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Site-Specific Coupling and Sterically Controlled Formation of Multimeric Antibody Fab Fragments with Unnatural Amino Acids

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Abstract

Immunoconjugates and multispecific antibodies are rapidly emerging as highly potent experimental therapeutics against cancer. We have developed a method to incorporate an unnatural amino acid, *p*-acetylphenylalanine (pAcPhe) into an antibody antigen binding fragment (Fab) targeting HER2 (human epidermal growth factor receptor 2), allowing site-specific labeling without disrupting antigen binding. Expression levels of the pAcPhe-containing proteins were comparable to that of wild-type protein in shake-flask and fermentation preparations. The pAcPhe–Fabs were labeled by reaction with hydroxylamine dye and biotin species to produce well–defined, singly conjugated Fabs. We then coupled a hydroxylamine biotin to the pAcPhe–Fab and demonstrated controlled assembly of Fabs in the presence of the tetrameric biotin-binding protein, NeutrAvidin. The position of Fab biotinylation dictates the geometry of multimer assembly, producing unique multimeric Fab structures. These assembled Fab multimers differentially attenuate Her2 phosphorylation in breast cancer cells that overexpress the Her2 receptor. Thus, an encoded unnatural amino acid produces a chemical “handle” by which immunoconjugates and multimers can be engineered.

Keywords

nonnatural amino acid; immunoconjugate; Fab multimer; directed protein assembly; controlled multimer; geometry

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Introduction

Recombinant antibodies are now a successful class of therapeutics, with over 20 products achieving regulatory approval. Immunoconjugates and bispecific antibodies have shown efficacy in many therapeutic animal systems, and two are approved for clinical use. Gemtuzumab ozogamicin (Mylotarg; Wyeth/Pfizer) is approved for acute myeloid leukemia and is composed of an anti-CD33 antibody conjugated to the toxin calicheamycin. Denileukin diftitox (Ontak; Eisai) is a diptheria toxin–IL-2 fusion protein approved for cutaneous T-cell lymphoma. A herceptin–maytansinoid drug has shown impressive clinical results,[1] as has an anti-CD19–anti-CD3 bispecific construct.[2] Thus, the ability to make improved antibody conjugates is important in biotechnology and medicine.

Small-molecule cross-linking to a protein is achieved through existing amino acid side chains, usually through coupling to lysine (amine) or cysteine (sulfhydryl) residues. Since most proteins contain several lysine or cysteine residues, heterogeneous coupling often occurs, resulting in labeling in unfavorable locations on the protein (i.e., active site, dimerization domain, or antigen binding site), which may affect the efficacy of the product. Furthermore, such nonhomogeneous mixtures of product are difficult, if not impossible, to biochemically characterize and could produce regulatory challenges.[3,4]

For bispecific proteins, the two proteins are typically produced genetically as a fusion protein. Thus, only two possible orientations are possible: A–B or B–A. Genetic fusions are two-dimensional in nature; a gene can only be fused to a second gene at either its 5′ or its 3′ end. Proteins, however, are three-dimensional, with several surface epitopes and/or active sites. Therefore, it would be advantageous to control the orientation of linkage of two proteins beyond simply N- or C-terminal fusions. If linkage could be accomplished through surface-exposed amino acid side chains, favorable steric orientations of the two proteins may be achieved, resulting in higher activity or stability. To accomplish surface linkage of two proteins, two important parameters are required: (i) a linkage “handle” that is specific to a single amino acid on each protein and not all other surface residues and (ii) an amino-acid-specific linkage chemistry that does not disrupt general protein structure and folding.

The ability to encode unnatural amino acids (UAAs) allows for a handle, providing a substrate for amino-acid-specific chemistry allowing for immunoconjugation. UAAs can be incorporated into recombinant proteins by supplying them in the media of cells containing engineered tRNA synthetases capable of charging an orthogonal tRNA, which then adds the UAA to the growing polypeptide chain. A UAA is encoded into a protein at an amber codon (TAG) in *Escherichia coli* through the use of an evolved *Methanococcus jannaschii* tyrosyl tRNA synthetase that charges a cognate *M. jannaschii* tRNA with the UAA.[5–8] Incorporating a cross-link-capable amino acid such as *p*-acetylphenylalanine (pAcPhe) by *in vivo* expression generates ready-to-react proteins without the need for further modification. Using this technology, one can site-specifically label an antibody, allowing highly controlled protein cross-linking or labeling.

Here, we demonstrate the incorporation of pAcPhe into the antigen binding fragment (Fab) of trastuzumab (Herceptin; Genentech/Roche), which binds specifically to the Her2

oncoprotein that is overexpressed in 25% of breast cancers. We validate the efficient expression and incorporation of the UAA at multiple constant region positions, analyze the coupling of that UAA on antibody function, and then utilize the UAA to multimerize Her2 and find that steric constraints imposed by UAA position affect the function of the Fab multimer on signal transduction of Her2+ cells. Thus, it is possible to create uniquely functional multimeric proteins through site-specific coupling mediated by UAAs.

Results

Design and preparation of pAcPhe-containing Fab

The amino acid sequence of the trastuzumab crystal structure[9] was used to generate the Fab V_H and V_L (variable region of immunoglobulin heavy and light chain, respectively) coding sequence, which were inserted into a vector containing the human gamma1 and kappa constant regions. A signal peptide (stII), appended to both the heavy and the light chains, directs secretion into the oxidizing periplasm for proper folding/disulfide formation. An amber codon (TAG) was engineered at one of several permissive sites in the light-chain constant region (shown in Fig. 1) in the anti-Her2 Fab. These TAG codons encoded pAcPhe at these sites with the evolved *M. jannaschii* pAcPhe tyrRS/tRNA machinery.[10,11] The five mutation sites selected for UAA incorporation were on solvent-exposed loops in disparate areas of the surface of the light-chain constant region (Fig. 1a), providing five different geometries for chemical coupling. The expression plasmid (pBAD-aHer2; Supplementary Fig. 1) was cotransformed with an evolved pAcPhe synthetase plasmid (pSup-pAcPhe)[7] into TOP10F′ *E. coli* for protein expression. After arabinose induction, overnight shake-flask expression, periplasmic lysis, and protein G purification, the Fab protein yield was 2 mg per liter of culture. UAA incorporation was confirmed by mass analysis (Table 1 and Supplementary Fig. 2). Surprisingly, wild-type and UAA-containing aHer2 expressed at similar levels (Table 1), indicating that amber suppression efficiency does not limit the protein yield under these conditions. High cell density fermentation was also used for aHer2 Fab expression. For these expressions, a plasmid encoding Fab (p4xH-aHer2; see Supplementary Fig. 1) and pSup-pAcPhe was cotransformed into 25F2 *E. coli* for fermentation in a 2-L reactor vessel.[12] Lysis and purification proceeded as in the shake-flask experiments, yielding 50–80 mg in each 2-L reaction, also independent of the presence of an encoded UAA.

Chemical coupling to pAcPhe-containing Fab

The incorporated UAA acts as a reactive chemical handle enabling site-specific direct covalent coupling to the Fab. The ketone reacts with hydrazines or aminooxy groups to produce a hydrazone or oxime adduct, respectively. These reactions occur slowly at physiological pH, but under mildly acidic conditions or in the presence of a soluble aniline catalyst, the rate and coupling efficiency can be increased.[13] We tested coupling to the pAcPhe-aHer2, using commercially available aminooxy and hydrazine dyes, through a variety of buffered pHs. Dye coupling was monitored by SDS-PAGE (Fig. 2b) and quantified by absorption spectroscopy as previously described.[14] Hydrazine–Alexa Fluor 488 coupling to the ketone–UAA was very inefficient (data not shown), consistent with previous attempts at protein hydrazide formation.[15] Conversely, aminooxy-Alexa Fluor 488

reacted efficiently with the pAcPhe-aHer2 at pH 4.5 (Table 1 and Supplementary Fig. 3), with less than 1% nonspecific labeling of the wild-type aHer2. Notably, the absolute coupling efficiency to pAcPhe-aHer2 was mutant specific, suggesting that the local protein or peptide context affects UAA reactivity. The UAA incorporation sites in this study were chosen for geometric position rather than local chemical context, and more studies on this local structure/reactivity effect are required.

After the optimal coupling conditions with dye reactions were established, pAcPhe-aHer2 was site-specifically biotinylated with aminooxy-biotin. Wild-type aHer2 Fab and three pAcPhe mutants (S156pAcPhe, K169pAcPhe, and S202pAcPhe) were coupled to aminooxy-biotin and analyzed by mass spectrometry (MS) (Table 1 and Supplementary Fig. 4) and streptavidin blot (Supplementary Fig. 6). Similar to the Fab–dye coupling reactions, only pAcPhe-aHer2 Fab reacted with the aminooxy-biotin, while wild-type aHer2 showed no measurable biotinylation. A wild-type aHer2 Fab was also labeled by reaction with a 10-molar excess of NHS-biotin (Pierce Biotechnology) under the recommended labeling conditions and analyzed by MS. Labeling by these methods yielded a heterogeneous protein product, shown by a range of observed mass peaks (Supplementary Fig. 4). These heterogeneously labeled Fabs bound Her2 less efficiently than unlabeled or site-specifically biotinylated Fabs (Supplementary Fig. 5).

To determine the effect of UAA incorporation and oxime ligation on antigen recognition, we tested pAcPhe-aHer2 Fabs (before and after biotinylation) by ELISA, using immobilized recombinant human epidermal growth factor receptor 2 (HER2) (Fig. 2b and Supplementary Fig. 5). No difference in antigen binding between UAA-containing and unlabeled wild-type Fab was observed. To further confirm the specificity for HER2, we analyzed the Fabs by FACS (fluorescence-activated cell sorting) of HER2-overexpressing cells (SK-BR-3) and HER2 low-expressing human cancer cells (M21L). The sorting profile (Fig. 2c) demonstrated that the pAcPhe–Fabs bind to HER2 specifically, comparable to the wild-type Fab. Alexa Fluor 488-conjugated UAA–Fab also showed selectivity for HER2, but the FACS mean intensity varied with UAA location (Fig. 2d). This variation tracks linearly with the dye–Fab coupling efficiency and can be attributed to competitive binding with residual unreacted pAcPhe UAA–Fab. Thus, sitespecific fluorescently labeled Fab maintains proper antigen recognition and can be used to directly bind antigen in a cellular context.

Construction of Fab multimers

With confirmation that antibody function is not disrupted by UAA incorporation or conjugation, these labeled Fabs were used for construction of more complex antibody constructs. To test the hypothesis that surface-linked (as opposed to genetically fused) multimers could have different activities based on linkage position, we used the UAA mutants in concert with site-specific biotinylation and NeutrAvidin-mediated multimerization. Through binding to each of four biotin-binding pockets, singly biotinylated Fabs can potentially be assembled into tetramers with the relative geometry defined by the biotinylation site. Tetrameric molecules are possible[16–20] but difficult to express and purify by genetic methods, and steric control is nearly impossible. Furthermore, chemical coupling is not easily amenable to producing multimers due to the heterogeneous coupling of typical

conjugation chemistries. Multimers were generated by reaction of excess biotinylated Fab with NeutrAvidin, and then purified by gel-filtration chromatography. An excess of Fab was required to assure that all available NeutrAvidin binding sites were saturated and not affected by the mutant-specific biotinylation efficiency described above. A native PAGE analysis showed that a 5:1 excess was sufficient for K169-biotin, but all multimeric samples were prepared with 10:1 excess (Supplementary Fig. 7a). Gel-filtration analysis (Supplementary Fig. 7b) showed a single preferred peak for each of the three biotinylated Fabs (S156X, K169X, and S202X), indicating that site saturation was successful. Notably, the apparent size of the NeutrAvidin-linked constructs depended on biotin position, indicating that a steric effect may play a role in assembly, or that some sites may produce more compact structures. NeutrAvidin is a deglycosylated form of avidin, a tetrameric protein with a pair of biotin binding sites situated in close proximity on one face and a similar pair on the opposite face.[21] Modeling studies indicate that the Fab biotinylation sites for positions 202 and 156 are located in accessible regions of the Fab kappa surface (Fig. 1), requiring minimal Fab–NeutrAvidin surface contact and therefore potentially allowing complete tetramer formation. The 169 mutant could potentially cause Fab–NeutrAvidin contacts that are sterically unfavorable (Supplementary Fig. 8), and cross-linking studies indicate that K169X may preferentially form dimers, whereas S156X and S202X form tetramers (Supplementary Fig. 9). Each of the mutants, however, could clearly generate higher-order constructs defined by a single peak on gel filtration. In competition binding studies with trastuzumab IgG, the S202X multimer competed for Her2 binding more effectively than did S156X or K169X, with the latter two having binding activities similar to that of trastuzumab (Supplemental Fig. 10).

While trastuzumab shows a therapeutic effect by a combination of mechanisms, one effect is reduced phosphorylation of Her2.[22] We reasoned that enhanced avidity of a tetrameric anti-Her2 might disrupt Her2 signal transduction more efficiently than monomeric Herceptin Fab. Furthermore, different multimers might have differential activities due to steric or conformational constraints produced by the surface position of the UAA–biotin (Fig. 3a). SK-BR-3 (Her2+) cells were treated with a biotinylated Fab or with purified Fab multimers over a wide range of concentrations and then analyzed for the amount of phospho-Her2 present in the cells. The inhibition of phosphorylation was quantified as an IC_{50} for each construct. First, monomeric Fab–biotin constructs (without added NeutrAvidin) had equivalent inhibitory activity to one another and to wild-type Herceptin Fab, as expected (Fig. 3b). The NeutrAvidin multimers, however, showed a dramatic difference compared to each monomeric Fab–biotin (Fig. 3b), in one case achieving a 50-fold decrease. Because treatment concentrations were normalized to Fab content, this effect can be attributed to the multimerization. Furthermore, S202X–bio-Neutrav showed a 10-fold improvement over the S156X–bio-Neutrav and K169X–bio-Neutrav, indicating that the mechanism is geometry specific. While the exact biophysical mechanisms behind these differences are not yet known, these data clearly show that geometric considerations are important in developing higher-order antibody constructs with optimized activity.

Discussion

The advent of biotechnology has resulted in several therapeutic recombinant protein products. As a next generation of therapeutics, it is clear that conjugated or otherwise multimeric proteins could have substantial clinical utility. However, the ability to produce multimeric or stable, well-defined, immunoconjugated proteins has been problematic. Furthermore, it has not been possible to create multimers in different spatial and geometrical orientations because of the severe restrictions imposed by N- and C-terminal genetic fusions. Here, we have generated the first multimeric protein construct with the ability to sterically control the protein's activity.

The therapeutically relevant trastuzumab Fab was expressed with the nonnatural amino acid pAcPhe in several diverse positions on the surface of the kappa constant region. This nonnatural amino acid served as a molecular handle through which single-position conjugates could be produced through reaction with an aminooxy group to produce an oxime immunoconjugate. We verified that pAcPhe incorporation in the trastuzumab constant region was efficient, resulting in yields (~2 mg/L) comparable to that of the wild-type trastuzumab Fab in *E. coli* shake flasks. Further, we confirmed single incorporation of pAcPhe by MS and by labeling of the protein by the fluorescent dye Alexa 488. With a single pAcPhe at different spatial positions on the constant region, we were able to couple an aminooxy functionalized biotin to each Fab and then produce multimers by reaction with NeutrAvidin. Since the biotin is linked at a different position in each constant region, the Fabs are expected to form differentially oriented tetramers when bound by NeutrAvidin. It might be expected, for example, that the K169 or S202 mutants, which are located near the V_L–C_κ domain interface, might result in more compact tetramers than the N152 mutant, which is at the most distal end of C_κ. The size-exclusion chromatography profile does show slower elution for multimersmade fromS202X–bio-Neutrav, which is consistent with this possibility.

The three Fab–NeutrAvidin multimers are nearly identical in every aspect except the position of the pAcPhe, which mediates tetrameric formation via the biotin–NeutrAvidin interaction. Thus, differences in activity must result from (i) differential formation or stability of the multimer or (ii) different orientations and steric constraints imposed through the linkage position. Size-exclusion chromatography and cross-linking SDS-PAGE analysis revealed mostly tetrameric species for S156X and S202X mutants, but potentially dimeric species for K169X (Supplementary Figs. 7–9). Each multimer was treated and isolated in the same manner; thus, these differences must be due to the position of the linking biotin. Cell-based assays of the multimers showed increased activity in reducing Her2 phosphorylation compared to their monomeric counterparts. Remarkably, the S202X mutant was an order of magnitude more potent than the S156X or the K169X mutant and was over 50-foldmore active than the corresponding biotinylated monomers. Thus, the orientation of binding sites in a multimer is an important factor regulating its biological activity.

The ability to site-specifically tag a protein with a nonnatural amino acid capable of mediating site-specific multimerization opens new possibilities in protein engineering. Bispecific proteins have typically been limited to N- or C-terminal fusions, which severely

restricts the potential orientations of each monomer within the complex. When any surface-exposed amino acid side chain can be used as a connection bridge between two proteins, the potential orientational diversity can be dramatically increased. For example, even two proteins with only 10 surface-exposed side chains per protein could be connected in 100 alternative ways, with different *x*, *y*, and *z* orientations. The orientational and steric control afforded by these possibilities can open up new opportunities in multimeric protein engineering. For example, the ability to simultaneously bind two proteins on the same cell, or different cells, could be controlled by selecting the optimal steric dimer amongst a multitude of different bispecific constructs. Multiple applications of this methodology could be envisioned, including in antibody drug conjugates, antibody-directed prodrug therapy (ADEPT), radioimmunoconjugates, bior multispecific antibodies, or in immuno-PCR.

Materials and Methods

UAA incorporation and Fab production

The plasmids pBad-aHer2 and pSup-pAcPhe were cotransformed into Top10F′ *E. coli*. Single colonies were picked and inoculated into 2YT (1.6% Bactotryptone, 1.0% yeast extract, 0.5% NaCl) medium (5 mL) supplemented with ampicillin (50 μg/mL), chloramphenicol (40 μg/mL), and tetracycline (25 μg/mL). The cells were grown to saturation and used to inoculate 2YT expression medium supplemented with antibiotics and 1 mM pAcPhe UAA (SynChem, Inc.) at 37 °C (250–270 rpm). At an OD_{600} of 1.0, the pBad promoter was induced with 0.2% arabinose and cells were grown at 30 °C for 15 h. The cells were then pelleted (6000***g***, 10 min) and resuspended in 80 mL of lysis buffer [20 mM Tris (pH 8.0), 2 mM EDTA (ethylenediaminetetraacetic acid), 20% sucrose] per 1 liter of culture. Periplasmic lysis occurred at 22 °C for 4 h (200 rpm), and the remaining cells were pelleted (6000***g***, 10 min). The supernatant was stored at 4 °C. The cells were resuspended in fresh lysis buffer and incubated for 12 h at 4 °C. The cells were again pelleted, and the supernatant was combined with the supernatant from the first round of lysis and then centrifuged to remove insoluble debris (14,000***g***, 20 min). The Fabs were purified from the supernatant by Protein G chromatography.[12] Larger-scale preparations were made with a 2-L fermentation system (Bioflo 3000, New Brunswick Scientific) using the plasmid p4xH-aHer2 (Supplementary Fig. 1), instead of pBad-aHer2, and the *E. coli* strain 25F2.[23] Overnight cultures of 5 mL were diluted to 25 mL with 2YT and grown for 2–3 h before inoculation in medium [ampicillin (50 μg/mL), 1 mM pAcPhe UAA, N-Z amine (12 g/L; Sigma), 125 μM $FeCl_3$, 2.4 mM isoleucine, 4.1 mM sodium citrate, 17 mM glucose, 47 mM $(NH_4)_2SO_4$, 12 mM $MgSO_4$, 3.3 mM NaH_2PO_4, 6 mM K_2HPO_4, and 20 μM each of $NaMoO_4$, $ZnSO_4$, $MnSO_4$, $CoCl_2$, H_3BO_3, and $CuSO_4$]. Cells were grown at 30 °C with controlled pH (7.0) and glucose feed (50% glucose) and were automatically induced when the phosphate in the medium was depleted. Cells grew overnight and were spun down at 6000***g*** for 10 min. Cells were lysed and Fab-purified with the same procedure as the shake flasks.

Site-specific conjugation

The anti-Her2 Fab was buffer-exchanged into 100 mM acetate buffer (pH 4.5) and concentrated to 20 μM [Amicon concentrator, 10,000 molecular weight cutoff (MWCO)].

The anti-Her2 Fab (20 μM) was reacted with 300 μMaminooxy-Alexa Fluor 488 (Invitrogen/Molecular Probes) and 100 mM methoxyaniline in acetate buffer (pH4.5) at 37 °C. After 16 h, the reaction was buffer-exchanged into phosphate-buffered saline [PBS (pH 7.0); Amicon concentrator, 10,000 MWCO], and the sample was washed four to five times to remove excess dye. The sample was resolved on an SDS-PAGE gel (4–20% Tris–glycine), and electrospray ionization MS (ESI-MS) was performed to confirm coupling. Coupling efficiencies were calculated by UV–vis (NanoDrop; dye absorbance=494 nm; protein absorbance=280 nm). Moles of aHer2 Fab and Alexa Fluor 488 were determined by absorbance. The ratio of Alexa Fluor 488 to aHer2 Fab determines the approximate coupling efficiency. Since the Alexa Fluor 488 dye also produces a small peak at 280 nm, an 11% correction was added to obtain the final coupling efficiency. The anti-Her2 Fab was coupled to aminooxy-biotin using the same procedure.

Phosphorylation assay

SKBR3 cells (American Type Culture Collection) were grown to 80% confluency in Dulbecco's modified Eagle's medium and 10% fetal bovine serum and detached with 1 mL of trypsin–EDTA (Invitrogen). Cells were diluted 1:10 in medium, and 100 μL (~10^4 cells) was plated onto white 96-well tissue culture plates (Corning). After overnight adherence, 10 μL of anti-Her2 Fab was added to cells at decreasing concentrations (Fig. 3b) and incubated at 37 °C for 45 min. The medium was aspirated and 100 μL of lysis buffer was added to each well [1 × IC Diluent #12 (R&D Systems, cat. no. DYC002), aprotinin (10 μg/mL), and leupeptin (10 μg/mL)] and incubated at 4 °C for 30 min. We previously prepared the ELISA plate (Nunc Maxisorp) by adding phospho-ErbB2 capture antibody (4 μg/mL; DuoSet IC Human Phospho-Erbb2 ELISA, R&D Systems, #DYC1768) at 100 μL per well and allowing it to adsorb overnight at room temperature. Wells were washed with PBST [PBS (pH 7.2), 0.05% Tween 20] five times and blocked with 300 μL of block buffer [1% BSA (bovine serum albumin), 0.05% NaN_3, PBS (pH 7.2)] for 1 h. Wells were washed immediately prior to addition of the cell lysate, which was incubated at room temperature for 2 h. The anti-phosphotyrosine–HRP (horseradish peroxidase) detection antibody was diluted to the working concentration specified on the vial in IC Diluent #14 [20 mM Tris, 137 mM NaCl, 0.05% Tween 20, 0.1% BSA (pH 7.2)]. The plate was washed five times and 100 μL of diluted detection antibody was added and incubated at room temperature for 2 h. The plate was again washed five times with wash buffer prior to addition of the detection reagent, QuantaBlue (Pierce). After 10 min, we measured relative fluorescence units (excitation 325 nm, emission 420 nm) in a spectrofluorometer (Molecular Devices). Each experimental test was performed in triplicate. Error bars were determined through standard deviation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

Fab	antigen binding fragment
HER2	human epidermal growth factor receptor 2
pAcPhe	*p*-acetylphenylalanine
UAA	unnatural amino acid
MS	mass spectrometry
ESI-MS	electrospray ionization mass spectrometry
PBS	phosphate-buffered saline

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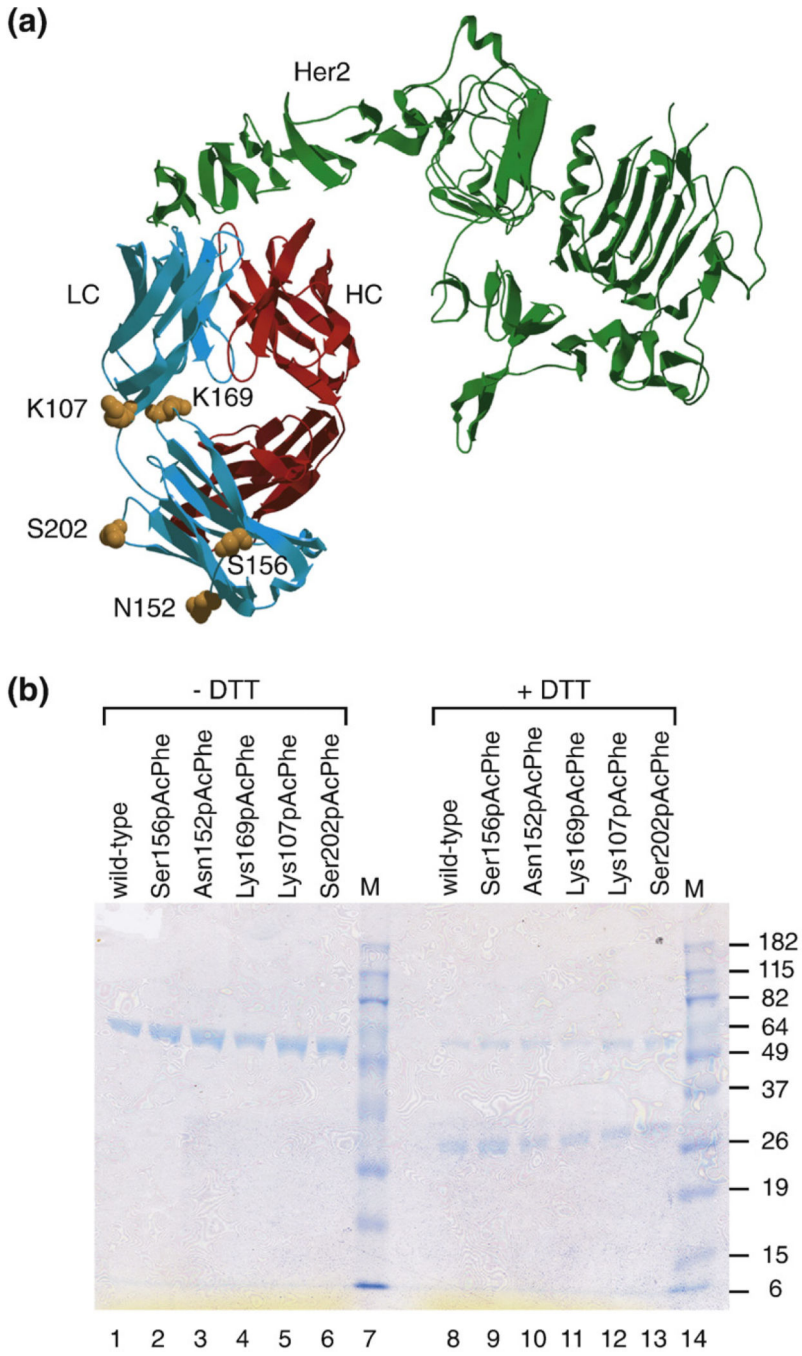

Fig. 1.

(a) A trastuzumab Fab bound to HER2 (green), showing the heavy (red) and light (blue) chains in ribbon format and positions of the five amino acids (orange) that were individually mutated in separate constructs to encode pAcPhe. Structures are derived from a reported crystal structure (Protein Data Bank ID 1N8Z).[9] (b) Wild-type Fab (lanes 1 and 8) and the mutants containing pAcPhe at each mutation site were purified and resolved by SDS-PAGE in the absence (lanes 1–6) or presence (lanes 8–13) of reducing agent dithiothreitol (DTT). Appropriate interchain disulfide formation producing a 50-kDa Fab is seen for each protein

(lanes 1–6). Reaction with DTT breaks the interchain disulfides to produce monomer heavy and light chains, which co-migrate around 30 kDa. Yields and further biochemical characterization are shown in Table 1.

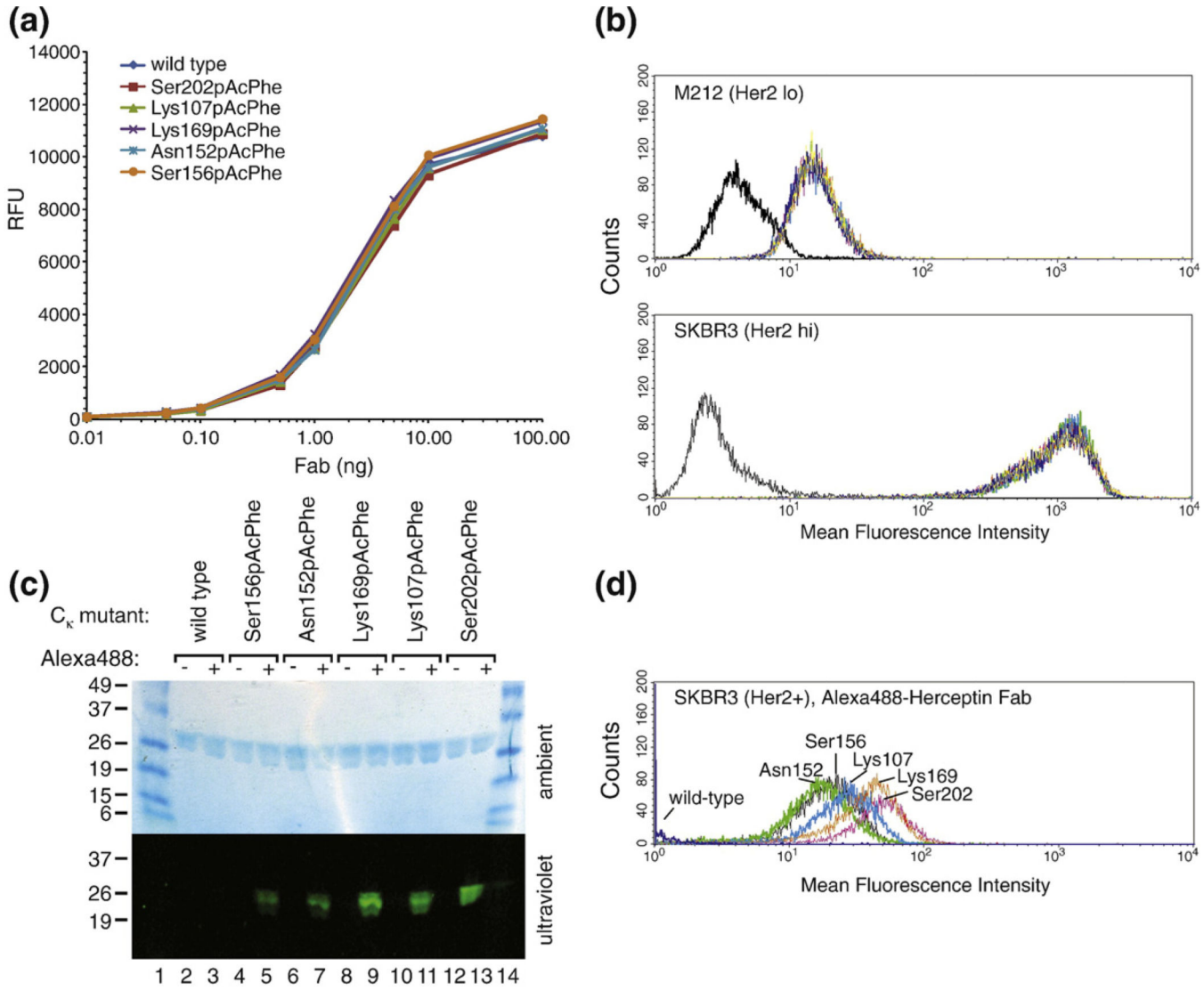

Fig. 2.

(a) ELISA of herceptin Fab mutants. Increasing amounts of Fab were added to 50 ng of Her2 (R&D Biosystems) and detected with anti-kappa HRP and QuantaBlu fluorescent substrate. A typical saturation binding curve is seen, with all mutants behaving nearly identically with the wild-type Fab. (b) The mutant Fabs were able to bind SKBR3 Her2+ cells (lower panel). The traces for wild type and mutant were overlaid and were nearly identical. Fluorescence was detected with an anti-kappa-fluorescein antibody. Little staining was seen for the Her2 cell line M212 (upper panel). (c) Wild type (lanes 2 and 3) and the mutants (lanes 4–13) were either reacted with (odd lanes) or without (even lanes) aminoxy-Alexa 488. The samples were resolved by SDS-PAGE and the gel was illuminated with ambient light (top) or UV light (bottom). (d) The fluorescent Fabs could directly stain the SKBR3 (Her2+) cell line. The amount of staining for each mutant (mean fluorescence intensity) was directly proportional to the labeling efficiency, as expected (Table 1).

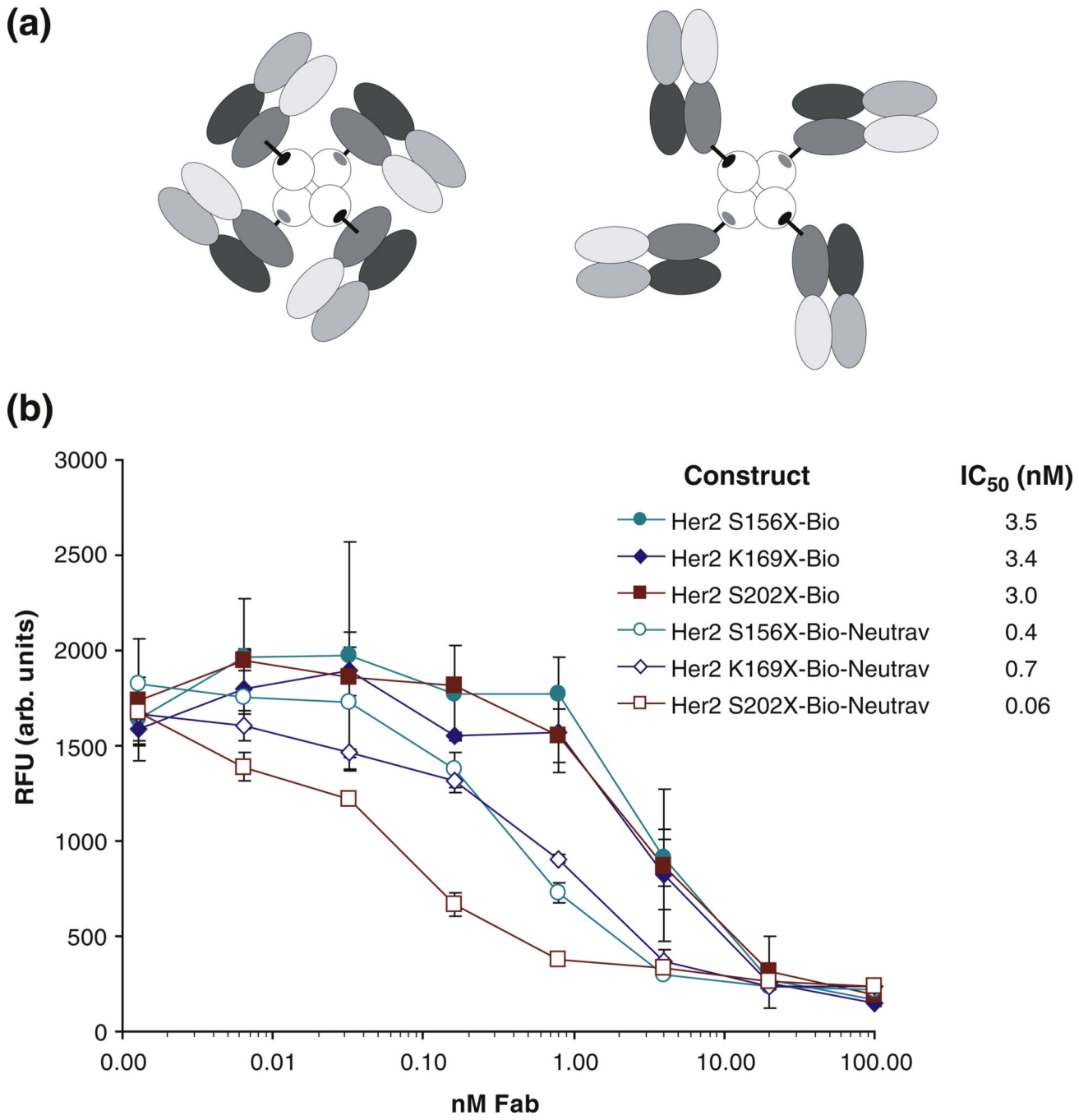

Fig. 3.

(a) Schematic structures of NeutrAvidin–Fab constructs. The mutation positions may alter the accessibility and steric favorability of the binding site of the individual subunits in the tetrameric Fab. For example, the S156X and K169X Fabs link near the V_L–C_κ interface (left) and may form a more compact structure, whereas the N152X mutant encodes pAcPhe near the distal region of C_κ (right). (b) ELISA analysis of phosphorylated HER2 levels in

SKBR-3 cells treated with biotinylated Fabs; NeutrAvidin–Fab constructs and their corresponding IC_{50} values.

Table 1

Expression and labeling results for Herceptin Fab mutants

C_κ mutant	Purified yield (mg/L)	ESI-MS aHer2 (expected)	Alexa 488 labeling efficiency (%)	ESI-MS Alexa 488-aHer2 (expected)
WT	1.51	47,758 (47,758.15)	1	47,761 (47,758.15)
S156pAcPhe	1.70	47,861 (47,860.35)	33	48,537 (48,535.08)
N152pAcPhe	1.13	47,833 (47,833.32)	27	48,511 (48,508.05)
K169pAcPhe	1.43	47,819 (47,819.17)	81	48,496 (48,493.90)
K107pAcPhe	1.42	47,823 (47,819.17)	47	48,495 (48,493.90)
S202pAcPhe	1.04	47,861 (47,860.35)	69	48.537 (48,535.08)