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Sol-Gel Materials Used in Phosphoproteomics and Glycoproteomics Applications

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Abstract

Glycosylation and phosphorylation are two of the most commonly seen and important post-translational modifications (PTMs) of proteins. Both play key role in many vital biological processes. Mass spectrometry is the most favored technique used for monitoring the dynamics of PTMs. Mass spectrometric analysis of phosphopeptide and glycopeptide is a crucial step in phosphoproteomics and glycoproteomics to understand the role of these modifications in the cellular pathways. Enrichment and purification of (phospho- and glyco-) peptides and glycans are recommended prior to mass spectrometric analysis because of the lower amount of modified peptides in a peptide mixture. Herein, we present titanium/silica and zirconium/silica sol-gel materials for the enrichment of (phospho- and glyco-) peptides and glycans to enhance MALDI-MS analysis performance. Enrichment of (phospho- and glyco-) peptides was successfully applied using standard proteins including β -casein, fetuin, and IgG as well as some complex medium. The sol-gel materials were compared with commercial metal oxides regarding their modified peptide enrichment performances.

Keywords: sol-gel materials, phosphoproteomics, glycoproteomics, mass spectrometry, enrichment

1. Introduction

The post-translational modification (PTM) of proteins plays a critical role in regulating many cellular pathways [1, 2]. There is a wide range of chemical modifications that have been observed in a protein after protein synthesis and these modifications are vital for protein function, localization, and turnover [3, 4]. The modifications that occur in protein structures after the translation step control many biological activities, so it is very important to examine the

roles of these modifications in cell regulation mechanisms. Functional diversity of proteins is increased by PTMs. Also, aberrant posttranslational modifications can cause several cellular diseases. Therefore, analysis of modified proteins is necessary to better understand the relationship between PTMs and functional changes. The detailed analysis of proteins in complex samples and the determination of the locations of modified amino acids are very important in terms of the understanding of cellular signaling mechanisms [5, 6].

Glycosylation and phosphorylation are two of the most commonly seen PTMs of proteins. Both are involved in many biological events, such as immune response, signal transduction, and cell-to-cell interaction [1, 6, 7]. Phosphoproteomics and glycoproteomics are the significant proteomics areas in which phosphorylated and glycosylated proteins are identified and characterized [2, 7, 8]. Many researches have been made in these fields in order to discover new biomarkers and drug targets [9]. Analysis performed for the characterization of (phospho- and glyco-) proteins needed huge efforts and intense operations. Mass spectrometry-based (phospho- and glyco-) proteomics studies using modern mass spectrometers enable fast and sensitive characterization of modified proteins [8, 10, 11]. Bottom-up approach is the most used application for the analysis of (phospho- and glyco-) proteins by mass spectrometry. By this approach, proteins of interest are enzymatically digested into complex peptide mixtures and then the peptide fragments are either separated using a chromatographic technique prior to mass spectrometric analysis or directly analyzed by a mass spectrometer [12]. Several difficulties are observed in the analysis of phosphorylated and glycosylated peptides by mass spectrometry. The abundance of (phospho- and glyco-) peptides in the peptide pool is usually less than ordinary peptides. Because the large amount of ordinary peptides generated by the proteolytic digestion of proteins, the ion signals of (phospho- and glyco-) peptides are suppressed. Moreover, the ionization efficiency of these modified peptides is low in mass spectrometric analysis.

Thus far, numerous enrichment and separation techniques have been developed in order to analyze both (phospho- and glyco-) peptides. Also, various approaches including covalent and noncovalent interaction-based methods can be used for the enrichment of (phospho- and glyco-) peptides. These analytical methods and strategies based on different interaction modes have been reviewed in detail [13–20]. Since these methods are complementary to each other, they can be applied together to improve enrichment and separation efficiency.

Sol-gel process is a chemical synthesis method based on inorganic polymerization reactions [21]. One of the advantages of sol-gel process is that little or no heating is required during reaction. The reaction is carried out in two steps: hydrolysis of metal alkoxides and polycondensation of hydroxyl groups. Consequently, three-dimensional sol-gel network is formed. The resulting sol-gel material is in the form of a solid polymeric network structure with chains having diameters in the micrometer size and chains with average lengths greater than a micrometer [22]. In a general sol-gel method, the precursors with lower molecular weight such as tetramethoxysilane (TMOS) or tetraethoxysilane (TEOS) are hydrolyzed in the presence of water, acid catalyst, and proper solvents. Titanium(IV) isopropoxide, tantalum(V) ethoxide, and zirconium(IV) ethoxide are widely used metal alkoxide precursors for producing metal-based sol-gel materials. Metal alkoxides of titanium, zirconium, and tantalum

are more reactive than alkoxy-silanes due to their lower electronegativity and higher Lewis acidity [23].

Sol-gel materials are of interest because of their potential to be produced with tailor-made pore size and shape. They have many advantages over organic polymers [24]. For example, the thermal stability of organic polymers is too low to compare with the sol-gel materials. In the sol-gel synthesis method, the control of the thickness, porosity, and surface area is easier [25, 26]. The sol-gel materials can be synthesized very purely in the appropriate temperature range and modified in mild conditions. The compositions of hybrid sol-gel materials can be well controlled. Sol-gel-based materials have been used for various bioapplications, such as enzyme encapsulation [27], immobilization [28], and biosensor [29]. Recently, our group has introduced tantalum-based and amine-functionalized sol-gel materials to enrich phosphopeptides from complex medium [30, 31]. Sol-gel materials that are fully compatible for the entire enrichment studies are not yet available. Methods and materials for enriching phosphopeptides and glycopeptides have to be selected depending on the sample to be analyzed.

In this chapter, we describe specific enrichment methods for the phosphopeptides and glycopeptides using titanium/silica and zirconium/silica sol-gel materials. Several standard (phospho- and glyco-) proteins including beta-casein, fetuin, and IgG were used to test enrichment efficiency of the materials prior to MALDI-MS analysis. Also, 2-AA-labeled human plasma N-glycans were analyzed by MALDI-MS after purification with titanium/silica and zirconium/silica sol-gel materials, allowing it to apply for further proteomics applications.

2. Materials and methods

2.1. Materials

If not otherwise stated, all the reagents, solvents, and proteins were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used without further purification. Sequencing grade trypsin was obtained from Roche Diagnostic GmbH (Mannheim, Germany). Human plasma (lyophilized, P9523) was bought from Sigma-Aldrich (St. Louis, MO, USA). Titanium(IV) oxide with a 21 nm primary particle size [transmission electron microscopy (TEM)] and $\geq 99.5\%$ purity (trace metals basis) and zirconium(IV) oxide powder, 5 μm , 99% (trace metals basis) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Purified water used in all experiments was obtained using an Expe-Ultrapure Water System (Mirae St. Co., Korea).

2.2. Methods

2.2.1. Synthesis of the titanium/silica and zirconium/silica sol-gel materials

A solution of ethanol and water was mixed (2.0 mL, ethanol/water, 5/1, v/v) in a reaction vessel. Titanium(IV) isopropoxide and TMOS (tetramethyl orthosilicate) were mixed with a molar ratio of 5:1 and added to the reaction vessel. After stirring for 20 min, 20 μL of fuming nitric

acid was added drop wise to the sol. The mixture was then stirred overnight to produce a wet gel. The wet gel was then washed with a mixture of water and ethanol to remove residues and dried at 50°C in vacuum prior to use. Some amount of the gel powder was calcined at 500°C for 2 h. The same protocol was also applied as described above to synthesize the zirconium/silica sol-gel material using zirconium(IV) ethoxide instead of titanium(IV) isopropoxide.

2.2.2. *Proteolytic digestion of standard proteins*

One milligram of fetuin, IgG, bovine serum albumin (BSA), and β -casein were separately dissolved in 78 μ L of 25 mM ammonium bicarbonate (ABC) prior to digestion. The reduction of cysteine residues was achieved by the addition of 2 μ L of DTT (1,4-dithiothreitol, 200 mM in 25 mM ABC) to these solutions and the samples were incubated at 60°C for 30 min. After adding 6.5 μ L of IAA (2-iodoacetamide, 200 mM in 25 mM ABC) to the solutions for alkylation of reduced cysteine residues, the samples were incubated in the dark at room temperature for 20 min. Finally, proteomics grade trypsin was added to these solutions (protein/enzyme, 30/1, w/w) and the samples were incubated overnight at 37 °C. The final solutions of the digested samples were diluted to a proper concentration for the enrichment studies.

2.2.3. *N-glycan release*

Human plasma (glyco-) proteins (3 mg) were denatured by the addition of 100 μ L of 2% SDS and the sample was incubated at 60°C for 10 min. Then, 40 μ L of 4% NP-40 and 4 U of PNGase F in 40 μ L of 5 \times phosphate buffered saline (PBS) were added to this solution. The sample was incubated overnight at 37°C in order to release N-glycans.

2.2.4. *Labeling of human plasma N-glycans with 2-aminobenzoic acid (2-AA)*

The released human plasma N-glycans were labeled by 2-AA as described previously with minor modifications [32]. The released glycan sample (50 μ L) was mixed with 25 μ L of 2-AA (47 mg/mL) prepared in DMSO/glacial acetic acid (17:3, v/v) and 25 μ L of 2-picoline borane (107 mg/mL in DMSO). The incubation was performed at 65°C for 2 h. The reaction mixture was then cooled to room temperature and used for 2-AA human N-glycan analysis by MALDI-TOF-MS after purification with titanium/silica and zirconium/silica sol-gel materials.

2.2.5. *Phosphopeptide enrichment by titanium/silica and zirconium/silica sol-gel materials*

Five milligram of the materials was washed three times with methanol prior to the enrichment process. The washed materials were then equilibrated three times with 1000 μ L of loading solution (ACN/water (1:1, (v/v) containing 1% (v/v) TFA). The mixture of the tryptic digests of BSA and β -casein at a 50:1 mole ratio (BSA: β -casein) was diluted in 50 μ L of the loading solution and mixed with the material for 30 min at room temperature. The supernatant was then collected for further processes and the sol-gel particulates were washed with 1000 μ L of the loading solution to remove unmodified peptides. The elution of phosphopeptides was achieved using 20 μ L of 50 mM ABC solution adjusted to pH 10–11 with the addition of ammonia.

2.2.6. Glycopeptide enrichment and N-glycan purification by titanium/silica and zirconium/silica sol-gel materials

Three milligram of the materials were washed two times with 800 μL of loading buffer, which is composed of ACN/water/TFA, 80:15:5, v/v/v containing 1 M glycolic acid. Subsequently, 5 μL of fetuin and IgG digests (from 1- $\mu\text{g}/\mu\text{L}$ digest samples) were individually diluted in 40 μL of the loading buffer and incubated with the materials for 20 min at room temperature. The supernatant was then collected for further processes and the sol-gel particulates were washed with 800 μL of the loading buffer, washing buffer 1 (ACN/water/TFA, 80:19:1, v/v/v) and washing buffer 2 (ACN/water/TFA, 20:79.9:0.1, v/v/v) to remove interferences. Before elution step, the sol-gel particulates were dried in a speedVac for 5 min. The elution of glycopeptides was achieved using 20 μL of 25 mM ABC solutions adjusted to pH 10–11 with ammonia.

The 2-AA-labeled N-glycans were purified by the materials (3 mg) with a similar method as described above. Briefly, 5 μL of 2-AA-labeled human plasma N-glycan sample was mixed with 40 μL of loading buffer (ACN/water/TFA, 88:11.8:0.2, v/v/v) and incubated with the materials for 10 min at room temperature. After the incubation step, the sol-gel particulates were washed with 800 μL of the loading buffer. Then, the sol-gel particulates were dried with a speedVac for 5 min. Finally, elution was then performed by the addition of 20 μL of 25 mM ABC solutions adjusted to pH 10–11 with ammonia.

2.2.7. MALDI-TOF-MS analysis

A Voyager-DE PRO MALDI-TOF-MS (Applied Biosystems, USA) containing a 337-nm nitrogen laser was used for the analysis. All spectra except for purified 2-AA-labeled N-glycan analysis were obtained in positive ion linear mode (mass range from m/z 1000 to 5000) using an acceleration potential of 20 kV. The instrument was externally calibrated using a standard peptide mixture. A 2,5-dihydroxybenzoic acid (DHB) matrix solution (10 mg/mL) was prepared in ACN:water (1:1, v/v) containing 0.1% orthophosphoric acid. Eluates (1 μL) were spotted onto MALDI target plate and co-crystallized with 1 μL of the DHB MALDI matrix.

3. Results and discussion

3.1. Enrichment of phosphopeptides from the mixture of β -casein/BSA digests

The noncovalent interaction of the phosphate group with metal cations or metal oxides is utilized in the commonly used phosphopeptide enrichment methods [33, 34]. Surfaces of the materials such as titanium dioxide (TiO_2) and zirconium dioxide (ZrO_2) become positively charged at low pH, making them suitable for binding of phosphopeptides. TiO_2 is the most commonly used metal oxide in the phosphopeptide enrichment methods [20, 35]. Oxides of various metals other than titanium are also used in the phosphopeptide enrichment studies [31, 36–41]. The phosphate groups of phosphopeptides form coordination with metal ions present on the metal oxide surface at acidic pH. Herein, the phosphate groups act as the electron pair donor bidentate ligand while metals are the corresponding electron

pair acceptor. The metal oxides used in the phosphopeptide enrichment studies are the oxides of the transition metals [42]. However, acidic nonphosphopeptides may also interact with the metal oxide surface at similar conditions. These undesired interactions lower the specificity of the phosphopeptide enrichment method. The interactions of phosphopeptides with metal oxides vary depending on the pH of the medium. By maintaining the pH at an appropriate value, the phosphopeptides can be retained on the surface while the other peptides are removed. The phosphopeptides act as Lewis bases at pH range 2.0–2.5 while the metal oxide surface tends to accept electrons. At this pH range, the carboxylic acid groups of the acidic nonphosphopeptides having pK_a ($-\log_{10}K_a$, K_a is the acid dissociation constant) values higher than phosphopeptides are in neutral state by retaining their protons. In this case, the phosphopeptides adhere to the metal oxide surface, while the surface does not interact with acidic nonphosphopeptides in the neutral state. When the pH is increased, the surface of the metal oxide material becomes neutral this time. The Lewis base effect of the phosphate groups of the phosphopeptides will be greatly reduced due to the increased amount of OH^- ions in the medium. Thus, phosphopeptides can easily be removed from the surface by breaking the interactions that support the formation of coordination between the species. The pH range is adjusted to 9.0–10.0 at the elution step of phosphopeptide enrichment studies using metal oxides.

The phosphopeptide enrichment performances of sol-gel materials containing titanium and zirconium metals were evaluated by using data obtained from MALDI-MS analysis. The phosphopeptides were attempted to enrich from tryptic digest mixtures of BSA and β -casein at a 50:1 mole ratio (BSA: β -casein). The peptide mixture of the proteins was analyzed using MALDI-MS without an enrichment step. None of the phosphopeptide signals could be observed in MALDI-MS analysis of the non-enriched sample (**Figure 1**).

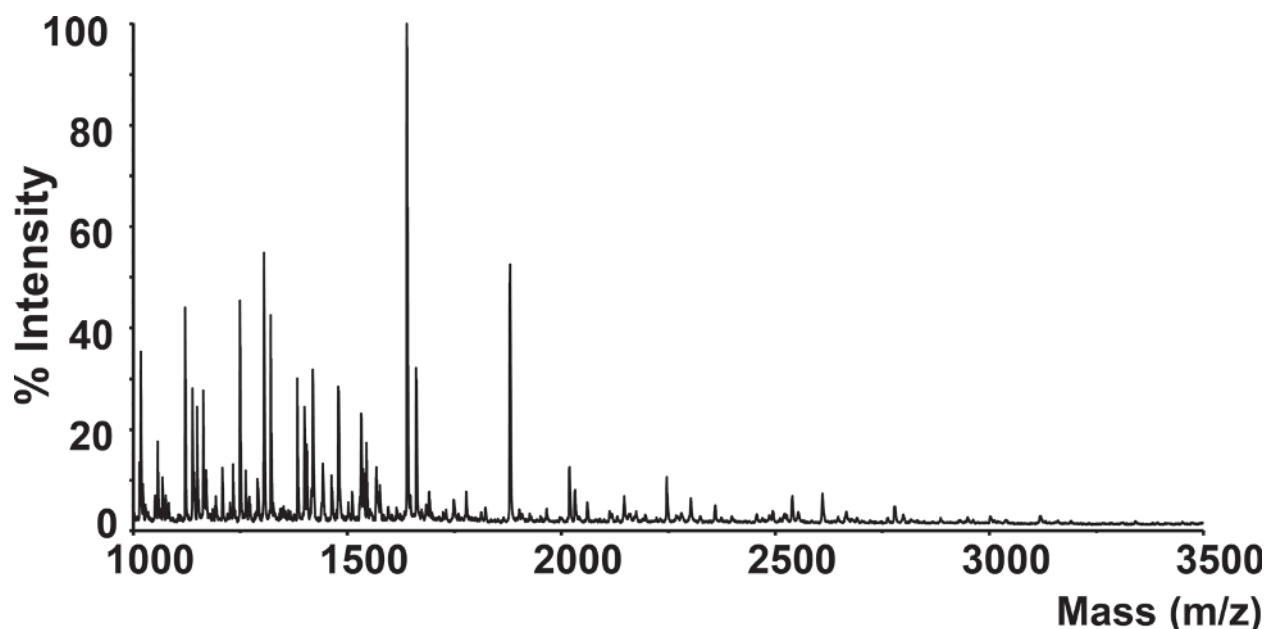


Figure 1. MALDI mass spectrum obtained from the direct analysis of the tryptic digest mixture of BSA and β -casein (in 50:1 mole ratio).

The phosphopeptide enrichment method was initially performed using commercial TiO_2 material. In the mass spectrum obtained from the MALDI-MS analysis of the enriched sample using TiO_2 , the signals of three phosphopeptides ($\beta 1$, $\beta 3$, and $\beta 4$) could be observed in the presence of intense nonphosphopeptide signals (Figure 2A). The list of phosphopeptides obtained from tryptic digestion of β -casein is given in Table 1. This result indicates that TiO_2 is not enough selective for phosphorylated peptides.

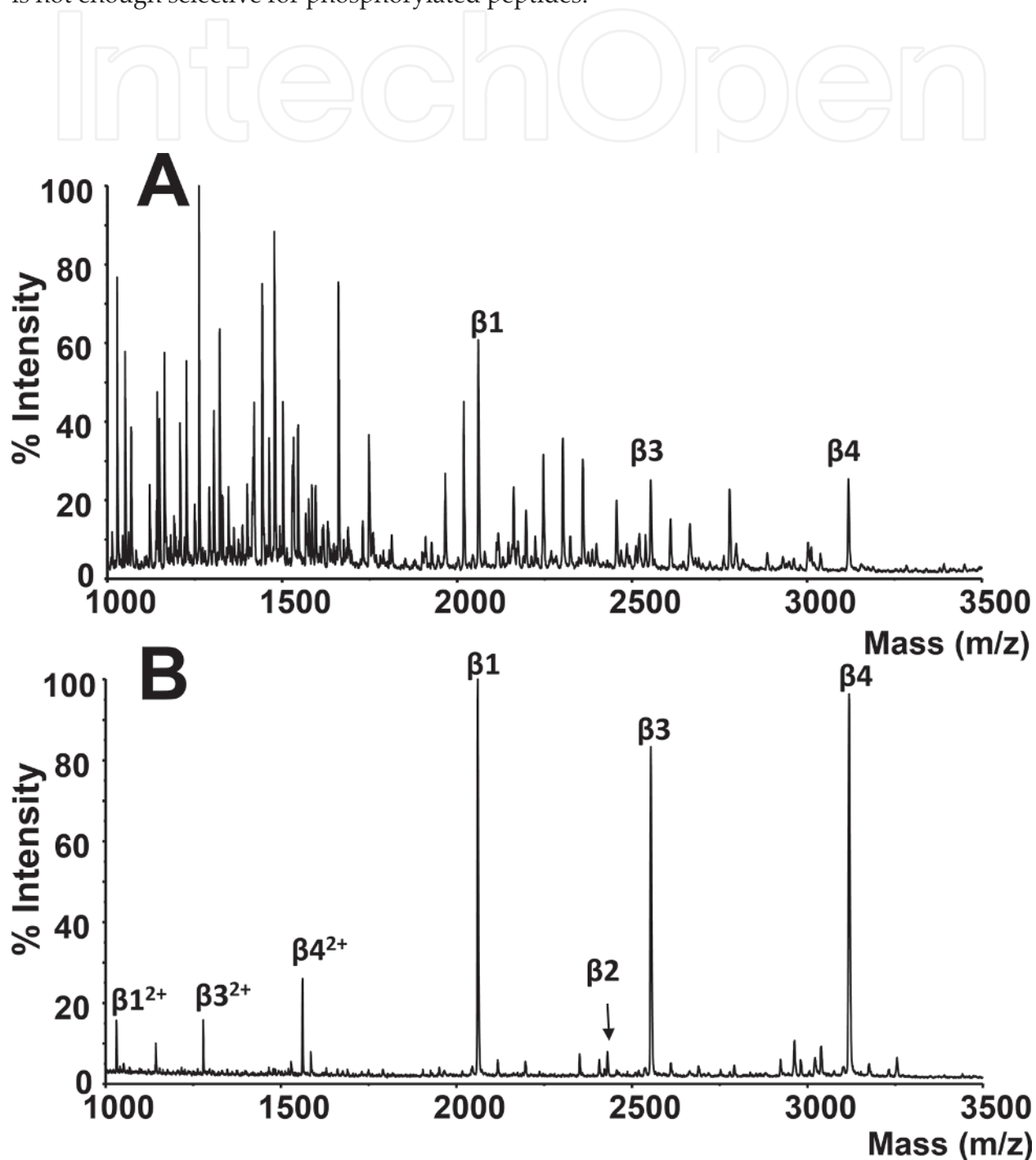


Figure 2. MALDI mass spectra obtained from the tryptic digest mixture of BSA and β -casein after enrichment with (A) commercial TiO_2 and (B) titanium/silica sol-gel material. The signals of the phosphopeptides are indicated by $\beta 1$, $\beta 2$, $\beta 3$, and $\beta 4$.

Phosphopeptides	Amino acid sequence	[M + H] ⁺
β1	FQ[pS]EEQQQTEDELQDK	2061.81
β2	IEKFQ[pS]EEQQQTEDELQDK	2432.08
β3	FQ[pS]EEQQQTEDELQDKIHPF	2556.03
β4	RELEELNVPGEIVE[pS]L[pS][pS][pS]EESITR	3122.26

Table 1. List of phosphopeptides obtained from tryptic digestion of β-casein.

The intense signals of four phosphopeptides were observed in the mass spectrum obtained from the analysis of the enriched sample using titanium/silica sol-gel material. Signals of doubly charged $[M + 2H]^{2+}$ molecular ions of three phosphopeptides (β1, β3, and β4) were also observed in the mass spectrum (**Figure 2B**). There were no signals of nonphosphopeptides or impurities other than phosphopeptides in the mass spectrum given in **Figure 2B**. The results show that the titanium/silica sol-gel material is far more successful than the commercial TiO₂ in phosphopeptide enrichment performance.

The digest mixture was also used to test the phosphopeptide enrichment efficiency of the zirconium/silica sol-gel material. The commercial ZrO₂ was compared with the zirconium/silica sol-gel in terms of the phosphopeptide enrichment performance. When the mass spectrum obtained from the analysis of the enriched sample using commercial ZrO₂ is evaluated, it is clear that the metal oxide is not selective for phosphopeptides (**Figure 3A**). The signals of three phosphopeptides (β1, β3, and β4) could be observed in the mass spectrum with the great complexity of the nonphosphopeptide signals.

The signals of four phosphopeptides could be clearly observed in the mass spectrum obtained from the analysis of the sample enriched using the zirconium/silica sol-gel material (**Figure 3B**). Signals of doubly charged $[M + 2H]^{2+}$ molecular ions of two phosphopeptides (β3 and β4) were also observed in the mass spectrum with low intensity. The signal at *m/z* 2351 represents dephosphorylation through neutral loss of HPO₃ (80 Da) from β2 (**Figure 3B**). The results show that the zirconium/silica sol-gel material has a rather high phosphopeptide selectivity compared to the commercial ZrO₂.

3.2. Enrichment of glycopeptides from the fetuin and IgG digests

Metal oxides are commonly used tools for the enrichment of sialylated glycopeptides as in phosphopeptide enrichments. The noncovalent interaction is formed by the way of coordination between the metal ions on the metal oxide and the oxygen atom or the nitrogen atom on the glycan. In addition, the carboxyl groups of sialic acid containing glycopeptides are interacted with positively charged ions on the metal-oxide surface at acidic pH [13]. In order to eliminate the interference effect of phosphopeptides, in the method, specific enzymes such as alkaline phosphatase are usually applied prior to the enrichment of glycopeptides. However, some acidic peptides containing glutamic acid and aspartic acid amino acid residues are also interacted with the surface of metal oxides, thereby decreasing the enrichment selectivity.

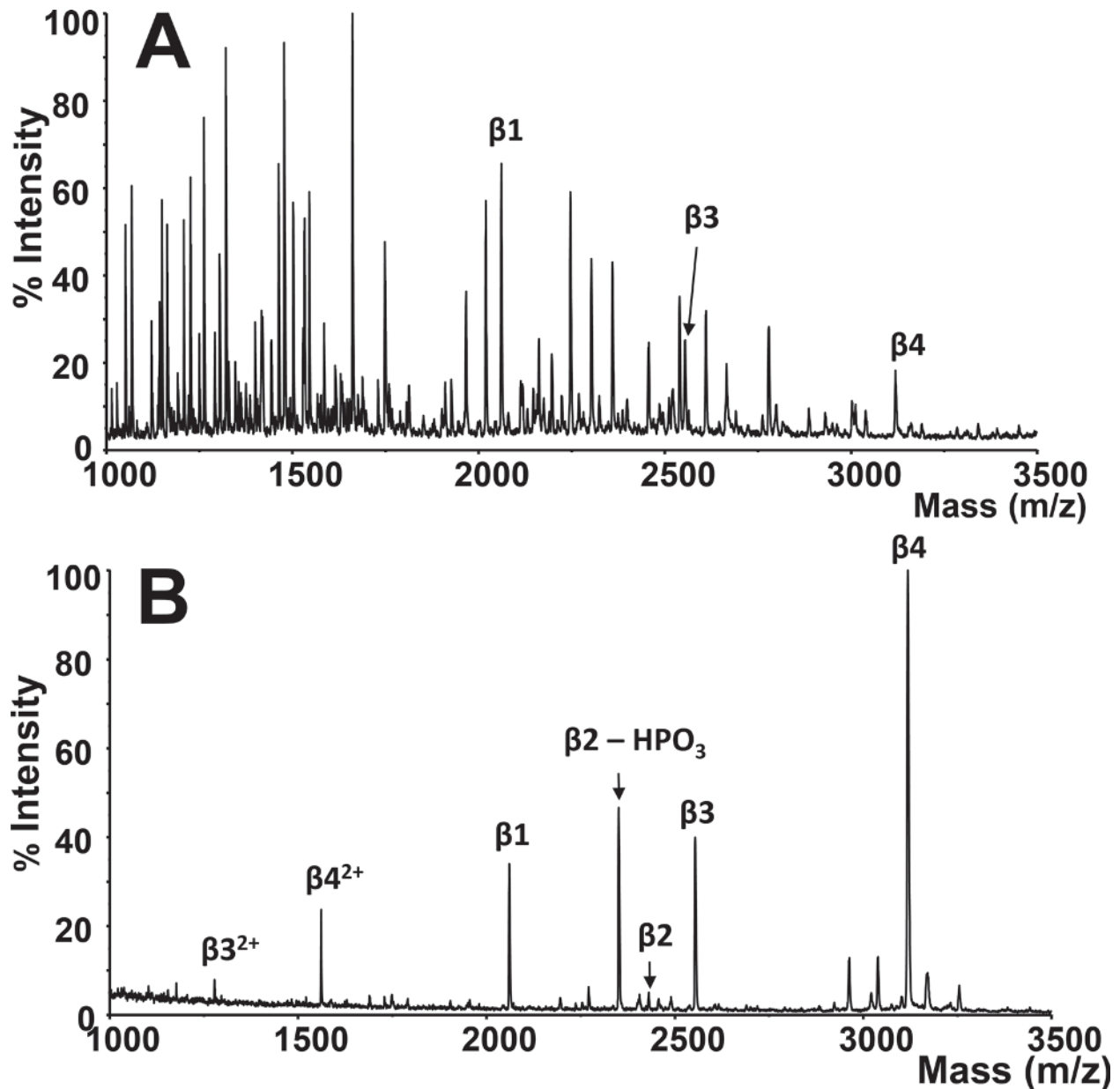


Figure 3. MALDI mass spectra obtained from the tryptic digest mixture of BSA and β -casein after enrichment with (A) commercial ZrO_2 and (B) zirconium/silica sol-gel material. The signals of the phosphopeptides are indicated by $\beta 1$, $\beta 2$, $\beta 3$, and $\beta 4$.

Elution of glycopeptides from the surface of metal oxides was achieved increasing the pH to 10–11 range.

In the study, fetuin and IgG proteins including sialylated and neutral glycans, respectively, were selected to evaluate glycopeptide enrichment performance of the sol-gel materials. Both proteins were enzymatically digested and the resulting peptide mixtures containing glycopeptides were used to identify their glycosylation sites. To achieve this, 5 μ g of fetuin and IgG digests were individually diluted with loading buffer and used for the enrichment

studies. Before the enrichment of sialylated glycopeptides of fetuin, direct analysis of tryptic products of fetuin was performed using MALDI-MS and no significant glycopeptide signal was observed in the mass spectrum (**Figure 4A**). When commercial ZrO_2 was used for the

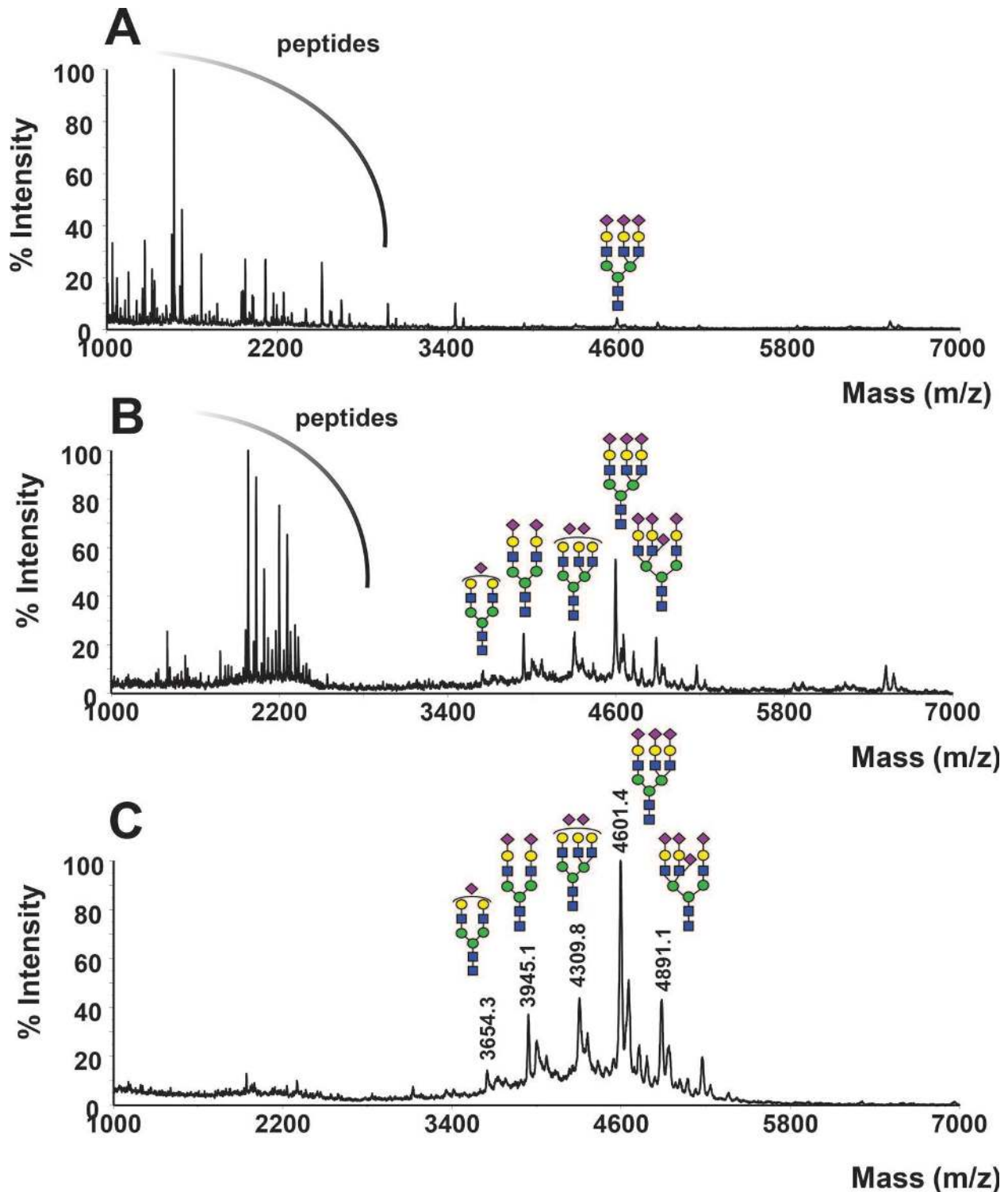


Figure 4. MALDI mass spectra obtained from the tryptic digest of fetuin. (A) Direct analysis, (B) after the enrichment with commercial ZrO_2 , and (C) after the enrichment with zirconium/silica sol-gel material.

enrichment of fetuin glycopeptides, the signals of non-glycosylated peptides were observed in the MALDI-MS spectrum together with glycosylated peptide signals (**Figure 4B**). The glycopeptides of fetuin belonging to Asn156 glycosylation site were identified. By the enrichment of glycopeptides using zirconium/silica sol-gel material, glycopeptides of fetuin were detected with improved signal intensities in MALDI-MS analysis (**Figure 4C**). This clearly shows that the zirconium/silica sol-gel material is more selective than commercial ZrO_2 for glycopeptides.

To evaluate the enrichment performance of the metal containing sol-gel materials in the presence of the glycopeptides including neutral glycans, tryptic (glyco-) peptides of IgG were enriched by titanium/silica sol-gel materials. In the direct analysis of tryptic products of IgG with MALDI-MS, a few glycopeptides were identified (**Figure 5A**) and non-glycosylated peptides dominated the spectrum.

In addition, commercial TiO_2 was also used to compare enrichment performance of the material with titanium/silica sol-gel material. When both spectra were obtained from the enrichment of glycopeptides by TiO_2 and titanium/silica sol-gel materials (**Figure 5B and C**), the enrichment selectivity and efficiency of titanium/silica sol-gel materials was found to be better than that commercial TiO_2 . These results also demonstrate that the glycopeptides containing neutral glycans were efficiently enriched by the sol-gel materials as well as sialylated glycopeptides.

It is stated in the literature that the salt removal is essential for the efficient analysis of glycopeptides by MALDI-MS [43]. Higher salt concentration causes decrease in ionization efficiency and suppresses glycopeptide signals. The developed sol-gel materials successfully remove salts and detergents derived from the reducing and alkylating reagents.

3.3. Purification of 2-AA-labeled N-glycans

N-glycan profiling of human plasma is very significant for clinical glycomics [44]. Glycan biomarker discovery studies focus on glycan analysis using high-throughput techniques [45]. 2-AA labeling of N-glycans is one of the commonly applied sample preparation methods for the analysis of N-glycans by MALDI-MS, HPLC-HILIC-Florescence, and CE-MS [46]. Purification of 2-AA-labeled N-glycans prior to MALDI-MS analysis is required and crucial in order to remove salts (PBS), detergents (SDS, NP-40), and solvents (DMSO).

In the study, N-glycans derived from human plasma were labeled with 2-AA and purified by both titanium/silica and zirconium/silica sol-gel materials, and thus MALDI-MS analysis of N-glycans was successfully achieved. However, the analysis of 2-AA-labeled N-glycans was not performed without a purification step. No N-glycan signals were observed in the mass spectrum (data not shown).

Figure 6A and B shows the MALDI-MS spectra of the purified 2-AA-labeled N-glycans after the purification step using zirconium/silica and titanium/silica sol-gel materials, respectively. As shown in **Figure 6A and B**, N-glycome profile was observed by the MALDI-MS analysis of purified 2-AA-labeled N-glycans carried out in negative ionization mode. By this approach, not only sialylated glycans but also neutral glycans were purified due to having florescence

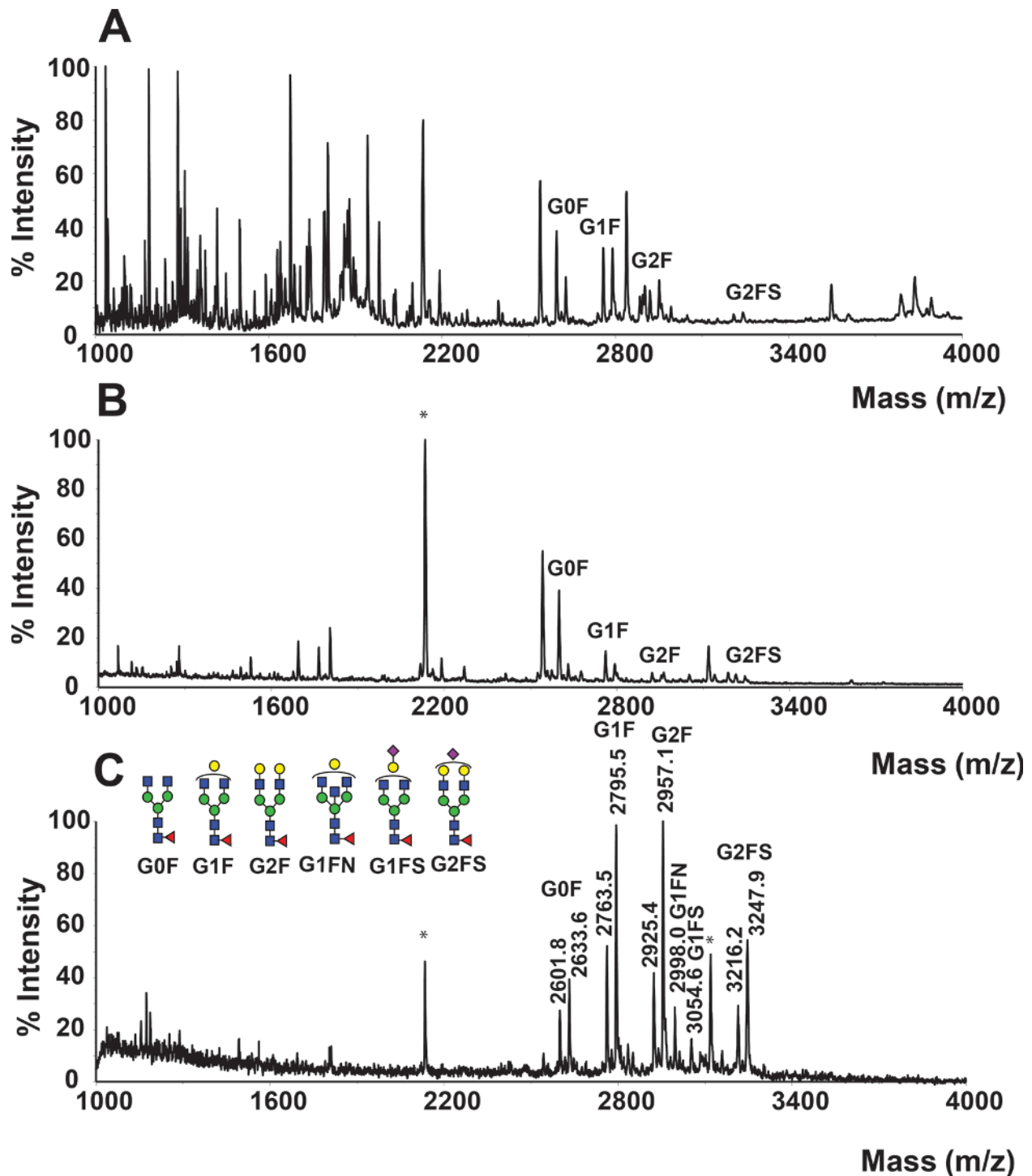


Figure 5. MALDI mass spectra obtained from the tryptic digest of IgG. (A) Direct analysis, (B) after the enrichment with commercial TiO_2 and (C) after the enrichment with titanium/silica sol-gel material.

tag of 2-AA, which has a carboxyl group. The additional negatively charged group on the glycans after 2-AA-labeled makes the interaction between 2-AA-labeled N-glycans and sol-gel particulates stronger.

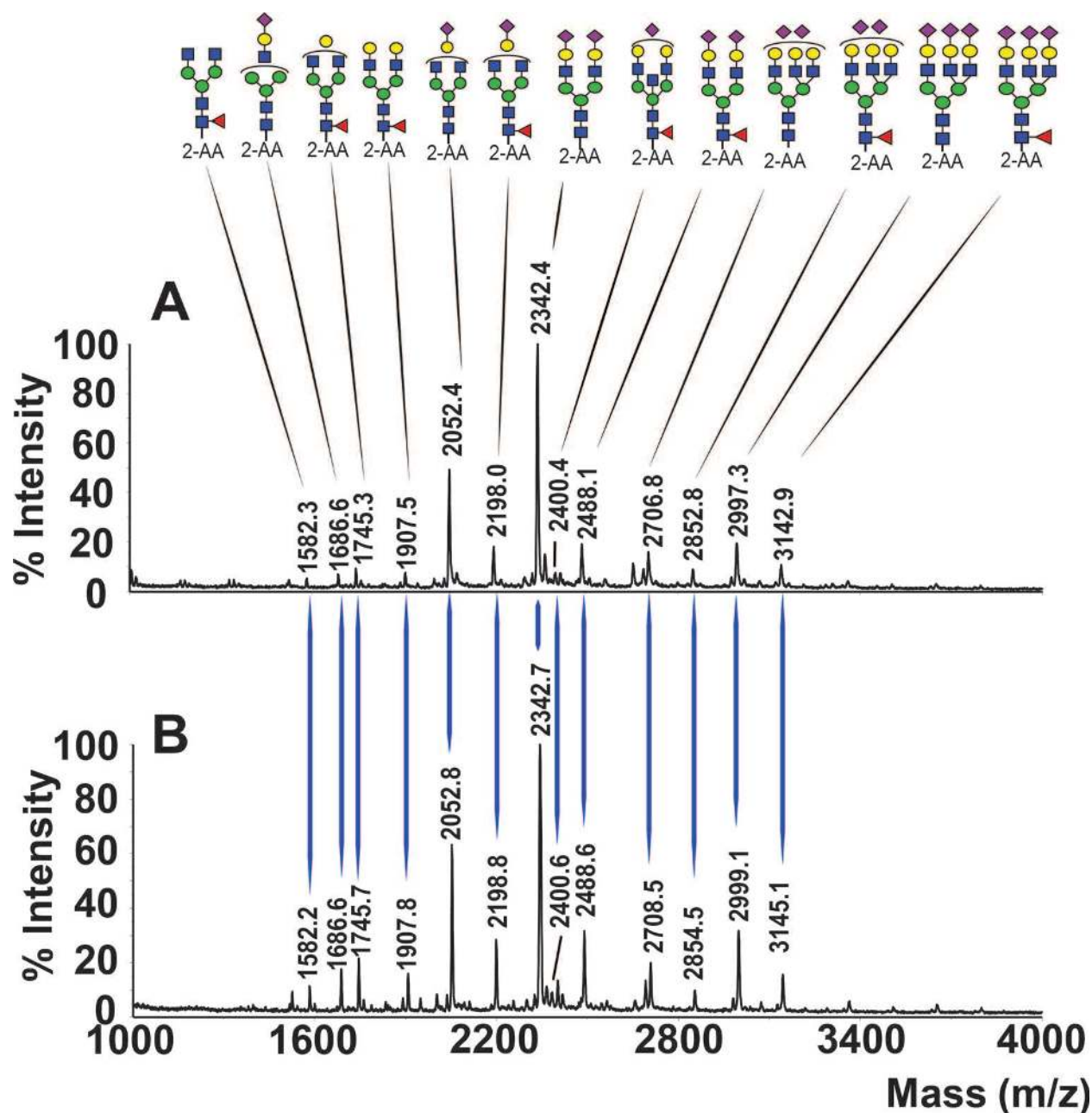


Figure 6. MALDI mass spectra of 2-AA-labeled N-glycans purified by (A) zirconium/silica sol-gel material and (B) titanium/silica sol-gel material.

4. Conclusions and future perspectives

Titanium/silica and zirconium/silica sol-gel materials were synthesized by a facile method and used for the enrichment of (phospho- and glyco-) peptides. Besides that, 2-AA-labeled N-glycans were purified using the sol-gel materials, thereby removing interferences such as salts, detergents, and solvents that are used in the sample preparation methods. This study shows that sol-gel materials promise advantages in phosphoproteomics and glycoproteomics

studies regarding sample preparation methods and provide improvements in the (phospho- and glyco-) peptide analysis.

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