(54) Title: SITE SPECIFIC PEGYLATION OF BROADLY-NEUTRALIZING ANTIBODIES AGAINST HUMAN IMMUNODEFICIENCY VIRUS SELECTED BY ENV-CD4-RECEPTOR COMPLEXES

(57) Abstract: The present invention relates to the pegylation of therapeutic antibodies to treat HIV infections. More specifically, the invention relates to site-specific pegylation of X5 scFv antibody fragments at specifically defined amino acid residue(s) that is optionally engineered away from the antigen binding sites. The pegylated-MAb derivatives show substantially improved pharmacokinetic and pharmacodynamic properties, and they are broadly-neutralizing against several HIV clinical isolates by selectively binding to a highly conserved CD4-inducible epitope of HIV gp120.
SITE SPECIFIC PEGYLATION OF BROADLY-NEUTRALIZING ANTIBODIES AGAINST HUMAN IMMUNODEFICIENCY VIRUS SELECTED BY ENV-CD4-RECEPTOR COMPLEXES

FIELD OF THE INVENTION

The present invention relates to site-directed pegylation of broadly-neutralizing MAb derivatives and their use as therapeutic drugs for the treatment of chronic human immunodeficiency viral (HIV) infections.

BACKGROUND OF THE INVENTION

The Human Immunodeficiency Virus (HIV) is the causative agent of Acquired Immunodeficiency Syndrome (AIDS). Existing therapeutic treatments involve drug combinations called highly active anti-retroviral therapy (HAART) that are not only expensive but have serious side effects (Carr, A., Nature Rev. Drug Dis. 2: 624-634, 2003). Most of the drugs are small molecules with short serum half-life and considerable toxicity. Upon repeated administration of these drugs HIV develops resistance and the therapy becomes ineffective. Perhaps part of the drug resistance issue is due to the short serum half-life (minutes to hours) of the existing HIV drugs. Given these deficiencies in the existing therapies, there is an immediate need for the development of potent and long-lasting therapies with novel mechanisms of action and minimal side effects to treat HIV infections.

Reports from EMEA (European Medicines Agency) warn that nearly 50-90% of the treatment-naïve patients do not respond to two commonly used HIV HAART therapies (EMEA Reports, July and October, 2003). Nearly 10% of the 1,633 patients from 17 countries across Europe were resistant to at least one of the three types of anti-retroviral drugs (Lange, J., Intl. AIDS Conference, Paris; July 2003).

Generally, monoclonal antibodies (MAbs) have emerged as "magic bullets" to treat chronic conditions such as cancers and rheumatoid arthritis because MAbs are highly selective to the targets (Gura, T., Nature 417: 584-586, 2002; Walsh, G., Nature Biotechnol. 21: 865-870, 2003). More significantly MAbs have a serum half-life of several days to few weeks. SynagisTM is the first market-validated MAb that inhibits viral entry, and is administered to treat pediatric patients infected with Respiratory Syncitial Virus (RSV) infections. Synagis has a serum half-life of 20 days. With the advent of human MAbs (HuMAbs), which are the most advanced generation of MAb drugs, the issue of immunogenicity or side effects upon repeated administrations of protein/MAb therapies is addressed. Taken these facts together, it is feasible to develop broadly-neutralizing and efficacious HuMAb therapies with longer serum half-life (several days to weeks) for treating chronic HIV infections.

Entry inhibition is the process of stopping and neutralizing the virus before it penetrates human cells. HIV Type I (HIV-I) must first penetrate host cells called CD4+ T cells, which are a critical component of the body's immune system. It has been well documented that discrete, step-wise sequence of events has to take place before the virus fuses with CD4+ T cells (Trkola et. al., J. Virol., 70: 1100-1108, 1996; Wu et. al., Nature 384: 179-183, 1996). First the glycoprotein gp120
binds to the receptor protein CD4, which leads to conformational changes in gp120, gp41 and CD4, and to this complex, a specific chemokine co-receptor (either CXCR4 or CCR5) latches on and forms a trimolecular complex (Trkola et al., supra; Wu et al., supra).

Neutralizing Abs are believed to act by binding to the exposed envelope (Env) surface of gp120/gp41 and arrest the subsequent set of events in the entry process (Parren et al., J. Virol. 72: 3512-3519; 1998). Extensive variations in the gp120 amino acid sequence of HIV clinical isolates presents a daunting task in designing an effective antibody that is broadly-neutralizing against diverse set of clinical isolates. HIV-1 has evolved a number of strategies to evade recognition by neutralizing Abs, particularly those directed to the "naturally exposed" conserved sites of Env to which CD4 and co-receptors bind. Thus, targeting a hidden and extremely conserved epitope in gp120, which opens up upon binding to CD4 receptor is considered a novel approach. A fragmented human antibody (FAb) called X5 has been recently isolated from a phage display library constructed from a seropositive individual and approximately fifty primary (clinical) isolates are shown to be effectively neutralized by this FAb in in vitro neutralization assays (Moulard et al., Proc. Natl. Acad. Sci. USA 99: 6913-6918, 2002; International Patent Application WO 03/03666).

X5 FAb binds with nanomolar affinity to a novel gp120 epitope that is exposed after binding to CD4, and is further enhanced by the binding of CCR5 to the complex. Cell fusion and infectivity assays with a number of HIV clinical isolates confirmed that X5 is a novel, broadly-neutralizing anti-HIV-1 antibody, which recognizes a unique, conserved, receptor-induced epitope on gp120. Therefore, the potential for neutralization escape is very minimal. This novel specificity for an entry intermediate state, combined with the broadly-neutralizing activity and high potency against many different HIV strains makes the X5 FAb an excellent drug candidate (Moulard et. al., supra; Zhang et. al., supra). There is a size-dependent steric hindrance for the X5 IgG, FAb, or scFv to bind the epitope effectively (Zhang et. al., supra). X5 scFvs, which are approximately half the size of FAbs, neutralize primary isolates much better compared to the FAb/IgG (Zhang et. al., supra). Contrarily, the whole immunoglobulin of X5 (X5 IgG) does not have comparable neutralizing activity (Labrijn et. al., J. Virol. 77: 10557-10565, 2003).

While the scFvs are considered effective in neutralizing HIV, unfortunately, because of their smaller size they are expected to have shorter serum half-life (in the order of minutes), and are cleared rapidly through glomerular filtration by kidneys. A novel way to address this problem is to pegylate scFvs such that the apparent molecular size of the pegylated scFvs is dramatically increased leading to extended serum half-life while the broadly-neutralizing property of scFvs is still retained. Pegylation is the process of covalently linking polyethylene glycol (PEG) of ca. 5-60 kiloDalton to antibodies or proteins, which leads to dramatic improvement in pharmacokinetic and pharmacodynamic properties (Harris and Chess, Nature Rev. Drug Discovery 2: 214-220, 2003). Recent examples of such improvements include pegylation of scFvs and FAbs (Chapman et. al. Nature Biotechnol. 17: 780-783, 1999; Yang et. al., Protein Eng. 16: 761-770, 2003).

Therefore, there remains a need for molecules which are not only highly potent and broadly-neutralizing but sufficiently large enough to escape glomerular filtration by kidneys. This invention provides such an antibody derivative, as well as methods of making and using pegylated scFvs to
inhibit HIV infection. These and other objects and advantages of the invention, as well as additional inventive features, will be apparent from the description of the invention provided herein.

**BRIEF SUMMARY OF THE INVENTION**

The present invention provides a substantially homogenous preparation of pegylated scFvs of X5 and derivatives thereof, and related methods. The method describes high yield of pegylated scFv derivative that is modified exclusively at defined sites, thereby providing several processing and therapeutic advantages as compared to other species involving random modification. The present invention stems from the observation that, as compared to unmodified native therapeutic scFvs, pegylated scFv derivatives are equally broadly-neutralizing, and their properties such as pharmacokinetics, pharmacodynamics, bioactivity and biocompatibility are substantially improved.

Pegylated scFv derivatives prepared from 5-100 kDa PEG polymers are highly efficacious. When the pegylated-X5 scFv derivatives are administered, preferably in a single subcutaneous dose, the HIV viral load may decrease for over 3-21 days. Correspondingly, the CD4+ T-cell count increased. The present invention also relates to optimization of X5 scFv by specific antibody engineering procedures wherein the affinity and broadly-neutralizing properties are significantly improved. Particularly, the amino acid residues of the complementarity determining residue (CDR) sequences of light and heavy chains of scFv are mutated, and that the affinity improvements are additive. For pegylation, a free cysteine residue is optionally engineered in the X5 scFv amino acid sequence, preferably away from the antigen binding site. The resultant recombinant X5 analogs are produced in high yield in heterologous expression system, for example bacterial expression systems such as *Escherichia coli*, purified, and then pegylated. Thus, in one aspect, the present invention relates to human X5 scFv having cysteine mutations engineered into positions away from the epitope binding sequences of X5 scFv.

The present invention also relates to pegylated X5 scFv derivatives wherein PEG is conjugated to the modified cysteine residue. Preferably the PEG has a molecular weight ranging from at least about 5 kDa to not more than about 100 kDa. A particularly preferred PEG is from at least about 20 to not more than about 60 kDa.

The present invention further relates to all of the pegylated X5 MAb derivatives as above, in a pharmaceutically acceptable carrier.

The present invention further relates to processes for preparing pegylated antibody derivatives as described above. The principal embodiment of the method for making the substantially homogenous preparation of pegylated-scFv comprises: (a) engineering a cysteine residue into a specific amino acid position within the amino acid sequence of X5 scFv to provide an analog of X5 scFv; (b) conjugating a polyethylene glycol to said analog at said cysteine residue to provide a mono-pegylated antibody conjugate; and (c) isolating said pegylated scFv conjugate.

The present invention also relates to methods of treating individuals using pegylated X5 scFv conjugates as above. The invention is directed to a method of inhibiting HIV infections in humans, which method comprises administering to humans in need thereof a pegylated scFv molecule comprising (a) a single chain variable fragment (scFv), and (b) a peg molecule, wherein the
pegylated scFv binds to an epitope of the HIV envelope protein that is inaccessible to whole immunoglobulin molecules due to molecular steric hindrance, whereupon the HIV infection is inhibited.

DESCRIPTION OF THE DRAWINGS

Figure 1: Amino acid sequence of X5 light chain. The variable region of light chain (VL) ends at the sequence LEIKRT of the Jk sequence, followed by the constant frame (CL). The complementarity determining regions (CDRs) of the light chain are set forth in SEQ ID No:2-4.

Figure 2: Amino acid sequence of X5 heavy chain. The variable region (VH) consists of the first 133 amino acid residues in the sequence, followed by the constant frame (CH1). The complementarity determining regions (CDRs) of the heavy chain are set forth in SEQ ID No:6-8.

Figure 3: Linkers. (G4S)n has a linear sequence of (SEQ ID NO:7)(Gly-Gly-Gly-Gly-Ser) in tandem repeats, where n = 3, 4, 6, or 8. The flexible linker (FL) relieves molecular steric hindrance due to pegylation, if any.

Figure 4: Engineering of X5 scFv by in vitro scanning saturation mutagenesis. In this example, 6th and 8th positions of CDR-3 of VL and VH, respectively, are replaced with nineteen other amino acids (only four variants are shown here for the sake of clarity), and the affinity measurements for variants measured. This illustration further exemplifies that the affinity improvements are additive when the variants are combined (see Table 1). For example, approximately 500-fold affinity improvement can be obtained when the mutated CDR-3 sequences of variants L-1 and H-4 are combined.

Figure 5: Pegylation of X5 scFv. The linear sequence of the pegylated scFvs can be constructed in different formats as shown in constructs A-D. VL, variable sequence of light chain; VH, variable sequence of heavy chain; CL and CH1 are the fragments of constant frames of light and heavy chains, respectively. Varying lengths of CL and CH1 are added to optimize the binding of the pegylated scFv to the CD4-inducible epitope of gp120. Engineered “free” cysteine (Cys) residue is placed away from the antigen binding sites of VL and VH, which are connected by a (G4S)n linker. The flexible linker (FL) relieves molecular steric hindrance due to pegylation, if any.

DEFINITIONS

In this specification and in the claims that follow, reference will be made to a number of terms which shall be defined to have the following meanings. It is to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

By “pegylation” is meant the process by which polyethylene glycol (peg) chains are attached to therapeutic drugs such as proteins, peptides, antibodies, or antibody fragments. Pegylation improves pharmacokinetics by increasing the molecular mass of proteins and shielding them from proteolytic enzymes. Upon pegylation these pegylated therapeutic drugs overcome such shortcomings as degradation to proteolytic enzymes, rapid clearance by the kidneys, and generation of neutralizing antibodies.
By "site specific pegylation" is meant that the process by which a peg molecule is attached to a specific amino acid residue such as cysteine in an antibody or antibody fragment.

By "pharmacokinetics" is meant that the movement of drugs throughout the body, including their absorption, distribution, metabolism and excretion, and the mathematical models that describe these actions.

By "pharmacodynamics" is meant that changes in measurable clinical parameters related to a drug, such as increase in CD4+ T cell count and decrease in viral load.

By "half-life" is meant that the amount of time it takes for one-half of the drug dose to be lost through biological processes.

By "shelf-life" is meant that the amount of time a stored drug retains its activity.

By "CD4-inducible epitope" is meant an antigenic site on HIV Env, gp120, or gp41, wherein specific binding to the antigenic site by an antibody of the invention is increased or augmented by the binding of CD4 to HIV Env, gp120, or gp41. Preferably this increase is by at least about 2-fold or greater.

By "CD4-inducible epitope on HIV Env that is distinct from the co-receptor binding site on gp120" is meant that an antibody of the invention does not compete with an HIV co-receptor for the co-receptor binding site on Env.

By "exposure of the epitope is enhanced" or "epitope that is enhanced" is meant that specific binding of an antibody of the invention to its cognate CD4-inducible epitope on HIV Env, gp120, or gp41 is further enhanced by the binding of HIV Env, gp120, or gp41 to a co-receptor such as CCR5 or CXCR4.

By "specifically binds", "specifically reacts with", and similar terms is meant that an anti-HIV antibody of the invention physically associates with its target molecule (e.g. gp120 of Env) to inhibit HIV entry into a cell and/or to inhibit or prevent HIV replication in humans. Preferably, the antibody does not substantially physically associate with other molecules.

By a "broadly-neutralizing" antibody against HIV, and similar terms is meant an antibody that can inhibit the activity (e.g., the ability to enter a target cell) of several HIV clinical (primary) isolates from more than one genetic subtype or clade.

By "selected" is meant that an antibody or antibody fragment of the invention is chosen or isolated from a group or library of candidate antibodies or antibody fragments using a screening assay for choosing or isolating antibodies with a desired characteristic (that is, the ability to bind a complex comprising HIV gp120, CD4, and a co-receptor for HIV; or the ability to specifically bind a CD4-inducible epitope on HIV Env that is enhanced by the binding of Env to a co-receptor for HIV, wherein the CD4-inducible epitope is distinct from the HIV co-receptor binding site on gp120), as would be understood by a skilled practitioner in the art.

The term "antibodies" is used herein in a broad sense includes monoclonal antibodies, fragments and multimers of immunoglobulin molecules (Hudson and Souriau, Nature Medicine 9: 129-134, 2003), single chain antibodies, and human or humanized versions of immunoglobulin molecules or fragments thereof, as long as they are chosen for their ability to broadly-neutralize HIV by binding a CD4-inducible HIV epitope that is enhanced by binding a HIV co-receptor, as described
The antibodies are tested for their desired activity using their in vitro assays described herein, or by analogous methods, after which their in vivo therapeutic and/or prophylactic activities are tested according to established clinical testing methods. Libraries of antibodies or active antibody fragments can also be generated and screened using phage display techniques, e.g., as described in U.S. Patent No. 5,604,440 (Burton et al.) and U.S. Patent No. 6,096,441 (Barbas, et al.), as well as in Proc. Natl. Acad. Sci. USA 101:9193-9198, 2004 (Harvey et al.). Recombinant antibodies and fragments can be expressed in vitro or in prokaryotic or eukaryotic cells, and further purified using well known methods (see, e.g., Sambrook et al., Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory Press (1989); and Ausubel et al., Current Protocols in Molecular Biology, John Wiley & Sons, New York, NY, 2001).

Any "antibody or antibody fragment" of the invention, whether attached to other sequences or not, can include insertions, deletions, substitutions, or other selected modifications of particular regions or specific amino acid residues, provided the activity of the antibody or antibody fragment is not significantly altered or impaired compared to the non-modified antibody or antibody fragment. These modifications can provide for some additional property, such as to remove or add amino acids capable of disulfide bonding, to increase its bioavailability, to alter its secretory characteristics, etc. However, the antibody or antibody fragment must possess a bioactive property, such as binding to its epitope or antigen. Functional or active regions of the antibody or antibody fragment may be identified and/or improved by mutagenesis of a specific region of the protein, followed by expression and testing of the expressed polypeptide. For example, amino acid sequence variants of antibodies or antibody fragments can be generated and those that display equivalent or improved affinity for antigen can be identified using standard techniques and/or those described herein. Methods for generating amino acid sequence variants are readily apparent to a skilled practitioner in the art and can include directed evolution technologies (US Patent No: 6,180,341) or random mutagenesis (e.g., by PCR) of the nucleic acid encoding the antibody or antibody fragment (Zoller, M.J. Curr. Opinion in Biotechnol. 3: 348-354, 1992). Both naturally occurring and non-naturally occurring amino acids may be used to generate amino acid sequence variants of the antibodies and antibody fragments of the invention.

By "isolated polypeptide" is meant a polypeptide (or a fragment thereof) that has been separated from the components that naturally accompany it. Typically, the polypeptide is substantially pure when it is at least 60%, at least about 70%, at least about 80%, at least about 90% or more by weight, free from the proteins and naturally occurring organic molecules with which it is naturally associated. A substantially pure polypeptide may be obtained, for example, by extraction from natural source by expression of a recombinant nucleic acid encoding the polypeptide, or by chemically synthesizing the polypeptide. Purity can be measured by appropriate methods such as column chromatography, polyacrylamide gel electrophoresis, or HPLC analyses.

A protein is substantially free of naturally associated components when it is separated from those contaminants, which accompany it in its natural state. Thus, a protein that is chemically synthesized or produced in a cellular system different from the cell from which it naturally originates will be substantially free from its naturally associated components. Accordingly, substantially pure
polypeptides not only include those derived from eukaryotic organisms but also those produced in E. coli or other prokaryotes.


Antibodies of the invention are preferably administered to a subject in a pharmaceutically acceptable carrier. Suitable carriers and their formulations are described elsewhere (Remington: The Science and Practice of Pharmacy [19th ed.] ed. A. R. Gennaro, Mack Publishing Company, Easton, PA 1995). Typically, an appropriate amount of a pharmaceutically acceptable salt is used in the formulation to render the formulation isotonic. Examples of the pharmaceutically acceptable carrier include, but are not limited to, saline, Ringer's solution and dextrose solution. The pH of the solution is preferably from about 5 to about 8, and more preferably from about 7 to about 7.5. It will be apparent to those persons skilled in the art that certain carriers may be more preferable depending upon, for instance, the route of administration and concentration of antibody being administered.

The antibodies can be administered to the subject or patient by injection (e.g., but not limited to, intravenous, intradermal, subcutaneous, intramuscular), or by other methods such as infusion that ensures its delivery to the bloodstream in an effective form. Local or intravenous injection is preferred.

Effective dosages and schedules for administering the antibodies may be determined empirically, and making such determinations is within the skill in the art. Those skilled in the art will understand that the dosage of antibodies that must be administered will vary depending on, for example, the subject that will receive the antibody, the route of administration, particular type of antibody used and other drugs being administered.

Following administration of an antibody for treating, inhibiting, or preventing an HIV infection, the efficacy of the therapeutic antibody can be assessed in various ways well known to the skilled practitioner. For instance, one of ordinary skill in the art will understand that an antibody of the invention is efficacious in treating or inhibiting HIV infections in a subject by observing that the antibody reduces viral load or delays or prevents a further increase in viral load. Viral loads can be measured by methods that are known in the art, for example, using PCR assays to detect the presence of HIV nucleic acid or antibody assays to detect the presence of HIV protein in a sample (e.g., but not limited to, blood or another body fluid) from a subject or patient, or by measuring the level of circulating anti-HIV antibodies in the patient. Efficacy of the antibody treatment may also be determined by measuring the number of CD4+ T cells in the HIV-infected subject. An antibody treatment that delays or inhibits an initial or further decrease in CD4+ T cells in an HIV-positive subject or patient, or that results in an increase in the number of CD4+ T cells in the HIV-positive subject, is an efficacious antibody treatment.
The broadly-neutralizing antibodies of the invention can also be administered prophylactically to patients or subjects who are at risk for being exposed to HIV or who have been newly exposed to HIV. Such patients include, but are not limited to, healthcare workers, fetuses, neonates, or infants (or nursing infants) whose mothers are infected or at risk for being infected, intravenous drug users, recipients of blood transfusions, blood products, or transplantation tissue, and other individuals who have been exposed to a body fluid that contains or may contain HIV.

In subjects who have been newly exposed to HIV but who have not yet displayed the presence of the virus (as measured by PCR or other assays for detecting the virus) in blood or other body fluid(s), efficacious treatment with an antibody of the invention partially or completely inhibits or delays the appearance of the virus or minimizes the level of the virus in the blood or other body fluid(s) of the exposed individual.

By “effective amount” is meant the amount of an anti-HIV antibody of the invention that is useful for treating, partially or completely inhibiting, or preventing an HIV infection in a patient or subject as described herein. Effective dosages and schedules for administering the antibodies of the invention may be determined empirically, and making such determinations is routine to one of ordinary skill in the art. An effective dose of HIV-antibody of the invention generally will range between about 1 μg/kg of body weight and 25 mg/kg of body weight.

**DETAILED DESCRIPTION OF THE INVENTION**

The invention is directed to a method of inhibiting HIV infection in humans, which method comprises administering to a human in need thereof a pegylated scFv fragment that comprises (a) single chain variable fragment comprising about 200 to about 400 amino acids, and (b) a polyethylene glycol molecule of size ranging from at least about 5 kDa to not more than about 100 kDa. The pegylated scFv fragment binds to an epitope of the HIV envelope protein that is inaccessible to whole immunoglobulin molecule due to molecular steric hindrance.

In one embodiment, the peptide or polypeptide is a pegylated scFv fragment. In this regard, the invention is directed to a method of inhibiting HIV infection in humans, which method comprises administering to humans in need thereof a pegylated scFv molecule comprising (a) an scFv fragment and (b) a peg molecule, wherein the pegylated scFv molecule binds to an epitope of the HIV envelope protein that is inaccessible to whole immunoglobulin molecules due to steric hindrance, whereupon the HIV infection is inhibited.

Inhibiting HIV infection refers to the inhibition in the onset of HIV infection, the inhibition of an increase in an existing viral infection, or a reduction in the severity of the infection. In this regard, one of ordinary skill in the art will appreciate that, while complete inhibition of the onset of HIV infection is desirable, any degree of inhibition of an increase in an existing HIV infection or any degree of a reduction of HIV infection is beneficial. Inhibition of HIV infection can be assayed by methods that are known in the art, such as by the assessment of viral load. HIV loads can be measured by methods that are known in the art, for example, using polymerase chain reaction assays to detect the presence of HIV RNA, or antibody assays to detect the presence of HIV protein.
in a sample (e.g. blood) from HIV-infected humans. Alternatively, an increase in the number of CD4+ T cells in HIV-infected subjects is considered an efficacious treatment.

Chronic infections of the Human Immunodeficiency Virus (HIV) lead to the development of Acquired Immunoodeficiency Syndrome (AIDS) in humans. HIV entry into cells involves formation of a complex between the HIV envelope glycoprotein (Env, which consists of a complex containing the HIV glycoprotein gp120 and gp41; gp120-gp41), a cell surface receptor (CD4), and a cell surface co-receptor (e.g., the chemokine receptor CCR5 or CXCR4). Binding of Env to CD4 and one of the co-receptors initiates a series of conformational changes that are heart of the fusion machinery leading to membrane rupturing, penetration and injection of HIV-RNA into the target cells (see e.g., Trkola et al., supra; and Wu et al., supra). Therefore efforts to develop special class of therapeutic drugs called “entry inhibitors” are underway. Entry inhibitors can be peptide or protein therapies, small molecule drugs, and neutralizing antibodies. However extensive variation of Env in the numerous isolates of HIV so far identified presents a major obstacle in designing an effective immunogen for the isolation of antibodies with broadly-neutralizing activity against multiple HIV clinical isolates.

MAbs with broadly-neutralizing activity are known in the art: the anti-gp120 MAbs b12 (Burton et al., Science 266: 1024-1027, 1994), 2G12 MAb that target the glyco component of gp120 (Trkola et al., supra), and the anti-gp41 MAb 2F5 (Conley et al., Proc. Natl. Acad. Sci. USA 91: 3348-3352, 1994). Recently a set of novel broadly-neutralizing antibodies and MAb derivatives that bind to an extremely conserved and hidden epitope of gp120 has been discovered (Mouland et al., supra; Zhang et al., supra). This CD4 inducible epitope of HIV Env is exposed by the binding of Env to a co-receptor for HIV. The binding sites for the MAbs, CD4 and the co-receptor(s) are distinct and non-overlapping. Of the known MAbs with broadly-neutralizing activity, X5 is the only MAb that is directed against the receptor-inducible and highly conserved epitope. X5 Fab was identified by screening a phage display library that was constructed from a HIV seropositive individual. The epitope recognized by the X5 antibody is inducible by CD4 and exposure of the epitope is enhanced by the major HIV-1 co-receptor CCR5 (WO 03/033666).

Therapeutic MAbs are beginning to find routine applications in therapies for human diseases, as demonstrated by the large number of MAbs approved for sale or in final stages of clinical trials (Walsh, G. Nature Biotechnol. 21: 865-870, 2003). Currently, therapeutic applications of these drugs are largely limited to acute indications such as rheumatoid arthritis and cancers because of shortcomings, which include their susceptibility to destruction by proteolytic enzymes, short circulating half-life, short shelf-life, low solubility, rapid kidney clearance and their propensity to generate neutralizing antibodies. In addition, most MAbs must be delivered by intravenous injections.

Chronic therapy usually requires large doses (3-5 mg/kg) of antibody repeatedly administered over months to years. Although high-level mammalian cell expression systems have been developed for producing MAbs (IgGs), large amounts needed to treat patients over several months or years make them prohibitively costly, and supplying sufficient quantities is difficult. A possible solution is to use antibody fragments, such as FAb or scFv, that can be readily expressed in microbial systems with the potential for large-scale and cost-efficient production. However, such
antibody fragments tend to have short in vivo half-life, making them unsuitable for clinical therapies that require antibody circulation over extended periods of time.

Microbial expression systems are attractive models for the expression of antibody fragments. The larger size of microbial fermentors, short process times and defined salt media contribute to addressing the capacity and cost constraints inherent in mammalian cell expression. E. coli is currently the host of choice for production of FAb and scFv fragments (Better et. al., Science 240: 1041-1043, 1988).

Generally, human immunoglobulins (IgGs) have long circulating half-lives in humans, with t1/2α (distribution-phase half-life) values of 18±22 h and t1/2β (terminal elimination-phase half-life) values of 21±23 days for human IgG1, 2 or 4 (Mariani, G. and Strober, W. in Fc Receptors and the Action of Antibodies (Metzger, H., ed.), pp.94-177, American Society for Microbiology, Washington DC). However, it has been found that the pharmacokinetics of human IgG is unusual in that the half-life varies with concentration. At very high IgG levels the serum half-life is decreased. This observation led to the notion that a receptor-mediated event is responsible for maintaining the long circulating half-life for IgG (Brambell et. al., Naure 203: 1352-1355 1964), by binding IgG, preventing degradation and recirculating the antibody back to the plasma. It is now known that this receptor-mediated recycling is mediated by the Fc region of the antibody. Isolated Fc has a half-life of similar duration to the whole IgG molecule, whereas FAbs and dimeric FAbs are cleared relatively rapidly. Site-directed mutagenesis studies have located the receptor-binding site to a region between the CH2 and CH3 domains (Kim et. al., Eur. J. Immunol. 24:542-548, 1994; Ghetie et. al., Eur. J. Immunol. 26: 690-696, 1996).

The cellular receptor responsible for maintaining half-life has been identified as the neonatal Fc receptor, FcRn (King, D.J., in Applications and Engineering of Monoclonal Antibodies, pp, 67-75, Taylor & Francis, London). Mice deficient in this receptor have reduced plasma IgG levels and clear administered IgG or Fc with an abnormally short half-life (Ghetie et. al., Eur. J. Immunol. 26: 690-696, 1996). Once internalized into cells, IgG is salvaged from the endosome during acidification through binding to FcRn, protecting the antibody from degradation. The IgG is then recycled to the cell surface where the higher pH leads to dissociation and return of the IgG to the circulation.

The half-lives of FAb fragments are relatively short compared with IgG. Pharmacokinetic experiments in rats show that a humanized IgG is relatively long-lived (t1/2α ~5.8 h, t1/2β ~104 h), whereas a FAb fragment derived from the same IgG is cleared much more rapidly (t1/2α ~0.3 h, t1/2β ~23 h). These results demonstrate the presence of a specific mechanism for maintaining serum levels of IgG, resulting in a marked difference in circulating half-life of IgG and FAb fragments. In fact, larger than 70 kDa is not a sufficient factor for longer serum half-life as shown for a dimeric FAb, a molecule with a molecular mass of ~100 kDa which is greater than the 60-70 kDa normally taken as the molecular mass cut-off for kidney filtration had a shorter serum half-life. Therefore, in order to exploit the potential benefits of controlled valency, rapid distribution to and penetration into tissues (Pedley et. al., Br. J. Cancer 68: 69-73, 1993; Lane et. al., Br. J. Cancer 70: 521-525, 1994) associated with therapeutic antibody fragments such as FAbs and scFvs, the rapid clearance of these molecules needs to be addressed.
Pegylation is an alternative method that overcomes these deficiencies by attaching polyethylene glycol (PEG) chains to scFvs and FAbs (Harris and Chess, supra). FDA has approved PEG for use as a vehicle or base in foods, cosmetics and pharmaceuticals, including injectable, topical, rectal and nasal formulations. PEG shows little toxicity, and is eliminated from the body intact by either kidneys if the PEGs are less than 30 kDa, or in the feces if the PEGs are larger than 20 kDa (Yamaoka et. al., J. Pharm. Sci. 83:601-606, 1994). PEG lacks immunogenicity (Working et. al., ACS Symposium Series 680:45-57, 1997). Generation of antibodies to PEG under routine clinical administration of pegylated proteins is not known.

Pegylation is a well established and validated approach for the modification of a range of MAbs, proteins and peptides (Chapman, A., Adv. Drug Deliv. Rev. 54: 531-545, 2002; and Harris and Chess, supra). The benefits include: (a) markedly improved circulating half-lives in vivo due to either evasion of renal clearance as a result of the polymer increasing the apparent size of the molecule to above the glomerular filtration limit, and/or through evasion of cellular clearance mechanisms; (b) reduced antigenicity and immunogenicity of the molecule to which PEG is attached; (c) improved pharmacokinetics and decreased viral load; (d) improved solubility—PEG has been found to be soluble in many different solvents, ranging from water to many organic solvents such as toluene, methylene chloride, ethanol and acetone (Harris and Chess, supra); (e) pegylated antibody fragments can be concentrated to 200 mg/mL and the ability to do so opens up formulation and dosing options such as subcutaneous administration of a high protein dose; this is in contrast to many other therapeutic antibodies which are typically administered intravenously; (f) enhanced proteolytic resistance of the conjugated protein (Cunningham-Rundles et.al., J. Immunol. Meth. 152:177-190, 1992); (g) improved bioavailability via reduced losses at subcutaneous injection sites; (h) reduced toxicity has been observed; for agents where toxicity is related to peak plasma level, a flatter pharmacokinetic profile achieved by sub-cutaneous administration of PEGylated protein is advantageous; proteins that elicit an immune response which has toxicity consequences may also benefit as a result of PEGylation; and (i) improved thermal and mechanical stability of the PEGylated molecule.

Earlier pegylation that enabled the protection of proteins from destruction during drug delivery were pioneered in early seventies (Davis et.al., Enzy. Engg. 4: 169-173, 1978), which led to the finding that pegylation also improves the pharmacokinetic and pharmacodynamic properties of polypeptide drugs by increasing water solubility, reducing renal clearance and limiting toxicity (Nucci et. al., Adv. Drug Delivery Rev. 6:133-151, 1991). Pegylation reduces kidney clearance simply by making the molecules larger, and as the kidneys filter substances basically according to size, larger molecules clear more slowly. The PEG is not degraded prior to elimination (Harris et. al., Clin. Pharmacokinetics 40: 539-551, 2001).

The first-generation pegylation methods were fraught with difficulties. With first generation pegylation, the PEG polymer was generally attached to the epsilon (ε) amino groups of lysine amino acid residues. This resulted in the modification of multiple lysines, and gave mixtures of PEG isomers with different molecular masses (Zaplinsky, S. Adv. Drug Delivery Rev. 16: 157-182, 1995). The existence of these isomers makes it difficult to reproduce drug batches, and can contribute to the
antigenicity of the drug and poor clinical outcomes. In addition, first generation methods mainly used linear PEG polymers with molecular masses of 12 kDa or less. Unstable bonds between the drug and PEG were also sometimes used, which led to degradation of the PEG–drug conjugate during manufacturing and injection (Harris, J.M., J. Macromol. Sci. Rev. C25: 325-373, 1985). An additional problem was that early pegylation was performed with methoxy–PEG (m–PEG), which was contaminated with PEG diol and which resulted in the cross linking of proteins to form inactive aggregates. Diol contamination can reach up to 10–15% (Dust et. al., Macromolecules 23: 3742-3746, 1990).

Second-generation pegylation chemistry strives to avoid the pitfalls associated with mixtures of isomers, diol contamination, unstable bonds and low molecular mass PEG. An overall goal of second-generation pegylation methods is to create larger PEG polymers to improve the pharmacokinetic and pharmacodynamic effects. In some cases the changes are dramatic, such as the pegylation of interleukin-6 (IL-6), which increases the half-life of IL-6 100-fold, which in turn results in 500-fold rise in its thrombopoietic potency (see Harris and Chess, supra).

Site-specific pegylation can minimize the loss of biological activity and reduce immunogenicity. For instance, because there are far fewer cysteine residues than lysine groups on polypeptides, the thiol groups of cysteine are ideal for specific modifications. Moreover, cysteines can be optionally added to polypeptides precisely where they are required by site directed mutagenesis. Although many proteins might not benefit from site-specific pegylation, in others, such as scFvs and FAbs, it is crucial that the PEG is attached at a site distant from the antigen binding site (Chapman et. al., Nature Biotechnol. 17: 780-783, 1999).

Another improvement in second generation PEG polymers is the use of branched structures, in contrast to the solely linear structures found in first generation PEGs (Harris and Chess, supra). Branched PEGs of greatly increased molecular masses—up to 60 kDa or more, compared with the 12 kDa or less found in first-generation PEGs—have been prepared. A branched PEG acts as if it were much larger than a corresponding linear PEG of the same molecular mass. Branched PEGs are also better at cloaking the attached polypeptide from the immune system and proteolytic enzymes, thereby reducing immunogenicity and likelihood of destruction.

The present invention provides substantially homogenous preparations of chemically modified proteins, and methods thereof. "Substantially homogenous" as used herein means that the only chemically modified proteins observed are those having one "modifier" (e.g., PEG) moiety. The preparation may contain unreacted (i.e., lacking modifier moiety) protein as ascertained by peptide mapping and N-terminal sequencing. As used herein, biologically active agents refers to recombinant or naturally occurring proteins, useful for prophylactic and therapeutic applications. One skilled in the art will readily be able to adapt a desired biologically active agent to the compositions of present invention.

The epitope of the HIV envelope protein to which the pegylated scFv molecule binds can be any epitope that is inaccessible to larger molecules, such as whole IgGs due to molecular steric hindrance. One of ordinary skill in the art is familiar with assays to determine whether molecular steric hindrance affects the binding of two molecules (Labrijn et. al., supra). The viral epitope is
preferably a conserved HIV epitope (i.e., the amino acid sequence of the epitope is shared by one or more strains and clades of HIV). Most preferably, the epitope is an epitope of HIV, such as an HIV envelope glycoprotein.

The pegylated scFv molecule can be any pegylated scFv molecule that can bind to an epitope of HIV envelope protein. The pegylated scFv molecule can comprise any suitable scFv, any suitable peg molecule, any suitable constant fragments of light and heavy chains, any suitable Fc region, and, optionally, any suitable long flexible linker. When the pegylated scFv molecule comprises a flexible linker (Figure 3), desirably and preferably the linker is positioned between the scFv fragment and peg (Figure 5).

The pegylated scFv fragment can be any suitable pegylated scFv that binds to an epitope of HIV envelope protein that is inaccessible to whole IgG molecules due to molecular steric hindrance. The pegylated scFv molecule is sufficiently flexible so as to avoid any steric, size, or orientation effects that prevent larger molecules (e.g., whole IgG molecules) from accessing the epitope of the HIV envelope protein. Preferably, the scFv comprises about 200 to about 400 amino acids.

The scFv molecule of the pegylated scFv fragment can be any suitable scFv fragment. As known in the art, an scFv fragment comprises a heavy chain variable domain (VH) joined via a short linker peptide to a light chain variable domain (VL), and is responsible for antigen binding. The scFv fragment to be used in the method of the invention is preferably broadly cross-reactive (e.g., can bind to a broad range of viral isolates from different strains and clades) with a high neutralization activity (e.g., typically with an IC50 of less than 100 μg/ml). Preferably, the scFv fragment is an scFv fragment of X5 (SEQ ID No: 1 and 5; see WO 03/033666). Alternatively, a variant of an aforementioned scFv fragment can be used. Desirably, the variant of the scFv fragment retains the ability to bind to the same epitope of the HIV envelope protein. A variant of an scFv fragment can be obtained by any suitable method, including random and site-directed mutagenesis of the nucleic acid encoding the scFv fragment and sequential antigen panning (see, e.g., PCT/US03/14292) and/or in vitro scanning saturation mutagenesis (US Patent No: 6,180,341). While a variant of the nucleic acid can be generated in vivo or in vitro and then isolated and purified, alternatively, a variant of the nucleic acid can be synthesized.

Any antibody or antibody fragment may be used according to the present invention. However, the preferred construct comprises a single chain antibody format, since no chain association event must take place following the translation. This facilitates the in vitro system for transcription/translation. "Antibody" or "antibody fragment" refers to any immunologic binding agent such as IgG, IgM, IgA, IgD and IgE or any antibody-like molecule that has an antigen binding region, and includes antibody fragments such as FAb, single domain antibodies (DAbs), Fv, scFv (single chain Fv) and the like. The techniques for preparing and using various antibody-based constructs and fragments are well known in the art.

The specificity of an antibody is determined by the complementarity determining regions (CDRs) within the light chain variable regions (VL) and the heavy chain variable regions (VH). The FAb fragment of an antibody, which is about one-third the size of a complete antibody contains the heavy and light chain variable regions, the complete light chain constant region and a portion of the
heavy chain constant region. FAb molecules are stable and associate well due to the contribution of the constant region sequences. However, the yield of functional FAb expressed in bacterial systems is lower than that of the smaller Fv fragment which contains only the variable regions of the heavy and light chains. The Fv fragment is the smallest portion of an antibody that still retains a functional antigen binding site. The Fv fragment has the same binding properties as the FAb, however without the stability conferred by the constant regions, the two chains of the Fv can dissociate relatively easily in dilute conditions. To overcome this problem, VH and VL regions may be fused via a polypeptide linker (Huston et. al., Meth. in Enzymol. pp. 46-99, 1991) to stabilize the antigen binding site. This single polypeptide Fv fragment is known as a single chain antibody (scFv). The VH and VL can be arranged with either domain first. The (G4S)n linker joins the carboxy terminus of the first chain to the amino terminus of the second chain (Figure 5).

In vitro scanning saturation mutagenesis (SSM) is particularly valuable for antibody engineering studies as a rapid way of identifying mutants with interesting properties that can be produced in large quantity and subjected to more detailed structural and functional characterization. SSM represents as a systematic new tool for exploring in vitro antibody affinity evolution, analogous to somatic hypermutation in vivo. Interesting single mutants can be used as a starting point for subsequent rounds of SSM at other sites, so that multiple mutations with synergistic effects on binding may be identified. This same sequential mutation approach should be useful to optimize properties such as affinity, potency, efficacy, altered specificity, reduced immunogenicity, and removal of proteolytic cleavage sites (US Patent No: 6,180,341).

The ability of the variants of the above-described scFv fragments to bind to the same epitope of the HIV envelope protein can be assessed by any suitable manner known in the art, such as by the enzyme-linked immunosorbent assay (ELISA). The variant of the above-described scFv fragment includes molecules that have about 50% or more identity to the above-described scFV fragments. More preferably, the variant includes molecules that have 85% to about 99% identity with the above-described scFv fragments. Ideally, the variant of the scFv fragment contains from 1 to about 40 amino acid substitutions, deletions, inversions, and/or insertions thereof. More preferably, the variant of the above-described scFv fragments contains from 1 to about 10 amino acid substitutions, deletions, inversions, and/or insertions thereof.

The substitutions, deletions, inversions, and/or insertions of the scFv fragment preferably occur in non-essential regions. The identification of essential and non-essential amino acids in the scFv fragment can be achieved by methods known in the art, such as by site-directed mutagenesis (for example, SSM) and AlaScan analysis (Moffison et. al., Chem. Biol. 5: 302-307, 2001). Essential amino acids have to be maintained or replaced by conservative substitutions in the variants of the scFv fragments, such that the pegylated scFv fragment maintains the ability to bind to an epitope of HIV envelope protein. Non-essential amino acids can be deleted, or replaced by a spacer or by conservative or non-conservative substitutions.

The variants can be obtained by substitution of any of the amino acids present in the scFv fragment. As can be appreciated, there are positions in the sequence that are more tolerant to substitutions than others, and some substitutions can improve the binding activity of the native scFv
fragment. The amino acids that are essential should either be identical to the amino acids present in the scFv fragment, or substituted by conservative substitutions. The amino acids that are non-essential can be identical to those in the scFv fragment, can be substituted by conservative or non-conservative substitutions, and/or can be deleted.

Conservative substitution refers to the replacement of an amino acid in the scFv fragment with a naturally or non-naturally occurring amino acid having similar steric properties. Where the side-chain of the amino acid to be replaced is either polar or hydrophobic, the conservative substitution should be with a naturally or non-naturally occurring amino acid that is also polar or hydrophobic.

A non-conservative substitution is a substitution in which the substituting amino acid (naturally or non-naturally occurring) has significantly different size, configuration and/or electronic properties compared to the amino acid being substituted. Thus, the side chain of the substituting amino acid can be significantly lower (or smaller) than the side chain of the native amino acid being substituted and/or can have functional groups with significantly different electronic properties than the amino acid being substituted.

The scFv or scFv fragment and peg are joined together by a long flexible linker (Figures 3 and 5). The linker can be any suitable long flexible linker, such that the scFv fragment of the pegylated scFv fragment can bind to the epitope of the HIV envelope protein (i.e., the molecule is not excluded from binding by molecular steric hindrance). The linker can be any suitable length, but is preferably at least about 15 to about 50 amino acids in length. Preferably, the long flexible linker is an amino acid sequence that is naturally present in IgG molecules of the host, such that the presence of the linker would not result in an immune response against the linker sequence by the mammal. Preferably, the linker is the long flexible linker of SEQ ID NO: 10 or SEQ ID NO: 11.

The pegylated scFv molecule encompassed by the invention comprise an scFv fragment, a peg molecule, and, optionally, a flexible linker. Preferably, the pegylated scFv molecules of the invention comprise SEQ ID NO: 1 and 5, or variants thereof, which retain the ability to bind to the same HIV epitope.

Variants of the pegylated scFv molecules can be obtained by any suitable method, including those methods discussed here. The ability of a variant to bind to the same epitope of the HIV envelope protein can be assessed by any suitable manner known in the art, such as by ELISA. The variants of the above-described pegylated scFv molecules include molecules that have about 90% or about 99% identity with the above-described pegylated scFV molecules. Preferably, the variants of the pegylated scFv molecules contain from 1 to about 30 amino acid substitutions, deletions, inversions, and/or insertions thereof. Preferably, the variants of the pegylated scFv molecules contain molecules of peg whose size range from at least about 5 kilodalton (kDa) to not more than about 100 kDa, usually not more than about 60 kDa, and at least about 10 kDa. The peg molecule may be linear or branched.

The pegylated scFv molecule preferably recognizes one or more strains of HIV-1. Preferably, the molecule is broadly cross-reactive and can bind to a wide range of isolates of HIV-1.
from different clades. More preferably, the pegylated scFv molecule binds to an epitope of a viral
envelope protein of clades A, B, C, D, E, EA, F, and/or G.

While the invention encompasses any pegylated scFv molecule that binds to an epitope of
HIV envelope protein, the molecule is preferably an antibody to HIV-1 envelope glycoprotein. The
binding of the pegylated scFv molecule preferably is enhanced by the presence of CD4 receptor and
a co-receptor (e.g., CXCR4 or CCR5). Preferably, the enhancement is exemplified by at least a two-
fold increase in the binding affinity, such as a two-fold decrease in EC50 as measured by ELISA.

One may prepare an analog of recombinant human X5 scFv by altering amino acid residues
of the scFv sequence, such as substituting the amino acids which diverge from the X5 scFv
sequence. Such derivatives are substantially homologous to X5 scFv, particularly at the CDR
regions of the light and heavy chains. Because the X5 scFv has biological activity in PBMC cells in
vitro, such an analog would likely be active in humans. Alternatively, one may use a natural variant
of X5 scFv (SEQ ID No: 1 and 5) derived from X5 phage library. Generally, the X5 scFv is expected
to be capable of pharmaceutical use in humans.

In X5 scFv, one can make use of a cysteine residue already present in the native sequence
as the site for pegylation. Conversely, a select cysteine mutation can be optionally introduced by
site-directed mutagenesis into the protein sequence (Figure 5). The purpose of the cysteine point
mutation is to allow a pegylation conjugation site. These "cysteine" protein analogs can be easily
prepared using conventional methods well known to one of ordinary skill in the art.

Subject to considerations for optimization as discussed below, the polymer may be of any
molecular weight, and may be branched or unbranched. For peg, the preferred molecular weight is
between about 10 kDa and about 100 kDa (the term "about" indicating that in preparations of
polyethylene glycol, some molecules will weigh more, some less, than the stated molecular weight).
Various sizes may be used, depending on the desired therapeutic profile (e.g., the duration of
sustained release desired, the effects, if any on biological activity, the ease in handling, and other
known effects of the polyethylene glycol to a therapeutic protein or analog).

Comprehended by the invention are pharmaceutical compositions comprising effective
amounts of chemically modified protein, or derivative products, together with pharmaceutically
acceptable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers needed for
administration. The optimal pharmaceutical formulation for a desired biologically active agent will be
determined by one skilled in the art depending upon the route of administration and desired dosage.
Exemplary pharmaceutical compositions are disclosed in Remington's Pharmaceutical Sciences
(Mack Publishing Co., 18th Ed., Easton, PA., pgs.1435-1712 (1990)).

One skilled in the art will be able to ascertain effective dosages by administration and
observing the desired therapeutic effect. Preferably, the formulation of the conjugate will be such
that between about 0.01 \( \mu \)g pegylated X5 scFv/kg body weight/day and 10 mg pegylated X5 scFv/kg
body weight/day will yield the desired therapeutic effect. The effective dosages may be determined
using diagnostic tools over time. The therapeutic dosages are determined depending on the severity
of the infection, viral load, CD4+ T-cell count over the course of therapy. The dosages may therefore
vary over the course of therapy, with, for example, a relatively high dosage being used initially, until therapeutic benefit is seen, and lower dosages used to maintain the therapeutic benefits.

**Experimental**

**EXAMPLE I: PEGYLATION OF SCFV FRAGMENT**

Site-directed mutagenesis is employed to introduce a cysteine residue at the C-terminal end of scFv (Yang et al. supra; Chapman et al., supra). Specific reactivity of PEG-maleimide (PEG-MAL; purchased from Nektar Therapeutics, San Carlos, CA; formerly Shearwater Corp.) with only free cysteines and the stability of these activated polymers in solution is validated first. Maleimide undergoes alkylation reactions with sulfhydryl groups to form stable thioether bonds. The availability of synthetic PEG polymers having one maleimide group at a single terminus allows thiol-directed pegylations. Using the defined reaction parameters, rapid and specific reaction with cysteine and not with lysine or histidine at pH 6.0 can be achieved (Yang et al., Protein Engg. 16: 761-770, 2003). It is further established that aqueous stability of maleimide in PEG-MAL polymers for optimization of reaction with scFv-thiol proteins. The PEG-MAL polymer maleimide moiety displays stability over 24h at 4 °C, pH 5-7 and over 2 h at 25 °C, pH 5-7, whereas, under these conditions, quantitative reaction with cysteine is completed within 5 min.

In pegylation reactions, total reduction of the engineered free cysteine is desired, while internal VL and VH disulfides must remain oxidized. An investigation of several reductants and reducing conditions demonstrate that 0.5-2 mM DTT reduction for 2 h at 37 °C followed by rapid separation on desalting columns prior to conjugation was successful for near quantitative reduction of one free thiol per scFv (Yang et al., supra). As determined by DTNB assays, the internal disulfides are not detectably reduced before or after reduction.

Functional group analysis is conducted in two steps: reaction of the MAL-PEG with excess cysteine and determination of unreacted cysteine by titration with DTNB (Creighton, (1989) T.E. In Creighton, T.E. (ed) Protein Structure: a Practical Approach, Oxford University Press, Oxford, pp. 155-167). Determination of active PEG-MAL is conducted at a reaction molar ratio of 1:3 (MAL-PEG:cysteine) in 50 mM sodium phosphate, pH 6.0, 1 mM EDTA. For cysteine titration by DTNB, the absorbance at 412 nm is recorded after 5 min at 25 °C using 13,300 l/mmol.cm as the extinction coefficient of DTNB.

Reduction and Pegylation: The free cysteine residue at the C-terminus or linker of the scFv proteins is reduced prior to reaction with MAL-PEG. The reduction solution contained 3 mg/ml scFv, 2 mM DTT, 2 mM EDTA, 100 mM sodium phosphate, pH 7.8. The reduction is performed at 37 °C for 2 h. Free DTT is removed on a HiPrep or PD-10 desalting column. The column is equilibrated with 100 mM sodium phosphate, pH 6.0, 2 mM EDTA. Near quantitative reduction of one thiol per scFv molecule is achieved as measured by DTNB assays (Creighton, 1989). The typical pegylation reaction buffer contains 1 mg/ml reduced scFv protein, 100 mM sodium phosphate pH 6.0, 2 mM EDTA and PEG-maleimide compound at a reaction molar ratio of 10:1 (PEG:scFv). The reaction is conducted at 25 °C under nitrogen atmosphere for 2 h. The typical conjugation yield is about 80% as
analyzed by SDS-PAGE. Unreacted scFv protein could be successfully reprocessed in a second reduction and conjugation reaction with comparable yields.

Pegylated scFv is then purified from native scFv, high molecular weight impurities, and unreacted free PEG by HS chromatography (see e.g., Yang et. al., supra). The column equilibration buffer contained 10 mM sodium phosphate, pH 5.0, and the gradient elution buffer is 10 mM sodium phosphate, pH 5.0, 1 M NaCl. Ion exchange chromatographic procedures are used to further purify the pegylated scFv.

Analytical characterization of pegylated scFv and scFv: Protein concentrations, Western blot analysis, SDS-PAGE analysis and staining procedures are according to established procedures (Yang et. al., supra). Mass values of pegylated scFv and scFv are determined by matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI-TOF-MS) (BrukerDaltronics OmniFlex NT) using an internal standard with a similar molecular weight. Apparent molecular weights (Stokes radius) of the scFv proteins are estimated using Superdex 200 HR 10/30 gel filtration column chromatography (Amersham Biosciences) in 50 mM sodium phosphate, pH 6.5, 150 mM NaCl.

Values for the association and dissociation rate constants (Kon and koff, respectively) are measured by Biacore analysis for the unpegylated and pegylated scFvs. In each case, care is taken to ensure that the kinetic values measured on the Biacore are free of artifacts due to rebinding or avidity effects arising from multimeric scFvs. Different concentrations of scFv or PEG-scFv are examined for association (3 min) and dissociation (2 or 5 min) and the data are analyzed for kinetic and affinity parameters using BiaEvaluation software (version 3.0).

**EXAMPLE II: IN VITRO NEUTRALIZATION ACTIVITY OF PEGYLATED SCFVS**

This example demonstrates that a pegylated scFv fragment has greater neutralization activity than the unpegylated scFv fragment. In this assay, single-round infectious molecular clones produced by envelope complementation will be used. Plasmids containing the env genes of HIV-1 strains JR-FL, AD8, and IIIB and recombinant pseudovirions will be produced as described in, for example, Labrijn et. al., supra. The degree of neutralization by antibody will be achieved by measuring luciferase activity. Briefly, 2 x 10⁶ of neuroglioma U87 cells that had been transfectected with CD4 and the chemokine receptors, CCR5 and CXCR4 (U87-CD4-CCR5-CXCR4 cells, Labrijn et. al., supra) in 100 µl of medium (DMEM containing 15% FBS, 1 µg of puromycin/ml, 300 µg of GL18/ml, glutamine, and penicillin-streptomycin) is added to microplate wells and incubated for 24 h at 37 °C in 5% CO2. One hundred microliters of medium containing viral isolates, JR-FL, AD8, or IIIB, were mixed with various amounts of one of the following antibodies: pegylated X5 scFv, non-pegylated X5 scFv, X5 FAB, X5 IgG (WO 03/033666). The mixtures will be incubated for 1 h at 37 °C, added to the cells, and incubated for a further 3 days. The wells are aspirated and washed once with PBS, and 60 µl of luciferase cell culture lysis reagent (Promega, Madison, WI) is added. The wells are scraped and the lysate mixed by pipeting, 50 µl is transferred to a round-bottom plate (Corning), and the plate was centrifuged at 1,800 x g for 10 min at 4° C, and then 20 µl is transferred to an opaque assay plate (Corning), and the luciferase activity was measured on a luminometer (Perkin Elmer,
Gaithersburg, MD) by using luciferase assay reagent (Promega). The percentage of maximal infection for the three HIV-1 isolates in U87 cells following administration of 100 µg/ml of pegylated X5 scFv, and the non-pegylated antibody fragments as mentioned above. The level of infection is compared to CD4 negative cells (CD4- cells), which are assumed to be non-infectable (i.e. 0% inhibition). Similarly, neutralization ability of these antibody fragments is compared in the presence of antibody formats at 100 µg/ml for the HIV-1 isolates mentioned.

EXAMPLE III: PHARMACOKINETIC AND PHARMACODYNAMIC STUDIES IN ANIMAL MODELS

For the pegylated scFv developed as the therapeutic lead, serum half-life, in vivo stability, pharmacokinetic studies, drug dosage cum time-course studies on the reduction of viral load, and the corresponding increase in CD4 T-cell count would be investigated in appropriate animal models. SCID-hu Thy/Liv mouse model uses conjoint implants of human fetal thymus and liver. These implants have been shown to be vascularized and to grow when implanted beneath the kidney capsule, eventually reaching a total mass of 10^7 to 10^8 human cells (Namikawa et. al., J. Exp. Med. 172: 1055-1063, 1990). Such growth occurs in 80-90% of recipients and is relatively unaffected by variable parameters in the SCID mouse colony. For periods of time as long as 12 months in vivo, the Thy/Liv organs sustain multipotential hematopoiesis and provide for a continuous source of normal human CD4+ T cells. Viral replication can be observed in a time- and dose-dependent manner after inoculation of HIV-1, and in the case of some virus isolates, thymocyte depletion is observed within a 3- to 5-week time interval. Stock preparation of PHA-activated PBMCs, stock preparation of HIV-1 isolates, TCID50 assay for HIV-1, p24 ELISA, construction of SCID-hu Thy/Liv mice, and preparation of drugs for administration to SCID-hu Mice, determination of drug levels in plasma, collection of human graft samples from SCID-hu mice, DNA PCR, FACS analysis for thymocyte depletion, and data analysis are done according to the published procedures (Rabin et. al., Antimicrob. Agents and Chemother. 40: 755-762, 1996). Although infection of the Thy/Liv implant can be achieved at low frequency after intravenous or intraperitoneal inoculation of virus, the most reliable means of infecting mice has proved to be direct intrathymic injection of small volumes of standardized viral stocks resulting in the infection of all animals. Dose-dependent levels of HIV-1 (as determined by p24 levels and HIV RNA) and CD4+ counts over a period of three to four weeks, as well as circulating plasma levels of the pegylated and non-pegylated scFvs will be evaluated in this mice (Rabin et. al., supra). The concentration of scFvs is determined by ELISA. The data are modeled using WinNonlin software (WinNonLin Pharsight, Mountain View, CA) to determine pharmacokinetic parameters using a two-compartment, bolus, first-order elimination model for the intravenous samples. Pharmacokinetic analysis of pegylated scFvs are carried out in cynomolgus monkeys (Chapman et. al., Nature Biotechnol. 17: 780-783, 1999). Blood samples are collected for 3-5 weeks after infusion and the concentration of scFvs is determined by ELISA.

Once-a-week dosing regimens are compared for pegylated scFv versus non-pegylated scFv. SCID Mice were administered subcutaneously with 2.5-25 mg/kg of pegylated scFv or 2.5-25 mg/kg of non-pegylated scFv on days 0, 7, 14 and 21. HIV-1 viral load and CD4+ T cell count were monitored relative to a buffer control over 1-3 weeks.
EXAMPLE IV: GENERATION OF X5 SCFV VARIANTS WITH INCREASED AFFINITY FOR ENV-CD4 CO-RECEPTOR COMPLEXES

The amino acid sequence of any antibody or antibody fragment of the invention may be varied in order to generate variant antibodies with equivalent or improved affinity for Env-CD4-co-receptor complexes (Figure 4). Such variant antibodies can be created and tested for their relative affinity using well-known methods and/or methods described herein (Burks et. al., Proc. Natl. Acad. Sci. USA 94: 412-417, 1997; Daugherty et. al., Proc. Natl. Acad. Sci. USA 97 (5): 2029-34, 2000; Zhang et. al., J. Mol. Biol. 335: 209-219, 2004).

For each chosen residue, in vitro scanning saturation mutagenesis (SSM) is carried out (US Patent No: 6,180,341). Briefly, at each site, twenty-one genes encoding all possible amino acid substitutions as well as a double stop codon (control) are constructed by overlap extension PCR. The final products of the overlap extension PCR reaction contain a T7 promoter and ribosome binding site in front of the scFv gene. An HSV sequence is also present at the C-terminal end of the scFv gene, so that the scFv protein can be detected by ELISA using an anti-HSV monoclonal antibody. The PCR overlap extension products are used as templates for coupled in vitro transcription-translation reactions to produce functional scFv proteins. An E. coli S30 ribosomal extract, as opposed to mammalian or plant cell extracts, is used for in vitro translation.

The protein products from the coupled in vitro transcription-translation step are analyzed by ELISA. In ELISA, 96-well microtiter plates are coated with the BSA conjugate of gp120/140-sCD4 complexes. The plates were then incubated with equal amounts from each of the in vitro synthesis reactions. In order to provide accurate calibration, the construct prepared with the wild-type sequence was used on each ELISA plate. The wild-type construct was produced by the overlapping PCR method alongside the mutants, thereby providing an accurate calibration for all stages of the procedure. The ELISA results for the different mutants are recorded. As shown in Figure 4, when the mutated variants of the CDRs are combined, the affinity improvements can be additive.

Table-1: Affinity Improvements of the Variants Can be Additive

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All references, including publications, patent applications, and patents, cited herein and hereby incorporated by reference to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein.

The use of the terms “a” and “an” and “the” and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms “comprising”, “having”, “including”, and “containing” are to be construed as open-ended terms.
(i.e., meaning "including, but not limited to," unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as") provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non claimed element as essential to the practice of the invention.

Preferred embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those preferred embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention included all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.
WHAT IS CLAIMED IS:

1. A pegylated antibody or antibody fragment that specifically binds a CD4-inducible epitope on Human Immunodeficiency Virus Type-1 (HIV-1) Env, wherein exposure of the CD4-inducible epitope is further enhanced by the binding of Env to a co-receptor for HIV-1, wherein the CD4-inducible epitope is distinct from the HIV-1 co-receptor binding site on gp120, wherein the antibody fragment is a fragmented antibody or a single chain variable fragment.

2. The pegylated antibody or antibody fragment of claim 1, wherein the antibody or antibody fragment is selected by virtue of its ability to specifically bind to a CD4-inducible epitope on HIV-1 that is enhanced by binding a co-receptor for HIV.

3. An isolated antibody or antibody fragment of claim 1, wherein the isolated antibody or antibody fragment is selected by virtue of its ability to specifically bind to a CD4-inducible epitope on HIV-1 gp120 that is enhanced by the binding of gp120 to a co-receptor for HIV, wherein the CD4-inducible epitope is distinct from the HIV-1 co-receptor binding site on gp120.

4. An isolated antibody or antibody fragment of claim 1, wherein the isolated antibody or antibody fragment is selected by virtue of its ability to specifically bind to a complex comprising HIV-1 gp120, CD4, and a co-receptor for HIV-1.

5. The isolated antibody or antibody fragment of claim 1 or 3, wherein the epitope is a HIV-1 gp120 epitope.

6. The isolated antibody or antibody fragment of claim 1 or 3, wherein the gp120 epitope is inducible upon binding to CD4 receptor.

7. The isolated antibody or antibody fragment of claim 1 or 3 is chemically modified by attaching polyethylene glycol (PEG) at specific sites in the antibody or antibody fragment by a process called pegylation.

8. The pegylated antibody or antibody fragment of claim 1 or 7 wherein said PEG moieties have a molecular weight from about 5 kiloDalton to about 60 kiloDalton.

9. The pegylated antibody or antibody fragment of claim 1 or 7 wherein said PEG molecule is linear or branched.

10. The pegylated antibody or antibody fragment of claim 1 or 7 wherein said PEG molecule is attached to a specific cysteine residue of the antibody or antibody fragment.
11. The isolated antibody or antibody fragment of claim 4, wherein the complex further comprises gp41.

12. The isolated antibody or antibody fragment of claim 1 wherein the HIV-1 co-receptor is CCR5 or CXCR4.

13. The isolated antibody or antibody fragment of claim 1 wherein the isolated antibody or antibody fragment has broadly-neutralizing activity against several clinical isolates of HIV-1.

14. The isolated antibody or antibody fragment of claim 1 wherein the isolated antibody or antibody fragment is monoclonal and pegylated.

15. The isolated antibody or antibody fragment of claim 1 wherein the isolated antibody or antibody fragment is human and pegylated.

16. The isolated antibody or antibody fragment of claim 1 or 13 wherein the isolated antibody or antibody fragment is isolated from a phage display library.

17. The isolated antibody or antibody fragment of claim 1 or 13 wherein the isolated antibody or antibody fragment comprises the heavy chain of antibody fragment X5 (SEQ ID No: 5) and is pegylated.

18. The isolated antibody or antibody fragment of claim 1 or 13 wherein the isolated antibody or antibody fragment comprises the light chain of antibody fragment X5 (SEQ ID No: 1) and is pegylated.

19. The isolated antibody or antibody fragment of claim 1 or 13, wherein the isolated antibody or antibody fragment comprises the heavy chain of antibody fragment X5 (SEQ ID No: 5) and the light chain of antibody fragment X5 (SEQ ID No: 1) and is pegylated.

20. The isolated antibody or antibody fragment of claim 1 or 19 wherein the isolated antibody or antibody fragment comprises the heavy chain of antibody fragment X5 (SEQ ID No: 5), wherein any particular amino acid residue is selectively substituted by nineteen other natural amino acids, and is pegylated.

21. The isolated antibody or antibody fragment of claim 20, wherein the isolated antibody or antibody fragment comprises the heavy chain of antibody fragment X5 (SEQ ID No: 5), wherein the amino acid substitutions are selectively made in CDR1, CDR2, or CDR3 regions, and is pegylated.
22. A method of selecting and identifying an antibody variant of claim 20, comprising the steps of:
   a) providing a DNA segment encoding a antibody, or antigen-binding fragment thereof;
   b) providing a set of primers that encode all nineteen amino acid variants at a single residue of said
   antibody or antigen-binding fragment thereof;
   c) performing PCR reactions on said DNA segment, using said set of primers, to generate a set of
   variant DNA segments encoding nineteen amino acid substitution variants at said single residue of
   said antibody or antigen binding fragments thereof;
   d) expressing each of said substitution variants using in vitro transcription/translation;
   e) identifying said antibody variant by antigen binding activity.

23. The method of claim 22, wherein said transcription/translation employs a prokaryotic
   expression system.

24. The method of claim 22, wherein said gene is under the transcriptional control of a phage
   promoter.

25. The method of claim 22, wherein said antigen binding activity is assessed by ELISA.

26. The isolated antibody or antibody fragment of claim 19 wherein the isolated antibody or
   antibody fragment comprises the light chain of antibody fragment X5 (SEQ ID No: 1), wherein any
   particular amino acid residue is selectively substituted by nineteen other natural amino acids, and is
   pegylated.

27. The isolated antibody or antibody fragment of claim 26, wherein the isolated antibody or
   antibody fragment comprises the light chain of antibody fragment X5 (SEQ ID No: 1), wherein the
   amino acid substitutions are selectively made in CDR1, CDR2, or CDR3 regions, and is pegylated.

28. A method of selecting and identifying an antibody variant of claim 26, comprising the steps
   of:
   a) providing a DNA segment encoding a antibody, or antigen-binding fragment thereof;
   b) providing a set of primers that encode all nineteen amino acid variants at a single residue of said
   antibody or antigen-binding fragment thereof;
   c) performing PCR reactions on said DNA segment, using said set of primers, to generate a set of
   variant DNA segments encoding nineteen amino acid substitution variants at said single residue of
   said antibody or antigen binding fragments thereof;
   d) expressing each of said substitution variants using in vitro transcription/translation;
   e) identifying said antibody variant by antigen binding activity.
29. The method of claim 28, wherein said transcription/translation employs a prokaryotic expression system.

30. The method of claim 28, wherein said gene is under the transcriptional control of a phage promoter.

31. The method of claim 28, wherein said antigen binding activity is assessed by ELISA.

32. The pharmaceutical composition is comprised of a peg molecule covalently attached to the antibody or antibody fragment of claim 1, 3, or 7, and a pharmaceutically acceptable carrier.

33. A method of inhibiting entry of HIV-1 into a cell, comprising administering to the cell an effective amount of the pegylated antibody or antibody fragment of claim 32, wherein the said antibody or antibody fragment specifically binds a CD4-inducible epitope of HIV-1 Env that is enhanced by the binding of Env to a co-receptor for HIV-1, wherein the CD4-inducible epitope is distinct from the HIV-1 co-receptor binding site on gp120, thereby inhibiting entry of HIV-1 into the cell.

34. The method of claim 33, wherein the cell is in human that is susceptible to infection by HIV and wherein the isolated antibody or antibody fragment is administered to human.

35. A method of inhibiting entry and replication of HIV-1 in humans susceptible to HIV-1 infection, comprising administering to the humans an effective amount of the pegylated antibody or antibody fragment of claim 32, wherein the said antibody or antibody fragment specifically binds a CD4-inducible epitope of HIV-1 Env that is enhanced by the binding of Env to a co-receptor for HIV-1, wherein the CD4-inducible epitope is distinct from the HIV-1 co-receptor binding site on gp120, thereby inhibiting HIV-1 entry and replication in humans.

36. The method of claim 33 or 35, wherein the co-receptor is CCR5.

37. The method of claim 33 or 35, wherein the co-receptor is CXCR4.

38. The isolated antibody or antibody fragment of claim 1 or 10, wherein said cysteine residue is an already existing residue of the antibody or antibody fragment (SEQ ID No: 1 and 5).

39. The isolated antibody or antibody fragment of claim 1 or 10, wherein said cysteine residue is optionally engineered in the antibody or antibody fragment.

40. The isolated antibody or antibody fragment of claim 1 or 10, wherein the heavy chain is additionally comprised of the constant fragment (CH1; SEQ ID No: 5).
41. The isolated antibody or antibody fragment of claim 40, wherein the heavy chain is additionally comprised of a fragment of the constant fragment (CH1; SEQ ID No: 5).

42. The isolated antibody or antibody fragment of claim 1 or 10, wherein the light chain is additionally comprised of the constant fragment (CL; SEQ ID No: 1).

43. The isolated antibody or antibody fragment of claim 42, wherein the light chain is additionally comprised of a fragment of the constant fragment (CL; SEQ ID No: 1).

44. The pegylated antibody or antibody fragment of claim 1 or 10, wherein the antibody fragment is comprised of heavy chain, light chain, linker, and flexible linkers.

45. The linker of claim 44, wherein the linker has the amino acid sequence Gly-Gly-Gly-Gly-Ser in tandem repeats (SEQ ID No: 9).

46. The tandem repeats of the linker in claim 45, wherein the G4S repeat is repeated three times, four times, six times, or eight times (SEQ ID No: 9).

47. The pegylated antibody or antibody fragment of claims 1 or 10, wherein the antibody fragment has a flexible linker (SEQ ID No: 10 or 11).

48. The isolated antibody or antibody fragment of claim 1 or 13, wherein the isolated antibody or antibody fragment is isolated from a bacterial display library.
X5 LIGHT CHAIN (SEQ ID NO: 1)

DIVLTQSPGT LSLSAGERAT LSCRASQSVS SGSLAWYQQK PGQAARLLIY GAEBRATGIP DRFSGSFFSGT DFTLTIGRLE PEDLAVYYCQ QGYGTSPYTFG

CDR Regions of X5 Light Chain:

CDR-1 (SEQ ID NO : 2) RASQSVSSGSLAW
CDR-2 (SEQ ID NO : 3) GAEBRATGI
CDR-3 (SEQ ID NO : 4) QQYGTSYTFQGGTKLEIKR
FIGURE 2

X5 HEAVY CHAIN (SEQ ID NO: 5)

MAVQLLEQSG AEVKKPGSSV QVSCKASGQT FSYMYGFNWR QAPGHGLEYW
GGIIPIFGTS NYAQRKFRGRV TFTADQTST AYMELTNLRS DDTAVVYCAR

CH1
DFGPDEWGDG SYDGSSGRGFF DFWGQAGTLVT VSSASTGKPS VFPLAPSSKS
TSGGTAAALGC LVKDYFPEPV TVSWNSGALT SGVHTFPAVL QSSGLYSLSS
VVTVPSSSLG TQTYCNVNH KPSNTKVDKK VEPKSCDKTS

CDR Regions of X5 Heavy Chain:

CDR-1 (SEQ ID NO : 6) MYGFN
CDR-2 (SEQ ID NO : 7) GGIIPIFGTSNYAQRKFRG
CDR-3 (SEQ ID NO : 8) DFGPDWEWDSYDGSSGRGFFDF
FIGURE 3: LINKERS

(SEQ ID NO:9)(G4)n LINKER: GLY-GLY-GLY-GLY-GLY-SER

FLEXIBLE LINKERS:

(SEQ ID NO:10) PDPEEPKSCDKHTCPPCP
(SEQ ID NO:11) EPKSCDKHTCPPCP PDPEEPKSCDKHTCPPCP
**FIGURE 4**

*in vitro* scanning saturation mutagenesis

CDR-3 $V_L$

QQYGTSYPYFGQGKLEIKR

6th position "S" mutated to 19 other amino acids

affinity increase

variant L-1 3x
variant L-2 5x
variant L-3 9x
variant L-4 6x

variants of light and heavy chain CDRs combined and affinity tested

CDR-3 $V_H$

DFGDWEDGDSYDGSGRGFFDF

8th position "D" mutated to 19 other amino acids

affinity increase

variant H-1 10x
variant H-2 1x
variant H-3 15x
variant H-4 20x
**FIGURE 5**
PEGYLATION OF X5 scFvs

**CONSTRUCT A:**

\[ V_L \rightarrow (G_4S)_n \rightarrow V_H \rightarrow Cys \rightarrow L_F \rightarrow PEG \]

**CONSTRUCT B:**

\[ V_L \rightarrow (G_4S)_n \rightarrow V_H \rightarrow C_{H1} \rightarrow Cys \rightarrow L_F \rightarrow PEG \]

**CONSTRUCT C:**

\[ V_L \rightarrow (G_4S)_n \rightarrow V_H \rightarrow C_{H1} \rightarrow Cys \rightarrow L_F \rightarrow PEG \]

**CONSTRUCT D:**

\[ V_L \rightarrow C_L \rightarrow (G_4S)_n \rightarrow V_H \rightarrow Cys \rightarrow L_F \rightarrow PEG \]
SITE SPECIFIC PEGYLATION OF
BROADLY-NEUTRALIZING ANTIBODIES AGAINST HUMAN
IMMUNODEFICIENCY VIRUS SELECTED BY ENV-CD4-RECEPTOR
COMPLEXES

VIRO-001WO

60/603,993
2004-08-25

FastSEQ for Windows Version 4.0

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