COMMUNICATION

Selection of optical biosensors from chemisynthetic antibody libraries

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We describe a method for creating antibodies with a fluorescent reporter integrated into the antigen-binding site. A reporter molecule was chemically linked to a hypervariable loop of an antibody repertoire displayed on phage, and this repertoire was selected for antigen binding. In one selected antibody, the fluorescence of the probe responded quantitatively to antigen binding. The method may have application for the engineering of homogeneous immunoassays.

Keywords: antibody repertoire/chemisynthetic/optical biosensor

Introduction

The ability of antibodies to bind strongly and specifically to antigens has led to the development of a wide range of immunoassays. In most immunoassays, the complex between antibody and antigen is detected and distinguished from free antigen and antibody in several steps, including sequential incubations of reagents and/or separation (by washing) steps. These can be time and labor intensive for immunodiagnostic services or too complex for consumers. Several homogeneous assays, such as EMIT, EMMIA, ARIS, CEDIA and others (for review, see Jenkins, 1992; Portsmann and Kiessig, 1992) avoid the use of wash steps, but are all based on competition of labelled antigen and analyte and usually require sequential additions and multiple incubations.

In principle, optical biosensors could provide direct and single-step immunoassays. For example, fluorophores have been built into proteins by covalent chemical attachment at cysteine residues close to binding sites. Changes in the environment of the fluorophore, such as occur on binding of a ligand, lead to quantitative changes in fluorescence (for review, see Hellinga and Marvin, 1998). The main challenge of optical biosensors has been the incorporation of reporter groups into binding sites close enough to the ligand to report a signal, but not so close as to compromise its binding. One strategy for antibody-based optical biosensors involved two steps (Pollack et al., 1988): first, the selective introduction of a nucleophilic thiol group at the periphery of hapten-binding site via an affinity label equipped with a cleavable thiol ester cross-link. Next, the peripheral thiol was used for incorporation of a fluorescent group in the antibody. This strategy is applicable to the functional modification of proteins in the absence of detailed structural information, but requires the synthesis of cleavable-affinity labels. Another approach exploiting antibodies involved the identification by mutagenesis of those amino acid residues bearing no functional importance for antigen-binding but adjacent to critical ones. Targeting these residues for reporter coupling proved successful in converting antibodies into reagentless fluorescent immunosensors (Renard et al., 2002, 2003). Our strategy for antibody-based optical biosensors differs from these two approaches: we have built the antigen-binding site around the reporter group so that it is fully integrated and may even engage in key contacts with the antigen. This was achieved by introducing a free cysteine into a hypervariable loop (CDR) of a synthetic antibody repertoire displayed on phage, linking this chemically to a fluorescent reporter group and selecting the phage repertoire for binding to the target antigen.

Materials and methods

Phage antibody library comprising Cys93 light chain

The synthetic Vh genes from the CDR3-5 and CDR3-8 phage antibody libraries (each comprising 107 clones; Hoogenboom and Winter, 1992) were excised by NcoI and NotI digestion, combined and ligated into the corresponding sites of pHEN-Vl3-Cys93, a derivative of pHEN-Vl3 (Hoogenboom and Winter, 1992) wherein Ser93 of Vl3 was mutated to Cys. The DNA was electroporated into Escherichia coli TG1 cells and plated on LB-agar plates supplemented with 2% (w/v) glucose and 100 μg/ml ampicillin (LBGA), giving a library of 2.6 × 107 clones (≥83% inserts). Phage particles were produced and purified by polyethylene-glycol (PEG) precipitation as described previously (Marks et al., 1991).

Chemical cross-linking of reporter group to phage antibody library and selection

Approximately 5 × 1012 phage particles were reacted with 1 mM dithiothreitol (DTT) for 30 min at room temperature, then PEG precipitated. After rinsing with water, the pellet was resuspended in 1 ml of reaction buffer (10 mM phosphate buffer, 1 mM EDTA, pH 7.8) and reacted with 50 μl of 1.6 mM N-[2-iodoacetoxy]ethyl]-N-methylamino-7-nitrobenz-2-oxa-1,3-diazole (NBDIA) (Molecular Probes) for 2 h at room temperature. The reaction was terminated by PEG precipitation of phage particles. Immunoaffibates (Nunc) were coated overnight with phOx conjugated to bovine serum albumin (100 μg/ml; 43 phOx molecules per BSA) (Mäkelä et al., 1978) in phosphate-buffered saline (PBS) and blocked for 1 h with PBS containing 2% (w/v) skimmed milk powder (PBSM). The reacted phage library was diluted in 4 ml of PBSM and incubated for 2 h in the immunoaffibate. After washing 15 times with PBS containing 0.1% Tween-20 (PBST), bound phage were eluted in 1 ml of 0.1 M triethylamine, neutralized.
with 0.5 ml of 1 M Tris, pH 7.4, and used to infect log-phase TG1 cells before plating on LBAG plates. After three such selection rounds, individual clones were analysed by phase ELISA for binding to phOx–BSA-coated microtiter wells. After washing with PBST, bound phage were detected using anti-M13 horseradish peroxidase conjugate (Amersham Biosciences) and o-phenylenediamine as substrate. Next, several positive phagemid clones were also transferred into non-suppressor E.coli HB2151 cells. Soluble expression of scFvs in bacterial supernatants (2 l scale) and purification on protein A-Sepharose were performed as described (Hoogenboom and Winter, 1992). Chemical modification with 1A-NBD (or other reagents) was performed on 0.5 ml of purified scFv (6.6 mM) in reaction buffer. A 5-fold molar excess of DTT was added and left to react for 1 h at room temperature. For affinity maturation of clone S34, the light-chain CDR1 and CDR3 were targeted for spliced mutagenesis: a library of V\text{\textsubscript{L}}3 genes (each encoding three amino acid mutations on average) was constructed by spliced-overlap-extension PCR (Horton et al, 1989), and ligated to the S34 synthetic VH gene on pHEN1 (Hoogenboom and Winter, 1992). The resulting library (7.9 × 10\textsuperscript{7} clones) was reacted with phOx–BSA and selected for binding to phOx–BSA as above.

Production of soluble labelled S348 scFv

The S348 gene was subcloned into pAB1 for periplasmic expression of the gene, with C-terminal c-myc and hexahistidine tags (Kontermann et al., 1997). Expression and purification were performed as described (Hoogenboom and Winter, 1992). For labelling, a 20 mM solution of single-chain Fv (scFv) in reaction buffer was first reacted with 2 mM DTT for 90 min at room temperature. Excess DTT was removed by gel filtration on a PD-10 column (Amersham Biosciences) and the scFv was incubated with a 30-fold molar excess of NBDIA for 2 h at room temperature. After reaction, the sample was diluted with 50 mM phosphate buffer, pH 7.4, (10 ml) containing 20 mM imidazole and 0.5 M NaCl and loaded on to a Hi-Trap Chelating column (Amersham Biosciences). Elution was performed with 200 mM imidazole and traces of dimeric scFv were removed by a final gel filtration on a Superdex-75 column (Amersham Biosciences). The extent of labelling was evaluated by determining the remaining free thiol concentration with Ellman’s reagent (Sigma-Aldrich) and by fluorescence analysis after SDS–PAGE.

Biophysical analysis

A 1 mg amount of phOx–BSA was biotinylated with the Ez-link NHS-SS-biotin reagent (Perbio) according to the manufacturer’s instructions, and immobilized at low density (≈800 resonance units) on a streptavidin-coated sensor chip in the BLAcore instrument. Sensograms were recorded upon injection of 100 μl of labelled S348 (0.1–1.0 μM in HBS-EP buffer, BLAcore) at 80 μl/min at 20°C and analysed using the 1:1 bimolecular equation for curve fitting. Conversely, an inverted assay was performed using biotinylated labelled S348 as ligand and phOx–BSA as analyte. Fluorescence measurements were obtained in triplicate on an F4500 fluorimeter (Hitachi) at 20°C with excitation and emission slit widths set to 5 and 10 nm at the wavelengths λ\textsubscript{ex} = 480 nm and λ\textsubscript{em} = 545 nm. The labelled S348 concentration was 0.25 μM in PBS, and 4 μl samples of concentrated phOx–BSA were added for titration up to 1.2 μM. After equilibration for 2 min, the data were recorded and corrected by subtraction of the signals recorded for buffer blank and for phOx–BSA alone at each concentration. The protein concentrations of phOx–BSA and labelled S348 were determined according to Mäkelä et al. (1978) and Renard et al. (2002). The fluorescence data were fitted to the following quadratic equation (Foote and Winter, 1992) to obtain the equilibrium constant for complex dissociation, K\textsubscript{d}:

\[
F = F_0(A_0) + f^2(-(L_0 - A_0 + K_d) - [(L_0 - A_0 + K_d)^2 + 4K_dA_0]^{1/2})/2
\]

Fluorescence data were also recorded after mixing labelled S348 (0.25 μM) with potassium iodide (KI, with concentrations ranging from 50 to 550 mM) in the presence or absence of 1.0 μM phOx–BSA. After correction for the dilution factor, the data were fitted to the Stern–Volmer equation to obtain the dynamic quenching constants, K\textsubscript{Q} (Lehrer and Leavis, 1978):

\[
F_0/F = 1 + K_0[\Gamma]
\]

Results and discussion

Building an antibody library comprising a single Cys93 light chain

Both CDR3s of antibody heavy and light chains lie at the centre of the antigen-binding site, but as the heavy chain CDR3 is the most diverse in sequence and length, we chose the light-chain CDR3 for incorporation of a thiol-specific reporter group, NBDIA. The Ser93 residue of a rearranged V\textsubscript{\text{h}} domain derived from the germline IGLV3S V\textsubscript{\text{h}} segment (Frippiat et al., 1990) was targeted for mutation into a cysteine. As shown by homology modelling to the KOL Fab (PDB entry: 1KOL; Marquart et al., 1980), the CDR3 loop of this variable light-chain domain extends the β-sheet framework with residues 92–95 forming a closing four-residue turn (Figure 1). Of these, Ser93 is the most solvent accessible and on mutation to Cys93 would be expected to provide an exposed nucleophilic thiol group at neutral pH, appropriate for specific chemical modification. The resulting Cys93-V\textsubscript{\text{h}} segment was then cloned for phage display with two libraries of rearranged human V\textsubscript{\text{h}} genes carrying a synthetic CDR3 of five or eight residues (Hoogenboom and Winter, 1992).

Phage selection on phOx–BSA

The synthetic phage antibody library comprising the single Cys93 light chain and diversity of human heavy chains was either selected directly for binding to phOx–BSA (Library I) or first coupled to the NBDIA reporter (Library II). After four rounds of selection (and prior chemical coupling at each round for Library II), the titer of eluted phage increased by ≈100-fold for both libraries. Sixteen randomly picked clones from each library were further analysed for binding to phOx–BSA before and after labelling with NBDIA and fluorescein–iodoacetamide (FIA). From Library I, the binding was either tolerant to coupling of either fluorophore (eight out of 16 clones) or inhibited by it (six out of 16 clones) (Figure 2). A different pattern emerged with Library II: in addition to tolerant clones...
(eight out of 16 clones), the binding of most of the other clones (seven out of 16 clones) was enhanced by 1.2–6.5-fold when coupled to NBDIA (but not to FIA) (Figure 2). This suggested that the NBDIA moiety had become an integral part of the antigen-binding site.

**Characterization of selected clones**

Individual clones with different patterns of binding by phage ELISA were selected for DNA sequencing (Table I). The V_H-gene segments and V_H-CDR3 sequences of the tolerant clones, S14, S20 and S30, are similar to αOx-18, αOx-1 and αOx-25 respectively, selected in the earlier study (Hoogenboom and Winter, 1992). By contrast, clone S34, which on coupling to NBDIA showed the greatest enhancement of binding (+650%), contains a short CDR3 sequence (Gly–Leu–Leu–Ser–Thr) fused to the segment V_H3-DP-53. Four clones (S14, S30, S32 and S34) were expressed and purified as soluble monomeric protein, and the binding patterns of the phage ELISA confirmed (Table I). The specificity of binding of S34 protein (with or without NBDIA moiety) to four different antigens was also determined by ELISA (Figure 3). NBDIA-labelled S34 did not bind to BSA or to any of the three phOx conjugates tested, suggesting that this antibody recognizes an epitope comprising both phOx and BSA. A preliminary account of some of this work has been reported (Bonnert, 1993; Jespers et al., 1995).

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**Fig. 1.** Ribbon representation of the Fv fragment of the KOL Fab (PDB entry: 1KOL; Marquart et al., 1980) using GRASP software (Nicholls et al., 1991). The molecule is shown from the antigen point of view with the variable light-chain domain (V_L) at the right and the variable heavy-chain domain (V_H) at the left. The third hypervariable loops of the V_L- and V_H-domains are colored in blue and red, respectively. The position at which an invariant cysteine has been engineered for chemical modification (V_L-Cys93) is highlighted in yellow. Amino acid positions highlighted in magenta, V_L-Ser33 and V_L-Ser92, were mutated to Asn and Arg, respectively, upon affinity maturation of scFv S34 labelled with NBDIA.

**Fig. 2.** Phage ELISA of selected clones to phOx–BSA. (A) Phage from 16 randomly picked clones after selection with untreated scFv library were either labelled with NBDIA (black bars), with FIA (shaded bars) or used as such (open bars) before incubation [~10^{10} transducing units (TU) per well] in phOx–BSA-coated microtiter plates (100 μg/ml). (B) Same as (A) but using 16 randomly picked clones after selection with NBDIA-labelled scFv library.
Table I. Sequences and modification tolerance of selected chemisynthetic clones

<table>
<thead>
<tr>
<th>Clone</th>
<th>Chemical modification</th>
<th>V\textsubscript{H}-CDR3 sequence</th>
<th>Germline V\textsubscript{H}-gene</th>
<th>Modification enhancement by NBDIA*</th>
<th>Phage scFv</th>
<th>Soluble scFv</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>a</td>
<td>RLPHT</td>
<td>V\textsubscript{H}-3-DP46</td>
<td>+/C0</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>8</td>
<td>b</td>
<td>AESRIFDY</td>
<td>V\textsubscript{H}-3-nd</td>
<td>−20</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>10</td>
<td>c</td>
<td>SIFPPFDY</td>
<td>V\textsubscript{H}-3-nd</td>
<td>−40</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>14</td>
<td>d</td>
<td>LSGVRFDY</td>
<td>V\textsubscript{H}-3-DP45</td>
<td>−45</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>19</td>
<td>e</td>
<td>RSGIRFDY</td>
<td>V\textsubscript{H}-3-DP45</td>
<td>+310</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>20</td>
<td>f</td>
<td>SMGAKFDY</td>
<td>V\textsubscript{H}-4-DP67</td>
<td>−10</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>30</td>
<td>g</td>
<td>TRADRFDY</td>
<td>V\textsubscript{H}-3-DP45</td>
<td>0</td>
<td>+10</td>
<td>+10</td>
</tr>
<tr>
<td>32</td>
<td>h</td>
<td>RRGLTFDY</td>
<td>V\textsubscript{H}-3-DP45</td>
<td>+120</td>
<td>+190</td>
<td>+190</td>
</tr>
<tr>
<td>34</td>
<td>i</td>
<td>GLLST</td>
<td>V\textsubscript{H}-3-DP53</td>
<td>+650</td>
<td>+1080</td>
<td>+1080</td>
</tr>
</tbody>
</table>

*Clone number as shown in Figure 2.

*aNo chemical modification prior to phage selection.

bDeduced germ-line V\textsubscript{H}-gene according to Tomlinson et al. (1992).

cCalculated from ELISA data on phOx–BSA-coated wells (in %): OD\textsubscript{490 nm} of labelled species/OD\textsubscript{490 nm} of unlabelled species. Normalization for labelled and unlabelled species by transducing units for the phage scFv and by OD\textsubscript{280 nm} for soluble scFv.

dCalculated from ELISA data on phOx–BSA-coated wells (in %): OD\textsubscript{490 nm} of labelled species/OD\textsubscript{490 nm} of unlabelled species. Normalization for labelled and unlabelled species by transducing units for the phage scFv and by OD\textsubscript{280 nm} for soluble scFv.

eIdentical with clone 33 in Figure 2.

fIdentical with clone 29 in Figure 2.

Characterization of S348

We attempted to measure the affinity of IANBD-labelled S348 for phOx–BSA using surface plasmon resonance (SPR) in the BIAcore but the signal was close to background, indicating a poor affinity. We therefore sought to increase the binding affinity by random mutation in CDR1 and CDR3 of the light chain and further rounds of chemisynthetic selection on immobilized phOx–BSA. After four rounds of selection, clones with stronger binding signals by phage ELISA emerged and also a consensus sequence pattern. We noted that residue Ser33 in CDR1 was preferentially mutated to Asn (six out of nine clones) and Ser92 to Arg mutation in CDR3 was frequently observed (eight out of nine clones). A clone (S348) carrying these two mutations was chosen for further study and expressed as soluble protein. As with the parent S34, binding was strongly dependent on coupling with NBDIA and diminished (>100-fold down) on coupling with FIA or iodoacetamide.

Moreover, concentrations up to 40 μM of BSA alone or phOx–thyroglobulin were unable to compete with binding of NBDIA-labelled S348 to immobilized phOx–BSA by ELISA (in comparison, an IC\textsubscript{50} of 20 nM was measured using soluble phOx–BSA). Furthermore, when the Cys93 residue was replaced by Ser in S348, binding activity to phOx–BSA was not restored upon incubation of the mutated scFv with NBDIA, thereby demonstrating that the fluorescent probe must be covalently attached to residue 93 to create a competent phOx–BSA-binding pocket. The apparent dissociation constant (K\textsubscript{d}) of the phOx–BSA:NBDIA–S348 complex was measured by SPR in the BIAcore at 20°C (data not shown). The affinities were the same irrespective of whether the antigen or antibody was immobilized on the Sensorchip (9 ± 2 and 10 ± 3 nM respectively).

Homogeneous assay with S348

We attempted to establish that the probe was able to report on binding of antigen. Since NBDIA is sensitive to change in the polarity of its microenvironment, the fluorescence of NBDIA-labelled S348 was followed by titration with increasing amounts of phOx–BSA (40 nM to 1.2 μM). As shown in Figure 4, the NBDIA fluorescence decreased by ~50% upon titration of phOx–BSA into a 0.25 μM solution of NBDIA–S348 at 20°C. By fitting the data of three independent titrations to the quadratic Equation 1 (see Materials and methods), we calculated a K\textsubscript{D} of 13 ± 4 nM (mean ± SEM), and this is consistent with the data derived from the BIAcore. Hence binding of phOx–BSA led to quenching of fluorescence. This contrasts with early studies of hydrophobic sites in ribosomes where the fluorescence of the NBD reporter was enhanced upon binding (Kenner and Aboderin, 1971; Huang and Cantor, 1975). Later, it was observed that not only is NBD a polarity probe also but that its spectral properties are sensitive to specific binding interactions such as the formation of a charge-transfer complex and hydrogen bonds (Lancet and Pecht, 1977). Thus upon changes in its microenvironment,
the NBD fluorescence can be either enhanced or quenched (Gilardi et al., 1994; Marvin et al., 1997; Marvin and Hellinga, 1998; Sloan and Hellinga, 1998; Renard et al., 2002, 2003). Presumably the use of different microenvironments (by using fluorophores and coupling to different locations on the antibody surface) would lead to different responses on antigen binding. The molecular environment around the NDBIA fluorophore was further characterized by determining the effect of iodide on the NDBIA–S348 fluorescence in the presence and absence of a saturating concentration of pHx–BSA (1.0 μM) (data not shown). Iodide selectively quenches solvent-exposed fluorophores according to the concentration-dependent Stern–Volmer Equation 2 (see Materials and methods). The quenching constants (KQ) for free and bound NDBIA–S348 were 4.2 and 2.1 M−1, respectively, indicating the partial burial of the fluorophore within the interface of the complex.

Conclusions

These results establish that we can create an antibody with good binding affinity and with a fluorescent probe fully integrated into the antigen-binding site. The probe was partially buried in the complex with antigen, and responded to antigen binding with changes in fluorescence. The methodology for making the chemisynthetic antibodies is very general and highly flexible. For example, it would have been possible to sample other exposed locations on the antigen-binding surface. It should also be possible to use a range of reporter groups as well as a wide range of ligand and antigens. Here we targeted the reporter molecule to the antibody fragment displayed on phage, but it would also have been possible to undertake the chemical coupling step on the free light chain and then to combine this in vitro with heavy chains displayed on phage (Figini et al., 1994). This would have helped to avoid the requirement for repeated chemical couplings, and also to avoid any complications due to chemical reactivity of the phage proteins, as reported by others (Soumilion et al., 1994; Pedersen et al., 1998; Jestin et al., 1999). Furthermore, although we focused on the characterization of a chemisynthetic antibody in which the probe contributes directly to antigen binding, the method was also found to elicit antibodies tolerant to coupling to fluorophores. As the fluorescence is expected to depend on the micro-environment of the probe, we cannot exclude the possibility that such antibodies are also endowed with biosensing properties, as demonstrated by others (Renard et al., 2002, 2003).

Chemisynthetic antibodies appear to offer a means towards direct single-step homogeneous immunoassays in solution. Presumably chemisynthetic antibodies would also be suitable as reagentless optical biosensors if immobilized on a surface. Indeed, the simplicity of optical biosensors based on chemisynthetic antibodies may offer an alternative to antibody biosensors based on tethered membranes and ion-channel switches (Cornell et al., 1997). The design may be particularly appropriate for high-throughput screening, including antibody arrays (de Wildt et al., 2000) in which binding of antigen could be detected directly by changes in fluorescence. It is also possible to envisage uses beyond fluorescence reporting; for example, the full integration of chemical synthetic moieties into antigen-binding sites may help to endow antibodies with nucleophilic, electrophilic or metal-binding properties or natural cofactors involved in electron-transfer mechanisms.

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References


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