Published in final edited form as:

Analyst. 2011 September 21; 136(18): . doi:10.1039/c1an15489c.

Bifunctional polymer brushes for low-bias enrichment of mono- and multi-phosphorylated peptides prior to mass spectrometry analysis[†]

Wei-Han Wang, Jin-Lan Dong, Gregory L. Baker, and Merlin L. Bruening*

Department of Chemistry, Michigan State University, East Lansing, Michigan 48824, USA. Fax: +1 517-353-1793; Tel: +1 517-355-9715 ext 237

Abstract

Polymer brushes orthogonally derivatized with oxotitanium and nitrilotriacetate-Fe(III) groups enrich both mono- and multi-phosphorylated peptides for mass spectrometry analysis.

Polymer brushes are assemblies of macromolecules that have one end tethered to an interface.¹ Due to their tunable thickness, architecture and functionality,^{1, 2} brushes are attractive for biological applications such as protein and peptide binding,^{3, 4} controlling cell adhesion,^{5, 6} and drug delivery.⁷ However, the complexity of biological systems frequently requires more than one functional group for a specific task.⁷⁻⁹ Copolymerization provides multiple functional units in a polymer chain,¹ and by post-polymerization modification of copolymer precursors, two orthogonal functional groups can be attached to side chains.¹⁰ Nevertheless, unmatched monomer reactivities lead to a functional group ratio different from that in the monomer solution.¹ Moreover, random copolymerization does not guarantee the close proximity of different functional groups on the polymer chain.

We report a post-polymerization derivatization scheme that appends two different functionalities on the same repeating unit of poly(glycidyl methacrylate) (PGMA) brushes (**f**, Figure 1A). Subsequently, the presence of carboxylate groups in these brushes allows formation of oxotitanium(IV) (oxoTi), where as nitrilotriacetate groups can bind Fe(III) (NTA-Fe) (**h**, Figure 1A). These two functionalities exhibit biased affinities toward different subsets of phosphopeptides,¹¹ and neighboring oxoTi and NTA-Fe moieties should reside in similar micro-environments for binding and releasing phosphopeptides.

In characterization of phosphoproteins with mass spectrometry (MS), a comprehensive isolation of mono- and multi-phosphorylated peptides is important for correlating protein phosphorylation with relevant cellular processes.¹² However, the most commonly used phosphopeptide-isolation materials, metal oxide particles¹³⁻¹⁶ (especially TiO₂) and immobilized metal ion affinity chromatography (IMAC) beads¹⁷⁻¹⁹ (especially Fe³⁺ complexes), enrich phosphopeptide subsets biased toward mono- and multi-phosphorylated species, respectively.^{20, 21} In contrast, bifunctional oxoTi-PGMA-Fe brushes (**h**, Fig. 1A) on matrix-assisted laser desorption/ionization (MALDI) substrates should isolate both subsets of phosphopeptides during on-plate enrichment prior to MS analysis.

[†]Electronic Supplementary Information (ESI) available: procedures for polymer synthesis and characterization, and protein digestion; phosphopeptide sequences; and additional mass spectra after phosphopeptide enrichment. See DOI: 10.1039/b000000x/bruening@chemistry.msu.edu.

Preparation of the bifunctional polymer (see ESI† for experimental details) starts with synthesis of poly(glycidyl methacrylate) (PGMA, **a**, Fig. 1A) on Au-coated Si wafers using surface-initiated atom transfer radical polymerization.²² Varying the polymerization time from 1 to 4 h yields homogeneous PGMA brushes with thicknesses from 20–70 nm ($< \pm 3$ nm variation in thickness across the wafer). Reaction of the PGMA epoxide with NaN_3 generates adjacent azide and hydroxyl groups (**b**, Fig. 1A),^{23, 24} and this structure allows introduction of oxoTi and NTA-Fe through orthogonal derivatization. Following our previous method for modifying poly(2-hydroxyethyl methacrylate),²⁵ we react the hydroxyl groups of **b** with succinic anhydride (SA) and activate the resulting carboxylic acids for reaction with aminobutyl NTA to give **e**. The NTA eventually serves as the Fe(III)-binding ligand. FTIR spectra confirm the addition of COO^- groups after reaction with SA (Fig. 2c), the subsequent formation of the activated NHS ester (Fig. 2d), and attachment of NTA (Fig. 2e). The key step in this orthogonal derivatization is the introduction of a second carboxylic acid group through the Cu(I)-catalyzed dipolar cycloaddition of an alkyne and azide, or “click” chemistry (Fig. 1A, f).²⁶ Importantly, the constant intensity of the azide absorbance after several derivatization steps (Fig. 2b–2e) shows that this group is stable during the NTA attachment.

The nearly complete disappearance of the N_3IR absorbance in Fig. 2f suggests an almost quantitative yield for the reaction of **e** with propiolic acid, even when the polymer is ~ 200 nm thick. Sequential reactions of **f** with Ti(IV) isopropoxide and Fe^{3+} give the bifunctional structure **h** (oxoTi-PGMA-Fe). For five films where the thickness of **f** ranged from 70 to 190 nm, the thickness-normalized metal coverages in oxoTi-PGMA-Fe were $0.28 \pm 0.02 \text{ nmol}/(\text{cm}^2\text{-nm})$ Fe and $0.40 \pm 0.06 \text{ nmol}/(\text{cm}^2\text{-nm})$ Ti. The molar ratio of Fe/Ti in these films was 0.72 ± 0.16 . (See ESI† for details.) Elemental analysis also suggests that minimal oxoTi forms at the NTA moiety (see Table S1, last column, ESI†), but we should note that hydrolysis of the sulfo-NHS ester may lead to incomplete derivatization with NTA, and some Fe(III) could complex with the COOH groups from propiolic acid. However, NTA-Fe and oxoTi groups should be in close proximity.

We enriched phosphopeptides directly on the bifunctional polymer brushes (see Fig. 1C and the footnote† for procedures). Previous studies suggest that preferences for enriching mono- or multi-phosphorylated peptides stem from (1) the intrinsic properties of the metal-ion complexes or metal oxide resins,^{17, 21} (2) the binding capacity,^{13, 27, 28} and (3) the sample acidity.^{11, 17} With these considerations in mind, we compared phosphopeptide enrichment on oxoTi-PGMA-Fe with enrichment on either PGMA-Fe(**i**, Fig. 1B) or oxoTi-PGMA(**g**, Fig. 1B). PGMA-Fe and oxoTi-PGMA share the oxoTi-PGMA-Fe polymer structure, but they have only one type of affinity group. With a 1- μL mixture of digested α -casein, β -casein and ovalbumin (200 fmol each in 1% trifluoroacetic acid, TFA), phosphopeptide enrichment on 220 nm-thick PGMA-Fe gives MALDI-MS signals primarily for multi-phosphorylated peptides (red labels in Fig. 3a). In contrast, enrichment on oxoTi-PGMA leads to dominant signals for mono-phosphorylated peptides (black labels in Fig. 3b). Finally, enrichment on 220 nm-thick polymer films that contain both oxoTi and Fe(III) (oxoTi-PGMA-Fe) yields more evenly distributed signals from mono-, di-, tetra-, and penta-phosphorylated peptides, without sacrificing signal-to-noise ratio (S/N) (Fig. 3c).

†For on-plate phosphopeptide enrichment (Fig. 1C), 2-mm circles were first scratched on the modified wafer surface to form sample wells. One μL of protein digest was added to each well for a 20-min incubation in a covered, humid petri dish. The wafer was then rinsed with ~ 3 mL of 50% acetonitrile (ACN) containing the same concentration of TFA as the sample solution. After drying with nitrogen, addition of 1 μL of 1% H_3PO_4 on the sample well (for phosphopeptide elution) was immediately followed by addition of 0.25 μL of 40 mg/mL 2,5-dihydroxybenzoic acid (matrix for MALDI, prepared in 1% H_3PO_4 in 50% ACN), and the entire solution was allowed to dry. The wafer with phosphopeptide/matrix spots was attached to a modified steel MALDI plate for MS analysis.

Binding capacity, which increases with film thickness, has a significant impact on phosphopeptide capture using oxoTi-PGMA-Fe. With a thinner (120 nm) film, enrichment on oxoTi-PGMA-Fe again yields a mass spectrum that presents an “average” of the spectra resulting from enrichment on PGMA-Fe and oxoTi-PGMA (Fig. S2a–c†). However, the preferences of PGMA-Fe, oxoTi-PGMA, and oxoTi-PGMA-Fe move toward peptides with more phosphate groups compared to enrichment on the corresponding 220 nm-thick polymers (compare Fig. S2a–c† with Fig. 3a–c). Insufficient binding capacity for the 120 nm-thick functional polymers in 1% TFA likely leads to increased bias for binding multi-phosphorylated peptides.

The binding capacity of oxoTi-PGMA-Fe also depends on sample acidity. High acidity will decrease the driving force for adsorption of deprotonated species and may cause metal leaching from the ligands.²⁹ When we decrease the TFA concentration from 1% to 0.1% in sample and washing solutions, enrichment of the 3 digested phosphoproteins (200 fmol each) on 120 nm-thick oxoTi-PGMA-Fe yields a mass spectrum similar to that obtained from enrichment in 1% TFA on the 220 nm-thick oxoTi-PGMA-Fe (compare Fig. 3d and 3c). In addition, in 0.1% TFA the 120 nm-thick PGMA-Fe and oxoTi-PGMA control films again present biased enrichment toward multiply and mono-phosphorylated peptides, respectively (Fig. S2d, e†). Further decreasing the thickness of oxoTi-PGMA-Fe to 60 nm for enrichment in 0.1% TFA generates a very similar MS pattern (Fig. S2f†) as in Fig. 3d. These data suggest that the binding capacity (film thickness) needed for low-bias enrichment of phosphopeptides greatly decreases with sample acidity.

Finally, we enriched phosphopeptides from samples with either less phosphoprotein or higher amounts of nonphosphoprotein. With only 50 fmol of the three digested proteins in 1% TFA, enrichment on the 220 nm-thick oxoTi-PGMA-Fe film still gives strong signals for both mono- and di-phosphorylated peptides because of the combination of affinity groups in the film (Fig. 3e, spectra for control polymers are in Fig. S2g, h†). Enrichment from a digest mixture containing 200 fmol each of the three phosphoproteins and 2 pmol of bovine serum albumin (BSA, a non-phosphoprotein) also leads to strong signals of mono- and multi-phosphorylated peptides (Fig. 3f), with similar peak patterns and S/N as for samples without BSA (e.g., Fig. 3c). These spectra clearly demonstrate the phosphopeptide enrichment selectivity and low bias in 1% TFA.

In conclusion, through an orthogonal post-polymerization derivatization strategy, we successfully introduced oxoTi and NTA-Fe groups in high-density polymer brushes. The complementary affinities of oxoTi and NTA-Fe in such brushes facilitate enrichment of both mono- and multi-phosphorylated peptides from samples with moderate acidity (0.1–1% TFA) and complexity (5–20 µg/mL of several phosphoproteins and <0.5 mg/mL of non-phosphoproteins). Increasing film thickness enhances binding capacity to promote unbiased enrichment and increase S/N. This bifunctional polymer film may be attractive for MALDI-MS or MALDI-MS/MS analysis of target phosphoproteins in small-volume samples from immuno-purified proteins, protein bands after gel electrophoresis, and high-throughput kinase assays. Moreover, the orthogonal derivatization strategy can introduce adjacent functional groups for other multi-functional applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the U.S. National Institutes of Health (GM080511).

Notes and references

1. Barbey R, Lavanant L, Paripovic D, Schuwer N, Sugnaux C, Tugulu S, Klok HA. *Chem Rev.* 2009; 109:5437–5527. [PubMed: 19845393]
2. Uhlmann P, Merlitz H, Sommer JU, Stamm M. *Macromolecular Rapid Communications.* 2009; 30:732–740. [PubMed: 21706659]
3. Jain P, Baker GL, Bruening ML. *Annual Review of Analytical Chemistry.* 2009; 2:387–408.
4. Zdyrko B, Hoy O, Luzinov I. *Biointerphases.* 2009; 4:FA17–FA21. [PubMed: 20408713]
5. Gautrot JE, Trappmann B, Ocegüera-Yanez F, Connelly J, He XM, Watt FM, Huck WTS. *Biomaterials.* 2010; 31:5030–5041. [PubMed: 20347135]
6. Raynor JE, Capadona JR, Collard DM, Petrie TA, Garcia AJ. *Biointerphases.* 2009; 4:FA3–FA16. [PubMed: 20408714]
7. Yang XQ, Grailer JJ, Rowland IJ, Javadi A, Hurley SA, Matson VZ, Steeber DA, Gong SQ. *ACS Nano.* 2010; 4:6805–6817. [PubMed: 20958084]
8. Qi M, O'Brien JP, Yang JJ. *Biopolymers.* 2008; 90:28–36. [PubMed: 17972282]
9. Motoyama A, Xu T, Ruse CI, Wohlschlegel JA, Yates JR. *Anal Chem.* 2007; 79:3623–3634. [PubMed: 17411013]
10. Gauthier MA, Gibson MI, Klok HA. *Angew Chem Int Ed.* 2009; 48:48–58.
11. Wang WH, Palumbo AM, Tan YL, Reid GE, Tepe JJ, Bruening ML. *J Proteome Res.* 2010; 9:3005–3015. [PubMed: 20380454]
12. Johnson LN, Lewis RJ. *Chem Rev.* 2001; 101:2209–2242. [PubMed: 11749371]
13. Li QR, Ning ZB, Tang JS, Nie S, Zeng R. *J Proteome Res.* 2009; 8:5375–5381. [PubMed: 19761217]
14. Li Y, Wu JS, Qi DW, Xu XQ, Deng CH, Yang PY, Zhang XM. *Chem Commun.* 2008:564–566.
15. Nelson CA, Szczech JR, Xu QG, Lawrence MJ, Jin S, Ge Y. *Chem Commun.* 2009:6607–6609.
16. Wu JH, Li XS, Zhao Y, Gao QA, Guo L, Feng YQ. *Chem Commun.* 2010; 46:9031–9033.
17. Thingholm TE, Jensen ON, Robinson PJ, Larsen MR. *Mol Cell Proteomics.* 2008; 7:661–671. [PubMed: 18039691]
18. Villen J, Beausoleil SA, Gerber SA, Gygi SP. *Proc Natl Acad Sci U S A.* 2007; 104:1488–1493. [PubMed: 17242355]
19. Hu LH, Zhou HJ, Li YH, Sun ST, Guo LH, Ye ML, Tian XF, Gu JR, Yang SL, Zou HF. *Anal Chem.* 2009; 81:94–104. [PubMed: 19117447]
20. Dunn JD, Reid GE, Bruening ML. *Mass Spectrom Rev.* 2010; 29:29–54. [PubMed: 19263479]
21. Thingholm TE, Jensen ON, Larsen MR. *Proteomics.* 2009; 9:1451–1468. [PubMed: 19235172]
22. Edmondson S, Huck WTS. *J Mater Chem.* 2004; 14:730–734.
23. Slater M, Snaiko M, Svec F, Frechet MJ. *Anal Chem.* 2006; 78:4969–4975. [PubMed: 16841919]
24. Tsarevsky NV, Bencherif SA, Matyjaszewski K. *Macromolecules.* 2007; 40:4439–4445.
25. Dunn JD, Igrisan EA, Palumbo AM, Reid GE, Bruening ML. *Anal Chem.* 2008; 80:5727–5735. [PubMed: 18578546]
26. Golas PL, Matyjaszewski K. *Chem Soc Rev.* 2010; 39:1338–1354. [PubMed: 20309490]
27. Qiao LA, Bi HY, Busnel JM, Hojeij M, Mendez M, Liu BH, Girault HH. *Chem Sci.* 2010; 1:374–382.
28. Wang WH, Bruening ML. *Analyst.* 2009; 134:512–518. [PubMed: 19238288]
29. Sharma S, Agarwal GP. *Sep Sci Technol.* 2002; 37:3491–3511.

Fig. 1. A. Scheme for orthogonal modification of PGMA brushes (see Fig. S1† for more details). B. PGMA-based control polymers (**i** and **g**). C. On-plate phosphopeptide enrichment for MALDI-MS analysis.

Fig. 2.

Reflectance FTIR spectra of a PGMA film on a Au-coated wafer before (a) and after sequential reactions with the following reagents: (b) NaN_3 ; (c) SA; (d) *N*-hydroxysulfosuccinimide (sulfo-NHS)/*N,N*-diisopropylcarbodiimide; (e) aminobutyl NTA; (f) propionic acid (PA). The polymer abbreviation and ellipsometric thickness of the film (dry form) after each step are noted above each spectrum. The ESI† further explains the IR spectra.

Fig. 3.

MALDI mass spectra after phosphopeptide enrichment from a 1- μ L solution containing three digested phosphoproteins (3pp), -casein, casein and ovalbumin, or a mixture of 3pp and BSA. The polymers employed for enrichment, their thicknesses (prior to addition of oxoTi or Fe(III)), and the sample acidity are noted above each spectrum. Each phosphopeptide is labeled as #p#, where the first # denotes the number of phosphates in that peptide and the second # denotes the peptide number in Table S2†. Black and red labels represent mono- and multi-phosphorylated peptides, respectively.