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Recent applications of metal nanoclusters for the analysis of nucleic acids.

Aplicacions recents dels "nanoclusters" de metalls per a l'anàlisi d'àcids nucleics.

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REPORT

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1. SUMMARY

micro ribonucleic acids (miRNAs) are analytes of great interest due to their role in gene regulation, more specifically in gene silencing. They are known to act as biomarkers for a variety of affections like cancers and neurodegenerative diseases, since they have been found overexpressed in affected cells. Therefore, its detection is key to diagnosis of early and advanced states of these diseases, so there is a need for quantitative and specific methods to target miRNAs. DNA templated fluorescent silver nanoclusters (DNA-AgNC) can meet the requirements, and its biocompatibility makes it suitable for analysis *in vivo*.

Nanoclusters (NCs) are tiny (below 2 nm) aggrupations of atoms of a metal (from a few to ten) that have quantized molecule-like orbitals, enabling fluorescence. Traditional detection methods of various analytes based on fluorescence and commonly used detection methods for miRNA detection are reviewed in this work with the objective of comparing them to DNA-AgNC. Several strategies involving DNA-AgNC for miRNA detection (isolated and from total RNA samples) are discussed, including generation and quenching of a fluorescent signal. Some signal amplification methods to enhance their sensitivity are mentioned as well.

The properties of gold nanoclusters (AuNCs) are also studied, along with some of their applications in the areas of imaging, drug carrying and loading, and catalysis.

Keywords: DNA, RNA, nanocluster, fluorescence, microRNA

2. RESUM

Els micro àcids ribonucleics (miARNs) són analits de gran interès degut al seu paper en la regulació de gens, més específicament en la inducció al silenci de la seva expressió. Actuen com a marcadors biològics d'afectacions com càncers i malalties neurodegeneratives, i és que s'ha comprovat que els miARN es troben sobreexpressats en les cèl·lules afectades. Per tant, la seva detecció és clau en la diagnosi d'aquestes malalties, tant en etapes avançades com en les primeres etapes, i un mètode que ofereixi un anàlisi quantitatiu i específic esdevé necessari. Els nanoclusters de plata estabilitzats per ADN (ADN-AgNC) compleixen els requisits, i a més, la seva biocompatibilitat els fa adequats per a l'anàlisi *in vivo*.

Els nanoclusters (NCs) són minúscules (de mida inferior als 2 nm) agrupacions d'àtoms de metall (des d'uns pocs fins a 10 àtoms) amb orbitals energètics quantitzats, com passa en les molècules. Aquesta característica fa possible la seva fluorescència. En aquest treball es revisen els mètodes tradicionals usats en la detecció de diferents analits mitjançant fluorescència i també els mètodes de detecció més utilitzats per a la detecció de miARNs, amb l'objectiu de comparar-los amb els ADN-AgNC. Diferents tècniques de detecció de miARN (aïllat o bé provinent de mostres d'ARN total) mitjançant aquest tipus de complex són analitzades, incloent tant la generació com l'extinció del senyal fluorescent. També es fa esment a algunes tècniques d'amplificació del senyal destinades a millorar la sensibilitat de la detecció.

Les propietats dels nanoclusters d'or (AuNCs) també són estudiades, juntament amb algunes de les seves aplicacions en els camps de la imatge mèdica, el transport i descàrrega de medicines, i la catàlisi.

Paraules clau: ADN, ARN, nanocluster, fluorescència, microARN

3. INTRODUCTION

Detection of nucleic acids such as micro ribonucleic acid (miRNA), other RNAs and DNA is becoming increasingly important for diagnostic applications, since they can work as biomarkers for a variety of affectations like cancers[1,2,3,4], hepatitis[5], cholesterol[6] and more. Biomarkers are quantifiable and reproducible indications of a medical state.[7] Fluorescence-based biosensors are the most commonly used for this purpose, and a need for biosensing methods which are biocompatible, have low or none toxicity, high quantum yield, significant fluorescence lifetime, possibility of multianalyte detection and good photostability has arisen. Some of fluorescence-based methods use quantum dots (QD), organic dyes and fluorescent proteins, but they have several limitations which make them deficient to be used in an *in vivo* analysis. Metal nanoclusters (NCs) are a novel material that can work as fluorescent labels, and they can respond to the mentioned needs if they are combined with a stabilizing agent like DNA[8] to from a fluorescent probe, so these complexes are currently being studied in depth.

3.1. BULK METALS, NANOPARTICLES AND NANOCLUSTERS

NCs are defined as an aggrupation of atoms (from a few to 10) with a diameter below 2 nm.[8] Metal atoms are more often found forming bulk metals or nanoparticles (from 11 to few hundreds of atoms with size above 2 nm). To understand the properties of these different structures, the band theory and the concept of surface plasmons must be considered.

Surface plasmons are the quantization of collective oscillations of the free electrons on a metal surface.[9] Only bulk metals and nanoparticles display plasmonic properties, that is, a continuous band structure (figure 1).

There is a sea of free moving electrons in the conduction band of bulk metals, which makes them good electrical conductors and optical reflectors. When light strikes on the surface of a bulk metal, those electrons reflect the totality of the light. If the size of the metal is reduced to form nanoparticles, free electrons are enclosed in a very small space and they can only vibrate at a determined frequency, so they can only absorb the frequency of the incoming light that coincides with their own frequency (surface plasmon resonance), reflecting the rest and thus

exhibiting intense coloring. It is when the size is further reduced to create NCs that the metal does no longer exhibit plasmonic behavior and presents quantum confinement: the size of the particles is so small that is comparable to the Fermi wavelength of electrons, and the band structure splits into discrete and quantized levels of energy so the movement of electrons is confined on those levels.[8] Due to this phenomenon, NCs interact with light differently than nanoparticles and bulk metals; they present molecule-like electronic transitions between energy levels, which results in absorption and emission of light, allowing their use as labels for fluorescence biosensing strategies.[10,11]

Considering this, aggregation of NCs to form nanoparticles should be avoided, since it results in the loss of these useful optical properties. Problematically, NCs have a great tendency to aggregate due to their high surface energy and small size[6], so they need a stabilizing agent like DNA.

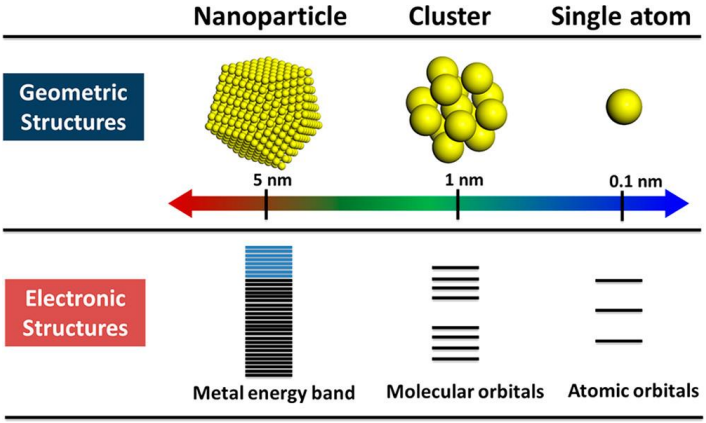


Figure 1. Different band structures of metals depending on their size. Movement of electrons is only quantized in NCs, allowing electronic transitions between molecular orbitals that display fluorescence.
(image extracted from Liu L., Corma A., ref. 10).

3.2. STABILIZING AGENTS FOR METAL NANOCLUSTERS

Different types of species, like polymers, dendrimers, thiols, proteins, glutathione (GSH) and DNA can be used as stabilizing agents[6,12], but DNA, apart from stabilizing, produces high fluorescence since it strongly absorbs UV light and transfers it to the NC.[6] A probe can be designed coupling DNA and metal NCs: the DNA sequence is designed to have a sequence for NC synthesis (scaffolding sequence) and a templating sequence for analyte recognition (DNA sequences can be prepared to recognize, for example, proteins, metallic ions, oligonucleotides,

and small molecules in general). This structure makes it easier to bring the NC near to the analyte avoiding aggregation, since NCs are embedded in the probe next to the target recognition sequence. It also controls the reduction of cationic silver species, avoiding particle aggregation due to an uncontrolled reduction.[6]

Double stranded DNA is often used as a template, since single stranded DNA is too flexible and can change its conformation.[13] Double stranded DNA has not only been used to detect the presence of certain analytes, but also to detect single nucleotide polymorphism (SNP) if gaps or abasic sites (AP sites) are introduced in its sequence.[14,15] SNP is a variation in duplex DNA occurring when the sequences of the two strands differ only in a nucleotide[16], and detecting these type of mutations is useful to diagnose cancers and heart diseases.[17]

Besides the wide variety of biosensing strategies that DNA probes permit, a crucial characteristic of templating with DNA is that it makes possible to synthetize NCs in aqueous solutions, which is convenient to study their behavior and applications in an environment like a real organism.[14,11]

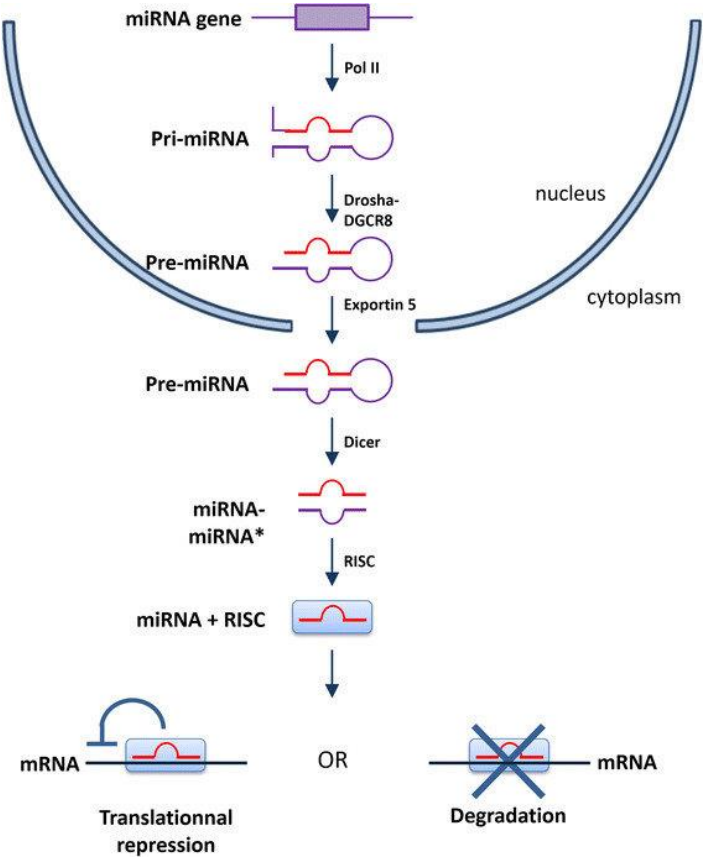
The most used biosensing methods using DNA-AgNC complexes are based on the enhancing or quenching of a fluorescent signal in the presence of the analyte. Different strategies of activation and quenching of NCs fluorescence will be discussed here, but in the classical approach, the NC, formed within the DNA structure, is fluorescent until a complementary strand (the target) hybridizes with it, quenching the fluorescence.[18,19]

3.3. MICRO RIBONUCLEIC ACIDS

miRNAs are a type of interference RNA consisting of short (from 19 to 22 nucleotides) single strands. These endogenous molecules are non-coding because they do not contain any information to synthetize proteins, as it happens with other types of RNA like transfer RNA (tRNA) or ribosomal RNA (rRNA). The main function of miRNA is to inhibit the expression of genes encoded in target messenger RNAs (mRNAs) through hybridization between complementary sequences, leading to target degradation or inhibition of its translation to a protein.[20,21,22,23] For mammalians, about 60% of genes are targeted by miRNAs (humans have approximately 1500 miRNA sequences in their genome).[1]

Gene silencing function of miRNA is carried out according to the following mechanism: RNA polymerase II transcribes miRNA genes that become part of a primary-microRNA (pri-miRNA),

which is a double-stranded RNA hairpin. Protein DGCR8 and enzyme Drosha associate to cleave the base of the RNA hairpin resulting in a precursor-microRNA (pre-miRNA). The protein Exportin-5 transports it from the nucleus to the cytoplasm of the cell, where Dicer enzymes cleaves the loop of the RNA hairpin. One of the strands becomes the mature miRNA whereas the other gets discarded. miRNA is then incorporated into a ribonucleoprotein complex called RNA-induced silencing complex (RISC), where it works as a template for target mRNA recognition. RISC is then attached to mRNA target strand and silence the gene, repressing its translation or degrading the mRNA (scheme 1).[17]



Scheme 1. microRNA biogenesis.
(image extracted from Devaux Y. et al, ref. 17)

The participation of miRNA in gene regulation and inhibition makes it useful for the study of diseases originated by genetic alterations like cancers. Consequently, its detection is being investigated with the objective of detecting, preventing and combating such pathologies, as well as viral infections (several viruses like Ebola hemorrhagic fever, common colds and hepatitis C use RNA as their genetic material) and neurodegenerative disorders (like Alzheimer and Multiple Sclerosis).[24,25] Cellular proliferation associated to cancers has been directly related with miRNA malfunctioning. Abnormal levels of miRNA-192 and miRNA-215 are associated to gastric cancer, miRNA-17 and miRNA-92 to colorectal cancer[26], miRNA-15 and miRNA-16 to chronic lymphatic leukemia, and overexpression of miRNA-1 to coronary artery disease.[25] Several miRNAs are overexpressed in patients with cancers, but there are other miRNAs that have a tumor suppressor function, like miRNA-34a, and they have a great potential to enhance cancer therapies. DNA-AgNCs can be used as nanocarriers to load miRNAs to cancer cells, designing the complexes to have a sequence to scaffold AgNC, a sequence complementary to the chosen miRNA, and an aptamer sequence for tumor-targeting.[27]

Therefore, developing methods for quantitative miRNA detection can be of major help in biomedical science, since it would eliminate the necessity for tumor biopsies. Unfortunately, detection of miRNA is problematic, given that they are short sequences with very little differences among them, so designed methods must have high selectivity, and they have low levels of expression in the organism, so signal-amplification is often necessary to detect miRNA in real samples.[28] The short nature of miRNA implies that used DNA probes must be short too, and short nucleotide sequences show less thermal stability than long ones.[29] Furthermore, RNA is less stable than DNA, so detection techniques must avoid degradation of the sample.[20,23,28] The majority of developed techniques until now require previous extraction and isolation of from the sample, so detection of miRNA in blood plasma, for example, presents a challenge. Given that miRNAs do not function alone but in a several component network, their detection *in situ* is crucial for diagnosis.[28]

A review on traditional detection methods for miRNA is done in this work, including northern blotting and microarrays, and the signal-amplification technique reverse-transcription polymerase chain reaction (RT-PCR) is explained as well. Finally, DNA-AgNCs are examined here as a novel material that have the potential to overcome the limitations of the abovementioned techniques.

4. OBJECTIVES

The aim of this project is to do a bibliographic research on metal NCs (of silver and gold mainly), focusing on miRNA detection using DNA-AgNC. The main goals are:

(1) Revising the concept of fluorescence and why NCs exhibit it.
(2) Studying the advantages and drawbacks of traditional fluorescent detection methods.
(3) Defining the properties and structure of AgNCs, and the stabilizing effect of DNA upon it.
(4) Determining the importance of miRNA detection and studying different methods for its detection, including DNA-AgNCs. Reviewing different detection strategies using DNA-AgNC, based on generation or quenching of a fluorescent signal and signal amplification techniques when necessary.
(5) Studying properties and applications of AuNCs.

5. METHODS

The scientific reports consulted for this work were published between 2006 and 2018, trying to keep the research in the more recent publications.

Data bases as, PubMed and PubChem from National Center for Biotechnology Information, Science Direct, Scopus and Research Gate were used to search the needed material.

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6. RESULTS AND DISCUSSION

6.1. Fluorescence

A brief review of the concept of fluorescence and the different magnitudes that define the process is done here before discussing further on fluorescent biosensors.

Electrons of a fluorescent material absorb a photon and achieve an excited state for a very short period of time. During the relaxation of these excited electrons the energy difference between the excited and fundamental state is emitted as a photon, in most of the cases with lower energy than that of the incident photon. Energy is inversely proportional to wavelength, so emission wavelength is usually longer than excitation wavelength.

There are many fluorescent processes with distinct characteristics that depend on a variety of factors: the chemical compound that is used as a fluorophore (compounds able to emit fluorescence, with aromatic groups or conjugated electron systems in their structure), environment conditions as pH or temperature, and interactions with other molecules (like it happens with metal NCs interacting with their stabilizing agents). The following magnitudes are meant to define fluorescent processes:

(1) Stokes shift: difference between the maximum wavelength of excitation and emission spectra. It's distinctive for each fluorophore.

(2) Quantum yield: efficiency of energy transfer from absorbed light to fluorescence. It is the ratio of emitted photons per absorbed photon.

(3) Brightness of the fluorophore: it results from the product of quantum yield by extinction coefficient. It indicates the relative intensity above the background. Systems with high brightness are useful to detect low-concentrated targets.

(4) Lifetime: time that a fluorophore remains in its excited state before relaxation.

Fluorescence is extensively used in the area of biosensing: detection of the autofluorescence of small molecules like tryptophan or NADH can be achieved. However, more interesting is the concept of "labelling", which involves the attachment of a fluorescent label (the most common being organic dyes, quantum dots and, in more recent times, metal NCs) into molecules like proteins, lipids or nucleic acids to use them as probes for detecting target molecules.

Metal NCs, stabilized by DNA and used as fluorescent labels, emit fluorescence when they are excited. This happens if they absorb visible light, or if UV light is coming to them transferred from the templating DNA.[6] There are two main output signals that DNA-AgNC produce: changes in the color of emission and changes in its intensity (that is, quenching or enhancement of the fluorescent signal). Changes in the emission color can be induced by several factors, but the most used for biosensing is the templating DNA sequence, given its tuneability. Other factors, like changes in the environment of the NC, compactness of the templating DNA bases, the use of a single metal or a mixture of them[6] can also change emission color. Even more than tunable emission color, changes in intensity of the fluorescent signal open a wide variety of useful strategies for detection of target molecules. This work will be focused on the interactions between DNA and AgNC that can produce or quench fluorescence.

6.2. Mostly used biosensing techniques based on fluorescence

Apart from DNA-AgNC, there are other fluorescence-based techniques that had been used, and are, in fact, still used, since a full comprehension of DNA-AgNC working mechanisms must still be acquired. Discoveries regarding NC characteristics and applications that have been made so far are only based on experimental observations, and we must be able to make theoretical predictions to extrapolate the gathered information.

Hence, the advantages and limitations of two of the most popular fluorophores, quantum dots and organic dyes, must be shortly discussed in order to compare them with each other and with metal NC.

(1) Quantum dots

This technique, as DNA-AgNC, works on a nanometer scale, using nanocrystals of semiconductor atoms. These are coated with an additional semiconductor shell and a polymeric shell over it, to make possible binding between QD and target biomolecules like proteins or oligonucleotides. To target oligonucleotides, polymeric shells of QDs are functionalized with ligands that can react with the target resulting in its attachment to the QD.[30]

QD are a great choice for multiplexing, since the wavelength of emission is directly related to the nanocrystal size, so several nanocrystals of distinct sizes can be synthetized to emit different wavelengths upon the same radiation.[31]

(2) Organic dyes

Organic dyes constitute the classical fluorophore label, used since the beginning of fluorescence biosensing. They can be water-soluble proteins, like phycoerythrin and allophycocyanine (two of the most used fluorescent labels currently)[33], xanthene derivatives such as fluorescein or rhodamine, naphthalene derivatives, and a wide variety of compounds with aromatic rings. They can be used to label proteins or nucleic acids.

Property	Quantum Dots	Organic dyes	AgNCs
Quantum Yield	0.1 – 0.8[30]	0.5 – 1.0[30]	~0.3 (depends on the probe) [18,36]
Fluorescence lifetime	10 – 100 ns[30]	1 – 10 ns[30]	~2.2 ns[18]
Photostability	High[30]	Poor[32]	Problematic[6,36]
Toxicity	Potential toxicity due to metal leakage[30]	Depends on the dye[30]	None[27]
Size[a]	10 – 20 nm[32]	0.5 nm[30]	<2 nm[8]
Simultaneous detection of several analytes	Possible[31]	Possible[30]	Possible[1,5]

a. Larger sizes contribute to a more cumbersome use of the labels.

Table 1. Comparison of properties of detection methods based on fluorescence

An important advantage of DNA scaffolding is that metal NCs bind naturally to DNA, getting to be a part of its structure, so no covalent modifications are needed to attach them to it. Other techniques such as quantum dots or organic dyes require chemical modifications, so DNA templated NC outruns these techniques in terms of cost-effectiveness as well.

6.3. DNA templated silver nanoclusters

6.3.1. Nanoclusters structure and binding to DNA

Mass spectrometry studies[34] have shown that there are approximately the same amounts of neutral Ag and Ag(I) in emitting AgNCs, so partial oxidation is necessary to achieve fluorescence. These studies have demonstrated that Ag atoms do not adopt planar nor globular structures within the DNA, but rod-like ones.[6] The length of these rod-like structures, which depends on the interactions between Ag atoms and DNA bases, determines the emission color.

Ag(I) has more affinity with DNA bases than with the phosphate backbone, so they interact with the bases, to which they bond covalently. Affinity between DNA bases and Ag(I) can be enhanced by adding Mg(II) to neutralize the negative charges of the phosphate groups (reducing ionic interactions between Ag(I) and negatively charged phosphate groups), altering DNA compactness around the NCs and thus altering the NC emissions.[15]

Metal ions can bind to any of the nucleotides in DNA, given that they all have at least one metal binding site (figure 2), but DNA sequences rich in cytosine are commonly used to template NCs, because its N3 position is deprotonated and ready to bind to a metal ion at neutral pH (pKa value of the N3 being around 4.2). Binding with thymine, for example, is only possible at high pH, because it requires the deprotonation of the N3. Adenine and guanine can also coordinate a metal ion effectively, at the N7 position or at other N sites. Therefore, it's not possible to predict the exact structure of AgNCs within a long chain of DNA because of the multiple binding sites, which makes design of the probes and prediction of its properties difficult. Some nucleotides have more than one binding site available, so several metal atoms can be found in one nucleotide.[14]

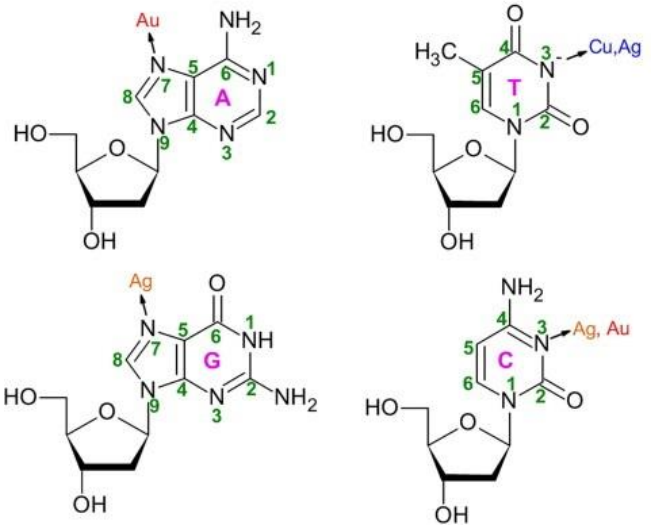


Figure 2. Chemical structures of A: adenine, T: thymine, G: guanine and C: cytosine. Commonly used metal binding sites are indicated in each nucleotide along with their respective metals. Adenine and guanine have more than one metal binding site: N1 and N3 can coordinate metals too.

(image extracted from Liu J., ref. 6)

6.3.2. DNA as a template: useful sequences

Just by modifying the templating DNA sequences it is possible to design -at some level- the structure of the NC or the emission wavelength. For example, size can be controlled if NC are AP site-directed[14,15] or gap-site directed[35] synthetized, and emission color can be changed by modifying an overhang sequence of the DNA[6], obtaining colors ranging from visible to near-IR. However, it must be said stated that the exact relationship between the DNA sequence and the final emission color cannot be predicted yet.

(1) DNA with AP sites

AP sites are locations in the DNA duplex strand without a purinic or pyrimidinic base in one of the strands, so they can be seen as binding pockets for directing the synthesis of metal NC. They allow size selective growing of metal NCs, providing a constrained environment in which Ag(I) concentration does not affect the size of the NC.[14]

Growing of NCs in AP sites is strongly dependent on the base sequences flanking it, so DNA containing AP sites can be used as scaffolds to detect SNP.[15]

(2) DNA with gaps

Gap sites are missing nucleotides, so they involve a discontinuity of one of the strands. Similarly to AP sites, gap sites in DNA sequences provide binding pockets for Ag(I), growing NC in a selective way when cytosine is in front of the gap in the DNA duplexes, so they can also be used for detecting SNP. Gap site-directed growth of AgNCs is cost-effective in front of AP site-directed, since they do not require modifications to be introduced into the DNA, whereas AP sites do.[35]

(3) DNA with different base-stacking environments

The wavelength of emission is affected by DNA base stacking environment surrounding the NC. An emission shift to longer wavelengths occurs when the base guanine is approaching the NC. The dipole moment of guanine, greater than other bases', was responsible: studies reported that the dipole moment of the excited state of a NC was greater than that of the ground state, so the excited state is stabilized by the dipole moment of the bases surrounding it.[15] Stabilizing of the excited state results in a decreased energetic band gap between ground and excited states, so wavelength is affected as well.

6.3.3. Photostability

To obtain good and reproducible fluorescent signals, the probe must be photostable. In most of the revised literature, photostability of AgNCs is assumed without further discussion, but other resources have reported that they are, in fact, sensitive to photobleaching (i.e., the photochemical destruction of the fluorophore) under UV light and under oxidation. Photobleaching by radiation is only observed under UV light, and no effect is observed with wavelengths longer than 400 nm.[6]

Fluorescence can be restored by addition of the reducing agent $NaBH_4$, so the short shelf-life of NCs has been attributed to oxidation by air. Oxidized NCs turn from red emitters to green, a shift that results problematic because green emitters have lower quantum yields than red emitters, and the wavelength of the signal overlaps with cellular and tissue autofluorescence.[6] Some studies[36] have found a specific DNA sequence (5'-ACCCGAACCTGGGCTACCACCCTTAATCCCC-3') that has longer shelf-life (retaining more than the 30% of the fluorescence after a year, in opposition to other NCs that only retain the 50% after a day). This sequence that has shown stability in front of oxidation also shows stability in front of pH and temperature. The reasons explaining these observations are not clear, but it is concluded that the DNA sequence plays an important role on photostability. However, a sequence which turns the NC completely resistant to oxidation will not produce a good probe, since partial oxidation is necessary to produce fluorescence.[34,37]

6.3.4. Synthesis and characterization

Typically, to synthetize DNA-AgNCs in aqueous solutions a micromolar solution of DNA is first mixed with $AgNO_3$ (with a ratio of cytosines to Ag(I) of 1:1 or 1:2) and then $NaBH_4$ (freshly prepared) is added as a reducing agent, so Ag(I) atoms are reduced to metallic Ag. DNA sequences have to be purified by desalting, and used $AgNO_3$ must be highly pure as well (99.9%).[6] The reaction can be carried out in water or in a neutral buffer like ammonium acetate or sodium phosphate.[8] The incubation of the DNA solution and Ag(I) can last for a few seconds to 15 minutes, and when the reducing agent is added, shaking the mixture for a minute is required. Typically, the probe must be kept in the dark for 15-18 hours before using it.[6,8]

The moment at which $AgNO_3$ and $NaBH_4$ are added plays an important role on the fluorescent signal to be obtained from the NCs. Studies have demonstrated that if the reactants

are added to a DNA probe previously mixed with the target miRNA the drop in the intensity of the fluorescence signal is much more significant than if they are added to the probe and then target miRNA is added. It has also been proved that higher intensities are achieved if $AgNO_3$ and $NaBH_4$ are added to the probe containing the scaffolding sequence and the templating sequence for target recognition than if they are added to the probe containing only the scaffolding sequence. The mechanism explaining this behavior is not clear.[18]

Apart from molecular fluorescence and absorption spectroscopy, other techniques such as mass spectrometry and X-ray crystallography are very useful to characterize metal NCs. This last technique is used with NCs stabilized by small molecules like thiolated compounds, whilst mass spectrometry is used when it is difficult to obtain single crystals of the sample, a fact which happens with DNA stabilized NCs.[6] Atomic Force Microscopy Imaging (AFM) in tapping mode can be used too to confirm the presence of AgNC.[38]

6.3.5. Signaling methods

DNA-AgNC can work as fluorescent labels following diverse strategies:

(1) Molecular beacons

To use DNA-AgNC as molecular beacons, the NC acting as the fluorophore and a fluorescence quencher are placed in each one of the ends of a single stranded DNA sequence in the form of a hairpin. When the hairpin is closed, the quencher is too close to the NC to let it produce any signal. The hairpin opens at the approach of the target DNA, and that is when the quencher moves away from the NC allowing fluorescence (figure 3).

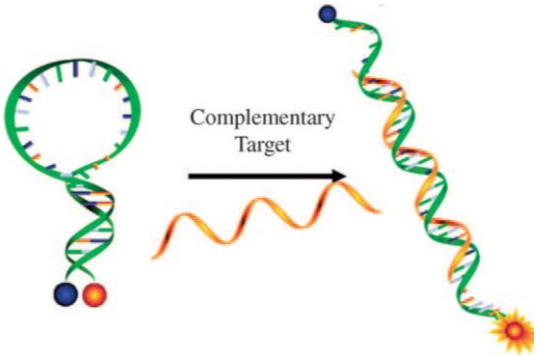


Figure 3. Mechanism of DNA-AgNCs working as molecular beacons. The quencher is represented by a blue sphere and the fluorophore by an orange one, which enlightens upon target hybridization.
(image extracted from Wang, K., Tang, Z. et al., Angew Chem Int Ed Engl. ***2009,*** *48: 856-870)*

Hemin-G4 complex (G4 is a secondary structure adopted by DNA or RNA strands which are rich in guanine bases) has been used as a quencher of NC (figure 4A), but not in the classical mechanism where it is attached to an end of the hairpin. A complementary strand of the target (probe sequence) is placed in one stem of the hairpin, and the hemin binding sequence is enclosed in the loop, so in the absence of the target the hairpin remains closed and there is no binding of the hemin, thus fluorescence remains high. In the presence of the target, the hairpin opens, freeing the hemin binding sequence, and hemin binds to the template, quenching the fluorescence (figure 4B).[39]

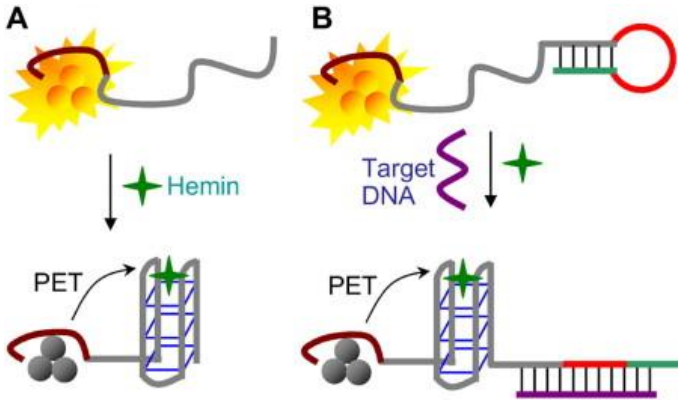
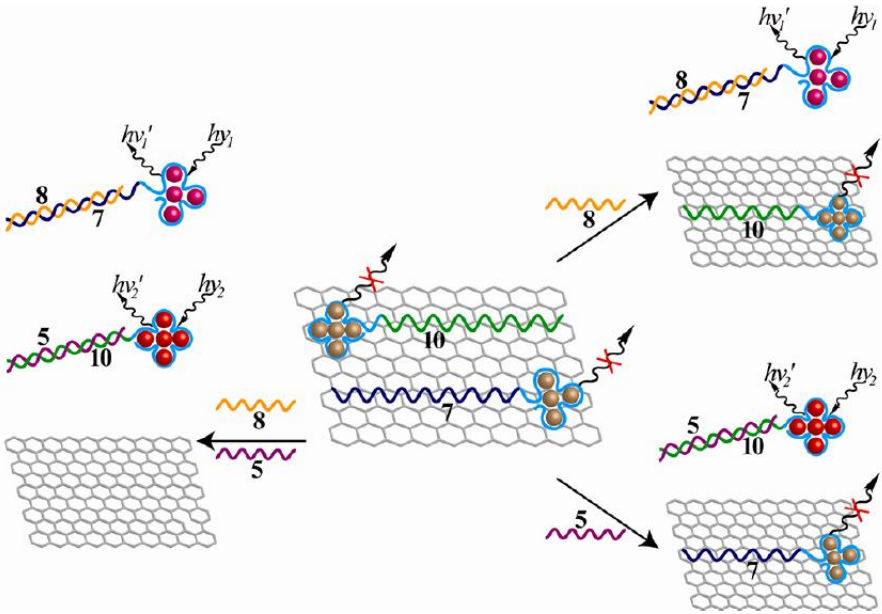


Figure 4. Hemin-G4 complex working as a molecular beacon. (A) Hemin-G4 complex acts as a fluorescence quencher. (B) Detection of a target DNA when hemin-G4 complex is formed.
(image extracted from Liu J., ref. 6)

Graphene oxide (GO) is also a well-known fluorescence quencher, and it can be used with DNA templated NC, since DNA absorbs in GO and desorbs in the presence of its complementary strand (the target) through hybridization.

GO acting as the matrix for DNA-AgNC probes allows simultaneous detection of several analytes in the same analysis, since two or more different probes can be adsorbed into GO and then selectively desorbed depending on the present target. With this strategy, detection of hepatitis B virus gene (HBV) and immunodeficiency virus gene (HIV) in the same experiment was possible (scheme 2).[5]



Scheme 2. Simultaneous detection of HIV and HBV genes in the same analysis by selective desorbing from a graphene oxide matrix. When the target DNA was HBV (sequence 5), the complementary sequence of probe 10, the duplex 5-10 was formed and the complex desorbed, leaving the GO matrix and producing fluorescence. The same happens with HIV (sequence 8) and probe 7. If HIV and HBV are present, they both leave the matrix and produce fluorescence. Each DNA-AgNC complex emits at a characteristic wavelength, so we can successfully detect if a DNA strand is expressing HBV, HIV or both genes.

(image extracted from Liu X. et al, ref. 5)

A similar strategy can be used for the detection of aptamers like ATP or thrombin, using their anti-aptamer sequences to form the complexes that desorb from GO allowing the NC to produce fluorescence.

(2) Detection based on DNA-AgNC synthesis

This strategy is based on the generation of the sequences or secondary structures of DNA needed for preparing fluorescent AgNCs, and it enables sequence selective detection and detection of mismatches through the detection of a fluorescent signal. To detect mismatches using this method, fluorescent AgNCs are scaffolded by a duplex DNA with a C_6 bulge in one of its strands. Perfect base pairing around the bulge is required to produce the signal, since AgNCs will not be synthetized if there is a mismatch. When the strand with the C_6 bulge

hybridizes with the target strand forming a duplex, and the reducing agent is added to synthetize AgNCs that will produce fluorescent signal if base pairing is the expected (figure 5).

This detection strategy has an important limitation that is the analysis time: synthesis of NC as an integrated step on detection may result in a process that lasts for a few hours.[6]

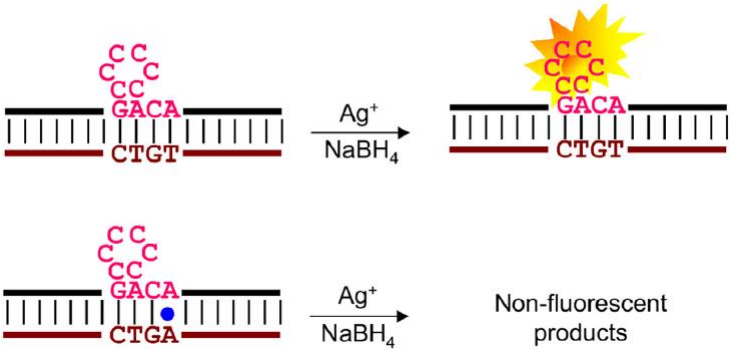


Figure 5. Sequence selective and mismatch detection using AgNC synthesis. If the target sequence hybridizes as expected with the strand containing the C_6 bulge, fluorescent NCs are synthetized. If there is a mismatch, fluorescent NCs are not synthetized.

(image extracted from Liu J., ref. 6)

(3) Hybridization activated fluorescence

It has been observed that color changes in emission can be induced by hybridization of DNA templating sequences with DNA strands that contain different overhang sequences. If this overhang contains poly-guanine (poly-G) sequences, emission shifts to red wavelengths and is enhanced 500-fold.[40] Strategies where the target brings close poly-G sequences and AgNCs have been developed to use the fluorescence enhancement as an output signal: thrombin and adenosine are examples of analytes that can be detected in this way (figure 6). Thrombin has two binding sites for aptamers, so two aptamers can be prepared, one with AgNC in its end and one with poly-G in its. In the absence of thrombin, the poly-G and the AgNC are too far to produce any signal, but when the target is present, it binds to the two aptamers bringing them close enough to produce a strong fluorescent signal. Adenosine, in the other hand, is detected placing its aptamer in a DNA strand between a poly-G sequence and a AgNC: in the absence of target they are too far to produce fluorescence, but when adenosine is present, the aptamer folds to bind to it, bringing the poly-G and the AgNC close and producing fluorescence.[6]

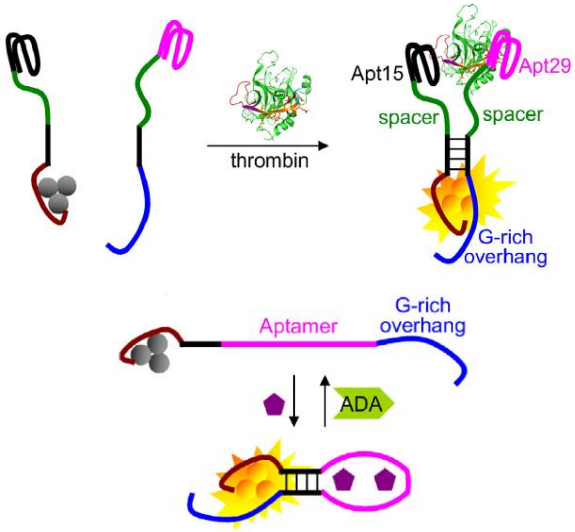


Figure 6. Detection of thrombin and adenosine through hybridization activated fluorescence strategy.
(image extracted from Liu J., ref. 6)

Hybridization-activated fluorescence strategy can be used to perform sequence selective detection too, since mismatches produce large wavelength shifts.

Following this same strategy, C_6 bulged DNA characteristics can be further exploited: fluorescent AgNCs are synthetized in DNA with a C_6 bulge, and overhang sequences are introduced in its complementary strand. When the target, a DNA sequence fully complementary to the strand with the overhang, is present, hybridization between these two strands will happen, leaving the C_6 bulge strand as a single non-fluorescent strand[6] (figure 7).

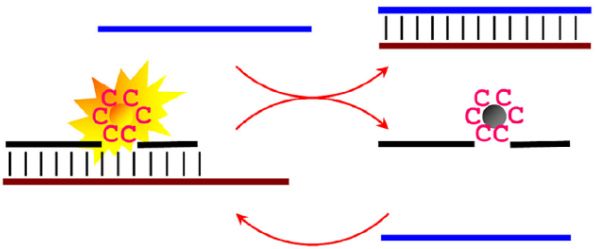


Figure 7. Fluorescence quenching induced by hybridization applied for sequence selective detection using NCs previously synthetized in C_6 bulges.
(image extracted from Liu J., ref. 6)

Hybridization can cause fluorescence quenching as well: this is the case of miRNA. A highly fluorescent DNA-AgNC is produced, and through hybridization with its complementary miRNA, fluorescence quenching is observed (figure 8). The quenching mechanism still lacks a clear explanation, but it is known that fluorescence intensity is related to the secondary structure of the DNA that scaffolds the NCs, so disrupting it through target hybridization leads to a drop in the intensity.[19]

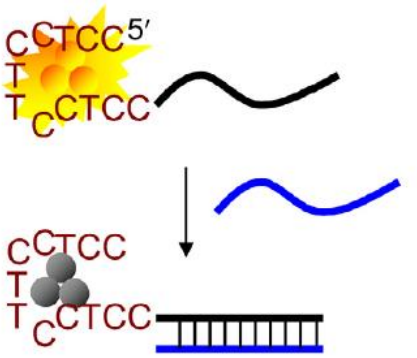


Figure 8. Fluorescence quenching induced by hybridization applied for detection of miRNA.
(image extracted from Liu J., ref. 6)

(4) Reactive quenchers

Since almost all the metal atoms present in a metal NC are exposed, they are sensitive to react with other species like heavy metal ions and thiolated compounds, which can result in the quenching of the fluorescence.

Metal ion quenching does not only make possible detection of metal ions themselves, but also of metal ion chelators. Oxidizing species like Cu(II) or Hg(II) can oxidize Ag atoms to Ag(I), quenching the fluorescence of AgNC as an output signal.[41] A chelator agent like guanosine tetraphosphate can be detected by its reaction with the heavy metal ions, which restores the fluorescence if the quenching reaction is reversible.

Thiolated compounds, quinones, and H_2O_2 usually act as quenchers, allowing detection of analytes such as biothiols (like cysteine or glutathione reductase), enzymes that generate quinones (like tyrosinase), and glucose and cholesterol.[6]

6.4. Gold nanoclusters

Apart from AgNCs, gold nanoclusters (AuNCs) are being studied as a material with potential on several areas like biosensing, imaging and cancer therapies. They are constituted by Au(0) and Au(I), and fluorescence comes from Au(I) species.[42] Fluorescence is emitted in the visible and near infra-red (NIR) region, and a long fluorescence lifetime is reported. The capacity of emitting in the NIR region is especially interesting because the interference of biomolecules is greatly reduced at those wavelengths.[43] Additionally, AuNCs have large Stokes shifts[42], which means that absorption and emission spectra do not overlap, resulting in an enhanced detection of its fluorescence. AuNCs have a variety of applications. In this work, their use as nanocarriers for drug delivery, as fluorescent labels to detect certain analytes, as a method for DNA quantification, and as catalysts are reviewed.

(1) AuNCs as nanocarriers

Like AgNCs, AuNCs can be useful to synthetize nanocarriers for anticancer drug delivery, achieving specificity to cancer cells. Tumor suppressor activity was confirmed *in vitro*. Further investigations regarding AuNC nanocarriers, especially in *in vivo* analysis, would be of utility to improve cancer therapies.[44]

Graphene oxide can also act as a scaffold for AuNC, and the resulting complex can be used to inhibit the expression of cells of human cancer cell lines. GO-AuNC complexes can be used to carry and load anticancer agents inside cells, crossing their membrane. Fluorescent properties of AuNCs are useful to follow the action of the complex against cancer cells.[45]

(2) AuNCs for imaging and quantification

AuNCs are a promising material because of their fluorescence brightness, simple synthesis in aqueous media, great photostability and biocompatibility.[46,47] AuNCs are expulsed of the organism through the urinary system[48], making possible *in vivo* analysis. Multiple ligands, including small molecules, polymers and DNA can be used to stabilize AuNCs. GSH stabilized AuNCs are useful to detect H_2S (an interesting target because of its role in neurotransmission and vasodilation processes). The polymer N-acryloxysuccinimide is used to stablilize probes composed of AuNCs, ovalbumin and folic acid for cancer cell imaging (ovalbumin serves as a protection for AuNCs and folic acid is used because folic acid receptors are overexpressed in certain cancer cells) through specific staining.[47]

Oligonucleotides can act as a stabilizing scaffold for AuNCs, although it is not as common as for AgNCs: in contrast to the high affinity between negatively charged DNA and Ag(I), $AuCl_4^-$ (which comes from $HAuCl_4$) does not interact so favorably with DNA due to its negative charge. However, synthesis of AuNC scaffolded by DNA is reported, typically through reduction with dimethylamine borane (DMAB) in acidic solutions[43], though these conditions make *in vivo* analysis challenging. A group of study[49] designed a "turn-on" method based on the synthesis of AuNCs, in which the intensity of the fluorescence emission is proportional to the amount of scaffolding DNA. This strategy has two main advantages: rapid analysis and no need for reducing agents, since only Au(III) and mercaptopropionic acid are used as reagents in the presence of the DNA. This way, certain DNA genes in cancers can be detected and quantified. It requires a PCR machine, but only one thermal cycle is needed, so detection can last for only two minutes (figure 9).

(3) AuNCs as catalysts

AuNCs possess electrochemical activity corresponding to oxidations and reductions of its Au(0) and Au(I) species. Therefore, they show catalytic activity, and they have been used to enhance the enzymatic reduction of O_2 that is carried out in enzymatic fuel cells, where fuel is oxidized in the anode and O_2 is reduced in the cathode. Multicopper oxidases are traditionally used to catalyze the reduction, but DNA templated AuNCs (DNA acts as a stabilizing ligand in the same way as it does for AgNCs) offer enhanced electron transfer between the enzymes that oxidize the fuel and the surface of the electrodes, resulting in higher efficiency.[42]

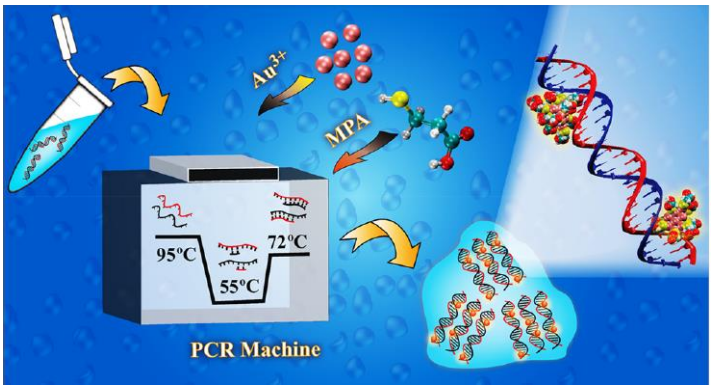


Figure 9. Representation of the quantification of DNA through AuNC synthesis ("turn-on" strategy). Isolated DNA sample is introduced in a PCR machine along with an Au(III) salt and MPA, and only one thermal cycle is needed to detect a fluorescent signal proportional to the amount of DNA present in the sample.
(image extracted from Sahoo AK. et al, ref 49)

6.5. Detection methods for microRNA

6.5.1. Traditional methods

(1) Northern blotting

Northern blotting[21,25,50] is a widely used technique for detection of miRNA and for comparing miRNA levels between samples. In fact, it is considered the "gold standard" technique for miRNA quantification. The miRNAs are separated by size through gel electrophoresis in agarose gels containing formaldehyde, which breaks hydrogen bonds between bases, disrupting the secondary structure of miRNA to ensure proper binding to the probe. miRNA is then transferred to a blotting membrane (nylon is usually used), which is positively charged to attract the negatively charged miRNAs. miRNA must be immobilized in the membrane (by heat or UV light) because DNA probes cannot penetrate the gel matrix. Finally, radiolabeled DNA probes complementary to target miRNA are introduced in the membrane and hybridization occurs. The position of the blot (output signal) on the membrane is directly related to target size, and its intensity to target concentration.

Northern blotting is the only technique able to determine both target size and relative abundance, allowing to compare miRNA abundance between samples on a single analysis.[22,23] Studies have demonstrated that simultaneous detection of several miRNA in a single membrane is possible.[22] However, northern blotting does not offer quantitative analysis (only relative comparison of miRNA abundance between samples) and it has low detection efficiency.[28] It involves radiolabeling of probes, toxic reactants like formaldehyde are necessary, and even though it is relatively inexpensive it is a complex and time consuming procedure.[23]

(2) microRNA microarrays

Detection methods involving microarrays[25,29] are based on hybridization between fluorescent labeled miRNA target molecules and miRNA probes that are covalently attached to a glass slide, forming a miRNA microarray. Thus, miRNA must be isolated from the sample and labeled with a fluorescent dye before their hybridization in the microarray. Types of miRNA present in the sample and their relative quantification can be studied.

This technique constitutes the best choice when expression levels of hundreds of miRNAs in a sample or comparison between samples must be studied. It shows good detection limits[28]

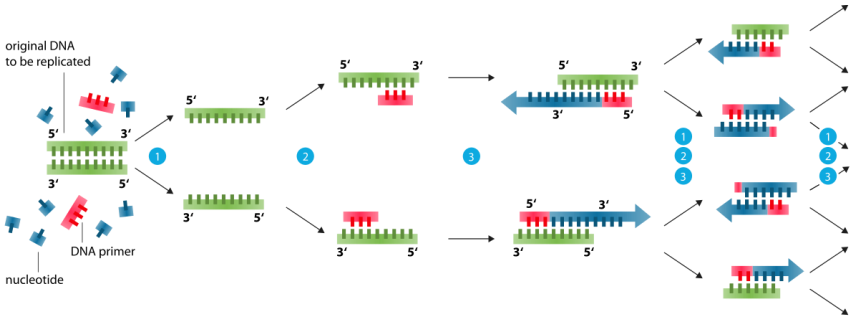
and offers rapid analysis[25], but the required instrumentation and materials are costly, and only relative quantification of miRNA is possible.

(3) Reverse-transcription polymerase chain reaction (RT-PCR)

This technique is based on the amplification of RNA target sequences, obtaining enough replications of them to detect low concentrated targets.[28] Reverse transcription step is required to detect RNA instead of DNA: reverse transcriptase enzyme generates the complementary DNA sequence to the target RNA, enabling its detection from this point following traditional PCR steps as if it was DNA (Scheme 3).[51]

Different temperatures are required throughout RT-PCR procedure: in denaturation (labeled as stage "1" in Scheme 3), high temperatures (95°C) are necessary to separate the double strands of target miRNA molecules in single strands. In the second stage (labeled as "2"), annealing, primer molecules (short sequences of nucleic acids that act as starting points for replication) bind to each of the single strands, so lower temperatures (5°C below primers' melting temperature) are applied. Replication of the single strands is achieved by a DNA polymerase in the third stage, extension (labeled as "3"). Thus, temperature needs to increase again to 72°C, which is the adequate temperature for DNA polymerases. These three stages are repeated in a cyclic manner to increase exponentially the concentration of target molecules, allowing detection through organic dyes.[51]

RT-PCR offers quantitative analysis and high specificity and sensitivity with low concentration of target needed, but it requires complex and costly instrumentation (especially the thermal cycler). Used polymerases must be thermostable to withstand the cycles.[52]



Scheme 3. Polymerase chain reaction mechanism. To detect miRNA, a DNA strand complementary to the target miRNA is used instead of "original DNA to be replicated".

(04/06/2019 via Wikimedia Commons, Creative Commons Attribution)

6.5.2. DNA templated silver nanoclusters

DNA-AgNCs have great potential as probes to improve miRNA detection. Modifications like labeling of target molecules or signal amplification methods are not needed, which makes this technique simple and inexpensive. DNA-AgNC probes enable rapid detection of miRNA, since fluorescent NCs may be formed within the probe in an hour[18] in the presence of $AgNO_3$ and $NaBH_4$. DNA-AgNC probes are biocompatible[27], meaning that they do not have toxic effects on the human organism. Though each DNA-AgNC probe – miRNA system has different quantum yields, the majority are reported to be high, around 0.3[18,36]. Aditionally, these probes constitute bright fluorophores with significant fluorescence lifetime.

Typically, DNA-AgNCs act as bright fluorescent labels until target miRNA complementary to the DNA templating sequence hybridizes with it resulting in a quenching of the fluorescence. This "turn-off" approach has showed good results, but it has worse sensitivity (more background noise) than a "turn-on" strategy (in which the presence of the target would result in an increase of the fluorescent signal) would have, so it is not a valid method to detect some human miRNAs (detection of human miRNA in real samples presents difficulties associated with interferences from the environment of the target). However, some simple signal amplification techniques can be applied to enhance sensitivity.[38,53]

In this work, when a probe in particular has to be mentioned, a specific and abbreviated nomenclature is used. The DNA sequence of DNA-AgNC probes, as it has been said, has two different regions: a sequence, complementary to the target, that recognizes it and hybridizes with it, and a sequence that scaffolds AgNC. To name a probe, the number of nucleotides for scaffolding AgNCs is indicated along with color of emission, followed by the sequence complementary to miRNA.

6.5.2.1. Detection of isolated microRNAs through fluorescence quenching

Detection is based on a drastic drop of the signal when analytes are present. This is the most used strategy when studying and analyzing isolated miRNAs.

To test the specificity of this method, the analyte miRNA-160 (a plant miRNA responsible for the development of Arabidopsis thaliana) is isolated and studied.[18] It has been found that high specificity can be achieved: experiments using the probe DNA-12nt-RED-160 have shown that fluorescence intensity drops drastically after an hour of having added $AgNO_3$ and $NaBH_4$ to solutions containing the probe mixed with the target. A slight drop of the fluorescence has been

observed as well in the presence of other miRNA sequences, but the effect is much more limited, allowing the detection of specific miRNA sequences (figure 10).

Quantification of miRNA targets is possible following this method, since the fall of the intensity of the signal is directly proportional to target concentration.[18,19]

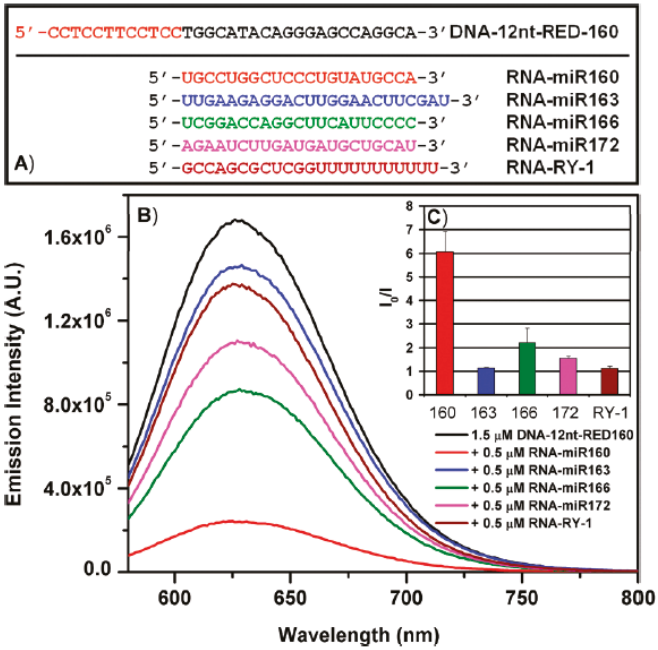


Figure 10. Example of the "turn-off" strategy. (A) Sequences of the probe DNA-12nt-RED-160, the specific target miRNA-160 and 4 non-specific targets: miRNA-163, miRNA-166, miRNA-172 and RNA-RY-1. (B) Emission spectra of 1.5 μM solution of the probe, and 1.5μM solution of the probe with additions of 0.5 μM of each target. Excitation was at 560 nm. (C) Io/I values corresponding to additions of 0.5 μM of each target to the 1.5 μM solution of the probe.

(image extracted from Wook Yang S. and Vosch T., ref. 18)

6.5.2.2. Detection of plant and human microRNAs from total RNA samples

miRNA analysis on total RNA samples (i.e., the complete RNA present in cells including mRNA, tRNA and rRNA) has been shown to be possible on samples extracted from the plant *Arabidopsis thaliana*[18], which paves the way for further investigations on miRNA analysis without previous isolation. By comparing expression levels of miRNA-160 from a whole RNA sample of a healthy (with normal levels of miRNA-160) and a mutant (with no detectable levels of miRNA by a previous northern blot analysis) specimen, distinction between the two

specimens was possible. The expected fluorescence drop upon target hybridization with the probe was much more drastic on the healthy specimen. A slight drop was observed on the mutant specimen too (much lower than that of the healthy specimen), which led to the conclusion that the probe hybridizes with other types of RNA apart from the target. Thus, a little part of the fluorescence quenching registered using this method on total RNA samples comes from non-specific hybridization, but it can be concluded that DNA-AgNC probes have good specificity in front of the other non-specific miRNA.

Despite the good results in detection of plant miRNAs, several important human miRNA targets for diagnosis like let-7a (which is a miRNA precursor that acts as a tumor suppressor in the organism) and miRNA-200c (which act as a biomarker for colorectal cancer)[2] cannot be detected using DNA-AgNC probes.[19] For let-7a, the DNA probe is not capable of scaffolding AgNC, and the probe designed for miRNA-200c cannot hybridize with the target. It is believed that the secondary structures of these target miRNA do not make possible proper hybridization (due to DNA sequence being too stable to promote hybridization) or adequate AgNC scaffolding (due to a lack of flexibility of the DNA backbone in the scaffolding sequence to accommodate AgNC). To solve this problem, DNA/RNA chimeras (mixtures of RNA and DNA nucleotides) have been used for templating instead of DNA, and new probes were designed to make possible detection of let-7a and miRNA-200c.

Experiments with let-7a substituted the target recognizing sequence of the DNA probe DNA-12nt-RED-let-7a by a sequence containing a mixture of DNA and RNA nucleotides, and the DNA of the scaffolding sequence was substituted by RNA too. The resulting probe was named D-12nt-R-22nt-let-7, and by using it, intensity of the signal was enhanced by a factor of 10, increasing the sensitivity of the method. The rotational freedom (leading to more flexibility) in the sugar backbone of RNA would permit easier embedding of AgNC than DNA. The new type of probes has shown great results detecting its target let-7a and specifically distinguishing it from other miRNA sequences.

A new probe was designed[19] to enhance sensitivity of miRNA-200c detection, substituting some of the DNA nucleotides of DNA-12nt-RED-200c with RNA ones. The resulting probe, D-12nt-R23nt-200c, showed great sensitivity towards its target, so it was deduced that DNA probes were too stable to promote target hybridization. It was concluded that probes less stable thermodynamically have better sensitivity. The new probe also showed good specificity, since

the presence of non-specific targets did not lead to fluorescence quenching but to an enhancement of it: specificity towards miRNA-160 has been tested using total human RNA from HT-29 cell line (which is a colon cancer cell line), and the experiment resulted in the expected drop of fluorescence. When the probe designed specifically for this target is used in presence of other cell lines like PANC-1 (pancreatic cancer cell line) or MIA-PaCa-2 (another pancreatic cell line), an enhancement of fluorescence is observed instead of the previous drop. The mechanism explaining this phenomenon of enhancement in the presence of non-specific targets is not clear yet.

6.5.2.3. Coupling of DNA templated silver nanoclusters with signal amplification techniques

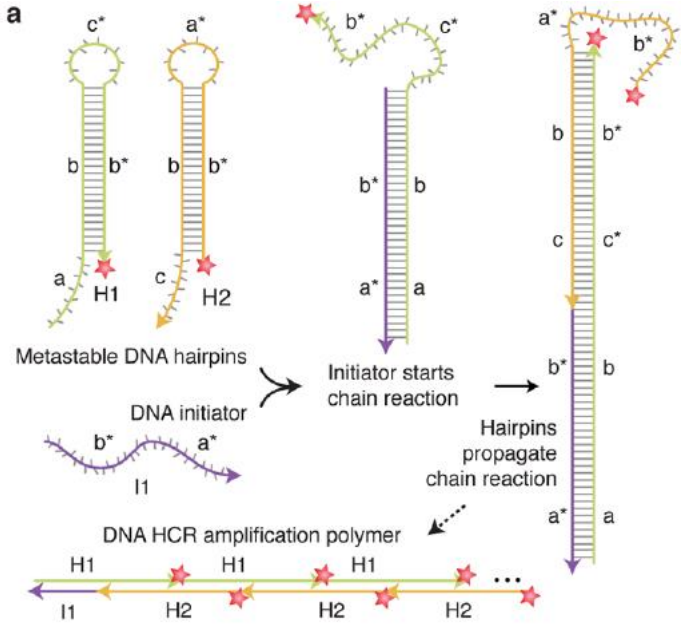
(1) Coupling with Hibridization Chain Reaction

DNA-AgNC probes have been coupled with Hybridization Chain Reaction (HCR) to improve their sensitivity towards miRNA targets, reaching a detection limit of 0.1 nM.[53] HCR amplifies the signal (as PCR does, but without the need of nicking enzymes) with the following mechanism: two hairpin structures, H1 and H2, are synthetized. Both contain overhang sequences that are complementary to the loop of the other hairpin, and H1 loop consists of a C6 sequence capable of embedding AgNC. H1 and H2 are in their closed form initially, and when AgNO3 and NaBH4 are added, AgNCs are formed in H1's loop and a fluorescence signal is detected. In the presence of miRNA target, H1 hybridizes with it opening its hairpin structure, and H2 opens as well to hybridize with the overhang of H1. Opening and hybridization occurs repeatedly in the presence of the target, resulting in a more significant drop of the fluorescence than when DNA-AgNC probes are used without HCR (scheme 4).

This method allows quantitative and selective analysis, since fluorescence intensity decreased proportionally with target concentration and distinction between complementary, non-complementary and mismatched targets is possible.[38]

Detection of target miRNA-145 (which can act as an Alzheimer disease biomarker) has been carried out in real human samples.[38] Blood extracted from a healthy woman was centrifuged to isolate the serum, and then miRNA targets were added to the serum to see if they could be detected in such an environment by using this method. Detection was possible but not as sensitive as when isolated and pure miRNA-145 was used as a sample: fluorescence intensity was high for the serum sample without target, and a drop was observed with the addition of miRNA-145, greater with the increase of target concentration (enabling quantitative

analysis). However, due to interference of other biomolecules, the decrease of fluorescence was significantly less pronounced than when isolated miRNA-145 was analyzed.

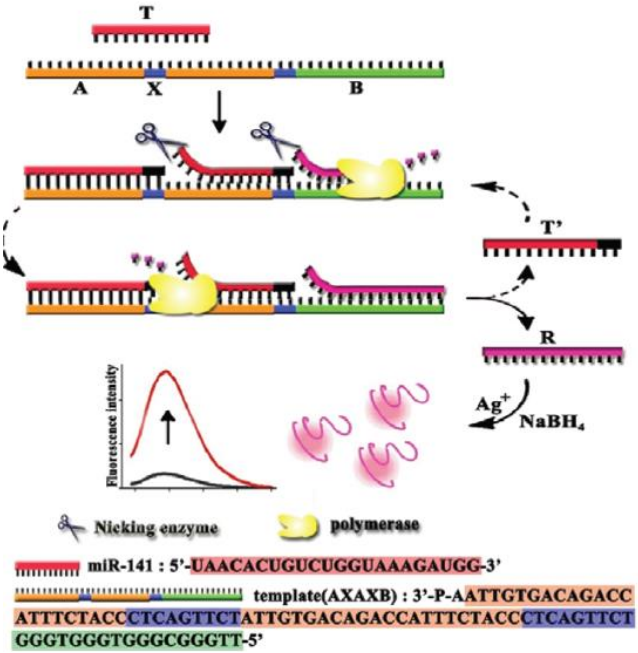


Scheme 4. Mechanism of Hybridization Chain Reaction coupled with DNA-AgNC probes.
(image extracted from Choi HMT. et al, ref. 53)

(2) Coupling with Target-Assisted Isothermal Exponential Amplification

To enhance sensitivity, DNA-AgNC probes have been coupled with an amplification technique called Target-Assisted Isothermal Exponential Amplification (TAIEA), which uses a DNA template, a DNA polymerase, nicking enzymes and dNTP to exponentially amplify both target molecules (T) and the DNA scaffolding sequence for AgNC formation. Template is designed to contain five regions (AXAXB): "A" sequences are complementary to target miRNA, "X" sequences are nicking sites and "B" sequence is complementary to a DNA scaffolding sequence. Upon target hybridization, the DNA polymerase synthetizes complementary sequences to the template, resulting in a strand consisting on five regions including target molecule (T), nicking sites (X), a DNA sequence complementary to target RNA (T') and a scaffolding sequence for AgNC (R): TXT'XR. When nicking enzymes cleave the sequence, T and T' sequences hybridize again with the template, and the repeated synthesis and cleavage

leads to an exponential amplification of the target, which means that scaffolding sequence is amplified too (scheme 5). [1,54]



Scheme 5. Mechanism of Target-Assisted Isothermal Exponential Amplification coupled with DNA-AgNC to detect target miRNA-141.

(image extracted from Liu Y. et al, ref. 1)

This method suggests a different approach from the classic "turn-off" strategy. Target presence leads to an amplification of scaffolding sequence, and upon addition of $AgNO_3$ and $NaBH_4$, a fluorescent signal proportional to scaffolding sequence concentration (so proportional to target miRNA as well, enabling target quantification) is registered. The increased sensitivity coming from amplification and the use of a "turn-on" strategy instead of a "turn-off" (which almost eliminates background noise) one can be of great help in diagnosis of cancers on its early stages.

The detection limit of this technique is 2 attomolar (10^{-18}M) when synthetic spike-in (short synthetic sequences of known concentration often used as a control for determining the sensitivity of a method[55]) miRNA are used as targets[1], and of 10 fM (the same as RT-PCR technique) when synthetic miRNA was analyzed.[26]

Simultaneous detection of several miRNA targets possible following this method[1], since it is possible to differentiate the emissions of four different DNA-AgNCs synthetized by four different scaffolding sequences corresponding to "B" sequences (figure 11).

Then, by changing sequences "B" to achieve different emission colors and sequences "A" to target different analytes, different wavelengths of emission corresponding to specific targets can be programmed allowing simultaneous detection. Synthetic miRNA-21 (a breast tumor biomarker) and miRNA-141 (a prostate cancer biomarker) have been detected in the same analysis following this method, and good specificity is achieved without the targets interfering with one another. When more than one target wants to be simultaneously detected, molar ratio of their respective templates must be optimized to obtain the maximum fluorescence intensities for both wavelengths.[26]

High selectivity toward miRNA targets has also been achieved by changing the target recognition sequence "A" on the template: highly homologous sequences of miRNA like miRNA-141, miRNA-429 and miRNA-200b were analyzed separately with a probe designed to target miRNA-141 and high fluorescence was only detected when target was present, whereas the presence of the other miRNA led to very low levels of fluorescence.[1]

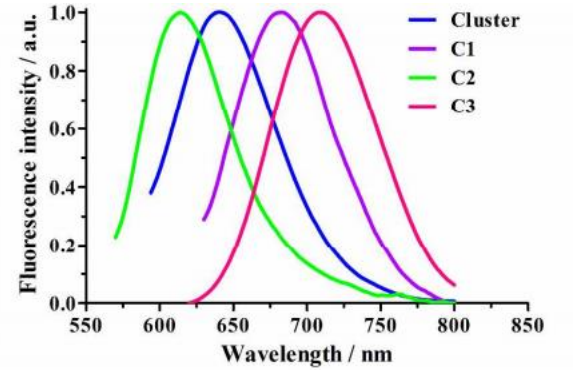


Figure 11. Fluorescence emission spectra of 4 DNA-AgNCs corresponding to 4 different scaffolding sequences: Cluster (5'-CCCACCCACCCGCCCAA-3'), C1 (5'-CCCTTAATCCCC-3'), C2 (5'-CCCTTACTCCCC-3') and C3 (5'-CCCTAACTCCCC-3'). Amplification of the signal through TAIEA makes possible to differentiate between each maximum wavelength of emission.

(image extracted from Liu Y. et al, ref. 1)

7. CONCLUSIONS

Fluorescent AgNCs probes constitute a good alternative to traditional miRNA detection methods, outrunning them because of their simple synthesis and rapid analysis, cost-effectiveness, tuneability to target different analytes when stabilized with DNA and possibility of simultaneous detection of several analytes, and because of their biocompatibility.

DNA-AgNC are promising complexes to improve miRNA detection, which is highly desirable due to their role in genetic regulation. The major drawback on DNA-AgNC probes is that they are not photostable. DNA-AuNC, on the other hand, have more photostability, but they are not as good probes as DNA-AgNC because $AuCl_4^-$ does not show high affinity with DNA.

There is a wide variety of methods to detect target miRNAs by using DNA-AgNCs. Detection based on fluorescence quenching are extensively used, as it provides specific and quantitative detection, but enhanced sensitivity can be achieved if the emission of fluorescence instead of its quenching is detected.

Most experiments involving miRNA detection through DNA-AgNC probes are carried out *in vitro*, so there is a lack of information about the efficiency of these strategies in real organisms. Further investigations need to be done to comprehend the mechanisms of action of AgNC, and the relationship between their structure and their properties, since almost all the information gathered to this point is based on experimental observations. Once these aspects are clear, application of these complexes to detect targets *in vivo* is expected to be done and protocolized like other detection methods are.

Studies on the photostability of DNA-AgNC probes would be of great help to make their use possible in *in vivo* analysis, as well as investigating ways to enhance AuNCs interactions with DNA. Other interesting pathways to direct future investigations turn to be enhancing of the sensitivity to overcome the interferences present in human samples analysis. Templating AgNC with mixtures of RNA and DNA or coupling the probes with signal amplification techniques can lead the way to achieve the objective.

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9. ACRONYMS

miRNA: micro Ribonucleic Acid

DNA-AgNC: DNA templated silver nanocluster

NC: Nanocluster

AuNC: Gold nanocluster

QD: Quantum Dots

GSH: Glutathione

SNP: Single Nucleotide Polymorphism

AP sites: Abasic

tRNA: transfer Ribonucleic Acid

rRNA: ribosomal Ribonucleic Acid

mRNA: messenger Ribonucleic Acid

pri-miRNA: primary Ribonucleic Acid

pre-miRNA: precursor Ribonucleic Acid

RISC: Ribonucleic acid-Induced Silencing Complex

RT-PCR: Reverse-Transcription Polymerase Chain Reaction

GO: Graphene Oxide

Poly-G: Poly-Guanine

NIR: Near Infra-Red