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Restricted Proteolysis and LC-MS/MS To Evaluate the Orientation of Surface-Immobilized Antibodies

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Abstract

The molecular orientation of antibodies immobilized on solid surfaces plays a significant role in the sensitivity of immunoassays and efficiency of protein isolation using antibody-decorated nanoparticles. Optimally, nearly all antibody binding sites should be available to bind. Here we report for the first time an LC-MS/MS approach to probe antibody orientation directly, utilizing sterically restricted proteolysis. Trypsin-decorated magnetic beads (MBs, 1.5 μm) were much larger than average antibody-free areas ($55 \times 55 \text{ nm}$) of oriented antibodies on MBs, restricting proteolysis to mainly Fab regions. Randomly attached antibodies on MB surfaces served as controls. The tryptic-hydrolyzed peptides were quantified using LC-MS/MS peptide analysis as markers for average positions of Fc and Fab of antibodies on the beads. Different patterns of digestion rates were found due to proteolysis of the oriented and nonoriented antibodies on MBs. For oriented antibodies, the peptides from outer Fab regions gave a much higher digestion rate than those from Fc regions, while for randomly immobilized antibodies digestion rates for Fab and Fc peptides were similar. This novel approach is a useful and convenient tool to characterize antibody orientation for immunoassays and other applications. The relative degree of orientation can be assessed using a metric R_o denoting amount of Fab marker peptides found divided by Fc + Fab marker peptides $\times 100\%$. Oriented antibodies on the MBs also provided more efficient antigen capture compared to randomly immobilized antibodies.

Graphical Abstract

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.9b01155.

Detailed experimental section; tables including UHPLC gradient and MRM transition list; figures including MS/MS spectra of tryptic peptide ions, assignments of Mascot software-predicted peptides, representative MRM chromatograms, XIC of MRM transitions, micro-BCA protein assays, free trypsin digestion kinetics, MP-trypsin digestion kinetics, and FITC-PSA binding study (PDF)

The authors declare no competing financial interest.

Antibodies are the key elements in all sorts of immunoassays. They are usually immobilized onto solid surfaces on biosensors and/or analyte-capture beads. The most commonly used antibodies in immunoassays are immunoglobulins (IgG, ~150 kDa), which have a characteristic Y-shape with two Fab fragments (antigen-binding) representing the branches of the Y and one Fc fragment (crystallizable) as the stem of the Y.^{1–3} In order to achieve optimal biorecognitions with antigens, the two Fab regions should be oriented mainly toward the solution containing the antigens to be determined while the Fc region should be directly anchored onto the surface.

Strategies for antibody immobilization include passive absorption and linking of amine/carboxyl group of antibodies onto the surface, which result in randomly immobilized antibodies. In a typical protein immunoassay surface prepared in this way, only a fraction of the Fab binding sites are available to bind the protein analytes.^{4–6} Thus, novel immobilization strategies have been developed to control IgG antibody orientation, aiming for an improved performance and sensitivity of immunoassays.^{3–7} Using Fc-binding proteins (e.g., with an intervening layer of protein A/G) is a good option since no chemical modification is needed on the antibody molecules, and they should retain the native binding properties.^{8,9} Protein A/G is a chimeric recombinant protein containing multiple Fc-binding domains from both protein A and protein G¹⁰ and binds strongly to Fc regions of IgGs from nearly all mammalian species.¹¹ It has much higher affinity under physiological conditions than either protein A or protein G alone,¹² which makes it an excellent platform to orient antibodies.

Although many reports have demonstrated that oriented immobilization of antibodies improves antigen binding capacities and efficiencies, few surface analysis tools are available to directly probe immobilized antibody orientation.^{3,13} Conventional surface characterization techniques, such as atomic force microscopy (AFM), dual-polarization interferometry (DPI), and spectroscopic ellipsometry (SE), infer degree of orientation by measuring dimensions of antibodies on the surface.^{14–16} Time-of-flight secondary ion mass spectrometry (TOF-SIMS) bombards the antibody layer with a pulsed primary ion beam to yield amino acid ions from the uppermost layer (1–3 nm).¹⁷ Antibody orientation assignments are extracted by analyzing the amino acid data with principal component analysis (PCA).^{18–20} The activity of a bound enzyme antigen was recently used to determine the binding capacity of IgGs on Au nanoparticles.⁹ However, direct molecular approaches to accurately characterize the orientation of surface-immobilized antibodies are thus far lacking.

In this paper, we report a general restricted proteolysis method to assess the degree of orientation of antibodies on surfaces. Restricted proteolysis was previously used for peptide

sequence identification in the Fab regions of monoclonal antibody (mAb) drugs oriented on nanoparticles by Iwamoto et al.²¹ Here, we report the first example of restricted trypsin proteolysis to generate peptides from the uppermost surface of known antibody layers at larger rates than from inner layers in order to characterize Ab orientation. The resulting tryptic peptides are characteristic of their positions in Fc or Fab regions of the immobilized IgG. Samples are analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) proteomics leading to a direct assessment of antibody orientation. For example, a high fraction of Fab peptides in the sample indicates a high degree of orientation with the Fab regions free for binding. Carboxylated magnetic beads (MB, 1.5 μm diam.) were used to immobilize IgGs since they are very effective for analyte capture and isolation.^{22–24} IgGs were oriented through protein A/G-mediated Fc binding, and amine-to-carboxyl IgG conjugation was used to achieve randomly oriented controls (Scheme 1). These two successful immobilization strategies are well accepted and thus used in this report as different immobilization models. Restricted proteolysis was then done using trypsin immobilized on MBs (MB-trypsin) of the same particle size, as MB-trypsin cannot gain entrance to the distance between immobilized IgGs. Comparisons of the rates of trypsin proteolysis of representative tryptic peptides in Fab and Fc regions were used to reveal the degree of antibody orientation. MB with oriented antibodies also exhibited a more efficient antigen capture compared to randomly immobilized antibodies.

EXPERIMENTAL SECTION

Chemicals and Reagents.

Monoclonal mouse IgG2a antibody (Ab) specific for human prostate specific antigen (PSA) was from R&D Systems, Inc. (Cat. No. MAB13442). Carboxylated magnetic beads (MB, 1.5 μm diameter, 20 mg mL^{-1}) were from Polysciences, Inc. Genetically engineered protein A/G (ProA/G) was from Biovision Inc. Sequencing-grade modified trypsin was from Promega Corp. Sources of other chemicals and full experimental details are in the Supporting Information (SI).

Preparation of MB–Biomolecule Conjugates.

MB-Ab, MB-ProA/G-Ab, and MB-trypsin conjugates were prepared according to the manufacturer protocols and our previously developed methods²⁵ with minor modifications (details in the SI). Carboxylated MBs were first washed and magnetically isolated 2 times using 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 5.2). MBs were activated for amidization using aqueous 400 mM 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDC) and 100 mM *N*-hydroxysulfosuccinimide (NHSS) for 15 min, followed by adding IgG2a Ab, ProA/G, or trypsin in 10 mM acetate buffer (pH 5.0) for amidization coupling. Unreacted EDC/NHSS derivatives were quenched with 100 mM ethanolamine (pH 8.0). MB-ProA/G conjugates were additionally incubated with IgG2a in 50 mM ammonium acetate (NH_4Ac) buffer (pH 8.0) to make MB-ProA/G-Ab conjugates. All MB conjugates were magnetically separated and washed 3 times with 50 mM NH_4Ac buffer (pH 8.0) before trypsin digestion. Numbers of protein molecules per MB were estimated using a Thermo Scientific Pierce Micro BCA Protein Assay Kit.²⁶

Trypsin Digestion under Nondenatured Conditions.

MB-Ab and MB-ProA/G-Ab conjugates were reconstituted in a 50 mM NH₄Ac buffer solution (pH 8.0) containing free trypsin or MB-trypsin with a trypsin/Ab (w/w) ratio of 1:1, respectively. The mixtures were then incubated at 37 °C with continuous stirring. At different times, trypsin digestion was stopped by adding formic acid. Internal standard peptide YAEGDVHATSKPARR was then spiked into the solutions. MBs were magnetically separated, while supernatant samples containing tryptic peptides were filtered through a 96-well filter plate (3 kDa molecular weight cut off, Pall Corp.) into a collection plate and subjected to LC-MS/MS analysis.

LC-MS/MS Analysis.

For the analysis of digest samples, a Thermo Scientific Dionex Ultimate 3000 UHPLC system was interfaced to either a SCIEX QSTAR Elite quadrupole time-of-flight mass spectrometer (Q-TOF, for peptide identification) or a Thermo Scientific TSQ Quantiva triple quadrupole mass spectrometer (QqQ, for peptide quantitation). Peptide separation was done on a Jupiter C18 column (150 × 0.50 mm, 5 μm, Phenomenex) with gradient elution (Table S1). Mass spectrometers were operated in positive ion mode. Information-dependent acquisition (IDA) was used on the Q-TOF for peptide identification and multiple reaction monitoring (MRM) on the QqQ for quantitation.²⁷ Key MS parameters are in the SI.

LC-MS/MS data from IDA (Q-TOF MS) were submitted to a local mascot server for MS/MS ion search using Mascot Daemon (version 2.3.0) against NCBIInr database.²⁸ Key identification parameters are in the SI. The Mascot-predicted peptide sequences and their MS/MS spectra are shown in Figure S1. Web-based bioinformatics tools, SIM Alignment Tool for protein sequences²⁹ and T-COFFEE Multiple Sequence Alignment Server,³⁰ were used to align the Mascot-predicted peptide sequences to a known mouse IgG2a antibody (PDB entry 1IGT). Results are summarized in Figure S2. An open-source bioinformatics tool, Skyline,³¹ was used to generate the transition list for MRM-MS (Table S2). The MRM data were then imported back to Skyline to obtain peak areas of peptides.

RESULTS

Tryptic Peptide Identification and Quantitation.

Unconjugated mouse monoclonal IgG2a was digested by trypsin under nondenaturing conditions. The resulting tryptic peptides were subjected to LC-MS/MS (Q-TOF) sequencing using a screening method called information-dependent acquisition (IDA). The acquired peptide MS/MS spectra were searched for peptide sequence match against the NCBIInr peptide database³² using the MASCOT MS/MS ions search engine. Eleven tryptic peptides (Pep 1–11) were successfully identified and further assigned into different domains and regions of a model mouse IgG2a antibody (PDB entry 1IGT) using alignment tools (Table 1, Figures 1, S1, and S2). Since proteolysis was done under nondenaturing, nonreducing condition, sequence coverage is comparatively lower than usual proteomic studies and no peptides from or near the hinge region of the antibody were found.

The IgG2a digest containing Pep 1–11 was spiked with internal standard YAEGDVHATSKPARR peptide and analyzed quantitatively by LC-MS/MS (QqQ) using multiple reaction monitoring (MRM). Three MRM transitions were used to identify each peptide, and the one with the largest peak area was selected for use in quantitation (Figure S3, Table S2). The peak areas of the peptides were normalized to the peak area of the internal standard in MRM chromatograms (Figure 2).

LC-MRM MS conditions were optimized so that the peptides chosen were well separated to provide a good basis for quantitation (Figure 2). Certain peptides were ruled out for further use to ensure the sensitivity, specificity, and robustness of peptide quantitation as they contained (1) methionine (M) which is prone to oxidation, (2) missed tryptic cleavage sites such as lysine–lysine (K–K), or (3) too many amino acids resulting in low analytical sensitivity. As a result, four representative peptides from four different antibody domains were selected for quantitation, i.e., Pep 2 (YASQSIGIPSR) from V_L domain, Fab region; Pep 5 (TSTSPIVK) from C_L domain, Fab region; Pep 8 (DLPAPIER) from C_H2 domain, Fc region; and Pep 11 (TELNYK) from C_H3 domain, Fc region. These four representative peptides all gave excellent linear calibration plots of peak area ratio of each representative peptide to internal standard vs peptide concentration (Figure 3). The different slopes are due to the different electrospray ionization efficiencies and variable fragmentation patterns of each peptide.³³

Trypsin and MB-Trypsin Digestion on Immobilized Antibodies.

Antibodies (Ab) were specifically captured by MB-ProA/G conjugates via Fc regions to give oriented antibodies (MB-ProA/G-Ab) or covalently conjugated to carboxylated MBs via EDC/NHSS-based amine-to-carboxyl nonoriented conjugation (MB-Ab, Scheme 1). Amounts of antibodies on MB-Ab and MB-ProA/G conjugates were estimated using Micro BCA Protein Assay (Figure S4A). The molecular weight of mouse IgG antibody is ~150 kDa, and 20 mg MB corresponds to 2.0×10^9 particles (according to vendor); $64\,000 \pm 6000$ antibody molecules were estimated on each MB-ProA/G-Ab, while $59\,000 \pm 7000$ antibody molecules were estimated on each MB-Ab, which is in good accord with a previous report.²⁵ No statistically significant difference of antibody numbers per bead was found between MB-ProA/G-Ab and MB-Ab using a *t*-test, $p > 0.05$.

We first used free trypsin in solution for proteolysis of MB-ProA/G-Ab and MB-Ab conjugates under nondenatured conditions, and the resulting peptide mixture was analyzed by LC-MS/MS. Plateaus in the MRM LC peak intensity were found between 6 and 12 h of reaction (Figure 4), indicating that digestion is complete. Thus, the peptide concentrations at each time interval were calculated using calibration plots (Figure 3) and compared with those at 12 h (defined as 100%). For the MB-ProA/G-Ab conjugates, a significantly larger digestion rate in the initial 3 h stage was found for Pep 2 (YASQSIGIPSR) from V_L domain, Fab region (Figures 4A and S5A), indicating the variable domains of the immobilized IgG2a antibody were oriented away from the MB and toward the trypsin solution due to Ab-stem linkage to the MB surface on the Fc-binding protein A/G. For MB-Ab conjugates, no significant difference among the digestion rates of the four representative

peptides was found (Figure 4B), consistent with randomly immobilized IgG2a similar opportunities for trypsin digestion of Fc and Fab domains.

Free trypsin enzymes are relatively small, and they can access inner parts of the antibody layer for proteolysis. Thus, we proceeded to restrict proteolysis to the outmost surfaces of the bead conjugates. To achieve this, trypsin was immobilized onto MBs of the same size as the MB-Ab materials. In this way, the effective range of MB-trypsin is more strictly confined to the outermost regions of the antibody layer due to steric effects. MB-trypsin will have a difficult time reaching the inner regions of the Ab layer, so that if Abs are 100% oriented, only Fab peptides should be found by LC-MS/MS. Thus, MB-trypsin digestion of MB-ProA/G-Ab resulted in much larger digestion rates from peptides 2 and 5 from the Fab regions, compared to Fc-stem peptides 8 and 11 (Figure 5A). The proteolysis rates of MB-Ab conjugates are more compressed and similar for the 4 peptides, even when a full 12 h reaction is observed (Figure S6). Results are consistent with EDC/NHSS linkage resulting in randomly immobilized antibodies on the surface. In addition, data from the first 6 h of reaction with MB-trypsin is clearly able to differentiate between oriented and nonoriented Abs on the bead surfaces.

It would be convenient to convert data in Figure 5A and 5B to a scale that indicates relatively how much antibody Fc-stem down orientation exists in a given system. We suggest that the ratio of the sum of the relative amounts of Fab peptides 2 and 5 divided by the sum of the relative amount of all four marker peptides 2, 5, 8, and 11 can serve this purpose (Table 2). After 1 h, this orientation ratio (R_o) is 87% for the MB-ProA/G-Ab-oriented beads and 66% for the EDC/NHSS-linked MB-Ab. The 87% R_o value is similar to the 91% of active antihorseradish peroxidase (HRP) antibodies that bound HRP at saturation to Au-ProA-Ab beads found by an enzyme activity assay. The 66% is relatively high compared to % activity of nonoriented Abs on surfaces,⁴ possibly reflecting the surface roughness of the beads used that can make more Fab regions available than on a flat surface.

At 2 h, R_o was 80% for Ab-oriented and 62% for nonoriented beads, while at 6 h, values were 70% for Ab-oriented and 53% for nonoriented beads. R_o trends downward as Fab peptides are freed, and relatively more peptides from Fc regions can be released as hydrolysis progresses. Thus, R_o measured for 1 h reactions is probably a more accurate metric of the original Ab orientation achieved.

PSA Capture Using MB-Ab Conjugates.

MB-Ab and MB-ProA/G-Ab conjugates were tested for the capture efficiency of antigen prostate-specific antigen (PSA) at 10 ng mL⁻¹ in 1 mL pH 7.4 solution with stirring using the anti-PSA property of mouse IgG2a. PSA captured on the beads was digested and quantified by LC-MS/MS MRM using the surrogate peptide SVILLGR from PSA.³⁴ The relative peak area of SVILLGR was proportional to the amount of PSA (Figure 6A). Using this calibration curve, the amounts of PSA captured by MB-Ab and MB-ProA/G-Ab conjugates was estimated. As expected, MB-ProA/G-Ab conjugates captured more PSA and at a faster rate (Figure 6B) than MB-Ab conjugates.

As a positive control for antibody availability for binding, fluorescein isothiocyanate (FITC)-labeled PSA was used to estimate PSA-binding fractions of antibodies on the MBs. At saturation binding levels of PSA, 85% of the antibodies bound PSA on the MB-ProA/G-Ab conjugates and 70% Abs bound PSA on MB-Ab ($\pm 15\%$, respectively, see SI). The 85% value is not statistically different from the $91 \pm 17\%$ for saturation PSA binding for IgG2a attached to Au nanoparticles by Protein A, estimated by an enzymatic method.⁹ This control suggests that the antibodies bound on MBs via protein A are intact and not significantly denatured.

DISCUSSION

The results above show that restricted proteolysis using MB-trypsin coupled with LC-MS/MS proteomic analysis permits direct evaluation of the orientation of surface-immobilized antibodies. Orientation evaluations are done in an aqueous, nondenaturing environment, which maintains the native conformation of antibodies. We restrict proteolysis to the outer layers of test MB-Ab systems by using trypsin attached to $1.5 \mu\text{m}$ diameter MBs to restrict access of the hydrolytic enzyme to the outer layers of the antibody layer. The orientation was monitored by identifying 2 position marker peptides on the Fab-binding regions of the test IgG antibody and 2 peptides on the Fc-stem region. LC-MS/MS analysis of these peptides gave linear calibration curves (Figure 3) suitable for quantitative evaluation of Ab orientation.

For the Fc-stem-down orientation of an Ab, important for antigen capture and immunoassay applications, the amounts and formation rates of the Fab peptides during proteolysis by MB-trypsin should be larger than those of the Fc peptides. Results show that this differentiation is achieved to some extent for free trypsin proteolysis (Figure 4), but Fc-Fab differentiation is larger and more informative for the restricted MB-trypsin proteolysis (Figure 5). A relative degree of orientation can be assessed by dividing the sum of amounts of the 2 Fab peptides by the sum of all four marker peptides and expressing as a percentage R_o . For our fully oriented MB-ProA/G-Ab at 1 h reaction, R_o was 87% and 66% for a nominally nonoriented Ab attached to MB by EDC chemistry.

The different proteolysis rates of peptides from different antibody regions directly reflect the circumstances of antibody orientation. The sequence specificity of peptides also makes this approach adaptable to assess the immobilized orientation of other proteins besides antibodies using appropriate peptides as a protein orientation interpreter. There is an average of 62 000 Abs per $\sim 1.5 \mu\text{m}$ diameter magnetic bead (MB), indicating that one antibody covers an average area of $11 \times 11 \text{ nm}^2$, comparable and consistent with IgG dimensions of $15 \times 9 \times 4 \text{ nm}$.³⁵ However, the irregular shape of the MBs provides an area 20–30 times that of the same size spherical particle according to the vendor, resulting in an average of one Ab in an area $55 \times 55 \text{ nm}^2$. Thus, 62 000 antibody molecules cover less than 5% of the total MB surface and on average are separated by $\sim 50 \text{ nm}$ if all are in the Fc-stem-down orientation. As trypsin is a relatively small enzyme ($\sim 23 \text{ kDa}$) compared to IgG2a ($\sim 150 \text{ kDa}$), free trypsin enzymes can diffuse through the gaps between antibodies and gain access to the inner regions of the antibody layer during proteolysis. The flexible structure of Abs and the linear shape of protein A/G containing multiple Fc-binding domains enhance this possibility.

Thus, free trypsin may hydrolyze both the Fc and the Fab regions on the MB, giving similar digestion rates for peptides from the Fab region and the peptides from Fc region (Figure 4A).

We chose the 1.5 μm beads to carry trypsin because they cannot enter into the $55 \times 55 \text{ nm}$ region between Abs on the beads, and proteolysis is thus restricted to the outermost regions of antibody layers. As a result, effective restrictive proteolysis makes it possible to minimize the complexity of the peptide mixture.²¹ Sample preparation is also simplified, as proteolysis is stopped by applying a magnetic field to remove the MBs, and supernatant solution that contains the tryptic peptides is collected. Contamination with trypsin or antibody is eliminated, and no further sample workup is required.³⁶

Improved antigen capture was observed when IgG antibodies were well oriented on the MB. Other reports using Fab fragment or single-chain Fv fragment (scFv) have a comparable impact in terms of antigen capture.³⁷ The Fab or scFv is smaller and more specific to antigen than whole IgG molecules; thus, they are emerging as novel biorecognition elements in biosensors. However, whole IgG antibodies are relatively cheap and well studied. No matter whether the immobilized protein is whole IgG antibody, Fab fragment, or scFv, we provide this general method to assess the degree of orientation of immobilized proteins on surfaces.

CONCLUSIONS

Restricted proteolysis using MB-trypsin of a diameter greatly exceeding that of the average distance between Ab molecules on a surface can be employed to directly evaluate the antibody orientation on surfaces of MBs and, by extension, flat surfaces. Oriented antibodies also showed improved antigen capture efficiency compared to randomly immobilized antibodies. Analysis of marker peptides from Fc and Fab regions of an IgG by LC-MS/MS confirm that protein A/G increases the fraction of Fc-stem-down-oriented Abs, while EDC/NHS linkage resulted in a more random Ab immobilization. In addition to antibodies, this approach can be extended in principle to analyze the orientation of virtually any type of protein immobilized on a surface.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Scheme 1. Antibody Orientation Estimation Using Restricted Trypsin Proteolysis^a

^aAntibodies were first immobilized onto the carboxylated magnetic bead (MB) surface in either oriented or randomly immobilized manner. Immobilized trypsin MBs (MB-trypsin) then perform restricted proteolysis onto the uppermost surface of antibody layer and result in certain representative tryptic peptides, leading to characterization of various orientation states using LC-MS/MS analysis. Here, EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) and NHSS (*N*-hydroxysulfosuccinimide) are commonly used for amine-to-carboxyl conjugation of proteins to carboxylated MBs.

Figure 1.
Locations of peptides 1–11 (Table 1) in IgG2a antibody (Protein database entry 1IGT).³²

Figure 2.

Representative extracted ion chromatograms (XIC) for 12 MRM transitions (peaks) for quantitation of 11 tryptic peptides from mouse IgG2a antibody with internal standard peptide: (1) DIVLTQSPATLSVTPGDR; (2) YASQSISGIPSR; (3) WKIDGSR; (4) QNGVLNSWTDQDSK; (5) TSTSPIVK; (6) LSISKDNSK; (7) VVSALPIQHQQDWMSGK; (8) DLPAPIER; (9) APQVYVLPPPEEEMTK; (10) APQVYVLPPPEEEMTKK; (11) TELNYK; (12) YAEGDVHATSKPARR internal standard. See Table S2 for MRM m/z transitions.

Figure 3. Calibration plots of normalized peak areas ratio for four representative peptides from mouse IgG2a vs peptide concentration. Error bars are standard deviations for $n = 3$.

Figure 4.

Free trypsin digestion kinetic results over 12 h for (A) MB-ProA/G-Ab and (B) MB-Ab conjugates. Error bars are standard deviations for $n = 3$. As indicated by asterisk (*), only statistically significant differences were found between Pep2 and Pep11 in (A) using t -test ($p < 0.01$). (C) Different locations of four representative peptides Pep 2, 5, 8, and 11 in an antibody, which results in different digestion kinetics in A.

Figure 5.

MB-digestion kinetic results using MB-trypsin for 6 h reactions: (A) MB-ProA/G-Ab and (B) MB-Ab conjugates. Error bars are standard deviations for $n = 3$. As indicated by asterisk (*), only statistically significant differences were found between Pep2 and Pep11 or Pep5 and Pep 11 in A using t -test ($p < 0.01$). (C) Different locations of four representative peptides Pep 2, 5, 8, and 11 in the antibody that result in different digestion kinetics in A.

Figure 6.

Analysis of PSA capture from 10 ng mL^{-1} solution, pH 7.4: (A) PSA samples of different masses were digested by trypsin, and resulting peptides were spiked with internal standard analyzed by MRM LC-MS/MS. Linear calibration curve is shown between the peak area of PSA peptide SVILLGR relative to internal standard and the mass of PSA. (Inset) Linearity at low PSA levels. (B) Amounts of PSA captured from 1 mL of 10 ng mL^{-1} PSA using MB-ProA/G-Ab (red) and MB-Ab (blue) conjugates. Error bars are standard deviations for $n = 3$.

Table 1.

Sequences of Mascot Software-Predicted-Peptides from Target IgG2a Antibody, with Assigned Domains and Regions

peptide no.	peptide sequence	domain	region
Pep 1	DIVLTQ_SPATLSVTPGDR	V _L	Fab
Pep 2	YASQSISGIPSR	V _L	Fab
Pep 3	WKIDGSR	C _L	Fab
Pep 4	QNGVLNSWTDQPSK	C _L	Fab
Pep 5	TSTSPIVK	C _L	Fab
Pep 6	LSISKDNSK	V _H	Fab
Pep 7	WSALPIQHQPWMSGK	C _{H2}	Fc
Pep 8	DLPAPIER	C _{H2}	Fc
Pep 9	APQVYVLPPPEEEMTK	C _{H3}	Fc
Pep 10	APQVYVLPPPEEEMTKK	C _{H3}	Fc
Pep 11	TELNYK	C _{H3}	Fc

Table 2.Orientation Ratio R_o Characterizing MB-Digestion Kinetics Using MB-Trypsin

reaction time (h)	MB-ProA/G-Ab	MB-Ab
1.0	87%	66%
2.0	80%	62%
3.0	78%	60%
4.0	77%	57%
5.0	74%	55%
6.0	70%	53%