Controlling Kinase Activities by Selective Inhibition of Peptide Substrates

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Supporting Information Placeholder

ABSTRACT: Phosphorylation is the most common reversible posttranslational modification of proteins. Because a given kinase often has many substrates in a cell and is involved in numerous functions, traditional inhibition of the enzyme leads to unintended consequences. Here we report synthetic receptors to manipulate kinase phosphorylation precisely for the first time, utilizing the receptors' abilities to bind peptides with high affinity and specificity. The inhibition enables selective phosphorylation of peptides with identical consensus motifs in a mixture. A particular phosphosite can be inhibited while other sites in the same substrate undergo phosphorylation. The receptors may work either individually on their targeted strands or in concert to protect segments of a long sequence. The binding-derived inhibition is able to compete with protein–protein interactions within a multidomain kinase, enabling controlled PTM to be performed in a previously unavailable manner.

Phosphorylation is the most common reversible posttranslational modification (PTM) of protein.[1] Because enhanced kinase activities often lead to cancer, kinases are regulated tightly by a number of mechanisms[2] and many kinases are established targets for anticancer drugs.[3,4]

The many proteins in a cell creates a vast number of potential phosphorylation sites, ~700,000 by one estimation.[2] Even if a large proportion of these sites are buried and kinases exhibit target preferences, a given kinase often has a wide range of substrates and controls numerous functions simultaneously.[1,2] The scope and diversity of kinase targets make traditional enzyme inhibition problematic, as unintended consequences frequently emerge when a kinase is shut down. Hence, although many small molecule kinase inhibitors are being pursued as anticancer drugs, lack of inhibitor selectivity remains a major challenge.[5]

An alternative approach in PTM manipulation is to inhibit the substrates instead. If a particular substrate can be selectively blocked from the action of a kinase, one can study and control phosphorylation-derived cellular processes with high precision. The strategy was considered "a nearly ideal solution to the manipulation of protein PTM",[6] but remains largely unexplored due to the lack of materials to bind short peptides with high selectivity and biologically competitive affinity. Not only so, different substrates of a kinase tend to have very similar or even identical motifs surrounding the phosphorylation sites, i.e., the consensus motifs.[7] Although many synthetic peptide-binding materials have been reported,[8,9] none could satisfy such stringent requirements for selective phosphorylation.

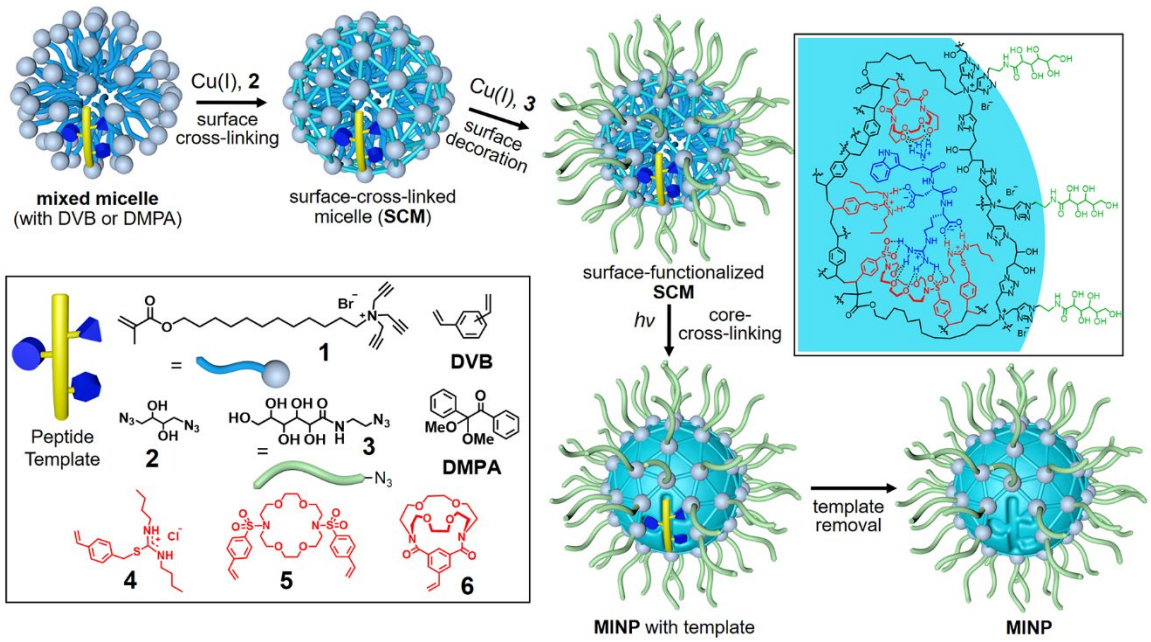
Our peptide-binding molecularly imprinted nanoparticles (MINPs) were prepared through molecular imprinting[10-12] of cross-linked micelles (Scheme 1).[13] The method involves solubilization of a peptide in the micelle of cross-linkable surfactant **1**, together with divinylbenzene (DVB), and 2,2-dimethoxy-2-phenylacetophenone (DMPA, a photoinitiator). The mixed micelle is cross-linked on the surface by click reaction with **2** and then functionalized with **3**. Core-cross-linking by free radical polymerization is key to the molecular imprinting, creating complementary, imprinted binding sites for both the hydrophobic side chains and acidic/basic groups if additional functional monomers (FMs) are used. FM **4** is particularly effective at imprinting carboxylic acids,[14] **5** the guanidinium of arginine,[15] and **6** amino groups.[16]

Cyclic AMP-dependent protein kinase (PKA) is the best understood kinase and has over 100 physiological substrates identified.[7,17] We chose four substrates that contained the most abundant consensus motif RRXS (X = a variable amino acid): Kemptide (**7**, LRRASLG),[18] β_2-adrenergic receptor peptide (**8**, TGHGLRRSSKFCLK),[19] pyruvate kinase peptide (**9**, PAGYLRRASVAQLT),[20] and cardiac myosin-binding protein-C peptide (**10**, FRRTSLAGGGRRISDSHE).[21] Peptides **7** and **9** have identical consensus motifs and peptide **10** has two phosphorylation sites. It should also be mentioned that, despite the attractiveness of substrate inhibition in kinase control,[6] only a single such example was found in the literature[22] and selective phosphorylation of highly similar sequences has never been achieved to the best of our knowledge.

We first measured the bindings of the MINPs for their corresponding peptides by isothermal titration calorimetry (ITC). For peptide **10**, since our goal was to selectively phosphorylate one of the two serine residues present, we prepared MINPs for **10a** (FRRTSLA) and **10b** (RRISDSHE), respectively, corresponding to the first and second halves of the full sequence, not counting the three glycines in between.

As shown in Table S1, nonfunctionalized MINP(**7**) could bind Kemptide with micromolar affinity; adding FMs **4**–**6** in the MINP preparation enhanced the binding by over one order of magnitude. The K_d values (19–550 nM) for functionalized MINP(**7**–**10**) were one to several orders of magnitude smaller than the K_m of typical kinases,[18] boding well for the inhibition.

Figure 1a shows the reaction profiles for the phosphorylation of peptide **7** by PKA. Figure 1b shows the HPLC chromatograms of the reaction mixtures at 120 min. Phosphorylation occurred rapidly in the buffer and was complete at 120 min. The nonimprinted nanoparticles (NINPs), prepared without the template, slowed down the reaction somewhat, but not nearly as much as 1 or 2 equivalents of MINP(**7**).



Scheme 1. Preparation of peptide-binding MINP from molecular imprinting of a cross-linked micelle, with a schematic representation of the cross-linked structure containing WDR bound by polymerized FMs.

Interestingly, when the phosphorylation yield was plotted against the MINP concentration, the curve resembled a typical 1:1 binding isotherm, with an apparent K_a of 1.2×10^6 M^{-1} (Figure 1c). This value was quite close to the actual binding constant (1.81×10^6 M^{-1}) obtained by ITC for the MINP (Table S1). Moreover, when inhibition experiments were performed with peptide **8** (Figures S22–S27) and peptide **9** (Figures S28–S34), similar correlation was observed (Figure 1d and Figure S34). These results strongly suggest that the inhibition was a direct result of binding. For all three peptides, the phosphorylation yield was linearly related ($R^2 \approx 0.99$) to the MINP concentration prior to the inflection point at 1 equiv MINP. The only difference was that the more tightly binding MINPs displayed a shaper transition at the inflection point of 1:1 MINP/peptide.

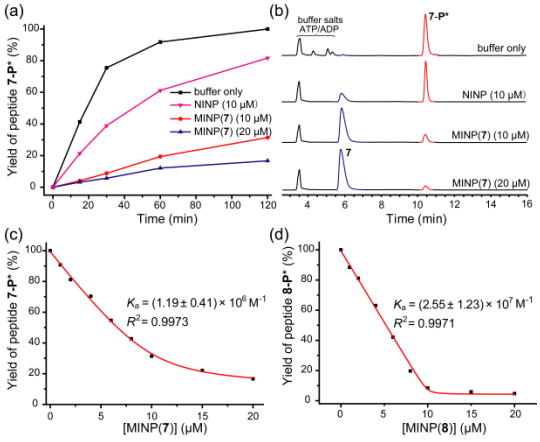


Figure 1. (a) Phosphorylation of peptide **7** by PKA in 10 mM Tris buffer (pH= 7.4) at 298 K. (b) HPLC analysis of phosphorylation of peptide **7** by PKA in 10 mM Tris buffer (pH= 7.4) at 120 min of reaction time. (c,d) Nonlinear least squares curve fitting of the phosphorylation yields of peptide **7** (c) and peptide **8** (d). [**7**] = [**8**] =10 μM. [ATP] = 40 μM.

The above results indicate that phosphorylation of a peptide by PKA could be reliably inhibited by the corresponding MINP and the degree of inhibition is completely predictable from the binding affinities. Encouraged by these results, we performed selective phosphorylation of peptide mixtures, from relatively simple to more complex ones.

Our initial mixture was **7** and **9**, two peptides with an identical recognition motif before the serine (i.e., RRA), even the leucine (L) in front of the serine. Nonetheless, we could suppress the reaction of either peptide, depending on the MINP used. Quantitative LC-MS analysis indicated that, at the end of 120 min, whereas the unprotected peptide had a conversion yield of >95%, the protected peptide was largely intact (Figure S43). The conversion yield in the binary mixture was 15% for **7** in the presence of 2 equiv MINP(**7**) and 4% for **9** with 2 equiv MINP(**9**). The stronger inhibition of **9** was expected from its stronger binding by its MINP protector (Table S1).

Multisite phosphorylation frequently happens in cells for signal integration and amplification.[23] Peptide **10** (FRRTSLAGGGRRISDSHE) is part of cardiac myosin-binding protein-C, whose phosphorylation is linked to modulation of cardiac contraction.[21] Phosphorylation of the second serine triggers kinase to act on two other sites including the first serine shown in the above sequence. The close distance between the two sites poses a large challenge to selective inhibition, given the nearly 5 nm diameter of typical MINP (Figures S6–S11). To our delight, LC-MS analysis indicated that bisphosphorylation was entirely absent when 2 equivalents of either MINP(**10a**) or MINP(**10b**) were present. Meanwhile, two different monophosphorylated products (with an identical m/z value but different retention times in the LC-MS) could be obtained, depending on the MINP used (Figures S35–S42).

Figure 2 shows the full product distribution curves for the phosphorylation of peptide **10** under different scenarios. Figure 2a,b shows that, even though NINP slowed down the enzyme reaction slightly, the reaction profiles were very similar, with the two monophosphorylated products showing up transiently only in the early stage of the reaction. Note that PKA phosphorylated the two serines with similar rates, evident from their nearly overlapping reaction profiles. Importantly, in the presence of either MINP(**10a**) or MINP(**10b**), the bisphosphorylated products disappeared while the desired monophosphorylated product

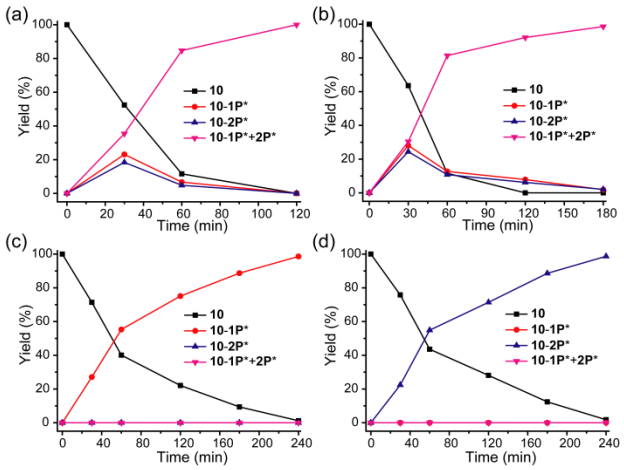


Figure 2. Product distribution curves in the PKA-catalyzed phosphorylation of peptide **10** in 10 mM Tris buffer (a) and in the presence of 1.0 equiv NINP (b), 2.0 equiv MINP(**10a**) (c), and 2.0 equiv MINP(**10b**) (d). [**10**] = 10 μM. [ATP] = 40 μM.

persisted. With either MINP bound to near half of **10**, phosphorylation of the unprotected serine slowed down. It is actually quite remarkable that the complete reaction in the "half-protected" peptide (Figure 2c,d) only took twice as long as the unprotected peptide (Figure 2a).

Having achieved selective phosphorylation of a peptide with two reactive sites, as well as binary/ternary mixtures (Figure S43 for **7** + **9**, Figure S44 for **8** + **9**, and Figure S45 for **7** + **8** + **9**), we attempted to control the phosphorylation of a mixture of all four peptides. As shown in Figure 3a, peptides **7**–**10** were completely phosphorylated by PKA in 240 min under our experimental conditions. When both MINP(**8**) and MINP(**9**) were added to the mixture, about 6% of peptide **8** and 5% of **9** were phosphorylated while **7** and **10** underwent complete reaction (Figure 3b). If only MINP(**10a**) or MINP(**10b**) was present, the bisphosphorylated product disappeared while the desired monophosphorylated products appeared (Figure 3c,d). Most interestingly, MINP(**10a**) or MINP(**10b**) could work together to protect the entire sequence of **10**: neither the mono- nor bisphosphorylated products could form when both MINPs were added to the reaction mixture (Figure 3e).

Biological phosphorylation frequently occurs within a protein complex, making the inhibition much more challenging because MINP binding has to compete with intramolecular protein–protein interactions. To demonstrate the utility of our MINP under such a scenario, we attempted to control a key phosphotransfer step in the activation of the proline-rich tyrosine kinase 2 (Pyk2), a regulator of leukocyte motility, bone remodeling, and neuronal development and a promising target for cancer therapeutics.[24] For *in vitro* activity assays, we employed a construct of Pyk2 encompassing the core regulatory domains (FERM-kinase).

The multistage Pyk2 activation is initiated by autophosphorylation of tyrosine Y402 in the linker between regulatory FERM and kinase domains.[25,26] We prepared MINPs using peptides **11a**–**c**, corresponding to AA373–383, 388–398, and 400–411 of the linker sequence, respectively (Figure S45). Although all three MINPs inhibited the phosphorylation (Figure 3f), MINP(**11a**) and MINP(**11b**) were significantly more potent than MINP(**11c**), even though it was the latter that directly impinged on the Y402 site.[27] The weaker inhibition of MINP(**11c**) may reflect a lower accessibility of the Y402 site in the FERM-kinase basal conformation. Whereas the conformation of the Pyk2 Y402 linker remains unknown, the structure of the FAK FERM-kinase revealed that the basal conformation constrains the autophosphorylation site in an abbreviated β sheet.[28] The differential impact between linker target sites thus suggests a potential for MINPs as conformational sensors.

In summary, precise control of peptide and protein phosphorylation is demonstrated for the first time using synthetic materials. Although peptide-binding molecularly imprinted materials have been reported,[29-39] controlled phosphorylation shown in this work was only possible with the nanodimension of MINP and its high binding affinity and selectivity. The most important feature of this method is its versatility—inhibition of a single peptide or multiple ones in a mixture, by the MINPs working individually on different substrates or cooperatively on the same substrate, for intermolecular and intramolecular phosphorylation to reveal the best sequence to target to control PTM. Given the importance of phosphorylation of proteins in biology and the straightforward one-pot preparation of MINPs, these materials are expected to enable researchers to control PTM and extract mechanistic insight associated with PTM in a previously impossible manner.

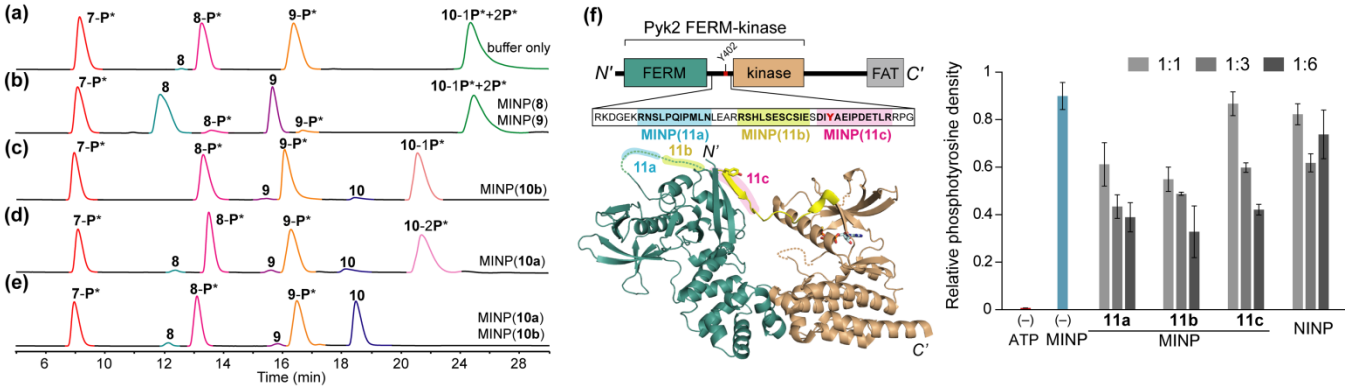


Figure 3. (a–e) HPLC analysis of phosphorylation of a mixture of peptides **7**, **8**, **9**, and **10** by PKA in 10 mM Tris buffer (pH= 7.4) at 240 min of reaction time under different conditions. [**7**] = [**8**] = [**9**] = [**10**] =10 μM. [MINP] = 20 μM. [ATP] = 100 μM. Identities of the peaks were confirmed by an HRMS detector coupled to the LC. (f) (left) Domain organization of Pyk2 and structural model depicting the Pyk2 FERM (PDB 4eku) and kinase (PDB 3fzp) aligned to the FAK FERM-kinase (PDB 2j0j). The FAK FERM--kinase linker is superimposed (yellow) to illustrate putative MINP binding sites. (right) Inhibition of Pyk2 autophosphorylation by MINPs at 1:1, 1:3, and 1:6 protein/MINP ratios, with the NINP as the control. [Pyk2] = 1.0 μM.

ASSOCIATED CONTENT

Supporting Information

Synthetic, protein purification, assay procedures, chromatographs, and NMR data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interests.

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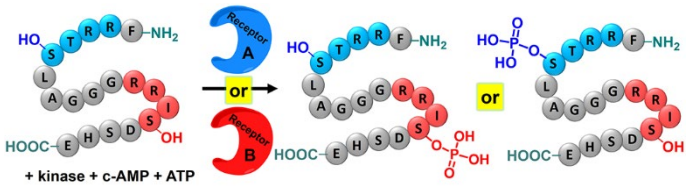
Receptor A
or
Receptor B
or
+ kinase + c-AMP + ATP