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Enhancing the Selectivity of Enzyme Detection by Using Tailor-Made Nanoparticles

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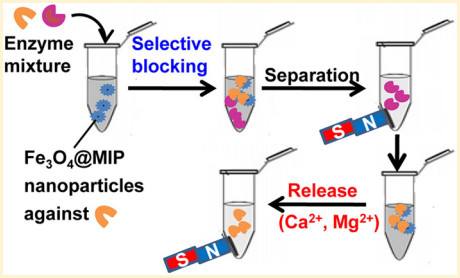
Supporting Information

ABSTRACT: Development of effective ways to specifically and reversibly block the activity of an enzyme is highly desirable for enhancing the selectivity of enzyme assays. Here we demonstrate a novel approach for selective detection of enzyme activities in complex biological samples by using tailor-made nanoparticles. Employing deoxyribonuclease I (DNase I) as a model enzyme template, we prepared surface imprinted polymers over magnetic nanoparticles with monomers screened out of commonly used functional monomers. The resultant Fe_3O_4@MIP nanoparticles can not only block the activity of the target enzyme via selective adsorption but also quantitatively release the bound enzyme under mild conditions with the assistance of metal ion cofactors, which offers a very useful tool for enhancing the selectivity in enzyme detection. The approach enables sequential detection of the activities of 3′-5′ exonuclease and DNase I in cell lysates. The strategy may be further extended to the detection of other enzyme proteins.



Enzymes play critical roles in maintaining normal cellular physiology by regulating the rates of biochemical reactions in cells. Abnormal activities of certain enzymes can be regarded as biomarkers for the disease diagnosis and drug discovery.[1] A number of fluorescent enzyme probes have been developed for the detection of enzyme activities.[2] However, in complex biological samples, other coexisting enzymes often cause side reactions of the probes and interfere with the detection.[3] Development of effective approaches to specifically and reversibly block the activity of the interfering enzymes is highly desirable for enhancing the selectivity of enzyme assays.

Enzyme activity can be inhibited by changing the local conditions such as pH, ionic strength, temperature, and addition of denaturants. However, these methods lack specificity and may cause other undesired effects.[1b] Some molecules can inhibit the enzymatic reaction by binding to the enzyme, but few of them can readily liberate the enzymes for further reaction.[2c] Molecular imprinting is a technique to create specific binding sites for predetermined target molecules in the polymer matrix.[4] Molecularly imprinted polymers (MIPs) have been widely used for separation,[5] biosensors,[6] mimicking enzymes,[7] and drug delivery.[8] In recent years, Shea and coworkers successfully obtained MIP for clearance toxic peptides (melittin).[9] Haupt et al. developed an MIP microgel for selective inhibition of the protease trypsin by using a polymerizable low-molecular-weight inhibitor (4-aminobenzamidine) as the functional monomer.[10] In comparison with natural enzyme inhibitors, MIPs have advantages of higher chemical and physical stability, easier availability, lower cost, and wider applicability.

In this study, we demonstrate the unique capability of molecularly imprinted nanoparticles for specific and reversible blocking of enzyme activities. Deoxyribonuclease I (DNase I) is a ubiquitous endonuclease that digests DNA without any sequence preference.[11] Employing DNase I as a model template, we prepared surface imprinted magnetic nanoparticles with monomers screened out of commonly used functional monomers (Figure 1A). The resultant MIP cannot only block the enzyme activity via selective adsorption but also quantitatively release the bound enzyme under very mild conditions with the assistance of metal ion cofactors, which offers a very useful tool for enhancing the selectivity in enzyme detection and other enzyme-related studies.

In our previous work, we have fabricated a surface imprinted hydrogel over silica microspheres by covalent immobilization of a water-soluble UV sensitive initiator 4,4′-azobis-(4-cyanopentanoic acid) onto the surface of silica beads.[12] In this work, we further grafted the initiator onto the surface of amino-functionalized superparamagnetic Fe_3O_4 nanoparticles, thus the resulting Fe_3O_4@MIP particles could be easily isolated from the solution by using an external magnetic field. DNase I is an acidic protein with an isoelectric point (p*I*) of 4.9 and molecular weight (MW) of 31 kDa.[11] To obtain water-compatible MIP nanoparticles, we tested several commonly used functional monomers. *N*-Isopropyl acrylamide (NIPAm) was chosen as the building-block monomer. *N*-[3-(Dimethylamino) propyl]methacrylamide (DMAPMA, pK_a = 8.9) and acrylic acid (AAc, pK_a = 4.7) were employed to provide electrostatic interactions with negatively and positively charged amino acid residues on DNase I, respectively. Methacrylamide (AAm) was used to provide hydrogen bonding. The total

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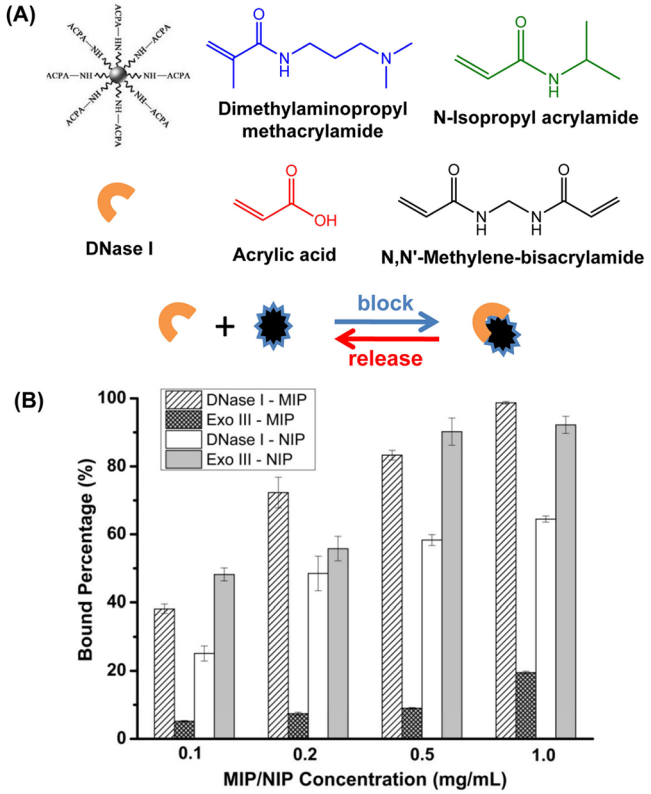

Figure 1. (A) Schematic illustration of the surface-imprinted nanoparticles for reversible blocking of the enzyme activities of DNase I. (B) Comparison of the adsorption of DNase I and Exo III on MIP-3 and NIP-3. 2.0 μg/mL DNase I/Exo III were incubated with 0.1, 0.2, 0.5, and 1.0 mg/mL MIP-3/NIP-3 at 25 °C for 15 min, respectively.

amount of the functional monomers were 98 mol %, while the rest was the water-soluble cross-linking monomer *N*,*N*′-methylene bisacrylamide (BIS). After the synthesis, the template was removed by washing with NaCl (1 M) solution at 50 °C under ultrasonication. Fluorescent oligonucleotide probes (see Table S1 in the Supporting Information) were employed for determination of the activity of DNase I and other reference nucleases.[13] The linear calibration curves for quantification of the activity of DNase I and other reference nucleases were shown in Figure S1 in the Supporting Information.

We synthesized eight different MIP nanoparticles (MIP-1−8) by using DNase I as a template and varying the composition of functional monomers (see Figure S2 in the Supporting Information for the detailed formula). Exonuclease III (Exo III, MW = 31 kDa, p*I* = 6.4) which has a double strand-specific, nonprocessive 3′ to 5′ exodeoxyrebonuclease activity[14] was employed as a reference nuclease to evaluate the selectivity of the obtained MIP nanoparticles. The binding capacities of the MIPs for DNase I and Exo III were determined through equilibrium binding assays. The sequence of the fluorescent probe used for detection of Exo III was listed in Table S1 in the Supporting Information. From Figure S2 in the Supporting Information, the binding capacities of MIP-1−4 for DNase I were all higher than those of MIP-5−8, suggesting that the basic functional monomers (DMAPMA) were essential for forming high-affinity binding sites for the templates. This is reasonable as DNase I are negatively charged under the polymerization reaction conditions. With the increase of AAm monomer, the binding capacities of DNase I and Exo III both increased, indicating lower selectivity of the MIP toward the target enzyme. By comparing the results of MIP-3 and MIP-8 with those of MIP-2 and MIP-7, respectively, it can be seen that addition of acidic functional monomer was beneficial for improving the specific binding affinity to DNase I. A possible reason for this is the surface charge distribution of the enzymes. In comparison with Exo III, there are more basic amino acids such as arginine and histidine in the active site of DNase I.[11] The pK_a of the imidazole ring is about 6 while that of the guanidine group is 12.48. So in the presence of DNase I, the carboxylic group of AAc may strongly interact with these basic groups and form well organized binding sites. For Exo III, though it has a similar molecular weight as DNase I, there are more acid amino acids such as glutamic acid and aspartic acid on the surface near the active site,[14] which are unfavorable to bind with the DNase I-imprinted binding sites. Taken together, a proper combination of DMAPMA and AAc offers the best binding capacity and selectivity of the MIP to DNase I.

On the basis of the above results, MIP-3 nanoparticles with a composition of 50% *N*-isopropyl acrylamide (NIPAm), 24% DMAPMA, 24% of AAc, and 2% cross-linker *N*,*N*′-methylene bisacrylamide (BIS) was used for further study (see Figure S3 in the Supporting Information for the morphology of the nanoparticles). Figure 1B compared the amount of DNase I and Exo III bound on MIP-3 and NIP-3 at different concentrations. As can be seen, NIP-3 bound more Exo III than DNase I, whereas MIP-3 bound much more DNase I than Exo III. This dramatic switch of binding selectivity should be attributed to the imprinting process. Besides, MIP-3 bound more DNase I than NIP-3 at all tested concentrations. Figure S4 in the Supporting Information showed the fast binding kinetics of DNase I to the nanoparticles.

To further confirm that the obtained MIP-3 nanoparticles can selectively bind DNase I even in a mixture system with other nucleases, we performed a competitive adsorption study by incubation MIP-3/NIP-3 with a mixture solution of DNase I and another reference protein, lambda Exonuclease (λ Exo, MW = 79 kDa, p*I* = 5.7) which preferably catalyzes the hydrolysis of one strand of DNA duplex from the 5′ end with a phosphate group.[15] First, we incubated λ Exo with MIP-3 or NIP-3 in the absence of DNase I and measured the residual activity of λ Exo in the supernatant. A small amount of λ Exo was retained by MIP-3, but much less than that bound to NIP-3 (see Figure S5A in the Supporting Information). Then we tested the adsorption of λ Exo on MIP-3 or NIP-3 in the presence of DNase I. For the two tested concentrations of λ Exo, it was observed that the activity of λ Exo in the supernatant remained nearly unchanged after incubation with MIP-3, whereas the nonspecific adsorption of λ Exo on NIP-3 was significant (see Figure S5B,C in the Supporting Information). By contrast, no DNase I was found in the supernatant, indicating that the target enzyme had been completely bound by MIP-3 after the incubation. These results demonstrated that MIP-3 has highly selective binding affinity for DNase I.

Then we tried to find out whether the DNase I bound to the MIP-3 nanoparticles are still active in digestion of the fluorescent oligonucleotide probes. In our preliminary experiments, we found that the fluorescent oligonucleotide probes that were used to assay the enzyme activity could be nonspecifically adsorbed and quenched by MIP-3, NIP-3, and

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the amino-functionalized Fe_3O_4 nanoparticles, as shown in Figure S6A in the Supporting Information. This is most likely because of the positively charged surfaces of the tested nanoparticles. So we optimized the amount of the probes added to the solution to ensure that the MIP polymers were saturated with the probes and there are sufficient free probes in the solution. The fluorescence intensity of the mixture solution containing DNase I bound to MIP-3 and free fluorescent oligonucleotide probes (referred to as solution 1) was shown in Figure S6B in the Supporting Information. Then we separated the supernatant of solution 1 and mixed it with a free DNase I solution. The resultant solution (referred to as solution 2) emits very strong fluorescence (see Figure S6B in the Supporting Information), indicating that the activity of DNase I in solution 1 has been significantly blocked after bound to MIP-3.

We next investigated how to release the enzymes from the MIP-3 nanoparticles without damaging their activity. Commonly used approaches to dissociate proteins from polymers include washing with high-concentration salt solution[16] or using denaturing reagent such as SDS[17] and urea.[18] Dialysis and electromigration are also reported for removal of the trapped proteins.[10] Shea et al.[19] successfully released bound lysozyme utilizing the temperature-responsive property of nanoparticles. Considering that DNase I is a metal ion cofactor-dependent enzyme and the MIP nanoparticles was prepared in the absence of metal ions, we tested the effects of different metal ions on the recovery of DNase I from the nanoparticles.

As shown in Figure 2A, Ca^{2+}, Mg^{2+}, and Mn^{2+} have considerable enhancing effect on the dissociation of DNase I from the nanoparticles, which agrees very well with the previously reported metal ion cofactors (Ca^{2+} plus Mg^{2+} or Mn^{2+}) for the endonucleolytic activity of DNase I.[11,20] Ca^{2+} has been found to be particularly important for the folding activation process of DNase I.[20] Inspired by these results, we attempted to use the DNase I buffer which has a proper pH and contains the required metal ions (Ca^{2+} and Mg^{2+}) as the dissociation reagent . We compared different concentrations of DNase I buffer (5×, 10×, 15×, and 20×). As shown in Figure S7 in the Supporting Information, 10× DNase I buffer exhibited the highest dissociation efficiency (close to 100%). Buffers with even higher concentrations may cause denaturation of the enzyme. To further understand the contributions of metal ions and Tris-HCl in the DNase I buffer to the high recovery of the enzyme, we prepared five different solutions. The detailed compositions and the recovery results are shown in Figure 2B. Clearly, the presence of Ca^{2+} and Mg^{2+} made the largest contribution. A further combination of the two metal ions with Tris-HCl provides the optimum conditions for the enzyme to release from the nanoparticles. These results demonstrate a fairly new strategy to release enzymes bound on polymers by using their metal ion cofactors. The underlying principle for these properties merits further investigation.

On the basis of above results, we isolated the MIP-3 nanoparticles from aforementioned solution 1 and mixed them with 50 μL of 10× DNase I buffer to release the DNase I from the polymer. After magnetic separation, we drew out 5.0 μL of the obtained supernatant that contained the released enzymes and mixed it with the previously obtained supernatant of solution 1 that contained excess free fluorescent oligonucleotide probes. The resultant solution (referred to as solution 3) gives out much stronger fluorescence than solution 1 (see Figure S6B in the Supporting Information). By comparison of

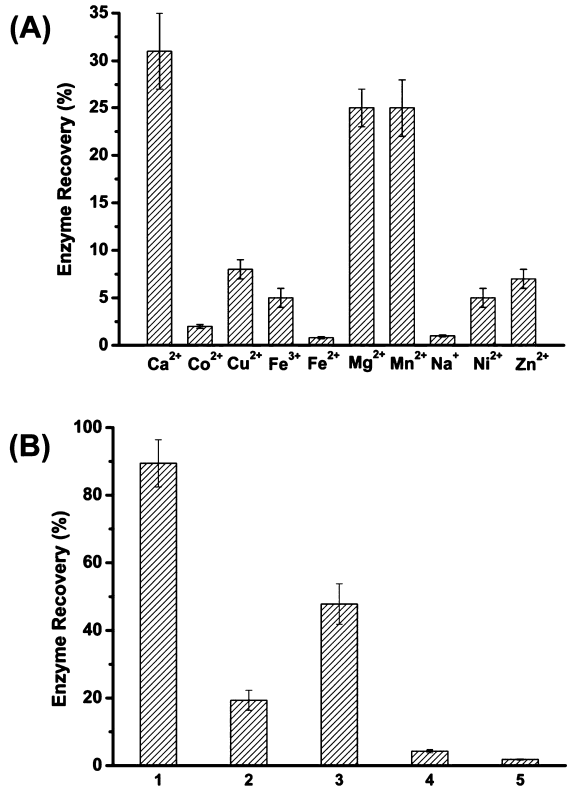

Figure 2. (A) Effects of metal ions on the enzyme recovery from MIP-3 nanoparticles. For 15 ng of DNase I blocked by 48.5 μg of MIP-3 nanoparticles, 50 μL of different metal ion solutions (0.1 M) were used to release the enzyme. (B) Comparison of the enzyme recoveries under different dissociation conditions: (1) 100 mM Tris-HCl containing 25 mM $MgCl_2$ and 5 mM $CaCl_2$; (2) 100 mM Tris-HCl; (3) 25 mM $MgCl_2$ and 5 mM $CaCl_2$; (4) 100 mM Tris-HCl containing 60 mM NaCl; (5) 60 mM NaCl. For 100 ng of DNase I blocked by 49.0 μg of MIP-3 nanoparticles, 50 μL of solutions 1−5 were used to release the enzyme, respectively.

the fluorescence intensities of the three solutions shown in Figure S6B in the Supporting Information, we concluded that MIP-3 can effectively and reversibly block the activity of DNase I.

Finally, we applied MIP-3 nanoparticles to block the DNase I activity in the lysates of MCF-7 cancer cells, thus allowing both 3′ to 5′ exonucleases and DNase I in the cell lysates being quantified. The whole process was illustrated in Scheme 1. The Exo III Probe shown in Table S1 in the Supporting Information is a universal probe for detection of 3′ to 5′ exonucleases.[13] However, without any protection, its backbone can also be nonspecifically digested by DNase I and gives out false positive signals. So it is necessary to block the activity of DNase I to prevent it from interfering with the detection of the activity of 3′ to 5′ exonucleases. By contrast, the DNase I probe we used is less affected by other coexisting exonucleases since the two ends of the probe were both protected.[13] So it can be directly used to measure the DNase I activity in a mixture solution such as the cell lysates.

Figure 3 summarizes the results measured by different probes before and after the isolation of DNase I. Curves 1 and 2 show the enzyme activity in the cell lysates directly measured by the DNase I probe and Exo III probe, respectively, which indicate the presence of a notable amount of DNase I in the cell lysates. Curves 3 and 4 show the enzyme activity measured by the

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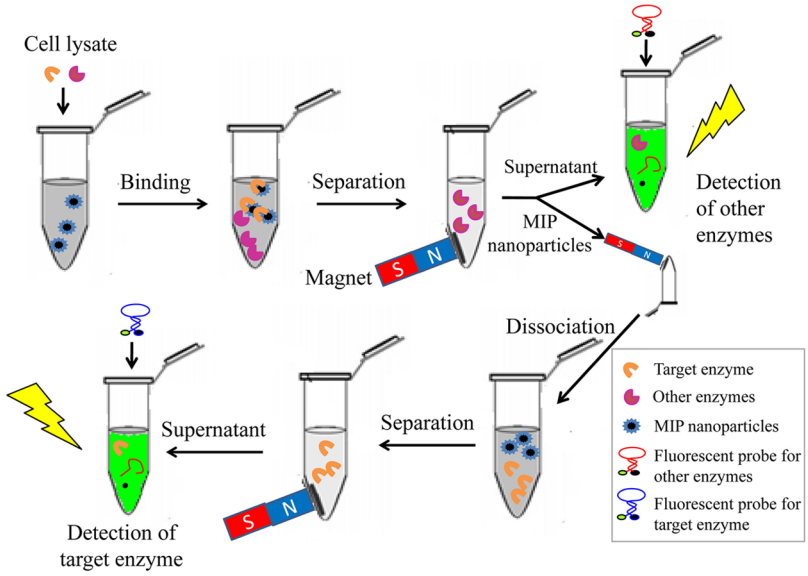
Scheme 1. Schematic Illustration of the Procedures for Selective Detection of the Activities of 3′-5′ Exonucleases and DNase I in Cell Lysates



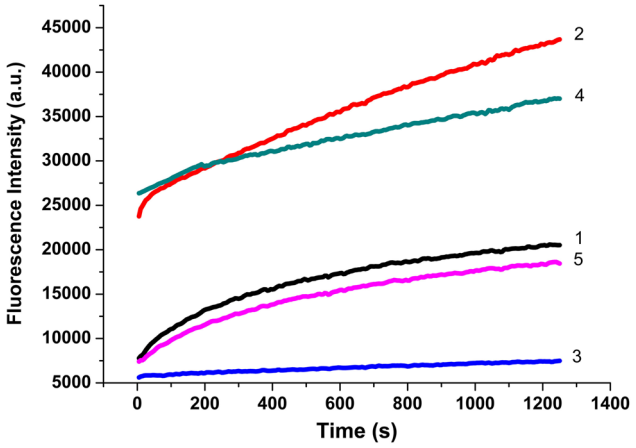


Figure 3. Time curves for measurement of the enzyme activity in cell lysates before and after the isolation of DNase I: curve 1, direct measurement of the cell lysates with the DNase I probe; curve 2, direct measurement of the cell lysates with the Exo III probe; curve 3, measurement of the supernatant of the cell lysate after incubation with the MIP and magnetic separation with the DNase I probe; curve 4, measurement of the supernatant of the cell lysate after incubation with the MIP and magnetic separation with Exo III probe; curve 5, measurement of the enzyme released from the MIP nanoparticles with the DNase I probe.

DNase I probe and Exo III probe in the supernatant after incubation with the MIP followed magnetic separation, which indicate the complete binding of DNase I by MIP-3 and the content of 3′-5′ exonucleases in the cell lysates. For further confirmation, the bound enzyme was released and analyzed with the DNase I probe (curve 5), which is very close to the amount observed in the original lysates (curve 1). These results prove that the MIP nanoparticles can selectively block DNase I in complex biological samples and allows reliable quantification of both 3′-5′ exonucleases and DNase I.

We also examined the stability and reusability of the MIP nanoparticles. As clearly demonstrated in Figure S8 in the Supporting Information, under the optimized adsorption and desorption conditions, MIP-3 could bind and release DNase I repeatedly and the performance remains stable after three regeneration cycles.

CONCLUSIONS

In conclusion, we have demonstrated a novel strategy for selective detection of enzyme activities in complex biological samples by using tailor-made nanoparticles. The nanoparticles act as a blocking reagent for the target enzyme by specifically binding and releasing the enzyme proteins under mild conditions. The nanoparticles are easy to isolate and the recovered enzymes maintain high activity for further reaction. The approach has been successfully applied for sequential quantification of both 3′-5′ exonucleases and DNase I in cell lysates without the need of other sample pretreatment steps. The mechanism that how the metal ions affect the interaction between MIP nanoparticles and the template enzyme will be further investigated. The results of this study provide new ways for enzyme activity regulation. The strategy may be further extended to other enzyme proteins.

ASSOCIATED CONTENT

Supporting Information

Experimental section and supplementary 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 interest.

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