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Copper-free click chemistry as an emerging tool for the programmed ligation of DNA-functionalised gold nanoparticles†

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We demonstrate a new method to program the ligation of single stranded DNA-modified gold nanoparticles using copper-free click chemistry. Gold nanoparticles functionalized with a discrete number of 3′-azide or 5′-alkyne modified oligonucleotides, can be brought together *via* a splint strand and covalently 'clicked', in a simple one-pot reaction. This new approach to the assembly of gold nanoparticles is inherently advantageous in comparison to the traditional enzymatic ligation. The chemical ligation is specific and takes place at room temperature by simply mixing the particles without the need for special enzymatic conditions. The yield of 'clicked' nanoparticles can be as high as 92%. The ease of the copper-free, 'click-ligation' method allows for its universal applicability and opens up new avenues in programmed nanoparticle organization.

In 1996, pioneering work by the groups of Alivisatos[1] and Mirkin[2] showed that individual gold nanoparticles (AuNPs) coated with one or more oligonucleotides can assemble into complex structures upon addition of a complementary single-stranded DNA (ssDNA). Since then, it has become evident that DNA is an excellent scaffold for the evolution of inorganic nanoparticles into complex functional materials.[3] Not surprisingly, DNA–gold nanostructures have become increasingly important units for applications in sensing and nanomedicine,[4] metamaterials,[5] nano-optics and nanoelectronics.[6]

In order to enrich the library of functional materials for specialized applications, the employment of new 'tools' to manipulate DNA–gold nanostructures is imperative, and of great interest to the scientific community. The utilization of a simple protocol to covalently bind two or more DNA coated particles, in an organized manner, yields products with higher stability towards increased temperatures or low salt concentrations than the regular DNA hybridization process. Moreover, the coupling of nanoparticles *via* a continuous DNA strand enables one to variably change the inter-particle distances, which is of high importance for several applications such as in sensing and imaging.[4] Among various strategies to covalently link nanoparticles, the use of 'biomolecular tools' has emerged as a unique approach to prepare gold nanostructures with a high level of programmability and complexity.[7] In those studies, restriction and ligation enzymes have been employed for the selective manipulation of DNA sites on gold nanoparticles. The utilization of these biomolecules has inherent advantages deriving from their specificity to cleave or ligate only the desired DNA sequences. This enables multistep nanostructure synthesis. However, these enzymes are only functional at specific temperatures and in a narrow range of pH and ionic strength conditions. Additionally, they can be easily denatured or interact with the surface of particles in a non-specific manner. Moreover, scale-up of enzymatic reactions brings further challenges. These limitations make their general applicability difficult.

In recent years, copper(I)-catalysed click chemistry has become an established method for the facile, fast and versatile linking of molecules, without the need for specific reaction conditions.[8] Due to its simplicity, it has been employed in many research areas such as DNA nanotechnology[9] and nanoparticle ligand modifications.[10] However, one of the downfalls of this method is the requirement of a copper(I) complex to catalyse the click reaction by bringing together the reactants at an intermediate structure.[8] Often this process is not straightforward, especially in aqueous solutions where copper(I) can be easily converted to the inactive copper(II) complex.

Here we show for the first time, the programmed ligation of DNA–gold nanoparticles using copper-free click chemistry. Unlike the use of enzymes, the utilization of this simple reaction allows the formation of ligated gold nanostructures

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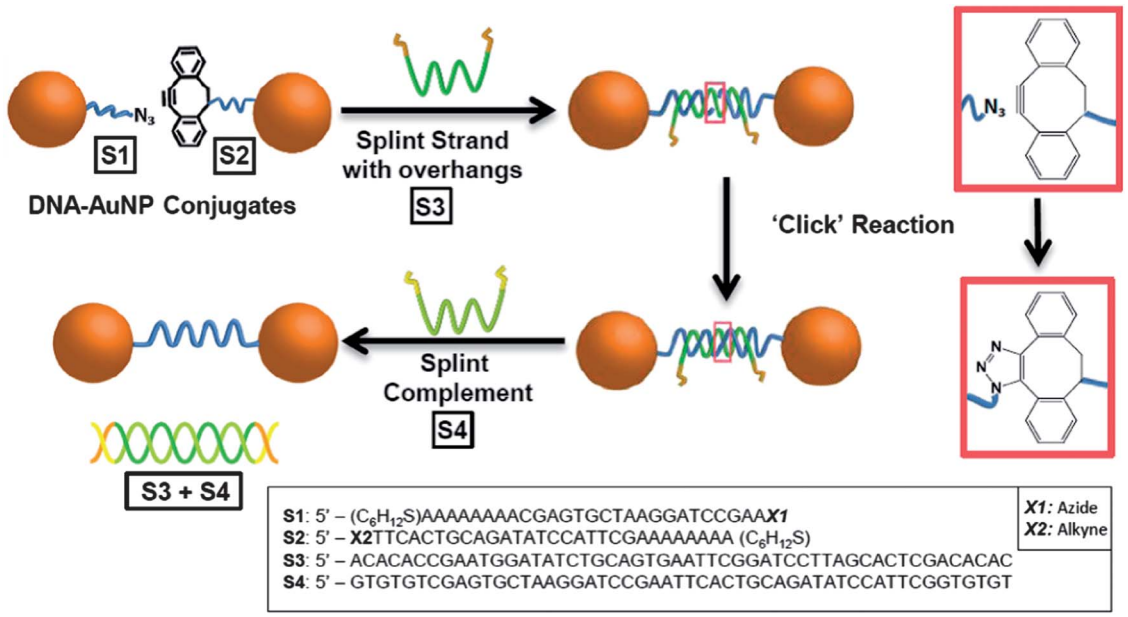

Scheme 1 Illustration of dimer formation using the method of DNA-click chemistry. 3′-azide (**S1**) and 5′-alkyne (**S2**) DNA–AuNP conjugates are brought into close proximity through a templating splint strand (**S3**) with non-complementary overhangs (orange). 'Clicking' occurs immediately after hybridisation. Addition of a single DNA strand (**S4**), fully complementary to **S3**, results in the removal of **S3** through competitive hybridisation, leaving a nanoparticle dimer system connected *via* a single continuous DNA strand.

without the need for specific temperature, pH or ionic strength conditions. It is a one-pot reaction, which reaches completion within five minutes at ambient temperature, and the yield of 'clicking' is extremely high (up to 92%). Employing copper free click chemistry to link DNA coated nanoparticles unravels an era of new capabilities in nanoparticle organization.

To demonstrate the effectiveness of copper-free click chemistry we programmed the formation of the simplest nanostructure, a dimer of nanoparticles. Scheme 1 shows an illustration of the experimental route. Initially, two batches of 5 nm gold nanoparticles were functionalized with two different types of oligonucleotides. Each type of oligonucleotide had a thiol modification (to link to the gold nanoparticle *via* a sulfur–Au bond) and either an azide (**S1**) or alkyne (**S2**) termination for the ligation. Using a strained cyclooctyne as the reactive alkyne allows for clicking *via* a ring-strain promoted alkyne-azide [3 + 2] cycloaddition.[11,12] Particles containing only one DNA strand were isolated using gel electrophoresis as previously described.[13] For dimer formation, equimolar amounts of mono-conjugates were hybridized with a DNA splint strand (**S3**). To maximize the yield of hybridization, the process was repeated by heating for 5 min at 65 °C and gradually cooling the sample to room temperature. Essentially, the **S3** strand catalyses the reaction; once hybridization happens, the clicking is spontaneous. The selectivity of the DNA splint strand allows clicking of only the azide and alkyne groups that are brought into close proximity through hybridization. This permits a multi-step synthesis – analogous to the uses of ligation and restriction enzymes, but without the need for specific enzymatic conditions.

Nanoparticle dimers were then purified by gel electrophoresis and extracted from the agarose *via* diffusion in tris-borate buffer, overnight. Having a DNA strand with overhangs, one is able to perform competitive hybridization with another DNA strand. In our experiments the **S3** splint strand was designed to be complementary to **S4** as shown in Scheme 1. Thus, treatment with an excess of **S4** allowed the isolation of dimers connected with a single stranded DNA.

Several techniques can be employed to show that the click reaction took place. The most popular is to use gel electrophoresis where the particles are separated according to their charge and volume. Fig. 1 shows the gel for our experiments. Lanes 1, 2, and 4 show control experiments, while lanes 3 and 5

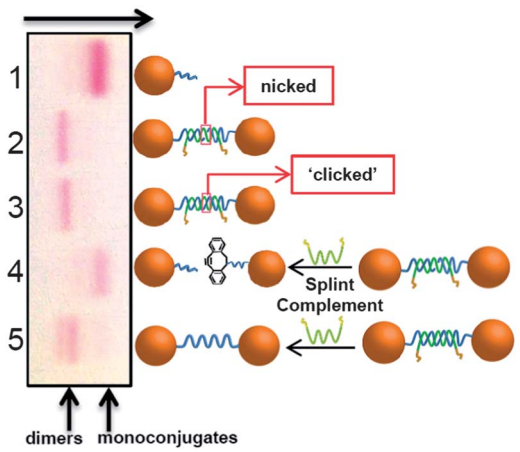

Fig. 1 Agarose gel electrophoresis demonstrates the success of the 'click' reaction. Lane 1: mono-conjugate used as reference; Lane 2: dimers unable to click, dimerized *via* splint; Lane 3: clickable dimers with splint; Lane 4: "nicked" dimers treated with splint complement (**S4**); Lane 5: clicked dimers treated with splint complement (**S4**).

show the clicked products. As the most representative control experiment the **S1** strand was synthesized without the azide group so that particles modified with this strand could not click to alkyne modified particles (see ESI†). These dimers have similar mobility in the gel in comparison to dimers with the clicked DNA (see lanes 2 and 3). However, after competitive hybridization with **S4**, the differences in mobility between ligated and non-ligated particles are clear (see lanes 4 and 5). The non-ligated particles, de-hybridize and run as monomers while the ligated ones remain as dimers presenting minor changes in mobility (possibly due to the extra flexibility allowed by the non-rigid single strand).

It is evident from the intensity of the bands in the gel that the yield of the clicked product is extremely high. Using the software ImageJ to compare optical densities for mono-conjugates and dimers, we were able to estimate that the reaction was completed up to 92%. This is significantly higher than the yields reported for the ligation of particles when enzymes were used (ranging from 50–72%).[7]

Transmission electron microscopy (TEM), also confirms our observations. Fig. 2A shows a representative TEM image of clicked nanoparticle dimers connected *via* a single DNA strand. We note the variation of inter-particle distances, which possibly derive from the flexibility of the ssDNA. However, as expected, the maximum distance between two particles does not exceed the maximum length of the ligated DNA strand (19.7 nm).

To test the versatility of our 'clicking' method, we applied it to different sized particles. For these experiments, we synthesised 13 nm AuNPs and functionalized them with DNA strands of **S1** or **S2** following previously established protocols.[7e] In this case, the experiment was slightly different. When 13 nm particles are linked to a short ssDNA, the separation in the gels between mono- di- or tri-oligonucleotide functionalized particles is poor. This happens due to only a small variation in the overall charge and volume between the different types of particles. Thus, in these experiments an alternative strategy was followed to make programmed assemblies. Dimers or even trimers of particles were formed by carefully controlling the ratio of **S3** (splint strand) to the oligonucleotide coated nanoparticles (see ESI†). This approach has significant advantages associated with the simplicity of the experimental steps (no need for gel separation of particles with discrete number of DNA strands) but it is more challenging regarding the optimization of the experimental conditions (*i.e.* appropriate molar ratios between oligonucleotide coated particles and splint strand are needed to program the formation of nanoparticle dimers or trimers).

After hybridization and subsequent clicking, **S4** was again used to remove the splint strand *via* competitive hybridization. Fig. 2B and C show representative TEM images of dimers and trimers of 13 nm particles connected *via* a single strand of clicked DNA.

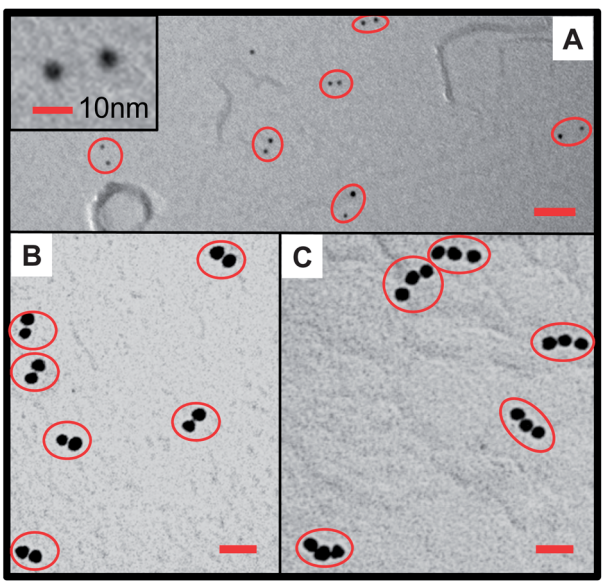

Fig. 2 TEM images of gold nanostructures derived from the clicking reaction, connected *via* a continuous single strand of ssDNA (A) 5 nm AuNP dimers, (B) 13 nm AuNP dimers and (C) 13 nm AuNP trimers. Scale bars for A, B, and C are 40 nm.

Conclusions

Over the past 15 years, nanotechnology has seen the development of DNA–AuNP systems driven by demands for implementation in biosensing, metamaterials and beyond. The introduction of copper-free click chemistry as a tool to organize nanocrystals in sophisticated structures represents a critical advancement of existing methods, in terms of processing speed, simplicity and efficiency. Furthermore it presents an easy way for multistep synthesis of nanostructures. We anticipate that the demonstrated approach for nanoparticle programming will enable the fabrication of nanostructures with a high degree of complexity for a variety of applications. Unlike enzymatic ligation, the methodology is applicable to chemically modified synthetic nucleic acids and analogues (*e.g.* PNA, LNA), where the use of enzymatic ligation is not possible.

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