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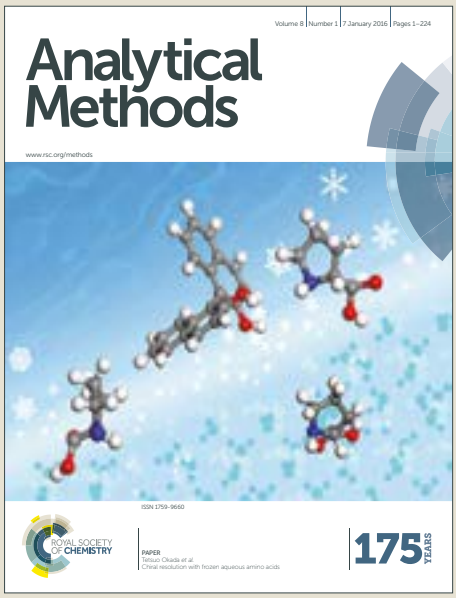

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Methods for preparing DNA-functionalized gold nanoparticles, a key reagent of bioanalytical chemistry

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Abstract

Gold nanoparticles (AuNPs) have excellent optical properties such as high extinction coefficients, distance-dependent color, strong fluorescence quenching, and localized surface plasmon resonance. At the same time, DNA has both programmable structures and molecular recognition properties. Functionalizing AuNPs with DNA has produced a diverse range of useful biosensors and stimuli-responsive materials. This article reviews a critical step required for all applications: conjugation of thiolated DNA to AuNPs. Since both DNA and AuNPs are negatively charged, a challenge is to overcome their charge repulsion while still maintaining the colloidal stability of AuNPs. Over the past twenty years, various strategies have been developed to achieve this goal, starting from the salt-aging method, where NaCl was added gradually over 1-2 days. The salt-aging method was accelerated by using acidic conditions, adding surfactants, and sonication. Depletion stabilization with a concentrated polymer solution was also tested as a new mechanism of avoiding AuNP aggregation. Finally, modified DNA was used to minimize charge repulsion. These research efforts have not only advanced the technology, but also allowed fundamental insights into the colloidal property of this system. For example, the three main colloidal stabilization mechanisms: charge, steric, and depletion have all been explored. This article describes both the experimental details as well as fundamental surface and colloid science.

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Introduction

In 1996, two seminal papers were published back-to-back in *Nature* by the labs of Mirkin and Alivisatos, respectively, for programmable assembly of DNA-functionalized gold nanoparticles (AuNPs).[1,2] The Mirkin group immobilized a high density of DNA on AuNPs, producing large aggregates with a linker DNA. Later, with more flexible linkers, directed crystallization of AuNPs was also achieved.[3,4] Alivisatos and co-workers on the other hand, limited the number of DNA on each AuNP to be just one or a few, mimicking the valency of atoms to organize AuNP 'molecules' with DNA bonds. Since then, these two ideas have been extensively developed for analytical, materials, and medicinal applications.

While many other materials, such as quantum dots,[5,6] magnetic nanoparticles,[7] silver and other noble nanoparticles,[8,9] hydrogels,[10] and proteins,[11] have also been functionalized by DNA, AuNPs still have a particularly important role for the following reasons. First, high quality and mono-dispersed AuNPs can be readily synthesized in most labs, allowing general accessibility of the material. AuNPs are highly stable and can be stored for years in clean buffers. Second, AuNPs have excellent optical properties ideal for biosensor development. For example, with surface plasmon resonance, the extinction coefficient of AuNPs are three to five orders of magnitude higher than the brightest small molecule dyes, allowing visual observation of AuNPs at low nM and even pM concentrations.[12] In addition, AuNPs have distance-dependent optical properties; dispersed AuNPs are red, while aggregated ones are blue or purple.[13] AuNPs are also strong fluorescence quenchers, which is useful for developing fluorescent sensors and molecular beacons.[14] Furthermore, the localized surface plasmon peak of AuNPs depends on its ligand adsorption or binding.[15,16] Therefore, AuNPs are very versatile in designing optical biosensors. Third, AuNPs have good biocompatibility and low toxicity.[17] Finally, AuNPs have well-established bioconjugation chemistry. Thiol containing molecules can be readily adsorbed by AuNPs forming self-assembled monolayers (SAM).[18,19] By varying the chemistry of the

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tail group, SAM can be used to control the surface property of AuNPs. The most popular and robust method of attaching DNA to AuNPs is by using thiol-modified DNA.

In the past three decades or so, many new functions of DNA have been developed, including making DNA nanostructures,[20-23] catalysis (DNAzymes),[24,25] and ligand binding (DNA aptamers).[26] As such, DNA-functionalized AuNPs allow molecular recognition and directed assembly, while AuNPs offer signal transduction for DNA-based biosensors[27-32] For the analytical community, a key for success is to prepare high quality DNA/AuNP conjugates. High quality is defined as high colloidal stability, and the attached DNA can hybridize to its complementary strand with fast kinetics.

While adsorption of small, thiol containing molecules on a gold surface is a spontaneous reaction, in the context of attaching DNA, it is actually quite a challenging task. Unlike bulk gold surfaces, one needs to maintain the colloidal stability of AuNPs during the conjugation process. The method initially developed by Mirkin and co-workers was called salt-aging.[33,34] This reliable method however takes more than a full day to complete. In the past 20 years, much progress has been made on this conjugation reaction, which in-turn has advanced our understanding of fundamental surface and colloid science. Reviews on fundamental DNA/AuNP interactions and their applications have been extensively published.[27-32,35-37] In this article, our focus is the conjugation reaction itself. Detailed conjugation methods, tips for experimentation, and related mechanistic insights are discussed.

DNA oligonucleotides.

Before reviewing the conjugation methods, the basic property of DNA and AuNPs are briefly described. Single-stranded DNA oligonucleotides are the most frequently used for the experiments discussed here. The structure of a four-nucleotide DNA with a sequence of 5′-ATCG-3′ is shown in Figure 1A. DNA has a phosphate backbone and four types of nucleobases. Even unmodified DNA adsorbs strongly with a gold surface via the bases with the adsorption energy ranking A>C>G>T>>phosphate.[38,39] The bases are charge neutral between pH 5 and 8. Protonated adenine and cytosine have pK_a values of 3.5 and 4.2,

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respectively. Therefore, the pH needs to drop to ~3 to protonate these two bases. Thymine and guanine cannot be protonated unless pH is lower than 2, which is outside the typical range of experimentation. Each phosphate carries one negative charge ($pK_a < 2$). As a result, DNA is a highly negatively charge polymer (i.e. a polyanion).

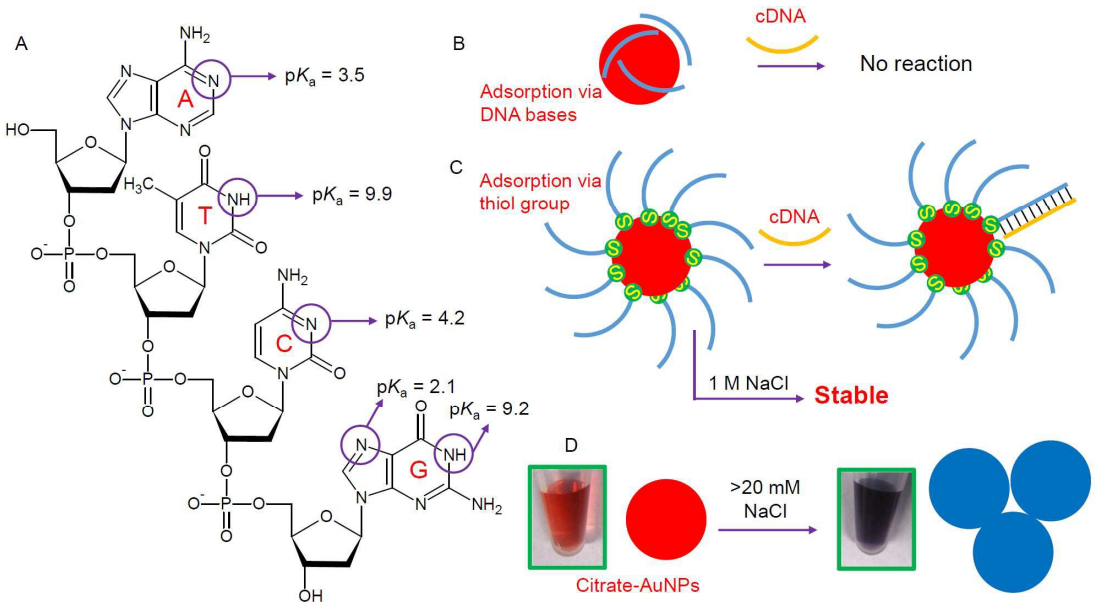

Figure 1. (A) The structure of a 4-mer DNA and the pK_a values of the bases. The pK_a's of the nitrogens without a proton refers its conjugate acid. The phosphate backbone is negatively charged. All the bases can strongly adsorb onto AuNPs via coordination interactions. (B) A scheme of an unmodified DNA adsorbing on DNA by wrapping around AuNPs using its bases. Since the bases are tightly adsorbed, it cannot react with its cDNA. (C) A thiolated DNA adsorbs via its thiol group, allowing the DNA to hybridize with its cDNA. After DNA attachment, the colloidal stability of AuNPs is significantly enhanced and can often survive 1 M NaCl. (D) Citrate-capped AuNPs are easily aggregated with even a low concentration of salt.

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Individual bases adsorb on a gold surface with energy greater than 100 kJ/mol in a vacuum, even for the weakest thymine. In water, the adsorption energy is lower, but still very strong. For example, if a DNA is stably adsorbed by AuNPs as shown in Figure 1B, adding its complementary DNA (cDNA) cannot desorb it.[40] The implication is that the energy of DNA hybridization is much lower than DNA base coordination energy on AuNPs. For comparison, adsorbed DNA can be easily desorbed by its cDNA from graphene oxide,[41,42] other nano carbons,[43,44] and many metal oxide surfaces.[45,46] An analytical implication is that unmodified DNA adsorbed onto AuNPs likely loses its molecular recognition function. Under special cases, it is still possible to achieve functional conjugates even with unmodified DNA.[47-51] This usually takes advantage of the different adsorption affinities of the bases and requires rational sequence design. For example, a poly-A is often used for anchoring on a gold surface with the rest of the DNA available for hybridization. More often, DNA adsorption is achieved via a thiol modification to prepare conjugates as shown in Figure 1C, where the DNA bases are free for hybridization.

Surface and colloid chemistry of AuNPs.

The colloidal property of AuNPs is strongly affected by its surface ligand. In this article, discussion is limited to AuNPs prepared by citrate reduction, and these AuNPs are called citrate-capped or citrate-stabilized AuNPs.[52] In a typical reaction, $HAuCl_4$ is reduced by trisodium citrate under reflux.[53-55] The amount of citrate added determines the size of the AuNPs, as larger AuNPs are produced with a lower concentration of citrate.[56] However, the lower size limit using this method is about 13 nm even with a high citrate concentration. Such 13 nm AuNPs can be readily prepared with a small size distribution and they are the most frequently used. It is more difficult to prepare AuNPs larger than 40 nm consistently with a narrow size distribution.

AuNPs have a very weak affinity for citrate, meaning that citrate can be easily displaced by stronger ligands. For such AuNPs, it is often easy to detect the Au-Cl bond using Raman

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spectroscopy,[57] while detecting citrate is more difficult. The chloride and citrate ligands give an overall negatively charged AuNP surface, rendering a moderate charge stabilization. By adding salt, the Debye length is shortened and AuNPs can approach each other more, entering an attractive van der Waals force region, which is responsible for its aggregation. AuNPs have a very large Hamaker constant (i.e. strong van der Waals force),[58] making them particularly susceptible to aggregation. For example, AuNPs experience about 70-fold stronger van der Waals forces than latex beads of the same size. Figure 1D shows a photograph of as-synthesized 13 nm AuNPs, and its intense red color turns blue due to aggregation upon addition of just >20 mM of NaCl. This color change generally irreversible. When such a color change happens, the DNA attachment experiment must start over again.

Since both DNA and AuNPs are negatively charged, salt is needed for DNA to overcome their long-ranged electrostatic repulsion. Since salt also causes AuNP aggregation, DNA needs to be attached with a sufficient density before AuNPs can approach each other. Once DNA is densely grafted, AuNPs can be stabilized also via steric stabilization and stronger electrostatic repulsion. The colloidal stability of such conjugates is typically quite high, able to survive even 1 M NaCl (Figure 1C).

DNA functionalization of bulk gold.

Before working on AuNPs, studies have already been carried out to functionalize gold electrodes and other bulk gold surfaces with DNA.[59,60] The process of thiolated DNA adsorption was carefully studied by Tarlov and co-workers using XPS spectroscopy.[61,62] In a typical experiment, 1 M salt was directly used since DNA does not adsorb without salt. The hybridization efficiency of such as-prepared samples was often quite poor, which was attributed to non-specific adsorption of DNA bases as mentioned above. To solve this problem, a small thiol ligand such as MCH was added afterwards to back fill the gold surface and displace DNA bases. Note that thiol adsorption is stronger than DNA base affinity. This helps DNA to adopt an upright confirmation for hybridization (Figure 2).

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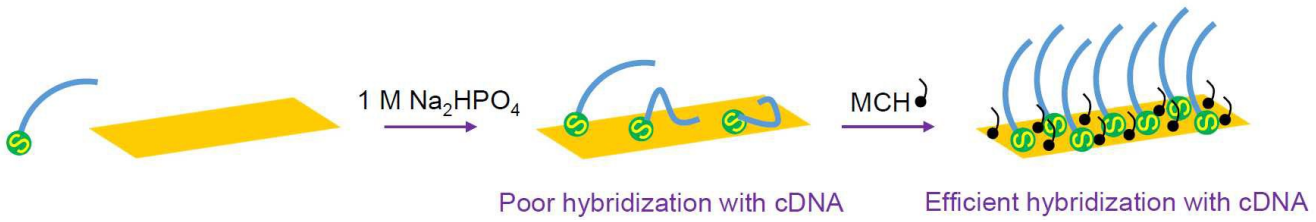

Figure 2. The process of DNA adsorption on a bulk planar gold surface. Without salt, DNA is not adsorbed on the gold surface. With a high salt concentration, DNA is adsorbed both via the thiol group and the bases, rendering a poor hybridization efficiency. Adding a low concentration of small thiol molecules such as mercaptohexanol (MCH) can displace DNA bases and improve hybridization. Adding too much MCH can fully desorb the DNA.

The salt-aging method.

Adding 1 M salt directly to screen charge repulsion, however, cannot be applied to AuNPs for DNA attachment due to the colloidal stability problem. To address this, the salt-aging method was developed by Mirkin and co-workers.[1,33,34] This method has been reliably reproduced in many labs and it works for essentially any DNA sequence. The basic idea is still to screen charge repulsion by adding salt, but salt is added slowly to retain the stability of the AuNPs. The full protocol for carrying out this has been published previously,[55] and will not be repeated in detail here. Briefly, the as-synthesized AuNPs are mixed with thiolated DNA. For the 13 nm AuNPs (~10 nM, A_{520} = ~2.7), thiolated DNA is mixed with AuNPs at a ratio of ~300:1. After incubation (typically for 1 h or longer), NaCl (1 M) is added dropwise with simultaneously shaking, to reach a final NaCl concentration of 50 mM. The sample is further incubated for 1 h or longer and then more salt is added, typically at increments of 50 mM NaCl. After 300 mM NaCl is reached, the sample is further incubated overnight. The final product is highly stable even in 1 M NaCl, and the attached DNA can hybridize to its cDNA.

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The reactions during the salt-aging process are presented in Figure 3A. Initially only a few DNA molecules are adsorbed, either via the thiol group or some via the bases. This is possible since the overall DNA density is very low. These initially adsorbed DNA molecules have increased the negative charge density of the AuNPs and repel other incoming DNA molecules greater than bare AuNPs. The stability of such conjugates is however better than that of bare AuNPs. As a side note, this increased stability was also used for developing label-free colorimetric biosensors.[63,64] With more NaCl added, a few more DNAs are adsorbed until a new electrostatic repulsion equilibrium is reached. These newly adsorbed DNA molecules further increase the ability to tolerate even more NaCl. During this salt-aging process, the thiol group gradually displaces the DNA bases adsorbed on AuNPs to make DNA stand up. In the later stage of the process, the main effect of the salt is to reduce the repulsion between the DNAs on AuNPs so that an ultrahigh density can be achieved. The high curvature of small AuNPs are also helpful for such a high density, higher than that on planar gold surfaces.[65]

Reaction containers. An important note is that the tubes and vials used for these reactions need to be very clean. AuNPs have a high surface energy and they tend to adsorb impurities, which may make it more difficult for DNA attachment. In addition, AuNPs may stick to the walls of containers, also spoiling the experiment. For AuNP synthesis, all the glassware needs to be cleaned with aqua regia (be very careful) in a fume hood.[55] After obtaining high quality AuNPs, the DNA conjugation reaction can be carried out in a glass scintillation vial or a polypropylene microcentrifuge tube (Axygen, catalog number: MCT-150-C worked well for us). It is also quite important to clean the glass vial with strong base (e.g. 12 M NaOH) before use. The plastic microcentrifuge tubes can be used directly.

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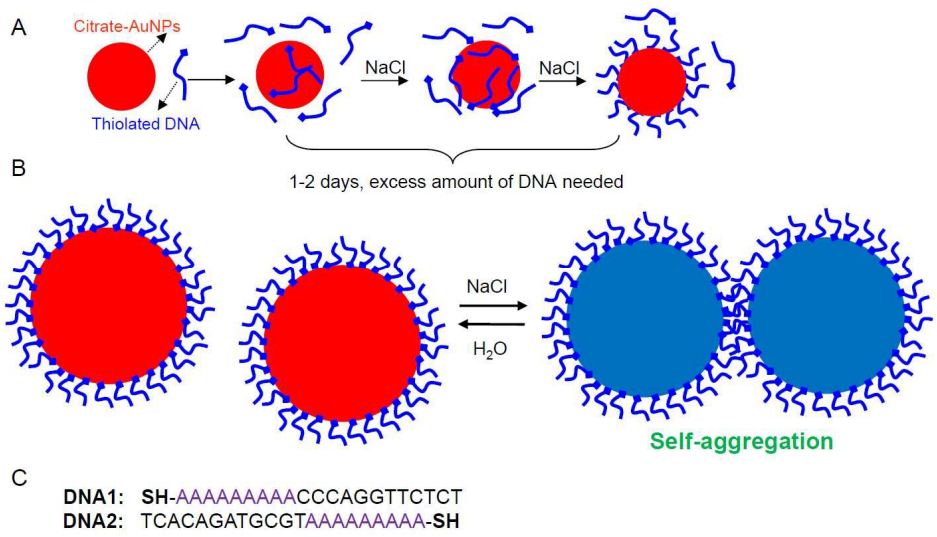

Figure 3. (A) The salt-aging process for DNA attachment to citrate-capped AuNPs. NaCl needs to be added in small increments to avoid irreversible AuNP aggregation. DNA needs to be used in excess (more than the adsorption capacity) to win the kinetics over AuNP aggregation. (B) A scheme showing DNA-mediated self-aggregation of large AuNPs. This process can be reversed by removing salt and adding water. (C) Two thiolated DNA sequences each with a poly-A spacer. DNA1 is resistant to self-aggregation, while DNA2 is prone to it.

Functionalizing larger AuNPs. For the most commonly used 13 nm AuNPs, this salt-aging method is very reliable. For larger AuNPs (e.g. >40 nm), the protocol is identical, but the incremental addition of salt is even smaller.[65-67] Despite that, the process often fails for such larger AuNPs. Based on our experience, the home-made AuNPs have variable performance, but 40-50 nm AuNPs from commercial sources, such as Ted Pella, work quite reliably and consistently. The quality of AuNPs can be judged based on the sharpness of the UV-vis spectrum, where a sharp surface plasmon peak is indicative of high quality AuNPs. Large AuNPs are important because of their much higher extinction coefficients. For example, 50 nm AuNPs are typically made at a final nanoparticle concentration of 0.075 nM, which still has an extinction value of ~1.0 at its surface plasmon peak. For comparison, the final

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nanoparticle concentration of 13 nm AuNPs (10 nM) is 133 times higher, while its extinction value is only ~3-fold higher. Despite a much lower AuNP concentration, the initial DNA concentration added still needs to be kept at around 3 μM. This is probably because larger AuNPs are even more prone to aggregation due to a much larger van der Waals force. In the end, more than 95% of the added DNA cannot be attached to AuNPs and are wasted.[68]

By collaborating with the Gill lab, we carefully studied the kinetics of DNA adsorption on AuNPs of different sizes.[68] The rate of DNA adsorption is not very dependent on the AuNP size, but it is linearly proportional to the concentration of DNA according to the mass action law. Since large AuNPs are often used at a much lower concentration, they have a lower capacity for DNA (despite each particle being able to adsorb more). If DNA is added close to its adsorption capacity, DNA adsorption cannot compete with AuNP aggregation. In this study, a method was proposed by centrifuging 40 nm AuNPs at 6000 g for 10 min and most of the supernatant was removed to concentrate the AuNPs 100 times. With a final DNA concentration of 10 μM, the conjugate survived 300 mM NaCl and more than 60% of the DNA attached to the AuNPs, which is a significant saving of the reagent. In the same work, sonication was also found to be helpful for attaching DNA to large AuNPs, consistent with a previous report.[69]

Aggregation of AuNPs during DNA attachment. For a typical successful experiment, the color of AuNPs should remain red during the whole process. Sometimes AuNPs aggregate immediately after mixing with DNA before even adding salt. This is an indication of impurities in the DNA and they can be removed by a desalting step to purify the DNA using a Sep-Pak column or other methods. Sometimes AuNPs change color to purple during the salt-aging process. In this case, there is a possibility of DNA-mediated self-aggregation. To check this, the aggregated sample can be centrifuged to remove the supernatant and then re-dispersed in pure water. If the AuNPs can fully go back to the same red color as the dispersed AuNPs, it means that the DNA sequence can self-hybridize. This is

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problematic especially for large AuNPs since they have a larger contact area for DNA to interact (Figure 3B).[69,70] Just three of four base pairs might be sufficient for this to occur. Therefore, careful sequence design is required to avoid such effects. For example, the two sequences shown in Figure 3C, DNA1 is resistant to such self-aggregation, while DNA2 is prone to it. Such AuNPs however are still useful for DNA-directed assembly when performed at a lower salt concentration to inhibit self-aggregation. One just needs to keep the salt concentration low enough (or at slightly higher temperature) so that self-aggregation cannot take place, but the intended DNA linker can still hybridize. After all, the designed linker is fully complementary to the adsorbed DNA and should be more stable than self-aggregation interactions. Sometimes, such self-aggregation can also be intentionally used for purification. For example, liposomes are very difficult to centrifuge. We used the self-aggregation of liposomes to remove free non-conjugated DNA at 4 °C, while also performing DNA-directed liposome assembly at room temperature.[71]

Effect of TCEP reduction. Thiolated DNA is not supplied as a free thiol. Typically, both the 5′-end and 3′-end of thiol are capped by a disulfide bond (Figure 4A, structures from Integrated DNA Technologies). In the initial protocol reported by Mirkin and co-workers, these DNAs were treated with an excess amount of DTT (Figure 4B), typically 10-50 mM, to cleave the disulfide bond and produce free terminal thiol on DNA. Since DTT is also a thiol containing molecule, the reduced DNA needs to be purified by a column to fully remove DTT. Alternatively, DTT immobilized on acrylamide resin was recommended by IDT to avoid purification (Reductacryl™, Calbiochem Inc. Cat. No. 233157).

Later, a new reducing agent called TCEP was used (Figure 4C). In this case, no separation step was needed since TCEP does not have a high affinity for AuNPs. TCEP was recommended to be added in great excess at a 100:1 ratio by IDT. While cleaving the disulfide bond was critical for conjugation of DNA to the maleimide group, we had second thoughts on its reaction to AuNPs. It has been noticed that the effect of TCEP was not obvious for attaching DNA to AuNPs.

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When TCEP is used, but without purification, the cleaved fragment is still in the same solution and will also adsorb on AuNPs. Since these are small, non-charged thiol molecules, they will adsorb on AuNPs prior to the DNA adsorption (Figure 4D). It is known that such disulfide bonds dissociatively adsorb on AuNPs, meaning that AuNPs can cleave the disulfide bond, which is the same effect as TCEP. On a 13 nm AuNP, ~1200 thiol groups can be adsorbed. However, it can only adsorb ~100 DNA molecules due to the bulky size of DNA. Therefore, there is sufficient space on AuNPs to adsorb the cleaved piece. Later, Gill and we systematically compared the effect of TCEP and found that it is not an absolutely required step.[68] It should be noted that for fully cleaved and purified DNA samples (Figure 4E), the performance might still be better, especially for very challenging samples. The small thiol molecule adsorbs more quickly to AuNPs and this adsorption may change the surface property of AuNPs.

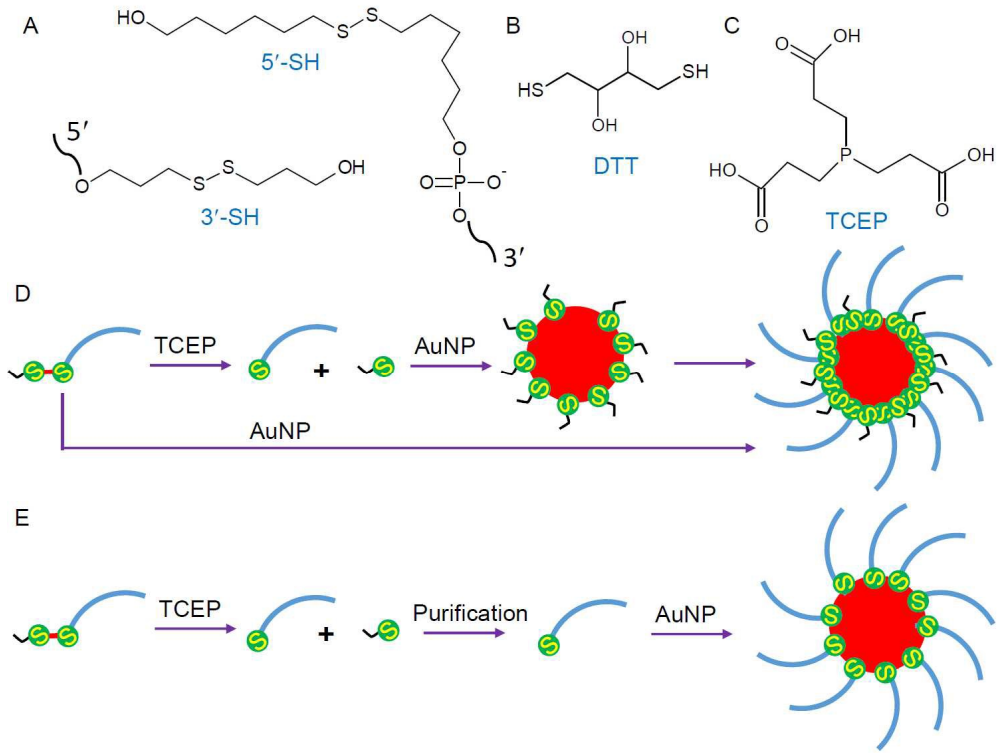

Figure 4. (A) The structure of commercial thiolated DNA with capping groups and a disulfide bond. The structures of (B) DTT and (C) TCEP. (D) A scheme of DNA adsorption by TCPE treated and non-treated DNA. (E) A scheme of TCEP treated and purified DNA adsorption on AuNPs.

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Calculation of DNA density. A standard parameter to report for such DNA conjugation experiments is the number of DNA on each AuNP. Typically a fluorophore and thiol dual-labeled DNA is used.[72] For non-fluorophore labeled DNA, then a DNA staining dye such as Oligreen or SYBR Green I can be used.[50] Recently, Baldock and Hutchison reported direct measurement of DNA absorbance at 260 nm.[73] In these methods, DNA is desorbed from AuNPs after adding a high concentration of thiol ligands, such as MCH, or by adding KCN to dissolve AuNPs. It is interesting to note that the reported density of DNA varies a lot from lab to lab. Even in our own lab, different students have reported different values for the 13 nm AuNPs. We have seen from literature that the density on each 13 nm AuNP can vary from ~70 to ~140. This is probably related to calibration, loss of DNA during washing and work-up, fluorescence quenching, and error in estimating the concentration of AuNPs or DNA. Therefore, the absolute value of these numbers does not carry much weight. The best way is to have a standard method side-by-side, such as the salt-aging method and then make comparisons between the new method with the standard method. This way, the systematic errors are minimized.

Surfactant-assisted DNA functionalization.

The direct salt-aging method is simple and quite reliable for small AuNPs. To solve the problem of attaching DNA to larger AuNPs, Mirkin and co-workers added surfactants, and AuNPs up to 250 nm could be consistently functionalized.[69] A few surfactant molecules were tested in that initial work, such as, sodium dodecyl sulfate (SDS), Tween 20, and Carbowax (Figure 5B). In a typical experiment, DTT-treated DNA was incubated with AuNPs in the presence of 0.01% SDS in phosphate buffer (10 mM, pH 7) for 20 min. Then the NaCl concentration was increased to 50 mM followed by another 20 min incubation. This process was repeated once more and then the NaCl concentration was increased by 100 mM up to 1 M. Finally, the mixture was incubated overnight at room temperature. Adding surfactants in the process was found to increase DNA loading capacity by 39% with a final concentration of 1 M NaCl.

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AuNPs capped by small surfactant molecules, such as SDS, are likely to form an interdigitated bilayer structure (similar to CTAB adsorption). For anionic surfactants, both charge and steric stabilization might be important. Non-ionic surfactants, such as Tween and Triton, are likely to exert only steric stabilization. The fluorinated surfactant might form quite rigid layers to allow a very high AuNP stability. In each case, the thiol on DNA can penetrate the surfactant layer and adsorb on AuNPs (Figure 5A). This penetration however is still quite slow, and typically more than 2 h is required. From the data, the type of surfactants had little effect on DNA loading.[69] In the same work, the effect of sonication was also studied. It was concluded that sonication in the salt-aging process can facilitate the DNA loading by decreasing the non-specific interaction between DNA bases and AuNP surface.

In 2009, Zu and Gao used nonionic fluorosurfactants (i.e., Zonyl FSN, Figure 5B) to protect AuNPs of different sizes (13 to 100 nm) against up to 1 M NaCl. It should be noted that the concentration of the surface needs to be between 0.02 to 0.2% for the protection effect. Then they mixed a thiolated DNA and the surfactant-protected AuNPs in 1 M NaCl in phosphate buffer (10 mM, pH 7.5). Stable conjugates were formed in 2 h. Therefore, this method has bypassed the salt-aging step due to the ultrahigh stability of AuNPs in the surfactant solution.[74] Xu and Wu compared the effect of four types of nonionic surfactants in protecting AuNPs (Tween 20, Tween 80, NP 40, and Triton-X 100). They found Tween 80 to be the most effective and it could also achieve a high tolerance to NaCl (1 M) and DNA loading was finished within 2-3 h.[75] Thiolated polymers were also combined with Tween 20 to protect AuNPs. For example, a low concentration (200 nM for 13 nm AuNPs) of thiolated methoxypoly(ethylene glycol) (mPEG-SH, MW ~5000) could provide additional steric protection for AuNPs in a high NaCl concentration (800 mM). The DNA conjugates were prepared in 1.5 h.[76]

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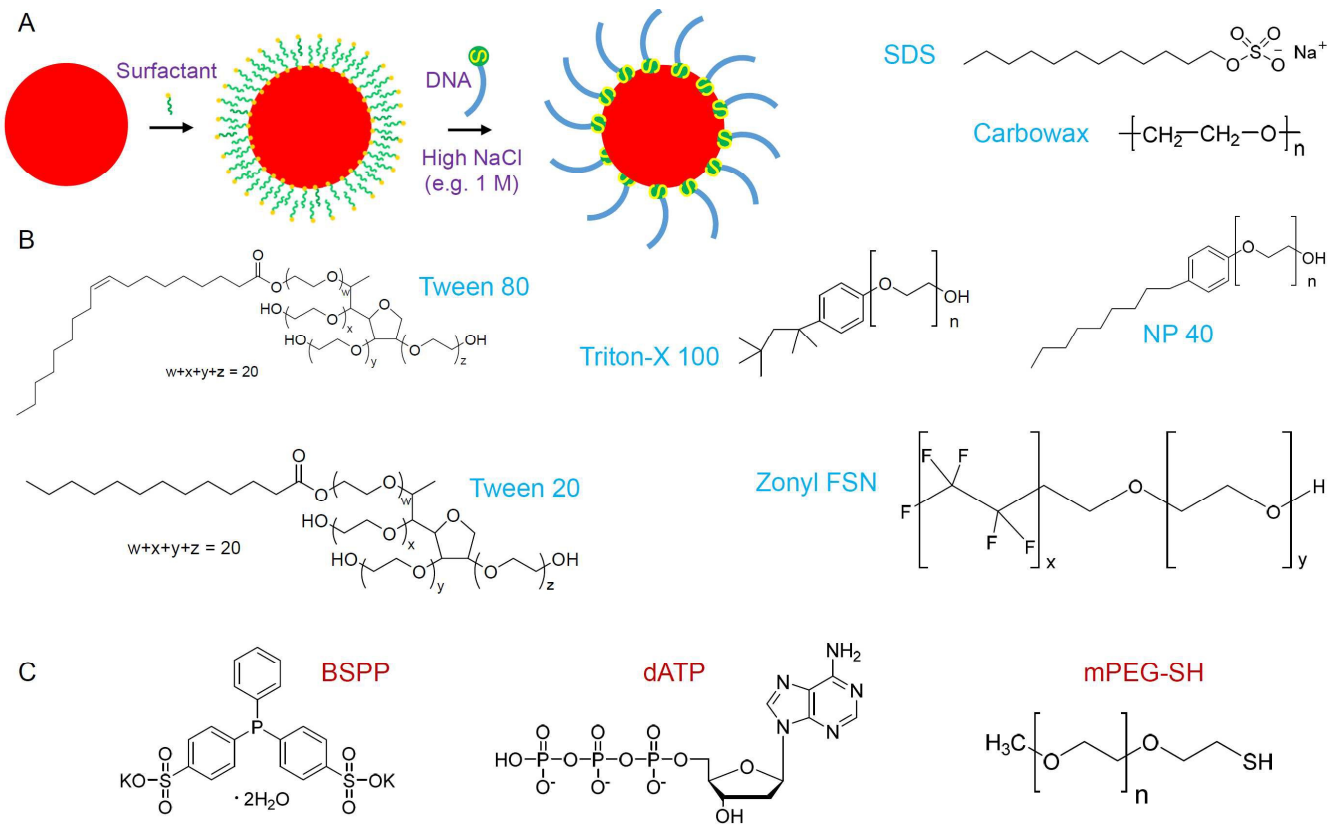

Figure 5. (A) A scheme of thiolated DNA attachment in the presence of surfactants. The thiol group needs to penetrate the surfactant (bi)layer for conjugation, resulting in a slow kinetics. (B) The structure of some surfactants used for stabilizing AuNPs for DNA attachment. (C) The structure of some small molecules and polymers used for helping functionalization of AuNPs by DNA.

Aside from surfactants, a few small molecules or polymers have also been applied to facilitate DNA conjugation while avoiding AuNP aggregation. For example, Alivisatos and co-workers displaced the citrate by dipotassium bis(p-sulfonatophenyl)phenylphosphine dihydrate (BSPP) ligand (Figure 5C).[77] Such ligand-protected AuNPs (5 nm) are stable in the presence of 0.15 M NaCl and 1 mM $MgCl_2$. However, the slightly larger 10 nm AuNPs are not stable when NaCl is higher than 0.15 M. Mononucleotide (e.g. dATP) was used by Hsing and co-workers to protect 20 nm AuNPs.[78] In a typical

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experiment, a mixture of thiolated DNA, AuNPs (ratio 500:1) was heated at 60 °C for 3 h in 10 mM phosphate buffer (pH 8.0) with 0.1 M NaCl. Finally, each AuNP was loaded with ~ 80 DNA strands.

Most of the surfactant related methods discussed above focused on improving the colloidal stability of AuNPs against NaCl. In this regard, a high concentration of NaCl is still necessary to achieve DNA loading. Alternatively, Sedighi and Krull incubated citrate-capped AuNPs and thiolated DNA with positively charged magnetic particles.[79] Due to the electrostatic interactions, AuNPs and DNA were pre-concentrated on the surface of magnetic particles. The reaction kinetics were enhanced by 1000-fold and a high DNA loading density was realized (e.g. 95 DNA on each 15 nm AuNP).

The low pH assisted method.

In 2012, we systematically studied the adsorption of DNA by AuNPs using fluorescently labeled oligonucleotides.[40] It confirmed the importance of screening for charge repulsion. A discovery was that DNA adsorption was much faster at acidic pH conditions, especially when the salt concentration was low. Based on this observation, we developed an improved method that takes only a few minutes for attaching DNA.[80] In a typical experiment, 13 nm AuNPs (e.g. 1 mL) are mixed with a thiolated DNA in a cleaned glass vial or microcentrifuge tube. Interestingly, there is no need to add excess amount of DNA. By using a 100:1 ratio (about the adsorption capacity), the system should work just as well. After a brief incubation, a final aliquot of 10 mM sodium citrate buffer was quickly added (stock solution of 500 mM sodium citrate, pH 3.0). Three minutes later, the sample was centrifuged to remove the non-conjugated free DNA and then re-dispersed in a pH 7 buffer for use (Figure 6A). Here, the role of acid is also to overcome the kinetic barrier of DNA attachment. Once the gold/thiol bond is formed, it is thermodynamically very stable and the pH can be adjusted back to neutral.

The quality of the AuNPs was characterized by UV-vis spectroscopy and DLS, both of which showed a comparable result with the salt-aging method. This method appears to be quite popular and has been reproduced by many researchers. At the same time, however, we also received inquiries

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regarding problems of this method. Taking this opportunity, some notes and recent developments are summarized.

DNA sequence requirement. The basis of this method is protonation of DNA bases. Namely, adenine and cytosine can be protonated at pH 3. Protonated bases reduce the repulsion between DNA and AuNPs, and more importantly, the repulsion between DNA strands on AuNPs. Therefore, if a DNA is rich in guanine and thymine, this low pH method is unlikely to work well. Most researchers would put a polynucleotide spacer between the thiol and the sequence intended for hybridization. The best spacer sequence is poly-A for the low pH method (even better than a poly-C spacer).[50,81]

We have been wondering why this method works so well, especially for DNA containing a poly-A spacer. If low pH just accelerates the overall adsorption kinetics, thiol displacement of adsorbed bases should still take time. In addition, even non-thiolated poly-A DNA could also be attached with a high density, while still retaining function.[48] Finally, non-thiolated poly-C DNA often lost function after attachment using this method, even though cytonsine is more easily protonated than adenine.[50] Therefore, some other factors beyond simple electrostatics might also play a role.

Parallel poly-A duplex. Recently, we articulated the importance of parallel poly-A structure.[81] At pH 3, two poly-A DNA strands can form a parallel duplex (e.g. 5′-end with 5′-end). As a result, the poly-A spacer part from two DNA molecules (the same sequence) can hybridize (Figure 6B).[82,83] In this duplex region, each base pair carries two positive charges, which neutralizes the negative charges on the phosphate backbone. As such, this part is overall charge neutral and it has no charge repulsion with AuNPs. The remaining non-poly-A sequences are however still negatively charged. This rigid poly-A duplex exposes the thiol groups to directly bind the AuNP surface (Figure 6A). This can also explain non-thiolated DNA adsorption through the one or few terminal adenine bases, resulting in a high DNA density.[48] At neutral pH, adsorption is achieved through all the adenine bases and the density is lower.[47] Note this is possible only by such a parallel duplex. Otherwise, the thiol groups are oriented in opposite

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ends in a normal anti-parallel duplex. To expose the thiol group at neutral pH, two different DNA sequences are needed.[84,85]

By considering the DNA conformation, we can also explain the poor performance of DNA with a poly-C spacer. While it has also a charge role, its conformation is the i-motif (Figure 6C), which cannot expose the thiol group as that in a poly-A. Therefore, the best conditions for this low pH method would be to have a poly-A block and then a random sequence for hybridization. For other sequences, we have not found a general rule to predict whether it will work or not by adding acid. We found it is also good to combine the low pH method and salt-aging (unpublished results). If the experiment is carried out at pH 4-5 using acetate or citrate buffer and then salt is gradually added, the salt-aging process can be shortened.

Acid requirement. In addition to being a useful method, this observation also has interesting fundamental implications. For example, we typically use a final aliquot of 10 mM sodium citrate (pH 3), but using 10 mM HCl does not work. The HCl sample can be rescued by an adding additional 30 mM of NaCl (note that 10 mM sodium citrate has also 30 mM Na^+). Therefore, for the low pH method, salt is still needed. This is reasonable since even at pH 3, both DNA and AuNPs are still negatively charged. Low salt alone or low pH alone is insufficient, and combining these two has a synergistic effect.

DNA stability at low pH. DNA is known to undergo depurination at acidic pH, which leads to loss of DNA bases and further cleavage of DNA.[86] This reaction might be undesirable for DNA attachment. To quantitatively understand this, we also studied the stability of DNA at pH 3 using gel electrophoresis and fluorescence-based assays.[81] Interestingly, depurination is not a concern for short poly-A sequences even after hours of incubation at pH below 3. Guanine is relatively easier to depurinate, but still needs to go to pH 3 for a few hours to observe the effect. Therefore, DNA has sufficient stability during this experimental process.

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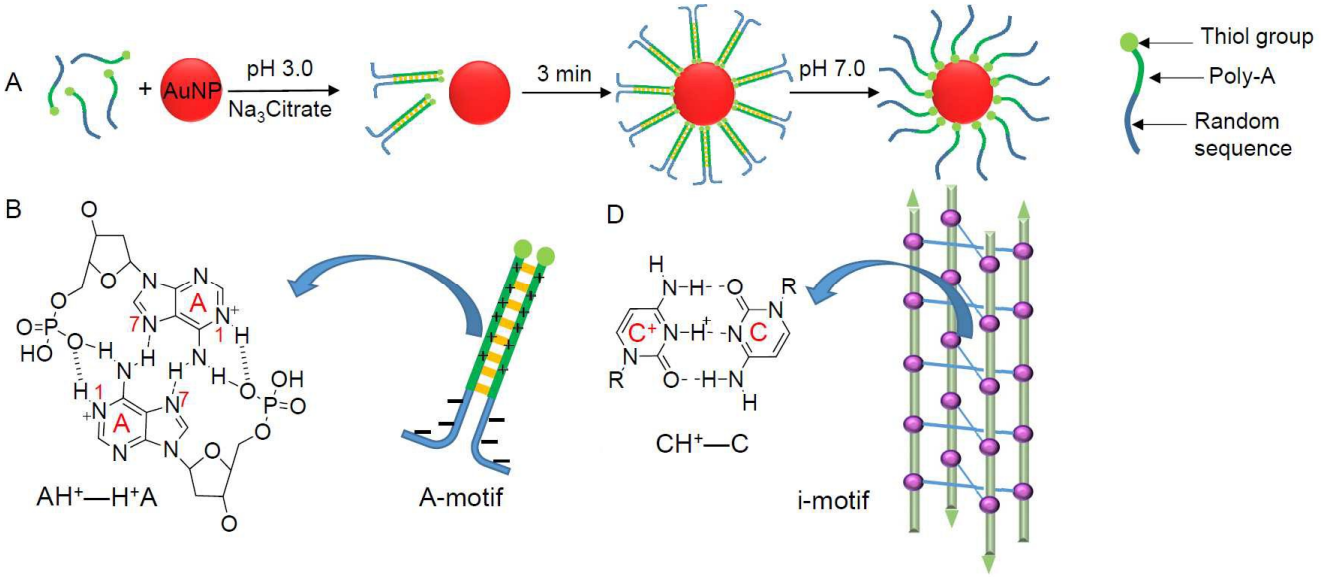

Figure 6. (A) A scheme of a thiolated DNA containing a poly-A spacer attaching to AuNPs at low pH. Such DNA can form parallel duplex at low pH, and the thiol groups are exposed directly for quick adsorption with the correct DNA orientation. Upon raising pH to neutral, the desired functional conjugates are formed. (B) The structure of protonated adenine forming a base pair responsible for the parallel-A duplex. The duplex region is charge neutral and is favorable for the thiol attachment. (C) The structure of CH^+-C base pair responsible for the i-motif, which is not desirable for DNA adsorption. Reproduced from ref. [81] with permission. Copyright 2016 American Chemical Society.

Functionalization by depletion stabilization.

In addition to maintaining AuNP stability by electrostatic or steric stabilization, another mechanism is depletion stabilization.[87] In this case, AuNPs (or other nanoparticles) are dispersed in a concentrated polymer solution passing through the semi-dilute region, where polymer chains start to overlap with each other. For AuNPs to aggregate, they must push the polymer chains away, which is energetically unfavorable. This is called depletion stabilization (Figure 7A). However, once the particles have enough energy to pass the barrier, the same depletion force becomes attractive for the particles and this

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is sometimes called depletion attraction (Figure 7B). The strength of depletion force is related to the particle size (R), the separation between particles (h), and the size of the polymer (σ) as shown in Figure 7A. In the field of biochemistry, this attraction increases ligand binding and is also called the macromolecular crowding effect.[88] Note that the polymer chains do not have to adsorb onto the AuNPs.

Using polyethylene glycol (PEG) as a stabilization agent, we demonstrated ultrahigh stability of AuNPs even in high molar concentrations of Mg^{2+}.[89] Detailed studies indicate that electrostatic repulsion, steric stabilization due to adsorbed PEG, and depletion stabilization are all in effect in this system.[90] Since the surface of the AuNPs has only weakly adsorbed PEG, it remains accessible. Thiolated DNA can then be added to functionalize AuNPs (Figure 7C). It is interesting that DNA-functionalized AuNPs are then easily aggregated in concentrated PEG solution.[91] Other than PEG, other polymers such as polyvinylpyrrolidone can also achieve a high stabilization effect.[92]

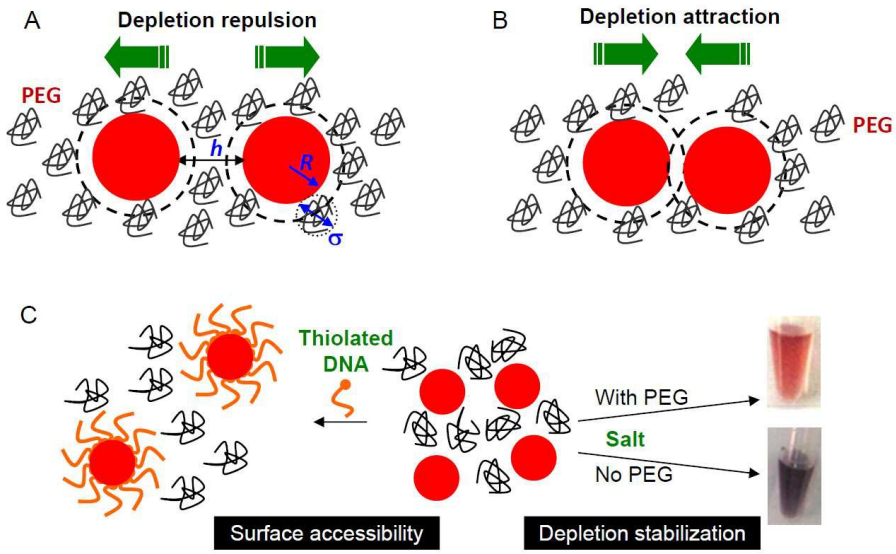

Figure 7. Schemes showing (A) depletion stabilization and (B) depletion attraction of AuNPs in a PEG solution. (C) AuNPs are highly stable against salt in PEG solution, while its surface accessibility allows

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DNA attachment. Figures reproduced from ref. [89,90] with permission. Copyright 2012 and 2013 American Chemical Society.

Using modified DNA.

Between the thiol group and the sequence intended for hybridization, a spacer is always inserted to relieve the steric effect near the AuNP surface. The use of polynucleotide spacers has been systematically studied by Mirkin and co-workers.[38,69] For example, a poly-T spacer allows a much higher DNA density on AuNPs and thus better stability against salt.[38] A poly-A spacer yielded a lower DNA density since adenine binds AuNPs more strongly and it may compete with the thiol, and the size of adenine is also larger than thymine. In our low pH method, we illustrated the importance of the poly-A spacer due to protonation and parallel duplex formation.[80,81]

In addition to using polynucleotide spacers, polymer spacers can also be used to overcome the electrostatic repulsion. Gill and co-workers used a cationic polyspermine-terminated DNA (Zip Nucleic Acid, ZNA, Figure 8A).[93] It was used to functionalize AuNPs of large sizes (40 nm and 80 nm). In a typical experiment, the ZNA was incubated with AuNPs for 5 min followed by the addition of NaCl up to 300 mM. Although this DNA did not contain a thiol, the obtained DNA-AuNP conjugates were still stable at such a high salt concentration. A recent report by Lou and co-workers used a PEG-modified DNA (Figure 8B).[94] Thiolated DNA with a PEG spacer has been proven to afford a high loading density on AuNPs of different sizes compared to DNA with A_{10} or T_{10} by Mirkin using the "salt-aging" method.[69] In their work, Lou et al found that NaCl can actually be added up to 1 M directly without the salt aging procedure. Since PEG is charge neutral and the thiol quickly reacts with the AuNP surface, the reaction kinetics was significantly enhanced due to the PEG spacer at physiological pH. We also tried a phosphorothioate (PS)-modified poly-A DNA to achieve enhanced conjugation by removing the

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thiol (Figure 8C). The PS-modified DNA allowed tighter binding compared to a normal nonthiolated poly-A DNA, but still the affinity was weaker than thiolated DNA (Figure 8D).[95]

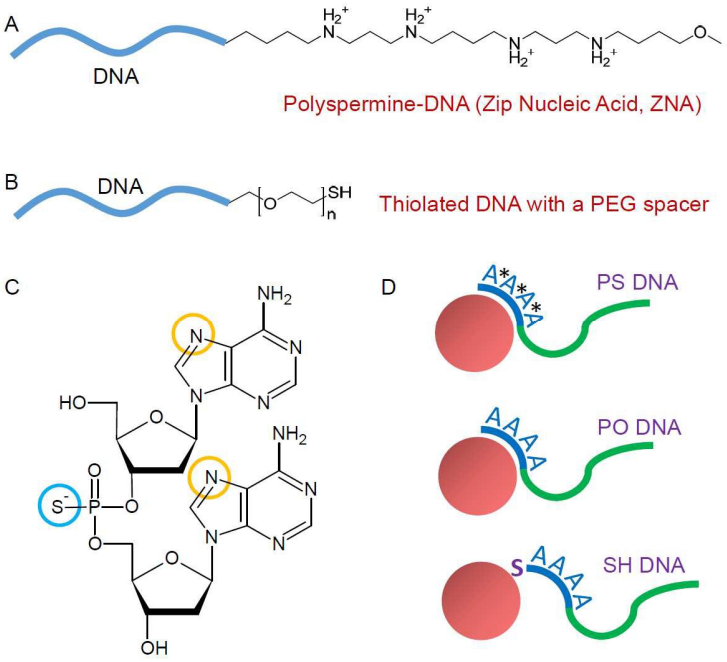

Figure 8. Using modified DNA for attaching to AuNPs: (A) modified with Polyspermine and the DNA is non-thiolated; (B) modified with a PEG spacer; and (C) modified with a PS DNA. (D) A scheme showing the adsorption of PS-modified DNA (denoted by the asterisks), normal phosphodiester DNA (PO) and thiolated DNA on AuNPs. Panels (C and D) are adapted from ref. [95] with permission. Copyright 2014 American Chemical Society.

Summary

In summary, we have reviewed various methods for attaching thiolated DNA to AuNPs. While thiol attachment to AuNPs is a very favorable thermodynamic reaction, the highly negatively charged and bulky DNA makes this process kinetically challenging, especially for the colloidal stability of AuNPs needs to be maintained in the whole conjugation process. In general, nanoparticles can be stabilized by

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charge, steric, and depletion mechanisms. The methods reviewed here have indeed covered all these mechanisms. While this conjugation reaction appears to be a technical problem, it still has quite profound implications in fundamental colloidal and surface science. The interplay between electrostatic repulsion, base adsorption and thiol adsorption, and the van der Waals force between AuNPs is very interesting.

The traditional salt-aging method is robust, but very slow. The low pH method does not work for all the sequences and it prefers those with a poly-A spacer. Introducing a covalent polymer spacer makes the synthesis even more expensive. With surfactants, the conjugation can be faster and more robust, but the surfactants need to be fully removed. The depletion stabilization method requires a high concentration of polymers and may take a long time for purification. In the ideal case, we want to have quantitative DNA adsorption (to avoid reagent waste) and fast reaction for highly functional conjugates. Therefore, we believe that there is still room for further improvement.

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