Selection of Human Metalloantibodies from a Combinatorial Phage Single-Chain Antibody Library

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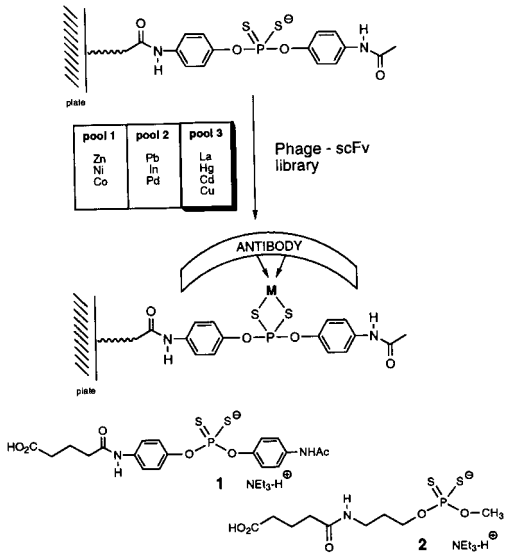
Metal-binding proteins occur widely in nature,[1] yet, metal ions alone are generally considered incapable of eliciting a specific antibody response.[2] The premise is that these small entities are not of sufficient size or complexity to induce formation of specific antibodies. However, metals are known to induce cell-mediated immunity, and exposure to heavy metals can induce autoimmune diseases in rodents.[3] On the basis of these findings, it might be argued that the presentation of the metal is key for immune recognition rather than the size of these molecules.

Multidentate metal chelate complexes have had success in eliciting monoclonal antibodies,[4] but a strategy for an immune response against a "naked" metal ion has only been achieved once with limited utility.[5] Consequently, antibodies have been "engineered" to bind metal ions via site-directed mutagenesis guided by molecular modeling.[6] Such a methodology, for the most part, has been quite triumphant; however, improvements could be made with regard to the repertoire of metals bound and their overall affinities. Given the inherent difficulties associated with trying to re-engineer an already-programmed antibody and antibody recognition of a metal epitope when encapsulated within a multidentate-complex hapten, we sought a more general strategy to select metalloantibodies. The foundation of this approach was grounded upon: (a) high-throughput screening of a naive antibody library and (b) metal presentation using coordinatively unsaturated metallo-panning agents.

Scheme 1 depicts our general strategy to isolate metal ion binding antibodies and consists of the following: (1) immobilization of a metallo-panning agent in a 96-well ELISA plate, (2) charging of these wells with pools of metal ions, (3) panning of the wells with a phage single-chain antibody (scFv) library, (4) removal of unbound phage-scFv antibodies with buffer, (5) release of selectively bound single-chain phage antibodies with acid, and (6) reiteration of this sequence. Presentation of metal ions in this format was two-pronged in nature as we hoped to not only accelerate the panning procedure but also increase the possibility of finding a metal's coordination sphere that would be the dominant epitope recognized by selected antibodies, that is, antibody binding energy would be directed toward metal stabilization rather than the "organic" portion of the panning agent. We anticipated such interactions to be possible within selected antibody combining sites as the metallo-panning agent, a phosphorodithioate−metal coordination complex, could be coordinatively unsaturated depending on the metal ion used.

Scheme 1. High-throughput Panning Strategy to Isolate Metalloantibodies and Structure of Metallo-Panning Agents 1 and 2.



The immobilized phosphorodithioate ligand **1**[7] was utilized as the parent metallo-panning agent (Scheme 1), while a single chain antibody library was constructed from the blood of 50 healthy volunteers (Supporting Information).[8] The resulting phage scFv antibody library was estimated to be 1×10^9 in diversity, based on transformation efficiency. The single-chain library was then panned against three metal pool mixtures and immobilized **1** (Scheme 1). The metals chosen for panning were based on the following criteria: (1) Biologically relevant metals that are nonthiophilic and Lewis acids (Zn^{2+}, Ni^{2+}, and Co^{2+}),[9] (2) thiophilic metals (Pb^{2+}, Pd^{2+}, Hg^{2+}, Cd^{2+}, and Cu^{2+}) that have been proven to be compatible with protein stablility[10] and the conditions used for panning, and (3) metals of synthetic utility that operate in aqueous media (La^{3+}, In^{3+}).[11] Clones from the single-chain antibody library were found to bind only metals from the combinatorial pool 3 (La^{3+}, Hg^{2+}, Cd^{2+}, Cu^{2+}). This was determined by a positive increase in the signal for phage-scFv ELISA to **1**-pool 3 and a steady increase in the output of the phage over four rounds of panning. Ten of these clones were randomly picked from pool 3 and were examined for their binding to microtiter wells of an amine surface strip plate (Costar) coated with **1** and to those of one coated with **1**-pool 3. Two clones (HM3 and HM5) were chosen for further examination as they showed the greatest affinity to **1**-pool 3 versus **1** alone, again, on the basis of phage-ELISA.

The scFv gene fragments of HM3 and HM5 were excised and inserted into the pIWPY vector, and soluble single chain was overexpressed and purified to homogeneity.[8] Affinity measure-

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Table 1. Dissociation Constants of HM3 and HM5 with Metal Ions[a]

panning agent	antibody	$K_D(La^{3+})$ (M)	$K_D(Y^{3+})$ (M)
1	scFv-HM3	2.0×10^{-7}	2.5×10^{-6}
1	scFv-HM5	5.0×10^{-7}	6.0×10^{-6}
2	scFv-HM3	8.5×10^{-7}	1.4×10^{-6}
2	scFv-HM5	4.6×10^{-7}	3.1×10^{-7}

[a] Dissociation constants (K_D) were determined by competition ELISA analysis.[12] The wells of an amine surface strip microtiter plate (Costar) were coated with the metallo-panning agents **1** or **2**, and a range of metal concentrations (5 mM to 10 pM) was used to inhibit antibody binding to **1**/**2**-metal. All other K_D values were found to be greater than 1.0×10^{-4} M (Zn^{2+}, Co^{2+}, Ni^{2+}, Hg^{2+}, Cd^{2+}, Cu^{2+}, In^{3+}, Ag^{+}, Gd^{3+}, Tb^{3+}, Lu^{3+}). All experiments were repeated in triplicate, experimental error, ±5%.

ments were carried out by competition ELISA.[12] The affinity of HM3 and HM5 to **1** was rather poor with both K_d values estimated to be greater than 2×10^{-4} M. To investigate whether antibodies HM3 and HM5 would selectively bind a metal ion, various dilutions of metal (5 mM to 10 pM) were used to inhibit antibody binding to an ELISA plate containing **1**-metal and, its simplified cousin, **2**-metal (Scheme 1). The initial four metals from pool 3 and nine additional metals (Y^{3+}, Ag^{+}, Lu^{3+}, Gd^{3+}, In^{3+}, Tb^{3+}, Zn^{2+}, Ni^{2+}, Co^{2+}) were individually examined to further test specificity and selectivity. The two best metals and their dissociation constants to HM3 and HM5 are shown in Table 1. Lanthanum and yttrium were found to bind HM3 and HM5 in the μM range, whereas the dissociation constants for all other metals examined were at least 2 orders of magnitude poorer.[13] This pattern of metal binding does not show absolute fidelity; however, it is noteworthy since only group IIIa metal ions were found to associate with either antibody with significant affinity. Interestingly, the organic nature of the panning agent that is used (**1** or **2**) does not influence the metal−antibody interaction significantly (Table 1). This strongly indicates that the recognition between **1**/**2**-metal and the antibody is mainly due to the phosphorodithioate moiety of the metallo-panning agent and not to the organic portion, i.e., the aromatic or alkyl residues of the molecule.

Inductively coupled plasma mass spectroscopy (ICPMS) analysis was utilized to detect the number of metal ions bound to HM3.[14] Yttrium was examined in this context as it was the most sensitive to this technique of the two metals listed in Table 1. ScFv-HM3 at concentrations of 1, 2.5, and 5 μM was found to have 1.2, 2.1, and 4.4 μM of yttrium associated with it, respectively. This would suggest that the scFv-HM3 contains a single metal ion combining site. To prove the unique affinity of the single-chain antibody, its affinity to yttrium was compared with those of two other proteins. ICP-MS analysis was performed with Bovine Serum Albumin and a randomly picked antibody (CPQ 15D8F, raised against a ferrocene-containing hapten).[15] At 2.5 μM of protein, both molecules showed weak nonspecific binding of Y^{3+} (<0.3 μM).

Sequencing of HM3 and HM5 revealed greater than 90% homology between the two light chains (Supporting Information). In contrast, their heavy chains differed significantly within the complementary determining regions (CDRs). With regards to potential metal-binding residues, the CDRs in both clones contained no cysteines and only one histidine in HM5 (LC CDR3) and two in HM3 (LC CDR3, HC CDR1). Aspartyl and glutamic acid residues were abundant in both clones. However, no contiguous stretches of amino acids were observed. Perhaps future crystallographic analysis will identify the metal-binding amino acids.

The results presented here demonstrate that the correct combination of a metallo-panning agent coupled with the screening of a large combinatorial antibody library can allow for the identification of antibodies that bind unique metal ions with excellent affinity. To our knowledge, this is the first time an antibody has been shown to bind metal ions such as yttrium and lanthanum. We propose that the metal is the dominant epitope, as antibodies HM3 and HM5 have comparable binding energies to **1** and the stripped cognate **2**. Provided a more complex metallo-panning agent can be designed, the incorporation of metals in an antibody combining site could augment the potential of catalytic antibodies[16] or the discovery of new biosensors.[17] Finally, because these antibodies were isolated from a human library, the technique we have described may hold promise for use in vivo as carriers of radionuclides for diagnosis and treatment of disease.[18] This is especially pertinent given the fact that both our single-chain antibody clones bind yttrium well, and ^{90}Y cytotoxic properties are of current clinical interest.[19]

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Supporting Information Available: Details of the construction of the human scFv phage display library, the phage panning, competition ELISA experiments, sequencing of HM3 and HM5, and metal-content measurement by ICP-MS. This material is available free of charge via the Internet at http://pubs.acs.org.

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