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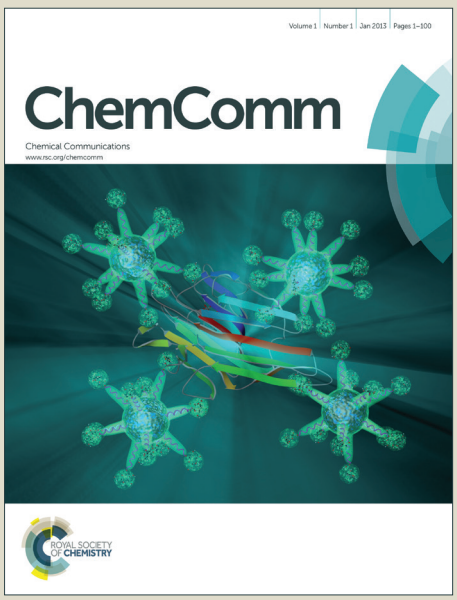

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DNA/RNA chimera indicates the flexibility of the backbone influences the encapsulation of fluorescent AgNCs emitters

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Abstract: Many DNA scaffolds efficiently encapsulate highly emissive silver nanoclusters (AgNCs). The secondary structures and the arrangement of sequences of DNA scaffolds are important factors by which the specific features of AgNCs emitters can be determined. By introducing DNA/RNA chimera scaffolds, we here explore another factor - the flexibility of backbone of nucleic acid-templates - in creating highly fluorescent AgNCs emitters.

The stable, strong, and spectrally diverse fluorescence of DNA-encapsulated silver nanoclusters (DNA/AgNCs) has increasingly been exploited for pragmatic sensors, including detection of single nucleotide polymorphism[1,2], target DNA sequences[3,4], proteins[5,6], miRNAs[7-9] among others. Simultaneously, the photo-luminescent potential of DNA/AgNCs, where the emission wavelength can be tuned by changing DNA sequence, has given rise to numerous recent studies, investigating the determinants of the emission features - color, intensity, and photo-stability. For instance, the sequences and secondary structures of the DNA-template have been extensively explored to understand the features. Several studies have reported that the correlation between sequences and their intrinsic secondary structures of DNA is crucial to determine the color and emission intensity of DNA encapsulated AgNCs[3,7,8,10-13]. We previously reported that the re-arrangement of a given sequence can make a non-emissive DNA-template become emissive, through an alteration of secondary structure of the DNA-template[8]. However, we have found that at least ~5 reconstituted DNA-templates of ~40 tested DNA-templates fail to become sufficiently fluorescent with our earlier approach, one of these being DNA-12nt-RED-let-7a presented in this communication, see Fig. 1. When comparing with the strong fluorescence of the well-structured DNA-12nt-RED-160 probe, which reaches an emission intensity reading of 1.5 x 10^6 (λex=560 nm), DNA-12nt-RED-let-7a and its reconstituted derivatives only encapsulated practically non-fluorescent AgNCs (Fig. 1, Fig. S1).

Here, we explore the concept that the conformational flexibility of single stranded nucleic acids is one of the essential factors for allowing nucleic acid-templated/AgNCs to form highly emissive structures. By comparing DNA vs RNA backbone and by adopting DNA/RNA chimera-templates, we demonstrate the significance of the rotational freedom of nucleic acid-templates for the emission properties of the formed AgNCs. In contrast to DNA, RNA contains ribose with a hydroxyl group at the 2' position of the ribose sugar that distinguishes its physical and chemical features from DNA. Because of the difference, RNA has in general much larger degrees of freedom for rotation of the backbone, which facilitates self-complementary base-paring in single-stranded RNA, resulting in the formation of structures such as hair-pins and pseudoknots. The secondary structure assumed by RNA is restricted to A-form, unlike DNA which displays greater secondary structural variability. The differences between RNA and DNA are complex, but a main difference is that RNA contains uracil, and that the rate of base-pair opening is much larger for A=U in RNA than for A-T in DNA, whereas the lifetime of G≡C base-pairs are comparable. The differences between rA-rU and dA-dT kinetics are related to carbohydrate backbone, not the methyl group of uracil[16]. However, uracil may form unusual G=U and U=A=U base-paring, and RNA is in general fragile to alkaline conditions. Because G=U base-pairs occur in a high probability, it also contributes the higher capacity of RNA for self-complementarity than DNA. RNA is reported to exist in a rapid equilibrium between numerous structurally excited states, which may have a profound impact on the chemistry of the oligonucleotide[17]. Shultz and Gwinn[14] showed that short RNA sequences (poly [rC_n] and [rG_n], n=6~11) without strong base-pairs for self-complementarity can be good templates for embedding emissive AgNCs but with distinctive trends in fluorophore populations to DNA/AgNCs, because of the differences of the carbohydrates. In bioinorganic chemistry studies on metalloproteins it is often discussed if the protein or the metal ion controls the structure of a given metal ion binding site. Hence, we here hypothesize that silver ion derived structure of nucleic acids can be an important factor for embedding highly emissive AgNCs, if the nucleic acids do not contain enough C≡G base-pairs for self-complementary structures. As an extension of these ideas, we believe that a more rigid DNA backbone is less susceptible to undergo transient folding, than a flexible RNA backbone, i.e. that latter is more liable to undergo a silver-driven restructuring. In other words, the flexibility of RNA backbone may be important to form a transient structure (a kinetic trap) that can be further stabilized by C-Ag^+-C bridge and lead to a facile situation for AgNCs formation. Accordingly, to test these ideas, we redesigned the non-emissive

DNA-12nt-RED-let-7a into RNA-12nt-RED-let-7a, and observed strong green (1.3 x 10^6) and red (3 x 10^6) fluorescence, when the sample was excited at 440 nm and 540 nm (Fig. 1 and Fig. S2). By contrast, the emissive DNA-12nt-RED-160 yielded a weakly emitting RNA analog RNA-12nt-RED-160 (33 nt) (Fig. 1). We tested the generation of red fluorescence in the case of the poly cytosine sequence [rC_n], n=24 with both DNA and RNA backbone (Fig S3), and obtained fluorescent probes in both cases, the RNA variant being slightly more intense and spectrally uniform. On the other hand, a number of short miRNAs (21 nt), and RNA-12nt-scaffolds (12 nt) were unable to form fluorescent emitters (Fig. S4, S5). These results showed that not all RNA-templates are compatible to encapsulate fluorescent emitters. As compared to the non-emissive RNAs, RNA-12nt-RED-let-7a (34 nt) is relatively longer than the shorter RNAs (21~22 nt and 12 nt) and has higher cytosine ratio than RNA-12nt-RED-160 without plausible self-complementary C≡G base pairing within the sequence. On this background, we propose three sequential steps in creating emissive AgNCs in RNA-12nt-RED-let-7a.

DNA-12nt-RED-160	$_dC_dC_dT_dC_dC_dT_dT_dC_dC_dT_dC_dC_dT_dG_dG_dC_dA_dT_dA_dC_dA_dG_dG_dA_dG_dC_dA_dG_dG_dC_dA$
RNA-12nt-RED-160	$_rC_rC_rU_rC_rC_rU_rU_rC_rC_rU_rC_rC_rU_rG_rG_rC_rA_rU_rA_rC_rA_rG_rG_rA_rG_rC_rC_rA_rG_rG_rC_rA$
DNA-12nt-RED-let-7a	$_dC_dC_dT_dC_dC_dT_dT_dC_dC_dT_dC_dC_dT_dA_dA_dC_dT_dA_dT_dA_dC_dA_dA_dC_dC_dT_dA_dC_dT_dA_dC_dA_dC_dA$
RNA-12nt-RED-let-7a	$_rC_rC_rU_rC_rC_rU_rU_rC_rC_rU_rC_rC_rA_rA_rC_rU_rA_rU_rA_rC_rA_rA_rC_rC_rU_rA_rC_rU_rA_rC_rC_rU_rC_rA$

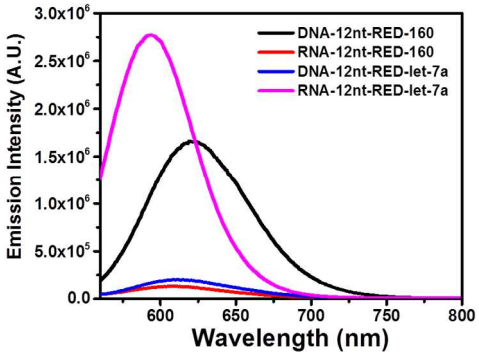
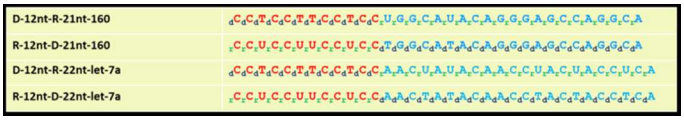


Figure 1: Sequences of DNA and RNA probes used in this study. Each nucleic acid sequence is given in two colours: red colored bases show cytosine-rich scaffold (DNA-12nt-RED) while blue colored bases show target miRNA sensing sequence (miR-160 or miR-Let7a). Prefix d-and r- refers to deoxyribose and ribose sugar bases of DNA and RNA, respectively. Emission spectra of the nucleic acid - AgNCs probes. The maximum emission spectrum (λ_{EX} around 540 nm) of each AgNCs modified nucleic acid probe was recorded.

These are 1) the flexible backbone of RNA-12nt-RED-let-7a may allow it to be entangled to have proximity between intrastrand cytosines - possibly assisted by a transient folding state (a kinetic trap) - and 2) the addition of Ag^+ induces formation of cytosine-Ag-cytosine bridging, and 3) subsequent stabilization of the unusual fold-back structure of RNA-12nt-RED-let-7a upon reduction and formation of AgNCs. This assumption is slightly different to the previous concept that a self-complementary structure of DNA has to precede the encapsulation of AgNCs to be highly emissive within an hour. In order to get an insight into the structure of the probes, we performed UV-CD measurements (Fig. S6), which are known to contain information on the nucleic acid folding. We do not see a clear-cut correlation between UV-CD signals and the emission properties of the probes. However, we note that DNA-12nt-RED-160 displays a quite altered signal compared to the RNA analog, where especially the positive peak at ca. 270 nm and the negative signal at 210 nm is characteristic of single stranded RNAs that form intramolecular base-pairing (hairpin or similar)[15]. It is noteworthy that RNA-12nt-RED-160 does not undergo any significant change upon formation of AgNCs (Fig. S6 top panel) apart from a red-shift in the near-UV band. The DNA-12nt-RED-160-AgNC probe that is highly emissive has a more marked red-shifted positive peak at ca. 290 nm. On the contrary, RNA-12nt-RED-let7a exhibits a major change upon AgNCs formation from a spectrum with weaker far-UV CD features to one exhibiting a strong negative peak at 210 nm (See Fig. S6 lower panel). This is an indication that with an RNA backbone, the presence of AgNCs's leads to a restructuring of the nucleic acid, RNA-12nt-RED-let-7a. This shift is not seen for DNA-12nt-RED-let-7a, which retains more or less that same CD spectrum with AgNCs albeit with a red-shift at 280 nm. It should be noted that the CD spectra contain contributions from free (non-AgNC-bound) nucleic acids, *vide infra*.

Next, capillary electrophoresis (CE) was used to further investigate the structural characteristics of the DNA and RNA-templates with/without AgNCs. A series of capillary electrophoresis experiments were performed to establish the homogeneity of the DNA/RNA samples and to investigate how effective the coupling procedure is between the DNA/RNA and the AgNCs probe. In Supplementary Figure S7 (A) the individual CE traces of DNA-12nt-RED-let-7a and RNA-12nt-RED-let-7a labelled with AgNCs are shown. In both cases we have two bands representing the modified and unmodified forms (the fastest moving form) as seen from the absorption at 400 nm. The similar peak shape of the modified and unmodified forms in the electropherograms in Fig. S7 (A) indicates that the modified and unmodified forms are equally structured, see also Fig. S9. In Supplementary Figure S7 (B), the individual capillary electrophoresis traces of DNA-12nt-RED-160 and RNA-12nt-RED-160 labelled with AgNCs are shown. These two probes have similar retention times, but it is evident that DNA-12nt-RED-160 and RNA-12nt-RED-160 at 254 nm give rise to one large and distinct peak overlaying one or two broader peaks in the electropherogram. Looking at the CE detection at 400 nm where contributions from the AgNCs are seen, we observe broad bands. This indicates that the major peak at 254 nm is due to unmodified DNA/RNA while the broader peaks that appear as shoulders could be due to the AgNCs modified forms. The broadness of the AgNCs modified forms compared to the unmodified form (see also Fig. S8) indicates a more flexible structure after modification. Important information obtained from Supplementary Figure S7 (A) and (B) is that only approximately 50% of the sample is modified by AgNCs using this procedure. Next, to further clarify whether the flexibility of RNA-backbone is important for the fluorescence of RNA-12nt-RED-let-7a, we converted DNA-12nt-RED-let-7a into 4 DNA/RNA chimera-templates; D-12nt-R-22nt-let-7a, D-17nt-R-17nt-let-7a, D-21nt-R-12nt-let-7a, and R-12nt-D-22nt-let-7a (see Fig. 2 and Fig S10). The spectra of each chimera-template were compared with DNA-12nt-RED-let-7a and RNA-12nt-RED-let-7a. Interestingly, when the chimera-templates have the longer stretch of DNA backbone the intensity of fluorescence is systematically decreased (Fig. S10). Fig. 2 shows that red fluorescence (1.2 x10^6) of D-12nt-R-22nt-let-7a is approximately 6 times higher than that of R-12nt-D-22nt-let-7a (2.0 x 10^5), confirming the importance of RNA backbone. In the case of the miR160 sensor, we similarly designed two chimera-templates with different ratios of RNA/DNA nucleotides, D-12nt-R-21nt-160 and R-12nt-D-21nt-160. The former showed the similar level of intensity to DNA-12nt-RED-160 (1.5 x 10^6) but the latter displayed an extremely high intensity of fluorescence up to 5 fold in maximum (7.0 x 10^6). It is perplexing to explain why the conversion of 12 nt's of the DNA-12nt-RED sequence into RNA enhanced the emission intensity, but it is evident that the partial exchange has a favourable effect on the ability of the probe to support an emissive AgNCs cluster. When examining the CD data in Supplementary Figure S11 the chimera sequences overall confirm the trends seen for the pure DNA/RNA scaffolds; a higher RNA content for both 160 and let-7a sequences tend to give a positive 270 nm band and a negative 210 nm band, indicative of

base-pairing in RNA. In Supplementary Figure S12 (A) the individual CE traces of D-12nt-R-21nt-let-7a and R-12nt-D-21nt-let-7a labelled with AgNCs are shown. In both cases we have two bands representing the modified and unmodified forms (the fastest moving form) as seen from the absorption at 400 nm. The similar peak shape of the modified and unmodified forms in the electropherograms in Fig. S12 (A) indicates that the modified and unmodified forms are equally structured, see also Figure S14. In Supplementary Figure S12 (B) the individual CE traces of D-12nt-R-21nt-160 and R-12nt-D-21nt-160 labelled with AgNCs are shown. The two probes have similar retention times, and we see that the electropherogram at 254 nm gives rise to one large and distinct peak overlaying one or two broader peaks in the electropherogram. Looking at detection at 400 nm where we only see broad peaks we conclude that the major peak is due to unmodified DNA-RNA chimera while the broader peaks that appears as shoulders are due to the AgNCs modified forms. The broadness of the AgNCs modified forms compared to the unmodified forms (see also Figure S13) indicates a more flexible structure after modification. Again it is clear from Fig. S12 (A) and (B) that only approximately 50% of the sample is modified with AgNCs.



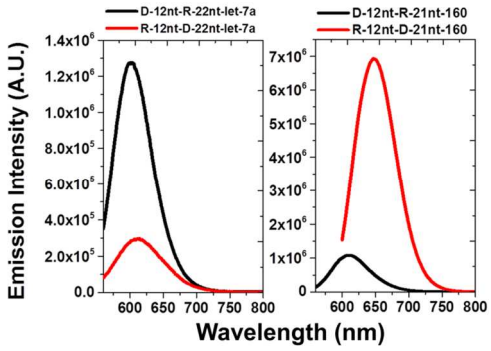


Figure 2: Sequence of DNA-RNA chimera/AgNCs probes used in this study. Each AgNCs-creating DNA-RNA chimera sequence is given in two colors: red colored bases show cytosine-rich scaffold (DNA-12nt-RED) while blue colored bases show target miRNA sensing sequence (miR-160 or miR-Let7a). Prefix d-and r- refers to deoxyribose and ribose sugar bases of DNA and RNA, respectively. Emission spectra of the AgNCs modified DNA-RNA chimera probes. The maximum emission spectrum of each DNA-RNA chimera - AgNCs probe was recorded. Left panel: DNA/RNA chimera of AgNC modified Let-7a, Right panel: AgNC modified DNA/RNA chimera of 160.

Next, we investigated whether the formation of a kinetic trap is important for a non-structural RNA template to encapsulate highly emissive AgNCs within an hour. In general, without base-pairs (A=U, or G≡C or G=U), it is unfavourable to form a kinetically trapped transient structure within a single stranded RNA. Therefore, we substituted all adenines of RNA-12nt-RED-let-7a with uracils and named this RNA-12nt-RED-let-7a (A→U). Indeed, without A-U base-pairing, even though the main silver embedding nucleotides, the cytosines were not altered, RNA-12nt-RED-let-7a (A→U) was unable to generate immediate and strong fluorescence (Figure S15). Reversely, to induce the formation of a self-complementary hair-pin or dimer structure, we changed cytosines into guanines in the complementary strand for let-7a and named RNA-12nt-RED-let-7a (C→G). The fluorescence of the modified strand was dramatically dropped (Figure S16). To confirm these results, we changed all guanines in RNA-12nt-RED-160 into cytosines and named RNA-12nt-RED-160 (G→C) (Figure S17). RNA-12nt-RED-160 (G→C) showed dramatically increased fluorescence. These results support the essentiality of RNA flexibility and its outcomes, kinetically trapped conformer and the possibility for C-Ag^{+}-C bridging, in the generation of highly emissive AgNCs in an RNA template. Furthermore, it was necessary to understand if the presence of the RNA-specific base U plays a role in the observed results. For this reason a uracil variant of DNA-12nt-RED-let-7a was prepared where all the thymines were substituted with uracils. As shown in Figure S18, DNA-12nt-RED-let-7a-Uracil was not suitable for the creation of emissive AgNCs, clearly precluding the possible role of uracils alone as a decisive factor in the creation of emissive AgNCs. The presented results for the let-7a sensor show that the more RNA in the backbone, the higher the emission (Figure S10). We interpret this to mean that the degree of freedom for backbone-rotation is important to yield emissive structure for the let-7a sequence. The sequence is rich in cytosines but without self-complementary guanine bases. As aforementioned, to be structured, the initial formation of a transient structure and subsequent C-Ag^{+}-C motifs could be important for RNA-12nt-RED-let-7a, along the lines illustrated in Supplementary Figure S19.

Conclusions

In conclusion, we have here demonstrated that the choice of backbone carbohydrate and thereby the rotational flexibility of backbone in nucleic acid-templates can be a factor by which the fluorescence of encapsulated AgNCs can be influenced. We believe that, when the nucleic acid-templates are unable to form self-complementary structures such as self-dimer and hair-pin, the backbone mobility of RNA can aid to form a transient pocket and subsequent C-Ag^{+}-C base pairing, favouring AgNCs formation on reduction. The described RNA-12nt-RED-let-7a sequence is such an example, yielding a much more fluorescent probe than its' DNA counterpart. By studying backbone-mixed chimera-templates, we found in the case of R-12nt-D-21nt-160 that only a partial DNA-RNA exchange was optimal in order to obtain a highly emissive species (6-fold higher emission intensity). These findings are highly relevant in terms of sequence design for target recognition, for instance for microRNA detection, as we know that the entire sequence is important for AgNCs formation. Where short, yet selective and efficient probes sequences are needed, the introduction of a (partial) RNA-backbone can be crucial, as it may allow the re-folding of a target recognition site that doesn't support emissive AgNC's with a DNA backbone. We foresee that the use of RNA-based designs for AgNCs encapsulation will facilitate creation of probes that have a high degree of selective target recognition as well as high emission.

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Notes and references

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