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[Continued on next page]

(54) Title: HUMAN ZONA PELLUCIDA GLYCOPROTEINS, THEIR OLIGOSACCHARIDES, AND USES THEREOF

(57) Abstract: The present invention relates to diagnosis of human infertility, more specifically, to methods and compositions for evaluating human sperm's ability to bind to a human oocyte.

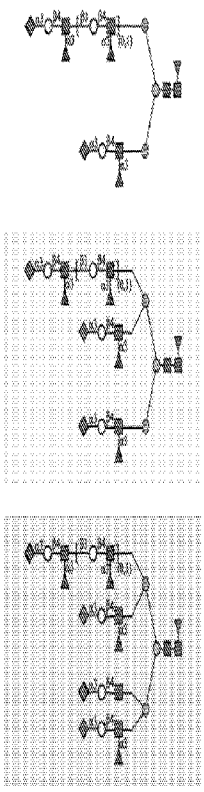

FIGURE 3

WO 2013/012749 A2

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HUMAN ZONA PELLUCIDA GLYCOPROTEINS, THEIR OLIGOSACCHARIDES, AND USES THEREOF

CROSS REFERENCE TO RELATED APPLICATION

[0001] This application claims the benefit of U.S. Provisional Application No. 61/572,450, filed July 15, 2011, which is hereby incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

[0002] The human oocyte is covered by a specialized extracellular matrix known as the zona pellucida (ZP). Human fertilization begins when spermatozoa bind to the ZP. The single spermatozoan that fertilizes the oocyte penetrates the ZP and fuses with the egg cell, generating a zygote.

[0003] The human ZP is composed of four major glycoproteins: huZP1, huZP2, huZP3, and huZP4. Data obtained in mammalian species indicate that sperm-egg binding is primarily mediated by the interaction of an egg binding protein on the sperm plasma membrane with carbohydrate sequences expressed on ZP glycoproteins (1, 2). Evidence that carbohydrate recognition plays a major role in human gamete binding was initially obtained when the polysaccharide fucoidan was shown to potently block this interaction (3). Fucoidan also inhibited leukocyte adhesion in the vascular and lymph systems in the same concentration range that it blocked human sperm interaction with ZP (3, 4). The binding of leukocytes to endothelial cells is mediated by C-type lectins known as selectins (5, 6). Therefore, human sperm to ZP binding was hypothesized to involve a binding specificity that overlaps with selectins (7). However, it has not been possible to confirm this hypothesis because the structures of the human ZP glycans are not well characterized due to challenges of the scarcity of human eggs for research and their microscopic size.

[0004] Sperm from 1 in about every 30 males do not bind to the human ZP and therefore do not fertilize oocytes. This situation can lead to problems during a standard IVF cycle, because the oocytes generated by ovarian stimulation remain unfertilized even in the presence of large numbers of sperm. Some IVF specialists have overcome this problem by routinely employing intracytoplasmic sperm injection (ICSI) to fertilize oocytes. However, there are concerns with aberrant genomic

imprinting associated with assisted reproductive technologies (ART). This has led some reproductive endocrinologists to conclude that they should make fertilization via IVF as natural as possible.

[0005] There remains a need to provide new and improved methods, compositions, and reagents, etc., to evaluate human sperm's functional ability to bind to oocytes.

SUMMARY OF THE INVENTION

[0006] The present invention is drawn to new and improved diagnostic and therapeutic methods, compositions, and reagents/apparatuses in the field of human fertility. In particular, certain embodiments of the invention are drawn to a method of evaluating the ability of human sperm to bind to a human oocyte.

[0007] Certain embodiments of the invention are drawn to beads that are surface-modified with a glycoconjugate comprising a selectin ligand. Certain embodiments of the invention are drawn to a method comprising the steps of 1) placing a bead that has been surface-modified with a glycoconjugate comprising a selectin ligand in a media, 2) adding a sample of human sperm to the media to allow at least a portion of the sperm to bind to the surface of the bead, 3) optionally removing the non-binding sperm, and 4) quantitating the sperm bound to at least a portion of the surface of the bead.

[0008] Certain embodiments of a method of the invention are drawn to performing steps comprising: (a) forming a combination that comprises - (i) a sample of human sperm, (ii) a media that is conducive to maintaining the physiological activity of sperm, and (iii) at least one matrix that comprises on at least a portion of its surface a glycoconjugate that comprises the sialyl-Lewis^x sequence, to allow for at least a portion of the sample of sperm to bind to the matrix; and (b) visualizing or quantitating the sperm bound to the matrix. In certain embodiments, the matrix comprises a bead. In certain embodiments, the matrix is attached to the surface of a specimen slide, dish, or well of a microtiter plate. In certain embodiments, the media is an *in vitro* fertilization (IVF) media.

[0009] Certain embodiments of the invention are drawn to a reagent for determining the ability of human sperm to bind to a human oocyte, the reagent comprising a matrix that comprises on at least a portion of its surface a glycoconjugate that comprises the sialy-Lewis^x sequence. In certain embodiments, the matrix comprises

a bead. In certain embodiments, the matrix comprises a specimen slide, dish, or well of a microtiter plate.

[0010] Certain embodiments of the invention are drawn to a kit for determining the ability of human sperm to bind to a human oocyte, the kit comprising an oligosaccharide that comprises the sialy-Lewis^x sequence and at least one other reagent selected from the group consisting of: (i) a matrix to which the oligosaccharide may be attached and (ii) at least one component of a media that is conducive to maintaining the physiological activity of sperm. In certain embodiments, the matrix comprises on at least a portion of its surface a glycoconjugate comprising the oligosaccharide. In certain embodiments, the media is *in vitro* fertilization (IVF) media. In certain embodiments, the matrix comprises a bead or comprises a specimen slide, dish, or well of a microtiter plate.

[0011] Certain embodiments of a method of the invention comprise performing the steps of: (a) contacting a sample of human sperm and a carrier-molecule, wherein the carrier-molecule is attached to a glycoconjugate that comprises the sialy-Lewis^x sequence, to allow for at least a portion of the sample of human sperm to bind to the carrier-molecule; and (b) detecting the carrier-molecule bound to the sperm. In certain embodiments, the carrier-molecule is a carrier-protein. In certain embodiments, the carrier-protein is bovine serum albumin or selected from the group consisting of the glycoproteins ZP1, ZP2, ZP3, and ZP4. In certain embodiments, the carrier-molecule bound to the sperm is visualized. In certain embodiments, the carrier-molecule is labeled with a detectable marker, such as a fluorescent label.

[0012] In certain embodiments, the detection of the carrier-molecule bound to the sperm is used to quantitate the amount of carrier-molecule bound in the sample of sperm. In certain embodiments, the detection of the carrier-molecule bound to the sperm is used to quantitate the amount of sperm in the sample with a certain affinity of binding to the glycoconjugate carbohydrate sequence. In certain embodiments, the detection of the carrier-molecule bound to the sperm is used to sort sperm cells based on the cells' ability to bind to the glycoconjugate carbohydrate sequence.

[0013] Certain embodiments of a method of the invention comprise passing a sample of sperm across a carrier-molecule that is fixed to a support and capturing at least a portion of the sperm sample that binds to the carrier-protein, wherein the portion of the sperm sample that does not bind the carrier-molecule is not captured.

[0014] Certain embodiments of a method of the invention comprise performing the steps of (a) contacting a sample of human sperm and an oligosaccharide, wherein the oligosaccharide comprises the sialy-Lewis^x sequence, to allow for at least a portion of the sample of human sperm to bind to the oligosaccharide; and (b) detecting the oligosaccharide bound to the sperm. In certain embodiments, the oligosaccharide bound to the sperm is visualized. In certain embodiments, the oligosaccharide is labeled with a detectable marker such as a fluorescent label.

[0015] In certain embodiments, the detection of the oligosaccharide bound to the sperm is used to quantitate the amount of oligosaccharide bound in the sample of sperm. In certain embodiments, the detection of the oligosaccharide bound to the sperm is used to quantitate the amount of sperm in the sample with a certain affinity of binding to the oligosaccharide carbohydrate sequence. In certain embodiments, the detection of the oligosaccharide bound to the sperm is used to sort sperm cells based on the cells' ability to bind to the oligosaccharide carbohydrate sequence.

[0016] Certain embodiments of a method of the invention comprise passing a sample of sperm across an oligosaccharide that is fixed to a support and capturing at least a portion of the sperm sample that binds to the oligosaccharide, wherein the portion of the sperm sample that does not bind the oligosaccharide is not captured.

BRIEF DESCRIPTION OF THE DRAWINGS

[0017] Figure 1. Figure 1 shows profiling of human ZP N- and O-glycans. The upper panel covers the m/z range from 2,700-4,500 and the lower, overlapping panel spans m/z 4,200-5,800. Each panel is normalized so that the most abundant peak is 100% intensity. Structural assignments area based on compositions assigned from molecular weights, complemented by MS/MS information and the results of the neuraminidase digest (Figure 6). Where more than one structure is shown, the upper structure is the more abundant.

[0018] Figure 2. Figure 2 shows partial MALDI-TOF-TOF fragment ion spectra obtained after collisional activation of the molecular ion at m/z 5733 in Figure 1 and m/z 4288 in Figure 5 (upper and lower panels, respectively), illustrating how the fragment ions arising from loss of antennae in sialylated and desialylated samples, respectively, define the antennae sequences. Isomeric glycans were identified which differ in fucose location.

[0019] Figure 3. Figure 3 shows structures of the core fucosylated, multivalent sialyl-Lewis^X members of each of the bi-, tri, and tetra-antennary families found on human ZP.

[0020] Figure 4. Figure 4 shows that sialyl-Lewis^X is involved in sperm-ZP binding. (A) Comparison of hemizona binding index (HZI) of capacitated spermatozoa incubated in the presence of sialyl-Lewis^X (SLEX), Lewis^X (LEX), and sialyl-N-acetyllactosamine (SLN) oligosaccharide or their BSA conjugates with medium alone (control). Each point represents the mean±SEM of the results of 10 hemizona assays. *$p<0.05$ when compared to the corresponding untreated control. †$p<0.05$ when compared with the desialyated counterpart. Representative photographs showing the binding of spermatozoa to hemizona are shown. (B) Representative fluorescent images of capacitated spermatozoa incubated with Alexa Fluor-594 labeled sialyl-Lewis^X-BSA, Lewis^X-BSA, sialyl-N-acetyllactosamine-BSA and BSA. N=5. (C) Immunostaining of sialyl-Lewis^X sequences on hemizona. Matching hemizona were incubated with anti- sialyl-Lewis^X (CSLEX1) or anti-6-sulfo lacNAc sequences on extended core 1 O-glycans (MECA-79) or anti-Lewis^X antibodies or pre-absorbed antibodies. N=5. (D) Left: Effect of CSLEX1 and anti-Lewis^X antibody on sperm-ZP binding in hemizona binding assays. Matching hemizona were incubated with the antibodies or pre-absorbed antibody. N=5. *$p<0.05$ when compared withteh control. Right: Representative photographs of binding of spermatozoa to hemizona following incubation with anti-sialyl-Lewis^X or Lewis^X antibody (10 µg/ml). (E) Left: Effect of desialylation of solubilized ZP (1 µg/ml) on sperm-ZP binding in hemizona binding assays. N=5. Right: Representative photographs of the binding of native and desialylated ZP to spermatozoa.

[0021] Figure 5: Figure 5 shows the MALDI-TOF-TOF fragment ion spectra obtained after collisional activation of (A) m/z 3664 and (B) m/z 4114 from the desialylated N-glycan sample (see Figure 6 for MS data), and (C) m/z 4575 from the sialylated N-glycan sample (see Fig. 1 for MS data). These data complement the spectra shown in Figure 2, further illustrating how the fragment ions define the antennae sequences. The cartoons show the sites of fragmentation within the antennae and the horizontal arrows show the moieties which are liberated from the molecular ion when each of the fragment ions is formed. Note the diagnostic

fragment ions at m/z 3458 (panel A) and 3908 (panel B) which arise specifically from elimination of the 3-linked fucose.

[0022] Figure 6: Figure 6 shows the MALDI-TOF mass spectrum of N-glycans after α2-3-specific sialidase digestion. All sialyl-Lewis^X antennae observed in Figure 1 have been converted to Lewis^X by the sialidase.

[0023] Figure 7: Figure 7 shows the identification of sulfated N-glycans in the human ZP sample. A portion of the released N-glycans was taken through permethylation and screened by MALDI-MS in negative ion mode for the presence of sulfated glycans as described (15). No significant peak was observed initially but several weak signals were detected and could be assigned as annotated if the sample was first desialylated to reduce the heterogeneity. The desialylated, sulfated N-glycans thus identified correspond in composition to the desialylated, sulfated counterparts of those multiantennary structures carrying multiple sialyl Lewis^x epitopes which are shown in Figure 1.

[0024] Figure 8: Figure 8 shows O-glycans found on human ZP. (A) MALDI-TOF mass spectrum of O-glycans obtained from reductive elimination of residual glycopeptides remaining after N-glycan release from trypsinised human ZP. Glycans were permethylated prior to MS analysis. Structural assignments take into account compositions assigned from molecular weights together with MS/MS-derived sequence information. Unlabelled signals are from the matrix used in the MALDI experiment. (B) Structures of O-glycans found on human ZP.

[0025] Figure 9: Figure 9 shows that NO immunoreactivity was observed on ZP using MECA-79 and anti-Lewis^x antibodies. Matching hemizona were incubated with 0.2 µg/ml anti-6-sulfo sialyl-Lewis^x (MECA-79) or anti-Lewis^x antibodies or antibodies preabsorbed with irrelevant antibody or Lewis^x-BSA for 3 hours at 37°C. The immunoreactivites were visualized using Alexa Fluor 594-conjugated goat anti-mouse IgG or anti-rat IgM. The results shown are representative of 5 replicate experiments. LEX: Lewis^x

[0026] Figure 10: Lewis^x and sialyl-N-acetyllactosamine is not involved in sperm-ZP binding. Representative photographs of ten replicate experiments showing the binding of capacitated spermatozoa to hemizona in the presence of Lewis^x-BSA and sialyl-N-acetyllactosamine-BSA neoglycoprotein or alternatively Lewis^x and sialyl-N-

acetyllactosamine-BSA oligosaccharide with medium alone (control). LEX: Lewis^x; SLN: sialyl-N-acetyllactosamine.

[0027] Figure 11: Fluorescently labeled sialyl-Lewis^x-BSA/Lewis^x-BSA/sialyl-N-acetyllactosamine-BSA did not bind to ZP. Matching hemizona were incubated with 2 µM Alexa-594 conjugated sialyl-Lewis^x-BSA/Lewis^x-BSA/sialyl-N-acetyllactosamine-BSA or BSA for 60 minutes at 37°C. Fluorescently-labeled sialyl Lewis^x/Lewis^x-BSA/sialyl-N-acetyllactosamine-BSA and BSA did not bind to the hemizona. The results shown are representative of 5 replicate experiments. SLEX: Sialyl-Lewis^x; LEX: Lewis^x; SLN: sialyl-N-acetyllactosamine.

[0028] Figure 12: Sialyl-Lewis^x/Lewis^x/sialyl-N-acetyllactosamine-BSA and sialyl-Lewis^x/Lewis^x/sialyl-N-acetyllactosamine oligosaccharides have no effect on the acrosomal status and motility parameters of human spermatozoa. (A) The effect of neoglycoproteins (0.01-2 µM) and oligosaccharides (0.1-500 µM) on the acrosomal status of spermatozoa. The fluorescence patterns of 300 fluorescein isothiocyanate-labeled *Pisum sativum* agglutinin-treated spermatozoa in randomly selected fields were determined. Data represent mean ± SEM of 5 separate experiments. (B) The effect of neoglycoproteins (2 µM) and oligosaccharides (500 µM) on motility were determined Hobson Sperm Tracker System. Parameters of spermatozoan motility measured: average path velocity (VAP), curvilinear velocity (VCL), straight line velocity (VSL), beat cross frequency (BCF), amplitude of lateral head displacement (ALH), linearity (LIN; VSL/VCL), straightness (STR; VSL/VAP), percentage hyperactivation (HYP; VCL ≥ 100 µm/s, LIN ≤ 60% and ALH ≥ 5.0 µm) and percentage progressive motility (VAP ≥ 25 µm/s). Data represent the mean ± SEM of five separate experiments using 5 different samples of spermatozoa. SLEX: Sialyl-Lewis^x; LEX: Lewis^x; SLN: sialyl-N-acetyllactosamine.

[0029] Figure 13: Figure 13 shows the results of shotgun proteomic analysis of the purified human ZP sample.

DETAILED DESCRIPTION

[0030] Headings are provided herein solely for ease of reading and should not be interpreted as limiting.

Definitions

[0031] As used herein, a "glycoconjugate" is any carbohydrate that is chemically coupled to an "aglycone (or aglycon)" wherein an aglycone/algycon is any substance or chemical that is not a carbohydrate. Also, as used herein, a "glycoconjugate" refers to a selectin ligand sequence that is attached to a carrier-molecule wherein such carrier-molecule is a higher molecular weight carbohydrate.

Overview

[0032] The present invention is drawn to uses of human zona pellucida (ZP) glycoproteins and their oligosaccharides in methods of determining the fitness of human sperm. Certain methods, compositions, reagents, etc., described herein may be employed in functional assays to determine if spermatozoa have a defect in their ability bind to the human ZP, such as in assays performed prior to IVF.

[0033] It has been discovered that human oocytes profusely express a specific, unusual carbohydrate sequence on their surfaces known as the sialy-Lewis^X sequence (NeuAcα2-3Galβ1-4(Fucα1-3)BlcNAc) (SLEX sequence) and that this sequence is the dominant antenna on the N- and O-glycans of human ZP. This epitope is present at densities that are very unusual compared with the levels of selectin-ligand expressed on human somatic cells. This carbohydrate sequence and polyvalent neo-glycoproteins bearing this sequence inhibit binding of human sperm to the ZP.

Sperm binding

[0034] One aspect of the invention relates to materials and methods for evaluating or predicting human sperm's ability to bind to a human oocyte.

[0035] In certain embodiments of a method of the invention, a sample of human sperm is combined with a media conducive to maintaining the physiological activity of sperm and at least one matrix that has on at least a portion of its surface a glycoconjugate that comprises a carbohydrate sequence that is a selectin ligand, to allow for at least a portion of the sample of sperm to bind to the matrix. It is understood that as used herein, reference to binding of "sperm to the matrix" encompasses binding of the sperm to the glycoconjugate on the surface of the matrix, such that sperm bound to the glycoconjugate on the surface of the matrix is

considered "bound to the matrix." One illustrative example of a carbohydrate sequence that is a selectin ligand is the sialyl-Lewis^x (SLEX) sequence. The media conducive to maintaining the physiological activity of sperm is a media such as *in vitro* fertilization (IVF) media. Further, the sperm in the sample may be capacitated before binding. The "matrix" can be one of any material with a surface that is suitable for attaching, either directly or indirectly, a carbohydrate sequence to. Illustrative examples of an indirect attachment may be via a linker or carrier-molecule such as a carrier-protein. Illustrative matrix materials include glass, plastic, agarose, and metal. Illustrative matrix surfaces include the surface of a bead and the surface of a specimen slide, dish, container, or well.

[0036] After the step of combining the sperm, media, and matrix to allow for at least a portion of the sample of sperm to bind to the matrix, sperm bound to the matrix may be visualized and/or the quantity of sperm bound to at least a portion of the matrix may be determined. In certain embodiments, unbound sperm may be removed, such as by washing, before visualizing and/or quantitating the bound sperm. Further, visualization and quantitation of the bound sperm may be enhanced by fixing and/or staining the bound sperm. In certain embodiments, cytochemical or immunohistochemical staining of the bound sperm may be used to enhance visualization and quantitation. Quantitation may be done by various methods, for example, by manual counting of sperm or by automated counting using imaging or flow cytometry methods. In certain embodiments, all of the matrix surface coated with the glycoconjugate may be examined. In certain embodiments, only a portion of the matrix surface may be examined to determine the amount of sperm bound to that portion. The value obtained may be used to determine another value, such as a number or approximate number of all of the sperm bound in the sample. The quantity of sperm bound to any amount of matrix may also be used to calculate, for example, the amount or approximate amount of sperm bound per area of matrix surface, or per concentration of matrix, or per density of glycoconjugate available for binding, etc. The quantity of sperm bound in one sample may also be compared to the quantity of sperm bound in one or more other samples, to determine a ratio. The quantity of sperm bound or the ratio of sperm bound may be useful in evaluating the ability of a sample of sperm to bind to the glycoconjugate carbohydrate sequence which may be representative and/or predictive of its ability to bind to an oocyte.

[0037] In one embodiment of a method comprising combining a sample of sperm, media, and a matrix, the matrix is first placed into the media and then the sample of sperm is added to the media containing the matrix. In another embodiment, the sperm is first placed into the media and then the matrix is added to the media containing the sperm. In another embodiment, the sample of sperm and matrix may be placed in the media simultaneously. One of skill in the art will appreciate that any combination of order of combining the sample of sperm, media, and matrix is contemplated and no particular order is limiting.

[0038] In certain embodiments where the matrix is a glycoconjugate-bead, such as a bead made from glass, plastic, agarose, or metal, one or more beads are placed into a dish, well, etc., and contacted with media and sperm to allow at least a portion of the sample of sperm to bind to the matrix. Sperm bound to the bead may then be visualized and/or quantitated.

[0039] In certain embodiments where the matrix comprises the surface of a specimen slide, container, dish, well, etc., the media is contacted with at least a portion of the matrix, thus combining the media and the matrix, and then the sample of sperm is placed in the media forming the combination of sperm, media and matrix. For example, at least a portion of the surface of a specimen slide comprises a glycoconjugate that comprises a carbohydrate sequence that is a selectin ligand, such as the sialyl-Lewis^x sequence. Media is contacted with some, or all, of the portion of the surface having the glycoconjugate. A sample of sperm is added to the media to allow for at least a portion of the sample of sperm to bind to the glycoconjugate. Sperm bound to the specimen slide, container, dish, well, etc., may then be visualized and/or quantitated.

[0040] In certain embodiments, at least a portion of one or more wells of a microtiter plate (6, 24, 96-wells, etc.) is coated with a glycoconjugate that comprises a carbohydrate sequence that binds to selectins, such as the sialyl-Lewis^x sequence. Media is placed in a well in contact with the coated portion and a sample of sperm is placed in the media to allow for at least a portion of the sample of sperm to bind to the glycoconjugate. Sperm bound in the well(s) may then be visualized and/or quantitated, for example, by using a microtiter plate reader for high throughput analysis.

[0041] Certain embodiments of the invention are drawn to reagents useful in evaluating the ability of human sperm to bind to a human oocyte. In certain such embodiments, the reagent comprises a matrix that is suitable for attaching a glycoconjugate to that has on at least a portion of its surface a glycoconjugate that comprises a carbohydrate sequence that is a selectin ligand. In certain embodiments, the selectin ligand is the sialyl-Lewis^x sequence. The glycoconjugate may be directly attached to the matrix or indirectly attached such as via a linker or carrier-molecule, for example, a carrier-molecule. An illustrative example of a commonly used carrier-molecule is the carrier-protein bovine serum albumin (BSA). Selectin ligands, such as the sialyl-Lewis^x sequence conjugated to BSA, are available for example from DEXTRA (Reading, United Kingdom). Other illustrative carrier-proteins include the ZP1, ZP2, ZP3, and ZP4 glycoproteins. In certain embodiments, the carrier-molecule is a high molecular weight carbohydrate such as dextran. In certain embodiments, the reagent is a bead, such as a bead comprising glass, plastic, agarose, or metal, that has on at least a portion of its surface a glycoconjugate that comprises a carbohydrate sequence that is a selectin ligand, such as the sialyl-Lewis^x sequence. In certain embodiments, the reagent is a specimen slide, a dish, a microtiter plate, or other plate, container, well, etc., capable of holding on or within a sample of sperm and media, that has on at least a portion of its surface a glycoconjugate that comprises a carbohydrate sequence that is a selectin ligand, such as the sialyl-Lewis^x sequence. In certain embodiments, the glycoconjugate may be applied or spotted onto a discreet area(s) of the surface of a specimen slide, plate, dish, well, etc.

[0042] Certain embodiments of the invention are drawn to kits useful in evaluating the ability of human sperm to bind to a human oocyte. In certain embodiments, a kit comprises a media that is conducive to maintaining the physiological activity of sperm, such as IVF media, or at least one or more of the components comprising such a media, and a matrix that comprises on its surface a glycoconjugate that is a selectin ligand such as the sialy-Lewis^x sequence. In certain embodiments, the matrix and carbohydrate sequence of the glycoconjugate may be provided in the kit as separate components to be combined before use. In certain embodiments, the kit comprises the carbohydrate sequence of the glycoconjugate for attaching to a matrix and media, but not the matrix itself, such that the matrix is provided

separately. The matrix can be one of any material with a surface that is suitable for attaching, either directly or indirectly, a carbohydrate sequence to. Illustrative matrix materials include glass, plastic, agarose, and metal. Illustrative matrix surfaces include the surface of a bead and the surface of a specimen slide, dish, container, or well.

[0043] In certain embodiments of a method of the invention, a sample of human sperm is contacted with a carrier-molecule that is attached to a glycoconjugate that comprises a carbohydrate sequence that is a selectin ligand, to allow for at least a portion of the sample of human sperm to bind to the carrier-molecule. Further, the sperm in the sample may be capacitated before binding. The carrier-molecule bound to the sperm can then be detected. It is understood that as used herein, reference to binding of "carrier-molecule to sperm" encompasses binding of the sperm to the glycoconjugate attached to the carrier-molecule, such that sperm bound to the glycoconjugate attached to the carrier-molecule is considered "bound to the carrier-molecule." One illustrative example of a carbohydrate sequence that is a selectin ligand is the sialyl-Lewis^x (SLEX) sequence. Numerous carrier-molecules are known to which a carbohydrate sequence may be attached. In certain embodiments, the carrier-molecule is a carrier-protein, such as bovine serum albumin (BSA). Other illustrative carrier-proteins include the ZP1, ZP2, ZP3, and ZP4 glycoproteins. In certain embodiments, the carrier-molecule is a high molecular weight carbohydrate such as dextran. The carrier-molecule may be attached to a single or a plurality of glycoconjugate carbohydrate sequences and thus a single carrier-molecule may be able to bind to one or a plurality of sperm cells.

[0044] In certain embodiments, detection of the carrier-molecule bound to sperm may be aided by labeling the carrier-molecule, either directly or indirectly, with a detectable marker. The carrier-molecule may be attached to a detectable marker before being contacted with a sperm sample, such as by attaching a fluorescent label to a carrier-molecule, for example, using fluorescently-labeled glycoconjugate-BSA as the carrier-molecule. The carrier-molecule may also be labeled with a detectable marker after it has been contacted with a sample of sperm, such as by contacting the carrier-molecule bound to sperm with a reagent, such as an antibody, that recognizes the carrier-molecule. Such reagent that recognizes the carrier-molecule may itself be labeled with a detectable label or may be detected by another

reagent, such as a secondary antibody, that is labeled with a detectable marker. The detectable marker may be any of those known in the art including, but not limited to, fluorescent labels, radiolabels, biotin, labels utilizing enzymatic amplification, etc. In certain embodiments, the sample of sperm bound to the carrier-molecule is washed and/or fixed according to standard cytochemical and/or immunohistochemical practices to remove unbound carrier-molecule, allow for binding of detector molecules, reduce background signal when detecting the carrier-molecule bound to sperm, etc.

[0045] In certain embodiments, the carrier-molecule bound to sperm is detected by visualization, such as by visualizing a fluorescent label attached or bound to the carrier-molecule. In certain embodiments, the detection of carrier-molecule bound to sperm is used to quantitate the amount or approximate amount of carrier-protein bound in the sample of sperm. Quantitation may be done by various methods, for example, by visual inspection of the detectable label or by automated quantitation using imaging or flow cytometry methods. Such quantitation may be used to determine affinity of the sperm in a sample to bind the selectin ligand attached to the carrier-molecule, which may be representative and/or predictive of its ability to bind to an oocyte. In certain embodiments, the detection of carrier-protein bound to sperm is used to quantitate the amount of sperm in the sample with at least a certain affinity of binding to the glycoconjugate carbohydrate sequence. A higher percentage of sperm in a sample capable of binding with a certain affinity to the glycoconjugate carbohydrate sequence may be representative and/or predictive of the ability of the sperm to bind to an oocyte.

[0046] In certain embodiments, the detection of carrier-molecule bound to sperm is used to sort the sperm based on the sperm's ability to bind to the glycoconjugate carbohydrate sequence. For example, flow cytometry can be used to sort labeled cells from unlabeled cells. Certain embodiments of a method of the invention are drawn to passing a sample of sperm across a carrier-molecule that is fixed to a support and capturing at least a portion of the sperm sample that binds to the carrier-molecule, wherein the portion of the sperm sample that does not bind the carrier-molecule is not captured.

[0047] In certain embodiments of a method of the invention, a sample of human sperm is contacted with an oligosaccharide that comprises a carbohydrate sequence

that is a selectin ligand, to allow for at least a portion of the sample of human sperm to bind to the oligosaccharide. Further, the sperm in the sample may be capacitated before binding. The oligosaccharide bound to the sperm may then be detected. One illustrative example of a carbohydrate sequence that is a selectin ligand is the sialyl-Lewis^x (SLEX) sequence.

[0048] In certain embodiments, detection of the oligosaccharide bound to sperm may be aided by labeling the oligosaccharide, either directly or indirectly, with a detectable marker. The oligosaccharide may be attached to a detectable marker before being contacted with a sperm sample, such as by attaching a fluorescent label to a carrier-molecule. The oligosaccharide may also be labeled with a detectable marker after it has been contacted with a sample of sperm, such as by contacting the oligosaccharide bound to sperm with a reagent, such as an antibody, that recognizes the oligosaccharide. Such reagent that recognizes the oligosaccharide may itself be labeled with a detectable label or may be detected by another reagent, such as a secondary antibody, that is labeled with a detectable marker. The detectable marker may be any of those know in the art including, but not limited to, fluorescent labels, radiolabels, and labels utilizing enzymatic amplification. In certain embodiments, the sample of sperm bound to the oligosaccharide is washed and/or fixed according to standard cytochemical and/or immunohistochemical practices to remove unbound oligosaccharide, allow for binding of detector molecules, reduce background signal when detecting the oligosaccharide bound to sperm, etc.

[0049] In certain embodiments, the oligosaccharide bound to sperm is detected by visualization, such as by visualizing a fluorescent label attached or bound to the oligosaccharide. In certain embodiments, the detection of oligosaccharide bound to sperm is used to quantitate the amount or approximate amount of oligosaccharide bound in the sample of sperm. Quantitation may be done by various methods, for example, by visual inspection of the detectable label or by automated quantitation using imaging or flow cytometry methods. Such quantitation may be used to determine affinity of the sperm in a sample to bind the selectin ligand carbohydrate sequence of the oligosaccharide, which may be representative and/or predictive of its ability to bind to an oocyte. In certain embodiments, the detection of oligosaccharide bound to sperm is used to quantitate the amount of sperm in the

sample with at least a certain affinity of binding to the selectin ligand carbohydrate sequence. A higher percentage of sperm in a sample capable of binding with a certain affinity to the selectin ligand carbohydrate sequence may be representative and/or predictive of the ability of the sperm to bind to an oocyte.

[0050] In certain embodiments, the detection of oligosaccharide bound to sperm is used to sort the sperm based on the sperm's ability to bind to the selectin ligand carbohydrate sequence. For example, flow cytometry can be used to sort labeled cells from unlabeled cells. Certain embodiments of a method of the invention are drawn to passing a sample of sperm across an oligosaccharide that is fixed to a support and capturing at least a portion of the sperm sample that binds to the oligosaccharide, wherein the portion of the sperm sample that does not bind the oligosaccharide is not captured.

Examples

[0051] The following disclosed embodiments are merely representative of the invention which may be embodied in various forms. Thus, specific structural, functional, and procedural details disclosed in the following examples are not to be interpreted as limiting.

Example 1:

[0052] Ultrasensitive mass spectrometric analyses was used to establish that the sialyl-Lewis^X sequence, a well-known selectin ligand, is the dominant antenna on the N- and O-glycans of human ZP. It was further shown that sperm-to-ZP binding was inhibited by 63-76% by glycoconjugates terminated with sialyl-Lewis^X sequences or by antibodies directed against this sequence.

[0053] ZP from 195 unfertilized human oocytes was isolated for glycan sequencing. Purity was assessed by proteomics analysis which identified human ZP4 as the top hit in the Mascot search (Figure 13). The other ZP glycoproteins (ZP1, ZP2, and ZP3) were third, fifth, and sixth on the Mascot list. At the second and fourth positions were haptoglobin and transthyretin, respectively, which are known constituents in follicular fluid (10).

[0054] The structures of the N- and O-glycans in the ZP sample were determined by glycomics analysis (8). Glycans were analyzed by Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) Mass Spectrometry (MS) as well

as by collisionally activated dissociation (CAD) on a MALDI-TOF-TOF instrument (MS/MS). Mixtures of N- and O-glycans were released from tryptic digests by peptide N-glycosidase F and reductive elimination, respectively, and were permethylated before MS and MS/MS analysis.

[0055] The MALDI-TOF N-glycan fingerprint (Figure 1) shows four families of bi-, tri-, and tetra-antennary structures, three of which display an unusually high density of sialyl-Lewis^X antennae (NeuAcα2-3Galβ1-4(Fucα1-3)GlcNAc). All members of the latter three families are core fucosylated, and each is fully sialylated. Heterogeneity is confined to differences in antenna fucosylation and length. Thus, bi-antennary glycans carry zero, one, or two sialyl-Lewis^X antennae (m/z 2966.6, 3140.7/359.9/3764.0, and 3314.8/3764.0/3938.1, respectively); tri-antennary glycans carry zero, one, two, or three sialyl-Lewis^X antennae (m/z 3777.1, 3951.2, 4125.2/4574.8/4748.6, and 4299.3/4748.6/4922.6, respectively); and tetra-antennary glycans carry zero, one, two, three, or four sialyl-Lewis^X antennae (m/z 4587.5, 4761.6, 4935.8/5384.9/5559.0, 5109.4/5559.0/5733.1 (major portion) and 5733.1 (minor portion), respectively).

[0056] Antennae compositions were defined by CAD-MS/MS. As shown in Figures 2 and 5, the most abundant fragment from the highest molecular weight species observed in Figure 1 (m/z 5733), together with its desialylated counterpart (m/z 4287.6, Figure 6) are shown in the upper and lower panels, respectively, of Figure 2. Collectively, these data demonstrate that m/z 5733 is a mixture of tetra-antennary glycans having one extended antenna and three or four sialyl-Lewis^X moieties. The extended antenna is largely composed of the sialyl-Lewis^X- Lewis^X sequence (NeuAcα2-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAc), although a minority of glycans have only a single fucose on this antenna (Figure 2).

[0057] Fucose was confirmed to be 3-linked in the sialyl-Lewis^X moiety via its diagnostic elimination in CAD-MS/MS experiments (Figure 2 and Figure 5). Linkages involving sialic acid were defined by MALDI analysis of an α2-3-specific neuraminidase digest of the N-glycans (Figure 6) which showed that α2-6 sialylation is confined to the aforementioned fourth family (Figure 1), that differs from the other families in having no core fucosylation. The human plasma glycome is characterized by the absence of core fucose plus high levels of α2-6 sialylation (11,

12). Therefore this fourth glycan family is largely derived from the follicular fluid constituents that co-purify with ZP (Figure 13).

[0058] An extended sialyl-Lewis^X-Lewis^X antenna, and/or its monofucosylated counterpart, that was identified in the m/s 5733 component (Figure 2), was additionally found in seven other members of the sialyl-Lewis^X-containing families (Figure 1: m/z 3589.9, 3764.0, 3938.1, 4574.8, 4748.6, 5384.9, and 5559.0). These extended sequences were firmly established by MS/MS analyses; examples of diagnostic fragment ions are illustrated in Figure 5 (Panel C).

[0059] ZP-associated N-glycans were also investigated to determine if any were sulfated, a modification that is known to play a key role in selectin-mediated leukocyte trafficking (13, 14). This study was done after desialylation by using ultrasensitive MS methodologies which have been optimized for sulfo-glycomics (15). Only trace levels of sulfated N-glycans were observed (Figure 7). Their compositions correspond to sulfated counterparts of the core fucosylated glycans shown in Figure 1.

[0060] ZP-associated O-glycans were released from the glycopeptides recovered from the peptide N-glycosidase F digestion, permethylated, and analyzed by MALDI-TOF-TOF. A limited number of core 1 and core 2 O-glycans were observed (Figure 8), the latter carrying a single sialyl-Lewis^X epitope. No sulfated O-glycans were detected in the MS experiments. Potential O-sulfation was also investigated by using the MECA-79 antibody, which recognizes 6-sulfated GlcNAc on extended core 1 sequences including those terminated by 6-sulfo sialyl-Lewis^X. No immunoreactivity was observed (Figure 9).

[0061] The most significant finding from the ZP glycomics is the presence of multivalent sialyl-Lewis^X N-glycans, representative examples of which are displayed in Figure 3. The high density of sialyl-Lewis^X antennae observed on the ZP N-glycans is unusual. This epitope is highly expressed in cells and tissues associated with many human cancers (16) and on orosomucoid in the sera of septic shock patients (17). However, in healthy humans, where sialyl-Lewis^X plays a vital role in leukocyte trafficking, glycomics studies have suggested that fewer than 1% of the N-glycans carry sialyl-Lewis^X and none has been found to carry more than one sialyl-Lewis^X antenna (18). Another feature of the ZP N-glycome is the presence of extended antennae carrying an internal Lewis^X sequence. This structure is found in

members of all three families and is a particularly abundant constituent of the tetra-antennary family. This extended sialyl-Lewis^X-Lewis^X sequence has previously been found only on tumor cells, but not on normal somatic cells (19).

[0062] The hemizona assay was employed to determine the effect of sialyl-Lewis^X terminated glycoconjugates on sperm-ZP binding (20). Compared with the controls, the number of spermatozoa bound to the hemizona was significantly decreased ($p<0.05$) after treatment with sialyl-Lewis^X-BSA at concentrations ≥ 1 μM and with sialyl-Lewis^X oligosaccharide at concentrations ≥ 100 μM (Figure 4A). Except for sialyl-N-acetyllactosamine oligosaccharide at the highest concentration, no significant inhibition was observed with Lewis^X, sialyl-N-acetyllactosamine oligosaccharide or BSA conjugates of these sequences (Figure 4A and Figure 10). Fluorescently-labeled sialyl-Lewis^X-BSA bound to the head of capacitated spermatozoa (Figure 4B), but not to the hemizona (Figure 11). In contrast, Lewis^X-BSA, sialyl-N-acetyllactosamine-BSA, and BSA did not bind to the sperm head (Figure 4B). These treatments did not affect the acrosomal status and motility of spermatozoa (Figure 12). Consistently, anti- sialyl-Lewis^X (Figure 4C), but not anti-Lewis^X antibody (Figure 9), bound strongly to ZP and suppressed sperm-ZP binding dose-dependently (Figure 4D). To confirm the importance of sialylation, the binding of fluorescence-labeled native and desialylated solubilized ZP to human spermatozoa was compared. Desialylation significantly reduced the binding of solubilized ZP to capacitated spermatozoa (Figure 4E).

[0063] Previous studies indicated that antibodies directed against sialyl-Lewis^a, sialyl-Lewis^X, and Lewis^b epitopes react with human ZP (21, 22). The anti-Lewis^b antibody blocked human sperm-ZP binding in the hemizona assay (21). Erythoragglutinating phytohemagglutin also binds to human ZP, indicating that bisecting type N-glycans are expressed on this matrix (23). However, these results establish that only the sialyl-Lewis^X antigen is expressed at physico-chemically confirmable levels on ZP. Based on assessments of signal to noise for detected molecular and fragment ions, it was estimated that other antigens must be substantially less than 1% of the glycome.

[0064] Figure 13 shows the results of shotgun proteomic analysis of the purified human ZP sample. The protein band corresponding to the purified human ZP sample was subjected to in-gel tryptic digestion and extracted peptides were further

de-*N*-glycosylated by PNGase F prior to LC-MS/MS analysis under data dependent acquisition mode. Full experimental conditions and peptide identification criteria were as described in Methods.

Material and Methods

Data Analysis

[0065] All the data were expressed as mean ± standard error of the mean (SEM). The data were analyzed by statistical software packages (SigmaPlot 8.02 and SigmaStat 2.03, Jandel Scientific). For all experiments, the non-parametric repeated measures ANOVA on Rank test for multiple comparisons were used. If the data were normally distributed, Tukey Test or Parametric Student t-test was used where appropriate as the post-test. A probability value $p<0.05$ was considered to be statistically significant.

Purification of solubilized zona pellucida

[0066] Unfertilized oocytes were obtained from the assisted reproduction program at Queen Mary Hospital, Hong Kong. The protocol of the study was approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster. Informed consent was obtained from patients donating their oocytes for the study. The purification of solubilized ZP was performed as described (20). Briefly, the purification involved the isolation of the ZP from the oocytes under a dissection microscope. The ZPs were then washed and heat-solubilized at 70°C in 5 mM NaH2PO4 buffer (pH 2.5) for 90 minutes.

Semen Samples

[0067] Informed consent was obtained from male donors for semen collection. The protocol for this collection was approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster. Spermatozoa from normal semen were processed by density gradient centrifugation on Percoll (Pharmacia, Uppsala, Sweden) (25, 26). After capacitation in Earle's balanced salt solution (EBSS; Flow Laboratories, Irvine, UK) supplemented with sodium pyruvate, penicillin G, streptomycin sulfate, and 3% bovine serum albumin for 3 hours, the spermatozoa were resuspended in EBSS containing 0.3% BSA (EBSS/BSA).

Proteomics analysis

[0068] NanoLC was performed on an nanoACQUITY UPLC System (Waters, Milford, USA) coupled to an LTQ-Orbitrap Velos hybrid mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a PicoView nanospray interface (New Objective, Woburn, USA). Peptide mixtures were loaded onto a 75-μm × 250-mm nanoACQUITY UPLC BEH130 column packed with C18 resin (Waters, Milford, USA) and were separated at a flow rate of 300 nl/min using a linear gradient of 5 to 40% solvent B (95% acetonitrile with 0.1% formic acid) in 30 min, followed by a sharp increase to 85% B in 1 min and held at 85% B for another 10 min. Solvent A was 0.1% formic acid in water. The mass spectrometer was operated in the data-dependent mode. Briefly, survey full-scan MS spectra were acquired in the Orbitrap (m/z 350–1600) with the resolution set to 60,000 at m/z 400 and automatic gain control target at 106. The 20 most intense ions were sequentially isolated for CID MS/MS fragmentation and detection in the linear ion trap (automatic gain control target at 5000) with previously selected ions dynamically excluded for 90 s. Ions with single and unrecognized charge states were also excluded. All the measurements in the Orbitrap were performed with the lock mass option for internal calibration.

[0069] All MS and MS/MS raw data were processed by Raw2MSM and searched against all entries in Swissprot database (vr 2010_11), or only the human subset in taxonomy, using the Mascot Daemon 2.2 server, with the target-decoy database search option enabled. Search criteria used were: trypsin digestion; variable modifications set as carbamidomethylation (Cys), oxidation (Met) and deamidation (NQ); up to two missed cleavages allowed; and mass accuracy of 10 ppm for the parent ion and 0.60 Da for the fragment ions. Returned peptide hits were further filtered by the built-in Percolator scoring option with significant threshold set at $p < 0.01$ (peptide ion score > 20), which resulted in a zero peptide false discovery rate.

Glycan sequencing by MALDI-TOF and MALDI-TOF/TOF

[0070] Purified human ZP were digested using trypsin (Sigma) and purified by reverse-phase Sep-Pak C18 cartridge (Waters Corp) as described *(27)*. The N-glycans were then released by N-glycosidase F (Roche Applied Science) and purified on a Sep-Pak C18 cartridge. The purified native N-glycans were

permethylated as described *(28)*, purified using a Sep-Pak C18 cartridge, dissolved in methanol and mixed with 20 mg/mL 2,5-dihydrobenzoic acid in 70% methanol at a 1:1 ratio (v/v). The glycan-matrix mixture (1 μL) was spotted on a stainless steel target plate and dried in vacuum. MALDI-TOF and -TOF/TOF data were obtained using a 4800 MALDI-TOF/TOF mass spectrometer (AB Sciex UK Limited). Argon was used as the collision gas with collision energy of 1 kV. The MS and MS/MS data obtained were analyzed using Data Explorer 4.9. The assignment of glycan sequence was done by manual annotation informed by knowledge of human biosynthetic pathways.

MALDI-MS screening of sulfated glycans

[0071] A portion of the released native N-glycans was additionally permethylated using the NaOH/dimethyl sulfoxide slurry method for 3 h at 4°C, followed by careful neutralization with 5% aqueous acetic acid on ice and then applied directly to a pre-washed and equilibrated C18 Sep-Pak cartridge (Waters), as described (28). For MALDI-MS analyses, the permethylated sample was redissolved in acetonitrile and mixed 1:1 with a 3,4-diaminobenzophenone matrix solution (10 mg/ml in 75% acetonitrile/0.1% trifluoroacetic acid) (Acros Organics) for spotting onto the MALDI target plate. MALDI-TOF MS analyses in negative ion mode were performed on a 4700 Proteomics Analyzer (Applied Biosystems), operated in the reflectron mode.

Determination of acrosomal status and motility of spermatozoa

[0072] Fluorescein isothiocyanate labeled peanut (Pisum sativum) agglutinin (FITC-PSA; Sigma) and Hoechst staining techniques were used to determine the acrosome reaction of spermatozoa (29). The fluorescence patterns of 300 spermatozoa in randomly selected fields were determined under a fluorescence microscope (Zeiss) with 400x magnification. Hobson Sperm Tracker System (Hobson Tracking Systems Ltd) was used to determine the motility of spermatozoa. The procedures and the set-up parameters of the system were described elsewhere (30).

Hemizona binding assay

[0073] The hemizona binding assay was performed as described previously (31). Unfertilized oocytes were micro-bisected into two identical hemizonae by a micromanipulator. Each hemizona was incubated with $2x10^6$ capacitated spermatozoa/ml in a 100 µl droplet of EBSS/BSA for 3 hours at 37°C in an atmosphere of 5% CO2 in air under mineral oil. The numbers of tightly bound spermatozoa on the outer surface of the hemizonae were counted. The hemizona binding index (HZI) was defined as the ratio of the number of bound spermatozoa in the test droplet to that in the control droplet times 100.

Effects of sialyl-Lewis^x-BSA/Lewis^x-BSA/sialyl-N-acetyllactosamine neoglycoprotein and sialyl-Lewis^x/Lewis^x/sialyl-N-acetyllactosamine oligosaccharide

[0074] Hemizona binding assay were performed as described (31) in the presence of different concentrations of sialyl-Lewis^x/Lewisx/sialyl-N-acetyllactosamine neoglycoprotein (0.01-2 µM) or sialyl-Lewis^x/Lewis^x/sialyl-N-acetyllactosamine oligosaccharide (Dextra; 0.1-500 µM) to determine their effects on the ZP binding capacity of capacitated spermatozoa. The effect of the neoglycoprotein and oligosaccharide on the acrosomal status, motility and viability of spermatozoa were also determined as described above.

[0075] The binding of sialyl-Lewis^x-BSA/Lewis^x-BSA/sialyl-N-acetyllactosamine-BSA to capacitated spermatozoa and hemizona was visualized by cytochemical staining. Sialyl-Lewis^x-BSA/Lewis^x-BSA/sialyl-N-acetyllactosamine-BSA (Dextra) was fluorescently labeled with Alexa Fluor-594 microscale fluorescence labeling kit (Invitrogen) according to the manufacturer's protocol. Motile processed spermatozoa (2×10^6 spermatozoa/ml) or hemizona were incubated with 0.5 µM Alexa Fluor-594-labeled sialyl-Lewis^x-BSA/Lewis^x-BSA/sialyl-N-acetyllactosamine-BSA in an atmosphere of 5% CO_2 in air at 37°C for 240 minutes. The treated spermatozoa or hemizona were washed with PBS containing 0.1% Triton-X 100 and examined under a phase-contrast microscope. Spermatozoa or hemizona incubated with labeled BSA were used as control. Image analysis was performed using Image-Pro Plus (Media Cybernetics).

Effects of anti-sialyl-Lewisx and Lewisx antibodies

[0076] For immunostaining, hemizona were incubated with 0.2 µg/ml of mouse monoclonal anti-sialyl-Lewisx or anti-Lewisx antibody (BD) for 3 hours at 37 °C. Three anti-sialyl-Lewis^x antibodies with different specificities were used: CSLEX1 binds sialyl-Lewis^x but not 6-sulfo sialyl-Lewis^x, MECA-79 only binds to 6-sulfo lacNAc on extended core 1 O-glycans and HECA-452 binds both sialyl-Lewis^x and 6-sulfo sialyl-Lewis^x (32). Matching hemizona treated with irrelevant antibody or antibody preabsorbed by the addition of 1:100 sialyl-Lewis^x-BSA or Lewis^x-BSA were used as controls. Bound antibodies were detected by Alexa Fluor-594-conjugated goat anti-mouse IgG or anti-rat IgM (Invitrogen).

[0077] To determine the effect of antibodies on the ZP binding capacity of capacitated spermatozoa, matching hemizona were pre-incubated either in various concentrations (0.1-10 µg/ml) of anti-sialyl-Lewis^x (CSLEX1)/Lewis^x antibody or preabsorbed antibody at 37°C for 3 hours. The hemizona were then washed with fresh EBSS/BSA. The hemizona binding assays were performed on these treated hemizona as described (31).

Binding of solubilized zona pellucida to spermatozoa

[0078] Solubilized ZP was desialylated by incubation with sialidase coated agarose beads (Sigma) in 1M Tris-HCl (pH 7) at 37 °C for 18 hours (33). The free sialic acid produced was removed by dialysis with 2 mM Tris-HCl, pH 7.5 at 4 °C. The success of desialylation was verified by the decreased binding of the treated solubilized ZP to wheat germ agglutinin which binds strongly to sialylated glycans and weakly to other glycoconjugates. Both native and desialylated solubilized ZP were fluorescently labeled with Alexa Fluor-488 microscale fluorescence labeling kit (Invitrogen).

[0079] The binding of native or desialylated solubilized ZP to spermatozoa was performed as described (34). Capacitated spermatozoa (2×10^6/ml) were mildly fixed in 0.5% paraformaldehyde for 10 minutes at room temperature and washed followed by the incubation with 1 µg/ml solubilized ZP at 4 °C with slow shaking. After 24 hours, the spermatozoa were washed and the fluorescent signals were quantified using a microplate reader (Dynatech MR5000, Dynatech Laboratories). The results were expressed as percentage of fluorescence intensity relative to the control using native ZP.

References

1. K. J. Mengerink, V. D. Vacquier, *Glycobiology* **11**, 37R (2001).
2. P. M. Wassarman, L. Jovine, E. S. Litscher, *Nat. Cell Biol.* **3**, E59 (2001).
3. T. T. F. Huang, E. Ohzu, R. Yanagimachi, *Gamete Res.* **5**, 355 (1982).
4. L. M. Stoolman, S. D. Rosen, *J. Cell Biol.* **96**, 722 (1983).
5. L. A. Lasky, *Science* **258**, 964 (1992).
6. M. Fukuda, N. Hiraoka, J. C. Yeh, *J. Cell Biol.* **147**, 467 (1999).
7. M. S. Patankar, S. Oehninger, T. Barnett, R. L. Williams, G. F. Clark, *J. Biol. Chem.* **268**, 21770 (1993).
8. S. J. North *et al.*, *Methods Enzymol.* **478**, 27 (2010).
9. P. C. Pang *et al.*, *J. Biol. Chem.* **282**, 36593 (2007).
10. F. J. Schweigert, B. Gericke, W. Wolfram, U. Kaisers, J. W. Dudenhausen, *Hum. Reprod.* **21**, 2960 (2006).
11. M. Ferens-Sieczkowska, M. Olczak, *Z. Naturforsch. [C]* **56**, 122 (2001).
12. Y. Mechref *et al.*, *J. Proteome Res.* **8**, 2656 (2009).
13. Y. Imai, L. A. Lasky, S. D. Rosen, *Nature* **361**, 555 (1993).
14. S. D. Rosen, *Annu. Rev. Immunol.* **22**, 129 (2004).
15. K. H. Khoo, S. Y. Yu, *Methods Enzymol* . 478, 3 (2010).
16. J. L. Magnani, *Glycobiology* **1**, 318 (1991).
17. E. C. Brinkman-van der Linden, E. C. van Ommen, W. van Dijk, *Glycoconj. J.* 13, 27 (1996).
18. P. Babu et al., *Glycoconj. J.* 26, 975 (2009).
19. M. Fukuda et al., *J. Biol. Chem.* 260, 12957 (1985).
20. P. C. Chiu et al., *Biol. Reprod.* 79, 869 (2008).

21. H. Lucas et al., *Hum. Reprod.* 9, 1532 (1994).

22. M. Jimenez-Movilla et al., *Hum. Reprod.* 19, 1842 (2004).

23. M. S. Patankar et al., *Mol. Hum. Reprod.* 3, 501 (1997).

24. G. F. Clark, M. S. Patankar, K. D. Hinsch, S. Oehninger, *Hum. Reprod.* 10 Suppl. 1, 31 (1995).

25. WHO, *World Health Organization Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction* (Cambridge University Press, Cambridge, UK, 1999).

26. P. C. Chiu *et al.*, *Biol. Reprod.* 69, 365 (2003).

27. J. Jang-Lee *et al.*, *Methods Enzymol.* 415, 59 (2006).

28. M. Sutton-Smith, A. Dell, in *Cell Biology: A Laboratory Handbook* J. E. Celis, Ed. (Academic Press, San Diego, 2006), vol. 4, pp. 415-425.

29. P. C. Chiu *et al.*, *J. Biol. Chem.* 280, 25580 (2005).

30. P. C. Chiu *et al.*, *Endocrinology* 151, 3336 (2010).

31. P. C. Chiu *et al.*, *J. Cell Sci.* 120, 33 (2007).

32. J. Mitoma *et al.*, *Glycoconj. J.* 26, 511 (2009).

33. C. L. Lee *et al.*, *J. Biol. Chem.* 284, 15084 (2009).

34. P. C. Chiu *et al.*, *Hum. Reprod.* 23, 1385 (2008).

CLAIMS

What is claimed is:

1. A method of evaluating the ability of human sperm to bind to a human oocyte, the method comprising:
(a) forming a combination that comprises -
(i) a sample of human sperm,
(ii) a media that is conducive to maintaining the physiological activity of sperm, and
(iii) at least one matrix that comprises on at least a portion of its surface a glycoconjugate that comprises the sialyl-Lewis^X sequence,
to allow for at least a portion of the sample of sperm to bind to the matrix; and
(b) visualizing or quantitating the sperm bound to the matrix.

2. The method of claim 1 wherein the matrix comprises a bead.

3. The method of claim 1 wherein the glycoconjugate is attached to the surface of a specimen slide, dish, or well of a microtiter plate.

4. The method of claim 1 wherein the media is an *in vitro* fertilization (IVF) media.

5. A reagent for determining the ability of human sperm to bind to a human oocyte, the reagent comprising a matrix that comprises on at least a portion of its surface a glycoconjugate that comprises the sialy-Lewis^x sequence.

6. The reagent of claim 5 wherein the matrix comprises a bead.

7. The reagent of claim 5 wherein the matrix comprises a specimen slide, dish, or well of a microtiter plate.

8. A kit for determining the ability of human sperm to bind to a human oocyte, the kit comprising an oligosaccharide that comprises the sialy-Lewis^x sequence and

at least one other reagent selected from the group consisting of: (i) a matrix to which the oligosaccharide may be attached and (ii) at least one component of a media that is conducive to maintaining the physiological activity of sperm.

9. The kit in claim 9 wherein the matrix comprises on at least a portion of its surface a glycoconjugate comprising the oligosaccharide.

10. The kit in claim 8 wherein the media is *in vitro* fertilization (IVF) media.

11. The kit in claim 8 wherein the matrix comprises a bead.

12. The kit in claim 8 wherein the matrix comprises a specimen slide, dish, or well of a microtiter plate.

13. A method of evaluating the ability of human sperm to bind to a human oocyte, the method comprising:
(a) contacting a sample of human sperm and a carrier-molecule, wherein the carrier-molecule is attached to a glycoconjugate that comprises the sialy-Lewis^x sequence, to allow for at least a portion of the sample of human sperm to bind to the carrier-molecule; and
(b) detecting the carrier-molecule bound to the sperm.

14. The method of claim 13 wherein the carrier-molecule is a carrier-protein.

15. The method of claim 14 wherein the carrier-protein is bovine serum albumin.

16. The method of claim 14 wherein the carrier-protein is selected from the group consisting of the glycoproteins ZP1, ZP2, ZP3, and ZP4.

17. The method of claim 13 wherein the carrier-molecule bound to the sperm is visualized.

18. The method of claim 13 wherein the carrier-molecule is labeled with a detectable marker.

19. The method of claim 18 wherein the detectable marker is a fluorescent label.

20. The method of claim 13 wherein the detection of the carrier-molecule bound to the sperm is used to quantitate the amount of carrier-molecule bound in the sample of sperm.

21. The method of claim 13 wherein the detection of the carrier-molecule bound to the sperm is used to quantitate the amount of sperm in the sample with a certain affinity of binding to the glycoconjugate carbohydrate sequence.

22. The method of claim 13 wherein the detection of the carrier-molecule bound to the sperm is used to sort sperm cells based on the cells' ability to bind to the glycoconjugate carbohydrate sequence.

23. The method of claim 13 wherein the carrier-molecule is a fluorescently labeled bovine serum albumin.

24. A method of evaluating the ability of human sperm to bind to a human oocyte, the method comprising passing a sample of sperm across a carrier-molecule that is fixed to a support and capturing at least a portion of the sperm sample that binds to the carrier-protein, wherein the portion of the sperm sample that does not bind the carrier-molecule is not captured.

25. A method of evaluating the ability of human sperm to bind to a human oocyte, the method comprising:

(a) contacting a sample of human sperm and an oligosaccharide, wherein the oligosaccharide comprises the sialy-Lewis^x sequence, to allow for at least a portion of the sample of human sperm to bind to the oligosaccharide; and

(b) detecting the oligosaccharide bound to the sperm.

26. The method of claim 25 wherein the oligosaccharide bound to the sperm is visualized.

27. The method of claim 25 wherein the oligosaccharide is labeled with a detectable marker.

28. The method of claim 27 wherein the detectable marker is a fluorescent label.

29. The method of claim 28 wherein the detection of the oligosaccharide bound to the sperm is used to quantitate the amount of oligosaccharide bound in the sample of sperm.

30. The method of claim 25 wherein the detection of the oligosaccharide bound to the sperm is used to quantitate the amount of sperm in the sample with a certain affinity of binding to the oligosaccharide carbohydrate sequence.

31. The method of claim 25 wherein the detection of the oligosaccharide bound to the sperm is used to sort sperm cells based on the cells' ability to bind to the oligosaccharide carbohydrate sequence.

32. A method of evaluating the ability of human sperm to bind to a human oocyte, the method comprising passing a sample of sperm across an oligosaccharide that is fixed to a support and capturing at least a portion of the sperm sample that binds to the oligosaccharide, wherein the portion of the sperm sample that does not bind the oligosaccharide is not captured.

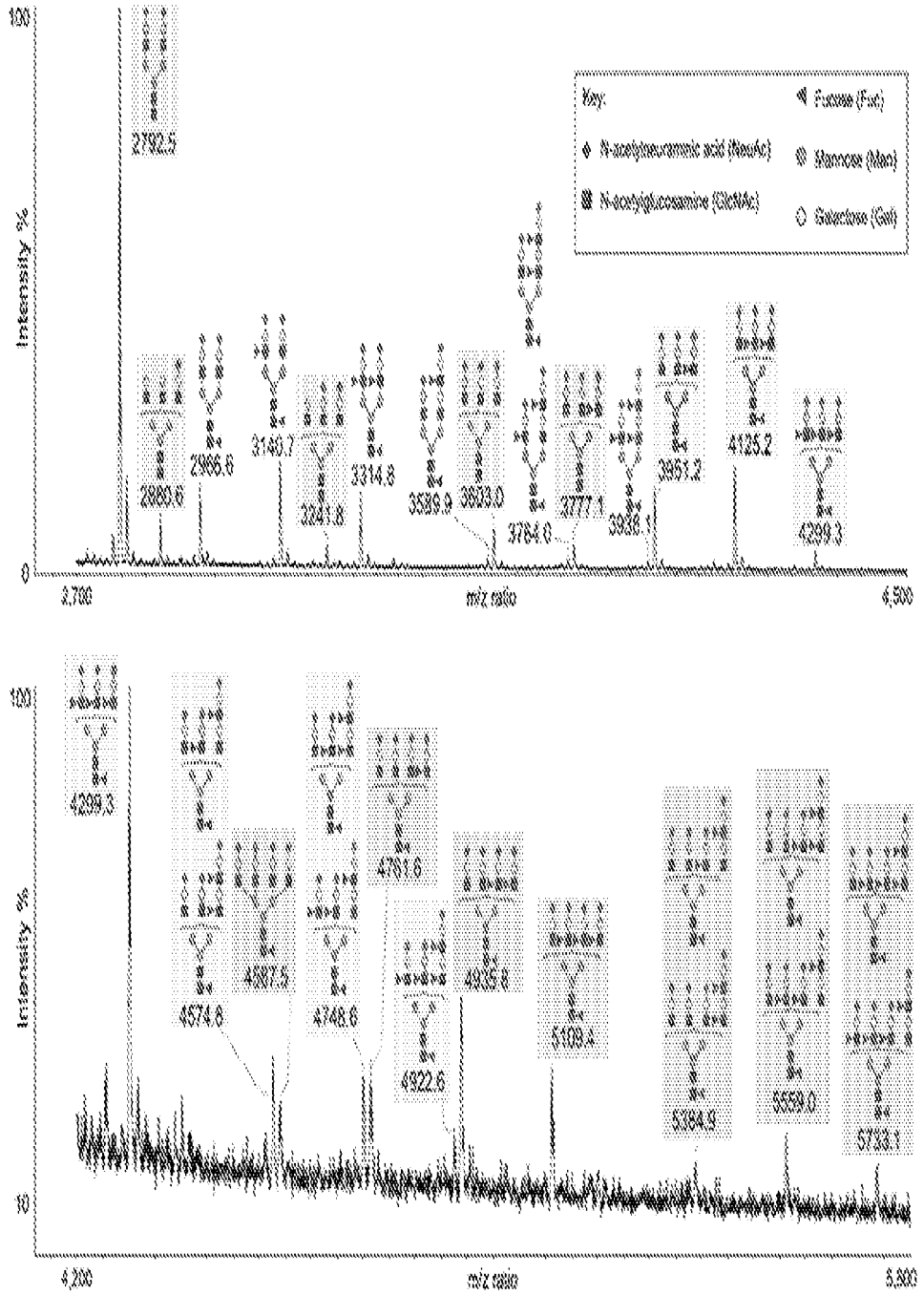

Key:
Fucose (Fuc)
N-acetylneuraminic acid (NeuAc)
Mannose (Man)
N-acetylglucosamine (GlcNAc)
Galactose (Gal)
Intensity %
m/z ratio
2792.5
2980.6
2986.6
3140.7
3241.8
3314.8
3539.9
3633.0
3784.0
3777.1
3938.1
3951.2
4125.2
4299.3
4574.8
4587.5
4748.6
4761.6
4922.6
4935.6
5108.3
5384.9
5559.0
5733.1

FIGURE 1

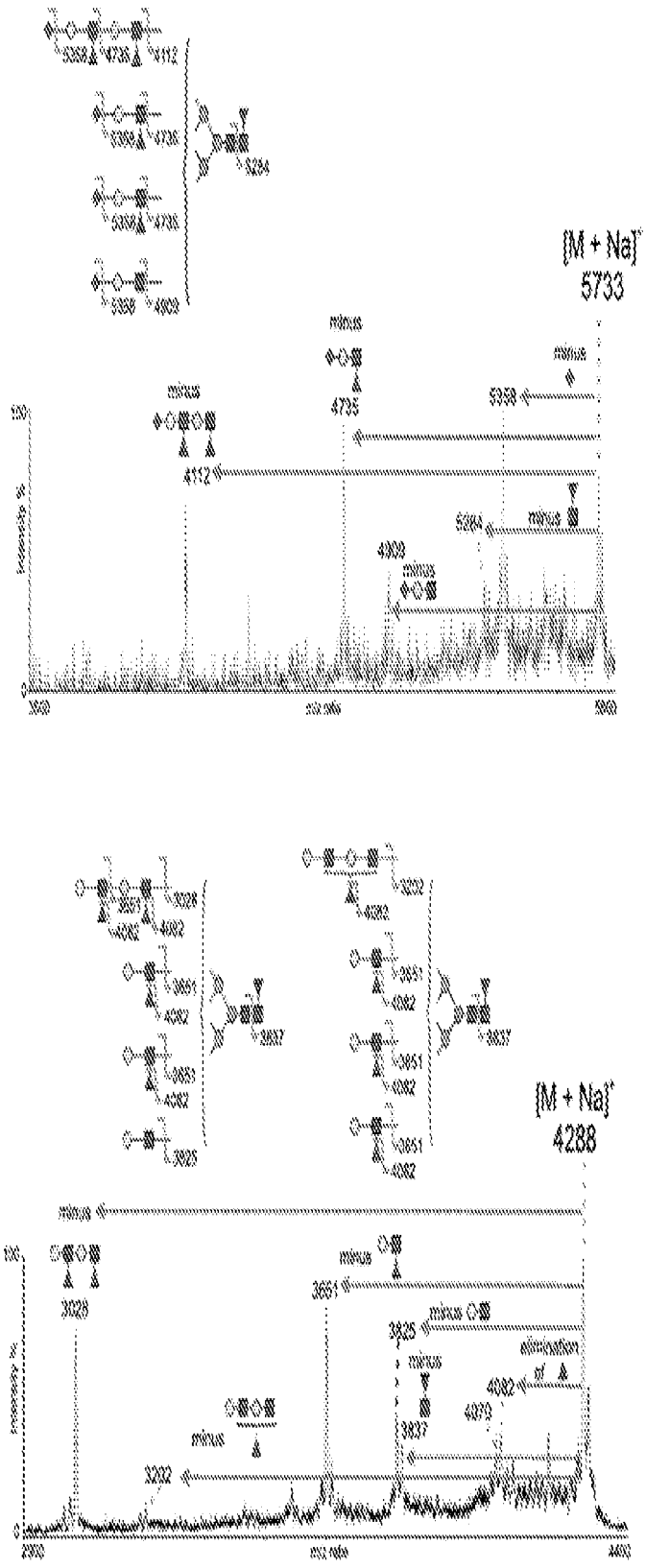


FIGURE 2

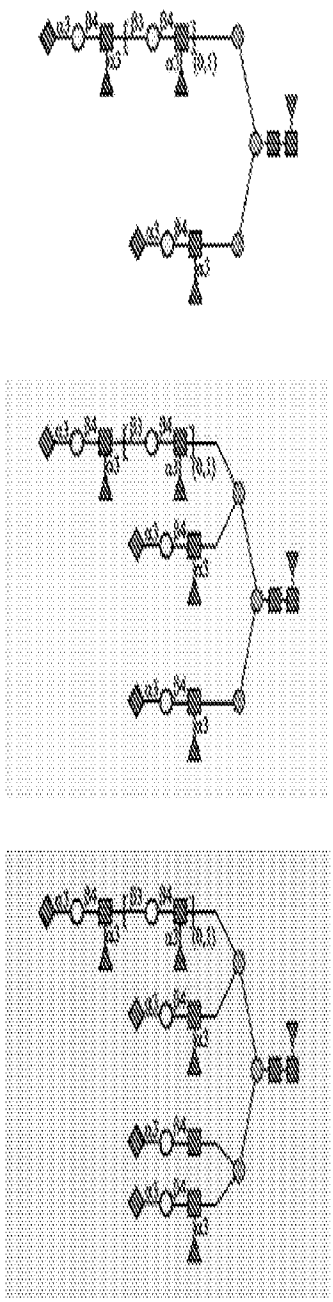


FIGURE 3

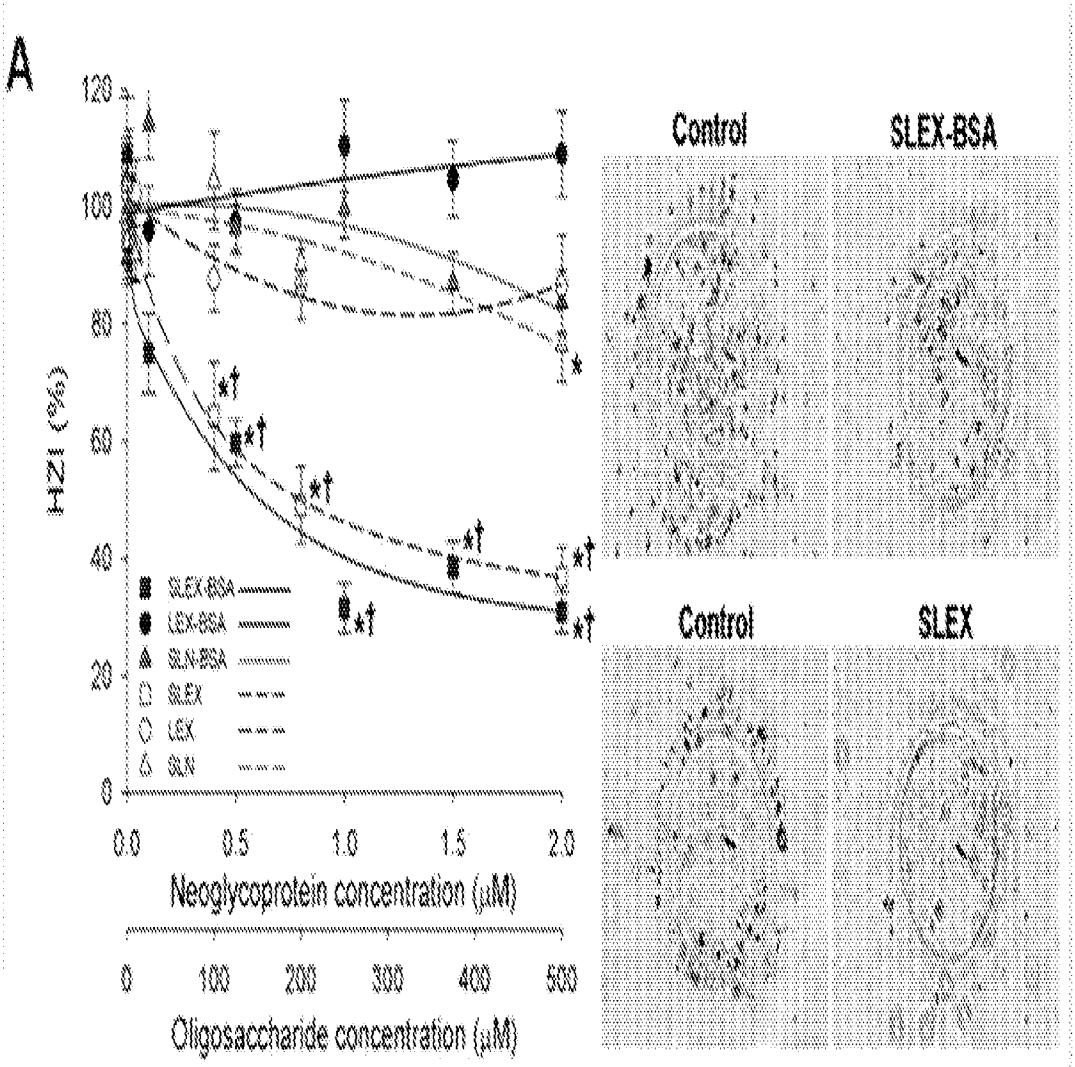

A
H2L (%)
120
100
80
60
40
20
0
SLEX-BSA
LEX-BSA
SLN-BSA
SLEX
LEX
SLN
0.0
0.5
1.0
1.5
2.0
Neoglycoprotein concentration (μM)
0
100
200
300
400
500
Oligosaccharide concentration (μM)
Control
SLEX-BSA
Control
SLEX

FIGURE 4A

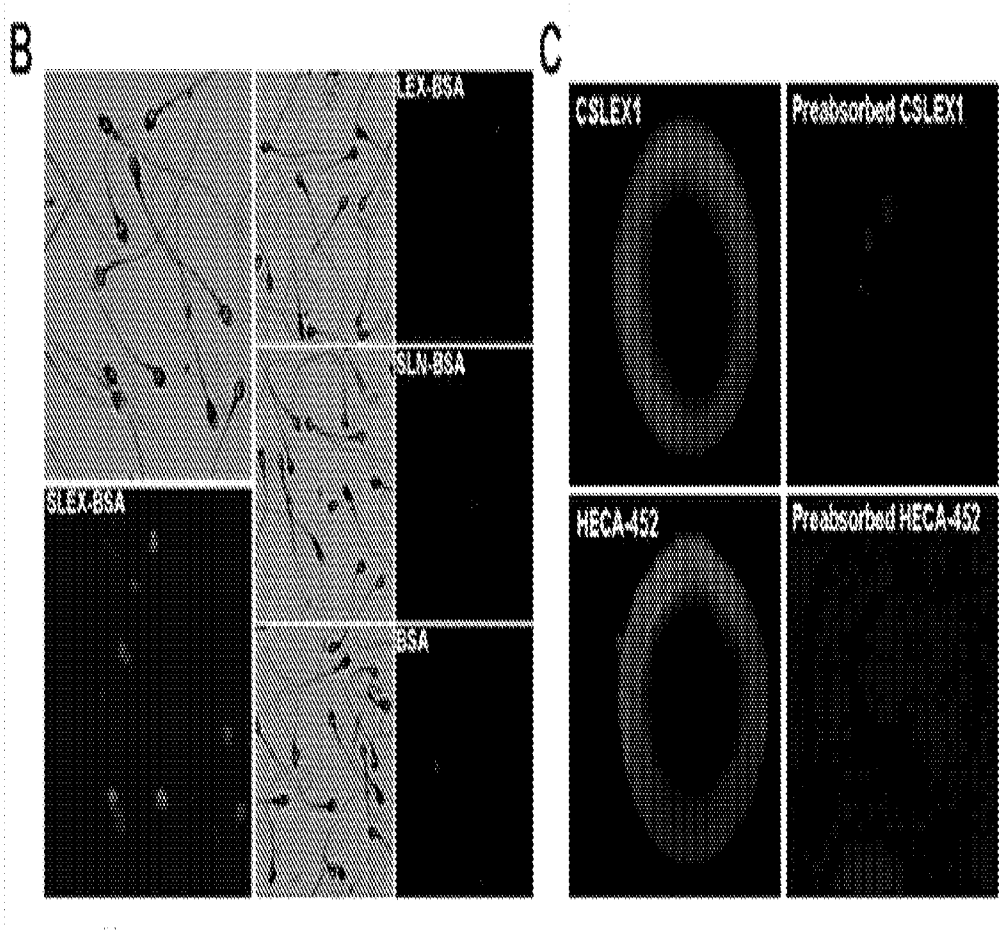

B
C
CSLEX1
Preabsorbed CSLEX1
HECA-452
Preabsorbed HECA-452

FIGURE 4B,C

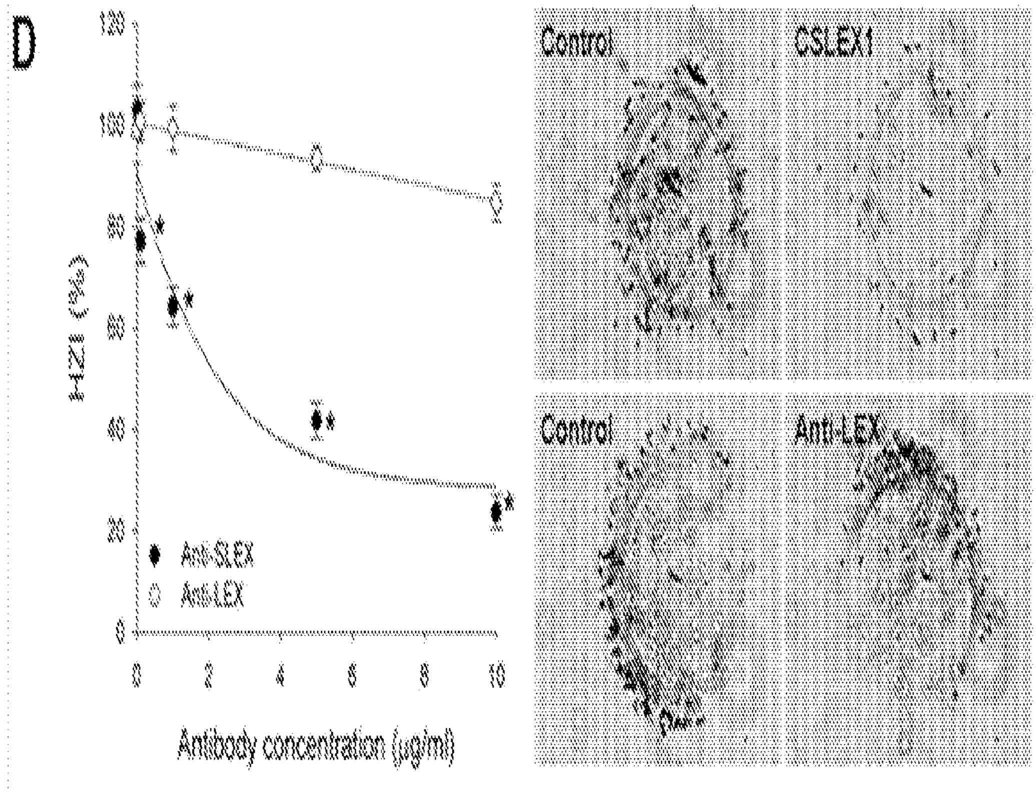

D
120
100
80
60
40
20
0
HZI (%)
0
2
4
6
8
10
Antibody concentration (µg/ml)
Anti-SLEX
Anti-LEX
Control
CSLEX1
Control
Anti-LEX

FIGURE 4D

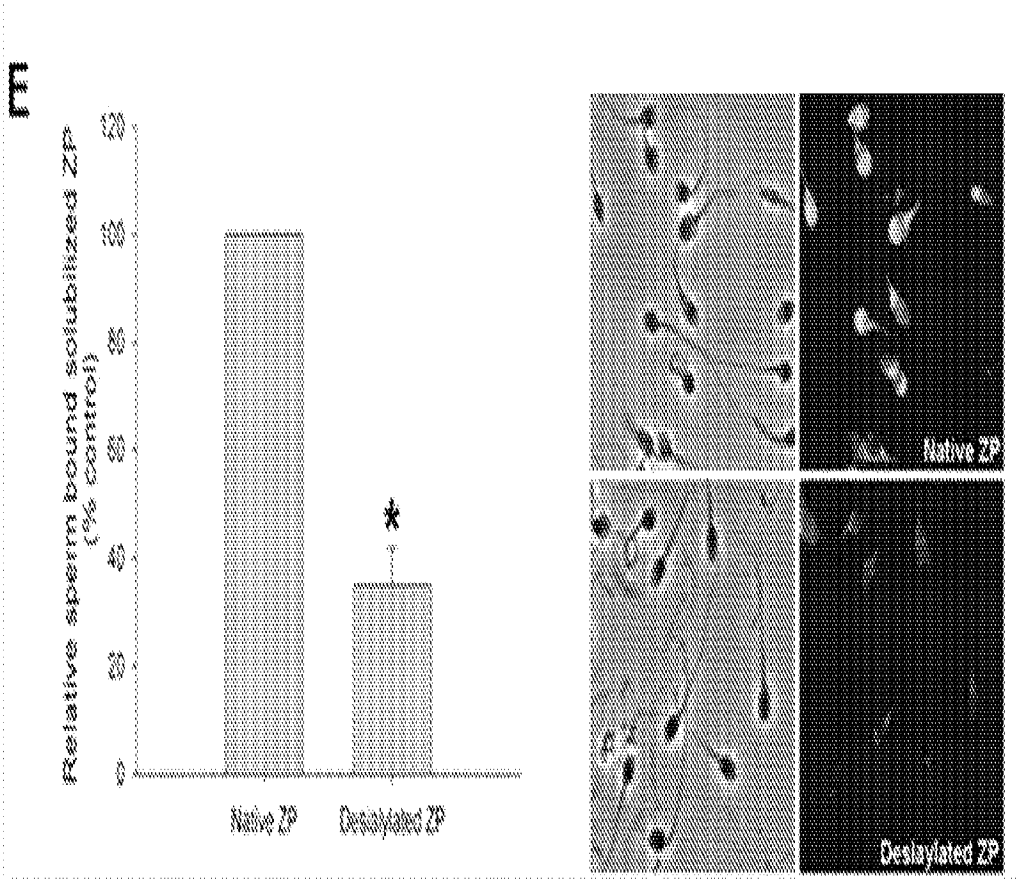

E
Relative sperm bound solubilized ZP (% control)
0
20
40
60
80
100
120
*
Native ZP
Deadpated ZP
Native ZP
Deadpated ZP

FIGURE 4E

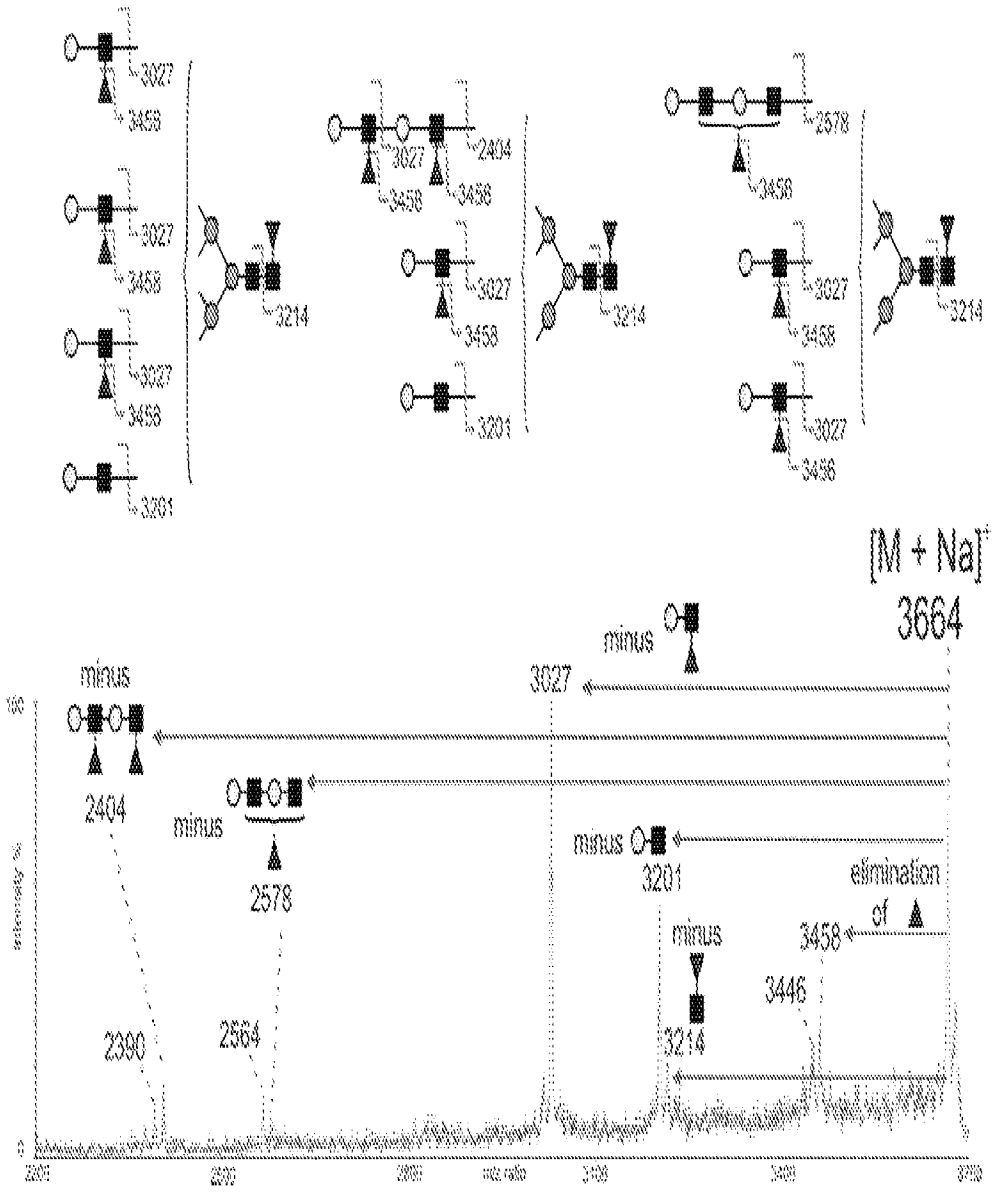

[M + Na]+
3664
minus
3027
minus
2404
minus
2578
minus
3201
elimination
of
3458
minus
3214
3446
2390
2564

FIGURE 5A

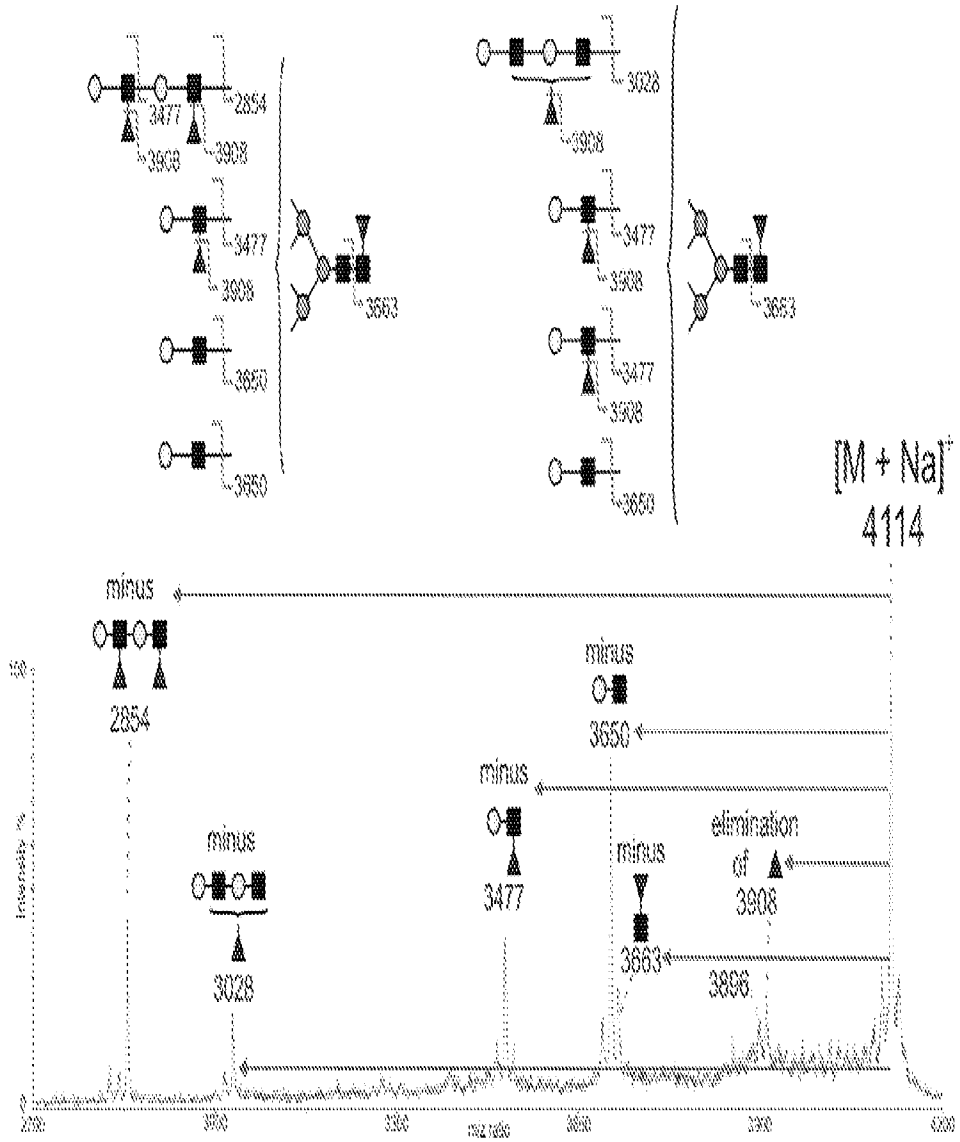

[M + Na]+
4114
minus
2854
minus
3028
minus
3477
minus
3650
minus
3663
elimination
of
3908
3886

FIGURE 5B

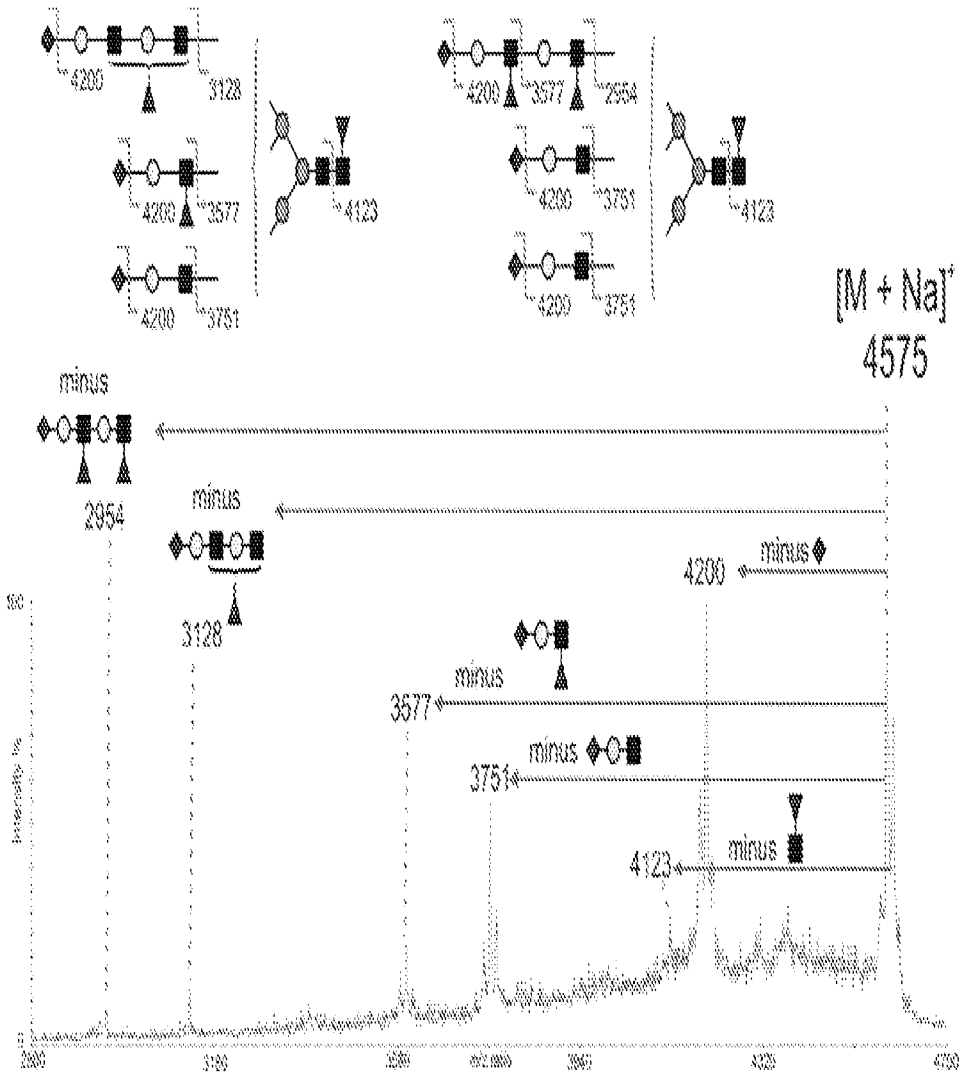

4200
3128
4200
3577
4123
4200
3751
4200
3577
2954
4200
3751
4123
4200
3751
[M + Na]+
4575
minus
2954
minus
3128
minus
4200
minus
3577
minus
3751
minus
4123

FIGURE 5C

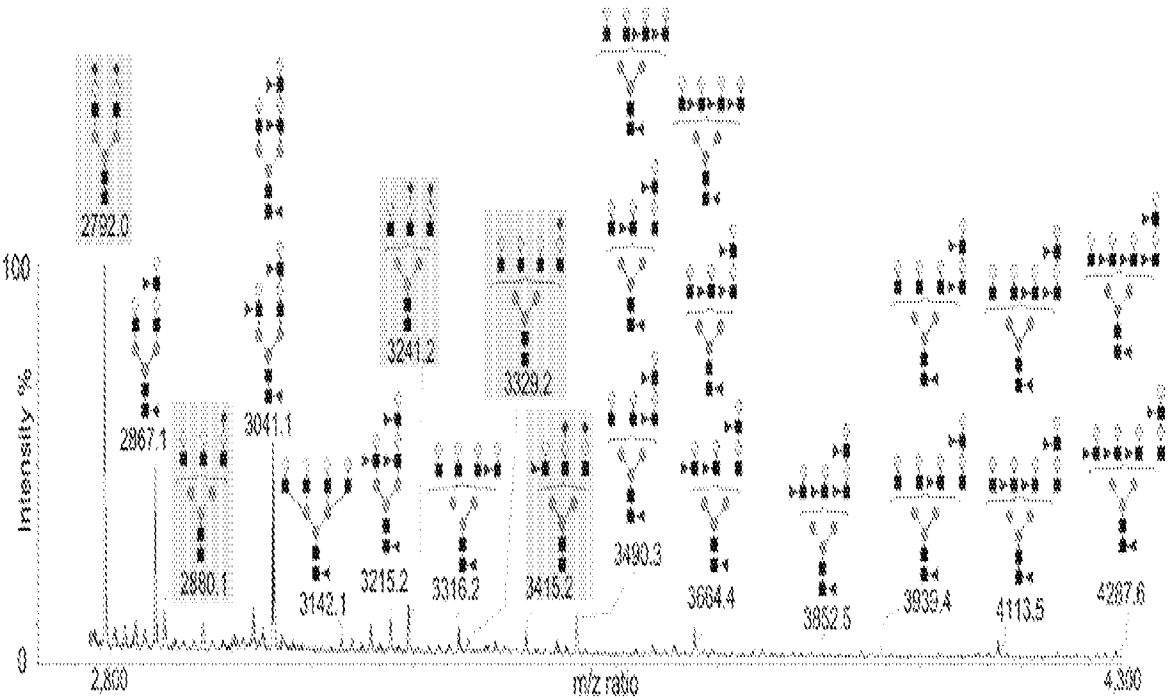

100
Intensity %
0
2732.0
2867.1
2880.1
3041.1
3142.1
3215.2
3241.2
3316.2
3329.2
3415.2
3490.3
3664.4
3852.5
3939.4
4113.5
4287.6
2,800
m/z ratio
4,350

FIGURE 6

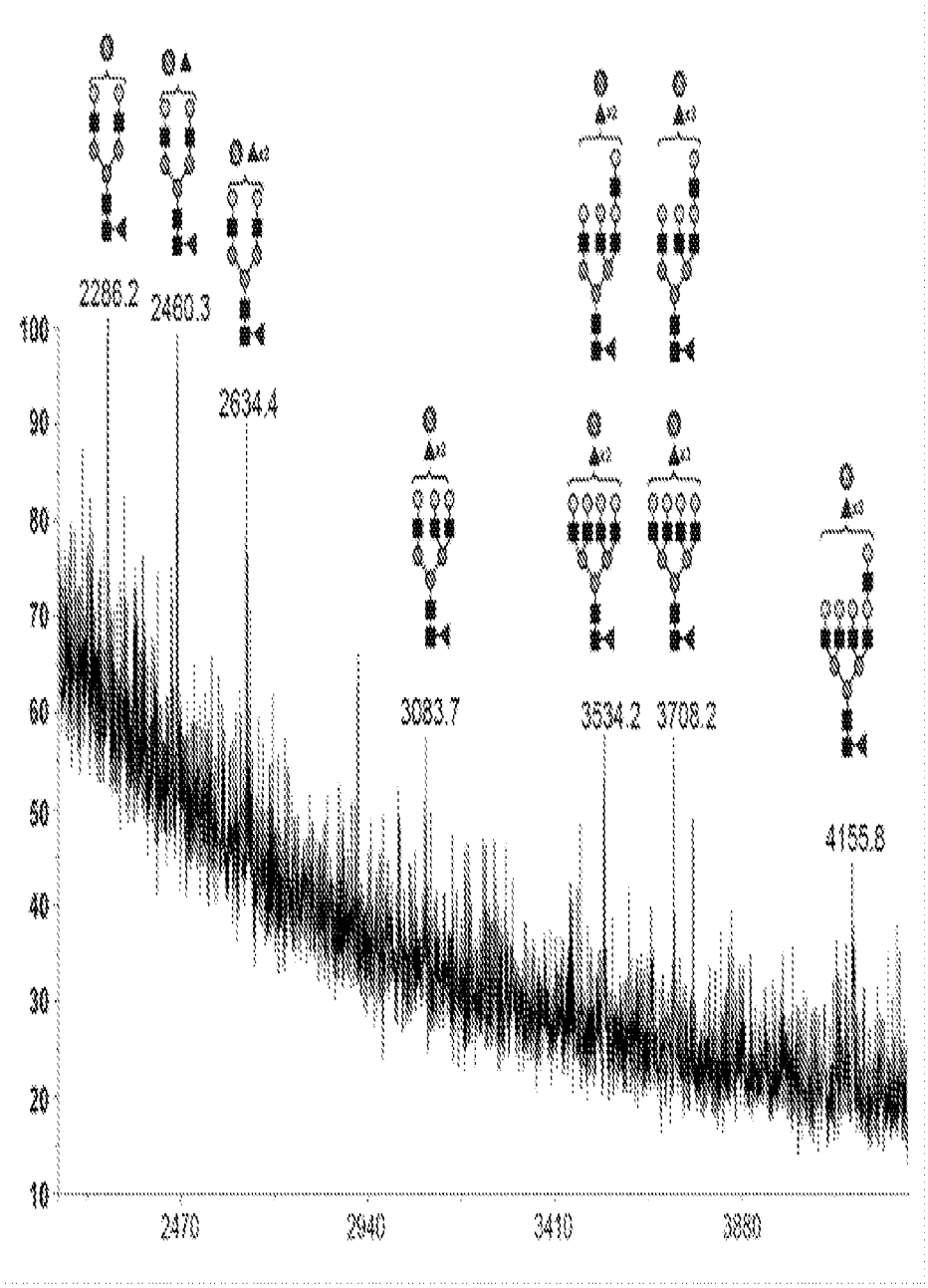
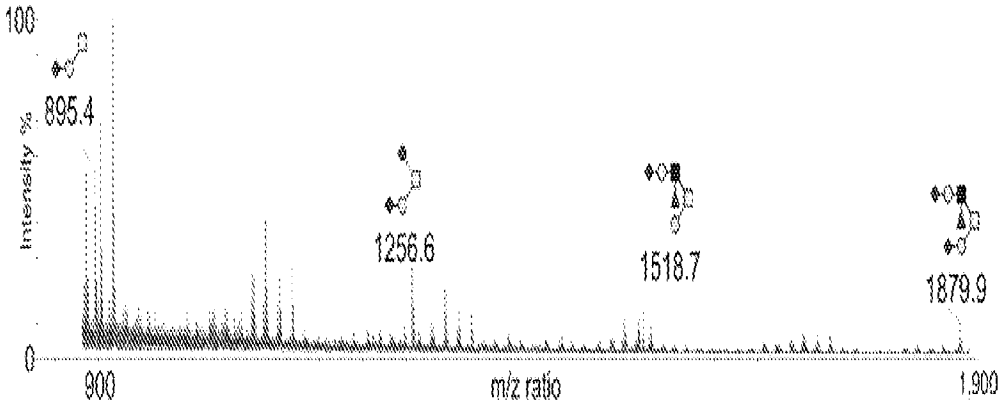

2286.2
2460.3
2634.4
3083.7
3534.2
3708.2
4155.8
100
90
80
70
60
50
40
30
20
10
2470
2940
3410
3880

FIGURE 7

A



B

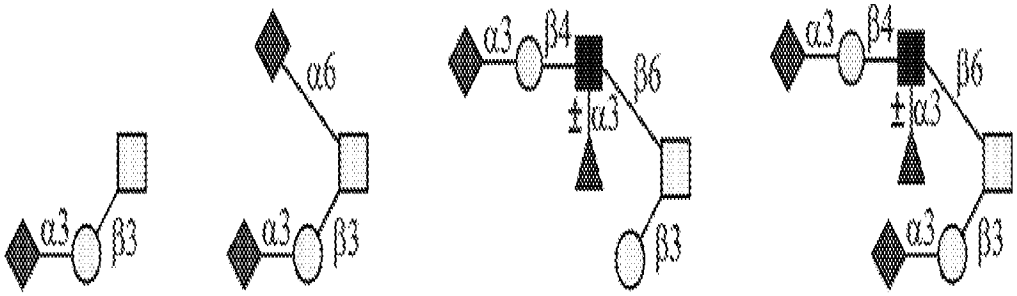


FIGURE 8 A,B

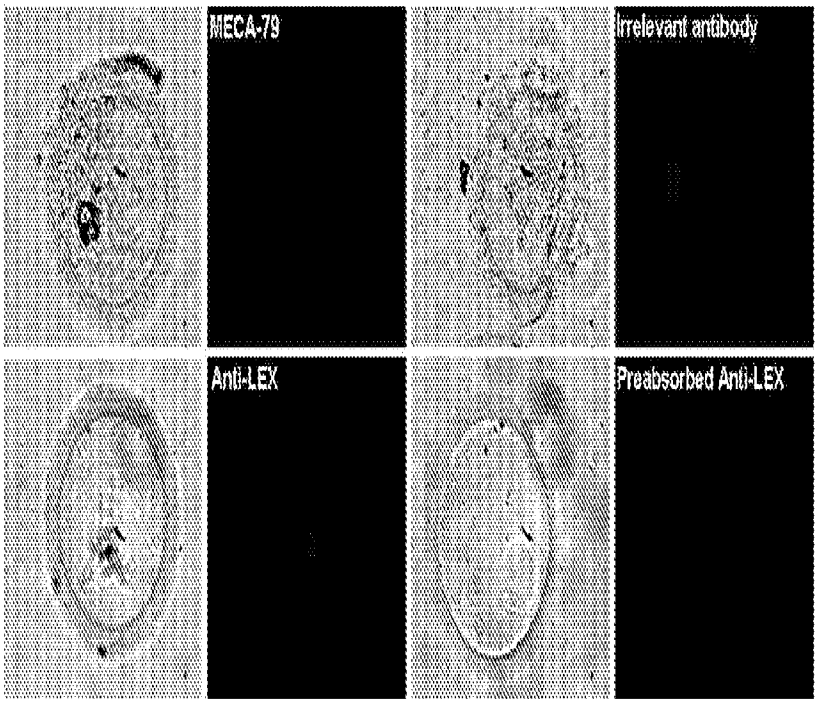


FIGURE 9

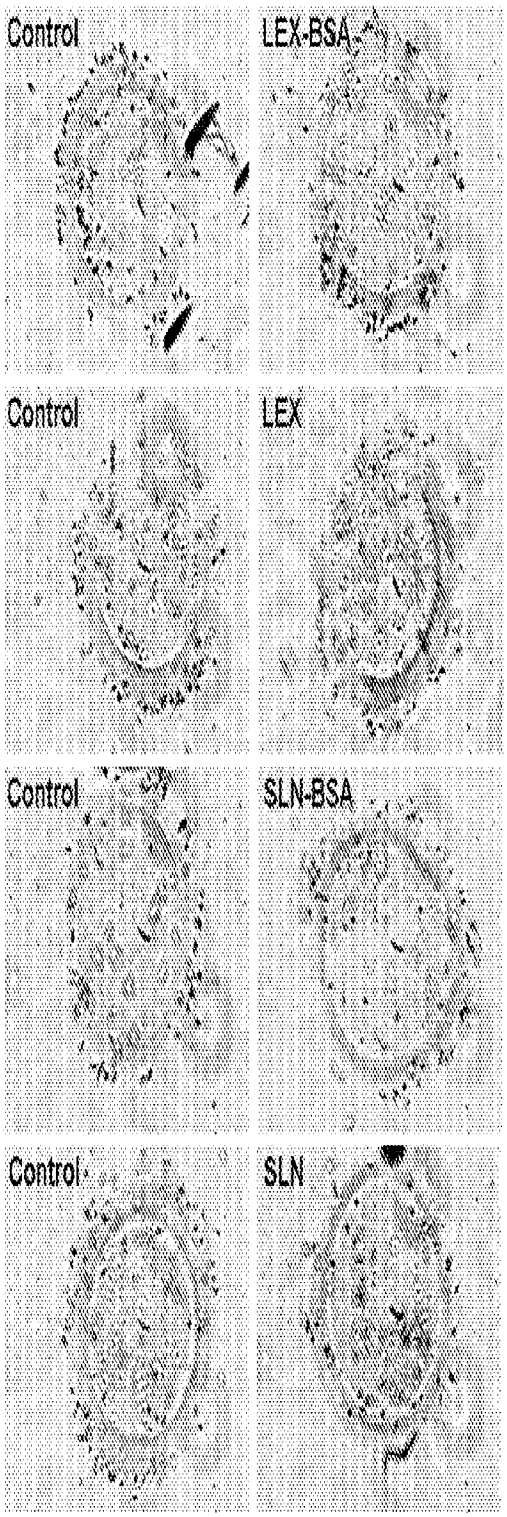


FIGURE 10

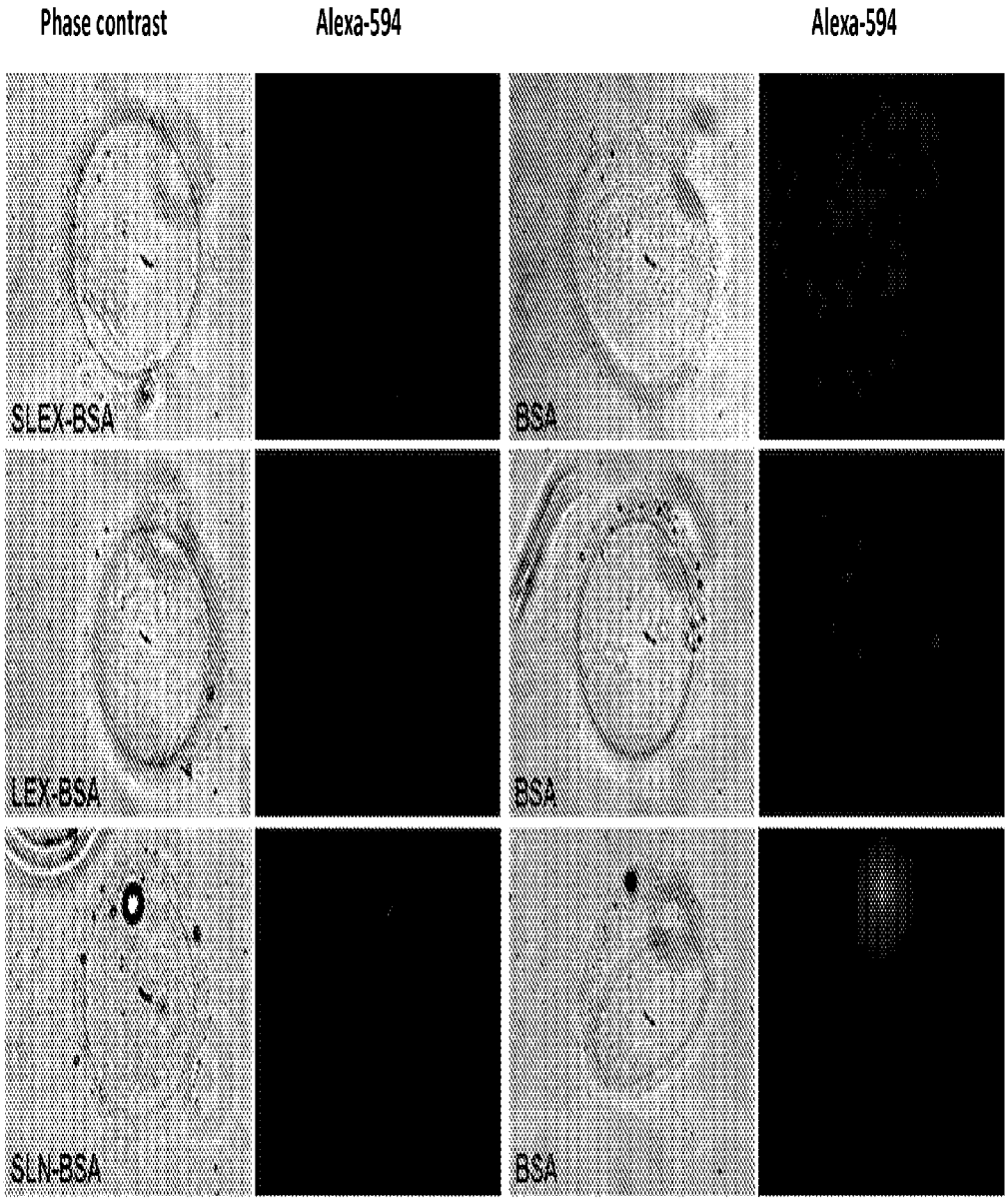
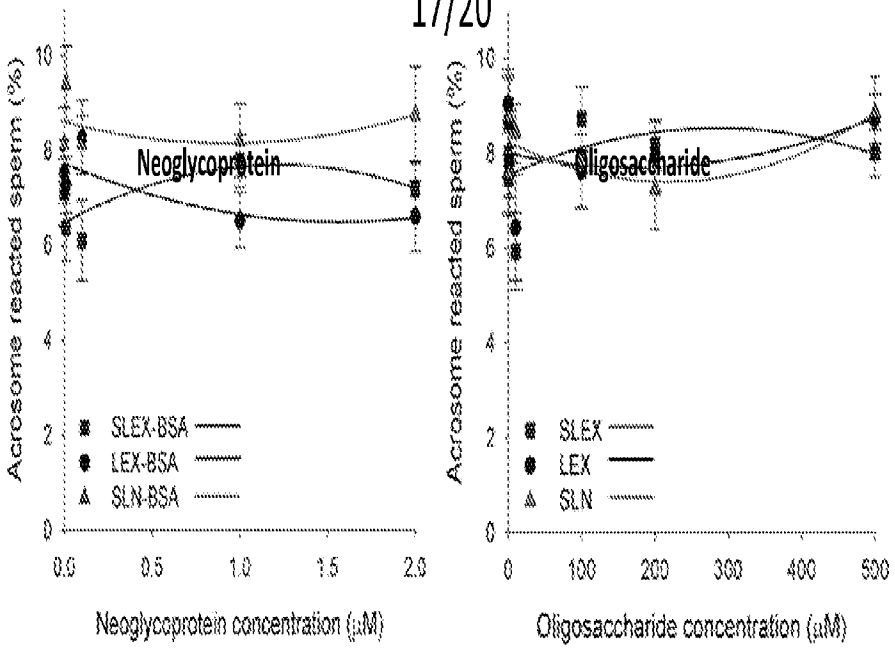

Phase contrast
Alexa-594
Alexa-594
SLEX-BSA
BSA
LEX-BSA
BSA
SLN-BSA
BSA

FIGURE 11

A



B

Treatment	Sperm motility parameters								
	VAP (μm/s)	VCL (μm/s)	VSL (μm/s)	BCF (Hz)	ALH (μm)	LIN (%)	STR (%)	HYP (%)	Progressive Motility (%)
Control	54.6 ± 2.3	80.1 ± 4.0	42.4 ± 2.7	10.4 ± 1.4	7.0 ± 1.0	53.4 ± 3.8	78.2 ± 6.2	6.6 ± 1.5	48.2 ± 4.3
Neoglycoprotein									
SLEX-BSA	57.6 ± 2.0	79.9 ± 5.7	40.4 ± 2.7	10.4 ± 0.8	7.0 ± 1.1	50.8 ± 2.0	70.4 ± 4.9	6.4 ± 1.2	55.6 ± 3.3
LEX-BSA	50.8 ± 3.0	79.6 ± 3.5	42.2 ± 3.4	10.6 ± 1.6	9.6 ± 1.4	53.6 ± 5.4	83.0 ± 4.3	7.8 ± 1.9	56.4 ± 3.9
SLN-BSA	50.2 ± 2.2	73.2 ± 3.8	41.0 ± 4.2	10.0 ± 0.7	7.6 ± 1.6	57.4 ± 8.3	81.8 ± 7.2	9.2 ± 2.4	47.9 ± 5.9
Oligosaccharide									
SLEX	55.6 ± 3.0	76.7 ± 4.5	39.0 ± 2.0	9.2 ± 1.5	8.6 ± 1.5	50.8 ± 1.6	71.1 ± 6.1	8.0 ± 3.0	56.7 ± 4.4
LEX	53.6 ± 3.0	78.6 ± 4.3	41.5 ± 3.7	9.0 ± 0.8	8.4 ± 1.2	53.4 ± 6.3	79.8 ± 12.0	8.0 ± 0.7	55.4 ± 4.1
SLN	54.0 ± 4.6	77.8 ± 5.9	40.8 ± 4.9	9.8 ± 0.8	8.6 ± 1.5	53.4 ± 7.8	79.4 ± 13.2	7.4 ± 0.8	50.6 ± 2.9

FIGURE 12 A,B

	Protein description	protein score	% protein coverage	charge state	peptide mw (theoretical)	peptide score	peptide sequence	peptide modifications
1	Zona pellucida sperm-binding protein 4	1023	32.8	2	1096.5698	68.73	LPCAPSPISR	Carbamidomethyl (C)
				2	1101.5667	38.33	EGHFSIAVSR	
				2	1263.6459	152.61	AVYENELVATR	
				2	1418.7718	48.33	NVTSPPLLLDSVR	Deamidated (NQ)
				2	1438.7773	140.53	DPIYWEVSILHR	
				3	1439.7773	44.45	DPIYWEVSILHR	
				2	1617.6729	53.82	DAPDTDWCDSIPAR	Carbamidomethyl (C)
				2	1679.7831	70.34	NYGSYYGYGDYPVYK	
				2	1761.8976	152.61	FSIFTFSFVNPTVEK	
				2	2093.0252	152.61	GCPYISDNYQTQLIPVQK	Carbamidomethyl (C)
				4	3246.4636	77.07	GPVHLHCSVSVCDPAETPSCVVTCPDLSR	4 Carbamidomethyl (C)
				3	3246.4636	152.61	GPVHLHCSVSVCDPAETPSCVVTCPDLSR	4 Carbamidomethyl (C)
				3	3647.3844	44.05	GDCEGLGCDYSSEEVNSCYYGNTVTLHCTR	5 Carbamidomethyl (C)
2	Haptoglobin	815	38.4	2	919.4552	61.88	GSFPWQAK	
				2	979.4876	145.46	VGYVSGWGR	
				2	1202.6295	152.61	VTSIQDWVQK	
				2	1344.6384	66.31	SCAVAEYGVYVK	Carbamidomethyl (C)
				2	1438.6576	45.17	TESDGVYTLNNEK	
				2	1459.8943	25.55	NLFLNHSENATAK	2 Deamidated (NQ)
				2	1722.8069	26.04	YVMLPVADQDQCIR	Carbamidomethyl (C) Oxidation (M)
				3	1794.0040	51.14	VVLHPNYSQVDIGLIK	
				3	1858.9124	144.1	AVGDKLPECEAVCGKPK	2 Carbamidomethyl (C)
				2	2187.0463	152.61	SPVGVQPILNEHTFCAGMSK	Carbamidomethyl (C) Oxidation (M)
				3	2895.3639	152.61	MVSHHNLTTGATLINEQWLLTTAK	Deamidated (NQ) Oxidation (M)
3	Zona pellucida sperm-binding protein 2	780	19.9	2	892.4587	32.62	VMIVISAALR	2 Deamidated (NQ) Oxidation (M)
				2	1114.5117	56.89	DRMSFSLPR	Oxidation (M)

FIGURE 13

	Protein description	protein score	% protein coverage	charge state	peptide mw (theoretical)	peptide score	peptide sequence	peptide modifications
				3	1271.6081	57.08	FHIPLNGDGTR	Carbamidomethyl (C) Deamidated (NQ)
				2	1350.6880	34.6	EITVEFPSSPGTK	
				2	1619.7089	63.94	VQMGWSIEVGDDAR	Oxidation (M)
				2	1660.8710	152.61	LSPDSPLCSVTCPVSSR	2 Carbamidomethyl (C)
				2	2189.1361	152.61	NDMLLMINVESLTPPVASVK	Oxidation (M)
				3	2795.2892	152.61	WHASWDPLGLDMPNCTYLDPEK	Carbamidomethyl (C) Deamidated (NQ) Oxidation (M)
				3	3402.6298	116.11	VIFSSQAICAPDPVTCNATHMTLTIPEFPGK	2 Carbamidomethyl (C) Deamidated (NQ)
4	Transthyretin	858	55.1	2	1365.7517	152.61	GSPAINVAVHVFR	
				2	2359.2311	152.61	YTIAALLSPYSYSTTAVVTNPK	
				3	2460.1979	26	ALGISPFHEHAEVVFTANDSGPR	
				2	2484.1438	152.61	TSESGELHGLTTEEEFVEGIYK	
				2	2488.2737	152.61	YTIAALLSPYSYSTTAVVTNPKE	
5	Zona pellucida sperm-binding protein 3	686	17.2	2	1276.5660	133.46	LMEEWWNAEK	Oxidation (M)
				2	1470.7202	152.61	VTLAEQDPDELNK	
				2	2803.2996	152.61	AADLTLGPEACEPLVSMDTEDVVR	Carbamidomethyl (C) Oxidation (M)
				2	2803.2996	74.5	AADLTLGPEACEPLVSMDTEDVVR	Carbamidomethyl (C) Oxidation (M)
				3	3035.2670	152.61	ACSFSKPSNSWFPVEGSADICQDCNK	4 Carbamidomethyl (C)
6	Zona pellucida sperm-binding protein 1	570	14.1	2	1611.8257	152.61	FTVATFALLDSGSQR	
				2	1963.8557	143.56	EVPCYYGNTATVQCFR	2 Carbamidomethyl (C)
				3	1980.9939	50.64	VDVAQDATLICPKPDPSR	Carbamidomethyl (C)
				2	2054.8745	152.61	DETFSSYYGEDDYPIVR	
				3	2767.3170	160.92	DVTGTHLSQEQCQVASGHLPDVR	2 Carbamidomethyl (C)
7	Hemopexin	483	15.6	2	1404.6456	143.71	SWPAVGNCSSALR	Carbamidomethyl (C) Deamidated (NQ)
				2	1484.6714	139.15	YYCFQGNQFLR	Carbamidomethyl (C)
				2	1499.6755	100.67	EWFWDLATGTMK	Oxidation (M)
				2	1711.7624	72.32	GEDQAEGVLFFQGDR	Carbamidomethyl (C)
				2	2363.1680	136.84	LLQDEFPGIPSPLDAAVECHR	Carbamidomethyl (C)

FIGURE 13 CONT.

	Protein description	protein score	% protein coverage	charge state	peptide mw (theoretical)	peptide score	peptide sequence	peptide modifications
8	Atamin	455	10	2	1589.8202	84.13	ESLLNHFLYEVAR	
				2	1621.8930	152.61	AESPEVCFNEESPK	Carbamidomethyl (C)
				2	1712.9196	152.61	IAPQLSTEELVSLGEK	
				2	1886.9302	73.35	SDVGFLPPFPTLDPEEK	
9	Zinc-alpha-2-glycoprotein	174	9.4	2	1460.6762	81.3	AYLEEECPATLR	Carbamidomethyl (C)
				2	1761.9352	112.24	EIPAWYPFDPAAQITK	
10	Ferritin light chain	163	8.6	2	1606.7991	152.61	LGGPEAGLGEYLFER	
11	Alpha-1-acid glycoprotein 1	153	7	2	1741.7981	152.61	EQLGEFYEALDCLR	Carbamidomethyl (C)
12	Alpha-1B-glycoprotein	153	3.4	2	1674.8924	152.61	VTLTCVAPLSGVDFQLR	Carbamidomethyl (C)
13	Major vault protein	134	1.8	2	1614.8464	133.87	LAQDPFRLYPGEVLEK	
14	Alpha-1-antitrypsin	123	4.3	2	1833.8996	122.61	VFSNGADLSGVTEEAPLK	Deamidated (NQ)
15	N-acetylmuramoyl-L-alanine amidase	117	2.3	2	1491.7028	117.27	TDCPGDALFDLLR	Carbamidomethyl (C)
16	Prostaglandin-H2 D-isomerase	105	10	2	1919.9000	105.34	SVVAPATDGGLNLTSTFLR	Deamidated (NQ)
17	Polyubiquitin-B	105	7	2	1786.9200	105.17	TITLEVEPSDTIENVK	
18	MAP kinase-activating death domain protein	60	3.3	4	6132.2975	49.71	VDIEVLPQELQPALTFALPDPSRFTLVDFPLHLP LELLGVDAQLQVLTCILLEHK	Deamidated (NQ)
19	Trypsin-1	38	4	2	1191.6057	38.37	TLNNDIMLIK	2 Deamidated (NQ) Oxidation (M)
20	Peripheral plasma membrane protein CASK	35	1.2	2	1298.6223	35.27	MNELNHQVAR	
21	Hemoglobin subunit beta	26	6.6	2	1273.7183	26.15	LLVVYPWTQR	

FIGURE 13 CONT.