

METHOD FOR MAKING ANTIBODY FC-HETERODIMERIC MOLECULES USING ELECTROSTATIC STEERING EFFECTS

BACKGROUND

5 Antibodies have become the modality of choice within the biopharma industry because they possess several characteristics that are attractive to those developing therapeutic molecules. Along with the ability to target specific structures or cells, antibodies make its target susceptible to Fc-receptor cell-mediated phagocytosis and killing (Raghavan and Bjorkman 1996). Further, antibodies ability to interact with neonatal Fc-receptor (FcRn) in a
10 pH dependent manner confers them with extended serum half-life (Ghetie and Ward 2000). This unique feature of antibodies allows extending the half-life of therapeutic protein or peptide in the serum by engineering Fc fusion molecules.

 Antibodies belong to immunoglobulin class of proteins which includes IgG, IgA, IgE, IgM, and IgD. The most abundant immunoglobulin class in human serum is IgG whose
15 schematic structure is shown in the Figure 1 (Deisenhofer 1981; Huber 1984; Roux 1999). The IgG structure has four chains, two light and two heavy chains, and each light chain has two domains and each heavy chain has four domains. The antigen binding site is located in the Fab region (Fragment antigen binding) which contains a variable light (VL) and heavy (VH) chain domains as well as constant light (LC) and heavy (CH1) chain domains. The
20 CH2 and CH3 domain region of the heavy chain is called Fc (Fragment crystallizable). The IgG molecule can be considered as a heterotetramer having two heavy chains that are held together by disulfide bonds (-S-S-) at the hinge region and two light chains. The number of hinge disulfide bonds varies among the immunoglobulin subclasses (Papadea and Check 1989). The FcRn binding site is located in the Fc region of the antibody (Martin, West et al.
25 2001), and thus extended serum half-life property of the antibody is retained in the Fc fragment. The Fc region alone can be thought of as a homodimer of heavy chains comprising CH2 and CH3 domains.

 In certain instances, it is desirable to create a molecule that contains the Fc portion of an antibody but comprises a heterodimer. An important application of Fc heterodimeric
30 molecules is the generation of bispecific antibodies (BsAbs). Bispecific antibodies refer to antibodies having specificities for at least two different antigens (Nolan and O'Kennedy 1990; de Leij, Molema et al. 1998; Carter 2001). Instead of having identical sequence in both the

Fabs, bispecific antibodies bear different sequences in the two Fabs so that each arm of Y-shaped molecule can bind to different antigens.

The use of bispecific antibodies for immunotherapy of cancer has been extensively reviewed in the literature (for example, see (Nolan and O'Kennedy 1990; de Leij, Molema et al. 1998; Carter 2001)). By having the ability to bind to two different epitopes or molecules, BsAbs provide means to both trigger an immune effector cell and bind a surface antigen on a tumor target cell. This helps to make use of the immune system to destroy cancer cells. Other applications of bispecific antibodies are extensively covered in U.S. Pat. Nos. 5,731,168 and 7,183,076.

The classical method of producing BsAbs by co-expressing two different IgGs in hybrid hybridomas leads to up to 10 possible combinations of heavy and light chains. This compromises the yield and imposes a purification challenge. Carter and co-workers engineered heavy chain for heterodimerization using a “knobs-into-holes” strategy (Ridgway, Presta et al. 1996; Atwell, Ridgway et al. 1997; Merchant, Zhu et al. 1998; Carter 2001). The knobs-into-holes concept was originally proposed by Crick as a model for packing of amino acid side chains between adjacent α -helices (Crick 1952). Carter and co-workers created a knob at the CH3 domain interface of the first chain by replacing a smaller amino acid side chain with a larger one (for example, T366Y); and a hole in the juxtaposed position at the CH3 interface of the second chain was created by replacing a larger amino acid side chain with a smaller one (for example, Y407T). The basis for creating knob and hole in the juxtaposed positions is that the knob and hole interaction will favor heterodimer formation, whereas the knob-knob and the hole-hole interaction will hinder homodimers formation due to the steric clash and deletion of favorable interactions, respectively. The knobs-into-holes mutations were also combined with inter-CH3 domain disulfide bond engineering to enhance heterodimer formation (Sowdhamini, Srinivasan et al. 1989; Atwell, Ridgway et al. 1997). In addition to these mutations, the input DNA ratio was also varied to maximize the yield (Merchant, Zhu et al. 1998). The “knobs-into-holes” technique is disclosed in U.S. Pat. Nos. 5,731,168 and 7,183,076.

SUMMARY

This application describes a strategy for altering a CH3 domain to reduce the ability of the domain to interact with itself, i.e., form homodimers. In particular, one of more residues that make up the CH3-CH3 interface is replaced with a charged amino acid such that interaction becomes electrostatically unfavorable. In preferred embodiments, a positive-

charged amino acid in the interface, such as a lysine, arginine, or histidine, is replaced with a negative charged amino acid, such as aspartic acid or glutamic acid. In other embodiments, a negative-charged amino acid in the interface is replaced with a positive-charged amino acid. In certain embodiments, the amino acid is replaced with an unnatural amino acid having the
5 desired charge characteristic.

Further described herein is a strategy for altering a pair of CH3 domains to reduce the ability of each domain to interact with itself but to increase the ability of the domains to interact with each other, i.e., form heterodimers. This can be achieved by replacing one or more residues that make up the CH3-CH3 interface in both CH3 domains with a charged
10 amino acid such that homodimer formation is electrostatically unfavorable but heterodimerization is electrostatically favorable. In certain embodiments, a charged amino acid in each CH3 domain is replaced with an amino acid with an opposite charge. For example, a positive-charged amino acid may be replaced with a negative charged amino acid in the first CH3 domain and a negative charged amino acid may be replaced with a positive-
15 charged amino acid in the second CH3 domain. By reversing the charge of the amino acid, homodimer formation is reduced. When the replacements are coordinated properly, the reversed charges create are electrostatically favorable, i.e., opposing charges in the interface, for heterodimerization formation.

In certain aspects, the invention provides a method of preparing a heterodimeric
20 protein. The heterodimer may comprise a first CH3-containing polypeptide and a second CH3-containing polypeptide that meet together to form an interface engineered to promote heterodimer formation. The first CH3-containing polypeptide and second CH3-containing polypeptide are engineered to comprise one or more charged amino acids within the interface that are electrostatically unfavorable to homodimer formation but electrostatically favorable
25 to heterodimer formation.

Such methods may include culturing a host cell comprising nucleic acids encoding the first and second CH3-containing polypeptides such that the polypeptides are co-expressed by the cell. In certain embodiments, the nucleic acids encoding the first and the second CH3-containing polypeptides are provided to the host cell at a ratio, for example 1:1, 1:2, 2:1, 1:3,
30 3:1, 1:4, 4:1, 1:5, 5:1, 1:6, 6:1, 1:7, 7:1, 1:8, 8:1, 1:9, 9:1, 1:10, 10:1. It is contemplated that altering the ratio of nucleic acids may increase the production of heterodimeric molecules versus homodimeric molecules.

The homodimeric molecules may be purified from the host-cell culture using standard techniques. For example, when the heterodimeric protein comprises an Fc, the protein may

be purified using a Protein A column. The purification techniques include but are not limited to chromatographic methods such as size exclusion, ion exchange and affinity-based chromatography and ultracentrifugation.

In certain embodiments, the Fc region is an IgG Fc region, preferably a wild-type human IgG Fc region. By “wild-type” human IgG Fc it is meant a sequence of amino acids that occurs naturally within the human population. Of course, as the Fc sequence may vary slightly between individuals, one or more alterations may be made to a wild-type sequence and still remain within the scope of the invention. For example, the Fc region may contain additional alterations that are not related to the present invention, such as a mutation in a glycosylation site, inclusion of a unnatural amino acid, or a “knobs-into-holes” mutation.

Human IgG Fc sequences include IgG1 (e.g., Swiss-Prot/TrEMBL accession number P01857), IgG2 (e.g., P01859), IgG3 (e.g., P01860), and IgG4(e.g., P01861).

The Fc region also may be an IgA (e.g., Swiss-Prot/TrEMBL accession number – P01876), IgD (e.g., P01880), IgE (e.g., P01854), and IgM (e.g., P01871) Fc region.

The polypeptide containing the CH3 region may be an antibody heavy chain and the host cell may further express one or more antibody light chains. In embodiments wherein more than one heavy chain and light chains are co-expressed (e.g., bivalent antibody), each heavy chain may comprise a mutation in the CH1 region and each light chain may comprise a mutation in the constant region to preferentially bind to each other but not bind to the other light or heavy chain, respectively. In preferred embodiments, such mutations involve altering the charge of one or more amino acids in the interface between the CH1 region and the constant region of a light chain.

Preferred embodiments of the invention include but are not limited to an antibody, a bispecific antibody, a monospecific monovalent antibody, a bispecific maxibody, a monobody, a peptibody, a bispecific peptibody, a monovalent peptibody (a peptide fused to one arm of a heterodimeric Fc molecule), and a receptor-Fc fusion protein. See FIG. 2.

Examples of mammalian host cells that may be used include but are not limited to CHO, 293, and myeloma cell lines. The host cell may also be yeast or a prokaryote, such as E. coli.

The heterodimeric proteins may be particularly useful in therapeutic compositions. In certain embodiments, a heterodimeric protein may be formulated in a composition that includes one or more pharmaceutically acceptable buffer or excipient. Such therapeutic composition may be administered to a subject to treat a disease or may be given to prevent a disease or prevent the symptoms of a disease from progressing.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Schematic diagram of IgG1 antibody with the domains indicated. The IgG1 antibody is a Y-shaped tetramer with two heavy chains (longer length) and two light chains (shorter length). The two heavy chains are linked together by disulfide bonds (-S-S-) at the hinge region. Fab – fragment antigen binding, Fc – fragment crystallizable, VL – variable light chain domain, VH – variable heavy chain domain, CL – constant (no sequence variation) light chain domain, CH1 – constant heavy chain domain 1, CH2 – constant heavy chain domain 2, CH3 – constant heavy chain domain 3.

Figure 2. Figure depicts some of the embodiments that include Fc-heterodimeric molecules. These include bispecific antibodies (have specificity for two or more antigens) to receptor-Fc fusion molecules. Preferably, the Fc retains its ability to interact with the FcRn receptor, even without the Fab domains, leading to longer serum half-life for proteins/domains that are fused to the Fc heavy chains. scFv – single chain fragment variable, Pep. – peptibody, A and B stands for proteins or receptors or domains.

Figure 3. CH3 domain interface structure with residues involved in the domain-domain interaction shown. The interface residues were identified using a distance cutoff method. Structurally conserved and buried (solvent accessible surface area < 10%) residues are shown in the ball-and-stick model. Solvent exposed or structurally not conserved residues are shown in the stick representation. The analysis is based on the IgG1 crystal structure (PDB code: 1L6X) which is determined at high-resolution (1.65Å) (Idusogie, Presta et al. 2000).

Figure 4. Comparison of IgG subclass sequences from (a) human and (b) mouse. Only the heavy chain sequence corresponding to the CH3 domain is shown. The star (*) indicates residue positions involved in the CH3-CH3 domain interaction identified based on the IgG1 human Fc crystal structure (1L6X). Positions marked with rectangles are preferred residues for mutation to enhance heterodimer formation. It may be noted here that charged residues are highly conserved among the IgGs. (c) CH3 domain sequence comparison of other class of antibodies (IgA, IgE, IgD, and IgM). The interface residue positions (indicated by “*”) in (b) and (c) were identified based on sequence comparison with Hu IgG1 sequence that is also shown.

Figure 5. Crystal structure of CH3 domain homodimer with one domain shown in ribbon representation and the other domain shown in wire model. The Lys409 (Lys409' in the second domain) and Asp399 (Asp399' in the second) residues are shown in ball-and-stick

model in order to illustrate each pair-wise interaction is represented twice in the structure. This is due to the two-fold symmetry present in the CH3-CH3 domain interaction. The figure was created using the 1L6X co-ordinates deposited in the PDB.

Figure 6. Schematics showing electrostatic interactions in the wild type and in the mutants designed as an example to enhance heterodimer formation and hinder homodimer formation. (a) In the case of WT, electrostatic interactions favor both heterodimer and homodimer formation giving them equal probability. (b) In the single mutant (K409E) case, one of the homodimer is discouraged by both the interactions and at the same time heterodimer is also discouraged by one of the interactions. (c) In the double mutant case, both the electrostatic interactions favor heterodimer and disfavor homodimer formation. (d) Additional mutations involving charge change (for example, K360E) could also be used to enhance the electrostatic steering effects on the formation of heterodimer and homodimer.

Figure 7. This figure shows that electrostatic interactions could also be used to favor homodimers and disfavor heterodimer formation, when two different chains are co-expressed.

Figure 8. Figure (a) shows the schematic drawing of the constructs used in the Example. The first chain of the Fc has a maxibody (single chain fragment variable, scFv) covalently linked, and the second chain called dummy Fc does not have any domain or functionality attached to it. (b) Illustration of expected relative mobility on the SDS-PAGE. Because the Fc chain attached to the maxibody has a higher molecular weight than the dummy Fc, homodimers and heterodimer have different mobility on the SDS-PAGE. The thickness of the band on the SDS-PAGE can be used as a measure of fraction of heterodimer and homodimer yield. The wild type is included as a control and to monitor relative improvement on the heterodimer yield due to various mutations.

Figure 9. SDS-PAGE analysis showing the effects of mutations on the D399'---K409 interaction pair.

Figure 10. SDS-PAGE analysis of charge residue mutations (listed in Table 4) in addition to D399'K --- K409D pair mutations. Wild type (first lane) and knobs-into-holes mutations (last lane) are also shown for comparison. 1:2 input DNA ratio of dummy Fc and M315 maxibody was used here.

Figure 11. Western blot demonstrating certain combinations of mutant achieve high selectivity for heterodimer formation. Fc molecules were detected using goat-anti-human Fc HRP conjugated at 1:10,000. 1:1 input DNA ratio of dummy Fc and M315 maxibody was used.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

A total of 48 antibody crystal structures which had co-ordinates corresponding to the Fc region were identified from the Protein Data Bank (PDB) (Bernstein, Koetzle et al. 1977) using a structure based search algorithm (Ye and Godzik 2004). Examination of the identified Fc crystal structures revealed that the structure determined at highest resolution corresponds to the Fc fragment of RITUXIMAB bound to a minimized version of the B-domain from protein A called Z34C (PDB code: 1L6X). The biological Fc homodimer structure for 1L6X was generated using the deposited Fc monomer co-ordinates and crystal symmetry. Two methods were used to identify the residues involved in the CH3-CH3 domain interaction: (i) contact as determined by distance limit criterion and (ii) solvent accessible surface area analysis.

According to the contact based method, interface residues are defined as residues whose side chain heavy atoms are positioned closer than a specified limit from the heavy atoms of any residues in the second chain. Though 4.5Å distance limit is preferred, one could also use longer distance limit (for example, 5.5Å) in order to identify the interface residues (Bahar and Jernigan 1997).

The second method involves calculating solvent accessible surface area (ASA) of the CH3 domain residues in the presence and absence of the second chain (Lee and Richards 1971). The residues that show difference ($>1\text{\AA}^2$) in ASA between the two calculations are identified as interface residues. Both the methods identified similar set of interface residues. Further, they were consistent with the published work (Miller 1990).

Table 1 lists twenty four interface residues identified based on the contact criterion method, using the distance limit of 4.5Å. These residues were further examined for structural conservation. For this purpose, 48 Fc crystal structures identified from the PDB were superimposed and analyzed by calculating root mean square deviation for the side chain heavy atoms. Figure 3 shows the CH3 domain interface along with the structurally conserved, buried (% ASA ≤ 10), and exposed (% ASA > 10) positions (% ASA refers to ratio of observed ASA to the standard ASA of amino acids; (Lee and Richards 1971)). Conservation of interface residues among Human and Mouse IgG subclasses as well as among other Ig classes was also examined through sequence comparisons (Figure 4).

Table 1: List of CH3 domain interface residues in the first chain (A) and their contacting residues in the second chain (B)^a

<i>Interface Res. in Chain A</i>	<i>Contacting Residues in Chain B</i>
GLN A 347	LYS B 360'
TYR A 349	SER B 354' ASP B 356' GLU B 357' LYS B 360'
THR A 350	SER B 354' ARG B 355'
LEU A 351	LEU B 351' PRO B 352' PRO B 353' SER B 354' THR B 366'
SER A 354	TYR B 349' THR B 350' LEU B 351'
<i>ARG A 355^b</i>	THR B 350'
ASP A 356	TYR B 349' LYS B 439'
GLU A 357	TYR B 349' LYS B 370'
<i>LYS A 360^b</i>	GLN B 347' TYR B 349'
SER A 364	LEU B 368' LYS B 370'
THR A 366	LEU B 351' TYR B 407'
LEU A 368	SER B 364' LYS B 409'
LYS A 370	GLU B 357' SER B 364'
ASN A 390	SER B 400'
LYS A 392	LEU B 398' ASP B 399' SER B 400' PHE B 405'
THR A 394	THR B 394' VAL B 397' PHE B 405' TYR B 407'
PRO A 395	VAL B 397'
VAL A 397	THR B 393' THR B 394' PRO B 395'
ASP A 399	LYS B 392' LYS B 409'
SER A 400	ASN B 390' LYS B 392'
PHE A 405	LYS B 392' THR B 394' LYS B 409'
TYR A 407	THR B 366' THR B 394' TYR B 407' SER B 408' LYS B 409'
LYS A 409	LEU B 368' ASP B 399' PHE B 405' TYR B 407'
LYS A 439	ASP B 356'

^aPositions involving interaction between oppositely charged residues are indicated in bold. Due to the 2-fold symmetry present in the CH3-CH3 domain interaction, each pair-wise interaction is represented twice in the structure (for example, Asp A 356 --- Lys B 439' & Lys A 439 --- Asp B 356'; Figure 5)

^bArg355 and Lys360 positions (shown in italics) could also be used for enhancing electrostatic steering effects though they are not involved in interaction with oppositely charged residues.

At neutral pH (=7.0), Asp and Glu residues are negatively charged and Lys, Arg and His are positively charged. These charged residues can be used to promote heterodimer formation and at the same time hinder homodimers. Attractive interaction takes place between opposite charges and repulsive interaction occurs between like charges. The method presented here makes use of the attractive and repulsive interactions for promoting heterodimer and hindering homodimer, respectively, by carrying out site directed mutagenesis of charged interface residues.

Examination of the identified CH3 domain interface residues (Table 1) reveals four unique charge residue pairs involved in the domain-domain interaction (Asp356---Lys439',

Glu357---Lys370', Lys392---Asp399', Asp399---Lys409'; residue numbering in the second chain is indicated by prime '). These charge pairs are not necessarily involved in charge-charge interaction in the crystal structure used here (1L6X), since crystal structure is an end product in the protein folding reaction pathway and it represents structure in the crystalline state. It is assumed here that in order to have electrostatic steering effects it is sufficient if the residues are close in space as defined by the distance limit criterion (4.5Å). It must also be noted here that due to the 2-fold symmetry present in the CH3-CH3 domain interaction, each unique interaction will be represented twice in the structure (for example, Asp399---Lys409' & Lys409---Asp399'; Figure 5).

The four pairs were ranked according to the extent of solvent accessibility (ASA analysis) (Lee and Richards 1971). In Lys409---Asp399' case, both the residues were structurally conserved as well as buried. In other three pairs case, at least one of the partner is solvent exposed (%ASA > 10). Therefore, for the Example herein, the Lys409---Asp399' pair was chosen for site directed mutagenesis. The strategy is schematically shown in Figure 6.

In the wild type, K409---D399' interaction favors both heterodimer and homodimer formation. A single mutation switching the charge polarity (K409E; positive to negative charge) in the first chain leads to unfavorable interactions for the formation of the first chain homodimer. The unfavorable interactions arise due to the repulsive interactions occurring between the same charges (negative---negative; D399---K409E & K409E---D399). A similar mutation switching the charge polarity (D399'K; negative to positive charge) in the second chain leads to unfavorable interactions (K409'---D399'K & D399'K---K409') for the second chain homodimer formation. But, at the same time, these two mutations (K409E & D399'K) lead to favorable interactions (K409E---D399'K & D399---K409') for the heterodimer formation.

The electrostatic steering effects on heterodimer formation and homodimer discouragement can be further enhanced by mutation of additional charge residues which may or may not be paired with an oppositely charged residue in the second chain, such as Arg355 and Lys360, as shown in Figure 6d. The mutations shown in Figure 6 are for the purpose of illustration only. Table 2 lists many possible mutations involving charge change, and the mutations can be combined to enhance the electrostatic effects.

Table 2a: List of some possible pair-wise charge residue mutations to enhance heterodimer formation^a

Position in the First Chain	Mutation in the First Chain	Interacting Position in the Second Chain	Corresponding Mutation in the Second Chain
Lys409	Asp or Glu	Asp399'	Lys or Arg ^b
Lys392	Asp or Glu	Asp399'	Lys or Arg ^b
Lys439	Asp or Glu	Asp356'	Lys or Arg ^b
Lys370	Asp or Glu	Glu357'	Lys or Arg ^b
Asp399	Lys or Arg ^b	Lys409'	Asp or Glu
Asp399	Lys or Arg ^b	Lys392'	Asp or Glu
Asp356	Lys or Arg ^b	Lys439'	Asp or Glu
Glu357	Lys or Arg ^b	Lys370'	Asp or Glu

^aCombinations of the above pair-wise charge residue mutations could also be used. For example Lys409 --- Asp399' interaction pair mutations could be combined with Lys439 --- Asp356' pair mutations.

- 5 ^bHistidine (His) could also be added to this list of positively charged residues, however, increase in side chain volume and pH dependency should be taken into account in the design.

Table 2b: Additional single charge residue mutations to enhance electrostatic steering effects^a

Position in Chain 1	Mutation	Position in Chain 2	Mutation
Arg355	Asp or Glu	Arg355'	Asp or Glu
Lys360	Asp or Glu	Lys360'	Asp or Glu

- 10 ^aThese single residue mutations could be combined with the Table 2a pair-wise mutations to enhance the heterodimer formation (Figure 6d).

Each positively charged residue (Lys and Arg) can be mutated to two negatively charged residues (Asp or Glu) and *vice versa*, and as a result the method described here provides numerous combinations. It must be stated here that different combinations will have diverse effect on the quaternary (homodimer/heterodimer) structure formation depending on surrounding residues at the mutation site and role of water molecules. The amino acid Histidine (His) is positively charged at neutral pH and therefore mutation to His is also contemplated. However, mutating negatively charged residues (Asp or Glu) to His will lead to increase in side chain volume which may cause steric issues. Further, Histidine proton donor- and acceptor-form depends on the localized environment. These issues should be taken into consideration during the design strategy.

Because the interface residues are highly conserved in Human and Mouse IgG subclasses, electrostatic steering effects can be applied to Human or Mouse IgG1, IgG2, IgG3, or IgG4. This strategy can also be extended to modifying uncharged residues to charged residues at the CH3 domain interface. A similar strategy involving charge residue

mutations can also be used to enhance homodimers and hinder heterodimer formation when two different heavy chains are co-expressed (Figure 7).

In order to assess the stability of the charge residue mutants, EGAD software was used to estimate the CH3-CH3 domain binding free energy. By optimizing parameters used in the calculation, Pokala and Handel could predict the effects of nearly 400 mutations on protein-protein complex formation within 1.0kcal/mol error (Pokala and Handel 2005). EGAD was used to roughly compare the binding free energy of various mutations made at the CH3 domain interface.

Table 3 lists computed binding free energy ($\Delta\Delta G$) for the interface charge residue mutants. The binding free energy of a mutant is defined as $\Delta\Delta G_{\text{mut}} = \mu (\Delta G_{\text{mut}} - \Delta G_{\text{wt}})$. Where, μ (≈ 0.1 , in general) is the scaling factor used to normalize the predicted changes in binding affinity to have a slope of 1 when comparing with the experimental energies (Pokala and Handel 2005). The free energy of dissociation (ΔG) is defined as the energy difference between the complex (ΔG_{bound}) and free states (ΔG_{free}). The comparison shows that charged residue mutations affect the stability to a much lesser extent compared to the knobs-into-holes mutations. For comparison, melting temperatures reported for the wild type and knobs-into-holes mutants are given. The melting temperatures were measured by Carter and coworkers using only the CH3 domain construct (Atwell, Ridgway et al. 1997). For the knobs-into-holes mutants, decrease in enthalpy was also observed in the differential scanning calorimetry experiments.

Table 3: CH3-CH3 domain binding free energy for various mutants designed to enhance heterodimer formation, calculated using the EGAD program (Pokala and Handel 2005)^a

Protein	Description	ΔG (in kcal/mol)	$\Delta\Delta G_{\text{mut}}$ (in kcal/mol)	Melting Temp. T_m (in °C)
WT	Wild Type	-30.69	0	80.4
T366W-Y407'A	Knob-Hole	-24.60	6.09	65.4
T366W-T366'S-L368'A-Y407'V	Knob-Hole	-28.57	2.12	69.4
K409E-D399'K	Charge-Charge	-29.56	1.13	ND
K409E-D399'R	Charge-Charge	-29.47	1.22	ND
K409D-D399'K	Charge-Charge	-28.16	2.53	ND
K409D-D399'R	Charge-Charge	-27.69	3.00	ND
K392E-D399'R	Charge-Charge	-29.27	1.42	ND
K392E-D399'K	Charge-Charge	-29.87	0.82	ND
K392D-D399'R	Charge-Charge	-28.82	1.87	ND
K392D-D399'K	Charge-Charge	-29.42	1.27	ND

^aNot all possible charge-charge pairs were considered for the binding free energy calculation. Wild type is listed for comparison. ΔG is defined as energy difference between the complex

and free states. The binding free energy of a mutant ($\Delta\Delta G_{\text{mut}}$) is defined as difference between the mutant (ΔG_{mut}) and wild type (ΔG_{WT}) free energies.

5 Figure 2 depicts several embodiments comprising Fc heterodimeric molecules, from
bispecific antibodies to heterodimeric receptor complexes. The two heavy chains of
heterodimeric Fc molecules can be fused with proteins and/or domains that have different
functionalities. For example, fusing Fabs that bind to different antigens will lead to bispecific
10 antibodies (BsAbs). Fusing two different single-chain Fv (scFv; variable light and heavy
chains joined by a flexible peptide linker) domains will lead to bispecific maxibodies.
Further, domains or proteins that interact for functional reasons can also be fused with
heterodimeric Fc for the purpose of developing functional assays or for therapeutic uses. For
instance, in the hematopoietic receptor family gp130 is known to interact with other receptors
15 such as Leukemia Inhibitory Factor Receptor (LIFR). The extra cellular domain (ECD) of
gp130 can be fused to the first heavy chain of Fc and the ECD of LIFR can be fused to the
second Fc heavy chain, which will lead to formation of gp130-LIFR complex that is likely to
mimic the biological state. Since FcRn binding site is located in the Fc region, Fc fusion
molecules are likely to have extended serum half-life - a feature that distinguishes Fc
heterodimeric molecules from other heterodimeric molecules such as leucine zipper fusion
20 proteins (Liu, Caderas et al. 2001). It is not essential to have different functionalities attached
to the two heavy chains of the Fc heterodimer. A monobody can also be created (Figure 2).

EXAMPLES

Example 1

25 This example demonstrates that CH3 domains can be engineered to favor
heterodimerization while disfavoring homodimerization using electrostatic steering effects.
A maxibody – dummy Fc construct as shown in Figure 8(a) was made having charge residue
mutations at the CH3 domain interface. The formation of homodimer and heterodimer yield
was assessed through SDS polyacrylamide gel electrophoresis. Because the maxibody has a
30 higher molecular weight compared to dummy Fc, the heterodimer (maxibody-dummy Fc) and
homodimers (maxibody-maxibody & dummy Fc-dummy Fc) have different mobility on the
SDS-PAGE facilitating the identification of the various pairings (FIG. 8(b)).

A rat anti-mouse NKG2D antibody, designated M315, was generated through
conventional hybridoma fusions and the DNA sequences encoding the variable heavy chain

(VH) and variable light chain (VL) were used to construct M315scFv-Fc using previously described method (Gilliland, L. K., N. A. Norris, et al. (1996). "Rapid and reliable cloning of antibody variable regions and generation of recombinant single chain antibody fragments." Tissue Antigens **47**(1): 1-20.)

5 The sequence of M315 scFv-Fc (SEQ ID NO:1) and huIgG1Fc (SEQ ID NO:2) were cloned into the pTT5 mammalian expression vector and the two constructs were used to co-transfect 293-6E cells to assess the formation Fc/scFv-Fc heterodimer relative to Fc homodimer and scFv-Fc homodimer.

10 SEQ ID NO: 1
M315scFv-huFc

HMAEVQLQQSGAELVKPGSSVKISCKASGYTFANNFMHWIKQQPGNGLEW
IGWIIYPGDGDTEYNQKFSGKATLTADKSSSTAYMQLNSLTSEDSAVYFCI
RLTEGTTYWGQGVMTVSSGGGGSGGGGSGGGGSGQFVLTQPNVSTNLGS
15 TVKLSCKRSTGNIGSNYVNWYQQHEGRSPTTMIYRDDKRPDGVPDFSGS
IDGSSNSALLTINNVQTEDEADYFCQSYSRGVSPVFGGGTKLTVLAAAEF
KSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVS
HEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGK
EYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTC
20 LVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRW
QQGNVFSCSVMHEALHNHYTQKSLSLSPGK

SEQ ID NO: 2
huIgG1-Fc

25 EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVS
HEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGK
EYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTC
LVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRW
QQGNVFSCSVMHEALHNHYTQKSLSLSPGK

30 (Shading corresponds to the Fc region)

The charge residue pairs in the CH3 region identified through computational analysis were changed to amino acid of opposite charge polarity on either human IgG1Fc (dummy) or M315 scFv-Fc (mxh) constructs. The mutations, which are listed in Table 4, were generated using the QuikChange® mutagenesis kit from Stratagene and verified by DNA sequencing.

35 The mutations are denoted by wild type residue followed by the position using the Kabat numbering system (Kabat et al., *Sequences of Proteins of Immunological Interest*, National Institutes of Health, Bethesda, Md., ed, 5, [1991]), which is consistent with the crystal structure (PDB code:1L6X) numbering scheme, and then the replacement residue in single letter code. The Fc sequence used in these two constructs was derived from human IgG1
40 non-(a) allotype, which has a Glu at position 356 and a Met at position 358. The CH3

sequences from the crystal structure are from a different IgG1 allotype, which has an Asp at position 356 and a Leu at position 368.

TABLE 4: List of charge residue mutations

huIgG1Fc (dummy)	M315 scFv-Fc(mxb)
Fc-WT	M315 scFv-Fc(WT)
K409D	D399'K
K409E	D399'R
K409D&K360D	D399'K&E356'K
K409D&K370D	D399'K&E357'K
K409D&K392D	D399'K&E356'K&E357'K
K409D&K439D	

5

DNA was transfected into human embryonic kidney cell line 293-6E using Lipofectamine™ 2000 reagent (Invitrogen). The cell culture supernatant was harvested 3-4 days after transfection and analyzed on SDS-PAGE Gels under non-reduced condition. The gel was then transferred to nitrocellulose membrane and subject to western analysis using peroxidase-conjugated goat anti-human IgG antibody (Jackson ImmunoResearch Laboratories) and results are shown in Fig 10.

Co-transfection of expression vector for M315 scFv-Fc (mxb) together with dummy Fc resulted in the formation of scFv-Fc/Fc heterodimer as well as scFv-Fc homodimer and Fc homodimer. The ratio of scFv-Fc/Fc heterodimer to scfv-Fc homodimer and Fc homodimer is close to 1:1:1 when the wild type CH3 sequence is used.

The introduction of one charge pair mutation K409D on dummy Fc and D399'K on M315 maxibody significantly increased the ratio of scFv-Fc/Fc heterodimer relative to scFv-Fc homodimer as well as Fc homodimer. Similar enhancement of heterodimer formation was also observed for other mutant variants such as K409D/D399'R, K409E/D399'K and K409E/D399'R (Fig.9), further underscore the importance of charge polarity complementation for the formation of Fc heterodimers. (The wild type M315 scFv-Fc construct used in this study has an extra tag at the carboxyl terminal of Fc, so it migrates slower on the SDS-PAGE gel.)

When additional mutations were introduced at charge residues that are located near K409 such as K360 and K392, a further increase of heterodimer formation was observed (Fig. 10). For example, the combination K409D;K392D on dummy Fc with D399'K on M315 maxibody showed increased ratio of heterodimer to homodimers, likely due to the

disruption of Fc homodimer. A 25KD band correspond to the size of Fc monomer was detected on all transfections using K409D;K392D dummy Fc (data not shown). Adding another mutation such as D356'K or D357'K on top of D399'K variant of M315 maxibody showed additional improvement. The combination of K409D;K392D on dummy Fc with

5 D399'K;D356'K on M315 maxibody resulted almost exclusive formation of heterodimer. Other combinations such as K409D;K392D/D399'K;D357'K and K409D;K370D/D399'K;D357'K also offered significant improvement over the K409D/D399'K variant.

10 Table 5: Quantification of percentage of homodimer and heterodimer yields for the SDS-PAGE shown in Figure 10.^a

Dummy Fc	WT	K409D;K360D	K409D;K392D	K409D;K392D	K409D;K392D	K409D;K439D	K409D;K370D	T366S;L368A;Y407V (Knob)
M315 scFv-Fc	WT	D399'K	D399'K	D399'K;E356'K	D399'K;E357'K	D399'K;E356'K	D399'K;E357'K	T366'W (Hole)
M315 scFv-Fc Homodimer	25.5	16.8	23.1	ND	ND	ND	ND	13.3
M315 scFv-Fc – Dummy Fc Heterodimer	32.4	55.1	76.9	100	79.1	92.3	85.2	86.7

Dummy Fc Homodimer	42.1	28.1	ND	ND	20.9	7.7	14.8	ND
-----------------------	------	------	----	----	------	-----	------	----

^a ND stands for Not Detectable in the density based analysis.

Example 2

This example demonstrates that CH3 domains containing certain triple charge-pair mutations were unable to form homodimers when expressed alone but were capable of forming heterodimers when co-expressed (Figure 11). Mutants were made and cells transfected as described in Example 1. When the constructs were co-transfected, a 1:1 ratio of plasmids were used. Heterodimer and homodimers were detected by Western blot using goat-anti-human Fc HRP conjugated antibody. Interestingly, Fc-containing molecules having triple mutations wherein positive-charged residues were changed to negative-charged residues (K409D,K392D,K370D or K409D,K392D,K439D) were unable to be detected when expressed alone. Similarly, Fc-containing molecules having triple mutations wherein negative-charged residues were changed to positive-charged residues (D399K,E356K,E357K) were unable to be detected when expressed alone. When co-expressed with an Fc-containing molecule having mutations of opposite charge polarity, however, heterodimers only were detected.

Throughout this invention application, it is to be understood that use of a term in the singular may imply, where appropriate, use of respective term in the plural, and *vice versa*. Atwell, S., J. B. Ridgway, et al. (1997). "Stable heterodimers from remodeling the domain interface of a homodimer using a phage display library." J Mol Biol **270**(1): 26-35.

Bahar, I. and R. L. Jernigan (1997). "Inter-residue potentials in globular proteins and the dominance of highly specific hydrophilic interactions at close separation." J Mol Biol **266**(1): 195-214.

Bernstein, F. C., T. F. Koetzle, et al. (1977). "The Protein Data Bank: a computer-based archival file for macromolecular structures." J Mol Biol **112**(3): 535-42.

Bogan, A. A. and K. S. Thorn (1998). "Anatomy of hot spots in protein interfaces." J Mol Biol **280**(1): 1-9.

- Carter, P. (2001). "Bispecific human IgG by design." J Immunol Methods **248**(1-2): 7-15.
- Crick, F. H. (1952). "Is alpha-keratin a coiled coil?" Nature **170**(4334): 882-3.
- de Leij, L., G. Molema, et al. (1998). "Bispecific antibodies for treatment of cancer in experimental animal models and man." Adv Drug Deliv Rev **31**(1-2): 105-129.
- 5 Deisenhofer, J. (1981). "Crystallographic refinement and atomic models of a human Fc fragment and its complex with fragment B of protein A from *Staphylococcus aureus* at 2.9- and 2.8-A resolution." Biochemistry **20**(9): 2361-70.
- Gabdoulline, R. R. and R. C. Wade (2002). "Biomolecular diffusional association." Curr Opin Struct Biol **12**(2): 204-13.
- 10 Ghetie, V. and E. S. Ward (2000). "Multiple roles for the major histocompatibility complex class I- related receptor FcRn." Annu Rev Immunol **18**: 739-66.
- Halperin, I., H. Wolfson, et al. (2004). "Protein-protein interactions; coupling of structurally conserved residues and of hot spots across interfaces. Implications for docking." Structure **12**(6): 1027-38.
- 15 Huber, R. (1984). "Three-dimensional structure of antibodies." Behring Inst Mitt(76): 1-14.
- Idusogie, E. E., L. G. Presta, et al. (2000). "Mapping of the C1q binding site on rituxan, a chimeric antibody with a human IgG1 Fc." J Immunol **164**(8): 4178-84.
- Joachimiak, L. A., T. Kortemme, et al. (2006). "Computational design of a new hydrogen bond network and at least a 300-fold specificity switch at a protein-protein interface." J Mol Biol **361**(1): 195-208.
- 20 King, D. J., J. R. Adair, et al. (1992). "Expression, purification and characterization of a mouse-human chimeric antibody and chimeric Fab' fragment." Biochem J **281** (Pt 2): 317-23.
- Kortemme, T. and D. Baker (2004). "Computational design of protein-protein interactions." Curr Opin Chem Biol **8**(1): 91-7.
- 25 Kortemme, T., L. A. Joachimiak, et al. (2004). "Computational redesign of protein-protein interaction specificity." Nat Struct Mol Biol **11**(4): 371-9.

- Laemmli, U. K. (1970). "Cleavage of structural proteins during the assembly of the head of bacteriophage T4." Nature **227**(5259): 680-5.
- Lee, B. and F. M. Richards (1971). "The interpretation of protein structures: estimation of static accessibility." J Mol Biol **55**(3): 379-400.
- 5 Liu, N., G. Caderas, et al. (2001). "Fusion proteins from artificial and natural structural modules." Curr Protein Pept Sci **2**(2): 107-21.
- Maizel, J. V., Jr., D. F. Summers, et al. (1970). "SDS-acrylamide gel electrophoresis and its application to the proteins of poliovirus- and adenovirus-infected human cells." J Cell Physiol **76**(3): 273-87.
- 10 Martin, W. L., A. P. West, Jr., et al. (2001). "Crystal structure at 2.8 Å of an FcRn/heterodimeric Fc complex: mechanism of pH-dependent binding." Mol Cell **7**(4): 867-77.
- Marvin, J. S. and H. B. Lowman (2003). "Redesigning an antibody fragment for faster association with its antigen." Biochemistry **42**(23): 7077-83.
- 15 Matthews, B. W. (1995). "Studies on protein stability with T4 lysozyme." Adv Protein Chem **46**: 249-78.
- Merchant, A. M., Z. Zhu, et al. (1998). "An efficient route to human bispecific IgG." Nat Biotechnol **16**(7): 677-81.
- Miller, S. (1990). "Protein-protein recognition and the association of immunoglobulin constant domains." J Mol Biol **216**(4): 965-73.
- 20 Nolan, O. and R. O'Kennedy (1990). "Bifunctional antibodies: concept, production and applications." Biochim Biophys Acta **1040**(1): 1-11.
- Papadea, C. and I. J. Check (1989). "Human immunoglobulin G and immunoglobulin G subclasses: biochemical, genetic, and clinical aspects." Crit Rev Clin Lab Sci **27**(1): 27-58.
- 25

- Pokala, N. and T. M. Handel (2005). "Energy functions for protein design: adjustment with protein-protein complex affinities, models for the unfolded state, and negative design of solubility and specificity." J Mol Biol **347**(1): 203-27.
- 5 Raghavan, M. and P. J. Bjorkman (1996). "Fc receptors and their interactions with immunoglobulins." Annu Rev Cell Dev Biol **12**: 181-220.
- Ridgway, J. B., L. G. Presta, et al. (1996). "'Knobs-into-holes' engineering of antibody CH3 domains for heavy chain heterodimerization." Protein Eng **9**(7): 617-21.
- Roux, K. H. (1999). "Immunoglobulin structure and function as revealed by electron microscopy." Int Arch Allergy Immunol **120**(2): 85-99.
- 10 Schreiber, G., Y. Shaul, et al. (2006). "Electrostatic design of protein-protein association rates." Methods Mol Biol **340**: 235-49.
- Selzer, T., S. Albeck, et al. (2000). "Rational design of faster associating and tighter binding protein complexes." Nat Struct Biol **7**(7): 537-41.
- 15 Sheinerman, F. B., R. Norel, et al. (2000). "Electrostatic aspects of protein-protein interactions." Curr Opin Struct Biol **10**(2): 153-9.
- Sondermann, P., R. Huber, et al. (2000). "The 3.2-A crystal structure of the human IgG1 Fc fragment-Fc gammaRIII complex." Nature **406**(6793): 267-73.
- 20 Sowdhamini, R., N. Srinivasan, et al. (1989). "Stereochemical modeling of disulfide bridges. Criteria for introduction into proteins by site-directed mutagenesis." Protein Eng **3**(2): 95-103.
- Szczepek, M., V. Brondani, et al. (2007). "Structure-based redesign of the dimerization interface reduces the toxicity of zinc-finger nucleases." Nat Biotechnol **25**(7): 786-93.
- 25 Ye, Y. and A. Godzik (2004). "FATCAT: a web server for flexible structure comparison and structure similarity searching." Nucleic Acids Res **32**(Web Server issue): W582-5.

CLAIMS

1. A method of preparing a heterodimeric protein comprising a first CH3-containing polypeptide and a second CH3-containing polypeptide that meet together to form an interface engineered to promote heterodimer formation, wherein said first CH3-containing polypeptide and said second CH3-containing polypeptide comprise one or more charged amino acids within the interface that are electrostatically unfavorable to homodimer formation but electrostatically favorable to heterodimer formation, the method comprising the steps of:
 - (a) culturing a host cell comprising a nucleic acid encoding the first CH3-containing polypeptide and a nucleic acid comprising the second CH3-containing polypeptide, wherein the cultured host cell expresses the first and second CH3-containing polypeptides; and
 - (b) recovering the heterodimeric protein from the host cell culture.
2. The method of claim 1, wherein the heterodimeric protein comprises an Fc region.
3. The method of claim 2, wherein the Fc region comprises an IgG Fc region.
4. The method of claim 3, wherein the IgG Fc region comprises a human IgG Fc region.
5. The method of claim 4, wherein the human IgG region comprises an IgG1 Fc region.
6. The method of claim 4, wherein the IgG region comprises an IgG2 Fc region.
7. The method of claim 4, wherein the IgG region comprises an IgG3 Fc region.
8. The method of claim 4, wherein the IgG region comprises an IgG4 Fc region.
9. The method of claim 2, wherein the Fc region comprises an IgA, IgE, IgD, or IgM Fc region.
10. The method of claim 4, wherein the first CH3-containing polypeptide or the second CH3-containing polypeptide comprises a polypeptide sequence differing from wild-type human IgG such that one or more positive-charged amino acids in a wild-type human IgG sequence are replaced with one or more negative-charged amino acids.

11. The method of claim 10, wherein the positive-charged amino acid is selected from the group consisting of lysine, histidine, and arginine.
12. The method of claim 10, wherein the negative-charged amino acid is selected from the group consisting of aspartic acid and glutamic acid.
13. The method of claim 10, wherein the first CH3-containing polypeptide or the second CH3-containing polypeptide comprises a polypeptide sequence differing from wild-type human IgG such that one or more negative-charged amino acids in a wild-type human IgG sequence is replaced with one or more positive-charged amino acids.
14. The method of claim 13, wherein the positive-charged amino acid is selected from the group consisting of lysine, histidine, and arginine.
15. The method of claim 13, wherein the negative-charged amino acid is selected from the group consisting of aspartic acid and glutamic acid.
16. The method of claim 5, wherein the IgG1 Fc comprises a first CH3-containing polypeptide or second CH3-containing polypeptide having a polypeptide sequence differing from wild-type human IgG1 such that one or more positive-charged amino acids in a wild-type human IgG1 sequence are replaced with one or more negative-charged amino acids.
17. The method of claim 16, wherein the first CH3-containing polypeptide comprises a replacement of a lysine with a negative-charged amino acid.
18. The method of claim 17, wherein the lysine is selected from the group consisting of Lys409, Lys392, Lys439, and Lys370.
19. The method of claim 5, wherein the IgG1 Fc comprises a first CH3-containing polypeptide or second CH3-containing polypeptide having a polypeptide sequence differing from wild-type human IgG1 such that one or more negative-charged amino acids in a wild-type human IgG1 sequence are replaced with one or more positive-charged amino acids.

20. The method of claim 19, wherein the first CH₃-containing polypeptide comprises a replacement of an aspartic acid with a positive-charged amino acid.
21. The method of claim 20, wherein the aspartic acid is selected from the group consisting of Asp399 and Asp356.
22. The method of claim 19, wherein the first CH₃-containing polypeptide comprises a replacement of a glutamic acid with a positive-charged amino acid.
23. The method of claim 22, wherein the glutamic acid is Glu357.
24. The method of claim 18, wherein the first CH₃-containing polypeptide comprises a replacement of Lys409 with a negative-charged amino acid and the second CH₃-containing polypeptide comprises a replacement of Asp399, Asp356, or Glu357 with a positive-charged amino acid.
25. The method of claim 18, wherein the first CH₃-containing polypeptide comprises a replacement of Lys392 with a negative-charged amino acid and the second CH₃-containing polypeptide comprises a replacement of Asp399, Asp356, or Glu357 with a positive-charged amino acid.
26. The method of claim 18, wherein the first CH₃-containing polypeptide comprises a replacement of Lys439 with a negative-charged amino acid and the second CH₃-containing polypeptide comprises a replacement of Asp399, Asp356, or Glu357 with a positive-charged amino acid.
27. The method of claim 18, wherein the first CH₃-containing polypeptide comprises a replacement of Lys370 with a negative-charged amino acid and the second CH₃-containing polypeptide comprises a replacement of Asp399, Asp356, or Glu357 with a positive-charged amino acid.
28. The method of claim 20, wherein the first CH₃-containing polypeptide comprises a replacement of Asp399 with a positive-charged amino acid and the second CH₃-

containing polypeptide comprises a replacement of Lys409, Lys439, Lys370, or Lys392 with a negative-charged amino acid.

29. The method of claim 20, wherein the first CH3-containing polypeptide comprises a replacement of Asp356 with a positive-charged amino acid and the second CH3-containing polypeptide comprises a replacement of Lys409, Lys439, Lys370, or Lys392 with a negative-charged amino acid.
30. The method of claim 23, wherein the first CH3-containing polypeptide comprises a replacement of Glu357 with a positive-charged amino acid and the second CH3-containing polypeptide comprises a replacement of Lys409, Lys439, Lys370, or Lys392 with a negative-charged amino acid.
31. The method of claim 1, wherein the first CH3-containing polypeptide is an antibody heavy chain.
32. The method of claim 1, wherein the second CH3-containing polypeptide is an antibody heavy chain.
33. The method of claim 1, wherein the host cell expresses one or more antibody light chains.
34. The method of claim 1, wherein the heterodimeric protein is selected from the group consisting of an antibody, a bispecific antibody, a monospecific monovalent antibody, a bispecific maxibody, a monobody, a peptibody, a bispecific peptibody, a monovalent peptibody, and a receptor fusion protein.
35. A host cell comprising a nucleic acid encoding a first CH3-containing polypeptide and a nucleic acid comprising a second CH3-containing polypeptide, wherein the first CH3-containing polypeptide and the second CH3-containing polypeptide meet together to form an interface engineered to promote heterodimer formation, and wherein said first CH3-containing polypeptide and said second CH3-containing polypeptide comprise one or more charged amino acids within the interface that are

electrostatically unfavorable to homodimer formation but electrostatically favorable to heterodimer formation.

36. A composition comprising a nucleic acid encoding a first CH3-containing polypeptide and a nucleic acid comprising a second CH3-containing polypeptide, wherein the first CH3-containing polypeptide and the second CH3-containing polypeptide meet together to form an interface engineered to promote heterodimer formation, and wherein said first CH3-containing polypeptide and said second CH3-containing polypeptide comprise one or more charged amino acids within the interface that are electrostatically unfavorable to homodimer formation but electrostatically favorable to heterodimer formation.
37. A heterodimeric protein comprising a first CH3-containing polypeptide and a second CH3-containing polypeptide that meet together to form an interface engineered to promote heterodimer formation, wherein said first CH3-containing polypeptide and said second CH3-containing polypeptide comprise one or more charged amino acids within the interface that are electrostatically unfavorable to homodimer formation but electrostatically favorable to heterodimer formation.
38. The heterodimeric protein of claim 37, wherein the heterodimeric protein comprises an Fc region.
39. The heterodimeric protein of claim 38, wherein the Fc region comprises an IgG Fc region.
40. The heterodimeric protein of claim 39, wherein the IgG Fc region comprises a human IgG Fc region.
41. The heterodimeric protein of claim 40, wherein the human IgG region comprises an IgG1 Fc region.
42. The heterodimeric protein of claim 40, wherein the IgG region comprises an IgG2 Fc region.

43. The heterodimeric protein of claim 40, wherein the IgG region comprises an IgG3 Fc region.
44. The heterodimeric protein of claim 40, wherein the IgG region comprises an IgG4 Fc region.
45. The heterodimeric protein of claim 38, wherein the Fc region comprises an IgA, IgE, IgD, or IgM Fc region.
46. The heterodimeric protein of claim 40, wherein the first CH3-containing polypeptide or the second CH3-containing polypeptide comprises a polypeptide sequence differing from wild-type human IgG such that one or more positive-charged amino acids in a wild-type human IgG sequence are replaced with one or more negative-charged amino acids.
47. The heterodimeric protein of claim 46, wherein the positive-charged amino acid is selected from the group consisting of lysine, histidine, and arginine.
48. The heterodimeric protein of claim 46, wherein the negative-charged amino acid is selected from the group consisting of aspartic acid and glutamic acid.
49. The heterodimeric protein of claim 46, wherein the first CH3-containing polypeptide or the second CH3-containing polypeptide comprises a polypeptide sequence differing from wild-type human IgG such that one or more negative-charged amino acids in a wild-type human IgG sequence is replaced with one or more positive-charged amino acids.
50. The heterodimeric protein of claim 49, wherein the positive-charged amino acid is selected from the group consisting of lysine, histidine, and arginine.
51. The heterodimeric protein of claim 49, wherein the negative-charged amino acid is selected from the group consisting of aspartic acid and glutamic acid.
52. The heterodimeric protein of claim 41, wherein the IgG1 Fc comprises a first CH3-containing polypeptide or second CH3-containing polypeptide having a polypeptide

sequence differing from wild-type human IgG1 such that one or more positive-charged amino acids in a wild-type human IgG1 sequence are replaced with one or more negative-charged amino acids.

53. The heterodimeric protein of claim 52, wherein the first CH3-containing polypeptide comprises a replacement of a lysine with a negative-charged amino acid.
54. The heterodimeric protein of claim 53, wherein the lysine is selected from the group consisting of Lys409, Lys392, Lys439, and Lys370.
55. The heterodimeric protein of claim 41, wherein the IgG1 Fc comprises a first CH3-containing polypeptide or second CH3-containing polypeptide having a polypeptide sequence differing from wild-type human IgG1 such that one or more negative-charged amino acids in a wild-type human IgG1 sequence are replaced with one or more positive-charged amino acids.
56. The heterodimeric protein of claim 55, wherein the first CH3-containing polypeptide comprises a replacement of an aspartic acid with a positive-charged amino acid.
57. The heterodimeric protein of claim 56, wherein the aspartic acid is selected from the group consisting of Asp399 and Asp356.
58. The heterodimeric protein of claim 55, wherein the first CH3-containing polypeptide comprises a replacement of a glutamic acid with a positive-charged amino acid.
59. The heterodimeric protein of claim 58, wherein the glutamic acid is Glu357.
60. The heterodimeric protein of claim 54, wherein the first CH3-containing polypeptide comprises a replacement of Lys409 with a negative-charged amino acid and the second CH3-containing polypeptide comprises a replacement of Asp399, Asp356, or Glu357 with a positive-charged amino acid.
61. The heterodimeric protein of claim 54, wherein the first CH3-containing polypeptide comprises a replacement of Lys392 with a negative-charged amino acid and the

- second CH3-containing polypeptide comprises a replacement of Asp399, Asp356, or Glu357 with a positive-charged amino acid.
62. The heterodimeric protein of claim 54, wherein the first CH3-containing polypeptide comprises a replacement of Lys439 with a negative-charged amino acid and the second CH3-containing polypeptide comprises a replacement of Asp399, Asp356, or Glu357 with a positive-charged amino acid.
63. The heterodimeric protein of claim 54, wherein the first CH3-containing polypeptide comprises a replacement of Lys370 with a negative-charged amino acid and the second CH3-containing polypeptide comprises a replacement of Asp399, Asp356, or Glu357 with a positive-charged amino acid.
64. The heterodimeric protein of claim 57, wherein the first CH3-containing polypeptide comprises a replacement of Asp399 with a positive-charged amino acid and the second CH3-containing polypeptide comprises a replacement of Lys409, Lys439, Lys370, or Lys392 with a negative-charged amino acid.
65. The heterodimeric protein of claim 57, wherein the first CH3-containing polypeptide comprises a replacement of Asp356 with a positive-charged amino acid and the second CH3-containing polypeptide comprises a replacement of Lys409, Lys439, Lys370, or Lys392 with a negative-charged amino acid.
66. The heterodimeric protein of claim 59, wherein the first CH3-containing polypeptide comprises a replacement of Glu357 with a positive-charged amino acid and the second CH3-containing polypeptide comprises a replacement of Lys409, Lys439, Lys370, or Lys392 with a negative-charged amino acid.
67. The heterodimeric protein of claim 37, wherein the first CH3-containing polypeptide is an antibody heavy chain.
68. The heterodimeric protein of claim 37, wherein the second CH3-containing polypeptide is an antibody heavy chain.

69. The heterodimeric protein of claim 37, wherein the heterodimeric protein further comprises one or more antibody light chains.
70. The heterodimeric protein of claim 37, wherein the heterodimeric protein is selected from the group consisting of an antibody, a bispecific antibody, a monospecific monovalent antibody, a bispecific maxibody, a monobody, a peptibody, a bispecific peptibody, a monovalent peptibody, and a receptor fusion protein.
71. A polypeptide comprising a CH3 region of an antibody, wherein the CH3 region comprises a polypeptide sequence differing from a wild-type CH3 region such that one or more positive-charged amino acids in wild-type CH3 are replaced with one or more negative-charged amino acids, wherein the polypeptide has decreased ability to form homodimers compared to a polypeptide comprising a wild-type CH3 region.
72. A polypeptide comprising a CH3 region of an antibody, wherein the CH3 region comprises a polypeptide sequence differing from a wild-type CH3 region such that one or more negative-charged amino acids in wild-type CH3 are replaced with one or more positive-charged amino acids, wherein the polypeptide has decreased ability to form homodimers compared to a polypeptide comprising a wild-type CH3 region.
73. The polypeptide of claim 71, further comprising a CH2 region.
74. The polypeptide of claim 72, further comprising a CH2 region.
75. The polypeptide of claim 73, wherein the polypeptide comprises an antibody heavy chain.
76. The polypeptide of claim 74, wherein the polypeptide comprises an antibody heavy chain.
77. An antibody comprising the polypeptide of claim 75.
78. An antibody comprising the polypeptide of claim 76.

- 79. An isolated nucleic acid encoding a polypeptide of any of claims 71-76.
- 80. An expression vector comprising the isolated nucleic acid of claim 79 operably linked to a promoter.
- 81. A host cell containing the expression vector of claim 80.
- 82. A pharmaceutical composition comprising a polypeptide of any of claim 71-76.

ABSTRACT

The invention relates to methods of making Fc-heterodimeric proteins or polypeptides. The invention also relates to the Fc-heterodimeric proteins or polypeptides themselves, including the individual polypeptide components that comprise the heterodimer. Nucleic acids encoding such polypeptides, expression vectors, and host cells. Moreover, the invention relates to pharmaceutical compositions comprising one of more Fc-heterodimeric proteins or polypeptides.

Electronic Patent Application Fee Transmittal				
Application Number:				
Filing Date:				
Title of Invention:		METHOD FOR MAKING ANTIBODY FC-HETERODIMERIC MOLECULES USING ELECTROSTATIC STEERING EFFECTS		
First Named Inventor/Applicant Name:		Gunasekaran Kannan		
Filer:		Thomas J. Wrona/Nanci M. Kertson		
Attorney Docket Number:		A-1392-US-PSP2		
Filed as Large Entity				
Provisional Filing Fees				
Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Basic Filing:				
Provisional application filing	1005	1	220	220
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Miscellaneous:				
Total in USD (\$)				220

Electronic Acknowledgement Receipt	
EFS ID:	4406134
Application Number:	61120305
International Application Number:	
Confirmation Number:	8756
Title of Invention:	METHOD FOR MAKING ANTIBODY FC-HETERODIMERIC MOLECULES USING ELECTROSTATIC STEERING EFFECTS
First Named Inventor/Applicant Name:	Gunasekaran Kannan
Customer Number:	37500
Filer:	Thomas J. Wrona/Nanci M. Kertson
Filer Authorized By:	Thomas J. Wrona
Attorney Docket Number:	A-1392-US-PSP2
Receipt Date:	05-DEC-2008
Filing Date:	
Time Stamp:	19:19:03
Application Type:	Provisional

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	\$220
RAM confirmation Number	2792
Deposit Account	010519
Authorized User	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
-----------------	----------------------	-----------	----------------------------------	------------------	------------------

1		A-1392-US-PSP2_specification.pdf	262345 51af30213049b188a68c598147416a9f56e20f10	yes	30
	Multipart Description/PDF files in .zip description				
	Document Description		Start	End	
	Specification		1	19	
	Claims		20	29	
	Abstract		30	30	
Warnings:					
Information:					
2	Drawings-only black and white line drawings	A1392USPSP2_Figures.pdf	2588218 a12a1bda59b53242a30c0902a4bc299d4ee8a729	no	13
Warnings:					
Information:					
3	Fee Worksheet (PTO-06)	fee-info.pdf	29927 0763500ef65ef8f6fdded19d6185eed39d7402d49	no	2
Warnings:					
Information:					
Total Files Size (in bytes):			2880490		
This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503. <u>New Applications Under 35 U.S.C. 111</u> If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application. <u>National Stage of an International Application under 35 U.S.C. 371</u> If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course. <u>New International Application Filed with the USPTO as a Receiving Office</u> If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.					

Electronic Acknowledgement Receipt	
EFS ID:	4407075
Application Number:	61120305
International Application Number:	
Confirmation Number:	8756
Title of Invention:	METHOD FOR MAKING ANTIBODY FC-HETERODIMERIC MOLECULES USING ELECTROSTATIC STEERING EFFECTS
First Named Inventor/Applicant Name:	Gunasekaran Kannan
Customer Number:	37500
Filer:	Thomas J. Wrona/Nanci M. Kertson
Filer Authorized By:	Thomas J. Wrona
Attorney Docket Number:	A-1392-US-PSP2
Receipt Date:	05-DEC-2008
Filing Date:	
Time Stamp:	19:39:06
Application Type:	Provisional

Payment information:

Submitted with Payment	no
------------------------	----

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Provisional Cover Sheet (SB16)	A-1392-US-PSP2_cover_letter.pdf	309725 f5f3f01965d93a8587e96581ff48ab6e6af8fb0	no	1

Warnings:

This is not a USPTO supplied Provisional Cover Sheet SB16 form.

Information:	
Total Files Size (in bytes):	309725
This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503. <u>New Applications Under 35 U.S.C. 111</u> If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application. <u>National Stage of an International Application under 35 U.S.C. 371</u> If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course. <u>New International Application Filed with the USPTO as a Receiving Office</u> If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.	

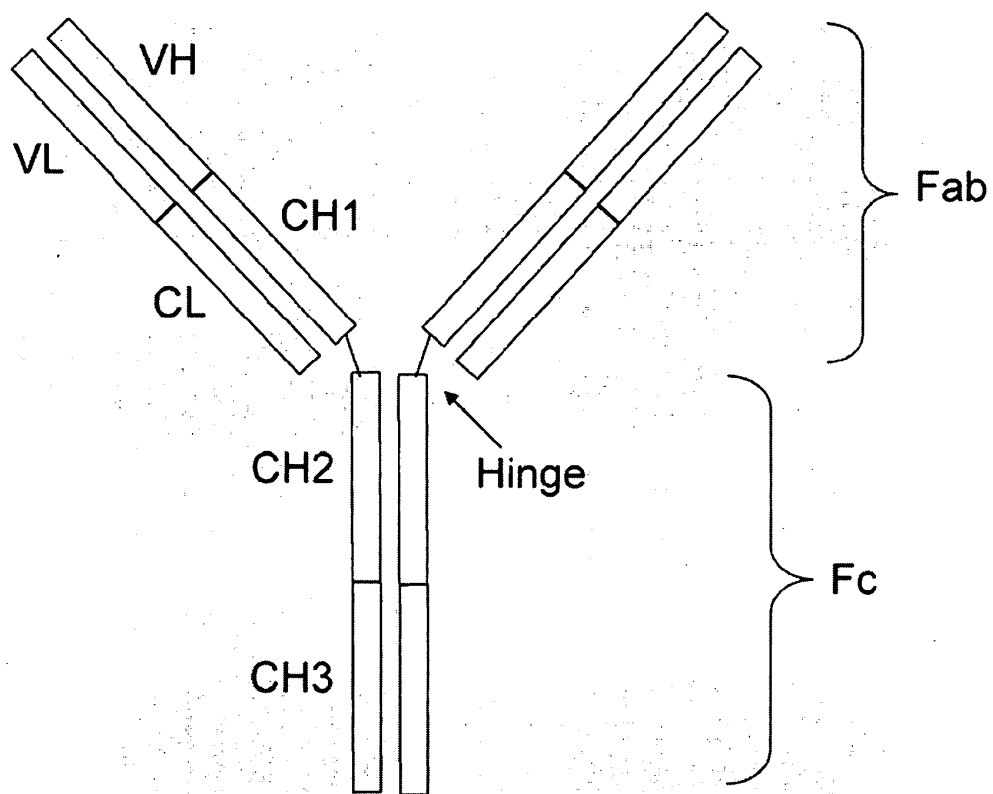


Figure 1

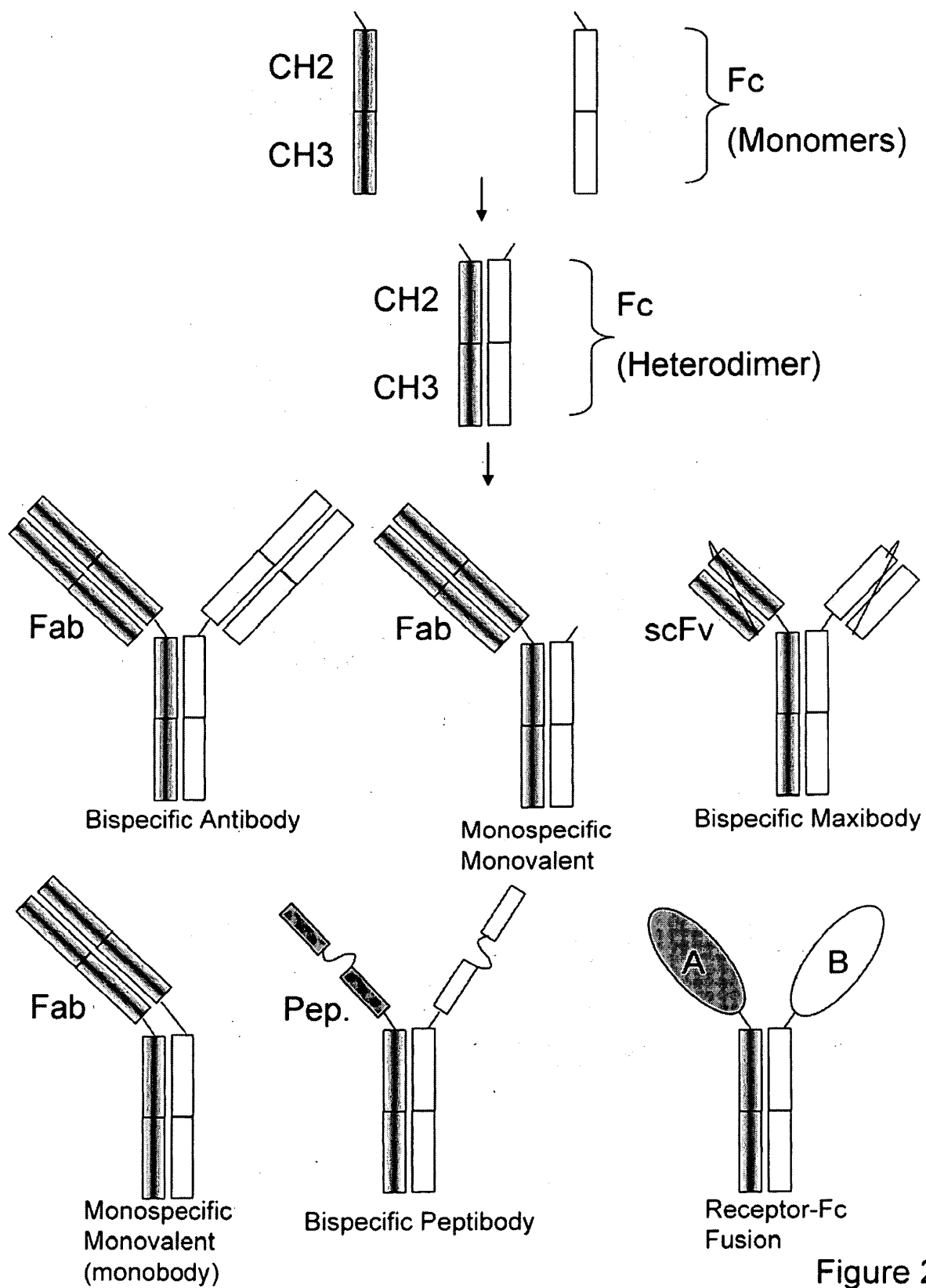


Figure 2

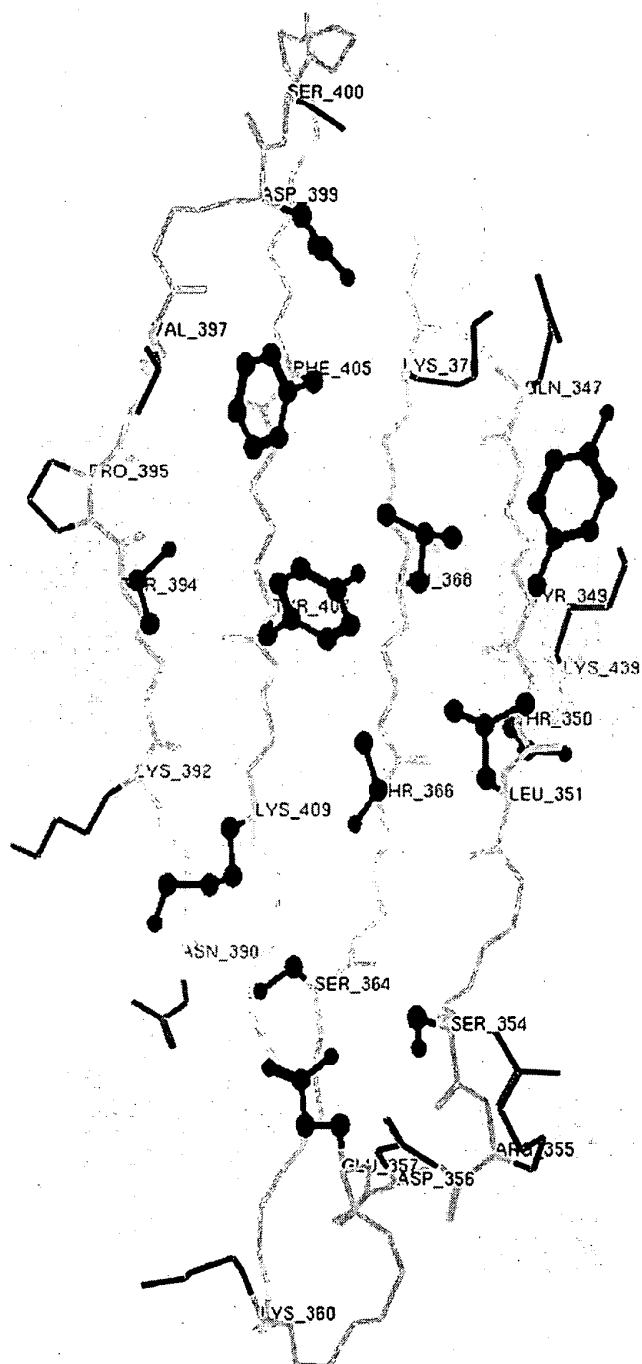


Figure 3

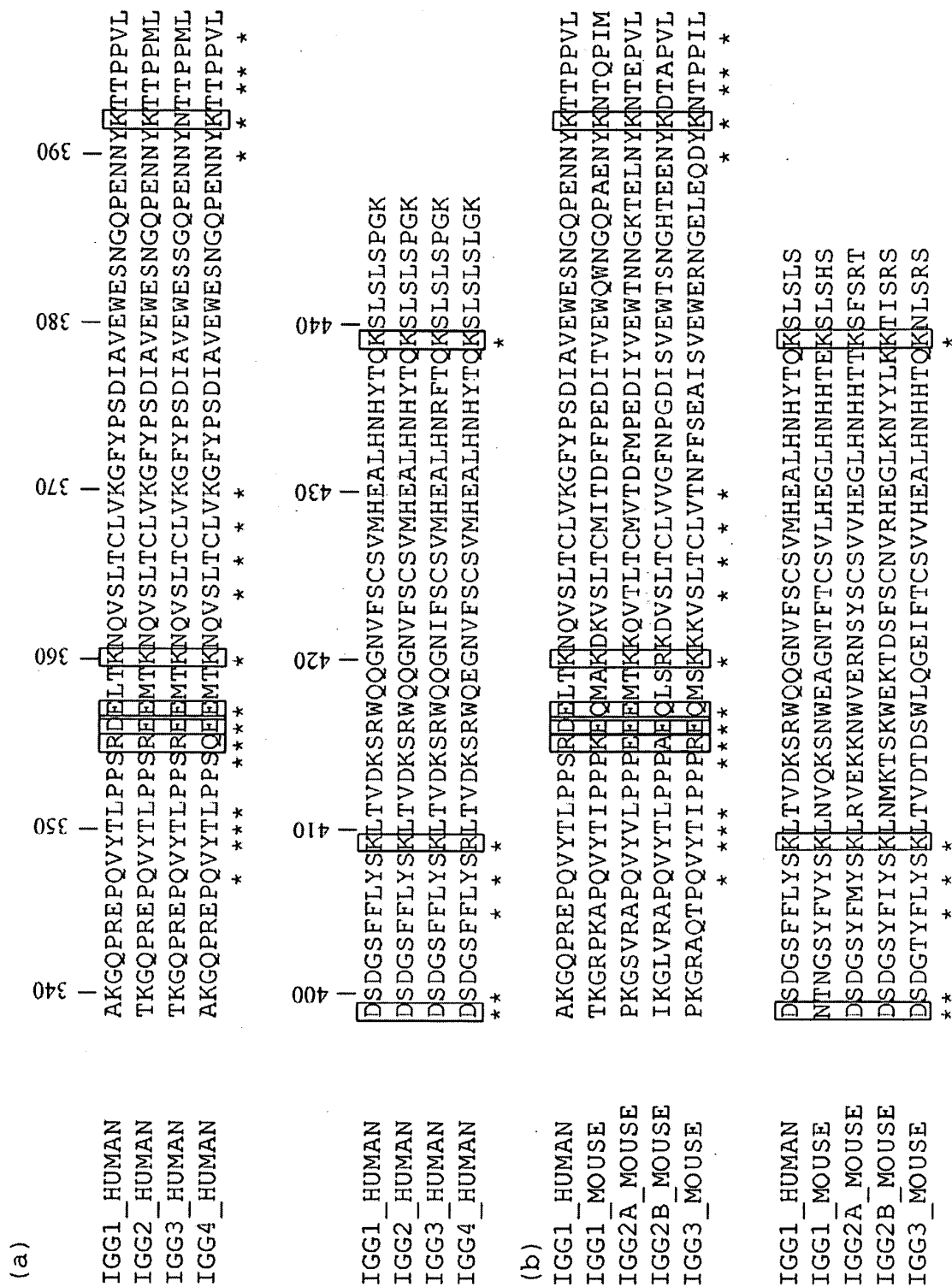


Figure 4

(C)

IGG1_HUMAN
IGA_HUMAN
IGE_HUMAN
IGD_HUMAN
IGM_HUMAN

AKQP-REPQVYTLPPSRDELTKNQ-VSLTCLVKGFYPSDIAVEWESNGQ--PENNYKTT
-SGNT-FRPEVHLLPPPSEELALNELVLTCLARGFSPKDVLRWLQGSQELPREKYLTW
TSGPR-AAPEVYAFATPEWPGSRDK-RTLACLIQNFMPEDISVQWLHNEVQLPDAHSHST
REPAA-QAPVKLSLNLASSDPPEAASWLLCEVSGFSPPNLLMWLEDQREVNTSGFAPA
PKGVALHRPDVYLLPPAREQNLRESATITCLVTGFSPADV FVQWMQRGQPLSPEKYVTS
* * * * *

IGG1_HUMAN
IGA_HUMAN
IGE_HUMAN
IGD_HUMAN
IGM_HUMAN

PPVLD---SDGSFFLYSKLTVDKSRWQQGNVFCSCVMHEAL-HNHYTQKSLSL----
ASRQEPSQGTTFFAVTSILRVAEDWKKGDTFSCVMGHEAL-PLAFTQKTI DRLAGK
QPRKT---KSGGFFVFSRLEVTRAWEQKDEFICRAVHEAASPQTQRAVSVNPGK
RPPQP--GSTTFWAVSVLRVPAPPSPQPATYTCVVSHEDSRTLINASRSLEVSVYT
APMPEP-QAPGRYFAHSILTVSEEEWNTGETYTCVVAHEAL-PNRVTERTVDKSTGK
* * * * *

Figure 4

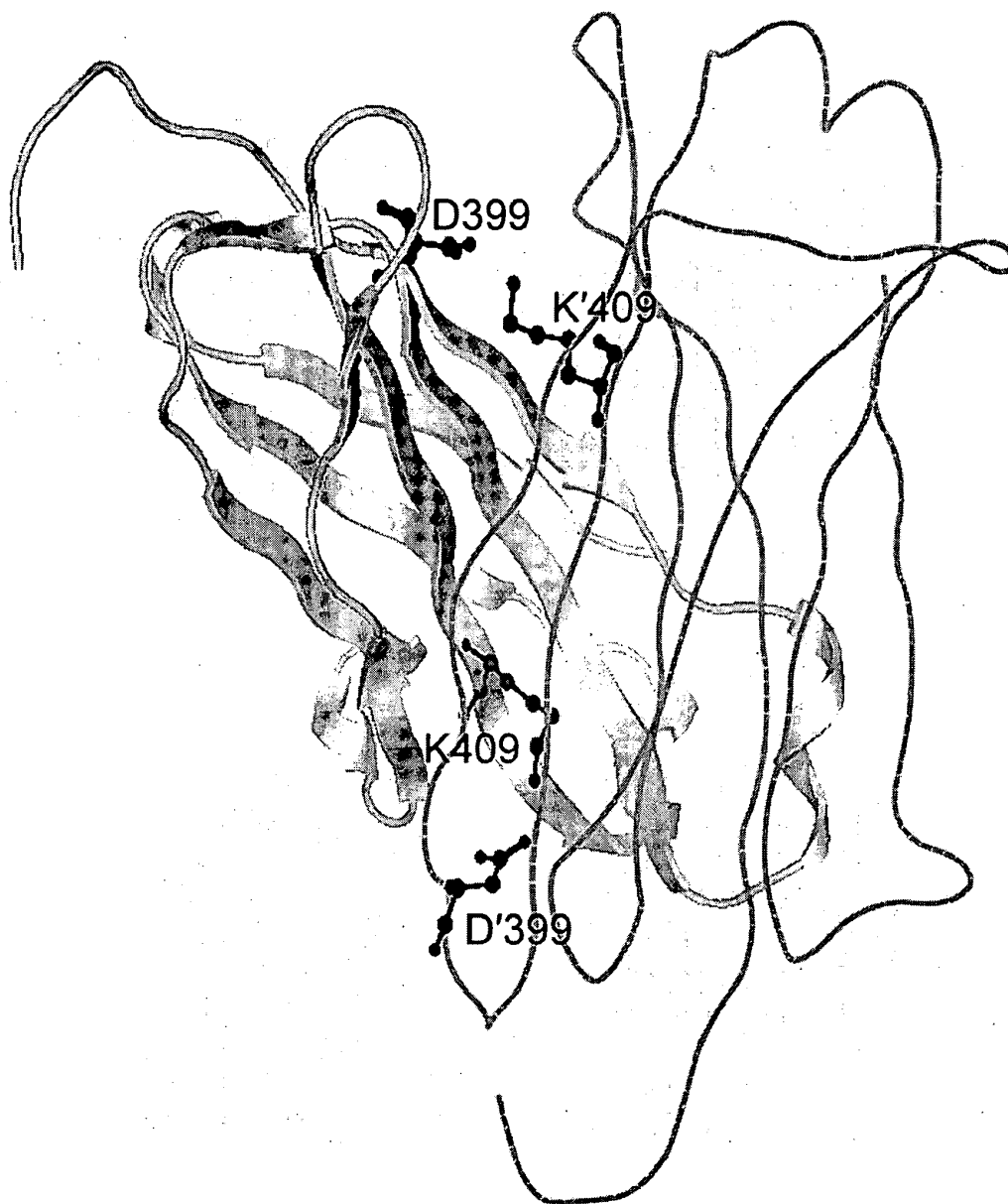


Figure 5

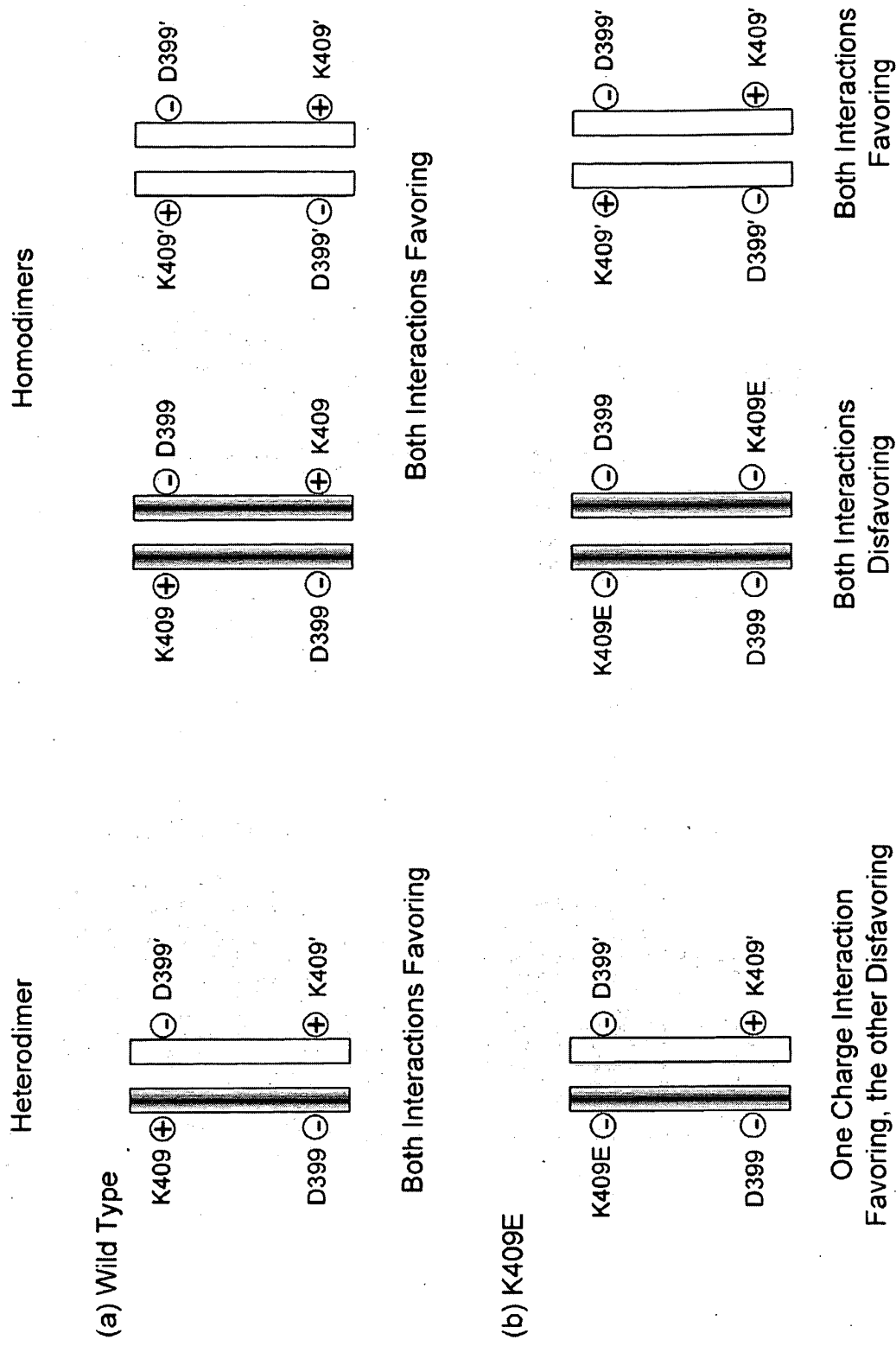
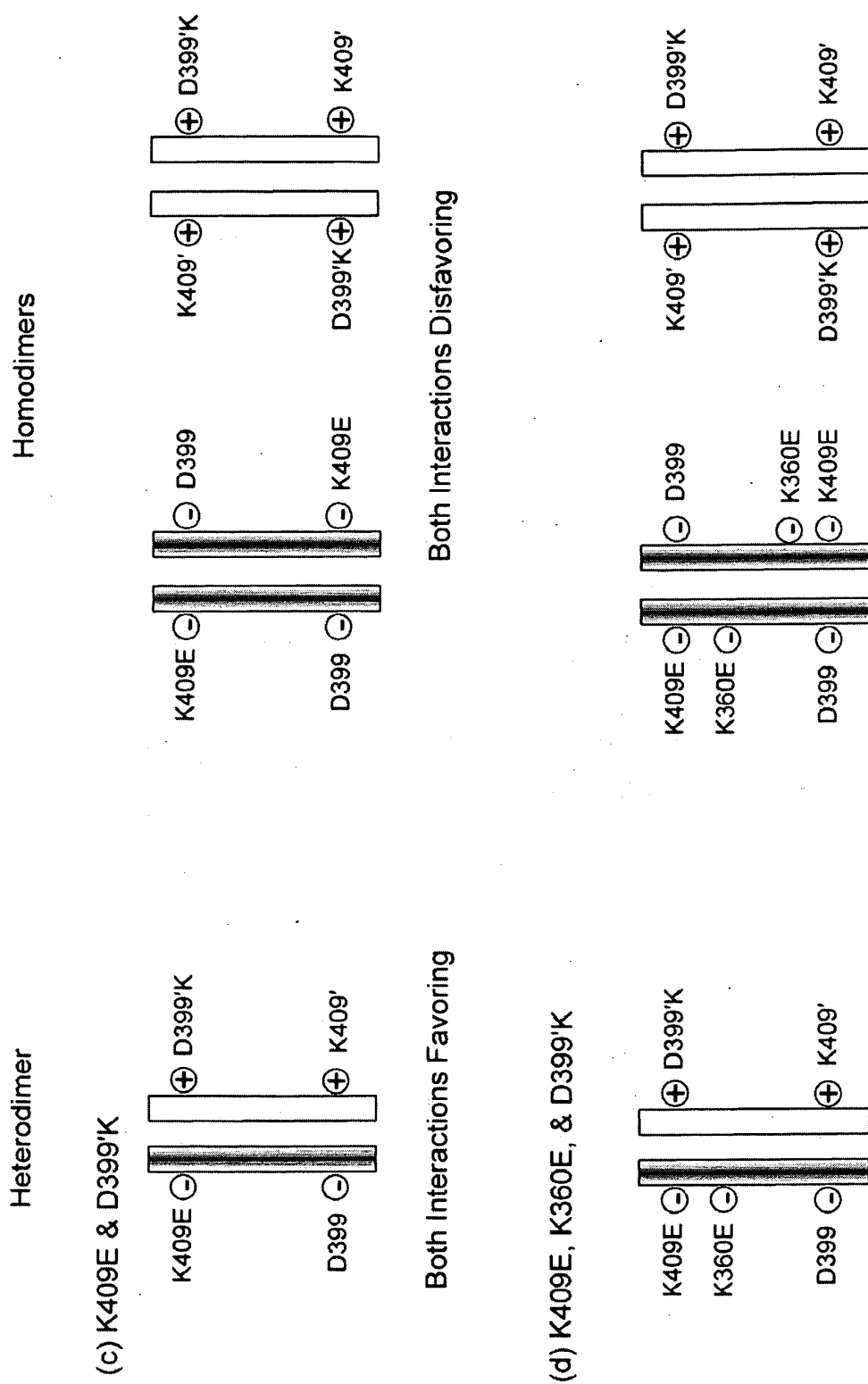


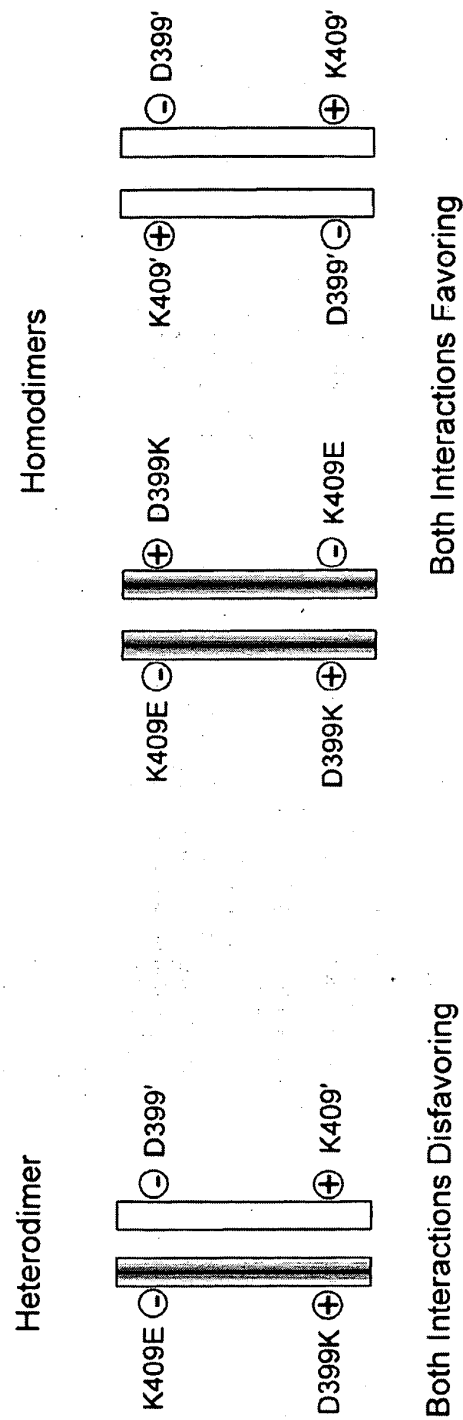
Figure 6



Electrostatic Interactions Favoring

Electrostatic Interactions Disfavoring (strongly in one homodimer case)

Figure 6



K409E & D399K Mutations as An Example for Promoting Homodimers

Figure 7

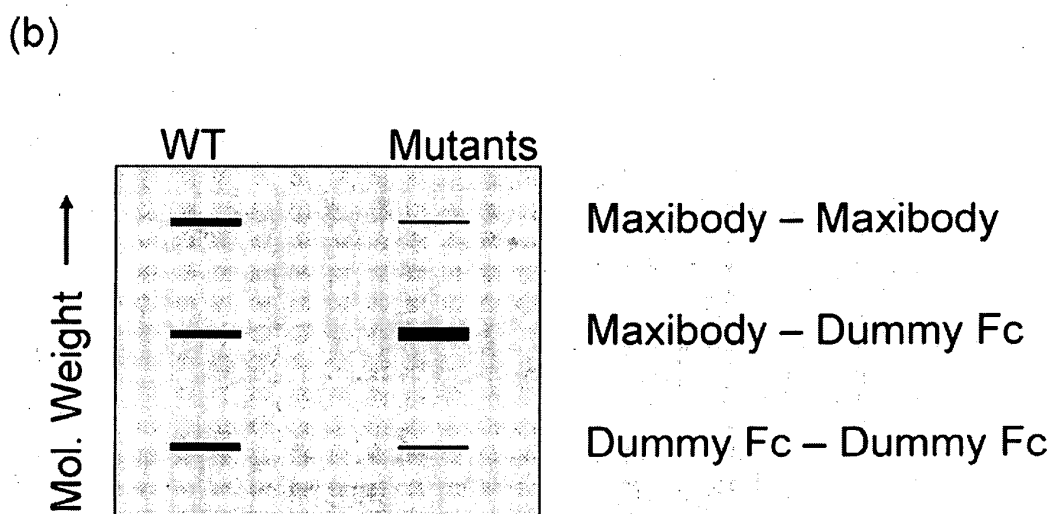
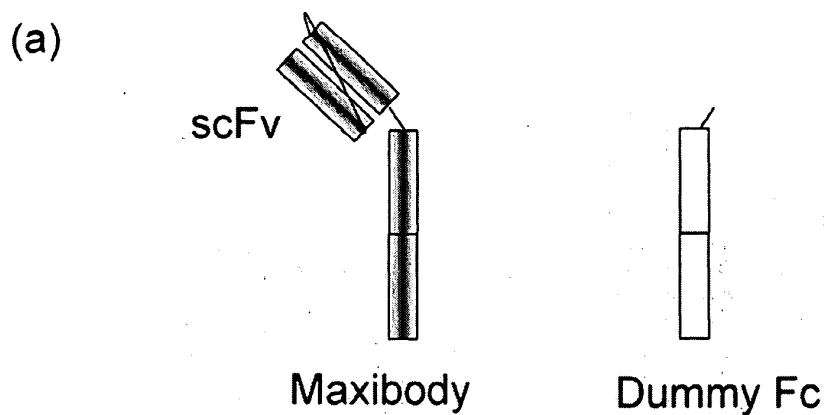


Figure 8

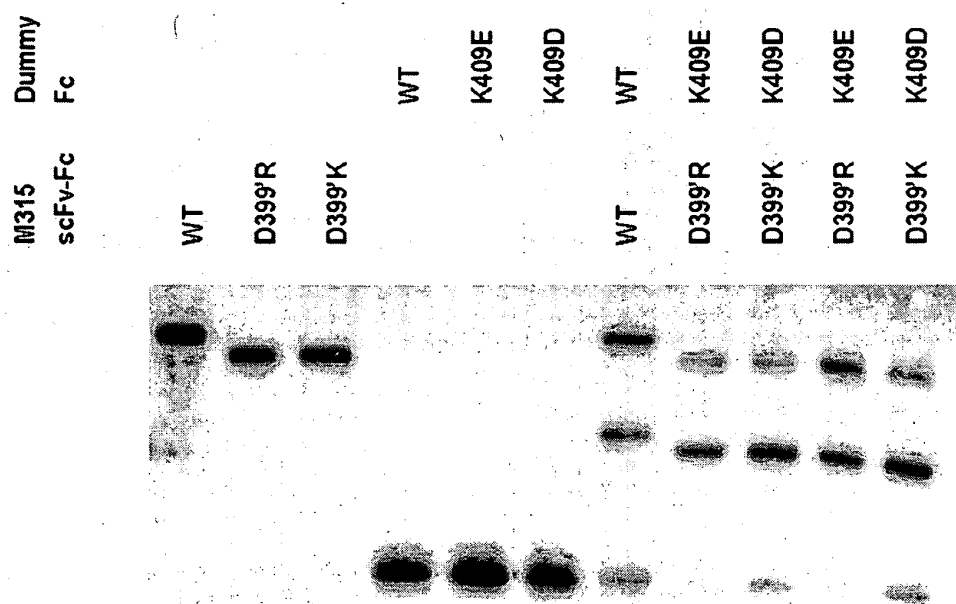


Figure 9

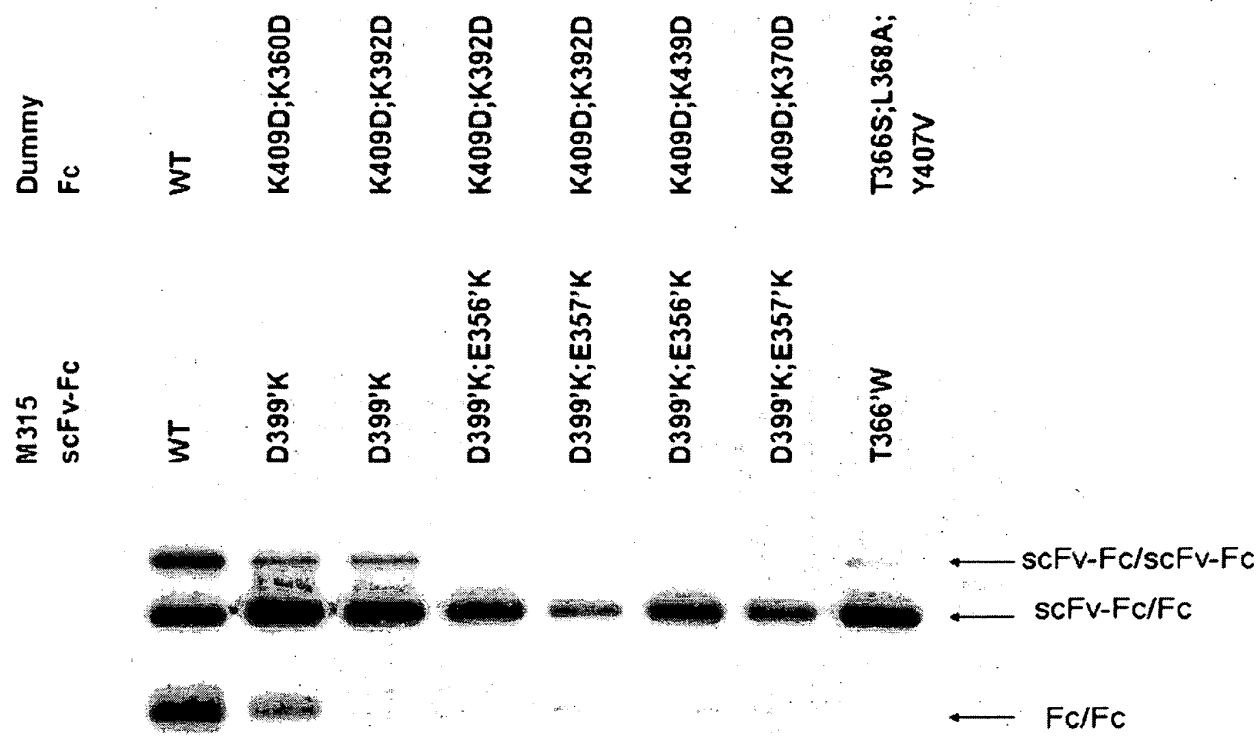


Figure 10

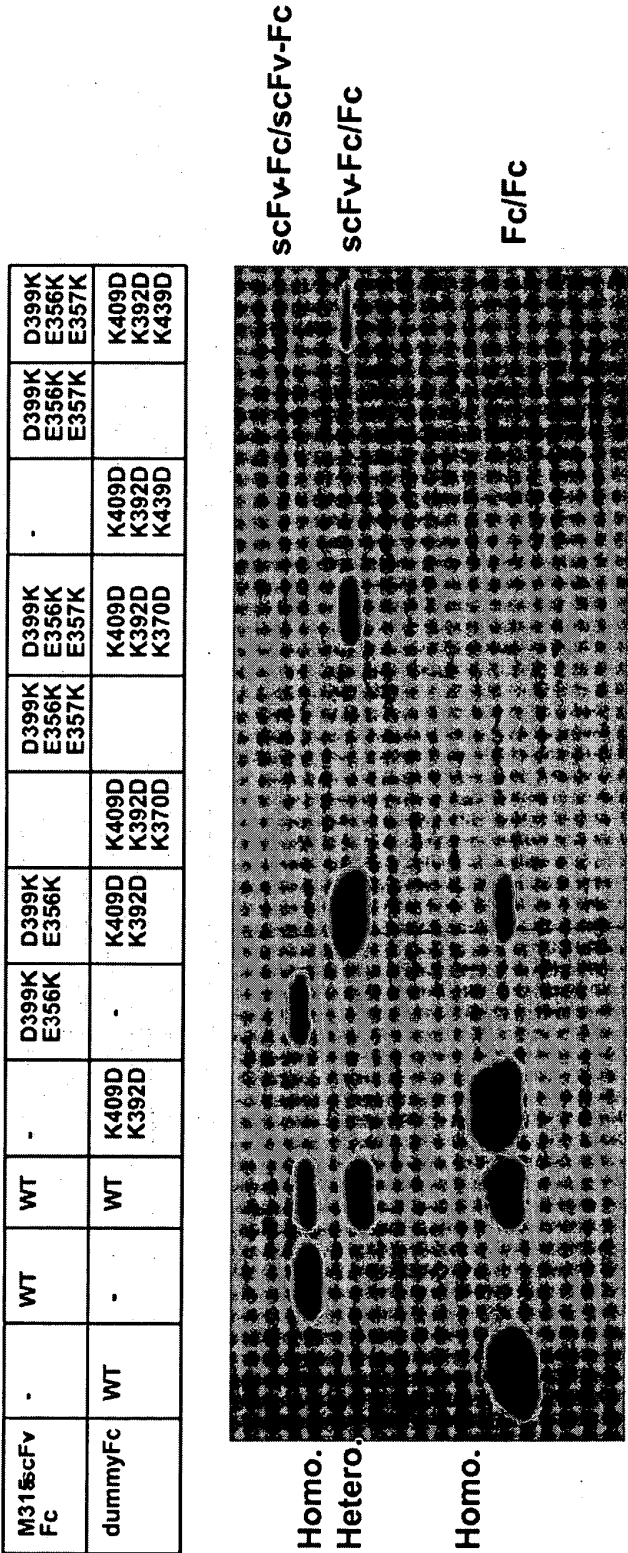
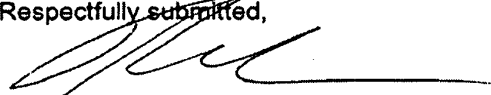
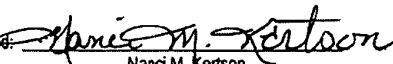


Figure 11

PROVISIONAL APPLICATION TRANSMITTAL LETTER			Docket No.: A-1392-US-PSP2			
To the Commissioner for Patents: This is a request for filing a PROVISIONAL APPLICATION under 37 CFR 1.53(c).						
Inventor(s)/Applicant(s)						
Last Name	First Name	Initial	Residence Address (City & either State or Foreign Country)			
KANNAN	Gunasekaran		Issaquah, Washington, USA			
WITTEKIND	Michael		Bainbridge Island, Washington, USA			
YAN	Wei		Sammamish, Washington, USA			
PENTONY	Martin		Seattle, Washington, USA			
Title of the Invention (280 characters max): METHOD FOR MAKING ANTIBODY FC-HETERODIMERIC MOLECULES USING ELECTROSTATIC STEERING EFFECTS						
Enclosed application parts are: ☒ 19 pages of specification, <u>10</u> pages of claim(s), <u>1</u> page of abstract, and <u>0</u> pages of sequence listing, totaling 31 pages ☒ 13 sheets of drawings ☐ Other:						
Fee Calculations: ☐ Provisional filing fee amount \$ ☐ Excess Page Fees:						
Total Pages		Number Extra		Number of sets of 50 pages or fractions thereof	Rate	Fee
43	- 100 =	0	/50 =	0	x \$260.00 =	\$ 210.00
Method of Payment:						
Respectfully submitted,  Thomas J. Wrona Attorney for Applicants Registration No.: 44,410 Phone: (206) 265-7374 Date: December 5, 2008 Please send all future correspondence to: 37500 Amgen Inc. Law Department 1201 Amgen Court West Seattle, Washington 98119-3105 (206) 265-7000						

CERTIFICATE OF ELECTRONIC TRANSMISSION

I hereby certify that the this correspondence, and all referenced documents, is being electronically transmitted to the United States Patent and Trademark Office, and is addressed to Commissioner for Patents, Arlington, Virginia 22313-1450, on the date appearing below.

Signed: 
Nanci M. Kertson

Date: Dec. 5, 2008



UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY. DOCKET NO	TOT CLAIMS	IND CLAIMS
61/120,305	12/05/2008		220	A-1392-US-PSP2		

CONFIRMATION NO. 8756

37500
AMGEN INC.
LAW DEPARTMENT
1201 AMGEN COURT WEST
SEATTLE, WA 98119

FILING RECEIPT



Date Mailed: 01/05/2009

Receipt is acknowledged of this provisional patent application. It will not be examined for patentability and will become abandoned not later than twelve months after its filing date. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Applicant(s)

Gunasekaran Kannan, Issaquah, WA;
Michael Wittekind, Bainbridge Island, WA;
Wei Yan, Sammamish, WA;
Martin Pentony, Seattle, WA;

Power of Attorney: None

If Required, Foreign Filing License Granted: 12/30/2008

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 61/120,305**

Projected Publication Date: None, application is not eligible for pre-grant publication

Non-Publication Request: No

Early Publication Request: No

Title

METHOD FOR MAKING ANTIBODY FC-HETERODIMERIC MOLECULES USING
ELECTROSTATIC STEERING EFFECTS

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing

of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at <http://www.uspto.gov/web/offices/pac/doc/general/index.html>.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, <http://www.stopfakes.gov>. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4158).

LICENSE FOR FOREIGN FILING UNDER

Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of

State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign Assets Control, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).