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Protease-Triggered Dispersion of Nanoparticle Assemblies

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Bioresponsive nanomaterials are of growing importance with potential applications in drug delivery and diagnostics.[1] DNA-, protein- or peptide-functionalized nanoparticle (NP) aggregates are particularly useful systems since triggered changes in their aggregation states may be readily monitored.[2] For example, dispersion of gold NP assemblies results in a change in the solution color from blue to red. A number systems have recently been described that exploit the catalytic action of enzymes to control the assembly state of NPs. Guarise et al. made use of enzyme-cleavable peptides that were modified at both termini with acylated Cys residues.[3] When gold NPs were added, the acylated thiol groups present in the Cys termini were able to cross-link them, leading to aggregates. When NPs were exposed to peptides that were pretreated with a target protease, NPs dispersed and a red-shift in the visible spectrum was observed. Zhao et al. used a similar two-stage approach involving exposure of peptides that were modified at both termini with biotin to avidin coated magnetic NPs. Aggregation/dispersion of NPs was detected as magnetic relaxation to detect protease activity.[4] These two-stage methods currently do not lend themselves to real-time monitoring of enzyme action. Brust et al. demonstrated that a number of DNA restriction and ligation enzymes could assemble different populations of DNA coated gold NPs.[5] Harris et al. demonstrated that magnetic NPs that carried either biotin or neutravidin capped with PEG chains via protease degradable linkers self-assembled upon exposure to the target enzyme.[6] In the last two approaches two separate populations of NPs are required.

In this Communication we describe a simpler conceptually novel approach to protease-responsive NPs whereby only a single population of NPs is required and real-time monitoring of protease action is possible. In addition, higher than previously reported sensitivity is achieved. The system uses NP tethered protease cleavable peptides consisting of three sections: (i) a thiol containing Cys for anchoring onto gold, (ii) a protease cleavable section, (iii) and an assembly directing actuator, in this case (*N*-(fluorenyl-9-methoxycarbonyl), (Fmoc)) that is known to control physical assembly through π-stacking interactions (Figure 1).[7] Upon enzymatic hydrolysis of peptide-NP conjugate, disassembly occurs through a combination of electrostatic repulsion between particle bound NH_3^+ groups and removal of hydrophobic interactions between Fmoc groups.

To demonstrate proof of concept, we designed a self-assembled peptide-functionalized NP network to be actuated by thermolysin from *B. Thermoproteolyticus rokko* using the tripeptide Fmoc-Gly-Phe-Cys-NH_2 (**1**). Thermolysin exhibits a preference for large hydrophobic residues at the amine side (*P*1′) of the scissile bond and is nonspecific for the amino acid residue on the carboxylic acid side (*P*1).[8] Hence, one would expect hydrolysis of the amide bond of Fmoc-Gly↓Phe-Cys-NH_2 specifically to be catalyzed by thermolysin and simultaneously result in the dispersion of the NPs and a visible color change.

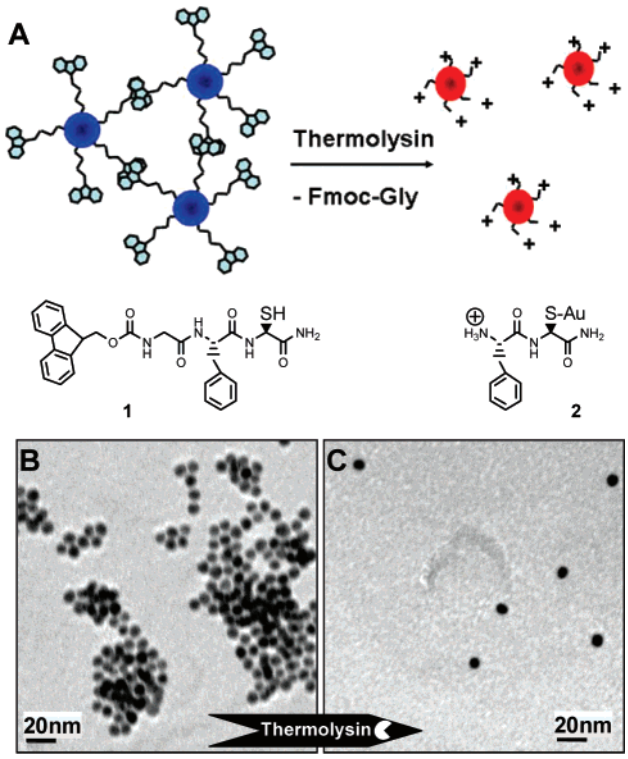

Figure 1. (A) Schematic representation of the protease-triggered NP dispersion approach; (B) TEM image of 8.5 nm gold NPs at pH 8 after functionalization with peptide **1** and C, following addition of thermolysin and generation of **2**.

Gold NPs functionalized with peptide **1** aggregated as observed by transmission electron microscopy (TEM)(Figure 1B) and a UV−vis surface plasmon resonance peak at 565 nm. A control peptide lacking Fmoc did not give rise to aggregation, indicating that π-stacking interactions are dominant in driving NP assembly. To test the enzyme responsiveness of this system, peptide **1**-NPs were incubated at 37 °C with concentrations of thermolysin ranging from 250 ng/mL to 90 zg/mL. TEM images following addition of thermolysin reveal a well-dispersed population of NPs (Figure 1C). The UV−vis spectra plasmon resonance peak shifted from 565 to 532 nm 6 h after the addition of 250 ng/mL thermolysin (Figure 2A). Similar UV−vis spectral shifts of around 30 nm were observed over different timescales for the range of thermolysin concentrations tested. HPLC analysis of the supernatant detected the presence of Fmoc-Gly only, thus confirming the site of enzymatic hydrolysis. In control experiments, 8.5 nm gold NPs functionalized with peptide **1** did not disassemble upon introduction of trypsin, an enzyme that

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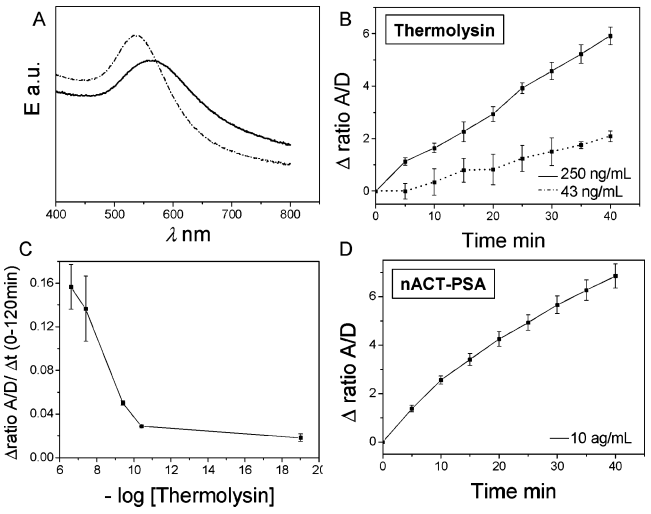

Figure 2. (A) UV−vis spectra of peptide **1**-NPs (solid line) preaddition of enzyme and **2** at 6 h (dotted line) postaddition of 250 ng/mL thermolysin; (B) Δ ratio A/D at 5 min intervals for 250 and 45 ng/mL thermolysin (see Figure SI-1); (C) {(Δ ratio A/D)/Δ*t*} at 2 h versus −log[thermolysin]; (D) Δ ratio A/D at 5 min intervals for peptide **3**-NPs postaddition of 10 ag/mL nACT-PSA.

exclusively cleaves peptides with Arg or Lys residues in the *P*1 position. NPs functionalized with Fmoc-Gly-D-Phe-D-Cys-NH_2 did not disperse upon introduction of thermolysin.

Monitoring of the hydrolysis reaction by direct observation of the plasmon resonance shift is only possible for large spectral shifts. A highly sensitive and accurate method to quantify change in spectral shape can be achieved by calculating the change in the ratio A/D (aggregated/dispersed area) for the area under the plasmon resonance peak.[9] Figure SI-1(Supporting Information) shows the region under which the integral was computed for the UV−vis spectra of all samples tested, with region D (dispersion) spanning from 490 to 540 nm and region A (aggregation) from 550 to 700 nm. The ratio A/D was monitored over time for all samples. Using this approach, NP dispersion could be followed in real time (Figure 2B). By measuring Δ ratio A/D over time, we achieved higher than previously reported sensitivity (detection of 90 zg/mL of thermolysin) (Figure 2C). This value corresponds to less than 380 molecules.[14] When the concentration was varied over 12 orders of magnitude, only a 1 order of magnitude difference in reaction rates is observed, suggesting that the kinetics of the system are limited to enzyme accessibility to the surface of aggregates.

The proposed modular approach (anchor, enzyme cleavable linker, actuator) obviates the need for major redesign when targeting other proteases. For example, we have applied the same design principles to the detection of nonbinding α_1-antichymotrypsin prostate specific antigen (nACT-PSA), a protease related to prostate cancer.[10] Prostate cancer is currently responsible for the second highest number of deaths in American men. The most widely used screening method for prostate cancer involves measurement of the total prostate specific antigen (PSA) level in serum.[11] Our system, unlike the conventionally used immunoassays, also monitors PSA complexed to α_2-macroglobulin (a form of PSA only enzymatically active toward relatively small proteins).[12]

We designed peptide **3** Fmoc-Ser-Ser-Phe↓Tyr-Ser-Gly-Gly-Gly-Cys-NH_2 with a nACT-PSA specific hydrolysis site.[13] Using peptide **3**-functionalized gold NPs, we were able to detect 10 ag/mL nACT-PSA at 37 °C (Figure 2D and Figure SI-2). To our knowledge this is the highest sensitivity published for PSA detection and may be valuable in early monitoring and detection of disease relapse after the surgical treatment of prostate cancer. In control experiments, NPs functionalized with the D-isomer of the peptide **3** did not disperse in the presence of nACT-PSA. Additionally, thrombin, a protease found in serum that selectively cleaves Arg-Gly, did not disassemble the NPs.

In conclusion, we have developed a simple and highly sensitive method to detect the presence of proteases. The efficacy of the design in generating NP dispersion upon enzyme action arises from a dual mechanism of simultaneous removal of attractive self-assembly groups and revelation of repulsive charged groups. The resulting high sensitivity is likely related to the more favorable thermodynamics of peptide hydrolysis when it is coupled to the irreversible process of charge-induced NP dispersion. The modular approach can be easily tailored for different proteases, as demonstrated here for thermolysin and nACT-PSA.

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Supporting Information Available: Experimental procedure, Figure SI-1 and Figure SI-2. This material is available free of charge via the Internet at http://pubs.acs.org.

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(14) The number of molecules present can be estimated from the molecular mass of thermolysin (35 kDa), the protein content of lyophilised powder (0.61 mg protein/mg solid), and the sample volume (400 μL).

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