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Delineating monoclonal antibody specificity by mass spectrometry

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ABSTRACT

Generation of monoclonal antibody (mAb) libraries against antigens in complex matrices can prove a valuable analytical tool. However, delineating the specificity of newly generated antibodies is the limiting step of the procedure. Here, we propose a strategy for mAb production by injecting mice with complex biological fluid and mAb characterization by coupling immunoaffinity techniques with Mass spectrometry (immuno-MS). Mice were immunized against fractionated seminal plasma and mAbs were produced. Different immuno-MS protocols based on four types of solid support (i.e. polystyrene microtiter plates, NHS-activated agarose beads, tosyl-activated magnetic beads and MSIA™ pipette tips) were established. A well-characterized mouse monoclonal anti-KLK3 (PSA) Ab was used as a model to evaluate each protocol's robustness and reproducibility and to establish a set of criteria which would allow antigen characterization of newly developed Abs. Three of the newly generated Abs were analyzed using our optimized protocols. Analysis revealed that all assay configurations used were capable of antibody characterization. Furthermore, low-abundance antigens (e.g. ribonuclease T2) could be identified as efficiently as the high-abundance ones. Our data suggest that complex biological samples can be used for the production of mAbs, which will facilitate the analysis of their proteome, while the established immuno-MS protocols can offer efficient mAb characterization.

Biological significance

The inoculation of animals with complex biological samples is aiming at the discovery of novel disease biomarkers, present in the biological specimens, as well as the production of

Abbreviations: ABC, Ammonium bicarbonate; BSA, Bovine serum albumin; D.A.R.T.'S, Disposable Automated Research Tips; DTT, Dithiotreitol; ELISA, Enzyme-linked immunosorbent assay; FDR, False-discovery rate; FPLC, Fast liquid chromatography; Fc, Constant region of immunoglobulin; HAT, Hypoxanthine-aminopterin-thymidine medium; HPLC, High performance liquid chromatography; KLK, Kallkrein; LFQ, Label-free quantification; MS, Mass spectrometry; MSIA, Mass spectrometric immunoassay; mAb, Monoclonal antibody; NHS, *N*-hydroxysuccinimide; PBS, Phosphate-buffered saline; PSA, Prostate-specific antigen; SRM, Selected reaction monitoring; SP, Seminal plasma; TBS, Tris-buffered saline; TCP, Toronto Centre for Phenogenomics; XIC, Extracted ion current.

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rare reagents that will facilitate the ultra-sensitive analysis of the biomolecules' native form. In the present study, we initially propose a general workflow concerning the handling of biological samples, as well as the monoclonal antibody production. Furthermore, we established protocols for the reliable and reproducible identification of antibody specificity using various immuno-affinity purification techniques coupled to mass spectrometry. Our data suggest that processed biological fluids can be used for the production of mAbs targeting proteins of varying abundance, and that various immuno-MS protocols can offer great capabilities for the mAb characterization procedure.

1. Introduction

Biological samples are deposits of numerous molecules—characteristic to a greater or lesser extent of the matrix and its functions [1]. In other words, these molecules could provide in-depth biological information and/or associate with disease development and progress. Although ever going technological progress, which promises better analytical procedures, polyclonal and monoclonal antibodies remain important tools for the analysis of biomolecules. Research antibodies are produced by inducing immune response to a host animal against an immunogen representing the human protein. It is evident that the utility of the Ab is related to the high resemblance of the immunogen to the native protein.

High throughput generation of large monoclonal antibody (mAb) libraries against diverse antigens in their native form has been proposed by immunization of mice with the complex biological sample the antigens exist [2]. Until recently, the large-scale specificity characterization of monoclonal antibodies presented a major bottleneck, limiting mAb development to single (recombinant) protein or peptide immunizations. This problem was recently tackled by the advent of immuno-precipitation methods coupled with MS-based proteomics (generally known as immuno-MS methods). These methods promise to revolutionize the field by allowing rapid characterization of the antigen specificity of multiple antibodies [3–6].

The success of an immuno-MS method depends on the choice of the affinity reagent, the proper separation of affinity reagent-target analyte complex from contaminants, and the suitable recovery of the bound analyte for MS identification. To date, several different immuno-MS formats have been reported, depending on the nature of the affinity-based enrichment used. Other than the classical antibody-based formats, several alternative affinity reagents have also been recently proposed, including DNA aptamers, engineered polypeptides and inorganic molecules [7]. Despite these advances, the classical antibody immobilization on a solid support (beads, plates, columns) still represents the most-established approach to affinity purification.

Several parameters can have a direct effect on the affinity of an individual antibody–antigen interaction, such as the nature of the solid support and the underlying chemistry of each interaction (e.g. Protein A/G–Fc, streptavidin–biotin or covalent binding via NH_2 or COOH groups, directed or random binding) [8–14]. Therefore, the selection of the appropriate set-up of an immuno-MS method should reflect the intended objective of each study. Towards Ab-antigen characterization, three main types of immuno-MS configurations have been described so far: i) polystyrene-based (e.g. microtiter plates), ii) bead-based (e.g. sepharose or magnetic-beads) and iii) MSIA™-tip format [5,15–20].

This study investigated the efficiency and robustness of these methods in determining the specificity of antibodies generated by immunizing mice with complex biological fluid. It should be noted that these configurations were used to pull down intact antigens.

As the biological matrix under investigation we decided to work with seminal plasma. Seminal plasma is a rather complex biological fluid, comparable to blood, which seems to be a source of promising biomarkers for the male reproductive system disorders [21]. Furthermore, our lab has worked extensively with seminal plasma, in a long-lasting attempt to decipher its proteome [22–24].

One of the most abundant components (>100 μg/ml) of seminal plasma is the prostate-specific antigen (PSA or KLK3)—a member of the kallikrein-related peptidases family. KLK3 is secreted by epithelial cells of the prostate gland and is involved in semen liquefaction [25]. We used a commercial anti-KLK3 (PSA) Ab as a model, in order to optimize the performance characteristics of each immuno-MS set-up, and to develop a set of decision rules, applicable to all configurations. We then successfully implemented these rules for the identification of the target antigens of three unknown antibodies, developed by immunizing mice with fractionated seminal plasma (SP). Technical insights on the innate characteristics of each of these immuno-MS methods are provided.

2. Methods

2.1. Fractionation of seminal plasma prior to animal immunization

Seminal plasma samples were pooled (N = 10). Samples were obtained after informed consent and institutional review board approval (Mount Sinai Hospital, Toronto, ON, Canada). Prior to animal injection, samples were subjected to a three-step chromatographic fractionation, including: i) initial FPLC fractionation using a HiTrap Q column (GE Healthcare Life Sciences, Baie d'Urfe, QC, Canada), ii) a higher-resolution fractionation step, using a Source 15Q column (GE Healthcare) and iii) HPLC size exclusion chromatography using a Tosoh Bioscience silica-based gel filtration chromatography column (TSK-GEL G3000SW, 7.5 mm × 60.0 cm; Tosoh Bioscience, Stuttgart, Germany) in an Agilent 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany). For more details on the technical description of each separation, see Supplementary Methods and Supplementary Figures S1 and S2.

2.2. Monoclonal antibody production

Female BALB/c mice were obtained from the Toronto Centre for Phenogenomics (TCP). All animal research (Animal Use Protocol #14-04-0119a-H) was approved by TCP Animal Care Committee. Mice were inoculated subcutaneously with 100 μg (total protein) of the selected fraction of seminal plasma, mixed 1:1 with Sigma Adjuvant System (Sigma-Aldrich, St. Louis, MO). Three booster injections with 20 μg of antigen in adjuvant were performed at 2-week intervals. Final boost was an intraperitoneal injection of 20 μg antigen in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4). Three days later, mouse spleen was excised aseptically and homogenized. Extracted spleen cells were fused with NSO murine myeloma cells (5:1 ratio) using polyethylene glycol (Sigma-Aldrich). Successfully fused cells were selected using HAT media (Invitrogen, Carlsbad, CA), supplemented with 20% fetal bovine serum (Hyclone, Thermo-Fisher Scientific, Waltman, MA). Cell culture supernatants were screened for the presence of IgG antibodies using ELISA, as previously described [26]. Subsequently, cells were further grown and transferred in serum-free media (Invitrogen). Supernatants were collected and purified using protein G according to the manufacturer's protocol (GammaBind Plus, GE Healthcare).

2.3. Immuno-enrichment

We used four immuno-MS methods to characterize the antigens bound to the IgG secreting clones. The antibodies used were a commercially available anti-KLK3 (PSA) mAb, as a positive control (Medix Biochemica, Kauniainen, Finland) and three newly developed (and uncharacterized) mAbs: Test Abs X1, X2 and X3.

2.3.1. Method 1: IgG coating onto polystyrene microtiter plate

Approximately 200 ng/well of the four IgGs (anti-KLK3 Ab + 3 test Abs) were coated on 96-well polystyrene plates in a final volume of 200 μl/well (coating buffer: 50 mM Tris–HCl, pH 7.8). Following overnight incubation at room temperature, the plate was washed five times (5×) using an automated plate washer (washing buffer: 0.1% BSA, 1% NaCl). Next, 100 μl of either non-fractionated (for anti-KLK3 Ab) or fractionated seminal plasma (same as immunogen; for unknown Abs) was loaded to each well, in quadruplicates (seminal plasma was used in various dilutions, see below). After 1 h incubation at room temperature with gently shaking, wells were washed 10 times (10×) with the same washing buffer. Prior to MS analysis, 120 μl of 50 mM ammonium bicarbonate (ABC) and 30 μl of 1.25 mM dithiothreitol (Sigma-Aldrich) were added to each well and kept at 60 °C for 30 min. Then, 20 μl of 11.5 mM iodoacetamide were added and samples were kept for 60 min (in the dark) at room temperature. Samples were then digested by addition of 10 μl methanol and 10 μl of 0.01 μg/μl of sequencing-grade modified porcine trypsin (Promega Cat. #V5111, Madison, WI), in 50 mM ABC. Quadruplicates were combined (final volume: 800 μl) and transferred to wells of a microcentrifuge plate. Trypsin inactivation was achieved with the addition of formic acid (10%, diluted in H_2O) (pH <5). In all KLK3 experiments, 50 fmol of heavy-labeled KLK3 proteotypic peptide LSEPAELTDAVK was spiked-in as an internal standard, prior to MS analysis.

2.3.2. Method 2: IgG coupling to N-hydroxysuccinimide (NHS)-activated Sepharose beads

The four IgGs were desalted using either Illustra NAP-5 Sepharose G-25 (GE Healthcare) or PD-10 (GE Healthcare) desalting columns and then concentrated (using 30 K filters) to a final concentration of 0.3 mg/ml in coupling buffer (0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3). After six washes in ice-cold equilibration buffer (1 mM HCl), NHS-activated Sepharose 4 Fast Flow (GE Healthcare; 1 ml of packed beads in 1.5 ml spin columns) was incubated with 0.3 mg/ml IgG solution for 2 h at 22 °C on a rotator, washed once with blocking buffer A (50 mM Tris–HCl, 1 M NaCl, pH 8.0) and two times with blocking buffer B (50 mM glycine, 1 M NaCl, pH 3.0). Using blocking buffer A, samples were subsequently incubated for 2 h at 22 °C on a rotator (blocking step), washed serially with buffer A and B and finally three times with binding buffer 1× TBS (50 mM Tris, 150 mM NaCl, pH 7.5). For antigen binding, 10 ml of seminal plasma, diluted in 0.1% BSA (50 mM Tris, 150 mM NaCl, pH 7.5) was mixed with beads for 2–4 h (at 22 °C on a rotator), washed in spin columns three to five times with high salt TBS buffer (50 mM Tris, 500 mM NaCl, pH 7.5), and eluted using 0.1 M glycine, pH 2.0. Eluates were pooled, neutralized using 1 M Tris, pH 9.0 and concentrated using a lyophilizer. In preparation for MS analysis, samples were diluted in 50 mM NH_4HCO_3 and dithiothreitol (Sigma-Aldrich) (final concentration of 10 mM DTT) and incubated for 30–40 min at 60 °C. Following incubation, fractions were incubated with iodoacetamide (final concentration of 30 mM) in the dark for 1 h at 22 °C. Subsequently, sequencing-grade modified porcine trypsin was added at 1/50 trypsin/protein ratio. Fractions were then trypsin-digested overnight at 37 °C. Trypsin inactivation was achieved with the addition of 10% formic acid. In all KLK3 experiments, 50 fmol of heavy-labeled KLK3 proteotypic peptide LSEPAELTDAVK was spiked-in as an internal standard, prior to MS analysis.

2.3.3. Method 3: IgG coupling to Tosyl-activated magnetic beads

The four IgGs were desalted as in Method 2 and concentrated to a final concentration of 0.3 mg/ml in coupling buffer (0.1 M borate buffer pH 9.5). Beads (Dynabeads M-280 Tosyl-activated, Life Technologies, Carlsbad, CA) were initially transferred to a glass tube, placed in a magnet for 1 min, and washed in borate buffer, as suggested by the manufacturer. Samples and buffer A (0.1 M borate buffer pH 9.5) were mixed to a final volume of 150 μl, followed by addition of buffer C (3 M ammonium sulphate in Buffer A) and incubation on a roller at 37 °C for 12–18 h. After 2 min in magnet, 1 ml of buffer D (PBS pH 7.4 with 0.5% (w/v) BSA) was added to the beads and was left at 37 °C for 1 h on a roller, followed by washing two times in buffer E (PBS pH 7.4 with 0.1% (w/v) BSA) with 2 min incubations. Beads were then washed in binding buffer (1× TBS; 50 mM Tris, 150 mM NaCl, pH 7.5). Serial seminal plasma dilutions (volume of 4 ml) were prepared in 0.1% BSA. The 0.1% BSA buffer and seminal plasma solutions were added to the beads to a total volume of 4 ml and incubated for 2–4 h at 22 °C in 15 ml conical tubes on a rotator. Samples were then transferred into glass tubes and washed in 1× TBS, followed by high salt TBS buffer (1×) in magnet (three to five times). Samples were then eluted in 1 ml elution buffer (0.1 M glycine, pH 2.0) and eluates were neutralized with 1 M Tris pH 9.0. Sample preparation for MS (reduction,

alkylation, trypsin digestion) was performed as described in Method 2. In all KLK3 experiments, 50 fmol of heavy-labeled KLK3 proteotypic peptide LSEPAELTDAVK was spiked-in as an internal standard, prior to MS analysis.

2.3.4. *Method 4: IgG coupling to MSIA™ pipette tips*

The immunoaffinity retrieval of protein targets from samples was also achieved with the use of the MSIA D.A.R.T.'S (Disposable Automated Research Tips) mounted onto the Versette Automated Liquid Handler (Thermo Scientific, Hudson, NH). After initially rinsing the MSIA D.A.R.T.'S with 25 cycles of 10 mM PBS (1 cycle consisting of a single aspiration and dispense of 150 μl volume), the MSIA D.A.R.T.'S were immersed into 500 μl samples (diluted seminal plasma) and 1000 aspirations and dispense cycles were performed (150 μl volumes per aspiration), allowing for affinity enrichment of each targeted protein. The MSIA D.A.R.T.'S were then rinsed with PBS (25 cycles) from another microplate, and twice with water (25 cycles each) from two more micorplates (150 μl volumes aspiration and dispenses, from 1800 μl in each well). Each wash cycle was performed using the same plate and buffers to allow equilibrium to be reached before proceeding to the next rinse and to minimize plates used during rinsing. The immuno-enriched protein targets were eluted into a microplate (Fisher Scientific, AB 1300) by aspirating and dispensing 30 μl of 30% acetonitrile/0.5% (v/v) formic acid, 250 times from a total of 50 μl volume, allowing for sufficient disruption of the antibody/antigen complex. The eluates were dried in a Speed Vac concentrator. In all KLK3 experiments, 50 fmol of heavy-labeled KLK3 proteotypic peptide LSEPAELTDAVK was spiked-in as an internal standard, prior to MS analysis.

2.4. *Mass spectrometric analysis*

In all cases, peptides were extracted from solution using OMIX C18 tips (Varian Inc., Lake Forest, CA) and eluted in 5 μl of elution buffer B (65% acetonitrile, 0.1% formic acid). Buffer A (80 μl of 0.1% formic acid) was added to sample tubes and transferred to a 96-well microplate (Axygen, Union City, CA). Using a 96-well microplate autosampler, 40 μl of each sample was loaded onto a 3 cm C18 trap column (inner diameter 150 μm; New Objective, Woburn, MA) that was packed in-house with 5 μm Pursuit C18 (Varian Inc.). An increasing concentration of Buffer B (0.1% formic acid in acetonitrile) was used to elute the peptides from the trap column onto a resolving analytical 5-cm PicoTip Emitter Column (inner diameter 75 μm, 8 μm tip; New Objective, Woburn, MA). This column was packed in-house using 3 μm Pursuit C18 (Varian). The EASY-nLC system (Proxeon Biosystems, Odense, Denmark) was coupled online to an LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Fisher Scientific, San Jose, California) and a nanoelectrospray ionization source (Proxeon) was used with a spray voltage of 2 kV and temperature of 160 °C. A data-dependent mode was utilized to analyze samples and a full MS1 scan was acquired from 450 to 1450 m/z in the mass analyzer (resolution of 60,000). This was followed by MS2 scan acquisition of the top three parent ions in the LTQ mass analyzer. The subsequent parameters were enabled: dynamic exclusion, charge state screening and monoisotopic precursor selection. Ions with charge states of +1, ≥+4 and unassigned charge states did not undergo MS2 fragmentation.

RAW files for each MS run were analyzed using MaxQuant 1.4.1.2 software (Max Planck Institute of Biochemistry) [27]. Protein searches were peformed against the non-redundant Human Swiss-Prot database (October 2013 release). The following parameters were used: fixed carbamidomethylation of cysteines; variable modification of methionine oxidation and N-terminal protein acetylation; maximum missed cleavages 2; trypsin as digestion enzyme; label-free quantification (LFQ) checked; first search of a small human database (embedded in MaxQuant) with 20 ppm precursor tolerance, final search against Human Swiss-Prot with 4.5 ppm precursor tolerance and fragment tolerance of 0.5 Da. Reverted decoy database was used to adjust false-discovery rate (FDR) to 1% at protein and peptide-sequence match levels. 'Intensity' columns in 'proteinGroup.txt' files represent Ion Current (XIC) intensities of peptides corresponding to protein they originate from. Protein 'Intensity' values were used for relative calculation of abundances.

3. Results

3.1. *Method optimization using a commercial anti-KLK3 monoclonal antibody*

Anti-KLK3 Ab and non-specific mouse IgG (negative control) were coupled onto the four different solid surfaces (polystyrene plate, NHS-activated sepharose beads, magnetic beads and MSIA™ pipette tips). Subsequently, the solid phases were incubated with serial dilutions of seminal plasma. Captured proteins were examined by mass spectrometry as described in Methods. In all cases, the same amount (50 fmol) of heavy-labeled KLK3 proteotypic peptide (LSEPAELTDAVK) was added as an internal standard.

Initially, the eXtracted Ion Current (XIC) values for light (636.838 m/z) and heavy (640.845 m/z) peptides were measured and the light/heavy (L/H) ratio for all sample dilutions following antibody capture was calculated. As shown in Fig. 1, the L/H KLK3 ratios show a clear dose–response pattern with decreasing sample dilutions in all set-ups, while very low or no signal was detected in the control anti-IgG experiments. Overall, the L/H KLK3 signal was significantly higher with the beads and MSIA™ format, compared to the polystyrene plate. For instance, at 10,000 fold seminal plasma dilution, the L/H KLK3 ratio in the polystyrene plate was only around 0.12, compared to 28 for MSIA™, 47 for NHS beads and 70 for magnetic beads. In general, the L/H ratios between 10^4 and 10^6-fold dilutions were in the linear range for all methods, hence this range was used in our subsequent analysis.

Max Quant software was used for the identification and relative quantification of proteins (including KLK3) in the eluted samples between 10^4 and 10^6-fold dilutions. The number of KLK3 unique peptides identified with each method and their respective ion intensities are depicted in Table 1. Overall, more unique peptides were detected with NHS-activated (9, 4 and 1 peptides with increasing dilution), or magnetic beads (8, 10 and 3 peptides), compared to polystyrene plate (only 1 peptide in all dilutions). As expected, other than KLK3, several non-specific

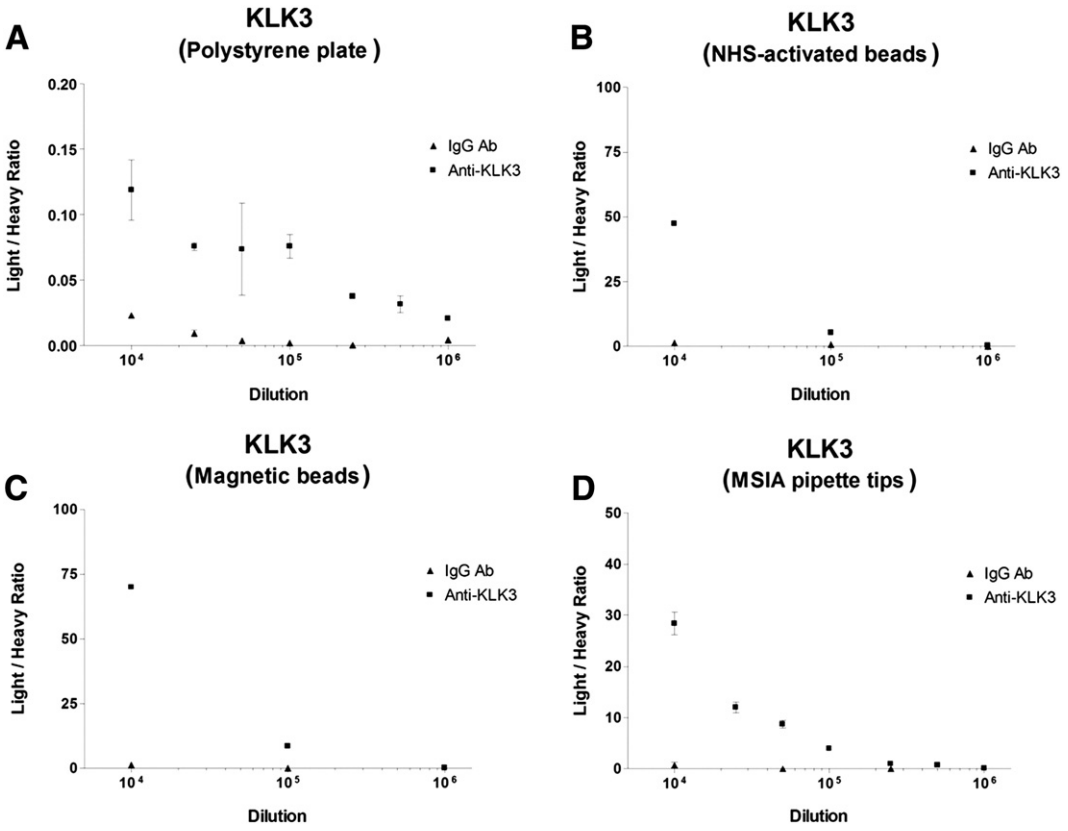

Fig. 1 – Ion intensity ratios of KLK3 proteotypic peptides (light/heavy) plotted against serial dilutions of fractionated seminal plasma. Eluates from different IgG (▲) and anti-KLK3 (■) antibody-coupled solid surfaces, as shown, were analyzed by mass spectrometry (for more details see text).

proteins were also identified in all experimental set-ups. Table 1 shows the total number of proteins identified in test and control (mouse IgG) samples between the 10^4 and 10^6-fold dilutions, with all methods. Clearly, the highest number of total identified proteins was observed with the MSIA™ tips (41, 41 and 37 proteins in increasing dilution), while the lowest was observed with the NHS-activated (17, 6 and 9 in the same order) or magnetic beads (26, 8 and 4). As an example, a representative list of all proteins identified in one specific dilution (10,000-fold), by using NHS-activated beads, accompanied by the number of unique peptides and ion intensity values are shown in supplementary Table S1.

Previous data underline that the unequivocal identification of the binding antigen could be problematic in the case of

Table 1 – Identification of specificity of monoclonal antibody against KLK3.

Method	Antigen dilution [1]	Anti-KLK3 capture			IgG capture (control)		
		Proteins identified	Unique peptides [2]	Ion Intensity [3]	Proteins identified	Unique peptides [2]	Ion intensity [3]
Polystyrene plate	10^4	17	1	11,310,000	19	1	1,468,100
	10^5	20	1	9,181,300	18	0	–
	10^6	23	1	864,610	11	0	–
NHS-activated beads	10^4	17	9	656,020,000	7	2	18,667,000
	10^5	6	4	42,125,000	4	2	6,332,500
	10^6	9	1	3,511,800	6	0	–
Magnetic beads	10^4	20	8	2,381,300,000	19	0	–
	10^5	6	10	844,480,000	11	0	–
	10^6	5	3	12,482,000	6	0	–
MSIA™ pipette tips	10^4	41	6	869,750,000	11	1	289,830
	10^5	41	3	34,779,000	28	0	–
	10^6	37	1	787,730	32	0	–

[1] Antigen is seminal plasma.
[2] Refers to unique peptides for KLK3.
[3] Refers to intensity of peptides unique to KLK3.

Table 2 – Identification of specificity of monoclonal antibody X1. This antibody binds to Ribonuclease T2.

Method	Antigen dilution [1]	X1 capture			IgG capture (control)		
		Proteins identified	Unique peptides [2]	Ion intensity [3]	Proteins identified	Unique peptides [2]	Ion intensity [3]
NHS-activated beads	10^3	15	11	721,170,000	8	0	–
	10^4	14	7	71,316,000	4	0	–
	10^5	28	1	279,930	13	0	–
Magnetic beads	10^3	15	10	168,770,000	31	0	–
	10^4	17	2	5,919,200	17	0	–
	10^5	12	0	–	9	0	–
MSIA™ pipette tips	10^1	47	11	111,030,000	52	0	–
	10^2	20	6	6,914,200	23	0	–
	10^3	34	2	192,670	10	0	–
	10^4	31	0	–	30	0	–
	10^5	26	0	–	28	0	–

[1] Antigen is seminal plasma.
[2] Refers to unique peptides for ribonuclease T2.
[3] Refers to intensity of peptides unique to ribonuclease T2.

unknown antibodies. Based on our data, we established a set of four criteria that could be applied for the selection of the most likely binding antigen, among the proteins identified by immuno-MS.

1) Remove from the list all proteins known to be common contaminants (e.g. keratins and high-abundance proteins in biological fluid [28]). 2) Remove proteins identified with less than two unique peptides. 3) Rank remaining proteins based on their total ion-intensity values. 4) Remove proteins whose ion intensity is less than 10-fold higher in the test sample compared to the negative controls (IgG capture). As a final confirmation, the predicted molecular mass of the candidate antigen should match the size of the protein detected on a Western blot, using the same sample and testing antibody.

On the basis of these criteria, KLK3 has been successfully identified as the most probable target antigen of the anti-KLK3 antibody in three immuno-MS formats (i.e. NHS-beads, magnetic beads and MSIA™ tips), but not the microtiter-plate (Table 1). Therefore, the microtiter plate format was not included in the characterization of antibodies of unknown specificity.

3.2. *Identification of target antigen of antibodies of unknown specificity*

We applied the aforementioned criteria to investigate the specificity of three unknown antibodies (Test Abs X1, X2 and X3). Test Abs were raised by immunizing mice with a seminal plasma fraction. Among the large number of clones we selected three IgG-secreting clones that detected a single distinct band each, on Western Blots. The three unknown monoclonal Abs (along with mouse IgG as negative control) were coupled onto the three different solid phases and incubated with serial dilutions of the immunogen.

Due to different binding capacity of each solid phase, different sample dilutions were used in each method. NHS-activated sepharose and magnetic beads were tested at dilutions from 10^3 to 10^5-fold, while MSIA™ pipette tips were tested in dilutions

Table 3 – Identification of specificity of monoclonal antibody X2. This antibody binds to zinc-alpha-2-glycoprotein.

Method	Antigen dilution [1]	X2 capture			IgG capture (control)		
		Proteins identified	Unique peptides [2]	Ion intensity [3]	Proteins identified	Unique peptides [2]	Ion intensity [3]
NHS-activated beads	10^3	21	16	543,340,000	9	0	–
	10^4	10	10	297,640,000	4	0	–
	10^5	7	9	45,944,000	16	0	–
Magnetic beads	10^3	17	15	931,050,000	17	0	–
	10^4	12	16	470,080,000	10	0	–
	10^5	17	6	24,554,000	6	0	–
MSIA™ pipette tips	10^1	45	19	128,580,000	48	0	–
	10^2	25	10	9,559,800	23	0	–
	10^3	21	11	5,979,800	11	0	–
	10^4	62	5	9,204,500	27	0	–
	10^5	47	0	–	27	0	–

[1] Antigen is seminal plasma.
[2] Refers to unique peptides for zinc-alpha-2-glycoprotein.
[3] Refers to intensity of peptides unique to zinc-alpha-2-glycoprotein.

Table 4 – Identification of specificity of monoclonal antibody X3. This antibody binds to lactoferrin.

Method	Antigen dilution [1]	X3 capture			IgG capture (control)		
		Proteins identified	Unique peptides [2]	Ion intensity [3]	Proteins identified	Unique peptides [2]	Ion intensity [3]
NHS-activated beads	10^3	16	37	481,690,000	30	0	–
	10^4	23	15	41,875,000	15	0	–
	10^5	11	0	–	9	2	1,605,500
Magnetic beads	10^3	32	26	396,470,000	31	0	–
	10^4	12	4	5,075,000	17	0	–
	10^5	42	0	–	9	0	–
MSIA™ pipette tips	10^1	32	59	1,948,700,000	48	8	7,623,500
	10^2	34	39	56,548,000	25	0	–
	10^3	17	11	4,820,600	23	0	–
	10^4	27	6	3,610,300	33	0	–
	10^5	25	2	476,730	26	0	–

[1] Antigen is seminal plasma.
[2] Refers to unique peptides for lactoferrin.
[3] Refers to intensity of peptides unique to lactoferrin.

from 10^1 to 10^5-fold. Similar to KLK3, LC-MS/MS analysis identified numerous candidate antigens for the three Abs in all solid-phase formats. The number of proteins identified in the eluents from different solid surfaces in both test and control samples are shown in Tables 2, 3 and 4. Application of our developed criteria resulted in unanimous suggestions for the unique candidate antigen in all three cases: ribonuclease T2 as the antigen for Test Ab X1, zinc-alpha-2-glycoprotein as the antigen for Test Ab X2, and lactoferrin as the antigen for Test Ab X3. The aforementioned tables (2, 3 and 4) also display the number of unique peptides and the total ion intensity identified for each of the candidate antigens in the same dilutions of test and control samples. Even though all three approaches identified the same antigens for the tested Abs, the highest number of unique peptides at the same dilution is consistently seen with NHS-activated and magnetic beads. Furthermore, the measured total ion intensities of all peptides representing these antigens were plotted against the serial sample dilutions used (Figs. 2–4). Again, highest ion intensities at the same dilution were observed with the bead formats. No intensity pattern was observed in any of the IgG negative controls. MSIA™ tips displayed good performance in more concentrated samples, especially in the case of lower-abundance antigens, like ribonuclease T2. Representative lists of all proteins identified in one specific dilution (1000-fold), by using NHS-activated beads, as well as the unique peptides numbers and ion intensity values, can be seen in supplementary Tables S2–S4.

4. Discussion

This study examines the feasibility and robustness of currently available MS-based technologies to determine the antigen specificity of newly generated Abs. As a proof-of-concept, we selected a commercial anti-KLK3 antibody, which was utilized for immuno-MS protocol optimization using: 1) polystyrene plate formats, 2) NHS- and magnetic beads, and 3) MSIA™ pipette tips. These protocols were then used for the characterization of new Abs, developed in mice by injecting a fraction of human seminal plasma. We selected seminal plasma (SP), based on our previous extensive proteomic work with this fluid [22–24]. Given that most of the relevant studies so far have been restricted to the characterization of antibodies against high- or medium-abundance proteins in biological fluids [17,29], SP was subjected to a three-step sequential fractionation, prior to immunization, to reduce its complexity.

A common problem of all immuno-MS methods for Ab-antigen characterization is the impurity of the eluted

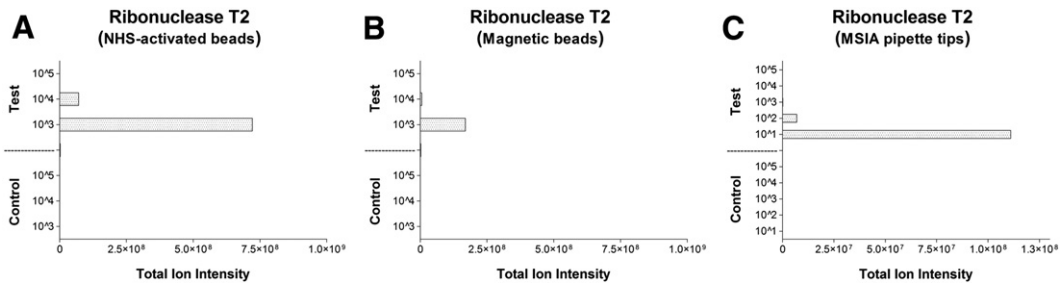

Fig. 2 – Total ion intensity of ribonuclease T2 peptides, plotted against serial dilutions of fractionated seminal plasma. Eluates from different IgG (control) and X1 (test) antibody-coupled solid surfaces, as shown, were analyzed by mass spectrometry.

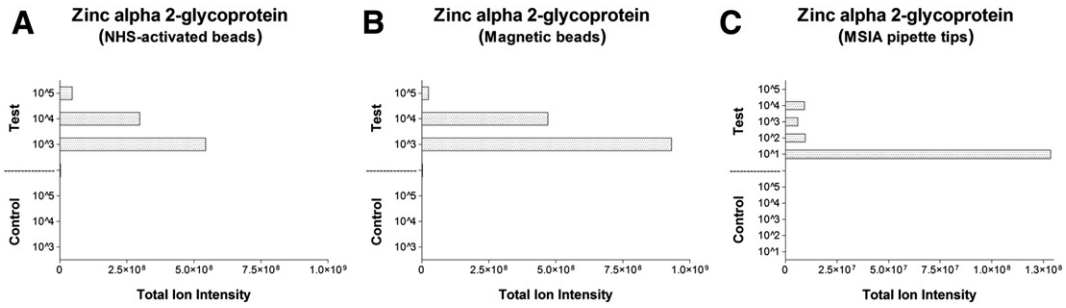

Fig. 3 – Total ion intensity of zinc-alpha-2-glycoprotein plotted against serial dilutions of fractionated seminal plasma. Eluates from different IgG (control) and X2 (test) antibody-coupled solid surfaces, as shown, were analyzed by mass spectrometry.

products, primarily due to non-specific protein binding. Unlike immuno-SRM methods (targeted MS analysis), in which absolute specificity of immuno-enrichment is not required, the use of shotgun immuno-MS for the characterization of the target antigen of an unknown antibody can be hampered by the concurrent enrichment and MS identification of non-specific protein binders.

For instance, as shown in Table 1, immuno-enrichment using anti-KLK3 antibody coupled onto MSIA™ pipette tips resulted in the identification of 41 candidate proteins (at 10^4-fold dilution), including non-specific proteins (false positives) and possibly true KLK3-binding partners (e.g.endogenous PSA inhibitors). In the literature, multiple causes of non-specific adsorption to the solid support have been described, including ionic or hydrophobic interaction and conformational occlusion [30]. Improved specificity can be achieved with a pre-elution procedure, using high pH or high ionic strength buffers [12,30]. Blocking (e.g. BSA) and harsh washing (e.g. with detergent-containing buffers) have also been shown to minimize non-specific binding, however, these processes may cause compatibility problems with downstream MS-analysis. In addition, immuno-extracting conditions cannot be too stringent as they may impact the sensitivity of the method and may present a particular problem for lower affinity antibodies.

A comparative look among the four approaches reveals that the weakest method in terms of sensitivity was the polystyrene plate set-up. For instance, only one KLK3 peptide was identified in all three dilutions with this configuration (LSEPAELTDAVK) compared to significantly more peptides identified when using the NHS-beads (9, 4 and 1), magnetic-beads (8, 10 and 3) and MSIA™ tips (6, 3 and 1). This is better illustrated by comparing the ratios of the eXtracted Ion Current (XIC) values for light (636.838) and heavy spiked (640.845 m/z) proteotypic PSA peptide (LSEPAELTDAVK) across the four different experimental approaches. As shown in Fig. 1, the L/H KLK3 ratios show a clear dose-response pattern with decreasing sample dilutions, with very low or absent signal for control anti-IgG antibody in all experimental conditions. Clearly, the capacity of the polystyrene plate to capture KLK3 antigen is very limited compared to the other approaches. For example, at 10^4 sample dilution, the L/H ratio for the polystyrene plate (Fig. 1A) ranged between 0.10 and 0.14 (among the different replicates) compared to 24 and 36, 47 and 70 for MSIA™, NHS beads, and magnetic beads, respectively (Fig. 1B, C and D, respectively). Apparently, this stems from the limited capacity of the plate for both Ab and antigen binding. We could use up to 10 ml and 4 ml of SP in the NHS and magnetic bead format, respectively, compared to a maximum of 1 ml in MSIA™ and a 0.2 ml/well in the 96-well polystyrene plate. Similarly, the capacity for total Ab immobilization was approximately 100 μg for the bead formats, compared to <5 μg/tip in MSIA™ format and <0.1 μg/well in the plate format (Supplementary data, Table S5). Therefore, it is evident that for the analysis of very low-abundance antigens, the use of beads is beneficial.

An important advantage of all methods, which was very helpful in the final confirmation of the candidates' identity, was the significant difference of peptide total ion intensities (more than one order of magnitude) between test and control samples. As shown in Tables 2–4 and Figs. 2–4, all verified antigens displayed significantly elevated ion intensity in each sample dilution for each method, compared to the control—if present at all in the latter. A high signal in the negative controls could be evidence of non-specific binding to the

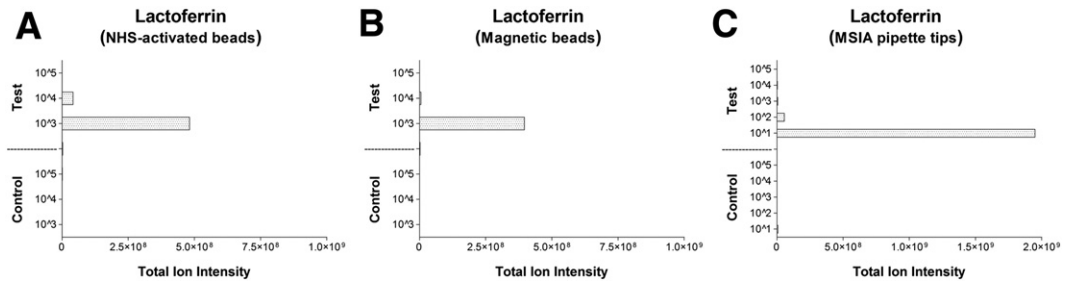

Fig. 4 – Total ion intensity of lactoferrin plotted against serial dilutions of fractionated seminal plasma. Eluates from different IgG (control) and X3 (test) antibody-coupled solid surfaces, as shown, were analyzed by mass spectrometry.

solid-phase. In these cases, titration of the total amount of Ab can be performed to exclude non-specific binders.

Additionally, Tables 2–4 and Figs. 2–4 also confirm what we discussed above concerning the assay's performance and each solid phase's antibody binding capacity. Results in all cases (referring to the same dilution factor) show very clearly that bead-based methodologies achieve >100-fold better performance; especially in the case of the alleged low-abundance antigen.

Recently, several immuno-MS-based platforms for high-throughput antibody characterization have been described. For example, Wang et al. recently reported the use of bead-based immuno-MS methods to characterize large libraries of Abs produced by direct injection of mice with a mixture of native glycoproteins, isolated from the blood of non-small cell lung cancer (NSCLC) patients [17]. In a related approach, Guergova-Kuras et al. used a global monoclonal antibody proteomic strategy to blindly screen for biomarkers that could detect early stages of lung cancer and then used immuno-MS to characterize the antigen specificities of those that exhibited significant diagnostic power. In the above studies, mostly medium and high abundance antigens were identified, probably as a result of the huge dynamic range of human plasma and often involved electrophoretic separation of eluted proteins [29]. To increase the probability of generating antibodies against lower abundance proteins, a common strategy is to deplete the plasma of common and highly abundant proteins. In a variation of this approach, Ning et al., immunized mice sequentially with complex fractions of subtracted human plasma, in which newly generated Abs were used to immunodeplete plasma from the same antigens prior to further immunizations [31].

In conclusion, it becomes clear that the advent of immuno-MS has brought ample new opportunities for the rapid development and characterization of large Ab libraries. This study evaluates four immuno-MS protocols for the characterization of monoclonal Abs, developed upon animal immunization with a complex biological fluid. In the absence of absolute validation, a set of four criteria was established, based on ion intensities, to help identify the most probable antigen for each mAb. According to our results, at least three out of the four immuno-MS configurations can be successfully used for Ab-antigen characterization.

Conflict of interest

The authors declare no competing financial interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2014.11.004.

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