

Detection of Enzyme Activity through Catalytic Signal Amplification with Functionalized Gold Nanoparticles**

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The detection of low levels of proteins and other biomarkers is of crucial importance for the early diagnosis of diseases.^[1] The development of chemical-sensing methodologies as an alternative to biological assays is of strong current interest, because such methods involve simple detection protocols. In addition, such systems can be adapted through straightforward structural modifications for use with a wide variety of targets.^[2–5] Nevertheless, a common feature of these assays is that the amount of generated signal is proportional to the amount of substrate converted by the enzyme. The sensitivity of such assays would be significantly increased if the enzymatic conversion of a single substrate molecule led to the formation of a multitude of reporter molecules through a cascade of chemical events, each of which magnified the previous event. Examples of chemical systems able to amplify originally weak input signals have been reported.^[6–9] Herein, we report the application of a catalytic amplification process for the detection of proteases. The strategy relies on a cascade of two catalytic events for signal generation, whereby a gold nanoparticle covered with a catalytic self-assembled organic monolayer (Au-MPC) has a crucial central role. In the first event, an enzyme hydrolyzes a peptide substrate, which acts as an inhibitor for the catalytic monolayer (Figure 1). Upon hydrolysis, the catalytic activity of the monolayer is restored, and large quantities of a yellow reporter molecule are produced. The Au nanoparticles are important for two reasons. First, they enable the facile, spontaneous formation of dinuclear catalytic sites on the periphery of the monolayer.^[10] Second, their multivalent nature permits the occurrence of multipoint interactions with (biological) targets.^[11] The latter aspect, together with the intrinsic physical and chemical properties of the nanoparticles and the ease of their preparation and functionalization,^[12] has led to extensive development of assays based on Au nanoparticles that also occasionally rely on various forms of signal amplification.^[13–20]

Previously, we showed that Au-MPC **1** catalyzes the transphosphorylation of 2-hydroxypropyl-4-nitrophenyl phosphate (HPNPP) highly efficiently.^[10] HPNPP is an activated RNA model substrate. Detailed kinetic studies revealed that catalysis results from the cooperative action of two triazacyclononane·Zn^{II} (TACN·Zn^{II}) complexes localized on the surface of the monolayer.^[21,22] Au-MPC **1**, which is fully covered with the TACN·Zn^{II} complex, displays enzyme-like saturation behavior with the “overall” values $k_{\text{cat}} = 6.7 \times 10^{-3} \text{ s}^{-1}$ and $K_{\text{M}} = 0.31 \text{ mM}$ at pH 7.5 in H₂O.^[23] This system is intriguing for the following reasons: a) under the experimental conditions, there is practically no background reaction, since k_{uncat} under the same conditions is of the order of 10^{-7} s^{-1} ; b) the reaction can be monitored visibly by measuring the absorbance of the *p*-nitrophenol product at 400 nm; c) a surprisingly high affinity is observed for the binding of HPNPP to **1**. Since Au-MPC **1** has a multitude of positively charged TACN·Zn^{II} complexes on its surface, we anticipated that the system would have a high affinity for oligoanions owing to multivalent interactions. This hypothesis was also supported by the contributions by Hamachi and co-workers, who demonstrated that oligophosphates and oligoaspartates bind a bis(zinc(II) dipicolylamine) complex with very high affinity.^[24–26] In our system, such oligoanions would act as competitive inhibitors for HPNPP and thus turn off the catalytic activity of the system.

To verify whether we could use the catalytic production of *p*-nitrophenol as a tool to detect binding events on the Au-MPC surface, we studied two series of biologically important oligoanions (peptides and phosphates) with negative charges increasing from 1 to 4. The peptide series comprised BocNH-Gly-OH (1–), AcNH-Asp-OH (2–), AcNH-Asp-Asp-OH (3–), and AcNH-Asp-Asp-Asp-OH (4–), and the phosphate series cAMP (1–), AMP (2–), ADP (3–) and ATP (4–; Figure 1). Increasing amounts of each compound were added to a solution of Au-MPC **1** in H₂O buffered at pH 7.0 at 40 °C with the concentration of TACN·Zn^{II} headgroups equal to 5 μM. This value implies a Au-MPC **1** concentration of around 100 nM, on the basis of the knowledge that a 1.6 nm sized nanoparticle contains roughly 50 thiols.^[27] A kinetic Zn^{II} titration confirmed that at these concentrations, the Zn^{II} ions are quantitatively bound to the TACN ligand (see the Supporting Information). Subsequently, HPNPP was added to give an initial concentration of 1 mM in the mixture, and the initial rate of cleavage, v_{init} , was measured for 30 min by monitoring the increase in absorbance at 400 nm.^[28] A plot of the initial rate (v_1), normalized with respect to the initial rate in the absence of an inhibitor (v_0), as a function of the concentration of added inhibitor, gave the inhibition curves depicted in Figure 2 for the peptides (the data for the

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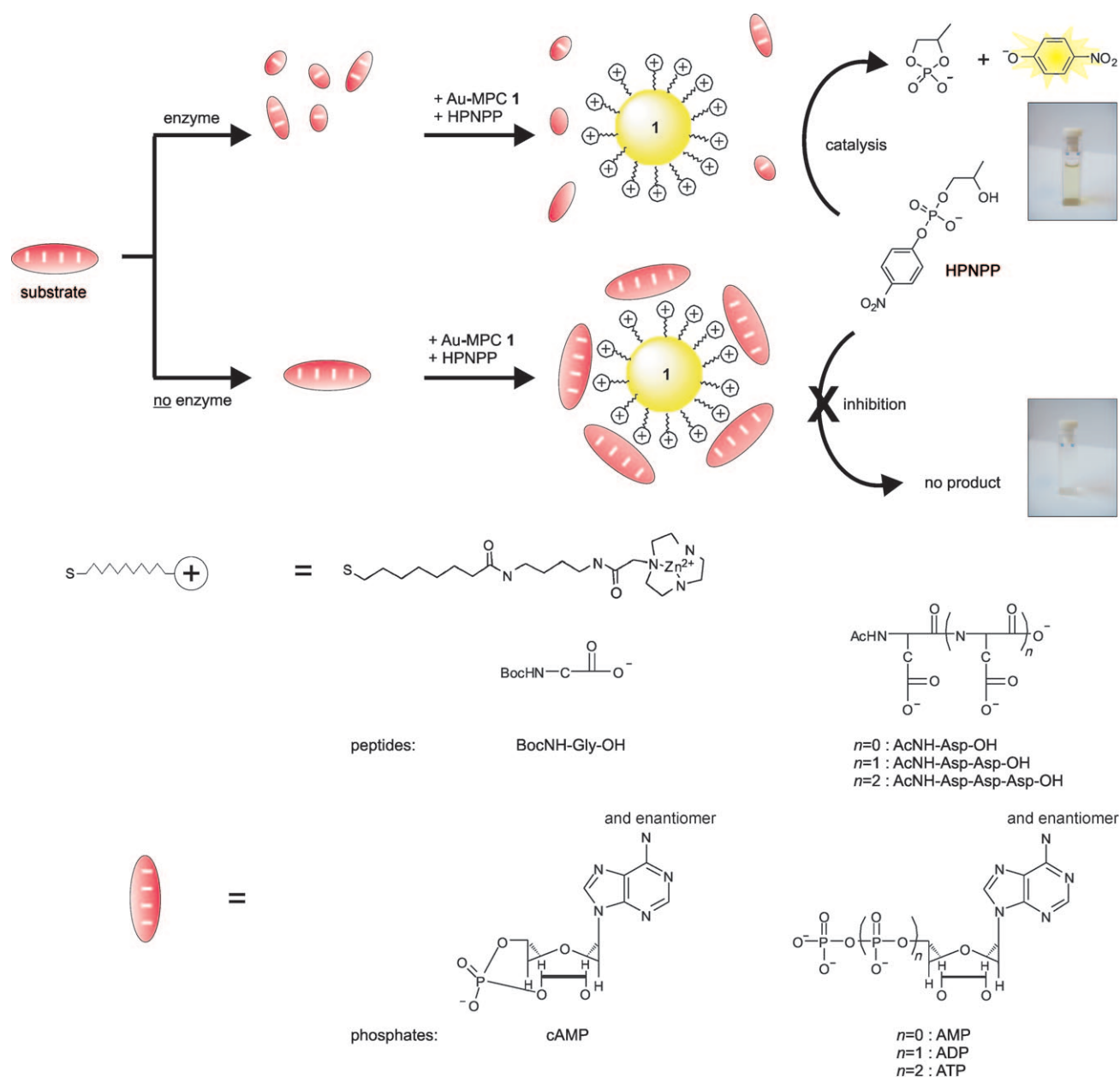


Figure 1. Schematic representation of the catalytic assay. The presence of an enzyme able to hydrolyze a substrate enables catalysis of the transphosphorylation of HPNPP by Au-MPC 1 resulting in the release of a yellow reporter molecule. In the absence of the enzyme, the catalytic activity of Au-MPC 1 is suppressed because the enzyme substrate acts as an inhibitor for Au-MPC 1. Boc = *tert*-butoxycarbonyl.

phosphate series are reported in the Supporting Information). In no case was precipitation observed, as confirmed by comparison of the absorption spectra before and after the addition of the inhibitors. Unambiguous proof that the catalytic activity is inhibited because of binding of the oligoanions to Au-MPC 1 was obtained from fluorescence titration studies with a fluorescent analogue of ATP (see the Supporting Information).^[29]

A first glance at the inhibition curves immediately reveals a striking difference in inhibitory power as a function of the negative charges present in the inhibitor, within both the peptide and the phosphate series. The singly charged anions BocNH-Gly-OH and cAMP inhibited catalytic activity at millimolar concentrations, whereas the quadruply charged

anions AcNH-Asp-Asp-Asp-OH and ATP were already effective in the low-micromolar region. Dissociation constants for the inhibitors were determined by fitting the curves to the following equation: $v_1/v_0 = 1/(1+[I]/K_I)$ (Table 1). We were unable to fit the curves for AcNH-Asp-Asp-Asp-OH, ADP, and ATP, because under these conditions the affinity of these inhibitors for Au-MPC 1 is too high. Repeating the inhibition experiments at a 2.5-fold lower concentration of TACN-Zn^{II} (2 μ M; see the Supporting Information) enabled determination of the dissociation constant for AcNH-Asp-Asp-OH, but still not for ADP and ATP.

The availability of the dissociation constants for the complete peptide series gives insight into the thermodynamics of binding. The data show that the dissociation constant is

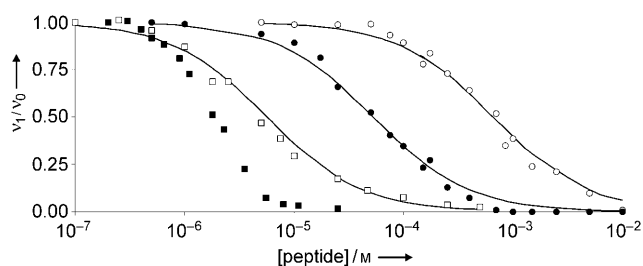


Figure 2. Initial rates (normalized with respect to the initial rate in the absence of analytes) as a function of the concentration of added AcNH-Asp-Asp-Asp-OH (■), AcNH-Asp-Asp-OH (□), AcNH-Asp-OH (●), and BocNH-Gly-OH (○). Experimental conditions: [TACN·Zn^{II}] = 5.0×10^{-6} M, [HEPES] = 1.0×10^{-2} M (except in the case with BocNH-Gly-OH, for which [HEPES] = 40 mM), pH 7.0, 40 °C. The solid lines are the best fit to the equation $v_1/v_0 = 1/(1+[I]/K_i)$. Analogous results were obtained for the phosphate series (see the Supporting Information). HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Table 1: Dissociation constants for inhibition by the analytes and corresponding changes in free energy.

Substrate	Log K_i	ΔG [kJ mol ⁻¹] ^[a]
Boc-Gly-OH	-3.18	-19.1
AcNH-Asp-OH	-4.28	-25.6
AcNH-Asp-Asp-OH	-5.25	-31.5
AcNH-Asp-Asp-Asp-OH ^[b]	-6.27	-37.6
cAMP	-3.50	-21.0
AMP	-4.70	-28.1
ADP ^[c]	≤ 6.3	≤ 38.0
ATP ^[c]	≤ 6.3	≤ 38.0

[a] $\Delta G = -RT \ln(1/K_i)$, in which $R = 8.314 \text{ J K}^{-1} \text{ mol}^{-1}$ and $T = 313 \text{ K}$.
 [b] The results for this peptide were obtained from an inhibition experiment at a lower Au-MPC concentration (see the Supporting Information). [c] Only the lower limits could be obtained for these analytes, since saturation occurs under the experimental conditions.

decreased by roughly a factor of 10 for each negative charge added to the analyte. Correspondingly, the change in the free energy of binding, ΔG , increases in a perfect linear manner as a function of the number of charges present in the oligoanion. This result implies that the contribution to the overall binding constant of each incremental anion does not interfere with the other contributions (i.e. $\Delta G_{\text{tot}} = \Delta G_i \times i$, in which i is the number of negative charges on the oligoanion), a behavior also observed in other systems.^[30] The increment per negative charge added corresponds to around -6 kJ mol^{-1} , which is very close to the value of -5 kJ mol^{-1} reported by Schneider et al. for a single ion pair in water.^[31] This similarity suggests that the binding of the oligoanions to Au-MPCs **1** is mostly driven by electrostatic interactions.^[32]

The ability to switch off the inhibitory activity of an oligoanion by its conversion into a shorter oligomer by an external effector implies that this system should be able to act as a sensor of the effector itself. For example, the enzymatic cleavage of a $5 \mu\text{M}$ solution of AcNH-Asp-Asp-Asp-OH should (at least partially) restore the catalytic activity of Au-MPC **1**, because the products of hydrolysis are worse

inhibitors at the resulting concentrations. The restored activity of Au-MPC **1** would signal the presence of the enzyme. From the point of view of developing an enzyme assay, the activation of a catalyst is highly attractive, because it enables the production of a multitude of reporter molecules per substrate molecule converted by the enzyme. As this process relies on a cascade of two catalytic events, the final strength of the output signal will depend on two time frames: first, the time during which the substrate is exposed to the enzyme (or, alternatively, and more importantly from an analytical point of view, the concentration of enzyme present), and second, the time allowed for production of the *p*-nitrophenol reporter molecule. The latter point creates a fundamental difference to conventional assays, in which the maximum output signal is determined only by the chromo- or fluorogenic properties of a probe at a fixed concentration. In our case, the maximum signal is determined by the amount of catalytic sites liberated multiplied by their turnover number. For Au-MPC **1**, under the experimental conditions, the turnover number amounts to around 75 molecules of *p*-nitrophenol produced per single TACN·Zn^{II} complex (see the Supporting Information).

To provide a proof-of-principle, we incubated AcNH-Asp-Asp-Asp-OH with subtilisin A, which is a nonspecific protease, at concentrations ranging from 66 nM to 42 μM . After 60 minutes, Au-MPC **1** ([TACN·Zn^{II}] = 5 μM) and HPNPP (1 mM) were added to the mixture, and the production of *p*-nitrophenol was measured for 30 minutes. The dependence of the observed absorbance at 405 nm on the initial concentration of the enzyme clearly indicates that the system is able to report on enzyme activity (Figure 3a). In fact, no recovery of catalytic activity at all was observed when subtilisin A was pretreated with the inhibitor phenylmethylsulfonyl fluoride (PMFS). The fact that the absorbance reaches a plateau indicates that the substrate AcNH-Asp-Asp-Asp-OH has been hydrolyzed to its maximum extent. However, the advantage of catalytic signal amplification becomes evident when the time interval for *p*-nitrophenol production by Au-MPC **1** is increased (Figure 3b). As a consequence of the catalytic production of *p*-nitrophenol, the absolute difference in absorbance with and without the enzyme increases as a function of time. As a result, even the small amount of substrate cleaved by a 66 nM solution of the enzyme in 1 hour is sufficient to generate an appreciable difference of around 0.04 absorbance units after incubation for 20 hours with HPNPP. The linearity of the plot of absorbance against enzyme concentration in the sub-micromolar regime (Figure 3c) indicates that the protocol is amenable to the quantitative determination of enzyme concentration even in the nanomolar range.

Although the above results validate the detection protocol, the absolute response values are rather poor, and the maximum difference in absorbance observed (0.12 AU; Figure 3b) corresponds to just a 15 μM difference in the concentration of *p*-nitrophenol. These factors indicate that the actual amount of substrate cleaved by subtilisin A is rather limited, and consequently, only a small amount of catalytic sites are activated. However, our detection protocol has general applicability. To illustrate this we studied the inhibitory

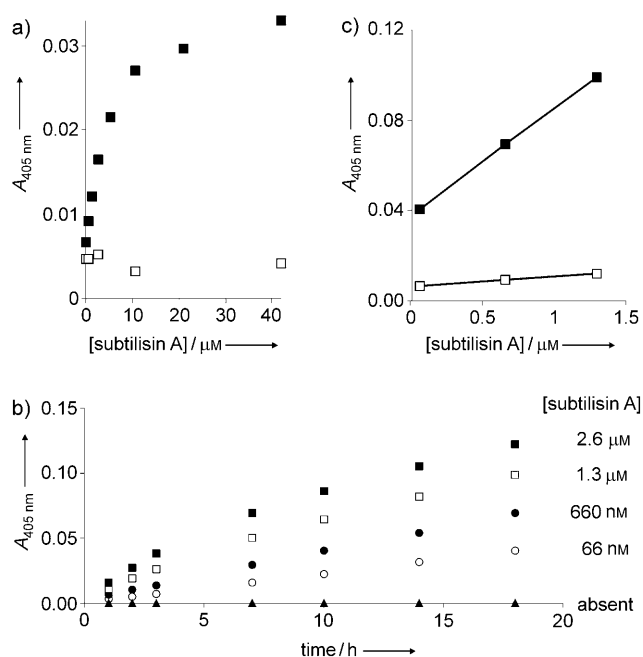


Figure 3. a) Absorbance at 405 nm measured 30 min after the addition of Au-MPC **1** and HPNPP to a solution of AcNH-Asp-Asp-Asp-OH, which had been previously incubated with subtilisin A at different concentrations for 1 h in the absence (■) and presence (□) of the enzyme inhibitor PMFS (H₂O buffered at pH 7.5, [HEPES]=10 mM, T=25 °C). b) Difference in the absorbance at 405 nm of AcNH-Asp-Asp-Asp-OH incubated for 1 h with subtilisin A at different concentrations and the reference sample containing AcNH-Asp-Asp-Asp-OH as a function of the assay time. c) Linearity of the response to enzyme concentrations in the nanomolar regime, and the positive effect of longer assay times (□ 20 min, ■ 20 h) on the strength of the output signal.

capacity of a series of substrates and products for several biologically important enzymes (Table 2; for the inhibition curves, see the Supporting Information). These enzymes were selected because their action changes the number of negative charges on the substrate. This effect renders their detection possible either through catalytic upconversion or downregulation of *p*-nitrophenol production by Au-MPC **1**. As expected, the substrate concentration at which the maximum

difference in inhibitory effect is observed depends on the number of charges on the substrate or product (Table 2, column 5). For asparaginase and glutamase, this concentration is in the low-millimolar region, and for GCPII and caspase 1, it is in the micromolar region.

As an illustration that this detection system can indeed be made enzyme-selective simply by changing the anionic probe, we performed preliminary studies on caspase 1. Caspase 1 belongs to the caspase family of cysteine proteases and plays an important role in cell apoptosis. Commonly, in colorimetric assays the substrate AcNH-YVAD-pNA is used, since caspase 1 cleaves peptides after the aspartic acid residue to release *p*-nitroaniline. We designed a probe containing an additional aspartic acid residue after the cleavage site; this residue increases the affinity of the probe, but not the product, for Au-MPC **1**. Caspase 1 (0.1 active units)^[33] was incubated with the substrate AcNH-YVADD-OH (5 μM) at 40 °C and pH 7.0 for 3 days, after which time Au-MPC **1** ([TACN·Zn^{II}]=5 μM) and HPNPP (1 mM) were added.^[34] The same experiment was also performed in the presence of the enzyme inhibitor AcNH-YVAD-CMK (CMK = chloromethylketone). The initial rate for *p*-nitrophenol production was significantly higher when the active enzyme was present ($\Delta v_{\text{init, meas}} = 0.30$, $\Delta v_{\text{init, expected}} = 0.42$; see Table 2) and correlated well with the expected rates based on the inhibition curves (see the Supporting Information). In analogy with the subtilisin study, the increase in absorbance was measured over an extended time interval and yielded a difference of 0.76 absorbance units after 24 hours between the two samples (see the Supporting Information), which corresponds to a difference in the *p*-nitrophenol concentration of 90 μM. Importantly, under the same conditions, a conventional colorimetric assay with AcNH-YVAD-pNA at a concentration of 5 μM would allow for a maximum difference in the *p*-nitroaniline concentration of 5 μM. The difference corresponds to a signal-amplification factor of 18 and is clear evidence for the advantage of catalytic signal amplification.^[35]

In conclusion, we have developed a new method to detect binding events between oligoanions and the surface of an Au-MPC through a catalytic readout mechanism.^[36] The advantage is that binding events that occur at low-micromolar concentrations of the analyte and the nanoparticle are amplified and affect the formation of the reporter molecule

Table 2: Substrate and product(s) (including dissociation constants) for several enzymes. For each enzyme, the substrate concentration is given at which the difference in the inhibitory power of the substrate and products is highest.^[a]

Enzyme	Role	Substrate (K_{dis} [M])	Product(s) (K_{dis} [M])	Optimal assay concentration [M] ^[b]
asparaginase	metabolism	asparagine (1.2×10^{-3})	aspartate (8.0×10^{-5})	2.5×10^{-4} (58)
Glutamase	metabolism	glutamine (2.4×10^{-3})	glutamate (8.0×10^{-5})	1.0×10^{-4} (52)
Glutamate carboxypeptidase II	neurotransmission	<i>N</i> -acetyl-L-aspartyl-L-glutamate (9.0×10^{-7})	<i>N</i> -acetyl-L-aspartate (6.0×10^{-5}) and glutamate (8.0×10^{-5})	5.0×10^{-6} (65)
caspase 1	cell apoptosis	AcNH-YVADD (5.0×10^{-6}) ^[b]	AcNH-YVAD (1.0×10^{-4}) ^[b] and aspartate (8.0×10^{-5})	5.0×10^{-6} (42)

[a] Inhibition experiments were performed by measuring the initial rate of transphosphorylation of HPNPP by Au-MPC **1** in the presence of increasing amounts of the anion (see Supporting Information). The dissociation constant is defined as the concentration at which $v_{\text{init}} = 0.5 v_0$. Experimental conditions: [TACN·Zn^{II}]= $5.0 \cdot 10^{-6}$ M, [HPNPP]= 1.0×10^{-3} M, [HEPES]= 1.0×10^{-2} M, pH 7.0, 40 °C. [b] AcNH-YVADD was synthesized by solid-phase peptide synthesis (see the Supporting Information). [b] The values between within the parentheses indicate the maximum difference in v_{init} that is expected upon complete hydrolysis of the substrate.

(in our case, *p*-nitrophenol) at much higher concentrations, which enables even visible detection. Importantly, no special requirement is needed for the analytes, such as the presence of a chromo- or fluorogenic leaving group. The gold-nanoparticle scaffold plays a fundamental role in the process, and the multivalent properties of its covering monolayer are exploited to their full extent. First, it preorganizes the single TACN·Zn^{II} complexes to form several highly active dinuclear catalytic sites. Second, it enables multiple interactions with negatively charged analytes. The linear increase in the free energy of binding as a function of the negative charges present in the analyte permits the use of this system for the detection of enzyme activity. Enzyme activity turns on the catalytic activity of Au-MPC **1**, which results in the production of *p*-nitrophenol. In comparison to conventional assays, this catalytic effect provides a much stronger signal and thus increases the sensitivity. Finally, the assay can be made enzyme-selective by changing the (negatively charged) peptide sequence.

Experimental Section

The synthesis and characterization of Au-MPC **1** and the assessment of catalytic activity in the transphosphorylation of HPNPP has been described elsewhere in detail.^[21] All oligoanions, except for AcNH-Asp-Asp-OH, AcNH-Asp-Asp-Asp-OH, AcNH-YVADD-OH, and AcNH-YVAD-OH, were obtained from commercial sources and were used as received. Other peptides were synthesized on a solid support according to standard 9-fluorenylmethoxycarbonyl-based peptide-synthesis protocols. ATP_F (2-fluoro-2-aminopurine riboside-5'-*O*-triphosphate) was obtained from commercial sources and used as received.

The inhibition experiments were performed at pH 7.0 ([HEPES] = 10 mM). The reaction was started by adding HPNPP (20 μL, 0.05 M) to a 1 mL cuvette containing the buffer, Au-MPC **1**, Zn(NO₃)₂, and the inhibitor in the reported concentrations. The cuvette was heated at 40 °C for 30 min, during which product formation was followed by measuring the absorbance at 400 nm.

The subtilisin enzyme assay^[34] was performed at pH 7.5 ([HEPES] = 10 mM). A solution of the peptide AcNH-Asp-Asp-Asp-OH (925 μL, 10.7 μM) was incubated with 10 μL of a solution of the enzyme subtilisin A at a concentration of 3–7 AU mL⁻¹. The mixture was kept at 25 °C for 20 min, after which Au-MPC **1**, Zn(NO₃)₂, and HPNPP were added to give the final concentrations reported. The cuvette was heated at 40 °C for 30 min, during which product formation was monitored by measuring the absorbance at 400 nm. The inhibition experiment with PMFS was performed in an analogous manner by adding the inhibitor (1 μM) to the enzyme solution 30 min before the addition of the tripeptide.

The caspase 1 enzyme assay^[34] was performed at pH 7.2 ([HEPES] = 10 mM). A solution of the peptide AcNH-YVADD-OH (5.0 μL, 1.0 mM) was incubated with 1.0 μL of a solution of the enzyme caspase 1 (human, recombinant, expressed in *Escherichia coli*; 100 AU were regenerated with 100 μL of a 0.5 M HEPES buffer solution) at a concentration of 0.1 AU μL⁻¹. The mixture was kept at 37 °C for 4 days, after which Au-MPC **1**, Zn(NO₃)₂, and HPNPP were added to give the final concentrations reported. The cuvette was heated at 40 °C for 48 h, during which product formation was monitored by measuring the absorbance at 400 nm. The inhibition experiment with Ac-Tyr-Val-Ala-Asp-CMK was performed in an analogous manner by adding the inhibitor (0.28 mM) to the enzyme

solution 40 min before the addition of the AcNH-YVADD-OH substrate.

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