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Bridging the gap: thymine segments to create single-stranded versions of DNA₂-[Ag₁₆Cl₂]⁸⁺

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Significant effort has been invested into unraveling the structure–property relationship of DNA–AgNCs using relatively short DNA sequences. Due to the limited sequence length, two or more strands are often required to stabilize a DNA–AgNC. Therefore, functionalization inherently introduces multiple reactive sites, hindering the implementation of single-site linking strategies. Here, we exploit the concept of using a thymine linking segment to connect two small DNA strands to develop a single-stranded version of DNA₂-[Ag₁₆Cl₂]⁸⁺. Our results demonstrate that these redesigned constructs preserve the core AgNC structure and photophysical properties while enabling future single-site functionalization. Furthermore, this approach allows for experimental verification that the DNA linking segments do not interfere with AgNC formation.

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Introduction

DNA-stabilized silver nanoclusters (DNA–AgNCs) are a unique class of fluorescent emitters that were first reported by Petty *et al.* in 2004.¹ These nanoclusters consist of a limited number of silver atoms and cations (typically 2–30), coordinated and stabilized with one or more single-stranded DNA oligomers.² DNA–AgNCs exhibit tunable emission spanning the visible to near-infrared (NIR) spectral range and are often characterized by high fluorescence quantum yields, large Stokes shifts, and good chemical and photostability.^{3–6} Due to their favourable optical properties, DNA–AgNCs are promising candidates for fluorescence imaging applications.^{7–9} Despite this potential, limited work has been done on developing and testing conjugation strategies of DNA–AgNCs for labelling applications.^{8,10,11} Such functionalization is essential for enabling specific target labelling and thereby unlocking the full potential of DNA–AgNCs as fluorophores. A key challenge lies in the possibility that introducing functional groups may interfere with the DNA–silver core interactions, potentially altering the photophysical properties or compromising the structural stability of the nanoclusters.

The current library of DNA sequences used for colour-specific stabilization of AgNCs consists mainly of strands that are about ten nucleobases long.^{12,13} As a result, most AgNCs are stabilized with two DNA strands.^{12,14–16} Although single, longer DNA strands can also stabilize AgNCs,¹⁷ using two shorter strands offers advantages, such as a more confined screening space for machine learning algorithms¹⁸ and the ability to introduce structural symmetry. A prominent example is the well-studied DNA₂-[Ag₁₆Cl₂]⁸⁺ nanocluster, which has become one of the best understood DNA–AgNC systems to date.^{19–21}

Rück *et al.* demonstrated the site-specific conjugation of the purified DNA₂-[Ag₁₆Cl₂]⁸⁺ nanocluster to three different peptides and a small protein using strain-promoted azide–alkyne cycloaddition (SPAAC), a copper-free click reaction eliminating the need for a copper catalyst.⁸ This approach enabled efficient and stable linkage of biomolecules to DNA₂-[Ag₁₆Cl₂]⁸⁺ without compromising their photophysical properties. In bioimaging experiments using Chinese hamster ovary (CHO) cells, specific labeling of human insulin receptors at the cell membrane was achieved.⁸ Notably, the nanoclusters maintained their spectral characteristics, highlighting their suitability for bioimaging applications. However, using two DNA strands inherently introduces two conjugation sites, which can cause potential cross-linking of binding sites and hence a non-linear fluorescence response. Thus, a single-site labeling strategy with one functional group to one binding site is more desirable.

Stabilizing [Ag₁₆Cl₂]⁸⁺ with a single DNA strand is challenging, and to the best of our knowledge, this has not yet been

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achieved for red- or NIR-emitting AgNCs. We note, however, that a few examples of smaller green emissive AgNCs have been reported in the literature.^{22,23}

In this work, we address this challenge by designing a single-stranded version of the DNA₂-[Ag₁₆Cl₂]⁸⁺ system, created by connecting the two original DNA sequences with thymine linkers of four, five and six nucleobases. These linker lengths were rationally chosen based on the available crystal structure.¹⁹ As a result, we obtained a single oligonucleotide that preserves the nanocluster's structural integrity and photophysical properties. Such single-stranded architecture enables controlled, single-site functionalization, enhances labeling specificity, and supports the development of targeted fluorescent probes for bioimaging.⁸

Two crystal structures show that the Ag₁₆ cluster core remains structurally consistent in these redesigned single-stranded variants. Together with mass spectroscopy analyses, these findings experimentally confirm that the small thymine bridge is suitable as a linking segment. This approach could also be used to screen other four- to six nucleotide segments to check if they do not interfere with AgNC formation.

Results and discussion

The original DNA₂-[Ag₁₆Cl₂]⁸⁺ structure is stabilized using two 5'-CACCTAGCGA-3' oligomers.¹⁹ Based on the crystal structure, we can see that the 3'-end of oligomer 1 and the 5'-end of oligomer 2 are relatively close. In order to connect these two ends, we used three different spacer lengths consisting of four, five and six thymines, which have been suggested in the literature to have the least affinity to the silver cations of the natural nucleobases.²⁴ These single-stranded sequences will be further referred to as 2xDNA(T₄), 2xDNA(T₅), and 2xDNA(T₆).

Synthesis of DNA-AgNCs using 24–26 nucleotide single-stranded sequences was performed as previously reported.¹⁹ Briefly, the oligomer strands were mixed with AgNO₃ in 10 mM ammonium acetate (NH₄OAc), and a freshly prepared NaBH₄ solution was added after an incubation time of 15 min to promote cluster formation. The final ratio between the components was [DNA]:[AgNO₃]:[NaBH₄] = 25 μM:187.5 μM:93.75 μM. After 7 days at 4 °C, the absorption spectra of the unpurified sample were recorded to verify the formation of the cluster. Fig. S1A shows that all three samples form the desired [Ag₁₆Cl₂]⁸⁺ cluster; however, the best yield is achieved with the T₆ spacer length.

Based on previous observations, the addition of sodium chloride (NaCl) can improve the yield of [Ag₁₆Cl₂]⁸⁺ cluster formation.^{21,25} Therefore, NaCl tests were carried out for the clusters with T₆ spacers. Different concentrations of NaCl (10, 30 and 50 mM) were added during the synthesis procedure. The best results were obtained with a NaCl concentration of 30 mM, see Fig. S1B. The addition of 50 mM NaCl did not result in a significantly higher yield than with 30 mM. Hence, a mixture of 10 mM NH₄OAc with 30 mM NaCl was used for the synthesis. After a 7-day reaction period, the mixture was purified by high-performance liquid chromatography (HPLC),

yielding successful separation for all three cases. Details on the HPLC purification and the corresponding chromatograms can be found in the SI (Fig. S2–S4).

The spectroscopic characterization of the purified 2xDNA(T₄)-[Ag₁₆Cl₂]⁸⁺, 2xDNA(T₅)-[Ag₁₆Cl₂]⁸⁺ and 2xDNA(T₆)-[Ag₁₆Cl₂]⁸⁺ nanoclusters shows that the photophysical properties remain nearly unaltered by the different spacer lengths, see Fig. 1 and Table 1. The absorption maximum remains at around 524 nm. However, compared to the original DNA₂-[Ag₁₆Cl₂]⁸⁺ nanocluster, the emission maxima are slightly red-shifted, now centred at around 750 nm. The average fluorescence decay time and fluorescence quantum yield are also very similar and seem not to be affected by the spacers (see Table 1). Time-resolved fluorescence anisotropy measurements were used to determine the hydrodynamic volume. Compared to DNA₂-[Ag₁₆Cl₂]⁸⁺, the hydrodynamic volume increases with increasing spacer length. While some increase is expected, the significant increase might indicate substantial drag of the thymine segment in solution.

Additionally, the single-stranded silver nanocluster versions were analysed by electrospray ionization mass spectrometry (ESI-MS) to verify whether the AgNCs are compositionally con-

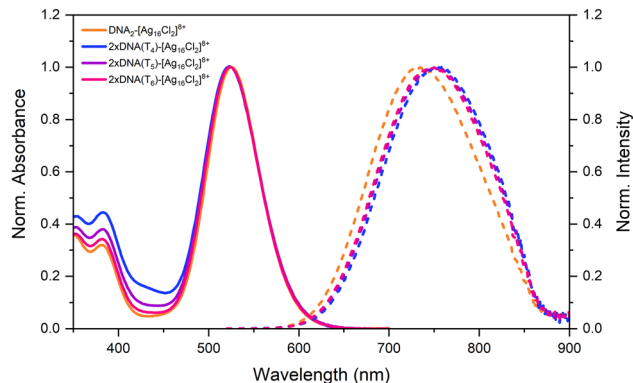


Fig. 1 Normalized absorption and emission spectra of DNA₂-[Ag₁₆Cl₂]⁸⁺, 2xDNA(T₄)-[Ag₁₆Cl₂]⁸⁺, 2xDNA(T₅)-[Ag₁₆Cl₂]⁸⁺ and 2xDNA(T₆)-[Ag₁₆Cl₂]⁸⁺ in 10 mM NH₄OAc at room temperature.

Table 1 Photophysical properties of the original DNA₂-[Ag₁₆Cl₂]⁸⁺ and single-stranded 2xDNA(T₄)-[Ag₁₆Cl₂]⁸⁺, 2xDNA(T₅)-[Ag₁₆Cl₂]⁸⁺ and 2xDNA(T₆)-[Ag₁₆Cl₂]⁸⁺ versions.

	Abs (nm)	Em (nm)	$\langle\tau\rangle$ (ns)	QY	ϕ_r (ns)	V_h (nm ³)
DNA ₂	525	736	3.26	0.26	2.19	10.14
2xDNA(T ₄)	524	753	3.31	0.21 ^a	3.09	14.31
2xDNA(T ₅)	524	750	3.46	0.23 ^a	3.45	15.97
2xDNA(T ₆)	523	747	3.44	0.24 ^a	3.67	16.99

DNA₂ sequence: 5'-CACCTAGCGA-3'. 2xDNA(T₄) sequence: 5'-CACCTAGCGATTTTCACCTAGCGA-3'. 2xDNA(T₅) sequence: 5'-CACCTAGCGATTTTTCACCTAGCGA-3'. 2xDNA(T₆) sequence: 5'-CACCTAGCGATTTTTCACCTAGCGA-3'. ^a Values were determined from a single point measurement (see the SI for details). For 2xDNA(T₆)-[Ag₁₆Cl₂]⁸⁺ a multi-point measurement as also available in the SI.



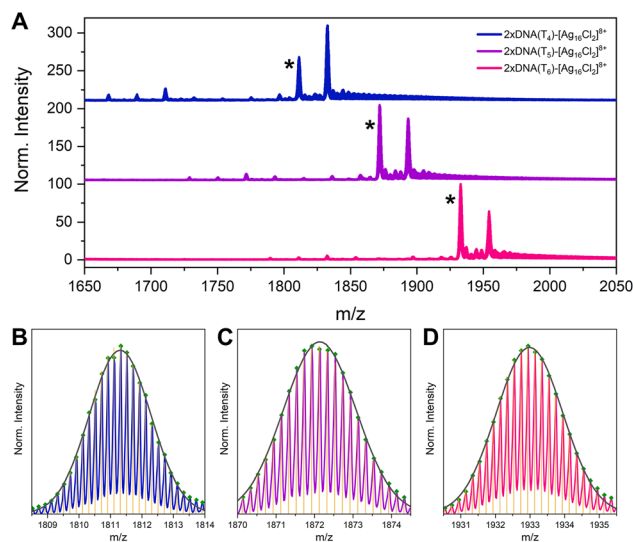


Fig. 2 (A) Mass spectra (zoom-in view) of the $z = 5^-$ regions of 2xDNA(T₄)-[Ag₁₆Cl₂]⁸⁺, 2xDNA(T₅)-[Ag₁₆Cl₂]⁸⁺ and 2xDNA(T₆)-[Ag₁₆Cl₂]⁸⁺ in 10 mM NH₄OAc at room temperature displayed with offsets. (B–D) Theoretical isotopic distribution fit (yellow) along with experimental data of 2xDNA(T₄)-[Ag₁₆Cl₂]⁸⁺ (blue), 2xDNA(T₅)-[Ag₁₆Cl₂]⁸⁺ (purple) and 2xDNA(T₆)-[Ag₁₆Cl₂]⁸⁺ (pink) for the highlighted peaks in (A). The dark gray line represents the Gaussian fit, while the green dots indicate the peaks used for the fit. The corresponding mean values are $\mu = 1811.3$, 1872.1, and 1933.0 for (B), (C), and (D), respectively.

sistent with the original DNA₂-[Ag₁₆Cl₂]⁸⁺ nanocluster. The mass spectra of all three modifications are reported in Fig. 2 and S5. The experimental mass data, highlighted in Fig. 2A, are aligned with the theoretical isotopic distribution of a compound comprising one DNA strand, 2 chloride atoms, and 16 silver atoms with an overall charge of +8. These findings are in line with the spectroscopic characterization that all three modifications form similar emitters. The second dominant molecular ion peaks correspond to a similar compound with one additional silver cation (see also Fig. S6). The presence of additional silver cations has been observed previously, and they are presumed not to be part of the AgNC core.^{8,19} These cations most likely coordinate with the 3'-terminal adenine, as observed in the crystal structure of the original DNA₂-[Ag₁₆Cl₂]⁸⁺ nanocluster.¹⁹ Interestingly, the mass spectroscopy data also suggest that no silver cations are appreciably bound to the thymine linker, or at least not strongly enough to be seen in the mass spectra.

The spectroscopic and mass spectrometry data indicated that the new single-stranded 2xDNA(T₄)-[Ag₁₆Cl₂]⁸⁺, 2xDNA(T₅)-[Ag₁₆Cl₂]⁸⁺ and 2xDNA(T₆)-[Ag₁₆Cl₂]⁸⁺ nanoclusters are similar to the original DNA₂-[Ag₁₆Cl₂]⁸⁺ nanocluster. In order to facilitate a structural comparison and gain insight into the thymine region, we crystallized all three single-stranded variants. The 2xDNA(T₄)-[Ag₁₆Cl₂]⁸⁺, 2xDNA(T₅)-[Ag₁₆Cl₂]⁸⁺ and 2xDNA(T₆)-[Ag₁₆Cl₂]⁸⁺ clusters all yielded pinkish crystals (see Fig. 3A–C and Fig. S10) using the hanging drop vapour method (see also the SI). All crystals displayed an emission maximum at around 740 nm (Fig. S11) and nanosecond-lived fluorescence decay

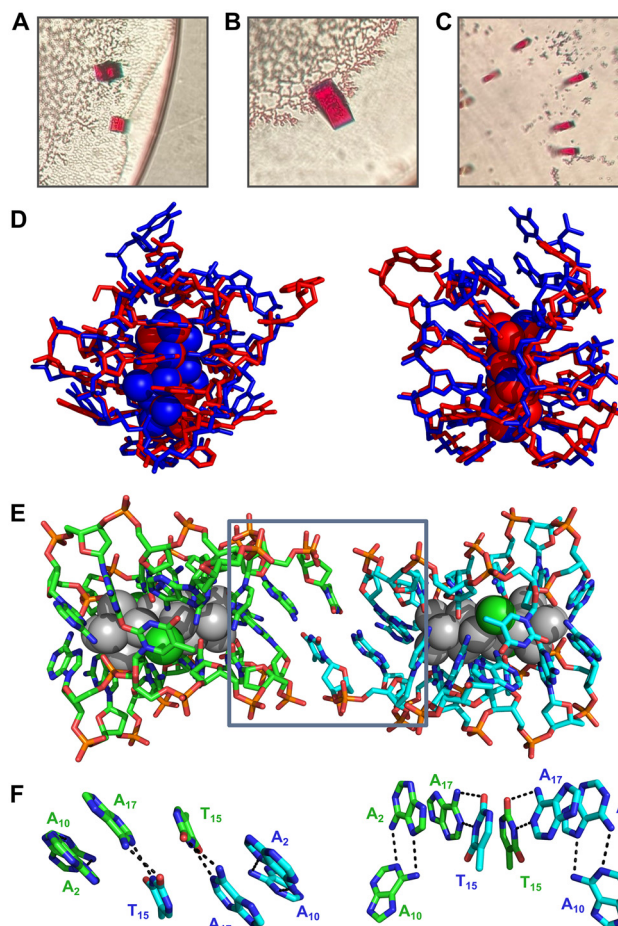


Fig. 3 Images of crystals of (A) 2xDNA(T₄)-[Ag₁₆Cl₂]⁸⁺, (B) 2xDNA(T₅)-[Ag₁₆Cl₂]⁸⁺ and (C) 2xDNA(T₆)-[Ag₁₆Cl₂]⁸⁺. (D) Crystal structure overlays of 2xDNA(T₅)-[Ag₁₆Cl₂]⁸⁺ (blue, PDB: 9XV9) and DNA₂-[Ag₁₆Cl₂]⁸⁺ units (red, PDB: 6JR4). (E and F) Detailed views of the crystal packing interactions in the 2xDNA(T₅)-[Ag₁₆Cl₂]⁸⁺ structure.

times (Fig. S12). We managed to solve the structures of two of the three variants (2xDNA(T₅)-[Ag₁₆Cl₂]⁸⁺ and 2xDNA(T₆)-[Ag₁₆Cl₂]⁸⁺), which are deposited at the PDB website under accession codes 9XV9 and 9XVA, respectively.

For 2xDNA(T₅)-[Ag₁₆Cl₂]⁸⁺, only T₁₅ (the fifth T of the thymine segment) is visible in the structure due to a Watson–Crick interaction with an A₁₇ of a neighbouring 2xDNA(T₅)-[Ag₁₆Cl₂]⁸⁺ structure in the unit cell. For the rest, the positions of the Ag atoms and most of the DNA sequence overlap very well with the original DNA₂-[Ag₁₆Cl₂]⁸⁺ nanocluster (see Fig. 3D). Fig. 3E and F show the intricate interactions between two 2xDNA(T₅)-[Ag₁₆Cl₂]⁸⁺ units in the crystal. Unfortunately, the T₁₁ to T₁₄ nucleobases are too disordered to be resolved. This is reasonable given that no complementary nucleobases are available to form stable interactions with the thymine section, apart from the T₁₅–A₁₇ interaction. For 2xDNA(T₆)-[Ag₁₆Cl₂]⁸⁺, similar interactions were found (Fig. S13). Here, T₁₅ and T₁₆ could be resolved in the structure, but like in 2xDNA(T₅)-[Ag₁₆Cl₂]⁸⁺, the T₁₁ to T₁₄ nucleobases could not be resolved. Results from Swasey *et al.* show that thymine strands



do not have a great affinity to form silver mediated duplexes,²⁶ while silver cations can easily replace a hydrogen to form adenine and thymine silver-mediated Watson–Crick-like interactions.^{26,27} Silver-mediated thymine–thymine interactions have been previously reported by Kondo *et al.* but require the deprotonation of one of the thymines.^{28,29}

Conclusions

In this work, we successfully designed and characterized single-stranded DNA-oligomer versions of the well-established DNA₂-[Ag₁₆Cl₂]⁸⁺ nanocluster. By connecting the two native stabilizing strands through thymine linkers of defined lengths (T₄, T₅ and T₆), we created a single oligonucleotide that retains the structural and photophysical integrity of the original system with the additional benefit of removing redundant labeling sites. This architecture opens the door for precise, site-specific future conjugation strategies. Single crystal X-ray diffraction of the 2xDNA(T₅)-[Ag₁₆Cl₂]⁸⁺ and 2xDNA(T₆)-[Ag₁₆Cl₂]⁸⁺ variants yielded two crystal structures that closely resemble the original DNA₂-[Ag₁₆Cl₂]⁸⁺ nanocluster, demonstrating that the overall [Ag₁₆Cl₂]⁸⁺ core remains preserved. Although the engineered thymine segment was not fully resolved, the combination of mass spectrometry, single crystal x-ray diffraction based structure determination and spectroscopic measurements indicate that it does not interfere with the metal–DNA coordination environment or alter the core geometry.

Together, these results validate our design concept and highlight the feasibility of stabilizing the Ag₁₆ cluster with a single DNA strand, thereby providing a robust and structurally conserved scaffold for controlled, one-site functionalization in future bioimaging applications. Furthermore, the presented approach could also be used as a testbed for other DNA sequences of limited length to investigate whether or not they interfere with AgNC formation.

Conflicts of interest

There are no conflicts to declare.

Data availability

The data supporting this article have been included as part of the supplementary information (SI). Supplementary information is available. See DOI: <https://doi.org/10.1039/d5qi02482j>.

Crystallographic data for 2xDNA(T₅)-[Ag₁₆Cl₂]⁸⁺ and 2xDNA(T₆)-[Ag₁₆Cl₂]⁸⁺ have been deposited at the PDB under accession codes 9XV9 and 9XVA.^{30,31}

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