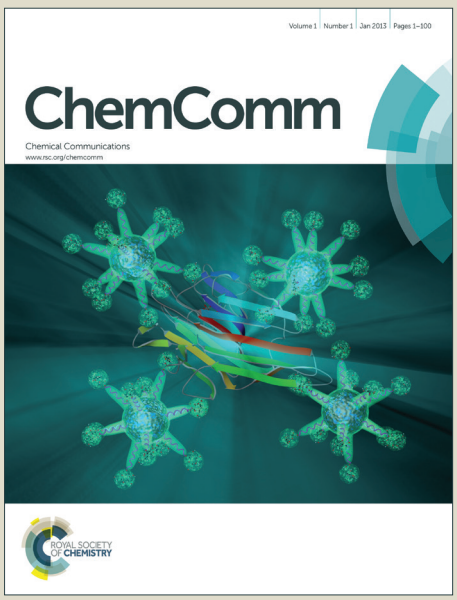
ChemComm

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/chemcomm

Chemical Communications ChemComm

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

COMMUNICATIONS

Guanidyl-Functionalized Graphene as a Bi-function Adsorbent for Selective Enrichment of Phosphopeptides

Lin-Nan Xu, Li-Ping Li, Liang Jin, Yu Bai*, and Hu-Wei Liu

Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX

DOI: 10.1039/b000000x

Guanidyl-functionalized graphene (GFG) was designed and synthesized. Through simple modulation of loading buffer, this novel bi-function adsorbent could provide two enriching performances, one is for global phosphopeptides, and the other is for multi- phosphopeptides with consecutive phosphorylated residues. It provides a promising perspective for consecutive phosphopeptide enrichment in phosphor-proteomic research and related biological processes study.

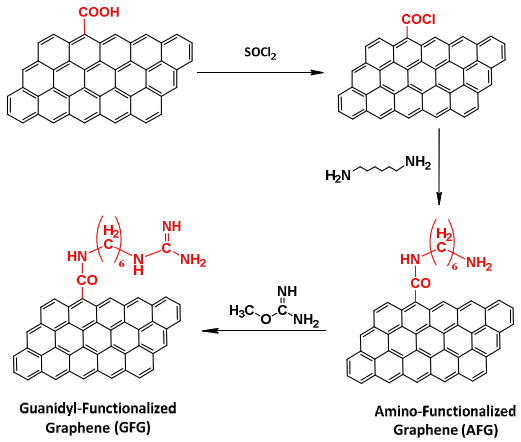
Protein phosphorylation, owing to its vital role in regulating numerous biological processes[1] and involvement in various diseases[2], has attracted extensive research interests. Although mass spectrometry (MS) has become a powerful tool for phosphopeptide analysis, low stoichiometry, poor ionization efficiency, and signal suppression by high-abundance non-phosphorylated peptides set hardly overstepped limitations for direct analysis of phosphopeptides by MS. Therefore, enrichment of phosphopeptides was often adopted prior to MS analysis.

Global phosphopeptide enrichment provides informations of both mono- and multi-phosphopeptides. As MS signal of multiphos-phopeptides with lower ionization efficiency would be severely suppressed in the presence of monophosphopeptides[3], selective enrichment of multiphosphopeptides was also required for more comprehensive phosphoproteomic analysis[4, 5]. Various materials, including SAX[6, 7], IMAC[8-11] and MOAC[12-17] with extensive investigations, have been developed for global or multiphospho-peptide enrichment[18]. However, few of them could meet both demands, and combination of different enrichment materials is often required for both global and specific multi- phosphopeptide analysis[12].

Recently, polyarginine (PA)-coated diamond nanoparticle was developed for selective enrichment of multiphosphopeptides[19] on the basis of the non-covalent complex formed by PA and phosphopeptides[20]. However, the affinity of PA towards phosphopeptides mainly originated from the interaction of phosphate groups with positively charged guanidyl groups rather than its peptide framework[21]. On the other hand, the introduction of consecutive arginine residues as well as peptide framework is not cost-efficient and may limit its applications in phosphor-peptide enrichment. Therefore, guanidyl could be considered as a functionalized group for the phosphopeptides enrichment[22].

Graphene-based materials have attracted extensive research interests in the past few years because of their exceptional properties in electrics, mechanics, optics, and thermotics. And graphene oxide, characterized by easy preparation, large surface area for adsorption, various functional groups for surface modification, and compatibility for direct matrix-assisted laser desorption ionization (MALDI) MS analysis[23-25], has become an ideal support for functionalization in developing novel graphene-based materials for sample preparation.

Herein, we designed a novel guanidyl-functionalized graphene (GFG) for selective enrichment of phosphopeptides. Guanidyl groups were modified on graphene substrate by functionalization of graphene oxide. GFG, as a bi-function adsorbent, could extract mono- and multi- phosphopeptides simultaneously or selectively enrich multiphosphopeptides through modulation of loading conditions. After loading and washing, phosphopeptides adsorbed on GFG could be directly analyzed by MALDI-TOF-MS without additional elution step. Comparison of the performance of GFG and amino-functionalized graphene (AFG) for phosphopeptide enrichment indicated the superior specificity of guanidyl group towards phosphopeptide over amino group. It is worth noting that multiphosphopeptides enriched by GFG under the optimized condition share the structural feature of existence of consecutive phosphorylated residues, and mechanism for this sequence-related selectivity was also discussed. Such unique selectivity of GFG could be considered as a potential path for specific sequence-recognition in phosphoproteomic and related biological processes research.



Scheme 1 Synthesis route of AFG and GFG.

Beijing National Laboratory for Molecular Sciences, Key Laboratory of Bioorganic Chemistry and Molecular Engineering of Ministry of Education, College of Chemistry and Molecular Engineering, Institute of Analytical Chemistry, Peking University, Beijing 100871, China. Tel: +86 10 6275 8198; E-mail: yu.bai@pku.edu.cn

† Electronic Supplementary Information (ESI) available: Experimental procedures, charactirazation of materials, detected phosphopeptides and their sequences. See DOI: 10.1039/b000000x/

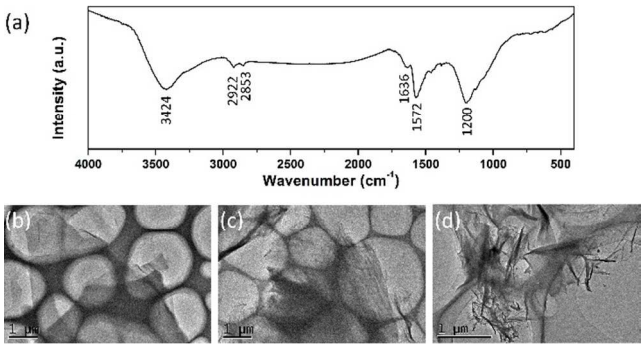


Fig. 1 FT-IR spectrum of GFG (a) and TEM images of GO (b), AFG (c) and GFG (d).

The synthesis method of AFG and GFG is illustrated in Scheme 1. In brief, carboxyl groups on the surface of GO was firstly acylated by $SOCl_2$. AFG was obtained by coupling –COCl on graphene with $–NH_2$ on one terminal of 1,6-hexanediamine through an amidation reaction. The alkyl chain of 1,6-hexanediamine could act as a spacer to improve flexibility and availability of guanidyl groups and avoid steric hindrance from graphene substrate during the extraction of phosphopeptides. For synthesis of GFG, amino groups on AFG were further guanidinated by O-methylisourea hemisulfate through a nucleophilic substitution for methoxyl group. Synthesized GFG was characterized by FT-IR, TEM and elemental analysis. Figure 1a shows the FT-IR absorbance spectrum of GFG. The broad absorbance peak centered at ~3400 cm^{-1} corresponds to stretching vibration of hydroxyl groups originated from GO substrate. Two minor absorbance peaks at 2922 cm^{-1} and 2853 cm^{-1} could be ascribed to C-H bonds on the alkyl chains of 1,6-hexanediamine moieties. The characteristic absorbance peaks of amide bonds appeared at 1636 cm^{-1} and 1572 cm^{-1}, and the peak at 1200 cm^{-1} was assigned to stretching vibration of C-N bonds from guanidyl groups. The TEM photographs of GO, AFG and GFG are shown in Figure 1b-d. GO shows a smooth surface and neat edge, while AFG appears to have a rough surface and GFG is characterized by wrinkles and irregular edge, which could be attributed to a series of surface modifications. The elemental analysis results (Table 1) show that AFG contained 5.54% N in weight, which was increased to 8.60% in GFG, thus further confirming the successful modification with amino groups and guanidyl groups in AFG and GFG, respectively.

Table 1 Elemental compositions of AFG and GFG.

ω%	C	H	N
AFG	75.87	4.07	5.54
GFG	70.41	3.99	8.60

By virtue of the electrostatic interaction and hydrogen bonds formed between guanidyl groups on GFG and phosphate groups on phosphopeptides[21], GFG could adsorb phosphopeptide and be applied for phosphopeptide enrichment. The performance of GFG for phosphopeptide enrichment was firstly studied using β-casein tryptic digests (4×10^{-7} M) as standard peptide mixture. Two loading buffers, including HAc buffer with 60% ACN, 0.1 M HAc, and TFA buffer with 66% ACN, 0.05% TFA, were adopted for phosphopeptide adsorption and adsorbent washing. The volume ratios of ACN/H_2O and concentrations of acids, as well as amount of GFG added, have been optimized to enhance signal intensity of phosphopeptides and eliminate adsorption of non-phosphopeptides. It was found that two monophosphopeptides (β1, β2) and one multiphosphopeptide (β4) could be clearly identified after enrichment by GFG in HAc buffer (Figure 2a), while no monophosphopeptides but two multiphosphopeptides (β3, β4) were detected after enrichment in TFA buffer (Figure 2b). The GFG enrichment selectivity for mono- and multi-phosphopeptides could be ascribed to different deprotonation degrees of phosphate groups on phosphopeptides in two loading buffers. In HAc buffer with lower acidity (pH=3.4), phosphate groups were highly deprotonated, which induced strong affinity between negatively charged phosphate groups and positively charged guanidyl groups on GFG, resulting in adsorption of both mono- and multiple phosphopeptides. However, the affinity between phosphate and guanidyl groups was weakened due to partially protonation of phosphate groups in higher acidic TFA buffer (pH=1.9). As a result, only phosphopeptides with multiple phosphate groups exhibited sufficient affinity to GFG, and the preference of GFG to multiphosphopeptides was presented. The detection limits for phosphopeptide enrichment by GFG were evaluated. As shown in Figure 2c and 2d, with 1×10^{-10} M β-casein tryptic digest, all three phosphopeptides could be detected with HAc buffer, while the multiphosphopeptide β4 could be identified under concentration of 4×10^{-10} M with TFA buffer.

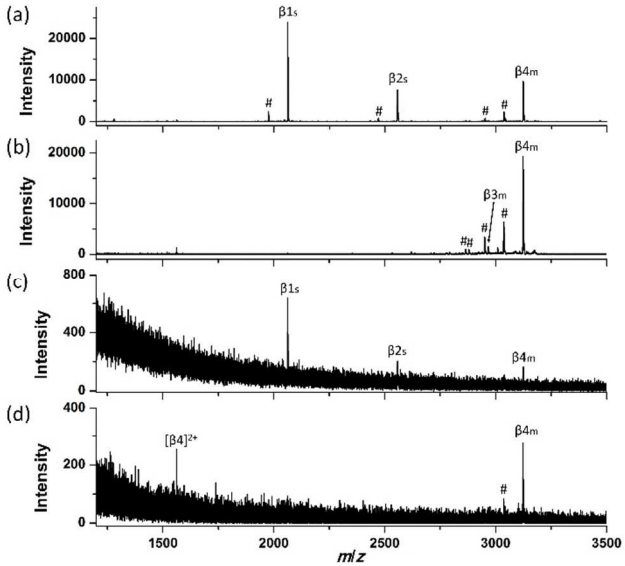


Fig. 2 Enrichment of phosphopeptides from digests of β-casein by GFG. Concentrations of digests of β-casein and compositions of loading buffers are listed as follow: (a) 4×10^{-7} M, 60% ACN with 0.1 M HAc; (b) 4×10^{-7} M, 66% ACN with 0.05% TFA; (c) 1×10^{-10} M, 60% ACN with 0.1 M HAc; (d) 4×10^{-10} M, 66% ACN with 0.05% TFA. (s: monophosphopeptide; m: multiphosphopeptide; #: dephosphorylated residue of phosphopeptide).

Because of structural similarity of amino and guanidyl groups and existence of electrostatic attraction between amino and phosphate groups, phosphopeptide enrichment by AFG was also studied for comparison. Unfortunately, a relatively strong MS signal for non-specific adsorption was observed after enrichment in HAc buffer as shown in Figure S1. This result proved the superior selectivity and specificity of GFG over AFG.

The different performances for phosphopeptide enrichment under HAc buffer and TFA buffer were further confirmed by using tryptic digests of α-casein (4×10^{-7} M) with more phosphorylation sites. As illustrated in Figure 3a and 3b, the peaks of 3 monophosphopeptides and 11 multiphosphopeptides (Table S1) were observed after enrichment by GFG in HAc buffer while 7 multiphosphopeptides in TFA buffer, which was in accordance with the GFG selectivity towards mono- and multi-

phosphopeptides in two buffers using β-casein tryptic digests.

It is noteworthy that not all of the multiphosphopeptides enriched by GFG in HAc buffer were presented after enrichment in TFA buffer. Especially, the dominant peak of a biphosphopeptide (α3) observed after enrichment in HAc buffer completely disappeared after enrichment in TFA buffer. With comparison of the sequences of multiphosphopeptides enriched from digests of α-casein and β-casein using two different buffers, it was found that only multiphosphopeptides with consecutive phosphorylated serine residues could be extracted by GFG in TFA buffer, while those ones with discrete phosphorylated sites could only be adsorbed in HAc buffer. Such distinctive structure selectivity has not been reported in phosphopeptide enrichment by any other adsorbent, as well as PA-coated diamond nanoparticles[19].

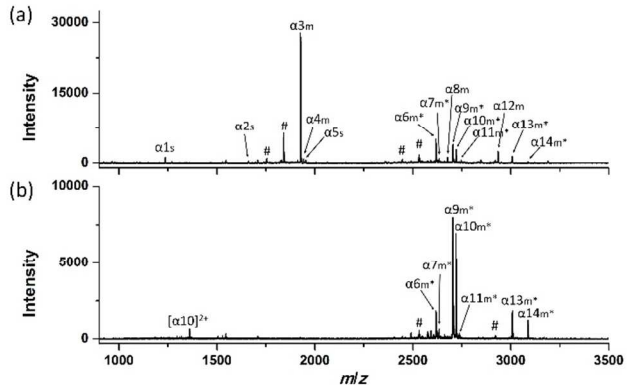


Fig. 3 Enrichment of phosphopeptides from digests of α-casein (4×10^{-7} M) by GFG. The loading buffers are (a) 60% ACN with 0.1 M HAc and (b) 66% ACN with 0.05% TFA, respectively. (s: monophosphopeptide; m: multiphosphopeptide; *: multiphosphopeptide with consecutive phosphorylated sites; #: dephosphorylated residue of phosphopeptide) .

This special structure selectivity could be elucidated by "salt bridge" interaction[26], which indicates a central charge mediated electrostatic interaction with charged-group arrangement of (-)·(+)·(-) or (+)·(-)·(+). The salt bridge interaction could stabilize non-covalent complex formed by guanidyl and phosphate groups[21, 27], and offset weakening effect on affinity between guanidyl and phosphate groups under a relatively strongly acidic condition, *i.e.* TFA buffer.. For GFG, guanidyl groups are scattered on the surface of graphene substrates and consecutive phosphorylated residues are needed for the formation of salt bridge of phosphate(-)·guanidyl(+)·phosphate(-), so that only phosphopeptides with consecutive phosphorylated residues could be extracted. As a comparison, owing to the appearance of adjacent arginine residues in PA, discrete phosphate groups on multiphosphopeptides are sufficient for the construction of salt bridge in the form of guanidyl(+)·phosphate(-)·guanidyl(+)[20], and multiphosphopeptide with discrete phosphoserines could also be adsorbed by PA-coated diamond nanoparticles as a result. Because adjacent phosphorylated residues are not rare in living systems and participate in many biologic processes, such as β-adrenergic receptor kinase-mediated desensitization of human α_{2A}-adrenergic receptor[28] and conformational transition in N-terminal region of cardiac troponin-I[29] *etc.*, GFG could act as a specific sequence-recognized affinity probe in research of such structure-related bio-processes.

In addition, to examine its applicability for real samples, GFG was further applied to trap phosphopeptides from non-fat milk digest with more complexity. As shown in Figure 4a and 4b, a total of 12 phosphopeptides with both mono- and multi-phosphorylated ones were detected in HAc buffer, and 8 multiphosphopeptides were identified after enrichment in TFA buffer (Table S1), exhibiting its capability for phosphoproteomic analysis in more complex real samples. As same as the results from digest of standard proteins, all of the multiphosphopeptides enriched in TFA buffer share the structural feature of consecutive phosphorylated residues, thus proving the practicality of GFG for sequence-recognized extraction of multiphosphopeptides in biological systems.

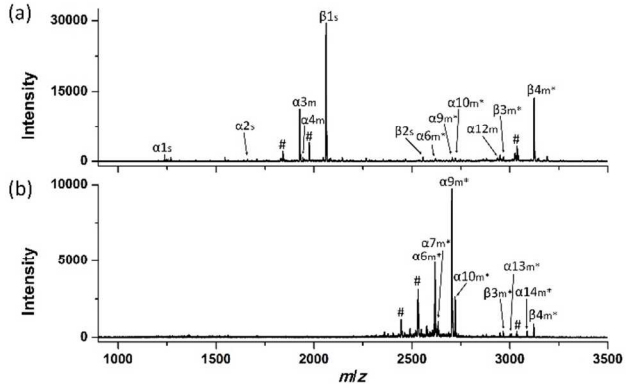


Fig. 4 Enrichment of phosphopeptides from digests of non-fat milk by GFG. The loading buffers are (a) 60% ACN with 0.1 M HAc and (b) 66% ACN with 0.05% TFA, respectively. (s: monophosphopeptide; m: multiphosphopeptide; *: multiphosphopeptide with consecutive phosphorylated sites; #: dephosphorylated residue of phosphopeptide) .

In summary, bi-function GFG was designed, prepared and applied in selective enrichment of phosphopeptides from digest of both standard proteins and real sample for the first time. As a novel bi-function adsorbent, GFG could enrich mono- and multi-phosphopeptides simultaneously in HAc buffer, while only multiphosphopeptides could be extracted by GFG in TFA buffer. In addition, all multiphosphopeptides extracted by GFG in TFA buffer contains structure of consecutive phosphorylated residues, and theoretical mechanism of such sequence-recognized selectivity was discussed. We believe this novel adsorbent would be of great value for phosphoproteomic analysis, and provides a promising perspective for sequence-recognized phosphopeptide enrichment in phosphoproteomic and related biological processes research.

This work was supported by the National Natural Science Foundation of China (21322505, 21175008) and Ministry of Science and Technology of China (2012IM030900) and The Project Sponsored by the Scientific Research Foundation for the Returned Overseas Chinese Scholars, State Education Ministry.

References

1. A. C. Newton, *Biochem. J.*, 2003, **370**, 361-371.
2. P. Viatour, M. P. Merville, V. Bours and A. Chariot, *Trends Biochem. Sci.*, 2005, **30**, 43-52.
3. L. Qiao, H. Bi, J.-M. Busnel, M. Hojeij, M. Mendez, B. Liu and H. H. Girault, *Chem. Sci.*, 2010, **1**, 374-382.
4. T. E. Thingholm, O. N. Jensen, P. J. Robinson and M. R. Larsen, *Mol. Cell. Proteomics*, 2008, **7**, 661-671.
5. J. Wan, K. Qian, L. Qiao, Y. Wang, J. Kong, P. Yang, B. Liu and C. Yu, *Chem-Eur. J.*, 2009, **15**, 2504-2508.
6. G. Han, M. Ye, H. Zhou, X. Jiang, S. Feng, X. Jiang, R. Tian, D.

Wan, H. Zou and J. Gu, *Proteomics*, 2008, **8**, 1346-1361.
7. M. Dong, M. Wu, F. Wang, H. Qin, G. Han, J. Gong, R. a. Wu, M. Ye, Z. Liu and H. Zou, *Anal. Chem.*, 2010, **82**, 2907-2915.
8. A. Stensballe and O. N. Jensen, *Rapid Comm. Mass Sp.*, 2004, **18**, 1721-1730.
9. H. Steen, A. Stensballe and O. N. Jensen, *CSH protoc.*, 2007, **2007**, pdb.prot4607-pdb.prot4607.
10. Z. Yu, G. Han, S. Sun, X. Jiang, R. Chen, F. Wang, R. a. Wu, M. Ye and H. Zou, *Anal. Chim. Acta*, 2009, **636**, 34-41.
11. S. Feng, M. Ye, H. Zhou, X. Jiang, X. Jiang, H. Zou and B. Gong, *Mol. Cell. Proteomics*, 2007, **6**, 1656-1665.
12. L.-P. Li, T. Zheng, L.-N. Xu, Z. Li, L.-D. Sun, Z.-X. Nie, Y. Bai and H.-W. Liu, *Chem. Comm.*, 2013, **49**, 1762-1764.
13. M. R. Larsen, T. E. Thingholm, O. N. Jensen, P. Roepstorff and T. J. D. Jorgensen, *Mol. Cell. Proteomics*, 2005, **4**, 873-886.
14. H. K. Kweon and K. Hakansson, *Anal. Chem.*, 2006, **78**, 1743-1749.
15. C.-T. Chen, W.-Y. Chen, P.-J. Tsai, K.-Y. Chien, J.-S. Yu and Y.-C. Chen, *J. Proteome Res.*, 2007, **6**, 316-325.
16. J. Lu, D. Qi, C. Deng, X. Zhang and P. Yang, *Nanoscale*, 2010, **2**, 1892-1900.
17. L. Han, Z. Shan, D. Chen, X. Yu, P. Yang, B. Tu and D. Zhao, *J. Colloid Interf. Sci.*, 2008, **318**, 315-321.
18. L. Li, L. Xu, Z. Li, Y. Bai and H. Liu, *Anal. Bioanal. Chem.*, 2014, **406**, 35-47.
19. C. K. Chang, C. C. Wu, Y. S. Wang and H. C. Chang, *Anal. Chem.*, 2008, **80**, 3791-3797.
20. A. S. Woods and S. Ferre, *J. Proteome Res.*, 2005, **4**, 1397-1402.
21. K. A. Schug and W. Lindner, *Chem. Rev.*, 2005, **105**, 67-113.
22. Q. Deng, J. Wu, Y. Chen, Z. Zhang, Y. Wang, G. Fang, S. Wang and Y. Zhang, *J. Mater. Chem. B*, 2014, **2**, 1048-1058.
23. D. R. Dreyer, S. Park, C. W. Bielawski and R. S. Ruoff, *Chem. Soc. Rev.*, 2010, **39**, 228-240.
24. Y. Zhu, S. Murali, W. Cai, X. Li, J. W. Suk, J. R. Potts and R. S. Ruoff, *Adv. Mater.*, 2010, **22**, 3906-3924.
25. Y. Liu, J. Liu, P. Yin, M. Gao, C. Deng and X. Zhang, *J. Mass Spectrom.*, 2011, **46**, 804-815.
26. M. F. Jarrold, *Annu. Rev. Phys. Chem.*, 2000, **51**, 179-207.
27. R. Luo, L. David, H. Hung, J. Devaney and M. K. Gilson, *J. Phys. Chem. B*, 1999, **103**, 727-736.
28. M. G. Eason, S. P. Moreira and S. B. Liggett, *J. Biol. Chem.*, 1995, **270**, 4681-4688.
29. P. G. Quirk, V. B. Patchell, Y. Gao, B. A. Levine and S. V. Perry, *FEBS Lett.*, 1995, **370**, 175-178.