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Short communication

Separation of deamidated peptides with mixed-mode chromatography using phospholipid-functionalized monolithic stationary phases

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ABSTRACT

Deamidation of asparagine (Asn) residues of monoclonal antibodies (mAbs) plays a pivotal role in the in vivo/vitro degradation or efficacy loss of biopharmaceuticals. However, a major challenge for MS analysis of deamidation of Asn-containing peptides in mAbs, is due to the fact that there is only a 1 Da mass shift between the native form (Asn residues) and deamidated forms (n-aspartyl (n-Asp) and isoaspartyl (isoAsp) residues with identical mass). Therefore, a chromatographic separation of the deamidated proteins and/or the peptides derived therefrom is needed prior to MS analysis. In this study, the monolithic column with various stationary phases, including reverse phase (RP), single phospholipid-functionalized and mixed phospholipid-functionalized monoliths, were prepared for the separation of the deamidation-sensitive signature peptide (IYPTNGYTR) of trastuzumab and its two deamidated products, n-Asp55 residue IYPTDGYTR and isoAsp55 residue IYPTisoDGYTR. Compared to the RP monolith, the phospholipid-functionalized monoliths provided mixed-mode interactions and exhibited better peak shape and separation selectivity. The effect of the parameters, including the type and concentration of buffer, temperature and pH value on the separation performance were investigated. Under the optimal conditions, the three peptides were fully separated on a mixed phosphocholine (PC) / phosphatidic acid (PA) functionalized monolith (poly (MDPC₆₀PA₄₀-co-EDMA)) due to the joint contribution of hydrophobic and electrostatic interactions. Therefore, the novel method based on the mixed phospholipids-functionalized monolithic column exhibited good potential for the analysis of deamidated peptides, which will be useful for the in-depth study of post-translational modifications of mAbs.

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1. Introduction

Monoclonal antibodies (mAbs) are favored to some therapeutic treatments over small molecule drugs due to their high target affinity and specificity, low off-target toxicity, and long clearance time [1]. However, some common post-translational modifications (PTMs) of proteins, such as the deamidation of asparagine (Asn), isomerization of aspartate (Asp), oxidation of methionine and tryptophan, and glycation of lysine residues, can occur during the production of mAbs as well as during storage and after administration [2,3]. The oxidation of methionine and deamidation of asparagine (Asn) could affect the biological activity, thereby potentially inducing an immunogenic response in patients or leading to a loss in binding affinity to the target protein, notably if the modified amino acids are present in the complementarity determining regions (CDRs) [1,2,4,5]. Therefore, the monitoring of these modifications is of paramount importance for therapeutic mAbs.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been well-established to study the structural modifications of mAbs in pharmaceutical formulations, such as for quality control purposes [4,5]. However, a major challenge for

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the MS analysis of the deamidation of Asn-containing residues in mAbs is due to the fact that there is only a single Dalton mass shift between the native form (Asn) and deamidated (n-Asp and isoAsp) forms (see **Fig. S1**), resulting in the overlap of mass peaks from the deamidated peptides and the ^{13}C isotopes of the corresponding native form peptides [6]. Hence, these native and modified peptides need to be chromatographically separated before MS analysis. Unfortunately, a baseline separation of the triad of peptides (peptides containing Asn, n-Asp, or isoAsp at the same position) by RPLC is not an easy task [7]. In order to overcome this challenge, Hao et al. developed a multidimensional method using RP as the first dimension to focus the three deamidation-related peptides in the same fraction and then directly transferring to ERLIC (electrostatic repulsion-hydrophilic interaction chromatography) for the second-dimension separation, prior to MS/MS detection and identification. These three deamidation-related peptides can be separated based on their *pI* values [7]. Mixed-mode chromatography has shown great potential for simultaneously separating peptides containing Asn residues in their native form and deamidated form [8].

Recently, a series of phospholipid-functionalized monoliths were developed for studying drug-membrane interactions [9–11] and predicting the phospholipidosis-inducing risk of drug candidates [12]. Moreover, these bio-membrane mimicking monoliths exhibited good separation ability for a wide range of compounds including small molecules, peptides, and intact proteins [9,11,13,14] owing to their mixed-mode interactions. Therefore, it is of interest to investigate the applicability of these phospholipid-functionalized monoliths for the separation of deamidation related residues of mAbs.

Trastuzumab is a humanized monoclonal immunoglobulin gamma 1 (IgG1) antibody directed against the human epidermal growth factor receptor-2 (HER2/neu). The Asn55 residue of trastuzumab is potentially susceptible to deamidation to either n-Asp55 or isoAsp55 [5]. This can cause a substantial decrease in potency due to a reduction in receptor binding affinity [5].

Therefore, the deamidation-sensitive signature peptide (IYPTNGYTR) of trastuzumab and its two deamidated products IYPTDGYTR and IYPTisoDGYTR were chosen as analytes in this study. Different single phospholipid or mixed phospholipid-functionalized monoliths were designed and fabricated via a facile one-step strategy using 12-methacryloyl dodecylphosphocholine (MDPC), 12-methacryloyl dodecylphosphoserine (MDPS) or 12-methacryloyl dodecyl-phosphatidic acid (MDPA) as functional monomers and ethylene dimethacrylate (EDMA) as cross-linker (**Fig. S2**). Three other RP monoliths with increasing alkyl chain length, i.e. butyl methacrylate (BMA, C4), stearyl methacrylate (SMA, C18) and 3-methylacryloyl-3-oxapropyl-3-(*N*, *N*-diocetadecylcarbamoyl)-propionate (AOD, C18 $\times$ 2) functionalized monoliths (**Fig. S2**), were also prepared for comparatively studying the effect of hydrophobicity on separation. The separation selectivity of all monoliths was evaluated using nanoLC-ESI-QTOF under the same conditions. The effect of several key parameters (type and concentration of buffer, column temperature and pH value) on the separation performance were systematically investigated. The separation mechanism of the mixed phospholipid-functionalized monoliths was further studied under the optimized conditions.

2. Experimental

2.1. LC and MS conditions and settings

The instrumentation detail was presented in supporting information, and the scheme of instrument system was shown in **Fig.**

S3. Basically, the injection volume was 1 μL . The loading pump was used to carry the sample from the sample loop to the trap column Acclaim® PepMap100 (75 μm $\times$ 2 cm, C18, 3 μm , 100 Å, Dionex) by using ACN/water (3/97, v/v, 0.1% FA) at a flow rate of 10 $\mu\text{L}/\text{min}$, the loading procedure was set to 3 min. Other chromatographic conditions for each experiment can be found in related figure caption. All buffer solutions were prepared by dissolving a certain amount of salt in water to reach the desired concentration. Buffer pH was adjusted by adding 25% formic acid or 25% ammonia to reach expected pH value. Three synthetic $^{13}\text{C}_6$ - $^{15}\text{N}_4$ -labeled internal standard peptides IYPTNGYTR, IYPTDGYTR, IYPTisoDGYTR were used as test analytes.

Mass spectrometric settings were optimized by infusion of the standard samples of the peptides into the mass spectrometer. MS scans from *m/z* 400 to 900 were recorded in positive ion mode. Analyte-specific mass spectrometer parameters are listed in **Table S3**. The analyses of the triad peptides were done in Parallel Reaction Monitoring (PRM) mode. Specifically, the $^{13}\text{C}_6$ - $^{15}\text{N}_4$ -labeled IYPTNGYTR was monitored at transition 547.8/818.4, while the $^{13}\text{C}_6$ - $^{15}\text{N}_4$ -labeled IYPTisoDGYTR and IYPTDGYTR were monitored at transition 548.3/819.4. Both transitions are presented as black and red trace, respectively, in all chromatograms throughout the entire paper.

In terms of data processing, chromatogram overlay and smoothing were performed using DataAnalysis 4.2 (Bruker, German). Then all raw data were exported as .mzData files that were transformed to .xls files with a custom-written python-based program. All Chromatograms were redrawn using Microcal Origin 8.5.

2.2. Preparation of monolithic columns

All monolithic columns reported in this study were fabricated in our lab [11,12,15–18], and their functional groups can be seen in **Fig. S2**. With regard to the nomenclature of mixed phospholipid-functionalized monoliths, the subscript number is referred to their molar ratio, e.g. MDPC₈₀PA₂₀ represents MDPC/MDPA (80/20, mol/mol). Column preparation is described in detail in supporting information.

3. Results and discussion

3.1. Comparison of separation selectivity of different monoliths

Since RP is the most often used separation mode for peptides and proteins, three RP monolithic columns, including poly (BMA-co-EDMA), poly (SMA-co-EDMA) and poly (AOD-co-EDMA) monoliths, were first employed for the separation of IYPTNGYTR, IYPTisoDGYTR and IYPTDGYTR. As can be seen in **Fig. 1a**, the three peptides could not be separated on all three RP monoliths. The extent of peak overlap for the three peptides increased with increasing alkyl chain length of the monolithic stationary phases. These results again indicated that it is difficult to separate IYPTNGYTR and its two deamidated products through a simple manipulation of hydrophobic interactions on the stationary phase. Considering that the side-chain *pK_a* of n-Asp is around 3.9, while the corresponding *pK_a* of isoAsp is around 3.1 [7], electrostatic interactions could contribute to their retention on charged stationary phases and be potentially useful for their separation. It has been reported that both hydrophobic and electrostatic interactions may contribute to the retention of charged analytes (such as charged drugs, intact proteins, etc.) on phospholipid-functionalized monoliths [9,11,12,18]. Therefore, several single phospholipid or mixed phospholipid-functionalized monoliths, including the poly (MDPC-co-EDMA), poly (MDPS-co-EDMA), poly (MDPC₉₀PS₁₀-co-EDMA) and poly (MDPC₆₀PA₄₀-co-EDMA) monoliths, were selected

(a)

(b)

Fig. 1. (a) Separation of peptides IYPTNGYTR (1), IYPTisoDGYTR (2) and IYPTDGYTR (3) on six different monoliths. Conditions: monolithic columns (100 μ m i.d. $\times$ 200 mm); flow rate, 300 nL/min; mobile phase component A, H₂O with 0.1% FA; mobile phase component B, ACN with 0.1% FA, gradient elution with the following settings: 0.0–3.0–67.0–68.0–69.0–70.0–71.0–72.0–80.0 min, 4%–4%–64%–75%–75%–90%–90%–4%–4% B; column oven, 35 $^{\circ}$ C; samples: 1. IYPTNGYTR (in black), 2. IYPTisoDGYTR (in red), 3. IYPTDGYTR (in red). (b) Effect of PA content in PC/PA monoliths on the separation of the triad peptides. Conditions are the same as in Fig. 1a, except gradient settings: 0.0–3.0–25.0–26.0–30.0–31.0–35.0 min, 4%–4%–25%–90%–90%–4%–4% B.

Fig. 2. Effect of the aqueous mobile phase pH on the separation of IYPTNGYTR (1), IYPTisoDGYTR (2) and IYPTDGYTR (3). Conditions: column, the poly (MDPC₆₀PA₄₀-co-EDMA) (100 μ m i.d. $\times$ 200 mm); other conditions as in Fig. 1b, except for the composition of mobile phase component A, 10 mM NH₄Ac at various pH values and mobile phase component B, ACN.

for the separation of the triad of peptides. Under the same separation conditions, the poly (MDPS-co-EDMA) monolith exhibited the strongest retention for all three peptides, while their retention on the poly (MDPC-co-EDMA) monolith was the weakest (Fig. 1a). These observations could be explained by the stronger electrostatic interaction on the phosphoserine (PS) functionalized monolith than on the phosphocholine (PC) functionalized monolith, although weak electrostatic interactions have been reported for the latter [11]. The same elution order as on the poly (MDPC-co-EDMA) monolith was observed on both the poly (MDPC₉₀PS₁₀-co-EDMA) and poly (MDPC₆₀PA₄₀-co-EDMA) monoliths. A likely reason is that there is a high percentage of PC groups in all the three monoliths, which play a main role for the retention of the three peptides. Better separation efficiency and peak shape were obtained on the poly (MDPC₆₀PA₄₀-co-EDMA) monolith. In order to further study the effect of the PA content in the PC/PA mixed monolith on separation performance, three other columns, i.e. the poly (MDPC₈₀PA₂₀-co-EDMA), poly (MDPC₇₀PA₃₀-co-EDMA) and poly (MDPA-co-EDMA) monoliths, were fabricated *via* a similar polymerization method. Fig. 1b shows the separation of the three peptides on the PC/PA mixed monoliths. With increasing the weight content of PA in the stationary phase from 20% to 40%, both the retention and resolution of the target peptides increased. This could be attributed to the increased cation-exchange interactions with the Arg residue and the N-terminal amino group on the mixed PC/PA monolith. When only PA was employed as the functional monomer, the electrostatic interactions between peptides and PA group were so strong that peptides cannot be eluted under the employed conditions. Finally, the poly (MDPC₆₀PA₄₀-co-EDMA) monolith was selected for further experiments.

3.2. Effect of column temperature on the chromatographic performance of the poly (MDPC₆₀PA₄₀-co-EDMA) monolith

The effect of column temperature on the separation performance was then investigated. As can be seen in Fig. S4, the resolution between the two aspartyl isomers (IYPTisoDGYTR, IYPTDGYTR) significantly decreased with the column temperature rising from 35 to 60 °C. Furthermore, it is worth mentioning that the peak height of IYPTNGYTR (Fig. S4, peak in black) was significantly reduced when the temperature increased. Therefore, 35 °C

(a)

(b)

Fig. 3. (a) Effect of NH₄Ac concentration on the resolution between IYPTisoDGYTR/IYPTDGYTR and IYPTDGYTR/IYPTNGYTR. Conditions: column, the poly (MDPC₆₀PA₄₀-co-EDMA) (100 μ m i.d. $\times$ 200 mm); mobile phase component A, H₂O with various amount of NH₄Ac (0.5, 1, 2, 5 or 10 mM) at pH 7.7; mobile phase component B, ACN; the others as in Fig. 2a. (b) Optimized separation of IYPTNGYTR, IYPTisoDGYTR, IYPTDGYTR. Conditions: flow rate, 1 μ L/min; mobile phase component A, H₂O with 2 mM NH₄Ac at pH 4.6; mobile phase component B, ACN; gradient elution with the following settings: 0.0–2.0 - 15.0–16.0 - 17.0–18.0 - 19.0–20.0 min, 4% - 4% - 64% - 75% - 90% - 90% - 4% - 4% B; column oven, 35 °C; the others as in Fig. 2a. Black and red trace represent transition of 547.8/818.4 and 548.3/819.4 (*m/z*) respectively.

was selected as the most favorable condition for both the separation and MS responses of the peptides.

3.3. Effect of buffer type and pH on the chromatographic performance of the poly (MDPC₆₀PA₄₀-co-EDMA) monolith

Ammonium acetate (NH₄Ac) and ammonium formate (AF) were selected as buffer components to adjust the pH value and to optimize the separation conditions. As depicted in Fig. S5, a higher ESI-MS response was obtained when using NH₄Ac as buffer compared to AF. The mobile phase pH can modify the charge state of both the stationary phase and the analytes, and thus affect the separation of charged analytes. Therefore, the effect of buffer pH on the separation of the three peptides was investigated by varying the pH of a 10 mM NH₄Ac aqueous buffer within a range of 3.0 to 7.7. From the chromatograms in Fig. 2, it appears clearly that the retention, selectivity and even the elution order of three

peptides were significantly affected by buffer pH. Their retention increased first and then dropped with increasing pH. At pH 3.0, IYPTNGYTR (peak 1) elutes before IYPTisoDGYTR (peak 2), but the elution order changes dramatically at higher pH values. At pH 3.6, IYPTNGYTR elutes between IYPTisoDGYTR and IYPTDGYTR (peak 3), while it elutes after IYPTisoDGYTR and IYPTDGYTR at pH $\geq$ 4.6. This might be explained by pH effect on electrostatic interaction with the monolith. pK_{a1} and pK_{a2} of phosphoric acid (PA) are 2.14 and 7.20, respectively. With increasing the buffer pH from 3.0 to 7.7, the negative charge of the PC/PA stationary phase (related to PA group) increased, which strengthens the electrostatic absorption of positively charged peptides. Oppositely, the net positive charge of the Asp-containing peptides decreases with increasing pH, which will weaken electrostatic absorption on the PC/PA stationary phase. These combined effects result in the observed retention behavior.

3.4. Effect of NH_4Ac concentration on the chromatographic performance of the poly (MDPC₆₀PA₄₀-co-EDMA) monolith

The influence of the NH_4Ac concentration on the selectivity and separation efficiency was finally evaluated. As shown in Fig. 3a, the resolution between IYPTisoDGYTR/IYPTDGYTR and IYPTDGYTR/IYPTNGYTR increased with increasing NH_4Ac concentration from 0.5 to 2 mM, while resolution decreased dramatically when further rising the NH_4Ac concentration from 2 to 10 mM, owing to the increasing competition between NH_4Ac and the peptides for electrostatic interactions between the charged residues and the stationary phase. Moreover, the peak height of IYPTNGYTR diminished with increasing NH_4Ac concentration, which might be ascribed to more ionization suppression at higher NH_4Ac concentrations (Fig. S6). Therefore, a 2 mM concentration of NH_4Ac was found to be most suitable with respect to chromatographic performance and MS response.

Using this optimized method, IYPTNGYTR and its deamidated products IYPTDGYTR and IYPTisoDGYTR were well separated on the poly (MDPC₆₀PA₄₀-co-EDMA) monolith employing a gradient elution with 2 mM NH_4Ac (pH 4.6) and ACN as mobile phase components at 35 °C at a flow rate of 1 μ L/min. (Fig. 3b)

4. Conclusions

In this study, various monolithic stationary phases, including RP (BMA, SMA, or AOD), single phospholipid (MDPC, MDPS, or MDPA) and mixed phospholipid (MDPC/MDPS or MDPC/MDPA) functionalized monoliths, have been designed and fabricated for the separation of the deamidation-sensitive signature peptide (IYPTNGYTR) of trastuzumab and its two deamidated products, IYPTDGYTR and IYPTisoDGYTR, by nano-LC-HRMS. In particular, the poly(MDPC₆₀PA₄₀-co-EDMA) monolith exhibited good selectivity, separation efficiency and peak shape due to the synergistic effect of hydrophobic and electrostatic interactions between the analytes and the stationary phase. Under optimal conditions, IYPTNGYTR and its deamidated products IYPTDGYTR and IYPTisoDGYTR were better separated (all $R_s > 4$) on the poly(MDPC₆₀PA₄₀-co-EDMA) monolith in comparison with their separation on a pure C18 column. Therefore, the separation based on the coupling of a mixed MDPC/MDPA monolithic capillary column to MS/MS could be applied to a more reliable characterization of the deamidation and isomerization sites of mAbs in the future.

Conflict of interest

The authors declared no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.chroma.2019.05.053>.

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