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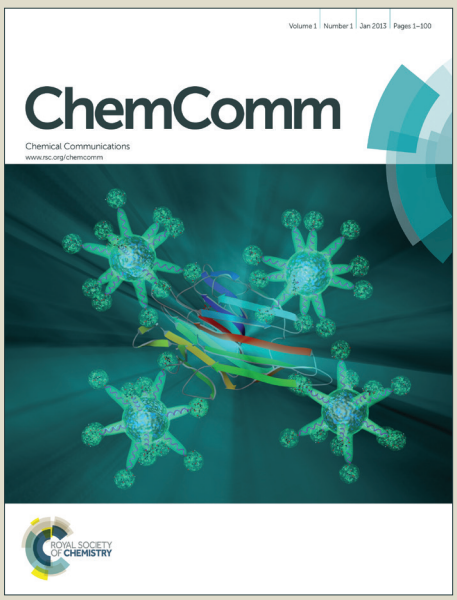

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An Artificial Receptor Synthesized by Surface-confined Imprinting for the Recognition of Acetylation on Histone H4 K16

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A novel artificial receptor has been synthesized by surface-confined imprinting for the recognition of lysine acetylation in histone H4. The material has high recognition fidelity and epitope affinity. It demonstrated that acetylated Lys plays roles in the binding sites creation and peptide imprinting can be performed in phosphate buffer.

The post-translational modification (PTM) is a critical step in the protein biosynthesis and becomes an important topic in the proteomics research.[1, 2] On the other hand, identification and quantification of proteins or peptides with PTM are often difficult because of the complexity of the biological samples and low abundance of the target compounds. Biological receptors are generally used in the specific enrichment. However, they are usually expensive and sensitive to the environment. Development of synthetic materials with high selectivity for the PTM determination is a pursuing goal in the material research.

Molecular imprinting is a technique of making synthetic material with recognition property.[3] Due to the advantages such as tailor-made selectivity, robustness and economic cost, molecularly imprinted polymers (MIPs) have become the attractive materials in the separations.[4] They also have application potential in protein and peptide analysis as artificial receptors.[5, 6] However, protein imprinting is still a challenge task.[7] The flexible conformation of the proteins is the obstacle for the construction of binding sites and for rebinding of the template. Meanwhile, large number of available functional groups in the protein surface may result in entrapment of the template and poor accessibility of the binding sites. The surface-confined imprinting using immobilized template is an approach to circumvent these problems.[8-10] By using this method, open-ended binding cavities with better accessibility can be created and homogeneity of the binding sites is improved due to the oriented immobilization of the template. The "epitope approach" is another strategy[11-14] that is based on the principle in nature in which antibody interact with an antigen by recognizing its epitope. Except selective extraction of target protein/peptide, the MIP synthesized by this approach can be used to extract different peptides carrying the same epitope, which is advantageous in the PTM studies. However, few papers have been published in this respect. Meanwhile, solvent selection in the peptide/protein imprinting is still an arguable question because aqueous solution may interfere hydrogen bonding between the template and monomers. On the other hand, peptides may have poor solubility in organic solvents and MIP synthesized in organic solvents may not be suitable for the recognition in biological environment. The protein/peptide imprinting in aqueous solution and its mechanism are still the subjects to be studied.[15]

Histones are alkaline proteins that have functions in the chromosome assembly and gene regulations. Different types of post-translational modifications have been found in the histone structure, playing important roles in the functions such as gene expression, DNA replication/repair and chromosome condensation. Disruption of these processes has been linked to the multistep process of carcinogenesis.[16] The histone acetylation has been proved to be important in the gene activity and transcriptional regulation.[17] The acetylation of histone H4 on lysine 16 (H4-K16ac) is of the particular interests because it modulates higher order chromatin structure and functional interactions between the chromatin fiber and a non-histone protein.[18] Histone H4 K16 acetylation also affects the cellular lifespan regulation[19] and marks active enhancers in embryonic stem cells.[20] In the H4-K16ac study, synthetic material with selectivity toward H4-K16ac is highly desirable.

Herein we report a research of creating molecularly imprinted polymer as artificial receptor for the H4-K16ac determination. The research demonstrates the peptide imprinted polymer synthesized in this research has good recognition fidelity for the template in biological environment and can be used to selectively extract peptides by epitope approach.

In the molecular imprinting, the template was selected from the N-terminus of the histone H4 with K16 acetylation. The GGAKacR was chosen as the imprinting template because it can be used as marker for H4-K16ac determination from the histone H4 trypsin digestion. To synthesize surface-confined imprinted polymers, totally porous silica was used as the sacrificial supporting material, which is not only the matrix for the template immobilization, but also a hard template in the synthesis of porous MIP material.[9] Because the acetyl group in Kac and amide structure in GGAKacR can form hydrogen bonding and side-chain

of arginine could have ionic interaction, functional monomers that are able to have these two interactions are selected for the binding side constructions. The imprint molecules were immobilized on the silica by reaction with aldehyde-bound silica (Fig. S1 in the Electronic Supplementary Information, ESI). The template-immobilized silica was soaked with polymerization solution containing functional monomer, cross-linker and initiator in phosphate buffer. After imprinting polymerization and silica removal by treatment with 40% aqueous HF, MIP with surface-confined cavities was obtained (Fig. S1 in the ESI). We anticipated that these binding sites not only can selectively bind the template peptide but also can bind the longer peptides by the epitope approach. The surface-confined imprinting, proposed template/functional monomer interactions in the imprinting process and selective rebinding of peptides on the MIP are demonstrated in the Fig.1. The detailed experimental conditions are described in the Supplementary Information.

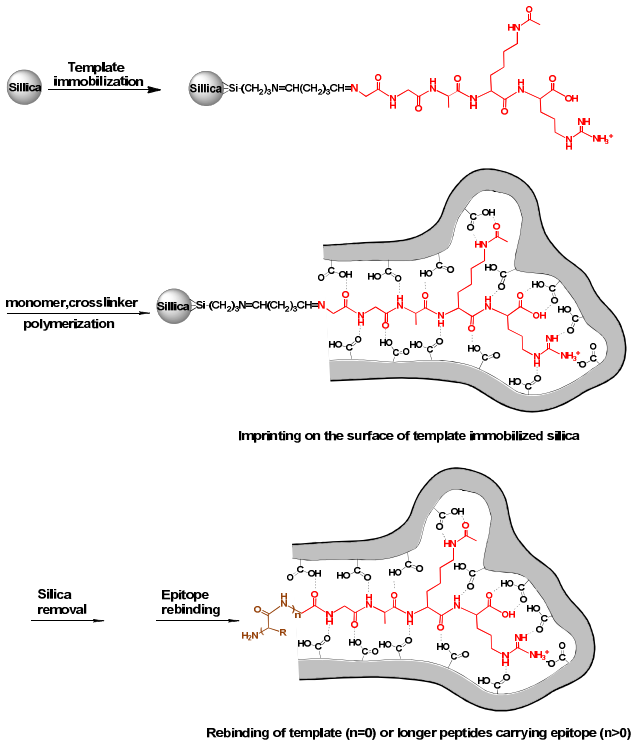

Fig. 1 Proposed imprinting process using surface immobilized GGAKacR as template and rebinding of peptides by epitope approach

Two functional monomers [methylacrylic acid (MAA) and 2-(trifluoromethyl) acrylic acid (TFMAA)] and two cross-linkers [*N,N*-ethylenebis(acrylamide), (EBA) and *N,N*-methylenebis(acrylamide) (MBA)], were compared in the selection of synthetic conditions (Table S1 in ESI). The phosphate buffer solution (PBS, 20 mM, pH 7.0) was used as the polymerization solvent, which is in favor of creating MIP suitable for rebinding in the biological solution. The result demonstrated that the MIP-3 synthesized using TFMAA as functional monomer and EBA as cross-linker has higher binding capacity and better imprinting factor (Fig. S2 in ESI). This situation was attributed to the higher possible crosslinking degree using EBA and stronger interaction between the TFMAA and arginine in the template. FT-IR spectroscopic study for the modified silica and MIP-3 indicated successful grafting of aminopropyl and aldehyde groups on the silica in the correlated silica modification and removal of the silica in the imprinted polymer (Fig. S3 in ESI). The MIP has similar size and spherical shape to the sacrificial silica gels (please see the scanning electron microscopic images in Fig. S4 in ESI), indicating that the MIP synthesis using porous silica as the hard template is successful.

The binding isotherms of GGAKacR on the MIP-3 and corresponding non-imprinted polymer (NIP-3) were compared to study the binding affinity of MIP (Fig. S5 in the ESI). The imprinting factor (IF) is 2.5 calculated by $Q_{s(MIP)}/Q_{s(NIP)}$, in which $Q_{s(MIP)}$ and $Q_{s(NIP)}$ are the saturated bound amount of GGAKacR on unit mass of MIP and NIP respectively. The Scachard analysis was performed to calculate the association constants and apparent maximum number of binding sites (Fig. S6 in the ESI). The binding affinities of the MIP-3 for the template and for the analogue peptides were compared to study the selectivity of the MIP. Five analogue peptides used in the evaluation are listed in the Table 1. The GGVKacR and GGAKR were used to study the sequence and side-chain recognition of the MIP, respectively. The GGAK is the product from the histone H4 digestion if K16 is not acetylated. Two peptides (H4-K12/16Ac and H4-K5/8/12/16Ac) were used to evaluate the epitope binding ability of the MIP. The H4-K12/16Ac can be produced from the histone H4 digestion if acetylation happens on both K12 and K16 and the H4-K5/8/12/16Ac can be obtained from the histone H4 trypsin digest if all the lysine residues in the N-terminus (amino acids 1- 17) are acetylated.

Table 1. The peptides used for MIP selectivity evaluation[a]

Peptide	The template and analogue peptides comparison
GGAKacR	Template and epitope
GGVKacR	Ala in template was replaced by Val
GGAKR	Same sequence as template except native Lys
GGAK	One amino acid less than the template with native Lys
H4-K12/16Ac	GLGKac<u>GGAKacR</u>, epitope-containing nonapeptide
H4K5/8/12/16Ac	GKacGGKacGLGKac<u>GGAKacR</u>, epitope-containing tetradecapeptide

[a]The underlined amino acid sequence in the Table is the epitope section.

The individual binding experiment for each peptide on the MIP and NIP was performed first. The initial concentration of peptide was 1.0 mmol·L^{-1}. The bound amount in unit mass of dry polymer (Q) and imprinted selectivity (IS, evaluated by Q_{MIP}/Q_{NIP}) for different peptides are shown in the Fig. 2A. The competitive binding experiment using peptide mixture was also carried out to investigate the recognition property in the environment with binding competitions (Fig. S7 in the ESI). The MIP has the highest imprinted selectivity for the GGAKacR in both experiments, which

demonstrated that the MIP has excellent binding selectivity. Meanwhile, the MIP has higher selectivity for H4-K12/16Ac and H4-K5/8/12/16Ac, which proved that it can bind peptides by the epitope approach. Compared with template, the H4-K12/16Ac and H4-K5/8/12/16Ac have higher non-specific interaction revealed from their higher bound amount Q on the NIP, which was attributed to their longer peptide chains out of the binding cavities. The other analogue peptides including GGVKacR, GGAKR and GGAK that only have side-chain difference and/or one amino acid shorter than the template, have lower Q and lower IS values. The results indicated that the MIP not only can distinguish one amino acid mismatch but also can discriminate small side-chain difference in one amino acid.

GGAKR imprinted polymer (GGAKR-MIP) was synthesized using the same method as GGAKacR imprinted polymer (GGAKacR-MIP). The binding experiment for GGAKacR and GGAKR demonstrated that both MIPs have selectivity for its own template (Fig. 2B). It provided a further evidence for the function of Lys side chain in the imprinting and recognition.

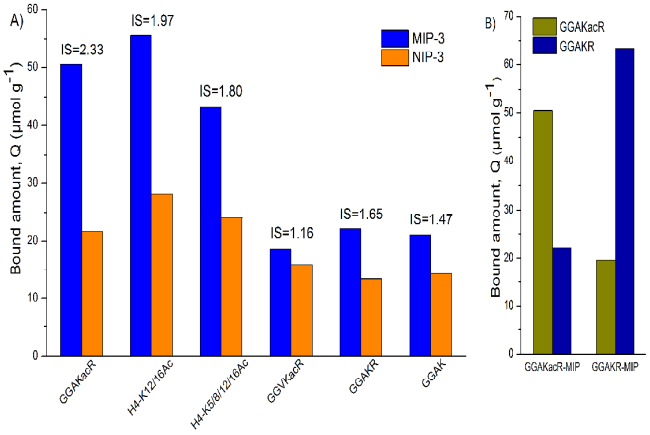

Fig. 2 A) Comparison of binding affinity and selectivity of MIP-3 for different peptides from the individual peptide binding experiments. B) The binding of GGAKacR and GGAKR on the GGAKacR-MIP and GGAKR-MIP. The concentration of analytes in the binding experiments in A) and B) was 1.0 mmol·L^{-1}. The experimental conditions are listed in the ESI. The IS was evaluated by Q_{MIP}/Q_{NIP}. The GGAKacR-MIP referred to MIP-3.

The selectivity of MIP-3 was also evaluated with HPLC analysis using MIP-3 packed short HPLC column (Fig. 3). The NIP-3 column was also used for comparison. The phosphate buffer (pH 7.0, 20 mM) was employed as the mobile phase. The highest retention of GGAKacR in the HPLC demonstrated that the MIP has good selectivity. The imprinted selectivity factors (k_{MIP}/k_{NIP}) and the separation factor (α, calculated by $k_{template}/k_{analogues}$) were used to evaluate the recognition ability and selectivity of the MIP. The sample concentrations were the same for all peptides solutions. The result has shown that the MIP has the highest imprinted selectivity (Table S5 in ESI) for the template, followed by H4-K12/16Ac and H4-K5/8/12/16Ac due to the epitope affinity. While other peptides have weaker retention and smaller selectivity factors. Meanwhile, the MIP-3 exhibited good separation ability indicated by its separation factor $\alpha \geq 1.7$ (Table S5 in ESI).

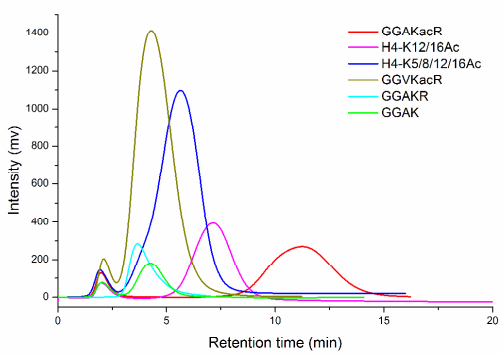

Fig. 3 The chromatographic profiles of the template and peptide analogues on the MIP-3 column. The column size was 20 mm × 4.6 mm i.d.. The mobile phase was PBS (20 mmol·L^{-1} and pH 7) with flow rate of 0.1 mL min^{-1}. The detection wavelength was 205 nm.

The influence of the mobile phase on the retention of template molecule in the HPLC was studied to investigate the recognition mechanism. We found that the template peptide has weak retention in the acetonitrile/H_2O and 100% H_2O mobile phases. Meanwhile, the retention versus water content in acetonitrile/H_2O mobile phase displayed an approximately bell-shaped curve (Fig. S8 in ESI). The situation can be explained by the change of peptide conformation and predominant peptide/MIP interactions in different environment. On the other hand, the retention factor of GGAKacR in the phosphate buffer (20 mM, pH 7.0) mobile phase was about 26 times as that in 100% H_2O (Fig. S9 in ESI). Meanwhile, the imprinted selectivity factor is 2.6 in the phosphate buffer while it is only 1.2 in the pure water. It is revealed that MIP has better recognition for GGAKacR in PBS, which was the imprinting solvent. In the circular dichroism (CD) analysis, GGAKacR in PBS displayed a negative peak in 200 nm. While the CD peak of GGAKacR shifted to 195 nm when the H_2O or ACN/H_2O (10/90, v/v) was the solvents (Fig. S10 in ESI). It demonstrated the backbone conformation of GGAKacR is different in various solutions, which is attributed to its binding behaviour. This result provided information about relation between the binding behaviours and environmental solution.

The performance of selective enrichment by the MIP from complex samples was evaluated using histone trypsin digestion as the sample matrix. For comparison, the same experiment was also conducted using NIP-3 as extraction material. The histone digestion was spiked with both GGAKacR and H4-K12/16Ac and incubated with the MIP-3 or NIP-3. After centrifugation, the MIP or NIP particles were washed to remove the non-specifically bound components. The extracted peptides were then eluted and analyzed by MALDI-TOF MS (Fig. 4) and HPLC analysis (Fig. 5). The results showed that the GGAKacR and H4-K12/16Ac have very small signals among other peptides in the spike histone digest. While they become the dominant components in the MIP extracted fraction (Fig. 4B). The HPLC trace was much cleaner in the MIP extracted fraction (Fig. 5, trace b), which further illustrated the specific selectivity and clean-up function of the imprinted polymer. The recoveries for the GGAKacR and H4-K12/16Ac were of 81% and 82% respectively, determined by the ratio of the extracted amount of peptide to the amount of the addition. The data demonstrated that both peptides were selectively extracted by MIP. It also showed that the surface imprinted polymer can be used to

selectively enrich the peptide by epitope approach. On the contrary, GGAKacR and H4-K12/16Ac can not be selectively enriched by the NIP-3 (Fig. S11 and Fig. S12 in the ESI), which further verified the imprinted selectivity of MIP.

The same experiment using MIP-3 for extraction was carried out for the GGAKacR spiked histone digestion. The MALDI-TOF MS (Fig. S13 in ESI) and HPLC analysis (Fig. S14 in ESI) also revealed good extraction selectivity of MIP. The recovery of GGAKacR from MIP extraction was 84%.

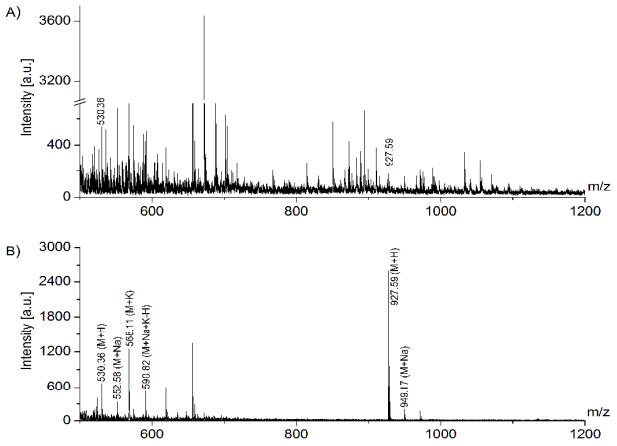

Fig. 4 MALDI-TOF MS spectra of the GGAKacR and H4-K12/16Ac spiked histone digest (A), and the extracted fractions from MIP-3 (B). The m/z 530.36 and 927.59 are signals of the GGAKacR+H and H4-K12/16Ac+H, respectively. The m/z 552.09, 568.01 and 590.60 are the signals of M+Na, M+K and M+Na+K (M is the GGAKacR) respectively produced in the positive-ion mode. The m/z 949.17 is from H4-K12/16Ac+Na.

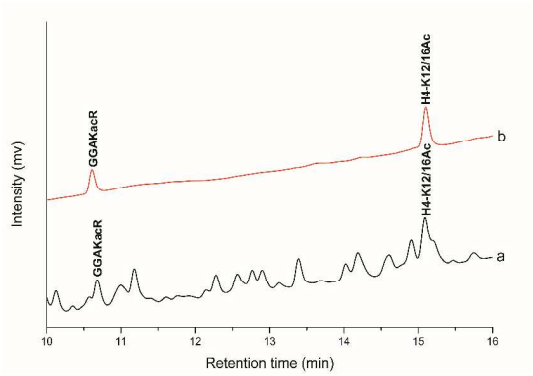

Fig. 5 Reversed-phase HPLC profiles of the GGAKacR and H4-K12/16Ac spiked histone digests before and after the extraction with MIP-3. (a) GGAKacR and H4-K12/16Ac spiked histone digest, (b) extracted fraction from the MIP-3. In the HPLC analysis, a C_{18} analytic column (250 × 4.6 mm i.d., Phenomenex) was used. The mobile phase A was 0.1% TFA in ACN and B was 0.1% TFA in H_2O. The gradient elution: 1-50 min (1% -95% B) was employed. The analytes was detected at 205 nm.

Conclusions

In conclusion, a molecularly imprinted polymer with surface confined binding sites has shown good selectivity. The MIP recognition fidelity demonstrated that the combination of acetylated side chain of Lys and its neighbouring amino acids can be used as imprinting structure and the MIP synthesis can be performed in phosphate buffer. The MIP can be used for the analysis of acetylation of histone H4 K16 from histone digest.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Experimental details and supporting figures. See DOI: 10.1039/c000000x/

1. M. Mann and O. N. Jensen, *Nat. Biotechnol.*, 2003, **21**, 255.
2. Q. L. Huang, J. H. Chang, M. K. Cheung, W. Y. Nong, L. Li, M. T. Lee and H. S. Kwan, *J. Proteome Res.*, 2014, **13**, 2735.
3. L. Chen, S. Xu and J. Li, *Chem. Soc. Rev.*, 2011, **40**, 2922.
4. J. Haginaka, *J. Sep. Sci.*, 2009, **32**, 1548.
5. M. J. Whitcombe, I. Chianella, L. Larcombe, S. A. Piletsky, J. Noble, R. Porter and A. Horgan, *Chem. Soc. Rev.*, 2011, **40**, 1547.
6. Y. Hoshino, H. Koide, T. Urakami, H. Kanazawa, T. Kodama, N. Oku and K. J. Shea, *J. Am. Chem. Soc.*, 2010, **132**, 6644.
7. E. Verheyen, J. P. Schillemans, M. van Wijk, M.-A. Demeniex, W. E. Hennink and C. F. van Nostrum, *Biomaterials*, 2011, **32**, 3008.
8. E. Yilmaz, K. Haupt and K. Mosbach, *Angew. Chem. Int. Ed.*, 2000, **39**, 2115.
9. M. M. Titirici and B. Sellergren, *Anal Bioanal Chem*, 2004, **378**, 1913.
10. D. Dechtrirat, K. J. Jetzschmann, W. F. M. Stoecklein, F. W. Scheller and N. Gajovic-Eichelmann, *Adv. Funct. Mater.*, 2012, **22**, 5231.
11. A. Rachkov and N. Minoura, *J. Chromatogr. A*, 2000, **889**, 111.
12. H. Nishino, C. S. Huang and K. J. Shea, *Angew. Chem. Int. Ed. Engl.*, 2006, **45**, 2392.
13. D.-F. Tai, M.-H. Jhang, G.-Y. Chen, S.-C. Wang, K.-H. Lu, Y.-D. Lee and H.-T. Liu, *Anal. Chem.*, 2010, **82**, 2290.
14. M. Emgenbroich, C. Borrelli, S. Shinde, I. Lazraq, F. Vilela, A. J. Hall, J. Oxelbark, E. De Lorenzi, J. Courtois, A. Simanova, J. Verhage, K. Irgum, K. Karim and B. Sellergren, *Chem.-Eur. J.*, 2008, **14**, 9516.
15. D. S. Janiak and P. Kofinas, *Anal Bioanal Chem*, 2007, **389**, 399.
16. J. Fullgrabe, E. Kavanagh and B. Joseph, *Oncogene*, 2011, **30**, 3391.
17. M. Grunstein, *Nature*, 1997, **389**, 349.
18. M. Shogren-Knaak, H. Ishii, J.-M. Sun, M. J. Pazin, J. R. Davie and C. L. Peterson, *Science*, 2006, **311**, 844.
19. W. Dang, K. K. Steffen, R. Perry, J. A. Dorsey, F. B. Johnson, A. Shilatifard, M. Kaeberlein, B. K. Kennedy and S. L. Berger, *Nature*, 2009, **459**, 802.
20. G. C. Taylor, R. Eskeland, B. Hekimoglu-Balkan, M. M. Pradeepa and W. A. Bickmore, *Genome Res.*, 2013, **23**, 2053.

