ChemComm

COMMUNICATION

Optimization of Fe_3O_4 nanozyme activity *via* single amino acid modification mimicking an enzyme active site†

Cite this: DOI: 10.1039/c6cc08542c

Received 24th October 2016,
Accepted 2nd December 2016

DOI: 10.1039/c6cc08542c

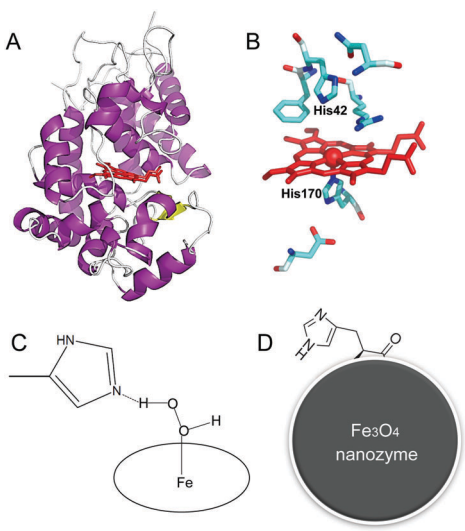
www.rsc.org/chemcomm

Kelong Fan,[a] Hui Wang,[b] Juqun Xi,[c] Qi Liu,[c] Xiangqin Meng,[ad] Demin Duan,[a] Lizeng Gao*[ac] and Xiyun Yan*[ad]

The Fe_3O_4 nanozyme was the first reported nanoparticle with intrinsic peroxidase-like activity and has been widely used in biomedicine. To optimize its catalytic activity, we introduced histidine residues onto the Fe_3O_4 nanoparticle surface in order to mimic the enzymatic microenvironment of natural peroxidase enzymes. Our results show that modification with a single amino acid could more than ten-fold improve the apparent affinity (K_M) of the Fe_3O_4 nanozyme for the substrate H_2O_2 and enhanced its catalytic efficiency (k_{cat}/K_M) up to twenty fold. Thus we not only optimized the activity of the Fe_3O_4 nanozyme, but also provide a new rationale for improving the efficiency of nanomaterial-based catalysts by utilizing strategies observed in nature.

Artificial enzymes, or enzyme mimetics have drawn considerable attention in the pursuit of alternatives for natural enzymes since the middle of the last century. However, constructing the ideal artificial enzyme is still challenging due to the low activity and selectivity of synthetic materials, which dramatically limits their applications. Nanomaterial-based enzyme mimetics, referred to as nanozymes, were recently discovered and represent a new generation of artificial enzymes.[1–7] Among them, the iron oxide (Fe_3O_4) nanozyme is a classical nanomaterial, with intrinsic peroxidase-like activity which was first reported by our group in 2007.[8] The Fe_3O_4 nanozyme was also found to have catalase-like activity at neutral/basic pH.[9] A multitude of new nanomaterial-based enzyme mimetics have subsequently been discovered and applied in disease diagnosis, virus detection, and environmental treatment.[10–16]

To study the catalytic efficiency and selectivity of a nanozyme, current methods mainly focus on changing their size, morphology, dopant, and surface. Here, we propose a new strategy to improve nanozyme activity through mimicking the enzymatic microenvironment of a natural enzyme. The active site of horseradish peroxidase (HRP) contains a heme cofactor in which iron plays a key role in the catalytic process (Scheme 1A).[17] Fe_3O_4 nanomaterials show a similar activity presumably because of the large area of ferric and ferrous iron available on its surface, which may catalyse the reaction in a similar way as the heme group within the active site of HRP. While showing comparable catalytic efficiency (k_{cat}) to HRP, the Fe_3O_4 nanozyme shows a much higher K_M for H_2O_2 than HRP, possibly due to the additional contributions to catalysis provided by the natural active site of



Scheme 1 Architecture of the active site in HRP and comparison with the histidine-modified Fe_3O_4 nanozyme. (A) Protein structure of HRP (PDB entry 1HCH); (B) architecture of active site in HRP; (C) H bond between histidine residual and H_2O_2 in the initial state of catalysis of HRP; (D) enhancement of Fe_3O_4 nanozyme activity by histidine modification.

[a] *Key Laboratory of Protein and Peptide Pharmaceuticals, CAS-University of Tokyo Joint Laboratory of Structural Virology and Immunology, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China. E-mail: lzgao@yzu.edu.cn, yanxy@ibp.ac.cn; Tel: +86-514-87797090, +86-10-64888583*

[b] *CAS Key Laboratory of Nanosystem and Hierarchical Fabrication, National Centre for Nanoscience and Technology, Beijing 100190, China*

[c] *School of Medicine, Yangzhou University, Yangzhou, Jiangsu 225001, China*

[d] *University of Chinese Academy of Sciences, Beijing 100049, China*

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c6cc08542c

HRP, including its porphyrin ring and proximal and distal histidine amino acid residues (Scheme 1A).

As shown in Scheme 1, two histidine residues, His42 and His170, are located distally and proximally to iron in the active site (Scheme 1B). According to the literature on the catalytic mechanism of HRP, the distal imidazole, His42, assists the location of H_2O_2 into the active site cavity through H-bond interaction and the formation of initial compound I (Scheme 1C), which plays an important role in holding H_2O_2 in place.[18,19] Inspired by this specific configuration of the active site of HRP, we hypothesized that introducing histidine on the surface of the Fe_3O_4 nanozyme might improve the affinity for H_2O_2 and therefore enhance its catalytic activity (Scheme 1D).

To verify this hypothesis, we synthesized Fe_3O_4 nanoparticles with histidine modification (His-Fe_3O_4) by the solvothermal method as described previously.[8,20] The modification was achieved by adding 0.1 g of histidine to the reaction solution during the synthetic process. Fe_3O_4 nanoparticles without modification (Naked-Fe_3O_4) and with Alanine (0.1 g) modification (Ala-Fe_3O_4) were also prepared as controls. As shown in Fig. 1A, the three types of synthesized Fe_3O_4 nanoparticles have consistent size and morphology, at around half a micron in diameter (characterized by TEM and SEM). The same size and morphology within these groups diminishes the interference from such parameters, which also affect catalytic activity.[21,22] XPS was used to verify the amino acid modification of these nanoparticles. The N1 signal (from nitrogen in the amino acid) was strongly present in His-Fe_3O_4 (3 N atoms/His) compared to Ala-Fe_3O_4 (1 N atom/Ala) and Naked-Fe_3O_4 (without N atom) which showed very minor to no signal, respectively (Fig. 1B). In addition, thermogravimetric analysis (TGA) showed that the ratio of histidine was about 3.1% (w/w), therefore the number of amino acid on each Fe_3O_4 nanozyme was estimated up to 4.2×10^7. These data indicate that histidine was successfully introduced onto Fe_3O_4 nanoparticles without affecting their size or morphology.

We next determined the peroxidase activity of Fe_3O_4 nanozymes modified with a single amino acid and compared their

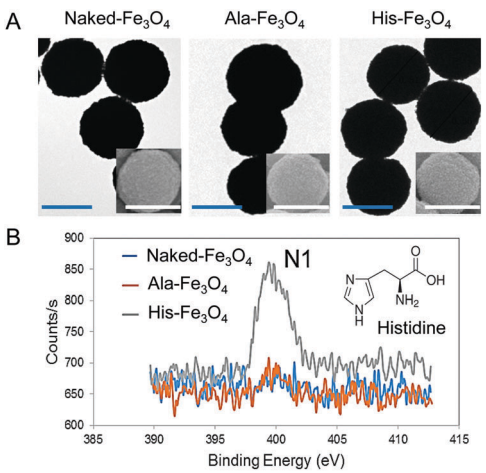


Fig. 1 Characterization of histidine modified Fe_3O_4 nanozyme. (A) TEM and SEM imaging for size and morphology characterization, scale bar is 500 nm; (B) XPS for amino acid modification analysis.

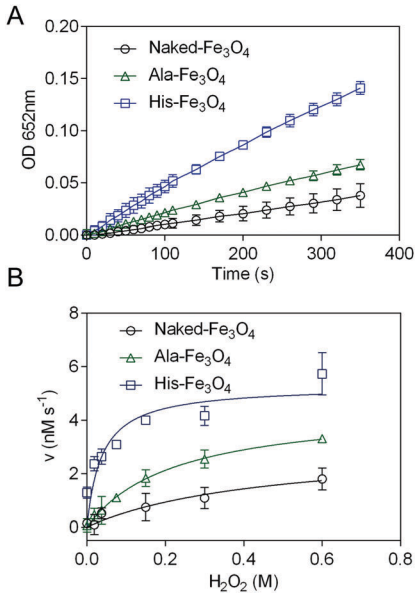


Fig. 2 Histidine modification significantly improved the K_M of H_2O_2 binding to Fe_3O_4 nanozyme in peroxidase-like catalysis. (A) Time course of catalysis under same reaction conditions; (B) Michaelis–Menten analysis.

catalytic parameters from Michaelis–Menten kinetic assays using a H_2O_2-3,3′,5,5′-tetramethylbenzidine (TMB) colorimetric system at acidic pH.[8] We first monitored the time course of the colorimetric reaction and found that His-Fe_3O_4 showed a significantly higher reaction velocity than Ala-Fe_3O_4 and Naked-Fe_3O_4, indicating histidine modification contributed to the improvement of Fe_3O_4 nanozyme activity (Fig. 2A). Further steady-state kinetic assay of H_2O_2 using the Michaelis–Menten model by fixing the enzyme and TMB concentration while varying H_2O_2 concentration demonstrated that the K_M of His-Fe_3O_4 was more than 10 times lower than that for Naked-Fe_3O_4, showing dramatically enhanced affinity for H_2O_2 *via* histidine modification (Fig. 2B and Table 1). To confirm that this enhancement is from the imidazole side-chain rather than amine or carboxyl groups in histidine, Ala-Fe_3O_4 (which has only a methyl group as the side chain) was introduced as a control and its K_M for H_2O_2 was found to be 226.6 ± 18.3 mM which was much higher than that for His-Fe_3O_4 (37.99 ± 7.6 mM), see Table 1. These results indicate that the improved affinity for H_2O_2 is from the side chain group (imidazole) of histidine. Actually, besides Alanine modification, other standard amino acids also could affect the K_M for H_2O_2, but were all much less impressive than histidine modification (Supplementary Table, ESI†). In addition, under the same reaction conditions, the V_{max} and k_{cat} of His-Fe_3O_4 were also higher than the other two types of Fe_3O_4 nanozymes, especially compared to the Naked-Fe_3O_4. Correspondingly, the k_{cat}/K_M, which reflects the catalytic efficiency of an enzymes for a given substrate, was compared to analyse the improvement of histidine modification for catalytic activity. Strikingly, the k_{cat}/K_M of His-Fe_3O_4 increased up to 7.1 fold *versus* Ala-Fe_3O_4 and 20.8 fold *versus* Naked-Fe_3O_4. These data from kinetic assays indicate that histidine modification of the Fe_3O_4 nanozyme could substantially increase the affinity of the nanozyme for its

Table 1 Parameters from steady-state kinetic with H_2O_2 as substrate

Michaelis–Menten	Naked-Fe_3O_4	Ala-Fe_3O_4	His-Fe_3O_4	HRP enzyme
$[E]$ (M)	9.8×10^{-15}	9.8×10^{-15}	9.8×10^{-15}	2.3×10^{-13}
V_{max} (nM s^{-1})	3 06 ± 0.54	4 45 ± 0.16	5.28 ± 0.71	0.689 ± 0.021
K_M (mM)	458.9 ± 29.1	226.6 ± 18.3	37.99 ± 7.8	10.35 ± 5.6
k_{cat} (10^5 s^{-1})	3.12 ± 0.55	4.54 ± 0.16	5.39 ± 0.73	0.03 ± 0.009
k_{cat}/K_M (10^6 s^{-1} M^{-1})	0.68 ± 0.12	2 ± 0.71	14.2 ± 1.89	0.29 ± 0.09

substrate H_2O_2 and further enhance the catalytic efficiency, providing strong evidence for our hypothesis that the activity of nanozymes can be improved by mimicking the architecture of the active site of natural enzymes.

To further verify the correlation between increased activity and histidine modification, we increased the amount of histidine present during the preparation of Fe_3O_4 nanozymes to 0.5 g or 1 g in order to introduce more imidazole groups onto the surface of the nanoparticles (TGA indicated the ratio (w/w) for histidine was 4.6% (6.2×10^7 per nanoparticle) and 6.5% (9.1×10^7 per nanoparticle), respectively). As expected, a greater amount of histidine produced higher activity, with 1 g > 0.5 g > 0.1 g (Fig. 3A). Kinetic assays showed that the K_M decreased with increasing histidine content, down to 23.75 mM with 1 g of histidine, which was close to that for the natural enzyme HRP. In addition to the enhanced apparent affinity for H_2O_2, the His modification also resulted in an increase the maximal velocity, V_{max}, with the value of $V_{max:1g}$ around 1.79 fold that for $V_{max:0.1g}$. The k_{cat} is proportional to V_{max} as a constant concentration of Fe_3O_4 nanozyme was used in the reaction.

Furthermore, the ratio k_{cat}/K_M for 1 g modification increased 2.85 fold compared to that for 0.1 g modification, indicating that the catalytic efficiency was dramatically improved. Based on these improvements in the catalytic activity and kinetic parameters, we concluded that histidine modification allows the Fe_3O_4 nanozyme to mimic HRP activity.

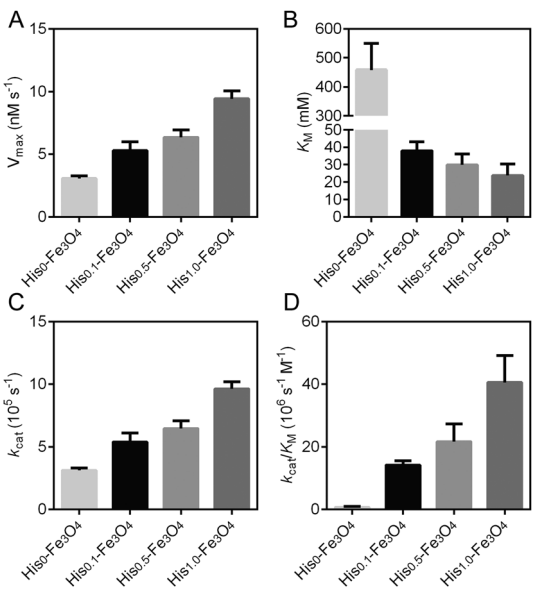


Fig. 3 Correlation between activity and degree of histidine modification. (A) V_{max}; (B) K_M; (C) k_{cat}; (D) k_{cat}/K_M.

Similarly, the steady-state kinetics for TMB were also investigated by fixing the nanozyme and H_2O_2 concentrations while varying the TMB concentration. Although His-Fe_3O_4 still showed the highest activity, the K_M values for His-Fe_3O_4 and Ala-Fe_3O_4 were similar, and were actually significantly higher than for Naked-Fe_3O_4 (Supplementary Figure, ESI†), indicating that histidine modification did not have a specific beneficial effect on the association of the Fe_3O_4 nanozyme with its substrate TMB. This may reflect that the reaction was carried out under acidic pH conditions, under which histidine has a net positive charge counteracting the binding of TMB which also possesses a positive charge from protonated primary amines.[23]

While the above assays demonstrating peroxidase-like activity were all performed under acidic pH, Fe_3O_4 also displays intrinsic catalase-like activity, directly decomposing H_2O_2 into O_2 and water under neutral or basic conditions (Fig. 4A).[9] Since histidine modification had significant impact on the apparent affinity (K_M) for H_2O_2, we speculated that the catalase-like activity might also be improved correspondingly. To verify this, we used a dissolved oxygen meter to detect the kinetics for H_2O_2 decomposition by monitoring the generation of O_2 under

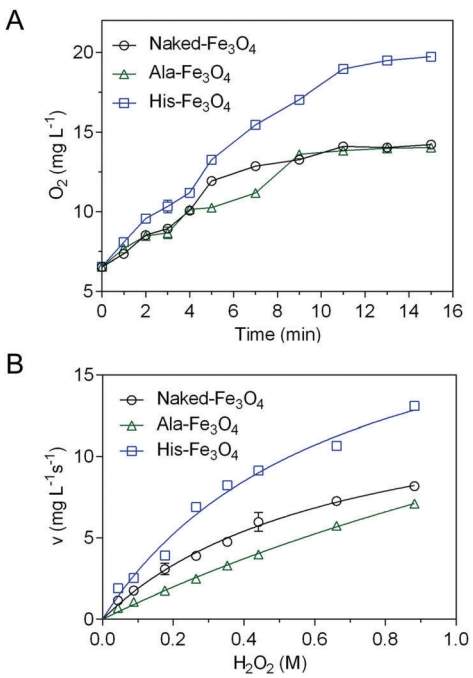
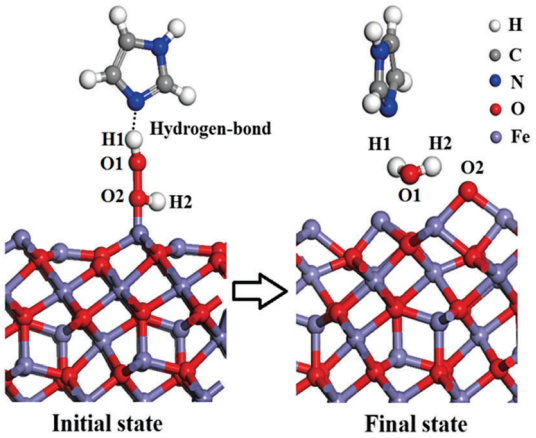


Fig. 4 The histidine modification significantly improved the catalase-like activity of the Fe_3O_4 nanozyme. (A) Time course for H_2O_2 decomposition; (B) Michaelis–Menten analysis.



Scheme 2 Simulation of the role of histidine in the catalytic process.

neutral pH. As shown in Fig. 4B, His-Fe_3O_4 showed significantly higher activity than Ala-Fe_3O_4 and Naked-Fe_3O_4. Correspondingly, the steady-state kinetic assay showed that His-Fe_3O_4 had the lowest K_M (571.6 mM) and the highest V_{max} (20.45 mg L^{-1} s^{-1} of O_2). These results indicate that histidine modification also improved the catalase-like activity of the Fe_3O_4 nanozyme.

To better understand why histidine modification could improve the enzymatic activities of the Fe_3O_4 nanozyme, we constructed a theoretical model based on the experimental data for the enhancement of peroxidase-like and catalase-like catalysis by the Fe_3O_4 nanozyme with a single histidine modification. We simulated the initial state of hydrogen peroxide in the adsorption and dissociation process on Fe_3O_4 nanoparticles[24,25] forming hydrogen bond with the coexistent histidine residue (Scheme 2 shows one of pathways). In the catalytic process, the hydrogen bond formed between histidine and the hydrogen peroxide (initial state) not only weakens the O–H bond strength but also leads O to become more negatively charged. The former process is beneficial for splitting the O–O bond of hydrogen peroxide and the latter step enhances its adsorption onto the Fe_3O_4 nanozyme (final state). It thus serves in a similar role as His42 in the active site of HRP.

In conclusion, we have demonstrated that a single amino acid modification can substantially enhance the catalytic activities of the Fe_3O_4 nanozyme by mimicking the architecture of the active site in natural HRP. Introduction of histidine improved the peroxidase-like activity and the catalytic efficiency of the Fe_3O_4 nanozyme by enhancing the affinity for H_2O_2 *via* hydrogen bond formation between the imidazole group of histidine and H_2O_2, which provides a similar configuration as in the active site of HRP. Besides peroxidase-like activity, the histidine modification also enhances catalase-like activity, also reflecting the enhanced affinity for H_2O_2 at the initial reaction step. These results not only demonstrate that a single amino acid modification can effectively improve Fe_3O_4 nanozyme activity, but also provide a new general strategy to design improved nanozymes by mimicking the architecture of the active site in naturally occurring enzymes.

We thank Dr Xuehui Chen for help in preparing the figure showing the HRP protein structure and architecture of its active site. We also thank the Testing and Analysis Centre at Yangzhou University for SEM and XPS analysis. We thank Prof. Sarah Perrett and Prof. Xingjie Liang for comments on the manuscript. This work was supported by the Foundation of the Thousand Talents Plan for Young Professionals and Jiangsu Specially-Appointed Professor, the Interdisciplinary Funding at Yangzhou University, Strategic Priority Research Program of the Chinese Academy of Sciences (Grant No. XDA09030306), National Natural Science Foundation of China (Grant No. 31530026), Key Research Program of Frontier Sciences, CAS (Grant No. QYZDB-SSW-SMC013), Beijing Natural Science Foundation (Grant No. 5164037), China Postdoctoral Science Foundation (Grant No. 2015M570158) and the China Postdoctoral Science Special Foundation (Grant No. 2016T90143).

Notes and references

1 L. Gao and X. Yan, *Sci. China: Life Sci.*, 2016, DOI: 10.1007/s11427-016-5044-3. Q3
2 H. Wei and E. K. Wang, *Chem. Soc. Rev.*, 2013, **42**, 6060–6093.
3 L. Z. Gao and X. Y. Yan, *Prog. Biochem. Biophys.*, 2013, **40**, 892–902.
4 Y. H. Lin, J. S. Ren and X. G. Qu, *Acc. Chem. Res.*, 2014, **47**, 1097–1105.
5 X. Y. Wang, Y. H. Hu and H. Wei, *Inorg. Chem. Front.*, 2016, **3**, 41–60.
6 R. Ragg, M. N. Tahir and W. Tremel, *Eur. J. Inorg. Chem.*, 2016, 1906–1915, DOI: 10.1002/ejic.201501237,.
7 H. Y. Shin, T. J. Park and M. I. Kim, *J. Nanomater.*, 2015, DOI: 10.1155/2015/756278.
8 L. Gao, J. Zhuang, L. Nie, J. Zhang, Y. Zhang, N. Gu, T. Wang, J. Feng, D. Yang, S. Perrett and X. Yan, *Nat. Nanotechnol.*, 2007, **2**, 577–583.
9 Z. W. Chen, J. J. Yin, Y. T. Zhou, Y. Zhang, L. Song, M. J. Song, S. L. Hu and N. Gu, *ACS Nano*, 2012, **6**, 4001–4012.
10 Y. Song, K. Qu, C. Zhao, J. Ren and X. Qu, *Adv. Mater.*, 2010, **22**, 2206–2210.
11 F. Natalio, R. Andre, A. F. Hartog, B. Stoll, K. P. Jochum, R. Wever and W. Tremel, *Nat. Nanotechnol.*, 2012, **7**, 530–535.
12 W. W. He, Y. Liu, J. S. Yuan, J. J. Yin, X. C. Wu, X. N. Hu, K. Zhang, J. B. Liu, C. Y. Chen, Y. L. Ji and Y. T. Guo, *Biomaterials*, 2011, **32**, 1139–1147.
13 K. L. Fan, C. Q. Cao, Y. X. Pan, D. Lu, D. L. Yang, J. Feng, L. N. Song, M. M. Liang and X. Y. Yan, *Nat. Nanotechnol.*, 2012, **7**, U833–U876.
14 H. Wei and E. Wang, *Anal. Chem.*, 2008, **80**, 2250–2254.
15 H. Cheng, L. Zhang, J. He, W. Guo, Z. Zhou, X. Zhang, S. Nie and H. Wei, *Anal. Chem.*, 2016, **88**, 5489–5497.
16 H. Cheng, S. Lin, F. Muhammad, Y.-W. Lin and H. Wei, *ACS Sens.*, 2016, DOI: 10.1021/acssensors.6b00500.
17 N. C. Veitch, *Phytochemistry*, 2004, **65**, 249–259.
18 G. I. Berglund, G. H. Carlsson, A. T. Smith, H. Szoke, A. Henriksen and J. Hajdu, *Nature*, 2002, **417**, 463–468.
19 E. Derat and S. Shaik, *J. Phys. Chem. B*, 2006, **110**, 10526–10533.
20 L. Z. Gao, J. M. Wu, S. Lyle, K. Zehr, L. L. Cao and D. Gao, *J. Phys. Chem. C*, 2008, **112**, 17357–17361.
21 S. Liu, F. Lu, R. Xing and J. J. Zhu, *Chem. – Eur. J.*, 2011, **17**, 620–625.
22 F. F. Peng, Y. Zhang and N. Gu, *Chin. Chem. Lett.*, 2008, **19**, 730–733.
23 F. Q. Yu, Y. Z. Huang, A. J. Cole and V. C. Yang, *Biomaterials*, 2009, **30**, 4716–4722.
24 X. K. Li and J. Paier, *J. Phys. Chem. C*, 2016, **120**, 1056–1065.
25 A. Kiejna, T. Ossowski and T. Pabisiak, *Phys. Rev. B: Condens. Matter Mater. Phys.*, 2012, **85**, 125414.

