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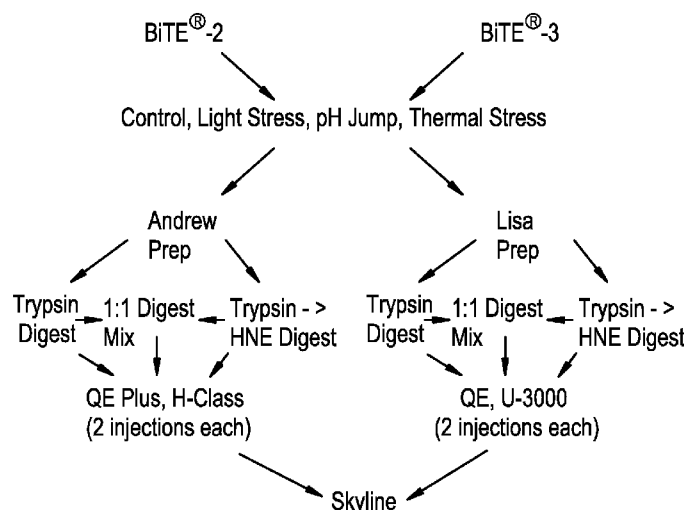
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(54) Title: METHODS FOR THE PREPARATION OF TRYPSIN-RESISTANT POLYPEPTIDES FOR MASS SPECTROMETRIC ANALYSIS

FIG. 6



(57) Abstract: The disclosed methods are directed to preparing and detecting polypeptides using neutrophil elastase, such as human neutrophil elastase. The polypeptides are optionally denatured, reduced, and/or alkylated before being subjected to a first digestion. A second digestion comprises the use of neutrophil elastase, such as human neutrophil 5 elastase. The prepared fragments are then analyzed chromatographically, electrophoretically, or spectrometrically, or a combination of these methods. The methods are especially useful for the preparation of therapeutic polypeptides for analysis, especially those that are not easily cleaved, such as some bi-specific T-cell engager (BiTE®) molecules.



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- *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*
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METHODS FOR THE PREPARATION OF TRYPSIN-RESISTANT POLYPEPTIDES FOR MASS SPECTROMETRIC ANALYSIS

RELATED APPLICATIONS

5 Priority is claimed to U.S. Provisional Patent Application No. 62/642,444, filed March 13, 2018, the entire contents of which are incorporated herein by reference.

SEQUENCE LISTING

10 The present application is being filed with a sequence listing in electronic format. The sequence listing provided as a file titled, "A-2151-WO-PCT sequence listing_ST25.txt," created 2019-03-12, and is 261 KB in size. The information in the electronic format of the sequence listing is incorporated herein by reference in its entirety.

FIELD

15 The presented subject matter relates to the field of polypeptide analysis. Specifically, the presented subject matter relates to the preparation of samples for the detection of polypeptides comprised therein, such as therapeutic polypeptides. The disclosed methods use sequential digestion in the preparation of the polypeptides, wherein a last digestion comprises the use of neutrophil elastase.

BACKGROUND

20 Multi-attribute method (MAM) relies on enzymatic digest of molecules followed by mass spectrometry-based characterization and quantitation of attributes of interest. Modifications in the complementary-determining regions (CDRs) are of particular concern because they may impact potency and/or safety of the molecule. Trypsin, which cleaves as the C-terminus of lysine and arginine residues, is typically utilized as the enzyme of choice for peptide mapping and MAM for many reasons, including high digestion specificity and the frequency of occurrence of lysine and arginine residues; trypsin digestion results in peptides with basic residues on the C-terminus which also typically have an optimal length for mass spectrometry-based analysis. However, many of the bi-specific T-cell engager (BiTE®) molecules currently being developed at Amgen contain a conserved α -CD3 domain with two CDRs in close proximity to a long linker region. One of these CDRs (HGNFGNSYISYWAY) contains two asparagine residues which may be susceptible to deamidation and a tryptophan residue that may be susceptible to

25

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oxidation. This region of the molecule contains no residues amenable to trypsin digestion (**Figure 1**). As such, attributes of interest in the CDR domains cannot be monitored by MAM due to the large size of this peptide (~8 kDa), the difficulty in chromatographically separating modified versions of the peptide, poor recovery and/or ionization of the peptide, and general challenges associated with interpreting the mass spectrometry data corresponding to peptides of this size. In addition, this linker region has no residues susceptible to commonly used secondary proteases such as Asp-N, Lys-C, and Glu-C. While chymotrypsin does cleave at tryptophan residues, utilization of this enzyme was not pursued both because of the enzyme's low specificity and because oxidation at tryptophan residues may affect quantitation of this attribute. Likewise, many BiTE[®] molecules also have a shorter linker peptide (~5-7 kDa) in close proximity to target-specific CDRs that is similarly difficult to monitor with trypsin digestion. For example, a potential CDR aspartic acid isomerization site is located within the 5.7 kDa linker peptide. Collectively, these attributes may impact target and/or CD3 binding, which necessitates MAM-based monitoring.

SUMMARY

In a first aspect, provided herein are methods of preparing a polypeptide for analysis, comprising cleaving the polypeptide in a sample in a first digestion, wherein the cleaving produces at least two fragments of the polypeptide, which the at least two fragments of the polypeptide are subsequently digested by a neutrophil elastase; and analyzing the sample after digesting the at least two fragments of the polypeptide with the neutrophil elastase.

In a second aspect, provided herein are methods of preparing a polypeptide for analysis, comprising cleaving the polypeptide in a sample in a first digestion, wherein the cleaving produces at least two fragments of the polypeptide; dividing the sample comprising the at least two fragments into a first aliquot and a second aliquot; digesting the at least two fragments of the first aliquot with a neutrophil elastase; and analyzing the first aliquot and the second aliquot. In some sub-aspects, after digesting the at least two polypeptide fragments with the neutrophil elastase, the first and second aliquots are combined; in such cases, the first and second aliquot can comprise approximately equal amounts of the polypeptide.

In both first and second aspects, cleaving the polypeptide in the first digestion comprises proteolytic or chemical cleavage. In those sub-aspect comprising proteolytic cleavage, such cleavage is accomplished by a protease, wherein the protease has an activity different from the neutrophil elastase. In some sub-aspects, the protease is selected from the group consisting of trypsin, endoproteinase Glu-

C, endoproteinase Arg-C, pepsin, chymotrypsin, chymotrypsin B, Lys-N protease, Lys-C protease, Glu-C protease, Asp-N protease, pancreatopeptidase, carboxypeptidase A, carboxypeptidase B, proteinase K, and thermolysin, and combinations thereof. In some sub-aspects of these first and second aspects, the protease is selected from the group consisting of trypsin, Asp-N, and Glu-C, and combinations thereof; in yet other sub-aspects, the protease is trypsin. However, when chemical cleavage is used to fragment the polypeptide in the sample in these first and second aspects, such cleavage is accomplished by a chemical, such as a chemical selected from the group consisting of cyanogen bromide, 2-Nitro-5-thiocyanobenzoate, hydroxylamine, and BNPS-skatole, and combinations thereof.

Furthermore, the polypeptide can be denatured, and/or reduced, and/or alkylated before cleaving the polypeptide in the sample in the first digestion. The neutrophil elastase can be, for example, human neutrophil elastase (EC 3.4.21.37). When analyzing the sample, the analysis can include at least one technique selected from the group consisting of chromatography, electrophoresis, spectroscopy, or spectrometry, and combinations thereof. For example, when the technique for analyzing the sample comprises chromatography, the chromatographic technique can be selected from the group consisting of gas chromatography, liquid chromatography, high performance liquid chromatography, ultra-performance liquid chromatography, size-exclusion chromatography, ion-exchange chromatography, affinity chromatography, expanded bed adsorption chromatography, reverse-phase chromatography, hydrophobic interaction chromatography, and combinations thereof; when the technique is electrophoresis, the electrophoretic technique can be selected from the group consisting of gel electrophoresis, free-flow electrophoresis, electrofocusing, isotachopheresis, affinity electrophoresis, immunoelectrophoresis, counterelectrophoresis, and capillary electrophoresis, capillary zone electrophoresis, and combinations thereof; and when the technique is spectroscopic (or spectrometric), the spectroscopic (or spectrometric) technique can be selected from the group consisting of mass spectrometry, ultraviolet spectroscopy, visible light spectroscopy, fluorescent spectroscopy, and ultraviolet-visible light spectroscopy, and combinations thereof. In some particular sub-aspects, the technique comprises liquid chromatography-mass spectrometry; in other cases, the technique comprises capillary zone electrophoresis coupled to mass spectrometry.

In a third aspect, disclosed herein are methods of preparing a polypeptide for analysis, comprising denaturing, reducing, and alkylating the polypeptide; digesting the polypeptide with trypsin to produce trypsin-cleaved polypeptide fragments; dividing the trypsin-cleaved polypeptide fragments into a first aliquot and a second aliquot; digesting the trypsin-cleaved polypeptide fragments of the first aliquot with neutrophil elastase; combining the first aliquot and second aliquot at about a 1:1 ratio; and

analyzing the combined aliquots. The neutrophil elastase can be, for example, human neutrophil elastase (EC 3.4.21.37). When analyzing the sample, the analysis can include at least one technique selected from the group consisting of chromatography, electrophoresis, spectroscopy, spectrometry, and combinations thereof. For example, when the technique for analyzing the sample comprises

5 chromatography, the chromatographic technique can be selected from the group consisting of gas chromatography, liquid chromatography, high performance liquid chromatography, ultra-performance liquid chromatography, size-exclusion chromatography, ion-exchange chromatography, affinity chromatography, expanded bed adsorption chromatography, reverse-phase chromatography, hydrophobic interaction chromatography, and combinations thereof; when the technique is

10 electrophoresis, the electrophoretic technique can be selected from the group consisting of gel electrophoresis, free-flow electrophoresis, electrofocusing, isotachopheresis, affinity electrophoresis, immunoelectrophoresis, counterelectrophoresis, and capillary electrophoresis, capillary zone electrophoresis, and combinations thereof; and when the technique is spectrometry, the spectroscopic (or spectrometric) technique can be selected from the group consisting of mass spectrometry,

15 ultraviolet spectroscopy, visible light spectroscopy, fluorescent spectroscopy, and ultraviolet-visible light spectroscopy, and combinations thereof. In some particular sub-aspects, the technique comprises liquid chromatography-mass spectrometry; in other cases, the technique comprises capillary zone electrophoresis coupled to mass spectrometry.

In a fourth aspect, disclosed herein are methods of preparing a polypeptide for analysis,

20 comprising providing a sample comprising the polypeptide; applying the sample to a filter having a molecular weight cut-off; digesting the sample on the filter with a first protease; digesting the sample on the filter with a second protease; and analyzing the sample, wherein the second protease is neutrophil elastase; and the first protease is different from the second protease. In this aspect, the first protease can comprise a protease selected from the group consisting of trypsin, Asp-N, and Glu-C; furthermore,

25 the polypeptide can be denatured, and/or reduced, and/or alkylated before digesting the polypeptide with the first protease. The neutrophil elastase can be, for example, human neutrophil elastase (EC 3.4.21.37). When analyzing the sample, the analysis can include at least one technique selected from the group consisting of chromatography, electrophoresis, spectroscopy, spectrometry, and combinations thereof. For example, when the technique for analyzing the sample comprises chromatography, the

30 chromatographic technique can be selected from the group consisting of gas chromatography, liquid chromatography, high performance liquid chromatography, ultra-performance liquid chromatography, size-exclusion chromatography, ion-exchange chromatography, affinity chromatography, expanded bed

adsorption chromatography, reverse-phase chromatography, hydrophobic interaction chromatography, and combinations thereof; when the technique is electrophoresis, the electrophoretic technique can be selected from the group consisting of gel electrophoresis, free-flow electrophoresis, electrofocusing, isotachopheresis, affinity electrophoresis, immunoelectrophoresis, counterelectrophoresis, and capillary
5 electrophoresis, capillary zone electrophoresis, and combinations thereof; and when the technique is spectroscopy or spectrometry, the spectroscopic (or spectrometric) technique can be selected from the group consisting of mass spectrometry, ultraviolet spectroscopy, visible light spectroscopy, fluorescent spectroscopy, and ultraviolet-visible light spectroscopy, and combinations thereof. In some particular sub-aspects, the technique comprises liquid chromatography-mass spectrometry; in other cases, the
10 technique comprises capillary zone electrophoresis coupled to mass spectrometry. The filter can have, for example, a molecular weight cut-off of 30 kDa.

In yet a fifth aspect, disclosed herein are methods of preparing a polypeptide for analysis by liquid chromatography, capillary zone electrophoresis, or mass spectrometry, comprising providing a sample comprising the polypeptide; applying the sample to a 30 kDa MWCO filter; capturing the sample
15 on the filter to produce a retentate; denaturing the sample retentate on the filter; reducing the sample retentate on the filter; alkylating the sample on the filter; digesting the sample on the filter with a first protease; filtering the sample through the same filter and retaining the filtrate; digesting the sample on the filter with a second protease; filtering the sample through the same filter into the previously retained filtrate; quenching the active proteases in the filtrate with guanidine; and analyzing the filtrate
20 by liquid chromatography, capillary zone electrophoresis, or mass spectrometry; wherein the second protease is neutrophil elastase; and the first protease is different from the second protease. In such aspect, the neutrophil elastase can be human neutrophil elastase (EC 3.4.21.37).

In these five aspects, the methods can be accomplished at least in part by automation. In some sub-aspects, the addition and exchange of solutions is handled by an automated liquid handling device
25 or robot.

Furthermore, in these five aspects, the disclosed methods can be used to prepare and analyze therapeutic polypeptides. Therapeutic polypeptides can be selected from the group consisting of an antibody or antigen-binding fragment thereof, a derivative of an antibody or antibody fragment, and a fusion polypeptide. Examples of antibodies and antibody construct include those selected from the
30 group consisting of infliximab, bevacizumab, cetuximab, ranibizumab, palivizumab, abagovomab, abciximab, actoxumab, adalimumab, afelimomab, afutuzumab, alacizumab, alacizumab pegol, ald518, alemtuzumab, alirocumab, altumomab, amatuximab, anatumomab mafenatox, anrukinzumab,

apolizumab, arcitumomab, aselizumab, altinumab, atlizumab, atorolimumab, tocilizumab,
 bapineuzumab, basiliximab, bavituximab, bectumomab, belimumab, benralizumab, bertilimumab,
 besilesomab, bevacizumab, bezlotoxumab, biciromab, bivatuzumab, bivatuzumab mertansine,
 blinatumomab, blosozumab, brentuximab vedotin, briakinumab, brodalumab, canakinumab,
 5 cantuzumab mertansine, cantuzumab mertansine, caplacizumab, capromab pendetide, carlumab,
 catumaxomab, cc49, cedelizumab, certolizumab pegol, cetuximab, citatuzumab bogatox, cixutumumab,
 clazakizumab, clenoliximab, clivatuzumab tetraxetan, conatumumab, crenezumab, cr6261,
 dacetuzumab, daclizumab, dalotuzumab, daratumumab, demcizumab, denosumab, detumomab,
 dorlimomab aritox, drozitumab, duligotumab, dupilumab, ecomeximab, eculizumab, edobacomab,
 10 edrecolomab, efalizumab, efungumab, elotuzumab, elsilimumab, enavatuzumab, enlimomab pegol,
 enokizumab, enoticumab, ensituximab, epitumomab cituxetan, epratuzumab, erenumab, erlizumab,
 ertumaxomab, etaracizumab, etrolizumab, evolocumab, exbivirumab, fanolesomab, faralimomab,
 farletuzumab, fasinumab, fbta05, felvizumab, fezakinumab, ficlatuzumab, figitumumab, flantovumab,
 fontolizumab, foralumab, foravirumab, fresolimumab, fulranumab, futuximab, galiximab, ganitumab,
 15 gantenerumab, gavilimumab, gentuzumab ozogamicin, gevokizumab, girentuximab, glembatumumab
 vedotin, golimumab, gomiliximab, gs6624, ibalizumab, ibritumomab tiuxetan, icrucumab, igovomab,
 imciromab, imgatuzumab, inclacumab, indatuximab ravtansine, infliximab, intetumumab, inolimumab,
 inotuzumab ozogamicin, ipilimumab, iratumumab, itolizumab, ixekizumab, keliximab, labetuzumab,
 lebrikizumab, lemalesomab, lerdelimomab, lexatumumab, libivirumab, ligelizumab, lintuzumab,
 20 lirilumab, lorvotuzumab mertansine, lucatumumab, lumiliximab, mapatumumab, maslimomab,
 mavrilimumab, matuzumab, mepolizumab, metelimomab, milatuzumab, minretumomab, mitumomab,
 mogamulizumab, morolimumab, motavizumab, moxetumomab pasudotox, muromonab-cd3,
 nacolomab tafenatox, namilumab, naptumomab estafenatox, narnatumab, natalizumab, nebacumab,
 necitumumab, nerelimomab, nesvacumab, nimotuzumab, nivolumab, nofetumomab merpentan,
 25 ocaratuzumab, ocrelizumab, odulimumab, ofatumumab, olaratumab, olokizumab, omalizumab,
 onartuzumab, oportuzumab monatox, oregovomab, orticumab, otelixizumab, oxelumab, ozanezumab,
 ozoralizumab, pagibaximab, palivizumab, panitumumab, panobacumab, parsatuzumab, pascolizumab,
 pateclizumab, patritumab, pentumomab, perakizumab, pertuzumab, pexelizumab, pidilizumab,
 pintumomab, placulumab, ponezumab, priliximab, pritumumab, PRO 140, quilizumab, racotumomab,
 30 radretumab, rafivirumab, ramucirumab, ranibizumab, raxibacumab, regavirumab, reslizumab,
 rilotumumab, rituximab, robatumumab, roledumab, romosozumab, rontalizumab, rovelizumab,
 ruplizumab, samalizumab, sarilumab, satumomab pendetide, secukinumab, sevirumab, sibrotuzumab,

sifalimumab, siltuximab, simtuzumab, sipilizumab, sirukumab, solanezumab, solitomab, sonepcizumab, sontuzumab, stamulumab, sulesomab, suvizumab, tabalumab, tacatuzumab tetraxetan, tadocizumab, talizumab, tanezumab, taplitumomab paptox, tefibazumab, telimomab aritox, tenatumomab, tefibazumab, teneliximab, teplizumab, teprotumumab, tezepelumab, TGN1412, tremelimumab, ticilimumab, tildrakizumab, tigatuzumab, TNX-650, tocilizumab, toralizumab, tositumomab, tralokinumab, trastuzumab, TRBS07, tregalizumab, tucotuzumab celmoleukin, tuvirumab, ublituximab, urelumab, urtoxazumab, ustekinumab, vapaliximab, vatelizumab, vedolizumab, veltuzumab, vepalimomab, vesencumab, visilizumab, volociximab, vorsetuzumab mafodotin, votumumab, zalutumumab, zanolimumab, zatuximab, ziralimumab, zolimomab aritox, and those antibodies shown in Table H. In yet other sub-aspects, the therapeutic polypeptide is a polypeptide selected from the group consisting a glycoprotein, CD polypeptide, a HER receptor polypeptide, a cell adhesion polypeptide, a growth factor polypeptide, an insulin polypeptide, an insulin-related polypeptide, a coagulation polypeptide, a coagulation-related polypeptide, albumin, IgE, a blood group antigen, a colony stimulating factor, a receptor, a neurotrophic factor, an interferon, an interleukin, a viral antigen, a lipoprotein, calcitonin, glucagon, atrial natriuretic factor, lung surfactant, tumor necrosis factor-alpha and -beta, enkephalinase, mouse gonadotropin-associated peptide, DNase, inhibin, activating, an integrin, protein A, protein D, a rheumatoid factor, an immunotoxin, a bone morphogenetic protein, a superoxide dismutase, a surface membrane polypeptide, a decay accelerating factor, an AIDS envelope, a transport polypeptide, a homing receptor, an addressin, a regulatory polypeptide, an immunoadhesin, a myostatin, a TALL polypeptide, an amyloid polypeptide, a thymic stromal lymphopoietin, a RANK ligand, a c-kit polypeptide, a TNF receptor, and an angiopoietin, and biologically active fragments, analogs or variants thereof.

In some cases, the therapeutic polypeptide is a BiTE® (bi-specific T-cell engager).

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows α -CD3 domain conserved among many BiTE® molecules (canonical and half-life extension). Tryptic digestion yields an 8 kDa peptide containing two complementarity determining regions (CDRs) with multiple hot spots.

Figure 2 shows enzyme specificity for human neutrophil elastase. P1 corresponds to the C-terminus of one amino acid, and P1' corresponds to the N-terminus of the subsequent residue. Relative font size corresponds to the likelihood of cleavage at that particular residue (adapted from MEROPS database; (Rawlings *et al.*, 2014))

Figure 3 shows extracted ion chromatograms (EIC) of BiTE[®]-1 unstressed and forced deamidation (pH 8.5, 50 °C, t=3 d) samples, showing unmodified and deamidated species after forced deamidation.

Figure 4 shows tandem mass spectrometry (MSMS) of the three deamidated species observed in Figure 3. The diagnostic b3 ion is shifted by +0.9838 Da in peak three relative to peaks 1 and 2. This mass shift corresponds to deamidation.

Figure 5 shows a schematic highlighting differences between the single digest, conventional MAM and one MAM approach for BiTE[®] molecules, which incorporates 1:1 mixing of sample digested with trypsin and sample digested with trypsin followed by neutrophil elastase digestion.

Figure 6 shows the experimental design used to evaluate effectiveness and robustness of Protocol 3 (see *Examples*, below, for further details).

Figure 7 shows a schematic over-sample preparation outlined in Protocol 4 (see *Examples*, below, for further details).

Figure 8 shows trypsin specificity of Protocol 4 for BiTE[®]-1, BiTE[®]-2, and BiTE[®]-3. Specificity was calculated with MassAnalyzer initially by specifying strict trypsin cleavage in search parameters. Data were then researched with cleavage at the C-terminus of residues KRVITAL (low specificity). Trypsin specificity was the total area of peptides identified with the strict tryptic search divided by total area of peptides identified with KRVITAL cleavage search. This value was expressed as a percent.

Figure 9 shows the percent of total area corresponding to the 8 kDa linker peptide for BiTE[®]-1, BiTE[®]-2, and BiTE[®]-3. Data were searched with MassAnalyzer with cleavage at the C-terminus of residues KRVITAL (low specificity). Total area of peptides corresponding to the 8 kDa linker peptide was divided by total area of all peptides identified with the KRVITAL cleavage search. This value was expressed as a percent. The percent of total area corresponding to the 8 kDa linker peptide is higher for BiTE[®]-2 because as a canonical BiTE[®] molecule, it does not contain the Fc domain added for half-life extension.

Figure 10 shows total ion chromatogram (TIC) overlays of replicate injections of BiTE[®]-3 samples prepped with Protocol 3. Injections were from the same vial and were separated by ~4 d.

Figure 11 shows TIC overlays of replicate injections of BiTE[®]-3 samples prepped with Protocol 4. Injections were from the same vial and were separated by ~4 d.

Figure 12 shows TIC overlays comparing BiTE[®]-2 sample prepped in triplicate with Protocols 4 and 5. Two peaks (arrow) observed in all three Protocol 4 samples correspond to non-specific alkylation.

DETAILED DESCRIPTION

In order to address the issue of cleavage-resistant polypeptides for MAM analysis, cleavage sites and specificity for a number of commercially available enzymes were examined. Human neutrophil elastase (HNE; EC 3.4.21.37, also known as elastase-2, human leukocyte elastase (HLE), bone marrow serine protease, medullasin, and PMN elastase) has been reported to cleave at the C-terminus of valine, isoleucine, threonine, alanine, and leucine residues (Fig. 2; (Doucet and Overall, 2011; Rawlings *et al.*, 2014)); however, the enzyme also cleaves non-specifically, a detriment which frequency decreases as substrate length decreases (Stein *et al.*, 1987). Given an amino acid sequence of a target polypeptide, digestion with HNE can serve as a means to monitor desired regions in the polypeptide but for HNE's propensity to non-specifically cleave polypeptides.

For example, the inventors observed that when bi-specific T-cell engager (BiTE[®]) molecules were digested only with HNE, resulting peptides were very numerous (due to nonspecific cleavage) and low-abundance. For example, Fig. 1 shows α -CD3 domain conserved among many BiTE[®] molecules (canonical and half-life extension). Tryptic digestion yields an 8 kDa peptide containing two complementarity determining regions (CDRs) with multiple hot spots that are difficult to identify and characterize using conventional mass spectrometry (MS)-based experiments. This peptide also lacks residues susceptible to cleavage by other widely-used enzymes such as Glu-C and Asp-N.

The inventors also unexpectedly observed that, following on-filter trypsin digestion, both the conserved 8 kDa linker peptide and molecule-specific linker peptides of BiTE[®] molecules were retained by 30 kDa molecular weight cut-off (MWCO) filters regardless of whether the filter membranes were composed of regenerated cellulose or polyethersulfone. Enhancing specificity of HNE by first digesting with a first protease that is not HNE and exploiting the unanticipated retention of the linker peptides resulting from the first protease digestion by 30 kDa MWCO (or other cut-off) filters allowed for the development of a novel, robust method for characterization of BiTE[®] molecules (and other polypeptides) and quantitation of critical attributes.

A description of a novel sample preparation method that uses a first digestion step followed by digestion with HNE is disclosed.

Definitions

Both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive. The use of the singular includes the plural unless specifically stated otherwise. The use of "or" means "and/or" unless stated otherwise. The use of the term

“including”, as well as other forms, such as “includes” and “included,” is not limiting. Terms such as “element” or “component” encompass both elements and components comprising one unit and elements and components that comprise more than one subunit unless specifically stated otherwise. The use of the term “portion” can include part of a moiety or the entire moiety. When a numerical range is mentioned, *e.g.*, 1-5, all intervening values are explicitly included, such as 1, 2, 3, 4, and 5, as well as fractions thereof, such as 1.5, 2.2, 3.4, and 4.1.

“About” or “~” means, when modifying a quantity (*e.g.*, “about” 3 mM), that variation around the modified quantity can occur. These variations can occur by a variety of means, such as typical measuring and handling procedures, inadvertent errors, ingredient purity, and the like.

“Comprising” and “comprises” are intended to mean that methods include the listed elements but do not exclude other unlisted elements. The terms “consisting essentially of” and “consists essentially of,” when used in the disclosed methods include the listed elements, exclude unlisted elements that alter the basic nature of the method, but do not exclude other unlisted elements. The terms “consisting of” and “consists of” when used to define methods exclude substantial method steps. Embodiments defined by each of these transition terms are within the scope of this disclosure.

“Coupled” means associated directly as well as indirectly. For example, a device or process can be directly associated with another device or process, or these devices and/or processes can be indirectly associated with each other, *e.g.*, via another device or process.

“Protein”, “peptide”, and “polypeptide” are used interchangeably to mean a chain of amino acids wherein each amino acid is connected to the next by a peptide bond.

“Denaturation,” “denaturing,” “denature,” and the like means a process in which polypeptides lose at least partially the quaternary structure, tertiary structure, and secondary structure that are found in the polypeptides in their native states by applying an external stress or reagent (*i.e.*, a denaturant).

“Denaturant” means any substance, composition, energy, or condition that can denature a polypeptide. Examples of denaturants include a strong acid or base, an inorganic salt, an organic solvent, radiation, a chaotropic agent, or heat, or combinations of these.

“Denatured polypeptide,” “denatured protein,” and the like means a polypeptide that has its secondary, tertiary, and/or quaternary structure changed from the native polypeptide. A polypeptide can be fully denatured or partially denatured. A “non-denatured polypeptide” or “non-denatured protein” (and similar terms) means a polypeptide that has maintained its secondary, tertiary, and as applicable, quaternary structure. A “native polypeptide” or “native protein” (and the like) refers to a

polypeptide as found in nature and has any primary reference sequence described in the UniProt Knowledgebase database (The UniProt, 2017) or the UniGene database (Pontius *et al.*, 2003).

“Reduced polypeptide” or “reduced protein” (and similar terms) means a polypeptide in which at least one of its interchain or intrachain disulfide bonds is broken. Such bonds can form between reduced thiol groups, such as those available on cysteine residues.

“Alkylated polypeptide” or “alkylated protein” (and the like) means a polypeptide to which an alkyl group has been transferred. Pragmatically, polypeptides are often alkylated on thiol groups (such as available on cysteine residues) to prevent reduced thiols from forming, or reforming after reduction, disulfide bonds or bridges.

“Digestion” and the like, in the context of polypeptides, means the fragmenting of a polypeptide into two or more fragments, which fragmentation is mediated by another substance, chemical, or enzyme.

“Proteolytic cleavage,” “proteolytic digestion,” and the like means the cleaving of a polypeptide by breaking the peptide bonds in a polypeptide, thus producing fragments. Proteolytic cleavage can be mediated by enzymes.

“Protease,” “peptidase,” “proteolytic cleavage enzyme,” “endoproteinase,” “proteinase,” and the like means an enzyme and means a polypeptide or fragment thereof that catalyzes hydrolysis of peptide bonds. Proteases include variants of an amino acid sequences of known proteases that catalyze peptide bond hydrolysis, even wherein such catalyzing activity is reduced in the variant.

Enzyme catalyzed reactions are classified according to the Enzyme Commission numbering system. Because proteases catalyze peptide bond hydrolysis, they are classified in class EC 3.4, which includes , EC 3.4.11 Aminopeptidases , EC 3.4.13 Dipeptidases , EC 3.4.14 Dipeptidyl peptidases and tripeptidyl peptidases , EC 3.4.15 Peptidyl dipeptidases , EC 3.4.16 Serine type carboxypeptidases , EC 3.4.17 Metallo carboxypeptidases , EC 3.4.18 Cysteine type carboxypeptidases , EC 3.4.19 Omega peptidases , EC 3.4.21: Serine proteases , EC 3.4.22 Cysteine proteases , EC 3.4.23 Aspartic endopeptidases , EC 3.4.24: Metallopeptidases , EC 3.4.25 Threonine endopeptidases , EC 3.4.99 Endopeptidases of unknown catalytic mechanism. Specific examples of proteases include trypsin (EC 3.4.21.4), endoproteinase Asp-N (EC 3.4.24.33), and endoproteinase Glu-C (EC 3.4.21.19).

Elastases are proteases that (except for those classified as EC 3.4.21.70) hydrolyze elastin, an elastic fiber polypeptide of the extracellular matrix. There are eight human genes that encode elastases (Table A):

Table A

The human elastases

Family	Gene	Name	EC number	Preferential cleavage
Neutrophil				
	ELANE (ELA2)	Neutrophil elastase (elastase 2)	3.4.21.37	Val- -Xaa; Ala- -Xaa; Thr- -Xaa; Ile- -Xaa; Leu- -Xaa
Chymotrypsin				
	CTRC (ELA4)	Chymotrypsin C (cadlecrin) (elastase 4)	3.4.21.2	Leu- -Xaa; Tyr- -Xaa; Phe- -Xaa; Met- -Xaa; Trp- -Xaa; Gln- -Xaa; Asn- -Xaa
Chymotrypsin-like				
	CELA1 (ELA1)	Chymotrypsin-like elastase family, member 1 (pancreatic elastase 1)	3.4.21.36	Ala- -Xaa
	CELA2A (ELA2A)	Chymotrypsin-like elastase family, member 2A (pancreatic elastase 2A)	3.4.21.71	Leu- -Xaa Met- -Xaa Phe- -Xaa
	CELA2B (ELA2B)	Chymotrypsin-like elastase family, member 2B (pancreatic elastase 2B)	3.4.21.71	Leu- -Xaa Met- -Xaa Phe- -Xaa
	CELA3A (ELA3A)	Chymotrypsin-like elastase family, member 3A (pancreatic elastase 3A)	3.4.21.70	Ala- -Xaa (Does not hydrolyze elastin)
	CELA3B (ELA3B)	Chymotrypsin-like elastase family, member 3B	3.4.21.70	Ala- -Xaa (Does not hydrolyze)

Family	Gene	Name	EC number	Preferential cleavage
Macrophage		(pancreatic elastase 3B)		elastin)
	MMP12 (HME)	Macrophage metalloelastase (macrophage elastase)	3.4.24.65	Hydrolysis of soluble and insoluble elastin. Specific cleavages are also produced at 14-Ala- -Leu-15 and 16-Tyr- -Leu-17 in the B chain of insulin

“Neutrophil elastase” (sometimes referred to as elastase-2, leukocyte elastase, bone marrow serine protease, medullasin, or PMN elastase) is an elastase that neutrophils and macrophages secrete during inflammation to destroy bacteria. Human neutrophil elastase (HNE) was first described in 1968 (Janoff and Scherer, 1968). HNE was shown to differ from pancreatic elastases in that soybean trypsin inhibitor and salivary kallikrein inhibitor inhibit HNE, but do not inhibit pancreatic elastase activity. HNE maintains activity at physiological salt concentrations, while pancreatic elastase activity decreases. HNE is at least ten-fold more resistant to serum elastase inhibitor (as defined by Janoff and Scherer, 1968) than pancreatic elastase. Finally, HNE is also more low pH-resistant than pancreatic elastase (Janoff and Scherer, 1968).

Sinha *et al.* (Sinha *et al.*, 1987) first described the amino acid sequence of HNE and showed that the polypeptide had only 43% sequence homology to pig pancreatic elastase. The UniProt database for UniProtKB – P08246 (ELNE_HUMAN) (The UniProt, 2017) describes ELANE as encoding HNE as a 267 amino acid precursor polypeptide consisting of a signal peptide (amino acid residues 1-27), a pro-peptide domain (residues 28-29), and the mature polypeptide (residues 30-267). Within the mature polypeptide resides the peptidase S1 domain (residues 30-247). Disulfide bonds can be found forming between residues 55 and 71, 151 and 208, 181 and 187, and 198-223. Asparagine residues at positions 88, 124, and 174 can be glycosylated (N-linked). The sequence for HNE is shown in Table B. In Table B, the signal peptides are **boldfaced**, the pro-peptide domain is single-underlined, the mature polypeptide is *italicized*, and the S1 peptidase domain is double-underlined, italicized, and boldfaced.

Table B

Amino acid sequence of human neutrophil elastase (HNE) (SEQ ID NO:1)

MTLGRRRLACL FLACVLPALL <u>LGGTALASEI</u> <u>VGGRRARPHA</u> <u>WPFMVSLQLR</u> <u>GGHFEGATLI</u>	60
<u>APNFVMSAAH</u> <u>CVANVNVRAV</u> <u>RVVLGAHNLS</u> <u>RREPTRQVFA</u> <u>VQRIFENGYD</u> <u>PVNLLNDIVI</u>	120
<u>LQLNGSATIN</u> <u>ANVQVAQLPA</u> <u>QGRRLGNGVQ</u> <u>CLAMGWLLG</u> <u>RNRGIASVLQ</u> <u>ELNVTVVVTSI</u>	180
<u>CRRSNVCTLV</u> <u>RGRQAGVCFG</u> <u>DSGSPLVCNG</u> <u>LIHGIASFVR</u> <u>GGCASGLYPD</u> <u>AFAPVAQFVN</u>	240
<u>WIDSIIQRSE</u> <u>DNPCPHPRDP</u> <u>DPASRTH</u>	267

HNE is capable of hydrolyzing almost every polypeptide member of the extracellular matrix, including several types of collagen, fibronectin, proteoglycans, heparin, and cross-linked fibrin.

HNE cleaves peptide bonds in which the P1 residue is a small alkyl group. Substrate specificity is summarized in Fig. 2.

“Chemical cleavage” in the context of producing fragments of a polypeptide, means the fragmenting of the polypeptide by a chemical. A “chemical” is a non-proteinaceous substance or compound. Chemicals can be organic or inorganic.

“Molecular weight cut-off” or “MWCO” means A membrane’s MWCO is a representation of membrane selectivity for solute molecules of different molecular weights (MWs), where the MW value (expressed in Daltons (Da)) is obtained from the solute molecule that gives a 90% rejection when a range of different MW solutes are filtered in the target solvent (which for most liquid based, pressure driven membrane applications is water), where rejection is defined as in Eq. (1):

$$Rejection, R(\%) = \left(1 - \frac{c_p}{c_f}\right) \times 100 \quad (1)$$

where C_p and C_f are the concentration of permeate and feed, respectively.

MWCO is determined experimentally using dextran, polyethylene glycol, and proteins of various molecular weights to rate the MWCO of membranes or filters. The rejection depends on many solute and process parameters like the type of solute, concentration, hydrodynamics, pressure, temperature and pH. MWCO measurements usually are carried out in separate experiments using different solutes, each with a certain MW.

For low range MW (approximately under 10 kDa), polyethylene glycols (PEGs), n-alkanes, and oligostyrenes can be used in solute rejection assays. For high range MW (approximately greater than 10 kDa), dextrans and sugars are often used (Rohani *et al.*, 2011).

Methods

Disclosed herein are methods directed to digesting polypeptide in a sample with a first protease and a second protease, wherein the first protease produces at least two fragments of the polypeptide, which the at least two fragments of the polypeptide are subsequently digested by the second protease; and analyzing the sample after digesting with the second protease, wherein the second protease is neutrophil elastase; and the first protease is different from the second protease. Other steps in the method can include dividing the sample into two aliquots after digesting the polypeptide in the sample with the first protease, and then digesting the polypeptide in one of the two aliquots with the second protease. These aliquots can then be combined before analysis. The polypeptide in the sample can be denatured and/or reduced and/or alkylated before digesting with the first protease. Analysis can include chromatography, electrophoresis, spectrometry, and combinations thereof.

In some embodiments, the methods include applying the sample to a filter having a MWCO before digesting the polypeptide in the sample on the filter with the first protease, digesting the

polypeptide in the sample with the second protease, and analyzing the sample. Such embodiments can further incorporate filtering steps. The MWCO filter, in some embodiments, retains a significant proportion of the polypeptide and polypeptide fragments, even though the size of these polypeptides or polypeptide fragments have a MW that is significantly smaller than the MWCO of the filter.

5

Method steps

Polypeptide denaturing

In some embodiments, the polypeptide that is prepared and analyzed according to the disclosed methods is denatured.

10 Polypeptides can be denatured using a variety of art-accepted techniques and denaturants. In some embodiments, multiple denaturants are used together, either simultaneously or in sequence. For example, the denaturants of SDS and heat can be combined to denature polypeptides.

Protein denaturation can be accomplished by any means that disrupts quaternary, tertiary, or secondary polypeptide structure. For example, the use of chaotropes, such as urea, and denaturing
15 detergents (*e.g.*, sodium dodecyl sulfate (SDS)), heat, reducing agents, and agents that inactivate reactive thiol groups to block disulfide reformation. The pH of polypeptide-containing samples can also be manipulated to encourage denaturation. These components are often used together to effectively unfold polypeptides.

Additional examples of chaotropes include, in addition to urea, n-butanol, ethanol, guanidinium
20 chloride, lithium perchlorate, lithium acetate, magnesium chloride, phenol, 2-propanol, and thiourea. Urea is preferred in most instances.

Detergents are classified in the form of the hydrophilic group: anionic, cationic, non-ionic, and zwitterionic. Anionic and cationic detergents are more likely to be denaturing, examples of which include: SDS, sodium cholate, sodium deoxycholate, sodium glycocholate, sodium taurocholate, sodium
25 taurodeoxycholate, N-lauroylsarcosine, lithium dodecyl sulfate (anionic) and hexadecyltrimethyl ammonium bromide (CTAB) and trimethyl(tetradecyl) ammonium bromide (TTAB) (cationic). In some cases, a zwitterionic detergent can be useful, examples include amidosulfobetaine-14 (ASB-14), amidosulfobetaine-16 (ASB-16), C7Bz0, CHAPS, CHAPSO, EMPIGEN® BB, 3-(N,N-dimethyloctylammonio)propanesulfonate inner salt (SB3-8), d (decyldimethylammonio)
30 propanesulfonate inner salt (SB3-10), *etc.* Anionic detergents are preferred, with SDS being particularly preferred.

A denaturant can be heat, such as an elevated temperature at or greater than 30 °C (for most polypeptides). Denaturants include agitation. In some embodiments, low salt, including essentially or substantially or no salt can denature polypeptides.

A denaturant can be a solvent, such as ethanol or other alcohols.

5 Denaturing of polypeptides has been extensively studied and described; for example, see (Tanford, 1968) for further details. A person of ordinary skill in the art understands how to denature polypeptides given the nature of the polypeptide and the many denaturants from which to choose.

Reduction of polypeptides

10 A reduced polypeptide is a polypeptide that is exposed to reducing conditions sufficient to reduce a reducible residue in the polypeptide structure, such as a cysteine. If the reduced polypeptide contains a thiol group, or sulfur-containing residue, then the thiol group in the reduced polypeptide is reduced. A reduced polypeptide comprising a cysteine residue has the sulfur atom of the cysteine residue reduced, which can be indicated as “-SH.” A reduced polypeptide can be a disulfide bond-
15 containing polypeptide. A disulfide bond-containing polypeptide can become a reduced polypeptide by exposure to reducing conditions that cause one or more disulfide bonds (disulfide bridges) in the disulfide bond- containing polypeptide to break.

A “reducing agent”, “reductant” or “reducer” is an element or compound that loses (or donates) an electron to another chemical species in a redox chemical reaction. A reducing agent allows disulfide
20 groups to become reactive by generating thiol (-SH) groups. Common polypeptide reducing reagents are shown in Table C.

Table C
Reducing reagents

Product	Notes (including alternative names and CAS entries)
2-Mercaptoethanol	β -mercaptoethanol (BME, 2BME, 2-ME, b-mer, CAS 60-24-2)
2-Mercaptoethylamine-HCl	2-aminoethanethiol (2-MEA-HCl, cysteamine-HCl, CAS 156-57-0), selectively reduces antibody hinge-region disulfide bonds
Dithiothreitol	Dithiothreitol (DTT, CAS 3483-12-3)
TCEP-HCl	Tris (2-carboxyethyl) phosphine hydrochloride (TCEP, CAS 5961-85-3) is a thiol-free reductant for polypeptide disulfide bonds

In some embodiments, polypeptide denaturation and reduction are carried out simultaneously. In other embodiments, the polypeptide denaturation and reduction are performed in discrete steps.

A person of ordinary skill in the art understands how to reduce polypeptides given the nature of the polypeptide and the reducing reagents from which to choose.

Alkylating a polypeptide comprised in a sample

“Inactivating reactive thiol groups” means blocking free thiol groups in a polypeptide to prevent unwanted thiol-disulfide exchange reactions. Alkylating agents are substances that cause the replacement of hydrogen by an alkyl group.

Alkylation of free cysteines, often following their reduction, prevents formation and reformation of disulfide bonds that might otherwise form between free thiols of cysteine residues. Commonly used alkylating agents include n-ethylmaleimide (NEM), iodoacetamide (IAA) and iodoacetic acid. Examples of other suitable alkylating agents include dithiobis(2-nitro)benzoic acid; acrylamide; 4-vinylpyridine; nitrogen mustards, such as chlorambucil and cyclophosphamide; cisplatin; nitrosoureas, such as carmustine, lomustine and semustine; alkyl sulfonates, such as busulfan; ethyleneimines, such as thiotepa; and triazines, such as dacarbazine. The person skilled in the art is aware of the reagents that can be used to protect sulfhydryl groups, as well as how to use such reagents.

Polypeptide digestion

The methods disclosed herein comprise cleaving the polypeptide to be analyzed in a sample in a first digestion, wherein the cleaving produces at least two fragments of the polypeptide. Any method of fragmenting the polypeptide can be used, provided that at least two fragments of the polypeptide are produced; furthermore, complete degradation of the polypeptide into, for example, single amino acids, is undesirable.

In such digestion step, convenient means of cleavage include using a protease (wherein the protease is not neutrophil elastase). Any protease can be used, as long as such protease cleaves the polypeptide into at least two fragments. The rationale supporting this first digestion before digesting with a neutrophil elastase is to provide polypeptide fragments to the neutrophil elastase (such as human neutrophil elastase), wherein the neutrophil elastase has an increased specificity, as compared to when presented with the intact polypeptide.

In some embodiments, mixtures of two or more proteases can be used. In other embodiments, the first digestion can include multiple sequential digestions. For example, a first digestion is performed with a first protease, and then in a subsequent reaction, a second digestion is performed with a second protease (wherein neither protease is neutrophil elastase). A second (or more) digestions are completed to improve the specificity of the neutrophil elastase used in the step of cleaving the polypeptide further with the neutrophil elastase.

Examples of useful proteases (but not at all inclusive) include trypsin, endoproteinase Glu-C, endoproteinase Arg-C, pepsin, chymotrypsin, chymotrypsin B, Lys-N protease, Lys-C protease, Glu-C protease, Asp-N protease, pancreatopeptidase, carboxypeptidase A, carboxypeptidase B, proteinase K, and thermolysin. In some embodiments, combinations of these proteases are used. In some embodiments, trypsin alone is used.

These and other proteases, including peptide bond selectivity and E.C. numbers, are shown in Table D, which is adapted from (Unspecified, 2007). The sources shown for each protease are exemplary only; many of these proteases are commercially available.

In some embodiments, a protein: protease ratio (w/w) of 10:1, 20:1, 25:1, 50:1, or 100:1 can be used. In some embodiments, the ratio is 20:1. In some embodiments, the neutrophil elastase used is at a concentration of about 100 ng/ml-1 mg/ml, or about 100 ng/ml-500 µg/ml, or about 100 ng/ml-100 µg/ml, or about 1 µg/ml-1 mg/ml, or about 1 µg/ml-500 µg/ml, or about 1 µg/ml-100 µg/ml, or about 10 µg/mg-1 mg/ml, or about 10 µg/mg-500 µg/ml, or about 10 µg/mg-100 µg/ml. In some embodiments, the digestion step is for 10 minutes to 48 hours, or 30 minutes to 48 hours, or 30 minutes to 24 hours, or

30 minutes to 16 hours, or 1 hour to 48 hours, or 1 hour to 24 hours, or 1 hour to 16 hours, or 1 to 8 hours, or 1 to 6 hours, or 1 to 4 hours. In some embodiments, the digestion step is incubated at a temperature between 20 °C and 45 °C, or between 20 °C and 40 °C, or between 22 °C and 40 °C, or between 25 °C and 37 °C. In some embodiments, the digestion step is incubated at 37 °C. One of skill in
5 the art can choose appropriate conditions (buffers, incubation times, amount of protease, volumes, etc.), as *in vitro* protease digestion is well understood in the art.

Table D

Proteases commonly used for protein fragmentation

Protease ^a	EC no.	Peptide bond selectivity	Exemplary Accession no. ^b
Trypsin (bovine)	3.4.21.4	P ₁ -P ₁ ¹⁻ (P ₁ = Lys, Arg)	P00760 ^S
Chymotrypsin (bovine)	3.4.21.1	P ₁ -P ₁ ¹⁻ (P ₁ = aromatic, P ₁ ¹⁻ = nonspecific)	P00766 ^S
Endoproteinase Asp-N (<i>Pseudomonas fragi</i>)	3.4.24.33	P ₁ -Asp- (and -P ₁ -cysteic acid)	φ
Endoproteinase Arg-C (mouse submaxillary gland)	φ	-Arg-P ₁ -	-
Endoproteinase Glu-C (V8 protease) (<i>Staphylococcus aureus</i>)	3.4.21.19	-Glu-P ₁ ¹⁻ (and -Asp-P ₁ ¹⁻) (2)	P04188 ^S
Endoproteinase Lys-C (<i>Lysobacter enzymogenes</i>)	3.4.21.50	-Lys-P ₁ ¹⁻	S77957 ^P
Pepsin (porcine)	3.4.23.1	P ₁ -P ₁ ¹⁻ (P ₁ = hydrophob pref.)	P00791 ^S
Thermolysin (<i>Bacillus thermo-proteolyticus</i>)	3.4.24.27	P ₁ -P ₁ ¹⁻ (P ₁ = Leu, Phe, Ile, Val, Met, Ala)	P00800 ^S
Elastase (porcine) (not neutrophil elastase)	3.4.21.36	P ₁ -P ₁ ¹⁻ (P ₁ = uncharged, nonaromatic)	P00772 ^S
Papain (<i>Carica papaya</i>)	3.4.22.2	P ₁ -P ₁ ¹⁻ (P ₁ = Arg, Lys pref.)	P00784 ^S
Proteinase K (<i>Tritirachium album</i>)	3.4.21.64	P ₁ -P ₁ ¹⁻ (P ₁ = aromatic, hydrophob pref.)	P06873 ^S
Subtilisin (<i>Bacillus subtilis</i>)	3.4.21.62	P ₁ -P ₁ ¹⁻ (P ₁ = neutral/acidic pref.)	P04189 ^S
Clostripain (endoproteinase-Arg-C) (<i>Clostridium histolyticum</i>)	3.4.22.8	-Arg-P ₁ - (P ₁ = Pro pref.)	P09870 ^S
Carboxypeptidase A (bovine)	3.4.17.1	P ₁ -P ₁ ¹⁻ (P ₁ cannot be Arg, Lys, Pro)	P00730 ^S
Carboxypeptidase B (porcine)	3.4.17.2	P ₁ -P ₁ ¹⁻ (P ₁ = Lys, Arg)	P00732 ^S
Carboxypeptidase P (<i>Penicillium janthinellum</i>)	φ	P ₁ -P ₁ ¹⁻ (nonspecific)	-
Carboxypeptidase Y (yeast)	3.4.16.5	P ₁ -P ₁ ¹⁻ (nonspecific)	P00729 ^S
Cathepsin C	3.4.14.1	X-P ₁ -P ₁ ¹⁻ (removes amino-terminal dipeptide)	-
Acylamino-acid-releasing enzyme (porcine)	3.4.19.1	Ac-P ₁ -P ₁ ¹⁻ (P ₁ = Ser, Ala, Met pref.)	P19205 ^{S+}

Protease ^a	EC no.	Peptide bond selectivity	Exemplary Accession no. ^b
Pyroglutamate aminopeptidase (bovine)	3.4.19.3	P ₁ -P ₁ ¹⁻ (P ₁ = 5-oxoproline or pyroglutamate)	-

^aExemplary source shown in parentheses

^bS = SwissProt; P = PIR; + = porcine sequence; φ = partial sequences of Asp-N; accession numbers: AAB35279, AAB35280, AAB35281, AAB35282

In some embodiments, the first digestion is accomplished using a chemical. Especially useful chemicals include those that cleave polypeptides in a site-specific manner. Such chemicals include cyanogen bromide (CNBr; carbononitridic bromide), which cleaves C-terminal of methionine residues; 2-nitro-5-thiocyanobenzoate (NTCB), which cleaves N-terminally of cysteine residues; asparagine-glycine
5 dipeptides can be cleaved using hydroxylamine; formic acid, which cleaves at aspartic acid-proline (Asp-Pro) peptide bonds, and BNPS-skatole (3-bromo-3-methyl-2-(2-nitrophenyl)sulfanylidole), which cleaves C-terminal of tryptophan residues. One of skill in the art understands how to select appropriate variables, including polypeptide concentration, chemical concentration, incubation time and temperature, *etc.* See also, for example, (Crimmins *et al.*, 2001; Li *et al.*, 2001; Tanabe *et al.*, 2014).

10 In some embodiments, the first digestion can include multiple sequential digestions, wherein at least one of such sequential digestions comprises using a chemical, such as CNBr, NTCB, hydroxylamine, formic acid, and BNPS-skatole. For example, a first digestion is performed with chemical that cleaves the polypeptide into at least two fragments, and then in a subsequent reaction, a second digestion is performed with a protease (wherein the protease is not neutrophil elastase), or *vice versa*. A second (or
15 more) digestions are completed to improve the specificity of the neutrophil elastase used in the step of cleaving the polypeptide further with the neutrophil elastase.

In the disclosed methods, a subsequent digestion is performed with a neutrophil elastase. In some embodiments, the neutrophil elastase is human.

In some embodiments, a protein: (human) neutrophil elastase ratio (w/w) of 10:1, 20:1, 25:1,
20 50:1, or 100:1 can be used. In some embodiments, the ratio is 20:1. In some embodiments, the neutrophil elastase used is at a concentration of about 100 ng/ml-1 mg/ml, or about 100 ng/ml-500 µg/ml, or about 100 ng/ml-100 µg/ml, or about 1 µg/ml-1 mg/ml, or about 1 µg/ml-500 µg/ml, or about 1 µg/ml-100 µg/ml, or about 10 µg/mg-1 mg/ml, or about 10 µg/mg-500 µg/ml, or about 10 µg/mg-100 µg/ml. In some embodiments, the digestion step is for 10 minutes to 48 hours, or 30 minutes to 48
25 hours, or 30 minutes to 24 hours, or 30 minutes to 16 hours, or 1 hour to 48 hours, or 1 hour to 24 hours, or 1 hour to 16 hours, or 1 to 8 hours, or 1 to 6 hours, or 1 to 4 hours. In some embodiments, the digestion step is incubated for 35 minutes. In some embodiments, the digestion step is incubated at a temperature between 20 °C and 45 °C, or between 20 °C and 40 °C, or between 22 °C and 40 °C, or between 25 °C and 37 °C. In some embodiments, the digestion step is incubated at 37 °C. One of skill in
30 the art is capable of understanding the various parameters and can select appropriate conditions for the digestion with neutrophil elastase.

Dividing sample into aliquots

In embodiments, before the sample comprising the polypeptide to be analyzed is digested with neutrophil elastase, the sample is divided into aliquots. In some cases, dividing the sample into aliquots is performed by simply taking half the volume of the sample before digesting with neutrophil elastase. In some cases, the aliquots can be equalized for polypeptide concentration using, for example, UV spectrophotometry.

Filter selection

When choosing the appropriate MWCO for specific applications, many factors are usually considered, including sample concentration, composition, molecular shape, and operating conditions such as temperature, pressure, and cross-flow velocity. Other variables regarding the flow of molecule passage are also factored in. For example, linear molecules, high transmembrane pressure (TMP) and low sample concentration can increase molecule passage, while low temperature and membrane fouling can decrease molecule passage. Qualification methods for MWCO are not always comparable, as they vary across manufacturers. In the art, it is commonly advised to select a MWCO that is at least two times smaller than the molecular weight of the solute that is being retained.

For the disclosed methods, filter shape is also considered. For 1-2 mL volumes, filter units where the filter is slanted when the filter unit is perpendicular to the ground are less desirable than those where the filter is horizontal when the filter unit is perpendicular to the ground.

Suitable materials for the MWCO membrane are hydrophilic. Suitable hydrophilic materials include polyethersulfone, polyvinylidene difluoride, and regenerated cellulose.

Suitable cut-off filters can be obtained commercially, such as from Pall Corporation (Port Washington, NY) or Millipore, Inc (Burlington, MA, such as Microcon® filters). For the disclosed methods, filters from these particular manufacturers have MWCO specifications that are suitable. Comparable filters from other manufacturers can also be used. The MWCO specifications provided by manufacturers can be relied upon because even accounting for differences between MWCO ratings between manufacturers, the disclosed methods take advantage in part of the surprising observation that small molecular weight molecules are retained by the filter even though these molecules have a molecular weight that is equal to or less than (and often substantially less than) the rated MWCO of the filter (see, for example, Example 11).

Filtering the sample using MWCO filters can use any suitable filter method, using any suitable filter device. Filtering can take place by gravity, capillary force, or commonly centrifugation, including ultracentrifugation.

5 Analyzing the sample

After the polypeptide has been digested with neutrophil elastase, the resulting polypeptide fragment can be analyzed by any suitable method. The proceeding discussion is not meant to limit in any way the methods that can be used to analyze the prepared polypeptides.

10 In general, suitable analytical methods can be chromatographic, electrophoretic, and spectrometric. Some of these methods can be combined.

One of skill in the art has access to, for example, handbooks, that facilitate the selection of appropriate analytical methods, as well as appropriate conditions to conduct those methods, including for example, (Gunzler and Williams, 2001).

15 Chromatographic methods are those methods that separate polypeptide fragments in a mobile phase, which phase is processed through a structure holding a stationary phase. Because the polypeptide fragments are of different sizes and compositions, each fragment has its own partition coefficient. Because of the different partition coefficients, the polypeptides are differentially retained on the stationary phase. Examples of such methods known in the art include gas chromatography, liquid chromatography, high performance liquid chromatography, ultra-performance liquid chromatography, 20 size-exclusion chromatography, ion-exchange chromatography, affinity chromatography, expanded bed adsorption chromatography, reverse-phase chromatography, and hydrophobic interaction chromatography.

A summary of some of the known chromatographic methods is shown in Table E.

Table E
Examples of chromatographic methods

Chromatographic type	Description
Adsorption	Adsorbent stationary phase
Affinity	Based on a highly specific interaction such as that between antigen and antibody or receptor and ligand, one such substance being immobilized and acting as the sorbent
Column	The various solutes of a solution travel down an absorptive column where the individual components are absorbed by the stationary phase. The most strongly adsorbed component will remain near the top of the column; the other components will pass to positions farther down the column according to their affinity for the adsorbent
Exclusion (including gel-filtration, gel-permeation, molecular exclusion, molecular sieve gel-filtration)	Stationary phase is a gel having a closely controlled pore size. Molecules are separated based on molecular size and shape; smaller molecules being temporarily retained in the pores
Expanded bed adsorption (EBA)	Useful for viscous and particulate solutions. Uses for the solid phase particles that are in a fluidized state, wherein a gradient of particle size is created.
Gas (GC)	An inert gas moves the vapors of the materials to be separated through a column of inert material
Gas-liquid (GLC)	Gas chromatography where the sorbent is a nonvolatile liquid coated on a solid support
Gas-solid (GSC)	Gas chromatography where the sorbent is an inert porous solid
High-performance liquid, high-pressure liquid (HPLC).	Mobile phase is a liquid which is forced under high pressure through a column packed with a sorbent
Hydrophobic interaction chromatography	Matrix is substituted with hydrophobic groups (such as methyl, ethyl, propyl, octyl, or phenyl). At high salt concentrations, non-polar sidechains on polypeptide surfaces interact

Chromatographic type	Description
Ion exchange	with the hydrophobic groups; that is, both types of groups are excluded by a polar solvent; elution accomplished with decreasing salt, increasing concentrations of detergent, and/or changes in pH. Stationary phase is an ion exchange resin to which are coupled either cations or anions that exchange with other cations or anions in the material passed through.
Paper	Paper is used for adsorption
Partition	The partition of the solutes occurs between two liquid phases (the original solvent and the film of solvent on an adsorption column)
Reverse-phase	Any liquid chromatography in which the mobile phase is significantly more polar than the stationary phase. Hydrophobic molecules in the mobile phase adsorb to the hydrophobic stationary phase; hydrophilic molecules in the mobile phase tend to elute first.
Thin-layer (TLC)	Chromatography through a thin layer of inert material, such as cellulose
Ultra-performance liquid (UPLC)	A liquid chromatographic technique that uses a solid phase with particles less than 2.5 μm (smaller than in HPLC) and has higher flow rates; pressure used is 2-3 times more than in HPLC.

Prepared polypeptides can be analyzed also using electrophoretic methods - gel electrophoresis, free-flow electrophoresis, electrofocusing, isotachopheresis, affinity electrophoresis, immunoelectrophoresis, counterelectrophoresis, capillary electrophoresis, and capillary zone electrophoresis. Overviews and handbooks are available to one of skill in the art, such as (Kurien and
5 Scofield, 2012; Lord, 2004).

Electrophoresis can be used to analyze charged molecules, such as polypeptides which are not at their isoelectric point, which are transported through a solvent by an electrical field. The polypeptides migrate at a rate proportional to their charge density. A polypeptide's mobility through an electric field depends on: field strength, net charge on the polypeptide, size and shape of the polypeptide, ionic
10 strength, and properties of the matrix through which the polypeptide migrates (*e.g.*, viscosity, pore size). Polyacrylamide and agarose are two common support matrices. These matrices serve as porous media and behave like a molecular sieve. Polyacrylamide forms supports with smaller pore sizes and is especially useful in the disclosed methods, being ideal for separating most polypeptide fragments.

Table F presents examples of polypeptide electrophoretic techniques.

Table F
Examples of electrophoretic methods

Technique	Description
Gel electrophoresis	Refers to electrophoretic techniques that use a gel as a matrix through which polypeptides travel. Many electrophoretic techniques use gels, including those based on polyacrylamide (polyacrylamide gel electrophoresis (PAGE), including denaturing and non-denaturing PAGE). Pore size of polyacrylamide gels is controlled by modulating the concentrations of acrylamide and bis-acrylamide (which cross-links the acrylamide monomers)
Free-flow electrophoresis (Carrier-free electrophoresis)	No matrices are used; instead, polypeptides migrate through a solution; fast, high reproducibility, compatible with downstream detection techniques; can be run under native or denaturing conditions; only small sample volumes required (although can be used as a preparative technique)
Electrofocusing (Isoelectrofocusing)	Polypeptides are separated by differences in their isoelectric point (pI), usually performed in gels and based on the principle that overall charge on the polypeptide is a function of pH. An ampholyte solution is used to make immobilized pH gradient (IPG) gels. The immobilized pH gradient is obtained by the continuous change in the ratio of immobilines (weak acid or base defined by pK). Polypeptides migrate through the pH gradient until its charge is 0. Very high resolution, separating polypeptides differing by a single charge
Isotachopheresis (ITP)	Orders and concentrates polypeptides of intermediate effective mobilities between an ion of high effective mobility and one of much lower effective mobility, followed by their migration at a uniform speed. A multianalyte sample is introduced between the leading electrolyte (LE, containing leading ion) and the terminating electrolyte (TE, containing terminating ion) where the leading

Technique	Description
	ion, the terminating ion, and the sample components have the same charge polarity, and the sample ions must have lower electrophoretic mobilities than the leading ion but larger than the terminating ion. After electrophoresis, the polypeptides move forward behind the leading ion and in front of the terminating ion, forming discrete, contiguous zones in order of their electrophoretic mobilities. Transient ITP includes an additional step of separating after ITP with zone electrophoresis.
Affinity electrophoresis	Based on changes in the electrophoretic pattern of molecules through specific interactions with other molecules or complex formation; examples include mobility shift, charge shift and affinity capillary electrophoresis. Various types are known, including those using agarose gel, rapid agarose gel, boronate affinity, affinity-trap polyacrylamide, and phosphate affinity electrophoresis
Immuno electrophoresis	Separates polypeptides based on electrophoresis and reaction with antibodies. Includes immuno electrophoretic analysis (one-dimensional immuno electrophoresis), crossed immuno electrophoresis (two-dimensional quantitative immuno electrophoresis), rocket-immuno electrophoresis (one-dimensional quantitative immuno electrophoresis), fused rocket immuno electrophoresis, and affinity immuno electrophoresis. Often uses agarose gels buffered at high pH
Counter electrophoresis (counter immuno electrophoresis)	Antibody and antigen migrate through a buffered diffusion medium. Antigens in a gel with a controlled pH are strongly negatively charged and migrate rapidly across the electric field toward the anode. The antibody in such a medium is less negatively charged and migrates in the opposite direction toward the cathode. If the antigen and antibody are specific for each other, they combine and form a distinct precipitin line.

Technique	Description
Capillary electrophoresis	Refers to electrokinetic separation methods performed in submillimeter diameter capillaries and in micro- and nanofluidic channels. Examples include capillary zone electrophoresis (CZE), capillary gel electrophoresis (CGE), capillary isoelectric focusing (CIEF), capillary isotachopheresis and micellar electrokinetic chromatography (MEKC).
Capillary zone electrophoresis	A type of capillary electrophoresis, CZE separates ions based on their charge and frictional forces within a fine bore capillary. Sensitive in the picomolar range

Prepared polypeptides can be analyzed also using spectrometric methods - mass spectrometry (Rubakhin and Sweedler, 2010), ultraviolet spectrometry, visible light spectrometry, fluorescent spectrometry, and ultraviolet-visible light spectrometry (Nowicka-Jankowska, 1986).

5 Table G presents examples of polypeptide electrophoretic techniques.

Table G
Examples of spectrometric methods

Technique	Description
Mass Spectrometry (MS)	Sample molecules are ionized by high energy electrons. The mass to charge ratio of these ions is measured by electrostatic acceleration and magnetic field perturbation, providing a precise molecular weight. Ion fragmentation patterns may be related to the structure of the molecular ion. Mass spectrum is a plot of the ion signal as a function of the mass-to-charge ratio. Analyzers include sector field mass, time-of-flight (TOF), and quadrupole mass analyzers. Ion traps include three-dimensional quadrupole, cylindrical, linear quadrupole, and Orbitrap ion traps. Detectors include electron multipliers, Faraday cups, and ion-to-photon detectors. Variations of MS include tandem MS. Mass spectrometers can be configured in a variety of ways, including matrix-assisted laser desorption/ionization source configured with a TOF analyzer (MALDI-TOF); electrospray ionization-mass spectrometry (ESI-MS), inductively coupled plasma-mass spectrometry (ICP-MS), accelerator mass spectrometry (AMS), thermal ionization-mass spectrometry (TIMS), and spark source mass spectrometry (SSMS).
Ultraviolet-Visible Spectroscopy	Absorption of high-energy UV light causes electronic excitation. Wavelengths of 200 to 800 nm show absorption if conjugated pi-electron systems are present
Infrared Spectroscopy	Absorption of infrared radiation causes vibrational and rotational excitation of groups of atoms within the polypeptide. Because of their characteristic absorptions, functional groups are identified

The principle enabling mass spectrometry (MS) consists of ionizing chemical compounds to generate charged molecules or molecule fragments, and then measuring their mass-to-charge ratios. In

an illustrative MS procedure, a sample is loaded onto the MS instrument and undergoes vaporization, the components of the sample are ionized by one of a variety of methods (*e.g.*, by impacting them with an electron beam), which results in the formation of positively charged particles, the positive ions are then accelerated by a magnetic field, computations are performed on the mass-to-charge ratio (m/z) of the particles based on the details of motion of the ions as they transit through electromagnetic fields, and, detection of the ions, which have been sorted according to their m/z ratios.

An illustrative MS instrument has three modules: an ion source, which converts gas phase sample molecules into ions (or, in the case of electrospray ionization, move ions that exist in solution into the gas phase); a mass analyzer, which sorts the ions by their mass-to-charge ratios by applying electromagnetic fields; and a detector, which measures the value of an indicator quantity and thus provides data for calculating the abundances of each ion present.

The MS technique has both qualitative and quantitative uses, including identifying unknown compounds, determining the isotopic composition of elements in a molecule, and determining the structure of a compound by observing its fragmentation. Included are gas chromatography-mass spectrometry (GC/MS or GC-MS), liquid chromatography mass spectrometry (LC/MS or LC-MS), and ion mobility spectrometry/mass spectrometry (IMS/MS or IMMS).

The analytical methods (chromatographic, electrophoretic, and spectrometric) can be combined. For example, combinations such as liquid chromatography-mass spectrometry, capillary zone electrophoresis coupled to mass spectrometry, and ion mobility spectrometry-mass spectrometry.

Automation

The various steps of the disclosed methods can be accomplished using liquid handling robots. Such robots dispense reagents, samples or other liquids to a designated container. Robots are controlled by software, either integrated directly into the robot itself, or by a connected computer. By automating liquid handling, the disclosed methods can be accomplished with high through-put, fewer errors, and reduced analyst hands-on time.

Liquid handling robots can be configured to use various laboratory instruments, such as centrifuges, PCR machines, colony pickers, shaking devices, heating devices, *etc.* Such customization permits adapting these machines to a particular method.

In some cases, such robots replace the use of pipettes and/or syringes by using sound to move liquids (acoustic liquid handling).

Currently, Agilent Technologies (Santa Clara, CA), Beckman Coulter, Inc. (Indianapolis, IN), Eppendorf North America (Hauppauge, NY), Hamilton Robotics (Reno, NV), Hudson Robotics, Inc. (Springfield, NJ), and Tecan AG (Männedorf, Switzerland) are some of the manufacturers of such robots.

5 *Therapeutic polypeptides*

Polypeptides, including those that bind to one or more of the following, can be prepared and analyzed in the disclosed methods. These include CD proteins, including CD3, CD4, CD8, CD19, CD20, CD22, CD30, and CD34; including those that interfere with receptor binding. HER receptor family proteins, including HER2, HER3, HER4, and the EGF receptor. Cell adhesion molecules, for example, LFA-
 10 I, Mol, p150, 95, VLA-4, ICAM-I, VCAM, and alpha v/beta 3 integrin. Growth factors, such as vascular endothelial growth factor ("VEGF"), growth hormone, thyroid stimulating hormone, follicle stimulating hormone, luteinizing hormone, growth hormone releasing factor, parathyroid hormone, Mullerian-inhibiting substance, human macrophage inflammatory protein (MIP-I -alpha), erythropoietin (EPO), nerve growth factor, such as NGF-beta, platelet-derived growth factor (PDGF), fibroblast growth factors,
 15 including, for instance, aFGF and bFGF, epidermal growth factor (EGF), transforming growth factors (TGF), including, among others, TGF- α and TGF- β , including TGF- β I, TGF- β 2, TGF- β 3, TGF- β 4, or TGF- β 5, insulin-like growth factors-I and -II (IGF-I and IGF-II), des(I-3)-IGF-I (brain IGF-I), and osteoinductive factors. Insulins and insulin-related proteins, including insulin, insulin A-chain, insulin B-chain, proinsulin, and insulin-like growth factor binding proteins. Coagulation and coagulation-related proteins, such as,
 20 among others, factor VIII, tissue factor, von Willebrands factor, protein C, alpha-1-antitrypsin, plasminogen activators, such as urokinase and tissue plasminogen activator ("t-PA"), bombazine, thrombin, and thrombopoietin; (vii) other blood and serum proteins, including but not limited to albumin, IgE, and blood group antigens. Colony stimulating factors and receptors thereof, including the following, among others, M-CSF, GM-CSF, and G-CSF, and receptors thereof, such as CSF-1 receptor (c-
 25 fms). Receptors and receptor-associated proteins, including, for example, flk2/flt3 receptor, obesity (OB) receptor, LDL receptor, growth hormone receptors, thrombopoietin receptors ("TPO-R," "c-mpl"), glucagon receptors, interleukin receptors, interferon receptors, T-cell receptors, stem cell factor receptors, such as c-Kit, and other receptors. Receptor ligands, including, for example, OX40L, the ligand for the OX40 receptor. Neurotrophic factors, including bone-derived neurotrophic factor (BDNF) and
 30 neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6). Relaxin A-chain, relaxin B-chain, and prorelaxin; interferons and interferon receptors, including for example, interferon- α , - β , and - γ , and their receptors. Interleukins and interleukin receptors, including IL-1 to IL-33 and IL-1 to IL-33 receptors, such as the IL-8

receptor, among others. Viral antigens, including an AIDS envelope viral antigen. Lipoproteins, calcitonin, glucagon, atrial natriuretic factor, lung surfactant, tumor necrosis factor-alpha and -beta, enkephalinase, RANTES (regulated on activation normally T-cell expressed and secreted), mouse gonadotropin-associated peptide, DNase, inhibin, and activin. Integrin, protein A or D, rheumatoid factors, immunotoxins, bone morphogenetic protein (BMP), superoxide dismutase, surface membrane proteins, decay accelerating factor (DAF), AIDS envelope, transport proteins, homing receptors, addressins, regulatory proteins, immunoadhesins, antibodies. Myostatin, TALL proteins, including TALL-1, amyloid proteins, including but not limited to amyloid-beta proteins, thymic stromal lymphopoietins ("TSLP"), RANK ligand ("OPGL"), c-kit, TNF receptors, including TNF Receptor Type 1, TRAIL-R2, angiopoietins, and biologically active fragments or analogs or variants of any of the foregoing.

Exemplary polypeptides and antibodies include Activase® (Alteplase); alirocumab, Aranesp® (Darbepoetin-alfa), Epogen® (Epoetin alfa, or erythropoietin); Avonex® (Interferon β -1a); Bexxar® (Tositumomab); Betaseron® (Interferon- β); bococizumab (anti-PCSK9 monoclonal antibody designated as L1L3, see US8080243); Campath® (Alemtuzumab); Dynepo® (Epoetin delta); Velcade® (bortezomib); MLN0002 (anti- α 4 β 7 mAb); MLN1202 (anti-CCR2 chemokine receptor mAb); Enbrel® (etanercept); Eprex® (Epoetin alfa); Erbitux® (Cetuximab); evolocumab; Genotropin® (Somatropin); Herceptin® (Trastuzumab); Humatrope® (somatropin [rDNA origin] for injection); Humira® (Adalimumab); Infergen® (Interferon Alfacon-1); Natrecor® (nesiritide); Kineret® (Anakinra), Leukine® (Sargamostim); LymphoCide® (Epratuzumab); Benlysta™ (Belimumab); Metalyse® (Tenecteplase); Mircera® (methoxy polyethylene glycol-epoetin beta); Mylotarg® (Gemtuzumab ozogamicin); Raptiva® (efalizumab); Cimzia® (certolizumab pegol); Soliris™ (Eculizumab); Pexelizumab (Anti-C5 Complement); MEDI-524 (Numax®); Lucentis® (Ranibizumab); Edrecolomab (Panorex®); Trabio® (Ierdelimumab); TheraCim hR3 (Nimotuzumab); Omnitarg (Pertuzumab, 2C4); Osidem® (IDM-I); OvaRex® (B43.13); Nuvion® (visilizumab); Cantuzumab mertansine (huC242-DML); NeoRecormon® (Epoetin beta); Neumega® (Oprelvekin); Neulasta® (Pegylated filgrastim, pegylated G-CSF, pegylated hu-Met-G-CSF); Neupogen® (Filgrastim); Orthoclone OKT3® (Muromonab-CD3), Procrit® (Epoetin alfa); Remicade® (Infliximab), Reopro® (Abciximab), Actemra® (anti-IL6 Receptor mAb), Avastin® (Bevacizumab), HuMax-CD4 (zanolimumab), Rituxan® (Rituximab); Tarceva® (Erlotinib); Roferon-A®-(Interferon alfa-2a); Simulect® (Basiliximab); Stelara™ (Ustekinumab); Prexige® (lumiracoxib); Synagis® (Palivizumab); 146B7-CHO (anti-IL15 antibody, see US7153507), Tysabri® (Natalizumab); Valortim® (MDX-1303, anti-B. anthracis Protective Antigen mAb); ABthrax™; Vectibix® (Panitumumab); Xolair® (Omalizumab), ETI211 (anti-MRSA mAb), IL-1 Trap (the Fc portion of human IgG1 and the extracellular domains of both IL-1 receptor

components (the Type I receptor and receptor accessory protein)), VEGF Trap (Ig domains of VEGFR1 fused to IgG1 Fc), Zenapax® (Daclizumab); Zenapax® (Daclizumab), Zevalin® (Ibritumomab tiuxetan), Zetia (ezetimibe), Atacicept (TACI-Ig), anti- $\alpha 4\beta 7$ mAb (vedolizumab); galiximab (anti-CD80 monoclonal antibody), anti-CD23 mAb (lumiliximab); BR2-Fc (huBR3 / huFc fusion protein, soluble BAFF antagonist);

5 Simponi™ (Golimumab); Mapatumumab (human anti-TRAIL Receptor-1 mAb); Ocrelizumab (anti-CD20 human mAb); HuMax-EGFR (zalutumumab); M200 (Volociximab, anti- $\alpha 5\beta 1$ integrin mAb); MDX-010 (Ipilimumab, anti-CTLA-4 mAb and VEGFR-1 (IMC-18F1); anti-BR3 mAb; anti-C. difficile Toxin A and Toxin B C mAbs MDX-066 (CDA-I) and MDX-1388); anti-CD22 dsFv-PE38 conjugates (CAT-3888 and CAT-8015); anti-CD25 mAb (HuMax-TAC); anti-TSLP antibodies; anti-TSLP receptor antibody (US8101182); anti-TSLP

10 antibody designated as A5 (US7982016); (anti-CD3 mAb (NI-0401); Adecatumumab (MT201, anti-EpCAM-CD326 mAb); MDX-060, SGN-30, SGN-35 (anti-CD30 mAbs); MDX-1333 (anti-IFNAR); HuMax CD38 (anti-CD38 mAb); anti-CD40L mAb; anti-Cripto mAb; anti-CTGF Idiopathic Pulmonary Fibrosis Phase I Fibrogen (FG-3019); anti-CTLA4 mAb; anti-eotaxin1 mAb (CAT-213); anti-FGF8 mAb; anti-ganglioside GD2 mAb; anti-sclerostin antibodies (see, US8715663 or US7592429) anti-sclerostin

15 antibody designated as Ab-5 (US8715663 or US7592429); anti-ganglioside GM2 mAb; anti-GDF-8 human mAb (MYO-029); anti-GM-CSF Receptor mAb (CAM-3001); anti-HepC mAb (HuMax HepC); MEDI-545, MDX-1103 (anti-IFN α mAb); anti-IGFIR mAb; anti-IGF-IR mAb (HuMax-Inflam); anti-IL12/IL23p40 mAb (Briakinumab); anti-IL-23p19 mAb (LY2525623); anti-IL13 mAb (CAT-354); anti-IL-17 mAb (AIN457); anti-IL2Ra mAb (HuMax-TAC); anti-IL5 Receptor mAb; anti-integrin receptors mAb (MDX-O18, CNTO 95); anti-

20 IPIO Ulcerative Colitis mAb (MDX- 1100); anti-LLY antibody; BMS-66513; anti-Mannose Receptor/hCG β mAb (MDX-1307); anti-mesothelin dsFv-PE38 conjugate (CAT-5001); anti-PD1mAb (MDX-1 106 (ONO-4538)); anti-PDGFR α antibody (IMC-3G3); anti-TGF β mAb (GC-1008); anti-TRAIL Receptor-2 human mAb (HGS-ETR2); anti-TWEAK mAb; anti-VEGFR/Flt-1 mAb; anti- ZP3 mAb (HuMax-ZP3); NVS Antibody #1; NVS Antibody #2; and an amyloid-beta monoclonal antibody.

25 Examples of antibodies suitable for the methods and pharmaceutical formulations include the antibodies shown in Table H. Other examples of suitable antibodies include infliximab, bevacizumab, cetuximab, ranibizumab, palivizumab, abagovomab, abciximab, actoxumab, adalimumab, afelimomab, afutuzumab, alacizumab, alacizumab pegol, ald518, alemtuzumab, alirocumab, altumomab, amatuximab, anatumomab mafenatox, anrukinzumab, apolizumab, arcitumomab, aselizumab,

30 altinumab, atlizumab, atorolimumab, tocilizumab, bapineuzumab, basiliximab, bavituximab, bectumomab, belimumab, benralizumab, bertilimumab, besilesomab, bevacizumab, bezlotoxumab, biciromab, bivatuzumab, bivatuzumab mertansine, blinatumomab, blosozumab, brentuximab vedotin,

briakinumab, brodalumab, canakinumab, cantuzumab mertansine, cantuzumab mertansine,
 caplacizumab, capromab pendetide, carlumab, catumaxomab, cc49, cedelizumab, certolizumab pegol,
 cetuximab, citatuzumab bogatox, cixutumumab, clazakizumab, clenoliximab, clivatuzumab tetraxetan,
 conatumumab, crenezumab, cr6261, dacetuzumab, daclizumab, dalotuzumab, daratumumab,
 5 demcizumab, denosumab, detumomab, dorlimomab aritox, drozitumab, duligotumab, dupilumab,
 ecomeximab, eculizumab, edobacomab, edrecolomab, efalizumab, efungumab, elotuzumab,
 elsilimomab, enavatuzumab, enlimomab pegol, enokizumab, enoticumab, ensituximab, epitumomab
 cituxetan, epratuzumab, erenumab, erlizumab, ertumaxomab, etaracizumab, etrolizumab, evolocumab,
 exbivirumab, fanolesomab, faralimomab, farletuzumab, fasinumab, fbta05, felvizumab, fezakinumab,
 10 ficlatuzumab, figitumumab, flanvotumab, fontolizumab, foralumab, foravirumab, fresolimumab,
 fulranumab, futuximab, galiximab, ganitumab, gantenerumab, gavalimomab, gemtuzumab ozogamicin,
 gevokizumab, girentuximab, glembatumumab vedotin, golimumab, gomiliximab, gs6624, ibalizumab,
 ibritumomab tiuxetan, icrucumab, igovomab, imciromab, imgatuzumab, inclacumab, indatuximab
 raptansine, infliximab, intetumumab, inolimomab, inotuzumab ozogamicin, ipilimumab, iratumumab,
 15 itolizumab, ixekizumab, keliximab, labetuzumab, lebrikizumab, lemalesomab, lerdelimomab,
 lexatumumab, libivirumab, ligelizumab, lintuzumab, lirilumab, lorvotuzumab mertansine, lucatumumab,
 lumiliximab, mapatumumab, maslimomab, mavrilimumab, matuzumab, mepolizumab, metelimomab,
 milatuzumab, minretumomab, mitumomab, mogamulizumab, morolimumab, motavizumab,
 moxetumomab pasudotox, muromonab-cd3, nacolomab tafenatox, namilumab, naptumomab
 20 estafenatox, narnatumab, natalizumab, nebacumab, necitumumab, nerelimomab, nesvacumab,
 nimotuzumab, nivolumab, nofetumomab merpentan, ocaratuzumab, ocrelizumab, odulimomab,
 ofatumumab, olaratumab, olokizumab, omalizumab, onartuzumab, oportuzumab monatox,
 oregovomab, orticumab, otelixizumab, oxelumab, ozanezumab, ozoralizumab, pagibaximab,
 palivizumab, panitumumab, panobacumab, parsatuzumab, pascolizumab, pateclizumab, patritumab,
 25 pentumomab, perakizumab, pertuzumab, pexelizumab, pidilizumab, pintumomab, placulumab,
 ponezumab, priliximab, pritumumab, PRO 140, quilizumab, racotumomab, radretumab, rafivirumab,
 ramucirumab, ranibizumab, raxibacumab, regavirumab, reslizumab, rilatumumab, rituximab,
 robatumumab, roledumab, romosozumab, rontalizumab, rovelizumab, ruplizumab, samalizumab,
 sarilumab, satumomab pendetide, secukinumab, sevirumab, sibrotuzumab, sifalimumab, siltuximab,
 30 simtuzumab, siplizumab, sirukumab, solanezumab, solitumab, sonenpcizumab, sontuzumab,
 stamulumab, sulesomab, suvizumab, tabalumab, tacatuzumab tetraxetan, tadocizumab, talizumab,
 tanezumab, taplitumomab paptox, tefibazumab, telimomab aritox, tenatumomab, tefibazumab,

teneliximab, teplizumab, teprotumumab, TGN1412, tremelimumab, ticilimumab, tildrakizumab, tigatuzumab, TNX-650, tocilizumab, toralizumab, tositumomab, tralokinumab, trastuzumab, TRBS07, tregalizumab, tucotuzumab celmoleukin, tuvirumab, ublituximab, urelumab, urtoxazumab, ustekinumab, vapaliximab, vatelizumab, vedolizumab, veltuzumab, vepalimomab, vesencumab, visilizumab, volociximab, vorsetuzumab mafodotin, votumumab, zalutumumab, zanolimumab, zatuximab, ziralimumab, zolimomab aritox.

Antibodies also include adalimumab, bevacizumab, blinatumomab, cetuximab, conatumumab, denosumab, eculizumab, erenumab, evolocumab, infliximab, natalizumab, panitumumab, rilotumumab, rituximab, romosozumab, tezepelumab, and trastuzumab, and antibodies selected from Table H.

10

Table HExamples of therapeutic antibodies

Target (informal name)	HC* Type (including allotypes)	LC* Type	pI	LC* SEQ ID NO:	HC* SEQ ID NO:
anti-amyloid	IgG1 (f) (R;EM)	Kappa	9.0	2	3
GMCSF (247)	IgG2	Kappa	8.7	4	5
CGRPR	IgG2	Lambda	8.6	6	7
RANKL	IgG2	Kappa	8.6	8	9
Sclerostin (27H6)	IgG2	Kappa	6.6	10	11
IL-1R1	IgG2	Kappa	7.4	12	13
Myostatin	IgG1 (z) (K;EM)	Kappa	8.7	14	15
B7RP1	IgG2	Kappa	7.7	16	17
Amyloid	IgG1 (za) (K;DL)	Kappa	8.7	18	19
GMCSF (3.112)	IgG2	Kappa	8.8	20	21
CGRP (32H7)	IgG2	Kappa	8.7	22	23
CGRP (3B6.2)	IgG2	Lambda	8.6	24	25
PCSK9 (8A3.1)	IgG2	Kappa	6.7	26	27
PCSK9 (492)	IgG2	Kappa	6.9	28	29
CGRP	IgG2	Lambda	8.8	30	31
Hepcidin	IgG2	Lambda	7.3	32	33
TNFR p55)	IgG2	Kappa	8.2	34	35
OX40L	IgG2	Kappa	8.7	36	37

Target (informal name)	HC* Type (including allotypes)	LC* Type	pI	LC* SEQ ID NO:	HC* SEQ ID NO:
HGF	IgG2	Kappa	8.1	38	39
GMCSF	IgG2	Kappa	8.1	40	41
Glucagon R	IgG2	Kappa	8.4	42	43
GMCSF (4.381)	IgG2	Kappa	8.4	44	45
Sclerostin (13F3)	IgG2	Kappa	7.8	46	47
CD-22	IgG1 (f) (R;EM)	Kappa	8.8	48	49
INFgR	IgG1 (za) (K;DL)	Kappa	8.8	50	51
Ang2	IgG2	Kappa	7.4	52	53
TRAILR2	IgG1 (f) (R;EM)	Kappa	8.7	54	55
EGFR	IgG2	Kappa	6.8	56	57
IL-4R	IgG2	Kappa	8.6	58	59
IL-15	IgG1 (f) (R;EM)	Kappa	8.8	60	61
IGF1R	IgG1 (za) (K;DL)	Kappa	8.6	62	63
IL-17R	IgG2	Kappa	8.6	64	65
Dkk1 (6.37.5)	IgG2	Kappa	8.2	66	67
Sclerostin	IgG2	Kappa	7.4	68	69
TSLP	IgG2	Lambda	7.2	70	71
Dkk1 (11H10)	IgG2	Kappa	8.2	72	73
PCSK9	IgG2	Lambda	8.1	74	75
GIPR (2G10.006)	IgG1 (z) (K;EM)	Kappa	8.1	76	77
Activin	IgG2	Lambda	7.0	78	79
Sclerostin (2B8)	IgG2	Lambda	6.7	80	81
Sclerostin	IgG2	Kappa	6.8	82	83
c-fms	IgG2	Kappa	6.6	84	85
$\alpha 4\beta 7$	IgG2	Kappa	6.5	86	87

*HC – antibody heavy chain; LC – antibody light chain.

In some embodiments, the therapeutic polypeptide is a BiTE[®] molecule. BiTE[®] molecules are engineered bispecific monoclonal antibodies which direct the cytotoxic activity of T cells against cancer cells. They are the fusion of two single-chain variable fragments (scFvs) of different antibodies, or amino

5

acid sequences from four different genes, on a single peptide chain of about 55 kilodaltons. One of the scFvs binds to T cells via the CD3 receptor, and the other to a tumor cell via a tumor specific molecule. Blinatumomab (BLINCYTO®) is an example of a BiTE® molecule, specific for CD19. BiTE® molecules that are modified, such as those modified to extend their half-lives, can also be used in the disclosed methods.

EXAMPLES

The following Examples section is given solely by way of example and are not set forth to limit the disclosure or claims in any way.

Example 1 – Reagent preparation for Examples 2-4

Denaturing buffer (7M guanidine HCl, 100 mM tris, 20 mM methionine, pH 8.3) was prepared by adding 10 mL 1 M (hydroxymethyl) aminomethane hydrochloride (tris), pH 7.8 (Teknova, Hollister, CA, P/N T1078) to 87.5 mL 8 M guanidine HCl (Pierce, Rockford, IL, P/N 24115) followed by addition of 299 mg L-methionine (J.T. Baker, P/N 2085-05). The pH of the solution was adjusted to pH 8.3 with 6 N hydrochloric acid (HCl) (Sigma, St. Louis, MO, P/N 84429). Volume was adjusted to 100 mL with HPLC-grade water. Reduction solution (500 mM DTT) was prepared by dissolving 7.7 mg pre-weighed dithiothreitol (DTT) (Pierce, Rockford, IL, P/N 20291) in 100 µL denaturing buffer. Alkylation solution (500 mM NaIAA) was prepared by dissolving 15-65 mg sodium iodoacetate (NaIAA) (Sigma, St. Louis, MO, P/N I-9148) in a volume of denaturing buffer sufficient to yield 500 mM NaIAA. Digestion buffer (100 mM tris, 20 mM methionine, pH 7.8) was prepared by dissolving 299 mg L-methionine in 10 mL 1 M tris, pH 7.8 and adding 100 mL HPLC-grade water. The pH of the solution was adjusted to pH 7.8 with HCl, and the volume was adjusted to 100 mL with HPLC-grade water. Enzyme solutions (1 mg/mL trypsin, 1 mg/mL HNE) were prepared by adding 100 µL digestion buffer to 100 µg trypsin (Roche, Basel, Switzerland, P/N 03708969001) or 100 µg HNE (Elastin Products Company, Owensville, MO, P/N SE563). Digest quenching solution (10% TFA) was prepared by adding 1.0 mL 100% trifluoroacetic acid (TFA) (Pierce, Rockford, IL, P/N 28904) to 9.0 mL HPLC-grade water

Example 2 – Trypsin and HNE independent digests

Samples were denatured and reduced by adding 57.1 µL denaturing buffer and 2 µL reducing solution to 42.9 µL sample (1-2 mg/mL in formulation buffer) followed by incubation at 37 °C for 35 min. Following reduction, 9 µL alkylation solution was added to each sample followed by incubation at room

temperature in dark for 20 min. Alkylation was quenching by addition of 7 μ L reducing solution. Samples were desalted with Zeba Spin Desalting Columns (Thermo Scientific, Waltham, MA, P/N 89883) in accordance with manufacturer's instructions using an Eppendorf 5430 centrifuge (Hamburg, Germany) with spins at 1,500 x g. Sample concentration was measured followed desalting using either an Implen NanoPhotometer Pearl (München, Germany) or a Thermo Scientific Nanodrop 2000c (Waltham, MA). Enzyme solution (either trypsin or HNE) was added to each sample at an enzyme:substrate ratio of 1:20. Samples were incubated in a water bath at 37 °C for 35 min. Digest was quenched by the addition of 6 μ L digestion quenching solution.

Example 3 – Trypsin and HNE sequential digests

Samples were denatured and reduced by adding 57.1 μ L denaturing buffer and 2 μ L reducing solution to 42.9 μ L sample (1-2 mg/mL in formulation buffer) followed by incubation at 37 °C for 35 min. Following reduction, 9 μ L alkylation solution was added to each sample followed by incubation at room temperature in dark for 20 min. Alkylation was quenching by addition of 7 μ L reducing solution. Samples were desalted with Zeba Spin Desalting Columns (Thermo Scientific, Waltham, MA, P/N 89883) in accordance with manufacturer's instructions using an Eppendorf 5430 centrifuge (Hamburg, Germany) with spins at 1,500 x g. Sample concentration was measured followed desalting using either an Implen NanoPhotometer Pearl (München, Germany) or a Thermo Scientific Nanodrop 2000c (Waltham, MA). Trypsin solution was added to each sample at an enzyme:substrate ratio of 1:20. Samples were incubated in a water bath at 37 °C for 35 min. HNE solution was added to each sample at an enzyme:substrate ratio of 1:20. Samples were incubated in a water bath at 37 °C for 30 min. Digest was quenched by the addition of 6 μ L digestion quenching solution.

Example 4 – Mixture of trypsin digest and trypsin-HNE sequential digest

Samples were denatured and reduced by adding 57.1 μ L denaturing buffer and 2 μ L reducing solution to 42.9 μ L sample (1 mg/mL in formulation buffer) followed by incubation at 37 °C for 35 min. Following reduction, 9 μ L alkylation solution was added to each sample followed by incubation at room temperature in dark for 20 min. Alkylation was quenching by addition of 7 μ L reducing solution. Samples were desalted with Zeba Desalting Columns in accordance with manufacturer's instructions using an Eppendorf 5430 centrifuge with spins at 1,500 x g. Sample concentration was measured followed desalting using either a NanoPhotometer Pearl or a Nanodrop 2000c. Trypsin solution was added to each sample at an enzyme:substrate ratio of 1:20. Samples were incubated in a water bath at 37 °C for

35 min. Following trypsin digestion, each sample was split into two equal aliquots (~55 μ L each). 3 μ L digestion quenching solution was added to the first aliquot, which was then set aside. To the second aliquot, HNE solution was added at an enzyme:substrate ratio of 1:20. This aliquot was incubated in a water bath at 37 °C for 30 min followed by addition of 3 μ L digestion quenching solution. The two aliquots were then combined at a 1:1 ratio followed by gentle mixing.

Example 5 – Reagent preparation for Examples 6 and 7

Denaturing buffer (6M guanidine HCl, 200 mM tris, 20 mM methionine, pH 8.3) was prepared by adding 20 mL 1 M (hydroxymethyl) aminomethane hydrochloride (tris), pH 8.3 (Teknova, St. Louis, MO, P/N T1083) to 87.5 mL 8 M guanidine HCl (Pierce, Rockford, IL, P/N 24115) followed by addition of 299 mg L-methionine (J.T. Baker, P/N 2085-05). The pH of the solution was adjusted to pH 8.3 with 1 N hydrochloric acid (HCl) (Ricca, Arlington, TX, P/N R3700100-120A) or 1 N Sodium hydroxide (NaOH) (Merck, Kenilworth, NJ, P/N 1.09137.100). Volume was adjusted to 100 mL with HPLC-grade water. Reduction solution (500 mM DTT) was prepared by dissolving 7.7 mg pre-weighed dithiothreitol (DTT) (Pierce, Rockford, IL, P/N 20291) in 100 μ L denaturing buffer. Alkylation solution (500 mM NaIAA) was prepared by dissolving 15-65 mg sodium iodoacetate (NaIAA) (Sigma, St. Louis, MO, P/N I-9148) in a volume of denaturing buffer sufficient to yield 500 mM NaIAA. Digestion buffer (50 mM tris, 20 mM methionine, pH 7.8) was prepared by dissolving 299 mg L-methionine in 10 mL 1 M tris, pH 7.8 and adding 100 mL HPLC-grade water. The pH of the solution was adjusted to pH 7.8 with 1 N hydrochloric acid (HCl) (Ricca, Arlington, TX, P/N R3700100-120A) or 1 N Sodium hydroxide (NaOH) (Merck, Kenilworth, NJ, P/N 1.09137.100), and the volume was adjusted to 100 mL with HPLC-grade water. Enzyme solutions (1 mg/mL trypsin, 1 mg/mL HNE) were prepared by adding 100 μ L digestion buffer to 100 μ g trypsin (Roche, Basel, Switzerland, P/N 03708969001) or 100 μ g HNE (Elastin Products Company, Owensville, MO, P/N SE563). Digest quenching solution (8 M guanidine HCl, 250 mM acetate, pH 4.7) was prepared by dissolving 76.4 g guanidine HCl (Sigma, St. Louis, MO, P/N 50933) and 1.0 g sodium acetate (Sigma, P/N 32319) in 95 mL HPLC grade water. 716 μ L glacial acetic acid (Sigma, St. Louis, MO, P/N 320099) was then added, and the pH was adjusted to pH 4.7 with either HCl or NaOH. Volume was then adjusted to 100 mL with HPLC grade water.

Example 6 – MWCO spin filter-aided sequential digest

100 μ g sample in formulation buffer was added to a 30 kDa molecular weight cut-off spin unit consisting of a membrane unit positioned inside a centrifuge tube for filtrate collection. (Millipore,

Billerica, MA, P/N MRCF0R030 or Pall, Port Washington, NY, P/N OD030C34). This unit was spun for 15 min at 14,000 x g using an Eppendorf 5430 centrifuge. Filtrate was discarded. 200 µL denaturing buffer was added to the sample, the filter unit was spun for 15 min at 14,000 x g, and the filtrate was discarded; this was repeated two additional times. For each sample, 3 µL reducing solution was added to 5 37 µL denaturing buffer, and 40 µL of this solution was added to the filter unit. Samples were denatured and reduced by incubating at 37 °C water bath for 45 min. Samples were then spun for 15 min at 14,000 x g, and the filtrate was discarded. For each sample 7 µL alkylation solution was added to 33 µL denaturing buffer, and 40 µL of this solution was added to the filter unit. Samples were alkylated by incubating at room temperature in dark for 20 min. Samples were then spun for 15 min at 14,000 x g, 10 and the filtrate was discarded. For each sample 4 µL denaturing solution was added to 36 µL denaturing buffer, and 40 µL of this solution was added to the filter unit to quench alkylation. Samples were then spun for 15 min at 14,000 x g, and the filtrate was discarded. 200 µL digest buffer was added to the sample, the filter unit was spun for 15 min at 14,000 x g, and the filtrate was discarded; this was repeated two additional times to remove denaturing, reducing, and alkylating agents. For each sample, 15 5 µL trypsin solution was added to 35 µL digest buffer, and 40 µL of this solution was added to the filter unit (1:20 enzyme:substrate ratio). Sample was incubated in a 37 °C water bath for 60 min. The filter unit was transferred to a new collection tube (Collection Tube 2). The initial collection tube (Collection Tube 1) was set aside. The filter unit was centrifuged for 15 min at 14,000 x g. Filtrate, which contained tryptic peptides, was retained in Collection Tube 2. 20 µL digest buffer was added to the filter unit, the filter 20 unit (in Collection Tube 2) was spun for 15 min at 14,000 x g, and the filtrate was retained in Collection Tube 2; this was repeated one additional time. The filter unit was transferred back to Collection Tube 1, and Collection Tube 2 was set aside. For each sample, 5 µL HNE solution was added to 35 µL digest buffer, and 40 µL of this solution was added to the filter unit (now in Collection Tube 1, 1:20 enzyme:substrate ratio based on starting material). Sample was incubated in a 37 °C water bath for 30 25 min. The filter unit was transferred to Collection Tube 2. Collection Tube 1 was discarded. The filter unit was centrifuged for 15 min at 14,000 x g. Filtrate, which contained peptides resulting from HNE digestion, was retained in Collection Tube 2 (along with tryptic peptides from previous steps). 20 µL digest buffer was added to the filter unit, the filter unit (in Collection Tube 2) was spun for 15 min at 14,000 x g, and the filtrate was retained in Collection Tube 2; this was repeated one additional time. 30 Digest was quenched by the addition of 160 µL digest quenching buffer to Collection Tube 2.

Example 7 – MWCO spin filter-aided sequential digest, shortened

200 µL denaturing buffer was added to a 30 kDa molecular weight cut-off spin unit consisting of a membrane unit positioned inside a centrifuge tube for filtrate collection. (Millipore, Billerica, MA, P/N MRCF0R030 or Pall, Port Washington, NY, P/N OD030C34). This unit was spun for 10 min at 14,000 x g using an Eppendorf 5430 centrifuge. Filtrate was discarded. 100 µg sample in formulation buffer was added to the filter unit and spun for 10 min at 14,000 x g. Filtrate was discarded. For each sample, 3 µL reducing solution was added to 37 µL denaturing buffer, and 40 µL of this solution was added to the filter unit. Samples were denatured and reduced by incubating at 37 °C water bath for 30 min. For each sample 7 µL alkylation solution was added to 33 µL denaturing buffer, and 40 µL of this solution was added to the filter unit. Samples were alkylated by incubating at room temperature in dark for 20 min.

For each sample 4 µL denaturing solution was added to 36 µL denaturing buffer, and 40 µL of this solution was added to the filter unit to quench alkylation. Samples were then spun for 15 min at 14,000 x g, and the filtrate was discarded. 200 µL digest buffer was added to the sample, the filter unit was spun for 15 min at 14,000 x g, and the filtrate was discarded; this was repeated two additional times to remove denaturing, reducing, and alkylating agents. For each sample, 5 µL trypsin solution was added to 35 µL digest buffer, and 40 µL of this solution was added to the filter unit (1:20 enzyme:substrate ratio). Sample was incubated in a 37 °C water bath for 60 min. The filter unit was transferred to a new collection tube (Collection Tube 2). The initial collection tube (Collection Tube 1) was set aside. The filter unit was centrifuged for 10 min at 14,000 x g. Filtrate, which contained tryptic peptides, was retained in Collection Tube 2. 20 µL digest buffer was added to the filter unit, the filter unit (in Collection Tube 2) was spun for 10 min at 14,000 x g, and the filtrate was retained in Collection Tube 2; this was repeated one additional time. The filter unit was transferred back to Collection Tube 1, and Collection Tube 2 was set aside. For each sample, 5 µL HNE solution was added to 35 µL digest buffer, and 40 µL of this solution was added to the filter unit (now in Collection Tube 1, 1:20 enzyme:substrate ratio based on starting material). Sample was incubated in a 37 °C water bath for 30 min. The filter unit was transferred to Collection Tube 2. Collection Tube 1 was discarded. The filter unit was centrifuged for 10 min at 14,000 x g. Filtrate, which contained peptides resulting from HNE digestion, was retained in Collection Tube 2 (along with tryptic peptides from previous steps). 20 µL digest buffer was added to the filter unit, the filter unit (in Collection Tube 2) was spun for 10 min at 14,000 x g, and the filtrate was retained in Collection Tube 2; this was repeated one additional time. Digest was quenched by the addition of 160 µL digest quenching buffer to Collection Tube 2. A comparison highlighting differences between Protocols 4 and 5 is collected in Table 9.

Example 8 – Ultra-Performance Liquid Chromatography (UPLC) conditions

For all samples, Mobile Phase A consisted of 0.1% formic acid in water, and Mobile Phase B consisted of 0.1% formic acid in acetonitrile. For initial experiments using Protocols 1 and 2, peptides were separated using a CSH C18 1.7 μ m, 2.1x150 mm UPLC column (Waters, Milford, MA, P/N 186005298). Following acquisition of bridging data, remaining experiments using the methods from Examples 2-4 were performed with a BEH C18 1.7 μ m, 2.1x150 mm UPLC column (Waters, Milford, MA, P/N 186003556). UPLC separations were performed using either a Thermo Scientific U-3000 system (Waltham, MA), a Waters Acquity H-Class system (Milford, MA), or and Agilent 1290 system (Santa Clara, CA) utilizing gradients outlined in Tables 1-3 (depending on the experiment). Based on starting material, ~3-4 μ g of sample was loaded on the column.

Example 9 – Mass spectrometry conditions

Peptides resulting from digestion were analyzed using a Thermo Scientific Q Exactive (Waltham, MA), a Thermo Scientific Q Exactive Plus (Waltham, MA), or a Thermo Scientific Q Exactive BioPharma (Waltham, MA). Because multiple instruments were used, data collection parameters varied slightly depending on the instrument. Instruments were operated in data-dependent mode (top 4-8) over a scan range of 200-2,000 m/z. The AGC target was set to 1E6 for MS1 scans and 5E5 for tandem mass spectrometry (MSMS) scans. MS1 scans were collected at a resolution of either 35,000 or 140,000, and MS2 scans were collected at a resolution of 17,500. An isolation window of 2-4 m/z was specified for MSMS scans. Peaks with unassigned charge states and charge states greater than 8 were excluded from MSMS. Dynamic exclusion was set to 10 s. Lock Mass of m/z 391.28430 was enabled.

Example 10 – Data analysis

MS data were searched with MassAnalyzer (data were collected over several months, so multiple versions of MassAnalyzer were used). Carboxymethylation was specified as a static modification. Depending on the experiment, cleavage was specified as either nonspecific or the C-terminus of amino acids KRVITAL amino acids. For all searches, signal-to-noise ratio was set to 20, mass accuracy of 15 ppm was specified, and confidence was set to 0.95. For sequence coverage maps, minimum peak area was set to 1% of the base peak, relative peak area threshold was set to 17%, minimum confidence was set to 0.95, and maximum peptide mass was set to 15,000.

Example 11 – Results

Initial experiments probing the efficacy of HNE for digestion of BiTE[®] molecules were direct comparisons of trypsin digestion to HNE digestion for BiTE[®]-3 using Protocol 1 and Gradient 1. Trypsin digestion did not yield peptides which could be used to characterize attributes in either CDR. On the other hand, digestion with HNE resulted in several peptides corresponding to the linker region. Many of these peptides were identified with low signal intensity ($<10^6$), and this lack of specificity (14 peptides identified) could potentially compromise quantitation of attributes in this region. However, HNE specificity was thought to be related to length of the substrate being digested (Stein *et al.*, 1987). This property of the enzyme was leveraged by first digesting BiTE[®]-3 with trypsin to produce potential substrates with shorter amino acid lengths followed by digestion with HNE (Protocol 2). This sequential digestion resulted in a single peptide with high signal intensity ($>1.5 \times 10^7$).

Having demonstrated feasibility of sample preparation utilizing trypsin-HNE digestion for monitoring attributes in the α -CD3 CDRs of interest, additional molecules (BiTE[®]-1, half-life extension BiTE[®]; BiTE[®]-2, canonical BiTE[®]; and CDH19, half-life extension BiTE[®]; in addition to BiTE[®]-3, half-life extension BiTE[®]) that had been subjected to photodegradation (1.2 million lux-h of cool white light, $t=3$ d) and forced deamidation (pH 8.5, 50 , $t=3$ d) in addition to unstressed samples were prepared using Protocol 2 and Gradient 1. For unstressed samples, sequence coverages for α -CD3 CDR 1 were very reproducible (**Figure 4**). One or two peptides corresponding to a potential asparagine deamidation sites in this CDR were consistently identified with high signal intensity ($>10^7$). Likewise, for all molecules except CDH19, a single peptide corresponding to a potential tryptophan oxidation site was identified with high signal intensity. Forced deamidation conditions resulted in three peaks corresponding to deamidation in the α -CD3 CDR 1 for all four molecules that were not observed in the unstressed samples (**Figure 3** illustrates BiTE[®]-1 as a representative example). MSMS (**Figure 4**) confirmed that the two peaks that elute first both corresponded to –GNS– motif deamidation while the peak that eluted last corresponded to –GNF– motif deamidation. A comparison of modification percentages for all four molecules (Table 11.1) highlights that both potential deamidation sites in this CDR were susceptible to modification and should be monitored. CDR tryptophan oxidation was also observed followed photodegradation at significantly lower percentages (Table 11.2). Increased deamidation was not observed for the second CDR in close proximity to the large linker peptide (α -CD3 CDR2). These initial results established that not only are labile sites present in the α -CD3 CDRs that cannot be monitored using trypsin digestion but also that trypsin-HNE sequential digestion can be used to identify and monitor these modifications.

Table 11.1Quantitation of asparagine deamidation observed for multiple BiTE® molecules

Molecule	Unstressed	Forced Deamidation*
BiTE®-1	1.0%	39.6%
BiTE®-3	1.8%	39.8%
BiTE®-2	1.8%	5.7%
CDH19	1.0%	42.4%

*Stressed conditions were pH 8.5, 50 °C for 3 d. Deamidation levels account for peak areas of all three deamidated species relative to the unmodified peak area.

Table 11.2Quantitation of tryptophan oxidation observed for multiple BiTE® molecules

Molecule	CDR Tryptophan Oxidation*		Non-CDR Tryptophan Oxidation*	
	Unstressed	Photodegradation^	Unstressed	Photodegradation^
BiTE®-1	0.1%	1.9%	0.1%	1.0%
BiTE®-3	0.4%	2.9%	0.0%	0.6%
BiTE®-2	0.3%	1.2%	0.3%	0.5%
CDH19	0.1%	0.9%	0.2%	0.3%

*Oxidation levels account for peak areas of oxidation and kynurenine relative to the unmodified peak area.

^Stressed conditions were 1.2 million lux-h of cool white light for 3 d.

While the trypsin-HNE digestion yielded high quality peptides for the two α -CD3 CDRs of interest, sequence coverage and modification quantitation for the rest of the molecule still required the tryptic peptides utilized by conventional MAM.

A single analysis was performed on a 1:1 mixture of trypsin-digested sample combined with an aliquot of sample digested with trypsin followed by HNE (**Figure 5**). An additional strength of this approach of analyzing a mixture of two digests lies in the fact that data for what is essentially two peptides maps is acquired in a single injection; not only does this allow for greater identified sequence coverage, but in the case of BiTE®-2, a peptide containing a critical attribute (CDR aspartic acid isomerization) resulting from trypsin-HNE digestion yielded better signal quality and reproducibility than the corresponding tryptic peptide. In addition, the UPLC gradient utilized for separation was switched to Gradient 2, and the column was switched from the Waters CSH C18 column to the Waters BEH C18

column. Robustness of this approach was evaluated by analyzing unstressed samples, photodegradation samples, pH jump samples, and thermal degradation samples corresponding to two molecules (BiTE[®]-2 and BiTE[®]-3). Trypsin digestion, trypsin-HNE sequential digestion, and a 1:1 mixture of trypsin digest and trypsin-HNE digest samples were prepared by two analysts and analyzed using two UPLC systems (Thermo U-3000 and Waters Acquity H-Class) connected to two different mass spectrometers (Thermo QExactive and Thermo QExactive Plus) (**Figure 8**). All samples were prepared in duplicate, so a total of four injections were acquired for each condition.

Table 11.3 collects quantitation results α -CD3 CDR 1 deamidation from both BiTE[®]-2 and BiTE[®]-3 as a representative peptide with asparagine deamidation. For the data presented in this table, photodegradation was performed with 192,000 lux-h for 2 d; pH jump degradation was performed at pH 8.4 at 37 °C for 7 d; and thermal degradation was performed at 40 °C for 4 weeks. These results indicate that mixing the trypsin digest with the trypsin-HNE digest had minimal impact on quantitation results. Reducing the gradient still resulted in clear separation of the three deamidation species (unmodified separated from –GNS– peak 1 by ~1.5 min, –GNS– peak 1 separated from –GNS– peak 2 by ~0.5 min, –GNS– peak 2 separated from –GNF– by ~1.3 min, data not shown). In addition, precision of the deamidation levels for both molecules was comparable regardless of whether trypsin-HNE digestion was run separately or mixed with the trypsin digest. Because deamidation and aspartic acid isomerization were typically the most challenging modifications to chromatographically separate from corresponding unmodified peptides, Table 11.4 collects quantitation results for BiTE[®]-3 D⁵¹⁰ isomerization as a representative peptide with aspartic acid isomerization. For the data presented in this table, photodegradation was performed with 192,000 lux-h for 2 d; pH jump degradation was performed at pH 8.4 at 37 °C for 7 d; and thermal degradation was performed at 40 °C for 4 weeks. Despite containing threonine and leucine residues, a portion of the sequence of BiTE[®]-3 undergoes minimal cleavage during sequential digestion with HNE, so it was possible to quantitate D⁵¹⁰ isomerization using a fully tryptic peptide regardless of whether the trypsin digest, the trypsin-HNE sequential digest, or the 1:1 mixture of the two digests was analyzed. In these results, D⁵¹⁰ isomerization levels were consistent regardless of the digest conditions, again reinforcing the observation that mixing these digests had minimal impact on attribute quantitation. These results demonstrate that mixing the two parallel digests is a reasonable approach for BiTE[®] molecules (as well as other polypeptides) and that doing so has minimal impact on modification quantitation regardless of whether tryptic peptides or trypsin-HNE peptides are used.

Table 11.3Quantitation of BiTE®-2 and BiTE®-3 asparagine deamidation

Condition	Digest	BiTE®-2 N ³⁶⁰ +N ³⁶³	BiTE®-3 N ³⁵⁹ +N ³⁶²
		Deamidation**	Deamidation**
Control	Trypsin → Neutrophil Elastase*	1.9% ± 0.2%	1.9% ± 0.1%
	Mix^	2.2% ± 0.6%	1.6% ± 0.3%
Photo Degradation	Trypsin → Neutrophil Elastase*	2.1% ± 0.4%	2.0% ± 0.3%
	Mix^	2.2% ± 0.7%	1.8% ± 0.2%
pH Jump Degradation	Trypsin → Neutrophil Elastase*	54.7% ± 0.3%	42.2% ± 1.1%
	Mix^	54.5% ± 1.0%	41.5% ± 1.2%
Thermal Degradation	Trypsin → Neutrophil Elastase	13.5% ± 0.4%	4.9% ± 0.3%
	Mix	13.6% ± 0.8%	4.5% ± 0.3%

*"Trypsin → Neutrophil Elastase" corresponds to BiTE®-2/ BiTE®-3 digested with trypsin followed by sequential digestion with neutrophil elastase.

^"Mix" corresponds to "Trypsin → Neutrophil Elastase" mixed (1:1) with BiTE®-2/ BiTE®-3 digested with trypsin.

**Deamidation levels are total and account for peak areas of all three deamidated species (–GNS– and –GNF–) relative to the unmodified peak area.

Table 11.4
Quantitation of BiTE®-3 aspartic acid isomerization

Condition	Digest	D ⁵¹⁰ Isomerization
Control	Trypsin*	0.2% ± 0.1%
	Trypsin → Neutrophil Elastase^	0.4% ± 0.1%
	Mix**	0.3% ± 0.1%
Photo Degradation	Trypsin*	0.3% ± 0.1%
	Trypsin → Neutrophil Elastase^	0.4% ± 0.1%
	Mix**	0.3% ± 0.1%
pH Jump Degradation	Trypsin*	0.8% ± 0.1%
	Trypsin → Neutrophil Elastase^	1.0% ± 0.2%
	Mix**	1.0% ± 0.2%
Thermal Degradation	Trypsin*	4.1% ± 0.4%
	Trypsin → Neutrophil Elastase^	4.1% ± 0.4%
	Mix**	3.9% ± 0.5%

*"Trypsin" indicates BiTE®-3 digested with trypsin.

^"Trypsin → Neutrophil Elastase" corresponds to BiTE®-2/ BiTE®-3 digested with trypsin followed by sequential digestion with neutrophil elastase.

**"Mix" corresponds to "Trypsin → Neutrophil Elastase" mixed (1:1) with BiTE®-2/ BiTE®-3 digested with trypsin

MAM utilizing the method described above (Protocol 3, Gradient 2) was qualified for BiTE®-2
 5 (ATM-000401) and BiTE®-3 (ATM-000391). However, during analysis, the new peak detection procedure
 (Sieve) incorporated into the method identified a number of new peaks. This resulted in system
 suitability failure of the sequences. After further evaluation, a number of the peaks corresponded to
 missed tryptic cleavages. However, some of these peaks were observed in final system suitability
 injection (but not in the initial pre-run injection). These findings suggested that trypsin efficiency was
 10 being somehow impeded, but they also suggested that one of the enzymes was still active despite
 acidification of the sample following digestion. However, attribute quantitation was not affected by the
 new peaks (only the system suitability checks). Troubleshooting indicated that the desalting columns
 utilized in Protocols 1-3 were not completely removing guanidine from the sample prior to digestion.
 Trypsin efficiency can be inhibited by low levels of guanidine, so it was likely that variable removal of
 15 guanidine by the desalting column resulted in inconsistent trypsin digestion. In addition, because acid

quenching of trypsin digestion has been reported, it was speculated that acidification was not sufficiently quenching HNE digestion. Enzymatic activity, though reduced due to acidification, would result in the observation of new peaks if a replicate injection was performed from a single vial after a period of time; this, in turn, would result in failure of system suitability assessment.

5 To address these issues, a thorough exploration of multiple preparation methods was conducted. These included evaluating multiple desalting columns (Zeba, Biospin, NAP-5), preparation methods that do not require guanidine for denaturation (Rapigest), and MWCO filter-based methods using multiple manufacturers (Pall and Microcon® (available from Millipore (Waltham, MA)) as well as multiple molecular weight cut-offs (30 kDa, 5 kDa, as specified by the manufacturers). In addition, the
10 digest quenching buffer was updated to include a high concentration of guanidine to inactivate both enzymes. In Protocols 1-3, acid quenching typically resulted in a final sample pH of ~2. The updated digest quenching buffer was low enough to inhibit trypsin and HNE activities but high enough to minimize artefactual modifications (*e.g.*, deamidation and isomerization) that may result due to extended exposure to low pH. Because this quenching buffer contained guanidine, the initial wash step
15 in the UPLC gradient was extended; all experiments utilizing the guanidine quenching buffer required the use of Gradient 3 to thoroughly desalt the samples prior to MS analysis.

The conclusion of this investigation was that a MWCO filter-based method (Protocol 4 above, adapted from the previously reported filter-aided sample preparation (FASP) method (Wisniewski *et al.*, 2009)) outperformed other preparation methods based on several metrics (*e.g.*, repeatability, recovery,
20 quantitation, sequence coverage, concentration range, *etc.*). A schematic of Protocol 4 is presented in **Figure 7**. In this method, the molecule is captured on top of the 30 kDa MWCO spin filter, and denaturation, reduction, alkylation, desalting, and digestion all occur on filter. Following digestion, resulting peptides are collected through centrifugation. In sequential digest outlined in Protocol 4, following trypsin digestion, the tryptic peptides are collected, and species that do not pass through the
25 filter are then subjected to digestion with HNE. One of the surprising observations of this method is that the 8 kDa linker peptide of interest was retained by the 30 kDa filter, despite the recommendations made by manufacturers Microcon® and Pall, respectively, of “using a membrane with a MWCO at least two times smaller than the molecular weight of the protein solute that one intends to concentrate)” and “a MWCO be selected that is three to six times smaller than the molecular weight of the solute being
30 retained.” While manufacturers recommend using a MWCO two to six times smaller than the species targeted for retention, surprisingly the 8 kDa peptide is being retained by a MWCO nearly four times larger than the peptide itself.

Retention of the 8 kDa peptide by 30 kDa MWCO filters with membranes consisting of different materials (Microcon® membranes are regenerated cellulose, Pall membranes are modified polyethersulfone) emphasizes that this is not a manufacturer-specific artefact. Not only did this novel use of a MWCO filter make a sequential digestion possible, but direct comparison of this method utilizing the 30 kDa MWCO filter to a different method using a 5 kDa MWCO filter showed that the 30 kDa filter method significantly outperformed the 5 kDa filter method in terms of trypsin digest efficiency, repeatability, precision of attribute quantitation, and recovery. The primary advantage of this method is that enzyme specificity afforded by trypsin digestion is preserved for the bulk of the molecule, and only the large peptides difficult to characterize by MS are further digested into smaller peptides because they are retained on-filter. This is illustrated by **Figure 8**, which highlights trypsin specificity of peptides identified for BiTE®-1, BiTE®-2, and BiTE®-3 (triplicate analysis for each molecule) using filters manufactured by both Microcon® and Pall. In these results, over 95% of identified peptides typically corresponded to trypsin digestion, while the remaining identified peptides primarily corresponded to the large 8 kDa linker peptide of interest (**Figure 9**). Attribute quantitation for BiTE®-2 using filters from both manufacturers was comparable to results for the parallel digestion (Protocol 3) (Table 11.5). Similar results were also observed for BiTE®-1 and BiTE®-3. This method resolved the new peak issue by removing guanidine more completely compared to the other method discussed above, and the enzymes were also inactivated either by the updated quenching buffer or by retention on the MWCO filter. In **Figure 10**, a BiTE®-3 sample prepared using Protocol 3 was reinjected after ~4 d. Several peaks were only present in the initial injection, which were absent in the second injection. In contrast, samples prepared using Protocol 4 were consistent even after 4 d had passed between injections (**Figure 11**). These results suggest that the MWCO filter-based preparation outline in Protocol 4 is robust, yields quantitation results consistent to those previously reported, and eliminates many of the root causes of new peaks observed with Protocol 3.

Table 11.5

Comparison of attribute quantitation for BiTE®-2 using 30 kDa MWCO filters manufactured by
Microcon® and Pall*

Attribute	Reference Protocol	Microcon® 30 kDa	Pall 30 kDa
M ²³³ Oxidation	0.254% ± 0.046%	0.14% ± 0.02%	0.22% ± 0.03%
D ⁵⁴ +D ⁵⁷ +D ⁶² Isomerization [^]	1.256% ± 0.298%	1.01% ± 0.04%	0.89% ± 0.00%
N ³⁶³ Deamidation	0.946% ± 0.375%	0.96% ± 0.02%	0.88% ± 0.02%

*The Microcon® prep was performed in triplicate, but due to instrument failure, only two preps for the Pall filters were analyzed.

[^]D⁵⁴+D⁵⁷+D⁶² isomerization was quantified using I⁵¹-R⁶⁵ for the Reference Protocol. C⁴⁴-R⁶⁵ was used for quantitation in filter-based experiments due to more efficient trypsin digestion.

One of the primary concerns about Protocol 4 was the length of time necessary to prepare the samples; a sequential digest utilizing Protocol 4 could take more than 8 h. In addition to analyst fatigue and other factors, the amount of time necessary to prepare samples using this method could introduce artefactual modifications resulting from extended exposure to ambient laboratory conditions. To address these concerns, a shortened filter-based preparation (Protocol 5) was developed. Table 11.6 highlights differences between Protocols 4 and 5. The bulk of time savings introduced by Protocol 5 were made possible by eliminating superfluous centrifugation steps, reducing centrifugation times, and reducing incubation times. These reductions theoretically yield roughly 2 h in time savings, but practically, time savings were typically greater than 3 h. While still slightly longer than solution-based preparation methods such as Protocol 3, these modifications reduced sample preparation time to a point where it is comparable to solution-based methods.

Table 11.6
Comparison of Protocols 4 and 5

<u>Protocol 4</u>			<u>Protocol 5</u>		
Step	Description	Time (min)	Step	Description	Time (min)
-	-	-	1	Equilibrate filter with denaturing buffer. Spin	10
1	Spin (remove formulation buffer)	15	2	Spin (remove formulation buffer)	10
2	Add denaturing buffer, spin	15	-	-	-
3	Add denaturing buffer, spin	15	-	-	-
4	Add denaturing/reducing solution. Incubate	45	3	Add denaturing/reducing solution. Incubate	30
5	Spin	15	-	-	-
6	Add alkylation solution. Incubate	20	4	Add alkylation solution. Incubate	20
7	Spin	15	-	-	-
8	Add reducing solution. Spin	15	5	Add reducing solution. Spin	15
9	Add digest buffer. Spin	15	6	Add digest buffer. Spin	15
10	Add digest buffer. Spin	15	7	Add digest buffer. Spin	15
11	Add digest buffer. Spin	15	8	Add digest buffer. Spin	15
12	Add trypsin solution. Incubate	60	9	Add trypsin solution. Incubate	60
13	Spin	15	10	Spin	10
14	Add digest buffer. Spin	15	11	Add digest buffer. Spin	10
15	Add digest buffer. Spin	15	12	Add digest buffer. Spin	10
16	Add HNE solution. Incubate	30	13	Add HNE solution. Incubate	30
17	Spin	15	14	Spin	10
18	Add digest buffer. Spin	15	15	Add digest buffer. Spin	10
19	Add digest buffer. Spin	15	-	-	-
Total Time		380	Total Time		270
Spin Steps		15	Spin Steps		9

Overlays comparing BiTE®-2 samples prepped with Protocols 4 and 5 are presented in **Figure 12** (each preparation performed in triplicate). The only noticeable peak differences observed were a shoulder at ~24 min and a peak at ~47 min that were present in all samples prepped with Protocol 4 but none of the samples prepped with Protocol 5. These peaks were attributed to nonspecific carboxymethylation resulting from overalkylation. Similarly, because denaturing/reduction incubation time was reduced and spin steps between reduction and alkylation were eliminated, alkylation levels for all cysteine residues were compared (Table 11.7). For the data in this table, alkylation percentages were determined by searching data with MassAnalyzer, specifying carboxymethylation as a variable modification (rather than a static modification, which is typically specified). Alkylation levels for all BiTE®-1 samples, regardless of whether samples were prepped with Protocol 4 or 5, were above 99.7%. Similar results were observed for BiTE®-2 and BiTE®-3, suggesting that time reductions introduced by Protocol 5 had no negative impact on alkylation levels. Attribute quantitation was comparable when samples were prepped with both Protocols 4 and 5, which in turn were consistent with modification levels historically observed (Table 11.8). For the data in this table, each preparation was performed in triplicate. D⁵⁴+D⁵⁷+D⁶² isomerization was quantified using I⁵¹-R⁶⁵ for MDR-001581. C⁴⁴-R⁶⁵ was used for quantitation in filter-based experiments due to more efficient trypsin digestion. These initial results for Protocol 5 were encouraging, so a limited robustness evaluation was performed (two analysts, BiTE®-2 samples prepped in triplicate). New peak detection passed for all samples (*e.g.*, no new peaks were detected), and attribute quantitation for all 6 injections was consistent with MDR-001581 (Table 11.9). Based on these results, along with comparison of other metrics such as trypsin specificity, total identified area, recovery of the 8 kDa linker peptide, and percent of identified area corresponding to enzymes used for digestion, Protocol 5 introduces significant time savings to preparation time with no significant negative impact on data quality.

Table 11.7Comparison of BiTE®-1 alkylation levels resulting from Protocols 4 and 5

Cys Residue	Protocol 4			Protocol 5		
	Prep 1	Prep 2	Prep 3	Prep 1	Prep 2	Prep 3
C22	99.95%	99.97%	99.96%	100.00%	100.00%	100.00%
C44	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%
C96	99.93%	99.95%	99.94%	99.99%	99.99%	99.99%
C162	99.93%	99.94%	99.95%	99.97%	99.98%	99.98%
C232	99.75%	99.73%	99.79%	99.94%	99.93%	99.94%
C244	99.95%	99.95%	99.96%	99.98%	99.98%	99.99%
C279	99.96%	99.97%	99.97%	99.99%	99.99%	100.00%
C355	99.92%	99.93%	99.94%	99.99%	99.98%	99.98%
C419	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%
C487	99.98%	99.99%	99.99%	100.00%	100.00%	100.00%

Table 11.8Comparison of attribute quantitation for AMG596 using Protocols 4 and 5

Attribute	Reference Protocol	Protocol 4	Protocol 5
M ²³³ Oxidation	0.254% ± 0.046%	0.14% ± 0.02%	0.13% ± 0.01%
D ⁵⁴ +D ⁵⁷ +D ⁶²	1.256% ± 0.298%	1.01% ± 0.04%	0.89% ± 0.02%
Isomerization			
N ³⁶³ Deamidation	0.946% ± 0.375%	0.96% ± 0.02%	0.75% ± 0.04%

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CLAIMS

1. A method of preparing a polypeptide for analysis, comprising:
 - a. cleaving the polypeptide in a sample in a first digestion, wherein the cleaving produces at least two fragments of the polypeptide,
 - 5 b. cleaving the at least two fragments of the polypeptide with neutrophil elastase; and
 - c. analyzing the sample after cleaving the at least two fragments of the polypeptide with the neutrophil elastase.
2. A method of preparing a polypeptide for analysis, comprising:
 - 10 a. cleaving the polypeptide in a sample in a first digestion, wherein the cleaving produces at least two fragments of the polypeptide;
 - b. dividing the sample comprising the at least two fragments of the polypeptide into a first aliquot and a second aliquot;
 - c. digesting the at least two fragments of the first aliquot with neutrophil elastase; and
 - 15 d. analyzing the first aliquot and the second aliquot.
3. The method of claim 2, wherein after digesting the at least two fragments of the polypeptide with the neutrophil elastase, the first and second aliquots are combined.
- 20 4. The method of claim 3, wherein the first and second aliquot comprise approximately equal amounts of the polypeptide.
5. The method of claim 1 or 2, wherein the cleaving the polypeptide in the first digestion comprises proteolytic or chemical cleavage.
- 25 6. The method of claim 5, wherein the cleaving is proteolytic cleavage accomplished by a protease, wherein the protease has an activity different from the neutrophil elastase.
7. The method of claim 6, wherein the protease is selected from the group consisting of trypsin, endoproteinase Glu-C, endoproteinase Arg-C, pepsin, chymotrypsin, chymotrypsin B, Lys-N protease, Lys-C protease, Glu-C protease, Asp-N protease, pancreatopeptidase,
- 30

carboxypeptidase A, carboxypeptidase B, proteinase K, and thermolysin, and combinations thereof.

8. The method of claim 7, wherein the protease is trypsin.

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9. The method of claim 5, wherein the cleaving is chemical cleavage accomplished by a chemical.

10. The method of claim 9, wherein the chemical is selected from the group consisting of cyanogen bromide, 2-Nitro-5-thiocyanobenzoate, hydroxylamine, and BNPS-skatole, and combinations thereof.

10

11. The method of claim 1 or 2, wherein the polypeptide is denatured before cleaving the polypeptide in the sample in the first digestion.

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12. The method of claim 1 or 2, wherein the polypeptide is alkylated before cleaving the polypeptide in the sample in the first digestion.

13. The method of claim 1 or 2, wherein the polypeptide is denatured and either alkylated or reduced before cleaving the polypeptide in the sample in the first digestion.

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14. The method of claim 1 or 2, wherein the polypeptide is denatured, reduced, and alkylated before cleaving the polypeptide in the sample in the first digestion.

15. The method of claim 1 or 2, wherein the neutrophil elastase is human neutrophil elastase (EC 3.4.21.37).

25

16. The method of claim 1 or 2, wherein analyzing comprises at least one technique selected from the group consisting of chromatography, electrophoresis, spectrometry, and combinations thereof.

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17. The method of claim 16, wherein the technique for analyzing the sample comprises:

- 5 a. chromatography and is selected from the group consisting of gas chromatography, liquid chromatography, high performance liquid chromatography, ultra-performance liquid chromatography, size-exclusion chromatography, ion-exchange chromatography, affinity chromatography, expanded bed adsorption chromatography, reverse-phase chromatography, hydrophobic interaction chromatography, and combinations thereof;
- b. electrophoresis and is selected from the group consisting of gel electrophoresis, free-flow electrophoresis, electrofocusing, isotachopheresis, affinity electrophoresis, immunoelectrophoresis, counterelectrophoresis, and capillary electrophoresis, capillary zone electrophoresis, and combinations thereof; or
- 10 c. spectroscopy or spectrometry and is selected from the group consisting of mass spectrometry, ultraviolet spectroscopy, visible light spectroscopy, fluorescent spectroscopy, and ultraviolet-visible light spectroscopy, and combinations thereof.

15 18. The method of claim 17, wherein the technique comprises liquid chromatography-mass spectrometry.

19. The method of claim 17, wherein the technique comprises capillary zone electrophoresis coupled to mass spectrometry.

20 20. A method of preparing a polypeptide for analysis, comprising:

- a. denaturing, reducing, and alkylating the polypeptide;
- b. digesting the polypeptide with trypsin to produce trypsin-cleaved polypeptide fragments;
- 25 c. dividing the trypsin-cleaved polypeptide fragments into a first aliquot and a second aliquot;
- d. digesting the trypsin-cleaved polypeptide fragments of the first aliquot with neutrophil elastase;
- e. combining the first aliquot and second aliquot at about a 1:1 ratio; and
- 30 f. analyzing the combined aliquots of step (e).

21. The method of claim 20, wherein the neutrophil elastase is human neutrophil elastase (EC 3.4.21.37).

22. The method of claim 20, wherein analyzing the sample comprises at least one technique selected from the group consisting of chromatography, electrophoresis, spectrometry, and combinations thereof.

5

23. The method of claim 22, wherein:

- a. the technique comprises chromatography and is selected from the group consisting of gas chromatography, liquid chromatography, high performance liquid chromatography, ultra-performance liquid chromatography, size-exclusion chromatography, ion-exchange chromatography, affinity chromatography, expanded bed adsorption chromatography, reverse-phase chromatography, hydrophobic interaction chromatography, and combinations thereof;
- b. the technique comprises electrophoresis and is selected from the group consisting of gel electrophoresis, free-flow electrophoresis, electrofocusing, isotachopheresis, affinity electrophoresis, immunoelectrophoresis, counterelectrophoresis, and capillary electrophoresis, and combinations thereof; or
- c. the technique comprises spectroscopy or spectrometry and is selected from the group consisting of mass spectrometry, ultraviolet spectroscopy, visible light spectroscopy, fluorescent spectroscopy, and ultraviolet-visible light spectroscopy, and combinations thereof.

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24. A method of preparing a polypeptide for analysis, comprising:

- a. providing a sample comprising the polypeptide;
- b. applying the sample to a filter having a molecular weight cut-off;
- c. digesting the polypeptide in the sample on the filter with a first protease;
- d. digesting the polypeptide in the sample on the filter with a second protease; and
- e. analyzing the sample,

wherein

- i. the second protease is neutrophil elastase; and
- ii. the first protease is different from the second protease.

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25. The method of claim 24, wherein the first protease comprises a protease selected from the group consisting of trypsin, Asp-N, and Glu-C.
26. The method of claim 24, wherein the polypeptide is denatured on the filter before digesting the polypeptide with the first protease.
27. The method of claim 24, wherein the polypeptide is alkylated on the filter before digesting the polypeptide with the first protease.
28. The method of claim 24, wherein the polypeptide is denatured and either reduced or alkylated on the filter before digesting the polypeptide with the first protease.
29. The method of claim 24, wherein the polypeptide is denatured, reduced, and alkylated on the filter before digesting the polypeptide with the first protease.
30. The method of claim 24, wherein the neutrophil elastase is human neutrophil elastase (EC 3.4.21.37).
31. The method of claim 24, wherein analyzing the sample comprises at least one technique selected from the group consisting of chromatography, electrophoresis, spectrometry, and combinations thereof.
32. The method of claim 31, wherein the technique for analyzing the sample comprises:
- a. chromatography and is selected from the group consisting of gas chromatography, liquid chromatography, high performance liquid chromatography, ultra-performance liquid chromatography, size-exclusion chromatography, ion-exchange chromatography, affinity chromatography, expanded bed adsorption chromatography, reverse-phase chromatography, hydrophobic interaction chromatography, and combinations thereof;
 - b. electrophoresis and is selected from the group consisting of gel electrophoresis, free-flow electrophoresis, electrofocusing, isotachopheresis, affinity electrophoresis, immunoelectrophoresis, counterelectrophoresis, and capillary electrophoresis, and combinations thereof; or

- c. spectroscopy or spectrometry and is selected from the group consisting of mass spectrometry, ultraviolet spectroscopy, visible light spectroscopy, fluorescent spectroscopy, and ultraviolet-visible light spectroscopy, and combinations thereof.

5 33. The method of claim 32, wherein the technique comprises liquid chromatography-mass spectrometry.

34. The method of claim 33, wherein the technique comprises capillary zone electrophoresis coupled to mass spectrometry.

10 35. The method of claim 34, wherein the filter having a molecular weight cut-off has a molecular weight cut-off of 30 kDa.

15 36. A method of preparing a polypeptide for analysis by liquid chromatography, capillary zone electrophoresis, or mass spectrometry, comprising:

- a. providing a sample comprising the polypeptide;
- b. applying the sample to a 30 kDa MWCO filter;
- c. filtering the sample through the filter to produce a retentate;
- d. denaturing the polypeptide in the sample retentate on the filter;
- 20 e. reducing the polypeptide in the sample retentate on the filter;
- f. alkylating the polypeptide in the sample on the filter;
- g. digesting the polypeptide in the sample on the filter with a first protease;
- h. filtering the sample through the same filter and retaining the filtrate;
- i. digesting the polypeptide in the sample on the filter with a second protease;
- 25 j. filtering the sample through the same filter into the filtrate of step (g);
- k. quenching the active proteases in the filtrate with guanidine; and
- l. analyzing the filtrate by liquid chromatography, capillary zone electrophoresis, or mass spectrometry,

wherein

- 30 i. the second protease is neutrophil elastase; and
- ii. the first protease is different from the second protease.

37. The method of claim 36, wherein the neutrophil elastase is human neutrophil elastase (EC 3.4.21.37).

38. The method of claim 36, wherein the technique comprises capillary zone electrophoresis coupled to mass spectrometry.

39. The method of any of claims 1, 2, 24, or 36, wherein the polypeptide is a therapeutic polypeptide.

40. The method of claim 39, wherein the therapeutic polypeptide is selected from the group consisting of an antibody or antigen-binding fragment thereof, a derivative of an antibody or antibody fragment, and a fusion polypeptide.

41. The method of claim 39, wherein the therapeutic polypeptide is selected from the group consisting of infliximab, bevacizumab, cetuximab, ranibizumab, palivizumab, abagovomab, abciximab, actoxumab, adalimumab, afelimomab, afutuzumab, alacizumab, alacizumab pegol, ald518, alemtuzumab, alirocumab, altumomab, amatuximab, anatumomab mafenatox, anrukizumab, apolizumab, arcitumomab, aselizumab, altinumab, atlizumab, atorolimumab, tocilizumab, bapineuzumab, basiliximab, bavituximab, bectumomab, belimumab, benralizumab, bertilimumab, besilesomab, bevacizumab, bezlotoxumab, biciromab, bivatumumab, bivatumumab mertansine, blinatumomab, blosozumab, brentuximab vedotin, briakinumab, brodalumab, canakinumab, cantuzumab mertansine, cantuzumab mertansine, caplacizumab, capromab pendetide, carlumab, catumaxomab, cc49, cedelizumab, certolizumab pegol, cetuximab, citatuzumab bogatox, cixutumumab, clazakizumab, clenoliximab, clivatuzumab tetraxetan, conatumumab, crenezumab, cr6261, dacetuzumab, daclizumab, dalotuzumab, daratumumab, demcizumab, denosumab, detumomab, dorlimomab aritox, drozitumab, duligotumab, dupilumab, ecromeximab, eculizumab, edobacomab, edrecolomab, efalizumab, efungumab, elotuzumab, elsilimomab, enavatuzumab, enlimomab pegol, enokizumab, enoticumab, ensituximab, epitumomab cituxetan, epratuzumab, erenumab, erlizumab, ertumaxomab, etaracizumab, etrolizumab, evolocumab, exbivirumab, fanolesomab, faralimomab, farletuzumab, fasinumab, fbta05, felvizumab, fezakinumab, ficlatuzumab, figitumumab, flinvotumab, fontolizumab, foralumab, foravirumab, fresolimumab, fulranumab, futuximab,

galiximab, ganitumab, gantenerumab, gavilimomab, gemtuzumab ozogamicin, gevokizumab, girentuximab, glembatumumab vedotin, golimumab, gomiliximab, gs6624, ibalizumab, ibritumomab tiuxetan, icrucumab, igovomab, imciromab, imgatuzumab, inclacumab, indatuximab ravtansine, infliximab, intetumumab, inolimomab, inotuzumab ozogamicin, ipilimumab, iratumumab, itolizumab, ixekizumab, keliximab, labetuzumab, lebrikizumab, lemalesomab, lerdelimumab, lexatumumab, libivirumab, ligelizumab, lintuzumab, lirilumab, lorvotuzumab mertansine, lucatumumab, lumiliximab, mapatumumab, maslimomab, mavrilimumab, matuzumab, mepolizumab, metelimumab, milatuzumab, minretumomab, mitumomab, mogamulizumab, morolimomab, motavizumab, moxetumomab pasudotox, muromonab-cd3, nacolomab tafenatox, namilumab, naptumomab estafenatox, narnatumab, natalizumab, nebacumab, necitumumab, nerelimomab, nesvacumab, nimotuzumab, nivolumab, nofetumomab merpentan, ocaratuzumab, ocrelizumab, odulimomab, ofatumumab, olaratumab, olokizumab, omalizumab, onartuzumab, oportuzumab monatox, oregovomab, orticumab, otelixizumab, oxelumab, ozanezumab, ozoralizumab, pagibaximab, palivizumab, panitumumab, panobacumab, parsatuzumab, pascolizumab, pateclizumab, patritumab, pemtumomab, perakizumab, pertuzumab, pexelizumab, pidilizumab, pintumomab, placulumab, ponezumab, priliximab, pritumumab, PRO 140, quilizumab, racotumomab, radretumab, rafivirumab, ramucirumab, ranibizumab, raxibacumab, regavirumab, reslizumab, rilotumumab, rituximab, robatumumab, roledumab, romosozumab, rontalizumab, rovelizumab, ruplizumab, samalizumab, sarilumab, satumomab pendetide, secukinumab, sevirumab, sibrotuzumab, sifalimumab, siltuximab, simtuzumab, siplizumab, sirukumab, solanezumab, solitomab, sonepcizumab, sontuzumab, stamulumab, sulesomab, suvizumab, tabalumab, tacatuzumab tetraxetan, tadocizumab, talizumab, tanezumab, taplitumomab paptox, tefibazumab, telimomab aritox, tenatumomab, tefibazumab, teneliximab, teplizumab, teprotumumab, tezepelumab, TGN1412, tremelimumab, ticilimumab, til-drakizumab, tigatuzumab, TNX-650, tocilizumab, toralizumab, tositumomab, tralokinumab, trastuzumab, TRBS07, tregalizumab, tucotuzumab celmoleukin, tuvirumab, ublituximab, urelumab, urtoxazumab, ustekinumab, vapaliximab, vatelizumab, vedolizumab, veltuzumab, vepalimomab, vesencumab, visilizumab, volociximab, vorsetuzumab mafodotin, votumumab, zalutumumab, zanolimumab, zatuximab, ziralimumab, zolimomab aritox, a glycoprotein, CD polypeptide, a HER receptor polypeptide, a cell adhesion polypeptide, a growth factor polypeptide, an insulin polypeptide, an insulin-related polypeptide, a coagulation polypeptide, a coagulation-related polypeptide, albumin, IgE,

a blood group antigen, a colony stimulating factor, a receptor, a neurotrophic factor, an interferon, an interleukin, a viral antigen, a lipoprotein, calcitonin, glucagon, atrial natriuretic factor, lung surfactant, tumor necrosis factor-alpha and -beta, enkephalinase, mouse gonadotropin-associated peptide, DNase, inhibin, activating, an integrin, protein A, protein D, a
5 rheumatoid factor, an immunotoxin, a bone morphogenetic protein, a superoxide dismutase, a surface membrane polypeptide, a decay accelerating factor, an AIDS envelope, a transport polypeptide, a homing receptor, an addressin, a regulatory polypeptide, an immunoadhesin, a myostatin, a TALL polypeptide, an amyloid polypeptide, a thymic stromal lymphopoietin, a RANK ligand, a c-kit polypeptide, a TNF receptor, and an angiopoietin, the antibodies shown in Table H
10 and biologically active fragments, analogs or variants thereof.

42. The method of claim 39, wherein the therapeutic polypeptide is a bi-specific T-cell engager molecule.

15 43. The method of any of claims 1, 2, 24, or 36, wherein the method is at least partially executed by an automated liquid handling device.

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FIG. 1

Conserved α -CD3 Domain

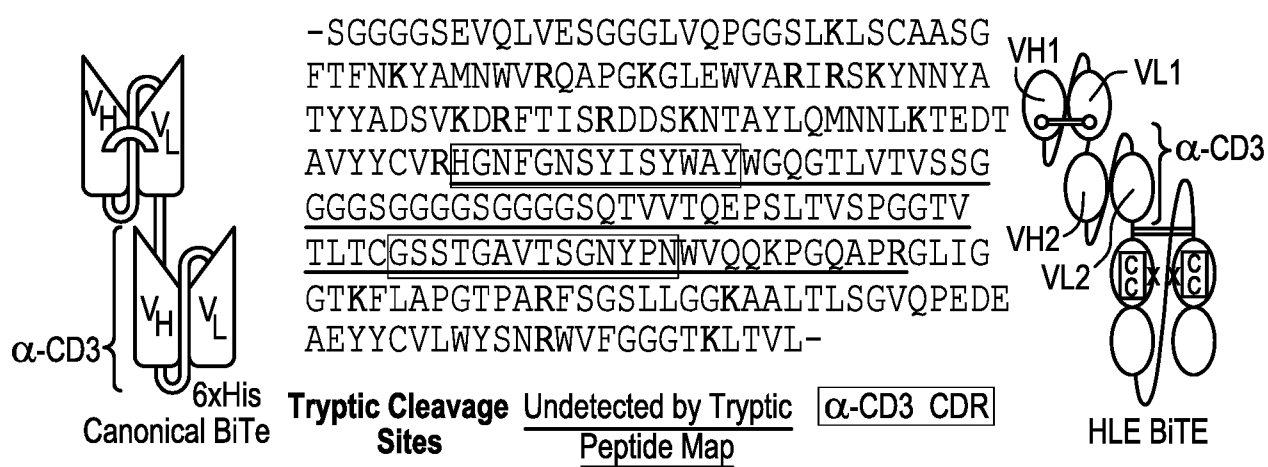
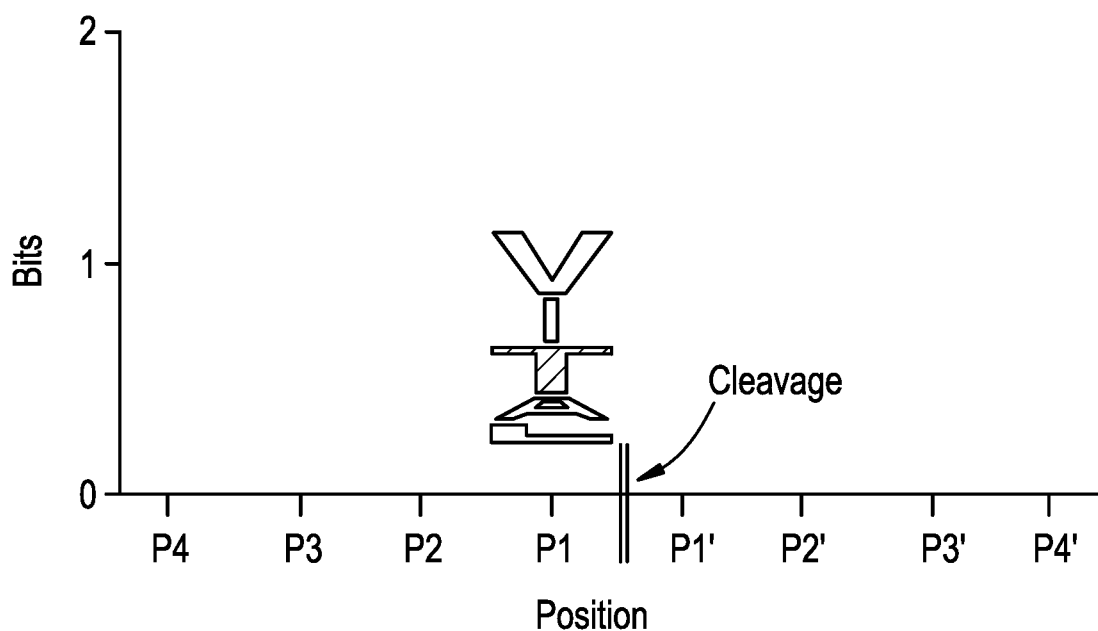
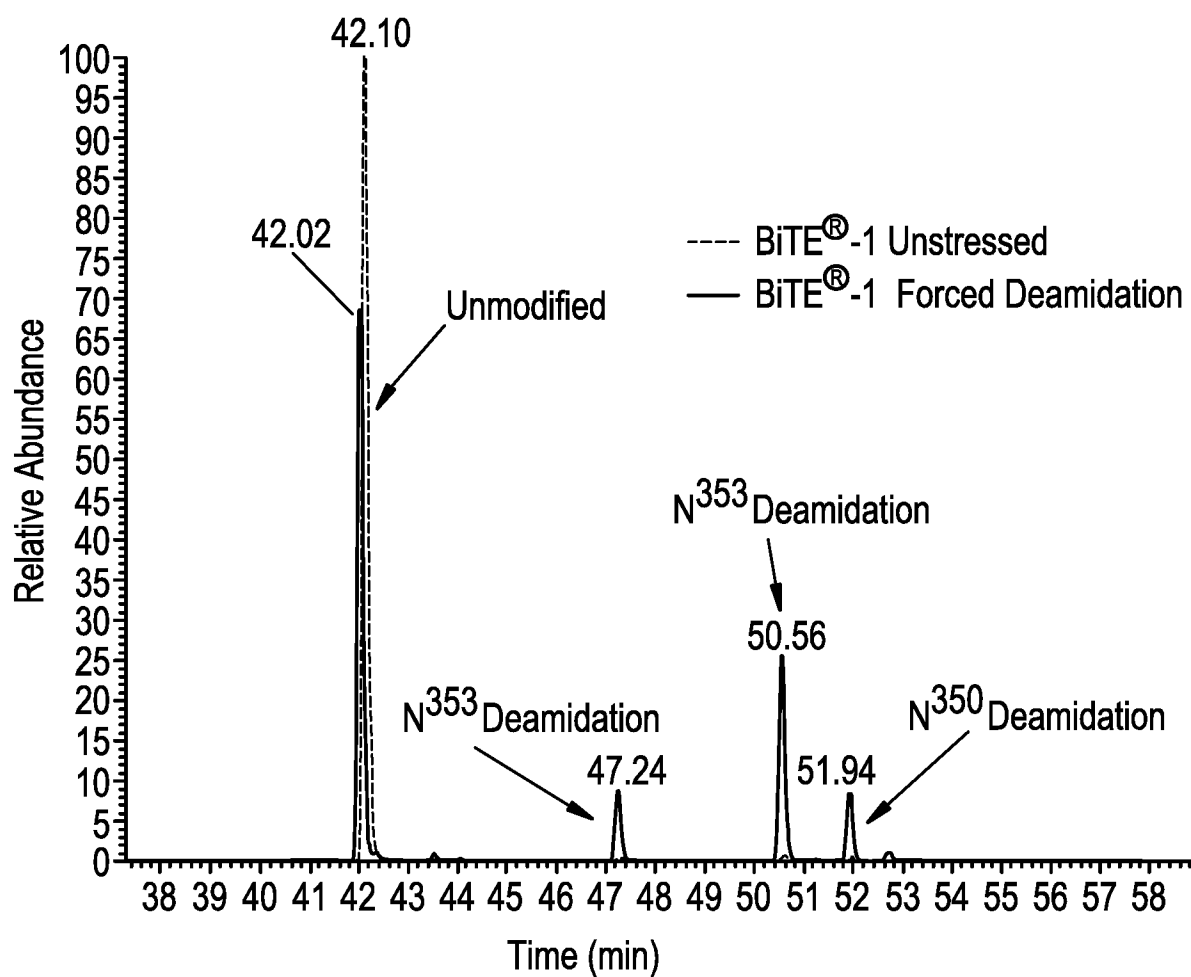


FIG. 2



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FIG. 3



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FIG. 4

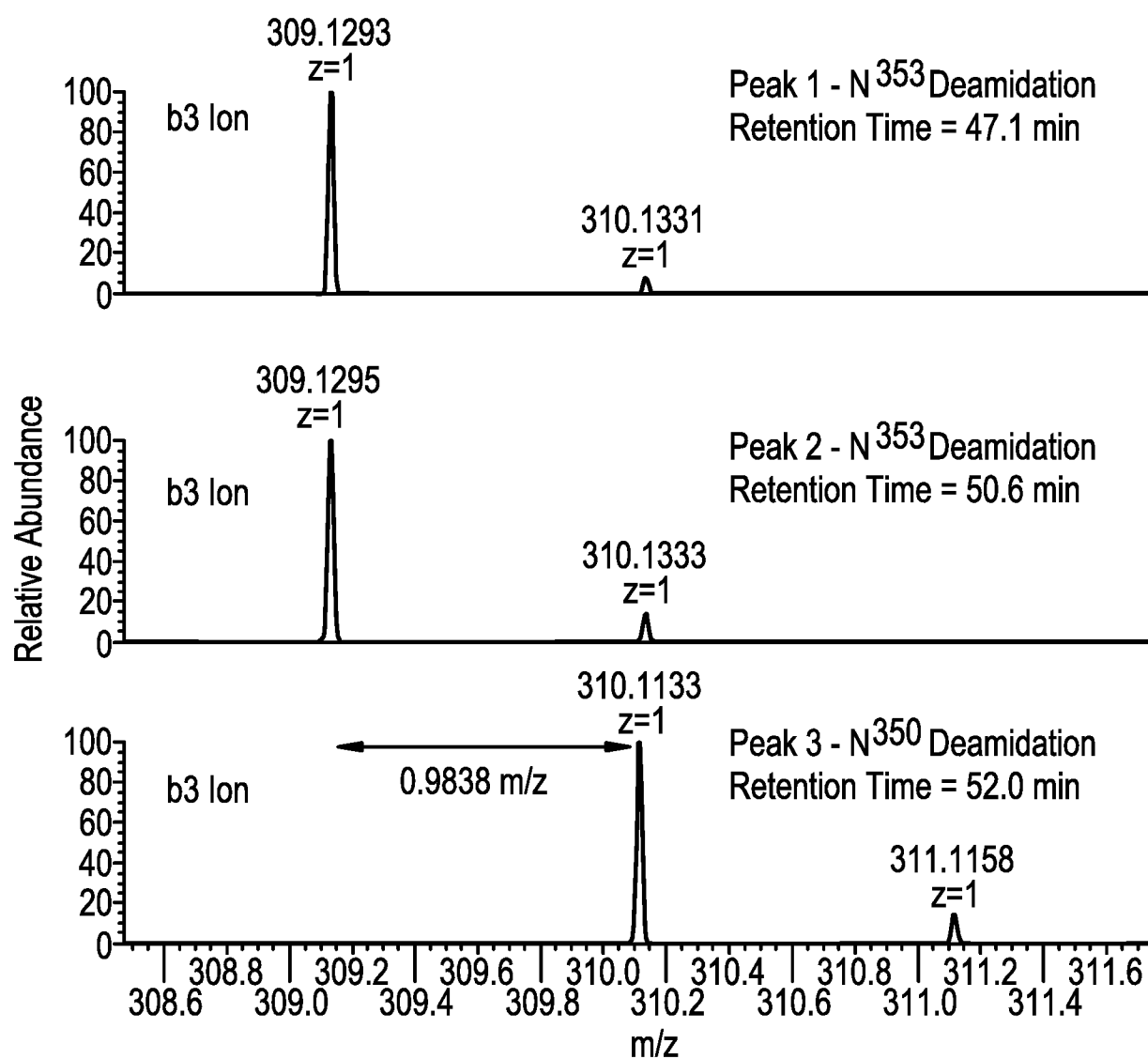
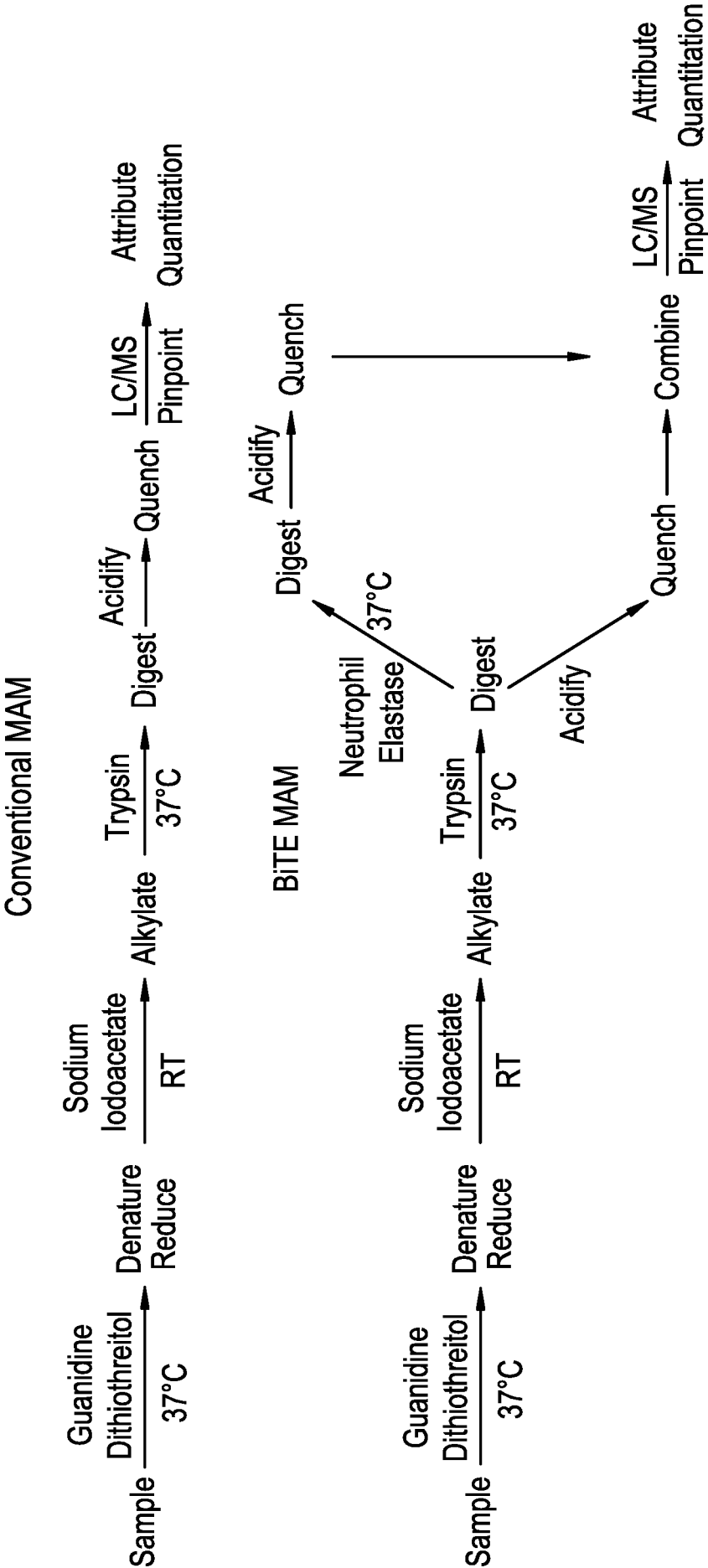


FIG. 5



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FIG. 6

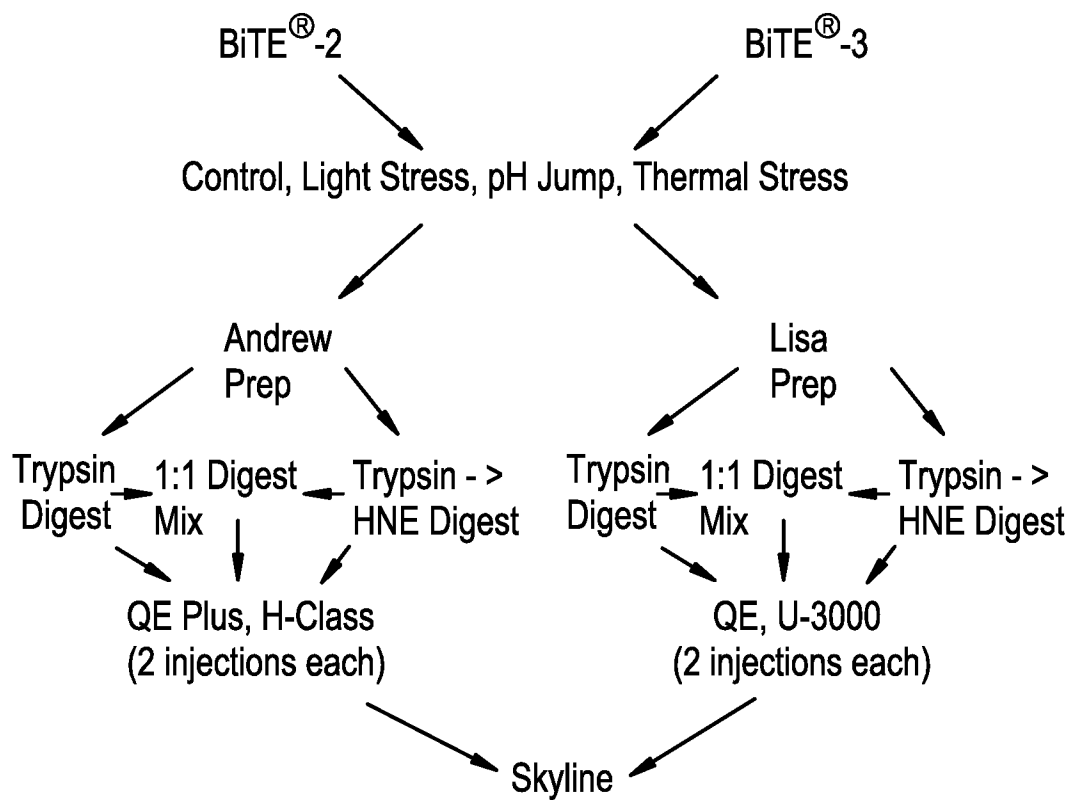
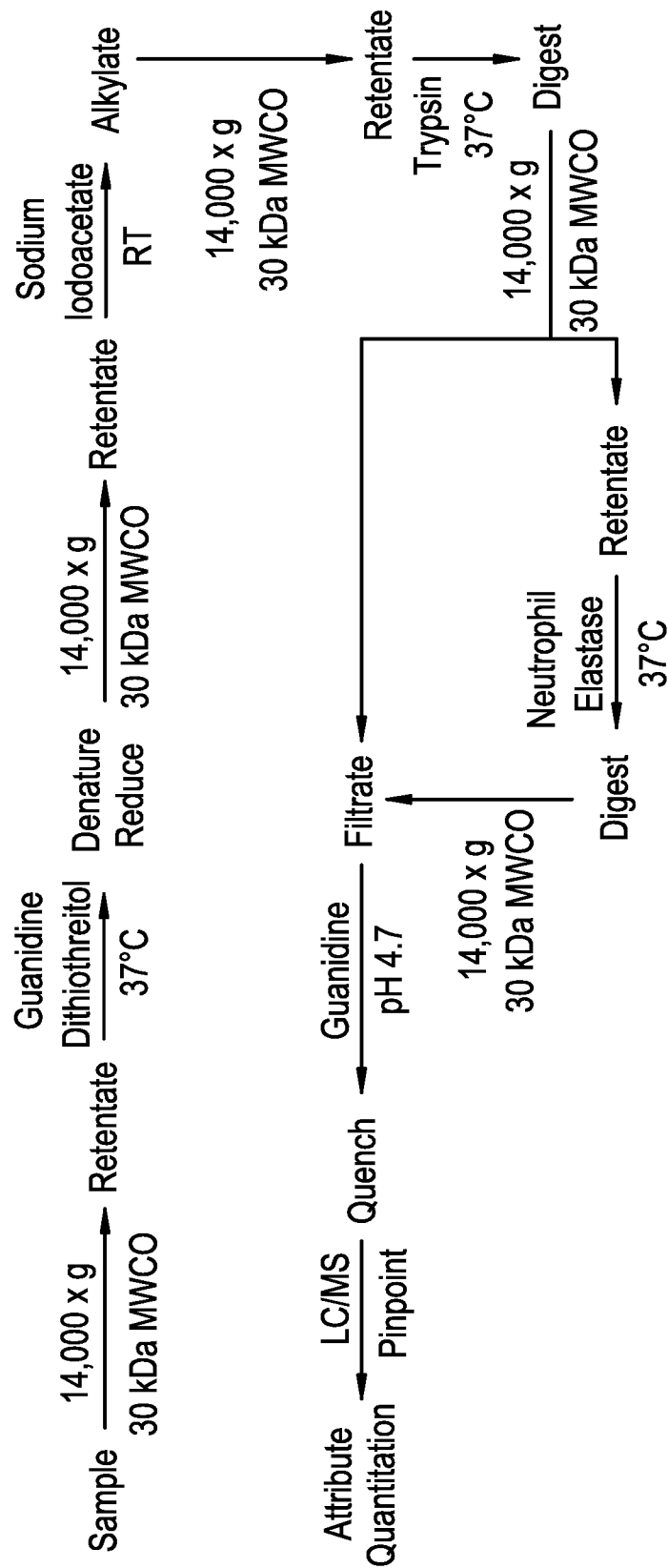
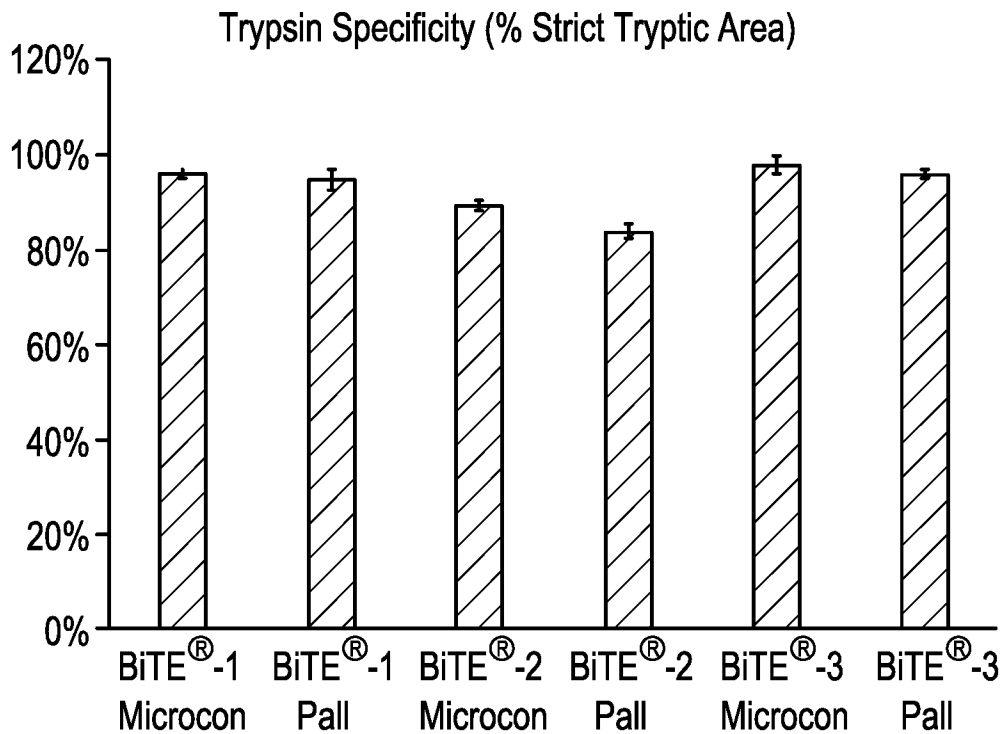


FIG. 7
Multi-Enzyme Filter-Based Sequential Digestion MAM



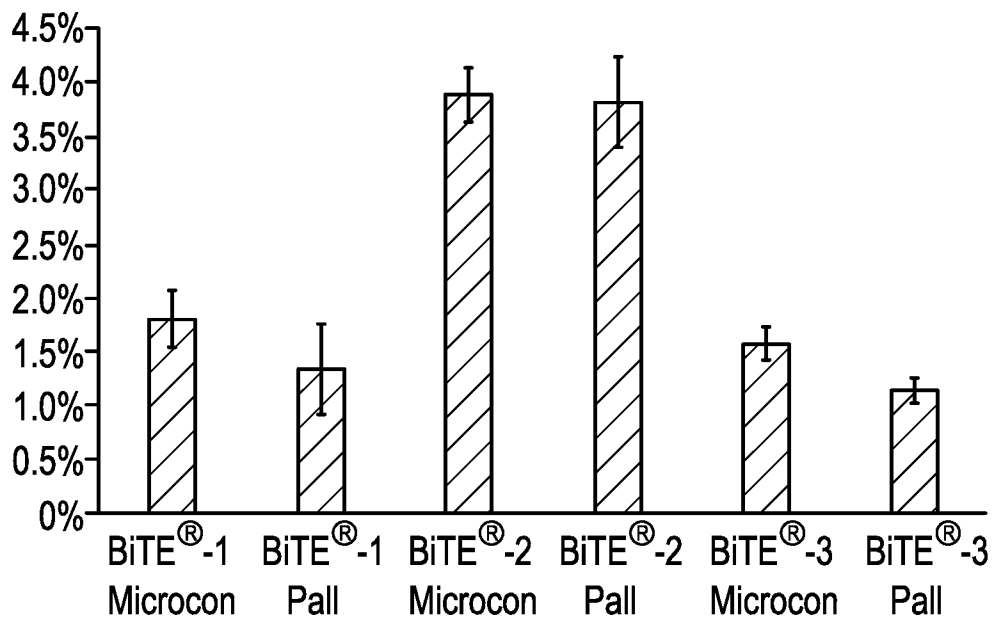
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FIG. 8

% Strict Tryptic Area = Total Area of Identified Peptides (Strict Tryptic Search)/
Total Area of Identified Peptides (KRVITAL Cleavage Search)

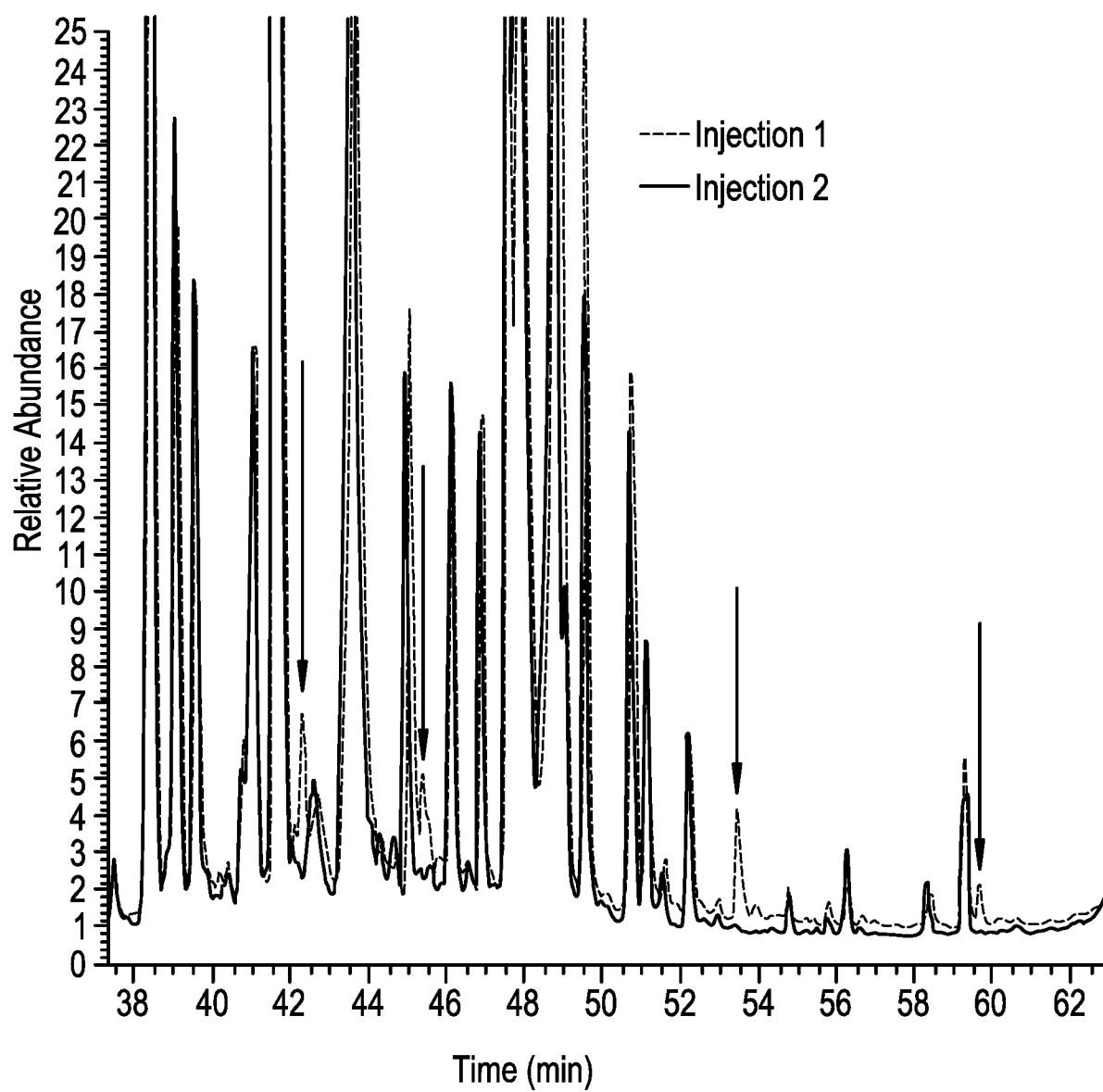
FIG. 9

% Total Area Corresponding to 8 kDa Linker



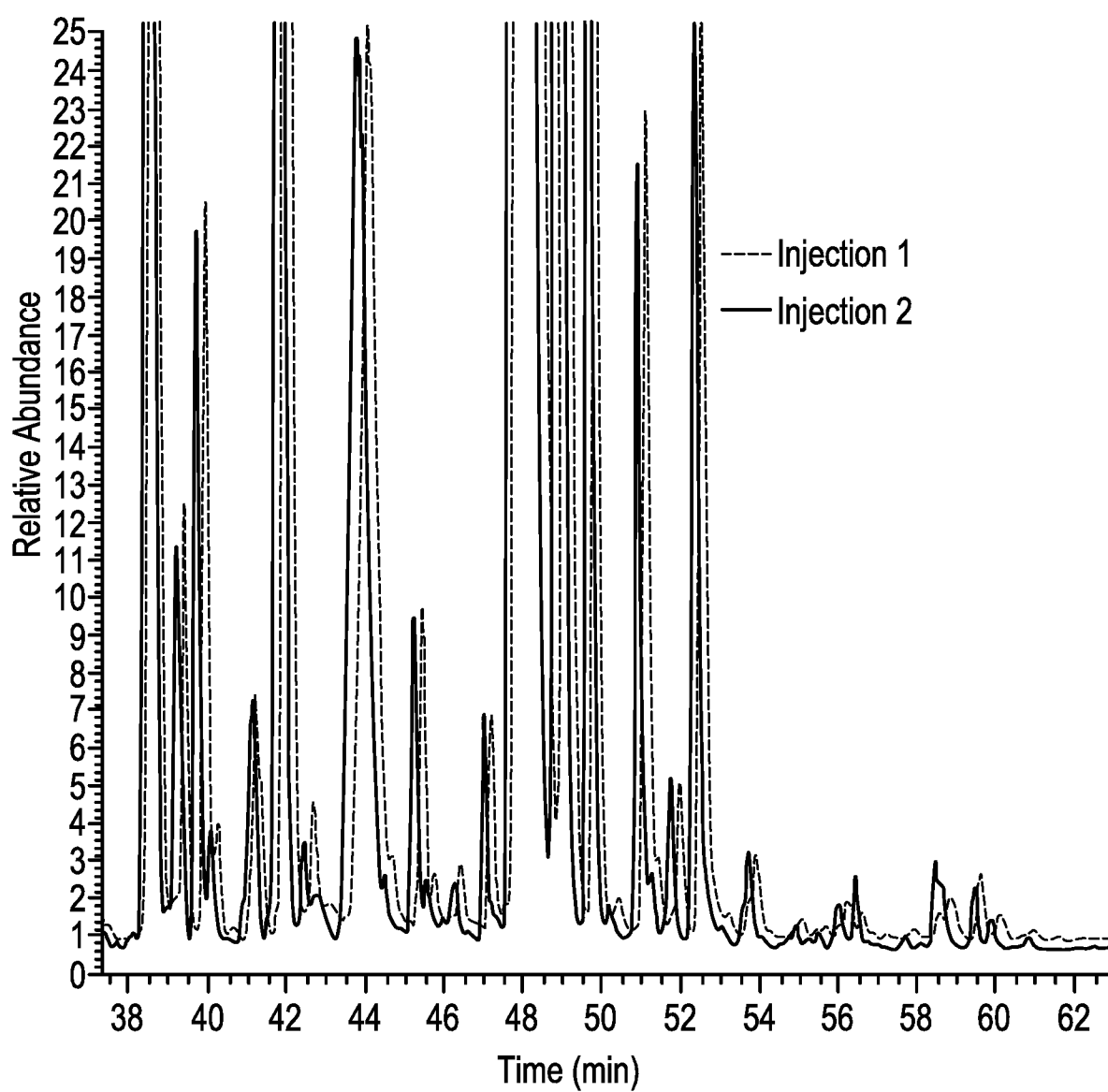
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FIG. 10



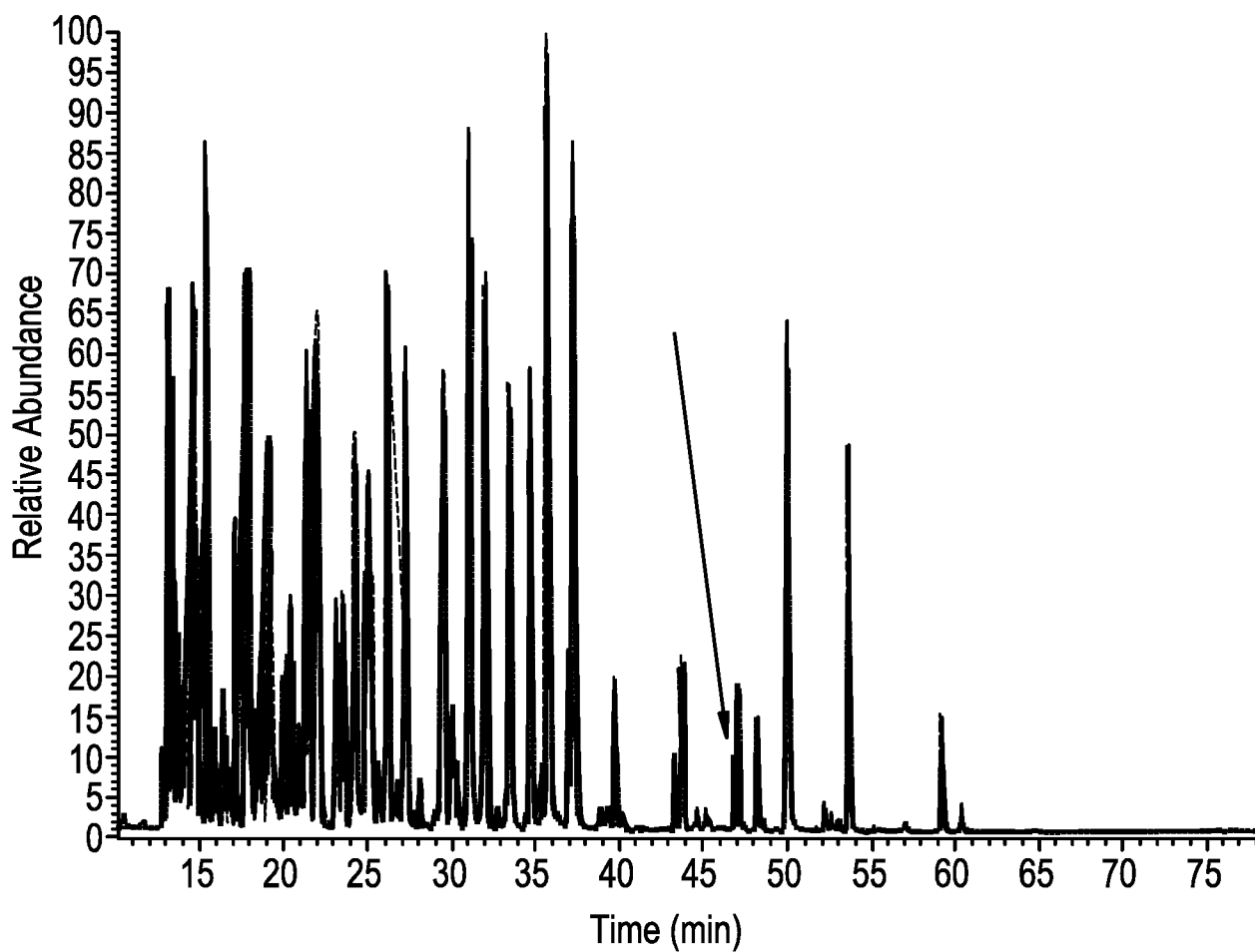
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FIG. 11



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FIG. 12



----- BiTE[®]-2 Protocol 4 Prep 1
—— BiTE[®]-2 Protocol 4 Prep 2
—— BiTE[®]-2 Protocol 4 Prep 3
- - - - BiTE[®]-2 Protocol 5 Prep 1
—— BiTE[®]-2 Protocol 5 Prep 2
—— BiTE[®]-2 Protocol 5 Prep 3

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2019/021927

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, INSPEC, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	C. A. HOBBS ET AL: "Identification of the SPLUNC1 ENaC-inhibitory domain yields novel strategies to treat sodium hyperabsorption in cystic fibrosis airway epithelial cultures", AMERICAN JOURNAL OF PHYSIOLOGY - LUNG CELLULAR AND MOLECULAR PHYSIOLOGY, vol. 305, no. 12, 15 December 2013 (2013-12-15), pages 990-1001, XP55411764, US	1-6, 11-19, 24-43
Y	ISSN: 1040-0605, DOI: 10.1152/ajplung.00103.2013 the whole document In particular: Title; Abstract; Experimental procedures; Figures 1-9; Table 1. ----- -/--	1-43



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Date of the actual completion of the international search

14 May 2019

Date of mailing of the international search report

21/05/2019

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INTERNATIONAL SEARCH REPORT

International application No
PCT/US2019/021927

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Xiaofeng Guot ET AL: "Confetti: A Multiprotease Map of the HeLa Proteome for Comprehensive Proteomics* - S", & Cellular Proteomics MCP Papers in Press, 24 February 2014 (2014-02-24), pages 1573-1584, XP55588192, Retrieved from the Internet: URL:https://www.mcponline.org/content/mcponline/13/6/1573.full.pdf the whole document In particular: Title; Abstract; Experimental procedures; Table I; Figures 1-6.	1-43
A	ALAN DOUCETTE ET AL: "Investigation of the applicability of a sequential digestion protocol using trypsin and leucine aminopeptidase M for protein identification by matrix-assisted laser desorption/ionization - time of flight mass spectrometry", PROTEOMICS, vol. 1, no. 8, 1 August 2001 (2001-08-01), pages 987-1000, XP55588187, DE ISSN: 1615-9853, DOI: 10.1002/1615-9853(200108)1:8<987::AID-PROT 987>3.0.CO;2-5 the whole document In particular: Title; Abstract; Materials and methods.	1-43
A	WO 2016/018978 A1 (MAYO FOUNDATION [US]) 4 February 2016 (2016-02-04) the whole document	1-43
X,P	Therese Dau ET AL: "Sequential digestion with Trypsin and Elastase in cross-linking/mass spectrometry", bioRxiv, 23 October 2018 (2018-10-23), XP55588186, DOI: 10.1101/450981 Retrieved from the Internet: URL:https://www.biorxiv.org/content/10.1101/450981v1 the whole document In particular: Title; Abstract; Material and methods; Figures 1-4.	1-43

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2019/021927

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		US 2018106815 A1	19-04-2018
		WO 2016018978 A1	04-02-2016
