Gold Nanoparticles

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Synthesis of Stable Peptide Nucleic Acid-Modified Gold Nanoparticles and their Assembly onto Gold Surfaces**

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DNA-based gold-nanoparticle (AuNP) systems are currently employed in a growing range of applications,[1] which include gene regulation,[2] nanofabrication,[3] sensing,[4] and plasmonic rulers.[5] However, a disadvantage of the assembly of DNA-modified NPs is the need for salt to keep their assemblies stable. Halogens are known to damage silver nanoparticles (AgNPs),[6] while the addition of salt in general destabilizes colloidal particles. Additionally, the presence of substantial amounts of ions is problematic for the study and use of physical phenomena that rely on electrostatics.[7] Thus, the functionalization of nanoparticles with the non-natural DNA analogue peptide nucleic acid (PNA), instead of DNA, is extremely attractive. PNA has many advantages over DNA including a higher stability against biodegradation, greater mismatch sensitivity, and higher binding efficiency to PNA, DNA, and RNA.[8] Hence, shorter oligonucleotide strands could potentially be used for the assembly of PNA-functionalized AuNPs compared to those required for stable DNA-based assembly. An increase in the resolution control of close-packed gold nanoparticles could therefore be observed. Additionally, the stability of PNA-DNA and PNA-PNA hybrids is independent of the ionic strength of the medium.[9] In comparison, DNA alone is not able to form assemblies under ion-free conditions as the electrostatic repulsion between the negatively charged strands is too high. Therefore, AuNP assemblies relying on PNA hybridization could be formed without the addition of salt, unlike DNA-AuNP hybrids. Despite the huge potential of PNA-based AuNPs, there are only a few articles reporting the direct attachment of PNA onto AuNPs.[10] Part of the reason for this is the strength of the interaction between gold and the neutral PNA, which is much stronger than with the negatively charged DNA.[11] The stronger tendency of PNA to adsorb flat onto the gold particle through direct interaction of the bases with the gold surface, rather than the preferred covalent linkage through a thiol–gold bond, results in a significantly thinner PNA layer and a strongly reduced PNA loading, leading to poor colloidal stabilization. This, combined with the lack of surface charge, results in inherently poor colloidal stability of PNA-modified metal nanoparticles.[12] This problem has not been sufficiently overcome with the PNA-functionalized AuNPs reported so far (see below). Herein, we use a conjugation technique that allows, for the first time, the synthesis of highly stable PNA-functionalized metal nanoparticles. We first functionalized the AuNPs with a stabilizing surfactant commonly used for DNA functionalization[13] and, in a second step, covalently attached the PNA to the AuNP surface. A variety of linkers were used, showing the wide applicability of the described method. Furthermore, we demonstrated the binding specificity of the PNA-functionalized nanoparticles to complementary oligomers, and the accessibility of the PNA strands, by directed assembly of PNA-AuNP conjugates to PNA-functionalized gold substrates. Scanning electron microscopy (SEM) imaging was used to show the sequence-specific binding to unstructured and micropatterned surfaces.

Functionalization of gold nanoparticles or surfaces with PNAs requires the modification of the oligomers with a terminal thiol group. Usually, either a cysteine or a similar monothiol-containing linker is inserted at the amino end of a PNA strand (Table 1, entry 1). Trithiol-capped DNA oligomers have been shown to bind more efficiently to

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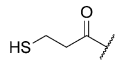
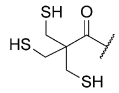
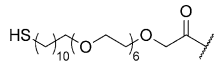
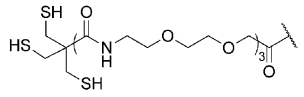
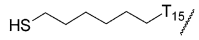
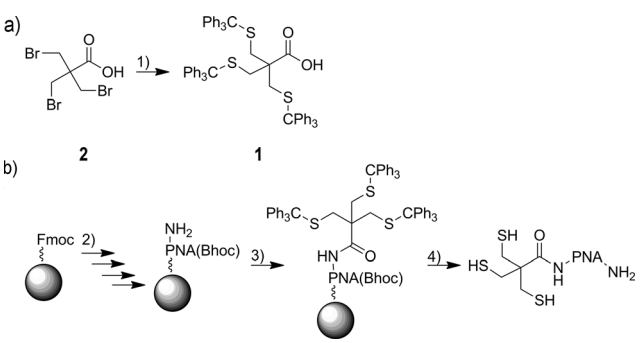
Table 1: Overview of the PNA and DNA conjugates with thiol linkers.

Entry	Linker	Sequence[a] a	Sequence[a] a′	Type	Corresponding NP[b]
1		**4**	**9**	PNA	**AuNP-4**
2		**5**	**10**	PNA	**AuNP-5**
3		**6**	**11**	PNA	**AuNP-6**
4		**7**	**12**	PNA	**AuNP-7**
5		**8**	**13**	DNA	**AuNP-8**

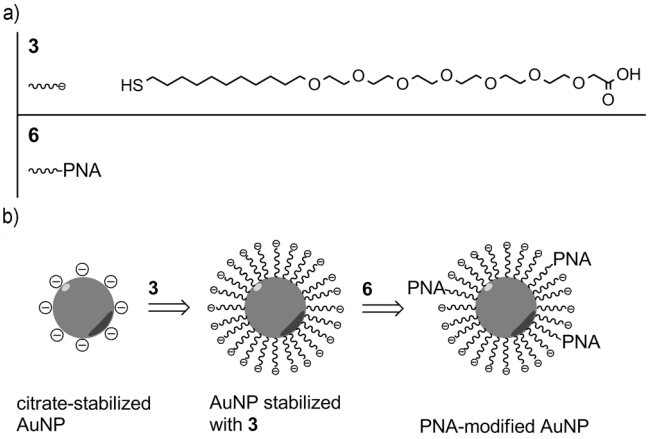
[a] The sequences, denoted N→C terminus (PNA), or 5′→3′ (DNA), respectively, are as follows: a: TCTCAGTATT; a′: AATACTGAGA; [b] 40 nm AuNPs, functionalized with **3** and the respective oligonucleotide.

AuNP surfaces and are displaced more slowly by other thiols compared to mono- and cyclic dithiol-capped derivatives.[14] As this stability towards subsequent ligand-exchange manipulation is of interest, for example, for sensing applications, we wanted to link the PNA strand in an analogous manner. However, to the best of our knowledge, trithiol ligands have not previously been used with solid-phase peptide/PNA synthesis (SPPS).[15] To fill this gap, we designed a novel and easy-to-prepare trithiol linker (**1**, Scheme 1). More specifically, the tribromide **2**[16] was converted into the trithioether **1** by reaction with triphenylmethanethiol in the presence of NaH. **1** could then be attached directly to different PNA sequences using standard SPPS procedures (Table 1, entry 2).[17] To assess the impact of the distance between the PNA and the AuNPs, PNA sequences containing ethylene glycol spacers between the PNA and the tripodal ligand were also synthesized (Table 1, entry 4). Importantly, the final step of SPPS, which necessitates trifluoroacetic acid (TFA) to cleave the PNA sequence from the solid support, also removes the trityl protecting groups of the thiols. An excess of a reducing agent such as tris(2-carboxyethyl)phosphine (TCEP) is required in this step to prevent formation of a disulfide bridge network.



Scheme 1. a) Synthesis of SPPS-compatible trithiol linker; b) Synthesis of a trithiol-linker-containing PNA oligomer. Conditions: 1) Ph_3CSH, NaH, THF, 50 °C, 62 h, 63%; 2) Fmoc-SPPS: i) 20% piperidine in DMF; ii) Fmoc-protected PNA-monomer (5 equiv), HATU (4.5 equiv), DIPEA (10 equiv), 2,6-lutidine (10 equiv), DMF; 3) **1** (3 equiv), HATU (2.75 equiv), DIPEA (13 equiv), 2,6-lutidine (13 equiv), procedure repeated again; 4) TFA:phenol:triisopropylsilane 38:2:1 (*v*/*v*/*v*), TCEP (1000 equiv). Fmoc = fluorenylmethoxycarbonyl; Bhoc = benzhydryloxycarbonyl; HATU = *O*-(7-azobenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; DIPEA = *N*,*N*-diisopropylethylamine.

With the thiol-containing PNA sequences made, we then investigated their ability to form stable PNA-functionalized AuNPs. Duy et al. reported the synthesis of PNA-modified particles in highly concentrated Tween 20 solutions.[10b] However, we found that the particles prepared using this method were not sufficiently stable for self-assembly purposes and therefore, did not provide the anticipated advantages of PNA on AuNPs. In the presence of Tween 20, we observed only non-selective aggregation. If the Tween 20 concentration was lowered, the PNA-functionalized NPs aggregated, even in the absence of ions. For DNA-modified particles, it was demonstrated that the additional attachment of 1-mercapto-6-hexyltri(ethylene glycol) improves salt stability through steric shielding without decreasing the hybridization efficiency.[18] Because PNA, in contrast to DNA, lacks negative charges, 32-mercapto-3,6,9,12,15,18,21-heptaoxadotriacontan-1-oic acid **3** was chosen for pre-functionalization of the AuNPs, because **3** incorporates steric shielding as well as additional electrostatic stabilization (Scheme 2).[13a] The final particles were, therefore, envisioned to remain stable in the presence of ions as well as under a wide range of pH values.[19]



Scheme 2. a) Illustrations of **3** and **6** and chemical formula of **3**; b) Overview of the two functionalization steps in the synthesis of PNA-modified AuNPs with stabilizing **3** and a representative PNA.

Additionally, **3** was used as a linker molecule for PNA (Table 1, entry 3). After in situ trityl protection of the thiol group of **3**, the carboxylate was coupled to the PNAs on the solid support (see Supporting Information for more details). This reaction did not proceed in the commonly used solvent for such coupling reactions, namely dimethylformamide (DMF). By switching to tetrahydrofuran (THF), which has a significantly lower dielectric constant, the desired product could be obtained.[20] We believe that the critical micelle concentration (CMC) of the PEG-derivative **3** and/or its

activated ester is too low in DMF, rendering the carboxylate and/or active ester site of the respective molecules inaccessible. For comparison, DNA-modified AuNPs were prepared using a linker with a 15-mer thymine spacer. Such linkers are often used for DNA-modified AuNPs because thymine has a very low affinity towards gold.[21]

The thermal stability of the PNA-PNA, PNA-DNA, and DNA-DNA hybrids in the absence of AuNPs was measured using UV/Vis spectroscopy (see Supporting Information). Consistent with previous reports,[22] the melting temperatures of the PNA-PNA duplexes were significantly higher than for the corresponding DNA-PNA or DNA-DNA duplexes, indicative of their greater stability. This highlights a major advantage of using PNA (as compared to DNA), because the PNA-modified particles have the potential to create assemblies with either shorter inter-particle distances or stronger hybridization than DNA-based systems, as fewer bases are required to achieve sufficient binding. Moreover, the PNA is not responsible for the stabilization of the particles and therefore the colloidal stability is largely independent of the oligomer length, unlike in the case of the common DNA-functionalized particles.

The preparation of PNA-modified AuNPs is described in detail in the Supporting Information. In brief, Tween 20,[23] phosphate buffer (pH 7.4), and **3** were successively added to a solution of AuNPs (40 nm diameter). After incubation for 12 hours, a sufficiently dense layer of **3** formed, protecting the AuNPs. The thiolated PNA was then added, and the incubation continued for another eight hours. Using this procedure, similar surface charges were predicted for all AuNPs, including the DNA modified **AuNP-8**.

The stability of particles functionalized with the different PNAs was investigated using UV/Vis spectroscopy. Partial aggregation results in a decrease of the extinction coefficient and/or red shifting/broadening of the localized surface plasmon resonance and can therefore easily be detected. In 1 mM phosphate buffer solutions, no changes in the UV/Vis spectra could be observed after 20 hours for any of the AuNPs (see Supporting Information for spectra). Similarly, in 10 mM buffer solutions, only minor decreases of λ_{max} were observed. At a phosphate buffer concentration of 0.1 M, aggregation and precipitation were visible within seconds for **AuNP-6** while the λ_{max} decreased less than 20% over the course of 15 hours for all other PNA-functionalized AuNPs.

Self-assembly experiments were carried out in the absence of additional ions or surfactants. Gold-coated substrates were modified with complementary PNA **7** or with non-complementary PNA **12**, as a negative control. After incubating 5 μL of NP solution on the PNA-modified substrates for four hours in a humidification chamber, the substrates were washed four times with water. After drying, SEM measurements were performed to determine the coverage densities of AuNPs on the substrate surface (see Figure 1 and Table 2). To the best of our knowledge, these are the first images proving the sequence-specific self-assembly of PNA modified nanoparticles.

All PNA- and DNA-functionalized nanoparticles selectively bound to the surfaces modified with complementary **7**. The PNA-modified AuNPs showed almost identical particle densities of about 40 μm^{-2} (i.e. 40 particles per μm^2). These observations along with the even distribution of particles on the surface suggest that the average distance between NPs adsorbed to the surface is dictated by the electrostatic repulsion forces between the negatively charged NPs. Also the trithiol-substituted AuNPs **AuNP-5** and **AuNP-7** reached this threshold and are expected to withstand oxidation or replacement by analytes if used, for example, for sensing applications in future studies.[14] Moreover, the similar surface coverage density of **AuNP-5** with **AuNP-6** demonstrates effective PNA hybridization, despite the use of a linker that is significantly shorter than the stabilizing surfactant **3**. Notably, the coverage density of the DNA-modified **AuNP-8** is lower compared to the PNA-modified AuNPs. This is in line with the results of the thermal denaturation experiments; different charge densities of the particles could also be a reason for this result, but this is unlikely because of the identical preparation conditions. Some non-complementary binding occurs in all cases, although slightly less in case of **AuNP-8**.

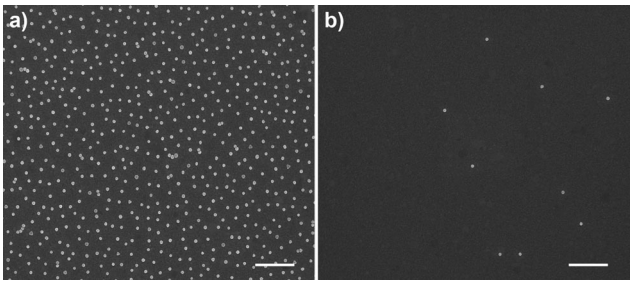


Figure 1. SEM images of surfaces modified with a) complementary PNA **7** and b) non-complementary PNA **12** after incubation with **AuNP-6**. Scale bars = 500 nm.

Table 2: Number of bound nanoparticles per μm^2.[a]

Nanoparticle	Complementary **7** [μm^{-2}]	Non-Complementary 12 [μm^{-2}]
AuNP-4	38	3.6
AuNP-5	39	5.1
AuNP-6	39	2.8
AuNP-7	43	5.5
AuNP-8	26	1.1

[a] As determined by particle counting in the SEM images.

DNA-based systems have been shown to be usable for specific deposition of AuNPs onto patterned substrates, a feature especially of interest in the context of nanoelectronics.[24] To test if our PNA-modified AuNPs were capable of fulfilling this task as well, we incubated **AuNP-6** on micropatterned substrates with lines of Au and silica. The substrates were first modified with hydrophilic PEGylated silanes or hydrophobic fluoroalkyl silanes and then with PNAs **7** or **12**, as described above for gold-coated substrates. **AuNP-6** assembles exclusively onto the areas with complementary PNA (Figure 2). Hence, the nanoparticles stick to neither hydrophilic nor hydrophobic surfaces but rather only to complementary oligonucleotide sequences. This further demonstrates the rigidity and practicality of our PNA-based system.

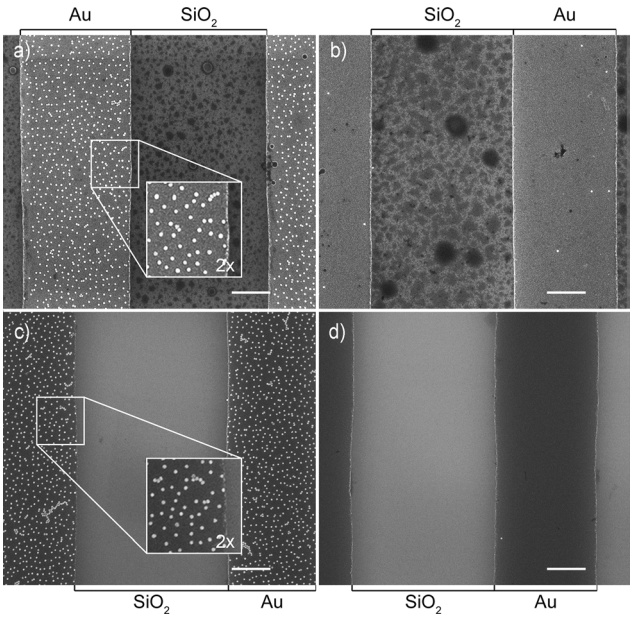


Figure 2. SEM images of micropatterned surfaces with a) complementary PNA **7** and PEG-modified silica, b) non-complementary PNA **12** and PEG-modified silica, c) complementary PNA **7** and fluoroalkyl-modified silica, d) non-complementary PNA **12** and fluoroalkyl-modified silica, after incubation with **AuNP-6**. Scale bars = 1 μm. The magnification of the insets is double that of the parent image.

In summary, we have demonstrated the preparation of stable PNA-modified particles through a novel approach involving the use of a thiolated alkyl PEG carboxylate surfactant. Standard, as well as novel, mono- and trithiol linkers were found to be compatible with this approach. The potential of these PNA-nanoparticles as new building blocks for self-assembling systems was confirmed by synthesizing particles that were able to self-assemble under additive-free conditions, an endeavor which has, to the best of our knowledge, not been directly shown with any other DNA/PNA-based systems. The superior properties of PNA compared to DNA make these particles highly interesting for future studies. Ion-free conditions are needed for investigation of electrostatic phenomena[7] and are not compatible with DNA hybridization. Sensing applications[4] could also benefit from the increased binding strength of PNA hybrids. This strength also allows for the investigation of optical phenomena at unprecedentedly small inter-particle distances.[5]

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