

**CHIMERIC DNA-TEMPLATED SILVER NANOLCLUSTERS AS
NEW FLUOROGENIC PROBES FOR BIOSENSING APPLICATIONS**

LEE SHI TING

THESIS SUBMITTED TO THE UNIVERSITY OF NOTTINGHAM
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

2019

ABSTRACT

Over a decade of research on DNA-templated silver nanoclusters (DNA-AgNCs), this material has now been recognised as an alternative to the conventional fluorescent probes (*i.e.* quantum dots and organic fluorophores). The DNA-AgNCs have been widely reported for biosensing application due to their design versatility and easy integration with functional DNA sequences. Notwithstanding, a good DNA-AgNCs design is crucial to generate a successful sensing result. Among all of the reported designs, chimeric DNA-AgNCs, *i.e.* placing AgNCs nucleation sequence and functional sequence in a single DNA strand, is relatively underexplored. This is because the chimeric DNA-AgNCs often exhibit fluorescence turn-off response whereby its accuracy could be affected by false results. However, the simplicity of the chimeric design deserves further investigation, considering it is more cost-effective and enables conclusive results on principles of detection.

In this thesis, the principles of chimeric DNA-AgNCs were applied on two test models of distinctive sizes, *i.e.* adenosine (small analyte) and telomerase (macromolecule). For both studies, an identical AgNCs nucleation sequence, termed Ct₉ was integrated with different functional sequences: (1) aptamer for specific recognition of adenosine; and (2) primer for telomerase binding. For adenosine study, the sensing performance of DNA-AgNCs was evaluated by systemically positioning the AgNCs nucleation sequence across the DNA template (*i.e.* 5'-end, 3'-end or in the middle of DNA template). Among the three formulations, only 5'-end design showed fluorescence enhancement upon binding to adenosine, which is attributable to the structural reformation of anti-parallel *G*-quadruplexes. In contrast, the 3'-end design

showed fluorescence quenching. The reason behind this quenching effect is not fully understood.

The study on detection of telomerase activity has indicated that parallel *G*-quadruplex conformation was able to induce a quenching effect. However, the microenvironment of complex buffer (*e.g.* TRAP buffer) is required to ensure a consistent turn-off signal, independent of the size of parallel *G*-quadruplex.

In summary, these studies have provided an insight on the principles of detection using DNA-AgNCs. The basic guidelines from the findings not only enable improvement on the formulation of chimeric DNA-AgNCs-derived aptasensors, but also expand their possible biosensing applications.

RESEARCH OUTCOMES

Publications

Lee, S.T., Rahman, R., Muthoosamy, K., Mohamed, N.A.H., Su, X.D., Tayyab, S., New, S.Y., 2019. Amplification-free and direct fluorometric detection of telomerase activity in cell lysate using chimeric DNA-templated silver nanoclusters. *Microchim. Acta*, 186, 81-90. (Impact factor: 5.705)
(As referred to Chapter 4)

Lee, S.T., Beaumont, D., Su, X.D., Muthoosamy, K., New, S.Y., 2018. Formulation of DNA chimera templates: Effects on emission behavior of silver nanoclusters and sensing. *Anal. Chim. Acta*, 1010, 62-68. (Impact factor: 4.513)
(As referred to Chapter 3)

New, S.Y., **Lee, S.T.**, Su, X.D., 2016. DNA-templated silver nanoclusters: structural correlation and fluorescence modulation. *Nanoscale*, 8, 17729-17746 (Impact factor: 7.367)
(As referred to Chapter 2)

Conferences

(Oral Presentation) 9th World Congress on Biosensors and Bioelectronics, Radisson Hotel, Japan, 28-29 August 2018

(Oral Presentation) UNMC LINK '17, Student-Led Interdisciplinary Research Conference, UNMC, 14 September 2017

(Oral Presentation) INFORMM's Second International Conference on Molecular Diagnostic and Biomarker Discovery, Hotel Equatorial Penang, 3-4 May 2017

(Poster Presentation) International Conference on Nanomedicine and Nanobiotechnology (ICONAN), University Pierre and Marie Curie, Paris, 28-30 September 2016

ACKNOWLEDGEMENT

First and foremost, I would like to express my deepest appreciation to my supervisor, Dr. New Siu Yee for her wise counsel, patience, and trusting in me throughout this PhD journey. Nonetheless, I am grateful to have Dr. Lim Kuan Hon and Dr. Su XiaoDi as my co-supervisors and getting advices from them throughout the years of research study.

Secondly, my sincere thanks to School of Pharmacy and MyBrain15 from Ministry of Higher Education to provide financial support on both tuition fee and living allowances, respectively. In addition, I would like to acknowledge the Fundamental Research Grant Scheme (FRGS) for the financial support on my research expenses.

I am grateful to the administration staffs (*i.e.* Radha and Fifi), lab technicians (*i.e.* Siak Chung, Siti and Asma) and my lab colleagues (*i.e.* Rayan and Sonia Wong) who have helped me to complete my experiments.

Last but not least, I would like to express my most humble thanks to my family members, especially my mother and my eldest sister for their tremendous supports on all my decisions made during this time.

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LIST OF ABBREVIATION

AgNCs	Silver nanoclusters
Ag ⁺	Silver ions
Ag ⁰	Silver atoms
AgNO ₃	Silver nitrate
A	Adenine
Apta	Aptamer
C	Cytosine
CD	Circular dichroism
DNA	Deoxyribonucleic acid
dsDNA	Double stranded deoxyribonucleic acid
ex	Excitation
em	Emission
G	Guanine
G-quadruplex	Guanine quadruplex
HCl	Hydrochloric acid
HOMO	Highest occupied molecular orbital
i-motif	intercalated motif
<i>i.e.</i>	id est
LOD	Limit of detection
LUMO	Lowest unoccupied molecular orbital
N	Nitrogen
N _{Ag}	Silver content
NaBH ₄	Sodium borohydride
NCB	NanoCluster Beacon
NC	Nucleation
NIR	Near infrared
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rTS	Reverse telomerase substrate
ssDNA	Single stranded deoxyribonucleic acid
TEM	Transmission electron microscope
TRAP	Telomerase Repeated Amplification Protocol
tsDNA	Triple stranded deoxyribonucleic acid
TS	Telomerase substrate
T	Thymine
WC	Watson-Crick
nm	nanometer
μM	Micromolar
nM	Nanomolar
mM	Millimolar
M	Molar
UV	Ultraviolet
w/v	Weight/volume
%	Percentage
–OH	Hydroxyl
λ	Wavelength
°C	degree Celsius

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Chapter 1. Introduction

Since the introduction of DNA-AgNCs by Petty *et al.* in 2004,¹ this fluorescent nanomaterial has become a vibrant field of research. Hitherto, studies related to DNA-AgNCs have rapidly grown with >3500 citations, assuredly implying their attractiveness in research communities (the number of citations was obtained using keywords “silver nanoclusters” and “DNA” when searching through Thomson Reuters (ISI) Web of Knowledge for the period of 2004-2018).

In the earlier stage, more studies were devoted to the preparation of DNA-AgNCs of different emission colours and with longer photostability, as well as their mechanism of formation and characterisation. Then, this has been further expanded to the employment of DNA-AgNCs in various application fields, such as bioimaging,^{2,3} biosensing,⁴⁻¹¹ nano-thermometer¹²⁻¹⁴ and antibacterial agents.¹⁵ Today, DNA-AgNCs have become an alternative fluorescent probe to the conventional quantum dots and organic fluorophores.¹⁶ This is mainly attributed to their design versatility, since the DNA template can be easily integrated with functional sequences, *e.g.* aptamers¹⁷ or complementary sequences.¹⁸ Hence, the DNA-AgNCs could serve dual roles as fluorescent probe and recognition element. Moreover, the DNA-AgNCs are low cost in production, small in size (*i.e.* 2 nm in diameter), highly photostable and have good biocompatibility.

This thesis will reveal the important design features of DNA templates used to prepare DNA-AgNCs and how they affect the performance of the nanoclusters in sensing application. The main focus of this study is chimeric DNA-AgNCs, which is a relatively underexplored design. In this context,

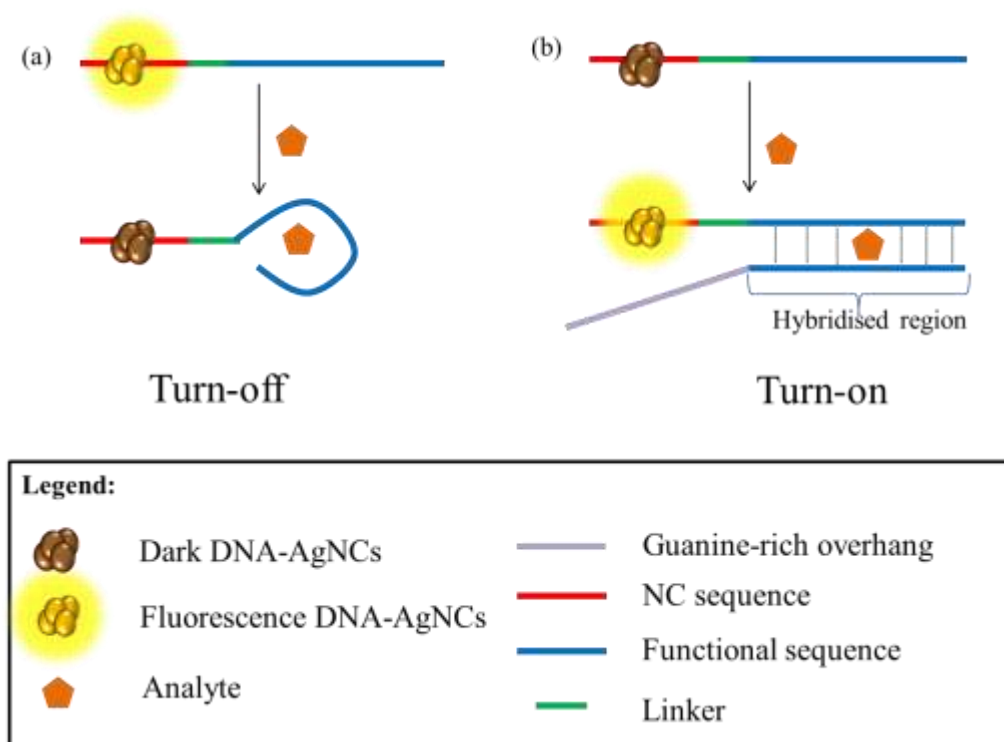
chimeric DNA refers to a single stranded DNA template (ssDNA) that consists of (1) an AgNCs nucleation sequence and (2) a functional DNA sequence that used to detect a target analyte. Currently, there is no known sensing mechanism associated to chimeric DNA-AgNCs, despite it is the simplest design of DNA-templated AgNCs. Hence, results outlined in this thesis are able to serve as basic guidelines when acquiring chimeric DNA-AgNCs as fluorescent probe for biosensing application.

1.1. Problem statement

Generally, the most commonly used designs of DNA-AgNCs are: (i) chimeric ssDNA and (ii) NanoCluster Beacon (NCB) (Scheme 1.1). By comparing the two models, NCB has been widely reported to detect various types of target analytes (*e.g.* nucleic acid,^{19,20} protein,^{21,22} enzyme activity,^{23,24} small biomolecule^{25,26} and heavy metal ions²⁵). The mechanism of detection in NCB design is well studied, *i.e.* target analyte will induce proximal location of AgNCs to guanine-rich overhang template, subsequently promote fluorescence enhancement through electron transfer from guanine bases to AgNCs.⁹ This design commonly employs a standard AgNCs nucleation sequence, *i.e.* C₃TTATC₄, because it has low signal-to-noise background and able to provide significant enhancement ratio.^{9,27}

On contrary, the chimeric design does not receive much attention, as reflected by its limited number of published reports. Generally, the cytosine-rich sequence listed in Table 2.2 (Chapter 2.6.1.1) are combined with other functional DNA sequence (*e.g.* aptamer¹⁷ or complementary DNA¹⁸) as chimeric ssDNA for biosensing application.^{17,18,28–32} Whilst its mechanism of detection remains unknown, the chimeric design often induces quenching

effect in the presence of target analytes.¹⁷ A turn-off effect is often undesirable due to possible false result. In order to generate turn-on signal, some studies have coupled the chimeric design with additional component,³¹ *e.g.* graphene¹⁸ or competitive quencher target analyte.³² Another possible reason for the unpopularity of chimeric DNA design is lack of standardisation of AgNCs nucleation sequence.^{17,18,31,32} For instance, despite there is a long list of sequences available in Table 2.2, only a few were employed as nucleation sequence of chimeric DNA, *e.g.* (C₃TTATC₄)^{17,28,30} and (C_n)^{29,33}. The possible combinations using other nucleation sequences are relatively unexplored. Interestingly, Huang *et al.*³⁴ and Liu *et al.*³⁵ have shown that through modulation of DNA bases in AgNCs nucleation sequence, a turn-on signal can be achieved. However, there is no continuance study on this, leading to inconclusive evidence to understand the underlying mechanism. Hence, more studies should be devoted on the chimeric design to investigate its principles of detection, so that a fundamental mean can be established to predict the sensing outcome (*i.e.* turn-on or -off signal). With this, not only the versatility of chimeric design enables one to construct a tailor-made biosensor by choosing nucleation sequence of desired emission colour and desired functional DNA, but its simplicity also makes the sensor more cost-effective.

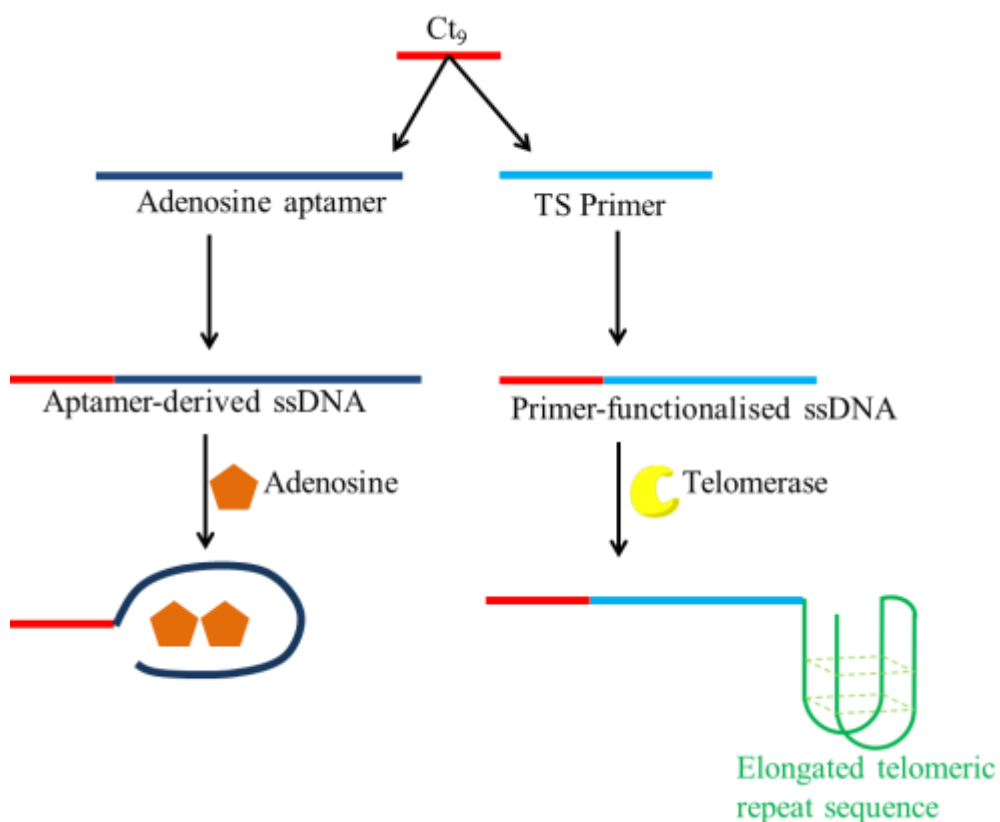


Scheme 1.1. Two main designs of DNA-AgNCs used in biosensing application, *i.e.* (a) chimeric-ssDNA that often results in turn-off effect and (b) NCB design that generates turn-on effect when a guanine-rich template is in proximity to AgNCs.

1.2. Project Design and Objectives

The research studies presented in this thesis are generally aim to provide a rationale associated to fluorescence changes of chimeric ssDNA-AgNCs in the presence of target analyte. In order to accomplish this, same AgNCs nucleation sequence, *i.e.* Ct₉, will be used throughout the sensing studies (Scheme 1.2). Two different test models, *i.e.* adenosine (small analytes) (Chapter 3) and telomerase activity (elongated target DNA) (Chapter 4) were chosen to study the applicability of the chimeric DNA-AgNCs on analytes of different types. For adenosine, target binding will induce aptamer template to undergo structural reformation into G-quadruplex, whilst the telomeric repeat sequence, *i.e.* (TTAGGG)_{*n*} elongated by telomerase has been widely known to form a G-quadruplex. Thus, the shared outcomes involving formation of G-quadruplex structures between the two different studies may enable meaningful

conclusion made on the principle of detection associated to chimeric DNA-AgNCs.



Scheme 1.2. Study designs presented in this thesis by using the same AgNCs nucleation sequence, Ct_9 as fluorescent probe to detect two target analyte of different sizes, *i.e.* adenosine and telomerase.

In summary, the specific objectives of this thesis are:

- i. To determine the principle of detection for chimeric ssDNA design.
- ii. To correlate the order of different *G*-quadruplexes confirmation with emission intensity
- iii. To determine the potential of chimeric ssDNA-AgNCs as generic nanobiosensor probe to different type of target analytes

Chapter 2. Literature review

2.1. Overview

This chapter will present a detail review on DNA-AgNCs, starting with their general aspects, *e.g.* size, electronic and energy levels. This is followed by two synthesis routes of DNA-AgNCs, the mechanism of formation, as well as their characterisation methods. Lastly, the chapter will highlight the influence of different DNA structural conformations on the emission properties of AgNCs.

2.2. The changing properties in metals: from bulk to nano

Metals of different size possess certain physical and chemical properties depending on their electronic situation and energy levels. In a bulk metal, free motion of electron in continuous energy band enables electrical conductivity and optical transmittance.³⁶ Comparatively, when the size of metal nanoparticles is smaller than the bulk electron mean path (*e.g.* 52 nm in the case of silver), the electron motion is more restricted and surface collision dominates the optical response. This results in surface plasmon resonance (SPR), as reflected by the intense colour of the material.^{37–39}

Metal nanoclusters are resulted from a further size reduction of metal nanoparticles (Figure 2.1). When the size of this nanomaterial approaches the Fermi wavelength of electrons (*i.e.* 2 nm in diameter), their energy structure further breaks down into discrete levels that is similar to those of molecules. Hence, it exhibits molecule-like properties, bridging the gap between single atom and plasmonic nanoparticles. Metal nanoclusters no longer possess SPR effect because the collective oscillation of electron is obstructed. Instead,

radiative decay is preferred owing to their sparser energy levels, rendering them with good fluorescence quantum yield (10–94 %).^{36–38,40}

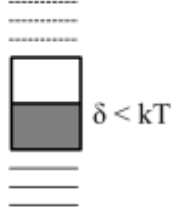
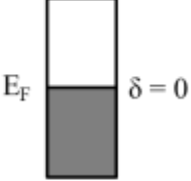
Metal atom (Single valence electron)	Metal cluster (Discrete energy levels)	Metal nanoparticle (Surface plasmon resonance)	Bulk metal (Free moving electrons)
 $\delta \gg kT$	 $\delta > kT$	 $\delta < kT$	 E_F $\delta = 0$
<0.1 nm	2 nm	<100 nm	>1 μm

Figure 2.1. Electronic situation and energy level with respect to different sizes of metal. Reproduced from ref ³⁶

2.3. DNA as biotemplate to silver nanoclusters

Despite metal nanoclusters have been studied over the past half century, they only found new potential applications after the introduction on the biocompatible synthesis route,¹ *i.e.* using DNA,^{41–43} protein,^{44–46} peptide^{47,48} or RNA⁴⁹ as capping agents. Since then, studies on this nanomaterial have rapidly grown and widely reported for various applications, such as catalytic reactions,^{50–52} biosensing,^{4–11} bioimaging,^{2,3} nano-thermometer^{12–14} and as antibacterial agents.¹⁵

DNA has gained the most popularity among aforementioned biotemplates as an encapsulating agent for AgNCs, mainly due to its tuneable emission properties range from violet to near-infrared (NIR) region.^{41,43,53–55} This can be commonly achieved by (i) modifying the experimental conditions (*e.g.* pH, temperature, buffer, salt, concentration of reactants, presence of metal ions or small molecule, *etc*)^{34,41,43,53,56–59} or (ii) varying DNA sequence, length

and/or design.^{60–63} The former method has less variability because the emission tuning is experimentally constrained. In comparison, the latter method enables better design versatility and material functionalisation. Perhaps due to this superiority, most studies focus on varying DNA templates and/or sequences for various applications. Further details on correlation between DNA structural changes and fluorescence modulation will be presented in Section 2.6.

2.4. Synthesis of DNA-AgNCs

In general, there are two methods to prepare DNA-AgNCs, namely direct preparation and cluster shuttle approach. In both cases, three reactants are essential, *i.e.* DNA template, silver salt (usually AgNO_3) and strong reductant (usually NaBH_4).

2.4.1 Direct Preparation

In a typical direct preparation approach, DNA-AgNCs are prepared in phosphate or acetate buffer, unless a specific pH is desired.^{1,64} This nanomaterial can be easily synthesised by mixing micromolar amount of the aforementioned reactants at an appropriate ratio. The formation of AgNCs templated by a linear ssDNA is illustrated in Figure 2.2. For a given ssDNA template, the silver ions (Ag^+):DNA ratio influences the population of fluorophores. The most commonly adopted ratio is 6:1 or more, but not too high as excess Ag^+ may favour the formation of plasmonic nanoparticles.^{64,65} An optimal ratio of NaBH_4 : Ag^+ is required as well.^{66–69} In most cases, an equal ratio of NaBH_4 and Ag^+ is used to generate the emissive AgNCs, despite one molecule of NaBH_4 is able to provide eight electrons for reduction.^{1,64,70–72} A lower molar ratio was found to shorten incubation times and generates AgNCs of lower emission intensity.^{67,73,74} It was plausibly due to the reduction of

positively charged AgNCs to non-fluorescent species or excess NaBH_4 acting as an electron donor that quenches AgNCs.⁶⁸ The lower concentration of NaBH_4 is especially adopted when using double-stranded DNA (dsDNA) as the template (Section 2.6.2, Table 2.5). In general, the incubation time after reduction step ranges from immediately right after addition⁷⁴ to two days,⁷⁵ likely due to the type of DNA template and affinity of Ag–DNA. Depending on the DNA templates, 50–100 mM of monovalent salt is mainly used in i-motif and G-quadruplex templates, whereas 1 mM divalent salt is widely employed in dsDNA templates.

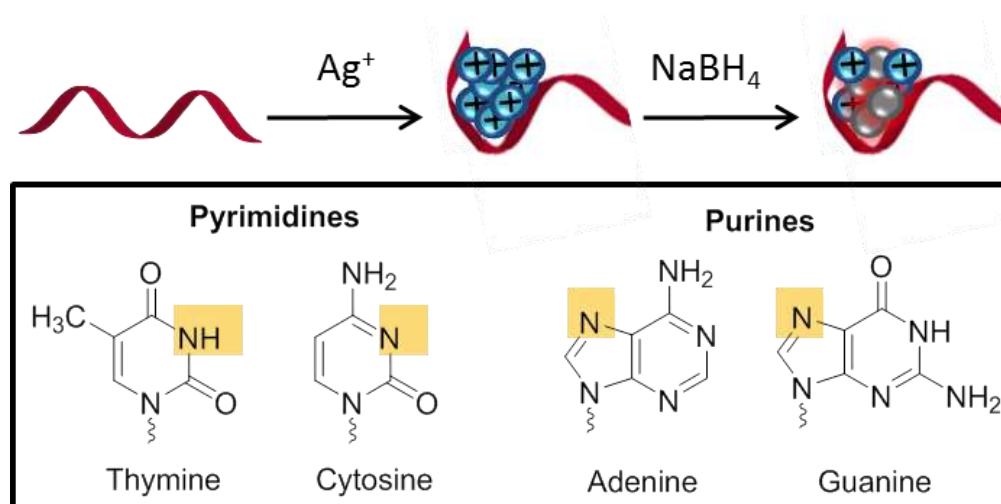


Figure 2.2. Formation of DNA-AgNCs with red strip, blue sphere and grey spheres representing the ssDNA template, Ag^+ and Ag^0 , respectively. The N3 of pyrimidines and N7 of purines that interact with Ag^+ are highlighted in yellow.

It is worth mentioning that the preparation of DNA-AgNCs usually yields a heterogenous mixture of dark (non-fluorescent) and bright (fluorescent) species.⁷² Essentially, the excess reactants do not interfere with the results. Therefore, the products are often not purified and are used directly for further application. However, such heterogeneity may affect the accuracy of analysis, as discussed in Section 2.5.

2.4.2 Cluster shuttle approach

As compared to direct preparation, cluster shuttle approach is unorthodoxly acquired. In this method, one needs to first mix silver salt with 3-(2-aminoethylamino)propyltrimethoxy silane (APTMOS) in methanol, followed by poly(acrylic acid) (PAA). Prior reaction with APTMOS is important to improve the product yield. Following the cluster stabilisation by PAA polymer, subsequent reduction generates PAA-AgNCs with low quantum yield. PAA-AgNCs are then added with desired ssDNA to enable cluster-transfer from PAA to the DNA scaffold (Figure 2.3). This is evidenced by a concomitant spectra shift.^{71,76} Of note, the transfer efficiency is controlled by the ratio of $\text{Ag}^+:\text{PAA}:\text{APTMOS}$. Experimentally, Dickson *et al.* demonstrated that a lower $\text{Ag}^+:\text{APTMOS}$ ratio favours lower $\text{Ag}^+:\text{PAA}$ and *vice versa*.⁷¹

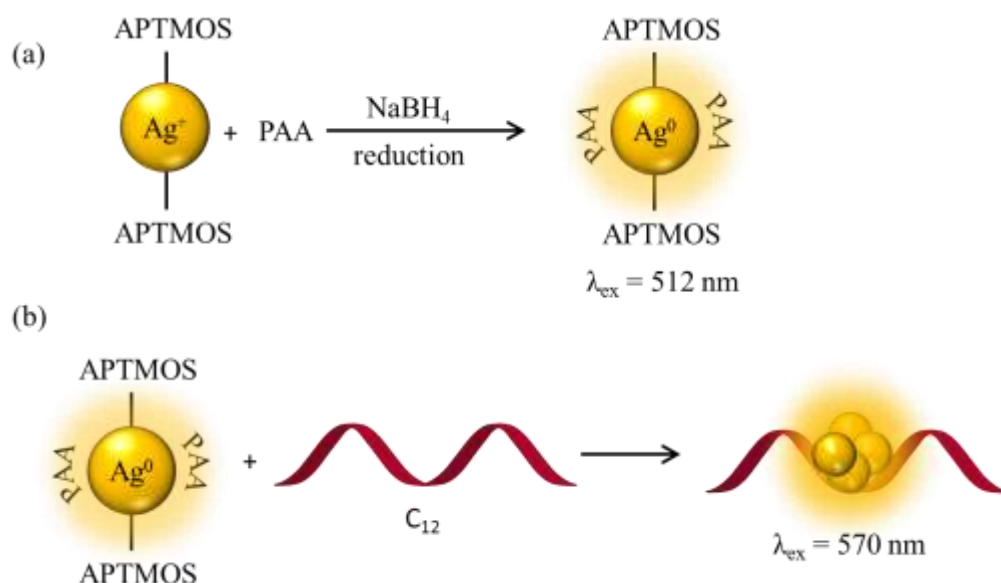


Figure 2.3. Preparation of AgNCs *via* cluster shuttle approach, using C_{12} (a ssDNA made of 12 cytosine bases) as DNA template. (a) Formation of AgNCs using PAA polymer, followed by (b) AgNCs transfer to DNA template. Peak shift on the excitation wavelength (λ_{ex}) indicates the success transfer of AgNCs from PAA polymer to C_{12} templates. Reproduced from ref.⁷¹

In terms of cost, simplicity and time, the cluster-shuttle approach is less advantageous than the first method. However, it allows easy scale-up of

clusters from micro- to milli-litres.⁷¹ Moreover, AgNCs acquired *via* this approach show sensitive and reversible pH response patterns. This behaviour is not seen in the corresponding adducts generated *via* direct preparation. Such merit may arise from the additional protection by the PAA polymer during cluster-transfer, when the DNA template is less folded at certain pH.⁷⁶ Notably, all the DNA-AgNCs in the experimental studies (Chapter 3 and 4) were prepared through direct preparation approach.

2.5. Mechanisms of DNA-AgNCs formation and characterisation

To understand the mechanism of DNA-AgNCs formation, one need to know (i) how the nucleation occurs, (ii) how the clusters grow and (iii) how the DNA stabilises the cluster adducts. This is then correlated to silver content (N_{Ag}) and cluster shape.

Following the mixing of silver salt and DNA template, Ag^+ associates strongly with DNA bases (affinities of $>10^5 M^{-1}$).⁷⁷ This facilitates the nucleation of neutral clusters. It is inferred that Ag^+ binds to DNA bases, specifically *via* doubly bonded endocyclic amines, *i.e.* N7 of purines and N3 of pyrimidines (Figure 2.2) with the affinity order of cytosine > guanine > adenine > thymine.^{1,49,77–81} Cytosine- and guanine-rich DNA are thus widely employed to synthesise AgNCs.^{7,28,82–84} More details on the interaction of Ag^+ with each DNA base type will be discussed in Section 2.6. The sugar-phosphate backbone of the oligonucleotide does not interact with Ag^+ , but it indirectly affects the formation of AgNCs. In the case of the backbone, for instance, an RNA is more flexible than a corresponding DNA. Such flexibility enables easy transient folding of the nucleic acid template and induces cytosine– Ag^+ –cytosine base pairing, thereby promoting the formation of

AgNCs.⁸⁵ Furthermore, the presence of additional –OH group in the ribose sugar of RNA introduces additional dipolar and steric interactions. This eventually influences the emission features and Stokes shift of the AgNCs.⁴⁹ Modifying the backbone to phosphorothioate (PS), however, is found to promote Ag–backbone interaction on top of Ag–base interaction. This is evident from the formation of bright AgNCs using PS-modified polythymine (T₁₅). The result is contrary to the unmodified T₁₅, which only gives fluoresces AgNCs at high pH.⁸⁶ The negatively charged phosphate group also affects the fluorescence evolution of AgNCs, especially those prepared from dsDNA containing gap/abasic/bulge sites.^{64,74,87}

Next, the multiple Ag–DNA ligations provide locally concentrated silver adducts. This promotes agglomerations of clusters when they are chemically reduced. Ag–Ag bonds are formed as well and they cooperatively enhance the stability of the clusters.⁸⁸ Overall, the AgNCs are framed by DNA template, as corroborated by their smaller hydrodynamic radius compared to that of native DNA alone.⁸⁹ This leads to consideration of N_{Ag} and cluster shape, which are responsible for the optical properties of AgNCs (detailed information will be presented in Section 2.6, Table 2.2–2.7).^{38,89,90}

As previously discussed, AgNCs are often used without purification. Notwithstanding, the heterogeneity of product affects the accuracy of reported N_{Ag}, which is generally of a smaller number (*i.e.* 2–3). Of note, N_{Ag} includes both Ag⁺ and neutral silver atom (Ag⁰). To mitigate this problem, Gwinn and co-workers isolated the bright species *via* high performance liquid chromatography (HPLC). Based on high-resolution electrospray ionisation

mass spectroscopy (ESI-MS) and simulation studies on the compositionally pure clusters, they claimed that:^{63,70,72}

- i. A rod-shaped neutral cluster core (*i.e.* Ag^0) is peripherally surrounded by base-bound Ag^+ (Figure 2.4a). The amount of Ag^+ and Ag^0 is similar, with N_{Ag} *ca.* 10–24.
- ii. The rod shape is the primary control of emission colour, where increasing the neutral size leads to a red-shift of emission wavelength (λ_{em}).
- iii. Affinity of Ag^+ for DNA bases, as well as geometry constraint imposed by DNA template, are determinants of emission colour.

Such model is also in agreement with a computational density functional theory (DFT) study that described AgNCs as elongated threadlike-shape. The bending angle of the silver chain in the core could cause a significant shift in the absorption band.⁹¹

Very recently, a cluster that absorbed in the violet region was found to have octahedral geometry (Figure 2.4b) based on collective evidence from ESI-MS, X-ray absorption spectroscopy and circular dichroism (CD). Such compact core is different from the previously proposed elongated shape, with suggested structures as detailed below:⁸⁹

- i. The metal-like core is composed of interconnected and short Ag–Ag bonds, with oxidised Ag^+ ions segregated to the periphery.
- ii. The ligand shell is dominated by Ag^+ –DNA in a long ranged and intermittent pattern.

- iii. The core (Ag–Ag) and the shell components (Ag⁺–DNA) are presumed to weakly interact with each other.

The two different models reflect the flexibility of AgNCs, but it is mentionable that this is based on ssDNA template. The underlying basis for other DNA counterparts is still unavailable at present.

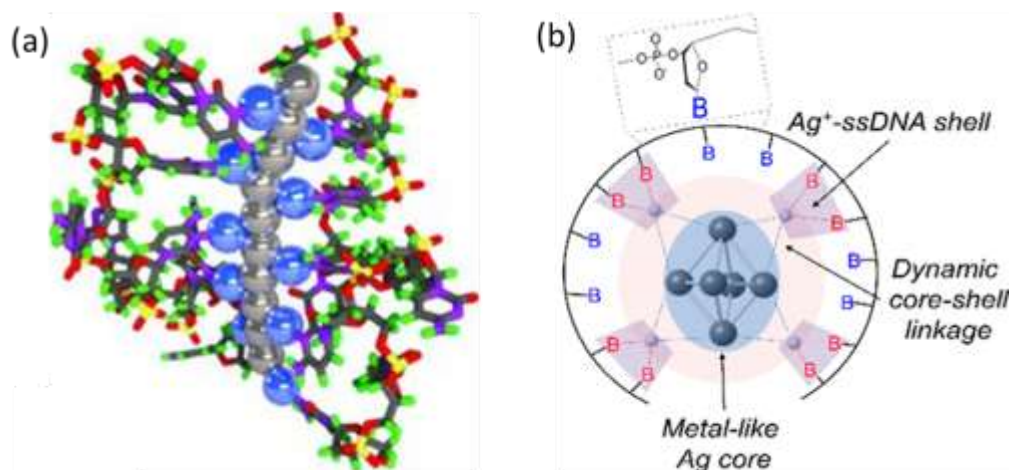


Figure 2.4. (a) A proposed rod-like model of AgNCs, with the neutral core (grey) peripherally surrounded by base-bound Ag⁺ (blue). Reprinted from ref.⁷⁰ with permission from John Wiley & Sons (Appendix I) (b) An illustrated octahedral Ag⁰ core that weakly links to an encapsulating Ag⁺–DNA shell. Reprinted with permission from ref.⁸⁹. Copyright (2016) American Chemical Society (Appendix II).

All the aforementioned findings were made upon structural analysis of the nanomaterial. This can be gleaned from various characterisation studies, including Fourier transform infrared spectroscopy (FTIR),⁷⁷ gel electrophoresis,⁹² X-ray crystallography,⁷⁸ nuclear magnetic resonance (NMR),^{1,93} ESI-MS,^{1,78} isothermal titration calorimetry (ITC),⁷⁹ DFT,⁸⁰ extended X-ray absorption fine structure (EXAFS),⁸⁸ inductively coupled plasma atomic emission spectroscopy (ICP-AES)⁵⁵ and pH-dependent studies.⁷⁸ Of note, DNA-AgNCs can be universally excited in the UV range (260–270 nm) and imaged with a UV transilluminator, despite their distinct emission colour. This suggests that UV excitation proceeds *via* DNA bases.^{94,95}

However, to measure the true excitation wavelength (λ_{ex}), one should use a short path length and low DNA concentration, as well as carefully choosing the right λ_{ex} to avoid second order diffraction of excitation light.

2.6. Correlation between DNA conformation and emission properties of AgNCs

14 years since the introduction of DNA-AgNCs,¹ this nanomaterial has achieved exciting progress due to the prominent excellence of its modulatable emission property. Yet, its emission behaviour is not fully understood. The huge possibilities of DNA sequence permutation have complicated the structural dependency of the emission behaviour. As an illustrated example, to construct AgNCs with a 10-mer oligonucleotide, there are >10 million distinct templates available.^{63,96} Additionally, this excludes the factor of DNA conformational polymorphism. Thus, substantial efforts have been devoted to deciphering the predictive colour code of DNA-AgNCs. Gwinn's and Yeh's group have done comprehensive studies on ssDNA and specific NanoCluster Beacon (NCB), respectively;^{9,63,70,96–98} whereas the other contribution summarised different DNA scaffolds to construct AgNCs.^{60–62,99} Crucially, an overall review on structural origin, particularly DNA sequence, as well as comparison among different DNA scaffolds is still lacking. To engineer AgNCs with desirable optical properties, it is important to establish a rational basis for their structural origins of emission

In this section, the content is organised based on the type of DNA strands used to construct AgNCs, ranging from ssDNA with primary and secondary structure, dsDNA, triple-stranded DNA (tsDNA) to DNA nanostructures (Table 2.1). In each category, the diverse DNA scaffolds are briefly and

structurally described, followed by their representative studies and highlights of coherent optical properties. For ease on correlation between AgNCs emission properties to DNA structural conformation, a compiled list of reported DNA templates, together with respective optical properties (represented by $\lambda_{\text{ex}}/\lambda_{\text{em}}$ and quantum yield) and N_{Ag} is presented (refer to Table 2.2 to 2.7). These tables also included experimental conditions (*i.e.* synthesis ratio and buffer used) and important remarks.

Table 2.1. Different DNA categories and the relevant components involved in modulating AgNCs emission properties and variety of application purposes

Category	Polymorphic DNA
ssDNA	Primary structure
	Secondary structure: - i-motif - G-quadruplex - Hairpin loop
dsDNA	Hybridised dsDNA Gap Abasic Mismatch site Bulge Loop NanoCluster Beacon (NCB)
tsDNA	
DNA nanostructure	Branch DNA: - X-shaped DNA - Y-shaped DNA Origami

2.6.1 ssDNA as template

As shown in Table 2.1, ssDNA can be divided into those with a primary or secondary structure. To avoid any confusion in defining the hierarchical structure of DNA, the primary structure is referred to the exact sequence of nucleobases in an oligonucleotide, in direct analogy with protein primary sequence.¹⁰⁰ Possible hairpins and slipped structures are excluded, unless it is clearly defined by the original reports. In contrast, secondary structure relates

to a structured motif stabilised by hydrogen bonding between units of primary structure.¹⁰⁰ The well-defined secondary structures, *i.e.* i-motif, G-quadruplexes and hairpin loops, are discussed individually.

ssDNA is the most commonly used oligomer for constructing AgNCs because its conformational flexibility enables better Ag⁺ or Ag⁰ incorporation. The common length of ssDNA template is 12–30 mer (Table 2.2).^{49,78,101–103} The yield for AgNCs is generally good, with the highest yield reported up to 94 % (Table 2.2; sequence (44)).⁷⁰ Chang and co-workers concluded that longer DNA length can produce AgNCs of better yield and stability than Cy5-conjugated DNA.⁶¹ AgNCs are stabilised by simultaneous binding to multiple bases. ssDNA shorter than 15-mer may result in steric constraints and prevent multi-base bonding. In this case, the strand dimer may dominate with two oligomer strands conjoined by the silver content. Comparatively, a long oligomer permits both intra- and inter-strand Ag⁺-base pairing and it tends to generate AgNCs of greater size, emitting in the higher energy region.^{54,70,72,98} Another alternative to enhance the yield of AgNCs is by preparing AgNCs under crowded conditions, as reported by Qu and co-workers. Crowding agents, such as polyethylene glycol (PEG), could induce the formation of more rigid DNA template which protects AgNCs from environmental quenching. Moreover, such molecular crowding was also found to increase emission intensity and shorten reaction time.¹⁰⁴

The flexibility of ssDNA and the possibilities of sequence permutations make the predictive design of AgNCs almost unfathomable. On the bad side, it may compromise product purity^{78,105}; whereas for the good side, a flexible ssDNA offers wider potential to be used for different application purposes.

2.6.1.1 Effect of base composition in ssDNA

A list of reported cluster-laden primary structure ssDNA is summarised in Table 2.2, starting from homo-oligonucleotides (sequence (2)–(6)). To embed AgNCs in a DNA scaffold, it is not surprising that Ag^+ will reconfigure the canonical Watson-Crick (WC) base pairing. This is substantiated with the reshaping of the UV peak after adding Ag^+ to bare DNA. The spectral shift implies DNA transformation from a random coil to a less fluctuational form.¹⁰⁶ The high affinity of cytosine– Ag^+ –cytosine pairing is particularly well recognised. Thus, a 1:2 ratio of Ag^+ :cytosine was adopted when preparing AgNCs with a homo-cytosine strand. Such combination is known to produce multiple emitter species (*i.e.* blue, green and red) (sequence (2) and (7)).^{78,107} As discussed, cytosine-rich DNA templates are predominantly used, with multiple cytosines at 5'- and/or 3'-end(s) assumed to be entropically favourable.^{41,53,108} Recently, it was reported that guanine– Ag^+ –guanine has comparable binding energy as well. In this case, Ag^+ binds to the N7 sites of guanines, which do not participate in WC base pairing.¹⁰⁹ The guanine– Ag^+ –guanine pairing are more desirable than other Ag^+ -mediated base pairs, presumably due to their greater base-stacking and coplanar arrangement. Polyadenine or polythymine generally gives no detectable fluorescence at neutral pH, similar to the mixed adenine/thymine strands with 20 – 86 % of adenine content (sequence (4)–(6)).⁴⁹ However, at alkaline pH, polythymine is able to form green-emitting AgNCs, delineating the association of its deprotonated N3 ($\text{pK}_a = 9.5$) with the silver content (sequences (8) and (9)).⁴²

Sequences (16)–(37) are oligomers that form chemopalettes (*i.e.* multicolour) of AgNCs emitting from blue to NIR region, in which those with

longer λ_{em} show better photo- and chemical-stability in buffer. Chemopalette-1 and Chemopalette-3 consists of 12 and 23 bases, respectively, whereas the rest are of diverse length, ranging from 15–46 mer. As can be seen, Chemopalette-1 to Chemopalette-4 are compositionally random and there is no clear predictive pattern for their emission behaviour. In this regard, Neidig *et al.* conducted an EXAFS study on AgNCs of different λ_{em} and hosted by oligomers of different lengths (sequence (22)–(25)). The results revealed that there is no correlation between cluster size, emission colour and Ag–Ag bond.^{88,89} However, one should note that the N_{Ag} of this and most studies may be affected by the heterogeneity of the products. Given the synthesis conditions and characterisation techniques were different, simple cross-comparison between studies may not be appropriate. Nevertheless, the purified bright species in Chemopalette-4 suggested that larger cluster emit at longer λ_{em} .⁷² Petty *et al.* also reported multiple IR-emitting species encapsulated in oligomers of similar lengths and base composition. They all contain similar N_{Ag} (sequence (38)–(40)).^{55,110}

To further demystify the relationship between sequence and emission colour, Gwinn and co-workers applied pattern recognition algorithm on AgNCs derived from a large array of 10-base oligomers. “Bright motifs”, named for DNA sequences that preferentially template bright AgNCs, have been identified. They generally possess the following sequences/features:⁹⁶

- i. Consist of 3 – 5 bases to make contact with AgNCs
- ii. Contain (a) multiple cytosine or guanine bases (*e.g.* CC_C, C_CC, GCG, CCG, GCC, CGC and CCC) and/or (b) adenine with flanking cytosine or guanine (*e.g.* GG_AC, G_GAA and

AGC_G); ‘_’ represent no base or any base. Thymine may act as termination site to limit cluster growth.

- iii. At least two bright motifs are prerequisite to stabilise the clusters
- iv. A higher number of bright motifs leads to larger AgNCs with longer λ_{em}

As listed in Table 2.2, the majority of the oligomers manifest a cytosine-rich motif. Another compelling observation is bimodal emission dominates at green (540 ± 20 nm) and red (630 ± 30 nm) regions, which corresponds to cluster with N_{Ag} of 4 and 6, respectively. The local maxima distributions at certain colour regions and number of atoms are termed as magic colour and magic number, respectively. Interestingly, the magic colour and magic number of DNA-AgNCs are generic, regardless of DNA lengths and distinct base composition.⁹⁸

In terms on stability, most DNA-AgNCs have short shelf-lives due to photobleaching (*i.e.* irreversible destruction of fluorophore) and facile oxidation by air, with some can be as short as hours.^{73,110} In certain cases, oxidation can further complicate the heterogeneity of the sample, mainly exemplified by AgNCs derived from ssDNA with four or more consecutive cytosine bases (*e.g.* sequence (7), i-motif in Section 2.6.1.2 and hairpin loop in Section 2.6.1.3).^{49,78,103,107} These samples often yield red and green emitters, with the red representing the reduced species and the green as oxidised one. Although the green oxidised emitters are relatively more stable, they suffer from low quantum yield and possibly get obscured by endogenous cellular autofluorescence. An intriguingly stable red emitter with long shelf-life (>1 year) that highly resistant to oxidation, pH and temperature has been exploited

(sequence (41)),⁵³ Gwinn *et al.* also described that pure AgNCs can last up to months upon storage at 10 °C.⁶³ Another solution to the temporal stability of DNA-AgNCs is through optical switching, which turns the dark species to bright species,⁹ which will be discussed in detail in Section 2.6.2.2 on NCBs. Another effort included under optical switching is using chameleon-like clusters, which absorb in violet region.^{111,112} These oligomers consist of (i) a clusters template that harbours emissively silent clusters and (ii) a recognition site for the complementary strand. Drastic spectral shift and emission enhancement occurred upon hybridisation, which Petty *et al.* postulated that the DNA conformational change (*i.e.* shape and structure of DNA host upon binding) triggers transformation of dark AgNCs to emissive counterparts (sequence (42) and (43)).^{111,112}

The third solution is *via* intergrowth of the emitter pair, which was recently discovered when placing two dark emitters at the termini of DNA with a spacer of decent length of an optimised 15 thymine bases in between (Table 2.2; ‘Intergrowth emitter pair’). In this case, the emission intensity can be remarkably enhanced by 850-fold.¹¹³ Such enhancement is highly affected by the base type and length of spacer as well as disruption through hybridisation. Of note, the resulting emission *via* intergrowth of an emitter pair can differ from that of the individual clusters. Ma *et al.* assigned this phenomenon by virtue of the plasmonic resonance and ligand-to-metal-metal charge transfer.^{113,114}

As compared to primary structure ssDNA, the emission properties of AgNCs nested in more confined structural elements (*e.g.* secondary structure

ssDNA, NCB, and dsDNA) seems more modulatable and will be discussed individually.

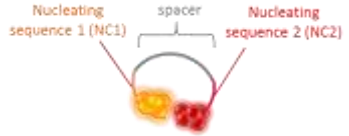
Table 2.2. Reported primary structure ssDNA used to form AgNCs

DNA sequence (5' → 3')	$\lambda_{\text{ex}}/\lambda_{\text{em}}$ (nm)	N _{Ag}	Quantum yield (%)	Synthesis ratio (DNA:Ag ⁺ :BH ₄ ⁻); buffer	Remarks	Ref.
(1) AG ₂ TCGC ₂ GC ₃	540–580/629–642	1–4	N/A	1:6:6; 5 mM PBS, pH 7.5 or 100 mM NaClO ₄	<u>First reported DNA-AgNCs</u> 1. N _{Ag} is determined from ESI-MS	¹
(2) C _n (n = 5–11)	350–850/500–950	10	N/A	1:11:6.1; 10 mM NH ₄ OAc, pH 6.8	<u>Homopolymers</u> 1. N _{Ag} is determined from ESI-MS 2. For C _n runs, n should be >4 3. For G _n runs, n should be >5 4. Terminal T in (3) is to increase synthesis yield and enhanced analysis for ESI-MS	49
(3) TG _n T (n = 3–6, 8, 9, 11)	350–850/500–900					
(4) A _n (n = 6, 9, 11)	No fluorescence					
(5) T _n (n = 6, 9, 11)	No fluorescence					
(6) Series of mixed A/T strands	No fluorescence					
(7) C ₁₂	340/485; 440/525; 280 or 580/665; 650/700	5; 4; 3; 2	1; N/A; 23; 17	1:6:6; 5 mM acetate or PBS	<u>Cytosine-rich or thymine-rich DNA</u> 1. N _{Ag} is determined from Job's plot of emission intensity vs [DNA]:[Ag ⁺]. 2. (7)-AgNCs in PBS prefer to stabilise NIR species 3. Low concentration of (10)	42,78,103,107
(8) T ₁₂	350/545	6	12	1:6:6; 5–10 mM borate, pH 10.5		
(9) T ₄ C ₄ T ₄	370/475	6	N/A			
(10) C ₄ T ₄ C ₄	340/495 or 580/850	8	N/A			

DNA sequence (5' → 3')	$\lambda_{\text{ex}}/\lambda_{\text{em}}$ (nm)	N_{Ag}	Quantum yield (%)	Synthesis ratio (DNA:Ag ⁺ :BH ₄ ⁻); buffer	Remarks	Ref.
					favour in blue/green species, while high concentration favour in red species. This is due to intramolecular vs intermolecular cluster template.	
(11) (CXG) _n , X = A, T, C or G; n = 6–9	512/600 (X = G/C; n = 6)	N/A	N/A	1:8:16; 20 mM PBS, pH 7.0 with 1 mM Mg(OAc) ₂	1. By varying synthesis ratio, type of middle base (X) or number of trinucleotide repeats (n), only emission intensity is affected 2. X = G gives the highest emission intensity followed by C 3. No fluorescence observed when X = A/T	⁶⁵
(12) n = 1	460/536	2	8	1:6:6; 20 mM PBS, pH 7.0	<u>Effect of C–Ag⁺–C pairs</u> $\begin{array}{c} 5'\text{-CCC}\left(\begin{array}{c} \text{TTCC} \\ \text{AACC} \end{array}\right)_n^{\text{T}} \\ 3'\text{-CCC}\left(\begin{array}{c} \text{TTCC} \\ \text{AACC} \end{array}\right)_n^{\text{T}} \end{array}$	¹⁰⁸
(13) n = 2	540/608	4–5	61			
(14) n = 5	540/620	5–6	21			
(15) n = 6	540/644	6	25			
					1. N_{Ag} is determined from	

DNA sequence (5' → 3')	$\lambda_{\text{ex}}/\lambda_{\text{em}}$ (nm)	N _{Ag}	Quantum yield (%)	Synthesis ratio (DNA:Ag ⁺ :BH ₄ ⁻); buffer	Remarks	Ref.
					ESI-MS 2. C ₃ at 5' and 3'-end helps to stabilises AgNCs 3. Higher number of A–T pairs in repeating units (n) may hinder the coordination of C–Ag ⁺ –C 4. Higher number of repeated CC bases results in larger AgNCs	
(16) C ₃ T ₃ A ₂ C ₄ (B)	340/485	N/A	N/A	1:6:6; water	Chemopalette-1^a 1. Preparation under crowded crowding conditions (<i>i.e.</i> in presence of PEG 200) has improved the quantum yield	41,104
(17) C ₃ TCT ₂ A ₂ C ₃ (G)	425/520		16 ± 3			
(18) C ₃ T ₂ A ₂ TC ₄ (Y)	475/572 ^c ; 475/560 ^{d,e}		38 ± 2 ^c ; 6.4 ^d ; 66.2 ^e	1:6:6; 20 mM PBS, pH 6.5–8 ^c ; 25 mM PBS, pH 7 ^d ; 25 mM PBS with 30 % PEG 200 ^e		
(19) A ₂ T ₂ C ₁₂ A ₂ T ₂	N/A (Yellow)		68	1:6:6; N/A		
(20) C ₂ TC ₂ T ₂ C ₂ TC ₂ (R)	543/620 ^c ; 580/616 ^{d,e}		32 ± 4; 24.5 ^c ; 67.6 ^d	1:6:6; 20 mM citrate, pH 5 ^c ; 25 mM PBS, pH 7.0 ^d ; 25 mM PBS with 30% PEG 200 ^e		

DNA sequence (5' → 3')	$\lambda_{\text{ex}}/\lambda_{\text{em}}$ (nm)	N _{Ag}	Quantum yield (%)	Synthesis ratio (DNA:Ag ⁺ :BH ₄ ⁻); buffer	Remarks	Ref.
(21) C ₃ TA ₂ CTC ₄ (N)	650/705		34 ± 5	1:6:6; 20 mM NH ₄ OAc, pH 6.5–8		
(22) T <u>G</u> ACTA ₄ C ₃ T ₂ A ₂ TC ₄	460/550	20–30	0.2	1:6:6; 20 mM PBS, pH 6.6 or water	<u>Chemopalette-2</u> 1. N _{Ag} is determined from Ag K-edge EXAFS 2. Ag–DNA ligation, cluster size and Ag–Ag bonding cooperatively modulate fluorescence properties of AgNCs	83,88
(23) AGTCAC ₄ A ₂ C ₂ TGC ₃ TAC ₂ ACG ₂ ACT	530/600	8–14	10			
(24) G ₂ CAG ₂ T ₂ G ₄ TGACTA ₅ C ₃ T ₂ A ₂ T C ₄	595/650	8–14	64			
(25) AGTC ₂ GTG ₂ TAG ₃ CAG ₂ T ₂ G ₄ TG ACTA ₅ C ₃ T ₂ A ₂ TC ₄	640/700	8	52			
(26) (CCCTA ₂) ₃ CCCTA	468/560	N/A	N/A	1:6:6, 10 mM Tris/HCl, pH 7.0	<u>Chemopalette-3</u> 1. Alternation of single C to G (as underlined) in (26) causes blue-shift in emission 2. Double and triple C to G mutation accounts for the remarkable red-shift.	35
(27) (CGCTA ₂) ₃ CGCTA	466/554					
(28) (CCGTA ₂) ₃ CCGTA	439/495					
(29) (GGCTA ₂) ₃ GGCTA	453/515					
(30) (GCGTA ₂) ₃ GCGTA	548/623					
(31) (GGGTA ₂) ₃ GGGTA	560/626					
(32) CGC ₆ T ₂ G ₂ CGT	270/558	21 ^b	N/A	1:10:2.4	<u>Chemopalette-4</u> 1. N _{Ag} is determined from HPLC-MS 2. Only N _{Ag} of bright species is shown.	55,72,11 0
(33) CGC ₆ TCG ₂ CGT	270/557	21 ^b		1:6:3		
(34) TGC ₂ T ₄ G ₄ ACG ₂ ATA	270/562	10		1:12.5:6.27		
(35) T ₂ C ₄ AC ₂ AC ₃ AG ₂ C ₄ GT ₂	270/632	16		1:12:6		
(36) T ₂ CGC ₆ GC ₄ AG ₂ CGT ₂	270/644	15		1:12:6		

DNA sequence (5' → 3')	$\lambda_{ex}/\lambda_{em}$ (nm)	N _{Ag}	Quantum yield (%)	Synthesis ratio (DNA:Ag ⁺ :BH ₄ ⁻); buffer	Remarks	Ref.
(37) C ₃ AC ₃ AC ₃ TC ₃ A	270/777	20 ^b		1:8:4	3. Larger cluster tend to emit at longer λ_{em} 4. Despite different ratio of synthesis, same buffer type: 10–40 mM NH ₄ OAc, pH 7 is used	
(38) C ₃ AC ₃ AC ₃ TC ₃ A	750/810	10	30 ± 2	1:8:4; 10 mM citrate, pH 7.0	IR emitting species 1. N _{Ag} is determined from ICP-AES 2. Based on general sequence C ₃ AC ₃ AC ₃ X _{C₃} Y 3. Chromatographically separated and identified with AES study	55,110
(39) C ₃ AC ₃ AC ₃ GC ₃ A	720/770	9–10	30 ± 5			
(40) C ₃ AC ₃ AC ₃ AC ₃ G	840/870	9	6 ± 2			
(41) AC ₃ GA ₂ C ₂ TG ₃ CTA ₂ A ₃ T ₂ A ₂ T ₄	535/615	N/A	30	1:6:6; 20 mM PBS, pH 6.8	AgNCs with enhanced stability	53
NC1 – X _n – NC2 NC1 and NC2: sequence (16) to (21) from “Chemopalette-1”; X = A, T, C or G; n = 0 – 70	λ_{em} ~604 (YN, NN, BN, RN and GN) λ_{em} ~630 for other combination	N/A	16.3 (for Y – T ₁₅ – N)	1:6:6; 5 mM PBS containing 50 mM NaNO ₃ , pH 7.0	Intergrowth emitter pair 	113

DNA sequence (5' → 3')	$\lambda_{\text{ex}}/\lambda_{\text{em}}$ (nm)	N _{Ag}	Quantum yield (%)	Synthesis ratio (DNA:Ag ⁺ :BH ₄ ⁻); buffer	Remarks	Ref.
(42) C ₃ AC ₃ AC ₃ TC ₃ AC ₃ GC ₂ GCTG ₂ A	N/A/790	11	N/A	1:8:4; 10 mM citrate/citric acid, pH 6.5	<u>Chameleon-like violet cluster</u> 1. N _{Ag} is determined from stoichiometry study from absorption spectra 2. (42) derives from (38) 3. (42)- and (43)-AgNCs transform the weakly emissive violet chromophore to strong NIR and green emitter respectively ($\Delta\lambda_{\text{abs}}$ 330 & 90 nm). 4. Violet cluster are favoured when Ag ⁺ :DNA = 4–10 and prepared at high oxygen pressure.	111,112
(43) C ₄ A ₂ CTC ₂ T ₂ C ₃ GC ₂ AC	490/550					
(44) T ₂ CC ₃ AC ₃ A ₄ G ₂ C ₃ GT ₂	571/635	24 ^b	94 ± 8	1:10:10; 10 mM NH ₄ OAc	<u>AgNCs with high yield</u>	⁷⁰

^a Abbreviation of sequences are given in (), with B, G, Y, R, N represent blue, green, yellow, red and NIR emitter respectively

^b N_{Ag} for strand dimer complexes

^{c,d,e} $\lambda_{\text{ex}}/\lambda_{\text{em}}$ (nm) and quantum yield of AgNCs formed at the corresponding buffer

2.6.1.2 i-motifs and G-quadruplexes

To study the effect of polymorphism DNA on AgNCs, Li *et al.* compared the binding affinity of Ag–DNA in different forms of clusters (Table 2.3). They found that the secondary structure regulates the binding affinity with decreasing order of coiled cytosine-rich strand > i-motif > duplex > G-quadruplex.¹¹⁵ The observed trend is in accordance with the previous studies, affirming that a flexible conformation facilitates nucleation and growth of AgNCs. Furthermore, the higher affinity of the first two structural elements elucidates that Ag interacts more strongly with cytosine than with guanine.

The i-motif is an intercalating topology, favoured by cytosine-rich sequences (Figure 2.5). Structurally, sequences carrying four stretches of cytosine (C₄) tend to form intramolecular i-motif folding. The structure forms at slightly acidic or neutral pH, where cytosines are hemiprotonated to stabilise the tetrametric structure.^{116,117} In the context of AgNCs synthesis, such balance protonation is crucial to maintain i-motif structure while providing necessary binding sites (*i.e.* N3 of cytosine) for Ag⁺. Using size exclusion chromatography (SEC), Petty *et al.* assessed that red-emitting AgNCs derived from C₄ i-motif have a similar global structure to the i-motif form of DNA alone. Its emission with red emitter as dominant species is the highest at pH 6, which is also an ideal pH for i-motif to form. This implies that the i-motif provides a stable template for the clusters. Interestingly, another green emitter was predominantly formed at pH 8–9, and its global structure was similar to that of an i-motif (sequences (45) and (46)).¹¹⁸ Four years after Petty's report, Waller *et al.* found that Ag⁺ can induce i-motif formation at physiological pH, possibly *via* cytosine–Ag⁺–cytosine base pairs. Considering the coordination is

different from the conventional proton-mediated i-motif, the conformation is unsurprisingly dissimilar, as depicted by CD spectra.¹¹⁷ A very recent study reported that the loop sequence of an i-motif may influence the loop flexibility and overall stability of the i-motif. Locating a single thymine next to C₄ preferably produces clusters of higher quantum yield, with X = ATAT gives the best yield and X = TATA has reduced the yield to 15 % (sequence (51)).¹¹⁹

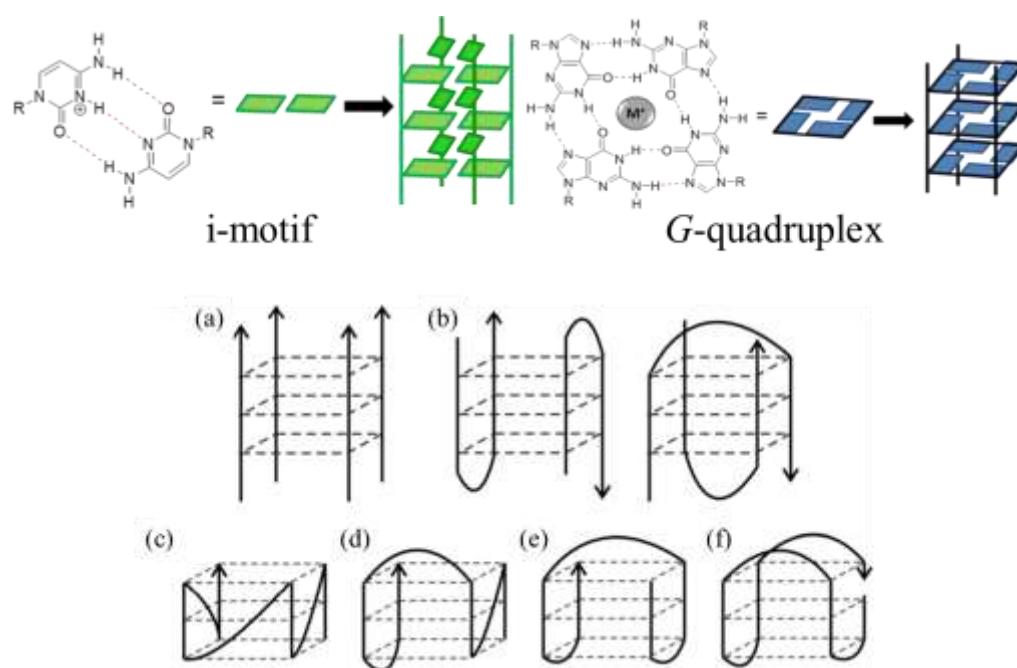


Figure 2.5. Polymorphism DNA tetraplex into i-motif or G-quadruplex conformation with different illustrative topologies as shown: (a) parallel intermolecular, (b) anti-parallel intermolecular, (c-f) intramolecular structure with different conformation. The arrow represents 5' $\rightarrow$ 3' orientation.¹²⁰

As compared to i-motif, G-quadruplexes may have more constraint when interacting with Ag⁺.¹¹⁵ Analogously, G-quadruplexes are square planar arrays made of guanine-rich sequences, with four Hoogsteen-paired guanines (*i.e.* tetrads) stacked on each other *via* π - π stacking (Figure 2.5). The structure is generally stabilised by monovalent cations (*e.g.* K⁺, Na⁺, NH₄⁺, *etc.*) located at the centre of the tetrads.¹²¹ Similar to i-motif, such folded topology offers a good matrix to protect AgNCs from aggregation. However, since N7 sites of

guanines participate in base pairings in the tetrads, it may attenuate the binding affinity with Ag^+ .¹¹⁵ One of the widely studied models of *G*-quadruplexes is the human telomeric sequence. Study showed that telomeric sequence can generate red- and green-emitting AgNCs, with the former primarily associated with guanine bases whereas the latter stabilised by adenine and guanine bases. In the presence of Na^+ , fluorescence was significantly quenched as a result of anti-parallel *G*-quadruplex formation (sequence (62)).⁸⁴ Wang and co-workers prepared AgNCs using AS1411, a *G*-quadruplex aptamer that binds to nucleolin (sequence (52)). Notwithstanding that the material was weakly fluorescence in solution, the signal was highly enhanced upon binding to nucleolin in cancer cell.² By incorporating both cytosine- and guanine-tracts into hairpin DNA named HP26 (sequences (63) and (64)), a logic device could be easily constructed by using H^+ and K^+ to trigger the formation of i-motifs and *G*-quadruplexes, respectively. Such a conformational change produces a distinct fluorescence response from different fluorescence species.⁵⁹

Recently, Tao *et al.* has indicated that *G*-quadruplexes with antiparallel configurations and with adjacent adenine-rich loop sequence tend to produce highly fluorescent AgNCs.¹²² Comparatively, *G*-quadruplexes with parallel configurations and with adjacent thymine-rich loop sequence generally leads to AgNCs with lower emission (sequence (65)–(71)).

Table 2.3. Reported i-motif and G-quadruplex templates that have been used to form AgNCs

DNA sequence (5' → 3')	$\lambda_{\text{ex}}/\lambda_{\text{em}}$	N _{Ag}	Quantum yield (%)	Synthesis ratio (DNA:Ag ⁺ :BH ₄ ⁻); buffer	Remarks	Ref.
(45) (TA ₂ C ₄) ₄	460/650 560/625	N/A	N/A	1:16:16; 10 mM formate or cacodylate or PBS or borate, pH 5–9	<u>i-motif</u> 1. N _{Ag} for (47)–(50) is determine from HPLC-MS 2. (47) & (48) is intermolecular i-motif 3. (49) & (50) is intramolecular i-motif	118
(46) (C ₄ A ₂) ₃ C ₄	500/570 560/625					
(47) C ₄ A ₄ C ₃	543/613 ^{a,b}	1–4 ^b	16 ± 2 ^a 37 ± 2 ^b	1:4:4 for (47) and (48) 1:6:6 for (49) and (50); MES containing 50 mM Na ⁺ , pH5 ^a ; PBS, pH 7 ^b ; 25 mM PBS, pH 7 ^c ; 25 mM PBS, pH7, 30 % PEG 200 ^d		104,115
(48) C ₄ A ₄ C ₄	575/635 ^{a,b} 540/600 ^{c,d}	N/A	11 ± 2 ^a 12 ± 1 ^b 14.5 ^c 32.1 ^d			
(49) (C ₃ TA ₂) ₂ C ₂ TA ₂ C ₃ T	463/538 ^{a,b} 520/575 ^{c,d}	5–8 ^a	10 ± 2 ^a 6.7 ^c 34.7 ^d			
(50) GC5(GC4)3T	582/655 ^{a,b}	N/A	11 ± 1 ^a			
(51) C ₄ –X ₁ X ₂ X ₃ X ₄ –C ₄ X = mixture of A/T	570–590/615–630	N/A	78 (X = ATAT)	1:6:1; 10 mM PBS, pH 7.6		119
(52) (G ₂ T) ₄ T ₂ G(TG ₂) ₄	325/420 510/680	2	N/A	1:6:6; 20 mM PBS containing 10 mM KCl, pH 8	<u>G-quadruplex</u> 1. (52)–(57): N _{Ag} is determined from MALDI-TOF MS 2. (58)–(61): N _{Ag} is determined from ESI-MS 3. (58) & (59) is intermolecular G-quadruplex	2
(53) (G ₃ T ₂ A) ₃ G ₃	390/435	1				
(54) (G ₄ T ₄) ₃ G ₄	430/565	2				
(55) G ₃ TAG ₃ CG ₃ T ₂ G ₃	435/565	2				
(56) (G ₃ T) ₄	425/560	2				
(57) GTG ₃ TAG ₃ CG ₃ T ₂ G ₂	240/390	3				

DNA sequence (5' → 3')	$\lambda_{\text{ex}}/\lambda_{\text{em}}$	N _{Ag}	Quantum yield (%)	Synthesis ratio (DNA:Ag ⁺ :BH ₄ ⁻); buffer	Remarks	Ref.
(58) G ₃ T ₄ G ₄	580/652	2–4 ^a	10 ± 2 ^a	1:4:4 for (58) & (59), 1:6:6 for (60) & (61); MES containing 50 mM Na ⁺ , pH 5 ^a ; PBS, pH 7 ^a	4. (60) & (61) is intramolecular G-quadruplex	115
(59) G ₄ T ₄ G ₄	580/648	N/A	8 ± 2 ^a			
(60) AG ₃ (T ₂ AG ₃) ₃	557/621	5–12	7 ± 2 ^a			
(61) A(G ₄ C) ₃ G ₅ C	610/670		10 ± 2 ^a			
(62) (AG ₃)(T ₂ AG ₃) ₃	445/520 560/620	N/A	N/A	1:6:6; deionised water		84
(63) G ₃ T ₂ AG ₃ T–C ₆ –AC ₃ T ₂ AC ₃	494/570 581/646 ^a 537/601 ^b	N/A	N/A	1:6:6; (63): 10 mM TrisOAc, pH 8 ^a or 10 mM TrisOAc containing 100 mM K ⁺ , pH 5 ^b (64): 10 mM TrisOAc, pH 8	<u>Logic gate using G-quadruplex & i-motif as templates</u> 1. (63) & (64) exist as hairpin structure without K ⁺ and H ⁺ inputs 2. In the presence of K ⁺ and H ⁺ , G- and C- tracts (shown in red and blue respectively) are convertible to G-quadruplex and i-motif 3. (63)-AgNCs show emission in orange region due to structural change; whereas (64)-AgNCs show different intensity in yellow and red region	59
(64) (G ₃ T) ₂ –C ₆ –(AC ₃) ₂	512/582 575/634					
(65) TG _n ATAG _n ATTAG _n ATAG _n T (n = 2 – 6)	575/500 (n = 2)	10	N/A	1:8:1.5; 10 mM NH ₄ OAc	<u>G-quadruplex with different topologies and loop sequence</u> 1. Addition of K ⁺ and other monovalent ions (e.g. Na ⁺ and Li ⁺) has drastically	122
	575/615 (n = 3)	6	81 ± 0.1			

DNA sequence (5' → 3')	$\lambda_{\text{ex}}/\lambda_{\text{em}}$	N _{Ag}	Quantum yield (%)	Synthesis ratio (DNA:Ag ⁺ :BH ₄ ⁻); buffer	Remarks	Ref.
	575/620 (n = 4)	8	85.4 ± 0.1		decrease the fluorescence of the AgNCs.	
	575/620 (n = 5)	10	69.4 ± 0.2		2. n = 4–6 has produce pure, mono-compositional AgNCs	
	575/620 (n = 6)	12	67.3 ± 0.1		3. n = 3, quadruplex structure is not distinct without presence of K ⁺	
(66) AG ₄ TATG ₄ TAATG ₄ TATG ₄ A	575/610	8	70.9 ± 0.1		4. n = 4: antiparallel G-quadruplex	
(67) AG ₄ TTAG ₄ TAATG ₄ TTAG ₄ A	575/615	8	69.4 ± 0.1		5. n = 5: hybrid G-quadruplex	
(68) AG ₄ AAAG ₄ AAAG ₄ AAAG ₄ A	575/610	8	69.9 ± 0.1		6. n = 6: parallel G-quadruplex	
(69) TG ₄ TTTG ₄ TTTG ₄ TTTG ₄ T	575/620	8	N/A		7. Loop sequence does not affect the composition of AgNCs	
(70) AG ₄ ATAG ₄ ATTAG ₄ ATAG ₄ A	575/620	8	81 ± 0.1		8. Emission intensity is the highest when length of side loop(s) (shown in red) is 3 nucleotides and central loop (shown in blue) is 4 nucleotides, whereas intensity in the lowest when side loop(s) is 2 nucleotides	
(71) TG ₄ TATG ₄ TAATG ₄ TATG ₄ T	575/615	8	N/A		9. Side loops possess more influence on emission intensity than middle loop.	

^{a, b, c, d} $\lambda_{\text{ex}}/\lambda_{\text{em}}$ (nm) and quantum yield of AgNCs formed at the corresponding buffer

2.6.1.3 Hairpin loop

A hairpin loop is another example of secondary structure of ssDNA. A hairpin loop is created when ssDNA folds by base-pairing at two ends, leaving certain unpaired bases as a U-shape loop (Figure 2.6). This structure has been widely incorporated into molecular beacon systems (refer to Section 2.6.2.2) for a multitude of sensing studies. A summary of ssDNA loop-hosted AgNCs is included in Table 2.4.

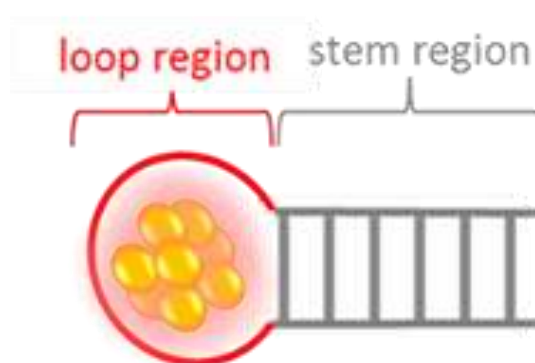


Figure 2.6. ssDNA hairpin structure with loop region is the primary site for nucleation and encapsulation of AgNCs.^{57,66,97,123} The loop size must be >3 bases to prevent steric hindrance effect that can influence formation of AgNCs.¹²⁴

ssDNA loop-hosted AgNCs were initiated by Gwinn *et al.* A corporation study was conducted among AgNCs formed using cytosine-rich ssDNA, C₅-loop strand (*i.e.* hairpin with a loop consists for five cytosine bases), guanine-rich ssDNA and G₅-loop strand, with each pair of cytosine- and guanine-templates differing only in seven bases. The emission intensity from cytosine/guanine loop samples is comparatively weaker than those from corresponding ssDNA; assignable to their geometric constraint that retard incorporation of Ag⁺/Ag⁰.⁹⁷

This also coincides with the finding on the effect of polymorphic DNA, as described earlier (Section 2.6.1.2). In comparison, cluster adducts derived

from thymine- and adenine-loops show negligible or weak fluorescence (sequence (72)–(75)). However, their N_{Ag} is larger than the bright cytosine- and guanine-analogues. The authors reasoned that when Ag^+ binds to a cytosine- or guanine-loop, it results in structural deformation and charge repulsion, which further retard the Ag–duplex stem interaction.⁹⁷

In a later study, the same group focused on the effect of loop size on AgNCs formation, particularly cytosine-loop (C_n). By increasing the loop size ($n = 3$ –12 cytosine bases), they observed loop-dependent fluorescence, with all except the smallest loop ($n = 3$) giving a mixture of green and red species (sequence (76)).⁶⁶ A similar study was done by Liu and co-workers using DNA of different stem-loops ($n = 5, 8, 11$, and 14 cytosine bases) (sequences (77)–(81)).⁷³ Dual emitters were also obtained in most samples. They have indicated that light exposure and $NaBH_4$ treatment have reversibly decreased and recovered fluorescence for red emitter, whereas an anti-correlation was observed for green emitter species. In this work, they highlight that red emitters from samples of larger loops are more sensitive to photobleaching and redox-induced quenching. Interestingly, in contrast to Gwinn's finding, they found that samples of larger loops ($n = 11$ and 14) formed pure red-emitters.⁷³ This likely implies that apart from loop region, base-pairs in the duplex stem also contribute to the formation of AgNCs. This is further exemplified in a study using templates consisting of thymine-loop and cytosine-/guanine-rich stems (sequences (82) and (83)).¹²³ While the thymine-loop is known as innocuous, Wang and co-workers inferred that the cytosine/guanine rich stem provides the binding sites for Ag^+/Ag^0 attachment, especially the N7 sites of guanine bases. The introduction of a thymine-loop possibly protects the

embedded AgNCs from bulk water and enhances the fluorescence emission.¹²³

In general, hairpin loop-derived AgNCs are often prepared in buffer only, considering that additional salt disfavours loop formation.¹²⁵

Table 2.4. A summary of ssDNA hairpin loop-hosted AgNCs

DNA sequence (5' → 3')	$\lambda_{\text{ex}}/\lambda_{\text{em}}$ (nm)	N _{Ag}	Quantum yield (%)	Synthesis ratio (DNA:Ag ⁺ :BH ₄ ⁻); buffer	Remarks	Ref.
TATC ₂ GT- X ₅ -ACG ₂ ATA (72) X = C	581/643	1	N/A	1:5.6:11.2; 40 mM NH ₄ OAc, pH 6.9	Loop of different base type 1. N _{Ag} is determined from ESI-MS	97
(73) X = G	544/641	1				
(74) X = T	N/A	2				
(75) X = A	471/534	2				
(76) TATC ₂ GT-C _n -ACG ₂ ATA n = 3–12	Group I: 553–600/614–668 (n = 3–8) Group II: 543–560/610–628 (n = 9–12) Group III: 450–483/534–596 (n = 4–8; 11–12) Group IV: 407–413/524–530 (n = 9–10)	N/A	34 (n = 7; red emitter); 3.7 (n = 9; green emitter)	1:6:2; 40 mM NH ₄ OAc, pH 6.9	C-loop of different size 1. Grouping (I – IV) is defined by abrupt fluorescence attenuation as loop size (n) increases 2. For group I and III, λ_{em} generally red-shift as loop size increases 3. All samples from (76), except n = 3, produce distinct red and green species 4. (78) and (79) produce distinct red and green species; with the green emitter being the oxidised species and red as reduced species.	66
(77) CCCCCC-C ₈ -GCCCCG	438/520 557/626	N/A	N/A	1:18:8; 20 mM HEPES, pH 7.6	5. Addition of Hg ²⁺ weakens the binding of DNA-Ag and	73
GCATATCG-C _n -CGATATGC (78) n = 5	466/522 555/649	N/A	N/A	1:18:18; 20 mM HEPES, pH 7.6		73

DNA sequence (5' → 3')	$\lambda_{\text{ex}}/\lambda_{\text{em}}$ (nm)	N _{Ag}	Quantum yield (%)	Synthesis ratio (DNA:Ag ⁺ :BH ₄ ⁻); buffer	Remarks	Ref.
(79) n= 8	450/550 530/630				causes irreversible quenching	
(80) n= 11	564/630					
(81) n= 14	570/635					
(82) (ACCC) ₃ -T _n -(GGGT) ₄ n = 0, 4, 5, 6, 8, 10	512/581 580/639	1-4 (n = 5)	N/A	1:6:6; 10 mM Tris-HNO ₃ , pH7	<u>T-loop with C-/G-rich stem</u> 1. Red fluorescence ($\lambda_{\text{em}} = 639$ nm) is 7x weaker than yellow ($\lambda_{\text{em}} = 581$ nm) 2. For (82) , when n = 0, excited-state lifetime is shorter and fluorescence intensity is 12.5x weaker 3. Fluorescence intensity increases as x increases, elucidating that longer stem may provide better protection environment. 4. Adding Mg ²⁺ , Pb ²⁺ or K ⁺ affect the stability of stem duplex or converts guanine-rich strand to G-quadruplex, thus influencing the emission intensity. This enables logic gate construction.	123
(83) (AACCC) _x -T ₅ -(GGGT) _x x = 1-4		N/A				

2.6.2 dsDNA strand as a more rigid template

When comparing a complementary pair of cytosine- and guanine-rich DNAs in the form of ssDNA and dsDNA, AgNCs generated by the latter showed negligible fluorescence signal. This is not surprising since the favourable binding sites of Ag^+ ions (*i.e.* heterocyclic bases) are rendered inaccessible in a fully complementary dsDNA as a result of WC base pairing.¹²⁶

Conversely, some works employing guanine-rich dsDNA were able to produce fluoresced AgNCs still, with N7 site of guanines located inside the major groove of duplex accounting for the proposed Ag^+ -binding (Table 2.5; sequence **(84)**–**(90)**).^{115,127,128} This duplex DNA structure binds more Ag^+ , comparing to individual cytosine- and guanine-rich strands. This also coincides with the aforementioned stem duplex of the hairpin loop (Table 2.4; sequences **(82)** and **(83)**).

In spite of this contradictory finding, dsDNA offers a good alternative to nest AgNCs because its rigid structure may impede the formation of multiple emitting species. In order to circumvent the poor accessibility of Ag^+ to dsDNA, many other structural derivatives have been introduced, including gap, abasic, mismatch site, bulge, loop and NCB. All these elements share the similarity of being imperfect duplexes, with the “imperfect site” serving to nucleate Ag^+ (Figure 2.7).

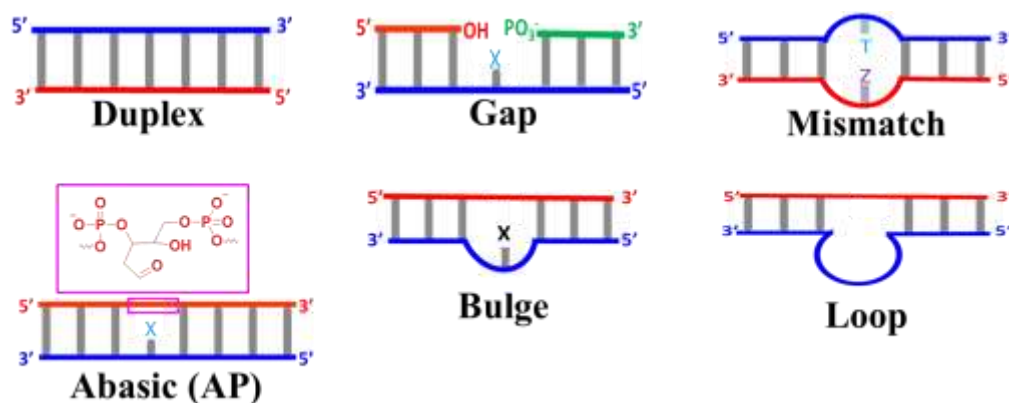


Figure 2.7. Structural derivatives (*i.e.* gap, mismatch, abasic (AP), bulge and loop) from dsDNA (duplex).

2.6.2.1 dsDNA containing gap, abasic, mismatch site, bulge and loop

Table 2.5 summarises the reported dsDNA containing gap, abasic, mismatched site, bulge and loop for AgNCs preparation.

A gap-site can be formed by hybridising a long ssDNA strand with two short complementary ssDNA strands, with one or more nucleotides eliminated from the resulting duplex. The vacant and constrained gap-site can serve to harbour AgNCs, particularly when having a cytosine base opposite to it (sequences **(91)** and **(92)**). Cui and co-workers elucidated that AgNCs were formed almost immediately after reduction (*i.e.* 1.5 h). They reasoned that without a phosphate group around the gap site, BH_4^- was able to approach cytosine-bound Ag^+ easily.⁷⁴

Comparatively, an abasic (AP) site contains one or more missing DNA bases with phosphodiester backbone remains intact. Therefore, its fluorescence evolution is longer by virtue of repulsion from the phosphate backbone. To form emissive AgNCs, an unpaired cytosine opposite an AP site and two flanking guanine bases are needed (sequence **(93)**). It was found that the Ag_2 dimer geometrically forms in the limited space of the AP site. When two

consecutive AP sites are introduced, λ_{em} blue-shifts, suggesting the formation of larger Ag_4 (sequences **(94)** and **(95)**).⁶⁷ Apart from the AP site, Ma *et al.* further advanced their investigation on adjacent flanking bases. Interesting, the flanking bases were invariably guanines in order to enhance the signal (sequence **(93)**).¹⁰⁵ As sequences located one base away from the AP site were modified to thymine, cytosine, adenine and guanine, the emission red-shifted (sequence **(96)**). They postulated that this was as a result of base stacking interaction with the band gap of AgNCs, thereby changing the matrix dipole of AgNCs.⁶⁷

Another way to construct an imperfect duplex is to introduce mismatched pairs, *i.e.* non-WC pairs (base pairs besides guanine–cytosine and thymine–adenine). In this manner, Ag^+ ions are proposed to bind at or near the mismatched site, then reduced and protected by the dsDNA scaffold. The fluorescence intensity decreases in the order of thymine–cytosine > thymine–guanine > thymine–thymine mismatches, which correlates well with the affinity of the nucleobases with Ag^+ . Moreover, the fluorescence intensity increases when the number of mismatched pairs increase, affirming that AgNCs form at the mismatched sites (sequences **(97)**–**(99)**).¹²⁶ Another study focused on the number of mismatched cytosine–cytosine pairs (n), given the strong binding of Ag^+ –cytosine (sequence **(100)**). It was found that incorporating four consecutive cytosine–cytosine pairs in dsDNA generates spectrally pure emission, assignable to Ag_4 . Comparatively, multiple emitters were formed when $n = 3$ or 5. The authors reasoned that the emission property of Ag_4 from the former is less dependent on their topological geometry.¹²⁹

A bulge site (*a.k.a* insertion mutation in genome DNA) is created when two complementary DNA strands hybridise, with one strand containing extra inserted base(s). An emissive AgNCs is formed by having a cytosine opposite to a bulge site is surrounded by guanine context (sequence **(101)**).⁶⁴ Contradictory to this, another study shows that a bulge site with unpaired thymine can distinctively fluoresces. Peng *et al.* explained that the bulged thymine is located in a more intrahelical state, so it is able to interact with Ag⁺ better (sequence **(102)**).⁶⁸ In either case, the authors hypothesised that the stacking reaction with flanking bases is responsible for the optical behaviours of AgNCs. Cross-comparison was made on AgNCs nested in bulge, gap and AP sites by using oligomers with identical sequences. In terms of fluorescence evolution, AgNCs on bulge sites display similar behaviour as AP site analogues, in which repulsion from the phosphate backbone prolongs the cluster growth. However, the bulge site-templated AgNCs exhibit more intense fluorescence than the rest (sequence **(101)**).^{64,68}

The dsDNA-derived loop structure resembles the hairpin loop in Section 2.6.1.3, except that the stem duplex is made of two individual DNA strands (*i.e.* S1 and S2, with S1 possessing an inserted loop; refer to Table 2.5). The loop structure can also be denoted as a bulge, but it contains more unpaired bases that are able to form a loop circle. Again, the dsDNA containing cytosine-loop is of particular interest as it facilitates the formation of AgNCs. Wang and co-workers reported a yellow-emitting species using a complementary duplex with an inserted cytosine-loop (sequence **(103)**–**(106)**). Notably, the fluorescence was quenched when the single-base mismatch was located less than three bases away from the loop. It is speculated that the mismatched site

alters the loop configuration, subsequently hampering the stability of the encapsulated AgNCs.¹³⁰ Another study demonstrated the use of cytosine-loop dsDNA for the strand exchange reaction (sequence **(107)**). Through the addition of another ssDNA with a longer complementary region to S2, the competitive hybridisation disrupts the stability of loop-hosted AgNCs, consequently turning off the fluorescence signal.¹³¹ Similarly, loop size shows an influence on the emission intensity and wavelength, but no detailed study has been reported to date.¹³⁰ The bases in the complementary stem contribute to the formation of AgNCs as well. As can be seen from sequences **(104)**, **(106)** and **(107)**, inserting a C₆-loop in stem duplexes of different compositions produces AgNCs of different emission colours.

Divalent salts, such as Mg²⁺, are commonly added during preparation of dsDNA-templated AgNCs. Such addition was found to enhance emission of AgNCs due to its efficient charge screening of the DNA phosphate backbone.⁵⁶

Table 2.5. Reported dsDNA templates that have been used to form AgNCs

DNA sequence	$\lambda_{\text{ex}}/\lambda_{\text{em}}$ (nm)	N_{Ag}	Quantum Yield (%)	Synthesis ratio (DNA:Ag ⁺ :BH ₄ ⁻); buffer	Remarks	Ref.
(84) 5'-G ₃ T ₄ G ₄ -3' 3'-C ₃ A ₄ C ₄ -5'	554/616	2–6	5 ± 1	1:4:4 for (77) and (78), 1:6:6 for (79); PBS, pH 7	Duplex 1. (84)–(86): N_{Ag} is determined from ESI-MS. 2. Longer cytosine in (89) & (90) stimulates higher fluorescence intensity 3. Longer guanine stimulates higher fluorescence intensity as compared to (89) 4. Hybridisation with guanine-rich strand can enhance the fluorescence.	115
(85) 5'-G ₄ T ₄ G ₄ -3' 3'-C ₄ A ₄ C ₄ -5'	635/706	N/A	7 ± 1			
(86) 5'-A(G ₄ C) ₃ G ₅ C-3' 3'-T(C ₄ G) ₃ C ₅ G-5'	597/666		10 ± 1			
(87) 5'-(C ₃ A) ₂ C ₃ GAGA(TGC) ₂ -3' 3'-(G ₃ T) ₂ G ₃ CTCT(ACG) ₂ -5'	517/583	N/A	N/A	1:10:10; 0.02 M PBS, pH 7.4		128
(88) 5'-(C ₃ A) ₂ C ₃ GAGA(TGC) ₂ -3' 3'-(TG ₃) ₄ CTCT(ACG) ₂ -5'	515/579					
(89) 5'-(C ₃ A) ₄ GAGA(TGC) ₂ -3' 3'-(G ₃ T) ₂ G ₃ CTCT(ACG) ₂ -5'	515/577					
(90) 5'-(C ₃ A) ₄ GAGA(TGC) ₂ -3' 3'-(TG ₃) ₄ CTCT(ACG) ₂ -5'	515/577		39.7			
(91) 5'-GCTCA(TG ₂) ₂ G ₂ CAGCGC ₂ TC-3' 3'-CGAGT(AC ₂) ₂ XC ₂ GTCGCG ₂ AG-5'	560/643 (X = C)	N/A	47.2	1:15:3; 20 mM PBS with 1 mM Mg ²⁺ , pH 7	Gap 1. Base opposite to gap site is represented as X. 2. Bright fluorescence only forms when X = C).	74
(92) 5'-C ₂ ACG ₂ ATCTGA G ₃ TGA ₃ TAT ₂ CTC-3' 3'-G ₂ TGC ₂ TAGACTXC ₃ ACT ₃ ATA ₂ GAG-5'	585/665 (X = C)					
(93) 5'-ATG ₂ TG GY GGCAGCG-3' 3'-TAC ₂ ACC X CCGTCGC-5'	588/670 (X = C)	N/A	N/A	1:10:11.6; 20 mM PBS, 1 mM Mg(OAc) ₂ , pH 7	Flanking bases & the base opposite to AP site 1. Flanking bases are shown in	105

DNA sequence	$\lambda_{\text{ex}}/\lambda_{\text{em}}$ (nm)	N_{Ag}	Quantum Yield (%)	Synthesis ratio (DNA:Ag ⁺ :BH ₄ ⁻); buffer	Remarks	Ref.
					red and base opposite to AP site is represented as X.	
(94) 5'-ATGT ₂ G ^G Y ^G GGTCAG ^G Y ^G GT ₂ ATG-3' 3'-TACA ₂ CCCCAGTCC ^C CA ₂ TAC-3'	588/670	2	N/A	DNA/Ag ⁺ varied; Ag ⁺ :BH ₄ ⁻ = 4.1; 20 mM PBS with 1 mM Mg ²⁺ , pH7	Number of AP sites 1. (94) with two distinct AP sites gives higher emission intensity than that with one AP site.	67
(95) 5'-ATG ₂ TG ^G Y ^G GCAGCG-3' 3'-TAC ₂ ACC ^C CCCGTCGC-5'	550/618	4				
(96) 5'-ATG ₂ TM ^G Y ^G PCAGCG-3' 3'-TAC ₂ AN ^C CC ^C QGTCTGC-5' M/P = A, T, C or G; N/Q = complementary to M/P	469– 585/ 546–667	2	N/A	DNA/Ag ⁺ varied; Ag ⁺ :BH ₄ ⁻ = 4.1; 20 mM PBS with 1 mM Mg ²⁺ , pH7	Sequences one base away from AP site 1. N_{Ag} is determined from Job's plot of emission intensity vs [DNA]:[Ag ⁺]. 2. When M = G and P is varied: λ_{em} red-shifts from 546 to 670, in the order of T < C < A < G. 3. When P = G and M is varied: λ_{em} red-shifts from 619 to 670 nm, in the order of T < C < A < G.	67
(97) 5'-C ₃ TA ₂ C ₃ TA ₂ C ₃ TA ₂ C ₃ T-3' 3'-G ₃ AT ₂ G ₃ ZT ₂ G ₃ AT ₂ G ₃ A-5' Z = A, T, C or G	520/570 (Z = T)	3–6 (Z = T)	8.1 %	1:6.6:6.6; 10 mM Tris-HNO ₃	Mismatched site with T 1. Mismatched base pairs are in blue and purple	126
(98) 5'-G ₂ CACA ₃ CACGCAC ₂ TCA ₂ -3' 3'-C ₂ GTGT ₃ GTTCTGTG ₂ AGT ₂ -5'	520/570	N/A	N/A		2. N_{Ag} is determined from ESI-MS.	

DNA sequence	$\lambda_{\text{ex}}/\lambda_{\text{em}}$ (nm)	N_{Ag}	Quantum Yield (%)	Synthesis ratio (DNA:Ag ⁺ :BH ₄ ⁻); buffer	Remarks	Ref.
(99) 5'-AG ₃ T ₂ TG ₃ T ₂ AG ₃ T ₂ TG ₃ -3' 3'-TC ₃ A ₂ TC ₃ A ₂ TC ₃ A ₂ TC ₃ -5'	520/570					
(100) 5'-GCATGTAC ₂ C _n G ₂ A ₂ GATCG-3' 3'-CGTACATG ₂ C _n C ₂ T ₂ CTAGC-5' n = 3, 4, 5	563/654 (n = 4)	4 (n = 4)	N/A	1:4:2; 20 mM PBS with 1 mM Mg ²⁺ , pH 7	<u>Mismatched site with C</u> 1. N_{Ag} is determined from Job's plot of emission intensity vs [DNA]:[Ag ⁺]. 2. One-size AgNCs form when n = 4; whereas n = 3 & 5 show large wavelength dependent emission ($\Delta\lambda_{\text{em}} = 150$ nm) 3. Presence of low concentration of halides can enhance the fluorescence.	¹²⁹
(101) 5'-ATG ₂ TG ₂ GG GGCAGCG-3' 3'-TAC ₂ ACCXCCGTCGC-5'	589/652 (X = C)	N/A	N/A	1:15:6; PBS with 1 mM Mg ²⁺ , pH 7	<u>Bulge C</u> 1. X = bulge base (A, T, C or G) and flanking bases are shown in red and blue. 2. (101)-AgNCs are 6.7x and 2x brighter than the corresponding gap- and AP-derived AgNCs	⁶⁴
(102) 5'-CGCTGCCXGCAC ₂ AT-3' 3'-GCGACGC CGTG ₂ TA-5'	565/624 (X = T)	N/A	N/A	1:15:6; 20 mM PBS with 1 mM Mg ²⁺ , pH 7	<u>Bulge T</u>	⁶⁸

DNA sequence	$\lambda_{\text{ex}}/\lambda_{\text{em}}$ (nm)	N _{Ag}	Quantum Yield (%)	Synthesis ratio (DNA:Ag ⁺ :BH ₄ ⁻); buffer	Remarks	Ref.
5'-GTGCAC ₂ TGACTC ₂ TG T G ₂ AGA ₂ G-3' 3'- CACGTG ₂ ACTGAG ₂ -C _n -C A C ₂ TCT ₂ C-5' (103) n = 4	485/560 560/630	N/A	N/A	1:6:6; 20 mM PBS with 1 mM Mg(OAc) ₂ , pH 7	<u>Effect of loop size and distance of mutation point</u> 1. Mutation points are shown in red . 2. Only fully matched duplex forms bright fluorescence 3. n = 6 gives the best differentiation between fully matched and mismatched stem region.	¹³⁰
(104) n = 6	520/572		34 ± 2			
(105) n = 8	560/620		N/A			
(106) 5'-G ₂ AT ₂ AT ₂ GT ₂ A ₃ T A T ₂ GATA ₂ G ₂ ATATA-3' 3'-C ₂ TA ₂ TA ₂ CA ₂ T ₂ -C ₆ - T A T ₂ CTAT ₂ C ₂ TATAT-5'	476/545	N/A	N/A	1:6:6; 20 mM PBS with 1 mM Mg(OAc) ₂ , pH 7	<u>Identity of mutation point</u> 1. Matched T/A gives the most distinctive signal than other mutation points. Other matched base pairs are distinguishable from their mutated analogues but with much weaker signal	¹³⁰
(107) 5'-CT ₂ CTC ₂ A-C ₆ -CAG ₂ AGTCAG ₂ TGCAC-3' (S1) 3'- GA ₂ GAG ₂ AGTC ₂ TCAGTC ₂ ACGTG ACT₂GAT₂ GT-5' (S2)	575/635	N/A	N/A	1:6:6; 20 mM PBS with 1 mM Mg(OAc) ₂ , pH 7	<u>Strand exchange reaction</u> 1. S1 and S2 are complementary (except extra sequences in S2 highlighted in orange)	¹³¹

2.6.2.2 Nanocluster Beacons (NCB)

Molecular beacons, stem-loop oligomers with each end tagged with a fluorophore and a quencher, have been extensively employed in the sensing field. As the molecule is internally quenched in the closed state, adding a target sequence that is complementary to the loop region can force the stem hybrid to open and restore its fluorescence signal.¹³² NCB is a nanocluster version of molecular beacon (Figure 2.8), which was devised and articulated by Yeh *et al.*⁹ The concept is that when a pendant overhang (*a.k.a* enhancer) is placed in proximity to another DNA strand bearing dark AgNCs (*a.k.a.* nucleation sequence), the fluorescence signal will be lit up. The design and the essential components of NCB are illustrated in Figure 2.8.

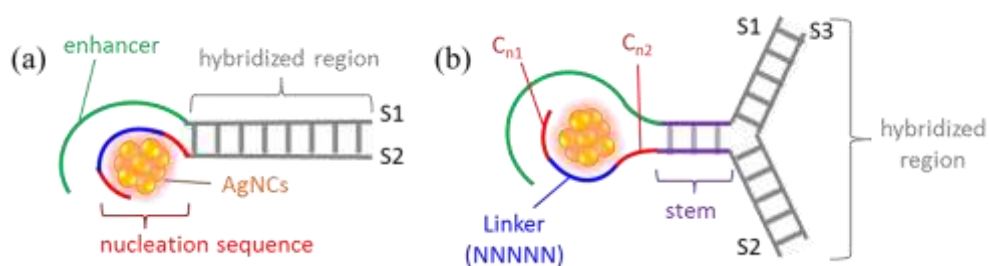


Figure 2.8. A schematic illustration of an NCB system made of (a) two and (b) three DNA strands. Each individual DNA strand is labelled as S1, S2 and S3. The structural components of NCB are shown in different colours, including nucleation sequences (red), linker (blue), enhancer (green), hybridised region (grey) and stem (purple).

The structural features and roles of each component in NCB are described below. First of all, an NCB possesses a nucleation sequence to harbour AgNCs. In most cases, dark AgNCs are preferred so that these adducts can morph into an optical switch. The general nucleation sequence is 5'-C_{n1}NNNNNC_{n2}-3', with two polycytosine heads (*i.e.* C_{n1} and C_{n2}) connected by a linker (*i.e.* NNNNN). The polycytosine serves to stabilise AgNCs and also as modulator of λ_{em} . As shown by sequences (108)–(115) in Table 2.6, changing the number of cytosine (n) from 2 to 8 can shift λ_{em} as much as 140

nm. On the other hand, the linker normally consists of adenine and/or thymine, which interact weakly with Ag^+ . It modifies the alignment between nucleation sequence and the enhancer; thereby regulating the enhancement ratio of the fluorescence intensity. The linker influences λ_{em} as well but to a lesser extent as compared to polycytosine heads (sequences **(116)**–**(125)**). In relation to the composition of the linker, placing a cytosine base adjacent to the C_n head favours green species formation (sequences **(126)**–**(130)**).¹³³

Secondly, another important component of an NCB is the enhancer that is responsible for lighting up the dark AgNCs. It has been suggested that the fluorescence enhancement is due to electron transfer from guanine bases to AgNCs.^{9,134,135} Thus, polyguanine is widely used as overhang pendant, with N7 of guanine is proposed to be responsible for the activation of red fluorescence.^{27,135} It was found that the red fluorescence doubles with every two guanines added and the optimal number of guanine bases reported to date is 15.⁹ In contrast, a cytosine-rich overhang results in a weaker emission owing to irreversible cluster-transfer from the nucleation sequence to the cytosine appendage. In addition, it was deduced that the larger size of guanine polarises AgNCs and conspicuously affects the emission, albeit cytosine has a stronger dipole.¹³⁶ While polyadenine shows innocuous result, the polythymine enhancer generally produces a green light-up colour (sequence **(131)**–**(133)**).^{9,27} It is also mentionable that alignment of the enhancer and the nucleation sequence is important for optimal interaction. Collectively, the NCB system allows tuning of emission colour from green to red, either by (i) using a different base composition of nucleation sequence; (ii) using an

enhancer of different base composition or length; or (iii) adjusting the relative position of the AgNCs to the enhancers.^{27,135}

Another structural feature of NCB is the hybridised region. Similar to a linker, this part mainly consists of adenine or thymine in order to give a lower fluorescence background, so that it enables large enhancement upon adding an enhancer. Alternatively, one can also incorporate another DNA strand to form a stem, which can tighten the interaction between the nucleation sequence and the enhancer. In this case, three individual DNA strands (*i.e.* S1, S2 and S3) are employed to construct the NCB (concept in Figure 2.4b). However, one may prefer to include NCB under the dsDNA section since its fundamental designs requires only two DNA templates (concept in Figure 2.4a). The ideal length of stem is 3 base pairs, as a longer stem has the drawbacks of a higher background signal.²⁷

The fluorescence of an NCB somehow decays fast due to photobleaching. This can be overcome since the dark AgNCs formed after reduction and before being hybridised with the enhancer are stable. Upon being activated by the enhancer, the sample generally has narrow time window of a few hours. Hence, one can use the enhancer instead of unstable NaBH_4 for signal activation making NCB an ideal design for field application.^{25,27,135} Furthermore, one can construct a logic device simply by adding a partially complementary strand that contains a cytosine- or thymine-rich overhang to a dark cluster-laden DNA. The hybridisation results in red or green fluorescence enhancement, thus acting as a signal transducer for DNA logic gates.¹³⁷

Table 2.6. A summary on design of NCB

DNA sequence ^a	$\lambda_{\text{ex}}/\lambda_{\text{em}}$ (nm)	Enhancement ratio	Synthesis ratio (DNA:Ag ⁺ :BH ₄ ⁻); buffer	Remarks	Ref.
(108) C ₂ TTATC ₂	580/650	1851 ± 686	1:12:6; 20 mM PBS, pH 6.7	<u>Polycytosine heads (C_{n1} and C_{n2})</u> 1. Longer C _n gives higher emission intensity 2. Emission peak blue-shifts as the length (n) increases	133
(109) C ₃ TTATC ₃	645/695	213 ± 8			
(110) C ₃ TTATC ₄	580/640	3465 ± 928			
(111) C ₄ TTATC ₄	580/635	760 ± 97			
(112) C ₅ TTATC ₅	525/585	408 ± 6			
(113) C ₆ TTATC ₆	525/590	242 ± 6			
(114) C ₇ TTATC ₇	525/590	62 ± 13			
(115) C ₈ TTATC ₈	460/555	24 ± 2			
(116) C ₃ TC ₄	649 ^b	356 ± 15	1:12:6; 20 mM PBS, pH 6.7	<u>Linker (NNNNN):</u> 1. Adding T to 3' side of linker cause red shift 2. Adding A to 3' side of linker cause blue shift 3. Placing C (underlined) close to PolyC heads tend to activate green emitting species	133
(117) C ₃ TT ₄	646 ^b	479 ± 28			
(118) C ₃ TTAC ₄	646 ^b	324 ± 7			
(119) C ₃ TTAAC ₄	633 ^b	824 ± 105			
(120) C ₃ TTAATC ₄	636 ^b	3465 ± 928			
(121) C ₃ TTAATTC ₄	650 ^b	238 ± 17			
(122) C ₃ TTAATTAC ₄	635 ^b	659 ± 87			
(123) C ₃ TTAATTAAAC ₄	632 ^b	527 ± 73			
(124) C ₃ TTAATTAAATC ₄	637 ^b	398 ± 17			
(125) C ₃ TTAATTAAATTC ₄	655 ^b	219 ± 11			
(126) C ₃ CTAATC ₄	640 ^b	161 ± 9			
(127) C ₃ TCAATC ₄	630 ^b	413 ± 79			
(128) C ₃ TTCAATC ₄	640 ^b	579 ± 27			
(129) C ₃ TTACTC ₄	620 ^b	132 ± 5			

DNA sequence ^a	$\lambda_{\text{ex}}/\lambda_{\text{em}}$ (nm)	Enhancement ratio	Synthesis ratio (DNA:Ag ⁺ :BH ₄ ⁻); buffer	Remarks	Ref.
(130) C ₃ TTAAC ₄	635 ^b	277 ± 18			
(131) T ₁₂	490/535	N/A	1:6:6; 20 mM PBS pH 6.6	<u>Enhancer (hanging region)</u>	9,27
(132) G ₃ (AG ₄) ₃	520/590	N/A			
(133) G ₃ (TG ₄) ₃	580/636	N/A			

^aSequence of the relevant component. Full sequence is not shown here

^b λ_{em}

2.6.3 tsDNA and DNA nanostructures

Apart from ssDNA and dsDNA, one can use a more complicated DNA scaffold to host AgNCs. This includes tsDNA and DNA nanostructure that are made of three or more DNA strands. Since the research on these two categories is relatively underexplored, both tsDNA and DNA nanostructures will be presented under the same section. The reported tsDNA templates are given in Table 2.7. Templates for DNA nanostructures are excluded since they are more complicated. The explicit illustration for these nanostructures can be found in ref.⁵⁷ and ¹³⁸.

tsDNA is formed when a third strand binds to the major groove of a B-form double helix. One of the common base triads is $CG \cdot C^+$, where there is a necessity of cytosine protonation (C^+) to form a *Hoogsteen*-based parallel triplex. Ihara *et al.* revealed that Ag^+ can displace the N3 proton of C^+ and form $CG \cdot CAg^+$, which stabilises the otherwise unstable triad at neutral pH (Figure 2.6).¹³⁹ By harnessing this feature as a useful template, Qu and co-workers demonstrate that two successive $CG \cdot C^+$ sites are indispensable for nucleating Ag_2 clusters.¹⁴⁰ As the number of $CG \cdot C^+$ sets increases, the emission intensity increase while λ_{em} remains unchanged, clearly illustrating the formation of site-specific and homogenous AgNCs. A profound diminution of emission intensity was observed when tsDNA consisting of only $TA \cdot T$ was used, further substantiated $CG \cdot C^+$ is the formation site for AgNCs. Apart from that, the relative position of $CG \cdot C^+$ is crucial, whereby discrete $CG \cdot C^+$ fails to nest AgNCs and three successive $CG \cdot C^+$ causes formation of multiple species (sequences (134)–(138)).

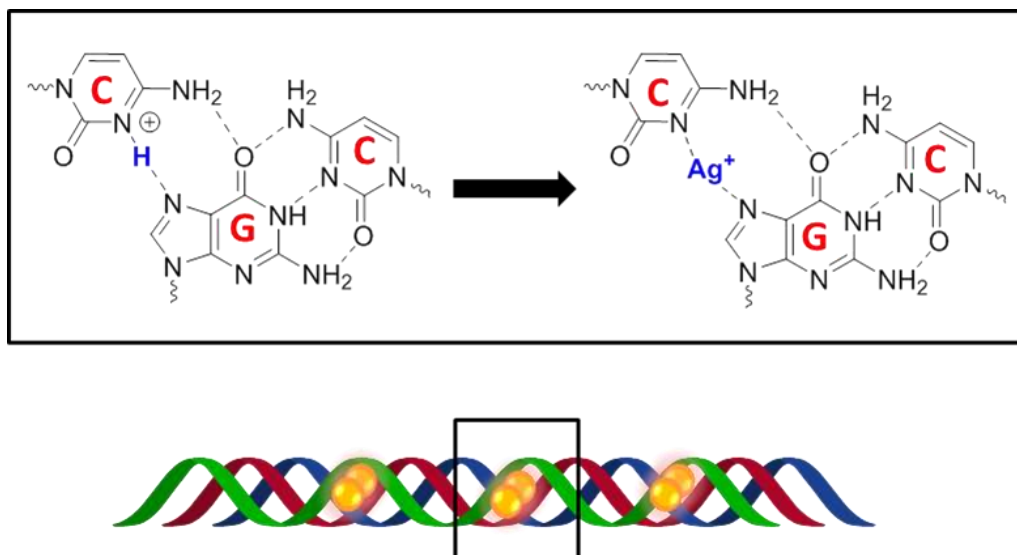


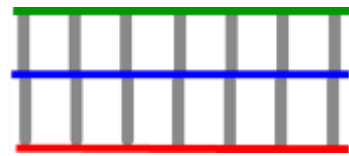
Figure 2.9. Schematic representation of AgNCs formation by using tsDNA

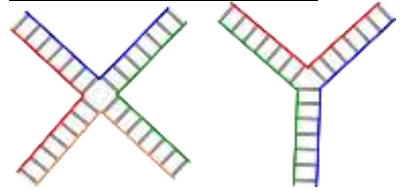
Multiple ssDNA can further assemble into branched DNA nanostructures, such as X- and Y-shaped DNA, which contain four and three arms, respectively. Since the structure is composed of dsDNA, its interaction with Ag^+ is more restricted and slower. The branch and junction within the structure may offer the matrix to encapsulate AgNCs. Since X-DNA has one additional arm as compared to Y-DNA, AgNCs of the former are brighter (sequences (139) and (140)).⁷⁵

A more intricate DNA structure termed DNA origami is significant breakthrough invented by Rothemund in 2006.¹⁴¹ The word “origami” originates from the Japanese art of paper folding. In the context of DNA origami, the three dimensional structure is formed *via* folding a long ssDNA into defined shaped, aided by hundreds of short DNA strands (*a.k.a* staple strands). The spatially addressable origami cage has been proven to template and even arbitrarily shape metal nanoparticles.^{141–144} To realise site specificity and uniform distribution of AgNCs on the DNA nanostructure, Yan *et al.* employed a triangular DNA origami with one sugar-modified arm. AgNCs were immobilised at these predefined sugar units *via* Tollens reaction, where

Ag^+ was reduced by aldehyde sugar molecules. The site-specific deposition of AgNCs on one are of the triangle was remarkably depicted by Atomic Force Microscope (AFM) image.¹³⁸ Notably, the λ_{em} is 418 nm, which is the shortest λ_{em} of DNA-AgNCs reported to date. Other work was done by Fyngson and co-workers on site-specific assembly of AgNCs on DNA nanotubes. To spatially control AgNCs along the nanostructure, the tube was assembled from DNA tiles with hairpin appendages. The hairpins were designed to protrude radially outwardly to nucleate AgNCs. The cluster adducts on hairpin tubes and freely diffusing hairpins were spectrally similar to each other, with both showing green and red emission.⁵⁷ Taken together, these nanoscaffolds provide a unique platform to site-specifically locate AgNCs. It is worth highlighting that although the nanostructure is structurally complicated, its clusters-laden DNA strand is similar as other DNA templates, *i.e.* 15-mer ssDNA and hairpin bearing cytosine-loop.

Table 2.7. Selected tsDNA templates used to form AgNCs

DNA sequence	$\lambda_{\text{ex}}/\lambda_{\text{em}}$ (nm)	N _{Ag}	Quantum Yield (%)	Synthesis ratio (DNA:Ag ⁺ :BH ₄ ⁻); buffer	Remarks	Ref.
(134) 5'-TCTCTCTCTCTT-3' 3'-AAGAGAGAGAGAGGA-5' 5'-TTCTCTCTCTCTCT-3'	N/A	N/A	N/A	1:6:24 or 1:8:32; 10 mM PBS containing 100 mM NaNO ₃	<u>tsDNA</u>  1. N_{Ag} is determined from ESI-MS 2. CG·C⁺ sites are shown in red 3. (138)-AgNCs give higher intensity than (137)-AgNCs	140
(135) 5'-TTCCTTCCTTCCTT-3' 3'-AAGGAAGGAAGGAA-5' 5'-TTCCTTCCTTCCTT-3'	480/534	2				
(136) 5'-TTCCTTTTTCCTT-3' 3'-AAGGGAAAAGGGAA-5' 5'-TTCCTTTTTCCTT-3'	420–480/ 530–550	3				
(137) 5'-TTCCTTTTTCCTT-3' 3'-AAGGAAAAAAGGAA-5' 5'-TTCCTTTTTCCTT-3'	480/534	N/A				
(138) 5'-TTCCTTCCTTCCTTCCTT-3' 3'-AAGGAAGGAAGGAAGGAA-5' 5'-TTCCTTCCTTCCTTCCTT-3'	480/534	N/A				

DNA sequence	$\lambda_{\text{ex}}/\lambda_{\text{em}}$ (nm)	N _{Ag}	Quantum Yield (%)	Synthesis ratio (DNA:Ag ⁺ :BH ₄ ⁻); buffer	Remarks	Ref.
(139) 5'-CGA CCG ATG AAT AGC GGT CAG ATC CGT ACC TAC TCG-3' 5'-CGA GTA GGT ACG GAT CTG CGT ATT GCG AAC GAC TCG -3' 5'-CGA GAC CAT ACG TAG AGC ACC GCT ATT CAT CGG TCG -3' 5'-CGA GTC GTT CGC AAT ACG GCT GTA CGT ATG GTC TCG -3'	520/621	N/A	19/8	1:7:14; 50 mM NH ₄ OAc	<u>X- and Y-shaped DNA</u>  (139) and (140) contains composition ratio of C & G bases of 55.6 % (140)-AgNCs shows slower reaction which may due to smaller number of base-pairs	75
(140) 5'-CGA CCG ATG AAT AGC GGT CAG ATC CGT ACC TAC TCG -3' 5'-CGA GTC GTT CGC AAT ACG ACC GCT ATT CAT CGG TCG -3' 5'-CGA GTA GGT ACG GAT CTG CGT ATT GCG AAC GAC TCG-3'	N/A/630	N/A	11.1			

2.7. Comparison among different DNA scaffolds

After going through all types of DNA scaffolds for AgNCs, this section will summarise the general DNA structural and sequence rules for AgNCs preparation, as well as the impact of different DNA scaffolds on the properties of AgNCs:

i. Short DNA vs long DNA

For all DNA templates, the common length ranges from 12 to 35 mer. Short DNA strands (<15 mer) commonly result in intramolecular reaction, with multiple DNA strands binding to the silver adducts, such as dimers, intermolecular i-motif and intermolecular G-quadruplexes.^{49,115} On the other hand, it was reported that longer ssDNA normally gives better yield and stability.⁶¹ Nonetheless, some DNA of moderate length, *e.g.* sequences (19) and (44) are only 20–22 mer, afford higher yields than many other longer strands.^{41,53,70} Strong evidence is still lacking so far and more efforts are needed to corroborate this finding

ii. Flexibility vs rigidity

As previously discussed, primary structure ssDNA is the most widely used template by virtue of its flexibility.^{8,60} However, its downsides, *i.e.* forming multiple species and causing wavelength-dependent emission shift, may balance against this advantage.^{78,107} Although one can obtain the pure species *via* purification, it complicates the preparation of AgNCs and a considerable amount of studies still focus on unpurified AgNCs and their application. A very recent solution reported by Vosch *et al.* is characterising the spectral

heterogeneity by means of time-correlated single photon counting (TCSPC).¹⁴⁵ Alternatively, one can choose more rigid DNA scaffold, which tend to produce single emitting species, such as dsDNA with a bulge site, dsDNA with an abasic site or tsDNA.^{67,68,140} Although the rigid scaffolds are synthetically more demanding, they allow direct usage of the as-synthesised solution without purification step.

iii. Versatility vs invariability

Simply by modifying the base composition of ssDNA, AgNCs of various emission colours can be generated, albeit their predictive code remains ambiguous. By investigating the accumulative ssDNA reported to date, a common observation is that they possess >2 motifs of consecutive guanine and/or cytosine, with alternate adenine and/thymine bases positioned in between.⁹⁶ Invariably, other scaffolds, such as i-motif, G-quadruplexes, hairpin loops and dsDNA derivatives, also require high guanine and/or cytosine contents. As compared to ssDNA, the more structured polymorphic DNA templates exhibit lower binding affinity to Ag⁺. Two independent studies have validated this point.^{97,115} Nevertheless, the restricted conformation of the structured scaffolds enable modulation of emission properties in a more controlled manner. AgNCs encapsulated by i-motifs and G-quadruplexes, for instance, are mainly governed by changing of pH and the presence of a cation⁵⁹² respectively. Another illustrative example is NCB, whose parameters such as pendant enhancer and alignment with nucleation sequence have been well defined.⁹ Another invariability to point out is that

most AgNCs emit in the green and red regions, albeit different DNA scaffolds are employed.^{66,73,98}

iv. Stability and yield

Most of the DNA-AgNCs have a shelf-life of hours to days. There are very few reported highly stable DNA-AgNCs that last for months.⁵³ Similar to other fluorophores, there is generally no valid rule to predict the photostability of DNA-AgNCs. Different fluorophores photobleach *via* different mechanism depending on their structural flexibility and local environment.¹⁴⁶ This is even more pertinent in the case of DNA-AgNCs. Zhang *et al.* reported that the temporal stability of AgNCs greatly correlates to the binding affinity of the Ag-DNA template, in which cytosine-rich templates give higher stability than other types of DNA templates.¹¹⁵ Paradoxically, as previous discussed, some cytosine-rich templates tend to generate multiple species that are inconvertible due to oxidation reaction.

Most DNA-AgNCs are stable at physiological pH. There are some exceptional cases, such as polythymine prefers high pH whereas i-motif templates are often stable at pH < 6.0.

In terms of quantum yield, since many did not report that, it is impossible to compare among different types of scaffolds. However, those with yield >50 % are mainly derived from ssDNA, which may attributed to its flexibility that promotes Ag-DNA binding (refer to Table 2.2). Of note, one can use molecular crowding to improve the quantum yield and fluorescence emission of AgNCs.¹⁰⁴ Another recent study demonstrated that GAG-containing sequences can act as

enhancers for AgNCs hosted by C₃T-rich DNA templates. Although the mechanism remains unknown, it raises profound questions concerning the possibility of improving the yield and perhaps the stability of AgNCs.¹⁴⁷

Chapter 3. Formulation of DNA chimera templates: Effects on sensing performance and emission behaviour of AgNCs

3.1. Overview

This chapter is majorly focused on the influence of AgNCs location in ssDNA chimeric template on sensing performance and emission behaviour. A total of three chimeric designs (*i.e.* containing AgNCs and aptamer sequence located at different positions) were formulated and tested for the selectiveness against adenosine molecules. Next, a control study was designed specifically to each chimeric design, in order to eliminate the possibility of false results. At the end of this chapter, an insight on mechanism of detection related to chimeric DNA-AgNCs is provided, which serves as basic guidelines to formulate aptasensors.

3.2. Introduction

Adenosine is a vital biological component presents in the body system.^{148,149} Interestingly, physiological function of adenosine differs, depending on the location and abundancy of adenosine receptors (*i.e.* A1, A2a, A2b and A3).^{150,151} To name a few examples: (i) in the central nervous system, adenosine regulates cerebral blood flow and modulates neurotransmission,¹⁵² (ii) in peripheral nervous system, adenosine involves in the control of smooth muscle contraction and regulation of cerebral and ocular blood flow,^{149,153} (iii) in renal, adenosine is involved in receptor-mediated effects,¹⁵⁰ and (iv) in tumor cells, high concentration of adenosine is accumulated in extracellular tissues, whereas for normal cells, the accumulation triggers apoptosis.^{148,149} Hence, adenosine becomes an impressive biomarker for disease detection. Of note, the levels of adenosine required to activate each receptor are varied from one to another.^{150,153}

Since the introduction of DNA-AgNCs, there are DNA-AgNCs derived designs reported to detect adenosine; *i.e.* through the incorporation of adenosine aptamer.^{18,22,25,154–156} This is because aptamer is now widely accepted as a synthetic version of an antibody and is applied in various application due to its high reproducibility, better stability, longer shelf-life and relatively small in size.^{157–159} Even though the reported DNA-AgNCs designs possess high sensitivity; they were unable to provide a wide range of detection (Table 3.1). Hence, there is a need of an assay that is able to achieve both in low detection limit and a wider linear range of adenosine detection.

Table 3.1. Comparison of dynamic range and response time for adenosine detection from other reported sensing studies.

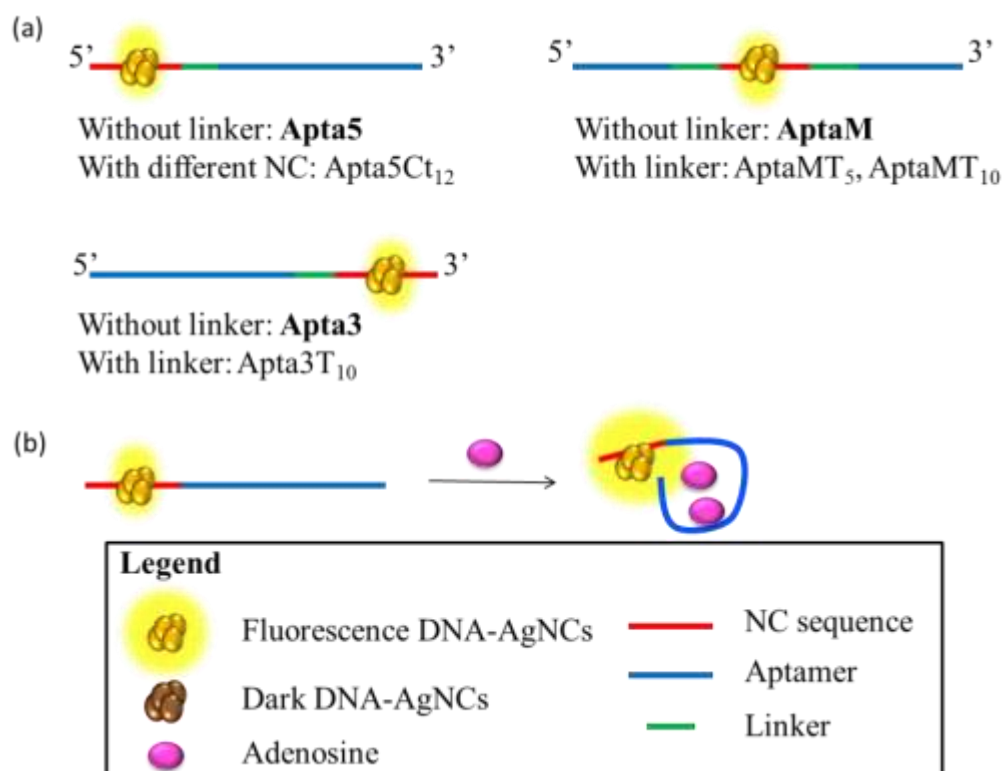
Mode of measurement	Linear range of detection	Response time	Ref.
Fluorescence (DNA-AgNCs)	0.5 – 7 μ M	1 h 30 min	²²
Fluorescence (DNA-AgNCs)	0 – 80 μ M	120 min	¹⁵⁴
Fluorescence (Fluorescent dye)	1 – 100 μ M	30 min	¹⁶⁰
Fluorescence (Fluorescent dye)	0 – 25 μ M Tunable dynamic range: 3.4 – 200 μ M 25 – 40 μ M 100 μ M – 4 mM 200 μ M – 4 mM	10 min	⁸⁷
Fluorescence (Fluorescent dye)	0 – 1400 μ M	40 min	¹⁶¹
Fluorescence polarisation	50 nM – 1 mM	30 min	¹⁶²
Chemiluminescence	10 nM – 10 μ M	2 h	¹⁶³
Colorimetric	0.3 – 0.4 mM	10 sec to 1 min	¹⁶⁴
Colorimetric	100 μ M – 1 mM	30 min	¹⁶⁵
Electrochemical	10 – 1000 nM	1 h	¹⁶⁶
Surface plasmon resonance (SPR)	0.5 – 50 pM	1 h	¹⁶⁷

In conjunction to the main objective of this thesis, an assay was constructed using chimeric design comprised of nucleation (NC) sequence and

adenosine aptamer (Apta). The three proposed chimeric conjugated designs were named as Apta5, Apta3 and AptaM, with the last letter implying the position of NC sequence at 5'-end, 3'-end and in the middle of ssDNA template (Scheme 3.1 and Table 3.2). The study was designed to address four important questions, which also serve as objectives of this chapter:

- i. Will the relative position of NC and Apta in chimeric template affect the AgNCs emission properties and sensing performance?
- ii. Is a simple chimeric derived design always induces turn-off signal upon analyte binding?
- iii. Is linker region between NC and Apta important to avoid unpredictable disturbance on AgNCs?
- iv. Will sensing performance change if
- v. Is a different NC sequence acquired will influence the sensing performance?

To answer the first two questions, NC and Apta were positioned in different location of a chimeric ssDNA) template. Considering the adenosine is small, the linker region (normally comprised of adenine and/or thymine) is omitted in all three proposed designs. This is because small molecular binding may have less or negligible disturbances on the adjacent AgNCs. To answer the last two questions, control sequences specific to each chimeric conjugated design were included for comparison, including bare NC (*i.e.* Ct₉), bare aptamer (*i.e.* Apta), chimeric conjugates with linker region (*i.e.* AptaMT₅, AptaMT₁₀ and Apta3T₁₀), as well as chimeric conjugate of different NC sequence (*i.e.* Apta5Ct₁₂) (details shown in Table 3.2).



Scheme 3.1. (a) Three different formulations of adenosine aptamer-derived AgNCs used in this study. NC sequence and adenosine aptamer are positioned in different locations of a DNA chimera. Linker region is inserted between NC sequence and aptamer if undesirable steric disturbance on aptamer binding is suspected. (b) The sensing procedure for adenosine using Apta5-AgNCs as model. The enhanced fluorescence is represented with larger yellow shadow.

3.3. Materials and methods

3.3.1 Materials and reagents

All BIO-RP purified DNA oligonucleotide (6 optical density ((OD) units) were purchased from Bioneer Inc. and dissolved in deionised water. Phosphate buffered saline (PBS) tablet, silver nitrate (AgNO₃), sodium borohydride (NaBH₄), adenosine and cytidine were purchased from Sigma-Aldrich; uridine and guanosine were purchased from Apollo Scientific. Mili-Q ultrapure water (Millipore, 18.2 MΩ) was used throughout the experiments.

3.3.2 Instrumentation

Fluorescence measurements were performed on a Tecan Infinite M-200 microplate reader (Tecan Group Ltd, Switzerland). The excitation and emission slit widths were set at 10 nm and 20 nm, respectively. Visible absorption spectra were acquired using an Epoch microplate reader (Biotek, U.S.A). Fluorescence images of DNA-AgNCs were visualised using a benchtop UV transilluminator (UVP, LLC, U.S.A) with short wavelength 302 nm and pictures were taken with a phone camera LG-H815 (LG, Korea). Scanning transmission electron microscopy (STEM) images were acquired using FEI Titan 80-300 S/TEM equipped with a high-angle angular dark field (HAADF) detector and operated at 200 kV. Circular dichroism (CD) measurements were conducted using a Jasco J-815 spectropolarimeter, with 1 cm cell path (Jasco Co. Ltd., Japan).

3.3.3 Preparation of DNA-AgNCs

DNA-AgNCs were prepared according to molar ratio of 1:6:6 (DNA:Ag⁺:BH₄⁻). Firstly, 15 µL of 100 µM DNA oligonucleotide was heated at 90 °C water bath for 5 min and cooled to room temperature for 10 min (0.1 °C/s cooling rate). Then, 25 µL of 10 mM PBS buffer (pH 7.4) and 30 µL of 300 µM AgNO₃ was added sequentially. The mixture was incubated in ice bath for 15 min. An equimolar of NaBH₄ was added to reach final volume of 100 µL and the mixture was incubated overnight before use. For subsequent studies, volume of reagents and buffer were scaled up accordingly. Unless otherwise stated, concentration of as-synthesised DNA-AgNCs was referred to the final concentration of DNA used.

3.3.4 Adenosine detection using DNA-AgNCs

The as-synthesised DNA-AgNCs were mixed with adenosine in 10 mM PBS buffer (pH 7.4), with volume (μL) ratio of 10:90:5 (DNA-AgNCs:PBS:adenosine). For control experiment, deionised water was used as blank. Cytidine, guanosine and uridine were used as negative control substrates. F_0 and F represent emission peak intensity in the absence and presence of analytes, respectively. Fluorescence spectra measurements ($\lambda_{\text{ex}} = 490 \text{ nm}$) were taken after 30 min of incubation.

Table 3.2. ssDNA chimeras used in this study. NC sequence, aptamer and linker region are shown in red, blue and green, respectively.

Name	Sequence (5' $\rightarrow$ 3')
Apta5	ACC CAC CCA ACC TGG GGG A(10)GT ATT GCG GAG GA(23)A GG
Apta3	ACC TGG GGG A(10)GT ATT GCG GAG G A(23)A GG ACC CAC CCA
AptaM	ACC TGG GGG A(10)GT AT ACC CAC CCA T GCG GAG G A(23)A GG
Ct ₉	ACC CAC CCA
Ct ₁₂	CCC TTA ATC CCC
Apta	ACC TGG GGG A(10)GT ATT GCG GAG G A(23)A GG
AptaMT ₅	ACC TGG GGG A(10)GT AT TTTT ACC CAC CCA TTTT T GCG GAG G A(23)A GG
AptaMT ₁₀	ACC TGG GGG A(10)GT AT TTTTTTTTTT ACC CAC CCA TTTTTTTTTT T GCG GAG G A(23)A GG
Apta3T ₁₀	ACC TGG GGG A(10)GT ATT GCG GAG G A(23)A GGT TTT TTT TTT ACC CAC CCA
Apta5Ct ₁₂	CCC TTA ATC CCC ACC TGG GGG A(10)GT ATT GCG GAG G A(23)A GG

*The highlighted A(10) and A(23) denotes adenine residue's position at 10 and 23.

3.4. Results and discussion

3.4.1 Characterisation of DNA-AgNCs

All three chimeric derived DNA-AgNCs (*i.e.* Apta5, Apta3 and AptaM) and Ct₉-AgNCs (*i.e.* control template) were characterised using STEM,

circular dichroism (CD) spectra and fluorescence spectra for physical and optical properties of AgNCs.

To characterise the nanostructure of AgNCs, high angular dark field (HAADF)-STEM was used, instead of the commonly used TEM.^{2,4,56,59,113} This is because the high energy electron beam from TEM will cause the aggregation of AgNCs.¹²² The HAADF-STEM images were obtained with camera length of 196 mm and a convergence angle of 25.6 mrad. STEM images showed comparable features at low magnification (Figure 3.1). The small isolated dots were identifiable as silver atoms, implying the absence of large nanoparticles and effective stabilisation by DNA templates. In contrast, the sparsely sampled image of Ct_9 -AgNCs implies that a short 9-mer DNA template is not an effective NC sequence to nucleate and stabilise AgNCs.

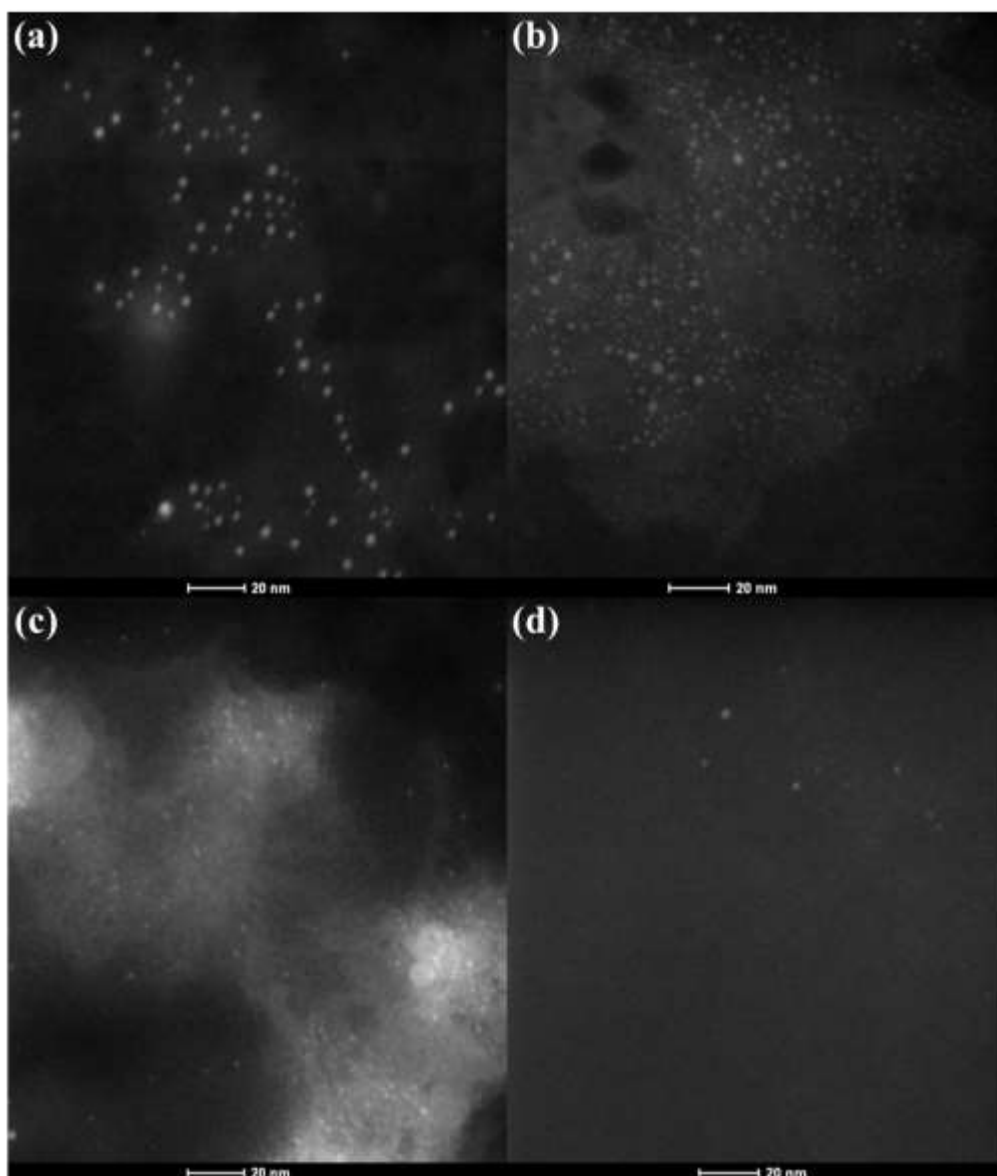


Figure 3.1. HAADF-STEM images of DNA-AgNCs from three chimeric derived-AgNCs conjugates, (a) Apta5-AgNCs, (b) Apta3-AgNCs, (c) AptaM-AgNCs and (d) control template, Ct₉-AgNCs.

To investigate the conformational change of DNA and its interaction with silver contents (Ag^+ or Ag^0), CD measurements were conducted to monitor DNA structures before and after addition of each reactant (*i.e.* Ag^+ and BH_4^-). All bare DNA templates adopted hairpin conformations, as evidenced by positive cotton effect between 260 nm and 280 nm and a negative band around 240 nm (Figure 3.2).^{168,169} Addition of Ag^+ to form DNA- Ag^+ complex does not affect the structural conformation of DNA templates.¹⁰⁶ However,

upon the formation of DNA-AgNCs, Apta3- and AptaM-AgNCs show hypsochromic shift in by 10 nm in 260 – 280 nm region, while the peak in 240 nm region remain unchanged (note that negative peak of Apta3-AgNCs is 238 nm, which is very similar to those at 240 nm). The blue-shift in long wavelength region may indicate structural change from hairpins to parallel *G*-quadruplexes, with the latter showing characteristic positive peak at 260 nm and negative peak at 240 nm. Besides that, it is possible that the peak shift is due to the ability of AgNCs to induce changes on DNA conformation.¹⁰⁶ In this case, this has signalled that encapsulation of AgNCs in 3'-end and middle region has more significant impact on the recognition binding with adenosine, as compared to that in 5'-end.

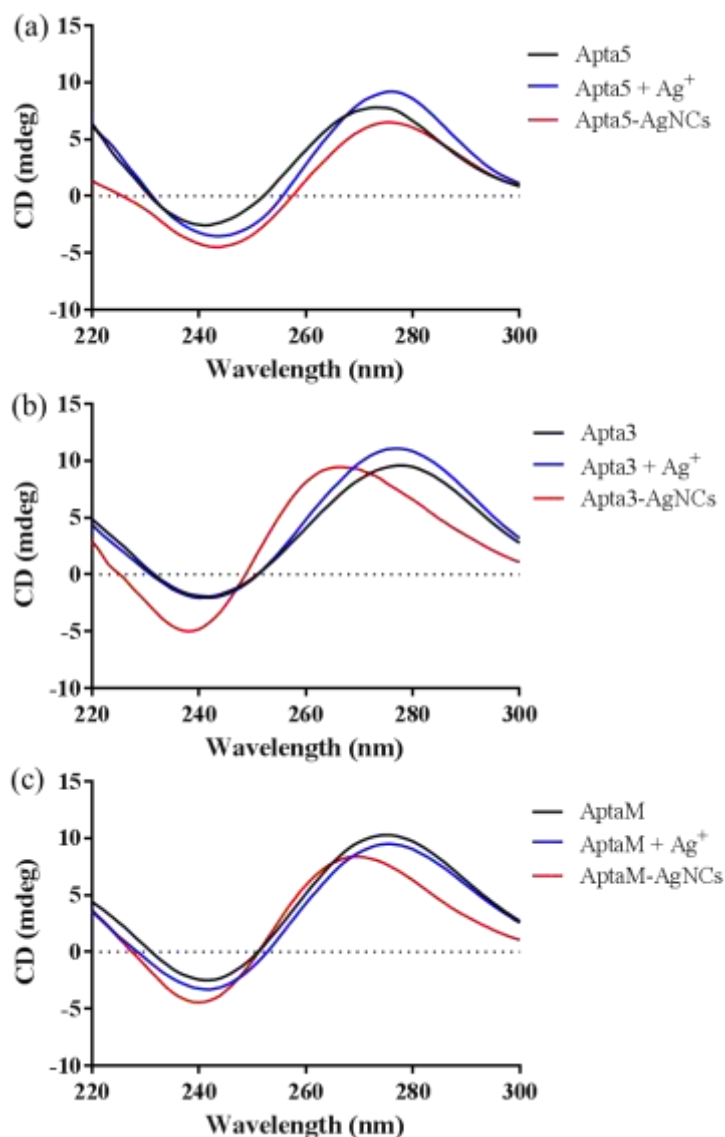


Figure 3.2. CD spectra of (a) Apt5, (b) Apt3 and (c) AptM before and after addition of silver salt and NaBH₄. 5 μ M of DNA was used for all measurements.

To study the emission properties of DNA-AgNCs, the emission spectra of bare DNA templates (*i.e.* Apt5, Apt3 and AptM) were compared to that of AgNCs. All bare DNA templates have no noticeable peak in the visible region (Figure 3.3). Upon the addition of silver salt and reduction by NaBH₄, colour of the sample solution changed from colourless to pale yellow (inset images in Figure 3.3). All Apt5-, Apt3- and AptM-AgNCs display primary emission peaks in yellow ($\lambda_{\text{ex}} = 490$ nm) and red ($\lambda_{\text{ex}} = 540$ nm) region. Ct₉, a

control sample that consists merely of NC sequence, produces only weakly fluoresced red species. Incorporating adenosine aptamer into the chimeric conjugates of Apta5, Apta3 and AptaM has changed the dominant species to yellow, as manifested by emission spectra and UV images in Figure 3.3. Such change is presumably due to multi-base bonding between AgNCs and neighbouring aptamer bases,⁶⁷ as well as intrinsic flexibility of longer Apta5, Apta3 and AptaM templates which may affect HOMO-LUMO gap.^{91,98}

In addition, AgNCs derived from all three chimeric conjugates give much higher emission intensity than that of Ct₉, which may be attributed to the presence of guanine-rich adenosine aptamer. It is worth mentioning that adenosine aptamer has approximately 52 % of guanine content and known to form *G*-quadruplex as a ready site for adenosine binding.^{158,170} The effect of emission enhancement in all chimeric designs has corresponded to a recent work reported by Wang's group, showing that *G*-quadruplex is able to enhance the fluorescence of AgNCs in a chimeric template.^{9,128} Notably, fluorescence enhancement was also observed in AptaM-AgNCs, indicating that split *G*-quadruplex segments can retain the same role on fluorescence enhancement too.

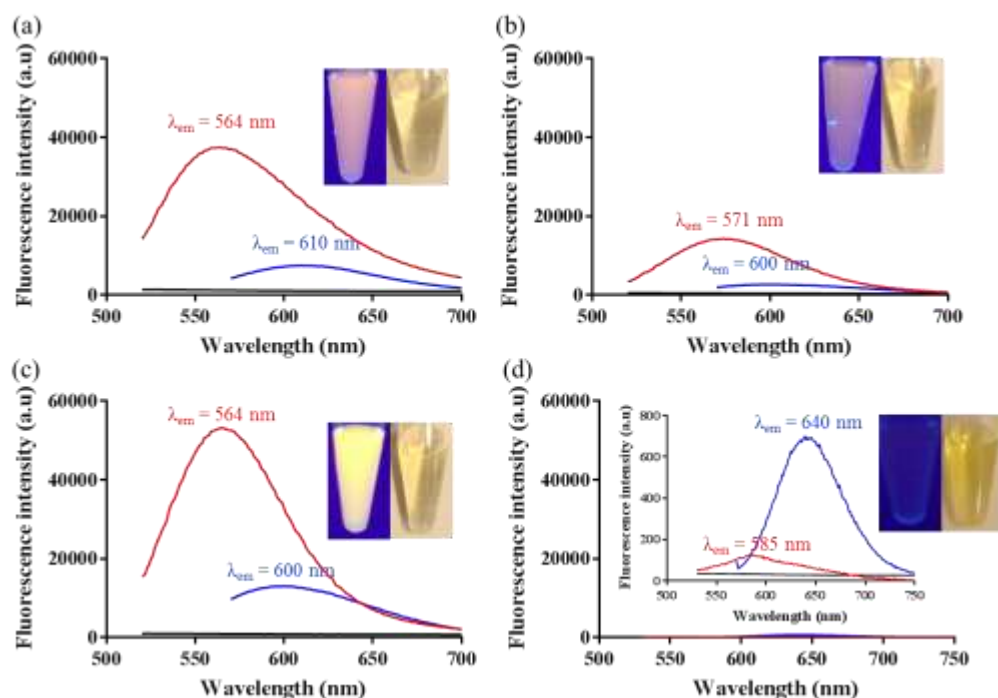


Figure 3.3. Emission spectra of (a) Apta-AgNCs, (b) Apta3-AgNCs, (c) AptaM-AgNCs and (d) Ct₉-AgNCs, excited at 490 nm (red) and 540 nm (blue). The bare DNA templates before AgNCs conjugation show no fluorescence (black). Both spectra and UV images of AgNCs (inset) were taken at 16 h after addition of NaBH₄.

Interestingly, the emission peaks of all three chimeric derived DNA-AgNCs are similar under various excitation wavelengths (Figure 3.4a - c). The similar emission peaks indicates that AgNCs of fixed size are selectively resides in the same region for all three samples.^{53,67,68,78,171,172} It is thus speculated that AgNCs are encapsulated in NC region, given all three chimeric conjugates formed the same emitting species as Ct₉-AgNCs. This is further supported by the attempt to prepare AgNCs using Apta as template, which contains only adenosine aptamer (Figure 3.4d). Comparatively, no obvious fluorescence was observed for Apta-AgNCs due to the absence of NC region. The emission bands shift significantly to longer wavelength as λ_{ex} increases, indicating a wide distribution of different emissive population. Apart from spectra heterogeneity, all emissive species are weakly fluoresced. Evidently, it strengthens the suggestion on the high affinity of NC region (*i.e.* Ct₉) to host

AgNCs, with the yellow-emitting species selectively and dominantly forms in NC region, despite the coexistence of aptamer region.

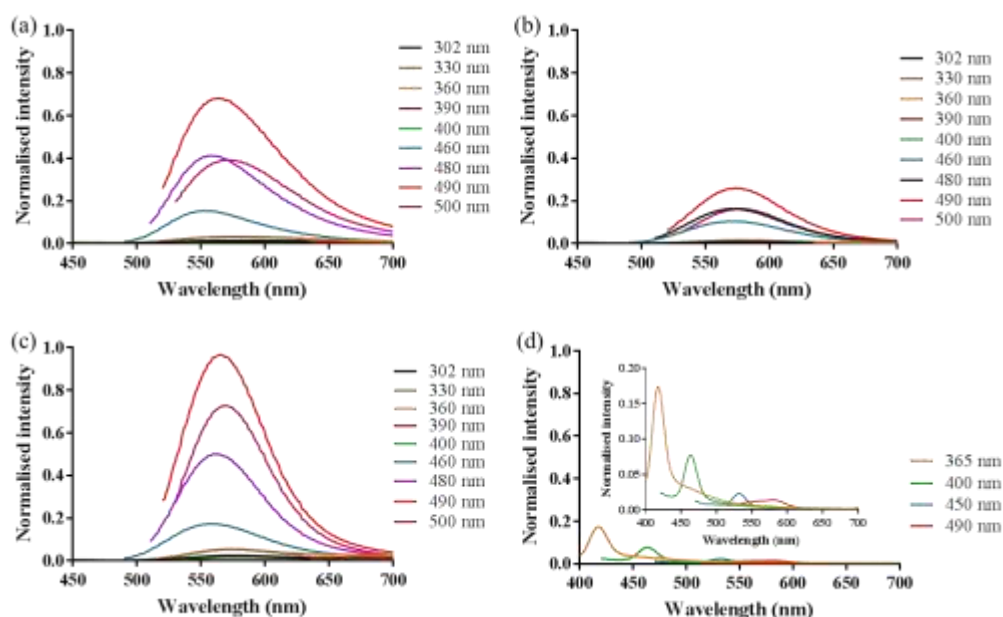


Figure 3.4. Emission spectra of three chimeric derived-AgNCs conjugates (a) Apta5-AgNCs, (b) Apta3-AgNCs and (c) AptaM-AgNCs excited from 302 nm to 500 nm. (d) Control study using Apta as DNA template for AgNCs preparation, with emission spectra excited from 365 nm to 490 nm. Concentration of DNA-AgNCs used for measurement was 15 μ M.

Notably, the red species for all samples decayed fast (<16 h). Hence, the following photostability and sensing studies focus only on yellow species (Figure 3.5). The instability of Ct₉-AgNCs is likely attributed to its short DNA template which unable to protect AgNCs effectively.^{49,107} Among the three chimeric conjugates, AptaM-AgNCs has considerably slower initial growth, but they showed the highest intensity and best photostability (retained 43 % of the initial fluorescence intensity after 122 h). This is followed by Apta5-AgNCs and Apta3-AgNCs. The different emission behaviour of AptaM-AgNCs may be attributed to dipole interaction between the cluster adducts nested in the middle region and the neighbouring bases on both sides. Ma *et al.*⁶⁷ reported that stacking interaction with flanking bases can modulate the dipole moment of a base, concomitantly affecting the emission energy of

AgNCs. Since the NC region of AptaM is sandwiched by split aptamer sequences, it is reasonable to assume that the as-formed AgNCs have more direct communication with the flanking bases, as compared to those located at the edge of Apta5 and Apta3. Such additional interaction may further stabilise AgNCs encapsulated in AptaM template.

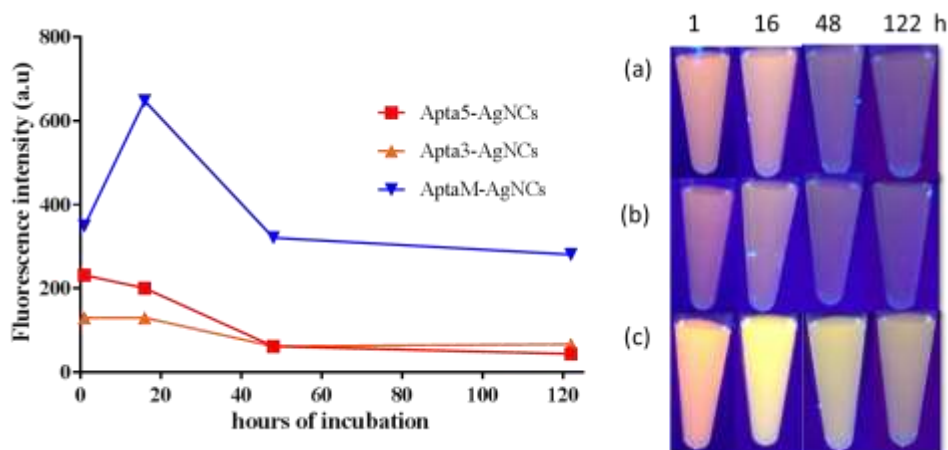


Figure 3.5. Fluorescence intensity evolution of (a) Apta5-AgNCs, (b) Apta3-AgNCs and (c) AptaM-AgNCs in yellow region after reduction with NaBH_4 . The corresponding UV images are shown as inset.

3.4.2 Comparison on fluorescence behaviour and sensing performance

The selectivity of Apta5-AgNCs, AptaM-AgNCs and Apta3-AgNCs was first tested after the characterisation of the nanoclusters. Note that the non-modified DNA aptamer to adenosine has low binding affinity (*i.e.* $6 \mu\text{M}$)¹⁵⁸ and the addition of extra 9-mer of NC sequence may affect this binding affinity. Thus, two different concentrations of analytes, *i.e.* 10 mM and 1 mM were used in selectivity tests. As depicted in Figure 3.6, only Apta5-AgNCs consistently show distinguishable fluorescence enhancement in the presence of adenosine, whilst none of the other three nucleosides (*i.e.* uridine, cytosine, and guanosine) produced statistically significant differences. In comparison, under the same experimental conditions, Apta3-AgNCs show moderate quenching upon adding adenosine, despite the relatively poor selectivity.

AptaM-AgNCs were not selective to adenosine, even though they were more photostable and strongly fluoresced, as discussed in Section 3.4.1. Therefore, in the subsequent optimisation and quantitative studies, only Apta5-AgNCs were chosen.

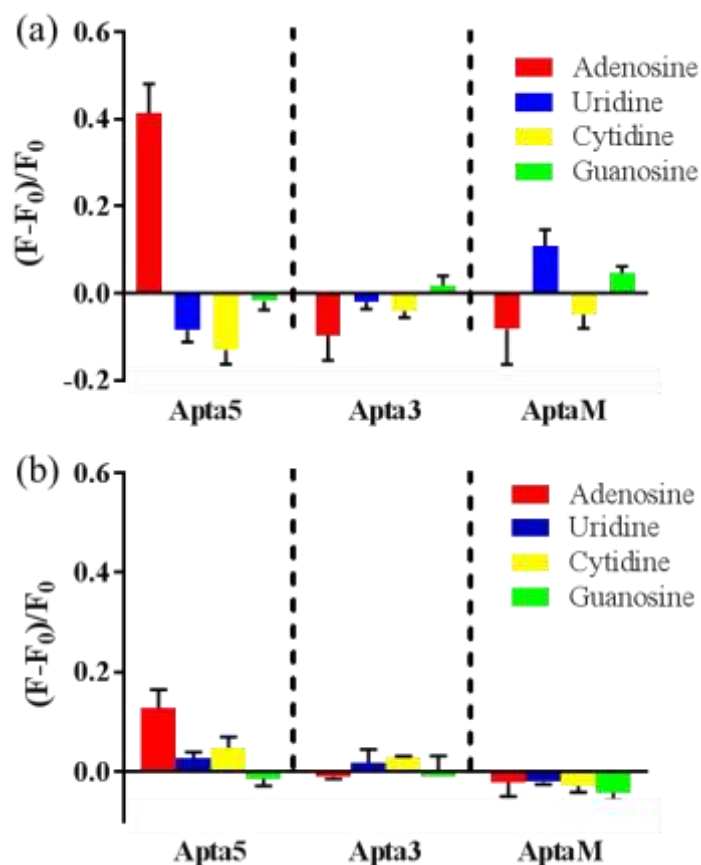


Figure 3.6. Selectivity of Apta5-AgNCs, Apta3-AgNCs and AptaM-AgNCs (15 μ M) for adenosine, relative to other control analytes at the concentration of (a) 10 mM and (b) 1 mM. Error bars represent standard deviations from three independent measurements.

To investigate why Apta5-AgNCs, AptaM-AgNCs and Apta3-AgNCs performed differently in the presence of adenosine, CD measurement was employed to study their conformation changes (Figure 3.7). Upon addition of adenosine, Apta5- and AptaM-AgNCs underwent conformational transition to anti-parallel G-quadruplex, as manifested by negative and positive bands at 260 nm and 290 nm,¹⁶⁸ respectively. Comparatively, Apta3-AgNCs retain its

original conformation structure (*i.e.* parallel *G*-quadruplex) despite the addition of adenosine.

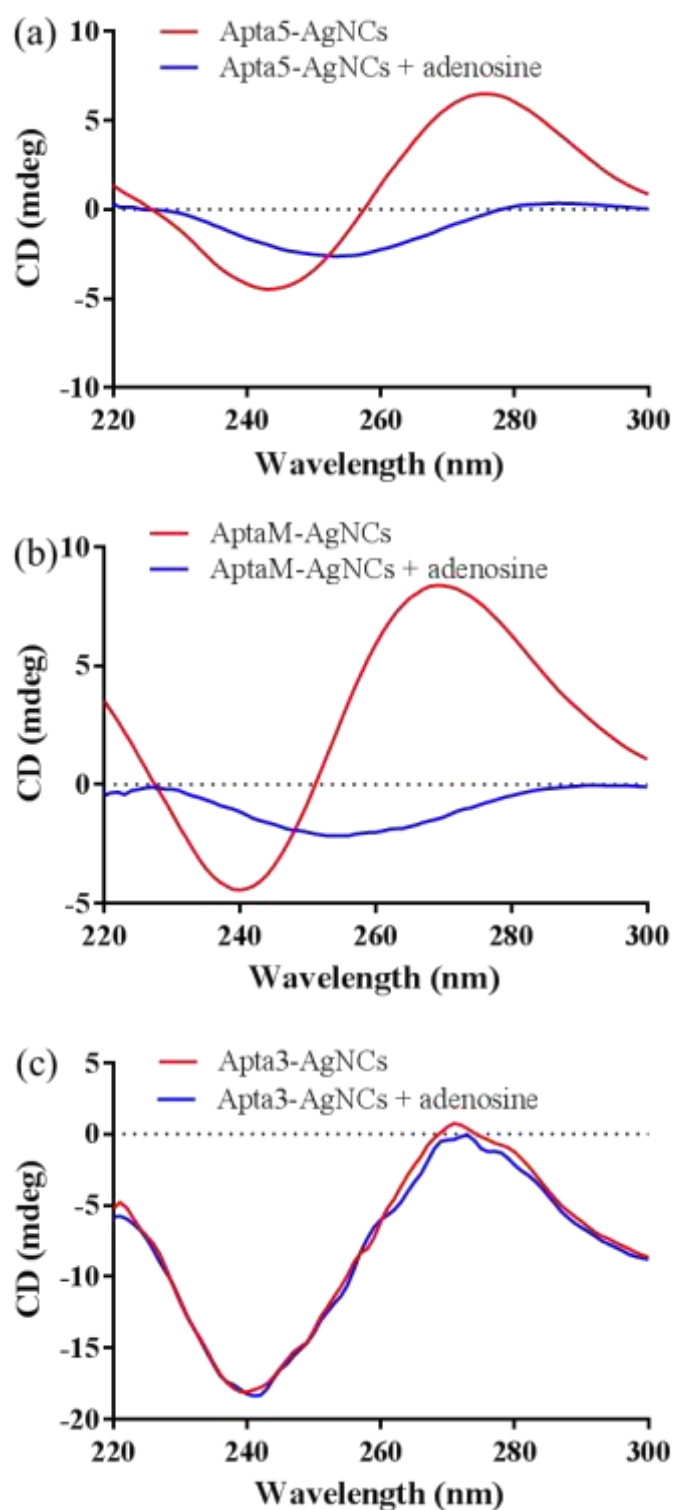


Figure 3.7. CD spectra of (a) Apt5-AgNCs (b) AptM-AgNCs and (c) Apt3-AgNCs before and after addition of adenosine.

To study the selectivity performance imposed by each chimeric design (*i.e.* Apta5-AgNCs, AptaM-AgNCs and Apta3-AgNCs) in presence of adenosine, one has to understand the binding mechanism of adenosine aptamer. Szostak and coworkers have claimed that A(10) and A(23) are responsible for the ligand-interaction sites, as well as an intact three-bases loop, –ATT– that is important to retain the binding activity of adenosine aptamer (Figure 3.8a, Table 3.2).¹⁷⁰ For Apta5 design, A(10), A(23) and –ATT– loop are conceivably distant from Ct₉ sequence; hence, the encapsulation of AgNCs in the nucleation sequence does not hinder the adenosine recognition event. In the presence of adenosine, Apta5-AgNCs underwent structural reformation into anti-parallel G-quadruplex (Figure 3.7a) which able to promotes higher emission intensity.¹²²

However, formation of anti-parallel G-quadruplex in AptaM design (Figure 3.7b) does not show the same fluorescence behaviour (*i.e.* increased in emission intensity) as Apta5. This is likely due to the dynamical behaviour of AptaM design (*i.e.* intramolecular split aptamer) with insertion of Ct₉ in the middle of –ATT– loop. Upon binding with adenosine *via* intramolecular split aptamer from both edges, it may destabilise the encapsulated AgNCs located in the middle position. Note that the CD results only provide information of DNA conformation but not to reflect the changes on the encapsulation of AgNCs. To address the poorest selectivity performance issue, a control study was carried out using AptaMT₅ and AptaMT₁₀, which contain linker regions of –T₅– and –T₁₀–, respectively, flanking on both sides of Ct₉ sequence. Normally, linker sequence is comprised of adenine and/or thymine bases because these two bases have low binding affinity to Ag⁺ and generate lower background

signal.^{9,79,133} However, the presence of any adenine base(s) in the linker region may influence the emission spectra properties.¹⁷³ Thus, linker with absolute thymine bases was used in these control studies to minimise the steric hindrance effect by AgNCs onto ligand-interaction sites, *i.e.* A(10) and A(23). However, AptaMT₅- and AptaMT₁₀-AgNCs shared similar results as that of AptaM-AgNCs (Figure 3.8b & c), thus weakening the aforementioned hypothesis.

As previously stated, one should note that two adenine residues, *i.e.* A(10) and A(23) in adenosine aptamer are responsible for ligand-interaction. While A(10) is conceivably distant from Ct₉ sequence of Apta3 design, A(23) is relatively nearer. Thus, the encapsulated AgNCs may affect the effective binding as one ligand-interaction site has been hindered for adenosine recognition. Thereby inhibit possible structural reformation upon addition of adenosine (Figure 3.7c). Notably, aptamer-target interaction is still possible in Apta3 design because the adenosine able to bind directly to the interaction sites of pre-formed *G*-quadruplex structure;^{158,170} apart from commonly known aptamer-target interaction induces conformational switches, as depicted by Apta5 and AptaM designs, as well as other similar reported studies.^{174,175} Considering the possible steric effect as discussed in AptaM design, another control study was carried out using Apta3T₁₀, by inserting a –T₁₀– linker to increase distance between A(23) and AgNCs. Interestingly, Apta3T₁₀-AgNCs show enhanced quenching capability by 20 % in the presence of adenosine (Figure 3.8d), as compared to Apta3-AgNCs (Figure 3.6). This finding indirectly supports the involvement of A(23) in adenosine binding, which is in agreement with Szostak's finding. At the moment, the underlying principle that

is responsible for the quenching of Apta3 remains unknown. However, some reported studies which employed similar design concept of placing NC sequence at 3'-end also yielded the same quenching results.^{17,28}

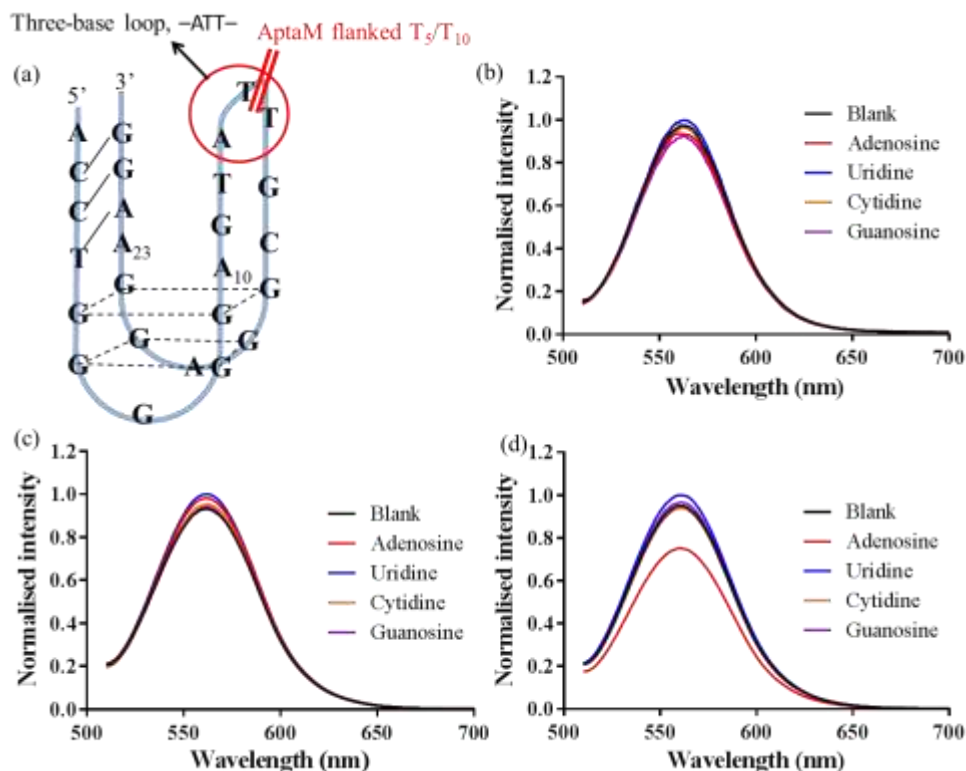


Figure 3.8. (a) Graphical illustration on the location of three-bases loop (–ATT–), A10 and A23 residues in the adenosine aptamer¹⁷⁰ as well as linker region T₅ and T₁₀ flanking both sides of AgNCs in AptaM design. Fluorescence emission spectra of (b) AptaMT₅-AgNCs, (c) AptaMT₁₀-AgNCs and (d) Apta3T₁₀-AgNCs in the presence of different analytes.

To answer the last question listed in Section 3.2, the control study continued to examine the effect on sensing performance by changing the NC sequence in Apta5. A widely reported NC sequence, Ct₁₂ was used to replace Ct₉ in Apta5 and named as Apta5Ct₁₂. Ct₁₂ is widely employed in NCB (as discussed in Chapter 2.6.2.2), given its low background signal which facilitates its uses as a turn-on sensor.^{9,27,133} However, Apta5Ct₁₂-AgNCs give neither selectivity nor turn-on signal (Figure 3.9). This finding establishes that despite AgNCs are confined in the NC region, they have direct interaction with adjacent aptamer and influence the aptamer-ligand binding. The interaction

may vary on AgNCs emitter species produced from different NC sequence (*i.e.* Ct₉ and Ct₁₂).

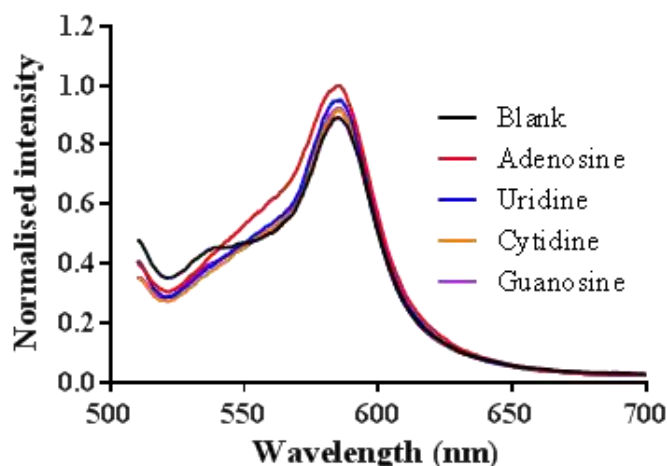


Figure 3.9. Fluorescence emission spectra of AptA5Ct₁₂-AgNCs in presence of different analytes.

3.4.3 Assay performance

Figure 3.10c shows the emission enhancement factor of AptA5-AgNCs in the presence of different concentration of adenosine based on optimised condition. Immediate analysis upon adding the analyte is possible because the emission signal does not differ much for up to 30 min (Figure 3.10a). The enhancement ratio increases linearly dependent on the concentration of adenosine added. This proposed assay shows a linear response range up to 2500 μM ($R^2 = 0.9816$), which is wider than that reported by most studies (refer to Table 3.1). As mentioned in Section 3.2, detecting a wide range of adenosine levels is necessary to meet different demands. The calculated detection limit (LOD) is 18.2 μM based on equation of $\text{LOD} = 3\sigma/s$, where σ is the standard deviation of lowest concentration of adenosine; s is the slope of the calibration curve (details on determination of LOD can be referred to Appendix III).

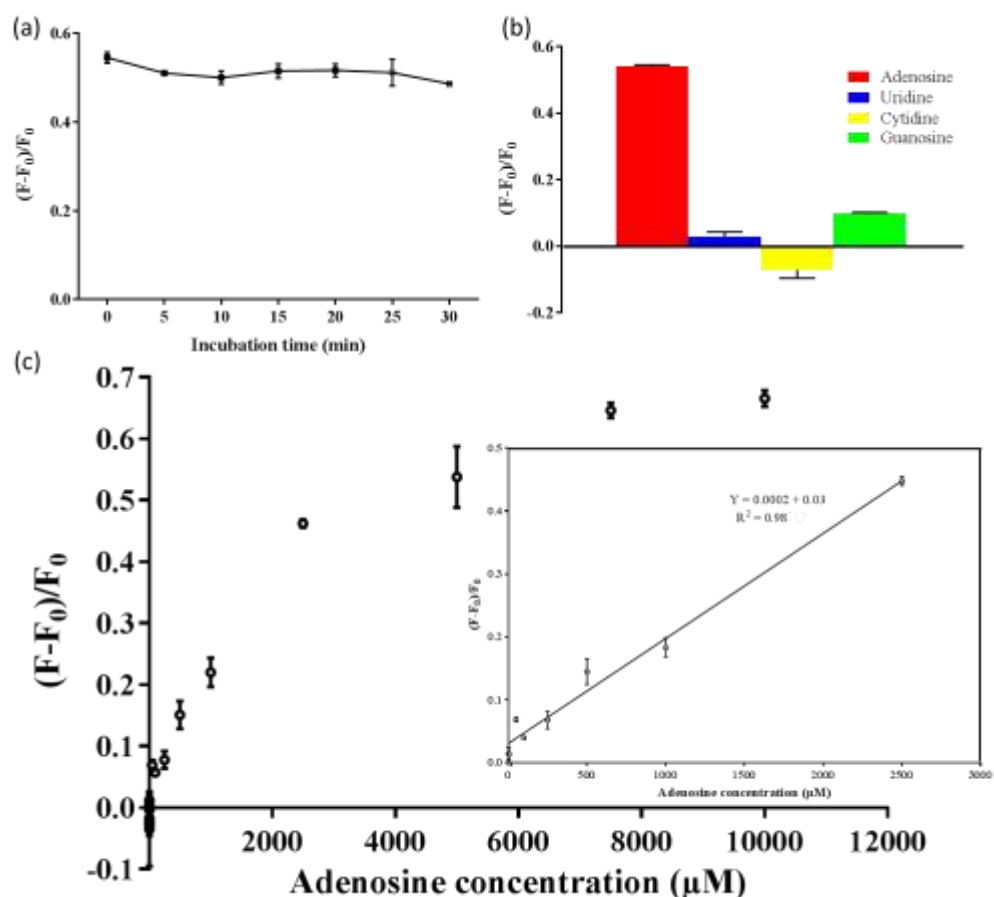


Figure 3.10. (a) Optimisation of reaction time upon addition of adenosine to Apta5-AgNCs. The fluorescence enhancement is instantaneous. (b) Selectivity test recorded immediately after adding 10 mM of analytes. (c) Plot of fluorescence enhancement of Apta5-AgNCs as a function of adenosine concentration. The inset shows the linear range of detection. Error bars represents standard deviation from three independent measurements.

3.5. Conclusion

In summary, AgNCs have been prepared using three DNA chimeras consisted of NC sequence (*i.e.* Ct₉) and adenosine aptamer located at different positions. The dominance of yellow emitting species was observed across all three chimeric conjugates, regardless of different position of Ct₉ in these templates. Interestingly, the relative position of adenosine aptamer affects the selectivity of chimeric derived-AgNCs towards adenosine, with Apta5-AgNCs giving the best response of fluorescence enhancement. Apta3-AgNCs, in contrast, were quenched and of moderate selectivity. It has been

experimentally proved that linker region is only required if AgNCs is in proximity with aptamer bases that are involved in ligand binding. In addition, different AgNCs confined in NC sequence can interact with adjacent aptamer region and alter its sensing performance. In short, using adenosine aptamer as test model, one can correlate the formulation of DNA chimera to its emission property and sensing performance of AgNCs-based aptasensor.

Chapter 4. Amplification-free and direct detection of telomerase activity in cell lysates

4.1. Overview

In this chapter, ssDNA chimeric design was further employed for the detection of telomerase activity in crude cell extracts. The content of this chapter focuses on design with NC sequence located at the 5'-end of telomerase substrate (TS) primer. This is because the 3'-end of TS primer needs to be intact for recognition and binding with telomerase enzyme. The telomerase enzyme then initiates elongation reaction by repetitively adding TTAGGG nucleotides to the 3'-end of TS primer. The results revealed in this chapter have continued to prove the potential of chimeric DNA-AgNCs in biosensing application. The end of this chapter will also reveal the important information on the possible principle of detection associated to chimeric design results in fluorescence turn-on/off.

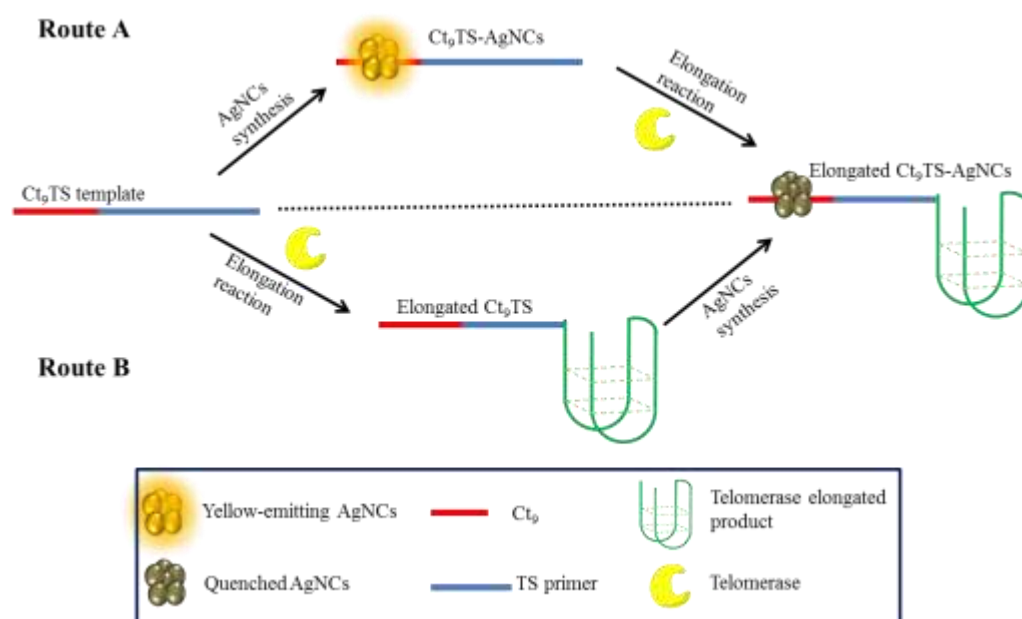
4.2. Introduction

Telomerase is a ribonucleoprotein that plays an important role in maintaining chromosome ends and preventing them from degradation, recombination and end-to-end fusion.^{176–179} Structurally, telomerase consists of (i) a recognition site, *i.e.* an RNA template that binds to telomerase repeats *in situ* and telomerase substrate (TS) primer *in vitro*, and (ii) a catalytic site, *i.e.* telomerase elongated reverse transcriptase (TERT) that adds repetitive TTAGGG nucleotides onto the 3'-end of telomeres *in situ* or the TS primer *in vitro*.^{180,181} While telomerase is absent in human somatic cells, it is highly expressed in 85 % of cancer cells,¹⁸² demonstrating its potential role as biomarker for cancer detection and prognosis.

The gold standard assay to detect telomerase *in vitro* is known as telomeric repeat amplification protocol (TRAP).¹⁸³ However, TRAP assay often suffers from polymerase chain reaction (PCR) associated artifacts and is relatively time-consuming. Thus, several PCR-free measurement methods have been developed to detect crude telomerase extract, which include electrochemical,^{184,185} chemiluminescence,^{186,187} colorimetric,^{188,189} and fluorescence.^{190–194} These methods are either clinically not feasible or have restricted practical applicability. For instance, catalytic activity of enzymes used in chemiluminescence assays is sensitive to enzyme degradation and often limited due to embedment of activity centre in the macromolecule.¹⁹⁵ Hence, the chemiluminescence often requires additional methods to enhance its operational stability and signal amplification, which subsequently increases the assay cost. Similarly, not only the dye-labeling in fluorescence methods increases production cost, the covalent attachment of reporter molecules may also affect the recognition event.^{196,197} In light of this, a new kind of fluorescent probe has been developed for direct detection of telomerase activity on cell lysates.^{23,134,198} Li *et al.*²³ and Huang *et al.*¹³⁴ have recently reported on using DNA-AgNCs for direct detection of telomerase activity through NanoCluster Beacon (NCB) approach. However, they required either long incubation time (*i.e.* 2 h)²³ or extreme high cell concentration (*i.e.* 20000 cells)¹³⁴ in order to be detected by DNA-AgNCs probe.

To further leverage the chimeric design for telomerase detection, a ssDNA template called ‘Ct₉TS’ that consist of (i) Ct₉¹⁹⁹ (*i.e.* the same NC sequence from Chapter 3) and (ii) TS primer for telomerase to bind and induce elongation reactions. The principle of detection is based on the fact that

formation of telomerase-elongated products, (TTAGGG) n can affect stability of the neighbouring AgNCs hosted in the same ssDNA. By measuring the emission intensity of AgNCs, one can selectively and quantitatively detect the telomerase activity in cell extracts, in a more rapid manner relative to conventional TRAP. To prove that AgNCs are confined in Ct₉, two different ways of detection were investigated: formation of AgNCs before and after telomerase elongation reaction, depicted as route A and route B, respectively in Scheme 4.1. In order to support the working principle of this assay, a proof-of-concept study was conducted using synthetically elongated DNA, *i.e.* Ct₉TSR n ($n = 3, 5$ and 7), where n represents different number of TTAGGG repeats.



Scheme 4.1 Two different ways to detect telomerase activity *via* formation of AgNCs before (route A) and after (route B) telomerase elongation reaction.

4.3. Materials and methods

4.3.1 Materials

All standard desalted DNA oligonucleotide (4 OD units) were purchased from Integrated DNA Technologies and dissolved in deionised water. Details of DNA sequences are listed in Table 4.1. The chemicals used include silver nitrate (AgNO₃), sodium borohydride (NaBH₄) and disodium dihydrogen

ethylenediaminetetraacetate dehydrate (EDTA) from Nacalai Tesque; ammonium persulfate (APS), *N,N,N',N'*-tetramethylethylene-1,2-diamine (TEMED), magnesium chloride (MgCl₂), potassium chloride (KCl) and boric acid from R&M Chemical; 40 % bis-acrylamide and acrylamide solution (29:1), Tris, deoxyribonucleotide triphosphate (dNTP), Taq buffer and Taq DNA polymerase from 1st Base; ethylene glycol-bis(2-aminoethylether)-*N, N, N',N'*-tetraacetic acid (EGTA), hydrochloric acid and phosphate buffered saline (PBS) pellets from Sigma Aldrich; 1X CHAPS lysis buffer (0.5 % CHAPS, 10 mM Tris-HCl, pH 7.5, 1 mM MgCl₂, 1 mM EGTA, 0.1 mM benzamidine, 5 mM 2-mercapthoethanol, 10 % glycerol) from Merck, Tween 20 from System Chemical, SYBR Safe DNA Gel stain from Invitrogen.

Table 4.1. DNA templates used in this study. NC sequence (Ct₉), primers (TS and rTS) and synthetically telomerase elongated products are shown in red, black and blue, respectively.

Name	Sequence (5' → 3')
Ct ₉ TS	ACC CAC CCA AAT CCG TCG AGC AGA GTT
Ct ₉ TSR3	ACC CAC CCA AAT CCG TCG AGC AGA GTT (AGGGTT) ₂ AGG G
Ct ₉ TSR5	ACC CAC CCA AAT CCG TCG AGC AGA GTT (AGGGTT) ₄ AGG G
Ct ₉ TSR7	ACC CAC CCA AAT CCG TCG AGC AGA GTT (AGGGTT) ₆ AGG G
Reverse TS (rTS)	CTA ACC CTA ACC CTA ACC

4.3.2 Instrumentation

Fluorescence measurements were conducted on a Hitachi F-7000 spectrofluorometer (Tokyo, Japan) with a Xenon lamp as excitation source. The excitation and emission slit widths were both set at 10 nm, with photomultiplier tube voltage at 950 V and scanning speed of 2400 nm/min. UV-visible absorption spectra were acquired using an Epoch Microplate Reader (Biotek, U.S.A). High-resolution transmission electron microscopy

(HR-TEM) images were acquired using FEI Tecnai G2-F20 operated at 200 kV with 0.2 nm resolution. Circular dichroism (CD) measurements were conducted on a Jasco J-815 spectropolarimeter (Tokyo, Japan). Centrifugation was performed on a Beckman Coulter (California, U.S.A). Telomerase extension reaction was accomplished using a Biorad thermal cycler (California, U.S.A). A ChemiDoc MP Imaging system (Bio-Rad, U.S.A) was used to capture fluorescence images.

4.3.3 Telomerase extract preparation

Telomerase was extracted using the CHAPS method.¹⁹⁴ Briefly, cells were grown to 90 % confluence and pelleted by centrifugation (900 rpm for 5 min). The cell pellet was washed twice with 1x ice-cold PBS buffer to remove residual culture medium. Then, the pellet was resuspended in 200 μ L 1X CHAPS lysis buffer by retropipeting five times before incubation in an ice bath for 30 min. The mixture was centrifuged at 14,000 rpm for 20 min at 4 °C. The concentration of telomerase extract was determined using the Bradford assay, where 200ng/ μ L corresponds to $\approx$ 1000 cell/ μ L.²⁰⁰ The supernatant containing telomerase was diluted with 1X CHAPS lysis buffer before use. Three different cancer cell types, namely, breast cancer (MCF7), colon cancer (HT29) and human nasal squamous cell carcinoma (RPMI 2650), were used in this study.

4.3.4 Detection of telomerase activity

For telomerase detection, samples of negative controls refer to telomerase extract heat-treated at 90 °C for 15 min. Emission spectra were recorded using optimised excitation wavelength (λ_{ex}) of 470 nm. F and F₀

represent emission peak intensity of AgNCs samples in the presence and absence of telomerase extracts, respectively. Unless otherwise stated, the concentration of as-synthesised DNA-AgNCs is referred to the final concentration of DNA used.

Route A: Ct₉TS-AgNCs were prepared according to a molar ratio of 1:10:10 (DNA:Ag⁺:BH₄⁻). Firstly, 20 µL of 100 µM Ct₉TS primer was heated in a 90 °C water bath for 5 min and left for 10 min to cool down to room temperature. Next, 120 µL of telomerase extension buffer (*a.k.a* TRAP buffer, containing 5 mM Tris-HCl, pH 8.3, 375 µM MgCl₂, 15.75 mM KCl, 0.0125 % Tween 20 and 250 µM EGTA) and 30 µL of 30 µM AgNO₃ were added sequentially. The mixture was incubated in ice bath for 15 min before NaBH₄ was added to reach a final volume of 200 µL. The resulting solution was further incubated for 1 h before mixed with 200 ng of telomerase extract (~1000 cells) and 200 µM dNTP for telomerase elongation reaction. The mixture was then incubated at 30 °C for 1 h. Volume of reagents and buffer used to synthesis Ct₉TS-AgNCs were scaled up accordingly in the subsequent study.

Route B: An amount of 10 µM of Ct₉TS primer was heat-treated and cooled as described as above before the addition of 200 ng of telomerase cell extract (~1000 cells) and 200 µM dNTP. The mixture was then incubated at 30 °C for 1 h. Next, the solution containing the telomerase elongated product was subjected to synthesis of DNA-AgNCs under similar conditions, as described above in section “Route A”.

4.3.5 Proof-of-concept

Ct₉TSR_n ($n = 3, 5$ and 7) was heat-treated at $90\text{ }^{\circ}\text{C}$ for 5 min , then incubated at $30\text{ }^{\circ}\text{C}$ for 1 h to mimic the condition of telomerase elongation reaction. Next, Ct₉TSR_n was subjected to preparation of AgNCs under similar conditions as described in section “Route A”. The resulting emission intensity was compared with that of Ct₉TS-AgNCs.

4.3.6 Conventional TRAP assay

A $10\text{ }\mu\text{L}$ of elongated Ct₉TS was added to $40\text{ }\mu\text{L}$ of solution containing $1\times$ Taq buffer, $200\text{ }\mu\text{M}$ dNTP, 2.5 U of Taq DNA polymerase and 200 nM of rTS. PCR was conducted with the following program: $95\text{ }^{\circ}\text{C}$ for 5 min ; 33 cycles at $95\text{ }^{\circ}\text{C}$ for 1 min , $60\text{ }^{\circ}\text{C}$ for 1 min , $72\text{ }^{\circ}\text{C}$ for 5 min ; $4\text{ }^{\circ}\text{C}$ hold. $25\text{ }\mu\text{L}$ of the PCR product was then loaded onto $4.5\text{ }\%$ (w/v) agarose gel. The gel was run at room temperature for 100 min at 80 V and visualised by SYBR Safe DNA gel staining.

4.4. Results and Discussion

4.4.1 Characterisation of Ct₉TS-AgNCs

The Ct₉TS-AgNCs was first characterised by means of HR-TEM, UV-vis and fluorescence spectroscopies (Figure 4.1). The TEM micrographs depict formation of spherical Ct₉TS-AgNCs ($\sim 3\text{ nm}$) and devoid of larger aggregates (Figure 4.1a). Similar to other DNA-AgNCs studies, both UV-vis and fluorescence results of Ct₉TS-AgNCs reveal the presence of multiple emitting species (Figure 4.1b & c).^{42,123,199,201} The UV-vis peaks at 370 and 547 nm correspond to the absorption of blue and red-emitting AgNCs. The band in $400\text{--}500\text{ nm}$ region is broad attributed to surface plasmon resonance of a trace amount of larger silver nanoparticles and absorption from the dominant

yellow-emitting AgNCs.¹³⁶ Given the response of both blue- and re-emitter toward telomerae elongation reaction is negligible (Section 4.4.2), the following studies focus only on the yellow species (optimised $\lambda_{\text{ex}}/\lambda_{\text{em}} = 470/557$ nm) that remains stable for at least 3 h (Figure 4.1d; Figure 4.2). Notably, the emission properties of Ct₉TS-AgNCs is very similar to chimeric DNA designs in Chapter 3 that used the same NC sequence, *i.e.* Ct₉.¹⁹⁹ This implies that AgNCs are also selectively confined in Ct₉ in this context, while TS primer site remains intact for telomerase binding. However, the λ_{ex} of Ct₉TS-AgNCs is more blue-shifted as compared to Ct₉Apta-AgNCs ($\lambda_{\text{ex}} = 490$ nm), possibly due to different multi-base bonding between AgNCs and the neighbouring region (TS vs adenosine aptamer).¹⁹⁹

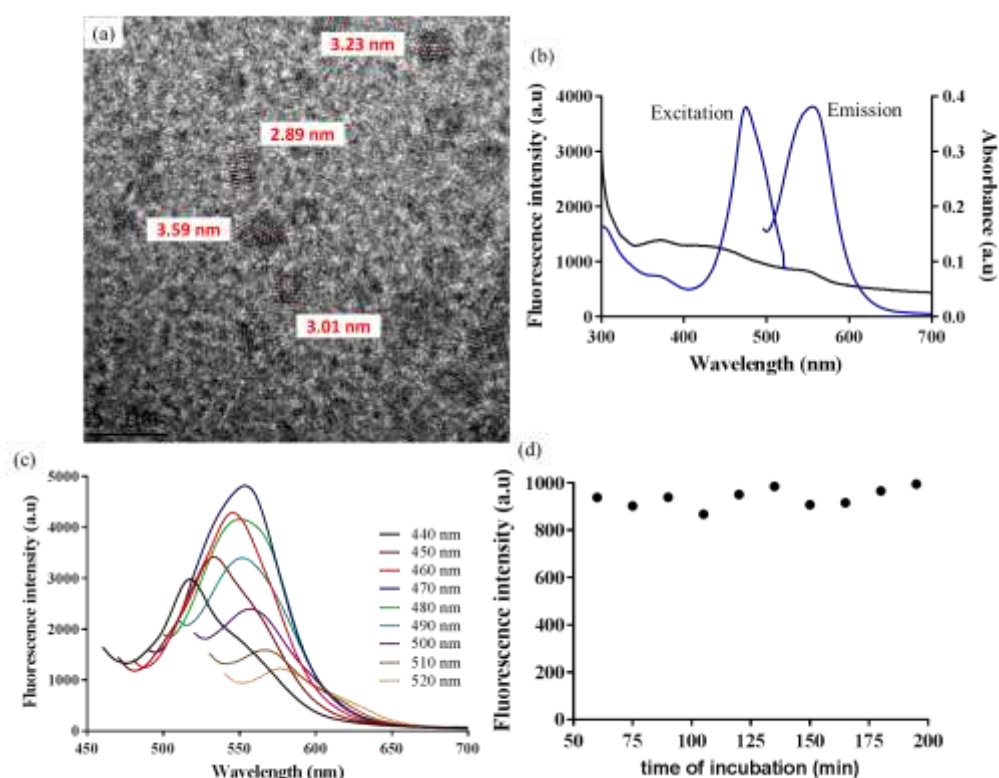


Figure 4.1. (a) HR-TEM images of Ct₉TS-AgNCs. (b) UV-visible (black) and fluorescence spectra (blue) of Ct₉TS-AgNCs. Only fluorescence data of yellow emitters are shown ($\lambda_{\text{ex}}/\lambda_{\text{em}} = 470/557$ nm). (c) Emission spectra of Ct₉TS-AgNCs excited from 440 to 520 nm. (d) Stability of emission intensity of yellow emitter. The DNA concentration used for fluorescence measurement was 450 nM.

4.4.2 Detection of telomerase activity in crude telomerase cell extract

The telomerase-elongated products, *i.e.* guanine-rich telomeric sequence, could render another possible region to host AgNCs or may induce cluster-transfer. To determine whether AgNCs preferably reside in the NC sequence (*i.e.* Ct₉) or in the elongated telomeric sequence, the emission spectra of Ct₉TS-AgNCs before (route A) and after (route B) telomerase elongation reaction was examined, using MCF7 as telomerase extract. The heat-treated MCF7 cells were used as negative controls, whereas 1X CHAPS lysis buffer served as blank to represent the absence of cell extracts. It is reasoned that if AgNCs are hosted in the elongated telomeric sequence, emission spectra collected from route A and route B will be different.

Interestingly, both routes produced the same types of emitters, implying neither cluster-transfer occurred⁷¹ nor additional emitting-species was formed²⁰² (Figure 4.2). Among the three emitters (*i.e.* blue, yellow and red species), only the major yellow species shows obvious quenching in the presence of telomerase-positive samples (Figure 4.2). Notwithstanding the quenching efficiency in route B is lower than that of route A. It is reasoned that adding Ag⁺ to the more complex synthesis medium in route B (*i.e.* containing telomerase cell extract, telomeric sequence and dNTP) has not only diminished the amount of AgNCs encapsulated in Ct₉ region, but the chelation of Ag⁺ to guanine-bases may also disrupt the G-quadruplexes of the elongated telomeric sequence.²⁰³ Considering greater practicability of a ready-made probe in the clinical setting, route A was thus chosen for the subsequent studies.

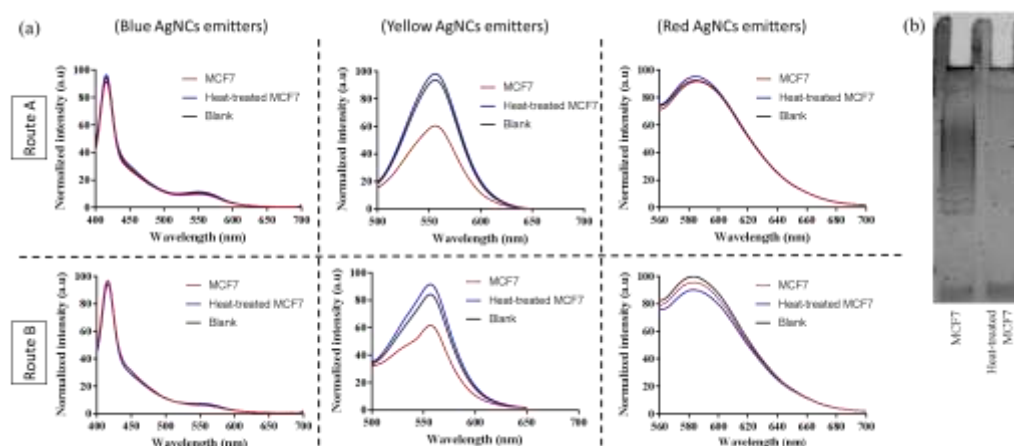


Figure 4.2. (a) Emission spectra of Ct₉TS-AgNCs prepared according to route A and route B using 250 MCF7 cells ($\lambda_{\text{ex}}/\lambda_{\text{em}}$ of blue emitters = 365/410 nm; $\lambda_{\text{ex}}/\lambda_{\text{em}}$ of yellow emitters = 470/557 nm; $\lambda_{\text{ex}}/\lambda_{\text{em}}$ of red emitters = 540/585 nm). (b) Comparison of telomerase activity on 250 MCF7 cells and heat-treated control sample. 15 % native PAGE gel was used for the gel analysis.

As indicated from the above finding, Ct₉TS-AgNCs were not only stable in cell extract, but also showed selective quenching in the presence of telomerase-positive samples. Control study proved that dNTP used in telomerase reaction does not contribute to the quenching of Ct₉TS-AgNCs, further re-affirming the quenching is specific to telomerase elongated products (Figure 4.3). To determine if Ct₉TS-AgNCs can be generally applied to different cancer cell lines, the assay is further evaluated on MCF7, HT29 and RPMI 2650 using different cell numbers. Figure 4.4 shows that all telomerase-positive samples display distinctive fluorescence quenching from the negative controls, suggesting the reliability and selectivity of our assay over a range of oncological cell lines.

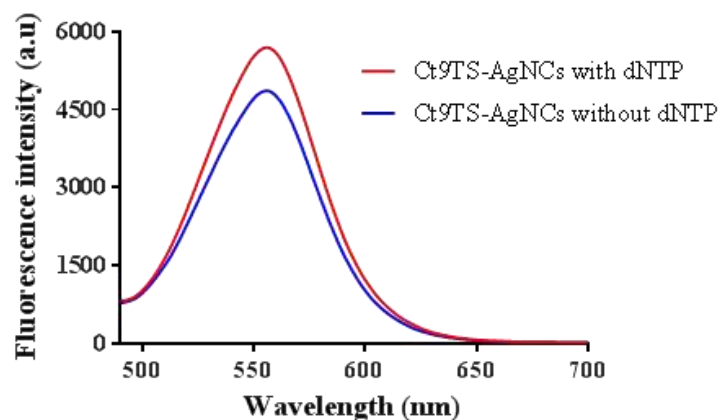


Figure 4.3. Emission spectra of Ct₉TS-AgNCs in the absence and presence of dNTP. The concentration of dNTP was 200 μ M.

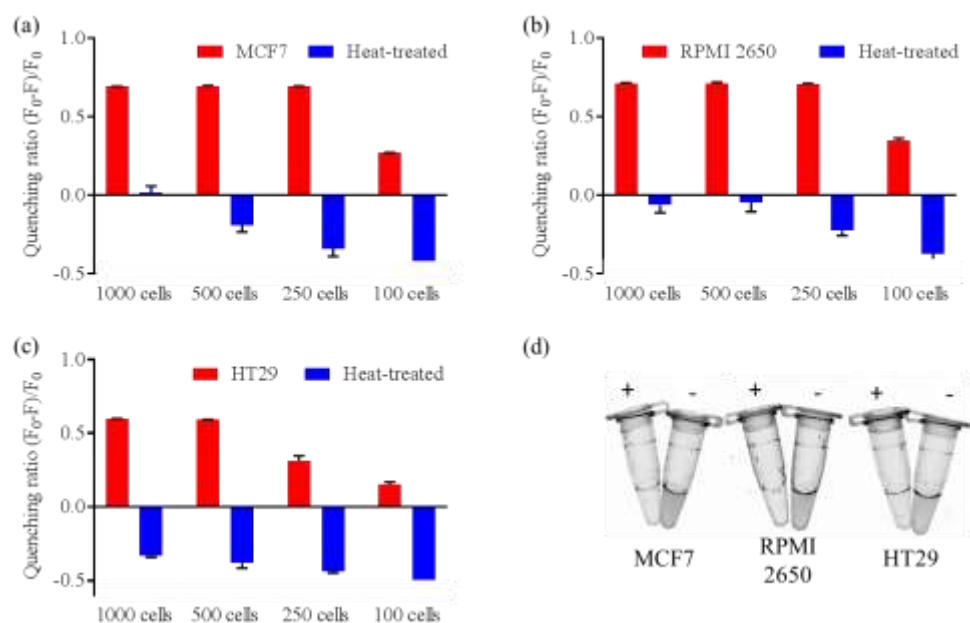


Figure 4.4. Emission response of Ct₉TS-AgNCs at 1000, 500, 250 and 100 cells of (a) MCF7, (b) RPMI 2650 and (c) HT29, with corresponding heat-denatured telomerase cell extracts as negative controls. (d) UV image of Ct₉TS-AgNCs in 250 cells of telomerase-positive (+) and -negative (-) samples. Error bars represent standard deviation from five independent measurements.

Interestingly, at the same number of cells (*i.e.* 250 cells), quenching ratios for RPMI 2650 and MCF7 are ~70 %, comparatively higher than that of HT29, implying that the former two cell lines might have higher telomerase activity than HT29 (Figure 4.4). The trend order is much clearer (*i.e.* RPMI 2650 > MCF7 > HT29) when compared at 100 cells. As an independent confirmation of the telomerase activity trend, the gold standard TRAP assay analysis was performed to crosscheck these findings. Based on the number and intensity of the telomerase-elongated PCR products, with reference to the 100 bp marker on an agarose gel (Figure 4.5a), an identical trend of telomerase activity was observed, with RPMI 2650 > MCF7 > HT29. Notably, gel analysis often requires higher amounts of elongated DNA to visualise bands in the gels, compared to this assay which needs only nanomolar concentration of elongated Ct₉TS (Figure 4.5b). Furthermore, the significant difference between emission intensity of telomerase-positive and that of negative controls was achieved without any amplification steps, indicating the higher sensitivity of chimeric DNA-templated AgNCs towards subtle changes (*i.e.* elongated Ct₉TS-AgNCs) in the subject.

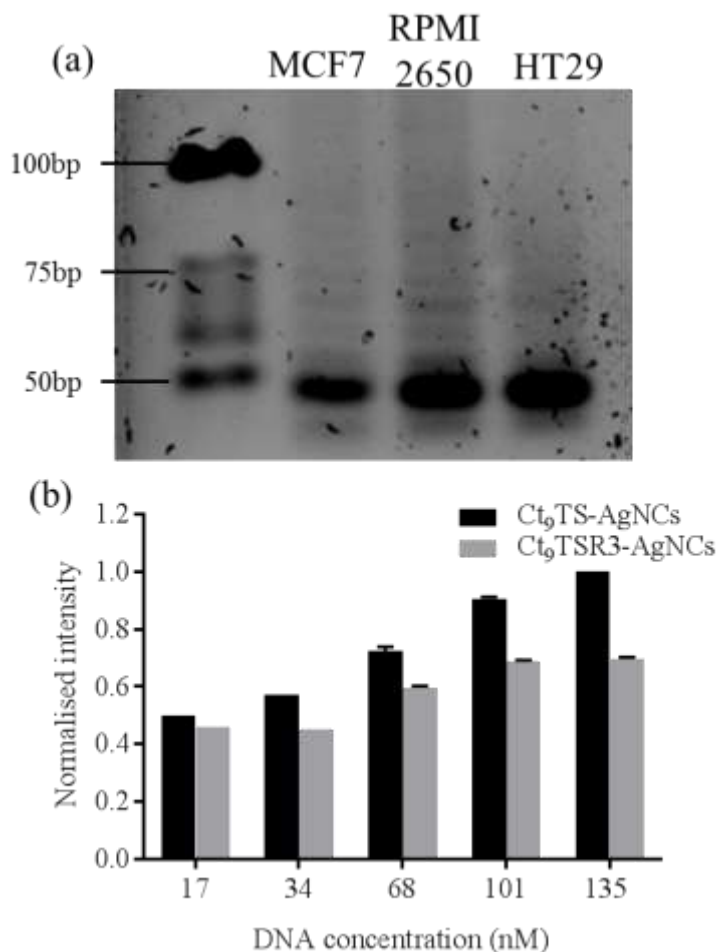


Figure 4.5. (a) Comparison of telomerase activity on three cancer cell lines based on band intensities of telomerase elongated product with reference to the 100 bp marker, which confirms the relatively higher telomerase activity detected in RPMI 2650 followed with MCF7 and HT29 cells. 250 cells were used for telomerase elongation reaction. 4.5 %(w/v) agarose gel in 1x Tris-acetate EDTA was used for gel analysis. (b) Fluorescence intensity with respect to different concentration of DNA used. Error bars represent standard deviation from three independent measurements.

Next, to examine the sensitivity of the proposed assay, all three types of telomerase cell extracts were serially diluted and the emission response of AgNCs in each sample was plotted as a function of concentration (*i.e.* number of cells) (Figure 4.6). A good linear correlation was observed in the range of 10 to 250 cells for MCF7, 25 to 250 cells for RPMI 2650; and 50 to 750 cells for HT29. The limit of detection (LOD) for MCF7, RPMI 2650 and HT29 are 15, 22 and 39 cells, respectively, calculated based on $3\sigma/m$, where σ is the

standard deviation of the lowest concentration and m is the slope of the calibration curve. These results surpass the optimal sensitivity performance of conventional TRAP assay and other PCR-free methods, in the context of LOD and assay time (Table 4.2). Additionally, with AgNCs being label-free probe, the whole assay involves only simple mixing steps, thus enabling low-cost fabrication.

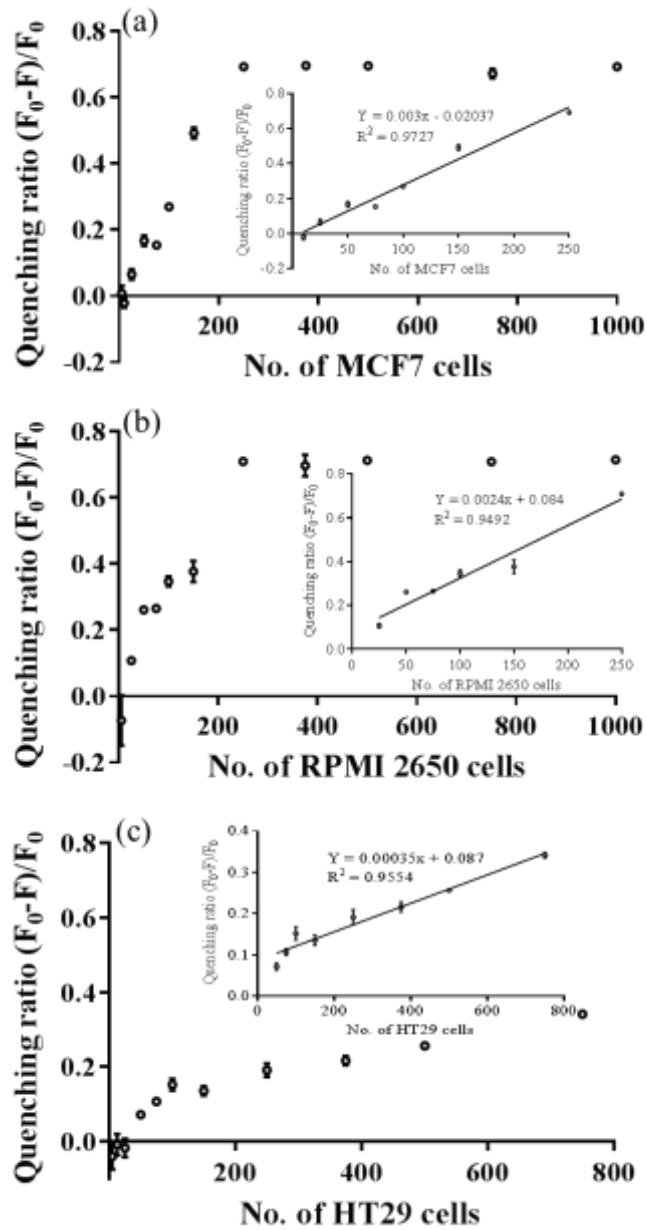


Figure 4.6. Plots of quenching ratio with respect to different numbers of cells for (a) MCF7, (b) RPMI 2650 and (c) HT29. Insets show as the respective linear range of detection for different cell types. Error bars represent standard deviation of five independent measurements.

Table 4.2. Comparison of assay performance for telomerase detection using the current assay, conventional TRAP and other reported amplification-free methods.

Mode of measurement	Linear range	Limit of detection	Reaction time	Ref.
Fluorescence (DNA-AgNCs)	10–250 cells (MCF7) 25–250 cells (RPMI 2650) 50–750 cells (HT29)	15 cells (MCF7) 22 cells (RPMI 2650) 39 cells (HT29)	1 h	This study
Conventional TRAP assay	100–1000 cells	N/A	At least 3 h	²⁰⁴
Fluorescence (DNA-AgNCs)	100–1000 cells	62 cells	2 h	²³
Fluorescence (DNA-AgNCs)	Log (50–50000) cells	50 cells	N/A	¹³⁴
Fluorescence (AuNP-TAMRA dye)	$0-8 \times 10^{-3}$ IU L ⁻¹	100 cells	40 min	¹⁹⁰
Fluorescence (TMR)	N/A	40–1000 cells	3 h	¹⁹²
Fluorescence resonance energy transfer (FRET)	5–2000 cells	5 cells	1 h 30 min	²⁰⁵
Chemiluminescence – single cell level	Synthetic telomerase product with 8 repeats 0.1 aM–1 nM	0.1 aM	1 h	¹⁸⁷
Chemiluminescence	10 to 400 cells	10 cells	N/A	¹⁸⁶
Colorimetric	1–8 cells/μL	100 cells	1 h 20 min	¹⁸⁸
Colorimetric	0–8 cells/μL	1 HeLa cell/μL	1 h 10 min	¹⁸⁹

4.4.3 Proof-of-concept

To gain insight into how the telomerase-positive samples quench the fluorescence of Ct₉TS-AgNCs, synthetically elongated DNA templates, *i.e.* Ct₉TSR_{*n*} (*n* = 3, 5 and 7) was employed to mimic the different length of elongated products. As illustrated in figure 4.7a, the emission intensity of Ct₉TSR3-, Ct₉TSR5- and Ct₉TSR7-AgNCs decreased by ≥ 50 % as compared to that of Ct₉TS-AgNCs without any telomeric sequence, which agrees well with the results in section 4.4.2. The CD spectra of Ct₉TSR_{*n*} templates and

Ct₉TSRn-AgNCs prepared in TRAP buffer reveal the presence of parallel G-quadruplex structures (Figure 4.7c & d). This is evidenced from characteristic signatures of positive bands at 210 and 260 nm, in addition to a less intense negative band at 240 nm. Comparatively, no characteristic positive band at 210 nm was observed for Ct₉TS and Ct₉TS-AgNCs, implying a B-form like structure.¹⁶⁸ Considering TRAP buffer contains additional components (*e.g.* EGTA and Tween 20) that may influence the optical properties of AgNCs, the CD spectra of Ct₉TSRn and Ct₉TSRn-AgNCs prepared in Tris-HCl buffer (pH 8) was also investigated for comparison. The CD results of Ct₉TSRn templates in Tris-HCl buffer are generally similar to that in TRAP buffer. However, upon formation of AgNCs, Ct₉TSRn-AgNCs in Tris-HCl demonstrate typical CD peaks of unfolded guanine-rich DNA (Figure 4.7e & f).^{206–208}

TRAP buffer could not be the main factor that responsible for the fluorescence quenching, as Ct₉TS-AgNCs remained fluoresced (Figure 4.7a & Appendix IV) Thus, the quenching effect observed in telomerase-positive samples is highly due to the formation of parallel G-quadruplex. This is also in accordance with the results reported by Tao *et al.*, where parallel G-quadruplex has weaker fluorescence intensity than that with anti-parallel conformation. On top of G-quadruplex topology, thymine-rich loop bases adjacent to G-tetrads (*i.e.* TTA in this context) was also found to diminish the fluorescence signal.¹²² At present, it is unclear on how TRAP buffer contributes to the consistency of G-topology, both in Ct₉TSRn-AgNCs containing different lengths of elongated products, as well as different telomerase cell extract with varying degrees of telomerase activity. Note that an active telomerase is capable of elongating TS primer by hundreds of TTAGGG nucleotides through multiple rounds of

repeat-addition.^{209,210} In comparison to those prepared in TRAP buffer, Ct₉TSR5- and Ct₉TSR7-AgNCs in Tris-HCl buffer show fluorescence enhancement, which could be due to the proximity of guanine-rich tail that is known to increase the emission of adjacent AgNCs,¹²⁸ similar as in NCB design. In contrast, the relatively shorter guanine-rich telomeric repeats of Ct₉TSR3 not only constraint their association with AgNCs hosted in Ct₉, but also undesirably disrupts the encapsulation of AgNCs.

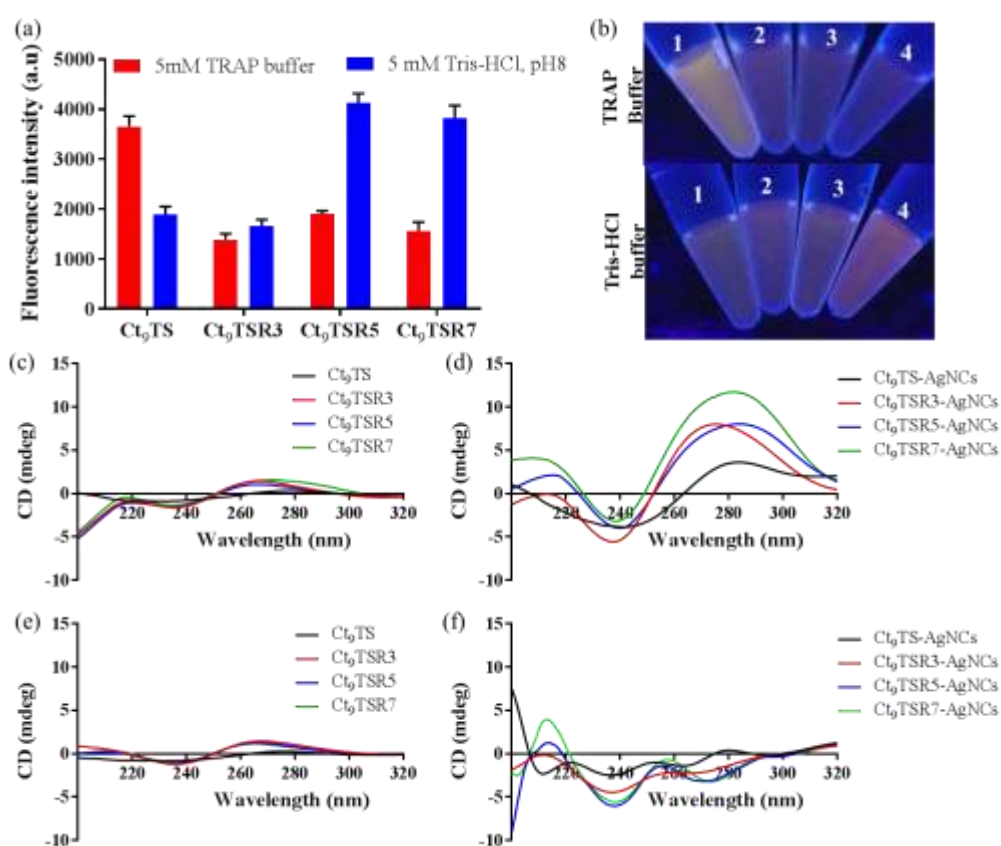


Figure 4.7. (a) Fluorescence intensity of Ct₉TS- and Ct₉TSR_n-AgNCs, representing the absence and presence of different lengths of telomeric repeats, respectively. Each set of samples were prepared in two different buffers (*i.e.* TRAP and Tris-HCl) to study to the effect of buffer. Error bars represent standard deviations from three repeated independent measurements. (b) The UV images of (1) Ct₉TS-, (2) Ct₉TSR3-, (3) Ct₉TSR5- and (4) Ct₉TSR7-AgNCs. DNA concentration used for fluorescence measurement was 135 nM. CD spectra of Ct₉TS and Ct₉TSR_n templates, as well as Ct₉TS-AgNCs and Ct₉TSR_n-AgNCs prepared in (c & d) TRAP buffer and (e & f) Tris-HCl.

4.5. Conclusion

In this chapter, a simple mix-and-detect assay analysis for telomerase activity using Ct₉TS-AgNCs has successfully been developed. It also revealed that the probes were stable in cell extract (tested up to 1000 cells) and showed selective quenching in telomerase-positive samples. More importantly, this assay has significantly outperformed the traditional TRAP assay, in terms of sensitivity, simplicity, time and cost. A meticulous study on the quenching mechanism has shed important light on the impacts of *G*-quadruplexes topologies and buffer on the optical properties of AgNCs. Despite the assay is stable in cell extracts, its applicability in more complex matrices such as tissue samples requires careful investigations to avoid false results.

Chapter 5. Conclusion

The biosensing studies presented in both chapter 3 and 4 have deciphered the detection principles of chimeric DNA-AgNCs which lead to either fluorescence turn-on/-off, by depending on the G-quadruplex topologies that involved in the sensing mechanism.

There are four principle of detection associated to chimeric DNA-AgNCs. First of all, the emission enhancement (*a.k.a* turn-on effect) occurs upon target trigger structural reformation into higher order of guanine-rich conformation, which is parallel G-quadruplex < unfolded guanine-rich < anti-parallel G-quadruplex. Secondly, the turn-off effect will be generated when the presence of analyte induces formation of parallel G-quadruplexes with –TTA– loop located next to G-tetrads. Thirdly, the position and emitter species of AgNCs within the chimeric DNA template can affect the sensing performance. This is based on the studies presented in chapter 3 and 4, that the location of AgNCs at 5'-end of adenosine aptamer provides better flexibility for structural reformation, whereas the yellow emitter species of better emission stability gives the best selectivity result when used to detect telomerase activity. Last but not least, the microenvironment of AgNCs may play an important role in determining the fluorescence turn-on/-off. This is because chapter 4 has shown that simple buffer with less components (*e.g.* detergents and chelating agents) is not enough to provide consistent turn-off effect by assuring the same conformation structure when a longer guanine-rich telomeric repeat is involved.

As an overall summary, a chimeric DNA provides a good template to develop AgNCs-nanobiosensor as presented in this thesis (*i.e.* Apt5-AgNCs

and Ct₉TS-AgNCs), where the sensors are highly sensitive, selective and able to generate result instantaneously.

Chapter 6. Future remarks

Lately, the progression in DNA-AgNCs is relatively slow as compared to the first few years since their discovery. This implies the urge to eliminate the bottlenecks in this field. As such, several aspects that are worthy of further investigation are listed as below:

- i. In relation to preparation, the cluster shuttle approach is barely adopted as compared to direct preparation, albeit NaBH_4 is known as harsh reductant that may impede the application of AgNCs in certain area. In fact, it was reported that the cluster shuttle approach generates AgNCs that are more responsive toward external stimuli and enables scale-up of DNA-AgNCs for commercialization purposes.
- ii. Three years after the concept of rod-shape AgNCs was introduced, another new model of octahedral geometry was recently proposed by a different group. While there are similarities between two models, it would be interesting to see how the differences would provide further insights on the mechanism of AgNCs formation, as well as the optical properties of the product.
- iii. Research on dsDNA or higher order oligomers has been relatively scarce, but deserves more attention. For example, there were contradicting findings on the ability of fully mismatched dsDNA to encapsulate AgNCs. This underline the need for more investigation of the ideal proportion of guanine/cytosine bases in a duplex to enable its use as cluster growth site. A very recent

and more definitive finding concluded that certain numbers of continuous guanine/cytosine pairs (ideally more than four pairs) can serve as growth-sites for rod-shaped AgNCs. Intermediate adenine/thymine pairs may quench the emissive AgNCs. Some other open questions include: will flanking sequences in gap/abasic/mismatch sites affect the emission property just like AP analogues? Will the rigidity of high order oligomers endow AgNCs with better stability in biological media? Are the rod and octahedral models of clusters applicable for AgNCs templated by other DNA scaffolds? These can be some key areas in further studies

- iv. There are impressive bioimaging studies employing DNA-AgNCs in live cell staining, Intracellular mRNA imaging, as well as simultaneous enhanced immune response and cell imaging. The success is mainly attributed to the easy integration of biofunctional sequences or cell specific internalization aptamers as part of the DNA template. Such versatility and simplicity is what positions DNA-AgNCs as credible competitors to standard fluorophores. One area that has not yet been addressed though is the long-term stability of this material, which may be also the primary reason that it is not ready to surpass the performance of quantum dots in long term cell labeling/tracking. As alluded to above, all existing fluorophores have their own drawbacks. Perhaps one solution to this end is learning from the solution for other fluorophores. For example, while adding photostabilising

additives or oxygen scavengers is known to enhance the photostability of organic dyes, a similar solution may be applied to DNA-AgNCs. Furthermore, will other DNA-templated metal analogues that are potentially more stable against photobleaching (*e.g.* gold-, gold-/silver- and copper-/silver-nanoclusters) further expand the repertoire of fluorophore that can be used in long term monitoring.

Appendix I

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Appendix II



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Publication: Journal of the American Chemical Society

Publisher: American Chemical Society

Date: Mar 1, 2016

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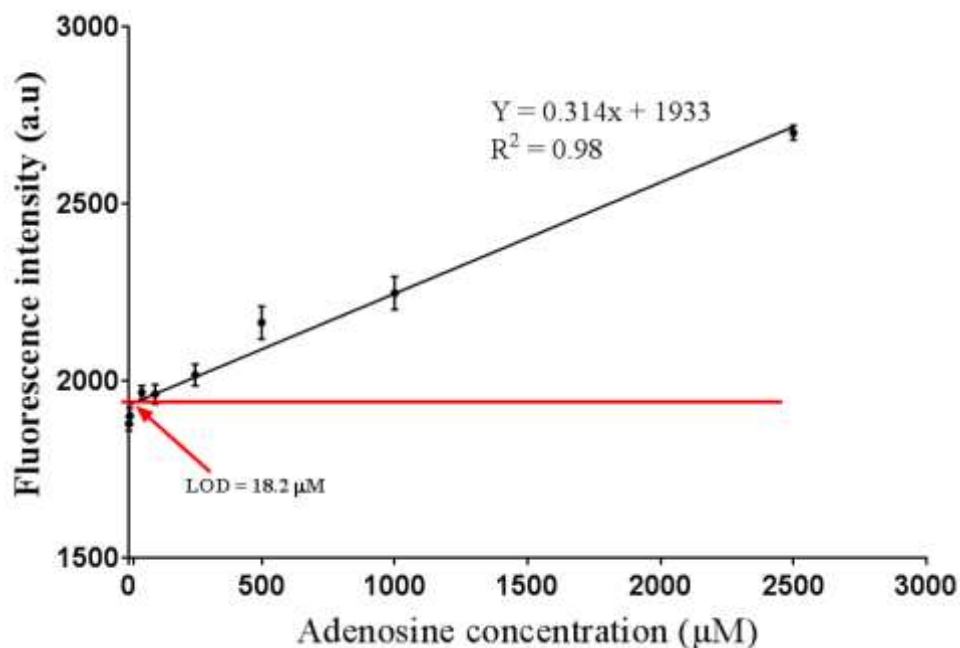
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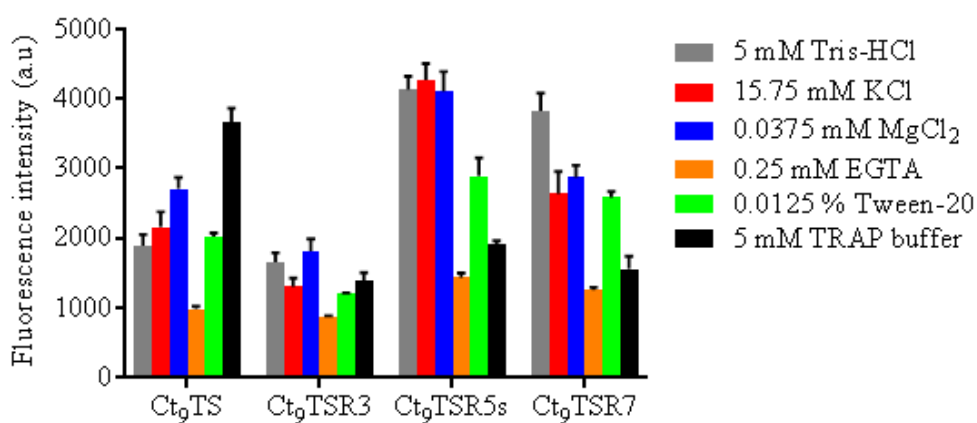
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Appendix III



LOD of Apt5-AgNCs, showing a good linear regression and threshold (red line) based on three times standard deviations from the low concentration to determine the LOD. Error bars represent standard deviation from three independent measurements.

Appendix IV



Emission intensity of Ct_9TS -AgNCs and Ct_9TSR_n ($n = 3, 5$ and 7) when prepared in five isolated TRAP components and combined components of TRAP buffer. DNA concentration used was 450 nM. Error bars represents standard deviation from three independent components.

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