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Antibodies with Infinite Affinity: Origins and Applications

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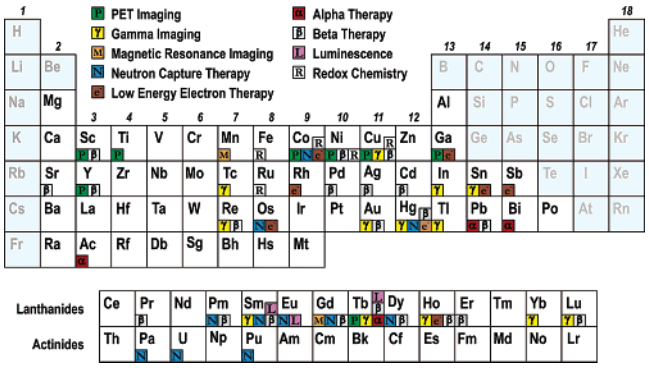
ABSTRACT

Antibodies with infinite affinity were developed with the aim of improving targeted delivery of metal complexes to sites of disease. This is part of a series of chemical technology developments for biomedical imaging and therapy. Using a combination of genetics and chemical synthesis, it addresses challenges in developing proteins that specifically bind synthetic molecules and do not release them. The result is a set of reagents that promise to capture any of a large variety of metallic elements under physiological conditions and hold them for long periods of time.

Introduction

The specific recognition of segments of biological molecules on cell surfaces or in solution plays an important role in experimental biology and medicine. This essentially chemical phenomenon involves structural complementarity between a targeting molecule, such as an antibody's binding site, and its target. The target may be a small collection of amino-acid residues or carbohydrate building blocks that are abundantly present on a particular cell type or uniquely represented on a soluble molecule. The development of specific binding molecules from peptides, polynucleotides, or related species has been raised to a very high level.[1] Antibodies (IgG,[40] MW 150 KDa) have been studied extensively; each possesses a unique binding site supported by a common protein framework. An example is the antibody Avastin, which binds to a site on the VEGF[40] protein that stimulates the formation of blood vessels.[2] Avastin is a highly engineered molecule that binds VEGF with equilibrium dissociation constant $K_D \approx 10^{-9}$ M under physiological conditions. The effectiveness of Avastin is dependent on its ability to compete effectively with the natural VEGF receptor, which binds with an even smaller dissociation constant.

Table 1. Metals That Can Form Stable Coordination Complexes with Probe Properties of Biomedical Interest Noted



In order to capture molecular probes *in vitro*, analytical biochemistry sometimes makes use of the much stronger interaction between the small molecule biotin and the protein avidin (or streptavidin), for which $K_D \approx 10^{-14}$ M.[3] Biotin has a carboxyl side chain that can be attached to fluorophores, proteins, and other interesting partners, and the conjugate can then be bound tightly to avidin. There are even some drugs in development that make use of the streptavidin−biotin binding pair for therapy. However, avidin and streptavidin are not of human origin, which may lead to immune responses that limit repeated therapeutic use. In contrast, engineered antibodies such as Avastin can be produced that are generally compatible with the human immune system. There are experimental approaches to develop antibodies with affinities approaching avidin−biotin,[4] but the applications described below may benefit from even stronger binding.

This led us to the notion that antibodies might be converted into molecules whose dissociation constants are literally zero. Such reagents might be used to trap, specifically and permanently, target molecules useful in biomedical applications. We began by trapping chelated metals inside engineered antibodies.

Metal Ions and Cancer

Metal ions and their radioisotopes have physical properties such as the emission of photons or particles that may be used in medical imaging and therapy (Table 1). A significant number of rare earth and transition metal isotopes such as ^{177}Lu and ^{90}Y decay by β-emission with energies useful for therapy.[5] Non-lanthanide elements that chelate effectively include the α-emitters ^{225}Ac and ^{213}Bi.[6,7] Three-dimensional medical imaging techniques rely on the distinctive properties of metals such as ^{99m}Tc for single-photon emission computed tomography (SPECT)[40] or ^{64}Cu for positron emission tomography (PET).[40] Emitters of medium energy γ-rays in the 100−300 keV range (^{111}In, ^{67}Cu, ^{117m}Sn, etc.) are also useful for more conventional imaging methods.

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Claude Meares was born in 1946 in Wilmington, NC. After graduating from the University of North Carolina at Chapel Hill, he obtained a Ph.D. from Stanford University. He has been a member of the Chemistry faculty at the University of California, Davis, since 1972. His research interests include the application of metal complexes to biological and biomedical problems.

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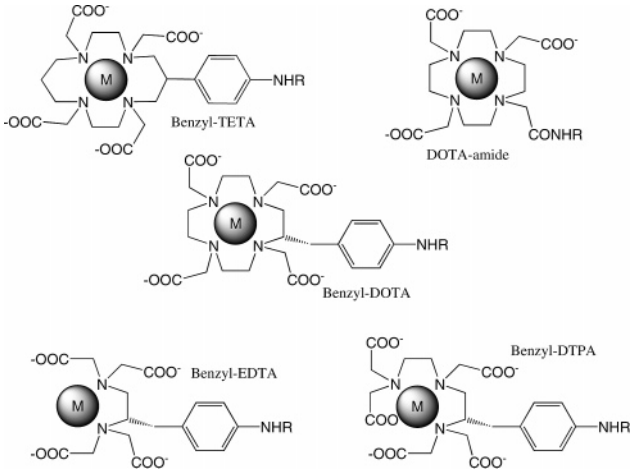
Chart 1. Bifunctional Chelating Agents



For many years, we have worked to develop bifunctional chelating agents and monoclonal antibodies for the diagnosis and therapy of cancer. Some of these chelating agents are shown in Chart 1; they have been found useful for holding a variety of metal ions, including the unusually labile copper(II), in human patients.[8−11]

Beyond our work in radiopharmaceuticals, DOTA-based[40] reagents have found uses in magnetic resonance contrast agents (ProHance, Bracco Diagnostics), element-coded affinity tags[12] for mass spectrometry, and lanthanide luminescence.[13] Likewise, DOTA-amide can be found in radiolabeled peptides such as somatostatin analogues for medical imaging and therapy. A methylated analogue of benzyl-DTPA[40] is used in the therapeutic drug Zevalin to treat non-Hodgkins lymphoma with β-radiation from ^{90}Y. The iron chelate of benzyl-EDTA[40] is an artificial protease/nuclease that maps interactions of proteins with other proteins and with nucleic acids.[14−16] The reagents in Chart 1 are a necessary first step to target metals to cancer in humans, because they carry the metal without releasing it to metal-binding biological molecules at significant rates.

Targeting Cancer with Radiation

Cancer cells are similar to normal cells in most ways, so targeting them selectively is not trivial. The most versatile tools for doing this are antibodies, which can be produced using a variety of technologies to bind molecules that are characteristic of cancer cells. Significant effort has been directed at developing new target-selective molecules with affinity and selectivity for cancer cell markers. This has been accomplished either through identifying new cancer-associated cell surface proteins for mAb[40] generation[17] or through the use of sequence optimization techniques to improve the affinity of previously isolated mAbs in engineered derivatives such as Fabs[40] or sFvs.[18,40]

Antibodies can be chemically conjugated with radionuclides for therapy (e.g., ^{131}I or ^{90}Y) or imaging (e.g., ^{111}In). Although there are several potential targeted radiotherapies in clinical trails, so far only two have been approved by the US Food and Drug Administration: Zevalin (^{90}Y, Biogen-Idec Pharmaceuticals) and Bexxar (^{131}I, Corixa). The radiolabeled antibody Zevalin binds the cell surface protein CD20 expressed on human B-cells, but as shown in Figure 1, it also accumulates in some normal tissues such as liver due to physiological clearance mechanisms.

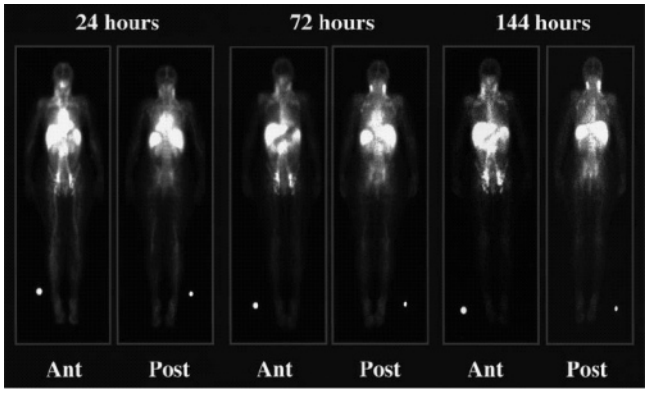


FIGURE 1. Serial whole-body gamma camera scans following ^{111}In−Zevalin injection. This antibody binds to B cells, and the image shows spleen and lymph nodes, some of which are enlarged. There is prominent, bright imaging of normal liver, as well as cancer sites. Reproduced with permission from ref 24, with the kind permission of Springer Science and Business Media. Copyright 2000 Springer-Verlag.

A fundamental limitation of radioimmunotherapy is the radiation dose to normal tissue. This nonspecific radiation results predominantly from the circulation of radiolabeled proteins that are not bound to a tumor site but can remain in the bloodstream for days and from the accumulation of the radionuclide in clearance organs such as liver and kidney. Not only do antibodies accumulate in these organs, but also metallic radionuclides can slowly dissociate from their attachment sites over the periods of several days needed for therapy and be transported there. For years, we have studied these problems and developed new approaches to solve them.

How to make targeting more effective? One might use small fragments of antibodies that permeate tissue and clear from the bloodstream quickly, but these typically have lower affinities and accumulate strongly in normal kidney.[19,20] Similar issues arise for some receptor-binding peptides such as the somatostatin analogues. We decided to investigate whether it might be possible to decouple the cancer-targeting step from the probe-binding step.

Pretargeting

With our long-term collaborator Dr. David Goodwin, we originated the concept of "pretargeting," where an antibody carries an artificial receptor to the surface of a tumor cell.[21,22] After excess antibody has cleared from the circulation and been sequestered in the cells of the liver and other clearance organs, a small probe molecule with affinity for the artificial receptor is administered. The probe molecule is chosen for efficient clearance through the kidney so that it either binds the receptor on the tumor cell surface or leaves the body within a few hours. Because of the need for high affinity, initial applications used the streptavidin−biotin system. An evolved example of this

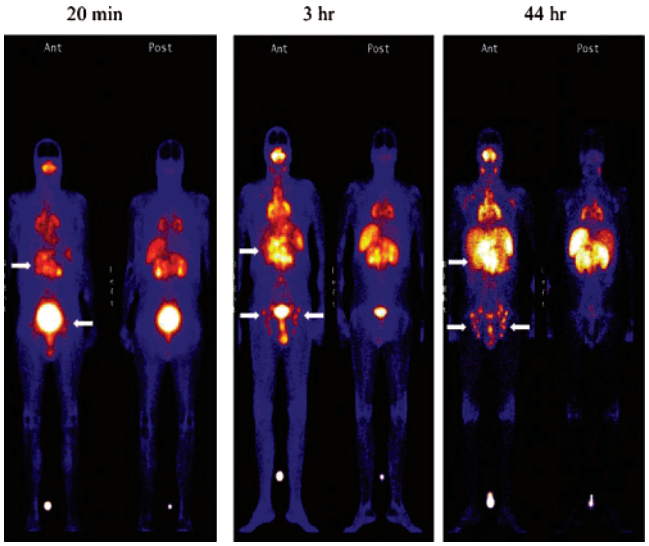


FIGURE 2. Images obtained at different times after pretargeting with B9E9—streptavidin (B9E9 sFv, anti-CD20) fusion followed by the administration of ^{111}In(5 mCi)/^{90}Y (15 mCi/m^2)—DOTA—biotin. Gamma camera whole-body scans of a patient obtained at various times after infusion of ^{111}In/^{90}Y—DOTA—biotin. Arrows show radionuclide localization to known sites of tumor involvement. Reproduced from ref 23 with permission. Copyright 2004 American Society of Hematology.

technology produced the images of a human patient in Figure 2, using an engineered streptavidin—sFv molecule that targets the same CD20 antigen as in Figure 1.[23,24] This pretargeting approach has also been extended to other targets such as tumor-associated glycoprotein 72 (TAG-72).[25] With pretargeting there is much less radiation uptake in normal organs and tissues.

Antibodies with Infinite Affinity

Pretargeting works very well under favorable circumstances; however, experience has shown that some aspects could be improved. Streptavidin is a bacterial protein, which limits repeated use in humans, biotin is an endogenous molecule, which can compete with the probe, and the binding of biotin to streptavidin is reversible, leading to slow dissociation of the probe from the target.

Therefore, there is an opportunity for a new generation of molecules for pretargeting. We conceived antibodies with infinite affinity to address this situation.[26] The surest way to prolong the lifetime of a complex is to make a stable covalent bond between its components. We hypothesized that it should be possible to change the properties of antibodies against metal chelates to prepare antibody/chelate pairs that exhibit the binding specificity of the antibody but do not dissociate. Chmura and coworkers achieved this by taking advantage of the slow dissociation of the correct ligand from the antibody's binding site, to form a covalent bond during the lifetime of the complex (Figure 3).

This is based on the principle of effective local concentration: two complementary reagents are brought into proximity in an antibody—antigen complex and subsequently form a covalent attachment. Scheme 1 compares

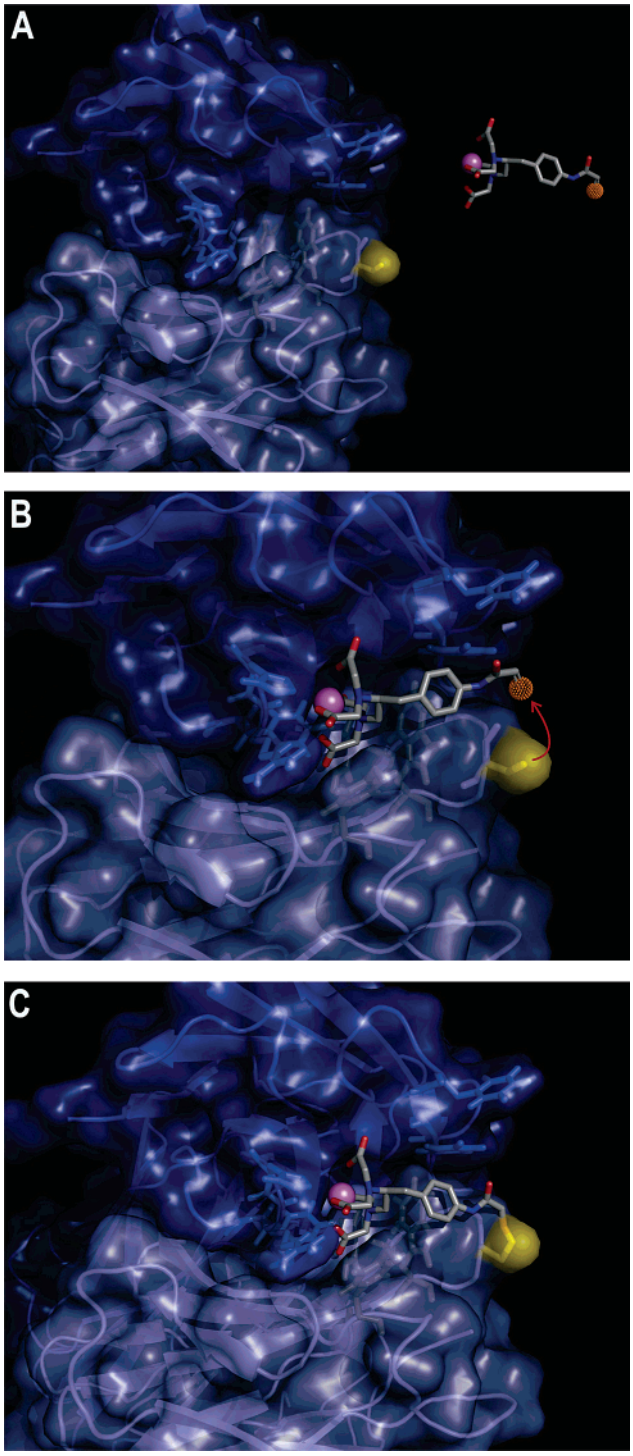


FIGURE 3. Covalent linkage of engineered Fab CHA255 and hapten In—AABE yields a complex with infinite affinity: (A) iFab and hapten associate due to noncovalent interactions of the iFab CDRs and the hapten; (B) following association, the effective concentrations of complementary reactive groups (cysteine in yellow, acryloyl reactive site in orange) are sharply elevated; (C) a covalent thioether link is formed. At this point, dissociation of the iFab and hapten cannot occur, even if the hapten were to leave the binding pocket. Figures prepared with PyMOL.[38]

a reaction between two molecules (A and B), each with one reactive group, to that of one molecule with two reactive groups. The effective local concentration of A in the presence of B may be defined as $[A]_{eff} = k_1/k_2$ mol/L,

Scheme 1

Proximity Accelerated Reaction

$$A + B \xrightarrow{k_2} A\text{-}B$$

$$A \quad B \xrightarrow{k_1} A\text{-}B$$

Scheme 2

Reversible Antibody-Ligand Interaction

$$L_{rev} + Fab \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} C_{rev}$$

$$K_A = k_{on} / k_{off}$$

Infinite Affinity Interaction

$$L_{irr} + iFab \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} C_{rev} \xrightarrow{k_{irr}} C_{irr}$$

$$If\ k_{irr} >> k_{off}\ apparent\ K_A \rightarrow \infty$$

which can be enormous ($>10^5$ M). This number is nonphysical, but it gives an idea of the magnitude of the rate acceleration available within a complex. In the context of antibody-target systems, this means we can use mild reagents (very small k_2) that nonetheless react (large k_1) when brought into close proximity by external forces (the binding of antibody to hapten).

The affinity of a typical antibody fragment (Fab) binding a target ligand (L) in a reversible manner is defined by the stability constant K_A, the quotient of the on and off rate constants. ($K_A = 1/K_D$). In an irreversible system, the off rate becomes zero and K_A approaches infinity (Scheme 2).

The successful development of this class of antibody with infinite affinity requires the simultaneous preparation and testing not only of mutant proteins but also of weakly reactive synthetic chelates. Such chelates must generally (i) form a highly stable covalent bond in the antibody's binding site under physiological conditions and (ii) not react significantly with naturally occurring biological molecules in circulation. In the cases that we have investigated, there is no suitable complementary reactive group around the antibody's binding site, so one must be placed there using site-directed mutagenesis. When engineering the protein, mutation site selection must take into account (iii) the candidate amino acid's relative distance and orientation to the reactive group of the chelate sidearm, (iv) the amino acid's direct role, if any, in chelate binding, and (v) the candidate residue's role in maintaining binding site integrity.

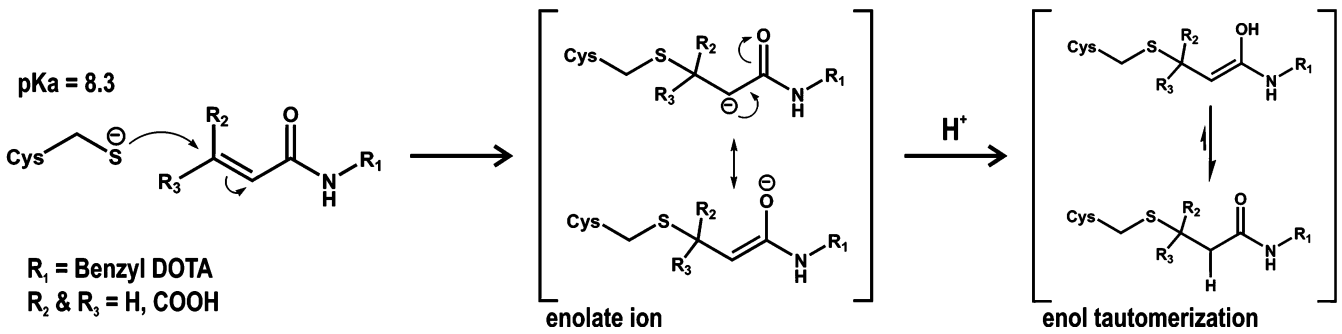
We have developed two infinite affinity antibody systems, each comprising engineered antibody fragments, reactive chelates, and their associated metal ions. The first system is based on the antibody CHA255, which has a high affinity for In−EDTA. The second system is based on the antibody 2D12.5 and the macrocyclic chelate DOTA, and it has high affinity for a wide range of metal ions. Both use the Michael addition for permanent association.

Electrophilic Chelates

The Michael addition satisfies our criteria for a reaction mechanism and practical reagents that form stable addition products under conditions of high local concentration at physiological temperature and pH (Scheme 3). Although Michael additions can be reversed in the laboratory with heat and base, physiological environments do not provide sufficient activation. We found the Michael addition useful for irreversible attachment of an acryloyl-substituted chelate to an engineered cysteine side chain.[26] Earlier work by other groups had demonstrated the reactivity, albeit low, of an acryloyl group toward Michael reaction with a natural glutamic acid residue of angiotensin-converting enzyme (ACE).[27] Additional reports have exploited cysteine residues on various receptors as targets for irreversible inhibition with a wide range of substituted vinyl reactive groups.[28−30] The combination of a cysteine side chain near the binding site and a mildly activated double bond appears to be a good choice for applications *in vivo*.

For use with antibody CHA255, Chmura and coworkers synthesized and studied the EDTA derivatives shown in Figure 4 to see which would be excreted quantitatively from animal models in a reasonable period.[31] We compared the acryloyl reagent with haloacetamide derivatives that span a range of reactivity and extensible length and rotational freedom; we found that AABE[40] was completely cleared from the body after 24 h, as was the parent amino compound ABE,[40] but not the

Scheme 3. Michael Addition of a Cysteine Side Chain to an Acryloyl Group



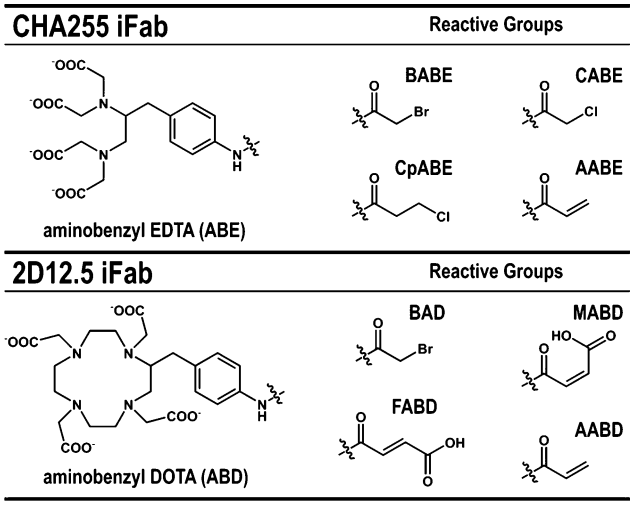


FIGURE 4. Electrophilic chelate derivatives tested for the CHA255 and 2D12.5 iFabs. The requirements for low intrinsic reactivity and high covalent bond formation efficiency were satisfied by the acryloyl compounds (AABE, AABD) and derivatives such as MABD.

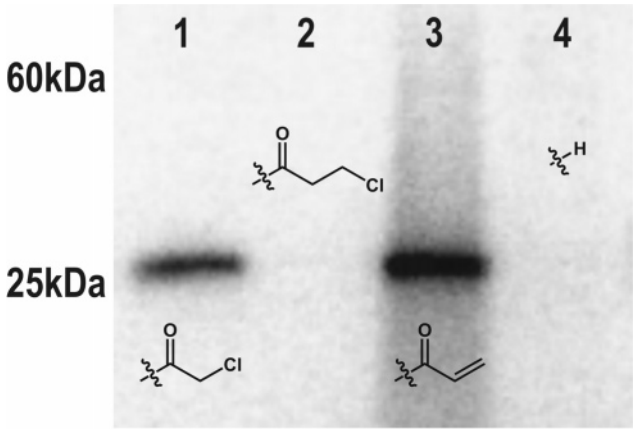


FIGURE 5. Conjugation of electrophilic ^{111}In−benzyl-EDTA derivatives (Figure 4) with engineered CHA255 iFab S95C. Phosphorimage of 10−20% SDS−PAGE gel of samples of complete culture media incubated with ^{111}In-labeled CABE (lane 1), CpABE (lane 2), AABE (lane 3), and ABE (lane 4). Reproduced with permission from ref 26. Copyright 2001 National Academy of Sciences of the U.S.A.

halogenated reagents. Alkene derivatives such as MABD and FABD[40] were investigated later as refinements for use with mAb 2D12.5 mutants.

Antibody CHA255: The First Example

Our first antibody with infinite affinity was the indium−EDTA binding murine antibody CHA255, initially developed by immunization of mice with a keyhole limpet hemocyanin−isothiocyanatobenzyl−EDTA(In^{3+}) conjugate, followed by development of a hybridoma cell line.[32] The crystal structure of the CHA255 complex was subsequently solved, providing a template for rational design of cysteine mutants that might confer infinite affinity.[33] Sequence positions 95 and 96 (Kabat positions 93 and 94) of the light chain CDR3[40] were mutated to cysteine residues in two separate constructs, producing mutants S95C and N96C. ^{111}In-labeled chelates (Figure 4) were incubated with the mutant antibodies *in vitro*; covalent adducts, which did not dissociate during denaturing gel electrophoresis, were formed between the mutant S95C and In−AABE (Figure 5, lane 3).

The desired combination of low background reactivity (rapid whole-body clearance in animals) and selective, irreversible bond formation was realized with In−AABE and the light chain S95C mutant of CHA255. These results paved the way for the irreversible DOTA-chelate capture antibody 2D12.5 and electrophilic DOTA derivatives, which greatly broadened the scope of applicable metallic elements.

2D12.5—A Multielement Antibody

We extended this concept to an antibody that binds a variety of metal ions as their DOTA complexes, discovering along the way that the mutation was needed in a very different position relative to CHA255. Antibody 2D12.5 possesses a loop in the heavy-chain CDR2 that is well located for attachment to a suitable DOTA chelate analogue.[35,36] In further contrast to antibody CHA255, which binds only indium or iron EDTA, it was determined through competitive ELISA[40] that 2D12.5 binds the entire lanthanide series (radioactive Pm was not tested) with high affinity (Figure 6A) in a pseudo-elastic manner dependent on ionic radius. Out of 15 metals tested in competitive assay, 6 were found to bind at least as well if not better than the original Y^{3+} used to raise the 2D12.5 antibody.[34] This relationship was paralleled in irreversible binding experiments that monitored the competitive radioactive labeling experiments of the 2D12.5 iFab[40] (Figure 6B,C).[35]

We also found that, in contrast to protein engineering techniques that increase affinity for a single target, infinite affinity can lead to permanent capture of probes that have only moderate affinity for the parent antibody, further broadening the number of metal−DOTA complexes that may be irreversibly captured.[39]

Capture of moderate affinity probes was demonstrated in competitive ^{90}Y irreversible labeling experiments with DOTA chelates of Y^{+3}, In^{+3}, and Cu^{+2} (Figure 7). Cold Y−AABD[40] competes with ^{90}Y−AABD very effectively for the 2D12.5 iFab binding site. Upon binding, a covalent linkage is formed, and no further chelate displacement can take place. Thus, at 5 min, cold Y−AABD has effectively saturated all sites, and therefore no ^{90}Y-AABD is able to form a covalent bond, resulting in no signal. Surprisingly, In−AABD, based on a reversible system with $K_D \approx 10^{-6}$ M, efficiently locks into the binding site in less than 20 min. Even more surprisingly, Cu−AABD, based on a system with $K_D \approx 10^{-4}$ M, irreversibly fills the sites after 2 h and prevents ^{90}Y−AABD from binding. The implications of this result are significant in that the absolute affinity of the metal−AABD species becomes unimportant for many practical applications. This promises to become a general approach for stably attaching any of the metals in Table 1 to proteins for use in living systems; however, pretargeting will probably be practical only with the stronger binders, due to the kinetic inef-

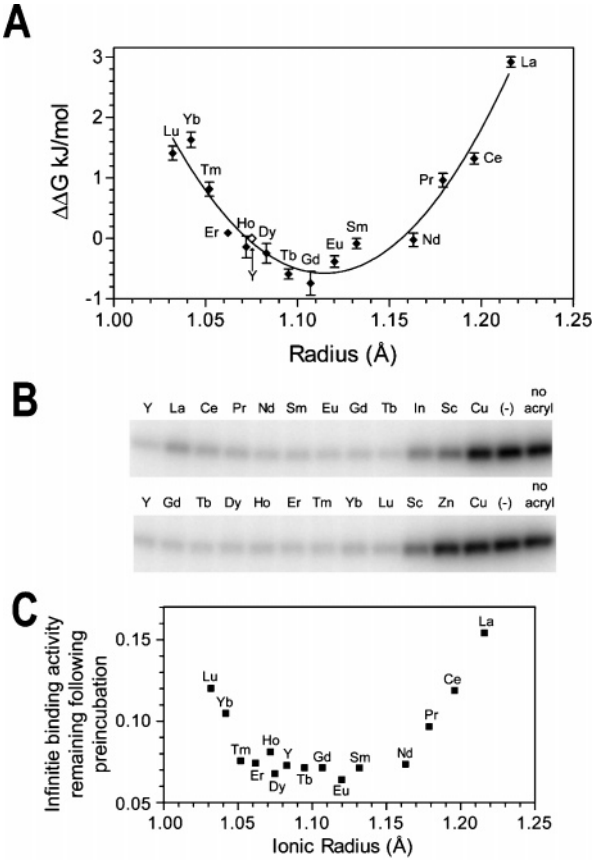


FIGURE 6. (A) Antibody 2D12.5 reversibly binds all the DOTA−lanthanides as determined by reversible competitive ELISA; (B) to test for irreversible binding, mutant 2D12.5 iFab G54C was preincubated with metal−AABD complexes followed by binding with ^{90}Y−AABD, which irreversibly attaches to free binding sites and results were measured by quantitative phosphorimaging following SDS-PAGE of 2D12.5 iFab complexes; (C) infinite binding results parallel the relationship in panel A. Reproduced with permission from refs 34 and 39. Copyright 2003 and 2004 American Chemical Society.

ficiency of binding and subsequent Michael addition relative to the rapid clearance of the metal−DOTA complexes *in vivo*.

Reactivity of Mutant Residues: Some Observations

The crystal structures of antibodies with bound haptens provide the starting points for determining candidate protein residues for mutation. However, factors such as side chain flexibility and rotational conformations may have predominant influence on the kinetics of covalent bond formation. Chemical reactivity data from the two different systems, CHA255/EDTA (pdb file 1IND) and 2D12.5/DOTA (pdb file 1NC2), each consisting of small sets of protein mutants and small libraries of reactive hapten derivatives, provide some useful insights.

The side-arm *p*-substituent of AABE sits snugly in a cleft formed by the interface of the heavy and light chains of CHA255 (Figure 8A). A limited range of motion exists for the AABE side arm, amounting to approximately 45° of rotation about the side-arm bond axis (Figure 8A, bond β). The original side-arm orientation from the crystal

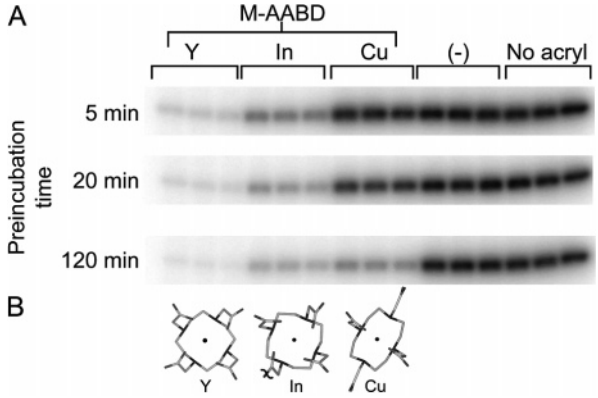


FIGURE 7. Weak binders become infinite binders. The G54C iFab was preincubated in triplicate with AABD complexes of Y^{3+}, In^{3+}, or Cu^{2+}. At the stated times, excess ^{90}Y−AABD was added to each solution to compete for free iFab: (A) quantitative phosphorimage of iFab−AABD irreversible complexes—the fainter the bands, the greater the permanent binding by the cold metal-chelate; (B) crystal structures of Y−DOTA, In−DOTA, and Cu−DOTA. Y−DOTA does not alter its structure when it binds to 2D12.5; however, the differences in chelate structure for Cu− and In−DOTA likely play a significant role in the reduced affinity observed for binding to 2D12.5. Reproduced with permission from ref 39. Copyright 2004 American Chemical Society.

structure is shown in gray (with terminal modification to model the vinyl group). An additional conformation is shown in green, corresponding to a small side-arm rotation within the binding cleft. Based on such a structural depiction, two residues from the light chain CDR3 were selected for mutation. Mutant proteins S95C and N96C were prepared, which appeared equally likely to react with the synthetic ligand. When we investigated them experimentally, we found that only the first mutant reacted with In−AABE; thus the original structure helped us choose a set of residues for mutation, but the final results depend on details that are difficult to predict.

In mAb 2D12.5, side-arm freedom is dictated by steric interaction of the side arm with the macrocycle itself (Figure 8B, gray side arm).[36,37] Some rotational freedom exists around the side-arm axis (Figure 8B, defined by aromatic carbon and *p*-substituent amide nitrogen), but rotation about axial bond α is highly constrained by clashes of the aromatic ring with metal coordinating arms.

Since the DOTA side arm is not involved in binding and the macrocycle itself possesses a four-fold rotational symmetry, DOTA can potentially bind mAb 2D12.5 in any of four orientations. In fact, two rotational isomers were observed in the two solved crystal structures (PDB files 1NC2 and 1NC4);[36] the remaining two rotational positions would involve steric clashes between side arm and protein.

In both the CHA255 and 2D12.5 iFabs, nonreactive or low-reactivity mutants (N96C for CHA255 and G55C for 2D12.5) have cysteine side chains generally oriented away from the bound ligand. In both cases, the cysteine side chain is largely shielded by the backbone carbonyl oxygen of the preceding amino acid (Figure 8A,B, red space-fills).

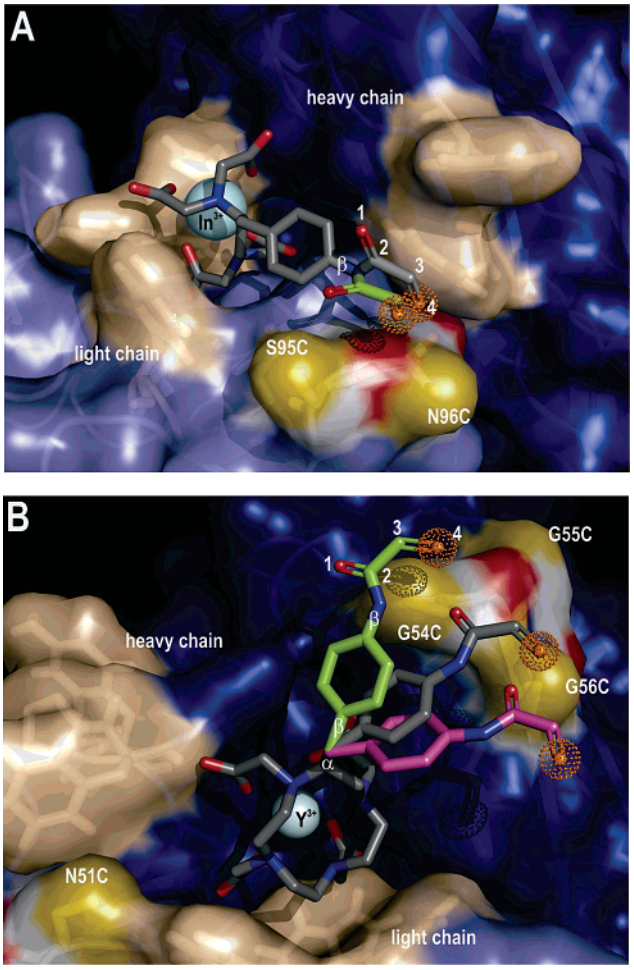


FIGURE 8. Ligand orientation and side-arm rotational freedom in CHA255 and 2D12.5 iFab complexes. Heavy and light chains are colored dark and light blue, respectively, with residues defining steric tolerances of the binding pocket colored tan. Electrophilic carbon is indicated in orange (dotted sphere). Atoms that are not manipulated relative to the original crystal structure are gray. Crystal structures are modified to depict cysteine substitutions. In panel A, the AABE side arm lies in a cleft between the CHA255 heavy and light chains. Rotation about carbon−nitrogen bond β, as well as *trans* orientation of the acryloyl unit gives modeled green side arm between the two cysteine mutant residues. The mutant S95C was reactive, while N96C was not. In panel B, side chains of 2D12.5 cysteine mutants G54C and G56C appear to be most available for reaction with Y−AABD. The G55C mutant is oriented away from the AABD and remains at significant distances from the side arm in all orientations.

Concluding Remarks

The use of antibodies that irreversibly capture small synthetic molecules is reminiscent of the use of streptavidin to capture biotinylated probes. We have recently designed and expressed fusion proteins that incorporate tumor-targeting moieties and irreversible probe capture. Their properties are now being studied *in vivo*. The next conceptual stage is to develop antibodies that bind selectively but irreversibly to natural targets such as proteins on cell surfaces. In contrast to the synthetic ligands above, we cannot easily modify the specific protein targets on cancer cells in human beings, so we must engineer the antibody to carry all the chemistry while maintaining its binding characteristics. The future may bring a new class of macromolecular affinity labeling reagents that retain the specificity of antibodies but undergo permanent attachment to their targets.

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(40) Abbreviations: AABE, (*S*)-1-(4-acrylamidobenzyl)-EDTA; ABD, (*S*)-2-(4-aminobenzyl)-DOTA; ABE, (*S*)-1-(4-aminobenzyl)-EDTA; CABE, (*S*)-1-(4-chloroacetamidobenzyl)-EDTA; CpABE, (*S*)-1-(4-chloropropionamidobenzyl)-EDTA; AABD, (*S*)-2-(4-acrylamidobenzyl)-DOTA; ABD, (*S*)-2-(4-aminobenzyl)-DOTA; BAD, (*S*)-2-(4-bromoacetamidobenzyl)-DOTA; CDR, complementarity determining region, one of six peptide loops that comprise an antibody's binding site; DOTA, 1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid; DTPA, diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; Fab, antibody fragment containing antigen-binding site and first constant domains, MW 50 kDa; FABD, (*S*)-2-(4-(*trans*-3-carboxy)-acrylamidobenzyl)-DOTA; iFab, Fab antibody fragment with infinite affinity; IgG, immunoglobulin G, a naturally occurring antibody molecule; mAb, monoclonal antibody; MABD, (*S*)-2-(4-(*cis*-3-carboxy)-acrylamidobenzyl)-DOTA; NBD, (*S*)-2-(4-nitrobenzyl)-DOTA; PDB, The RCSB Protein Data Bank; PET, positron emission tomography; RIT, radioimmunotherapy; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; sFv, single-chain antibody fragment; SPECT, single-photon emission computed tomography; VEGF, vascular endothelial growth factor.

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