Biosensors

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Multivalent Nanoparticle Networks as Ultrasensitive Enzyme Sensors**

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Nanoparticles are becoming extremely useful building blocks for the fabrication of ultrasensitive optical[1] and electrochemical[2] nanosensors. Among these applications, the use of Au nanoparticles as colorimetric sensors is gaining much attention owing to the possibility of attaining high sensitivity with unsophisticated transducers.[3] A common strategy for biodetection with Au nanoparticles is to modify their surface with bioresponsive ligands, which trigger the assembly or disassembly of the nanoparticles upon recognition by the target biomolecule, for example, an enzyme.[4] These phenomena shift the plasmon resonance band of the optical probes to yield a spectral change in the visible region of light that is proportional to the amount of the effector biomolecule. Despite the great success of this approach, it would be desirable to find an intrinsic amplification mechanism to boost the sensitivity of this detection scheme so that tiny amounts of the target molecule could be detected in less time and without an additional analytical step.[5]

Herein we report dramatic enhancement of the sensitivity of a model nanoparticle bioassay by modulation of the valency of the interactions that cause the assembly of the nanoprobes (Figure 1). The key step of this method is to bind the bioresponsive ligands to the surface of the nanoparticles through weak host–guest interactions; stronger multivalent interactions between nanoparticles then lead to the formation of nanoparticle clusters. Recognition by the effector biomolecule disrupts the multivalent network, and competition with a monovalent guest amplifies the dispersion of the nanoparticles as the signal of the bioassay.

To prove the validity of the concept of this ultrasensitive detection scheme, Au nanoparticles were decorated with perthiolated β-cyclodextrin (βCD, Figure 1a) and assembled into clusters with ligands **1** (diFc, Scheme 1) bearing two ferrocenyl groups,[6] which can interact weakly with the

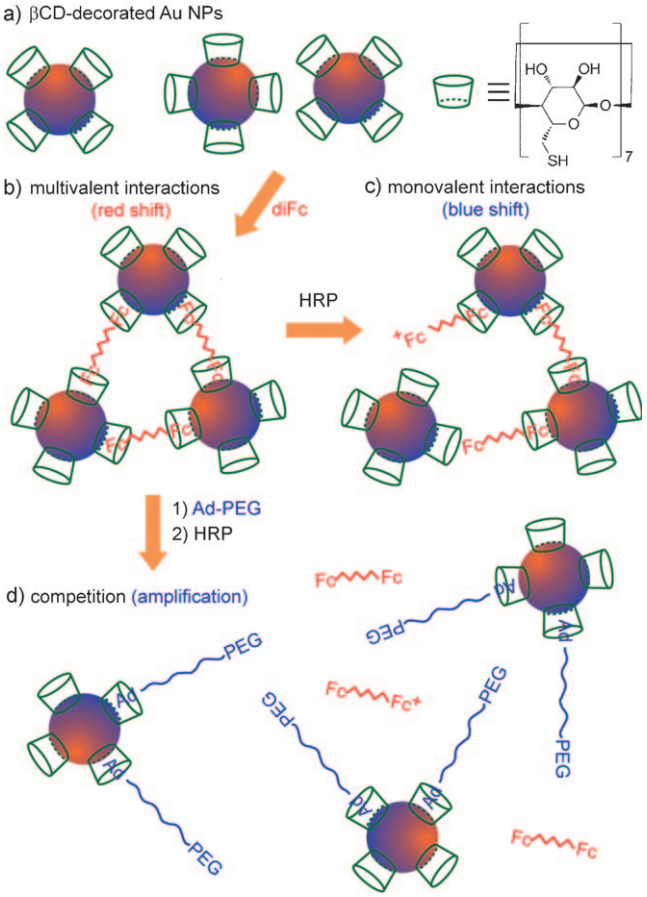
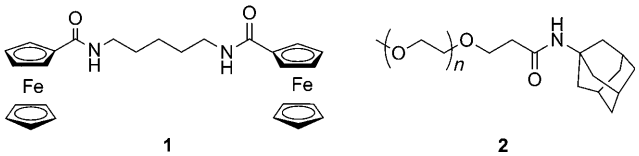


Figure 1. Principle of ultrasensitive biodetection with gold-nanoparticle optical probes: a) Au nanoparticles (NPs) are decorated with perthiolated β-cyclodextrin (βCD); b) the addition of the ferrocene dimer **1** (diFc) leads to the assembly of the nanoparticles into clusters through multivalent host–guest interactions; c) diFc is released from βCD after oxidation by horseradish peroxidase (HRP); the resulting monovalent interactions are weaker, and the network disassembles; d) the signal of the assay is enhanced by the addition of the competing guest molecule **2** (Ad-PEG), which favors the formation of monovalent interactions for more efficient nanoparticle disassembly.



Scheme 1. Structures of the ferrocene dimer **1** (diFc) and the monovalent guest molecule **2** (Ad-PEG). PEG = poly(ethylene glycol).

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hydrophobic cavity of the macrocycle (Figure 1b).[7] In the clusters, the nanoparticles are linked by multiple host–guest connections. The overall strength of the multivalent interactions is higher than that of their monovalent counterparts.[8] Oxidation of the ferrocenyl moieties by the biocatalytic action of horseradish peroxidase (HRP)[9] decreases the binding affinity of diFc for βCD[10] and triggers the disassembly of the clusters. The resulting blue shift of the plasmon resonance band of the Au nanoparticles is used as the signal of the bioassay (Figure 1c). Moreover, oxidation by HRP decreases the amount of host–guest connections and thus favors the formation of weaker monovalent interactions. This phenomenon can be exploited to amplify the colorimetric signal by the addition of the monovalent guest molecule **2** (Ad-PEG) to the nanoparticle assemblies. The adamantyl groups in **2** have a higher binding affinity than the ferrocenyl moieties for βCD,[9] but they are not strong enough to disassemble the clusters linked by multivalent interactions.[11] Hence, although the addition of Ad-PEG does not perturb the assemblies, the Ad-PEG molecules can compete efficiently for interaction with βCD upon the oxidation of even a small amount of the diFc ligands. They help to disassemble the clusters through the formation of monovalent inclusion complexes and thus amplify the signal of the bioassay (Figure 1d). By using this methodology, we achieved a detection limit as low as approximately 23 HRP molecules within a detection time of 30 min, without the requirement of further analytical steps, such as Ag deposition,[2a] for signal amplification.

To prove the assembly of βCD-decorated nanoparticles with diFc (Figure 1b), we mixed a solution of nanoparticles (0.1 mg mL^{-1}) containing surface-bound βCD (0.02 mM) with diFc (0.01 mM). Figure 2a shows the UV/Vis spectra of the nanoparticle solutions before (black line) and after (red line) the addition of diFc. As expected, the absorption peak was red-shifted in the presence of the ligand owing to the formation of nanoparticle assemblies. Moreover, at this stage, the addition of Ad-PEG (0.1 mM) to the solution of nanoparticles assembled by diFc did not change the optical properties of the solution (blue line). This result indicates that the nanoparticle assemblies are linked by strong multivalent interactions. Dynamic light scattering (DLS) experiments confirmed this outcome by revealing large nanoparticle aggregates in the presence of diFc as well as in the presence of diFc and Ad-PEG (Figure 2b). These results demonstrate that diFc can assemble βCD-decorated Au nanoparticles into clusters, and that the addition of Ad-PEG does not perturb the nanoparticle assemblies.

Next, we tested the ability of HRP to disassemble the clusters through the biocatalytic oxidation of diFc with H_2O_2 as the cosubstrate in the absence of Ad-PEG (Figure 1c). Figure 3a shows a transmission electron microscopy (TEM) image of the nanoparticle assemblies, which were dispersed upon incubation with HRP (1 pg mL^{-1}) for 1 h (Figure 3b). The UV/Vis spectrum of the nanoparticle solution was blue-shifted after this treatment, as expected following the enzyme-triggered dispersion of the assemblies (Figure 3c).

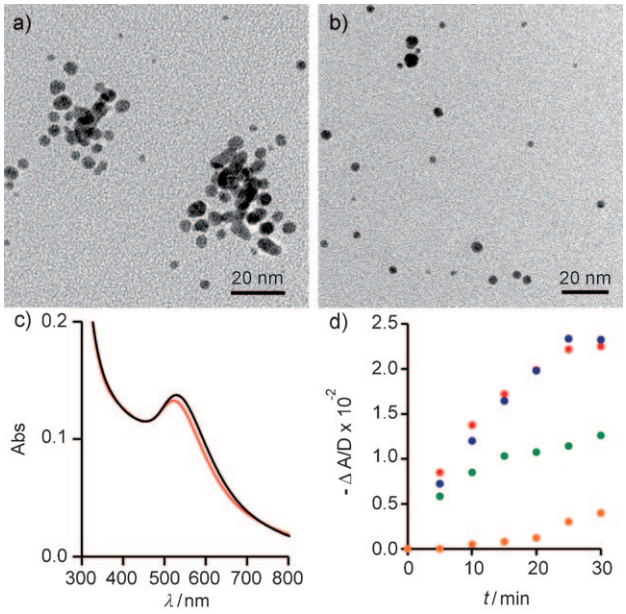

Figure 3. Dispersion of gold-nanoparticle assemblies by HRP: a) TEM image of the assemblies; b) TEM image after incubation with HRP (1 pg mL^{-1}) for 1 h; c) UV/Vis spectra before (black) and after (red) the addition of HRP; d) change in the spectral integral ratio ΔA/D with time after the addition of HRP at a concentration of 1 (red), 0.1 (blue), 0.01 (green), and 0.001 pg mL^{-1} (orange).

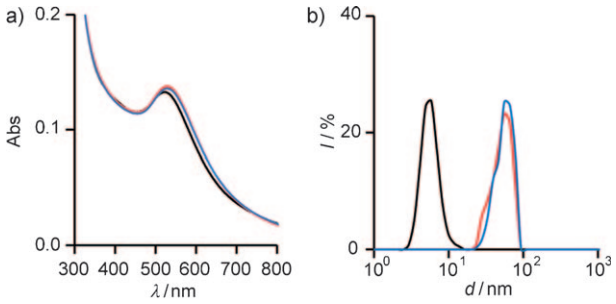

Figure 2. Assembly of βCD-decorated Au nanoparticles by diFc: a) UV/Vis spectra and b) size distribution of the particles as determined by DLS experiments for βCD-decorated Au nanoparticles before the addition of diFc (black), after the addition of diFc (red), and after the addition of diFc and Ad-PEG (blue).

These experiments demonstrate qualitatively the disassembly of Au nanoparticles linked by multivalent host–guest interactions. However, it can be difficult to monitor this process for samples containing ultralow amounts of the enzyme unless an adequate data-treatment process is used for the quantification of spectral changes. In fact, changes in the spectroscopic properties of nanoparticle solutions can be monitored with high sensitivity by calculating the ratio between the integral of the spectrum in the range $\lambda = 550$–700 nm (A) and the integral of the spectrum in the range $\lambda =$ 490–540 nm (D; see Section S2 in the Supporting Information for details).[3b,4b] Values of ΔA/D calculated from the spectra of samples containing HRP at different concentrations were plotted as a function of time (Figure 3d). For an assay time of 30 min, the signal depends on the enzyme concentration in the

range 0.001–0.1 pg mL^{-1} and is saturated at higher concentrations. These results demonstrate that the nanoparticle assemblies can be disassembled by the catalytic action of peroxidase, and that the extent of this phenomenon depends on the concentration of HRP, which validates the proposed methodology for the fabrication of HRP sensors.

According to our hypothesis, the addition of the competing guest molecule Ad-PEG could amplify the signal generated by the disassembly of Au nanoparticles through the formation of competing monovalent interactions. To prove this intrinsic amplification mechanism, we repeated the experiments in Figure 3d after adding Ad-PEG (0.1 mM) to the solution containing the nanoparticle assemblies. In this case, the signal was saturated at HRP concentrations higher than 10 ag mL^{-1}: a value 10^4 times lower than the HRP concentration in the equivalent experiment in the absence of Ad-PEG (Figure 4a). Moreover, the signal decreased as the

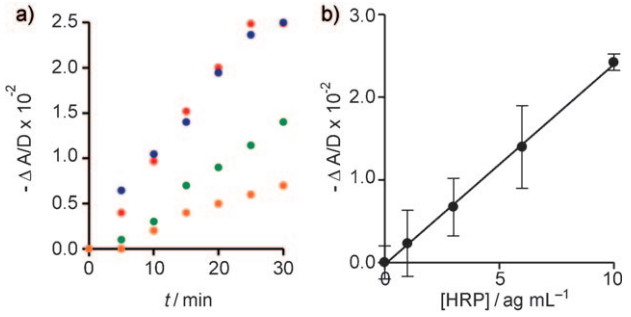

Figure 4. Signal amplification through the formation of competing monovalent interactions with Ad-PEG (0.1 mM): a) Variation of the nanoparticle signal with time after the addition of HRP at a concentration of 100 (red), 10 (blue), 6 (green), and 3 ag mL^{-1} (orange); b) variation of the nanoparticle signal with HRP concentration at $t = 30$ min (linear fit: $y = 0.24x + 0.02$, $r^2 = 0.999$; error bars correspond to the standard deviation ($n = 3$)).

concentration of HRP decreased from 10 to 3 ag mL^{-1}. In comparison with the results in Figure 3d, it is evident that the dynamic range of the bioassay decreased in exchange for higher sensitivity. The upper limit of detection is the same in both cases ($-\Delta A/D \approx 2.5 \times 10^{-2}$), and therefore a higher sensitivity implies a larger variation in the signal with smaller variations in the target concentration, which results in a smaller dynamic range. This feature could be extremely useful for tuning the dynamic range and the sensitivity of the bioassay to solve a particular analytical problem through simple modification of the concentration of Ad-PEG. In other words, when ultrahigh sensitivity is crucial, the experiment can be performed in the presence of highly concentrated Ad-PEG, but when ultrahigh sensitivity is not required, the experiment should be carried out with a lower concentration of the competing molecule for a larger dynamic range.

To test the final performance of the nanoparticle biosensor under conditions of ultrahigh sensitivity, we repeated each of the experiments described by Figure 4a three times and plotted the parameter $\Delta A/D$ at $t = 30$ min as a function of the HRP concentration (Figure 4b). A linear correlation was observed between the signal and the concentration of the enzyme with a fixed concentration of H_2O_2 as the cosubstrate. The absence of a signal in the blank experiment without the enzyme ([HRP] = 0) demonstrates that the biocatalytic action of peroxidase rather than the presence of H_2O_2 triggers the disassembly of the nanoparticle networks. The assay shows a dynamic range between 10 and 1 ag mL^{-1} with a detection limit calculated as two times the standard deviation of the blank of 1.75 ag mL^{-1} (95% confidence level). This detection limit corresponds to approximately 23 HRP molecules. These experiments demonstrate that the addition of a monovalent guest can increase the sensitivity of the assay; thus, the signal is amplified without additional analytical steps.

In summary, we have shown that the sensitivity of nanoparticle biosensors can be boosted by assembling the building blocks through supramolecular multivalent interactions and adding a competing monovalent guest that helps to disperse the nanoparticles in the detection step. By this method, it is possible to control the relevant parameters of the bioassay (limit of detection and dynamic range) as desired for particular analytical requirements. Since HRP is widely used as an enzymatic label for enzyme-linked immunoassays,[12] it may be possible to use multivalent nanoparticle assemblies as universal probes for biodetection through immunoreaction. Moreover, the proposed amplification mechanism could be applied to the detection of other enzymes, such as endonucleases[4a] and proteases,[4b] by simply binding the bioresponsive ligands (oligonucleotides and peptides) to the surface of the nanoparticles through supramolecular host–guest interactions instead of commonly used covalent linkages or thiolate chemisorption. Finally, the methodology could be readily translated to other relevant optical nanoprobes, such as Ag nanoparticles[13] and quantum dots,[14] which would also greatly benefit from an amplification mechanism based on the assembly of multivalent networks as ultrasensitive enzyme sensors.

Experimental Section

βCD-decorated Au nanoparticles were synthesized according to a previously reported procedure.[15] The size of the nanoparticles as calculated from TEM images ranged from 3 to 7 nm (see Figure S2 in the Supporting Information). The amount of surface-bound βCD was estimated by thermogravimetric analysis.[16] Details of the synthesis and characterization of diFc can be found in Section S1 of the Supporting Information. The procedure for the synthesis of Ad-PEG has been reported previously.[17] Prior to use, the nanoparticles were incubated with bis(*p*-sulfonatophenyl)phenylphosphane dihydrate dipotassium salt (0.5 mg mL^{-1}; Sigma) for 24 h and subsequently washed with deionized water, whereby they were collected by centrifugation at 14000 rpm for 30 min and redispersed in deionized water by ultrasonication; this washing procedure was carried out three times. This treatment makes the nanoparticles insensitive to changes in the salt content of the solution[18,4b] (see Section S4 in the Supporting Information). All experiments were performed in acetate buffer (10 mM, pH 6). For the detection of HRP, the enzyme was mixed with solutions containing βCD-decorated Au nanoparticles (0.1 mg mL^{-1}) assembled into clusters by diFc (0.01 mM) and H_2O_2 (0.05% v/v) in a final volume of 1 mL. To increase the sensitivity of the assay, Ad-PEG (0.1 mM) was added to the reaction mixture. UV/Vis spectra were recorded with a Perkin–Elmer Lambda 850 spectrometer. DLS experiments were performed with a Microtrac Zetatrac particle analyzer; for the determination of the size of the

objects, it was assumed that they were spherical. TEM images were collected with a Phillips CM300 microscope operating at 300 kV.

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