination geometry of the metal ion. This reduction in the number of coordination sites can lead to premature breakthrough of the tagged recombinant protein. More critical, however, is the fact that NTA has recently been classified within some national jurisdictions as a potential carcinogen [9]. These concerns on metal leakage and ligand toxicity have resulted in limited application of IMAC adsorbents in several industrial sectors, e.g. the biopharmaceutical industry.

The synthesis and properties of some members of the family of binuclear metal chelating ligands, based on the macrocyclic 1,4,7-triazacyclo-nonane (tacn), have been described and their ability to bind histidine-containing oligopeptides and tagged and untagged proteins investigated [10–16]. Compared to conventional IMAC ligands, significantly higher metal ion stability constants were observed with Cu^{2+}, Ni^{2+} and Zn^{2+} ions especially for the *bis*(tacn) ligands, such as *bis*(tacn)ethane (dtne) or *bis*(tacn)-propane (dtnp). Even though tacn and *bis*(tacn) ligands are tridentate chelators,

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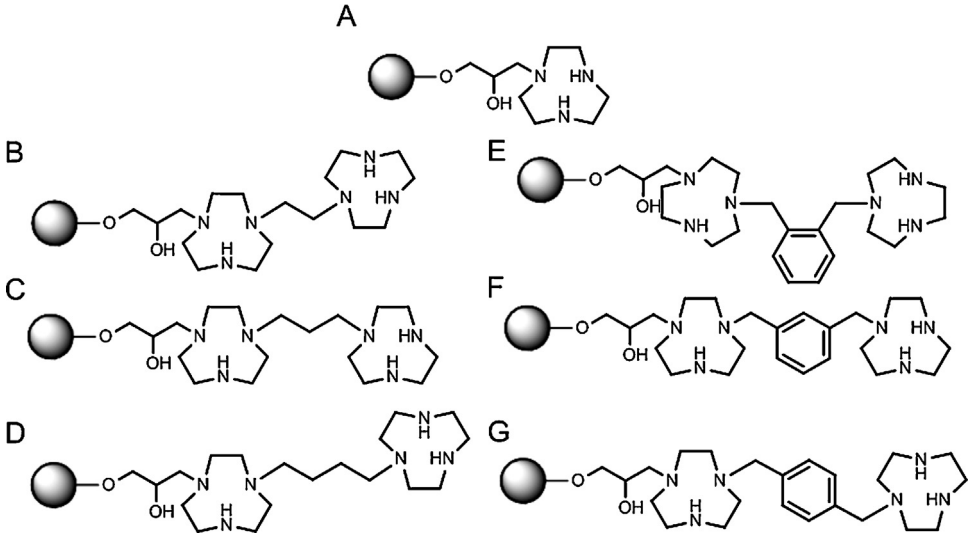

Fig. 1. Structures of the 1,4,7-triazacyclononane (tacn) and bis(tacn) metal coordinating ligands immobilized onto a solid support material, e.g. Sepharose™ 6 FF, via epichlorohydrin activation chemistry. (A) tacn; (B) 1,2-bis(tacn)ethane (dtne); (C) 1,3-bis(tacn)propane (dtnp); (D) 1,4-bis(tacn)butane (dtnb); (E) *ortho*-bis(1,4,7-triazacyclonon-1-ylmethyl)benzene (L^{ox}); (F) *meta*-bis(1,4,7-triazacyclonon-1-ylmethyl)benzene (L^{mx}); and (G) *para*-bis(1,4,7-triazacyclonon-1-ylmethyl)benzene (L^{px}).

the ligand-metal ion stability constants for Cu^{2+}, Ni^{2+} and Zn^{2+} are between three and ten orders of magnitude higher than those found with NTA [7,17–19], making the tacn family virtually free of MIT effects. Moreover, *bis*(tacn) ligands are capable of immobilizing two metal ions simultaneously, which allows even stronger protein retention to be achieved due to the potential for multipoint interactions.

In characterizing the performance of chromatographic adsorbents, measurement of their adsorption isotherms and binding kinetics is particularly useful [20–24]. Typically, static (batch) adsorption experiments are used to deliver this isotherm and kinetic information and to facilitate prediction of the binding behavior of the same proteins in dynamic column formats, operated under different conditions of ionic strength, pH, temperature flow rate and sample loading in packed or fluidized beds [25–31]. Until recently, the generation of such information has often been time consuming and labour intensive. For example, in a previous study on the interaction between several immobilized metal-loaded chelating ligands and different untagged proteins, such as hen egg white lysozyme, bovine myoglobin and bovine cytochrome *c* [13,21], many thousands of sample and buffer dilutions and metal ion replacement experiments were manually carried out, taking over ~18 months, to obtained the required sets of experimental data. The emergence [32–34] of high throughput techniques, based on robotic microfluidic liquid handling systems, for the screening of chromatographic resins, such as ion exchange, hydroxyapatite or hydrophobic interaction materials, has provided an opportunity to circumvent these limitations with IMAC procedures.

In this study, we describe the application of such robotic procedures in the determination of the adsorption isotherms and binding kinetics of tagged proteins in IMAC systems under static (batch) binding conditions. To this end, different tacn-derived ligands, chemically immobilized onto Sepharose™ 6 FF (Fig. 1) and loaded with Cu^{2+}, Ni^{2+} or Zn^{2+} ions, have been evaluated. Compared to the manual methods hitherto employed, the application of similar automated liquid handling systems with these IMAC adsorbents was not only expected to significantly reduce the consumption of reagents but also to increase throughput via miniaturization, The performance of these IMAC resins was investigated with a recombinant protein, namely N-terminally tagged glutathione S-transferase (GST), containing a new type of tag, called NT1A, specifically designed for enhanced molecular recognition with this class of IMAC resins. Key binding parameters (maximum capacity, Q_{max} association constant, K_a, and adsorption kinetics, k_i) were determined and compared to those obtained with a conventional tag system, represented by His_6, and a commercial IMAC resin with the same recombinant protein. A further objective of this study was to determine the influence of the spacer group linking the two tacn units on the interaction with the NT1A- and His_6-tagged protein in terms of their Q_{max} and K_a, values with these IMAC systems.

2. Experimental

2.1. Materials and reagents

Glutathione Sepharose 4B and Ni Sepharose™ 6 FF were purchased from GE Healthcare (Uppsala, Sweden). The synthesis of the tacn and *bis*(tacn) ligands (Fig. 1) and their immobilization onto Sepharose™ 6 FF (GE Healthcare, Uppsala, Sweden), activated with epichlorohydrin, was performed according to our published procedures [11,13]. Available X-ray crystallographic data [14] and established energy minimization/docking algorithms [35] based on software programs developed by Molecular Simulations Inc. (San Diego, USA), were employed to generate a model for the interaction of the histidinyl groups of the NT1A tag in its α-helical or β-sheet conformations with the $(Cu^{2+})_2$-Ligand complexes. Metal salts ($Ni(NO_3)_2{\cdot}6H_2O$, $Cu(NO_3)_2{\cdot}1H_2O$ and $Zn(NO_3)_2{\cdot}6H_2O$) were obtained from Sigma-Aldrich (St Louis, MO, USA).

2.2. Protein expression and purification

The fusion proteins were expressed using *E. coli* strain BL21 cells containing the plasmid vector pTrc99A [36]. Cell pellets were re-suspended in 1× PBS buffer, pH 7.2, and the cells lyzed by sonication with a Sonicator 3000 (Misonix, Farmingdale, NY, USA). The filtered cell lysate was then purified by affinity chromatography using Glutathione Sepharose 4B with an ÄKTA Explorer 100 FPLC platform (GE Healthcare, Uppsala, Sweden) with 1× PBS buffer as the loading and wash buffers. Step elution of the tagged proteins was achieved with 10 mM reduced glutathione (Sigma-Aldrich, St Louis, MO, USA) in 50 mM Tris-HCl, pH 8.0. The collected eluate was dialyzed into 1× PBS, pH 7.2. From SDS-PAGE analysis, the eluted tagged proteins were of very high purity (nearly 100% by densitometry). The final sample buffer was adjusted to 20 mM sodium