Instructable Nanoparticles Using Dynamic Combinatorial Chemistry
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Supporting Information

**ABSTRACT:** The application of nanoparticles to the multivalent recognition of biomacromolecules or programmed self-assembly requires control over the relative placement of chemical groups on their surface. We have developed a method to direct the functionalization of surfaces of aldehyde-equipped gold nanoparticles using a DNA template. An error-correction mechanism is built into the functionalization process thanks to the thermodynamic control enabled by the hydrazone exchange reaction. This reversible reaction can be conveniently switched off by removing the catalyst, preserving the functionalization.

**INTRODUCTION**

The chemical arrangement and composition of surfaces on the nanoscale determine their physical and chemical properties. Increasing the resolution of the features on functionalized nanoparticles down to the nanometer level should facilitate their application in the multivalent recognition of (biomacromolecules) or the programmed self-assembly of patchy nanoparticles. Unfortunately, most methods for the functionalization of nanoparticles with multiple different functional units result in their uniform or statistical arrangement on the surface. A convenient way to control the relative positioning of components in a system can be provided by self-assembly. Performing the assembly process under thermodynamic control ensures that the final system corresponds to the Gibbs energy minimum, and the reversibility of the reactions provides an error-correction mechanism.

The key requirement for self-assembly is the reversibility of the involved processes. It can be achieved using either supramolecular chemistry (i.e., through noncovalent interactions) or dynamic covalent chemistry. The latter method provides an opportunity to switch off the exchange if this reaction needs particular conditions (e.g., pH or a catalyst) to proceed. Thus, it is possible to equilibrate the system and then to preserve a particular molecular snapshot by switching off the exchange reaction (e.g., by changing the pH or removing the catalyst). If multiple different components (building blocks) can reversibly react with each other in a single system, then a large number of library members can be formed and amplified using template molecules. Such an approach is called dynamic combinatorial chemistry. When a template molecule can interact with one or more library members, it stabilizes them by forming a supramolecular complex. The methodology has been successfully utilized in the discovery of hosts for small molecules, ligands for biomacromolecules, interlocked molecules, catalysts, and even self-replicating systems.

Recently, dynamic covalent/combinatorial chemistry (DCC) has been used in the functionalization of flat liposomes and nanoparticle surfaces.

**RESULTS AND DISCUSSION**

In our previous work, we showed that the functionalization of nanoparticles using dynamic imine chemistry can be controlled with multivalent DNA templates. Imine bonds are stable only in the presence of the template, demanding a method to stop the exchange to preserve the DNA imprint on the nanoparticles. We hypothesized that the reduction of dynamic imines to static amines would be suitable for this purpose. Unfortunately, performing this reaction on gold nanoparticles proved challenging. We noticed that upon addition of reducing agents the nanoparticles lose their colloidal stability, likely due to the displacement of thiolate ligands with hydrides. To overcome this problem, we decided to use hydrazone chemistry instead. In contrast to imine exchange, hydrazones are kinetically stable under neutral or basic conditions and require either acidic or nucleophilic catalysis. Thus, the nanoparticles can be functionalized in the presence of the catalyst and should remain stable after its removal.

To check if hydrazone chemistry can be used in the template-directed functionalization of nanoparticle surfaces, we first synthesized and characterized aldehyde-equipped gold nanoparticles (AuNPs) and studied the characteristics of hydrazone formation and exchange on these nanoparticles.

Our experience with >10 nm, positively charged nanoparticles suggested two possible improvements to the nanoparticle design. First, AuNPs should be much smaller for a higher surface to volume ratio, simplifying their analysis and increasing the number of template-bound nanoparticles.

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Second, they should be formally neutral, as high positive surface charge density may result in nonspecific binding, ionic strength dependence, and aggregation upon binding polyanions such as DNA.

Sub-10-nm gold nanoparticles are usually prepared following the Brust–Schiﬀrin protocol, requiring the addition of aqueous NaBH₄ to a toluene solution of surfactant-stabilized Au(III) and thiolate ligands. We prepared our nanoparticles using a modiﬁed Brust–Schiﬀrin protocol. Because our L₂ ligand (Scheme 1) bears an aldehyde group, it can be reduced under the reaction conditions. Therefore, we had to remove all of the remaining hydrides by extraction before adding the thiolate ligands. Most of the resulting nanoparticles were 3–5 nm in diameter (Figure 1).

To provide the nanoparticles with colloidal stability, we decided to equip them with PEG-based ligands. The resulting steric stabilization was suﬃcient to provide solubility in water for ligands based on PEG-600 (or longer). In order to minimize the mobility of the ligands on the AuNP surface, we used lipoic acid, a disulﬁde-based anchor.

Treatment of the nanoparticles with an equimolar mixture of ligands L₁ and L₂ resulted in a higher surface coverage of aldehydes (approximately 1.4 nm⁻²) than in our previous work (approximately 0.5 nm⁻²), providing a promising platform for multivalent functionalization/recognition. The resulting value, measured by titration with nicotinic hydrazide (SI, page S3) is roughly 2 times smaller than the surface density of lipoic acid on gold nanoparticles (2.3 nm⁻²), with the difference presumably comprising ligand L₁. Because of the hydrophobicity of the benzaldehyde group, nanoparticles prepared using higher L₂ to L₁ ratios were poorly soluble in water.

The acylhydrazone exchange is signiﬁcantly slower than the imine exchange used previously in DCC on AuNPs. To determine if the reaction with acylhydrazides and their exchange can occur eﬃciently on aldehyde-modiﬁed AuNPs, we performed the experiment summarized in Figure 2. Because of the small size of the nanoparticles (and therefore the large surface area available for functionalization), we could monitor the process directly using ¹H NMR of the AuNPs rather than resorting to the analysis of the supernatant only (Figure 2C). First, we mixed the nanoparticles with an excess of either H₁ or H₂ with acetic acid as a catalyst. Before the NMR measurements, we neutralized the libraries and washed them thoroughly with water to ensure that we observed only hydrazones on the nanoparticles, unobscured by the hydrazides in the bulk medium. The washing step also removes acetic acid, preventing the hydrolysis of the hydrazones.

The disappearance of the aldehyde signal and the appearance of the new peaks between 8 and 9 ppm indicate that hydrazones form eﬃciently on the nanoparticle surfaces after 1 day. Furthermore, hydrazone functionalization is indeed persistent when the acid catalyst is removed; i.e., the AuNPs do not reequilibrate to free aldehydes and hydrazides. The absence

Scheme 1. Synthesis of the Aldehyde-Functionalized Nanoparticles

The first step is based on the Brust–Schiﬀrin method where the nanoparticles are initially stabilized with tetraoctylammonium bromide. Afterwards, they are washed with water to remove remaining hydrides, aged for 1 h, and mixed with an equimolar mixture of L₁ and L₂.

Figure 1. (A) Size distribution of the nanoparticles functionalized with L₁ and L₂. (B) TEM image of the nanoparticles. (C) Average stoichiometry of the nanoparticles with standard deviations.
of sharp signals indicates that all building blocks in the sample are attached to the nanoparticles. After confirming the formation of acylhydrazones, we performed an exchange experiment. We added H2 to the nanoparticles functionalized with H1 and H1 to H2-modified AuNPs, subsequently acidifying the libraries. The exchange was complete when an excess of H1 was used. However, in the opposite process, a significant amount of H1 remained attached to the nanoparticles, even though a 10-fold excess of H2 was allowed to react for 20 days, signifying a higher relative thermodynamic stability of the H1-based hydrazone.

In order to quantitatively study the thermodynamic properties of AuNP−hydrazone-based libraries, 1H NMR is not ideal because of broad signals and significant overlap between neighboring hydrazone peaks. We approached the problem by analyzing the DCLs using a filtration−cleavage−filtration HPLC protocol as shown in Figure 3D. The first filtration step removes solutes which are not attached to the nanoparticles. Subsequent treatment with hydroxylamine hydrochloride solution leads to the displacement of the hydrazides from the nanoparticles due to the formation of more stable oximes. The second round of filtration isolates the hydrazides liberated by NH2OH. Alternatively, the quantitative cleavage of hydrazides can be performed by hydrazine treatment.59 Because of the high stability of the hydrazones in the absence of the catalysts as proven by the NMR studies, the nanoparticles can be washed thoroughly after the first filtration, removing the excess hydrazides, catalysts, and templates and any impurities. Thus, the second round of filtration quantitatively yields only the hydrazides which were attached to the nanoparticles, allowing an easy determination of the composition of AuNP surfaces using HPLC.

To ensure that thermodynamic control can be maintained without an acidic pH, we evaluated aniline catalysis31,52 using DCLs composed of AuNPs and hydrazides H1, H3, and H4 (Figure 3A). The four libraries had identical building block compositions, but the hydrazides were added in different orders (Figure 3B). In library I, all hydrazides were added simultaneously, whereas in DCLs II–IV we allowed the first hydrazide to equilibrate with the nanoparticles for 48 h before adding the remaining two hydrazides. In all cases we observed similar hydrazone functionalization (Figure 3C), suggesting that aldehyde-equipped nanoparticles can be functionalized under thermodynamic control in water without the need for an acidic pH. Importantly, we noticed that even with an approximately 2.5-fold excess of hydrazides over aldehydes we obtained complete functionalization of the nanoparticle surface. This observation stands in a stark contrast to the imine chemistry we studied previously, which yields surface functionalization only in the presence of a proper template macromolecule.44

Figure 3. HPLC analysis probing the thermodynamic control over the exchange reaction. (A) Hydrazides used in these experiments. (B) Four libraries prepared by adding the hydrazones in four different sequences (I–IV). (C) Final concentrations of the hydrazones on the nanoparticle surfaces. (D) Analytical procedure used to quantify the concentration of the hydrazones on the nanoparticles.
Encouraged by the ease of functionalization of the AuNPs under biocompatible conditions, we embarked on a study of localized functionalization using template stabilization. For the experiments with double-stranded DNA templates we chose H1 and H4 hydrazides because of the better reproducibility of their behavior in the equilibration experiments. We added 16-meric CG-rich and AT-rich DNAs to two DCLs and compared them with an untemplated library using the same analytical protocol as that shown in Figure 3D. We discovered that the nanoparticles in a nontemplated library were mostly functionalized with H1, whereas H4 was the dominant hydrazone on the surface of nanoparticles in a DCL templated by GC-rich DNA (Figure 4). This preference can be explained by the electrostatic attraction between the negatively charged DNA backbone and the positively charged ammonium group of H4. The increase in the amount of H4 on the surface of AuNPs is much more pronounced for CG- than for AT-rich DNA (amplification factors of 1.6 and 1.2, respectively). At this temperature the AT-rich DNA exists mainly in a single-stranded form, and it has a much smaller charge density than the corresponding double-stranded GC-rich DNA. The difference in the amount of H4 attached to the nanoparticles under the influence of the GC-rich template is approximately 8 times greater than the concentration of the double-stranded DNA, suggesting that most of the binding sites on the DNA are involved in H4 binding. Thus, the template not only shifts the composition of the nanoparticle surface but also ensures the localization of the preferred hydrazone next to itself, effectively creating a molecular imprint on the nanoparticle.

CONCLUSIONS

The results presented here point to the conclusion that the combination of hydrazone exchange with neutral, aldehyde-functionalized nanoparticles leads to a promising platform for the directed functionalization of nanoparticle surfaces using biomacromolecular templates in water. With this approach we not only achieved localization of the functional groups driven by the interactions between a multivalent template and small building blocks but also showed that such nonstatistical functionalization can be efficiently preserved even in the absence of the chemical stimulus which induced it. Such behavior can be compared to learning (recognition of the molecular pattern exhibited by the template), followed by subsequent memorization of the new information (preservation of the chemical information after the removal of the acid or aniline catalyst and the DNA template).

We believe that the strategy presented here can become a useful method for yielding selective, multivalent binders for biomacromolecules used to instruct the nanoparticles. The approach might also be valuable for material scientists interested in the self-assembly of nanoparticles with precisely located functional patches.

METHODS

Preparation of Neutral, Aldehyde-Functionalized AuNPs. HAuCl4 (0.30 mmol) was dissolved in 20 mL of water and added to a toluene solution of tetraoctylammonium bromide (TOABr, 1.2 mmol, 50 mL). The mixture was vigorously shaken until all of the tetrachloroaurate was transferred to the organic layer, leaving a colorless aqueous layer. The yellow organic layer was separated for further reduction. NaBH4 (3.0 mmol) in 20 mL of water was added over 1 min, and the biphasic mixture was stirred vigorously for 1 h. The reaction mixture changed from yellow to dark purple upon addition of NaBH4. The water layer was removed, and the organic layer was washed twice with water to remove the unreacted hydrazides. The mixture of disulfide ligands L1 and L2 (each 0.15 mmol) in toluene (10 mL) was then added to the toluene solution of TOABr-stabilized gold nanoparticles, and the mixture was incubated for 24 h. AuNPs were obtained as precipitates in toluene after incubation. The solvent with free ligands was removed after centrifugation. Toluene was added to wash the AuNPs. The washing–centrifugation process was repeated three times. Purified L1,L2-functionalized AuNPs were dissolved in 20 mL of water for the following study, either as the original solution or diluted.

Hydrazone Formation and Exchange Monitored by 1H NMR. The libraries shown in Figure 2 were prepared by mixing 5.0 mL of stock nanoparticle solution (~8 μM) with 0.80 mL of 20 mM hydrazide solution (either nicotinic hydrazide H1 or Girard’s T reagent H2) and 2.0 μL of acetic acid. The samples were shaken for 2 days, filtered using centrifugal filters, and washed thoroughly with water to remove acetic acid and excess hydrazide. Afterward, the nanoparticles were analyzed with 1H NMR. The washed H1- and H2-modified nanoparticle samples were then treated with 0.80 mL of H2 and H1, respectively, together with 2.0 μL of acetic acid. After 20 days to ensure full equilibration, the samples were filtered, washed, and reanalyzed by NMR.

Hydrazone Equilibration Monitored by HPLC. The libraries shown in Figure 3 (I–IV) were prepared by mixing 1.0 mL of stock nanoparticle solution (~5 μM) with 50 μL of 10 mM solutions of hydrazides H1, H3, and H4 (in different orders) and 5 μL of aniline as a catalyst. For libraries II–IV, the two other hydrazides were added 48 h after the first one, whereas for DCL I, all hydrazides were added...
simultaneously. All library samples were subjected to the following analytical protocol: (1) centrifugal filtration of a 1.0 mL solution of nanoparticles, followed by a triple wash with water to remove free hydrazides; (2) treatment with 100 μL of a 1.0 M aqueous hydroxylamine hydrochloride solution for 24 h to displace hydrazides with more stable oximes; (3) a second round of centrifugal filtration to separate the nanoparticles from liberated hydrazides; and (4) HPLC analysis of the hydrazides, which gives the concentrations of the hydrazones on the nanoparticles before the treatment with hydroxylamine.

DNA-Templated Libraries. DCLs showing the influence of DNA templates (Figure 4) were prepared by mixing 1.0 mL of stock nanoparticle solution (~8 μM) with 50 μL of 10 mM solutions of hydrazides H1 and H4, 10 μL of aniline as a catalyst, and 800 μL of 25 μM double-stranded DNA solutions in 10 mM NaCl. The nontemplated library had exactly the same composition but did not contain DNA. DNA solutions were thermally annealed before addition to the libraries. Hydrazone H3 was excluded from the libraries due to poor reproducibility of its reaction with the nanoparticles; in its absence the differences between separately set libraries were below 5%. The libraries were analyzed using the protocol described above.

We observed that the nanoparticles in libraries templated with DNA did not aggregate, likely because of the insignificant charge of the nanoparticles themselves. The only positively charged groups were provided by the attached hydrazides. Conversely, the positively charged nanoparticles used in our previous studies showed significant aggregation, in some cases even precipitation.44 Transmission electron micrographs of libraries with and without DNA templates are shown in the Supporting Information.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.langmuir.5b03673.

Experimental procedures and characterization for L1, L2, and H4; characterization of the nanoparticles; HPLC method; detailed information on the composition of the libraries; additional transmission electron micrographs; information on the materials and instruments used in the study (PDF).

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Notes
The authors declare no competing financial interest.

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